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POTENCIAL BIOTECNOLÓGICO DE EXTRATOS DE MACRÓFITAS AQUÁTICAS NO CONTROLE DA BIOINCRUSTAÇÃO

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Tese apresentada ao Programa de Pós-Graduação em Oceanologia, como parte dos requisitos para a obtenção do Título de Doutor.

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A

AAS – Água artificial de salinidade

ABNT – Associação Brasileira de Normas Técnicas (ABNT)

AI – Auto indutoras (Moléculas sinalizadoras)

C

CC – Cloreto de cobre II

CORSAN – Companhia de Saneamento do Rio Grande

CEOB – Concentração de efeito observável baixo

CENO – Concentração de efeito não observável

CE50 – Concentração efetiva para 50% da população

CL50 – Concentração letal para 50% da população

D

DMSO – Dimetilsulfóxido

E

EBB – Erradicação do biofilme bacteriano

F

FP – Frequência de pulsação

FTIR – Espectroscopia de infravermelho de transmissão de Fourier

G

GC/MS – Cromatografia gasosa acoplada à espectroscopia de massa

GLM – Modelos lineares generalizados (GLM)

I

IFB – Inibição da formação do biofilme

IV – Inibição da violaceína

IC – Inibição do crescimento

ICP – Inibição do crescimento de bactérias planctônicas

IQS – Inibição do *quorum sensing*

IMO – *International Maritime Organization*

L

LC/MS – Cromatografia líquida acoplada a espectroscopia de

massa

M

MAC – Macroincrustação

MIC – Microincrustação

MPE – Matriz polimérica extracelular

N

NMDS – Escalonamento multidimensional não métrico

O

OECD – *Organization for Economic Co-operation and Development*

OTU – Unidades taxonômicas operacionais

P

PERMANOVA – Análise permutacional de variância multivariada

Q

QS – *Quorum sensing*

S

SDS – Surfactante dodecil sulfato de sódio

SIMPER – Análise percentual de similaridade

SZ – Sulfato de zinco

T

TBT – Tributilestanho

TPT – Trifenilestanho

U

USEPA – *United States Environmental Protection Agency*

Resumo

A bioincrustação pode causar grandes prejuízos econômicos e ecológicos em estruturas artificiais expostas ao ambiente aquático, deste modo, sendo necessárias estratégias para prevenir esse processo. Um dos métodos mais difundidos para prevenir a bioincrustação são as tintas anti-incrustantes, entretanto, esse tipo de revestimento de superfícies já apresentou inúmeros impactos ambientais. Por este motivo, existe a busca por alternativas anti-incrustantes menos impactantes para o meio ambiente, sendo estas a base de produtos naturais com uma possível maior biodegradabilidade e potencialmente menor toxicidade para organismos não-alvo. Nesse sentido, o presente estudo teve como objetivo avaliar o potencial biotecnológico de alternativas anti-incrustantes naturais formuladas a partir de extratos de macrófitas aquáticas, para ambientes límnicos, estuarinos e marinhos, a partir de experimentos de laboratório e campo. Um total de 25 extratos aquosos de macrófitas foram investigados quanto a capacidade em inibir a formação do biofilme bacteriano, bem como a sua comunicação por *quorum sensing,* além da adesão de invertebrados usando como modelos mexilhões da espécie invasora *Limnoperna fortunei* (mexilhão dourado) e pólipos do cnidário *Aurelia coerulea*. Ainda, foram realizados ensaios toxicológicos com diferentes organismos não-alvo (microalgas, microcrustáceos e peixes). Por fim, foram realizados experimentos de campo para verificar a capacidade anti-incrustante dos extratos associados a cobetura epóxi. Os extratos de *Cabomba caroliniana* e *Schoenoplectus californicus* foram os mais eficientes para inibir a bioincrustação marinha e estuarina. Esses extratos inibiram mais de 70% a formação do biofilme bacteriano estuarino uni e multiespécies e a adesão de pólipos de *A. coerulea* (marinho). Enquanto os extratos de *Pontederia crassipes* e *Typha domingensis* foram os mais promissores para inibir a bioincrustação límnica, inibindo mais de 70% do biofilme bacteriano límnico uni e multiespécies e a adesão de *L. fortunei*. Quanto a toxicidade, a diluição segura dos extratos de *P. crassipes* e *T. domingensis* foi de até 35% para os organismos não-alvo límnicos: a microalga *Pseudopediastrum boryanum*, o cladócero *Daphnia magna* e o peixe *Pimephales promelas*. Enquanto que para *C. caroliniana* e *S. californicus* a diluição segura foi de 20% e 5% para os organismos e fase não-alvo, respectivamente, para a

microalga *Thalassiosira pseudonana* (marinha)*, o copépodo Nitokra* sp. (estuarino) e o cnidário *A. coerulea* (fase planctônica não-alvo - marinho). Os compostos presentes nos extratos mais promissores foram caracterizados quimicamente por cromatografia gasosa (GC-MS) e líquida (LC-MS) acopalhada à espectroscopia de massas. Os compostos mais abundantes nos extratos de *P. crassipes*, *T. domingensis*, *C. caroliniana* e *S. californicus* foram Eicosano e 4-metilfenetilamina, entretanto, cada extrato apresentou compostos específicos. Os experimentos de campo com os extratos de *P. crassipes* e *T. domingensis*, formulados com cobertura epóxi, corroboraram com os resultados laboratoriais, apesar da complexidade encontrada nas interações do ambiente natural. Isso se deu pela diminuição de frequência de taxa e inibição da adesão de bactérias heterotróficas, organismos autotróficos e macro-organismos. Deste modo, os resultados destacam o potencial biotecnológico de *P. crassipes* e *T. domingensis* para combater a bioincrustação límnica e *C. caroliniana* e *S. californicus* para a bioincrustação estuarina e marinha. Também destacamos sua adequação e utilização para o desenvolvimento de aplicações anti-incrustantes ecologicamente mais seguras para o meio ambiente.

Palavras-Chave: Antibiofilme; antiadesão; compostos naturais; inibição do *quorum sensing*; plantas aquáticas.

Abstract

Biofouling can cause significant economic and ecological damage to artificial structures exposed to the aquatic environments, needing the development of strategies to prevent this process. One of the most widespread methods for preventing biofouling is the use of antifouling paints. However, these surface coatings have historically caused numerous environmental impacts. Consequently, there is a search for antifouling alternatives that have less impact on the environment, these being based on natural products with possible greater biodegradability and potentially less toxicity to non-target organisms. The present study aimed to evaluate the biotechnological potential of natural antifouling alternatives formulated from extracts of aquatic macrophytes, for use limnic, estuarine and marine environments. Laboratory and field experiments were conducted to assess these alternatives. A total of 25 aqueous extracts of macrophytes were investigated for their ability to inhibit bacterial biofilm formation and quorum sensing, as well as their effect on the attachment of invertebrates, including the invasive species *Limnoperna fortunei* (golden mussel) and polyps of the cnidarian *Aurelia coerulea*. Additionally, toxicological assays were performed with various non-target organisms, including microalgae, microcrustaceans, and fish. Field experiments further evaluated the antifouling efficacy of the extracts when incorporated into an epoxy coating. The extracts of *Cabomba caroliniana* and *Schoenoplectus californicus* were the most efficient to inhibit marine and estuarine biofouling. These extracts inhibited more than 70% the formation of single and multispecies estuarine bacterial biofilm and the attachment of *A. coerulea* (marine) polyps. While the extracts of *Pontederia crassipes* and *Typha domingensis* were the most promising to inhibit limnic biofouling, inhibiting more than 70% of single and multispecies limnic bacterial biofilm and the adhesion of *L. fortunei*. Regarding toxicity, the safe dilution of the extracts of *P. crassipes* and *T. domingensis* was up to 35% for the non-target limnic organisms: the microalgae *Pseudopediastrum boryanum*, the cladoceran *Daphnia magna* and the fish *Pimephales promelas*. While for *C. caroliniana* and

S. californicus the safe dilution was 20% and 5% for the non-target organisms and phase, respectively, for the microalgae *Thalassiosira pseudonana* (marine), the copepod *Nitokra* sp. (estuarine) and the cnidarian *A. coerulea* (non-target planktonic phase - marine). The compounds present in the most promising extracts were chemically characterized by gas chromatography (GC-MS) and liquid chromatography (LC-MS) coupled with mass spectroscopy. The most abundant compounds present in the extracts *of P. crassipes*, *T. domingensis*, *C. caroliniana* and *S. californicus* were Eicosan and 4-methylphenethylamine, although each extract contained specific compounds unique to its composition. Field experiments using epoxy coatings formulated with *P. crassipes* and *T. domingensis* extracts supported the laboratory findings, despite the complexity of interactions in natural environments. These experiments demonstrated reduced rates of adhesion by heterotrophic bacteria, autotrophic organisms, and macroorganisms. Overall, the results underscore the biotechnological potential of *P. crassipes* and *T. domingensis* for combat limnic biofouling and *C. caroliniana* and *S. californicus* against estuarine and marine biofouling. These findings highlight the suitability of these extracts for the development of ecologically safer antifouling applications.

Keywords: Aquatic plants; antibiofilm; anti-attachment; inhibition of quorum sensing; natural compounds.

Prefácio

A presente tese está estruturada em forma de capítulos, conforme as normas propostas pelo Programa de Pós-Graduação em Oceanologia da Universidade Federal do Rio Grande. Os artigos publicados e/ou submetidos em revistas, encontram-se em sua forma completa e nas normas exigidas por cada periódico. O primeiro artigo (Capítulo VII), publicado na revista internacional INNOTEC, foi desenvolvido para dar subsídio à proposta da Tese, buscando compreender as principais alternativas de controle da bioincrustação de mexilhões invasores. O segundo artigo da Tese (Capítulo VIII), publicado na revista internacional *Chemistry and Ecology,* avaliou a atividade anti-incrustante de extratos de macrófitas aquáticas contra a formação do biofilme bacteriano estuarino, bem como a sua toxicidade em organismos não-alvo. O terceiro artigo da Tese (Capítulo IX), publicado na revista internacional *Environmental Science and Pollution Research,* avaliou a capacidade de extratos macrófitas em inibir a micro e macroincrustação límnica, bem como a toxicidade para organismos não-alvo. O quarto artigo da Tese (Capítulo X) foi submetido na revista internacional *Environmental Toxicology,* avaliou a capacidade de extratos de macrófitas em inibir a adesão de pólipos do cnidário *Aurelia* coerulea, bem como a sua toxicidade para éfiras (fase não-alvo) da mesma espécie. O quinto artigo da Tese (Capítulo XI), submetido na revista internacional *Environmental Pollution*, avaliou em campo o potencial anti-incrustante dos extratos das macrófitas que apresentam resultados mais promissores quando testados em laboratório frente às espécies límnicas. Ressalta-se que a presente Tese teve colaborações internacionais da University of KwaZulu da África do Sul, da Fundación del

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Capítulo I: Hipóteses

O presente trabalho de Tese é norteado por quatro hipóteses principais:

(i) Os extratos aquosos de macrófitas aquáticas irão apresentar atividade anti-incrustante contra a microincrustação pela inibição do quorum sensing e/ou também contra a macroincrustação;

(ii) O efeito anti-incrustante e toxicológico irá variar de espécie para espécie de macrófita aquática, dependendo da composição química presente em cada extrato e também em relação ao ambiente límnico, estuarino e marinho.

Capítulo II: Introdução

Bioincrustação

A bioincrustação ou incrustação biológica (do inglês "*biofouling*"), é um processo sucessional natural que envolve a adesão e o acúmulo de organismos em superfícies expostas ao ambiente aquático [Scheer 1945]. A bioincrustação é amplamente observada em ambientes límnicos, estuarinos e marinhos, e pode ocorrer em estruturas artificiais ou naturais, incluindo embarcações, dutos e estruturas submarinas, pilares de plataformas, sistemas de cultivo na aquicultura, rochas, conchas e até animais [Dobretsov & Rittschof 2020]. Ainda, a sequência da composição biológica dos organismos incrustados varia da interação entre os ambientes aquáticos visto a interação entre a disponibilidade do plâncton e do bentos [Agostini *et al*. 2018].

O processo ocorre de maneira progressiva e pode ser dividido em quatro etapas principais [Martín-Rodríguez *et al*. 2015, Agostini *et al*. 2018]. A primeira etapa ocorre imediatamente após a submersão do substrato, onde acontece a adsorção de íons e moléculas orgânicas e inorgânicas (*e.g*., água, proteínas e polissacarídeos) pela superfície, formando um filme condicionante (Figura 1A), tornando o meio mais atrativo para etapa posterior [Martín-Rodríguez *et al*. 2015]. Em sequência, ocorre a segunda etapa, quando bactérias planctônicas pioneiras tornam-se sésseis e colonizam a superfície formando um biofilme (Figura 2B) [[Martín-Rodríguez *et al*. 2015]. Ainda nesta fase, outros micro-organismos como microalgas, fungos e protozoários se aderem a superfície [Martín-Rodríguez *et al*. 2015, Agostini *et al*. 2018].

Na terceira etapa inicia-se a colonização secundária, em que organismos

macroscópicos (*e.g.*, macroalgas e larvas de invertebrados) aderem-se à superfície (Figura 1C) [Martín-Rodríguez *et al*. 2015, Agostini *et al*. 2018]. Por fim, na última etapa ocorre o crescimento e amadurecimento da bioincrustação [Martín-Rodríguez *et al*. 2015], onde há um aumento significativo da sua biomassa e complexidade estrutural, incluindo camadas aderentes de colonizadores terciários como mexilhões, cracas e outros invertebrados adultos (Figura 3D) [Agostini *et al*. 2018].

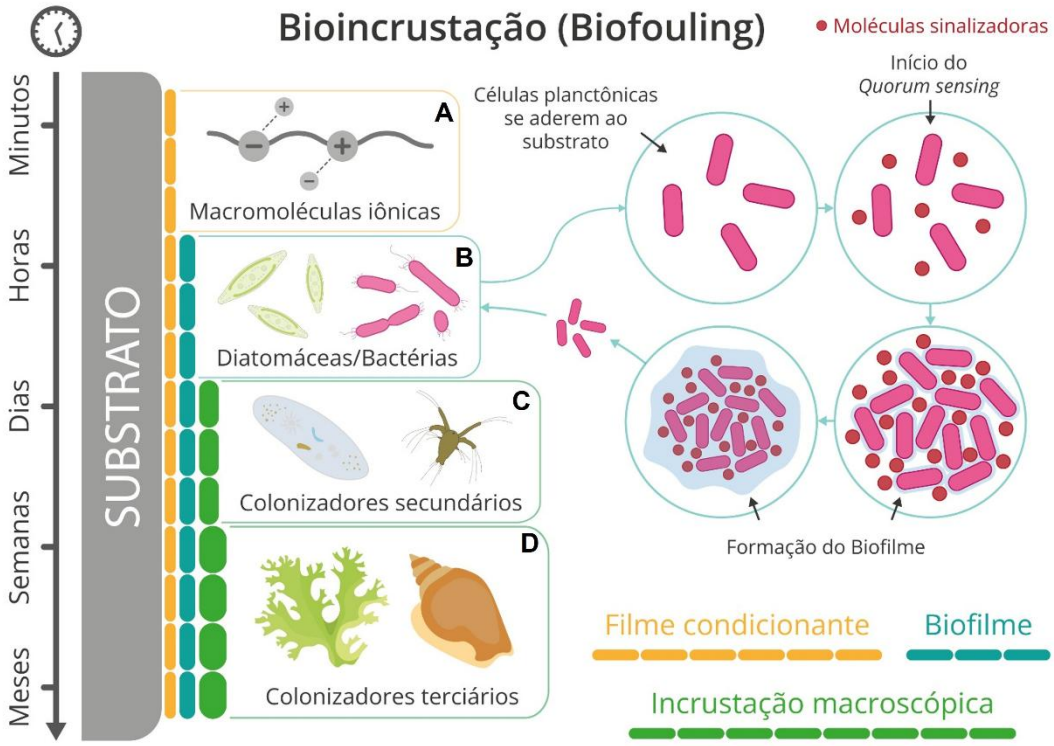

Figura 1: Etapas de formação do processo de bioincrustação. A – Filme condicionante; B – Formação do biofilme; C e D – Incrustação macroscópica por colonizadores secundários e terciários. Fonte: Autoral.

A formação do biofilme inicia-se imediatamente após as bactérias aderirem de maneira irreversível ao substrato, onde estas secretam uma matriz polimérica extracelular (MPE) composta de substâncias poliméricas e água [Flemming & Wingender 2010]. A MPE consiste principalmente de exopolissacarídeos, exoDNA, água, exoproteínas e exolipídios [Flemming &

Wingender 2010] e possui a função de revestimento do biofilme, permitindo a sua formação, multiplicação e o seu espalhamento [Agostini *et al*. 2018]. Com o biofilme formado, as bactérias podem suportar condições ambientais extremas, como a baixa disponibilidade de nutrientes, mudanças de pH, e ação de agentes químicos externos [Davey & O'toole 2000, Chattopadhyay *et al*. 2022].

Ainda, em conjunto com a formação do biofilme, há um aumento na produção, liberação e detecção de moléculas sinalizadoras auto indutoras (AI) que regulam a formação deste complexo de bactérias através do processo de comunicação intercelular (Figura 1), denominado de *Quorum sensing* (QS) [Chattopadhyay *et al*. 2022]. O QS permite que as bactérias cooperem entre si (de forma interespecífica e intraespecífica), coordenando a expressão de fenótipos específicos e regulando atividades fisiológicas [Borges & Simões 2019]. Dentre as principais funções do QS está a secreção de exopolissacarídos e evolução do biofilme (produção da MPE e formação do biofilme) [Jefferson 2004, Chattopadhyay et al. 2022]. Devido as AI regularem o QS e a MPE, uma interrupção e/ou modificação no reconhecimento das AI pode desencadear uma cascata de eventos que inibam a transcrição de genes e consequentemente iniba o processo de formação do biofilme ou seu espalhamento [Borges & Simões 2019].

Por desencadear mudanças químicas e físicas na superfície do substrato, deixando-o mais atrativo, os biofilmes bacterianos são capazes de influenciar na adesão de algas, protozoários, fungos, invertebrados e urocordados [Peng *et al*. 2020, Muras *et al*. 2021, Agostini *et al*. 2021, Ma *et al*. 2023]. Por exemplo, AI específicas promovem a adesão de macroalgas, cirripédios e diatomáceas [Peng *et al*. 2020, Muras *et al*. 2021]. Além disso, bactérias gram-negativas,

principalmente Proteobacteria e Bacteroidetes são altamente correlacionadas com o estabelecimento de organismos macroscópicos [Agostini *et al*. 2021].

Impactos negativos da bioincrustação

Em substratos artificiais, o processo de bioincrustação pode comprometer a funcionalidade e eficiência das estruturas, ocasionando diversas consequências econômicas. Para o setor naval, esse processo gera um aumento da rugosidade em cascos de embarcações, acarretando aumento do atrito e consequentemente a redução da sua hidrodinâmica e flutuabilidade [Campos *et al*. 2022]. Dessa forma, necessitando de um maior consumo de combustível e consequentemente, uma maior liberação de gases de efeito estufa [Campos *et al*. 2022]. Em usinas hidrelétricas a bioincrustação pode ocasionar o entupimento de tubulações, sistemas de resfriamento de turbinas e câmaras, e ainda obstruir sensores hidráulicos [Brugnoli *et al*. 2005, Brugnolli *et al*. 2011].

Em estações de tratamento de água, refinarias, sistemas agroindustriais de aquicultura e silvicultura também ocorre o desgaste e alterações na conformação de suas estruturas, principalmente relacionado ao seu entupimento, tornando-as mais frágeis e reduzindo sua durabilidade [Boltovskoy & Correa 2015, Maranhão & Stori 2019]. Ainda, a bioincrustação pode deformar redes de pisciculturas e impedir a troca de água e nutrientes, reduzindo a produção pesqueira [Bloecher & Floerl 2020]. Dessa forma, todos esses impactos acarretam em prejuízos para a indústria aquática, com despesa global anual estimada em 340 milhões de dólares, incluindo os gastos com manutenção e prevenção [Cuthbert *et al*. 2021].

A bioincrustação também pode desenvolver problemas ambientais,

atuando como um dos principais vetores para a disseminação de espécies invasoras [Dobretsov & Rittschof 2020], influenciando na dinâmica de invasões biológicas. Essas espécies podem ser transportadas em cascos de embarcações ou outras estruturas, ou até mesmo serem carregadas pela água de lastro de embarcações [Jagerbrand *et al*. 2019]. Além disso, esses organismos competem com espécies nativas por recursos, resultando em impactos negativos à biodiversidade e alterações na dinâmica dos ecossistemas locais [Cataldo *et al*. 2012, Boltovskoy & Correa 2015]. Dessa forma, a complexidade do problema da bioincrustação é magnificado quando associado a espécies invasoras [Morales *et al*. 2024b], como por exemplo o mexilhão dourado *Limnoperna fortunei* Dunker, 1857), invasor em diversas regiões da América Latina e Ásia.

Soluções anti-incrustantes

A fim de evitar a bioincrustação e seus problemas econômicos e ecológicos, tornou-se necessário o desenvolvimento de estratégias para recobrir os cascos de embarcações e outras estruturas artificiais imersas, sendo desenvolvidas as tintas anti-incrustantes (Figura 2) [Amara *et al*. 2018]. O registro mais antigo do uso destas tintas é de 2 mil anos atrás, onde se utilizava chumbo em misturas com óleo de baleia, enxofre e arsênio para revestir cascos de embarcações de madeira [Castro *et al*. 2011, Dafforn *et al*. 2011]. Em 1625, na Inglaterra, foi registrada a primeira patente de tinta anti-incrustante, uma mistura de arsênio, cobre e goma em pó [Castro *et al*. 2011].

No século XVII, os cascos das embarcações passaram a ser substituídos por cascos metálicos, o que acrescentou problemas associados à corrosão aos

já conhecidos efeitos da bioincrustação em madeira [Yebra *et al*. 2004, Castro *et al*. 2011]. Em meados do século XX, surgiu a primeira geração moderna de tintas anti-incrustantes, composta por óxidos de cobre e de zinco, comos primeiros biocidas [Godoi *et al*. 2003]. Entretanto, apesar de apresentar eficácia contra a maioria dos organismos incrustantes (*e.g.*, moluscos, cirripédios e ascídias), sua durabilidade era curta [Fernandez & Pinheiro 2007], perdendo sua eficiência em até um ano após sua aplicação [Castro *et al*. 2011], o que ocasionava aumento nos custos de manutenção das embarcações [Godoi *et al*. 2003]. Além disso, algumas espécies de algas demonstraram tolerância fisiológica ao cobre, diminuindo a eficácia dessas tintas [Almeida *et al*. 2007].

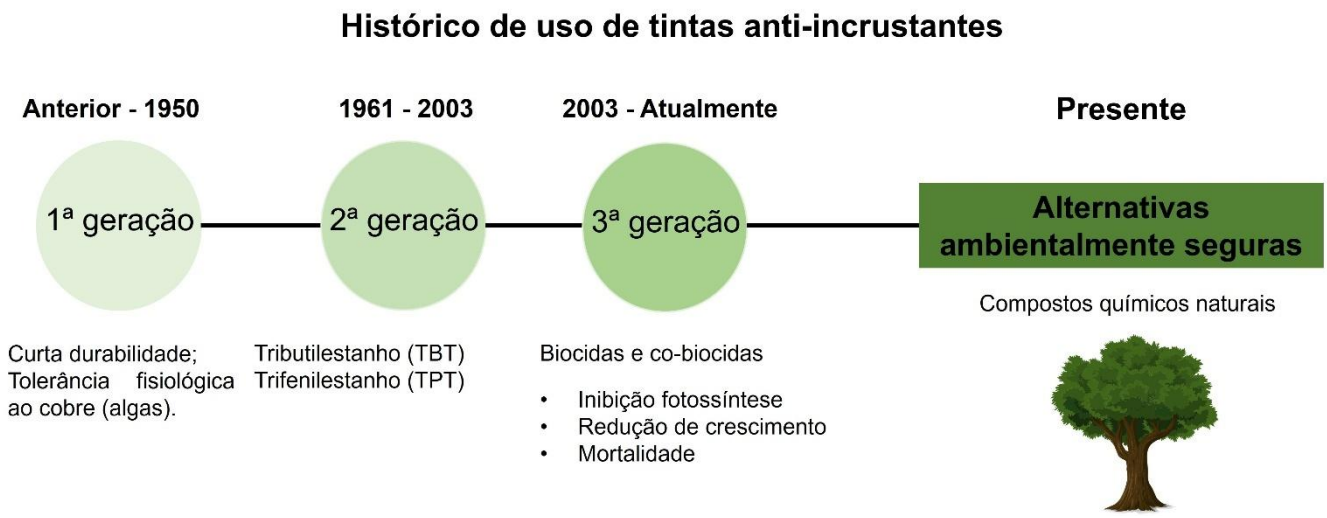

Figura 2: Linha do tempo das gerações de estratégias anti-incrustantes.

Como consequência desta baixa durabilidade, em 1961 foi iniciada a utilização de substâncias organoestânicas na composição das tintas anti-incrustantes [Fent 2003]. Essas tintas foram denominadas de anti-incrustantes de segunda geração [Fernandez & Pinheiro 2007], apresentando como princípio ativo os compostos tributilestanho (TBT) e/ou trifenilestanho (TPT), que possuíam alta eficiência e durabilidade [Almeida *et al*. 2007]. Com o passar dos

anos, também houve o desenvolvimento de tecnologias de copolímeros em tintas anti-incrustantes, permitindo a liberação de compostos organoestânicos em taxas mais lentas e constantes [Hugget *et al*. 1992]. Dessa maneira, em 1970 o TBT foi o biocida anti-incrustante mais utilizado no mundo, chegando em 1990 a um consumo mundial de 35.000 t ano^{-1}, e em 1999, aproximadamente 70% de todas embarcações do mundo utilizavam revestimentos com TBT [Godoi *et al*. 2003].

No entanto, foram descobertos diversos problemas ambientais relacionados a essas coberturas de segunda geração, devido a sua elevada toxicidade para organismos não-alvo e persistência ambiental [Dafforn *et al*. 2011, Gittens *et al*. 2013, Castro *et al*. 2018]. Dentre os efeitos deletérios causados por esses anti-incrustantes podem ser citadas as anomalias no desenvolvimento de larvas, alterações de conchas (balling) [Alzieu 2000], surgimento de órgãos sexuais masculinos em fêmeas de moluscos prosobrânquios (imposex) [Ketata *et al*. 2008] e problemas de imunossupressão em mamíferos [Tanabe 1999]. Por estas razões, em setembro de 2008, a *International Maritime Organization* (IMO), baniu o uso de tintas anti-incrustantes de segunda geração [IMO 2021].

Antes mesmo do banimento das tintas de segunda geração, a indústria desenvolveu a terceira geração de tintas anti-incrustantes, tendo como base em sua composição biocidas metálicos, como óxido cuproso e zinco [Chen *et al*. 2021] e co-biocidas orgânicos de reforço [Peres *et al*. 2015, Liu *et al*. 2020], podendo uma única formulação utilizar mais de um biocida e co-biocida. Dentre os biocidas de terceira geração tem-se os compostos orgânicos (*e.g*., Diuron, Irgarol e DCOIT); e organometálicos e/ou inorgânicos (*e.g*., Óxido de cobre,

Maneb, Ziram) [Castro *et al*. 2011, Paz-Villarraga *et al*. 2022].

Entretanto, mesmo com a utilização de biocidas e co-biocidas, a utilização destas tintas ainda apresentam limitações, como menor eficácia em estágios sucessionais avançados da bioincrustação [Agostini *et al*. 2019] e alta toxicidade para organismos não-alvo [Soroldoni *et al*. 2018]. Alguns desses impactos incluem a inibição da fotossíntese pelo bloqueio de elétrons no fotossistema II, redução do crescimento em peixes e mortalidade em crustáceos planctônicos e bentônicos [Martins *et al*. 2018, Mansano *et al*. 2018, Soroldoni *et al*. 2020, Campos *et al*. 2022]. Desta forma, o desenvolvimento de alternativas ambientalmente amigaveis passaram a ser consideradas.

Anti-incrustantes naturais

A fim de contrapor as inúmeras desvantagens das tintas anti-incrustantes de terceira geração, formuladas a partir de biocidas metálicos e co-biocidas, a busca por alternativas anti-incrustantes ecologicamente mais seguras cresceu nos últimos anos, deste modo a utilização de compostos naturais têm ganhado destaque na literatura científica [Agostini *et al*. 2021b, Hamidi *et al*. 2022]. Essas alternativas denominadas anti-incrustantes “verdes” são menos prejudiciais ao meio ambiente [Hamidi *et al*. 2022], devido a sua maior biodegradabilidade e potencial de menor toxicidade para organismos não-alvo [Pérez *et al*. 2021].

Os compostos naturais são produzidos pelos organismos como mecanismo de defesa que podem interferir na fixação, crescimento e/ou desenvolvimento de outros organismos, fenômeno conhecido como alelopatia [Gross *et al*. 2007, Hamidi *et al*. 2022]. Essas substâncias podem se originar de desde micro-organismos (*e.g*., bactérias, fungos e algas) até plantas e animais,

sejam eles de ambiente terrestre, marinho ou de água doce [Kyei *et al*. 2020; Hamidi *et al*. 2022]. Assim, esse fenômeno é um fator importante na determinação de interações bióticas nos ecossistemas, visto que pode influenciar na diversidade de espécies e na estrutura das comunidades biológicas [Borella *et al*. 2011].

Muitos produtos naturais marinhos têm sido relatados a partir de uma variedade de invertebrados (*e.g.*, esponjas, corais, briozoários), diatomáceas, bactérias e macroalgas [Salta *et al*. 2013; Kyei *et al*. 2020; Gu *et al.* 2020]. Entretanto, quando comparado a plantas, a maioria dos organismos marinhos são difíceis de obter em grandes quantidade de forma sustentável [Pérez *et al*. 2014]. Assim, a busca por alternativas anti-incrustantes à base de produtos naturais de plantas vem aumentando nos últimos anos [Agostini *et al*. 2021b].

Compostos químicos anti-incrustantes a base de plantas

Além de serem utilizados como anti-incrustantes, os compostos vegetais são conhecidos por combater doenças, devido às suas propriedades anti-inflamatórias [Nunes *et al*. 2020], anticancerígenas *[Khan et al*. 2019], antimicrobianas [Chassagne *et al*. 2021] e antioxidantes [Unuofin & Lebelo 2020]. As plantas destacam-se por possuírem grande número de compostos químicos que são distribuídos quali e quantitativamente em diferentes órgãos vegetais da planta [Hamidi *et al*. 2022]. Ainda, os mesmos podem variar quanto aos fatores abióticos (*e.g*., temperatura e regimes hidrológicos) e bióticos (*e.g*., herbivoria, espécie vegetal e aspectos reprodutivos) [Ramos *et al*. 2022].

A atividade anti-incrustante das plantas está relacionada principalmente à presença de compostos químicos da classe dos alcaloides, flavonoides, taninos,

álcoois, aldeídos e alcenos [Hamidi *et al*. 2022, Mangoba & Guzman Alvindia 2023, Rambaran *et al*. 2024]. Ainda, sabe-se que os taninos são os principais agentes anti-incrustantes, devido aos polifenóis possuírem propriedades antimicrobianas e anticorrosivas [Kyei *et al*. 2020]. Dentre esses compostos, se destacam os terpenos de *Ceriops tagal* [Chen *et al*. 2011], indol de árvores de Guatambú [Pérez *et al*. 2019], taninos de árvores de acácia negra [Peres *et al*. 2015a] e derivados de capsaicina de pimentas [Xu *et al*. 2005].

No entanto, os estudos relacionados aos anti-incrustantes naturais, estão focados principalmente na atividade de plantas terrestres [Agostini *et al*. 2021b; Hamidi *et al*. 2022], não sendo encontrados trabalhos desenvolvidos com macrófitas aquáticas. Em contrapartida, as macrófitas têm atraído grande interesse dos pesquisadores por demonstrarem-se promissoras como agentes antimicrobianos, no controle da eutrofização, e como fonte para o desenvolvimento de biogás [Batistote & Mascarenhas 2023, Amarilla *et al*. 2024, Dilshad *et al*. 2024]. Dentre essas têm-se os gêneros *Ludwigia*, *Typha*, *Schoenoplectus*, *Cabomba*, *Eichhornia* (Atualmente *Pontederia*), *Nymphoides* e *Salvinia* [Takao *et al*. 2011, Chicalote-Castillo *et al*. 2017, Jiménez 2020].

Macrófitas aquáticas

As macrófitas aquáticas são caracterizadas como organismos fotossintéticos que se desenvolvem periodicamente ou permanentemente flutuando, submerso ou na superfície da água [Chambers *et al*. 2008]. Elas desempenham um papel fundamental na estruturação e manutenção em sistemas de água doce [Son *et al*. 2021], contribuindo para a produtividade primária e participando da estocagem e ciclagem de nutrientes e no controle da

poluição como por exemplo na diminuição da eutrofização e remoção de metais pesados nos ecossistemas [Thomaz & Esteves 2011, Jeppesen *et al*. 2012, Schneider *et al*. 2012].

Por apresentarem uma grande variedade de formas biológicas, essas plantas são classificadas em grupos ecológicos de acordo com a localização de seus órgãos vegetais em relação à água [Pedralli 2000, Pompêo & Moschini-Carlos 2003, Thomaz & Esteves 2011]: (*i*) submersas: plantas com raízes pouco desenvolvidas que flutuam submersas na água ou totalmente enraizadas e submersas; (*ii*) flutuantes: plantas enraizadas ou com raízes livres abaixo da coluna da água e que flutuam na coluna da água; (*iii*) emersas: plantas enraizadas no sedimento com folhas acima da coluna da água; (*iv*) anfíbias: plantas que se desenvolvem na interface entre ambiente terrestre e aquático, adaptadas a condições de seca e alagamento.

As plantas aquáticas retornaram ao ambiente aquático, e sofreram uma série de adaptações que permitiram o seu reestabelecimento no ecossistema aquático [Trindade *et al*. 2010]. As principais adaptações foram em relação a sua anatomia, como a redução da cutícula e do sistema de sustenção, estômatos (quando presentes) e cloroplastos passaram a se encontrar na parte superior das folhas [Thomaz & Esteves 2011]. Além dessas, algumas macrófitas passaram a possuir o aerênquima bem desenvolvidos, auxiliando no armazenamento de gases (e.g., CO_2 e O_2) no seu interior [Thomaz & Esteves 2011].

Ainda, de forma adaptativa, devido a competição de recursos no ambiente aquático (*e.g*., nutrientes, luz e espaço no habitat) e da proteção contra a herbivoria, as plantas aquáticas produzem compostos químicos (aleloquímicos)

[Gross *et al*. 2003]. Devido ao habitat aquático, os aleloquímicos liberados por essas plantas devem apresentar concentrações eficientes suficientes para atingirem o organismo alvo) [Gross *et al*. 2003]. Dessa forma, espera-se encontrar mais compostos hidrofílicos em plantas aquáticas do que em plantas terrestres [Gross *et al*. 2003]. Nesse contexto, os compostos hidrofílicos atuam sobre espécies fitoplânctonicas, enquanto compostos lipofílicos atuam sobre organismos que tem contato direto com a planta, sendo mais relacionado a herbivoria [Gross *et al*. 2003].

A defesa química frente a herbivoria é considerada como a principal característica que determina a palatabilidade em plantas aquáticas [Cronin *et al*. 2002, Sotka *et al*. 2009]. Assim, dada a pressão seletiva exercida pela herbivoria [Lodge 1991] e a diversidade de aleloquímicos defensivos produzidos [Ramesh *et al*. 2014], os compostos químicos desempenham papéis ecológicos fundamentais na estruturação do ambiente aquático [Pereira *et al*. 2021]. Devido a isso, as plantas aquáticas são conhecidas por produzir uma grande variedade de aleloquímicos, como fenóis, terpenos, alcaloides, entre outros [Ramesh *et al*. 2014].

A exemplo disso, têm-se a macrófita emergente *Typha domingensis* que pode inibir a predação de lagostins devido a presença de fenóis e ácidos graxos em sua composição [Gallardo-Williams *et al*. 2002, Bolser *et al*. 1998]. A macrófita flutuante *Eichhornia crassipes* produz os compostos de fenalenona e esteróis, ambos fenóis que também influenciam a predação [Lalitha *et al*. 2012]. O *Potamogeton* (macrófita submersa), possui altos níveis de fenóis, que exercem atividade inibitória contra as microalgas *Selenastrum capricornutum* e *Taphidocelis subcapitata* [Zhang *et al*. 2011, Waridel *et al*. 2003, Spencer &

Ksander, 1994]. Já a defesa química de *Cabomba caroliniana* também uma macrófita submersa, é ativada pela presença do caracol *Pomacea canaliculate*, que reduz a palatabilidade da planta e suprime o crescimento de fungos co-ocorrentes em sua estrutura [Morrison & Hay, 2011].

Devido as plantas emergentes e flutuantes serem mais acessíveis a herbívoros terrestres e aquáticos, e plantas submersas possuem apenas o contato com os organismos aquáticos [Groos & Baker 2012], existe uma diferença na composição química entre essas plantas. Plantas submersas possuem níveis mais baixos de compostos fenólicos em comparação com plantas emergentes ou flutuantes [Smolders *et al*. 2000], porém, em outros estudos o mesmo padrão não é evidenciado [Pereira *et al*. 2021]. Assim, apesar do conhecimento de que as defesas químicas de plantas aquáticas são extensas, elas ainda permanecem inexploradas [Gross & Baker 2012], principalmente relacionado a atividade anti-incrustante, nunca explorada em macrófitas aquáticas.

Mecanismos para ação anti-incrustantes

A procura por anti-incrustantes de origem natural não só auxiliam a fornecer o desenvolvimento de anti-incrustantes menos tóxicos para o meio ambiente, mas também ajudam a compreender os mecanismos de adesão dos organismos bioincrustantes. Em geral, os revestimentos anti-incrustantes ambientalmente seguros agem de diversas maneiras para prevenir a bioincrustação. Os mecanismos de inibição da microincrustação focam principalmente em inibir a formação do biofilme, e podem ser divididos em [Trentin *et al*. 2013]: (*i*) inibição direta da formação do biofilme; (*ii*) inibição por

bloqueadores do quorum sensing; (*iii*) erradicação do biofilme já formado. Já a macroincrustação pode ser inibida por [Kyei *et al*. 2020]: (*i*) inibidores da produção e/ou liberação de bissos; (*ii*) reguladores da expressão proteica; (*iii*) bloqueadores da neutrotransmissão; (*iv*) e também inibição da formação do biofilme, que consequentemente inibe a adesão de organismos macroscópicos.

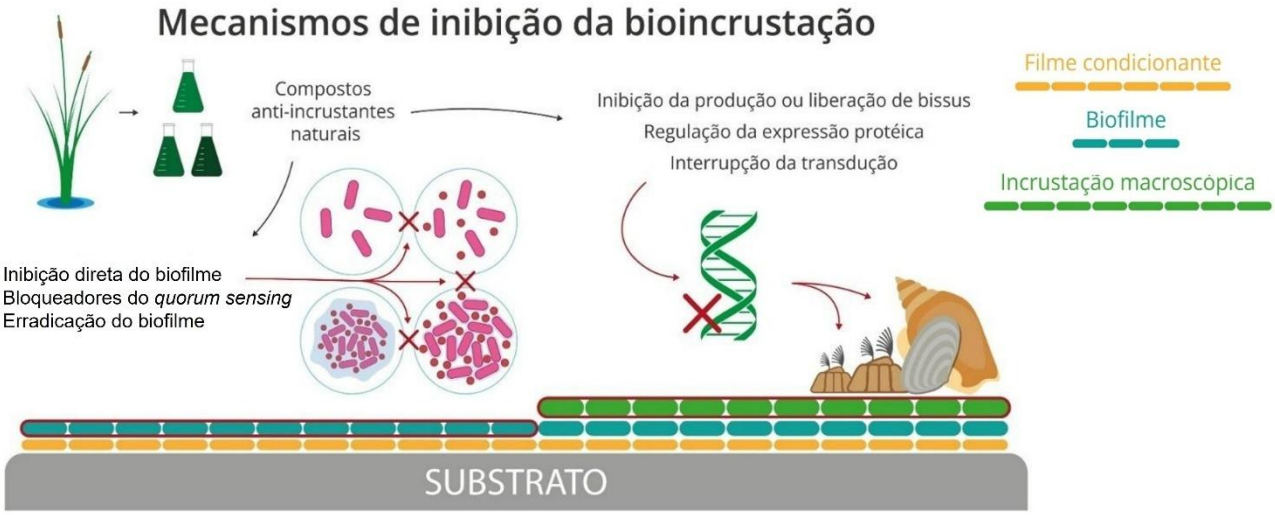

Figura 3: Mecanismos de inibição da adesão de organismos incrustantes.

Ainda existe outra via de ação através da toxicidade por letalidade [Jin *et al*. 2022], em que os compostos previnem a bioincrustação matando os organismos incrustantes, devido a problemas fisiológicos e moleculares causados por estresse oxidativo, quebras de fitas de DNA, inibição da fotossíntese dentre outros [Chen & Qian 2017]. Entretanto, esse modo de ação não é interessante, uma vez que as chances são altas de apresentar toxicidade também para organismos não-alvo.

Com isso, estudos atuais para o desenvolvimento de novas alternativas anti-incrustante buscam que o efeito anti-incrustante aconteça de modo precoce pela inibição do biofilme bacteriano, pois esta etapa é uma condição crítica para as proximas etapas da bioincrustação [Peng *et al*. 2020, Agostini *et al*. 2021a, Ma *et al*. 2023]. Ainda, para reduzir a resistência bacteriana [Agostini *et al*.

2019a], esses efeitos buscam o mecanismo de ação de modo a não afetar o crescimento planctônico bacteriano [Neves *et al*. 2024], e sim afetar o QS, pela sua inibição [Martín-Rodríguez *et al*. 2015].

Ensaios anti-incrustantes e toxicológicos

Para o desenvolvimento de novos anti-incrustantes, testes preliminares em condições controladas de laboratório são realizados, seguido de testes em campo. Ensaios anti-incrustantes *in vitro* envolvendo a microincrustação, especialmente biofilmes bacterianos, são bem estabelecidos, sendo na sua grande maioria realizados testes com comunidades bacterianas ao invés de espécies únicas [Agostini *et al*. 2021b]. Entretanto, a busca por um agente anti-incrustante que não afete o crescimento planctônico bacteriano ainda não é tão explorada [Agostini *et al*. 2021b], sendo na maioria das vezes realizados ensaios sem avaliar esse parâmetro, principalmente relacionado ao QS.

Já para os ensaios de macroincrustação existe uma grande variedade de organismos utilizados, no entanto, restringem-se principalmente a espécies de vermes tubulares, mexilhões e cirripédios [Almeida & Vasconcelos 2015, Agostini *et al*. 2021b]. Para isso, as espécies são coletadas em campo e expostas a processos físicos e químicos para liberação de ovos e/ou larvas. Esses procedimentos podem levar dias (~10 dias) para obter a fase necessária do seu ciclo de vida para realização dos testes [Pinteus *et al*. 2020]. Com base nesses desafios, por não necessitar de metamorfose para obtenção dos organismos-teste, os pólipos da água viva *Aurelia* vem chamando atenção [Pinteus *et al*. 2020].

Após comprovada a sua eficácia em laboratório, para a sua aplicação

comercial, testes *in situ* são necessários. Esses ensaios permitem que os estudos sejam realizados em interações e condições ambientais complexas entre os organismos incrustantes e a hidrodinâmica do ambiente [Romeu & Mergulhão 2023]. Para a sua validação em campo, os compostos naturais promissores nos ensaios de laboratório podem ser adicionados em tintas, funcionando como aditivos em suas formulações [Hamidi *et al*. 2022].

Junto aos efeitos anti-incrustantes, as novas soluções devem apresentar baixa ou nenhuma toxicidade para organismos não-alvo [Pérez *et al*. 2021]. Visto isso, para desenvolver novos anti-incrustantes, também deve-se realizar ensaios de toxicidade com organismos não-alvo representantes de diferentes níveis tróficos, a fim de avaliar seus efeitos com maior precisão [Pane *et al*. 2008]. Ainda, testes de toxicidade são úteis para avaliar novos produtos químicos para estabelecer seus limites no ambiente aquático [Morales *et al*. 2024b].

Os ensaios toxicológicos possuem diferentes organismos modelo, sendo a sua escolha de extrema relevância com o parâmetro ambiental que se encontra a substância química testada. Dessa forma, organizações e agências internacionais como a *Organization for Economic Co-operation and Development* (OECD), a *United States Environmental Protection Agency* (USEPA) e a Associação Brasileira de Normas Técnicas (ABNT) estabelecem e padronizam testes com diferentes espécies com o intuito de fornecerem uma maior confiabilidade dos dados.

Neste mesmo contexto, nos testes toxicológicos, para identificar a relação entre uma substância e seu efeito específico, existem os descritores de concentrações [Amara *et al*. 2018]. Esses determinam o perigo de cada substância testada e podem ser expressos em concentração letal média para

50% da população (CL_{50}), concentração efetiva mediana para 50% da população (CE_{50}), concentração de efeito não observável (CENO) e concentração de efeito observável baixo (CEOB) [Amara *et al*. 2018].

Capítulo III: Objetivos

Objetivo Geral

Avaliar o potencial biotecnólogico de extratos aquosos de macrófitas aquáticas no controle da micro e macroincrustação em ambiente límnico, estuarino e marinho.

Objetivos específicos

(i) A partir de um estudo de revisão, verificar o status sobre as principais alternativas no controle de incrustações de mexilhões invasores;

(ii) Verificar o potencial dos extratos aquosos de macrófitas aquáticas em inibir a micro e macroincrustação límnica, estuarina e marinha;

(iii) Avaliar a toxicidade dos extratos aquosos de macrófitas aquáticas em organismos e fase de desenvolvimento não-alvo;

(iv) Avaliar a eficácia anti-incrustante dos extratos aquosos associados a cobertura epóxi de tintas comerciais em ambiente límnico natural.

Capítulo IV: Área de Estudo

Os ensaios de avaliação da atividade anti-incrustante dos extratos aquosos de macrófitas aquáticas em campo (Capítulo X – Artigo 5) foram desenvolvidos na Hidroelétrica de Salto Grande Uruguai-Argentina. Salto Grande é um reservatório fluvial subtropical de aproximadamente 750 km² com múltiplos braços localizado ao longo de 100 km do canal principal do rio Uruguai, com profundidade média de 6,4 m, máxima de 35 m, temperatura média anual de 19 ºC e precipitação anual de 1.260 mm. É caracterizado por um período de vazante de dezembro a março e cheias de abril a novembro, com vazão máxima de 22.000 $m^3\ s^{-1}$ e mínima de 5.563 $m^3\ s^{-1}$ [O'Farrell et al. 2012]. O reservatório é usado principalmente para a geração de energia, mas também para o abastecimento de água potável e atividades recreativas, incluindo esportes e pesca.

O reservatório também é caracterizado pela proliferação de florações de cianobactérias, que varia de acordo com as condições hidrológicas de vazante e nível d'água, e atinge maiores abundâncias na margem direita do reservatório e nas áreas costeiras mais próximas da barragem [O'Farrell et al. 2012]. Os principais sistemas hídricos (rio Paraná e rio da Prata) que ligam a região de estudo apresentam alta abundância de *Limnoperna fortunei* (mexilhão dourado) [Fabián *et al*. 2021, Silva *et al*. 2021]. Esta espécie é uma das macroincrustações mais prejudiciais para as indústrias aquáticas [Pereira *et al*. 2022]. Com isso, a área de gestão ambiental de Salto Grande vem enfrentando desafios para combater a bioincrustação na região.

Capítulo V: Material e Métodos

As metodologias utilizadas no presente trabalho estão descritas abaixo, sendo separadas de acordo com cada artigo científico desenvolvido.

4.1. Estratégias de controle de mexilhões invasores: uma revisão (Artigo 1)

A busca na literatura foi realizada na base de dados da *Web of Science*, da *Scopus* e do *Science Direct*. A busca foi realizada considerando o período entre 1990 e 2020 (junho), considerando a combinação de palavras em inglês: "(*invasive mussel biofouling*) *AND control*". A literatura cinza (teses, dissertações e resumos de congressos não foram consideradas nesta revisão. Após a busca, os artigos sobre o tema de interesse foram selecionados, identificados e quantificados. A análise de dados foi realizada nos diferentes artigos, considerando: (*i*) autor, (*ii*) país de desenvolvimento da pesquisa, (*iii*) ambiente aquático estudado (marinho, dulcícola ou estuarino), (*iv*) experimento realizado em campo ou laboratório, (*v*) tipo de controle (biológico, químico ou físico). Para determinar os parâmetros de classificação do controle biológico, químico e físico, foram levadas em consideração as seguintes regras: *biológico*, artigos que tratavam de predação e/ou competição por recurso alimentar para o controle de mexilhões; *químico*, artigos sobre utilização de compostos químicos isolados ou brutos, e/ou fatores abióticos (*e.g.*, pH, oxigênio e salinidade), físico, artigos sobre o uso de barreiras físicas como filtros, diferentes tipos de substratos e/ou hidrodinâmicas e temperaturas. Para a nomenclatura atual de espécies marinhas e dulcícolas foi utilizado o registro mundial de espécies WoRMS

(https://www.marinespecies.org/).

4.2. Atividade anti-incrustante de extratos de macrófitas aquáticas em biofilmes bacterianos estuarinos (Artigo 2)

4.2.1 Preparação dos extratos de macrófitas

Para o preparo dos extratos, macrófitas aquáticas foram coletadas em lagos permanentes (32°09’23,3”S 52°05’57,6”W) no sul do Brasil localizados na Universidade Federal do Rio Grande (FURG). A região apresenta clima úmido subtropical com verões quentes [Alvares *et al*. 2013] e a coleta foi realizada no inverno de 2020 e verão de 2021. A identificação das espécies foi realizada através da análise morfológica de estruturas vegetativas e reprodutivas com o auxílio de chaves de identificação indicadas pela literatura [Pott & Pott 2000, Souza and Lorenzi 2012], resultando em um total de 11 espécies.

Cada planta foi cuidadosamente cortada em órgãos vegetais distintos, variando de raízes a flores/inflorescências, resultando em um total de 25 extratos vegetais diferentes (Tabela 1). A fim de garantir amostras representativas e mitigar a variabilidade sazonal dos compostos químicos presentes nos extratos, os materiais coletados durante o inverno e verão foram combinados [Ramos *et al.* 2022]. Para a obtenção da biomassa vegetal seca, o material vegetal foi seco a 60-80 °C até atingir massa constante, e triturado manualmente com pilão e almofariz.

Para a preparação dos extratos, 6 g da biomassa seca foram adicionados a 300 mL de água estuarina estéril (25). A mistura foi mantida no escuro a 22 °C por 24 h [Agostini *et al*. 2019a]. Posteriormente, a mistura foi centrifugada (1000

rpm por 10 min), e seu sobrenadante foi filtrado-esterilizado (0,2 µm) (filtro de acetato de celulose, Sartorius Biolab Products) [Agostini *et al*. 2019a]. A preparação resultou na solução estoque de 100% que foi diluída com água estuarina estéril para concentrações de 5, 10, 20 e 40% para utilização nos experimentos. O tratamento controle consistiu em água estuarina estéril (0%).

Tabela 1: Lista dos extratos vegetais testados nos bioensaios. Fonte: Morales et al., 2024b.

Grupo	Família	Espécie e Autor	Órgão da Planta
Angiosperma	Cabombaceae	*Cabomba caroliniana* A. Gray	Folha
			Caule
Angiosperma	Cyperaceae	*Schoenoplectus californicus* (C. A. Mey.) Soják	Caule
			Inflorescência
Angiosperma	Menyanthaceae	*Nymphoides humboldtiana* (Kunth) Kuntze	Folha
			Caule
			Flores
Angiosperma	Onagraceae	*Ludwigia hexapetala* (Hook. & Arn.) Zardini et al.	Folha
			Caule
Angiosperma	Onagraceae	*Ludwigia multinervia* (Hook. & Arn.) Ramamoorthy	Folha
			Caule
Angiosperma	Pontederiaceae	*Eichhornia azurea* (Sw.) Kunth	Folha
			Caule
Angiosperma	Pontederiaceae	*Eichhornia crassipes* (Mart.) Solms	Folha
			Caule
			Raiz
			Flores
Angiosperma	Potamogetonaceae	*Stuckenia pectinata* (L.) Börner	Mix
Samambaias e Licófitas	Salvinaceae	*Salvinia minima* Baker	Folha
			Rizoide
Samambaias e Licófitas	Salvinaceae	*Salvinia herzogii* De la Sota	Folha
			Rizoide
Angiosperma	Typhaceae	*Typha domingensis* Pers.	Parte aérea superior
			Parte aérea inferior
			Inflorescência

4.2.2. Isolados bacterianos

Para obtenção dos isolados bacterianos, foi coletada água do estuário da Laguna dos Patos (32°09’23.3”S, 52°05’57.6”W), com temperatura de 13 °C e salinidade 25 no dia da coleta. A água coletada foi transportada para o laboratório e colocada em um recipiente plástico de 56 L. Para obtenção das bactérias formadoras de biofilme, substratos de acrílico, compensado naval, aço carbono ASTM-36 (25 cm^2) e concreto (36 cm^2) foram colocados no recipiente plástico contendo água estuarina, por 24 h com fotoperíodo de 12 h: 12 h (claro:escuro). Foram feitas três unidades experimentais (recipientes de 56 L) contendo cada três repetições de cada substrato. Após a exposição, os substratos foram lavados com solução salina estéril (0,4%) para remover células planctônicas. Em câmara de fluxo laminar, o biofilme foi raspado dos substratos com o auxílio de um swab estéril, que foi então espalhado em meio ágar nutriente (Kasvi K25-610036, Laboratórios Conda S.A., Espanha). As culturas foram incubadas no escuro durante sete dias a 25 °C.

Colônias morfologicamente distintas foram selecionadas e isoladas 3x por meio de semeadura em estrias (método streak plate). Em seguida, cada isolado foi transferido para um meio caldo nutriente (Kasvi, Laboratórios Conda S.A., Espanha). Os isolados foram criopreservados (-80 °C) com 15% de glicerol e resazurina (0,010 M) como indicador. Então criou-se o Banco de Microincrustação no Laboratório de Microcontaminantes Orgânicos e Ecotoxicologia Aquática (CONECO) do Instituto de Oceanografia (IO) da FURG, e armazou-se os isolados a fim de fornecê-los para pesquisas futuras. Todos os isolados foram rastreados para confirmar sua capacidade de formar biofilmes, através do teste do cristal violeta [O’Toole 2011].

Foram escolhidos doze isolados morfologicamente distintos para serem sequenciados através do gene 16S rRNA. Para isso, a extração de DNA foi realizada usando água de Milli-Q estéril (filtrada em 0,2 µm) e esferas lavadas em ácido (Sigma-Aldrich). A lise celular foi realizada por vortex (30 s), seguida de aumento da temperatura (90 °C) e diminuição (-20 °C) por 15 s cada. A amplificação da quase totalidade do gene bacteriano 16S foi realizada com os primers 27F (AGAGTTTGATCMTGGCTCAG) e 1492R (TACGGYTACCTTGTTACGACTT) utiliando a enzima Promega Tag G2. . A construção das bibliotecas moleculares e o sequenciamento de DNA foram feitas na plataforma ABI 3730 (Sanger) pelo Laboratório de Biotecnologia da Empresa de Serviço Nacional de Aprendizagem Industrial (SENAI).

As sequências consenso entre as reads forward e reverseforam geradas com a ajuda dos softwares Seqtrace 0.90 e BioEdit 7.2. As sequências foram submetidas à plataforma Silva (https://www.arb-silva.de/) para alinhamento múltiplo com relação às sequências filogeneticamente mais próximas e classificadas usando o banco de dados Silva SSU r138.1. Para identificação de espécies, os critérios foram >99% de identidade, e para gênero >95%. As sequências foram submetidas ao GenBank sob o número de acesso SUB13384869. Essas bactérias foram utilizadas para realizar ensaios de inibição e erradicação do biofilme e inibição do crescimento bacteriano planctônico.

4.2.3. Comunidade bacteriana

Para obter a comunidade bacteriana, a água foi coletada do Estuário da Laguna dos Patos (salinidade 25, temperatura 19 °C) (32°09’44.6”S 52°06’ 04.4”W). Antes da realização dos ensaios, a água coletada foi pré-filtrada (7 µm)

para remover organismos fitoplanctônicos e zooplanctônicos [Agostini et al. 2019b].

4.2.4 Ensaio de inibição do biofilme

Os ensaios antibiofilme foram conduzidos separadamente para cada isolado bacteriano e para a comunidade bacteriana. Os ensaios foram conduzidos em placas multipoços de acrílico (Citotest Labware Manufacturing CO. LTD, Jiangsu, China), com oito replicações por tratamento. Para realização dos ensaios foi utilizada uma suspensão de inóculo bacteriano de 10^6 bactérias mL^{-1} em caldo nutriente (Kasvi K25-1216, Laboratórios Conda S.A., Espanha) [Agostini *et al*. 2019a]. Para ensaios de inibição da formação do biofilme (IFB), 100 µL do inóculo bacteriano foram misturados com 100 µL de tratamentos (diluições de 0, 5, 10, 20 e 40%) e incubados no escuro a 25 °C por 48 h. Os extratos que apresentaram resultados de IFB ≥ 60% em comparação aos controles foram usados para realizar ensaios de erradicação do biofilme bacteriano (EBB) e bactérias em sua forma planctônica (inibição do crescimento de bactérias planctônicas- ICP).

Para o ensaio EBB, 200 µL do inóculo bacteriano foram adicionados aos poços, e a placa foi então incubada no escuro a 25 °C por 24 h. Após a remoção do sobrenadante, 200 µL de tratamentos (0, 5, 10, 20 e 40%) foram adicionados. As placas foram então incubadas no escuro (25 °C) por mais 48 h. Para medir a densidade do biofilme de ambos os ensaios (IBF e EBB), o sobrenadante foi removido, e o biofilme foi seco (60 °C) por 1 h, corado com cristal violeta (0,4%) por 15 min, e solubilizado com etanol 99,5% por 30 min. A densidade óptica foi medida em um leitor de microplacas de fluorescência (filtermax F5, dispositivos

moleculares) (550 nm) [O'Toole 2011, Agostini *et al*. 2019a]. Para o ensaio ICP, foi usada como um ponto final a diferença na densidade óptica (620 nm) no início e no fim da incubação (no escuro a 25 °C) após 48 h [Agostini *et al*. 2019a; Vale *et al*. 2019]. Os controles dos ensaios IFB, EBB ou ICP foram considerados como representando 100% da biomassa. Os resultados foram expressos como uma porcentagem de IBF, EBB ou ICP.

4.2.5. Ensaios toxicológicos

Para os extratos e suas diluições que apresentavam efeito inibitório na formação de biofilme ≥ 60%, foram realizados ensaios toxicológicos com os organismos não-alvo *Thalassiosira pseudonana* (Hasle & Heimdal, 1970) (microalga planctônica marinha) e *Nitokra* sp. (Boeck, 1865) (copépode epibentônico).

Para *T. Pseudonana,* foi realizado um ensaio crônico com quatro repetições por tratamento [ABNT 2021]. O ensaio foi realizado em erlenmeyers de 50 mL (49 mL de diluições de extrato e 1 mL de microalgas) com inóculo a uma densidade de 10^4 células mL^{-1} por 72 h a 24 °C. O ensaio continha iluminação contínua de 7.000 lux, agitação constante e um pH inicial de 6,60-7,0. Tanto no início (0 h) quanto no final (72 h) do ensaio, uma alíquota (1 mL) foi removida e 200 µL de formaldeído a 0,4% foram adicionados para interromper o crescimento de cultura. A densidade celular (células mL^{-1}) foi estimada pela contagem de células usando uma câmara de Neubauer, através da diferença nas contagens de células entre 0 e 72 h. Ao final do tempo de exposição, alíquotas de 5 mL foram retiradas para estimar a concentração de clorofila-a (µg L^{-1}). As alíquotas foram centrifugadas, o sobrenadante foi removido e 3 mL de

metanol (100%) foram adicionados, sendo então refrigeradas a -14 °C por 2 h. A absorbância do sobrenadante foi lida em espectrofotômetro em 663 e 750 nm. A concentração de clorofila-a foi calculada de acordo com Mackinney [1941].

Para *Nitokra* sp. foi realizado um ensaio agudo com três repetições por tratamento [Nascimento *et al*. 2002]. Dez espécimes adultos não segurando sacos de ovos por repetição foram expostas a 20 mL de cada tratamento selecionado. O ensaio teve duração de 96 h a 25 °C, a um fotoperíodo de 16 h: 8 h (claro: escuro), pH inicial de 6,6-7,0. Após o tempo de exposição, organismos vivos e mortos foram contados, sendo copépodos completamente imóveis contados como mortos. Os resultados foram expressos como porcentagem de sobrevivência em relação ao controle (0%).

4.2.5. Análise de dados

Para observar diferenças potenciais entre cada um dos tratamentos em comparação com o controle nos ensaios com as bactérias, o test T de Student foi utilizado com correção de Bonferroni para comparações múltiplas. Para os ensaios com *T. pseudonana* após verificar a normalidade dos resíduos e homocedasticidade, foi utilizada ANOVA *one-way*. Quando aceita a hipótese alternativa a um alfa de 0,05 ($p<0,05$), o teste post hoc de Tukey foi utilizado. Devido a natureza discreta dos dados no ensaio de Nitokra sp., um modelo linear generalizado (GLM) com distribuição binomial foi utilizado aplicando a função de ligação logit. Todas as análises foram realizadasno software R 4.2.2 (R Development Core Team, 2024).

4.3. Macrófitas aquáticas como fonte de anti-incrustante não tóxico contra

biofilmes bacterianos e adesão do mexilhão dourado: um possível papel da interferência do quorum sensing (Artigo 3)

4.3.1. Preparação dos extratos vegetais

Devido a este capítulo utilizar organismos dulcícolas para o seu desenvolvimento, foi utilizado água natural estéril (salinidade 0) de um lago (32° 09' 44.6"S 52° 06' 04.4"W) para diluição e preparação dos extratos. O método de coleta, identificação e preparação dos extratos vegetais para este capítulo foi o mesmo descrito anteriormente (seção 4.2.1). Resumidamente, uma amostra de 6 g de biomassa vegetal seca (seca em estufa a 60 °C) foi adicionada a 300 mL de água natural estéril (filtrada −0,22 µm e autoclavada) sem salinidade (salinidade 0) [Agostini *et al*. 2019]. A preparação resultou em uma solução estoque, considerada 100%, que foi diluída para 5, 10, 20 e 40%. O tratamento controle foi água natural estéril (0% e salinidade 0).

4.3.2. Isolamento bacteriano

Os isolados bacterianos foram obtidos de acordo com metodologia da seção 4.3.1, sendo as amostras de água coletadas em uma lagoa (32° 04' 56.9"S 52°14' 05.2" W) em agosto de 2021, quando a temperatura era de 18 °C, salinidade zero.

4.3.3. Comunidade bacteriana

Para a obtenção da comunidade bacteriana, amostras de água foram coletadas em uma lagoa (32° 09' 44.6"S 52° 06' 04.4"W) a uma temperatura de 18 °C. Posteriormente, as amostras foram filtradas (7 µm) para remoção de

organismos fitoplanctônicos e zooplanctônicos [Agostini et al. 2021a].

4.3.4. Ensaios de densidade bacteriana

Os ensaios de densidade bacteriana foram realizados utilizando a mesma metodologia descrita na seção 4.2.4 e resumidas na figura 4.

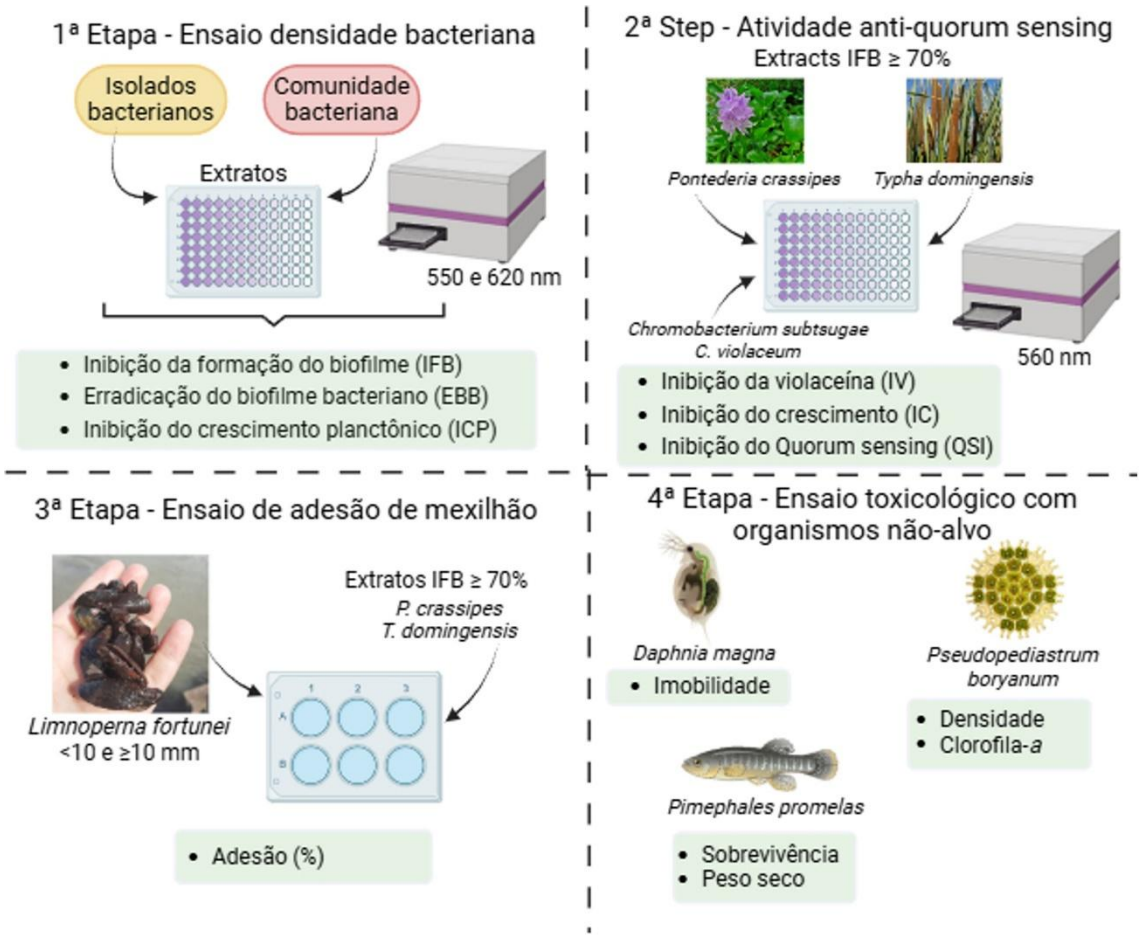

Figura 4: Esquema dos procedimentos experimentais realizados com os extratos das macrófitas aquáticas.

4.3.5. Ensaio de detecção da atividade anti-quorum sensing: inibição da violaceína

Para investigarum dos possíveis mecanismos da atividade antibiofilme dos extratos, foi realizado o ensaio da inibição da violaceína pela quantificação

das atividades de inibição do *quorum sensing* (IQS) (Figura 4) dos mesmos extratos aquosos de macrófitas aquáticas usados nos ensaios toxicológicos (*E. crassipes* e *T. domingensis*) (descritos na seção 4.3.7). Para detectar a inibição da lactona acil-homoserina de cadeia curta (C4-C6), foi utilizada a bactéria *Chromobacterium subtsugae* CV017 e para detectar a inibição de AHL de cadeia longa (C10+) foi utilizada a bactéria *C. violaceum* ATCC 12472 (Chernin *et al*. 1998, Morohoshi *et al*. 2008].

As cepas foram inoculadas em tubos de ensaio contendo 3 mL de caldo Luria-Bertani, que foram expostos a diluições variadas (0,07, 0,15, 0,30, 0,60 e 1,20%) dos respectivos extratos e incubados a 30° C por 18 h com agitação (150 rpm) em um misturador de suspensão rotativa (SM-3600-0018, Lab YIHDER Technology CO, Taiwan) [Chenia 2013]. Essas diluições foram utilizadas por apresentarem efeitos antibiofilme e anti-adesão e não apresentarem efeito toxicológico em organismos não-alvo. O controle de crescimento foi o caldo Luria-Bertani com as cepas e sem extrato, e o controle positivo foi a vanilina (Sigma-Aldrich) nas mesmas diluições dos extratos.

Após a incubação, as leituras de densidade óptica a 600 nm da cultura foram obtidas com um leitor de placas de microtitulação Glomax Multi+ Detection System (Promega). Para isso, 1 mL das culturas foi submetido a centrifugação (13.000 rpm) por 10 min (microcentrífuga Labnet Prism), precipitando assim a violaceína insolúvel. O sobrenadante foi descartado e os pellets foram ressuspensos em 1 mL de dimetilsulfóxido (DMSO) [Chenia 2013]. Após, foram centrifugadas novamente e as soluções foram quantificadas a 560 nm usando o mesmo leitor de placas [Chenia 2013]. Para serem considerados bons inibidores do quorum sensing, os extratos devem apresentar inibição da violaceína (IV)

≥50% e inibição do crescimento (IC) <40%. Caso a IV seja ≥50% e o IC ≥40%, a atividade é considerada bactericida ao invés de anti-*quorum sensing* [Rambaran *et al*. 2024].

4.3.6. Ensaio de adesão de mexilhão

Os extratos que apresentaram efeito inibitório ≥70% nos ensaios de IFB para bactérias únicas e multiespécies foram utilizados para avaliar a inibição da adesão do mexilhão dourado (Figura 4). Espécimes de *L. fortunei* foram coletadas de paredes de concreto do canal da primeira elevação da Companhia de Saneamento do Rio Grande (CORSAN) (32° 3′ 14.39"S 52° 22' 18.28" W) e transportados em recipientes plásticos sem água e sem aeração até o laboratório. Em laboratório, os mexilhões foram separados, triados e armazenados em tanques de plástico preto de 80 L contendo água desclorada acoplada a sistema de recirculação de água contendo um filtro biológico com conchas, a uma temperatura controlada de 20 °C e fotoperíodo de 12C:12E. Os mexilhões foram aclimatados durante duas semanas nessas condições e alimentados duas vezes ao dia com concentrado comercial de *Chlorella vulgaris* (Beyerinck (Beijerinck), 1890) (ChloFresh, Algasul, Rio Grande, Brasil) a 10^9 células mL^{-1}.

Os ensaios foram realizados em dois experimentos de acordo com o tamanho do mexilhão: < 10 mm e ≥ 10 mm [Cataldo *et al*. 2005]. Antes da realização dos ensaios, para a seleção dos mexilhões, foi realizada a sua transferência do tanque de cultivo para um recipiente de plástico transparente (5 L) para verificação do seu comportamento de exploração do substrato *[Longo et al.* 2021]. Os indivíduos selecionados para os ensaios foram apenas aqueles que

apresentavam comportamento de exploração. Os tratamentos utilizados foram de 0, 5, 10, 20 e 40% de cada extrato vegetal selecionado e o tratamento controle foi composto apenas por água natural estéril (salinidade 0). Os ensaios foram realizados em placas de 6 poços (Barloworld Scientific Ltd., Stone, Reino Unido), com cada poço contendo um mexilhão e 10 mL da solução teste [Longo *et al.* 2021]. As placas foram armazenadas em uma câmara de BOD no escuro com temperatura de 20 °C durante 72 h [Longo *et al.* 2021]. Ao final do tempo de exposição, os mexilhões aderidos às paredes dos poços foram contados e os resultados foram expressos em porcentagem de adesão.

4.3.7. Ensaios toxicológicos em organismos não-alvo

Devido a todos os diferentes órgãos das plantas inibirem a adesão do mexilhão dourado, misturas de partes de *E. crassipes* e *T. domingensis* foram utilizadas para realização dos ensaios toxicológicos (Figura 4). Para isso, os extratos foram liofilizados e em seguida utilizados para preparar soluções com diluições de 0, 6,25, 12,50, 25, 50 e 100% de cada extrato. Para realização dos testes toxicológicos, foram utilizados três organismos modelo não-alvo de água doce: a microalga *Pseudopediastrum boryanum* [(Turpin) E.Hegewald 2005] (Chlorophyta, Hydrodictyaceae), o crustáceo *Daphnia magna* (Straus, 1820) (Arthropoda, Daphniidae) e o peixe *Pimephales promelas* (Chordata, Cyprinidae) (Rafinesque, 1820).

4.3.7.1 Ensaio com microalga

Para realização do ensaio com *P. boryanum,* os extratos foram enriquecidos com o meio de cultura WC [Guillard & Lorenzen 1972]. O ensaio foi

realizado em frascos erlenmeyer de 50 mL contendo 49 mL de solução teste (diluições dos extratos) e 1 mL do inóculo da microalga *P. boryanum* em uma densidade de 10^4 células mL^{-1}. Os frascos foram mantidos em uma mesa vibratória sob agitação constante, iluminação contínua de 7.000 lux a 24 °C durante 72 h (± 2 h) [OECD 2011]. Cada tratamento teve quatro repetições. O tratamento controle foi realizado com o meio WC e inoculação, sem adição do extrato.

Ao final do teste foi analisada a densidade celular (células mL^{-1}) e a concentração de clorofila-*a*. Para estimar a densidade celular, no início do teste (0 h) e no final (72 h), uma alíquota de 1 mL foi retirada e armazenada em eppendorf contendo 200 µL de formaldeído (utilizado para fixar a cultura de algas) com concentração final de 0,4%. A diferença entre a contagem de células entre 72 h e 0 h foi utilizada para calcular a densidade celular. Já para a clorofila-a, após o tempo de exposição, as alíquotas de 5 mL foram removidas e centrifugadas (4.000 rpm) por 10 min. O seu sobrenadante foi descartado e 3 mL de metanol (99,9%) foram adicionados ao pellet e em seguida armazenadas sob refrigeração (5 °C) no escuro por 12 h. Após, as amostras foram centrifugadas novamente e a densidade óptica do sobrenadante foi determinada usando um espectrofotômetro UV-VIS (UV mini-1240, Shimadzu, Kyoto, Japão) em absorbância de 663 e 750 nm [Mackinney 1941]. A concentração de clorofila foi estimada pela leitura das absorbâncias de acordo com Mackinney [1941].

4.3.7.2 Ensaio com cladócero

Para esse ensaio foi realizado um teste de toxicidade aguda com neonatos de *D. magna* em placas de 6 poços (Barloworld Scientific Ltd., Stone,

Reino Unido), com quatro repetições, cada uma contendo cinco organismos e 10 mL da solução teste (2 mL organismo^{-1}) [ABNT 2022]. As placas foram armazenadas em câmara BOD a uma temperatura de 20 °C (± 2 °C), fotoperíodo de 12h:12h por 48 h (± 1 h). Durante a realização do ensaio, os organismos não foram alimentados. Ao final do tempo de exposição, observou-se o efeito dos extratos em influenciar a mobilidade dos organismos, sendo os resultados expressos em porcentagem de imobilidade para cada tratamento [ABNT 2022].

4.3.7.2 Ensaio com peixe

Larvas de *P. promelas* com idade de 0 a 24 h foram expostas aos tratamentos em um teste estático de 7 dias com renovação diária da água. Cada tratamento teve quatro repetições com 10 larvas cada. Em beckérs de 500 mL foram colocados 250 mL da solução teste contendo 10 larvas, que foram incubadas a 25 °C, fotoperíodo de 16h:8h, incidência de radiação luminosa de 500-1000 lux [EPA 2002]. As larvas foram alimentadas 3x ao dia com náuplios de *Artemia* a uma concentração de 700 a 1000 indivíduos mL^{-1}. Diariamente foi determinada a sobrevivência das larvas e ao final do tempo de exposição foi estimada a massa seca por secagem em estufa (60 °C) por 24 h [EPA 2002].

4.3.8. Análise estatística

Para observar potenciais diferenças entre os tratamentos e respectivos controles nos ensaios IFB, EBB e ICP de bactérias únicas e multiespécies, foram utilizados testes t-student com correção de bonferroni para comparações múltiplas. Para as respostas toxicológicas, também foram calculados os valores da menor diluição com efeito observado (CEOB), da maior diluição em que não

se observou qualquer efeito (CSEO) e da diluição segura dos extratos, através da média aritmética entre CEOB e CSEO (Zagatto and Bertoletti 2008). Para verificar diferenças significativas entre os tratamentos nos ensaios de *P. boryanum*, *P. promelas* (massa seca) e inibição da violaceína foram verificados os pressupostos de normalidade dos resíduos pelo teste de Shapiro-Wilk e homocedasticidade pelo teste de Levene. Com os pressupostos dentro dos parâmetros estabelecidos, foram realizadas ANOVA-*one way*, seguida do teste post-hoc de Tukey quando hipóteses alternativas com alfa = 0.05 foram aceitas. Para a sobrevivência de *D. magna* e *P. promelas* e a adesão do mexilhão foram analisados com Modelos Lineares Generalizados (GLM) com distribuição binomial (função logit). As análises estatísticas foram realizadas no programa GraphPad Prism 8.4 (GraphPad, EUA).

4.4. Macróftias aquáticas como candidatas anti-incrustantes: efeitos anti-adesão e toxicológicos em Aurelia coerulea (Cnidaria, Scyphozoa) (Artigo 4)

4.4.1. Manutenção do cultivo da espécie Aurelia

Pólipos da espécie *Aurelia* sp. foram adquiridos em parceria de uma cultura pré-estabelecida do Laboratório de Zooplâncton (LABZOO) da Universidade Federal do Rio Grande (FURG). Para manutenção do cultivo, os pólipos foram mantidos em aquários de vidro com água do mar natural filtrada (0,45 µm e salinidade 32-35) a 20-23 °C sob fotoperíodo de 12L:12D h. A cada dois dias, os indivíduos foram alimentados *ad libitum* com náuplios recém-eclodidos (<2 dias de idade) de *Artemia franciscana*. Para evitar a proliferação

excessiva de microorganismos, a água do cultivo foi renovada sempre no dia subsequente à alimentação.

Para obter as éfiras de *Aurelia*, os pólipos foram induzidos à estrobilização por aclimatação em temperatura reduzida a ~15 °C com água artificial de salinidade 35 (AAS) e fotoperíodo de 12C:12E (adaptado de Costa et al. 2020). Após a liberação das éfiras (0 a 5 dias de idade), estas foram imediatamente colocadas separadamente em recipientes de vidro para realização dos ensaios toxicológicos. Para aumentar a atividade de natação, as éfiras foram alimentadas *ad libitum* com náuplios de *A. franciscana* 24 h antes dos testes.

4.4.2. Identificação molecular da espécie de Aurelia

Para identificar a espécie de *Aurelia*, o DNA total foi extraído de três pólipos inteiros com um protocolo de acetato de amônio em triplicatas [Fetzner 1999]. Os marcadores moleculares selecionados foram amplificados e sequenciados: um fragmento de ~650 pb da subunidade COI I codificadora de proteínas do genoma mitocondrial e ~650 pb do gene 16S rRNA, subunidade menor ribossomal mitocondrial [Lawley *et al*. 2016]; de seu genoma nuclear, um fragmento de ~650 pb da grande subunidade ribossomal 28S rRNA [Bayha *et al*. 2010].

Os protocolos de reação em cadeia de polimerase e condições de termociclador foram realizados conforme Lawley *et al*. [2021]. Os produtos de PCR foram purificados usando o kit Agencourt AMPure XP (B37419AB) e as reações BigDye usaram os mesmos primers e condições das PCRs originais. Finalmente, esses amplicons foram precipitados (acetato de sódio e etanol) e sequenciados usando um analisador genético ABI PRISM ®3100 Hitachi. s

cromatogramas foram montados, aparados, alinhados e as sequências de consenso finais foram comparadas com os dados disponíveis no GenBank para identificar as espécies de Aurelia usando o software Geneious® 9.540. As sequências foram depositadas no banco de dados NCBI, sendo o código de depósito fornecido após o aceite de publicação do artigo.

4.4.3. Preparo das soluções teste

Para verificar a sensibilidade dos dois estágios de vida de *Aurelia* (pólipo e éfira), foram realizados ensaios toxicológicos com três substâncias referência: o surfactante dodecil sulfato de sódio (SDS - $NaC_{12}H_{25}SO_4$), sulfato de zinco (SZ - $ZnSO_4$) e cloreto de cobre II (CC - $CuCl_2$) (Labsynth). Para isso, os compostos foram diluídos em ASS com salinidade 35. As concentrações utilizadas para os ensaios foram para SDS: 5, 15, 45 e 135 mg L^{-1} para pólipos e 0,5, 1, 2,5, 5 e 15 mg L^{-1} para éfiras; ZS: 1, 2, 3, 4 e 5 mg L^{-1} para pólipos e 0,4, 0,8, 1,6, 2,4 e 3,2 mg L^{-1} para éfiras e CC: 0,1, 0,25, 0,5, 1 e 2,5 mg L^{-1} para pólipos e 0,02, 0,05, 0,10, 0,15 e 0,20 mg L^{-1} para éfiras.

Os extratos aquosos de *Cabomba caroliniana* (caule e folha) e *Schoenoplectus californicus* (inflorescência e parte aérea) foram preparados conforme descrito na seção 4.2.1. Essas macrófitas aquáticas foram escolhidas para desenvolver este capítulo devido a alta inibição do biofilme bacteriano (resultados apresentados no capítulo VI – artigo 2). Os tratamentos com SDS, ZS, CC e extratos de macrófitas foram utilizados para realizar ensaios toxicológicos e de fixação em condições experimentais de 20 °C, 12L:12D. Para ambos os experimentos, os controles foram definidos por AAS com salinidade 35. Todos os procedimentos realizados estão resumidos na figura 5.

Figura 5: Resumo de procedimentos experimentais realizados com os extratos de macrófitas aquáticas e pólipos e éfiras de *Aurelia coerulea*. Adaptado de Morales et al. (2025a).

4.4.4. Ensaios toxicológicos

4.4.4.1. Ensaios com pólipos

Os ensaios foram realizados em placas de 6 poços, contendo cada poço um pólipo e 10 mL do tratamento. Para cada tratamento foram realizadas três repetições, cada uma contendo quatro pólipos. As respostas crônicas (subletais) e agudas foram observadas com o auxílio de um um microscópio estereoscópico Olympus SZX9 com aumento de 40× nos tempos de 1, 6, 24, 48, 72 e 96 h após o início da exposição (Tabela 2).

A contração dos tentáculos dos pólipos e sua mudança na ingestão de presas foram observadas como respostas crônicas (Tabela 2). A contração dos tentáculos se deu quando todos os tentáculos estavam destendidos (mesmo que ligeiramente curvados) ou tentáculos contraídos (quando todos os tentáculos se encontravam totalmente contraídos) (Figura 6). A mudança na ingestão de presas se deu pela observação, durante 30 s, do potencial dos pólipos em capturar e ingerir o alimento (% de indivíduos que ingeriram presas). A

alimentação *ad libitum* foi realizada pela adição de 1 mL de concentrado de náuplios de *A. franciscana* de forma controlada e progressiva. A observação da mudança na dieta foi realizada em 48 e 96 h. A resposta aguda foi determinada pela mortalidade dada pela observação da desintegração total ou parcial dos pólipos (Figura 6). Considerou-se a desintegração dos pólipos quando ocorreu a perda de sua forma corporal típica, resultando na fragmentação de seus tecidos (Figura 6).

Tabela 2: *Endpoints* observados para *Aurelia coerulea.* Adaptado de Morales et al. (2025a).

Estágio de vida	Ensaio		Tempo de observação (h)
	Agudo	Crônico	
Pólipo	Desintegração	Contração dos tentáculos	1, 6, 24, 48, 72 e 96
		Captura e ingestão	48 e 96
Éfira	Imobilidade	Frequência de pulsação	24 e 48

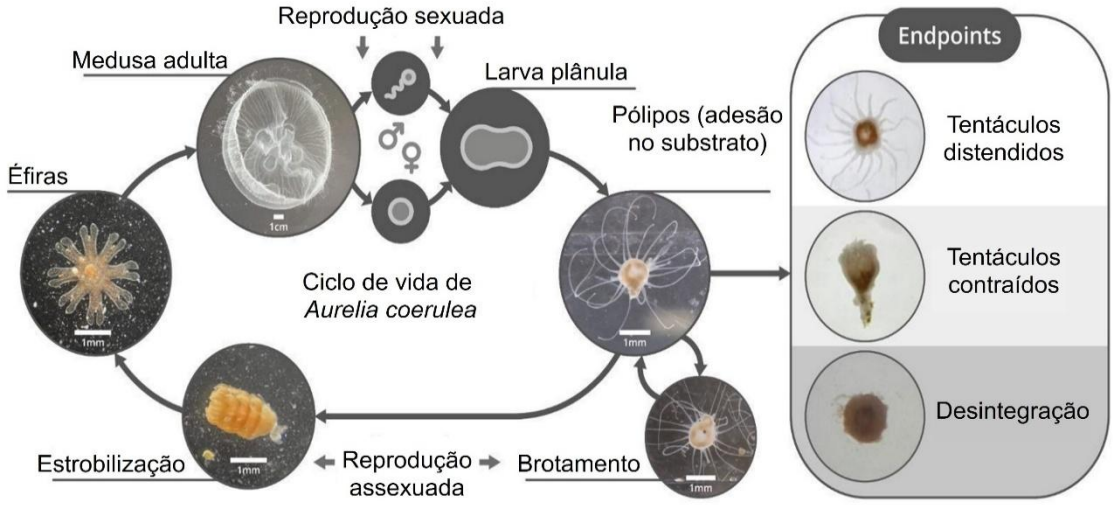

Figura 6: Ciclo de vida da Scyphomedusa *Aurelia coerulea* (Cnidaria, Scyphozoa) e seus endpoints analisados nos testes toxicológicos com pólipos. Adaptado de Morales et al. (2025a).

4.4.4.2. Ensaios com éfiras

As éfiras foram expostas a cada tratamento em placas de 24 poços, contendo cada poço 2 mL do tratamento e um indivíduo por poço, para evitar

interações entre os organismos [Faimali *et al*. 2014]. Para cada tratamento, foram realizadas três repetições, cada uma contendo quatro indivíduos. Após 24 e 48 h de exposição, foram observados os efeitos crônicos (subletais) e agudos (Tabela 2). A resposta aguda foi observada através da mobilidade dos organismos, pela observação por 10s do movimento do indivíduo após a estimulação com o auxílio de uma pipeta Pasteur sob visualização em microscópio estereoscópico (Olympus SZX9, aumento de 40×). Éfiras totalmente incapazes de mudar sua posição do baricentro (imóveis) foram consideradas como organismos imóveis, e a porcentagem de imobilidade para cada tratamento foi determinada em comparação ao tratamento controle. A resposta crônica foi dada pela frequência de pulsação (FP) de cada indivíduo medida pela observação em microscópio estereoscópico (Olympus SZX9, aumento de 40×). Para cada indivíduo foram realizadas três medidas de FP de 10s cada, e a média das três medidas foi utilizada para calcular a FP por minuto para cada tratamento.

4.4.5. Ensaios de adesão

Os pólipos foram expostos aos dois tratamentos de extratos e às três substâncias referência em placas de 6 poços contendo 10 mL de tratamento e um indivíduo por poço. Para cada tratamento foram realizadas três repetições, contendo cada uma quatro pólipos. A adesão dos pólipos foi avaliada nos períodos de 24, 48, 72 e 96 h de exposição pela resistência dos pólipos ao desprendimento do substrato quando submetidos a fluxos leves de água (realizados com pipeta Pasteur). A confirmação da adesão dos pólipos foi dada pela observação em microscópio estereoscópico (Olympus SZX9, aumento de

40×). A adesão de cada pólipo foi contabilizada para todos os tratamentos e os resultados foram expressos em porcentagem de adesão (%).

4.4.6. Caracterização química dos extratos

4.4.6.1. Cromatografia gasosa – espectroscopia de massa (GC – MS)

Para verificar a composição química dos extratos, em colaboração com a *School of Life Sciences* da *University of KwaZulu-Natal*, Campus Durban da África do Sul, os extratos foram submetidos à cromatografia gasosa acoplada à espectroscopia de massas (GC – MS). Para isso, uma solução de 1 µL do extrato foi injetada no GC – MS Shimadzu (série AOC-20i) (GCMS-QP2010 SE). O hélio foi usado como gás transportador com vazão de 0,68 mL/min. A temperatura do forno foi programada em 50 °C, enquanto a temperatura de injeção foi de 260 °C. O início da análise foi de 3 min, enquanto o horário de término foi de 32 min. Os espectros foram ajustados em 20 a 1000 m/z para evitar a captura de moléculas de água e outros voláteis. A coluna capilar utilizada foi a coluna Zebron ZB-5MSplus 0,25 × 30 m (comprimento) × 0,25 µm (df). Uma proporção de divisão de 50/50 foi usada para a amostra de injeção em um tempo inicial de espera de um minuto e, posteriormente, em 10 minutos.

A quantidade relativa de cada composto presente nos extratos foi expressa em porcentagem com base na área do pico (%) produzido no cromatógrafo. Os espectros de massa registrados dos constituintes dos extratos brutos foram identificados usando os espectros de massa padrão do Instituto Nacional de Padrões e Tecnologia – África do Sul (NIST05. LIB) fornecidos pelo software do sistema GC-MS [Naicker 2024, Sukreem 2024].

4.4.6.2. Cromatografia líquida – espectroscopia de massa (LC – MS)

As análises de cromatografia líquida – espectrometria de massa (LC – MS) foram realizadas em colaboração com a *School of Life Sciences* da *University of KwaZulu-Natal*, Campus Durban na África do Sul. Os dados foram obtidos utilizando um LCMS2020 Shimadzu com uma coluna Shimadzu Shim-Pak GIST HP C18 3 µm 4,5 x 150 mm equipada com um detector UV usando um gradiente de fase móvel de 10% de água, 90% de acetonitrila, ambos contendo 1,1% de ácido fórmico a uma taxa de fluxo de 1 mL / min. Os espectros de massa registrados dos constituintes dos extratos brutos foram identificados usando os espectros de massa padrão dos dados analíticos MASSBANK (http://www.massbank.jp/index-e.html) [Tohge & Fernie 2009].

4.4.6.3. Espectroscopia de infravermelho de transmissão de Fourier (FTIR)

Também junto a *University of KwaZulu-Natal*, os extratos foram submetidos a espectroscopia de infravermelho por transmissão de Fourier (FTIR) para identificar a presença dos grupos funcionais. Após a preparação dos extratos conforme descrito em 4.2.1, eles foram liofilizados. Amostras liofilizadas (2 mg) foram misturadas com 200 mg KBr. Os extratos brutos foram analisados usando a espectroscopia de infravermelho Bruker Alpha II (Parâmetros: ATR Diamond-1 Bounce, 24 varreduras de fundo, 24 varreduras de amostragem, faixa de 4000 - 400 cm^{-1} com resolução de 4 cm^{-1}) e processados com o software de espectroscopia Opus. Os grupos funcionais dos extratos brutos foram identificados usando tabelas de espectro de infravermelho fornecidas pela Sigma-Aldrich [Naicker 2024, Sukreem 2024].

4.4.7. Análises estatísticas

Para as respostas agudas dos testes toxicológicos, os valores da concentração letal para 50% da população (CL_{50}) foram calculados em um período de 96 h para pólipos e para a concentração de efeito para 50% da população (CE_{50}) de 48 h para éfiras foram através do método Probit (Finney 1952). Para testar possíveis diferenças entre os tratamentos em cada tempo de exposição para os ensaios dos pólipos e mobilidade de éfiras, foram utilizados modelos lineares generalizados (GLM), com um modelo de distribuição binomial. Para a frequência de pulsação das éfiras (variável contínua), a normalidade e homocedasticidade dos resíduos foram verificadas (Testes de Shapiro-Wilk e Levene, respectivamente). Com a aceitação das hipóteses alternativas, foi utilizada uma ANOVA *one-way*, seguido do teste post-hoc de Tukey para comparações múltiplas para testar diferenças significativas entre os níveis dos fatores. As análises estatísticas foram realizadas por meio do software GraphPad Prism 8.4 para Windows (San Diego, EUA).

4.5 Avaliação do potencial anti-incrustante de extratos de macrófitas aquáticas como aditivos em tintas: um primeiro experimento in situ (Artigo 5)

4.5.1. Preparação da tinta

Para preparação da tinta anti-incrustante, extratos aquosos de *Pontederia crassipes* e *Typha domingensis* foram preparados de acordo com a seção 4.3.1. Esses extratos foram escolhidos porque apresentaram uma alta inibição da

formação do biofilme bacteriano, inibição do *quorum sensing* e inibição da adesão do mexilhão dourado, conforme relatado no capítulo VII (artigo 3) desta tese. Após a preparação dos extratos, eles foram liofilizados (LIOTOP, L101), e sua massa seca remanescente foi então misturada com tinta epóxi Hempadur Base 15579 (Hempel – 15570) para obter os tratamentos 2,5, 5 e 10 g L^{-1} (g de liofilizado L^{-1} de tinta). Esta tinta epóxi é amplamente utilizada por profissionais da indústria aquática na América do Sul e sua composição, além de resina epóxi, apresenta xileno, talco, dióxido de titânio, bultan-1-ol, bezeno 1,3-bis, acetato de n-butila e tolueno (conforme descrição do produto. Além desses tratamentos, também foram utilizados dois tratamentos controle: o controle A – com o substrato “virgem”, sem revestimento; e o controle B – substrato revestido apenas com tinta epóxi sem adição de extrato.

4.5.2. Procedimento experimental

Os tratamentos foram utilizados para revestir substratos de aço inoxidável (12,5 cm^2) com o auxílio de um rolo de espuma de poliéster. O tempo de duração da secagem da tinta foi de ±48 h em tempeartura de ambiente natural (20 – 25 °C). Os substratos foram distribuídos em 7 *frames* (Figura 7B), retirados diariamente durante 7 dias, de modo a possuir três substratos (três repetições) para cada análise explicada posteriormente. Esses substratos foram expostos verticalmente [Agostini *et al*. 2019b] nas proximidades da usina hidrelétrica de Salto Grande – Uruguai (31°16'11.8" S 57°56'44.7" W) (Figura 7A) a 1 m de profundidade durante 165 h (Figura 7C) em abril de 2023. Três repetições (substratos) para cada análise foram coletadas no período de 23, 46, 70, 94, 118, 142 e 165 h, para analisar a comunidade bioincrustante.

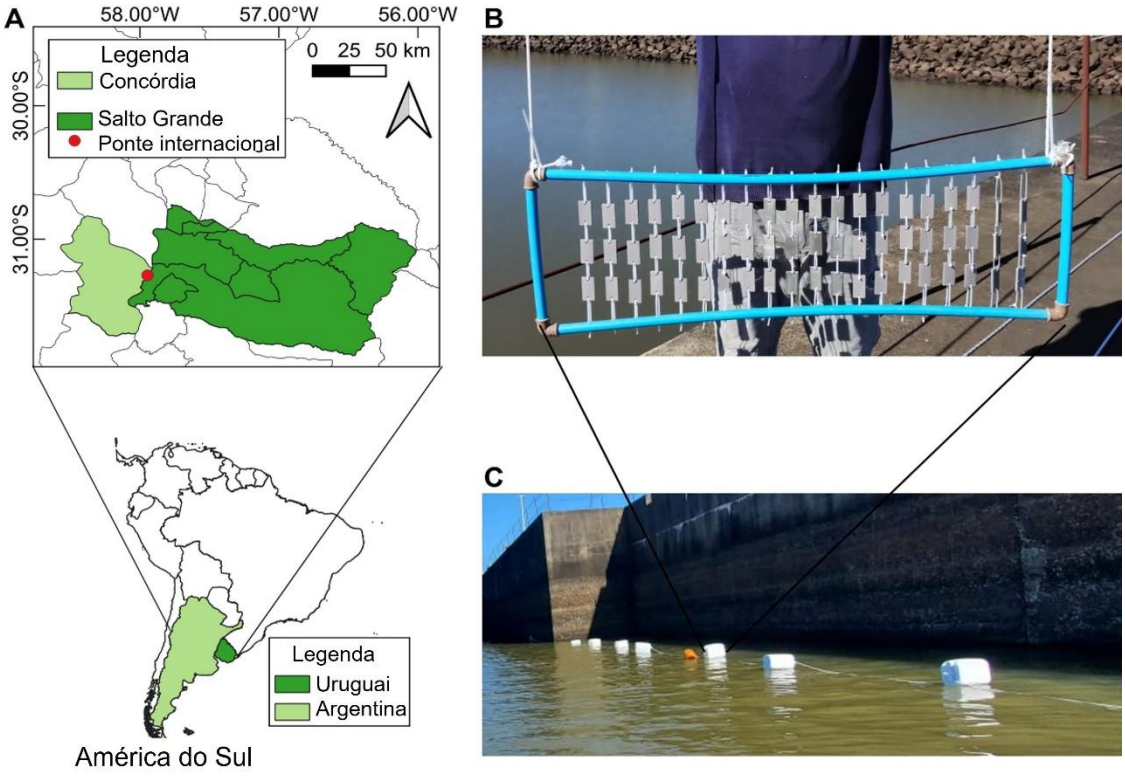

Figura 7: Local experimental, nas proximidades da Ponte Internacional Salto Grande – Uruguai/Argentina (A) e estruturas que foram submersas com os substratos pintados com tratamentos anti-incrustantes (B-C). Adaptado de Morales et al. (2025b).

4.5.2. Monitoramento de dados ambientais e biológicos

Em cada período de amostragem (23, 46, 70, 94, 118, 142 e 165 h) foram avaliadas as variáveis ambientais: temperatura (°C), condutividade elétrica (µS cm^{-1}), pH, turbidez (NTU) e oxigênio dissolvido (mg L^{-1}) com o auxílio de sonda multiparâmetro (YSI DSpro). Também foi avaliada a transparência da água (cm) usando um disco de Sechi (30 cm ⌀). A abundância de *Microcystis* spp. (cel mL^{-1}), *Dolichospermum* spp. (cel mL^{-1}) e cianobactérias (cel mL^{-1}), e as concentrações de fósforo total fósforo total (mg L^{-1}), nitrogênio total (mg L^{-1}), nitrogênio amoniacal total (TAN) (mg L^{-1}), dióxido de nitrogênio (mg L^{-1}), nitrato (mg L^{-1}), fosfato (mg L^{-1}), sólidos suspensos (mg L^{-1}) e clorofila-a (µg L^{-1}) foram fornecidas semanalmente a partir de dados do monitoramento realizado pela Gestão Ambiental da Comissão Técnica Mixta de Salto Grande

(https://www.saltogrande.org/organizacion.php).

4.5.3. Análise da bioincrustação

A bioincrustação foi analisada nos tempos de exposição de 23, 46, 70, 94, 118, 142 e 165 h, nos quais três substratos (três repetições) foram removidas para cada análise de microincrustação (MIC) e macroincrustação (MAC). Após a coleta dos substratos, estes foram lavados 3x com solução salina estéril (0,4%) para remover material solto ou organismos planctônicos das amostras (Agostini et al. 2019b). Em seguida, as amostras foram armazenadas em tubos falcon contendo 40 mL de formaldeído a 4% (concentração final) para MAC e 40 mL de solução salina estéril 0,4% para MIC. As amostras de MIC foram armazenadas no escuro e refrigeradas a – 18 °C e processadas imediatamente, enquanto as amostras de MAC foram refrigeradas a – 18 °C e processadas posteriormente.

4.5.3.1. Análise de microincrustação

Para análise da MIC, imediatamente após a coleta, os substratos foram raspados com o auxílio de uma alça microbiológica de metal esterilizada por incineração com bico de bunsen, e agitados manualmente. Para cada análise, foram realizadas 3 repetições por tratamento. Para analisar as bactérias heterotróficas totais por citometria de fluxo, uma alíquota de 1,8 mL foi coletada e armazenada em paraformaldeído/glutaraldeído (1:0,05%) e congeladas por aproximadamente 1-2 semanas a – 20 °C [Krock *et al.* 2015].

A fim de melhorar a dispersão celular, as amostras foram sonicadas por cinco minutos (40 hz) em ultrassom SB 3200 DTN após o descongelamento. Em seguida, as células foram coradas durante 15 minutos em temperatura ambiente

com SYBR-Green I (SYBR-I, diluição 1:30 do estoque comercial; Invitrogen, EUA) diluído em dimetilsulfóxido (DMSO, Merck, Alemanha) [Marie *et al*. 2005] na proporção de 0,001:1 corante:amostra. O total de bactérias foi contado por citômetro de fluxo Apogee-A40 (Apogee Flow Systems, Reino Unido) equipado com laser de argônio (488 nm), e delimitado a população citométrica a um gráfico de dispersão de 488Green (pico) x 488Red (pico) usando o software FLowJo (v.10.10). Os resultados foram expressos em células cm^{-2} para cada tratamento.

Posteriormente, as amostras remanescentes da metodologia de raspagem foram homogeneizadas para imediata análise de organismos autotróficos por meio da concentração de clorofila total (µg L^{-1}), clorofila-a (µg L^{-1}) e atividade fotossintética (%) de algas verdes, azuis-esverdeadas (cianobactérias), diatomáceas/dinoflageladas, criptófitas com o equipamento Algae Online Analyser bbe Moldaenke. A atividade fotossintética foi medida avaliando a atividade do fotossistema II.

4.5.3.2. Análise da macroincrustação

Para cada período de amostragem, foram retiradas três repetições (três substratos) de cada tratamento para verificar a presença de macroorganismos. Esses substratos foram analisados individualmente em estereomicroscópio (aumento de 40x). Para evitar a perda de organismos na solução de armazenamento do substrato, o líquido também foi analisado em uma câmara de Bogorov sob o estereoscópio. Os organismos encontrados foram identificados, fotografados e quantificados. As espécies foram identificadas por meio de literatura especializada descritas em Agostini *et al*. [2019b, 2021a].

4.5.4. Composição da comunidade bioincrustante - Metabarcoding

Para analisar a composição da comunidade biológica (bactérias, fungos, algas e invertebrados), foi realizada a análise metabarcoding para os substratos removidos no tempo de 165 h. Para isso, três substratos (três repetições) de cada tratamento foram removidos e armazenados em solução salina estéril (0,4%). As três repetições de cada tratamento foram misturadas em partes iguais para formar amostras compostas (pools). Posteriormente, foram sequenciados os genes 16S, ITS e 18S pela empresa especializada Neoprospecta Microbiome Tecnologia, Brasil. A amplificação da região 16S rRNA v3/v4 foi realizada usando os primers 341F (5'-CCTACGGRSGCAGCAG-3') e 806R (5'-GGACTACHVGGGTWTCTAAT-3') [Christoff *et al*. 2017]. A amplificação da região ITS1 foi realizada usando os primers ITS1 (GAACCWGCGGARGGATCA-3') e ITS 2 (5'-GCTGCGTTCTTCATCGATGC-3') [Schmidt *et al*. 2013]. A amplificação da região 18S rRNA v9 foi realizada usando o primer 1510r (5'-CCTTCYGCAGGTTCACCTAC-3') [Bradley *et al*. 2016].

As bibliotecas moleculares foram sequenciadas usando o sistema de sequenciamento NextSeq 1000/2000TM (Illumina Inc., EUA) com o Kit NextSeq 1000/2000 P1 600-Cycle. A remoção de sequências quiméricas e o agrupamento de unidades taxonômicas operacionais (OTU) foram realizados usando UPARSE [Edgar 2013]. As identificações taxonômicas foram realizadas pelo Blastn v.2.6.0 [Altschul *et al*. 1990], utilizando como referência as bases de dados Silva (v 138.2) [Quast *et al*. 2012] e Greengenes [De Santis *et al*. 2006]. A rarefação (normalização) dos dados metagenômicos (OTUs) foi realizada utilizando como base a amostra com menor número de sequências. Em seguida, com base na abundância relativa de sequências em cada amostra, foi calculada a frequência

de táxons e a riqueza para cada tratamento.

4.5.5. Caracterização química dos extratos

Para identificar a composição química, os extratos de *P. crassipes* e *T. domingensis* (preparados conforme item 4.3.1) foram submetidos à cromatografia líquida (LC-MS) e gasosa (GC-MS) acoplada à espectroscopia de massas, além da espectroscopia de infravermelho por transmissão de Fourier (FTIR). Todas as análises foram desenvolvidas na *School of Life Sciences* da *University of KwaZulu-Natal*, Campus Durban da África do Sul, sendo toda metodologia descrita na seção 4.4.6 desta tese.

4.5.6. Análise estatística

Para verificar a diferença entre os tratamentos (Controle A, Controle B, 2,5, 5 e 10 g L^{-1}) em cada tinta (*P. crassipes* ou *T. domingensis*) e os tempos de exposição (23, 46, 70, 94, 118, 142 e 165 h) nos ensaios de microincrustação, foi utilizada uma ANOVA-*two way*. Em todos os casos, foram verificados os pressupostos de distribuição normal dos resíduos e homocedasticidade. Quando as hipóteses alternativas com alfa = 0.05 foram aceitas, foi utilizado o teste post-hoc de Tukey. Análises exploratórias foram realizadas com a riqueza de grupos taxonômicos (metabarcoding, matriz de similaridade de Bray-Curtis) e bactérias totais e dados de organismos autotróficos (distância euclidiana) por meio de escalonamento multidimensional não métrico (NMDS). A análise permutacional de variância multivariada (PERMANOVA) foi realizada para verificar diferenças entre os grupos de tratamentos para dados multivariados. A análise percentual de similaridade (SIMPER) também foi realizada para verificar a contribuição

individual das variáveis para os grupos de tratamento. A análise estatística foi realizada com o uso do software R (R Core Development Team, 2024).

Capítulo VI: Artigo 1

O primeiro artigo científico proveniente desta Tese de Doutorado é apresentado neste capítulo. O manuscrito, de autoria de Mikael Luiz Morales Pereira, Ivna Maria Bastos Vasconcelos, Alexandre José Macedo, Erik Muxagata, Grasiela Lopes Leães Pinho e Vanessa Ochi Agostini, intitula-se "***Estrategias de control de mejillones invasores: una revisión***" e foi publicado no periódico "***INNOTEC***" em 2022. O manuscrito encontra-se disponível pelo link https://doi.org/10.26461/23.08.

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REVISIONES

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Estrategias de control de mejillones invasores: una revisión

Strategies to control invasive mussels: a review

Estratégias de controle de mexilhões invasores: uma revisão

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RESUMEN

El proceso de bioincrustación puede dañar varias estructuras construidas por el hombre y, junto con el agua de lastre, es el principal vector para la transferencia de especies invasoras en todo el mundo. El control de las especies invasoras, principalmente mejillones, se ha debatido durante mucho tiempo, pero las diferentes técnicas utilizadas aún tienen limitaciones. Por lo tanto, el propósito de esta revisión fue recopilar información sobre los avances en el control de la incrustación de mejillones invasores. Se utilizó la base de datos del portal de publicaciones Periódicos CAPES (Brasil), con la búsqueda de palabras clave: "(invasive mussel biofouling) AND control". Se analizaron 53 artículos publicados entre 1999 y 2020. Se identificó que se han realizado más estudios experimentales en agua dulce que en otros sistemas acuáticos. La mayoría se llevó a cabo en laboratorios, involucrando las fases del mejillón juvenil y adulto. El control químico fue el más discutido en la literatura analizada en comparación con los controles físicos y biológicos. Entre los tipos de controles químicos se destaca el uso de extractos naturales por ser efectivo y causar menos daño al ambiente.
Palabras clave: antiincrustante, depredación, extractos naturales, luz ultravioleta, macroincrustación.

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ABSTRACT

The biofouling process can damage various man-made structures, and together with ballast water are the main vectors for the transfer of organisms around the world. The control of invasive species, especially mussels, has been debated for a long time, but all techniques have limitations. Therefore, the purpose of this review was to collect information on the advances in research on invasive mussel control. The database Periódicos CAPES was used, searching for the keywords: "(invasive mussel biofouling) AND control". Fifty-three articles were analyzed from 1999 to 2020. It was identified that more experimental studies have been carried out in fresh water than in other systems. Most were performed in the laboratory compared to the field, involving the juvenile and adult phases of the mussel. The chemical control was the most discussed in the analyzed literature in comparison with the physical and biological controls. Among the types of chemical control, the use of natural extracts stands out, as it is effective and causes less damage to the environment.
Keywords: antifouling, predation, natural extracts, UV light, macrofouling.

RESUMO

O processo de bioincrustação pode danificar várias estruturas feitas pelo homem e, junto com a água de lastro, é o principal vetor de transferência de espécies invasoras em todo o mundo. O controle de espécies invasoras, principalmente mexilhões, é debatido há muito tempo, mas as diferentes técnicas utilizadas ainda apresentam limitações. Portanto, o objetivo desta revisão foi coletar informações sobre os avanços no controle de incrustação de mexilhões invasores. Foi utilizada a base de dados do portal de publicações Periódicos CAPES (Brasil), com a busca das palavras-chave: "(invasive mussel biofouling) AND control". Foram analisados 53 artigos publicados entre 1999 e 2020. Identificou-se que mais estudos experimentais foram realizados em água doce do que em outros sistemas. A maioria desses estudos foi realizada em laboratórios, envolvendo as fases juvenil e adulta do mexilhão. O controle químico foi o mais discutido na literatura analisada em relação aos controles físicos e biológicos. Dentre os tipos de controles químicos, o uso de extratos naturais destaca-se por ser eficaz e causar menos danos ao meio ambiente.
Palavras-chave: anti-incrustante, predação, extratos naturais, luz ultravioleta, macroincrustação.

INTRODUCCIÓN

En el ambiente acuático, la bioincrustación puede definirse como la acumulación no deseada de depósitos biológicos en superficies sumergidas (sustratos), ya sea en ambientes naturales o artificiales (Agostini, et al., 2018). Este es un proceso natural que se inicia con la adsorción de moléculas orgánicas en un sustrato sumergido y su posterior colonización por especies microbianas pioneras. Con la progresión de la sucesión ecológica ocurre el asentamiento de organismos macroscópicos, facilitado por los colonizadores pioneros (Agostini, et al., 2018).

La bioincrustación puede causar varios efectos: en los cascos de los barcos, la superficie se vuelve irregular, incrementando la resistencia y provocando un mayor consumo de combustible (Schultz, 2007). Además promueve la corrosión y aumenta el peso de las estructuras artificiales, modificando su formato original (Hertiani, et al., 2010) y causando

serios problemas al obstruir tuberías de conducción y otros componentes (Schaefer, et al., 2010). Esto supone un gasto global superior a los 340 mil millones de dólares por año para la industria acuática (Cuthbert, et al., 2021), que incluye el costo de la prevención de la bioincrustación, el mantenimiento de las estructuras dañadas y el aumento del consumo de combustible en barcos.

La bioincrustación, junto con el agua de lastre y sus sedimentos, es el vector más importante en la transferencia de organismos invasores marinos en todo el mundo. La colonización en los cascos de los barcos, plataformas, boyas y otras estructuras está directamente relacionada con la dispersión y propagación a nivel global de especies exóticas e invasoras en los ecosistemas marinos (Uliano-Silva, et al., 2018). Las especies exóticas invasoras colonizan hábitats diferentes al ambiente natural, reproduciéndose fácilmente y superando la densidad de las especies nativas, lo que causa daños en la biodiversidad, y en las relaciones sociales y económicas (Boltovskoy y Correa, 2015). Los mejillones son exitosos en la colonización de ambientes acuáticos, principalmente las especies de las familias Dreissenidae y Mytilidae (Boltovskoy y Correa, 2015). Estos organismos tienen características fisiológicas -como alta tasa de dispersión, plasticidad fenotípica, polifagia y alta variabilidad genética- que garantizan su establecimiento en diferentes ambientes (Lockwood y Somero, 2011). Controlar la propagación e invasión de especies exóticas, principalmente de mejillones, ha sido un desafío mundial durante las últimas décadas, incrementado especialmente con la invasión de *Limnoperna fortunei* en América del Sur y Norte, y de *Dreissena polymorpha* en Europa (Silva, et al., 2021).

Como estrategia para controlar la incrustación de dichos mejillones invasores, a lo largo de los años se han utilizado controles poblacionales que presentan diferentes metodologías (Boltovskoy y Correa, 2015). Estos controles se pueden dividir en tres: biológicos, físicos y químicos. El control biológico consiste en la utilización de un organismo para la depredación de las especies invasoras. Esta metodología puede implicar una amenaza para el ambiente, y existen controversias en torno a la selección de las especies utilizadas para la depredación de la especie objetivo (invasora) (Rosa, et al., 2019). El control físico implica el uso de cambios de temperatura y luces ultravioleta que pueden generar importantes consumos de energía eléctrica, lo que hace que el método sea costoso (Boltovskoy y Correa, 2015). El control químico implica el uso de productos químicos para controlar las incrustaciones, y a menudo es necesario desintoxicar el agua después de aplicar la metodologia; además puede causar contaminación en el ambiente receptor (Boltovskoy y Correa, 2015; Rosa, et al., 2019). Para el control químico se destaca el uso de cloro, pinturas antiincrustantes, sustancias naturales con fines antiincrustantes y el control de pH (Davis, et al., 2015; Matsui, et al., 2018; Lepoutre, et al., 2018).

El propósito de esta revisión fue recopilar información sobre el progreso de la investigación en el control de la incrustación de mejillones, con enfoque en las siguientes preguntas: (i) ¿Ha aumentado el número de trabajos con los años?; (ii) ¿Qué países estudiaron el mayor número de alternativas de control?; (iii) ¿Qué tipo de ambiente acuático fue el más estudiado?; (iv) ¿Los trabajos se realizaron mayormente en el laboratorio o *in situ*?; (v) ¿El propósito de los trabajos fue controlar larvas, juveniles o adultos?; (vi) ¿Qué especie, orden y familia fueron los más estudiados?; (vii) ¿Cuál es el mejor método de control utilizado: biológico, físico o químico?

Se evaluaron los estudios publicados con el objetivo de resaltar la forma de control de mejillones invasores más eficiente y ambientalmente segura.

MATERIALES Y MÉTODOS

Se desarrolló una revisión de artículos científicos considerando la base de datos del portal de publicaciones periódicas de la Coordinación de Perfeccionamiento del Personal de Educación Superior (CAPES), del Ministerio de Educación de Brasil, que incluye colecciones de Scopus, Web of Science y Science Direct Journals. La búsqueda fue realizada considerando el período entre 1990 y 2020 (junio). Se consideró la combinación de palabras en inglés: "(invasive mussel biofouling) AND control". Se identificó y cuantificó el número de publicaciones, y se seleccionaron los artículos de los temas de interés. Posteriormente se realizó el análisis de los datos en los diferentes artículos, considerando autor, año, país del autor, país de desarrollo de la investigación, revista, estudio de agua dulce (marina o estuarina), experimento de campo o laboratorio, especie considerada, tipo de control (biológico, químico o físico). No se consideró en esta revisión la literatura gris (tesis y disertaciones, resúmenes de congresos y artículos de revisión).

Para la determinación de los factores "control biológico, químico o físico" se tomaron en cuenta las siguientes reglas de clasificación: (i) control biológico, artículos que trataban de depredación o competencia por algún recurso alimenticio para control de los mejillones; (ii) control químico, artículos sobre la utilización de compuestos químicos aislados o brutos, o de factores abióticos (pH, oxígeno y salinidad); (iii) control físico, artículos sobre la utilización de barreras físicas como filtros, diferentes tipos de sustratos o distintos tipos de olas o temperaturas. Para la nomenclatura actual de especies marinas y dulceacuícola se utilizó el registro mundial de especies marinas WoRMS (WoRMS, 2020).

RESULTADOS Y DISCUSIÓN

Se encontró un total de 453 artículos, del cual se seleccionaron 53 para su análisis. Los resultados se presentan a continuación, de acuerdo con el orden de las preguntas planteadas en la presente revisión:

¿Ha aumentado el número de trabajos con los años?

De los 53 artículos seleccionados, el primero fue publicado en 1999 y el segundo en 2002 (Figura 1). Desde fines de la década de los noventa hasta la actualidad se han explorado alternativas para controlar los impactos de mejillones invasores, con una explosión máxima de generación de información sobre el tema a partir de 2009. Desde ese año se registró al menos una investigación anual (Figura 1).

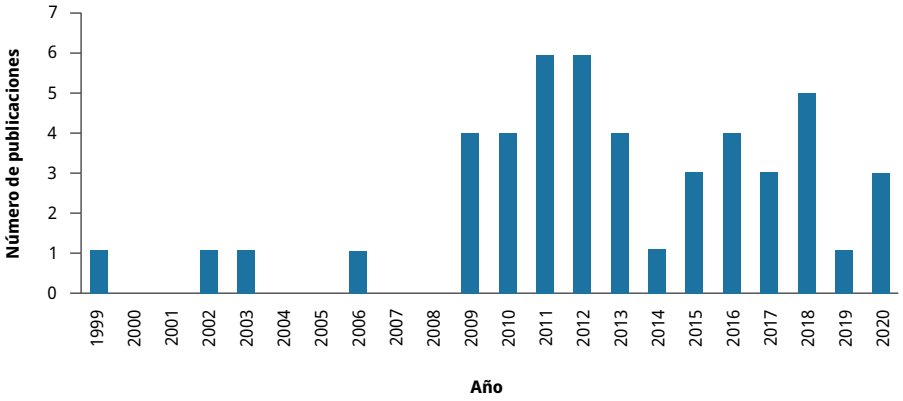

FIGURA 1. Número de trabajos desarrollados a lo largo de los años sobre estrategias de control de mejillones invasores (1999-2020).

También se analizaron los principales investigadores de las especies de mejillón estudiadas y el origen (continente) de los autores. Quienes se destacaron fueron: Perepelizin y Boltovskoy en América del Sur para la especie *L. fortunei*; Costa y Kobak en Europa para *D. polymorpha*; y Hicks y Mcmahon en América del Norte para *P. perna*.

¿Qué países estudiaron el mayor número de alternativas de control?

Los países en los que se identificó un mayor número de publicaciones referidas al control de mejillones invasores fueron Estados Unidos (20,8%); seguido de Argentina (11,3%); Brasil (9,4%); Holanda y Japón (7,5% cada uno); China, India, Polonia y Reino Unido (5,7% cada uno); Irlanda y Canadá (3,8% cada uno); Alemania, Australia, Francia, Corea, Nueva Zelanda, Portugal y Túnez (1,9% cada uno).

¿Qué tipo de ambiente acuático fue el más estudiado?

De los 53 artículos analizados, el 51% se ocupó de estudios sobre mejillones de agua dulce, seguido de agua marina (41%) y finalmente de agua salobre (estuario) (8%), dejando en evidencia que los mejillones pueden causar problemas en diferentes tipos de ambientes acuáticos. Según Schultz y otros (2011) y Ozkan y Berberoglu (2013), la bioincrustación puede causar daños a diferentes sustratos como plataformas marítimas y portuarias, tuberías, cables submarinos y cascos de barcos. En este sentido, a partir de los datos recopilados podemos observar que los trabajos en el entorno de agua dulce se realizaron en plantas hidroeléctricas, vías fluviales de construcción, plantas de tratamiento de agua, represas y fábricas (Nakano, et al., 2010; Schaefer, et al., 2010; Costa, et al., 2011; Matsui, et al., 2018). Los estudios en agua marina se realizaron en los sistemas de agua de lastre, acuicultura y enfriamiento de agua (Rajagopal, et al., 2006; Piola y Hopkins, 2012).

¿Los trabajos se realizaron mayormente en el laboratorio o *in situ*?

El 73% de los trabajos correspondió a experimentos de laboratorio y el 27% a trabajos realizados *in situ*. En tres de los estudios se realizaron tanto experimentos de laboratorio como *in situ* (Matsuo, et al., 2009; Piola y Hopkins, 2012; Comeau, et al., 2017). Específicamente, Comeau y otros (2017) observaron que el hidróxido de calcio causa un efecto agudo y un estrés conductual a corto plazo en *Mytilus edulis* (Linnaeus, 1758) tanto en un experimento en tanques acrílicos (laboratorio) como en barcos de pesca (campo). Por otro lado, Piola y Hopkins (2012) analizaron la mortalidad de *Perna canaliculus* (Gmelin, 1791) y *Mytilus galloprovincialis* (Lamarck, 1819) frente a la temperatura, y observaron que temperaturas entre 40 y 60 °C controlan estas especies de mejillones en el campo (en botes) y en el laboratorio (en tanques). Matsuo y otros (2009) utilizaron un sistema de fibra óptica llamado técnica de emisión acústica (AE) para analizar la incrustación por *M. galloprovincialis* en el laboratorio y en el campo (en las tuberías). En los tres estudios fue posible notar el potencial del control analizado tanto *in situ* como en el laboratorio. Por lo tanto, se demuestra que es importante realizar experimentos en ambos lugares ya que el laboratorio ofrece condiciones más controladas, mientras que el campo refleja las condiciones del ambiente con las diferentes variables que regulan la ocurrencia de mejillones, complejas de verificar en el laboratorio.

¿El propósito de los trabajos fue controlar larvas o mejillones juveniles y adultos?

De los estudios analizados, el 80% realizó bioensayos para el control de mejillones en su estado juvenil y adulto, mientras que el 20% analizó mejillones en su estado larvario. De acuerdo con Murray y otros (2011) y Crego-Prieto y otros (2015), las especies invasoras pueden transportarse en su fase larval en agua de lastre y en los cascos de los barcos en su fase juvenil o adulta. Por lo tanto, llevar a cabo una metodología para controlar estos organismos en diferentes etapas de la vida es de suma importancia. En seis estudios analizados se abordaron las diferentes etapas de vida de los mejillones (Folino-Rorem, et al., 2006; Matsuo, et al., 2009; Schaefer, et al., 2010; Sahu, et al., 2013; Yuan, et al., 2016).

¿Qué especie, orden y familia fueron los más estudiados?

Las especies más estudiadas fueron el mejillón cebra *Dreissena polymorpha* (Pallas, 1771) de la familia Dreissenidae (35%) y el mejillón dorado *Limnoperna fortunei* (Dunker, 1857) de la familia Mytilidae (65%). Estas especies se encuentran en hábitats de agua dulce y salobres, corroborando los resultados presentados previamente sobre el ambiente acuático más estudiado (Figura 2). Además, los órdenes más abundantes en los artículos fueron Mytiloida (38%) y Mytilida (25%), de la familia Mytilidae; seguidos por Veneroida (25%) y Myida (12%), de la familia Dreissenidae.

En algunos estudios se encontraron especies cuya clasificación taxonómica fue modificada con el paso del tiempo. La especie *Xenostrobus securis* (Lamarck, 1819) es actualmente sinónimo de *Limnoperna fortunei.* Del mismo modo, *Modiolus metcalfei* (Hanley, 1843) y *Perna indica (*Kuriakose y Nair, 1976) ahora se clasifican como *Modiolus modulaides* (Röding, 1798)

y *P. perna,* respectivamente. En este estudio se adoptó la nomenclatura actualizada en el WoRMS (WoRMS, 2020).

Las especies *D. polymorpha*, *D. bugensis*, *M. leucophaeata* y *M. sallei* -de la familia Dreissenidae- se describen en la literatura como invasoras (Schaefer, et al., 2010; Cai, et al., 2014; Gaag, et al., 2017). La especie *L. fortunei* es una de las especies invasoras más estudiadas (Xu, et al., 2015) en América del Sur (Schwindt y Bortolus, 2017). El género *Perna*, compuesto por las especies *P. perna* y *P. viridis* (Linnaeus, 1758), y el género *Modiolus,* compuesto por las especies *M. modulaides, M. modiolus (*Linnaeus, 1758*), M. philippinarum (*Hanley, 1843*)* y *M. edulis*, también se consideran mejillones invasores (WoRMS, 2020). Las especies *Mytella charruana* (d'Orbigny, 1842) y *M. galloprovincialis* se identifican en algunas regiones como no nativas (Yuan, et al., 2016; Olabarria, et al., 2016). La especie *P. canaliculus* (Gmelin, 1791) es endémica de Nueva Zelanda; sin embargo, se introdujo en Australia (WoRMS, 2020) y se encontró en artículos junto a *M. galloprovincialis*, discutiendo el control de la bioincrustación de ambas especies invasoras (Piola y Hopkins, 2012). Por lo tanto, consideramos que todas las especies en este estudio son invasoras.

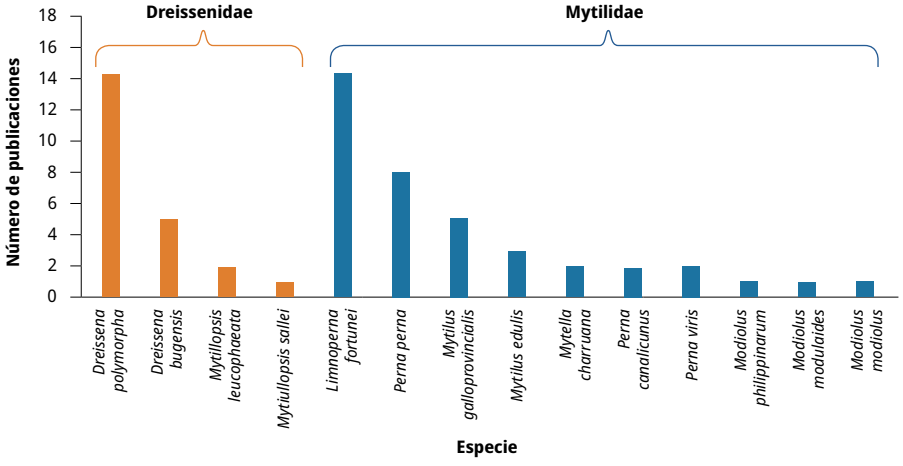

FIGURA 2. Número de publicaciones por especie de mejillones encontradas en los artículos analizados entre 1999 y 2020.

¿Cuál fue el tipo de control más utilizado?

De los artículos analizados, el 13% estudió el control biológico; del cual el 50% resultó efectivo para el control del mejillón. En tanto, el 20% analizó el control físico, del cual el 86% obtuvo resultados satisfactorios. El control químico presentó un mayor número de reportes (67%), del cual el 84% mostró efectividad en el control de mejillones (Figura 3). En el Anexo 1 se detallan los tipos de control de mejillones invasores encontrados que han demostrado ser satisfactorios.

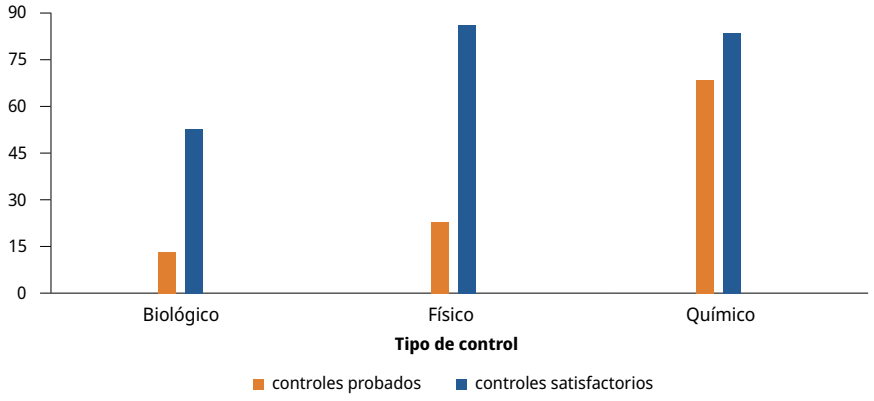

FIGURA 3. Número de publicaciones por tipo de control de mejillones testeadas en los artículos analizados entre 1999 y 2020.

¿Cuál fue el mejor control biológico?

El primer artículo donde se estudió el control biológico fue publicado en 2009, en el cual se comprobó la selectividad alimentaria del pez *Prochilodus lineatus* (Valenciennes, 1837) sobre larvas de *L. fortunei* (Paolucci, et al., 2009). Vieira y Lopes (2013) investigaron el contenido estomacal de *Pimelodus pintado (*Azpelicueta, Lundberg y Loureiro, 2008) para verificar la presencia de *L. fortunei* en su dieta, y encontraron una preferencia por los mejillones durante la primavera. En el 60% de los peces estudiados se comprobó la presencia del mejillón dorado en su contenido estomacal. Otro trabajo también evidenció la capacidad de los peces de consumir *L. fortunei*: de 81 especies de peces, 28 de ellas presentaron mejillón dorado en el estómago (González-Bergonzoni, et al., 2019). Estos peces podrían usarse como un posible depredador del mejillón *L. fortunei*; sin embargo, sería necesario realizar un mayor número de estudios sobre dicho potencial.

En tanto, Kobak y otros (2012) analizaron el potencial de depredación de los anfípodos *Dikerogam marusvillosus* (Sowinsky, 1894), *Pontogammarus robustoides* (Sars, 1894) y *Gammarus fossarum* (Koch, 1836) y de los peces *Corydoras paleatus* (Jenyns, 1842) sobre el mejillón *D. polymorpha,* no siendo exitosos en su depredación.

Otros estudios utilizaron la coexistencia de especies en el mismo hábitat para generar una competencia entre ellos (Olabarria, et al., 2016; Cai, et al., 2014). Estos estudios concluyeron que ambas especies de mejillones analizadas -*L. fortunei* y *Mytilopsis sallei* (Recluz, 1849)- son más resistentes a las variaciones ambientales y tienden a impactar especies que ya existen en el agua, pudiendo disminuir la existencia de especies nativas en el ambiente.

Los métodos biológicos se basan en el uso de organismos que actúan con depredación sobre el mejillón invasor. Sin embargo, de los trabajos que utilizaron esta metodología solo el 50% mostró efectividad (Figura 3). Esta alternativa para el control del mejillón es muy delicada cuando se utilizan especies no nativas como depredadores debido a que pueden afectar no solo a la especie objetivo, sino también a otras que habitan en el medio; sumado a su alto costo (Kobak, et al., 2012; Rosa, et al., 2019). No obstante, cuando se utilizan especies nativas como agente de control, el costo puede ser menor y el efecto ambiental puede disminuir, causando un menor riesgo para el ambiente acuático (Rosa, et al., 2019).

La solución más efectiva es el uso de peces que depreden mejillones. Sin embargo, debido a las consecuencias que puede ocasionar en los ecosistemas, este método debe ser estudiado en mayor profundidad para conocer sus interacciones con el medio en el que se aplica y evitar amenazas en el ecosistema receptor (Rosa, et al., 2019). Según los datos analizados sobre las consecuencias ambientales que puede ocasionar este tipo de control, se recomienda su uso en entornos industriales como empresas hidroeléctricas y plantas de energía. En estos lugares es posible tener un mayor control de las especies introducidas para actuar como depredadores y controlar los mejillones, y en consecuencia, causar un menor impacto al medio ambiente.

¿Cuál fue el mejor control físico?

Como una forma de inhibir el asentamiento se identificaron estudios que evaluaron el tipo de sustrato (material) y el formato del sustrato, la efectividad de la luminosidad y la gravedad, la luz ultravioleta, la presión para reducir la bioincrustación y los sensores de fibra óptica para detectar el mejillón en el agua. A excepción de la obra de Kobak y otros (2008), donde se evaluó la luminosidad y la gravedad en el laboratorio, las otras metodologías identificadas de control físico se realizaron *in situ*. Además, se llevaron a cabo estudios sobre el control del mejillón a través del uso de diferentes temperaturas del agua (Perepelizin y Boltvoskoy, 2011; Piola y Hopkins, 2012; Yuan, et al., 2016; Gaag, et al., 2018; Liu, et al., 2020). De hecho, la temperatura fue identificada entre los factores con mayor capacidad de control: las temperaturas superiores a 20 °C afectan la fijación del viso de los mejillones *Mytillopsis leucophaeata* (Conrad, 1831) (Gaag, et al., 2018) y *M. edulis* (Clements, et al., 2018), y las superiores a 35 °C pueden matar a *L. fortunei* (Liu, et al., 2020) y *M. galloprovincialis* (Piola y Hopkins, 2012). Sin embargo, otros estudios encontraron que *M. charruana, P. viridis* (Yuan, et al., 2016) y *L. fortunei* (Perepelizin y Boltvoskoy, 2011) son tolerantes a las variaciones de temperatura, y dependiendo de la localización geográfica de la especie y su abundancia en el medio ambiente, la temperatura no resulta eficaz para controlar estos mejillones.

Como forma de controlar la incrustación del mejillón cebra (*D. polymorpha*), un estudio analizó diferentes tipos y formas de sustratos utilizando *in situ* placas de policloruro de vinilo (PVC), con y sin filamentos de polipropileno (PP), durante seis semanas. Los resultados sugirieron que los filamentos artificiales facilitan el reclutamiento, principalmente al aumentar la superficie disponible para la fijación (Folino-Rorem, et al., 2006). Estos resultados corroboran lo encontrado por Nakano y otros (2010) al investigar la distribución de poslarvas de *L. fortunei,* donde las densidades de mejillones en las jaulas huecas con malla de 5 mm fueron mayores que en jaulas no huecas en todas las profundidades analizadas. En base a dichos estudios es posible señalar que un área de superficie más pequeña y poco filamentosa se vuelve más ventajosa para controlar la bioincrustación de esta especie. Con respecto al tipo de revestimiento de sustrato utilizado, se comparó el uso de hormigón, madera y piedra caliza como forma de repeler el asentamiento del mejillón. Entre estos sustratos, el concreto no presentó efectos que rechacen significativamente el asentamiento del mejillón dorado *L. fortunei* (Matsui, et al., 2018). Por lo tanto, las construcciones de hormigón en los ríos pueden ser el sustrato preferido para la colonización del mejillón dorado. Otro estudio mostró que el movimiento de fuerza de fijación del asentamiento del mejillón cebra *D. polymorpha* puede cambiar según los parámetros físicos de luminosidad y gravedad (Kobak, et al., 2008).

Pucherelli y otros (2018) instalaron durante dos años en sistemas de refrigeración un generador de luz ultravioleta hidro óptica (HOD UV) para monitorear el impacto de HOD UV en el asentamiento del mejillón *Dreissena bugensis* (Andrusov, 1897). Al comparar los pesos secos de bioincrustación entre las placas expuestas al agua tratada con HOD UV y el control (sin usar HOD UV), se encontró una reducción significativa después de la exposición: el asentamiento de mejillones *D. bugensis* y la formación de biopelículas bacterianas se redujeron constantemente en las cámaras de prueba. Además, se confirmó que el mantenimiento del enfriador relacionado con la bioincrustación se redujo un 75% después del primer año de funcionamiento del HOD UV, eliminándose en el segundo y tercer año después de la implementación. Las pruebas de campo usando pulsos de presión (sistema *sparker*) mostraron un uso efectivo para el control de los mejillones cebra (Schaefer, et al., 2010). Estos resultados indicaron que más pulsos de presión pueden erradicar los mejillones adultos existentes y evitar el establecimiento de estadios larvarios posteriores (Schaefer, et al., 2010). La detección temprana de la obstrucción por mejillones ha sido estudiada utilizando un sistema de fibra óptica llamado técnica de emisión acústica (AE) (Matsuo, et al., 2009). En este trabajo se desarrolló un sensor para la percepción de señales AE de ondas procedentes de mejillones, donde solo se pueden detectar conchas de mejillón con el tamaño de más de 11 mm. Esta metodología no es necesariamente una forma de control ya que no evita la inhibición del asentamiento de macroinvertebrados.

Entre las formas de control físico analizadas, los estudios reflejaron que los sustratos de madera y piedra caliza tienen resultados significativos en disminuir y/o impedir la bioincrustación de los mejillones. A su vez, la luz ultravioleta y los pulsos de presión mostraron un resultado prometedor. Para algunas especies, la temperatura fue un factor que afectó la fijación del viso y consecuentemente aumentó la mortalidad de los mejillones. Finalmente, como medida de prevención fue posible detectar la fijación de mejillones a través de un sistema de fibra óptica AE.

De los trabajos que utilizaron el control físico, el 86% fue eficaz para controlar los mejillones invasores. Sin embargo, este control se vuelve costoso ya que depende del uso de equipos. En ese sentido, el costo del sistema térmico alcanza los 300 mil dólares (Phillips, et al., 2005), y el sistema *sparker* tiene un costo promedio de 65 mil dólares (instalación, formación de profesionales, electricidad y mantenimiento de equipos). La lámpara ultravioleta tiene un costo más alto que el sistema *sparker* (Schaefer, et al., 2010) ya que el gasto de electricidad es mucho mayor: las lámparas UV funcionan continuamente, con un mayor consumo de correspondencias eléctricas. Además, las lámparas UV deben reemplazarse cada mil horas, y si se rompen agregan mercurio al agua, creando un riesgo para el suministro de agua (Schaefer, et al., 2010). Por lo tanto, al comparar estas diferentes tecnologías, el sistema *sparker* tiene mayor potencial ya que es menos costoso y posiblemente menos contaminante que otras tecnologías de control físico.

Para identificar el control físico a utilizar se deben considerar previamente el costo y el daño ambiental, el tiempo de residencia de los organismos en el sistema acuático y otros factores ambientales en el ecosistema. A través del tiempo de residencia del mejillón es posible estimar el período de reproducción del organismo, la abundancia y la dispersión, siendo un factor muy importante para mitigar los impactos que provocan los mejillones en las estructuras artificiales (Somma, et al., 2021).

¿Cuál fue el mejor control químico?

La mayoría de los estudios realizados con control químico fue efectivo para combatir la macroincrustación (84%). En este tipo de control se destacan las modificaciones de pH en el agua (Comeau, et al., 2017; Liu, et al., 2020), la salinidad (Barbosa y Melo, 2009; Yuan, et al., 2016) y el uso de diferentes tipos de reactivos químicos. Entre estos últimos se encuentran el cloro (Rajagopal, et al., 2006), el cloruro de sodio (Davis, et al., 2015), el óxido de cobre (Kojima, et al., 2016; Matsui, et al., 2018) y los compuestos extraídos de material biológico (Lepoutre, et al., 2018).

Yuan y otros (2016) analizaron diferentes temperaturas en conjunto con la influencia de la salinidad para el control de *M. charruana* y *P. viridi*s. En este trabajo, la salinidad fue un factor tolerante para ambas especies, por lo que no mostró un control sobre los mejillones. Rice y otros (2016) señalaron que *M. charruana* se adapta a diferentes salinidades. Barbosa y Melo (2009) predijeron que *L. fortunei* podría sobrevivir a una salinidad de hasta 5 y que a medida que aumenta la salinidad la supervivencia disminuye. De esta manera, salinidades superiores a 5 podrían usarse para controlar el mejillón dorado, disminuyendo su supervivencia.

También se estudió la exposición del mejillón dorado a la variación de hipoclorito de sodio (NaClO), permanganato de potasio ($KMnO_4$) o peróxido de hidrógeno (H_2O_2) (Li, et al., 2019). Únicamente el NaClO debilitó la fijación de los filamentos del viso del mejillón, con baja mortalidad y alta tasa de apertura de la concha en estas condiciones (Li, et al., 2019). Además, de las tres especies reportadas previamente la fijación puede verse comprometida por la concentración de oxígeno disuelto por debajo de 1.4 $\mu g/mL^{-1}$, un pH por encima de 9.7, y puede causar la expulsión del sustrato con una iluminación fuerte y continua (Liu, et al., 2020). La exposición del mejillón al cloruro de sodio (NaCl) también fue un factor limitante para la mortalidad de la especie *D. polymorpha:* la mortalidad puede alcanzar el 100% a concentraciones de 10.000 y 30.000 $\mu g/mL^{-1}$ (Davis, et al., 2015).

Hicks y Mcmahon (2005) evaluaron en el mejillón *P. perna* los efectos de la presión parcial de O_2 (PO_2) en concentraciones de 0, 1, 2, 4 y 6 kPa, junto con la temperatura del agua. Encontraron que la especie tolera PO_2 en concentraciones de 4 y 6 kPa a temperaturas de 15 y 20 °C, mientras que expuesta a una temperatura de 25 °C presenta una mortalidad del 50%. Estos resultados confirmaron que la presión parcial de oxígeno (PO_2) presenta menores efectos sobre la supervivencia del mejillón que la temperatura (Hicks y Mcmahon, 2005).

Por otro lado, Clements y otros (2018) evaluaron la presión parcial de CO_2 (PCO_2), junto con la temperatura del agua, sobre la especie *M. edulis*. Dichos autores encontraron que la especie es tolerante a diferentes concentraciones de PCO_2; sin embargo, a altas temperaturas existe una disminución en la fijación del viso y, en consecuencia, un aumento de la mortalidad corporal. Los mencionados estudios que evaluaron oxígeno disuelto, pH, presión parcial de oxígeno y CO_2 no obtuvieron resultados positivos para el control de los mejillones analizados (Hicks y Mcmahon, 2005; Clements, et al., 2018).

Solo el trabajo realizado por Liu y otros (2020) sobre las variaciones en el oxígeno disuelto y el pH obtuvo resultados positivos para el control del mejillón, específicamente en su estudio con *L. fortunei*. Es necesario aclarar que estos factores se analizaron junto con la iluminación fuerte y continua del sustrato, lo que refleja la importancia de utilizar factores físicos y químicos para controlar el mejillón dorado. Aunque usar diferentes metodologías

sería un enfoque costoso de aplicar y mantener, lo que afectaría la elección de este método por parte de los usuarios.

En la presente revisión se encontraron trabajos que utilizaron productos antiincrustantes. Un experimento de inmersión de cuatro años de duración usó sustratos a base de cobre que demostraron tener un fuerte efecto repelente sobre la bioincrustación de *L. fortunei* (Matsui, et al., 2018). Y para *M. Galloprovincialis* tuvo su fijación en sustrato inhibido a medida que aumentó la concentración de la pintura en base de óxido de cobre (CuO) (Kojima, et al., 2016). Costa y otros (2011) administraron toxinas que contienen biocidas en partículas comestibles para *D. polymorpha,* encontrando una mortalidad del 90% en dicha especie en un tratamiento de 12 horas.

De los estudios realizados con control químico, el 84% demostró efectividad (Barbosa y Melo, 2009; Costa, et al., 2011; Davis, et al., 2015; Kojima, et al., 2016; Matsui, et al., 2018; Li, et al., 2019; Liu, et al., 2020), mientras que el 16% restante no presentó resultados satisfactorios (Hicks y Mcmahon, 2005; Yuan, et al., 2016; Clements, et al., 2018). Sin embargo, los estudios que mostraron efectividad utilizaron en su mayoría metodologías que implican altas concentraciones de productos químicos en el agua (Barbosa y Melo, 2009; Davis, et al., 2015; Kojima, et al., 2016; Matsui, et al., 2018; Li, et al., 2019).

En el ambiente acuático, el uso de sustancias químicas podría afectar no solo a las especies objetivo sino también a las especies no objetivo (Dafforn, et al., 2011; Turner, 2010), y tener un impacto en la calidad del agua (Soroldoni, et al., 2017), causando pérdidas de la biodiversidad y ocasionando un desequilibrio en los diferentes niveles tróficos del ecosistema. Además, el hipoclorito de sodio (NaClO) puede ser efectivo (Li, et al., 2019), pero se estima que el gasto por su uso alcanza los 100 mil dólares (entre instalación, operación y mantenimiento de la metodología) (Phillips, et al., 2005), lo que hace que el método sea costoso.

Entre las formas de control de bioincrustación, el control químico más utilizado corresponde a las pinturas antiincrustantes; sin embargo, puede causar efectos tóxicos a diferentes organismos que se encuentran en el medio acuático (Soroldoni, et al., 2017; Agostini, et al., 2021a). Además, los efectos de las pinturas antiincrustantes pueden prolongarse a largo plazo debido a su acumulación en el sedimento (Thomas, et al., 2003). Por eso se han desarrollado alternativas con productos naturales como forma de control de la bioincrustación, siendo más seguras para el ecosistema (Agostini, et al., 2021b).

En esta revisión se encontraron artículos sobre alternativas antiincustrantes naturales, en los que los autores utilizaron compuestos químicos extraídos de diferentes tipos de materiales biológicos con fines antiincrustantes. Mabrouk y otros (2020) observaron que los extractos de los tallos de la hierba marina *Halophila stipulacea* (Forsskal Ascherson, 1867), usando el diluyente de hexano en la concentración de 11.3 $\mu g/mL^{-1}$ y metanol de las hojas con 17 $\mu g/mL^{-1}$, mostraron un efecto inhibidor de menos del 50% en el mejillón *M. galloprovincialis*.

El uso de extractos crudos de plantas naturales también se registró en otro trabajo, en el cual se evidenció la acción antiincrustante del extracto de *Streptomyces fradiae* (Waksman y Curtis, 1916; Waksman y Henrici, 1948) en el mejillón *P. indica*, consiguiendo un resultado satisfactorio para el mejillón estudiado (Prakash, et al., 2015). Los autores informan que la investigación se desarrolló en el laboratorio y para estudios posteriores se debe realizar un experimento *in situ*. El uso de extractos también fue analizado por Ribeiro y otros (2013), quienes utilizaron diferentes extractos elaborados a partir de doce especies de esponjas de Brasil contra la fijación de *P. perna*. Solo el extracto de dos de las especies inhibió significativamente

el asentamiento del mejillón en más del 10%: *Hymeniacidon heliophila* (Wilson, 1911) y *Petromica citrina* (Muricy, Hajdu, Minervino, Madeira y Peixinho, 2001).

Otros autores utilizaron extractos de compuestos específicos extraídos de organismos naturales para verificar su potencial antiincrustante. Lepoutre y otros (2018) evaluaron el impacto de la neurotoxina ambiental en los hemocitos en relación con la mortalidad celular, fagocitosis e integridad del ADN de *D. polymorpha*. En el estudio se demostró que una concentración de 0.75 $\mu g/mL^{-1}$ está contenida en la hemolinfa e induce daño transitorio al ADN, en consecuencia, conduce a la muerte celular y al daño de otras estructuras del mejillón. En esta línea, Siless y otros (2017) utilizaron cuatro compuestos naturales aislados del alga *Dictyota dichotoma* (Hudson, JVLamouroux, 1809), en los que los compuestos Paquidictyol A y Dictyoxide -en una concentración de 4.7 $\mu g/mL^{-1}$- llevaron a la supresión del viso para su fijación en el sustrato por *L. fortunei*.

En esta revisión, solo se encontraron cinco trabajos de control químico que utilizaron sustancias naturales como alternativa para el control de mejillones (Ribeiro, et al., 2013; Prakash, et al., 2015; Siless, et al., 2017; Lepoutre, et al., 2018; Mabrouk, et al., 2020), y todas han demostrado ser efectivas para controlar la bioincrustación ocasionada por estas especies. Por lo tanto, a la hora de buscar una alternativa eficaz y menos dañina para el ambiente la mejor opción de control químico sería el uso de sustancias naturales como los extractos crudos de organismos o compuestos específicos extraídos de los materiales biológicos, los cuales han mostrado resultados prometedores tanto para el control de la microincrustación como de la macroincrustación (Agostini, et al., 2021b).

CONCLUSIONES

El número de trabajos publicados sobre el control de mejillones invasores ha variado a lo largo de los años: a partir de 2009 oscila entre 1 y 6 estudios anuales. El país que investigó más alternativas de control fue Estados Unidos. El ambiente acuático más estudiado fue el agua dulce, y la mayoría de las investigaciones se llevó a cabo en el laboratorio -comparado con trabajos *in situ*-, centrándose en el control de mejillones en su fase juvenil y adulta. Las principales familias evaluadas fueron Mytilidae y Dreissenidae, con las especies *L. fortunei* y *D. polymorpha*, respectivamente.

El control químico fue el más utilizado y mostró una alta eficacia en el control de mejillones. Las metodologías efectivas fueron el uso de salinidad, hipoclorito de sodio, óxido de cobre, pinturas, oxígeno disuelto y extractos naturales. El control físico, a pesar de tener pocos trabajos publicados, también fue muy efectivo: tanto el uso de la luz UV como los pulsos de presión y alta temperatura lograron inhibir la incrustación del mejillón. Sin embargo, tienen un alto costo ya que depende del uso de equipos caros. El control biológico fue el menos ensayado hasta la fecha y también el menos efectivo. Además, este control solo se puede utilizar con el uso de peces como especie de depredación.

Dentro del control químico se identifica el problema de usar productos químicos como una forma de controlar los mejillones debido a su posible contaminación al ecosistema acuático y daño a las especies objetivo y no objetivo. Sin embargo, fue demostrado que el uso de extractos naturales tiene un gran potencial para el control de los mejillones, además de ser menos nocivo para el ecosistema. Por esta razón, se destaca el control químico mediante el uso de extractos naturales ya que es efectivo y genera menos daño al ambiente acuático.

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ANEXOS

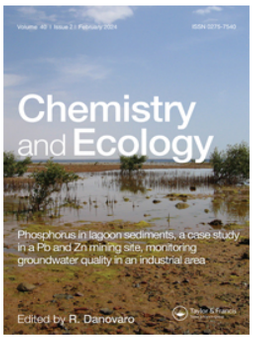
ANEXO 1. Experimentos probados con resultados satisfactorios sobre el uso de métodos de control de mejillones invasores, según familia y especie de mejillón. La información bibliográfica completa se puede encontrar en el documento principal.

Familia	*Especies*	Control	Ambiente	Fase	Detalle de control	Efecto	Autor
Mytilidae	*L. fortunei*	Químico y físico	Agua dulce	Juvenil-adulto	Oxígeno disuelto menos de 1.4 µg/mL^{-1}; pH superior a 9,7; Iluminación continua y fuerte; temperatura encima de 35 °C	Expulsión del sustrato y aumento de la mortalidad	Liu, et al., 2020
		Químico	Agua dulce	Juvenil-adulto	Hipoclorito de sodio (NaClO)	Disminución de la fijación; baja mortalidad	Li, et al., 2019
		Químico y físico	Agua dulce	Juvenil-adulto	Pintura a base de cobre (Cu)	Asentamiento inhibido	Matsui, et al., 2018
		Químico	Agua dulce	Juvenil-adulto	Uso de extracto de *Dictyola dichotoma*	Supresión de viso	Siless, et al., 2017
		Biológico	Agua dulce	Juvenil-adulto	Pez *Pinelodus pintado*	Durante la primavera	Vieira y Lopes, 2013
		Físico	Agua dulce	Juvenil-adulto	Relación área por superficie filamentosa	Superficie pequeña y menos filamentosa	Nakano, et al., 2010
		Químico	Agua dulce	Juvenil-adulto	Salinidad encima de 5 PSU	Disminuye la supervivencia	Barbosa y Melo, 2009
		Biológico	Agua dulce	Larva	Pez *Prochilodus lineatus*	Depredación de mejillones	Paolucci, et al., 2009
	M. galloprovincialis	Químico	Agua dulce	Juvenil-adulto	Extracto de tallo en hexano (11.3 µg/mL^{-1}) y metanol en hojas (17 µg/mL^{-1}) en *Halophila stipulacea*	Efecto inhibidor inferior al 50%	Mabrouk, et al., 2020
		Químico	Agua marina	Juvenil-adulto	Sustratos de óxido de cobre (CuO)	A medida que aumenta la concentración de CuO aumenta la inhibición del asentamiento	Kojima, et al., 2016
		Físico	Agua marina	Juvenil-adulto	Temperatura superior a 35 °C	Causa la muerte	Piola y Hopkins, 2012
	M. edulis	Físico	Agua marina	Juvenil-adulto	Temperatura superior a 20 °C	Afecta la fijación del viso	Clements, et al., 2018
	M. leucophaeata	Físico	Agua de estuario	Juvenil-adulto	Temperatura superior a 20 °C	Afecta la fijación del viso	Gaag, et al., 2018

Familia	*Especies*	Control	Ambiente	Fase	Detalle de control	Efecto	Autor
	P. indica	Químico	Agua marina	Juvenil-adulto	Extracto de *Streptomyces fradiae*	Potencial antiincrustante y baja toxicidad	Prakash, et al., 2015
	P. perna	Químico	Agua marina	Juvenil-adulto	Extracto de *Hymeniacidon heliophila* y *Petromica citrina*	Asentamiento inhibido en más del 10%	Ribeiro, et al., 2013
		Físico	Agua marina	Juvenil-adulto	Temperatura 25 °C	50% de mortalidad	Hicks y Mcmahon, 2005
Dreissenidae	*D. bugensis*	Físico	Agua dulce	Juvenil-adulto	Sistema hidro-óptico ultravioleta (UV HOD)	Asentamiento reducido	Pucherelli, et al., 2018
	D. polymorpha	Químico	Agua dulce	Juvenil-adulto	Neurotoxina ambiental a una concentración de 0.75 $\mu g/mL^{-1}$	Muerte celular, fagocitosis y daño al ADN	Lepoutre, et al., 2018
		Químico	Agua dulce	Juvenil-adulto	Cloruro de sodio (NaCl) en concentraciones de 10.000 y 30.000 $\mu g/mL^{-1}$	Mortalidad del 100%	Davis, et al., 2015
		Químico	Agua dulce	Juvenil-adulto	Partículas comestibles con toxinas biocidas	90% de mortalidad	Costa, et al., 2011
		Físico	Agua dulce	Larva y Juvenil-adulto	Sistema de presión de sparker	Asentamiento reducido	Schaefer, et al., 2010

Capítulo VII: Artigo 2

O segundo artigo científico proveniente desta Tese de Doutorado é apresentado neste capítulo. O manuscrito, de autoria de Mikael Luiz Pereira Morales, Gabrielle Paulsen Figurelli, Bárbara Oleinski, Grasiela Lopes Leães Pinho, Ng Haig They e Vanessa Ochi Agostini, intitula-se "***Antifouling activity of aquatic macrophyte extracts on estuarine bacterial biofilms***" e foi publicado no periódico "***Chemistry and Ecology***" em 2024. O manuscrito encontra-se disponível pelo link https://doi.org/10.1080/02757540.2024.2321990.



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Antifouling activity of aquatic macrophyte extracts on estuarine bacterial biofilms

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ABSTRACT

Ecologically safe antifouling solutions have received growing attention since the acknowledgment of the environmental risks of traditional biocides used currently. Given that bacteria constitute the initial stage of the biofouling process, this work aims to evaluate the antifouling potential of aquatic macrophyte extracts in inhibiting biofilm-forming bacteria and to evaluate their ecotoxicological effects on target and nontarget organisms (*Thalassiosira pseudonana* and *Nitokra* sp.). For this, the ability of 25 macrophyte extracts to inhibit and eradicate single and multispecies estuarine biofilms was evaluated. The extracts and respective serial dilutions that showed a biofilm inhibitory effect $\geq 60\%$ were evaluated in ecotoxicological assays. Of the 25 extracts, only 6 extracts inhibited the biofilm $\geq$ 60%. The extracts of *Cabomba caroliniana* and *Schoenoplectus californicus* stood out as having a biofilm inhibitory effect $\geq 80\%$ ($p < 0.05$) for bacterial isolates and $\geq 70\%$ ($p < 0.05$) for multispecies biofilms. Furthermore, these extracts showed no toxic effects on nontarget organisms. These results demonstrate the biotechnological potential of macrophyte compounds.

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Aquatic plants; antibiofilm activity; bacterial adhesion; biofouling; biotechnology; natural compounds

1. Introduction

Biofouling is a complex process of ecological succession that occurs when biological deposits accumulate on artificial structures (eg vessels and platforms) or natural hard substrates (eg rocks and shells) exposed to the aquatic environment [1]. This process begins with the adsorption of organic and inorganic particles on the submerged surface [1]. Immediately after, bacteria adhere to surfaces and start secreting a matrix composed of water and extracellular polymeric substances (EPSs), thereby forming a biofilm [2]. EPSs consist mostly of exopolysaccharides, exoDNA, exoproteins, exolipids, and water [3]. It

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has long been recognised that the presence of biofilms and EPSs is a critical condition for the later settlement of secondary colonisers such as algae, protozoans and fungi, followed by tertiary colonisers including invertebrates and urochordates [2,4–6].

Biofouling has several negative aspects, primarily linked to economic losses on artificial surfaces (eg vessel hulls, submarine tubes, and cables). In vessel hulls, it increases friction and weight and decreases buoyancy and hydrodynamics, resulting in increased fuel consumption and greater release of greenhouse gases [7]. Additionally, it causes clogging in submarine tubes and a reduction in the durability of cables, making them more brittle [8]. The annual global expenditure for biofouling is estimated to exceed US$ 340 billion, encompassing costs associated with prevention and surface maintenance [9,10].

Over the years, to combat biofouling, antifouling coatings have been developed for application to these structures. Currently, we are in the third generation of antifouling paints composed of different biocides [11], such as copper oxide, diuron and chlorothalonil [12–14]. However, it is known that these paints have negative environmental impacts on the aquatic environment due to their high toxicity against nontarget organisms. Some of these impacts include the inhibition of photosynthesis by blocking photosystem-II electron transport and toxicity to crustaceans, freshwater fish and amphibians [7,15–17].

To address the various drawbacks of antifouling paints, there has been an increasing search for ecologically safer alternatives in recent years [18–22]. 'Green' antifouling has garnered significant attention from the scientific community due to its low toxicity to nontarget organisms and easier degradation than traditional antifouling [20,22]. Several naturally occurring compounds have been identified as promising candidates against microfouling and macrofouling [18,19,22], with a particular focus on bacteria, polychaetas, crustaceans, and mollusks [12].

Natural compounds can interfere with the settlement, growth, and/or development of other organisms, a phenomenon known as allelopathy [21,23]. These substances can originate from various organisms, including bacteria, fungi, algae, plants, and animals. Among the plants possessing chemical compounds for this purpose, aquatic macrophytes stand out. Aquatic macrophytes are characterised as photosynthetic organisms that develop permanently or periodically submerged, floating, or on the aquatic surface [24]. They play a pivotal role in the structure and maintenance of aquatic systems, contributing to primary production and participating in nutrient cycling, among other ecological processes [25,26]. Due to the wide variety of biological forms, these plants are classified into ecological groups based on the location of plant organs in relation to water [27–29], with the main biological forms are submerged, floating, emerged, and amphibians.

The availability and quality of allelopathic compounds (allelochemicals) present in aquatic macrophytes can vary depending on the species and plant organ studied [30,31]. These allelochemicals are produced as an effective defense strategy against other organisms that compete for space, nutrients and light (eg periphyton and phytoplankton) [32,33]. As a result, macrophytes have attracted the interest of researchers and have been shown to be a promising source of antimicrobial agents for biotechnological use [34,35]. Genera like *Ludwigia*, *Typha*, *Schoenoplectus*, *Cabomba*, *Eichhornia*, *Nymphoides*, *Salvinia,* and *Potamogeton*, for example, have been report to exhibit allelopathic effects against bacteria, plants, and other organisms [36–38].

The use of substances inhibiting microbial growth can effectively reduce microfouling and consequently prevent invertebrate settlement, inhibiting the initial stage of the

colonisation process. The bacterial biofilm response to these substances can be influenced by several factors: 1: the composition and concentration of chemical compounds per unit of plant mass; 2: antagonistic relationships among plant constituents; and 3: the individual responses of bacterial species in the biofilm and/or interspecies competition [19]. This highlights the need to investigate the effect of chemical compounds present in plants both at the species level and at the community levels bacterial.

To ensure the safety of new antifouling products, ecotoxicological tests need to be conducted on nontarget organisms from different trophic levels. In this sense, plant extracts are good candidates because of the low potential for toxicity to nontarget organisms and lower biodegradability when compared to traditional antifouling biocides [20,22]. Thus, in this study, we tested the antimicrofouling activity of aqueous extracts of aquatic macrophytes in inhibiting single and multispecies biofilm bacteria and evaluated their ecotoxicological effects on nontarget organisms.

2. Material and methods

2.1. Preparation of plant extracts

Aquatic macrophytes were collected from permanent lakes in southern Brazil (32° 09′ 23.3″ S 52° 05′ 57.6″ W) (subtropical climate) during the winter of 2020 and summer of 2021. The collection was carried out with authorisation by the Brazilian Agency SisBio (process number 77095-1). Species identification involved morphological analysis of reproductive and vegetative structures using identification keys resulting in a total of 11 species. Each plant was carefully cut into distinct organs (roots/rhizoids, stems, leaves, and flowers/inflorescences), resulting in a total of 25 different plant extracts (Table 1). To ensure representative samples and mitigate seasonal variability of the

Table 1. Lists of plant extracts tested in bioassays.

Family	Species	Plant organs
Cabombaceae	*Cabomba caroliniana*	Leaf talk
Cyperaceae	*Schoenoplectus californicus*	Stalk
		Influorescence
Menyanthaceae	*Nymphoides humboldtiana*	Leaf
		Stalk
		Flower
Onagraceae	*Ludwigia hexapetala*	Leaf
		Stalk
	Ludwigia multinervia	Leaf
		Stalk
Pontederiaceae	*Eichhornia azurea*	Leaf
		Stalk
	Eichhornia crassipes	Leaf
		Stalk
		Root
		Flower
Potamogetonaceae	*Stuckenia pectinata*	Mixed
Salvinaceae	*Salvinia minima*	Leaf
		Rhizoid
	Salvinia herzogii	Leaf
		Rhizoid
Typhaceae	*Typha dominguensis*	Upper aerial part
		Lower Aerial part
		Inflorescence

chemical compounds present in the extracts, materials collected during both winter and summer were combined [39]. Subsequently the biomass was dried at 60–80 °C until reaching a constant mass and manually crushed with a mortar and pestle.

For the preparation of the extracts, 6 g of dry biomass was mixed with 300 mL of sterile estuarine water with salinity of 25. The mixture was kept in the dark at 22 °C for 24 h [19]. Then, the mixture was centrifuged (1000 rpm for 10 min), and its supernatant was filtered-sterilised (0.2 µm) (cellulose acetate filter, Sartorius Biolab Products) [19]. This preparation resulted in a stock solution of 100%, which was further diluted to concentrations of 5, 10, 20 and 40%. The control treatment consisted of sterile water (0).

2.2. Bacterial isolates

To obtain bacterial isolates, water was collected from the Patos Lagoon Estuary (32° 09′ 23.3″ S, 52° 05′ 57.6″ W) in August 2021. The salinity was 25, and the temperature was 13 °C. The collected water was transported to the laboratory and placed in a 56 L plastic container. Subsequenthly, acrylic, marine plywood, carbon steel ASTM-36 (25 cm^2), and concrete (36 cm^2) substrates were placed introduced into the plastic container for 24 h with a photoperiod of 12 h:12 h (light:dark).

After exposure, the substrates were washed with a sterile saline solution to remove planktonic cells. Then, the biofilm was scraped from substrates with the help of a sterile swab, which was then spread plated onto nutrient agar media (Kasvi K25-610036, Laboratórios Conda S.A., Espanha). Cultures were incubated in the dark in a BOD incubator for seven days at 25 °C. Morphologically distinct colonies were selected and isolated by streak plating three times, and then transferred to nutrient broth media (Kasvi, Laboratórios Conda S.A., Espanha). Finally, the isolates were cryopreserved (- 80 °C) with 15% glycerol and resazurin (0.010 M) as an indicator. The isolates were included in the Microfouling Bank from *Laboratório de Microcontaminantes Orgânicos e Ecotoxicologia Aquática* (CONECO) of the Institute of Oceanography (IO) from the Federal University of Rio Grande (FURG). All isolates were screened to confirm their ability to form biofilms [40].

The selected isolates were submitted to 16S rRNA gene sequencing. For this, DNA extraction was carried out using sterile Milli-Q water (filtered in 0.2 µm) and acid-washed glass beads (Sigma–Aldrich). Cell lysis was performed by vortexing (30 s), followed by increasing the temperature (90 °C for 15 min), and decreasing (−20 °C for 15 min). Molecular preparation, construction of sequencing libraries, and DNA sequencing were conducted using the ABI 3730 platform (Sanger) at the *Laboratório de Biotecnologia da Empresa de Serviço Nacional de Aprendizagem Industrial* – SENAI. The enzyme Promega Tag G2 with the primers 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (TACGGYTACCTTGTTACGACTT) amplified the bacterial 16S gene. The PCR product was purified using SV PCR and gel cleanup columns (Promega). The sequencing reaction was performed with BigDye terminator 3.1. After the addition of ddNTPS, the DNA was resuspended in formamide and sequenced in a 3730XL instrument with the use of a 50 cm capillary.

The sequences were inspected and corrected for sequence errors and base calls, and the consensus sequences between forward and reverse reads were generated with the help of Seqtrace 0.90 and BioEdit 7.2 software. The curated sequences were submitted to the platform Silva (https://www.arb-silva.de/) for multiple alignment with respect to phylogenetically

closest sequences and classification using the database Silva SSU r138.1. Multiple alignment was used to resolve ambiguous base calls that were conserved across all closest relatives. The Silva database provides comprehensive, quality-checked and regularly updated datasets of sequences from rRNA aligned for all three domains of life (Bacteria, Archaea and Eukarya). The criteria were > 99% identity for species and > 95% for genus identification. Table 1 (Supplementary material) shows the taxonomic information from bacterial isolates.

The sequences were submitted to GenBank under the accession number SUB13384869. These bacteria were used to carry out biofilm inhibition and eradication assays and potential inhibition of planktonic bacterial growth.

2.3. Bacterial community

To obtain the bacterial community, water was collected from the Patos Lagoon Estuary (salinity 25 and temperature 19 °C) (32° 09′ 44.6″ S 52° 06′ 04.4″ W). The collected water was prefiltered (7 µm) to remove the majority of the phytoplankton and zooplankton organisms [41].

2.4. Biofilm inhibition assays

Antibiofilm assays were conducted separately for each bacterial isolate and the bacterial community. These assays took place in 96 acrylic multiwell plates (Citotest Labware Manufacturing CO. LTD, Jiangsu, China), with eight replications per treatment. A bacterial inoculum suspension of 10^6 bacteria mL^{-1} in nutrient broth (Kasvi K25-1216, Laboratórios Conda S.A., Espanha) was used [41].

For biofilm formation inhibition (BFI) assays, 100 µL of the bacterial inoculum was mixed with 100 µL treatments (0, 5, 10, 20 and 40% dilutions) and incubated in the dark at 25 °C for 48 h. The extracts that showed BIF results ≥ 60% compared to controls were used to carry out bacterial biofilm eradication (BBE) and bacteria in their planktonic form (planktonic bacteria growth inhibition PBGI) assays.

For the BBE assay, 200 µL of the bacterial inoculum was added in the wells and incubated in the dark at 25 °C for 24 h. After removing the supernatant, and 200 µL of treatments (0, 5, 10, 20 and 40%) were added. The plates were then incubated in the dark (25 °C) for another 48 h. To measure the biofilm density of both assays (BFI and BBE), the supernatant was removed, and the biofilm was dried (60 °C) per 1 h, stained with violet crystal (0.4%) per 15 min, and solubilised with ethanol 99.5% per 30 min. The optical density was measured in a fluorescence microplate reader (filtermax F5, molecular devices) (550 nm) [19,40].

For the PBGI assay, the planktonic bacteria growth inhibition was used as an endpoint by calculating the difference in optical density (620 nm) at the beginning and end of the incubation (in the dark at 25 °C) after 48 h [19,42]. Controls from BFI, BBE, or PBGI assays were considered to represent 100% of the biomass. The results were expressed as a percentage of BFI, BBE, or PBGI.

2.5. Ecotoxicological assays

For the ecotoxicological assays, the extracts that presented an inhibitory effect on biofilm formation ≥ 60% were selected for evaluation. Two nontarget model organisms were

chosen: the marine planktonic microalgae *Thalassiosira pseudonana* (Hasle & Heimdal, 1970) obtained from the Freshwater Microalgae Bank (https://ccmd.furg.br/), and the epibenthic copepod *Nitokra* sp. (Boeck, 1865), sourced from cultures of CONECO, IO, both from FURG.

The *T. pseudonana* chronic assay was performed following the ABNT [43], with four replicates per treatment. The assay was carried out in 50 mL transparent glass (49 mL of extract dilutions and 1 mL microalgae) with inoculum at a density of 10^4 cells mL^{-1} for 72 h ± 2 h at 24 °C, continuous lighting of 7.000 lux, constant agitation, and an initial pH of 6.60–7.0. At both the beginning (0 h) and the end (72 h) of the assay, an aliquot (1 mL) was removed, and 200 µL of 0.4% formaldehyde was added to stop the culture growth. The cell density (cells mL^{-1}) was estimated by counting cells using the Neubauer chamber, through the difference in cell counts between 0 and 72 h. Additionally, concentration of chlorophyll-a was also measured and expressed in µg L^{-1}. For each treatment, 5 mL aliquots were centrifuged for 10 min, the supernatant was removed, and 3 mL of methanol (100%) was added, which was refrigerated at −14 °C for 2 h. After that, the samples were centrifuged again for 10 min, and their supernatant was read in a spectrophotometer at wavelengths of 663 and 750 nm. The chlorophyll-*a* concentration was calculated according to Mackinney [44].

The *Nitokra* sp. acute toxicity assay was conducted following the methodology described by Nascimento et al. [45], with three replicates per treatment. In glass beakers, ten organisms were exposed to 20 mL of the extract treatments. Adult specimens, without sex distinction and not holding egg sacks (ie mature females), were exposed to the treatment for 96 h at 25 °C, a photoperiod of 16 h:8 h (light:dark), and an initial pH of 6.6–7.0. At the end of the exposure time, live and dead organisms were counted, and the results were expressed as a percentage of survival relative to the control. Copepods that were completely motionless were counted as dead.

2.6. Statistical analysis

Student's t tests were performed with Bonferroni correction for multiple comparisons to observe potential differences between each of the treatments compared to the respective controls in the BFI, BBE, and PBGI assays. One-way ANOVA was used to test for statistical significance among treatments in the assays with *T. pseudonana* after checking for the test assumptions (normal distribution of residuals and homoscedasticity). Upon accepting alternative hypotheses with a 95% confidence level, the Tukey post hoc test was used to check where the difference among averages was located. Given the discrete nature of the data in the *Nitokra* sp. assay, a generalised linear model (GLM) with a binomial distribution was utilised applying the logit link function. All analyses were performed in R software 4.2.2.

3. Results

3.1. Bacterial isolates identified

The bacterial isolation process resulted in a total of 10 species identified (Table 1 – supplementary material). For each substrate, 3 species were found. *Pseudoalteromonas tunicata* and *Albirhodobacter marinus* were common species found in both marine plywood

and acrylic substrates. Exclusive species were observed on the concrete and ASTM-a36 substrates, each exhibiting three different biofilm-forming bacterial species.

3.2. Biofilm assays

3.2.1. Bacterial isolates

Of the 25 different extracts tested, six showed inhibitions ≥ 60% for at least one of the tested bacteria (Figure 1): *Salvinia minima* leaf; *Cabomba caroliniana* leaf; *C. caroliniana* stalk; *Schoenoplectus californicus* stalk; *S. californicus* inflorescence; and *Nymphoides humbolftiana* flower. Extracts from *C. caroliniana* and *S. californicus* were particularly effective, showing inhibition > 80% and inhibiting six and five out of the 10 bacteria tested, respectively (Figure 1(c-f)). The bacteria *Exiguobacterium* sp. and *Microbacterium marinilacus* were the most representative in terms of inhibition of biofilm formation. Since the 6 extracts presented in some dilutions a > 70% inhibition for *Exiguobacterium* sp. and for *M. marinilacus,* only the *N. humboldtiana* flower extract did not show this inhibition (Figure 1(a-f)).

In the biofilm eradication assay, only the 10% treatment extract of *S. californicus* stalk showed a significant effect >60% against *Psychrobacter alimentarius* (Figure 1(e)). Interestingly, all extracts showed induction of planktonic growth instead of inhibition for at least two species of bacteria, varying according to the tested dilution (Figure 2). The bacterium *Pseudoalteromonas tunicata* stood out from the other bacteria tested, as it presented the greatest induction of planktonic growth.

3.2.2. Bacterial community

The formation of bacterial community biofilms was inhibited (≥ 60%) by four extracts: *C. caroliniana* leaf and stalk, *S. californicus* stalk, and inflorescence (Figure 3). Two extracts were highly effective: *C. caroliniana* stalk 20% and *S. californicus* stalk 5%, both with inhibition greater than 70%. For the planktonic growth assays, only the extracts of the leaves of *C. caroliniana* (40%), stalks of *S. californicus* (5%) and inflorescences of *S. californicus* (40%) influenced growth, all presenting growth induction (Figure 3). For biofilm eradication, of the extracts that yielded satisfactory results on bacterial isolates (*C. caroliniana* and *S. californicus*), none showed eradication capacity on bacterial community biofilms.

3.3. Ecotoxicological tests

In terms of the toxicity of macrophyte extracts, compared to the control group, only the extract of *S. minima* (leaf) 20% decreased the chlorophyll-*a* content and the growth of *Thalassiosira pseudonana* (Figure 4(a,b)). The survival of *Nitokra* sp. was not affected by any aquatic macrophyte extract tested (Figure 4(c)).

4. Discussion

In our study, we observed that the type of plant organ did not influence the biofilm inhibition response, which contrasts with findings by Cardoso et al. [46] and Ramos et al. [39], who evaluated different plant organs of the species *Piper mollicomum*, *Camellia sinensis*, and *Smallanthus sonchifolius*. Our results underscore the significance of different organs

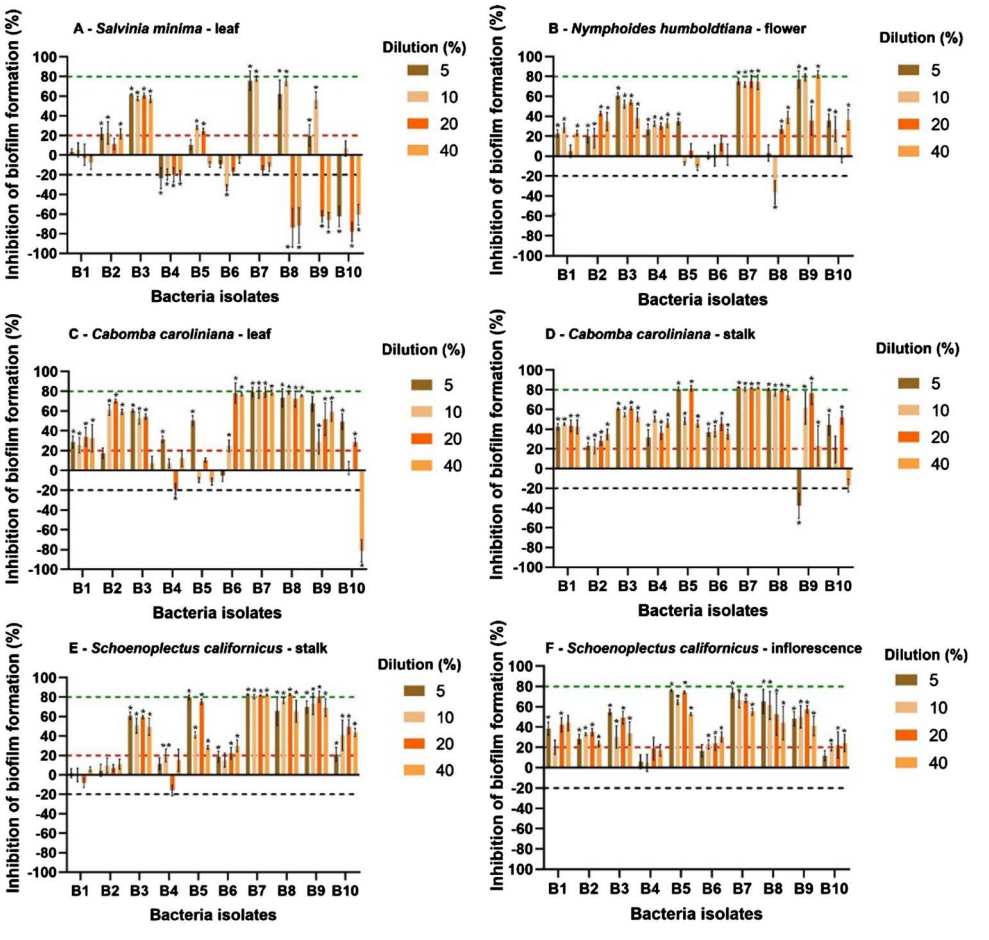

Figure 1. Inhibition of bacterial isolate biofilm formation (%). The six extracts with at least one significant result (>60% inhibition) are shown. B1 = *Psychrobacter* sp.; B2 = *Pseudomonas fulva*; B3 = *Dietzia maris*; B4 = *Psychrobacter alimentarius*; B5 = *Pseudoalteromonas tunicata*; B6 = *Albirhodobacter marinus*; B7 = *Exiguobacterium* sp.; B8 = *Microbacterium marinilacus*; B9 = *Kocuria palustris*; B10 = *Psychrobacter nivimaris*. A – F = Different extracts of aquatic macrophytes. * = significant difference when compared to the control group. ** = eradication of biofilm. Red line = inhibition of biofilm formation activity (antibiofilm activity); black line = biofilm formation induction activity; green line = inhibition of biofilm formation ≥ 80%.

of macrophytes are important for the effectiveness of biofilm inhibition, with notable emphasis on the extracts of *Schoenoplectus californicus* (stalk and inflorescence) and *Cabomba caroliniana* (leaf and stalk) that showed greater effects (inhibition ≥ 70%). In a review study on the antifouling potential of plants, Agostini et al. [12], indicated that leaves and reproductive organs are the most investigated and presented more satisfactory results against biofouling. However, in our case, we did not see this difference, noting that other factors may have influenced the concentration and quality of allelochemicals.

Plant species are an important component to be analysed, as differences in the composition of allelochemicals, along with variations in the quality and concentration of these

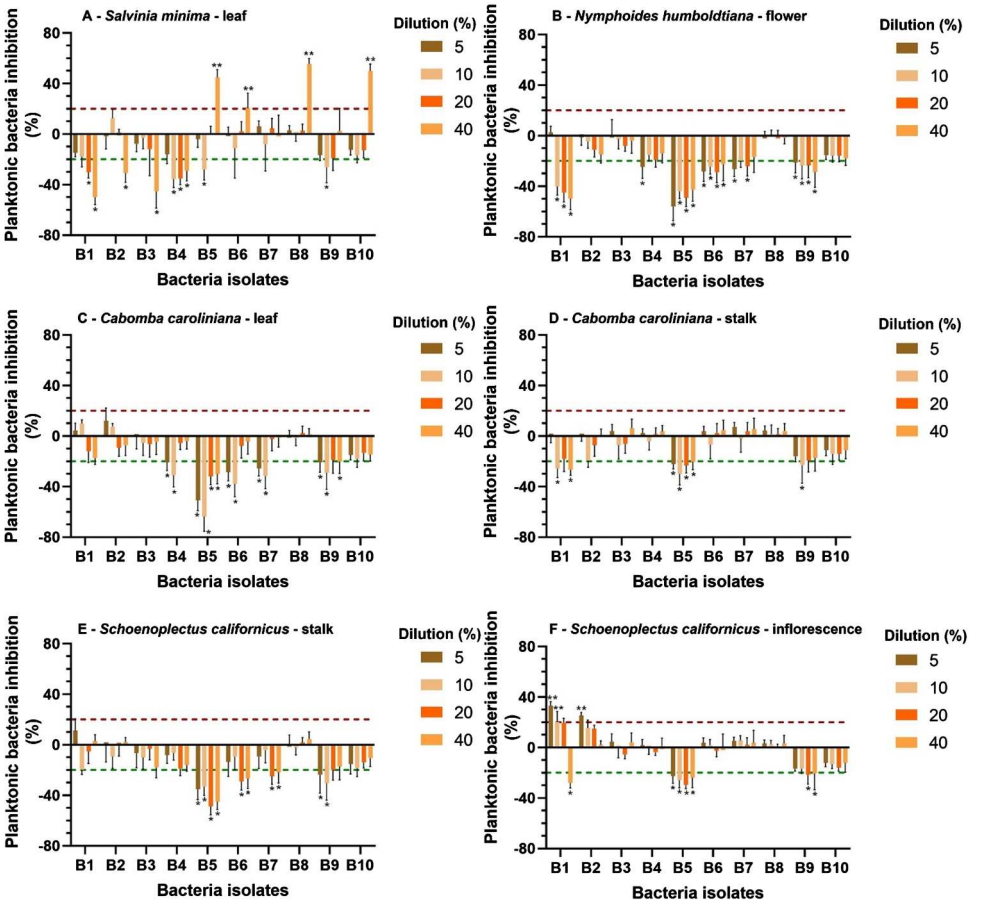

Figure 2. Bacterial isolate planktonic inhibition (%). B1 = *Psychrobacter* sp.; B2 = *Pseudomonas fulva*; B3 = *Dietzia maris*; B4 = *Psychrobacter alimentarius*; B5 = *Pseudoalteromonas tunicata*; B6 = *Albirhodobacter marinus*; B7 = *Exiguobacterium* sp.; B8 = *Microbacterium marinilacus*; B9 = *Kocuria palustris*; B10 = *Psychrobacter nivimaris*. A – F = Different extracts of aquatic macrophytes. ** or * indicates a significant difference when compared to the control group. * = effect of inducing planktonic growth, from the green dotted line; ** = antibiotic effect (reduced planktonic growth, from the red dotted line). Dilutions that showed no difference when compared to the control group = antibiofilm activity.

compounds, can arise due to abiotic factors (eg temperature, rainfall, and seasonality) and biotic (eg herbivory) factors [21,39,47]. In our study, among the total number of tested aquatic macrophytes species, four exhibited an effect on bacterial biofilm inhibition. However, in the literature, all eleven species evaluated here are reported to influence the growth of bacteria, algae, and plants [36–38]. This suggests that the exposure of the plants to different abiotic and biotic factors may have contributed to the variations in the concentration and quality of allelochemicals present.

When evaluating the allelopathic effects of macrophytes, the biological form of these plants becomes a significant factor to consider [48–50]. In our study, we considered the biotypes submerged, floating, and emergent. Among the biological forms whose extracts have greater inhibitory effects are *C. caroliniana* and *S. californicus*. *C. caroliniana* develops

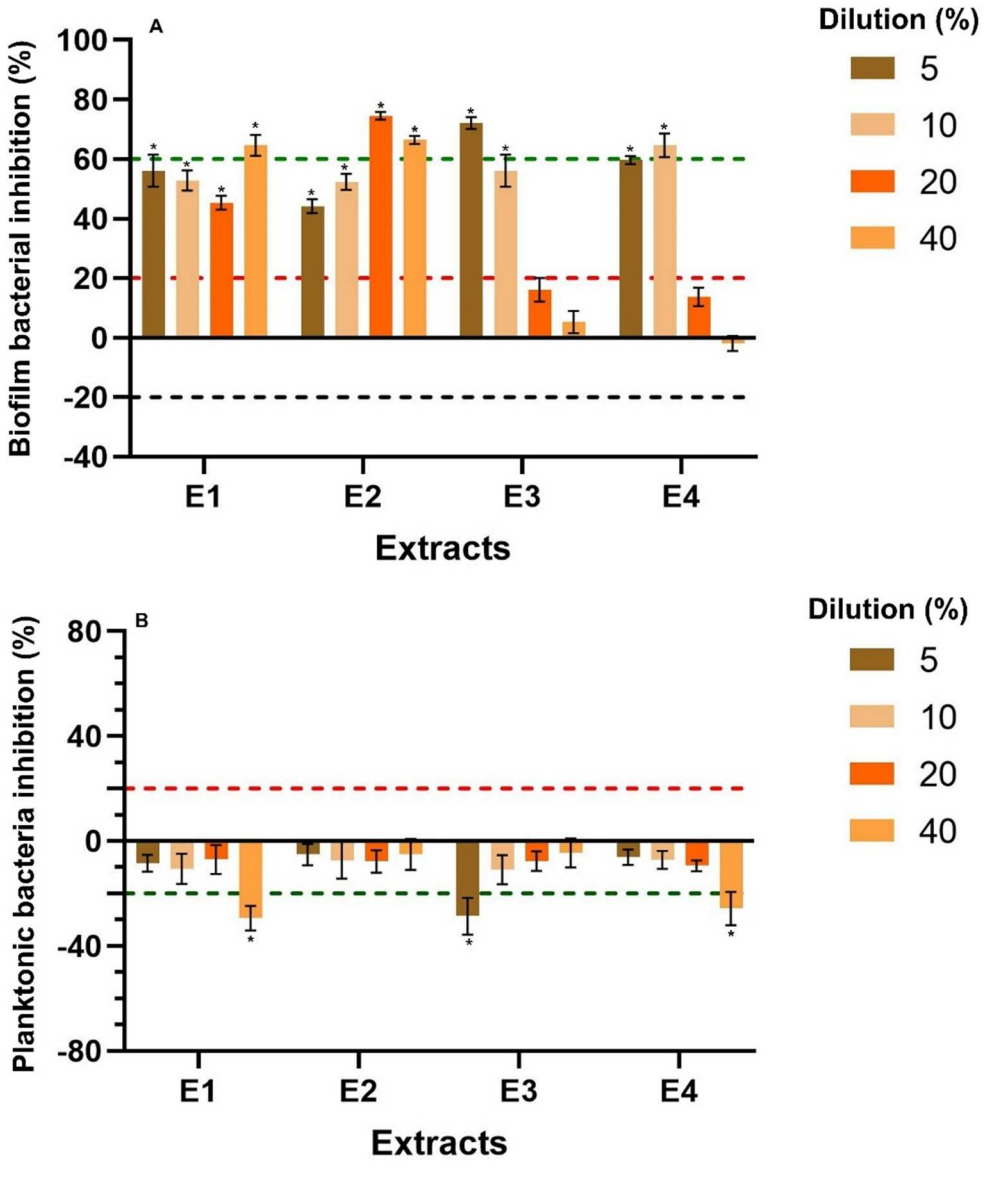

Figure 3. Bacterial biofilm inhibition (%) (A) and planktonic bacteria inhibition (%) of the bacterial community. E1 = *Cabomba caroliniana* leaf; E2 = C. caroliniana stalk; E3 = *Schoenoplectus californicus* stalk; E4 = *S. californicus* inflorescence. * = presents a significant difference when compared to the control group. In 'A', red line = inhibition of biofilm formation activity (antibiofilm activity); black line = induction of biofilm formation activity; green line = inhibition of biofilm formation ≥ 60%. In 'B', green line = induction of planktonic growth; red line = antibiotic effect (reduction in planktonic growth).

completely submerged in water [29,51], while *S. californicus* is emergent, with roots that are fixed in the sediment and leaves outside the water column [29,51]. According to Trindade et al. [52], submerged macrophytes generally display greater sensitivity to water quality than emergent ones, potentially due to heightened competition for nutrients

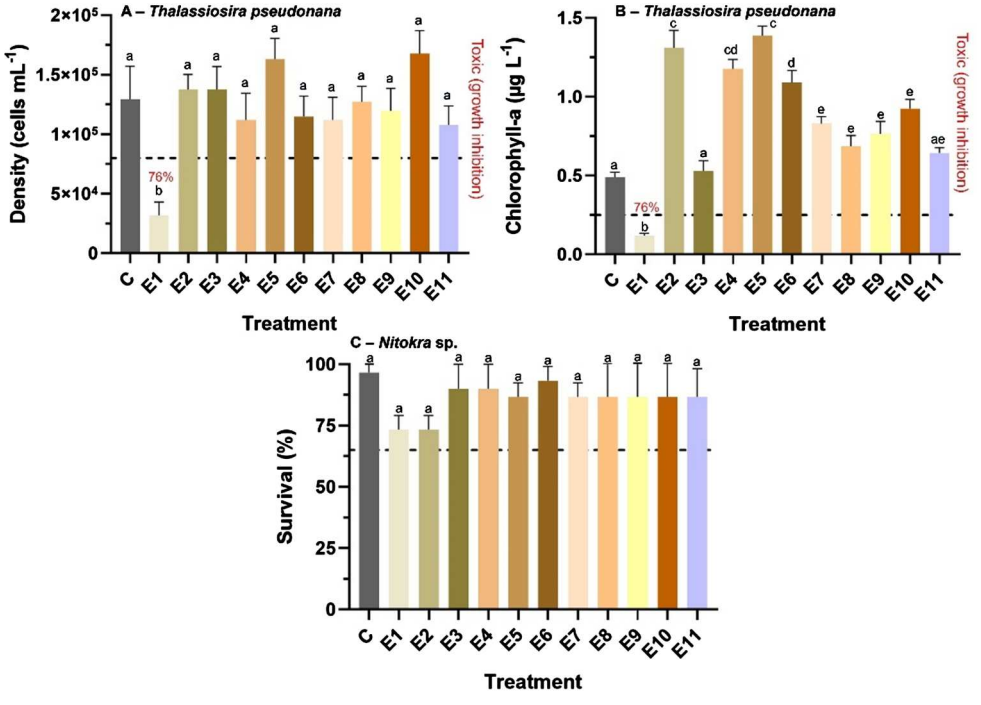

Figure 4. A – B = Response of *Thalassiosira pseudonana* exposed to treatments with aqueous extracts of aquatic macrophytes. A = Density (cells mL^{-1}). B = Chlorophyll a concentration (µg L^{-1}). C – Response of *Nitokra* sp. exposed to treatments with aqueous extracts of aquatic macrophytes. Percentage in red = percentage of toxicity when compared to the control group. E1 = *Salvinia minima* leaf – dilution 20x; E2 = *S. minima* leaf – dilution 40x; E3 = *Cabomba caroliniana* leaf – dilution 5x; E4 = *C. caroliniana* stalk – dilution 5x; E5 = *C. caroliniana* stalk – dilution 10x; E6 = *C. caroliniana* stalk – dilution 20x; E7 = *Schoenoplectus californicus* stalk – dilution 5x; E8 = *S. californicus* stalk – dilution 10x; E9 = *S. californicus* inflorescence – dilution 5x; E10 = *S. californicus* inflorescence – dilution 10x; E11 = *Nymphoides humbolftiana* flower – dilution 5x. Different letters = means when compared, they present a significant difference ($p < 0.05$).

and light with phytoplankton, since these organisms occupy the same stratum in the water column [53]. However, our results indicated that submerged and emergent macrophyte species had similar effects on the inhibition of single and multispecies bacteria.

The most recurrent allelochemicals in aquatic macrophytes are phenolics, terpenoids, alkaloids and fatty acids [54], frequently reported in the literature with the potential to inhibit algae growth [54]. Specifically, flavonoids are reported due to their antifouling properties [12]; however, in this work, we did not identify which types of compounds are present in the tested macrophytes. Nevertheless, there is ample evidence in the literature reporting the presence of allelochemicals in these plants [55–58].

The species *C. caroliniana* is recognised as an effective competitor for nutrients, and the secondary compounds present in the macrophyte (eg humic acids and possibly other allelochemicals) possess the ability to reduce the growth of algae and cyanobacterial biomass [37,50,55,56]. The present work is the first report on the inhibition of biofilm formation by heterotrophic bacteria. While the effects on autotrophic bacteria and microalgae are typically attributed to photosystem II inhibition, primarily due to polyphenols

[57], the impacts on heterotrophic bacteria remain unclear and necessitate further investigated. The species *S. californicus* is known in the literature for its antimicrobial properties due to the high concentration of secondary compounds such as flavonoids and phenols [58]. These compounds primarily function is defense against herbivores and pathogens.

Among the bacteria identified in our work, we observed the predominance of the phylum Proteobacteria, including five species of the Gamma-Proteobacteria class and one species of the Alpha-Proteobacteria class. The phylum is recognised for its high diversity and phenotypic and phylogenetic versatility, enabling the colonisation of different environments [59,60]. In the literature, the phylum Proteobacteria is reported to be abundant in natural waters, with a predominance in substrates that are ligno, calcarenitic, acrylic and sediments from aquatic environments [4,61,62]. These organisms actively contribute to biofilms on various surfaces including rocky substrates, ship hulls and their interactions with coral reefs and the water column [61,63,64]. This underscore that the species used in our study are representative and naturally occurring in aquatic bacterial biofilms.

Microbacterium marinilacus (gram-positive) and *Exiguobacterium* sp. (gram-negative) bacteria were the most affected by the inhibition of macrophyte extracts. Gram-positive bacteria do not have an outer membrane and, therefore, are more sensitive to external factors, where consequently, most of the allelochemicals act efficiently on bacteria of this type [65,66]. On the other hand, gram-negative bacteria, with their outer membrane, exhibit greater selectivity and are generally less sensitive to external factors [65,66]. However, according to our results, there was no systematic response considering the type of gram cell wall. Thus, we suggest that inhibition may be associated with other mechanisms unrelated to cell permeability.

The assay used to eradicate the already formed biofilm revealed that the extracts were not as effective, in which we observed eradication $\geq$ 60% only in the extract from the stalk of *S. californicus* (10% dilution) on *Psychrobacter alimentarius* bacteria. This finding suggests that eradicating bacterial biofilms is more challenging than inhibiting their formation [19]. This is not entirely unexpected, considering that biofilms are organised as complex colonies of microorganisms wrapped in an extracellular polymeric matrix (EPM) [1]. Along with the EPM, there is an increase in the production, release, and detection of signaling molecules that regulate the formation of the biofilm complex (quorum sensing process (QS)) [67]. Consequently, bacteria in biofilms can withstand extreme environmental conditions such as pH changes, low nutrient availability and the action of chemical agents, such as chemical compounds present in extracts [67,68].

In the planktonic growth inhibition assays, three types of responses were observed. The first type involved the reduction of planktonic growth, indicating an antibiotic effect. The second type demonstrated increased planktonic growth, representing an inductive effect of growth. The third type showed no effect on the growth of planktonic bacteria but exhibited inhibition of the biofilm, characterising the antibiofilm effect. When considering the objective of new antifouling agents, the desired response would be the antibiofilm effect, as it is considered environmentally safer than the antibiotic effect [19]. Responses that specifically target the antibiofilm effect reduce the risk of bacterial resistance and may be associated with the inhibition of biofilm formation through QS inhibition [69].

Allelochemicals with QS inhibition potential is crucial in developing of new antibiofilm agents. In our study, we observed that the extracts of *C. caroliniana* and S. *californicus*

showed a biofilm inhibition effect, but they did not show effects on the eradication and inhibition of planktonic growth, suggesting that the action of the extracts did not cause toxicity. Furthermore, we believe that the extracts reduced enzymatic activity and interfered with QS. A study by They et al. [50], verified the effects of extracts from the submerged macrophyte *Potamogeton pectinatus* on alkaline phosphatase activity in bacterial communities, where it was found that the extracts have the potential to reduce the activity of this enzyme. Our study did not evaluate the action of extracts on QS and enzymatic activity; however, we suggest the development of these analyses to better understand the potential effect of these extracts.

Although it is recognised that aquatic macrophytes have allelochemical effects on target organisms, such as phytoplankton and cyanobacteria [50,54], the use of high concentrations can generate toxic effects for nontarget organisms. Previous studies have highlighted the toxicity of allelochemicals, such as linoleic acid and salicylic acid extracted from macrophytes, towards nontarget organisms like as *Moina macrocopa* (Straus, 1820), *Danio rerio* (Hamilton, 1822) and *Daphnia magna* (Straus, 1820) [70–72]. Notably, among the macrophyte extracts tested in our study, only *Salvinia minima* showed a toxic effect on the species *Thalassiosira pseudonana*, causing effects on the chlorophyll-*a* content and growth.

None of our extracts showed toxic effects on the copepod *Nitokra* sp. Thus, for these two organisms, most of the evaluated extracts did not show toxicological effects. The pattern found in our study was also observed by other authors. When evaluating the effect of different extracts from plants of the Fabaceae family, Agostini et al. [19] found that most of the extracts tested were not toxic to the nontarget organisms *Chaetoceros calcitrans*, *Nitokra* sp. and *Artemia salina*. In another study, Agostini et al. [18] also found no toxic effects on the nontarget organisms *C. calcitrans* and *A. salina*.

The quest for plant-derived components in surface coatings has gained momentum in recent years, driven by their potential in various functionalities such as anticorrosion, antifouling, and antimicrobial properties [21]. Chemical compounds found in plant extracts hold the capability to deter biofouling by inhibiting motility, adhesion and/or biofilm formation on substrates [21]. Despite the extensive knowledge of the ecological dynamics of aquatic macrophytes in freshwater aquatic systems, their antifouling characteristics remain relatively unexplored. Aquatic plants have important potential for prospecting sustainable antifouling, as they have a range of chemical compounds in their different compositions, concentrations, and qualities, and in general, the extracts have low toxicity to nontarget organisms. In our study, we highlighted the potential of *C. caroliniana* and *S. californicus* species, emphasising their promise for the development of antifouling alternatives. Furthermore, for the development of new natural antifouling agents, we highlight the importance of identifying the compounds present in extracts, as well as antifouling tests in the field.

5. Conclusion

Our work demonstrates the biotechnological potential of aquatic macrophyte extracts for incorporation into natural antifouling coatings. *C. caroliniana* and *S. californicus* extracts are promising alternatives to traditional antifouling biocides, showing efficacy in biofilm control and safety for nontarget organisms. These plants can be considered

strong candidates for the development of natural products, giving hope for a new perspective on the topic of antifouling paints. Our next efforts will be aimed at evaluating the inhibition of macrofouling settlement, in addition to carrying out field tests to assess biofouling, as well as the chemical characterisation of extracts and isolation of bioactive compounds, for incorporation as additives in antifouling paints. This is already an ongoing trend due to its greater biodegradability and possible lower toxicological action against nontarget organisms.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

All authors contributed to the study conception and design. The author Mikael Luiz Pereira Morales and Bárbara Oleinski contributed to writing, conceptualisation, methodology, laboratory analysis, statistical analysis, revision, and editing. Gabrielle Paulsen Figurelli realized the methodology, laboratory analysis. Grasiela Lopes Leães Pinho, Ng Haig They and Vanessa Ochi Agostini contributed the conceptualisation, methodology, statistical analysis, revision, and editing. The first draft of the manuscript was written by Mikael Luiz Morales Pereira and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Supplementary material

Table 1: Taxonomy information of the selected biofilm-forming bacterial isolates for each type of substrate.

Class	Family	Genus	Species	Substrates
Gama-Proteobacteria	Moraxellaceae	*Psychrobacter* sp.	*Psychrobacter* sp.	concrete
Gama-Proteobacteria	Pseudomonadaceae	*Pseudomonas* sp.	*Pseudomonas fulva*	concrete
Actinomycetia	Dietziaceae	*Dietzia* sp.	*Dietzia maris*	concrete
Gama-Proteobacteria	Moraxellaceae	*Psychrobacter* sp.	*Psychrobacter alimentarius*	marine plywood
Gama-Proteobacteria	Pseudoalteromonadaceae	*Pseudoalteromonas* sp.	*Pseudoalteromonas tunicata*	marine plywood and acrylic
Alpha-Proteobacteria	Rhodobacteraceae	*Albirhodobacter* sp.	*Albirhodobacter marinus*	marine plywood and acrylic
Bacilli	Bacillaceae	*Exiguobacterium* sp.	*Exiguobacterium* sp.	ASTM-36 carbon steel
Actinomycetia	Microbacteriaceae	*Microbacterium* sp.	*Microbacterium marinilacus*	ASTM-36 carbon steel
Actinomycetia	Micrococcaceae	*Kocuria* sp.	*Kocuria palustris*	ASTM-36 carbon steel
Gama-Proteobacteria	Moraxellaceae	*Psychrobacter* sp.	*Psychrobacter nivimaris*	Acrylic

Capítulo VIII: Artigo 3

O terceiro artigo científico proveniente desta Tese de Doutorado é apresentado neste capítulo. O manuscrito, de autoria de Mikael Luiz Pereira Morales, Pablo Santos Guimarães, Camila de Martinez Gaspar Martins, Diana Míguez, Fabiana Rey Bentos, Lucía Boccardi, Ernesto Brugnoli, Ayman Shaik, Hafizah Chenia, Ronaldo Olivera Cavalli, Ng Haig They, Grasiela Lopes Leães Pinho e Vanessa Ochi Agostini, intitula-se "***Aquatic macrophytes as a source antifouling non-toxic, against bacterial biofilms and golden mussel attachment: a possible role of quorum sensing interference***", foi publicado no periódico "***Environmental Science and Pollution Research***" em 2024. O manuscrito encontra-se disponível no link https://doi.org/10.1007/s11356-024-35744-y.

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RESEARCH ARTICLE

Aquatic macrophytes as a source of antifouling non-toxic against bacterial biofilms and golden mussel attachment: a possible role of quorum-sensing interference

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Abstract

Biofouling in freshwater and marine environments developed on man-made aquatic surfaces causes significant economic losses. Still, this problem is magnified when it comes to invasive species, such as the golden mussel. One of the alternatives to combat it is the use of antifouling solutions; however, the vast majority focus on solutions for the marine environment. In this same context, natural antifouling solutions from macrophytes have been reported as promising to combat estuarine biofouling; however, trials with freshwater organisms are still incipient. Thus, this study evaluated the performance of 25 macrophyte extracts in inhibiting the formation and/or eradication of bacterial biofilms, settlement of the golden mussel (*Limnoperna fortunei*), as well as its toxicity effect on three different non-target model organisms of three trophic levels. Among the 25 extracts, nine demonstrated ≥ 60% inhibition of biofilm formation, with only the extracts *of Typha domingensis* and *Eichhornia crassipes* having a biofilm inhibitory effect of ≥ 70% for bacterial isolates and ≥ 60% for multispecies biofilms. Planktonic growth had distinct responses, ranging from induction, inhibition, and no effect on growth. The *T. domingensis* extract showed quorum sensing inhibition (QSI) with a dose-dependent relationship, while the *E. crassipes* extract showed QSI only at a dilution of 1.2%. These same extracts prevented the golden mussel from attaching and showed safe concentrations of 35.35% for *Pseudopediastrum boryanum* and *Daphnia magna* and 70.71% for *Pimephales promelas*. This study highlights the biotechnological potential of macrophyte extracts as a sustainable and environmentally harmless alternative for the control of micro and macrofouling in freshwater environments.

Keywords Antibiofilm · Anti-attachment · Aquatic plants · Freshwater antifouling · Quorum sensing inhibition · Natural compounds

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Introduction

Biofouling is the accumulation of biological deposits on practically all submerged surfaces in marine and freshwater environments that occurs due to the activity of a diverse community because of a complex ecological succession (Martín-Rodríguez et al. 2015). A crucial step in this succession is the initial colonization by bacteria (microfouling), which forms a biofilm associated with extrapolymeric substances and influences the attachment of other fouling organisms (*e.g.*, algae and invertebrates) (Peng et al. 2020; Ma et al. 2023). Biofouling is considered a serious economic problem for man-made aquatic structures (*e.g.*, platforms, ducts, and piers), particularly when it involves the attachment of mussels and barnacles (i.e., macrofouling) (Dobretsov and Rittschof 2020). The complexity of this problem is further magnified when associated with invasive species, like the golden mussel.

The golden mussel *Limnoperna fortunei* (Dunker, 1857) is one of the most studied invasive mussel species with a plethora of studies searching for control strategies (Boltovskoy and Correa 2015; Pereira et al. 2022). Native to mainland Southeast Asia, *L. fortunei* was accidentally introduced from the ballast water of transoceanic ships into South America in the 1990s, where it quickly invaded continental water bodies (Darrigran et al. 2020). Perhaps more importantly, the colonization of this invasive mussel into aquatic ecosystems leads to severe ecological impacts, such as competition with native species for food and space (for the consumption of phyto and zooplankton), increased cyanobacteria proliferation, and the introduction of new fish parasites, therefore causing changes in local chains and aquatic habitat structures (Cataldo et al. 2012; Boltovskoy and Correa 2015). The economic impacts are mostly related to the obstruction of hydroelectric power plants as mussels colonize hydraulic sensors and clog pipes, turbine cooling systems, chambers, and other submerged structures (Brugnoli et al. 2005; Brugnolli et al. 2011). They are also implicated in wear and changes in the conformation of hydroelectric and nuclear plant structures, water treatment plants, refineries, steel mills, and aquaculture and forestry agro-industrial systems (Boltovskoy and Correa 2015; Maranhão and Stori 2019; Fabián et al. 2021).

Over the years, several biological, physical, and chemical strategies have been attempted to control invasive mussels (Pereira et al. 2022). The most common method is the use of antifouling paints to combat biofouling. This method is well applied; however, currently, even third-generation biocides added to antifouling paint formulations have negative impacts on the aquatic environment due to their toxicity on non-target organisms (*e.g.*, inhibition of photosynthesis in microalgae, mortality in planktonic crustaceans, reduced growth of fish) (Martins et al. 2018; Mansano et al. 2018; Campos et al. 2022; Perina et al. 2023). An alternative approach that has been investigated is the use of natural products (Pereira et al. 2022) that are ecologically safer and less harmful to the environment (Agostini et al. 2021b; Pérez et al. 2021; Hamidi et al. 2022) due to their greater biodegradability and potentially lower toxicity against non-target organisms (Pérez et al. 2021).

Plant compounds have long been used to combat numerous diseases due to their antioxidant (Unuofin and Lebelo 2020), antimicrobial (Chassagne et al. 2021), anti-inflammatory (Nunes et al. 2020), anti-cancer (Khan et al. 2019), and antifouling properties (Agostini et al. 2021b). Furthermore, the use of plant compounds stands out due to the large number of chemicals present in different plant organs (Hamidi et al. 2022). Many of these compounds act as allelochemicals for their capacity to interfere with the settlement, growth, and/or development of several organisms (Hamidi et al. 2022).

The antifouling activity of plants is related to the presence of allelochemicals from the class of alkaloids, flavonoids, and tannins (Hamidi et al. 2022), which are used as additives in antifouling formulations (Hamidi et al. 2022; Neves et al. 2024). However, studies related to this topic are mainly focused on the activity of terrestrial plants (Agostini et al. 2021b; Hamidi et al. 2022), and there are still few studied with aquatic macrophytes, with only the study by Morales et al. (2024) being observed. In their study, Morales et al. (2024) observed only the antifouling effect of aqueous macrophyte extracts on the growth and formation of estuarine bacterial biofilm, and there are no reports in the literature for the effect of these plants on freshwater biofouling, especially the golden mussel, a highly invasive species with an emerging concern for its control.

Still in the literature, these plants are reported to have alkaloids, phenolic compounds, tannins, and flavonoids (Shanab et al. 2010; Silva et al. 2010), which have an influence on bacterial growth in lakes; however, their antifouling purpose in freshwater environments for the formation of bacterial biofilm and inhibition of the attachment of the golden mussel have not yet been investigated. Besides, for the development of new antifouling alternatives, current studies seek that the antifouling effects on bacterial biofilm are without affecting bacterial growth (Neves et al. 2024) but rather have an inhibitory action on quorum sensing (QS), a genetic signaling process that regulates bacterial establishment (Martín-Rodríguez et al. 2015), thus reducing bacterial resistance (Agostini et al. 2019) and making it a better alternative to inhibit biofilm.

Thereby, to assist in the development of a freshwater antifouling alternative, the present study evaluated the potential of macrophyte extracts to inhibit the formation of bacterial biofilms (microfouling) and golden mussel (*L. fortunei*) attachment (macrofouling). To assess the toxicity of macrophyte extracts more accurately, three different trophic levels were used (Pane et al. 2008): a microalga (*Pseudopediastrum*

boryanum), a crustacean (*Daphnia magna*), and a fish (*Pimephales promelas*). These species were used because they are bioindicators and have well-established toxicity tests standardized by international organizations and agencies, such as the Organization for Economic Co-operation and Development (OECD), the United States Environmental Protection Agency (USEPA), and the Brazilian Association of Technical Standards (ABNT). These tests are useful because they help to establish limits of chemical substances in the aquatic environment (Cetesb 2008), as well as evaluate new chemicals.

Materials and methods

Preparation of plant extracts

Eleven macrophytes were collected in southern Brazil (32°09′23.3″ S 52°05′57.6″ W) during the austral winter of 2020 and summer of 2021. Species identification was performed through morphological analysis of reproductive and vegetative structures using identification keys (Pott and Pott 2000; Souza and Lorenzi 2012). The preparation of plant extracts followed Morales et al. (2024). In total, 25 aqueous extracts were obtained from different organs of the macrophytes (Table 1). The separation into different plant organs of the plant occurred due to the chemical compounds being distributed qualitatively and quantitatively according to the plant organ (Cardoso et al. 2019; Ramos et al. 2022). Materials collected during both summer and winter were combined to ensure representative samples and reduce seasonal variability of the chemical compounds present in the extracts (Ramos et al. 2022). A 6 g sample of dry plant biomass (oven-dried at 60 °C until constant mass) was added to 300 mL of sterile (filtered −0.22 µm and autoclaved) natural water without salinity (0 salinity) (Agostini et al. 2019). The preparation resulted in a stock solution, considered 100%, that was diluted to 5, 10, 20, and 40%. The control treatment was sterile natural water (0% and 0 salinity).

Bacterial isolates

Bacterial isolates were obtained according to Morales et al. (2024), from biofilm-forming bacteria on acrylic, marine plywood, carbon steel (ASTM-36), and concrete substrates.

Table 1 List of plant extracts tested in bioassays

Group	Family	Species and author	Plant organ
Angiosperms	Cabombaceae	*Cabomba caroliniana* A. Gray	Leaf Stalk
Angiosperms	Cyperaceae	*Schoenoplectus californicus* (C. A. Mey.) Soják	Stalk Influorescence
Angiosperms	Menyanthaceae	*Nymphoides humboldtiana* (Kunth) Kuntze	Leaf Stalk Flower
Angiosperms	Onagraceae	*Ludwigia hexapetala* (Hook. & Arn.) Zardini et al	Leaf Stalk
Angiosperms	Onagraceae	*Ludwigia multinervia* (Hook. & Arn.) Ramamoorthy	Leaf Stalk
Angiosperms	Pontederiaceae	*Eichhornia azurea* (Sw.) Kunth	Leaf Stalk
Angiosperms	Pontederiaceae	*Eichhornia crassipes* (Mart.) Solms	Leaf Stalk Root Flower
Angiosperms	Potamogetonaceae	*Stuckenia pectinata* (L.) Börner	Mixed
Ferns and lycophytes	Salvinaceae	*Salvinia minima* Baker	Leaf Rhizoid
Ferns and lycophytes	Salvinaceae	*Salvinia herzogii* De la Sota	Leaf Rhizoid
Angiosperms	Typhaceae	*Typha domingensis* Pers	Aerial part upper Aerial part lower Inflorescence

The water samples were collected from a lagoon (32°04′56.9″ S 52°14′05.2″ W) in August 2021, when the temperature was 18 °C, and salinity zero. All isolates were screened to confirm their ability to form a biofilm (O'Toole 2011). Acquisition and identification of the bacterial isolates by sequencing were performed as described in Morales et al. (2024). Sequences were submitted to the Genbank (accession number SUB 13564963 (OR000433-OR000442)). Isolates were added to the *Microfouling Bank* of the Laboratory of Organic Microcontaminants and Aquatic Ecotoxicology—CONECO, Institute of Oceanography – IO at the Federal University of Rio Grande (FURG).

Isolated bacteria were used to carry out the biofilm inhibition and eradication assays, as well as the potential growth inhibition of planktonic bacteria. Taxonomic information of all bacterial isolates can be seen in Supplementary Table S1.

Bacterial community

Water samples were collected from a lagoon (32°09′44.6″ S 52°06′04.4″ W) at a temperature of 18 °C to obtain the multispecies bacterial community inoculate for antibiofilm assays. Water samples were filtered (7 µm) to remove phytoplankton and zooplankton organisms (Agostini et al. 2021a).

Bacterial density assays

Each bacterial isolate and multispecies bacterial community sample was used separately to perform bacterial density assays (Fig. 1). These were carried out in 96 acrylic multi-well plates (Citotest Labware Manufacturing Co. Ltd, Jiangsu, China) with eight replicates per treatment. A suspension of bacterial inoculum in nutrient broth (K25-1216, Laboratories Conda S.A., Madrid, Spain) with an initial density of 10^6 bacteria mL^{-1} was used (Agostini et al. 2019). The nutrient broth was chosen to avoid misinterpretation and not to mask the results, eliminating a confounding factor from the results.

To test the inhibition of biofilm formation (IBF), an initial screening assay containing 100 µL of plant extracts at increasing dilutions (0, 5, 10, 20, and 40%) was mixed with the bacterial inoculum and incubated in the dark at 25 °C for 48 h (Agostini et al. 2019). Measurements of the biofilm density for the IBF were performed by removing the supernatant, drying the biofilm (60 °C for 1 h), staining

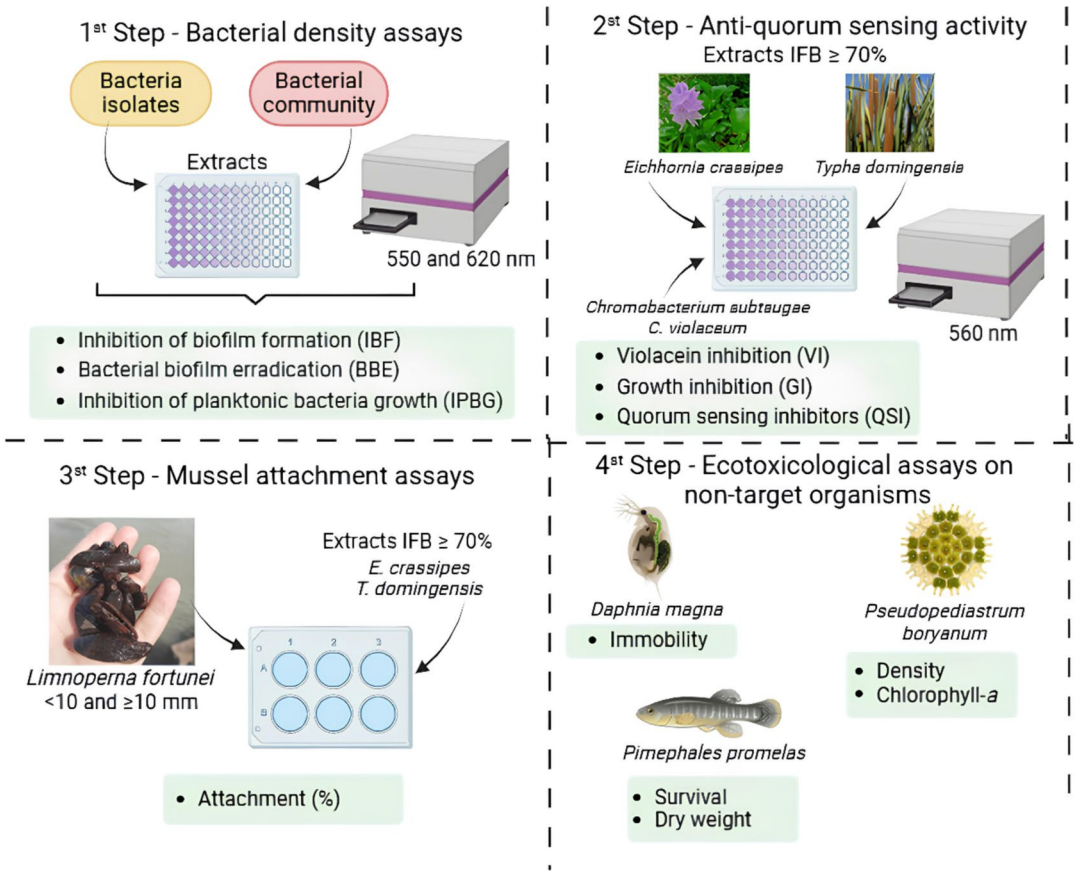

Fig. 1 Scheme of procedures adopted to evaluate the antifouling effect of aqueous extracts of macrophytes

with violet crystal (0.4%) for 15 min, discarding the violet crystal solution and rinsing the wells three times with sterile Milli-Q water, and solubilizing with ethanol (Abs.) for 30 min. The optical density was measured on a microplate reader (Filtermax F5, Molecular Devices, San Jose, USA) at 550 nm (O'Toole 2011; Agostini et al. 2019).

Plant extracts producing IBF ≥ 60% were then used to carry out the bacterial biofilm eradication (BBE) and inhibition of planktonic bacteria growth (IPBG) assays. For the BBE assays, biofilms were established from 200 µL of bacterial inoculum incubated in the dark for 24 h. Subsequently, the biofilm was exposed to 200 µL of the test solutions (0, 5, 10, 20, and 40%) for 48 h in the dark at 25 °C (Morales et al. 2024). Biofilm density for BBE was analyzed in the same way as for IPBG.

The IPBG assay was performed to ascertain whether the biofilm inhibition activity was due to antibiofilm (no effect on growth and single effect on the biofilm) or antibiotic activity (growth reduction) (Agostini et al. 2019). For the IPBG assay, the growth inhibition of planktonic bacteria was used as the endpoint, thus estimating the difference in optical density (620 nm) at the beginning and end of the incubation period (48 h in the dark at 25 °C) (Agostini et al. 2019; Do Vale et al. 2021). Controls for the IBF, BBE, or IPBG assays were considered 100%. Results were, therefore, expressed as a percentage of inhibition (IBF or IPBG) or eradication (BBE) compared to the control.

Anti-quorum sensing activity: violacein inhibition

To confirm the antibiofilm effect of the extracts, the violacein inhibition assay was used to quantify the quorum sensing inhibition (QSI) activities (Fig. 1) of the same aqueous macrophyte extracts used in the toxicological assays (*E. crassipes* and *T. domingensis*) (described in the "Ecotoxicological assays on non-target organisms" section), against two Gram-negative biosensor microorganisms: *Chromobacterium subtsugae* CV017 to detect short chain (C4-C6) acyl-homoserine lactone (AHL) inhibition (Chernin et al. 1998) and *C. violaceum* ATCC 12472 to detect long chain (C10 +) AHL inhibition (Morohoshi et al. 2008). Extracts were resuspended in sterile, deionized water to a stock of 20 mg.mL^{-1}, considered 100%. One hundred microliters of an overnight *C. subtsugae* CV017 and *C. violaceum* ATCC 12472 culture, grown in 5 mL Luria–Bertani (LB) broth, was used to inoculate test tubes containing 3 mL LB broth, which were exposed to varying dilutions (0.07, 0.15, 0.30, 0.60, 1.20%) of respective extracts and incubated at 30 °C for 18 h with agitation (150 rpm) in a rotary suspension mixer (SM-3600–0018, Lab YIHDER Technology CO, Taiwan) (Chenia 2013). These dilutions were used because they presented antibiofilm and anti-attachment effects in the previous stages and did not present a toxicological effect on non-target organisms. The growth control was LB broth inoculated with only *C. subtsugae* CV017 and *C. violaceum* ATCC 12472 culture (no extract). Vanillin (Sigma-Aldrich) was used as QSI-positive control and tested at same dilution range as extracts.

Following incubation, culture growth readings were obtained with a Glomax Multi + Detection System microtiter plate reader at OD_{600} nm (Promega). One mL of the cultures was subjected to centrifugation at 13,000 rpm for 10 min (Labnet Prism microcentrifuge), thus precipitating the insoluble violacein. Culture supernatants were discarded, and pellets were resuspended in 1 mL of dimethyl sulfoxide (DMSO) (Chenia 2013). Following centrifugation at 13,000 rpm for 10 min, cells were pelleted, and the violacein-containing solutions were quantified at OD_{560} nm using the Glomax Multi + Detection System microtiter plate reader (Chenia 2013). Violacein inhibition was assessed in triplicate on two separate occasions. The percentage of violacein inhibition was calculated according to Chenia (2013). If extracts demonstrated violacein inhibition (%VI) and growth inhibition (%GI) of ≥ 50% and < 40%, respectively, they were considered good quorum sensing (QS) inhibitors. However, a %VI and %GI of ≥ 50% and ≥ 40%, respectively, were considered bactericidal activity rather than QSI (Rambaran et al. 2024).

Mussel attachment assays

The extracts that showed an inhibitory effect ≥ 70% in the IBF (single and multispecies bacteria) assays were used to evaluate the ability to inhibit golden mussel attachment (Fig. 1). Specimens of *L. fortunei* were collected from the channel walls of the first elevation of the Rio Grande Sanitation Company (CORSAN) (32°3′14.39″ S 52°22′18.28″ W) and transported in plastic containers without water to the laboratory. In the laboratory, the mussels were separated with scissors, cleaned with dechlorinated water, and transferred to an 80 L black plastic tank containing dechlorinated water circulating through a biological filter at a controlled temperature of 20 °C and photoperiod 12L:12D. Mussels were fed twice a day with a commercial concentrate of *Chlorella vulgaris* (Beyerinck (Beijerinck), 1890) (ChloFresh, Algasul, Rio Grande, Brazil) at 10^9 cells mL^{-1}. Mussels were kept acclimatized under these conditions for 2 weeks.

Two separate assays were performed according to mussel size: < 10 mm and ≥ 10 mm (Cataldo et al. 2005). Mussels were transferred from the maintenance tank to a transparent plastic container (5 L) to verify their substrate exploration behavior (Longo et al. 2021). Only individuals showing such behavior were selected for the attachment assays. Treatments were composed of 0, 5, 10, 20, and 40% of the test solution of each selected plant extract, with 30 individuals per

treatment. Assays were carried out in 6-well plates (Barloworld Scientific Ltd., Stone, UK), each well containing one mussel and 10 mL of the test solution (Longo et al. 2021). Exposure time was 72 h at 20 °C in the dark (Longo et al. 2021). At the end of the exposure, mussels adhered to the well walls were counted, and results were expressed as a percentage of attachment.

Ecotoxicological assays on non-target organisms

As all the different organs of the plants were successful in inhibiting the attachment of the golden mussel, mixtures of parts of *E. crassipes* and *T. domingensis* were used for ecotoxicological assays (Fig. 1). Extracts were freeze-dried and then used to prepare solutions with dilutions of 0, 6.25, 12.50, 25, 50, and 100% of each extract. Three freshwater non-target model organisms were selected: the microalga *Pseudopediastrum boryanum* [(Turpin) E.Hegewald 2005] (Chlorophyta, Hydrodictyaceae), the crustacean *Daphnia magna* (Straus, 1820) (Arthropoda, Daphniidae), and the fish *Pimephales promelas* (Chordata, Cyprinidae) (Rafinesque, 1820).

Microalgae assay: density and chlorophyll-a

The assay with *P. boryanum* was done following OECD guidelines 201 (2011), with some modifications, such as the use of a WC culture medium (Guillard and Lorenzen 1972) to enrich the test solutions. The assay was carried out in 50 mL Erlenmeyer flasks containing 49 mL of the test solution and 1 mL of microalgae inoculum at a density of 10^4 cells mL^{-1} at 24 °C, continuous lighting of 7000 lx, and constant agitation on a shaking table. The duration of the test was 72 h (±2 h), with an initial pH of 6.6–7.0. Each treatment had four replicates. At the beginning of the test (0 h) and at the end (72 h), an aliquot of 1 mL was taken, and 200 µL of formaldehyde was added to fix the algal culture (final concentration 0.4%). The endpoint analyzed was cell concentration (cells mL^{-1}) for each treatment, estimated as the difference in cell count between 72 and 0 h with a Neubauer counting chamber. The control treatment used uninoculated WC culture medium.

After 72 h, 5 mL aliquots were removed and centrifuged (4000 rpm) for 10 min. The supernatant was discarded, and 3 mL of methanol (99.9%) was added. The samples were kept under refrigeration (5 °C) for 12 h in the dark. The samples were then centrifuged (4000 rpm), and the optical density of the supernatant was determined using a UV–VIS spectrophotometer (UV mini-1240, Shimadzu, Kyoto, Japan) at 663 and 750 nm. Chlorophyll-*a* (µg L^{-1}) extraction and concentration estimation were performed according to Mackinney (1941).

Cladoceran assay

The acute toxicity test with *D. magna* was carried out following ABNT NBR 12713 (2022) guidelines in 6-well plates (Barloworld Scientific Ltd., Stone, UK), with four replicates, each containing five organisms and 10 mL of the test solution (test solution ratio of 2 mL $organism^{-1}$). Plates were incubated for 48 h (±1 h) at a temperature of 20 °C (±2 °C) and a photoperiod of 12 h:12 h. The organisms were not fed during the assay. At the end of the exposure time, the effect of the extracts on the mobility of the organisms was observed, and results were expressed as immobility organisms (%) per treatment.

Fish assay: survival and dry weight

From 0 to 24 h after hatching, *P. promelas* larvae were subjected to a 7-day long static test with daily water renewal. Survival and larval dry weight were determined at the end of the test according to EPA—821 R—02—013—Method 1000.0 (2002). Each treatment had 4 replicates with 10 larvae and 250 mL of test solution placed within 500 mL containers. These were incubated at 25 °C (±1 °C), with a photoperiod of 16 h:8 h, and a light incidence of 500–1000 lx. Larvae were fed with *Artemia nauplii* at a concentration of 700 to 1000 ind mL^{-1}, three times a day. Larval survival was estimated daily. At the end of the exposure time, the dry weight of larvae was determined by drying in an oven (60 °C) for 24 h.

Statistical analysis

For toxicological responses, the values of the lowest concentration (or dilution) with observed effect (LOEC), the highest concentration (or dilution) at which no effect was observed (NOEC), and the safe concentration (or dilution) of extracts through the arithmetic mean between LOEC and NOEC were also calculated (Zagatto and Bertoletti 2008). *T*-Student tests were performed, using Bonferroni correction for multiple comparisons, to observe potential differences between treatments and respective controls in BFI, BBE, and PBGI assays for single and multispecies bacteria. One-way ANOVA was run to verify significant differences between treatments in the assays with *P. boryanum*, *P. promelas* (dry weight), and violacein inhibition (both *Chromobacterium* species biosensors). In all cases, the assumptions of normal distribution of residues (Shapiro–Wilk test) and homoscedasticity (Levene test) were verified. When alternative hypotheses with a confidence level of 95% were accepted, the Tukey post-hoc test was run. Survival of *D. magna* and *P. promelas* and mussel attachment data were analyzed with Generalized Linear Models (GLM) with binomial distribution with logit link function. Statistical analysis was conducted with the use of GraphPad Prism 8.4 (GraphPad, USA).

Results

Identification of bacterial isolates

The isolation of bacteria resulted in the identification of nine taxa (Supplementary Table S1). For each of the four substrates tested (concrete, marine plywood, acrylic, and ASTM-36 carbon steel), three species were identified (Supplementary Table S1), with *Pseudomonas* species being isolated from all the substrates. Each substrate displayed two other unique species of biofilm-forming bacteria.

Bacterial density assays

Bacterial isolates

Of the 25 different extracts initially tested, nine showed IBF ≥ 60% for at least one of the tested bacteria ($p < 0.05$) (Fig. 2): flower, leaf, stalk, and root of *Eichhornia crassipes*; leaf of *Salvinia herzogii*; inflorescence of *Schoenoplectus californicus*; stalk of *Nymphoides humboldtiana*; and inflorescence and the upper aerial part of *Typha domingensis*. Extracts from *E. crassipes* (Fig. 2A–D) and

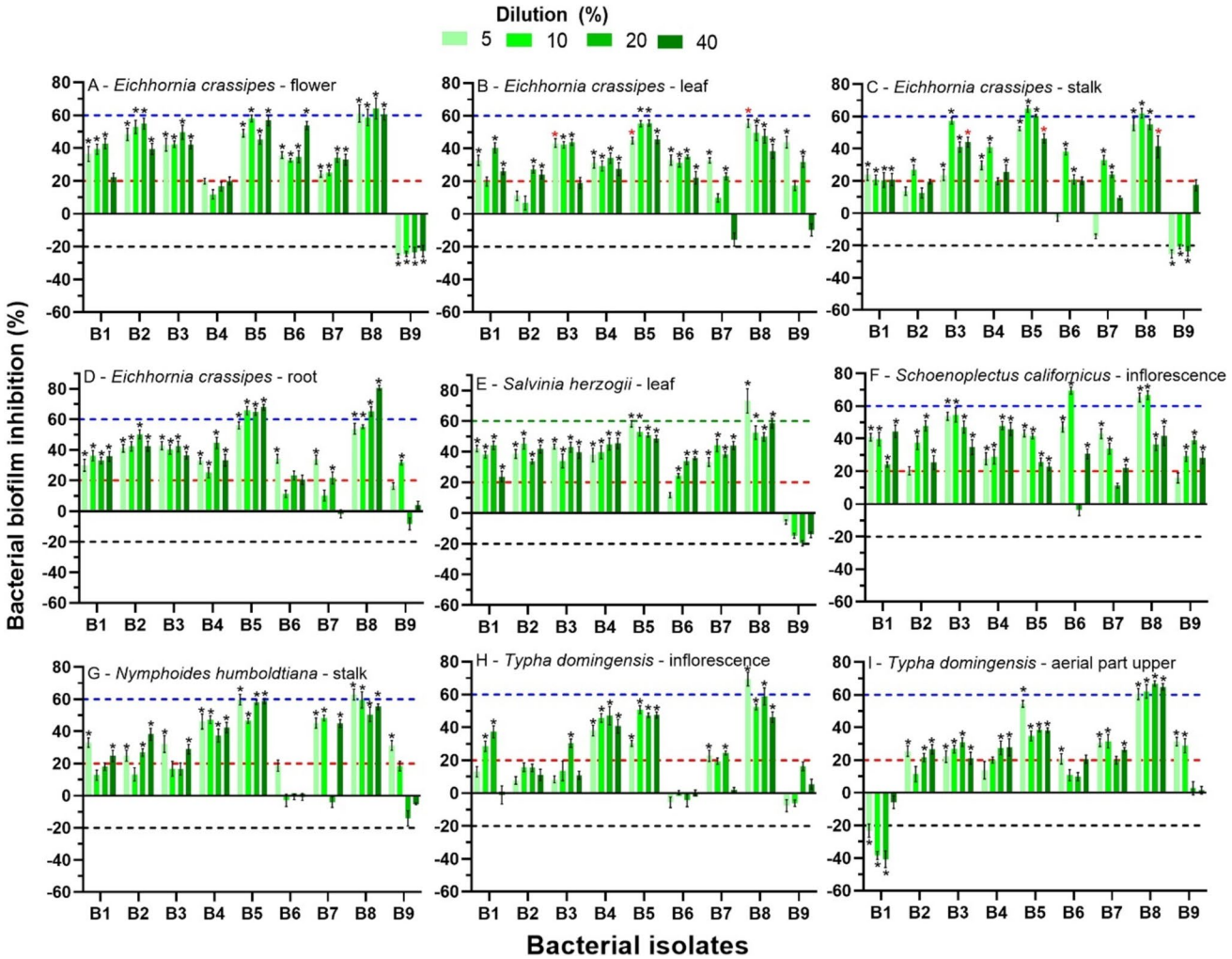

Fig. 2 Mean (±SD) inhibition of biofilm formation (IBF; %) of nine biofilm-forming bacterial isolates (B1 = *Pseudomonas* sp.; B2 = *Psychrobacillus psychrodurans*; B3 = *Citricoccus nitrophenolicus*; B4 = *Pseudomonas putida*; B5 = *Paeniglutamicibacter kerguelensis*; B6 = *Acinetobacter haemolyticus*; B7 = *Bacillus vietnamensis*; B8 = *Acinetobacter bohemicus*; B9 = *Pseudomonas rhodesiae*) following exposure to nine (**A**–**I**) selected extracts of aquatic macrophytes. *black asterisk denotes significant differences compared to the control group ($p < 0.05$). *red asterisk indicates the results of biofilm eradication assays ($p < 0.05$), combined in the same figure e. Red line = inhibition of biofilm formation; black line = biofilm formation induction activity; blue line = inhibition of biofilm formation ≥ 60% (inhibition in all cases is read as positive values)

T. domingensis (Fig. 2H, I) were the most effective, resulting in IBF > 70% for at least one dilution for each of the nine bacterial isolates ($p < 0.05$) (Fig. 2). In terms of IBF, the bacterium *Acinetobacter bohemicus* showed greater inhibition by 88.89% (8/9) of plant extracts (IBF > 60%) at least one dilution ($p < 0.05$). All data regarding biofilm inhibitory effects of extracts are summarized in Supplementary Table S2.

For the BBE assay, only extracts of leaves (5%) and stalks (40%) of *E. crassipes* (Fig. 2B, C) had an eradication effect ($p < 0.05$). For both extracts, this effect was observed only against *Citricoccus nitrophenolicus*, *Paeniglutamicibacter kerguelensis*, and *A. bohemicus*. Six of the selected nine extracts demonstrated a bactericidal effect against at least one bacterial species in some dilution ($p < 0.05$). An antibiofilm effect ($p > 0.05$) was also observed for some dilutions of *E. crassipes*, *N. humboldtiana*, and *T. domingensis* (Fig. 3). However, for most bacteria and dilutions tested, extracts had a growth-inducing effect (Fig. 3; $p > 0.05$).

Bacterial community

Biofilm formation by the multispecies bacterial community was inhibited (≥ 60%; $p < 0.05$) by nine extracts: flower, stalk, and roots of *E. crassipes*; leaves of *S. herzogii*; inflorescence of *S. californicus*; flower and stalk *N. humboldtiana*; and inflorescence and upper aerial part of *T. domingensis* (Fig. 4A). Two extracts were highly effective (inhibition > 70%), i.e., the roots of *E. crassipes* at the 40% dilution and the upper aerial part of *T. domingensis* at 20% dilution ($p < 0.05$) (Fig. 4A).

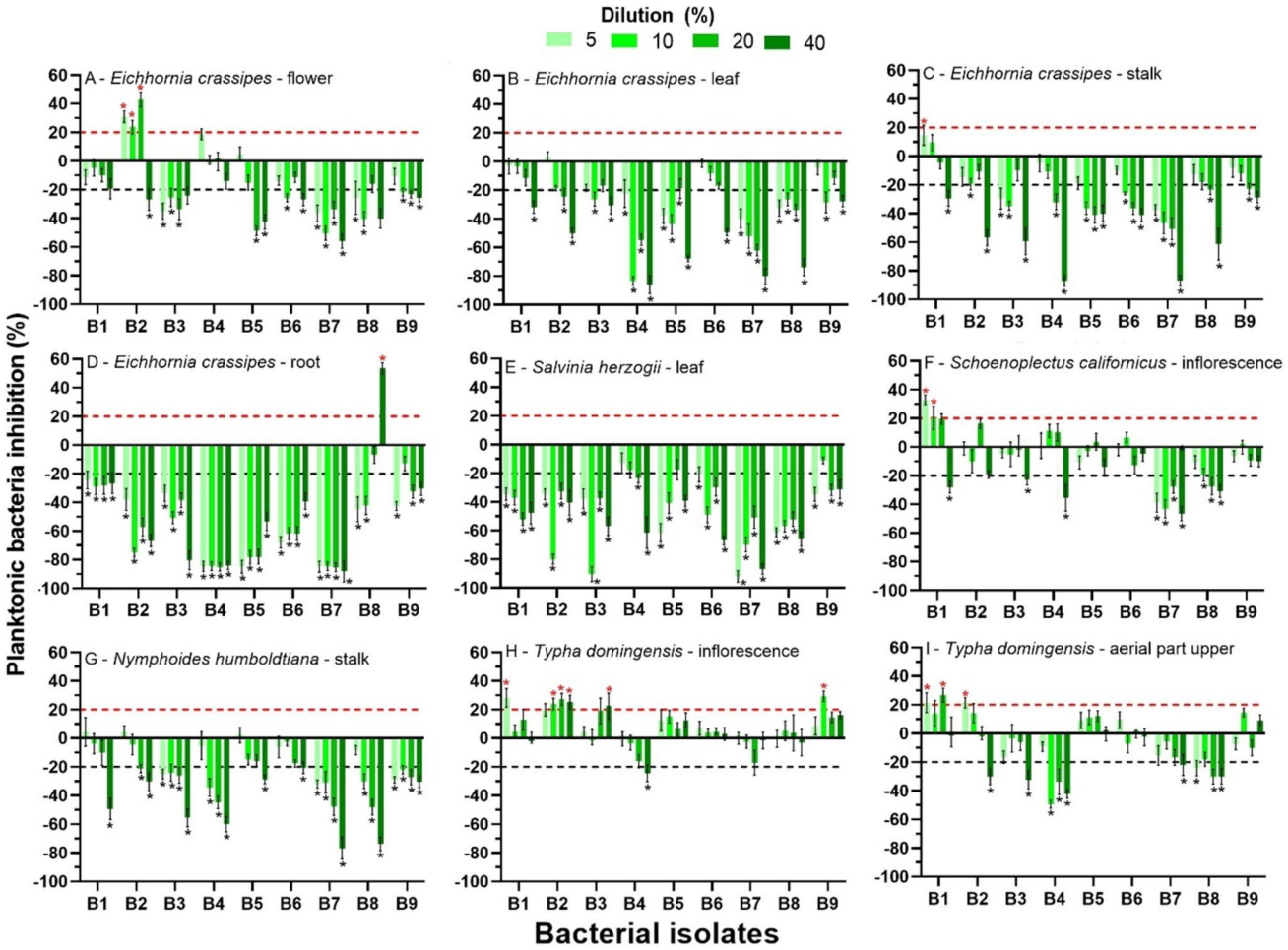

Fig. 3 Mean (±SD) response (% planktonic bacteria inhibition) of nine bacterial isolates (B1 = *Pseudomonas* sp.; B2 = *Psychrobacillus psychrodurans*; B3 = *Citricoccus nitrophenolicus*; B4 = *Pseudomonas putida*; B5 = *Paeniglutamicibacter kerguelensis*; B6 = *Acinetobacter haemolyticus*; B7 = *Bacillus vietnamensis*; B8 = *Acinetobacter bohemicus*; B9 = *Pseudomonas rhodesiae*) to the exposure to nine (**A–I**) selected extracts of aquatic macrophytes. Asterisk (*) = presents statistical difference when compared to the control group; red asterisk (*) = antibiotic effect; black asterisk (*) = inducing planktonic growth. Dilutions that showed no difference when compared to the control group = antibiofilm activity. Red line = antibiotic effect (reduced planktonic growth); black line = effect of inducing planktonic growth

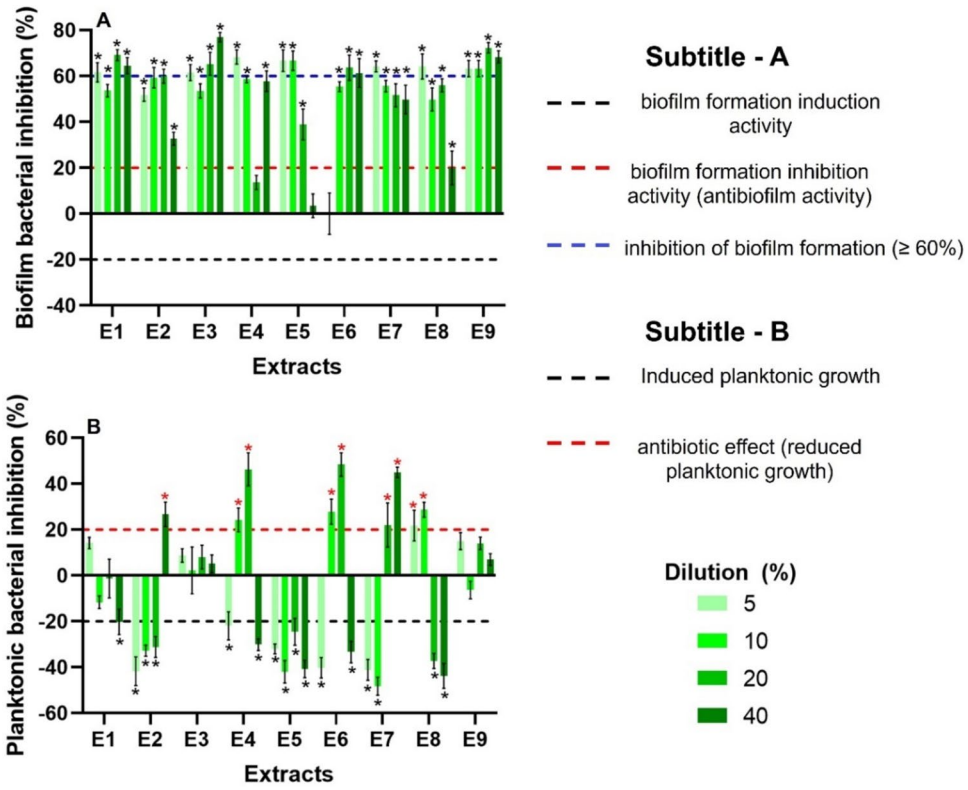

Fig. 4 Mean (± SD) response biofilm bacterial inhibition (%) (**A**) and planktonic bacterial inhibition (%) (**B**) of the bacterial community. E1 = *Eichhornia crassipes* flower; E2 = *E. crassipes* stalk; E3 = *E. crassipes* root; E4 = *Salvinia herzogii* leaf; E5 = *Schoenoplectus californicus* influorescence; E6 = *Nymphoides humboldtiana* flower; E7 = *N. humboldtiana* stalk; E8 = *Typha domingensis* influorescence; E9 = *T. domingensis* aerial part upper. Asterisk (*) = presents statistical difference when compared to the control group; red asterisk (*) = antibiotic effect

As for the planktonic growth assays, only the extracts of flowers and roots of *E. crassipes* and *T. domingensis* upper aerial part demonstrated an antibiofilm effect ($p > 0.05$) on planktonic growth (Fig. 4B). The remaining extracts varied in their response depending on their dilution in terms of induction ($p < 0.05$) or inhibition ($p < 0.05$) of planktonic growth (Fig. 4B). None of the extracts had a biofilm eradication effect on the multispecies bacterial community (Fig. 4B). None of the extracts that present satisfactory results with bacterial isolates (*E. crassipes* and *T. domingensis*) demonstrated the same ability to eradicate biofilms of the multispecies bacterial community (Fig. 4B).

Anti-quorum sensing activity

For short-chain AHL-producing *C. subtsugae* 017, QSI was observed with the two extracts tested, i.e., *E. crassipes* extract demonstrated 50–67.30% VI from 0.15 to 1.20%, while the *T. domingensis* extract demonstrated a 66.70% VI at 1.20% only (Fig. 5). The QSI positive control vanillin also demonstrated 67.82% VI at 1.20%. Extracts significantly influenced the %VI of *C. subtsugae* 017 in a dose-dependent manner ($p < 0.05$).

For the long-chain AHL-producing *C. violaceum* ATCC 12472, a 57.29% VI was obtained with the *T. domingensis* extract at 1.20%. For *E. crassipes*, no note-worthy QSI. The vanillin-positive control had a 78.30% VI at 0.60% but was bactericidal at 1.20%. Although only *T. domingensis* reached the target 50% VI, a dose-dependent relationship was also observed between extracts and *C. violaceum* ATCC 12472 (Fig. 5). All data regarding QSI and %VI are summarized in Supplementary Table S3.

Mussel attachment assays

All six extracts tested inhibited the attachment of golden mussels (Fig. 6). The proportion of successfully attached mussels (average) varied more in relation to the different dilutions ($p < 0.05$) than due to the size of the mussels ($p > 0.05$). The extract from the inflorescence of *T. domingensis* was the most effective in reducing mussel attachment (Fig. 6), with an ≥ 80% reduction in attachment ($p < 0.05$).

Ecotoxicological assays

Regarding the toxicity of *T. domingensis* extract, the 50 and 100% dilution had a significant negative effect on cell

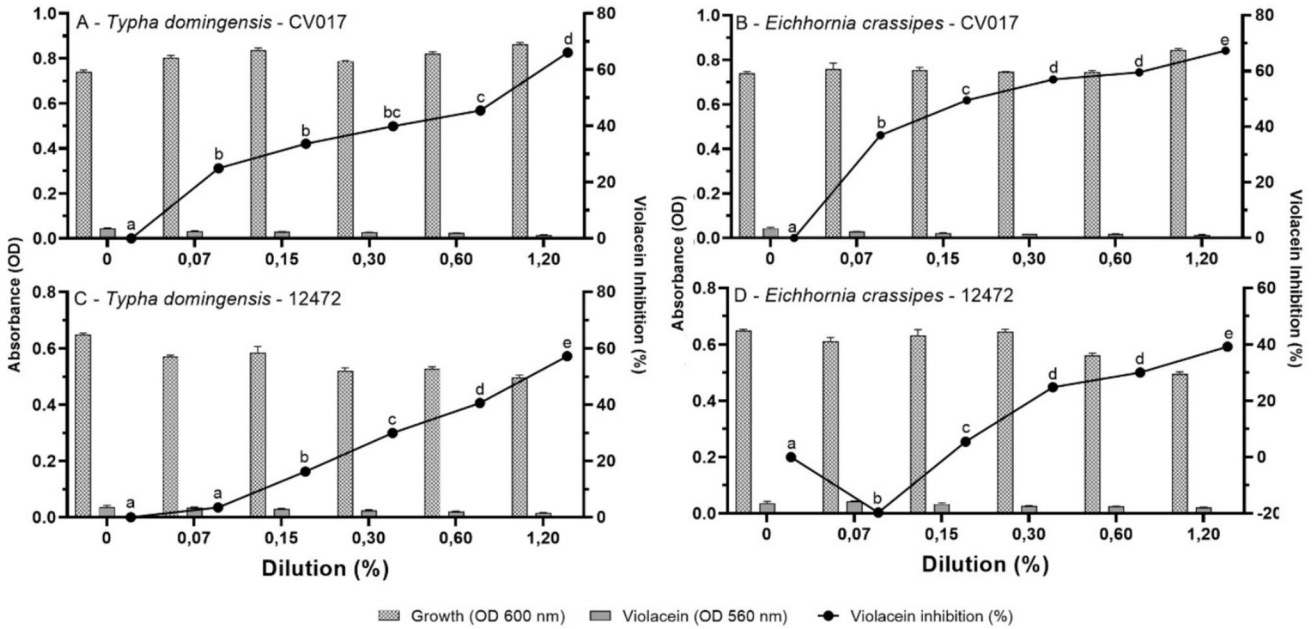

Fig. 5 Quantitative analysis of the dilution inhibitory effects (Mean (± SD)) of aqueous extracts *Typha domingensis* (**A** and **D**) and *Eichhornia crassipes* (**B** and **C**), on growth and violacein production by two biosensors *Chromobacterium subtsugae* CV017 (short chain) and *Chromobacterium violaceum* ATCC 12472 (long chain). Different letters of violacein inhibition = denote significantly different means ($p < 0.05$) between dilutions in each extract

density and chlorophyll-*a* content of *P. boryanum* ($p < 0.05$) and on the mobility of *D. magna* ($p < 0.05$) (Table 2). The survival and weight of *P. promelas* (Table 2) were only negatively affected at 100% dilution (undiluted extract; $p < 0.05$). A similar response pattern was observed for the *E. crassipes* extract in relation to the three species (Table 2). The 50 and 100% dilution had a significant negative effect on the cell density of *P. boryanum* ($p < 0.05$) and the mobility of *D. magna* ($p < 0.05$). For *P. boryanum* chlorophyll-*a*, only the 100% dilution showed negative effects ($p < 0.05$). The survival and weight of *P. promelas* (Table 2) were also only negatively affected at 100% dilution ($p < 0.05$).

For the *T. domingensis* extract, the NOEC for *D. magna* and *P. boryanum* was 25%, LOEC 50%, and a safe dilution of 35.35% was estimated for these organisms, while for *P. promelas*, the NOEC was 50%, LOEC 100%, with a safe dilution of 70.71% (Table 3). For the *E. crassipes* extract toxicity, the results were similar (Table 3). All data regarding *T. domingensis* and *E. crassipes* extracts are presented in Tables 2 and 3 and Supplementary Fig. S1.

Discussion

Several studies indicate that the eleven species of aquatic macrophytes species tested in the present study had a negative effect on the growth of algae, plants, bacteria, and bacterial biofilm (Takao et al. 2011; Chicalote-Castillo et al. 2017; Jiménez 2020; Morales et al. 2024). The results of the present study demonstrated that only extracts of five species (*E. crassipes*, *S. herzogii*, *S. californicus*, *N. humboldtiana*, and *T. domingensis*) were considered effective (> 60%) in inhibiting freshwater bacterial biofilm. The *E. crassipes* and *T. domingensis* extracts resulted in bacterial biofilm inhibition of > 70%. It is thus apparent that the inhibition potential of allelochemicals may vary depending on macrophyte species and organs, an effect also observed by Cardoso et al. (2019) and Ramos et al. (2022).

The antibiofilm potential of the macrophyte extracts was assessed against selected bacterial strains as well as naturally occurring multispecies bacterial communities. Most of the strains tested are from the phylum Proteobacteria, specifically the Gamma-Proteobacteria class. Proteobacteria are recognized for their high phylogenetic diversity and phenotypic versatility, which enables the colonization of different habitats (Kersters et al. 2006; Zinger et al. 2011). This phylum is reported to be abundant in natural waters, with many representatives that are able to grow in substrates such as lignin, calcarenites, acrylics, marine plywood, ASTM-36 cable carbon steel and sediments from aquatic environments (Agostini et al. 2021a; Gusmão et al. 2023; Morales et al. 2024), and can be isolated from biofilms on ship hulls and rocky substrates (Muthusamy et al. 2017; Bergo et al. 2021; Ferreira et al. 2022). This confirms that the species used in the present study are representative of bacterial biofilms found on different substrates in the aquatic environment.

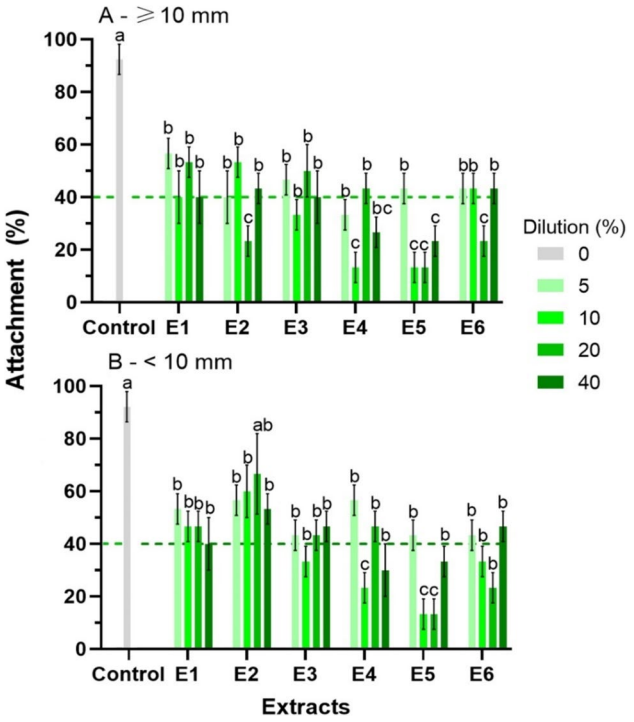

Fig. 6 Mean (± SD) attachment (%) of the golden mussel *Limnoperna fortunei* after 72 h exposure to extracts of different aquatic macrophytes (E1 = *Eichhornia crassipes* flower; E2 = *E. crassipes* leaf; E3 = *E. crassipes* stalk; E4 = *E. crassipes* root; E5 = *Typha domingensis* inflorescence; and E6 = *T. domingensis* upper aerial part) at varying dilutions (0 to 40%): **A** Assays with individuals ≥ 10 mm. **B** Assays with individuals < 10 mm. Percent in green = percentage inhibition of attachment ≥ 60% when compared to the control. Different superscript letters denote significant differences ($p < 0.05$)

Table 2 Summary of ecotoxicity results following exposure to aquatic macrophyte extracts

Extract/ Dilution (%)	*Pseudopediastrum boryanum*		*Daphnia magna*		*Pimephales promelas*
	Density	**Chlorophyll-*a***	**Immobility**	**Survival**	**Weight**
Typha domingensis					
6.25	No	No	No	No	No
12.50	No	No	No	No	No
25	No	No	No	No	No
50	Yes	Yes	Yes	No	No
100	Yes	Yes	Yes	Yes	Yes
Eichhornia crassipes					
6.25	No	No	No	No	No
12.50	No	No	No	No	No
25	No	No	No	No	No
50	Yes	Yes	Yes	No	No
100	Yes	Yes	Yes	Yes	Yes

Yes = showed toxic effect; No = showed no toxic effect. Dilutions here are read as dilutions from the 100% original extracts

The Gram-negative bacterium *A. bohemicus* was inhibited by most of the macrophyte extracts, unlike the Gram-positive bacteria *Psychrobacillus psychrodurans*, *Bacillus vietnamensis,* and *C. nitrophenolicus* which had little inhibition. Due to their external lipopolysaccharide membrane, Gram-negative bacteria have greater selectivity and consequently less sensitivity to external factors, such as exposure to natural extracts (Awolola et al. 2014; Seibert et al. 2019). In the present study, however, *A. bohemicus* did not present the expected systematic response according to its cell wall Gram reaction. The biofilm inhibition strategies are multifactorial and may be related to other mechanisms not necessarily related to cellular permeability, such as the inhibition of quorum sensing (QS) between biofilm-forming bacteria, surface modulation of bacterial adhesion, or degradation of the biofilm matrix (Srinivasan et al. 2021; Rambaran et al. 2024).

Biofilms are structured as complex colonies of microorganisms enveloped in an extracellular polymeric matrix (EPM) that provides protection against external chemical agents (Agostini et al. 2018). Along with EPM, there is an increase in the detection, production, and release of signaling molecules that help regulate biofilm formation (QS process) (Chattopadhyay et al. 2022). The QS process coordinates population behavior and regulates gene expression through bacterial cell-to-cell communication (Xiao et al. 2022). Thus, QS-inhibiting phytochemicals arouse considerable interest among researchers (Martínez et al. 2019; Mulat et al. 2019; Rambaran et al. 2024).

Based on the results of eradication, planktonic growth, and QS inhibition assays, some hypotheses can be raised and will be discussed later throughout the text. For the planktonic growth inhibition assay, three types of response were observed: antibiotic (growth reduction), inductive (growth induction), and antibiofilm (no effect on growth and single effect on the biofilm). The inductive effect was observed for all extracts tested at least one of the dilutions (5, 10, 20, and 40%) for the bacterial species and bacterial community. However, the *T. domingensis* extracts resulted in a lower growth induction when compared to the other extracts, showing a greater antibiofilm effect. The induction of bacterial growth may have occurred due to the chemical composition of the extracts and their respective dilutions, where some treatments may have more compounds to induce growth than to inhibit it. These differential effects between inhibition and induction are already reported and explained by the specificity of the chemical composition of each macrophyte species (Santonja et al. 2018). Further, they can be attributed to differences in strains due to attached bacterial biofilm, cell wall properties, and induced oxidative stress (Mulderij et al. 2005).

The extracts from macrophytes were not as effective in the biofilm eradication assays as they were at inhibiting biofilm formation. Eradication rates ≥ 60% were only

Table 3 Ecotoxicological responses to *Typha domingensis* and *Eichhornia crassipes* extracts

Macrophyte extract	Test-organism	Observed-response	LOEC (%)	NOEC (%)	Mean ± SD
Typha domingensis	*P. boryanum*	Density	50	25	35.35 ± 12.5
		Chlrophyll-*a*	50	25	35.35 ± 12.5
	D. magna	Immobility	50	25	35.35 ± 12.5
	P. promelas	Survival	100	50	70.71 ± 25
		Weight	100	50	70.71 ± 25
Eichhornia crassipes	*P. boryanum*	Density	50	25	35.35 ± 12.5
		Chlrophyll-*a*	100	50	70.71 ± 25
	D. magna	Immobility	50	25	35.35 ± 12.5
	P. promelas	Survival	100	50	70.71 ± 25
		Weight	100	50	70.71 ± 25

LOEC = observed effect dilution (lowest dilution at which effects are observed); NOEC = no observed effect dilution (highest dilution at which no effect is observed); mean = safe dilution (geometric mean between OEC and NOEC). Dilutions here are read as dilutions from the 100% original extracts

observed with *E. crassipes* leaf (5% dilution) and stalk (40% dilution) extracts against the bacteria *C. nitrophenolicus*, *Paeniglutamicibacter kerguelensis*, and *A. bohemicus*. When evaluating Caatinga plant extracts, Agostini et al. (2020) and (2019) also found that the rate of inhibition of marine biofilms was much higher than their eradication. This response is not entirely unexpected as biofilm architecture can improve the defense against external chemical agents (Agostini et al. 2018, 2019). Biofilm-forming bacteria can withstand and resist different environmental conditions, such as low nutrient availability and the action of chemical agents such as the compounds present in extracts (Davey and O'toole 2000; Srinivasan et al. 2021; Chattopadhyay et al. 2022).

The low eradication efficacy combined with the non-inhibition of planktonic growth suggests that macrophyte extracts have mechanisms other than toxicity for inhibiting biofilm formation. One possible explanation is that the presence of chemical substances in the extracts may interfere with the QS process. These results were mainly evidenced by the *T. domingensis* extract, as it had a specific inhibitory effect on biofilm formation. This is corroborated by the QSI assay results. The *T. domingensis* extract demonstrated inhibition of both short- and long-chain AHL-producing biosensor strains, suggesting broad-spectrum QSI activity. The *E. crassipes* extract, however, was only effective for short-chain AHL QSI inhibition.

Chemical substances with QSI potential are extremely relevant in the development of new antibiofilm agents, as they reduce the risk of bacterial resistance (Chenia 2013; Borges and Simões 2019). However, other mechanisms may be involved in the biofilm inhibition process, such as the reduction of enzymatic activity. Aqueous and methanolic extracts of the macrophyte *Stuckenia pectinatus* (formerly *Potamogeton pectinatus*) have been reported to reduce the alkaline phosphatase activity of bacterial communities, probably due to enzyme complexation by humic acids (They et al. 2015).

Reproductive organs and leaves are the most investigated plant parts as they usually present the most satisfactory results against biofouling (Agostini et al. 2021b). In the present study, extracts from different plant organs had a significant effect against bacterial biofilm and mussel attachment, especially the *E. crassipes* (flower, leaf, stem, and roots) and *T. domingensis* (inflorescence and upper aerial part) extracts. In fact, there is an extreme consensus in the literature that the quantity and quality of chemical compounds can vary among plants due to biotic and abiotic factors, which vary between species (Reigosa et al. 2013; Ramos et al. 2022). However, in our study, we observed that the plant species under investigation is the predominant factor affecting the composition of allelochemicals. A similar response pattern was reported by Morales et al. (2024), who observed that the antibiofilm effect of aquatic macrophyte extracts on marine bacteria varied more between the species studied than between plant organs of the same species.

The concentration and quality of allelochemicals can also vary according to factors such as herbivory, temperature, precipitation, and seasonality, as well as the spatial and biological form of aquatic plants (They et al. 2015; Álvarez-Martínez et al. 2020; Hamidi et al. 2022; Ramos et al. 2022). In the present study, all biotypes of aquatic macrophytes: submerged, floating, and emerging, were assessed. *E. crassipes* is a floating macrophyte with roots below the water surface (Trindade et al. 2010; Thomaz and Esteves 2011), while *T. domingensis* is an emerging species with roots in the sediment and leaves reaching great heights above the water level (Trindade et al. 2010; Thomaz and Esteves 2011). Floating and submerged aquatic macrophytes are generally more sensitive to water quality than emerging ones (Trindade et al. 2018). Sensitivity may be associated with high growth rates and competition for nutrients and light

(Reynolds 2006; Tang et al. 2017). Results from the present study indicate that the extracts from floating and emerging macrophytes had similar effects against individual bacterial species and multispecies community inhibition. The present results are, therefore, in line with Morales et al. (2024), who observed that submerged and emerging aquatic macrophytes had similar effects on estuarine bacteria.

Macrophytes have a wide range of allelochemicals, most frequently phenolic compounds, alkaloids, terpenoids, and fatty acids, which are regularly reported in the literature as having the potential to inhibit algal growth (Li et al. 2021). Although flavonoids are also reported to have antifouling properties (Agostini et al. 2021b), the chemical composition of the macrophyte extracts was not determined. Nonetheless, there is wide evidence in the literature reporting the presence of allelochemicals in these plants (Shanab et al. 2010; Silva et al. 2010; Patel 2012; Lobo et al. 2013). The main allelochemicals present in *E. crassipes* are alkaloids, phenolic compounds, and terpenoids, which have been used as antimicrobial agents against some pathogenic strains of bacteria, fungi, and algae (Shanab et al. 2010; Patel 2012). The macrophyte *T. domingensis* is known for its antimicrobial and anthelmintic properties due to its high concentration of secondary compounds such as flavonoids, tannins, and phenols (Silva et al. 2010; Lobo et al. 2013).

Although several studies have investigated the effects of plant extracts against macrofouling (Feng et al. 2018; Agostini et al. 2021b, 2022; Pérez et al. 2021), those specifically addressing golden mussel are scarce (Agostini et al. 2021b). This study is the first to evaluate the effect of macrophyte extracts on golden mussel byssal attachment. The *E. crassipes* and *T. domingensis* extracts showed attachment inhibition $\geq 80\%$ for this species in terms of size, smaller or larger than 10 mm, which is in line with other studies on the antifouling effect of plant extracts on macrofouling. Pérez et al. (2021) observed the effect of extracts of *Verbena bonariensis* and *Tillandsia tenuifolia* on macrofouling by the adult mussel *Mytilus edulis* (Linnaeus, 1758), while Feng et al. (2018) reported that 15 alkaloids extracted from terrestrial plants were effective in inhibiting the attachment of the larvae of the barnacle *Fistulobalaus albicostatus* (Pilsbry, 1916) and the larvae of bryozoan *Bugula neritina* (Linnaeus, 1758). It must be emphasized that the macrofouling assay carried out with the golden mussel in the present study identified an anti-attachment response; however, this attachment inhibition may have occurred due to the behavioral toxicity of the extracts or other mechanisms of action. Therefore, in future research, mortality tests should be conducted alongside anti-attachment assays.

Natural compounds, in addition to having an antifouling effect for both micro- and macrofouling, must also have low toxicity for non-target organisms (Pérez et al. 2021). In this study, toxicity tests were carried out using organisms representing three different trophic levels, establishing a broad ecological scenario in the aquatic ecosystem. It was observed that for both extracts, there was a difference in the safe dilutions between trophic levels. For the species at the base of the food chain, *P. boryanum*, there was a decrease in cell density and chlorophyll at 50 and 100% dilutions, with a safe dilution of 35%, and the primary consumer (*D. magna*) followed the same pattern. For the species at the highest trophic level, *P. promelas*, the effect of reducing survival and weight was only at 100% dilution, presenting a safe dilution of 70%.

Furthermore, it was observed that microalgae and Cladocera were more sensitive than fish. This was to be expected, as the size of the organism, as well as the study species, can affect sensitivity to chemical substances (Costa et al. 2008). The toxicity of microalgae may be attributed to the mode of action of the chemical substances, mainly involving the inhibition of photosynthesis, also triggering the inhibition of growth (Silva et al. 2024), as found in the present study. Microalgae are important primary producers in aquatic ecosystems, and effects at this level can compromise not only the survival of microalgae but also that of primary and secondary consumers, triggering disturbances at the community level in the ecosystem (Cedervall et al. 2012).

The toxic effects on the cladoceran *D. magna* are also worrying as they are intermediate organisms in the trophic chain, where while they feed on bacteria and algae, they also serve as food for invertebrate and vertebrate predators (e.g., fish) (Thorp and Covich 2009), thus also causing disturbances at the community level. The importance of carrying out toxicity tests at different trophic levels is emphasized as each species may present different sensitivity to chemical substances tested (Costa et al. 2008), besides helping to simulate a natural ecosystem (Pane et al. 2008).

In the present study, it was observed that dilutions of macrophyte extracts $> 35\%$ had toxicological effects on non-target organisms and were dependent on the non-target species. Other studies (Zheng et al. 2010; Techer et al. 2016; Huang et al. 2016) also reported toxic effects at low dilutions of macrophyte extracts on non-target organisms. Specifically, high concentrations of the allelochemicals linoleic acid and salicylic acid extracted from macrophytes were found to be toxic to *Danio rerio* (Hamilton, 1822), *D. magna*, and *Moina macrocopa* (Straus, 1820).

The search for natural antifouling alternatives has increased significantly over the years, especially for those seeking the use of phytochemicals as antifouling agents (Agostini et al. 2021b; Hamidi et al. 2022). Despite this, studies that use extracts from macrophytes as an alternative to control biofouling are still incipient. In fact, although the ecological dynamics of aquatic macrophytes in freshwater ecosystems are very well known, little is known about their antifouling effects. Results from this study suggest that these

plants, especially *E. crassipes* and *T. domingensis*, have great potential to control biofilm formation, inhibiting QS communication between bacteria and attachment of the golden mussel. Improved antifouling activity might be obtained by organic solvent-based extraction with ethanol, ethyl acetate, and/or methanol which would target polar compounds and lipophilic and hydrophilic molecules, respectively. Therefore, chemical compounds present in the macrophytes extracts could be incorporated into paints to develop natural antifouling alternatives that are less harmful to the environment (Pérez et al. 2021; Hamidi et al. 2022).

We reinforce that our study was developed only in the laboratory with tests with individual biofouling organisms, having limitations and not providing insights into the effects of macrophyte extracts on the entire biofouling process at different stages of development. This limitation can be circumvented through future studies with antifouling evaluations in the field, which allow tests to be carried out under environmental conditions of complex interactions between fouling organisms and hydrodynamics of the environment, making the test more similar to natural conditions (Romeu and Mergulhão 2023). In addition, for the incorporation of the compounds present in macrophyte extracts into antifouling solutions (Hamidi et al. 2022), it is necessary to carry out the chemical identification of these extracts in order to search for the main bioactive molecule responsible for this effect. As a result, our efforts are currently already directed to ongoing research on the validation of antifouling effectiveness under in situ conditions and chemical characterization of extracts. Besides, we emphasize that in our study, we did not provide information on the durability of the antifouling effect of the extracts and that studies are needed to understand the half-life of the effects of these extracts.

Conclusions

Of the 25 plant extracts tested here, those from *E. crassipes* and *T. domingensis* were the most promising as they efficiently inhibited > 70% of biofilm formation, inhibited quorum sensing, and inhibited the attachment of golden mussels while being considered safe for non-target organisms at dilutions of 35%. Thus, the potential of aquatic macrophyte extracts to inhibit biofilm through inhibition of QS and inhibition of golden mussel attachment was demonstrated, even at low extract dilutions (≤ 35%). Therefore, extracts from these macrophytes offer a new perspective on developing natural antifouling paints and should eventually be further explored such as the identification of the chemical compounds present in the extracts with the antifouling effect, half-life tests of the extracts to understand the durability of their effect, and field tests to verify the antifouling effect under natural conditions.

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Author contribution All authors contributed to the study conception and design. The authors MLPM and BO contributed to writing, conceptualization, methodology, laboratory analysis, statistical analysis, revision, and editing. PSG, CdMGM, DM, FRB, LB, EB, AS, HC, ROC, NHT, GLLP, and VOA contributed to the conceptualization, methodology, statistical analysis, revision, and editing. The first draft of the manuscript was written by MLPM, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format, they are available from the corresponding author upon reasonable request.

Declarations

Ethical approval According to Brazilian legislation (Law 11,794 of October 8, 2008), research with invertebrates does not require authorization from any ethics or animal welfare committee. The collection of invertebrates was approved by the Brazilian Agency SisBio (process no. 77095–1). The assay with *P. promelas* was approved by the Consejo Nacional del Ética Animal (CNEA) – Ata n° 17/11.

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Supplementary material

Table S1: Taxonomic information on selected biofilm-forming bacterial isolates for each type of substrate.

Class	Family	Genus	Species	Substrates
Gama-Proteobacteria	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas* sp.	Concrete, marine plywood, acrylic and ASTM-36 carbon steel
Bacilli	Bacillaceae	*Psychrobacillus*	*Psychrobacillus psychrodurans*	Concrete
Actinomycetia	Micrococcaceae	*Citricoccus*	*Citricoccus nitrophenolicus*	Concrete
Gama-Proteobacteria	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas putida*	Marine plywood
Actinomycetia	Micrococcaceae	*Paeniglutamicibacter*	*Paeniglutamicibacter kerguelensis*	Marine plywood
Gama-Proteobacteria	Moraxellaceae	*Acinetobacter*	*Acinetobacter haenolyticus*	ASTM-36 carbon steel
Bacilli	Bacillaceae	*Bacillus*	*Bacillus vietnamensis*	ASTM-36 carbon steel
Gama-Proteobacteria	Moraxellaceae	*Acinetobacter*	*Acinetobacter bohemicus*	Acrylic
Gama-Proteobacteria	Pseudomonadaceae	*Pseudomonas*	*Pseudomonas rhodesiae*	Acrylic

Table S2: Summary of results of aquatic macrophyte extracts for biofilm and anti-attachment assays. - = not evaluated for this step; Yes = presented this effect for this step; No = did not show this effect for this step; Antibiotic = reduced planktonic growth; Antibiofilm activity = Dilutions that showed no difference when compared to the control group.

Extract/Dilution (%)	Biofilm bacteria				Planktonic bacteria						Golden mussel	
	Isolates		Community		Isolates			Community			< 10 mm	> 10 mm
	Inhibition	Eradication	Inhibition	Eradication	Inducing	Antibiotic	Antibiofilm	Inducing	Antibiotic	Antibiofilm	Anti-attachment	Anti-attachment
Eichhornia crassipes flower												
5	6 bacteria	No	Yes	No	3 bacteria	1 bacteria	5 bacteria	No	No	Yes	Yes	Yes
10	6 bacteria	No	Yes	No	5 bacteria	1 bacteria	3 bacteria	No	No	Yes	Yes	Yes
20	8 bacteria	No	Yes	No	4 bacteria	1 bacteria	4 bacteria	No	No	Yes	Yes	Yes
40	6 bacteria	No	Yes	No	7 bacteria	No	2 bacteria	Yes	No	No	Yes	Yes
Eichhornia crassipes leaf												
5	8 bacteria	3 bacteria	-	-	4 bacteria	No	5 bacteria	-	-	-	Yes	Yes
10	5 bacteria	No	-	-	6 bacteria	No	3 bacteria	-	-	-	Yes	Yes
20	9 bacteria	No	-	-	5 bacteria	No	4 bacteria	-	-	-	Yes	Yes
40	6 bacteria	No	-	-	9 bacteria	No	No	-	-	-	Yes	Yes
Eichhornia crassipes stalk												
5	6 bacteria	No	Yes	No	2 bacteria	1 bacteria	6 bacteria	Yes	No	No	Yes	Yes
10	8 bacteria	No	Yes	No	5 bacteria	No	4 bacteria	Yes	No	No	Yes	Yes
20	6 bacteria	No	Yes	No	6 bacteria	No	3 bacteria	Yes	No	No	Yes	Yes
40	5 bacteria	3 bacteria	Yes	No	9 bacteria	No	No	No	Yes	No	Yes	Yes
Eichhornia crassipes root												
5	8 bacteria	No	Yes	No	9 bacteria	No	No	No	No	Yes	Yes	Yes
10	7 bacteria	No	Yes	No	8 bacteria	No	1 bacteria	No	No	Yes	Yes	Yes
20	7 bacteria	No	Yes	No	8 bacteria	No	1 bacteria	No	No	Yes	Yes	Yes

40	7 bacteria	No	Yes	No	8 bacteria	1 bacteria	No	No	No	Yes	Yes	Yes
Salvinia herzogii leaf												
5	7 bacteria	No	Yes	No	8 bacteria	No	1 bacteria	Yes	No	No	Yes	Yes
10	8 bacteria	No	Yes	No	7 bacteria	No	2 bacteria	No	Yes	No	Yes	Yes
20	8 bacteria	No	No	No	8 bacteria	No	1 bacteria	No	Yes	No	Yes	Yes
40	8 bacteria	No	Yes	No	9 bacteria	No	No	Yes	No	No	Yes	Yes
Schoenoplectus californicus inflorescence												
5	7 bacteria	No	Yes	No	1 bacteria	1 bacteria	7 bacteria	Yes	No	No	Yes	Yes
10	9 bacteria	No	Yes	No	2 bacteria	1 bacteria	6 bacteria	Yes	No	No	Yes	Yes
20	7 bacteria	No	Yes	No	2 bacteria	No	7 bacteria	Yes	No	No	Yes	Yes
40	9 bacteria	No	Yes	No	5 bacteria	No	4 bacteria	Yes	No	No	Yes	Yes
Nymphoides humboldtiana flower												
5	-	-	No	No	-	-	-	Yes	No	No	-	-
10	-	-	Yes	No	-	-	-	No	Yes	No	-	-
20	-	-	Yes	No	-	-	-	No	Yes	No	-	-
40	-	-	Yes	No	-	-	-	Yes	No	No	-	-
Nymphoides humboldtiana stalk												
5	8 bacteria	No	Yes	No	3 bacteria	No	6 bacteria	Yes	No	No	Yes	Yes
10	4 bacteria	No	Yes	No	5 bacteria	No	4 bacteria	Yes	No	No	Yes	Yes
20	4 bacteria	No	Yes	No	6 bacteria	No	3 bacteria	No	Yes	No	Yes	Yes
40	7 bacteria	No	Yes	No	9 bacteria	No	No	No	Yes	No	Yes	Yes
Typha domingensis inflorescence												
5	4 bacteria	No	Yes	No	No	1 bacteria	8 bacteria	No	Yes	No	Yes	Yes

10	4 bacteria	No	Yes	No	No	2 bacteria	7 bacteria	No	Yes	No	Yes	Yes
20	6 bacteria	No	Yes	No	No	1 bacteria	8 bacteria	Yes	No	No	Yes	Yes
40	3 bacteria	No	Yes	No	No	2 bacteria	7 bacteria	Yes	No	No	Yes	Yes
Typha domingensis aerial part upper												
5	7 bacteria	No	Yes	No	1 bacteria	2 bacteria	6 bacteria	No	No	Yes	Yes	Yes
10	5 bacteria	No	Yes	No	1 bacteria	No	8 bacteria	No	No	Yes	Yes	Yes
20	5 bacteria	No	Yes	No	2 bacteria	1 bacteria	6 bacteria	No	No	Yes	Yes	Yes
40	6 bacteria	No	Yes	No	5 bacteria	No	4 bacteria	No	No	Yes	Yes	Yes

Table S3: Quantification the quorum sensing inhibition (QSI) effect of *Eichhornia crassipes* and *Typha domingensis* extracts against two biosensors *Chromobacterium subtsugae* CV017 (short chain) and *Chromobacterium violaceum* ATCC 12472 (long chain). Red = ≥50% Violacein inhibition (VI) and ≤40% growth inhibition (GI) (Good QSI activity); Different letters = denote significantly different means ($p < 0.05$) between dilutions in each extract.

Extract	***Chromobacterium subtsugae* 017**									
	0.07%		**0.15%**		**0.30%**		**0.60%**		**1.20%**	
	%GI	**%VI**	**%GI**	**%VI**	**%GI**	**%VI**	**%GI**	**%VI**	**%GI**	**%VI**
Typha domingensis	-8,54a	24,97a	-13,09a	33,66a	-6,5a	39,92ab	-11,08a	45,52b	-16,65b	66,07c
Eichhornia crassipes	-0,52a	36,95a	-1,89a	49,46b	-0,82a	56,95c	-0,73a	59,54c	-13,96a	67,3d
Vanillin	-4,2a	12,86a	2,14a	22,68a	5,1a	37,89b	17,17b	48,67c	18,1b	67,82d
Extract	***Chromobacterium violaceum* ATCC 12472**									
	0.07%		**0.15%**		**0.30%**		**0.60%**		**1.20%**	
	%GI	**%VI**	**%GI**	**%VI**	**%GI**	**%VI**	**%GI**	**%VI**	**%GI**	**%VI**
Typha domingensis	11,22a	3,53a	13,2a	16,28b	18,14a	29,96c	20,53a	40,62d	23,47a	57,29e
Eichhornia crassipes	-1,54a	-19,65a	-0,4a	5,51b	1,44a	24,86c	13,72b	30,07c	23,67c	39,27d
Vanillin	-3,04a	-2,74a	2,98a	16,5b	8,96a	40,99c	37,44b	78,3d	72,8c	131,38e

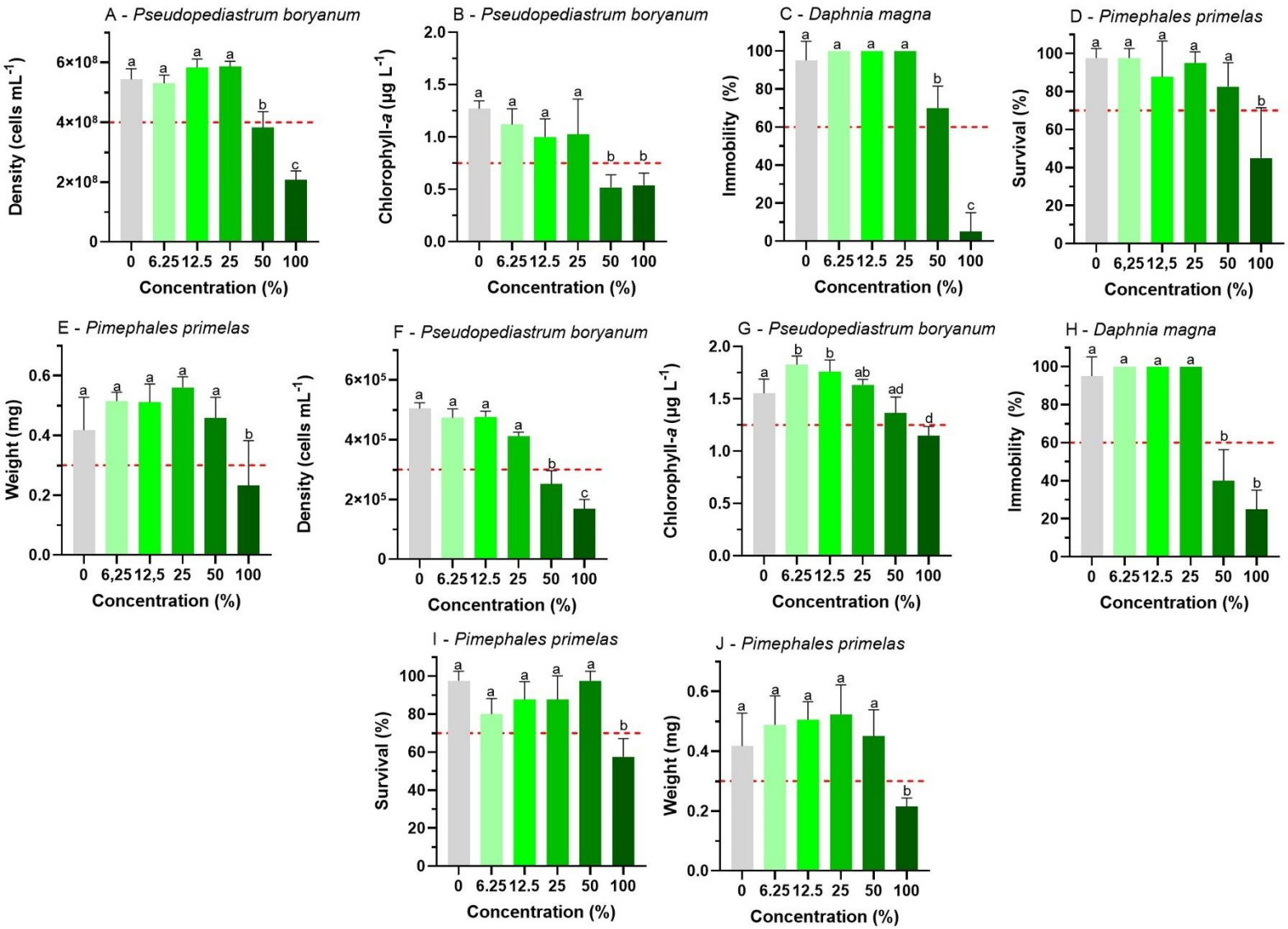
A - Pseudopediastrum boryanum
Density (cells mL-1)
6×10⁸
4×10⁸
2×10⁸
0
0 6.25 12.5 25 50 100
Concentration (%)
B - Pseudopediastrum boryanum
Chlorophyll-a (µg L-1)
2.0
1.5
1.0
0.5
0.0
0 6.25 12.5 25 50 100
Concentration (%)
C - Daphnia magna
Immobility (%)
100
80
60
40
20
0
0 6.25 12.5 25 50 100
Concentration (%)
D - Pimephales primelas
Survival (%)
100
80
60
40
20
0
0 6,25 12,5 25 50 100
Concentration (%)
E - Pimephales primelas
Weight (mg)
0.6
0.4
0.2
0.0
0 6,25 12,5 25 50 100
Concentration (%)
F - Pseudopediastrum boryanum
Density (cells mL-1)
6×10⁵
4×10⁵
2×10⁵
0
0 6.25 12.5 25 50 100
Concentration (%)
G - Pseudopediastrum boryanum
Chlorophyll-a (µg L-1)
2.0
1.5
1.0
0.5
0.0
0 6.25 12.5 25 50 100
Concentration (%)
H - Daphnia magna
Immobility (%)
100
80
60
40
20
0
0 6.25 12.5 25 50 100
Concentration (%)
I - Pimephales primelas
Survival (%)
100
80
60
40
20
0
0 6.25 12.5 25 50 100
Concentration (%)
J - Pimephales primelas
Weight (mg)
0.6
0.4
0.2
0.0
0 6.25 12.5 25 50 100
Concentration (%)

Figure 1: Mean (± SD), A – E = Response of *Pseudopediastrum boryanum* (A – B), *Daphnia magna* (C) and *Pimephales promelas* (D – E) exposed to treatments with aqueous extracts of *Typha domingensis*. F – J = Response of *P. boryanum* (F – G), *D. magna* (H) and *P. promelas* (I – J) exposed to treatments with aqueous extracts of *Eichhornia crassipes*. A and F = Density (cells m^{-1}). B and G = Chlorophyll *a* concentration (µg L^{-1}). C and H = Immobility (%). D and I = Survival (%). E and J = Weight (mg). Red line = threshold of toxicity when compared to the control group. Different letters = denote significantly different means ($p < 0.05$).

Capítulo IX: Artigo 4

O quarto artigo científico proveniente desta Tese de Doutorado é apresentado neste capítulo. O manuscrito, de autoria de Mikael Luiz Pereira Morales, Laís Olivera das Neves, Ayman Shaik, Hafizah Chenia, Maximiliano Manuel Maronna, Sanye Soroldoni, Renato Mitsuo Nagata, Ng Haig They, Vanessa Ochi Agostini e Grasiela Lopes Leães Pinho, intitulado-se "***Aquatic macrophytes as antifouling candidates: anti-attachment and toxicological effects in Aurelia coerulea (Cnidaria, Scyphozoa)***", foi submetido no periódico "***Environmental Toxicology***" onde encontra-se em processo de revisão.

Aquatic macrophytes as antifouling candidates: anti-attachment and toxicological effects in *Aurelia coerulea* (Cnidaria, Scyphozoa)

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest disclosure

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Abstract

Biofouling on artificial surfaces in aquatic ecosystems leads to significant economic losses. Current antifouling paints, while effective, often harm the aquatic environment. This study explores ecologically safe antifouling alternatives derived from plants, focusing on the aquatic macrophytes *Cabomba caroliniana* (CC) and *Schoenoplectus californicus* (SC). While these macrophytes have shown promise against microfouling, their effectiveness against marine macrofouling remains underexplored. For marine macrofouling tests, *Aurelia* polyps have been recommended due to their availability and handling. Using the marine cnidarian *Aurelia coerulea* (AC) as a model organism, the ability of CC and SC extracts to inhibit polyp attachment was evaluated as well as their toxicological effects on polyps and ephyrae. Additionally, the sensitivity of AC to reference substances (surfactant, zinc and copper) was assessed to determine its sensitivity compared to other organisms. A dose-dependent inhibition of polyp attachment was observed, with up to 65% efficacy. Toxicity tests indicated low toxicity at concentrations of up to 5% for CC and 20% for SC. The main compounds identified were n-nonadecanol-1 for CC and eicosane for SC. Additionally, AC proved to be a versatile, anti-attachment assay model, offering advantages such as sensitivity to chronic and acute tests, dual life stages and short assay times of up to 72 hours. These results suggest the biotechnological potential of CC and SC as natural antifouling agents and highlight their suitability for developing environmentally friendly antifouling applications.

Keywords: Macrophytes, natural products, antifouling, *Aurelia*, attachment, toxicology.

1. Introduction

Biofouling is a complex process of ecological succession where biological deposits accumulate on submerged surfaces in aquatic ecosystems[1]. It begins with the adsorption of organic and inorganic molecules, followed by bacterial adhesion and biofilm formation[1]. Over time, this leads to colonization by other microorganisms (*e.g.*, protozoa, fungi and microalgae), and adhesion of macroorganisms (*e.g.*, macroalgae, mussels, barnacles, cnidarians and urochordates)[1,2].

As fouling communities grow on artificial surfaces, particularly involving macrofouling[3], they alter the weight, integrity and conformity of these structures[4]. This results in issues such as increased friction, reduced hydrodynamics, vessel buoyancy[5], and clogged water collection systems[4]. Consequently, there are large economic losses for the aquatic industries, with the estimated expense for vessel maintenance and biofouling prevention at about US$340 million[6]. Additionally, biofouling is an important vector for the spread of invasive species[3], influencing the dynamics of biological invasions.

Currently, third generation antifouling paints, containing biocides like diuron, chlorothalonil and copper oxide[7,8], are widely used. Limitations include being less effective at the later stages of biofouling[9] and negatively impact aquatic ecosystems[10]. Non-target organisms, including planktonic crustaceans and fish[5,11], often experience toxic effects. There is thus a great need for the development of eco-friendly “green” antifouling solutions which are safer and less harmful to the environment[12,13], with higher biodegradability and potential lower toxicity against non-target organisms[12].

Aquatic macrophytes are a promising source of natural compounds with anti-fouling potential[14,15]. Species such as *C. caroliniana* and *S. californicus* have shown promise due to their secondary compounds like humic acids and polyphenols[16,17]. Morales et al.[14] reported that of 11 tested macrophyte species extracts, those from *C. caroliniana* and *S. californicus* inhibit bacterial biofilms by over 70% without harming non-target organisms like *Thalassiosira pseudonana* and *Nitokra* species. However, their study did not evaluate the toxicity of a wide range of concentrations, only concentrations of up to 10% for *C. caroliniana* and 20% for *S. californicus*[14]. While they evaluated the anti-microfouling effect, their efficacy against macrofouling was only studied with freshwater organisms (*Limnoperna fortunei*)[15] and was not evaluated with marine organisms.

Antifouling tests involving microfouling, particularly with bacterial biofilms, are well established[18], however, although a wide variety of organisms have been reported for macrofouling tests, most studies have been restricted to species of tubular worms and mussels[18,19]. These species are collected in the field and exposed to chemical and physical procedures for the release of eggs and/or larvae. These procedures may take several days (~10 days) to obtain the necessary phase of their life cycle to carry out experimental tests[20]. In view of these challenges, the use of polyps of the *Aurelia* jellyfish has been gaining attention because it does not require metamorphosis to perform the anti-attachment tests[20].

Cnidarian species of the genus *Aurelia* (Lamarck, 1816) are effectrive biofoulers[21], colonizing natural substrates (*e.g*., rocks and shells) and artificial substrates (*e.g*. boats and concrete structures)[22]. *Aurelia aurita* and *Aurelia coerulea* are, however, easy to cultivate with minimal laboratory conditions and are maintained in public aquariums and in laboratories around the world. These animals have been used for decades as model organisms in developmental[23], ecological[24], biomechanical[25], and ecotoxicology[26–28] studies. The availability of genetic data for multiple strains of cultures[29] along with the accumulated knowledge of physiological, reproductive and ecological traits in various *Aurelia* species makes them excellent models for experimental biology. Furthermore, due to climate change and anthropogenic activities, in recent years the proliferation of cnidarians has been increasing, especially in estuarine regions[29].

Aurelia coerulea, the moon jellyfish (Figure 1), offers a unique opportunity for antifouling studies due to its metogenic life cycle, with benthic (polyps) and planktonic (ephyrae and medusae) stages[30], making them ideal for testing antifouling substances. *Aurelia* ephyrae, from *A. aurita*[26–28] and *A. coerulea*[31,32], have been widely used in toxicological studies. Studies with polyps are still incipient in relation to toxicity and anti-attachment[20,32]. Unlike other macrofouling organisms, *Aurelia* polyps are easy to cultivate and allow for rapid anti-attachment testing[20]. Additionally, ephyrae and polyps exhibit varying sensitivity to stressors, enabling a comprehensive evaluation of antifouling efficacy[33].

Given the environmental concerns surrounding the use of chemical antifouling, the present study evaluates the antifouling potential and toxicological effects of *C. caroliniana* and *S. californicus* extracts on *Aurelia coerulea* polyps and ephyrae. We also aimed to compare the sensitivity of *A. coerulea* to three reference substances—sodium dodecyl sulfate (SDS), zinc sulfate (ZS), and copper chloride (CC)—to assess their effectiveness as natural, eco-friendly antifouling. These three reference substances have been used to estimate the accuracy and reliability of data produced in the laboratory[34]. SDS is a surfactant generally used to assess the relative sensitivity of test organisms, as it is less toxic and easy to handle in the laboratory. Copper and zinc are commonly used reference substances in toxicology, as they are essential elements for organisms, but in high concentrations they are toxic[35]. These substances are widely used as base compounds for antifouling systems, resulting in toxicity to non-target organisms[36].

2. Methodology

2.1. Aurelia species maintenance

Polyps of *Aurelia* species were acquired through a pre-established culture from the Zooplankton Laboratory (LABZOO) at the Universidade Federal do Rio Grande (FURG), Brazil. Polyps were maintained in plastic containers with filtered natural seawater (salinity between 32 and 35, 0.45 µm) at 20° to 23 °C under a 12L:12D photoperiod and fed *ad libitum* every two days with newly (<2 days old) hatched *Artemia franciscana* nauplii. One day after feeding, the cultivation water was renewed to avoid the excessive proliferation of microorganisms. To minimize stress related to removing the polyps from the substrate, polyps were removed from the

substrates 24 h before initiation of the assays. Polyps that did not show a stress response were selected for the assays.

To obtain ephyrae from *Aurelia* polyps, strobilation was induced by acclimation in decreased temperature to ~15 °C in 1.5 L plastic containers with artificial 35 salinity water (ASW) and a photoperiod of 12L:12D (adapted from[26]). Once the ephyrae (0 to 5 d old) were released, they were immediately collected and placed in glass beakers to carry out toxicological tests. The ephyrae were fed *ad libitum* with *A. franciscana* nauplii, 24 h before the tests, to increase swimming activity.

2.2. Molecular species determination

Total DNA was extracted from three whole-body polyps cultivated at LABZOO at FURG with an ammonium acetate protocol (triplicates;[37]). For taxonomic validation of analyzed cultures, selected molecular markers were amplified and sequenced: from the mitochondrial genome, a ~650 bp fragment of the large ribosomal 16S rRNA subunit and ~650 bp of the protein-coding COI I subunit[38]; from their nuclear genome, a ~650 bp fragment of the large ribosomal 28S rRNA subunit[39]. Amplification based on Polymerase Chain Reaction (PCR) protocols followed standard procedure and thermocycler reaction conditions were conducted as described by Lawley et al.[30]. PCR products were purified using the Agencourt AMPure XP kit (B37419AB) and the BigDye reactions used the same primers and Tm conditions as original PCRs. Finally, these amplicons were precipitated (sodium acetate and ethanol) and sequenced using an ABI PRISM ®3100 Hitachi genetic analyzer. Using Geneious® 9.5[40], chromatograms were assembled, trimmed, aligned and final consensus sequences were compared with data available in GenBank to identify the *Aurelia* species[30]. Sequence data was deposited in the NCBI database.

2.3. Test solutions

To verify the sensitivity of the two *Aurelia* life stages (polyp and ephyrae), toxicology assays were carried out with the reference substance the surfactant sodium dodecyl sulfate (SDS - $NaC_{12}H_{25}SO_4$), zinc sulfate (ZS - $ZnSO_4$) and copper chloride II (CC - $CuCl_2$) (Labsynth). For this, SDS in its powder form was used to prepare diluted treatments in ASW with salinity 35 (Marine salt VeroSal Corais). The concentrations were 5, 15, 45 and 135 $mg.L^{-1}$ of SDS for polyps and 0.5, 1, 2.5, 5, and 15 $mg.L^{-1}$ for ephyrae. Powdered ZS and CC were also used to prepare treatments with ASW. The defined concentrations were as follows: ZS - 1, 2, 3, 4 and 5 $mg.L^{-1}$ for polyps and 0.4, 0.8, 1.6, 2.4 and 3.2 $mg.L^{-1}$ for ephyrae and CC - 0.1, 0.25, 0.5, 1 and 2.5 $mg.L^{-1}$ for polyps and 0.02, 0.05, 0.10, 0.15 and 0.20 $mg.L^{-1}$ for ephyrae.

Aqueous extracts of *C. caroliniana* (stalk and leaf) and *S. californicus* (inflorescence and stalk) were prepared according to the methodology described by Morales et al.[14]. Plant biomass was collected in permanent lakes in southern Brazil (32° 09' 23.3" S 52° 05' 57.6" W), dried at 60-80 °C until it reached a constant mass and manually crushed with a pestle and mortar. To prepare the extracts, 6 g of dry biomass were mixed with 300 mL of ASW (salinity 35) and left to rest for 24 h at 22 °C. Thereafter, the mixture was centrifuged and sterilized by filtration (0.2 µm)

(cellulose acetate filter, Sartorius Biolab Products). Preparation of the extracts resulted in a 100% stock solution, which was used to obtain test concentrations through dilution in sterile ASW. The concentrations defined for the tests were 5, 10, 20, 40 and 80%. Treatments with SDS, ZS, CC and aquatic macrophyte extracts were used to carry out attachment and toxicological assays under experimental conditions of 20 °C, 12L:12D. For both experiments, controls were set by ASW with salinity 35.

2.4. Toxicological assays

2.4.1. Assays with polyps

Polyps were individually isolated in 6-well plates, each well containing 10 mL of treatment. For each treatment, three replicates were prepared, each containing four polyps. After 1, 6, 24, 48, 72, 96 h of exposure, chronic (sublethal) and acute responses were observed with the aid of a stereoscopic microscope Olympus SZX9, magnification 40× (Table 1).

The following chronic endpoints were observed: tentacle contraction by *Aurelia* species was indicated distended (all tentacles were distended, even if slightly curved; Fig. 1) or contracted tentacles (when all tentacles were absolutely contracted; Fig. 1); change in prey ingestion was observed during 30 s of food capture and ingestion (% of individuals who ingest prey). Feeding *ad libitum* was done by adding 1 mL of solution containing *A. franciscana* nauplii in a controlled and progressive manner, using a Pasteur pipette. Observation of the change in diet was carried out at 48 and 96 h. The acute response was indicated by mortality determined by the observation of total or partial disintegration of the polyps (Fig. 1). A polyp is considered to disintegrate when it loses its typical body shape, which results in tissue fragmentation (Fig. 1).

2.4.2. Assays with ephyrae

Ephyrae were exposed to each treatment in 24-well plates, each well containing 2 mL of the treatment (one individual per well). For each treatment, three replicates were prepared, each containing four individuals, one per well to avoid interactions between organisms[27]. After 24 and 48 h, acute and chronic (sublethal) effects were observed (Table 1).

The acute response for each treatment was observed through the mobility of the organisms, by observation of the individual's movement for 10s after stimulation with the aid of a Pasteur pipette under a stereoscopic microscope (Olympus SZX9, magnification 40×). Completely immobile ephyra (unable to change their barycenter position during 10s) were counted as immobile organisms, and the percentage of immobility (%) was calculated for each treatment compared to the control treatment. The chronic response was given by the pulsation frequency (PF) of each ephyra, measured by observing it under a stereoscopic microscope. For each ephyra, three PF measurements of 10 s each were performed. The average of the three measurements was calculated and the data transformed to PF per minute for each treatment.

2.5. Attachment assays

Polyps were individually exposed to the three reference substances and two extract treatments in 6-well plates containing 10 mL of treatment and one individual per well. For each treatment, three replicates were prepared, each assessing four polyps. Polyp attachment was evaluated over periods of 24, 48, 72 and 96 h by the polyps' resistance to detaching from the substrate when subjected to light water flows performed with the aid of a Pasteur pipette. In addition, we confirmed the attachment of the organisms with the aid of a stereoscope microscope (Olympus SZX9, magnification 40×). Each individual's attachment was counted for all treatments and control, and the results were expressed as percentage of attachment (%).

2.6. Chemical characterization of extracts

2.6.1 Gas chromatography-mass spectroscopy (GC-MS)

The two crude aqueous macrophyte extracts were subjected to gas chromatography-mass spectroscopy (GC-MS) to identify their composition. A 1 µl solution was injected into a Shimadzu gas chromatograph (series AOC-20i)–coupled mass spectrophotometer (GCMS-QP2010 SE). Helium was used as the carrier gas. The column oven temperature was programmed at 50 °C and the injection temperature was 260 °C, while the flow rate was 0.68 ml/min. The capillary column used was Zebron ZB-5MSplus column 0.25 × 30 m (length) × 0.25 µm (df). A 50/50 split ratio was used for the injection sample at an initial holding time of one min then later at 10 minutes. The start time for analysis was 3 min, while the end time was 32 min. The spectra were set at 20 to 1000 m/z to avoid capturing water molecules and other volatiles by the GC. Analysis commenced after 3 min and ended after 32 min, with the spectra set at 20 – 1000 m/z to prevent interference by water molecules and other volatiles by the gas chromatograph. The relative amount of each phyto-component present in extracts was expressed as a percentage based on peak height (%) produced in the chromatograph. The recorded mass spectra of the constituents of the crude extracts were identified using the standard mass spectra from the National Institute of Standards and Technology (NIST05.LIB) library data provided by the GC-MS system software[41,42].

2.6.2 Liquid chromatography-mass spectroscopy (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) data was obtained using a Shimadzu LCMS2020 with a Shimadzu Shim-Pak GIST HP C18 3 µm 4.5 x 150 mm column equipped with a UV detector using a mobile phase of 20-90% acetonitrile, 10% water and both containing 1.1% formic acid at a flow rate of 1 mL/min. The recorded mass spectra of the constituents of the crude extracts were identified using the standard mass spectra from the analytical data MASSBANK (http://www.massbank.jp/index-e.html)[43].

2.6.3 *Fourier transmission infrared spectroscopy (FTIR)*

Fourier transmission infrared spectroscopy (FTIR) was performed to identify the functional groups present in the two crude extracts. Crude extract samples were prepared for analysis by drying 2 mg of the extracts which were mixed with 200 mg KBr. Crude extracts were analyzed

using the Bruker Alpha II Infrared Spectrophotometer (Parameters: ATR Diamond^{-1} Bounce, 24 background scans, 24 sampling scans, 4000 - 400 cm^{-1} range with a resolution of 4 cm^{-1}) and processed with Opus Spectroscopy Software. Functional groups of the crude biosurfactants were identified using IR spectrum tables provided by Sigma-Aldrich[41,42].

2.7. Statistical analysis

For acute responses, LC_{50} (lethal concentration for 50% of the population) values were calculated over a period of 96 h for polyps and EC_{50} (effect concentration for 50% of the population) of 48 h for ephyrae, using Probit Analysis[44]. To test possible differences between treatments at each exposure time for polyps and mobility of ephyrae, generalized linear models (GLM) were used. Because these variables are discrete, a model with a binomial distribution was used. As for the ephyrae pulsation frequency, as this variable is continuous, the normality and homoscedasticity of the residuals were verified using the Shapiro-Wilk and Levene tests, respectively. With the acceptance of the premises, an ANOVA one-way was used. When a significant effect of a variable was detected, a *post hoc* Tukey comparison was performed to detect significant differences between levels of the factors. Statistical analyzes were performed using GraphPad Prism 8.4 software for Windows (San Diego, USA).

3. Results

3.1. Molecular identification

Genetic sequences confirmed that the *Aurelia* culture used belongs to *Aurelia coerulea* von Lendenfeld, 1884 (+98% DNA sequence homology identity for all three molecular markers with samples from the Mediterranean Sea).

3.2. Reference substances

3.2.1. Attachment assays

For all reference test solutions, polyp attachment began after 48 h, stabilizing after 72 h (Fig. 2A-C). Attachment was inhibited from 15 mg.L^{-1} ($p<0.05$) when testing the surfactant SDS (Fig. 2A). With ZS and CC (Fig. 2B-C), exposure to 1 and 0.1 mg.L^{-1}, respectively, did not inhibit attachment ($p>0.05$), however, the remaining higher concentrations inhibited attachment in a dose-dependent manner ($p<0.05$).

3.2.2. Toxicological assays with polyps

After 1 h, polyps achieved 100% tentacle contraction at all SDS concentrations (Fig. 2D) except the control ($p<0.05$). This response was observed at all subsequent exposure times. For ZS (Fig. 2E), tentacle contraction occurred in a dose-dependent manner after 6 h of exposure ($p<0.05$). For CC, the tentacle contraction response pattern was the same as observed for SDS (Fig. 2F). From 72 h onwards, all ZS concentrations (Fig. 2E) showed 100% contraction ($p<0.05$).

The responses of the prey ingestion assay were similar between 48 and 96 h (Fig. 3) for all test solutions. For SDS (Fig. 3A), the percentage of prey ingested decreased from ≥ 5 $mg.L^{-1}$ ($p<0.05$). For ZS and CC, prey ingestion decreased with increasing concentration ($p<0.05$), demonstrating a dose-dependent response (Figs. 3B-C).

For all test solutions, the disintegration response increased after 48 h of exposure, remaining constant at 72 and 96 h (Fig. 3). For SDS at ≥ 15 $mg.L^{-1}$, disintegration was 100% after 24 h (Fig. 3D). For ZS and CC (Figs. 3E-F), the disintegration response also occurred in a dose-dependent manner after 24 h ($p<0.05$). The LC_{50} for SDS, ZS and CC was 8.66, 3.33 and 0.30 $mg.L^{-1}$, respectively (Table 2).

3.2.3. Toxicological assays with ephyrae

Pulsation frequency responses were similar for 24 and 48 h exposure to all reference solutions, presenting a dose-dependent response (Fig. 4). For SDS (Fig. 4A), the PF decreased from the concentration of 1 $mg.L^{-1}$ ($p<0.05$). For ZS (Fig. 4B), the frequency of pulsation decreased from the concentration of 0.4 $mg.L^{-1}$ and for CC (Fig. 4C) to a concentration of 0.02 $mg.L^{-1}$.

Immobility responses were similar for 24 and 48 h across all tested reference solutions (Fig. 4). For SDS and ZS (Fig. 4D-E), the immobility response was dose-dependent ($p<0.05$). For CC (Fig. 4F), immobility was significantly higher than the control for all concentrations tested (0.02 mg/L). The EC_{50} values were 1.00, 2.72 and 0.023 $mg.L^{-1}$ for the SDS, ZS and CC, respectively (Table 2).

3.3. Extracts of aquatic macrophytes

3.3.1. Attachment assays

For the *C. caroliniana* extract, the 80% concentration inhibited attachment at all exposure times (Fig. 5A; $p<0.05$), while the other concentrations showed attachment inhibition ≥65% after 48 h ($p<0.05$). The attachment of polyps exposed to *S. californicus* demonstrated a dose-dependent response ($p<0.05$), decreasing under higher concentrations (Fig. 5B).

3.3.2 Toxicological assays with polyps

For *C. caroliniana*, there was an increase in tentacle contraction with increasing extract concentrations ($p<0.05$) at 24 and 48 h (Fig. 5C). However, at 96 h (Fig. 5C), there was a decrease in tentacle contraction (≤25%) at concentrations of 5 and 10%, compared to the control ($p<0.05$). For the *S. californicus* extract (Fig. 5D), the response remained constant after 6 h of exposure, with 100% contraction at concentrations of 10, 20, 40 and 80% ($p<0.05$). For the *C. caroliniana* extract (Fig. 5E), prey ingestion decreased by 50% only at the 80% concentration ($p<0.05$). For the *S. californicus* extract, prey ingestion decreased with increasing concentrations ($p<0.05$), demonstrating a dose-dependent response (Fig. 5F).

For the *C. caroliniana* extract (Fig. 5G), disintegration occurred only at 40 and 80% concentrations ($p<0.05$). As for the *S. californicus* extract, disintegration increased with increasing

extract concentration after 48 h of exposure ($p<0.05$), reaching 100% disintegration at concentrations of 40 and 80% (Fig. 5H). This response remained constant for the 72 and 96h periods (Fig. 5G-H). The LC_{50} for the *C. caroliniana* extract was 62.92%, while that of *S. californicus* was 10.76% (Table 2).

3.3.3. Toxicological assays with ephyrae

For the *C. caroliniana* extract (Fig. 6A), concentrations of 5, 10 and 20% had no effect on the PF compared to the control ($p>0.05$). For the *S. californicus* extract (Fig. 6B), a dose-dependent response was observed with the PF decreasing with increasing concentrations ($p<0.05$).

Immobility responses were similar for 24 and 48 h exposures to all test solutions (Fig. 6C-D). For *C. caroliniana* and *S. californicus* extract (Fig. 6C-D), the immobility response was dose-dependent ($p<0.05$). The EC_{50} for the *C. caroliniana* extract was 62.92%, while that of the *S. californicus* extract was 10.14% (Table 2).

3.4. Chemical characterization of extracts

3.4.1 Gas chromatography-mass spectroscopy (GC-MS)

The three most abundant compounds identified in the *C. caroliniana* extract, were n-nonadecanol-1 with 24.39% area, cyclohexadecane with 19.42% and eicosane with 12.78% (Table 3A). The three most abundant compounds in the *S. californicus* extract were eicosane with 32.90%, E-15-heptadecenal with 16.08%, and 9-tricosene, (Z) with 13.94% (Table 3A). Complete data such as retention time and chromatographic peaks are presented in the supplementary material (Table S1 and Fig. S1).

3.4.2 Liquid chromatography-mass spectroscopy (LC-MS)

The three compounds abundant in the *C. caroliniana* extract were 4-methylphenethylamine with mean 77.85% area, diethanolamine with mean 19.35%, and lysine hydrochloride with mean 1.04% (Table 3B). The abundant compounds in the *S. californicus* extract were 4-methylphenethylamine with mean 73.83%, L-lysine monohydrochloride with mean 13.79%, piperidinic acid with 6.36% (Table 3B). Complete data such as retention time and chromatographic peaks are presented in the supplementary material (Table S2, S2 and Fig. S2).

3.4.3 Fourier transmission infrared spectroscopy (FTIR)

FTIR indicated different functional groups for the two extracts (Supplementary Table S3). For the *C. caroliniana* extract, the functional groups identified included primary amine, aldehyde, amide and fluor compounds. The *S. californicus* extract mainly presented functional groups such as amide, alcohol-hydrogen bonded, alkyne, alcohol, aliphatic ether and halo compound.

4. Discussion

Overall, the results of this study underscore the potential of aquatic macrophyte-derived extracts as effective and environmentally friendly antifouling agents. Interest in plant-derived

antifouling alternatives has grown significantly, with a particular focus on phytochemicals as potential antifouling agents[18]. However, research on aquatic macrophyte extracts for biofouling control remains in its early stages, particularly regarding their effects on macrofouling organisms[14,15]. In the present study, the antifouling efficacy of aqueous extracts of *C. caroliniana* and *S. californicus* was evaluated by examining their ability to inhibit the attachment of *A. coerulea* polyps, as well as their toxicity to polyps and ephyrae.

Recent studies have shown promising antifouling effects of macrophyte extracts. For instance, these *C. caroliniana* and *S. californicus* extracts have demonstrated up to 70% biofilm inhibition for single- and multi-species estuarine bacteria[14]. For this study, a dose-dependent anti-attachment response was observed for *C. caroliniana* and *S. californicus* extracts, aligning with other studies investigating plant-derived anti-macrofouling agents. Feng et al.[45] observed that 15 alkaloids extracted from plants inhibited the attachment of *Bugula neritina* and *Fistulobalanus albicostatus* larvae. Similarly, *Verbena bonariensis* and *Tillandsia tenuifolia* extracts effectively deterred macrofouling of adult *Mytilus edulis*[12]. Moreover, *Halophila stipulacea* extracts have shown antifouling activity against bacteria and *Mytilus galloprovincialis*, with a dose-dependent effect[46]. Additionally, *Posidonia oceanica* extracts reduced diatom and bacterial biofilms while also inhibiting polychaete *Ficopomatus enigmaticus* adhesion[47].

Unlike barnacles, mussels and polychaetes, the use of polyps in antifouling research is relatively novel. To date, only studies by Pinteus et al.[20] and the present work have focused on this application. By analyzing the DNA sequence, it was possible to verify the identity of the study polyps as *A. coerulea*. This species is known for its wide geographic distribution and numerous introductions, as detailed by Lawley et al.[30]. For assays with *A. coerulea*, it is not necessary to perform a metamorphosis step, since laboratory cultures are usually kept in the polyp phase. Thus, obtaining data through macrofouling tests with this species can be much faster (3 days of testing), compared to tests performed with other macrofouling species. Moreover, *A. coerulea* polyps multiply readily *via* asexual reproduction, allowing for a rapid increase in available specimens under adequate feeding and space conditions[48].

In our study, *A. coerulea* polyp attachment stabilized within 72 h, making assays with this species significantly faster than with others, such as *A. aurita* or *Phyllorhiza punctata*, which require up to 120 and 144 h, respectively[20]. This efficiency, coupled with the straightforward cultivation of *A. coerulea* polyps, underscores their practical advantages for antifouling research. In contrast, the utilization of mussel and barnacle larvae involve time-consuming and often unsuccessful procedures to release and develop larvae in the pre-nesting stage, causing a delay of at least a few days (e.g., 6 days for barnacles) to start experiments[49]. For barnacles, after obtaining the larvae, antifouling tests usually last three days[48], often requiring up to 9 days to complete the trial.

We emphasize that in addition to having endpoints to be used in *Aurelia* anti-attachment tests, due to its metagenic life cycle, it is also possible to use them in toxicological tests with different life stages (polyps and ephyrae), which guarantees its scope in the performance of tests for the development of new antifouling agents. Toxicological tests during the settlement stage are

crucial to confirm that attachment inhibition results from attachment inhibition rather than extract toxicity. In this study, the *S. californicus* extract demonstrated significantly higher toxicity than *C. caroliniana* extract at both life stages. The non-toxic concentration for *S. californicus* extract was 5%, compared to 20% for *C. caroliniana*. This was evident in chronic behavioral responses such as feeding and tentacle contraction in polyps, PF responses in ephyrae and acute lethality responses. These results align with previous findings on these extracts' effects when evaluating cell density and chlorophyll-a content of *T. pseudonana* and *Nitokra* species survival[14]. In the present study, at non-toxic concentrations the extracts inhibited *A. coerulea* polyp attachment by ≥ 65%.

The antifouling effects of *C. caroliniana* and *S. californicus* may stem from their chemical compositions, with anti-adhesion and toxicological effects. Both extracts contained eicosane and 4-methylphenethylamine, known for their antimicrobial properties[50]. While these compounds likely play a role in antifouling activity, further investigation is required to clarify their specific contributions to fixation inhibition and toxicity. Unique compounds in the *C. caroliniana* extract such as N-nonadecanol-1, cyclohexadecane, and diethanolamine[51], as well as exclusive compounds in the *S. californicus* extract, such as heptadecenal E-15, 9-triccosene-(Z), and piperidine likely contributed to the antibacterial activity[52], the observed antifouling and toxicological effects. Lysine derivatives, widely found in higher plants, were identified in both extracts and may also have antimicrobial activity[53].

The main chemical groups present, i.e., alkanes, aldehydes, alcohols, amines, and amide, play critical roles in plant physiology, influencing plant-herbivore interactions (pollinator attraction and repellency) and potentially affecting macrofouling organisms' behavior and metabolism[54,55]. In addition, aldehydes that are part of cellular metabolism and the growth of organisms can be toxic in high concentrations[55]. Therefore, these compounds potentially influence the toxicity and binding of *A. coerulea* through behavioral changes or more complex metabolic changes. For *Aurelia*, little is known about these changes, but in general, for macro-invertebrates, attachment inhibition may involve neurotransmission disruption, oxidative stress and inhibition of adhesive production and release[56]. Further studies isolating and testing these compounds individually are necessary to clarify their roles in antifouling efficacy and toxicity. The discrepancy in toxicity levels between these two macrophytes may be attributed to differences in their chemical composition and concentration of specific metabolites[13].

In addition to the antifouling results of the macrophytes *C. caroliniana* and *S. californicus*, through the susceptibility tests with different reference substances, we were also able to observe promising results for the use of *A. coerulea* in toxicological tests. For the chronic toxicity tests with the polyps (tentacle contraction), high values of tentacle contraction were observed in the first observation period with ZS and CC, in addition to the extracts of *C. caroliniana* and *S. californicus*. This response, however, decreased later, with stabilization at 72 and 96 h. At these times, the polyps decreased their maximal contraction responses, especially at lower concentrations. The observed decrease in tentacle contraction may indicate that the polyps were acclimatizing to the conditions of exposure. This phenomenon known as "functional tolerance" suggests that polyps

may be adapting to prolonged exposure, potentially improving their tolerance over time[57]. In other words, exposure to low concentrations can trigger a response that improves tolerance or functional capacity, resulting in an improvement in chronic response over the time of exposure.

In the acute toxicity tests (disintegration), the responses of polyps exposed to the reference substances (SDS, ZC and CC) and to the *S. californicus* extract reached stability after 48 h, while for the *C. caroliniana* extract it was only after 96 h. Using the *C. caroliniana* extract, it was possible to verify the importance of performing acute tests within 96 h to evaluate the development of new antifouling agents. This acute disintegration response was also observed by Massaro and Rocha[58]. The authors observed that, for the acute toxicity of adults of the hydrozoan *Hydra viridissima*, disintegration only occurred after 96 h of exposure to potassium dichromate at concentrations of 2.5 and 5 mg. L^{-1}.

The disparity observed in the LC_{50} ratios between ephyrae and polyps can be attributed to physiological differences between the life cycle stages of this organism. *Aurelia* ephyrae, being at an earlier stage of development, may have different metabolic and detoxification capabilities than polyps, thus influencing their sensitivity to toxic substances. Ephyrae would, therefore, be more sensitive than polyps to certain stressors, such as the microalgae *Ostreopsis* cf. *ovata*[59]. This sensitivity disparity was also observed in trials with saponins and atrazine[33,60]. In addition, we highlight that in the present study tests with polyps were performed for 96 h, while the tests with ephyrae were performed for 48 h. This discrepancy in the duration of the trials is due to the use of different methodologies, justified by the divergent nature of the two life stages of *A. coerulea*, given the differences in morphology and behavior in the life stages.

When comparing the sensitivity of *A. coerulea* to other organisms, it is important to consider the overlapping confidence intervals for LC_{50}/EC_{50} values. This comparison allows the contextualization of *A. coerulea* sensitivity within a broader ecological and toxicological framework. Our study highlights the distinct advantages of using *A. coerulea* polyps and ephyrae in antifouling and toxicological tests. Both *A. coerulea* life stages demonstrated high sensitivity to SDS, ZS and CC in comparison with other organisms used in toxicological tests (Table 2). The polyps' high sensitivity to SDS was 1.5× to 3.5× greater than that of widely used organisms like the planktonic crustacean *A. salina* (nauplio stage)[61,62], 5.5× more sensitive than the benthic crustacean *Monokaliapseudes schubarti*[11]. Similarly, polyps were 5.8× more sensitive than the benthic crustacean *Nitokra spinipes* (test with benthic phase; adult) to CC[36]. *Nitokra* species and *M. schubarti* are widely recommended for toxicological testing with water, elutriates, or sediments[63,64]. These findings suggest that *A. coerulea* is a highly promising benthic model organism for toxicological studies, offering a broader ecological and toxicological perspective.

The *A. coerulea* ephyrae (planktonic phase) also demonstrated good sensitivity when compared to other organisms. It demonstrated 46× greater sensitivity for SDS than *M. schubarti* (adult)[11], 28× higher than *A. salina* (nauplius)[61], 7× higher than *A. amphitrite* (larvae)[65], 6× higher than *A. coerulea* (polyp) (this study). For ZS, the sensitivity of *A. coerulea* ephyra was 3.65× higher than that of *A. salina*[62] and similar to *A. coerulea* polyps (present study). For CC, *A.*

coerulea (ephyrae) was 15× more sensitive than polyps (present study), 12× more sensitive than *A. amphitrite* (larvae)[66] and 6×more sensitive than *A. tonsa*[67].

Therefore, we demonstrate that the two stages of the life cycle of *A. coerulea* have different sensitivities, which vary depending on the reference substance used, with greater sensitivity to CC than other tested solutions. This was to be expected, since copper accumulates to a greater degree in kyphozoans than zinc[68], due to its ability to regulate intracellular copper concentrations[69]. While this can happen, metals may not be metabolically available, causing oxidative stress in your tissues, such as increased activity of reactive oxygen species (ROS) due to cellular damage[29]. This may have occurred in the tests performed with copper in the present study, as indicated by the disintegration at low concentrations of this metal. Analyses of ROS activity and lipid peroxidation should, however, be performed to confirm this statement. It is important to note that both the polyps and the ephyrae of *A. coerulea* demonstrate a more pronounced sensitivity than *M. schubarti* and *A. salina*, with a more pronounced sensitivity in the ephyrae.

The results of this study emphasize the potential of the aquatic macrophytes *C. caroliniana* and *S. californicus* as natural antifouling agents. Their extracts effectively inhibited *A. coerulea* polyp attachment without toxicity at practical concentrations (up to 60% for *C. caroliniana* and 15% for *S. californicus*). These compounds can be further extracted, isolated, and incorporated into environmentally friendly antifouling paints[12]. Additionally, the efficiency of *A. coerulea* polyps and ephyrae as test organisms (as shown in this study) offers significant advantages over traditional species, both in terms of assay duration and sensitivity. *A. coerulea* polyps are a better alternative than other macrofouling species, because both polyp and ephyrae stages can also be used in toxicological tests. For toxicological tests, *A. coerulea* demonstrated higher sensitivity compared to other widely used model toxicology study organisms, ephyrae sensitivity being more pronounced than that of polyps. By leveraging their unique life cycle, researchers can conduct rapid and comprehensive tests, paving the way for developing less harmful antifouling solutions.

5. Conclusion

Research on aquatic macrophyte extracts for biofouling control remains in its early stages, particularly regarding their effects on macrofouling organisms. This study evaluated the antifouling efficacy of aqueous extracts of *C. caroliniana* and *S. californicus* by examining their ability to inhibit the attachment of *A. coerulea* polyps, as well as their toxicity to polyps and ephyrae. It provides critical insights into the antifouling potential of macrophyte extracts, establishing *A. coerulea* as a promising model organism for antifouling and toxicological tests. Extracts from *C. caroliniana* and *S. californicus* inhibited up to 65% of *A. coerulea* polyp attachment while exhibiting no toxicity at practical concentrations (5% for *C. caroliniana* and 20% for *S. californicus*). These results underscore the potential of these extracts as environmentally friendly antifouling agents, paving the way for their use in developing natural antifouling paints.

We also highlight the potential use of *A. coerulea* as a test organism for the evaluation of new antifouling agents, since it can be used for anti-attachment and toxicological tests (two life cycle stages). Furthermore, *A. coerulea* demonstrated high sensitivity and rapid response times,

making it an efficient organism for assessing antifouling and toxicity. Its ease of cultivation and maintenance as well as reproducibility in tests further enhance its utility. For optimal results, we recommend chronic toxicity tests with polyps over 48 h and acute toxicity assessments over 96 h. The *A. coerulea* polyps are suitable for attachment assays, with their maximum response observable within 72 h. This rapid response time could streamline the process of evaluating antifouling agents.

Future research should focus on elucidating the mechanisms behind attachment inhibition through biomolecular studies, field-testing antifouling efficacy, and evaluating extract stability for incorporation as additives in antifouling paints. Additionally, isolating and characterizing bioactive compounds from the extracts could advance their incorporation into sustainable antifouling solutions. These efforts will contribute significantly to mitigating marine pollution and improving biofouling management in industrial applications.

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6. Author contributions

All authors contributed to the study conception and design. The first draft of the manuscript was written by Mikael Luiz Pereira Morales and all authors commented on previous versions of the manuscript. All authors read and approved of the final manuscript.

7. References

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Tables

Table 1: *Aurelia coerulea* selected endpoints.

Life stage	Assays		Observation time (h)
	Acute	**Chronic (sublethal)**	
Polyps	Disintegration	Contraction of the tentacles	1, 6, 24, 48, 72 and 96
		Capture and ingestion	48 and 96
Ephyrae	Immobility	Pulsation frequency	24 and 48

Table 2: LC_{50} or EC_{50} values for different marine test organisms following exposure to sodium dodecyl sulfate (SDS), zinc sulfate, copper chloride II and aqueous aquatic macrophyte extracts (*Cabomba caroliniana* and *Schoenoplectus californicus*).

Marine organisms	Species	Habitat	Substance	LC_{50} or EC_{50} (mg.L^{-1})	Confidence interval (95%)		Exposure time (h)	Authors
					Minimum	Maximum		
Crustacea	*Amphibalanus amphitrite* (larvae)	Planktonic	SDS	7.49	no information	no information	48	Greco et al., 2006
Mollusca	*Perna perna* (larvae)	Planktonic	SDS	0.86	0.79	0.91	48	Jorge e Moreira, 2005
Echinodermata	*Paracentrotus lividus* (sperm)	Planktonic	SDS	3.18	3.18	3.89	24	Mariani et al., 2006
Crustacea	*Artemia salina* (nauplius)	Planktonic	SDS	34.15	28.7	40.64	24	Rotini et al., 2015
Crustacea	*Tigriopus fulvus* (nauplius)	Benthic	SDS	8.52	8.00	9.17	96	Mariani et al., 2006
Fish	*Dicentrarchus labrax* (juveniles)	Planktonic	SDS	7.88	7.10	8.19	48	Mariani et al., 2006
Cnidaria	*Aurelia* sp. (ephyra)	Planktonic	SDS	1.55	no information	no information	48	Faimali et al., 2014
Cnidaria	*Aurelia coerulea* (ephyra)	Planktonic	SDS	1.00	0.28	2.56	48	This study
Crustacea	*Monokaliapseudes schubarti* (adult)	Planktonic	SDS	56.57	46.55	68.74	96	Perina et al., 2023
Crustacea	*Acartia tonsa* (adult)	Planktonic	SDS	2.08	1.84	3.34	48	Perina et al., 2023
Crustacea	*Tiburonella viscana* (adult)	Benthic	SDS	12.84	9.93	16.60	96	Perina et al., 2023
Cnidaria	*Aurelia coerulea* (polyp)	Benthic	SDS	8.66	6.24	12.00	96	This study
Mollusca	*Perna perna* (larvae)	Planktonic	Zinc Sulfate	0.54	0.50	0.57	48	Jorge e Moreira, 2005
Crustacea	*Parhyale hawaiensis* (neonates)	Planktonic	Zinc Sulfate	1.73	1.42	2.09	96	Artal et al., 2019
Crustacea	*Artemia salina* (nauplius)	Planktonic	Zinc Sulfate	10.00	5.00	13.00	48	Dobretsov et al., 2020

Cnidaria	*Aurelia coerulea* (ephyra)	Planktonic	Zinc Sulfate	2.72	2.24	3.31	48	This study
Crustacea	*Nitokra* sp.(adult)	Benthic	Zinc Sulfate	0.69	0.60	0.78	168	Artal et al., 2019
Cnidaria	*Aurelia* sp. (polyp)	Benthic	Zinc Sulfate	3.33	2.98	3.72	96	This study
Crustacea	*Eurytemora affinis* (nauplius)	Planktonic	Copper Chloride II	0.15	0.01	0.34	96	Heuschele et al., 2022
Crustacea	*Tigriopus fulvus* (nauplius)	Benthic	Copper Chloride II	0.05	0.01	0.01	96	Rinna et al., 2011
Cnidaria	*Aurelia coerulea* (ephyra)	Planktonic	Copper Chloride II	0.023	0.016	0.033	48	This study
Crustacea	*Acartia tonsa* (adult)	Planktonic	Copper Chloride II	0.12	0.05	0.20	48	Pinho et al., 2010
Crustacea	*Nitokra spinipes* (adult)	Benthic	Copper Chloride II	1.95	1.74	2.16	96	Heuschele et al., 2022
Crustacea	*Eurytemora affinis* (adult)	Benthic	Copper Chloride II	0.09	0.01	0.18	96	Heuschele et al., 2022
Cnidaria	*Aurelia coerulea* (polyp)	Benthic	Copper Chloride II	0.30	0.18	0.48	96	This study
Cnidaria	*Aurelia coerulea* (ephyra)	Planktonic	*C. caroliniana*	62.92	51.45	79.07	48	This study
Cnidaria	*Aurelia coerulea* (ephyra)	Planktonic	*S. californicus*	10.14	6.14	16.74	48	This study
Cnidaria	*Aurelia coerulea* (polyp)	Benthic	*C. caroliniana*	87.49	74.68	101.51	96	This study
Cnidaria	*Aurelia coerulea* (polyp)	Benthic	*S. californicus*	10.76	6.18	18.72	96	This study

Table 3: GC-MS (A) and LC-MS (B) analysis of aqueous extract of aquatic macrophytes (*Cabomba caroliniana* and *Schoenoplectus californicus*).

A- GC-MS Analysis				
		***C. caroliniana* extract**	***S. californicus* extract**	
Compound	**Molecular Formula**	**Area %**	**Area %**	**Chemical group**
n-Nonadecanol-1	$C_{19}H_{40}O$	24.39	3.42	Alcohol
Cyclohexadecane	$C_{16}H_{32}$	19.42	-	Ciclic Alkane
Eicosane	$C_{20}H_{42}$	12.78	32.90	Alkane
E-15-heptadecenal	$C_{17}H_{32}O$	-	16.08	Aldehyde
9-tricosene	$C_{23}H_{46}$	-	13.94	Alkene
B - LC-MS Analysis				
		***C. caroliniana* extract**	***S. californicus* extract**	
Compound	**Molecular Formula**	**Area %**	**Area %**	**Chemical group**
4-Methylphenethylamine	$C_9H_{13}N$	77.85	73.83	Aromatic compound
Diethanolamine	$C_4H_{11}NO_2$	19.35	-	Amines
Lysine hydrochloride	$C_6H_{14}N_2O_2ClH$	1.04	0.03	Amino acid
L-Lysine monohydrochloride	$C_6H_{15}ClN_2O_2$	-	13.79	Amino acid
Piperidinic acid	$C_4H_9NO_2$	-	6.36	Carboxylic acid

Figures legends

Fig. 1: Life cycle of the scyphomedusae *Aurelia coerulea* (Cnidaria, Scyphozoa) and endpoints of toxicological tests with polyps. Credit: Hugo Custódio.

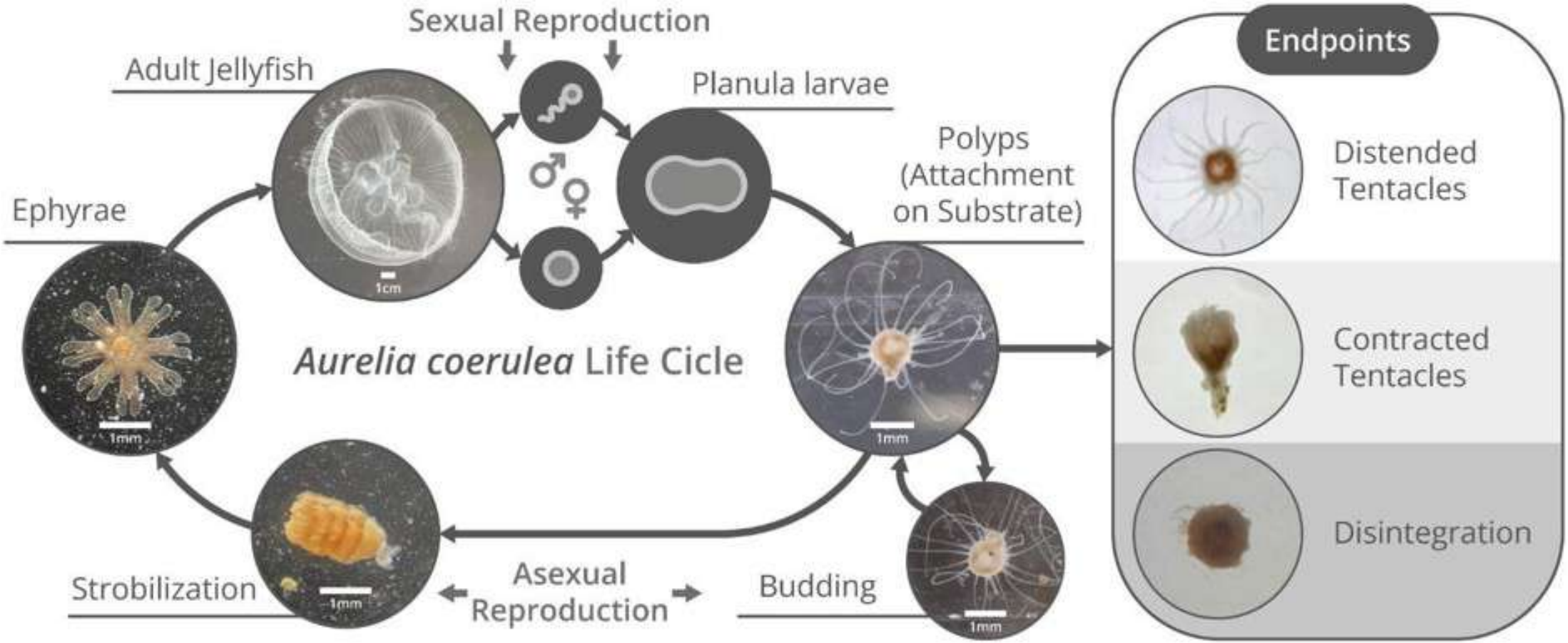
Fig. 2: Mean (± SD) of attachment (A-C) or tentacle contraction (D-F) of *Aurelia coerulea* polyps exposed to reference substance at different times. Different letters within each observation period: statistical difference between concentrations ($p<0.05$). ns= There was no significant difference between treatments.

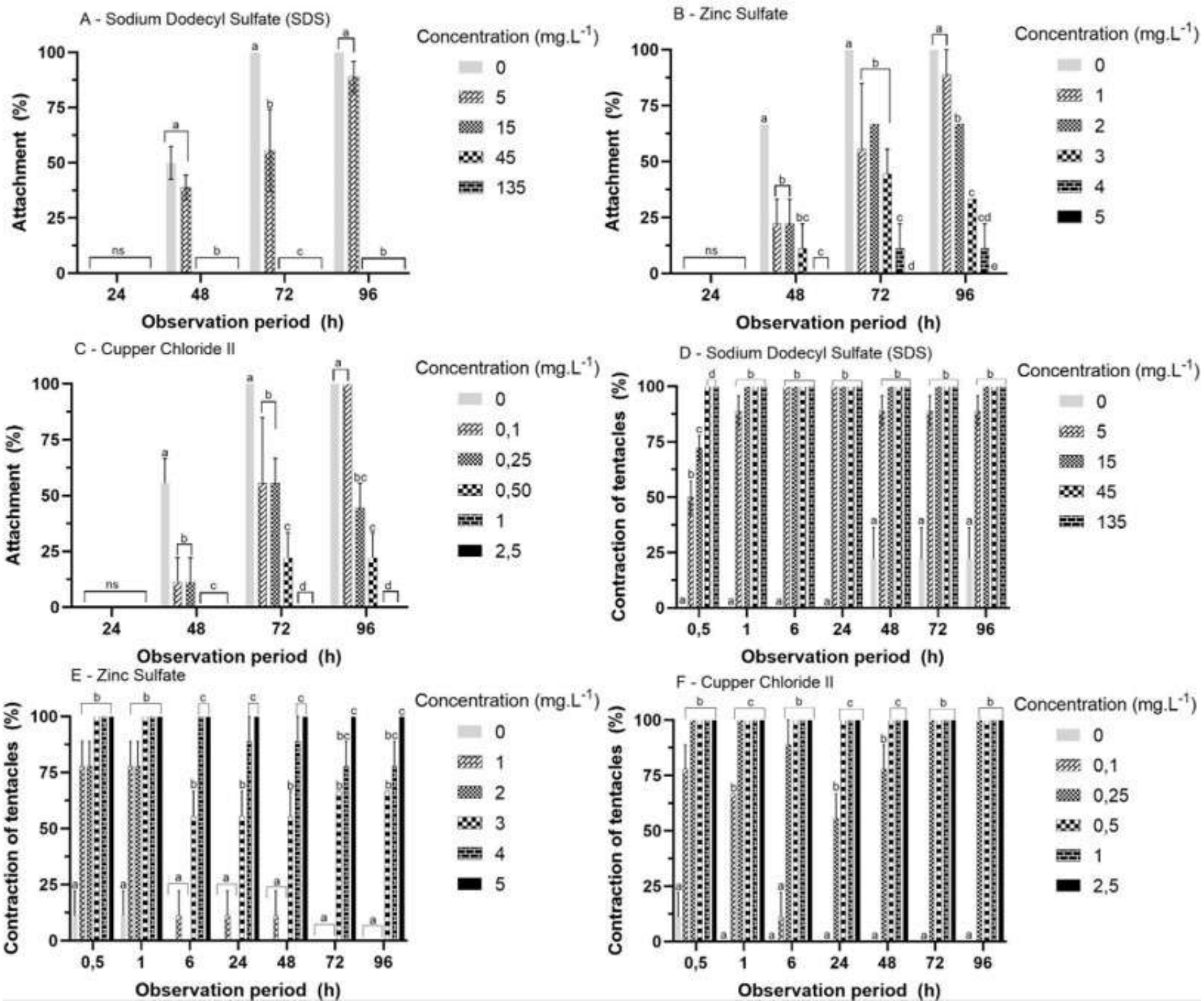
Fig. 3: Mean (± SD) of the response prey ingestion (A-C) and disintegration (D-F) of polyps of *Aurelia coerulea* following exposure to reference substance. Different letters within each observation period: statistical difference between concentrations ($p<0.05$).

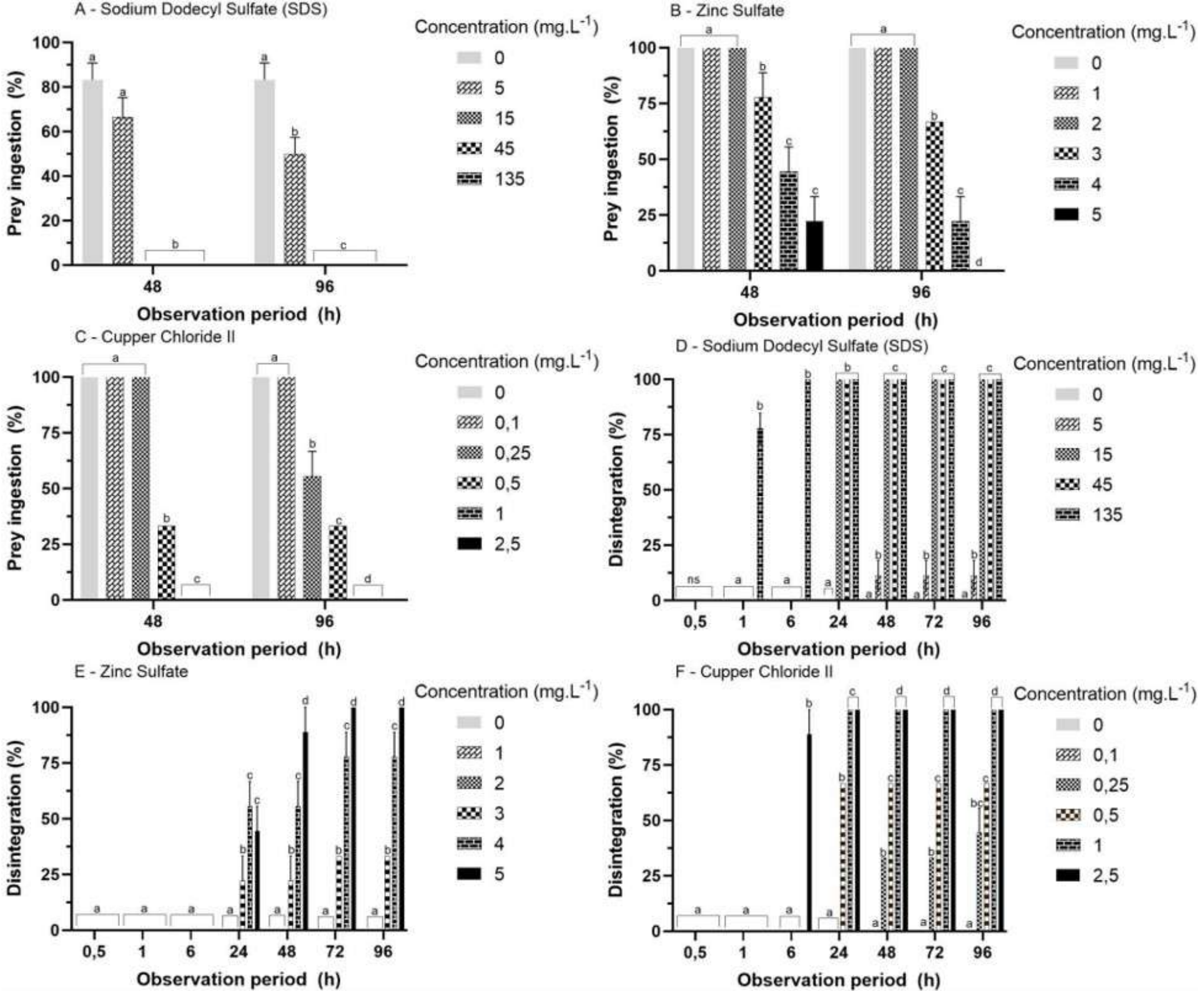
Fig. 4: Mean (± SD) of the frequency of pulsation (A-C) and immobility (D-F) response of ephyrae of *Aurelia coerulea* exposed to different reference substances. Different letters within each observation period: different between concentrations ($p<0.05$).

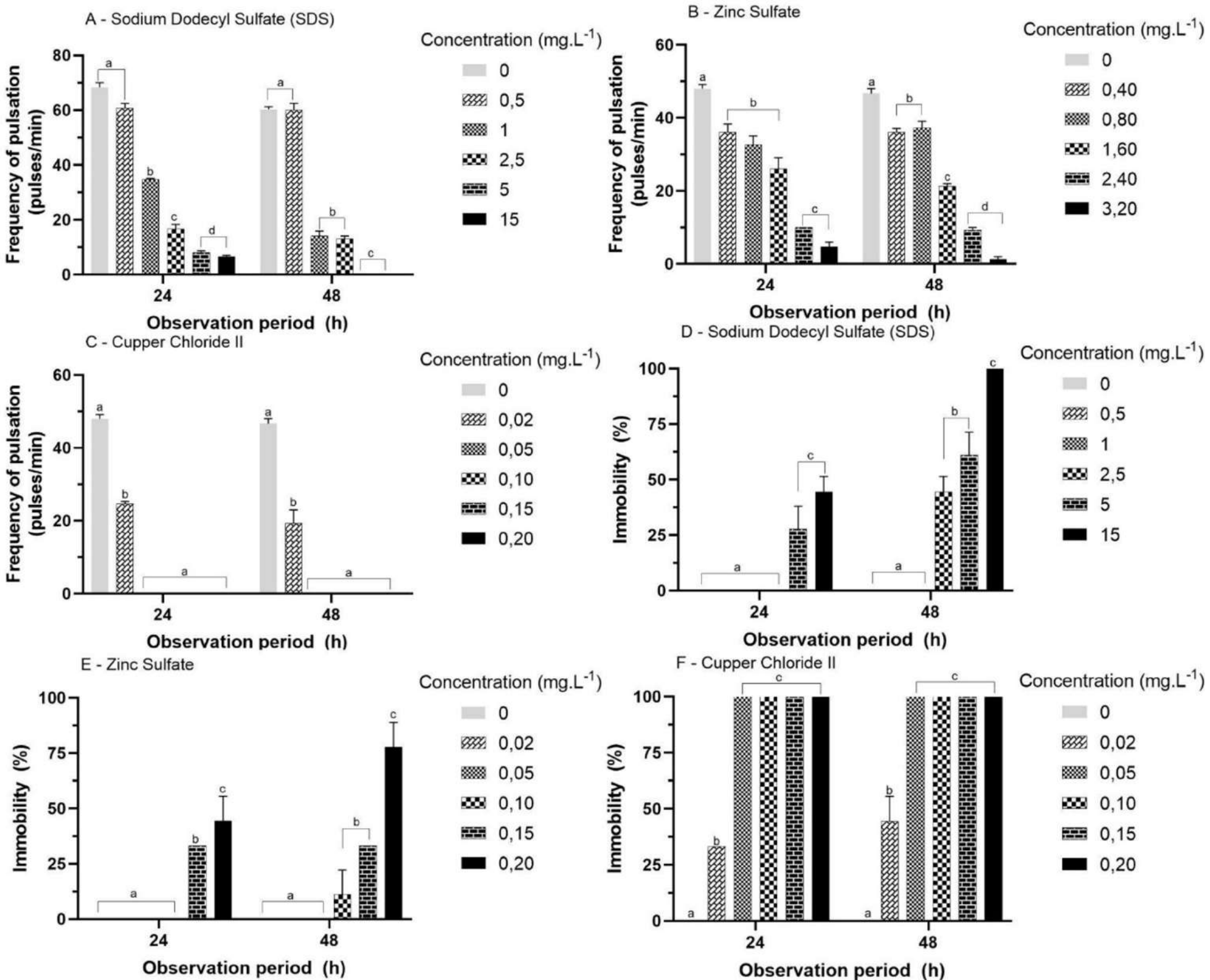
Fig. 5: Mean (± SD) of the attachment (A-B) contraction of tentacles (C-D), prey ingestion (E-F) response of polyps of *Aurelia coerulea* exposed to aqueous extracts of aquatic macrophytes *Cabomba caroliniana* and *Schoenoplectus californicus*. Different letters within each observation period: statistical difference between concentrations ($p<0.05$).

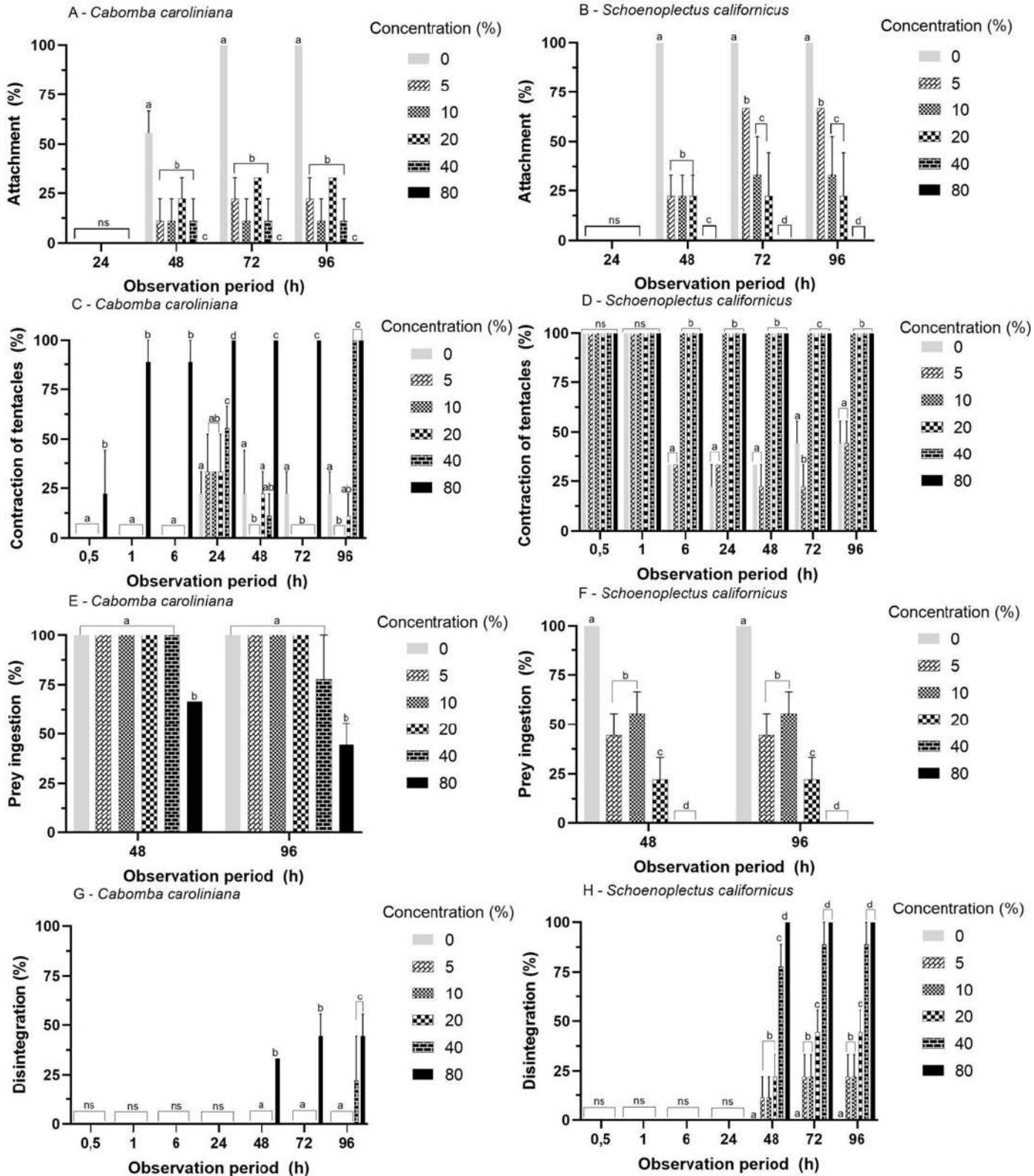
Fig. 6: Mean (± SD) of the frequency of pulsation (A-B) and immobility (C-D) response of ephyrae of *Aurelia coerulea* exposed to different extracts of aquatic macrophytes *Cabomba caroliniana* and *Schoenoplectus californicus*. Different letters within each observation period: statistical difference between concentrations ($p<0.05$).

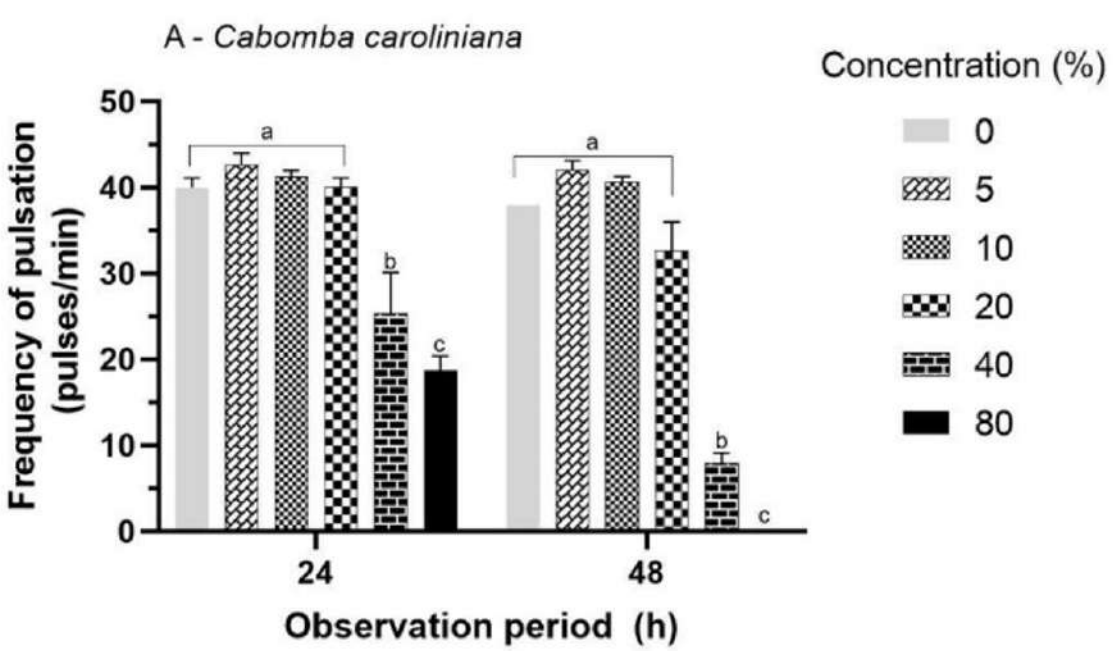

Sexual Reproduction
Adult Jellyfish
Planula larvae
Polyps
(Attachment
on Substrate)
Ephyrae
1cm
1mm
1mm
1mm
1mm
Aurelia coerulea Life Cicle
Strobilization
Asexual
Reproduction
Budding
Endpoints
Distended
Tentacles
Contracted
Tentacles
Disintegration

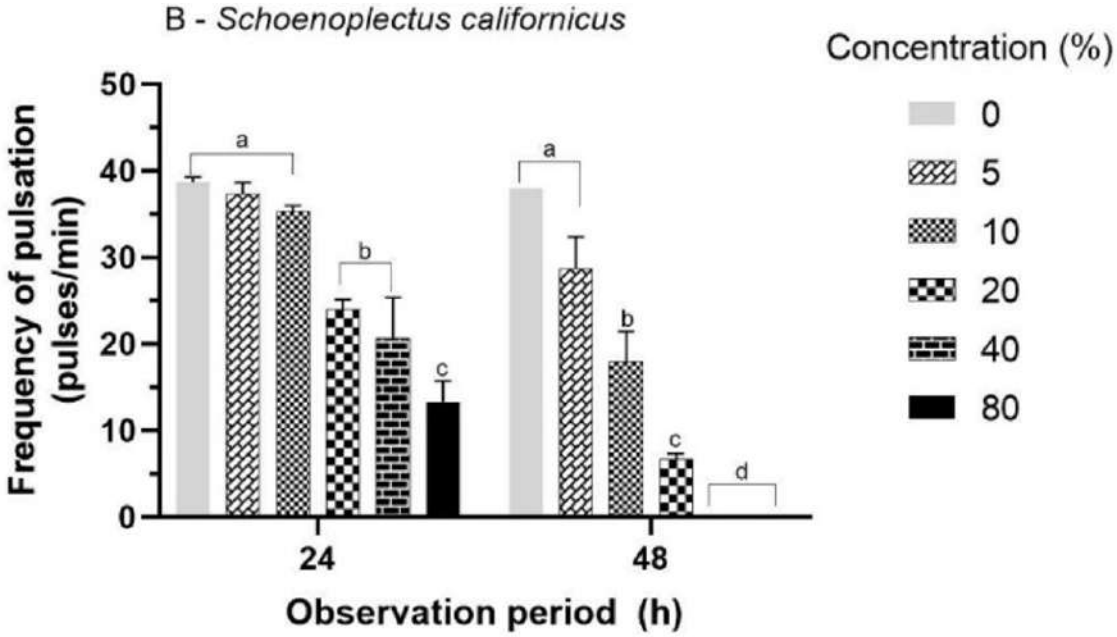

A - Sodium Dodecyl Sulfate (SDS)
Attachment (%)
Observation period (h)
Concentration (mg.L-1)
0
5
15
45
135
B - Zinc Sulfate
Attachment (%)
Observation period (h)
Concentration (mg.L-1)
0
1
2
3
4
5
C - Cupper Chloride II
Attachment (%)
Observation period (h)
Concentration (mg.L-1)
0
0,1
0,25
0,50
1
2,5
D - Sodium Dodecyl Sulfate (SDS)
Contraction of tentacles (%)
Observation period (h)
Concentration (mg.L-1)
0
5
15
45
135
E - Zinc Sulfate
Contraction of tentacles (%)
Observation period (h)
Concentration (mg.L-1)
0
1
2
3
4
5
F - Cupper Chloride II
Contraction of tentacles (%)
Observation period (h)
Concentration (mg.L-1)
0
0,1
0,25
0,5
1
2,5

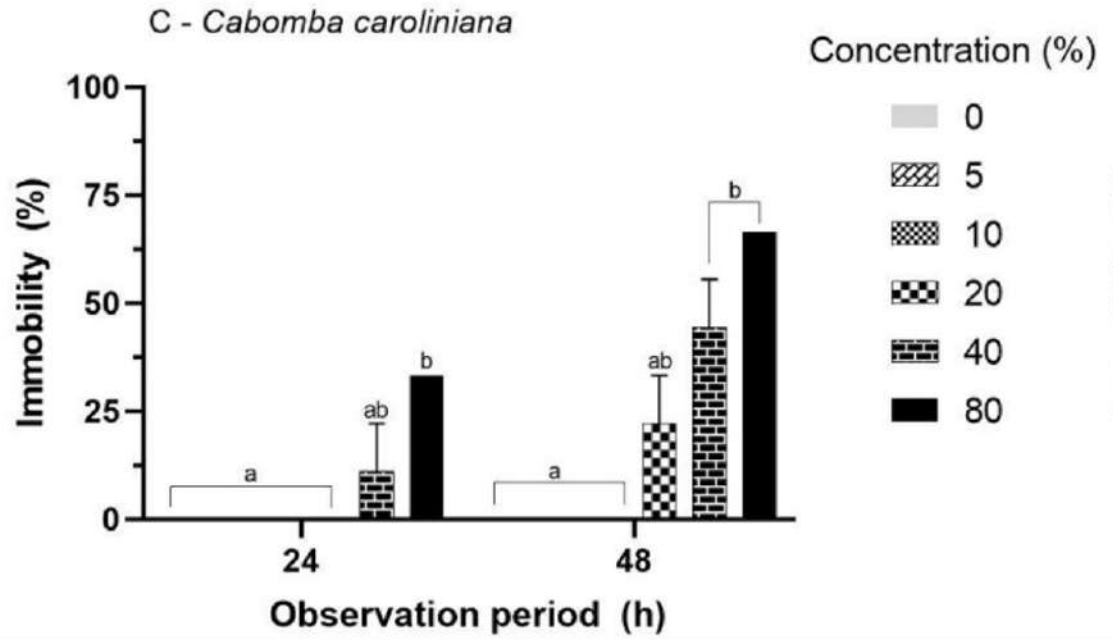

A - Sodium Dodecyl Sulfate (SDS)
Prey ingestion (%)
Observation period (h)
48
96
Concentration (mg.L-1)
0
5
15
45
135
B - Zinc Sulfate
Prey ingestion (%)
Observation period (h)
Concentration (mg.L-1)
0
1
2
3
4
5
C - Cupper Chloride II
Prey ingestion (%)
Observation period (h)
Concentration (mg.L-1)
0
0,1
0,25
0,5
1
2,5
D - Sodium Dodecyl Sulfate (SDS)
Disintegration (%)
0,5
1
6
24
48
72
96
Observation period (h)
Concentration (mg.L-1)
0
5
15
45
135
E - Zinc Sulfate
Disintegration (%)
Observation period (h)
Concentration (mg.L-1)
0
1
2
3
4
5
F - Cupper Chloride II
Disintegration (%)
Observation period (h)
Concentration (mg.L-1)
0
0,1
0,25
0,5
1
2,5

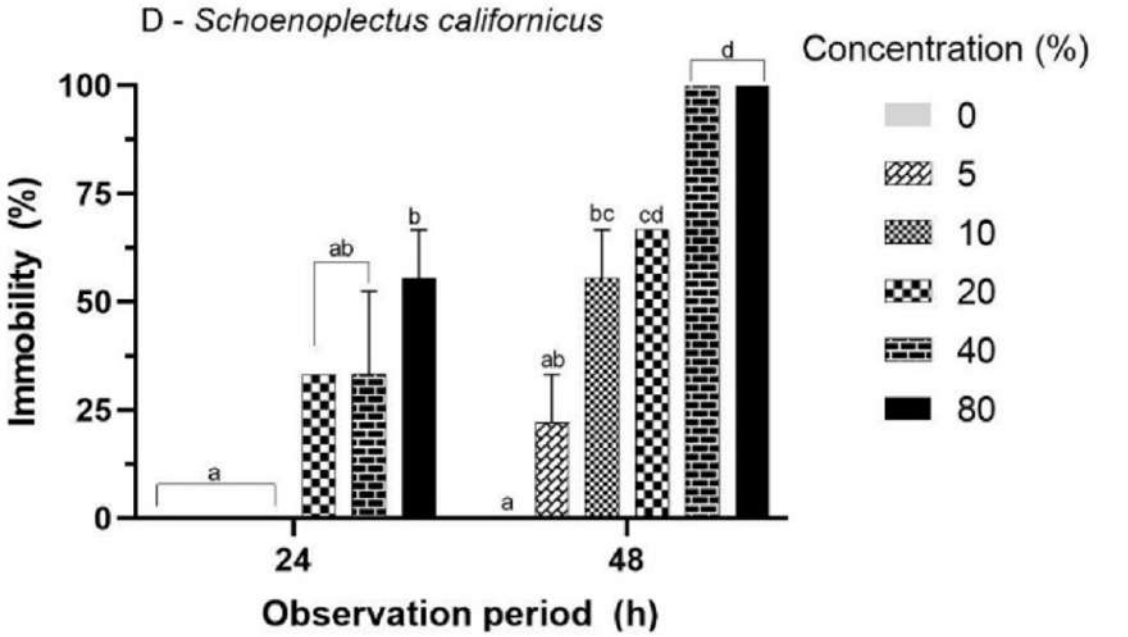

A - Sodium Dodecyl Sulfate (SDS)
Frequency of pulsation (pulses/min)
Observation period (h)
Concentration (mg.L-1)
0
0,5
1
2,5
5
15
B - Zinc Sulfate
Frequency of pulsation (pulses/min)
Observation period (h)
Concentration (mg.L-1)
0
0,40
0,80
1,60
2,40
3,20
C - Cupper Chloride II
Frequency of pulsation (pulses/min)
Observation period (h)
Concentration (mg.L-1)
0
0,02
0,05
0,10
0,15
0,20
D - Sodium Dodecyl Sulfate (SDS)
Immobility (%)
Observation period (h)
Concentration (mg.L-1)
0
0,5
1
2,5
5
15
E - Zinc Sulfate
Immobility (%)
Observation period (h)
Concentration (mg.L-1)
0
0,02
0,05
0,10
0,15
0,20
F - Cupper Chloride II
Immobility (%)
Observation period (h)
Concentration (mg.L-1)
0
0,02
0,05
0,10
0,15
0,20


A - Cabomba caroliniana
B - Schoenoplectus californicus
C - Cabomba caroliniana
D - Schoenoplectus californicus
E - Cabomba caroliniana
F - Schoenoplectus californicus
G - Cabomba caroliniana
H - Schoenoplectus californicus
Attachment (%)
Contraction of tentacles (%)
Prey ingestion (%)
Disintegration (%)
Observation period (h)
Concentration (%)
0
5
10
20
40
80
ns
24
48
72
96
0,5
1
6


A - Cabomba caroliniana
Frequency of pulsation (pulses/min)
Observation period (h)
24
48
Concentration (%)
0
5
10
20
40
80


B - Schoenoplectus californicus
Frequency of pulsation (pulses/min)
Observation period (h)
24
48
Concentration (%)
0
5
10
20
40
80


C - Cabomba caroliniana
Immobility (%)
Observation period (h)
24
48
Concentration (%)
0
5
10
20
40
80


D - Schoenoplectus californicus
Immobility (%)
Observation period (h)
24
48
Concentration (%)
0
5
10
20
40
80

Appendices (Supplementary material)

Table S1: GC-MS analysis of aqueous extract of aquatic macrophytes (*Cabomba caroliniana* and *Schoenoplectus californicus*).

GC-MS Analysis						
		***C. caroliniana* extract**		***S. californicus* extract**		
Compound	**Molecular Formula**	**R.Time**	**Area %**	**R.Time**	**Area %**	**Chemical group**
1-Dodecen-3-ol	$C_{12}H_{24}O$	-	-	21.310	0.34	Alcohol
1-Hexen-4-ol, 4-cyclohexyl-3-methyl	$C_{13}H_{24}O$	-	-	11.310	0.88	Alcohol
1H-Tetrazole-1-ethano	$C_3H_6N_4O$	13.435	3.16	-	-	Alcohol
1-Pentadecene	$C_{15}H_{30}$	-	-	11.249	4.32	Alkene
1-Tetradecene	$C_{14}H_{28}$	11.266	7.08	-	-	Alkene
5,5-Diethylpentadecane	$C_{19}H_{40}$	14.746	3.48	14.050	0.39	Alkane
5-Hydroxy-6-methyl-12,13-dioxa-tricyclo	$C_{14}H_{22}O_5$	18.495	2.15	-	-	Alcohol
5-Methyldodecane	$C_{13}H_{28}$	-	-	7.103	1.47	Alkane
9-Tricosene	$C_{23}H_{46}$	-	-	15.321	13.94	Alkene
alpha.-D-Xylofuranose, cyclic 1,2:3,5-bis(methylboronate)	$C_7H_{12}B_2O_5$	21.395	1.97	-	-	Boronated carbohydrate
Cyclohexadecane	$C_{16}H_{32}$	13.334	19.42	-	-	Ciclic Alkane
Decane, 5,6-dimethyl-	$C_{12}H_{26}$	-	-	15.465	0.54	Alkane
Dodecane, 2,6,11-trimethyl	$C_{15}H_{32}$	9.860	3.32	9.846	5.41	Alkane
		-	-	10.405	2.11	
		-	-	11.945	1.52	
E-15-Heptadecenal	$C_{17}H_{32}O$	-	-	13.325	16.08	Aldehyde
Eicosane	$C_{20}H_{42}$	12.230	5.08	12.220	8.37	Alkane
		14.317	5.07	12.319	1.50	
		17.536	2.63	12.694	8.01	

		-	-	14.313	6.96	
		-	-	14.744	3.08	
		-	-	16.819	3.11	
		-	-	17.528	1.87	
Heptane	C_7H_{16}	-	-	4.302	0.59	Alkane
Hexadecane, 1-iodo-	$C_{16}H_{33}Eu$	-	-	12.805	2.10	Alkane
Hexadecane, 2,6,11,15-tetramethyl-	$C_{20}H_{42}$	12.699	5.18	-	-	Alkane
Methoxyacetic acid, 4-hexadecyl ester	$C_{19}H_{38}O_3$	-	-	21.383	2.73	Ester
Methyl 13,16-docosadienoate	$C_{23}H4_2O_2$	18.065	3.58	-	-	Methyl ester
N-Methyl-10-hydroxydecahydroquinoline	$C_{10}H_{19}NO$	16.876	1.87	-	-	Quinoline
n-Nonadecanol-1	$C_{19}H_{40}O$	15.331	16.67	18.366	3.42	Alcohol
		18.376	7.72	-	-	
Nonane, 5-methyl-5-propyl	$C_{13}H_{28}$	-	-	10.515	0.79	Alkane
Octadecane, 5-methyl	$C_{18}H_{38}$	16.830	2.33	12.900	0.49	Alkane
Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	12.597	5.49	12.586	4.51	Fenol
Piperidine, 1-(1,2,3,4-tetrahydro-2-naphthaleny	$C_{23}H_{26}FNO$	18.284	1.77	-	-	Piperidine
Toluene	C_7H_8	-	-	3.886	1.48	Aromatic compounds
Tridecanol, 2-ethyl-2-methyl-	$C_{16}H_{34}O$	-	-	14.976	1.07	Alcohol
Undecane, 2-methyl-	$C_{12}H_{26}$	-	-	13.435	2.01	Alkane
Z-5-Methyl-6-heneicosen-11-one	$C_{22}H_{42}O$	17.280	2.00	-	-	Ketone

Table S2: LC-MS analysis of aqueous extract of aquatic macrophytes (*Cabomba caroliniana* and *Schoenoplectus californicus*).

		***C. caroliniana* extract**			***S. californicus* extract**			
Compound	**Molecular Formula**	**R.Time**	**Area %**	**Base Peak m/z**	**R.Time**	**Area %**	**Base Peak m/z**	**Chemical group**
2-Trifluoromethyl-benzenesulfonamide	$C_7H_6F_3NO_2S$	5.097	0.56	224.80	5.142	0.28	224.80	Aromatic compound
4-Methylphenethylamine	$C_9H_{13}N$	4.694	32.75	135.55	4.783	41.84	135.55	Aromatic compound
		5.109	45.10		5.133	31.99		
Acridone	$C_{13}H_9NO$	4.492	0.34	194.90	4.473	0.37	194.90	Aromatic compound
		4.768	0.27					
Benzamidine hydrochloride	$C_7H_9ClN_2$	0.494	0.13	121.10	-	-	-	Aromatic compound
D-cysteine	$C_3H_7NO_2S$	4.325	0.44	121.15	-	-	-	Amino acid
Diethanolamine	$C_4H_{11}NO_2$	5.342	3.78	105.20	-	-	-	Amines
		5.501	15.51					
		35.580	0.06					
Lysine Hydrochloride	$C_6H_{14}N_2O_2ClH$	33.434	0.10	146.20	6.367	0.03	146.20	Amino acid
		35.168	0.11		22.855	0.06		
		37.267	0.10					
		37.512	0.12					
		37.967	0.35					
		38.343	0.09					
		39.023	0.17					
Benzaldehyde	C_7H_6O	-	-	-	5.425	3.52	106.25	Aromatic compound
Isonicotinic acid	$C_6H_5NO_2$	-	-	-	5.367	1.76	123.15	Carboxylic acid
L-Lysine monohydrochloride	$C_6H_{15}ClN_2O_2$	-	-	-	5.608	13.42	146.15	Amino acid
					28.118	0.03		
					18.300	0.05		
					28.571	0.04		

					29.213	0.09		
					32.372	0.06		
					33.300	0.04		
					33.706	0.06		
Piperidinic acid	$C_4H_9NO_2$	-	-	-	4.373	6.36	103.30	Carboxylic acid

Table S3: FTIR analysis of aqueous extract of aquatic macrophytes (*Cabomba caroliniana* and *Schoenoplectus californicus*).

Phytocompounds identified	Functional groups	***C. caroliniana* extract**	***S. californicus* extract**
		Wave number (cm^{-1})	**Wave number (cm^{-1})**
Primary Amine	N-H stretch	3334.58	-
Aldehyde	C-H	2977.48	-
Amide	C=O stretch/ Aliphatic C=C stretching	1636.38	1633,9
Fluoro Compound	C-F stretch	1100.11	-
Alcohol-Hydrogen bonded	O-H stretch	-	3353,08
Alkyne	C≡C stretch	-	2105,96
Alcohol	O-H Bend / N-H deformation	-	1418,2
Aliphatic Ether	C-F Stretch/ C-O	-	1106,67
Halo Compound	C-Br	-	603,8

Figure S1: GC-MS analysis, peaks of aqueous extract of aquatic macrophytes. A - *Cabomba caroliniana*; B - *Schoenoplectus californicus*.

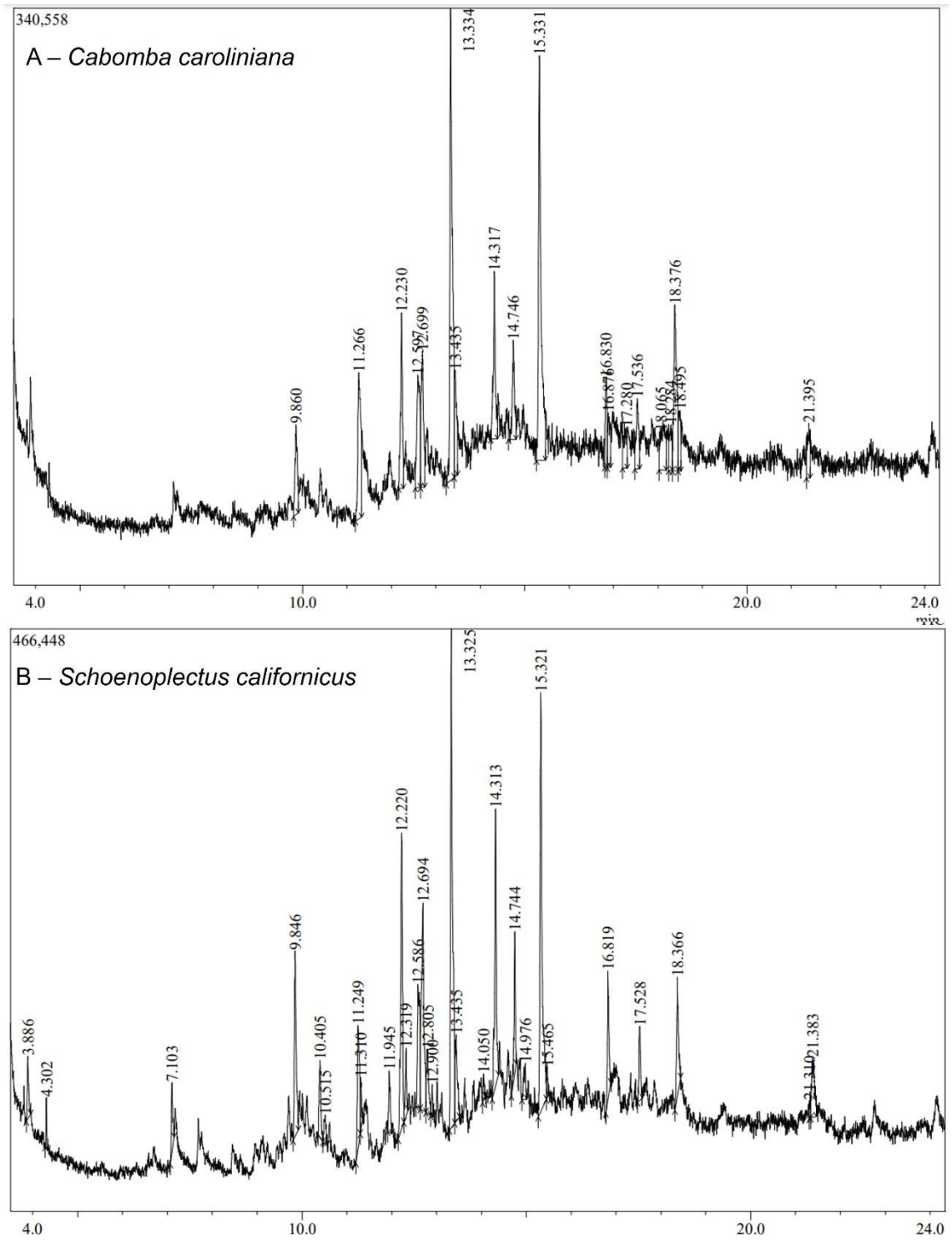
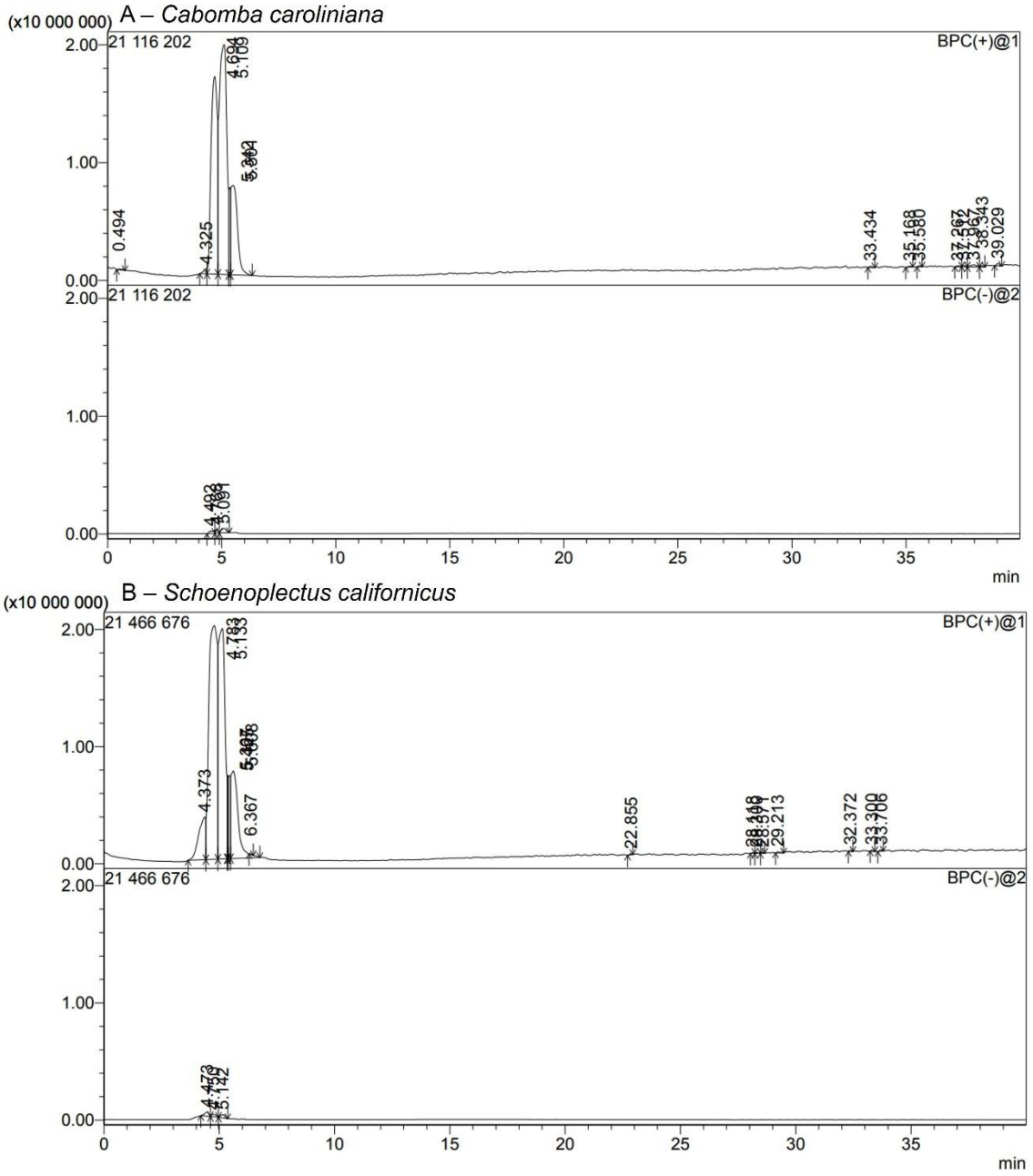


Figure S2: LC-MS analysis, peaks of aqueous extract of aquatic macrophytes. A - *Cabomba caroliniana*; B - *Schoenoplectus californicus*.



Capítulo X: Artigo 5

O quinto artigo científico proveniente desta Tese de Doutorado é apresentado neste capítulo. O manuscrito, de autoria de Mikael Luiz Pereira Morales, Leandro Capurro, Facundo Bordert, Hafizah Chenia, Cecília Alonso, Fabiana Rey Bentos, Lucía Boccardi, Ernesto Brugnoli, Ng Haig They, Grasiela Lopes Leães Pinho e Vanessa Ochi Agostini, intitulado-se "***Evaluating macrophyte extracts as eco-friendly antifouling additives for freshwater made-man structures paints: a in situ experiment***", será submetido no periódico "***Environmental Pollution***".

Evaluating macrophyte extracts as eco-friendly antifouling additives for freshwater made-man structures paints: a in situ experiment

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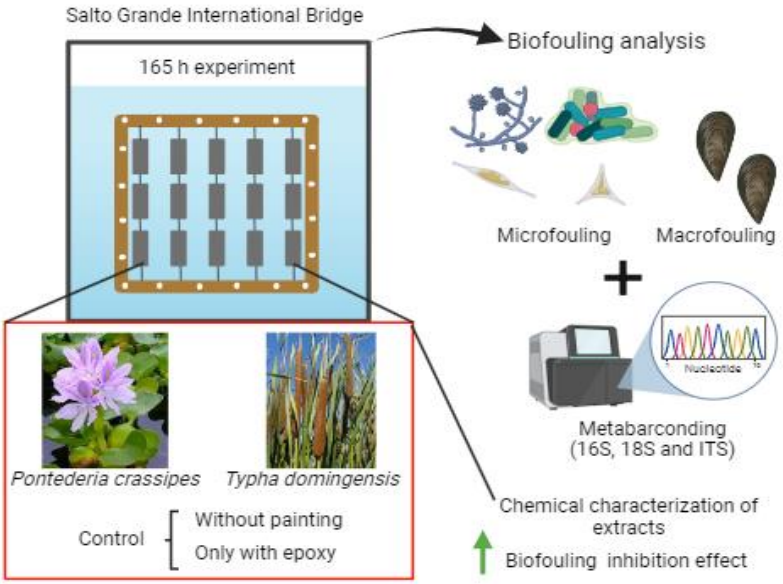
Abstract

Biofouling developed on made-man aquatic surfaces causes significant economic losses. In view of this, an environmentally safe alternative to combat biofouling is the use of natural compounds with antifouling action. In this context, extracts of the aquatic macrophytes *Pontederia crassipes* and *Typha domingensis* have been reported as promising antifouling sources. However, studies to evaluate their antifouling efficacy in the field have not yet been reported. Thus, this study evaluated the antifouling efficacy of their extracts combined with epoxy coating in a natural environment. For this, stainless steel substrates were coated with concentrations of 2.5, 5 and 10 g. $^{L-1}$ of *P. crassipes* and *T. domingensis* lyophilized extracts with the epoxy base, and two control treatments (uncoated and epoxy-coated). The ability of the different treatments to inhibit biofouling was evaluated for 165 h through analyses of chlorophyll, total bacteria, presence of macroorganisms and their frequency of taxa. We also identified the compounds present in the extracts by gas and liquid chromatography, and fourier transmission infrared spectroscopy. The painting with *P. crassipes* was more effective in inhibiting biofouling, which was better at concentrations of 5 and 10 g. $^{L-1}$. This effect was seen by the decrease in the frequency of bacteria, algae, fungi and macroorganisms, and an increase in the frequency of some more opportunistic taxa in the face of exposure to treatments containing the extracts. With this, we have proven the antifouling effect in the field of extracts of these macrophytes mixed with the epoxy coating, *P. crassipes* being more effective, making them excellent candidates for the development of natural antifouling alternatives. We also highlight that these plants are easy to obtain a large volume of biomass, facilitating their industrial development as an antifouling.

Keywords: Aquatic plants, biotechnology, freshwater antifouling, natural compounds.

Graphical abstract



Highlights

Pontederia crassipes macrophyte extracts were more effective than *Typha domingensis* to inhibit micro- and macrofouling.

For both extracts, biofouling inhibition occurred for micro and macrofouling.

Biofouling inhibition occurred for bacteria, algae, fungi and macroorganisms.

Macrophyte extracts induced an increase of opportunistic and strategist taxa.

1. Introduction

Biofouling causes major industrial (Xiao et al., 2020) and economics (Dobretsov and Rittschof, 2020) losses on artificial surfaces exposed to aquatic environments, especially when it involves the attachment of invertebrates and invasive species such as the golden mussel (*Limnoperna fortunei*). In hydroelectric power plants, freshwater mussels colonize hydraulic sensors, turbine cooling systems, and chambers, causing the obstruction of these structures (Brugnoli et al., 2005; Brugnolli et al., 2011). They also cause wear and tear and changes in the conformation of structures of water treatment plants, refineries, steel mills and aquaculture and forestry agro-industrial systems (Boltovskoy and Correa, 2015; Fabián et al., 2021; Maranhão and Stori, 2019). As a result, it is estimated that the industry dependent on water resources has a global expenditure of more than 340 million dollars per year (Cuthbert et al., 2021), including prevention of biofouling and maintenance of damaged structures.

Nowadays natural antifouling alternatives have gained great attention from researchers (Agostini et al., 2021b) to combat biofouling, since these can be ecologically safer and less harmful to the environment (Morales et al., 2024a, 2024b), due to their higher biodegradability and potential lower toxicity to non-target organisms (Pérez et al., 2021). Recent studies in laboratory experiments pointed to the aquatic macrophytes *Pontederia crassipes* Mart. (formerly *Eichhornia crassipes* Mart. Solms) and *Typha domingensis* Pers. as strong candidates as a source of natural compounds with antifouling potential for freshwater environments (Morales et al., 2024b).

P. crassipes (water hyacinth) species are native to the Amazon basin in South America (Ayanda et al., 2020) and later introduced in all continents (Guimarães et al., 2017). *T. domingensis* (cattail) is native to Brazil, but is currently distributed worldwide (Cruz et al., 2023), in tropical and temperate climate regions (Gomes et al., 2014). Both species have high growth and development rates, which guarantees success in the invasion of different types of aquatic ecosystems and environmental conditions, being considered invasive in many places around the world (Ayanda et al., 2020; Hegazy et al., 2011). Despite the threat to ecosystems, due to their rapid proliferation and easy obtention of large biomass (Amarilla et al., 2024; Cruz et al., 2023; Guimarães et al., 2017; Su et al., 2018), *P. crassipes* e *T. domingensis* are receiving attention for use in biotechnology, such as bioremediation, biogas and biofuel production, and as an antimicrobial and antifouling agent (Amarilla et al., 2024; Dilshad et al., 2024; Morales et al., 2024b; Su et al., 2018).

Morales et al. (2024b) found that aqueous extracts of these plants have antifouling activity against the formation of single and multispecies bacterial biofilms, as well as the adhesion of the golden mussel (*Limnoperna fortunei*), without causing toxicity to model organisms *Pseudopediastrum boryanum*, *Daphnia magna* e *Pimephales promelas* at concentrations of up to 35%. However, the antifouling effect of *P. crassipes* and *T. domingensis* in a natural

environment has not yet been explored and validated. *In situ* validation tests allow studies to be carried out under environmental conditions and complex interactions between fouling organisms and the hydrodynamics of the environment (Romeu and Mergulhão, 2023), then *preliminary in vitro* tests.

To carry out these tests and their commercial application, natural compounds can be incorporated into paints (Hamidi et al., 2022). Among these, epoxy coatings are widely used for this purpose, due to their excellent mechanical properties, chemical resistance (Vijayan et al., 2022) and release of the antifouling compounds over time through leaching (Pereira et al., 2024). Thus, the present study aimed to evaluate the antifouling efficacy of extracts of *P. crassipes* and *T. domingensis* associated with epoxy coating in a natural environment, the Salto Grande Hydroelectric dam, Uruguay.

2. Materials and methods

2.1. Painting preparation

Aqueous extracts of *P. crassipes* and *T. domingensis* were prepared according to Morales et al. (2024b). In summary, 6 g of dry plant mass was mixed with 300 mL of sterile natural water and then the solution was lyophilized (LIOTOP, L101). The remaining dry mass was then mixed with Hempadur Base 15579 epoxy paint (Hempel - 15570) to obtain the treatments 2.5, 5 and 10 g L^{-1} (g of lyophilizate L^{-1} of paint). This paint base is widely used by professionals in the aquatic industry in South America and in its composition in addition to epoxy resin, it features xylene, talc, titanium dioxide, bultan-1-ol, n-butyl acetate, 1,3-bis benzene, and toluene (as per product description). In addition to these treatments, two control treatments were also used: the control with the original substrate, without coating (control A); and the control coated only with epoxy paint (control B).

2.2. Experimental procedure

With the aid of a polyester foam roller, these treatments were used to coat stainless steel substrates (12.5 cm^2). The substrates were placed in frames (Fig. 1B) and exposed vertically (Agostini et al., 2019) in the aquatic environment (Fig. 1A) near to the Salto Grande hydroelectric power plant – Uruguay (31°16'11.8" S 57°56'44.7" W) for 165 h, at a depth of 1 m (Fig. 1C) in April 2023. Samples were taken daily to analyze different variables (see below).

2.3. Study area

Salto Grande is a subtropical fluvial reservoir of approximately 750 km^2 with multiple arms located along 100 km of the main channel of the Uruguay River, with an average depth of 6.4 m, a maximum of 35 m, an average annual temperature of 19 ºC and annual precipitation of 1,260 mm. It is characterized by a period of ebb and flow from December to March and floods from April to November, with a maximum discharge of 5,563 $m^3\ s^{-1}$ and a minimum of 22,000 $m^3\ s^{-1}$ (O'Farrell et al., 2012). The reservoir is primarily used for power generation,

but also for drinking water supply and recreational activities, including sports and fishing. The reservoir is also characterized by the proliferation of cyanobacterial blooms, which varies according to the hydrological conditions of ebb and water level and attains greatest abundances on the right bank of the reservoir and in the coastal areas closest to the dam (O'Farrell et al., 2012). The main water systems (Paraná River and Prata River) that connect the study region have a high abundance of *Limnoperna fortunei* (golden mussel) (Fabián et al., 2021; Silva et al., 2021). This species is one of the most harmful macrofouling for aquatic industries (Pereira et al., 2022). As a result, the environmental management area of Salto Grande has been facing challenges to combat biofouling in the region.

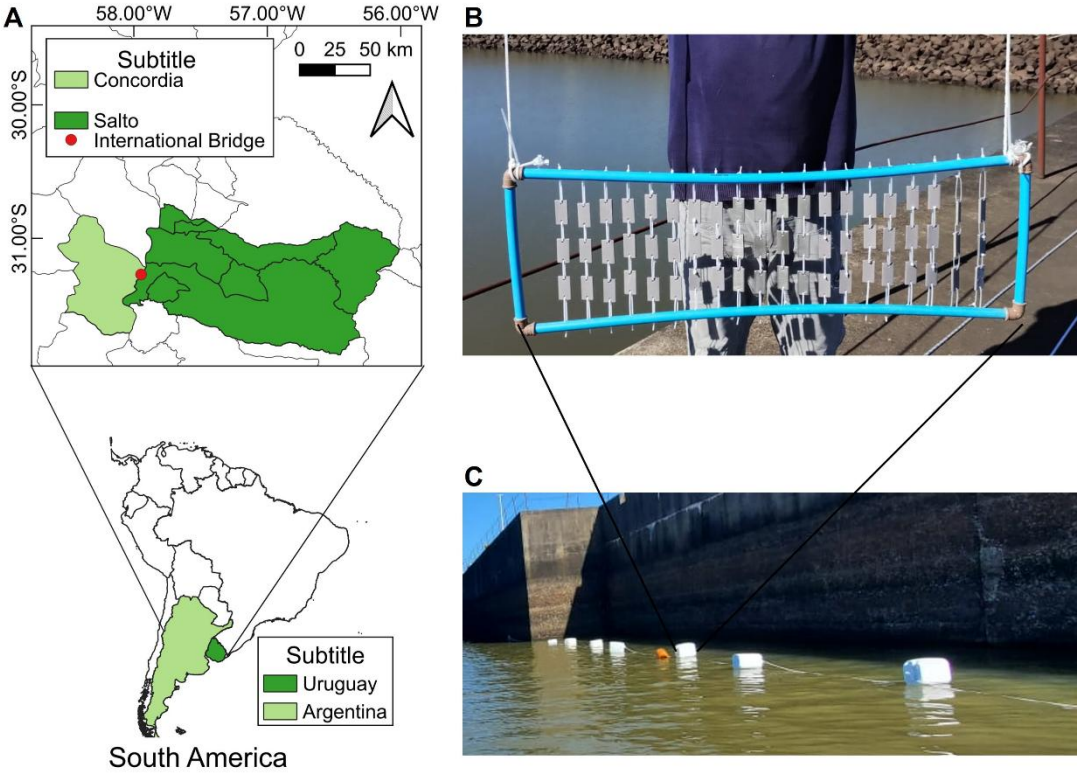


Fig. 1: Experimental site location, surroundings of the Salto Grande International Bridge – Uruguay/Argentina (A) and submerged frames with the substrates painted with antifouling treatments (B-C).

2.3. Monitoring of environmental and biological data

For each sampling period, the environmental variables temperature (°C), dissolved oxygen (mg L^{-1}), electrical conductivity (µS cm^{-1}), pH, and turbidity (NTU) through a multiparameter probe (YSI DSpro). Water transparency (cm) was also evaluated using the Sechi method (30 cm ⌀). Chlorophyll-*a* (mg m^3), suspended solids (mg L^{-1}), phosphate (mg L^{-1}), total phosphorus (mg L^{-1}), total nitrogen (mg L^{-1}), total ammoniacal nitrogen (TAN) (mg L^{-1}), nitrogen dioxide (mg L^{-1}), nitrate (mg L^{-1}), and abundances of *Microcystis* spp. (cel mL^{-1}), *Dolichospermum* spp. (cel mL^{-1}) and Cyanobacteria (cel mL^{-1}), were obtained from data from the ecology sector of the Environmental Management area of the

Mixta Grande Technical Commission (https://www.saltogrande.org/organizacion.php).

2.4. Biofouling analysis

For the analysis, at exposure times of 23, 46, 70, 94, 118, 142 and 165 h, three replicates (three substrates) per treatment were removed for each microfouling (MIC) and macrofouling (MAC) analysis and stored in falcon tubes with 40 mL of 0.4% sterile saline solution for MIC and tubes containing 40 mL of 4% formaldehyde for MAC. Before storing the substrates in the container, they were washed 3x with sterile saline solution (0.4%) to remove loose material or planktonic organisms from the samples. The MIC samples were stored in the dark and refrigerated at -18 °C and processed immediately, while the MAC samples were refrigerated at -18 °C and processed later.

2.4.1. Microfouling analysis

For microfouling analysis, immediately after collection the substrates were scraped with the aid of a sterile metal microbiological loop in the falcon tube and manually stirred. For each analysis, 3 replicates were performed per treatment. An aliquot (1.8 mL) of the samples was taken and stored in a cryotube containing paraformaldehyde/glutaraldehyde (1:0.05%) and for approximately 1-2 weeks at -20 °C (Krock et al., 2015) to analyze total heterotrophic bacteria by flow cytometry.

After thawing, samples were sonicated to improve cell dispersion for five minutes (40 hz) in an SB 3200 DTN ultrasonic cleaner. Prior to downstream analyses, the cells were stained with SYBR-Green I (SYBR-I, 1:30 dilution of commercial stock; Invitrogen, USA) diluted in dimethyl sulfoxide (DMSO, Merck, Germany) (Marie et al., 2005) in a ratio of 0.001:1 stain:sample for 15 minutes in the dark at room temperature. The analyses were performed on the Apogee-A40 flow cytometer (Apogee Flow Systems, UK) equipped with argon laser (488 nm). The total bacteria were counted by identifying and delimiting the cytometric population to a 488Green (peak) x 488Red (peak) scatter plot using the FLowJo software (v.10.10). The results were expressed as cell cm^{-2} for each treatment.

Subsequently, the remaining samples of the falcon tube were homogenized for the analysis of autotrophic organisms by measuring total chlorophyll (µg L^{-1}), chlorophyll-*a* (µg L^{-1}) and photosynthetic activity (%) of Green algae, Bluegreen (Cyanobacteria), Diatoms/Dinoflagellates, Cryptophytes with the Algae Online Analyser bbe Moldaenke equipment. Photosynthetic activity was measured by evaluating photosystem II activation.

2.4.2 Macrofouling analysis

To verify the presence of macroorganisms on the surface of the substrates, three replicates of each treatment were analyzed individually under a stereomicroscope (magnification 40x). To avoid the loss of organisms in the substrate storage solution, the liquid was also analyzed in a Bogorov chamber under the stereoscope. Macrorganisms found were identified, photographed and

quantified. The species were identified using specialized literature described in Agostini et al. (2021a, 2019).

2.5. Fouling community composition

Metabarcoding was performed only for substrates removed at 165 h, where they were stored in sterile 0.4% saline solution. To investigate the composition of bacteria, fungi, algae and invertebrates, the sequencing of the 16S, ITS and 18S genes was performed by the specialized company Neoprospecta Microbiome Tecnologia, Brazil. Composite samples (pools) were made by mixing in equal parts the three replicates per treatment. Amplification of the 16S rRNA v3/v4 region was performed using 341F (5´-CCTACGGRSGCAGCAG-3´) and 806R (5´-GGACTACHVGGGTWTCTAAT-3´) primers (Christoff et al., 2017). Amplification of the ITS1 region was performed using the primers ITS1 (GAACCWGCGGARGGATCA-3´) and ITS 2 (5´-GCTGCGTTCTTCATCGATGC-3´) (Schmidt et al., 2013). Amplification of the 18S rRNA v9 region was performed using the 1510r primer (5´-CCTTCYGCAGGTTCACCTAC-3´) (Bradley et al., 2016).

Libraries were sequenced using the NextSeq 1000/2000TM sequencing system (Illumina Inc., EUA) with NextSeq 1000/2000 P1 600-Cycle Kit. The removal of chimeric sequences and grouping of operational taxonomic units (OTU) were performed using UPARSE (Edgar, 2013). The taxonomic identifications were carried out by the Blastn v.2.6.0 (Altschul et al., 1990), using as a reference Silva (v 138.2) database (Quast et al., 2012) and Greengenes (De Santis et al., 2006). The rarefaction (normalization) of the metagenomic data (OTUs) was performed using the sample with the lowest number of sequences as a basis. Then, based on the relative abundance of sequences in each sample, the frequency of taxa and the richness were calculated for each treatment.

2.6. Chemical characterization of extracts

To identify the chemical composition of the extracts, the crude extracts of *P. crassipes* and *T. domingensis* were submitted to gas chromatography (GC-MS) and liquid chromatography (LC-MS) coupled to mass spectroscopy, as well as Fourier transmission infrared spectroscopy (FTIR). For GC-MS, 1 µL of the solution was injected into a mass spectrophotometer coupled to the Shimadzu gas chromatograph (AOC-20i series) (GCMS-QP2010 SE). After the chromatography procedure, the relative amount of each phytocomponent present in the extracts was expressed as a percentage based on the height of the peak (%) produced in the chromatograph. The recorded mass spectrum was identified by the GC-MS system software (Naicker, 2024; Sukreem, 2024) using the standard mass spectra of the National Institute of Standards and Technology (NIST05. LIB). For the LC-MS with a Shimadzu Shim-Pak GIST HP C18 column equipped with a UV detector was used. The recorded mass spectra were identified using the standard MASSBANK (http://www.massbank.jp/index-e.html) mass spectra (Tohge and Fernie, 2009). For the FTIR, was also used, using a

Bruker Alpha II infrared spectrophotometer and processed with the Opus spectroscopy software. The functional groups were identified by the infrared spectrum table provided by Sigma-Aldrich (Naicker, 2024; Sukreem, 2024).

2.7. Statistical analysis

In each paint (*P. crassipes* or *T. domingensis*), to verify the difference between the treatments (Control A, Control B, 2.5, 5 and 10 g L^{-1}) and the exposure times (23, 46, 70, 94, 118, 142 and 165 h) in the microfouling assays, a two-way ANOVA was used. In all cases, the assumptions of normal distribution of the residues (Shapiro-Wilk test) and homoscedasticity (Levene test) were verified. When alternative hypotheses with a significance level of 95% were accepted, Tukey's post-hoc test was performed. Explanatory analyses were performed with the frequency of taxonomic groups (metabarcoding, Bray-Curtis similarity matrix) and total bacteria and autotrophic data (Euclidean distance) through non-metric multidimensional scaling (NMDS). Permutational analysis of multivariate variance (PERMANOVA) was performed to verify differences among groups of treatments for multivariate data. Similarity percentage analysis (SIMPER) was also performed to verify the individual contribution of variables for treatment groups. Statistical analysis was conducted with the use of software Past 4.03.

3. Results

3.1. Monitoring of environmental and biological data

The environmental variables showed little variation between the experimental period (Table 1). The water temperature remained at an average of 21.24 °C (± 0.56), dissolved oxygen 8.35 mg L^{-1} (± 0.56), chlorophyll-*a* 9.40 mg m^{-3} and concentrations below the detection level were found for *Microcystis* spp., *Dolichospermum* spp. and Cyanobacteria (Table 1).

Table 1: Mean ± SD of environmental and biological variables at the point where the field experiment was carried out. <LQ = below detection limit. TAN = total ammoniacal nitrogen; DL = detection limit.

Environmental variables	Mean	SD	Biological variables	Mean	SD
Temperature (°C)	21.24	0.56	Chlorophyll-*a* (µg L^{-1})	9.40	8.53
Dissolved oxygen (mg L^{-1})	8.35	0.18	*Microcystis* spp. (cel mL^{-1})	<DL	<DL
Electrical conductivity (µS cm^{-1})	66.14	1.77	Cyanobacteria (cel. mL^{-1})	<DL	<DL
pH	7.68	0.09	*Dolichospermum* spp (cel. mL^{-1})	<DL	<DL
Turbidity (NTU)	11.58	1.03			
Water transparency (cm)	87	7			
Suspended solids (mg L^{-1})	5.70	0.50			
Total phosphorus (mg L^{-1})	0.04	0.00			
Total nitrogen (mg L^{-1})	0.63	0.16			
Phosphate (mg L^{-1})	0.03	0.01			

TAN (mg L^{-1})	0.12	0.02
Nitrogen dioxide (mg. L^{-1})	<DL	<DL
Nitrate (mg. L^{-1})	0,38	0,32

3.2. Microfouling

Results of microfouling are summarized in Table 2. The chlorophyll concentration of the analyzed groups (µg L^{-1}) varied during the exposure time and the treatments (Fig. S1). Total chlorophyll (Fig. S1A-F) and green algae (Fig. 2B-G) increased in concentration from 118h on ($p<0.05$), Diatoms/Dinoflagellates (Fig. S1D-I) from 70 h on ($p<0.05$) and the Cryptophytes (Fig. S1E-J) from 142 h on ($p<0.05$). Cyanobacteria (Fig. S1C-H) showed a decrease in their concentration between 40 and 70 h ($p<0.05$), but then increased from 94 h on ($p<0.05$). The Diatoms/Dinoflagellates showed a higher concentration of chlorophyll in relation to the other groups analyzed (Fig. S1D-I).

Table 2: Summary of the effects on biofouling in substrates painted with aquatic macrophyte extracts at the end of the experiment (165 h). - = decrease, + = increase in comparison to control B.

Endpoints	***P. crassipes* (g L^{-1})**			***T. domingensis* (g L^{-1})**		
	2.5	**5**	**10**	**2.5**	**5**	**10**
Total *Chlorophyll (µg. L^{-1})*						
	no effect	-	-	no effect	+	no effect
Green Algae chlorophyll	-	-	no effect	no effect	-	+
Cyanobacteria chlorophyll	-	-	-	+	no effect	no effect
Diatoms/Dinoflagellates chlorophyll	+	no effect	no effect	+	+	no effect
Cryptophytes chlorophyl	+	+	+	+	+	no effect
Total bacteria (cells/cm^2)	+	-	+	no effect	+	no effect
Presence of L. fortunei larvae	Present	present	absent	absent	absent	absent
Frequency (%);richness						
Algae	-;-	-;-	-;-	-;-	-;-	-;-
Bacteria	+;-	+;no effect	+;-	+;-	+;+	+;+
Fungi	+;-	+;-	+;-	+;-	+;-	+;-
Presence of Mollusca (*Sinomytilus harmandi*)	Absent	+	absent	absent	absent	+

For *P. crassipes* extracts, at the end of the experiment (165 h), the concentration of total chlorophyll (Fig. S1A) was lower in the 10 g L^{-1} treatment ($p<0.05$), while the chlorophyll of green algae was lower in the 2.5 g L^{-1} treatment compared to the control B (Table 2; Fig. S1B) ($p<0.05$). For cyanobacteria chlorophyll (Fig. S1C), there was a decrease with the increase in extract concentrations ($p<0.05$). For the Diatoms/Dinoflagellates chlorophyll (Fig. S1D) there was an increase in the 2.5 g L^{-1} treatment compared to the control B

treatment (p>0.05) and for Cryptophytes chlorophyll (Fig. S1E) there was an increase for all extracts concentration (p<0.05), particularly for 5.0 g L^{-1}.

On the other hand, treatments with different concentrations of *T. domingensis* extracts, when compared to the control group B at 165 h (Table 2), the total chlorophyll (Fig. S1F) show an increase in the 5.0 g L^{-1} (p>0.05), the green algae chlorophyll (Fig. S1G) decreased in the treatment of 5 g L^{-1}, but an increase in the 10 g L^{-1} treatment (p<0.05), the cyanobacteria chlorophyll (Fig. S1H) showed an increase in the 2.5 g L^{-1} (p>0.05). For Diatoms/Dinoflagellates (Fig. S1I) and Cryptophytes (Fig. S1J), both 2.5 and 5.0 g L^{-1} treatments showed an increase compared to control B treatment (p>0.05).

Photosynthetic activity was detected only for the Diatoms/Dinoflagellates group for treatments containing macrophyte extracts (*P. crassipes* and *T. domingensis*) from 118 h and for control B only at 165 h. The treatments with the extracts of *P. crassipes* and *T. domingensis* at 165 h, when compared to control B, the photosynthetic activity did not show any difference (p>0.05) (Table S1).

Regarding the results of total bacteria (cells cm^{-2}) (Fig. 2 and Table 2), we observed in general an increase in total heterotrophic bacteria from 70 h on (p<0.05), with a decrease in 142 h and an increase again in 165 h (p<0.05). For the treatments with *P. crassipes* (Fig 2A), in the period of 165 h, when compared to the control B treatment, the treatments of 2.5 and 10 g L^{-1} showed an increase in total bacteria (p<0.05), while in the treatment of 5 g L^{-1} there was a decrease (p<0.05). For the treatments with *T. domingensis* (Fig. 2B), the treatments of 2.5 and 10 g L^{-1} remained the same as the control treatment (p>0.05), on the other hand, the treatment of 5 g L^{-1} increased total bacteria (p<0.05).

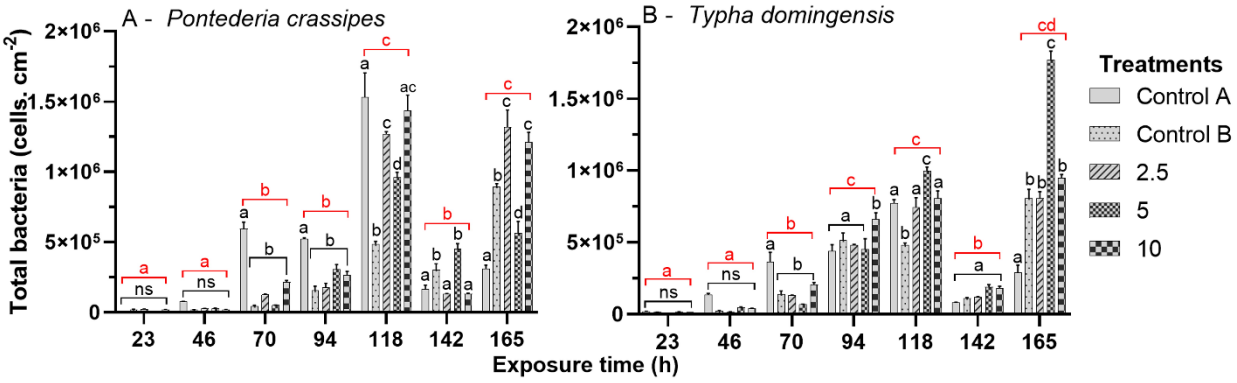


Fig. 2: Mean (± SD) of total bacteria (cell cm^{-2}) of substrates painted with different treatments of aquatic macrophyte. Different letters within each exposure time – statistical difference between concentrations (p<0.05); Different letters for each exposure time (in red) – statistical difference between exposure times (p<0.05); ns – did not show significant difference (p>0.05).

3.3 Macrofouling

Regarding macroorganisms, it was found only one representative of the pediveliger larva of *Limnoperna fortunei* at 165 h in the 2.5 g L^{-1} treatment of *P. crassipes* (Fig. 3A); and an umbonate larva of *L. fortunei* in the 5 g L^{-1} treatment at the end of the experiment (Fig 3B), also for *P. crassipes*. For other treatments and exposure times, no representative of macroorganisms were found (Table 2).

We also present the org cm^{-2} ratio for each treatment in Figure S2, where at 165 h, *P. crassipes* 2.5 and 5 g L^{-1} presented the maximum of 0.08 org cm^{-2}.

Fig. 3: Macroorganisms found in biofouling samples of different treatments of *Pontederia crassipes* at the exposure at the time of 165 h by analysis under a stereoscope microscope.

3.4. Fouling community composition

A total of 12 taxa were recorded, with the Bacteria, Fungi and Algae being the most representative of all samples (Fig. 4A). The control B treatment presented greater richness with 83 taxonomic groups, while the treatments of 10 g L^{-1} of *P. crassipes* and 2.5 g L^{-1} of *T. domingensis* were less rich with only 20 groups each (Fig. 4A). Among bacteria (Fig. 4B), Bacilli was the most frequent for all treatments, followed by Desulfovibrione and Alphaproteobacteria. The 2.5 g L^{-1} treatment of *T. domingensis* and 10 g L^{-1} of *P. crassipes* had less richness when compared to the control B treatment (Fig. 4B).

For the Fungi, eight different classes were found, with respectively Microbotryomycetes, Eurotiomycetes and Tremellomycetes being the most frequent (Fig. 4C). The control B treatment presented greater richness with 7 different classes, while the 10 g L^{-1} treatment of *T. domingensis* was less rich, with only three classes, and a high predominance of Microbotryomycetes (Fig. 4C). For algae (Fig. 4D), the Bacillariophyceae class was the most frequent for all treatments, being the only class present in the treatments of 10 g L^{-1} of *P. crassipes*, 2.5 and 10 g L^{-1} of *T. domingensis* (Fig. 4D). Again, control B was the presented greater richness, with 38 taxonomic groups found, while for the other treatments there was a decrease in richness (Fig. 4D). The abundance of taxa for each treatment is presented in table S2.

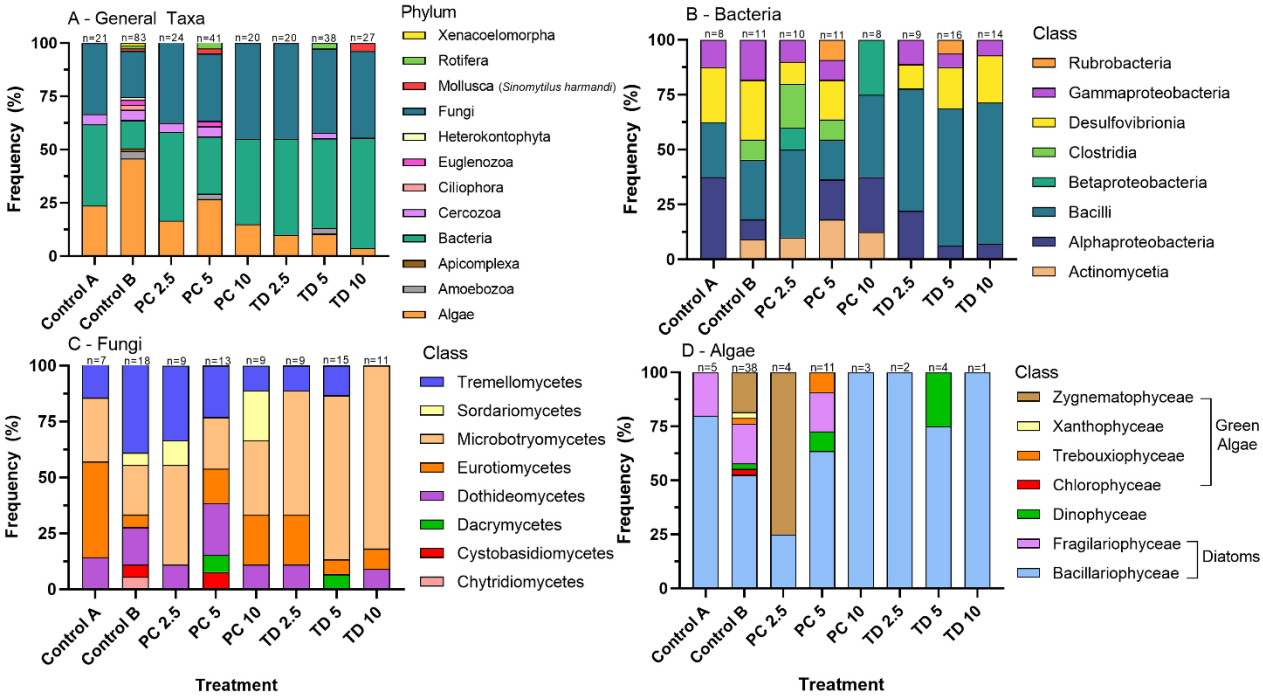


Fig. 4: Frequency of taxa (%) in biofouling samples of different treatments at the exposure time of 164 h. A – 16S, 18S and ITS rRNA, B – 16S rRNA, C – ITS and 18S rRNA, D – 18S rRNA, n – number of taxa in each treatment, PC – *Pontederia crassipes*; TD – *Typha domingensis*.

3.5. Biofouling community analyses

The NMDS generated for the abundance of taxa (Fig. S3A) revealed eight groups for the treatments tested (STRESS = 0.17), which were confirmed by the PERMANOVA ($p<0.001$). However, we can observe the similarity of the treatments of *T. domingensis* 5 with 10 g L^{-1}, and control A with *P. crassipes* 5 and 10 g L^{-1} (Fig. S3A). We also observed that Bacillariophyceae, Microbotryomycetes and Bacilli contributed 14.02%, 11.60% and 11.41% respectively to the formation of this group (Table S3A). For the NMDS with the data of total bacteria and chlorophyll (Fig. S3B), eight groups were also generated (STRESS=0.05), confirmed by PERMANOVA ($p<0.001$). But we can also observe some similarities between the treatments, as seen for *T. domingensis* 2.5 g. L^{-1}, *P. crassipes* 2.5, 5 and 10 g L^{-1}, and control B (Fig. S3B). We also observed that in this case, total chlorophyll and chlorophyll from Diatoms/Dinophyceae contributed 55.25% and 32.06% respectively, to the formation of this grouping (Table S3B).

3.6. Chemical characterization of extracts

The three most abundant compounds identified in GC-MS in the *T. dominguensis* extract, were E-15 Heptadecenal with 32.03% area, eicosane with 21.33% and 2,6,11-Trimethyldodecane with 12.54% (Table S4A). The three most abundant compounds in GC-MS in the *P. crassipes* extract were eicosane with 22.84%, E-14-hexadecenal with 17.32%, and n-Nonadecanol-1 with 13.70%

(Table S4A). Complete data such as retention time and chromatographic peaks are presented in the supplementary material (Table S5 and Fig. S4).

For LC-MS, for *T. domingensis* extract, the three main compounds identified were 4-Methylphenethylamine with 55.45%, D-cysteine with 15.59% and L-Lysine Hydrochloride with 10.73% (Table S4B). And for *P. crassipes* extract it was 4-Methylphenethylamine with 63.00%, betaine-aldehyde with 18.21% and carbamoyl-DL-aspartic acid with 14.81% (Table S4B). Complete data such as retention time and chromatographic peaks are presented in the supplementary material (Table S6 and Fig. S5).

FTIR indicated different functional groups for the two extracts (Table S7). For the *T. domingensis* extract, the functional groups identified included amine, alcohol, aliphatic nitro, aromatic ether and halo compound. The *P. crassipes* extract presented mainly the functional groups such as alcohol, halo compound, nitro compound, aromatic ether, halide and alkane.

4. Discussion

Current antifouling alternatives seek solutions that inhibit the formation of bacterial biofilms, as this step influences the adhesion of other organisms in the successional process of biofouling (Agostini et al., 2021a). In our study, both *P. crassipes* and *T. domingensis* extracts added to the epoxy coating were successful in inhibiting heterotrophic and autotrophic components of microbial biofilms. This effect was less pronounced at the end of the experiment (165 h), which may be due to the loss of antibiofilm effect. It is important to stress that the half-life of the bioactives present in the extracts are unknown, and loss of efficacy cannot be ruled out. It is also possible that biofilms, once developed and mature, confer protection against chemicals and other abiotic stresses (Chattopadhyay et al., 2022; Davey and O'toole, 2000), reducing the efficacy of the bioactives even if they are still active.

In addition, we observed that the *P. crassipes* treatments showed greater inhibition of heterotrophic bacteria density, and autotrophic bacteria and algae chlorophyll than the extracts of *T. domingensis*. In laboratory studies, extracts from these aquatic macrophytes are reported to have negative effects on the formation of heterotrophic bacterial biofilms, by interfering in bacterial quorum sensing signaling (Morales et al., 2024b). Our results showed that the antifouling effect of these plants is also applicable in the field.

We observed that autotrophic bacteria (bluegreen) were the first to grow, but soon after they slowed their growth and grew again. This same pattern was observed for total bacteria, suggesting that most of the bacteria analyzed in the flow cytometry assays are bluegreen. In this same context, when compared to control A, the treatments with the extracts showed a delay in the lag phase of microbial growth, where for *P. crassipes* the growth occurred from 94 h and for *T. domingensis* from 70 h. The lag phase is a latency phase of microbial growth, that is, without stable growth, and is followed by exponential growth (log phase) (Reischke et al., 2015, 2014).

In addition, the substrates with the paintings with *P. crassipes* and *T. domingensis* showed a dose-dependent response in relation to the results of bluegreen chlorophyll, which was not observed for other photosynthetic organisms. This specific response of inhibition effect on cyanobacteria is reported in other studies with the competition between macrophytes and cyanobacteria (Neilen et al., 2017; Yu et al., 2019). The chemical compounds released by macrophytes can cause changes in cell morphology, enzymatic activity and inhibition of photosynthetic activity, by decreasing the concentration of chlorophyll-*a* and damage to photosystem II (Eigemann et al., 2013; Liu et al., 2021; Tan et al., 2019). This could explain bluegreen low chlorophyll concentration and its non-detection of photosynthetic activity.

We also observed that diatoms increased their density in the period of 70 h, the period after the adhesion of autotrophic (bluegreen) and heterotrophic bacteria. Soon after this period, we observed the adhesion of green algae (118 h) and cryptophytes (142 h) respectively, where there are already reports that they adhere only after the adhesion of diatoms (França et al., 2011). However, we emphasize that the successional stage of biofilm formation may vary due to interspecific species competitions and environmental variables (Zhu et al., 2024). It is known that in the first 48 hours of biofouling there is a conditioning phase of the surface with the adsorption of nutrients and that, together with temperature, this influences the process of bacterial adhesion and biofilm formation (Rao, 2010). In addition, there is a positive relationship between the concentration of chlorophyll and fouling algae, and also between particulate matter and biofouling organisms (Rao, 2010). This was not the focus of our study, but we observed that the environmental variables showed little variation, which were within the parameters found in the study region.

The extracts of *P. crassipes* and *T. domingensis* also showed effects on the frequency of taxa and species richness. This effect was observed by the increased frequency and decreased richness of taxa in these treatments with aquatic macrophytes. This pattern has already been observed on surfaces coated with chemical substances, where bacterial biofilm communities tend to decrease their taxonomic richness (Agostini et al., 2021a; Ammon et al., 2018; Flach et al., 2017). We also observed that for the bacteria the extracts caused an increase in the frequency of Bacilli, Alphaproteobacteria and Desulfovibrionia and a decrease in Betaproteobacteria and Gammaproteobacteria. These overrepresented taxa are considered tolerant to environmental stressors such as heavy metals and increased temperature (W. Huang et al., 2024; Y. Huang et al., 2024; Keshri et al., 2024), which may explain their high frequency in the treatments. In addition, Proteobacteria are recognized for their high diversity and abundance in natural and artificial substrates (Agostini et al., 2021a; Bergo et al., 2021; Morales et al., 2024b). Antibiofilm effects of P. crassipes and *T. domingensis* extracts on Gammaproteobacteria have already been reported by Morales et al. (2024b). Gammaproteobacteria are reported to be favored for their enrichment in substrates with antifouling coatings, resulting in high abundance

(Ammon et al., 2018; Flach et al., 2017), however, in our study we saw a decrease in this taxon in the substrates.

Most biofouling studies focus mostly on bacteria or invertebrates (Sérvulo et al., 2023; Watson et al., 2016), with research on fungi and algae still incipient, especially when there are class-level responses. In our study we not only observed effects on richness and frequency in the bacterial community, but also in fungi and algae. This may have probably occurred due to the inhibition of some bacteria, which may have favored the increase in opportunistic organisms (Agostini et al., 2021a). For example, observed the increase in the frequency of Microbotryomycetes and diatoms fungi (Fragilariophyceae and Bacillariophyceae) and a decrease in Dothideomycetes and green algae. The high predominance of diatoms, observed by metabarcoding data and chlorophyll concentrations, can be explained by the fact that they are extremely opportunistic, strategist and have different adaptations that facilitate their adhesion, making them the most abundant benthic organisms in substrates (Felisberto and Rodrigues, 2012; França et al., 2011).

The bacterial richness inhibition effects present in the biofilm may also have led to inhibitory effects on the adhesion of macrorganisms. That is, surfaces with chemical coatings could have unique biofilm communities, which leads to the selection of also unique communities of macrorganisms (McNamara et al., 2009). According to Agostini et al. (2021a), Gammaproteobacteria to be involved positively with macrofouling. In our study, we found few macrorganisms adhered to the substrates (0.08 org. cm^{-2}) for *P. crassipes* 2.5 and 5 $g.L^{-1}$ in the time of 165 h, which could be possibly explained by the effect of *P. crassipes* and *T. domingensis* extracts on the decrease in the frequency of Gammaproteobacteria. Another explanation is a low abundance of macroorganism larvae in the environment that due to the absence of macroorganisms also in control treatments could be presented. During the period of the experiment, the site presented high precipitation rates, which may have caused a decrease in macroinvertebrates in aquatic environments (Sousa et al., 2012; Torres et al., 2024). However, tests to verify the larval abundance in the environment must be done for such a statement. We also emphasize that the experiment was carried out at a time of high abundance of larvae of *Limnoperna fortunei* (Cataldo et al., 2022, 2012)

Treatments containing only epoxy paint (without the addition of extracts) were more prone to biofouling when compared to the treatment without painting (only with stainless steel). Stainless steel is composed of zinc, which in turn is essential for the metabolism for the formation of bacterial biofilm, ensuring greater attractiveness for organisms (Bongo et al., 2010). However, in high concentrations zinc can be toxic to organisms (Eisler, 1998). In addition, the roughness of stainless steel facilitates the adsorption and absorption of organic and inorganic particles (Patil and Anil, 2005). Thus, this effect can be attributed not to stainless steel, but rather to epoxy paint that may have changed its roughness and chemical composition (by slow zinc leaching), making it more

attractive to colonizers. Epoxy bases are a category of polymeric materials containing high molecular weight binders that make up contact leaching coatings (Pereira et al., 2024). They have been used as a basis for antifouling additives (Pereira et al., 2024; Vijayan et al., 2022), due to their mechanical, anticorrosive property, chemical resistance, and their potential for constant release of antifouling agents (Vijayan et al., 2022).

On the other hand, epoxy paint-based treatments with *P. crassipes* were more effective against biofouling than *T. domingensis*, being more evident in the concentration of 5 g L^{-1} of *P. crassipes*. The observed dose-dependent non-response can be explained by the fact that some microbial taxa survive at higher and lower concentrations, and at intermediate concentrations end up decreasing their density, also known as the eagle effect (Eagle, 1948; Eagle and Musselman, 1948; Prasetyoputri et al., 2019).

Besides, the chemical compounds that macrophytes possess may have provided biofouling inhibition effects. Both extracts contained eicosane and 4-Methylphenethylamine, which are known for their antimicrobial properties (Rambaran et al., 2024), and for being present in high concentrations in aquatic macrophyte extracts (*Cabomba caroliniana* and *Schoenoplectus californicus*) with antifouling properties (Morales et al., 2025). E-14-Hexadecenal and n-Nonadecanol-1 were exclusive to *P. crassipes* extracts, with also previously reported antimicrobial activities (Mangoba and Guzman Alvindia, 2023; Ullah et al., 2024). E-15-Heptadecenal and 2,6,11-Trimethyldodecane were unique to *T. domingensis* and showed antibacterial and anti-quorum sensing activity (Abdelshaheed et al., 2021; Li et al., 2020). These compounds likely contributed to the observed antifouling effects, although their specific roles in inhibiting biofouling have yet to be investigated further.

In addition, the main chemical groups of the compounds present in the extracts were predominantly alkanes and alcohols, which are related to plant constituents and play critical roles in their physiology, mainly related to plant-herbivore interactions, such as pollinator attraction or repellency (Lal and Biswas, 2023). We also emphasize that identifying the compounds present in the extracts is a fundamental step in the development of new antifouling agents. This step provides information for the isolation of effective bioactive compounds. Therefore, new tests should be developed for the incorporation of the isolated compounds directly into the paints, making this alternative more efficient for a final antifouling product (Hamidi et al., 2022). In this context, antifouling efficacy can also be improved by extracting the compounds using organic solvents (*e.g*., ethanol, ethyl acetate, and/or methanol) that would target polar compounds and lipophilic and hydrophilic molecules (Hamidi et al., 2022).

Although the search for antifouling alternatives that are less harmful to the environment has increased over the years, especially those derived from natural plant compounds (Agostini et al., 2021b; Hamidi et al., 2022), studies from natural compounds of aquatic macrophytes are incipient (Morales et al., 2025, 2024b, 2024a). Still, there are few studies that seek to evaluate the antifouling efficacy

of natural plant compounds in the field (Pérez et al., 2021). Our study is the first to test the antifouling activity of aquatic macrophyte extracts mixed with epoxy paint in a natural environment. The information obtained through our study contributes not only to the knowledge of the antifouling activity of aquatic macrophytes, but also provides an avenue for the development of new antifouling that is less harmful to the environment.

5. Conclusion

Our study was the first to validate the antifouling efficacy of aquatic macrophyte extracts associated with epoxy coating in the natural environment. Throughout 165 h, the paintings with treatments of 5 and 10 g. L^{-1} of *P. crassipes* were more effective to combat biofouling. The inhibition of biofouling was proven for all groups analyzed, with a decrease in bacterial biofilm, chlorophyll content, the frequency of some taxa (Mollusca, Clostridia, Actinomycetia, Tremellomycetes, Sordariomycetes, Green Algae and Dinophyceae) and an increase in tolerant taxa such as Bacilli, Alphaproteobacteria, Desulfovibrionia, Microbotryomycetes and Diatoms. Thus, *P. crassipes* is a strong candidate for the development of new antifouling paints based on natural products, giving hope for a new perspective on antifouling paints. The durability of the antifouling effect was promising during the 165 h analyzed, however, we emphasize that to verify its durability, more studies are needed to understand the half-life of the antifouling effects of these paints. In addition, we recommend carrying out long-term tests in the field, in order to establish a complete understanding of the durability and effectiveness of the paintings. Also, to increase effectiveness, we suggest the isolation of the antifouling compounds present in the extracts, by its purification through chromatographic data, making it a more efficient product for the direct incorporation into the epoxy base. These plants can be considered strong candidates for the development of natural products, giving hope for a new perspective on the topic of antifouling paints.

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7. Authorship contributions statement

All authors contributed to the study conception and design. The first draft of the manuscript was written by Mikael Luiz Pereira Morales and all authors commented on previous versions of the manuscript. All authors read and approved of the final manuscript.

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Supplementary materials

Table S1: Mean (± SD) of the photosynthetic activity (%) of the microbial communities scrapped from substrates coatedwith different concentrations of lyophilized aquatic macrophyte extracts added to epoxy paint. Different letters within each exposure time - statistical difference between concentrations ($p<0.05$).

Pontederia crassipes

Exposure time (h)	Green Algae Chlorophyll (%)					Bluegreen Chlorophyll (%)				
	Control A	**Control B**	**2.5**	**5**	**10**	**Control A**	**Control B**	**2.5**	**5**	**10**
23	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
46	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
70	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
94	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
118	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
142	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
165	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
Exposure time (h)	**Diatoms/Dinophytes Chlorophyll (%)**					**Cryptophytes Chlorophyll (%)**				
	Control A	**Control B**	**2.5**	**5**	**10**	**Control A**	**Control B**	**2.5**	**5**	**10**
23	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
46	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
70	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
94	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
118a	0,00a	0,00a	61.38 ± 2.65b	57.18 ± 5.11b	62.55 ± 1.17b	0,00a	0,00a	0,00a	0,00a	0,00a
142b	0,00a	0,00a	48.09 ± 2.28b	0,00a	45.31 ± 3.43b	0,00a	0,00a	0,00a	0,00a	0,00a
165c	0,00a	54.74 ± 8.72b	51.29 ± 6.22b	47.95 ± 5.59b	54.52 ± 2.76b	0,00a	0,00a	0,00a	0,00a	0,00a

Typha domingensis

Treatments	Green Algae Chlorophyll (%)					Bluegreen Chlorophyll (%)				
	Control A	**Control B**	**2.5**	**5**	**10**	**Control A**	**Control B**	**2.5**	**5**	**10**
23	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
46	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a

70	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
94	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
118	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
142	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
165	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
	Diatoms/Dinophyceae Chlorophyll (%)					**Cryptophyta Chlorophyll (%)**				
Treatments	**Control A**	**Control B**	**2.5**	**5**	**10**	**Control A**	**Control B**	**2.5**	**5**	**10**
23	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
46	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
70	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
94	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
118a	0,00a	58.05 ± 5.90b	0,00a	60.51 ± 3.05b	59.63 ± 2.24b	0,00a	0,00a	0,00a	0,00a	0,00a
142b	0,00a	0,00a	0,00a	43,72 ± 4.87b	0,00a	0,00a	0,00a	0,00a	0,00a	0,00a
165a	0,00a	51.05 ± 2.70b	60.87 ± 1.00c	59.89 ± 5.08bc	52.08 ± 2.73b	0,00a	0,00a	0,00a	0,00a	0,00a

Table S2: Abundance of fouling organisms found in substrates painted with different treatments of *Pontederia crassipes* and *Typha domingensis* by metabarconding analysis. 0 = absent. Total N = total number of taxafound for treatment.

	Control		*P. crassipes*			*T. domingensis*		
Taxa	Control A	Control B	2.5 g. L^{-1}	5 g. L^{-1}	10 g. L^{-1}	2.5 g. L^{-1}	5 g. L^{-1}	10 g. L^{-1}
Bacteria								
Actinomycetia	0	1	1	2	1	0	0	0
Alphaproteobacteria	3	1	0	2	2	2	1	1
Bacilli	2	3	4	2	3	5	10	9
Betaproteobacteria	0	0	1	0	2	0	0	0
Clostridia	0	1	2	1	0	0	0	0
Desulfovibrionia	2	3	1	2	0	1	3	3
Gammaproteobacteria	1	2	1	1	0	1	1	1
Rubrobacteria	0	0	0	1	0	0	1	0
N total off bacteria	8	11	10	11	8	9	16	14

Fungi								
Chytridiomycetes	0	1	0	0	0	0	0	0
Cystobasidiomycetes	0	1	0	1	0	0	0	0
Dacrymycetes	0	0	0	1	0	0	1	0
Dothideomycetes	1	3	1	3	1	1	0	1
Eurotiomycetes	3	1	0	2	2	2	1	1
Microbotryomycetes	2	4	4	3	3	5	11	9
Sordariomycetes	0	1	1	0	2	0	0	0
Tremellomycetes	1	7	3	3	1	1	2	0
N total off fungi	7	18	9	13	9	9	15	11
Eukaryotic **Algae**								
Bacillariophyceae	4	20	1	7	3	2	3	1
Dinophyceae	0	1	0	0	0	0	0	0
Fragilariophyceae	0	1	0	1	0	0	1	0
Chlrophyceae	1	7	0	2	0	0	0	0
Trebouxiophyceae	0	1	0	1	0	0	0	0
Xanthophyceae	0	1	0	0	0	0	0	0
Zygnematophyceae	0	7	3	0	0	0	0	0
N total off algae	5	38	4	11	3	2	4	1
Other taxa								
Amoebozoa	0	3	0	1	0	0	1	0
Apicomplexa	0	1	0	0	0	0	0	0
Cercozoa	1	4	1	2	0	0	1	0
Ciliophora	0	2	0	0	0	0	0	0
Euglenozoa	0	2	0	1	0	0	0	0
Heterokontophyta	0	1	0	0	0	0	0	0
Sinomytilus harmadi	0	1	0	1	0	0	0	1
Rotifera	0	1	0	1	0	0	1	0
Xenacoelomorpha	0	1	0	0	0	0	0	0

Table S3: Similarity percentage analysis (SIMPER) with Bray-Curtis similarity for the abundance of biofouling communities by metabarcoding data (A) and Euclidean similarity for chlorophyll and total bacteria data (B).

A - Bray-Curtis similarity

Bacteria		**Fungi**	
Taxon	**Contrib. %**	**Taxon**	**Contrib. %**
Bacilli	11.41	Microbotryomycetes	11.6
Desulfovibrionia	4.41	Tremellomycetes	6.10
Alphaproteobacteria	3.72	Eurotiomycetes	3.72
Actinomycetia	2.60	Dothideomycetes	2.84
Betaproteobacteria	2.53	Sordariomycetes	2.71
Clostridia	2.52	Dacrymycetes	1.27
Gammaproteobacteria	1.42	Cystobasidiomycetes	1.05
Rubrobacteria	1.28	Chytridiomycetes	0.45
Eukaryotic **Algae**		**Other taxa**	
Taxon	**Contrib. %**	**Taxon**	**Contrib. %**
Bacillariophyceae	14.02	Cercozoa	3.81
Zygnematophyceae	5.50	Amoebozoa	2.40
Chlrophyceae	4.95	Mollusca	1.58
Fragilariophyceae	1.49	Rotifera	1.49
Trebouxiophyceae	1,053	Euglenozoa	0.90
Xanthophyceae	0.45	Ciliophora	0.90
Dinophyceae	0.45	Heterokontophyta	0.45
		Apicomplexa	0.45
		Xenacoelomorpha	0.45

B - Euclidean similarity

Data	**Contrib. %**
Total Chl	55.25
Diatoms/Dynophytes Chl	32.06
Green Algae Chl	5.02
Cryptophyta Chl	4.48
Total heterotrophic Bacteria	2.24
Cyanobacteria Chl	0.95

Table S4: GC-MS (A) and LC-MS (B) analysis of aqueous extract of aquatic macrophytes (*Typha domingensis e Pontederia crassipes*).

A- GC-MS Analysis

Compound	Molecular Formula	*T. domingensis* extract Area %	*P. crassipes* extract Area %	Chemical group
E-15 Heptadecenal	$C_{17}H_{32}O$	32.03	5.89	Aldehyde
Eicosane	$C_{20}H_{42}$	21.33	22.84	Alkane
2,6,11-Trimethyldodecane	$C_{15}H_{32}$	12.54	-	Alkane
E-14-Hexadecenal	$C_{16}H_{30}O$	-	17.32	Aldehyde
n-Nonadecanol-1	$C_{19}H_{40}O$	-	13.70	Alcohol

B - LC-MS Analysis

Compound	Molecular Formula	*T. domingensis* extract Area %	*P. crassipes* extract Area %	Chemical group
4-Methylphenethylamine	$C_9H_{13}N$	55.45	63.00	Aromatic compound
D-cysteine	$C_3H_7NO_2S$	15.59	-	Amino acid
L-Lysine Hydrochloride	$C_6H_{15}ClN_2O_2$	10.73	-	Amino acid
Betaine-Aldehyde	$C_5H_{12}NO$	8.32	18.21	Aldehyde
Carbamoyl-DL-aspartic acid	$C_5H_8N_2O_5$	-	14.81	Amino

Table S5: GC-MS analysis of aqueous extract of aquatic macrophytes (*Pontederia crassipes* and *Typha domingensis*).

GC-MS Analysis

Compound	Molecular Formula	*P. crassipes* extract		*T. domingensis* extract		Chemical group
		R.Time	Area %	R.Time	Area %	
3,7,11-Trimethyldodeca-2,6,10-enoic acid	$C_{16}H_{30}O_2$	-	-	15,460	1,03	Lipids
1,4-Dimethoxydecahydronaphthalene	$C_{12}H_{22}O_2$	-	-	15,445	2,47	Ether
1,54-Dibromotetrapentacontane	$C_{54}H_{108}Br_2$	14,391	1,23			Alkane
1,5-Heptadien-3-yne	C_7H_8	-	-	3,886	2,02	Alkene
1-Bromo-11-iodoundecane	$C_{11}H_{22}BrEu$	-	-	14,435	2,28	Alkane
1-Pentacosanol	$C_{25}H_{52}O$	-	-	24,108	3,39	Alcohol
2,3-Dimethyldodecane	$C_{14}H_{30}$	-	-	9,840	3,58	Alkane
2,6,10-Trimethyltetradecane	$C_{17}H_{36}$	-	-	13,010	1,98	Alkane
2,6,11-Trimethyldodecane	$C_{15}H_{32}$	9,849	4,43	-	-	Alkane
		10,394	2,38	-	-	
		12,690	5,73	-	-	
2-Bromopropionic acid, 6-ethyl-3-octyl ester	$C_{13}H_{25}BrO_2$	-	-	15,792	1,79	Ester
3,6-Dimethyldecane	$C_{12}H_{26}$	11,937	1,21	-	-	Alkane
3-Phenylmethoxybutane-1,2,4-triol	$C_{11}H_{16}O_4$	3,802	0,49	-	-	Alcohol
3-Tetradecene, (Z)-	$C_{14}H_{28}$	-	-	11,265	2,30	Alkene
4,5-Dimethylnonane	$C_{11}H_{24}$	-	-	7,099	1,14	Alkane
4-Methyldodecane	$C_{13}H_{28}$	-	-	12,313	1,09	Alkane
	C_9H_{20}	-	-	4,299	0,46	
5,5-Diethylpentadecane	$C_{19}H_{40}$	-	-	16,814	2,17	Alkane
		-	-	17,511	1,60	
5-Ethyl-5-methyldecane	$C_{13}H_{28}$	-	-	10,390	1,60	Alkane
5-Methyloctadecane	$C_{18}H_{38}$	12,311	1,20	-	-	Alkane
		13,440	1,61	-	-	

		14,580	2,12	-	-	
		14,790	0,68	-	-	
5-Methyltetradecane	$C_{15}H_{32}$	-	-	13,430	1,81	Alkane
9-Tricosene	$C_{23}H_{46}$	18,365	5,24	-	-	Alkene
Bromoacetic acid, 4-tridecyl ester	$C_{15}H_{29}BrO_2$	24,134	0,84	-	-	Ester
Cyclohexylidenecyclohexane	$C_{12}H_{20}$	-	-	16,290	1,28	Ciclic Alkane
Decane, 4-ethyl-	$C_{12}H_{26}$	9,700	1,28	-	-	Alkane
Diethylene glycol monododecyl ether	$C_{16}H_{34}O_3$	13,618	1,04	-	-	Ether
E-14-Hexadecenal	$C_{16}H_{30}O$	11,260	5,93	13,316	17,32	Aldehyde
E-15-Heptadecenal	$C_{17}H_{32}O$	13,324	17,78	18,347	5,89	Aldehyde
		15,320	14,25	-	-	
Eicosane	$C_{20}H_{42}$	12,221	6,71	12,213	6,31	Alkane
		14,312	7,07	12,685	5,04	
		14,740	4,12	14,304	8,23	
		16,825	1,86	14,731	3,27	
		17,530	1,57	-	-	
Hexacontane	$C_{60}H_{122}$	21,391	1,11	-	-	Alkane
Isonipecotic acid	$C_6H_{11}NO_2$	-	-	14,685	0,89	Carboxylic acid
n-Nonadecanol-1	$C_{19}H_{40}O$	-	-	15,311	13,70	Alcohol
Octyldodecanol	$C_{20}H_{42}O$	12,799	0,79	-	-	Alcohol
Oxalic acid, 6-ethyloct-3-yl propyl ester	$C_{15}H_{28}O_4$	14,956	1,25	-	-	Ester
Oxalic acid, hexadecyl isohexyl ester	$C_{24}H_{46}O_4$	-	-	14,585	0,94	Ester
Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	12,585	4,25	12,576	5,83	Phenol
Solasonine	$C_{45}H_{73}NO_{16}$	13,470	0,67	-	-	Alkaloid
tert-Hexadecanethiol	$C_{48}H_{99}AuS_3$	-	-	11,933	0,59	Alkane
Toluene	$C_6H_5CH_3$	3,884	1,75	-	-	Aromatic hydrocarbon
Undecyl chloroacetate	$C_{13}H_{25}ClO_2$	15,450	1,38	-	-	Ester

Table S6: LC-MS analysis of aqueous extract of aquatic macrophytes (*Pontederia crassipes* and Typha domingensis).

LC-MS Analysis								
		***P. crassipes* extract**			***T. domingensis* extract**			
Compound	**Molecular Formula**	**R.Time**	**Area %**	**Base Peak m/z**	**R.Time**	**Area %**	**Base Peak m/z**	**Chemical group**
Betaine-Aldehyde	$C_5H_{12}NO$	4.492	8.32	102.25	4.308	8.31	102.25	Aldehyde
		-	-	-	4.433	3.50	-	
		-	-	-	4.648	6.40	-	
4-Methylphenethylamine	$C_9H_{13}N$	4.741	17.60	135.50	5.065	63.00	135.50	Aromatic compound
		5.053	37.85	-	-	-	-	
		7.428	0.78	-	-	-	-	
D-cysteine	$C_3H_7NO_2S$	5.498	15.59	121.15	-	-	-	Amino acid
L-Lysine monohydrochloride	$C_6H_{15}ClN_2O_2$	5.817	10.73	146.20	-	-	-	Amino acid
4-Pyridoxylic acid	$C_8H_9NO_4$	4.683	0.26	183.00	-	-	-	Carboxylic acid
Nicotinoylcholine	$C_{11}H_{17}N_2O_2$	4.865	0.35	209.05	-	-	-	Pyrimidine
3-Methyladenine	$C_6H_7N_5$	5.213	4.52	149.15	5.053	1.12	149.10	Aromatic compound
Homopiperidinic acid	$C_5H_{11}NO_2$	5.534	3.44	117.15	5.383	2.86	117.15	Carboxylic acid
Chloromethyltriphenylphosphonium	$C_{19}H_{17}CIP$	6.296	0.55	311.00	-	-	-	Phosphonium
Carbamoyl-DL-aspartic acid	$C_5H_8N_2O_5$	-	-	-	5.363	14.81	176.00	Amino acid

Table S7: FTIR analysis of aqueous extract of aquatic macrophytes (*Typha domingensis* and *Pontederia crassipes*).

Phytocompounds identified	Functional groups	***T. domingensis* extract**	***P. crassipes* extract**
		Wave number (cm^{-1})	**Wave number (cm^{-1})**
Amine/Alcohol	N-H stretch/ O-H stretch	3254.45	-
Aliphatic Nitro	NO_2 stretch	1561.21	-
Aliphatic Nitro	NO_2 stretch	1401.30	
Aromatic Ether	CO-stretch	1277.30	-
Primary Alcohol	C-O	1065.03	1064.19
Halo compound	C-Br	652.15	609.05
Alcohol	O-H stretch		3239.88
Nitro compound	NO_2 stretch		1561.07
Aromatic/Nitro compound	C=C/N-O		1401.40
Aromatic Ether/Alkyl Halide	C-O/C-H		1275.68
Alkene	C-H		652.08
Halo compound	C-I		532.53

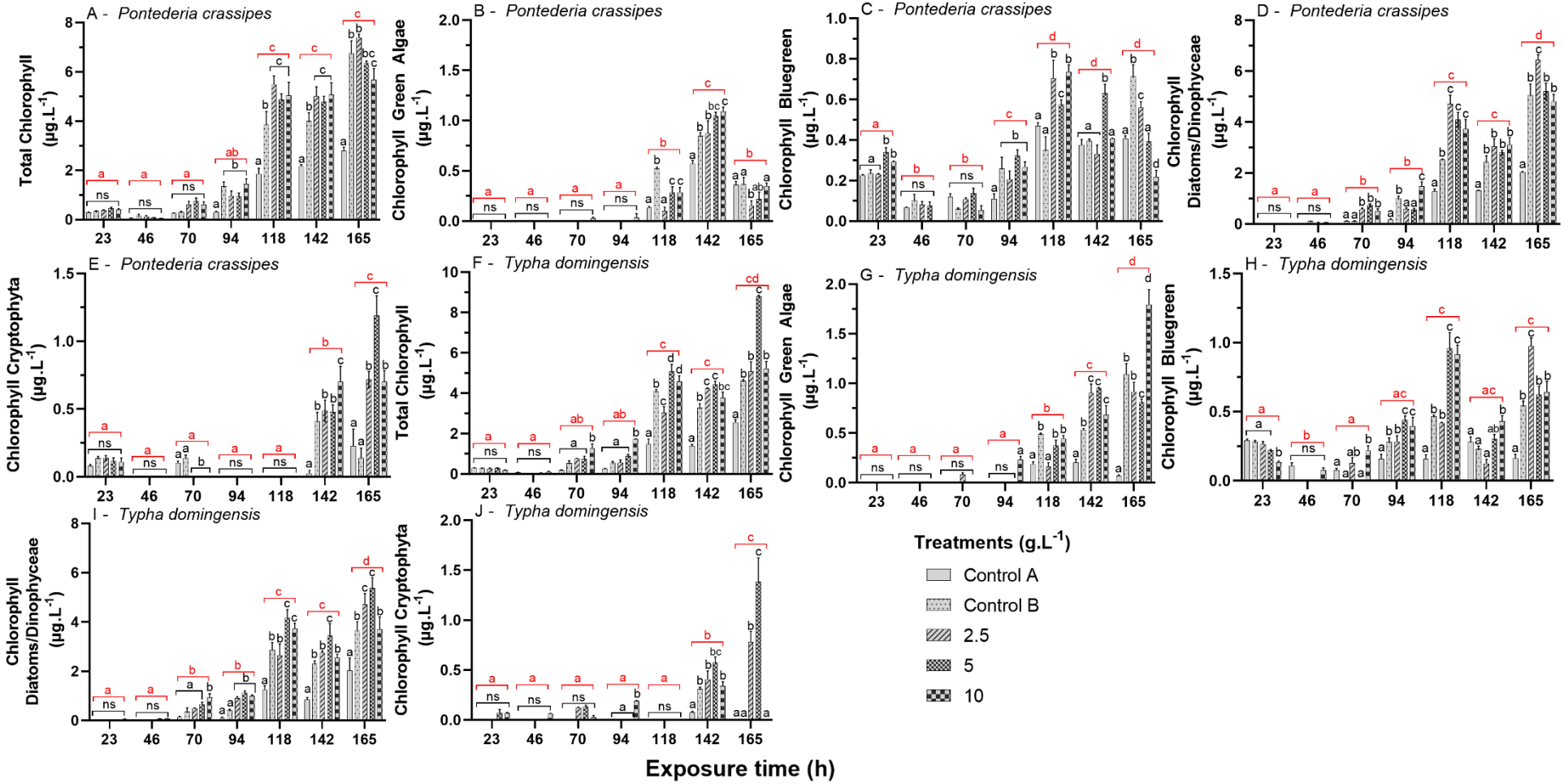


Fig. S1: Mean (± SD) of the chlorophyll content (µg L-1) of substrates coated with different treatments of aquatic macrophyte. Interaction between exposure time and treatments p<0.05. Different letters within each exposure time indicate statistical difference among treatments (p<0.05); Different letters for each exposure time (in red) – statistical difference among exposure times (p<0.05); ns – did not show significative difference (p>0.05).

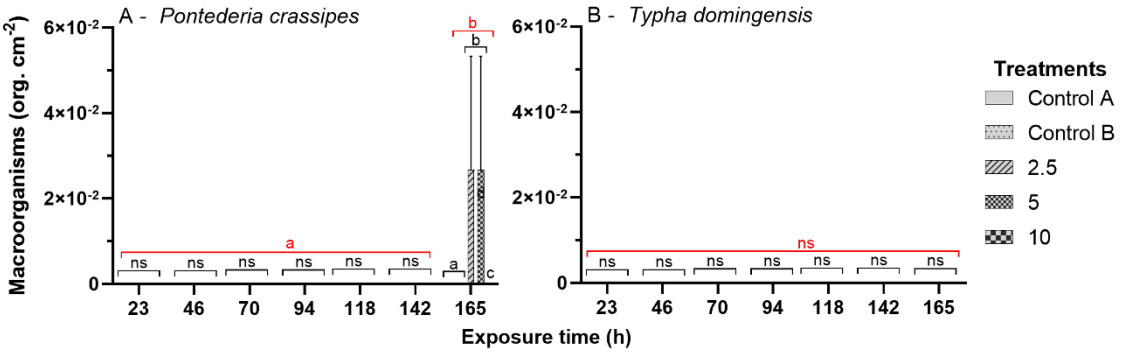


Fig. S2: Macrofouling (org cm^{-2}) found in substrates exposed to epoxy treatments containing aquatic macrophyte extracts. Interaction between exposure time and treatments $p<0.05$. Different letters within each exposure time – statistical difference between concentrations ($p<0.05$); Different letters for each exposure time (in red) – statistical difference between exposure times ($p<0.05$); ns – did not show significative difference ($p>0.05$).

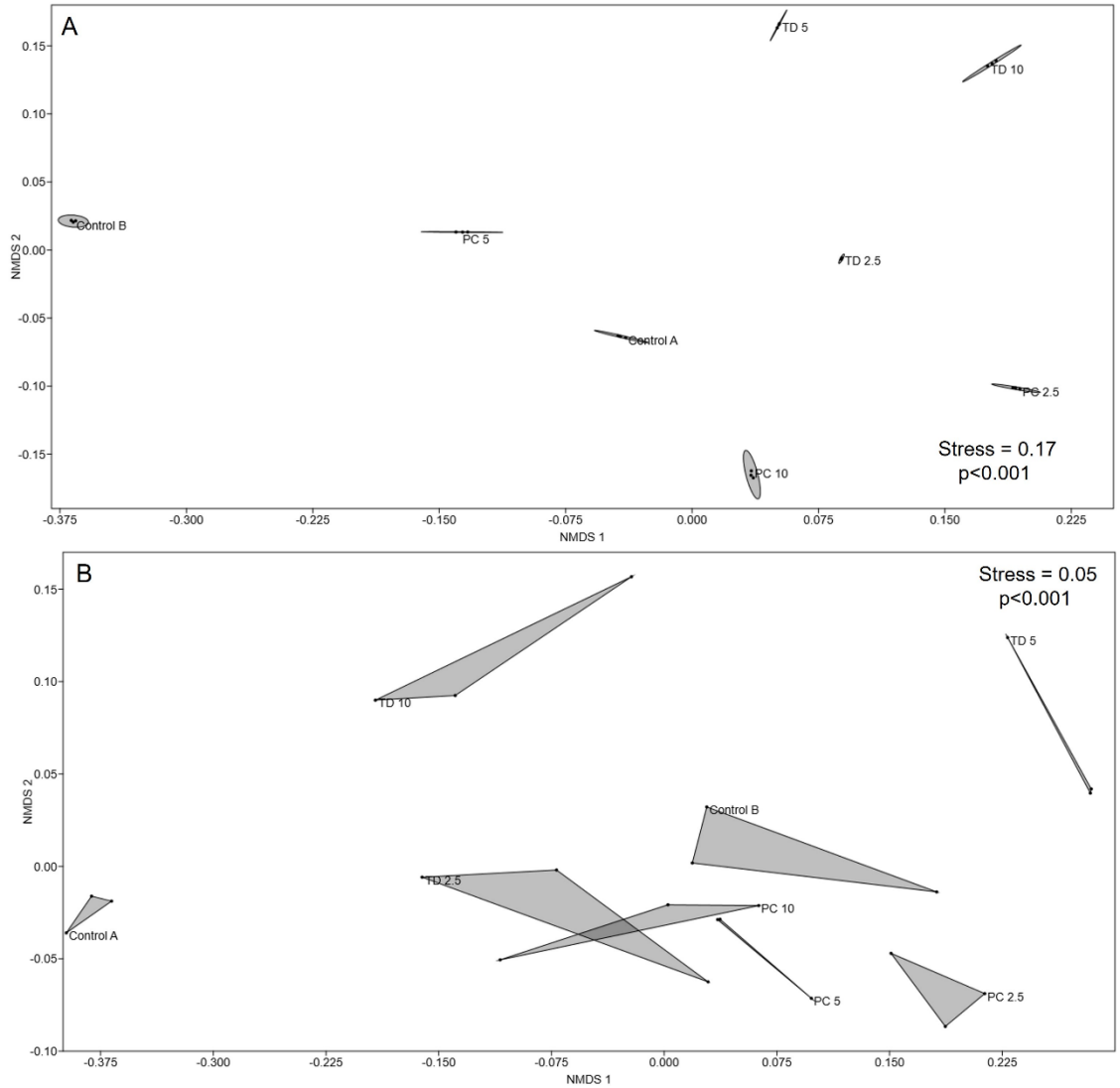


Fig. S3: Non-metric multidimensional scale ordering (NMDS) with Bray-Curtis similarity for the abundance of biofouling communities by metabarcoding data (A) and Euclidean similarity for chlorophyll and total bacteria data (B). PC – *Ponteria crassipes*; TD – *Typha domingensis*.

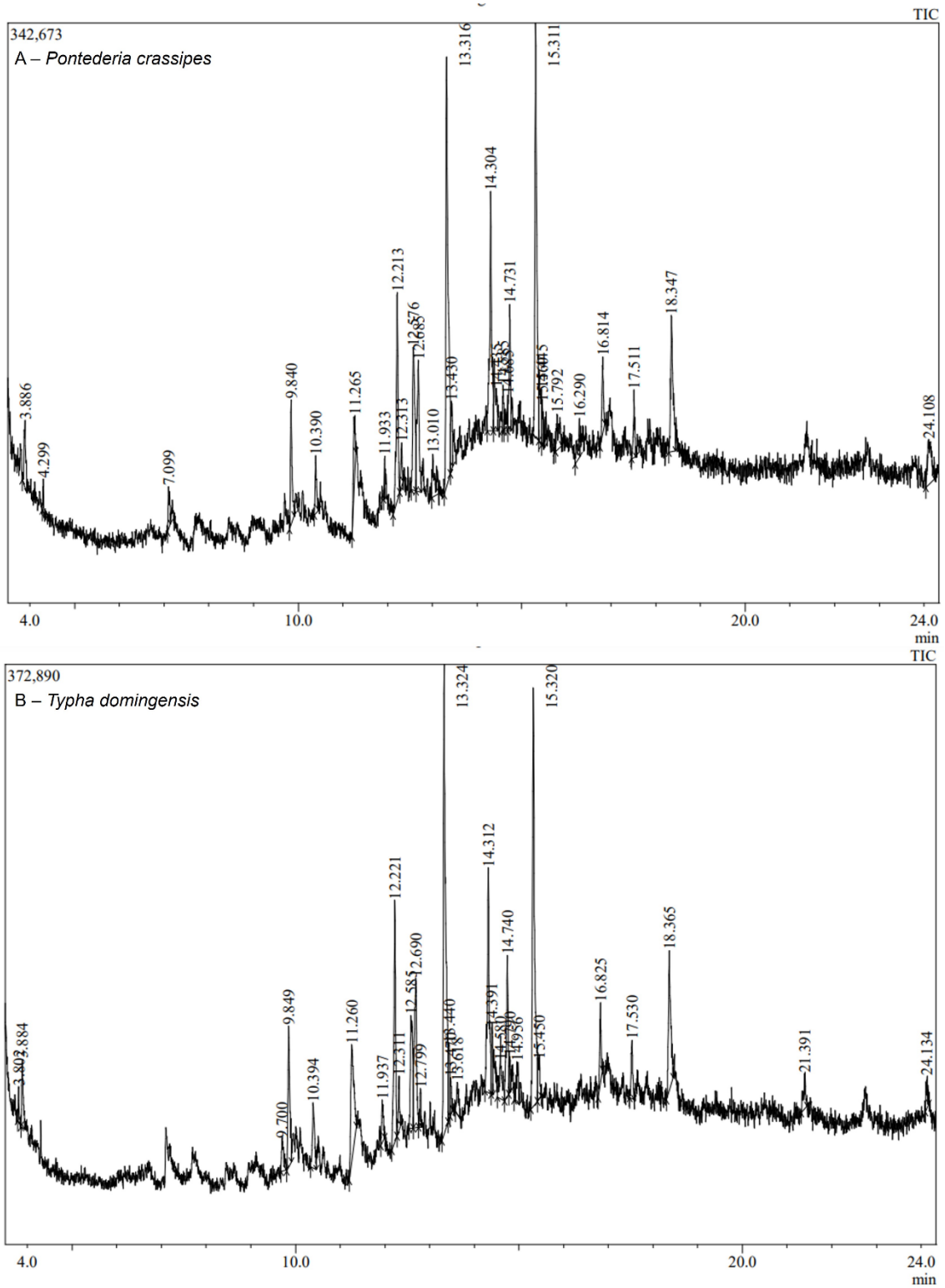
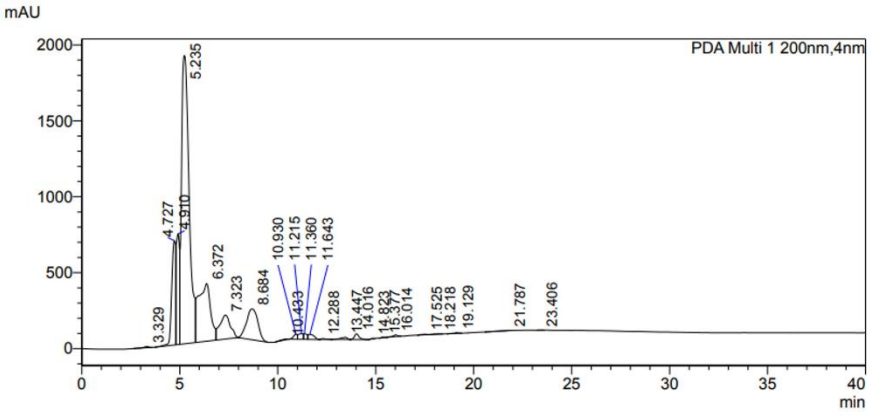
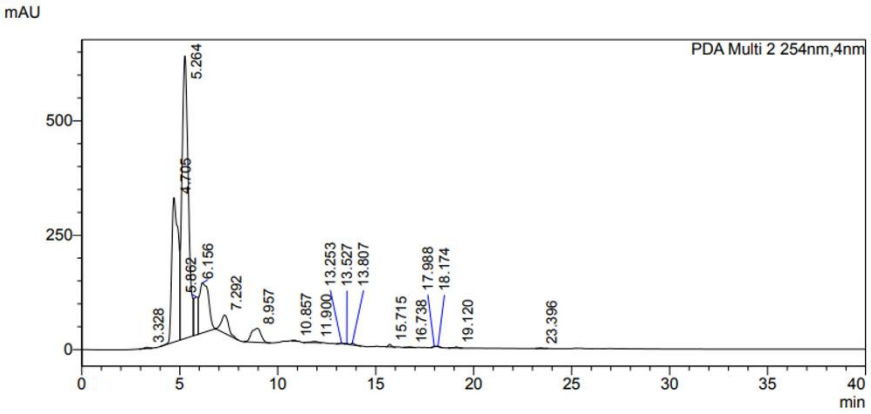


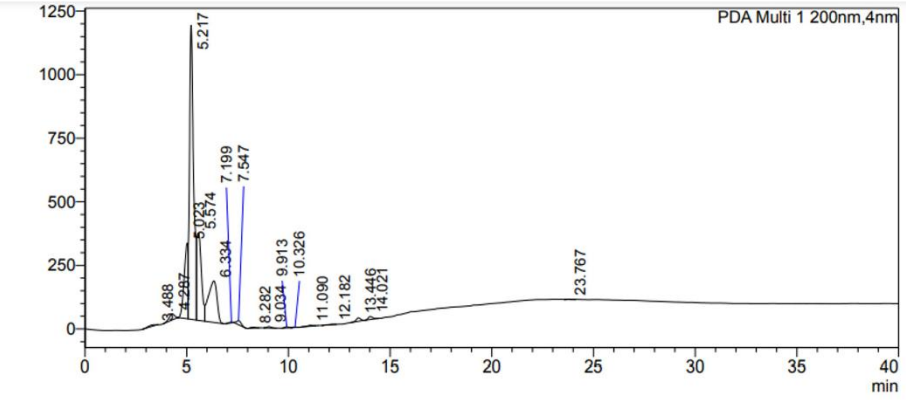
Fig. S4: GC-MS analysis, peaks of aqueous extract of aquatic macrophytes. A – *P. crassipes*; B – *T. domingensis*.

A – *Pontederia crassipes*





B – *Typha domingensis*



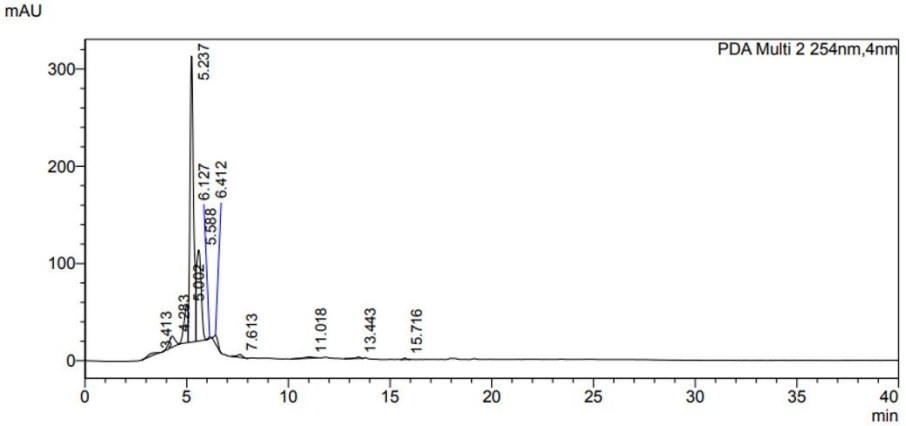


Fig. S5: GC-MS analysis, peaks of aqueous extract of aquatic macrophytes. A – *P. crassipes*; B – *T. domingensis*.

Capítulo XI: Síntese da discussão

O interesse por alternativas anti-incrustantes derivadas de plantas cresceu significativamente, com foco particular em plantas terrestres com a presença de compostos químicos com propriedades anti-incrustantes [Agostini *et al*. 2021b]. No entanto, a pesquisa sobre extratos de macrófitas para o controle da bioincrustação ainda permanece em seus estágios iniciais, sendo este o primeiro estudo nessa temática. Na presente Tese, os resultados ressaltam a eficácia anti-incrustante de extratos de macrófitas, tornando-as promissoras no desenvolvimento de agentes anti-incrustantes menos impactantes para o meio ambiente que os produtos anti-incrustantes atualmente comercializados.

As onze espécies de macrófitas utilizadas na presente Tese, foram escolhidas com base no seu efeito negativo no crescimento de algas, plantas e bactérias [Takao *et al*. 2011, Chicalote-Castillo *et al*. 2017, Jiménez 2020]. Entretanto, foi aqui demonstrado que destes, apenas os extratos de *Cabomba caroliniana* e *Schoenoplectus californicus* foram capazes de inibir ≥70% da formação do biofilme bacteriano estuarino uni e multiespécies [Morales *et al*. 2024a]. Para biofilmes bacterianos uni e multiespécie límnicos, apenas os extratos de *Pontederia crassipes* e *Typha domingensis* foram capazes de inibir ≥70% [Morales *et al*. 2024b]. Dessa forma, percebe-se que o potencial de inibição dos extratos das macrófitas pode variar de acordo com a espécie vegetal [Cardoso *et al*. 2019, Ramos *et al*. 2022], e também a partir do organismo teste (limnico, estuarino ou marinho) utilizado.

Ao avaliar os efeitos fitoquímicos das macrófitas, um fator importante a ser considerado é a sua forma biológica [They *et al*. 2015, Taleb *et al*. 2016,

Álvarez-Martínez *et al*. 2020]. Na presente Tese consideramos os biótipos submerso, flutuante, anfíbia e emergente, entretanto, esses não aparesentaram diferença sistemática no potencial inibitório. Os extratos com maiores efeitos inibitórios variaram entre submerso, flutuante e emergente. *C. caroliniana* se desenvolve completamente submersa na água, enquanto *S. californicus* e *T. domingensis* são emergentes com suas raízes fixadas no sedimento e suas folhas fora da água. *P. crassipes, por sua vez,* é flutuante com suas raízes abaixo e suas folhas acima da coluna d' água [Trindade *et al*. 2010, Thomaz & Esteves 2011].

Ao ocupar o mesmo estrato na coluna da água, as macrófitas submersas e flutuantes apresentam maior competição por nutrientes e luz com o fitoplâncton [Reynolds 2006], e o que propiciam grande parte dos casos uma maior sensibilidade à qualidade da água do que as macrófitas emergentes [Trindade *et al*. 2018]. Ainda neste sentido, sabe-se que as macrófitas aquáticas competem com cianobactérias e algas, através da liberação de compostos químicos na água [Neilen *et al*. 2017, Yu *et al*. 2019]. A ação desses compostos podem causar alterações na morofologia celular, atividade enzimática e inibição da atividade fotossíntetica, pela diminuição na concentração de clorofila-*a* e dano no fotossistema II [Eigemann *et al*. 2013, Tan *et al*. 2019, Liu *et al*. 2021]. No entanto, por mais que se reconheça os mecanismos de ação dos compostos químicos na comunidade autotrófica [Neilen *et al*. 2017, Yu *et al*. 2019, Liu *et al*. 2021], os seus efeitos em bactérias heterotróficas ainda não é esclarecido.

O potencial antibiofilme dos extratos de macrófitas foi avaliado contra cepas bacterianas únicas e comunidades bacterianas multiespécies de ocorrência natural. A maioria das cepas avaliadas foram do Filo Proteobacteria,

especificamente da classe Gamma-Proteobacteria e Alpha-Proteobacteria. O filo é reconhecido por apresentar alta diversidade filogenética e versatilidade fenotípica, permitindo a colonização de diferentes estruturas [Kersters *et al*. 2006, Zinger *et al*. 2011]. Proteobactérias são abundantes em águas naturais com muitos representantes que são capazes de aderir em substratos como acrílicos, compensados navais, aço carbono ASTM-36, sedimentos, rochas e lignina [Muthusamy *et al*. 2017, Agostini *et al*. 2021a, Bergo *et al*. 2021, Ferreira *et al*. 2022, Gusmão *et al*. 2023]. Isso confirma que as espécies estudadas na presente tese são representativas de biofilmes bacterianos e ocorrem em diferentes ambientes aquáticos.

Para os ensaios com bactérias límnicas, *Acinetobacter bohemicus* (gram-negativa) foi inibida pela maioria dos extratos e bactérias gram-positivas (*Psychrobacillus psychrodurans* e *Bacillus vietnamensis*) tiveram pouca inibição. Enquanto para os ensaios estuarinos, as bactérias gram-positivas (*Exiguobacerium* sp. e *Microbacterium marinilacus*) foram as mais afetadas pela ação dos extratos. Devido à sua membrana externa, as bactérias gram-negativas apresentam maior seletividade e são geralmente menos sensíveis a fatores externos, como a exposição a extratos naturais [Awolola *et al*. 2014, Seibert *et al*. 2019]. Por outro lado, as bactérias gram-positivas não possuem membrana externa e consequentemente são mais sensíveis a fatores externos [Awolola *et al*. 2014, Seibert *et al*. 2019].

No entanto, na presente tese, não foi observada uma resposta sistêmica considerando o tipo de parede celular. As estratégias de inibição do biofilme são multifatoriais e podem estar relacionadas a outros mecanismos não necessariamente relacionados à permeabilidade celular. Como por exemplo a

inibição do *Quorum Sensing* (QS) entre bactérias formadoras de biofilme, modulação superficial de adesão bacteriana ou degradação da matriz do biofilme [Srinivasan *et al*. 2021, Rambaran *et al*. 2024].

Com base nos resultados dos ensaios de erradicação, crescimento planctônico e inibição do QS (somente para bactérias límnicas), algumas hipóteses podem ser levantadas e discutidas. Quanto aos ensaios de erradicação, tanto para as bactérias límnicas quanto estuarinas, os extratos não foram tão eficazes para erradicar o biofilme quando comparados com os resultados de inibição da formação do biofilme. Essa resposta não é totalmente inesperada, uma vez que, a complexidade do biofilme pode melhorar a defesa contra agentes químicos externos [Srinivasan *et al*. 2021, Chattopadhyay *et al*. 2022].

Para o ensaio de inibição do crescimento planctônico, foram observados três tipos de respostas: indutiva (indução do crescimento), antibiótica (redução do crescimento) e antibiofilme (sem efeito no crescimento planctônico e efeito único no biofilme). A indução do crescimento bacteriano pode ter ocorrido devido à composição química dos extratos e suas respectivas diluições, pois alguns tratamentos podem ter mais compostos capazes de induzir o crescimento do que inibi-lo. Esses efeitos diferenciais entre inibição e indução já são relatados e explicados pela especificidade da composição química de cada espécie de macrófita [Santonja *et al*. 2018].

Ao considerar a busca de novos agentes anti-incrustantes, o efeito antibiofilme seria a resposta desejada, uma vez que ele é considerado ambientalmente mais seguro do que o efeito antibiótico [Agostini *et al*. 2020]. Além disso, as respostas que buscam o efeito antibiofilme reduzem o risco de

resistência bacteriana e podem estar associadas à inibição da formação do biofilme por meio da inibição do QS [Chenia 2013]. Assim, substâncias químicas com o potencial de inibição do QS são de extrema relevância para o desenvolvimento de novos agentes antibiofilme [Chenia 2013, Borges & Simões 2019].

A baixa eficácia de erradicação combinada com a não inibição do crescimento planctônico sugere que os extratos das macrófitas possuem outros mecanismos além da toxicidade para inibir a formação do biofilme. Uma possível explicação é que os compostos químicos presentes nos extratos podem interferir no processo de QS. Esses resultados foram evidenciados nos ensaios (límnicos) de inibição do QS para os extratos de *T. domingensis* e *P. crassipes.* T. domingensis apresentou um efeito inibitório de cepas biossensores produtoras de AHL de cadeia longa e curta, sugerindo um amplo espectro de atividade de inibição do QS. Enquanto o extrato de *P. crassipes* apresentou eficácia apenas para inibição do QS AHL de cadeia curta. Para os extratos de C. caroliniana e S. californicus não foi realizado o ensaio de inibição do QS. Assim, recomenda-se a realização deste ensaio para verificar a existência deste mesmo mecanismo mecanismo de ação dos extratos destas plantas sobre a inibição do biofilme.

Embora vários estudos tenham investigado os efeitos de extratos vegetais contra a macroincrustação [Feng *et al*. 2018, Pérez *et al*. 2019, Bel Mabrouk *et al*. 2020], aqueles que abordam o efeito de extratos de macrófitas aquáticas ainda são incipientes. A presente tese foi a primeira a avaliar o efeito extratos de macrófitas na adesão do mexilhão dourado (*Limnoperna fortunei*) e pólipos do Cifozoário *Aurelia coerulea*. Os extratos de *P. crassipes* e *T. domingensis* apresentaram inibição da adesão ≥ 80% para *L. fortunei* (de até 10 mm), e *C.*

caroliniana e *S. californicus* apresentaram inibição da adesão ≥ 70% de pólipos de *A. coerulea*. Ambos os resultados demonstraram eficácia de inibição da adesão de macroorganismos, o que está de acordo com outros estudos sobre o efeito de extratos vegetais em macroincrustantes.

Por exemplo, Feng *et al*. [2018] relataram que 15 alcaloides extraídos de plantas terrestres foram eficazes na inibição da fixação das larvas da craca *Fistulobalaus albicostatus* e das larvas do briozoário *Bugula neritina*, enquanto Pérez *et al*. [2021] observaram o efeito de extratos de *Verbena bonariensis* e *Tillandsia tenuifolia* na macroincrustação pelo mexilhão adulto *Mytilus edulis*. Já Oliva *et al*. [2022] observaram que os extratos de *Posidonia oceanica* reduziram os biofilmes de diatomáceas e bactérias, ao mesmo tempo em que inibiram a adesão do poliqueta *Ficopomatus enigmaticus.*

Deve-se ressaltar que, embora o ensaio realizado com o mexilhão dourado na presente tese tenha identificado uma resposta anti-adesão, essa inibição de fixação pode ter ocorrido devido à toxicidade comportamental dos extratos, ou outro mecanismo de ação. Portanto, em pesquisas futuras, os testes de mortalidade devem ser realizados juntamente com os ensaios anti-fixação. Nesse contexto, além de analisar a adesão dos pólipos de *A. coerulea,* também mensuramos sua mortalidade através de ensaios de toxicidade. Ainda, reforçamos que é importante a realização de testes anti-adesão com larvas plantígradas, entretanto, os estágios larvais são de díficil obtenção diretamente do ambiente natural e cultivo em laboratório.

Para *A. coerulea* foi constatado que os extratos testados nas concentrações mais baixas, inibiram a sua adesão sem causar toxicidade. Portanto, os compostos presentes nos extratos potencialmente influenciaram na

sua adesão por meio de alterações comportamentais ou vias metabólicas mais complexas. Para *Aurelia* pouco se sabe sobre essas alterações, mas para macroinvertebrados em geral, a inibição da adesão pode ocorrer pela interrupção da neurotransmissão, estresse oxidativo e inibição da produção de bissos [Chen & Qian 2017, Kyei *et al*. 2020].

Os compostos naturais, além de terem um efeito anti-incrustante tanto para micro quanto para macroincrustação, também devem ter baixa toxicidade para organismos não-alvo [Pérez *et al*. 2021]. Embora seja reconhecido que as macrófitas aquáticas possuem efeitos em organismos-alvo como fitoplâncton e cianobactérias [They *et al*. 2015, Li *et al*. 2021], o uso de altas concentrações pode gerar efeitos tóxicos para organismos não-alvo. Assim, para os extratos mais eficazes, foi avalaida a toxicidade utilizando diferentes organismos modelo não-alvo.

Os extratos de *C. caroliniana* e *S. californicus* não apresentaram toxicidade em diluições de até 20% no teor de clorofila-*a* e crescimento da microalga *Thalassiosira pseudonana* e sobrevivência do copépodo *Nitokra* sp. Entretanto, não foi calculada a sua CL_{50}/CE_{50} para esses organismos, ou sua concentração segura. Visto isso, nos ensaios de toxicidade com éfiras de *A. coerulea*, fase de vida não-alvo, foi calculada sua CE_{50}, sendo segura para o organismo em diluições de até 5% do extrato de *S. californicus* e 20% de *C. caroliniana*. Ressalta-se que as éfiras de *A. coerulea* são mais sensíveis do que *Nitokra* sp., como discutido no artigo 4 desta tese.

Para os extratos de *P. crassipes* e *T. domingensis,* os testes de toxicidade foram realizados com organismos não-alvo de três níveis tróficos diferentes, estabelecendo um amplo cenário ecológico natural [Pane *et al*. 2008]. Observou-

se diferença entre as respostas toxicológicas e suas diluições seguras nos diferentes níveis tróficos. Para a base da cadeia alimentar, *Pseudopediastrum boryanum* (microalga) sua diluição segura foi de 35%, sendo para o consumidor primário, *Daphnia magna* (cladocera), foi observado o mesmo padrão. Enquanto que para a espécie de maior nível trófico, *Pimephales promelas* (peixe), observou-se uma concentração segura de 70%. Dessa forma, observou que a microalga e o Cladocera foram mais sensíveis que os peixes, isso pode ser explicado devido ao tamanho dos organismos, assim como a espécie utilizada serem os principais fatores que influenciam a sensibilidade de substâncias químicas [Costa *et al*. 2008] .

Os efeitos anti-incrustantes e toxicológicos dos extratos de *C. caroliniana*, *S. californicus*, *P. crassipes* e *T. domingensis* podem decorrer de suas composições químicas. Todos esses extratos continham eicosano e 4-metilfenetilamina, conhecidos por suas propriedades antimicrobianas [Rambaran *et al*. 2024]. Além disso, foram encontrados compostos exclusivos, sendo para o extrato de *C. caroliniana* o ciclohexadecano, em *S. californicus*, o composto heptadecenal E-15. Já para *P. crassipes*, foram encontrados E-14-hexadecenal e para *T. domingensis* E-15-Heptadecenal. Todos esses compostos são relatados por possuírem atividade antimicrobiana [Abdelshaheed *et al*. 2021; Mangoba & Guzman Alvindia 2023, Ullah *et al*. 2024], e provavelmente contribuíram para a anti-incrustante e toxicológica observada. Entretanto, é necessária uma investigação mais aprofundada para esclarecer suas contribuições específicas para a inibição da adesão e toxicidade.

Após a realização de ensaios *in vitro*, é importante realizar testes de *in situ*, pois estes permitem a realização de estudos em condições ambientais e de

interações complexas entre os organismos incrustantes e a hidrodinâmica do ambiente [Romeu & Mergulhão 2023]. Para isso, os compostos naturais são utilizados como aditivos através de sua incorporação em revestimentos [Hamidi *et al*. 2022]. Dentre estes, os revestimentos de epóxi são amplamente utilizados para esse fim, devido às suas excelentes propriedades mecânicas, resistência química [Vijayan *et al*. 2022] e liberação dos compostos ao longo do tempo [Pereira *et al*. 2024].

Ao adicionar os extratos de *P. crassipes* e *T. domingensis* em revestimento epóxi, foi observado que essas coberturas foram bem sucedidas em inibir organismos heterotróficos (bactérias totais) e autotróficos (concentração de clorofila dos diferentes grupos taxonômicos) presentes nos biofilmes microbianos. Entretanto, este efeito foi menos pronunciado no final do tempo experimental (165 h), o que pode indicar a perda do seu efeito antibiofilme. É importante ressaltar que as meias-vidas dos bioativos presentes nos extratos são desconhecidas, e a perda de eficácia não pode ser descartada. Também é possível que os biofilmes, uma vez desenvolvidos e maduros, confiram proteção contra produtos químicos e outros estresses abióticos [Srinivasan *et al*. 2021; Chattopadhyay *et al*. 2022], reduzindo a eficácia dos bioativos mesmo que ainda estejam ativos. No entanto, neste estudo não avaliamos a meia-vida das soluções anti-incrustantes, uma lacuna importante a ser preenchida futuramente.

Observou-se que os tratamentos de *P. crassipes* apresentaram maior inibição de bactérias heterotróficas e clorofila de algas do que com os extratos de *T. domingensis*. Anteriormente, mostramos que ambos os extratos apresentam efeito antibiofilme em condições de laboratório, especificamente interferindo no processo QS. Assim os resultados do ensaio em campo mostram

que o efeito anti-incrustante dessas plantas também é aplicável em ambiente natural. Além disso, esses extratos apresentaram efeitos em táxons específicos, diminuindo sua diversidade e aumentando a sua frequência nesses tratamentos. Esse padrão já foi observado em superfícies revestidas com substâncias químicas, nas quais as comunidades de biofilme bacteriano tenderam a diminuir sua diversidade taxonômica [Flach *et al*. 2017, Ammon *et al*. 2018, Agostini *et al*. 2021a].

Observou-se também que para as bactérias, que os tratamentos dos extratos causaram um aumento na frequência de bacilos, Alfaproteobacteria e Dessulfovibrionia e uma diminuição de Betaproteobacteria e Gammaproteobacteria. Esses táxons super-representados são considerados tolerantes a estressores ambientais, como metais pesados e aumento da temperatura [Huang *et al*. 2024b, a, Keshri *et al*. 2024], o que pode explicar sua alta frequência nos tratamentos com extratos. Além disso, as Proteobacteria são reconhecidas por sua alta diversidade e abundância em substratos naturais e artificiais [Agostini *et al*. 2021a, Bergo *et al*. 2021].

Os estudos sobre anti-incrustantes se concentram principalmente em invertebrados e bactérias [Agostini *et al*. 2021a, b], com pesquisas sobre fungos e algas ainda incipientes, especialmente quando há respostas em nível de classe. Na presente tese, observamos não apenas efeitos na frequência da comunidade bacteriana, mas também em fungos e algas. Isso provavelmente pode ter ocorrido devido à inibição de algumas bactérias, o que pode ter favorecido o aumento de organismos oportunistas [Agostini *et al*. 2021a]. Por exemplo, o aumento da frequência de fungos Microbotryomycetes e diatomáceas (Fragilariophyceae e Bacillariophyceae) e uma diminuição de

Dothideomycetes e algas verdes. A alta predominância de diatomáceas, pode ser explicada pelo fato desses indivíduos serem extremamente oportunistas, estrategistas e possuírem diferentes adaptações que facilitam sua adesão [França *et al.* 2011, Felisberto & Rodrigues 2012].

Os efeitos de inibição da frequência bacteriana presentes no biofilme também podem ter levado a efeitos sobre a adesão de macroorganismos. Superfícies com revestimentos químicos possuem a capacidade de ter comunidades de biofilme únicas, o que acarreta na seleção específica de comunidades de macroorganismos [McNamara *et al*. 2009]. Em nossos resultados encontramos poucos (um indivíduo em uma repetição) ou nenhum macro-organismo aderidos aos substratos, o que poderia ser possivelmente explicado pelo efeito dos extratos na diminuição de frequência de Gammaproteobacteria. Bactérias dessa classe possuem uma correlação positiva com organismos macroincrustantes [Agostini *et al*. 2021a].

Embora a busca por alternativas anti-incrustantes menos prejudiciais ao meio ambiente tenha aumentado ao longo dos anos, especialmente aquelas derivadas de compostos naturais de plantas [Agostini *et al*. 2021b, Hamidi *et al*. 2022], estudos de compostos naturais de macrófitas aquáticas com esse intuito foram desenvolvidos apenas na presente Tese. Ainda assim, são poucos os estudos que buscam avaliar a eficácia anti-incrustante de compostos naturais de plantas no campo [Pérez *et al*. 2021]. As informações obtidas na presente Tese contribuem não apenas para o conhecimento da atividade anti-incrustante das macrófitas aquáticas, mas também fornecem subsídios para o desenvolvimento de novos anti-incrustantes menos prejudiciais ao meio ambiente.

Capítulo XII: Conclusões

Ao longo da presente Tese foram utilizadas diferentes abordagens e metodologias a fim de atingir os objetivos propostos. A partir disso, são apresentadas as principais conclusões para cada capítulo (artigo), seguido de conclusões finais da Tese.

- ✓ Os principais estudos que buscam alternativas para o controle de mexilhões invasores estão relacionados principalmente ao controle dos mexilhões *Limnoperna fortunei* e *Dreissena polymorpha*;
- ✓ Como forma de combater a bioincrustação de mexilhões invasores, o controle químico (*e.g*, salinidade, hipoclorito de sódio, cobre, tintas anti-incrustantes, extratos naturais) é o mais utilizado, entretanto, apesar do controle físico com utilização de luz ultravioleta e pulsos de pressão serem pouco utilizados, eles apresentaram alta eficácia;
- ✓ Dos 25 extratos utilizados nos bioensaios, foi observado que para combater a adesão de pólipos de *Aurelia coerulea* e a formação do biofilme uni e multiespécies estuarino, os oriundos de *Cabomba caroliniana* e *Schoenoplectus californicus* foram mais promissores;
- ✓ Os extratos de *C. caroliniana* e *S. californicus* não apresentaram toxicidade para os organismos não-alvo (*Thalassiosira pseudonana* e *Nitokra* sp.), bem como para a fase planctônica não-alvo de *A. coerulea* (éfiras), indicando possivelmente uma ação inibitória por via atóxica;
- ✓ Para os bioensaios anti-incrustantes límnicos, foi observado que *Pontederia crassipes* e *Typha domingensis* foram os mais promissores. Esses extratos apresentaram inibição da formação do biofilme uni e

multiespécies pela via de inibição do Q*uorum Sensing* e também inibição da adesão do mexilhão dourado (*Limnoperna fortunei*) de até 10 mm;

- ✓ Quanto aos ensaios toxicológicos, *P. crassipes* e *T. domingensis* foram considerados seguros em diluições de até 35% para a microalga *Pseudopediastrum boryanum*, o microcrustáceo *Daphnia magna* e o peixe *Pimephales promelas*;
- ✓ A partir do teste em campo durante 165h, observamos que os extratos de *P. crassipes* e *T. domingensis* incorporados em revestimento epóxi inibem a bioincrustação pela diminuição do biofilme, teor de clorofila e frequência de taxa.
- ✓ Também foi observado o aumento de organismos tolerantes a agentes químicos como fungos Microbotryomycetes e diatomáceas;
- ✓ O efeito anti-incrustante dos extratos de *C. caroliniana*, *S. californicus, P. crassipes* e *T. domingensis* pode estar relacionado principalmente à alta concentração dos compostos Eicosano e 4-metilfenetilamina.

Os estudos realizados na presente tese demonstraram o potencial biotecnológico de extratos de macrófitas, especialmente para as espécies *C. caroliniana* e *S. californicus* para o controle da bioincrustação marinha e estuarina, e *P. crassipes* e *T. domingensis* para bioincrustação límnica. A partir disso, aceitamos as nossas duas hipóteses, fornecendo a informação de que a atividade anti-incrustante foi encontrada ao menos em um dos extratos aquosos testados, com *P. crassipes* e *T. domingensis* inibindo o biofilme através da inibição do *quorum sensing*. Além disso, observamos que a atividade anti-incrustante variou em decorrência da composição química presente em cada espécie de macrófita e também o ambiente límnico, estuarino e marinho.

Com isso, essas plantas são consideradas promissoras para o desenvolvimento de anti-incrustantes naturais, na forma de alternativas ecologicamente mais amigáveis para o meio ambiente. Dessa maneira, a presente tese traz avanços e perspectivas que contribuem para mitigar a poluição marinha e melhorar o gerenciamento da bioincrustação e espécies invasoras.

Capítulo XIII: Perspectivas futuras

Apesar do efeito anti-incrustante comprovado dos extratos de macrófitas em inibir a micro e macroincrustação límnica, estuarina e marinha, para dar seguimento no desenvolvimento de alternativas anti-incrustantes à base de macrófitas, o presente estudo deixa alguns insights que ainda devem ser explorados quanto a sua capacidade de inibição. Com isso, pesquisas futuras devem se concentrar em elucidar:

- ✓ O mecanismo de ação dos extratos de *C. caroliniana* e *S. californicus* na inibição da formação do biofilme, deve ser investigado através de ensaios de inibição do *quorum sensing,* medidas de taxa de crescimento, atividade enzimática, viabilidade celular e concentração mínima inibitória;
- ✓ O efeito anti-incrustante no ambiente natural, com a realização de ensaios em campo para os extratos de *C. caroliniana* e *S. californicus*, com a sua incorporação como aditivos em revestimentos;
- ✓ O efeito anti-incrustante em outros organismos bioincrustantes, como fungos, algas e bivalves;
- ✓ O tempo de meia-vida dos extratos, buscando compreender a durabilidade do seu efeito anti-incrustante;
- ✓ À busca de uma maior atividade anti-incrustante, através do isolamento e caracterização dos compostos bioativos presentes nos extratos, bem como avaliar sua capacidade anti-incrustante e também a extração dos compostos por outros solventes (*e.g*., hexano, tolueno, metanol e acetato de etila).

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