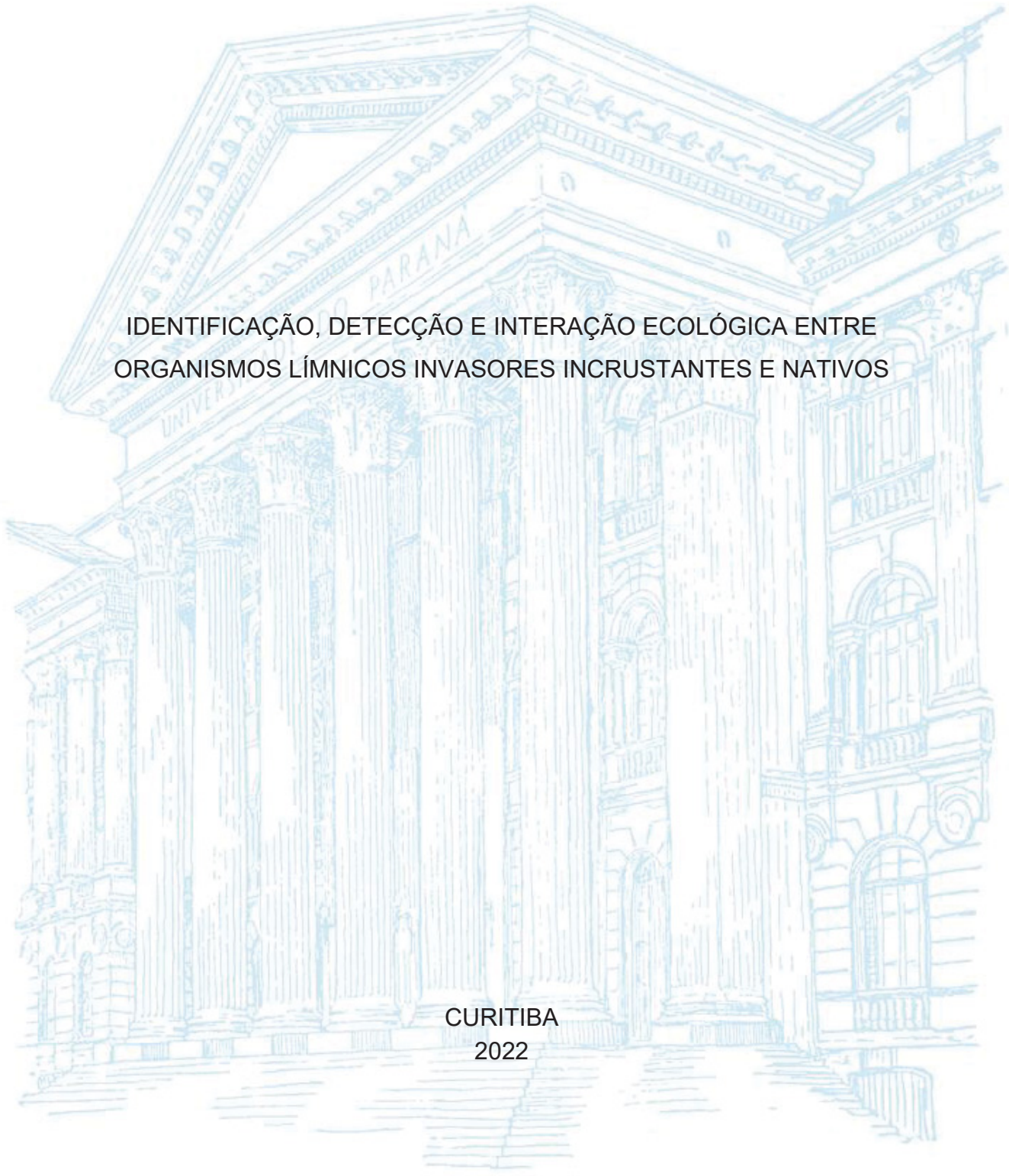
ANA PAULA DA SILVA BERTÃO



IDENTIFICAÇÃO, DETECÇÃO E INTERAÇÃO ECOLÓGICA ENTRE ORGANISMOS LÍMNICOS INVASORES INCRUSTANTES E NATIVOS

CURITIBA
2022

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IDENTIFICAÇÃO, DETECÇÃO E INTERAÇÃO ECOLÓGICA ENTRE ORGANISMOS LÍMNICOS INVASORES INCRUSTANTES E NATIVOS

Tese apresentada como requisito à obtenção do título de Doutora. Curso de Pós-Graduação em Zootecnia, área de meio ambiente, melhoramento e modelagem animal do setor de Ciências Agrárias da Universidade Federal do Paraná.

Orientador: Prof. Antonio Ostrensky.

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação ZOOTECNIA da Universidade Federal do Paraná foram convocados para realizar a arguição da tese de Doutorado de **ANA PAULA DA SILVA BERTÃO** intitulada: **Identificação, detecção e interação ecológica entre organismos límnicos invasores incrustrantes e nativos** , sob orientação do Prof. Dr. ANTONIO OSTRENSKY NETO, que após terem inquirido a aluna e realizada a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de doutora está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

CURITIBA, 27 de Junho de 2022.

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RESUMO

O trabalho aqui apresentado tem como foco central a compreensão dos processos biológicos e desafios associados à ocorrência de espécies incrustantes invasoras em ambientes límnicos. Primeiramente, identificamos, em uma escala global e por meio de uma revisão sistemática, as principais espécies invasoras incrustantes de ambientes límnicos bem como as informações disponíveis sobre suas interações (capítulo I). Após constatada a relativa escassez de estudos abordando tais organismos, dedicamo-nos à realização de estudos empíricos para avaliar a interação entre espécies incrustantes invasoras e nativas em um reservatório de usina hidrelétrica (UHE) no estado do Paraná, Brasil (capítulo II). Um dos desafios envolvidos na detecção precoce de espécies invasoras é a amostragem, e uma das alternativas que vêm sendo empregada para tentar contornar o problema envolve a identificação e quantificação do DNA que é liberado no ambiente por essas espécies, também conhecido como DNA ambiental ou eDNA. Por isso, o passo seguinte foi desvendar experimentalmente como o DNA se espalha na coluna da água (capítulo III). Também abordamos os desafios envolvendo a detecção de duas espécies aquáticas invasoras incrustantes em dois reservatórios de UHE, utilizando o DNA (extrato e ambiental) e investigamos como fatores abióticos influenciam na degradação desse DNA (capítulo IV). Por fim, para tornar nossos dados acessíveis ao público em geral, apresentamos os resultados desta tese em um artigo de divulgação científica (capítulo V).

Palavras-chave: Interação ecológica; degradação de moléculas; monitoramento; DNA ambiental; qPCR.

ABSTRACT

The work presented here focuses on understanding the biological processes and challenges associated with the occurrence of invasive fouling species in limnetic environments. First, we used a systematic review to identify the most important invasive fouling species in limnetic environments at a global scale and compiled the available information on their interactions (Chapter I). After finding that there are relatively few studies on these organisms, we turned to conducting empirical studies to assess the interaction between invasive and native fouling species in a hydroelectric power plant (UHE) in the Brazilian state of Paraná (Chapter II). One of the challenges in early detection of invasive species is sampling. Thus, a relevant alternative that have been tried to circumvent the problem is to identify and quantify the DNA released by these species into the environment, also known as environmental DNA or eDNA. Therefore, the next step was to experimentally decipher how DNA spreads in the water column (chapter III). We also addressed the challenges associated with detecting two invasive aquatic fouling species in two UHE reservoirs by using DNA (extract and environmental DNA) and investigating how abiotic factors influence the degradation of this DNA (chapter IV). In order to make our data available to the general public, we present the results of this work in a popular science article (Chapter V).

Keyword: Ecological interaction; molecule degradation; monitoring; environmental DNA; qPCR.

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Agradecimentos
A todas as energias que favoreceram a minha chegada até ao final deste trabalho.

1 APRESENTAÇÃO GERAL

Espécies aquáticas invasoras, são aquelas que invadem e se estabelecem em áreas onde historicamente não eram encontradas e passam a impactar negativamente os ecossistemas colonizados ou as atividades econômicas associadas aos ambientes aquáticos invadidos (Kolar e Lodge, 2001; Mcneely *et al.*, 2005; Russell e Blackburn, 2017). Entre as espécies aquáticas consideradas invasoras destacam-se aquelas que são incrustantes e que possuem estruturas de adesão que facilitam sua fixação em diferentes substratos (Nicklisch e Waite, 2012; Waite, 2017).

A disseminação e o estabelecimento destas espécies têm sido percebidos em escala global pela sociedade, através dos impactos econômicos diretos causados, principalmente às empresas de setores envolvidos na captação, tratamento e uso de água bruta, como indústrias de geração de energia hidrelétrica e tratamento de água para abastecimento humano (Callow, 1993; Coetser e Cloete, 2005; Rajagopal e Van Der Velde, 2012; Simberloff e Vitule, 2014; Mansur *et al.*, 2016).

A incrustação geralmente começa afetando as estruturas hidráulicas de captação de água (Elliott *et al.*, 2005; Karatayev *et al.*, 2015), canais de distribuição, redes, dutos e sistemas de refrigeração (Ricciardi e Reiswig, 1994; Ricciardi, 1996; Karatayev *et al.*, 2015). As incrustações provocadas por espécies aquáticas invasoras costumam ainda causar impactos ecológicos severos para espécies nativas (Richardson e Pyšek, 2008; Keller *et al.*, 2011), muitas vezes ocasionados até mesmo pelas interações ecológicas estabelecidas entre dois ou mais organismos invasores de um mesmo ambiente. Tais espécies costumam causar mudanças nos ambientes, interferindo nas condições físicas do habitat, ciclagem de nutrientes e nichos ecológicos (Clavero e García-Berthou, 2005; Burlakova *et al.*, 2014; Ricciardi, 2015).

Por muito tempo, a comunidade científica só contou com técnicas baseadas na observação direta de formas larvais, juvenis e adultas para o monitoramento dos organismos invasores incrustantes (Coste, 1982). Os métodos tradicionais associados a tal técnica costumam ser caros, demorados, pouco precisos, podendo até mesmo causar impactos negativos sobre as

próprias populações de organismos nativos nas áreas estudadas (Taberlet e Luikart, 1999).

Uma técnica relativamente recente, baseadas em DNA ambiental (eDNA) têm ganhado espaço nas pesquisas, no monitoramento de espécies aquáticas invasoras (Ficetola *et al.*, 2008; Barnes *et al.*, 2014) tanto em ambientes terrestres (Johnson, 2022) quanto aquáticos (Sassoubre *et al.*, 2016; Sansom e Sassoubre, 2017; Taberlet *et al.*, 2018; Dugal *et al.*, 2022).

A liberação de eDNA pelos organismos ocorre naturalmente, por meio de suas atividades biológicas básicas (alimentação, respiração, excreção, reprodução e morte), por meio de muco, pele, escamas, fezes, ovos, sangue e secreções, entre outras fontes (Thomsen *et al.*, 2012; Sassoubre *et al.*, 2016; Taberlet *et al.*, 2018). A coleta desse DNA pode ser realizada em ambientes aquáticos por meio de uma simples amostra de água, que posteriormente é submetida ao processo molecular de identificação e quantificação, possibilitando o monitoramento das espécies-alvo mesmo sem que haja um contato direto com as mesmas (Goldberg *et al.*, 2016; Tsuji *et al.*, 2019; Ray e Umapathy, 2022).

No entanto, até mesmo por envolver técnicas recentes, os estudos envolvendo eDNA ainda enfrentam alguns desafios metodológicos importantes. Dois dos grandes obstáculos a serem superados dizem respeito à compreensão e ao domínio dos fatores que levam à degradação do eDNA, tanto no ambiente, após a sua liberação, quanto nas amostras coletadas, ou seja, desde o campo até o momento em que serão analisadas em laboratório (Lindahl, 1993; Sassoubre *et al.*, 2016; Sansom e Sassoubre, 2017; Collins *et al.*, 2018). A degradação total ou parcial da fita de DNA pode comprometer os resultados da análise, gerando assim falsos negativos ou impedindo a quantificação do DNA (Saito, 2021).

A presente tese teve como objetivo identificar e caracterizar as principais espécies límnicas invasoras registradas atualmente no mundo; verificar quais delas estariam presentes em corpos hídricos no estado do Paraná; investigar suas interações ecológicas; e aprimorar as técnicas de monitoramento dessas espécies através do eDNA. Para isso, o trabalho foi subdividido em quatro

perguntas principais, que, por sua vez, deram origem aos cinco capítulos aqui apresentados. Tais perguntas iniciais foram:

- Quais são a principais espécies invasoras de água doce no mundo e quais informações existem sobre suas interações ecológicas? (Capítulo 1);
- Quais interações ecológicas existem entre espécies nativas, invasoras e incrustantes registradas em uma das mais importantes usinas hidrelétricas do estado do Paraná? (Capítulo 2);
- Como o DNA se distribuí ao longo da coluna de água? (Capítulo 3);
- Quais os efeitos de variáveis abióticas na degradação do DNA em um ambiente límnico? (Capítulo 4);

Por fim, com o intuito de apresentar para sociedade em geral o conhecimento científico gerado nos quatro capítulos iniciais da presente tese e utilizando uma linguagem simples, direta e de fácil compreensão, apresentamos um texto de divulgação científica sobre espécies invasoras incrustantes, seus desafios e principais resultados obtidos no trabalho aqui realizado (Capítulo 5).

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3 CAPÍTULO I - ECOLOGICAL INTERACTIONS BETWEEN FRESHWATER INVASIVE FOULING SPECIES WORLDWIDE

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Abstract: The successful colonization of limnic ecosystems by fouling invasive species has generated several negative impacts, causing severe and, sometimes, irreversible ecological and economic damage. These species usually establish complex biological interactions, which include the occupation of space and acquisition of food. This study aimed to identify the main fouling invasive species in limnic environments worldwide and to evaluate their ecological interactions. This review was based on criteria delineated by PRISMA (Preferred Reporting Items for Systematic Review and Meta-Analyses) using the Google Scholar platform. Following the search phase, a total of 7,915 relevant documents were identified. After applying eligibility criteria to include only studies on ecological interactions among invasive fouling species, only 44 documents, met all search requirements and were selected. This analysis allowed us to uncover that dreissenids (*Dreissena polymorpha* and *Dreissena bugensis*) make up the dominant group of invasive invertebrates that cause ecological impacts in limnic environments. Documented studies were strongly dominated by the USA, which was also the country with the highest number of reported species. In addition, a total of 13 invasive fouling species were identified and their interactions studied. Direct positive (mutualism), indirect positive (epibiosis, predation and cannibalism), and purely negative (competition) interactions were identified. These interactions have implications for the processes of biological invasion, which directly affect the dispersal and establishment of these species in their habitats. Competition was the most frequently documented interaction, which facilitated the process of colonization, establishment, and propagation of fouling invasive aquatic species in limnic environments.

Keywords: dreissenids; biofouling; bioinvasion; freshwater.

3.1 Introduction

Exotic species are defined as those species that establish themselves outside their original distribution area, where they can reproduce, and yet do not cause severe impacts to the environments they have colonized (Inoue et al. 2015; Rejmánek et al. 2002; Ribeiro-Silva and Manête 2021; Richardson et al. 2000). Invasive aquatic species, on the other hand, besides invading and settling in areas where they were not historically found, also negatively affect the colonized ecosystems or economic activities that depend on these environments (Kolar and Lodge 2001; McNeely et al. 2005; Russell and Blackburn 2017). There are still those species with little historical data on their occurrence, and this ends up causing a lack of information, these species can be classified as cryptogenic (Carlton, 1996).

The spread and establishment of fouling invasive aquatic species have been perceived by society through the direct economic impacts caused, mainly to companies in sectors involved with raw water catchment, treatment and use, such as hydroelectric power generating industries and water treatment for human supply around the world (Callow 1993; Coetser and Cloete 2005; Mansur et al. 2016; Rajagopal and van der Velde 2012; Simberloff and Vitule 2014). Fouling species usually affects hydraulic water intake structure (Elliott et al. 2005; Karatayev et al. 2015; Melo and Bott 1997; Perepelizin and Boltovskoy 2011), water channels, grids, pipelines and cooling systems (Karatayev et al. 2015; Ricciardi 1996; Ricciardi and Reiswig 1994) by blocking pipes (Barton et al. 2008; Pucherelli et al. 2016), reduce the speed of water flow (Darrigran 2002), cause filter occlusion (Darrigran 2002; Nakano and Strayer 2014), and increase the corrosion of hydraulic structures (Effler et al. 1996; Nakano and Strayer 2014; Nelson 2009).

Fouling invasive aquatic species also often cause severe ecological impacts for native species (Keller et al. 2011; Richardson and Pyšek 2008). These organisms cause environments changes, interfering on the habitat physical conditions, nutrient cycling and ecological niches (Burlakova et al. 2014; Clavero and García-Berthou 2005; Ricciardi 2004; Ricciardi 2015). Furthermore, ecological communities are established through complex sets of direct and indirect interactions between species, which are mediated by changes in their abundance (Beshai et al. 2021; Mittelbach and McGill 2019; Somaweera et al. 2019; Utsumi et al. 2010). Interactions between native species are often replaced after the introduction or establishment of invasive species, which can change the structure and ecological properties of invaded habitats (Didham et al. 2007; Dobler and Geist 2022; Hellmann et al. 2008; Tilman 1996). As a consequence, invasive species have also facilitated and contributed to the establishment of other potentially invasive species over the course of a bioinvasion process (Ricciardi 2005; Simberloff 2009; Simberloff and Von Holle 1999).

The interactions between invasive species can be classified according to the characteristics of their effects (Burkholder 1952; Kueffer and Daehler 2009; Lidicker Jr 1979; Malcolm 1966; Odum 1959), i.e direct positive interactions, when individuals of the species involved mutually benefit from that may include access to food, shelter or substrate (Crego et al. 2016), indirect negatives interactions, when only one of the individuals in the interaction has positive benefits and the others do not have and purely negative, when the population or organisms depend on other individuals, and at least one species involved in this relationship is harmed (Lidicker Jr 1979).

Understanding the complexity of ecological interactions among invasive fouling species is fundamental to establishing management strategies aimed at the protection and conservation of freshwater environments. Besides interacting with each other, invasive

species also interact with native species. Often these relationships cause cumulative impacts, as with facilitating interactions (mutualism), which can occur concomitantly with negative ones (competition, predation and epibiosis) (Altieri et al. 2010; Beshai et al. 2021; Bulleri and Benedetti-Cecchi 2008; Callaway 2007; Ricciardi 2005; Rodriguez 2006; Smith et al. 2004). The result may be the replacement of the ecological niche of native species, besides facilitating the entry of pathogens and diseases into the environment, with direct effects on the biodiversity of native communities (Ricciardi 2001; Ricciardi 2004; Ricciardi 2005; Ricciardi and MacIsaac 2011b; Simberloff 2009; Simberloff and Alexander 1998; Simberloff and Von Holle 1999; Wong and Gerstenberger 2015). Due to the complexity of biological invasions in limnic environments and their potential negative impacts on biodiversity and economics, here, we identify the main fouling invasive species in limnic environments worldwide and their interactions through a systematic review.

3.2 Materials and Methods

The identification and characterization of interactions between limnic invasive aquatic species were performed after a systematic literature review, using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) method (Moher et al. 2009), based on the Google Scholar platform. The aim was to search for literature sources that presented information about the invasive limnic fouling species and their ecological interactions in hydropower reservoirs. However, due to the limited number of these studies (just four), we then amplified the selection to include interaction of invasive species in limnic environments beyond hydropower reservoirs.

Three criteria were adopted for the inclusion of studies (i): the approach focusing on ecological interactions among fouling invasive species; (ii) evidence of facilitation

between these species; (iii) a description, by the authors, of the biological characteristics of the fouling species, including type, strategies or reproductive period, habitat, feeding habits or biological characteristics of the fouling, as well as the effects of interactions. For this, we searched for scientific articles, books, theses and reports published until January 2019, which presented in their title, abstract, or keywords the terms listed in Table 1. The searches were performed both in English and in Portuguese.

Table 1. Terms and combinations used to search for and obtain bibliographic data for identification of ecological interactions between fouling invasive species in limnic environments

Terms and combinations
Interactions + Facilitation + Ecological + Hydroelectric + Power + Invasive + Biotic
Interactions + Facilitation + Ecological + Hydroelectric + Power + Non indigenous + Fouling + Biotic
Ecological + Succession + Hydroelectric + Power + Invasive + Fouling + Biotic

The initial searches, using the combination of cited terms, identified a total of 7,915 documents. Of those, 4,971 were pre-selected after elimination of those with some non-adherence to the established criteria or because they were duplicates. At the end of the second phase, 1,501 documents were selected for presenting in their title information directly related to the research object. After careful reading the abstracts, the number of documents was reduced to 156. In the case of books and book chapters, which did not have a structured abstract, the relevance of themes addressed by authors was evaluated. After full reading of the texts, 44 documents (27 scientific articles, 8 books and 9 theses and dissertations) were selected for meeting the objectives of identification and characterisation of ecological interactions between fouling aquatic species [Table 2].

Table 2. Evaluation and identification of documents used for evaluation and characterization of interactions between invasive fouling species, detailed in the four phases of the systematic review

Phase 1: Pre-Identification	**Number of Documents**
References pre-identified in Google Scholar	7,911
Documents identified from other sources	04
Duplicated documents	2,944
Phase 2-Selection	
Exclusion of duplicates	4,971
Exclusions for not meeting the criteria (i; ii, and iii presented at the Materials and Methods section)	3,470
Selected after title review	1,501
Phase 3: Eligibility	
Assessed for eligibility	1,501
Excluded	1,345
Selected for full document reading	156
Final results (included documents)	44

The 44 documents selected (40 in English and 4 in Portuguese) were saved in PDF format and, using the software R (R Core Team 2015), word mining was performed on the words used by the respective authors in the selected English texts. We plot the studies and their species in a map with QGis v.3.6.2 (https://www.qgis.org/) licensed under CC-BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0/).

Additionally, we classified the studies into a categorical traits matrix, characterizing the species, phylum, ecological interactions, locality (country), and metadata (authors and publication year) of each study. On the studies with more than one locality were included again on the categorical matrix. We then used the traits species, ecological interactions, and locality in a multiple correspondence analysis (MCA) to identify distribution of the traits in a bi-dimensional space as implemented in the clustrd v. 1.3.7-2 R package (Markos et al. 2019).

Finally, the ecological interactions described in each study were investigated, as well as the ecological niche and trophic levels of the invading limnic species involved. The biological

characteristics of the species and the types of environments assessed by the authors were also described.

3.3 Results

3.3.1 Exploratory data analysis

The studies analysed were concentrated in North America (n=11), followed by Europe (n=7), South America (n=6) and Oceania (n=3) [Table 4]. In 17 of the cases (38.6% of the total), essentially literature reviews, it was not possible to specify the location of the episodes reported by the authors. The distribution of fouling limnic invasive species are concentrated on three main continents, represented by North America (n = 18), South America (n = 12), and Europe (n = 8, fig. 1). Documents studies were strongly dominated by the USA (n = 15), which was also the country with the highest number of reported species (*Cordylophora caspia* (n = 1); *Dreissena. polymorpha* (n = 14), *Dreissena bugesnsis* (n = 3), *Corbicula fluminea* (n = 1), *Ephidatia fluviatilis* (n = 1), *Plumatella fungosa* (n = 1), *Lophopodella carteri* (n = 1), and *Hidrilla vertillatlata* (n = 1). Interactions related to invasive species in the USA was competition (n = 10; 61.5 %) followed by epiosis and predation (both with n = 2; 12.5 %). The second country with the highest report of interactions was Brazil, being represented by competition (n = 8; 61.5%), mutualism (n = 3; 23.1%) and epiosis (n = 2; 15.4%).

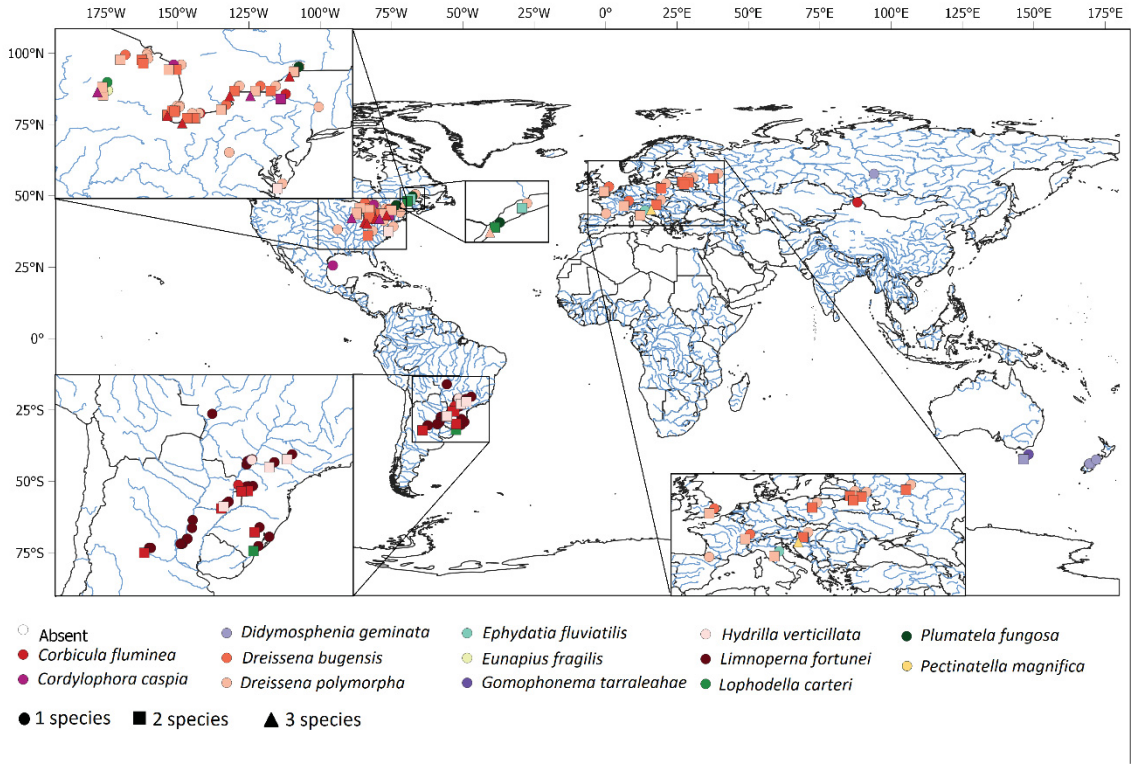

Fig. 1 Worldwide distribution of the 13 fouling limnic invasive species reported in the reviewed studies. Circle symbols represent the rating of one species, squares rating with two species and triangles represent rating including three species.

The multiple correspondence analysis showed significant relationships between ecological interactions, species, and study sites ($p < 0.05$). The first axis explained 8.5% of the data variation, and the competition interaction, followed by *D. polymorpha* were the terms that most contributed to this axis. The second axis explained 8.3%, represented mainly by epibiosis and *L. carteri*. It is possible to observe that the sponges (*E. fragilis* and *E. fluviatillis*) and the interaction (facilitation) are found in a more dispersed way in the bi-dimensional space. Some studies cite groups of organisms but do not report the species studied in the evaluation of ecological interaction, contributing to some data as absent in the analysis.

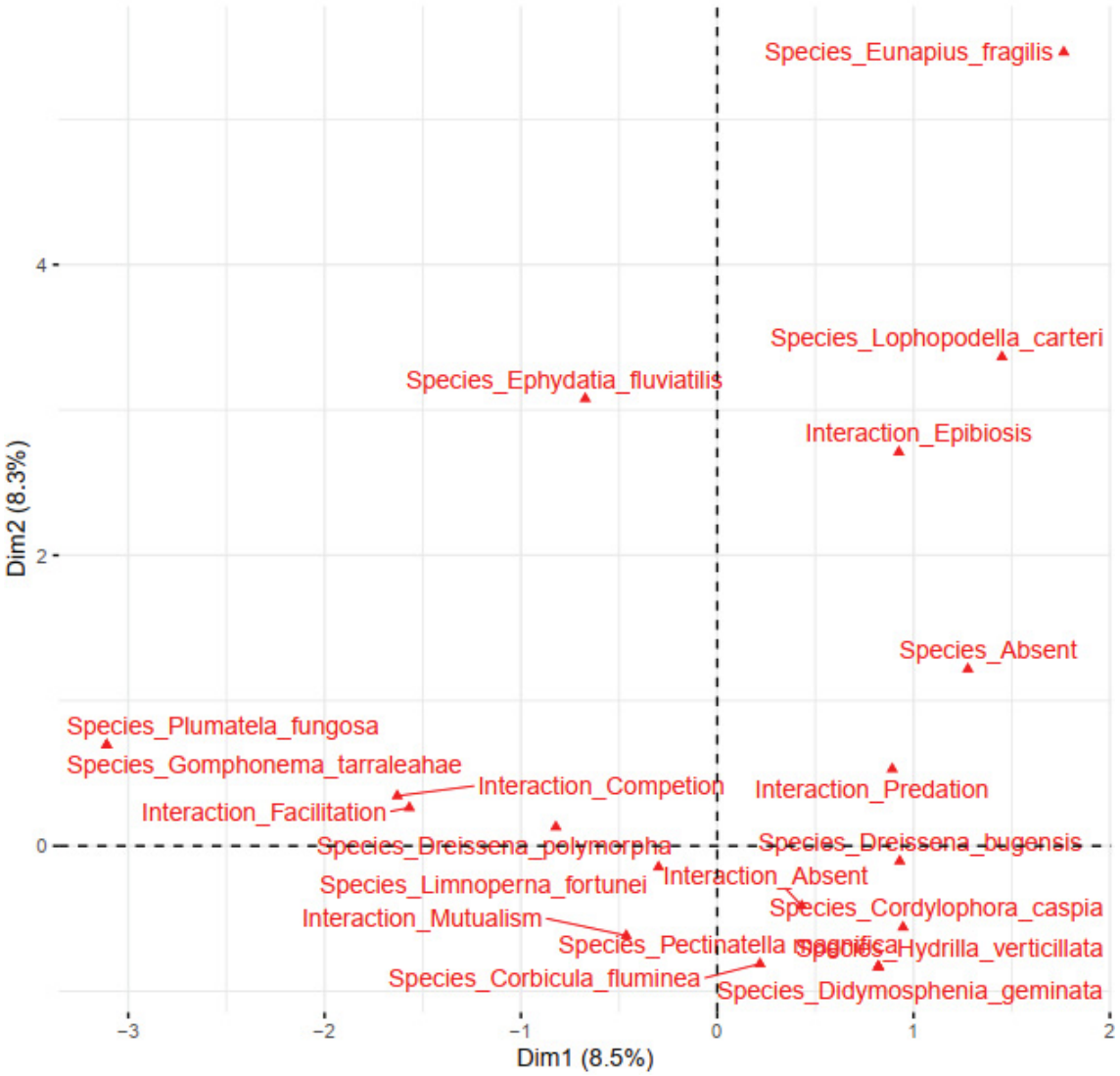

Fig. 2 The results of the multiple correspondence analysis with fouling invasive limnic species and their ecological interactions. The triangles symbolize all 44 references included in this study.

3.3.2 The taxonomic relationship between freshwater invasive species

The 13 freshwater fouling invasive species identified in this study have representatives in both the Plantae and Animalia kingdoms and are divided into seven classes, eight orders, ten families and 12 genera, as summarised in Fig. *3*.

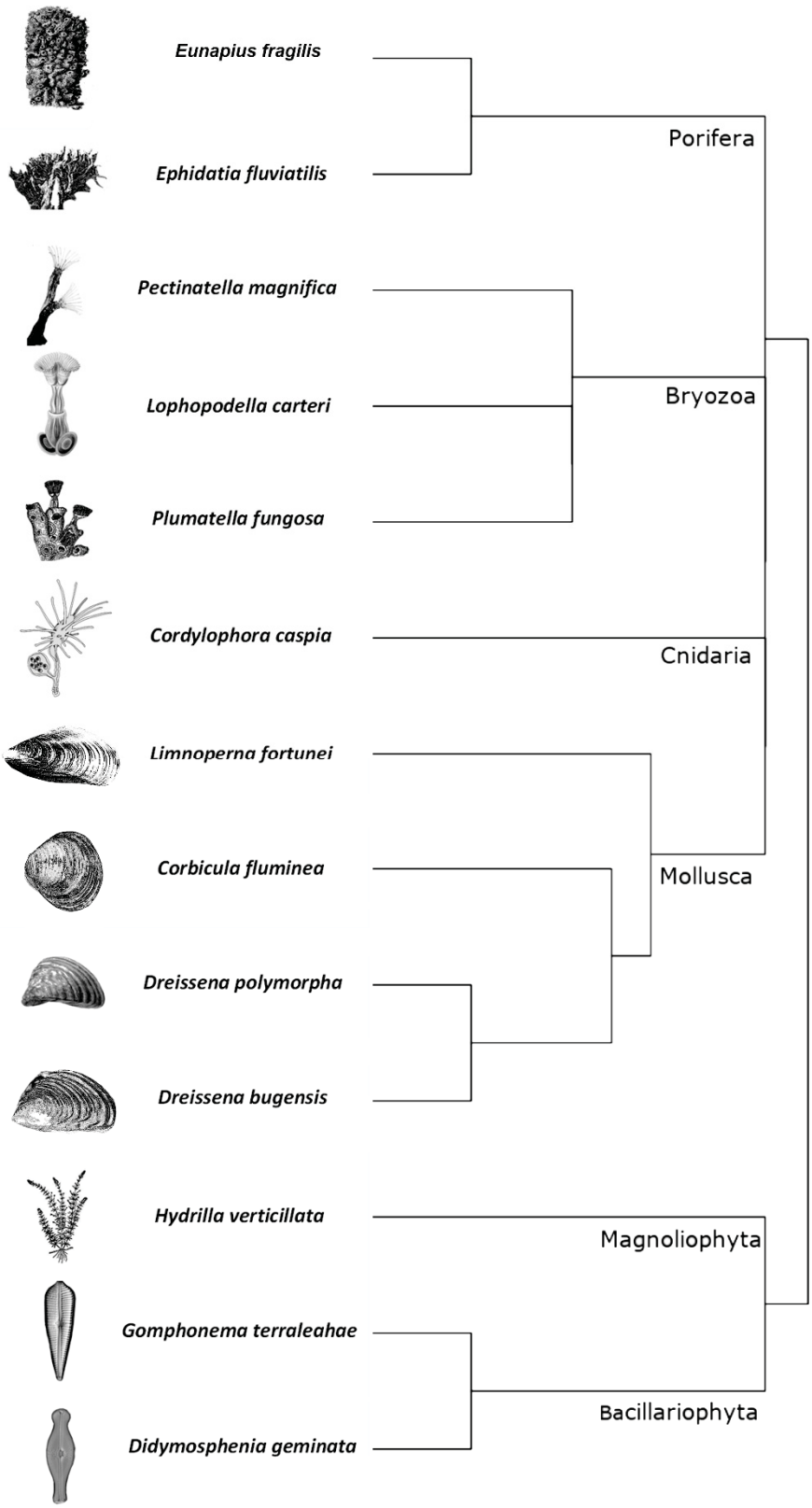

Fig. 3 Dendrogram constructed from cluster analysis, using the Jaccard similarity index, relating the 13 main freshwater fouling invasive species around the world

The most significant number of freshwater invasive fouling species ever identified are of Asian origin (7 species), followed by North America (3 species). The phylum Mollusca (*Limnoperna fortunei*, *Dreissena polymorpha*, *Dreissena bugensis*, and *Corbicula fluminea* (Of Muller, 1774)), Cnidaria (*Cordylophora caspia* (Pallas, 1771), Magnoliophyta (*Hydrilla verticillata* (L.f. Royle)) and Bryozoa (*Lophopodella carteri* (Hyatt) , 1886) shared the same continent of origin: Asia. Most identified species were filtering feeding, plankton-feeding or photosynthetic eating habitats. However, there are few records indicating their origin, so by definition they can be classified as cryptogenic. [Table 3].

Table 3. Principal freshwater fouling invasive species, their feeding habitat and origin.

Species	**Feeding habit**	**Continent of origin**	**References**
Limnoperna fortunei	Filter feeding	Asia	(Boltovskoy and Correa 2015)
Dreissena polymorpha	Filter feeding	Asia	(Karatayev et al. 2007)
Dreissena rostriformis bugensis	Filter feeding	Asia	(Ram et al. 2012)
Corbicula fluminea	Filter feeding	Asia	(Sousa et al. 2008)
Cordylophora caspia	Planktophagous/ Carnivorous	Asia	(Folino-Rorem 2000)
Eunapius fragilis	Filter feeding	North America	(Braekman and Daloze 1986; Williams et al. 1985)
Ephydatia fluviatilis	Filter feeding	Netherlands	(Van 1977; Manconi 2008; Manconi and Pronzato 2008)
Lophopodella carteri	Planktophagous	Asia	(Lacourt 1968)
Pectinatella magnifica	Planktophagous	North America	(Jullien 1885; Kafka 1887)
Plumatella fungosa	Planktophagous	Europe	(Wood and Okamura 2005)
Hydrilla verticillata	Autotrophic	Asia and Oceania	(Cook and Lüönd 1982)
Gomphonema tarraleahae	Autotrophic	Oceania	(Andrewartha et al. 2007)
Didymosphenia geminata	Autotrophic	North America and Europe	(Blanco and Ector 2009; Kelly 2009)

Table 4. Geographic regions and natural environments reported in studies of ecological interactions between invasive aquatic species. NR -Not Reported

Country	River	Lake	Hydroelectric Power Plants (HPP) reservoir	Reference
North America				
Canada	NR*	Erie	NR	(Cummings and Graf 2010)
		Erie and Ontario		(Garton et al. 2013; Wood 2013)
EUA	Hudson	Michigan	NR	(Lauer et al. 1999b; Lauer and Spacie 2004)
	Verdigris and Arkansas	Great lakes	Oologah and Sooner	(Ricciardi 2001; Wong and Gerstenberger 2015)
EUA and Canada	Detroit	Erie and Ontario	NR	(Burlakova et al. 2014)
	Lawrence	Erie, Ontario, St. Clair, Burt, Douglas and Paradise		(Ricciardi and Reiswig 1994; Ricciardi et al. 1996)
United Kingdom	Wraysbury	NR	NR	(Aldridge et al. 2014)
South America				
Argentina	Tercero	NR	NR	(Tokumon and Boltovskoy 2018)
Brazil	Piratini Paranapanema, Tietê and Paraná	NR	NR	(Reis 2014) (Michelan et al. 2014)
Argentina and Brazil	Paraná	NR	Itaipu	(Borges 2014)
	Tercero and Paraná		NR	(Ernandes-Silva et al. 2016; Montalto and Molina 2014)
Europe				
Spain	Ebro	NR	NR	(Araujo et al. 2010)
Italia	NR	Maggiore	NR	(Kamburska et al. 2013)
		Trasimeno		(Gaino 2005)
NR	NR	Great lakes	NR	(Cairns and Bidwell 1996; Velde and Rajagopal 2006; Wormer 2009)
		Batalon and Saint Clair		(Molloy et al. 1997)
Oceania				
New Zealand	NR	NR	Krasnoyarsk	(Whitton et al. 2009)
	Waitaki		NR	(Lagerstedt 2007)
Australia	NR	NR	Tasmania	(Ravizza 2015)

3.3.3 Reproductive characteristics

Some of the reproductive characteristics of fouling invasive species were common to each other. For example, *D. polymorpha, D. bugensis, L. fortunei*, *C. fluminea, C. caspia, E. fragilis* (Leidy, 1851)*, E. fluviatilis* (Linnaeus, 1759)*, L. carteri, P. magnifica* (Leidy, 1951), and *P. fungosa* (Pallas, 1768) are dioecious and eventually hermaphroditic. The reproductive process of these species is evidenced with various tactics and characteristics invasive species (Callil et al. 2012; Cataldo and Boltovskoy 1999; Joo et al. 1992; Paulus and Weissenfels 1986; Pereira et al. 2018; Perkins et al. 2009). The molluscs *D. polymorpha*, *D. bugensis*, *L. fortunei*, and *C. fluminea* reach sexual maturity within one year, have high fecundity, life cycle divided into several planktonic larval stages, juveniles and adults are sessile (except for *C. fluminea*), but they can detach from the substrate and actively disperse. *Corbicula fluminea* has a different strategy from other mollusc species. This species performs internal fertilization with the release of its larvae at a later stage (pediveliger). *Dreissena polymorpha, D. bugensis,* and *L. fortunei* release gametes into the environment, and the fertilization is external. The reproduction of *D. polymorpha*, *L. fortunei,* and *C. fluminea* occurs in spring and summer, and *D. bugensis* reproduces mainly in summer, at temperatures between 5 and 18 ºC [Table 5]. Reproduction of *C. caspia* happens at temperatures between 16 and 30 ºC, through budding, forming new colonies and producing menonts that are fragments of tissue with regenerative capacity, highly resistant to drought and high temperatures. The species has planktonic larvae. Juveniles and adults are sessile. In adulthood, individuals can reach 5 to 400 mm in length.

The cryptogenic sponges *E. fragilis* and *E. fluviatilis* reproduce during spring and summer, after about 2 and 4 months of life, respectively. This group has one of the most effective strategies for reproduction, such as the ability to regenerate through the budding process, fragmentation and resistance propagules, called gemmules, which are formed by a rigid wrap inside the sponges. The bryozoan *Lophopodella carteri* reproduces at temperatures between 15 and 35 ºC, whereas *P. magnifica* reproduces between 18 and 24 ºC and *P. fungosa* between 19 and 20 ºC. These organisms use as reproductive strategy the formation of statoblasts (hard and rounded structures), which are released in capsule forms, called ovoids, and that function as seeds for the generation

of future individuals. *Hydrilla verticillata* macrophyte reproduces at temperatures between 20 and 30ºC, through the production of tubercules (or nodules), which can remain viable for up to 4 years, and through the fragmentation of the stems, which is the primary mechanism of dispersal of this species to different aquatic environments. Diatoms *D. geminata* and *G. tarraleahae* reproduce by cell division, with separation of the frustules resulting in daughter cells. *Didymosphenia geminata* (Schmidt, 1899) reproduces at temperatures between 11.5 and 14.6 °C during spring, and *G. tarraleahae* (Perkins et al. 2009) during spring and summer.

Table 5. Reproductive characteristics and sexual maturity of freshwater fouling invasive species

Species	**Reproduction**				**Time to sexual maturity (months)**	**References**
	Type	**Strategy***	**Period**	**Temperature (ºC)**		
Dreissena polymorpha	Di; He	HF	Late spring and early summer	12 - 13	3-11	(Nichols 1996; Sprung 1991); (Wong and Cross 2015)
Dreissena bugensis	Di; He	HF	Summer	5 - 18	4-5	(Nalepa et al. 2009; Wilson et al. 1999); (Ianniello 2013); (Ackerman et al. 1994)
Limnoperna fortunei	Di; He	HF	Spring and summer	11 - 35	3-4	(Callil et al. 2012; Cataldo and Boltovskoy 1999; Darrigran et al. 1999; Oliveira 2003);(Callil et al. 2012)
Corbicula fluminea	Di; He	JD	Spring and late summer	16 - 22	3-9	(Britton 1979) (Cataldo and Boltovskoy 1999; Park and Chung 2004)
Cordylophora caspia	Di; He	Bu; Me	Spring	16 – 30	-	(Jormalainen et al. 1994)

Species	Reproduction				Time to sexual maturity (months)	References
	Type	Strategy*	Period	Temperature (ºC)		
						(Borges 2013; Pereira et al. 2018; Schuchert 2004)
Eunapius fragilis	Di; He	Bu; Ge	Spring and summer	15-25	2	(Williamson and Fitter 1996)
Ephydatia fluviatilis	Di; He	Bu; Ge	Spring and summer	-	4	(Paulus and Weissenfels 1986); (Manconi et al. 2008)
Lophopodella carteri	Di; He	St	Spring and summer	15-35	-	(Bushnell and Rao 1974; Ricciardi and Reiswig 1994)
Pectinatella magnifica	Di; He	St	Spring	19-20	-	(Joo et al. 1992; Ricciardi and Reiswig 1994)
Plumatella fungosa	Di; He	St	Spring and summer	18-24	-	(Bushnell and Rao 1974; Jónasson 1963; Wesenberg-Lund 1896)
Hydrilla verticillata	Di; He	SF; Tu	Spring and summer	20-30	-	(Sousa 2011; Van et al. 1978; Van and Steward 1990)
Didymosphenia geminata	Di; VC	-	Spring	11,5 - 14,6	-	(Kawecka and Sanecki 2003; Round et al. 1990)
Gomphonema tarraleahae	VC	-	Spring and summer	-	-	(Perkins et al. 2009)

* Di - Dioecious; He - Hermaphrodite; VC - Vegetative cell division; HF - High fecundity; JD - Juvenile development in demibranchs; Bu - Budding; Me - Menont; Ge - Gemmulation; St – Statoblasts; SF; Stem Fragmentation; Tu – Tubercle.

3.3.4 Ecological interactions

The main ecological interactions identified among the freshwater invasive fouling species were: mutualism (direct positive interaction), competition (negative interaction), and epibiosis, predation, cannibalism (indirect positive interactions). A mutualistic relationship was described between *L. fortunei* and *H. verticillata*. The golden mussel uses the macrophyte as a substrate for its fixation. *Hydrilla verticillata*, in turn, benefits from the fact that the mussel is a filtering organism, increasing the water transparency and, thus, favouring the realization of photosynthesis by macrophyte.

Predation was recorded among *C. caspia*, *D. bugensis* and *D. polymorpha*. *Cordylophora caspia* can be settle on these molluscs and feed on their larvae. Some species are used as basibionts, that is, they are used as substrate by species considered epibionts, such as *D. polymorpha* which hosts *E. fluviatilis*, *E. fragilis*, *C. caspia*, *D. bugensis*, *L. carteri*, *P. fungosa,* and *P. magnifica*; *C. fluminea* wich hosts *L. fortunei;* and *H. verticillata* which hosts *D. polymorpha* and *L. fortunei*. Cannibalism has been reported among adult, larval and juvenile of *D. polymorpha*. This species is considered a strong competitor for space with *E. fluviatilis, E. fragilis*; *C. fluminea*, *D. polymorpha* (juvenile), *D. bugensis*, *C. caspia,* and *P. magnifica* (Fig *4*). The competition was the most frequently reported type of ecological interaction among fouling freshwater invasive species in the literature, followed by epibiosis, predation, mutualism, and cannibalism.

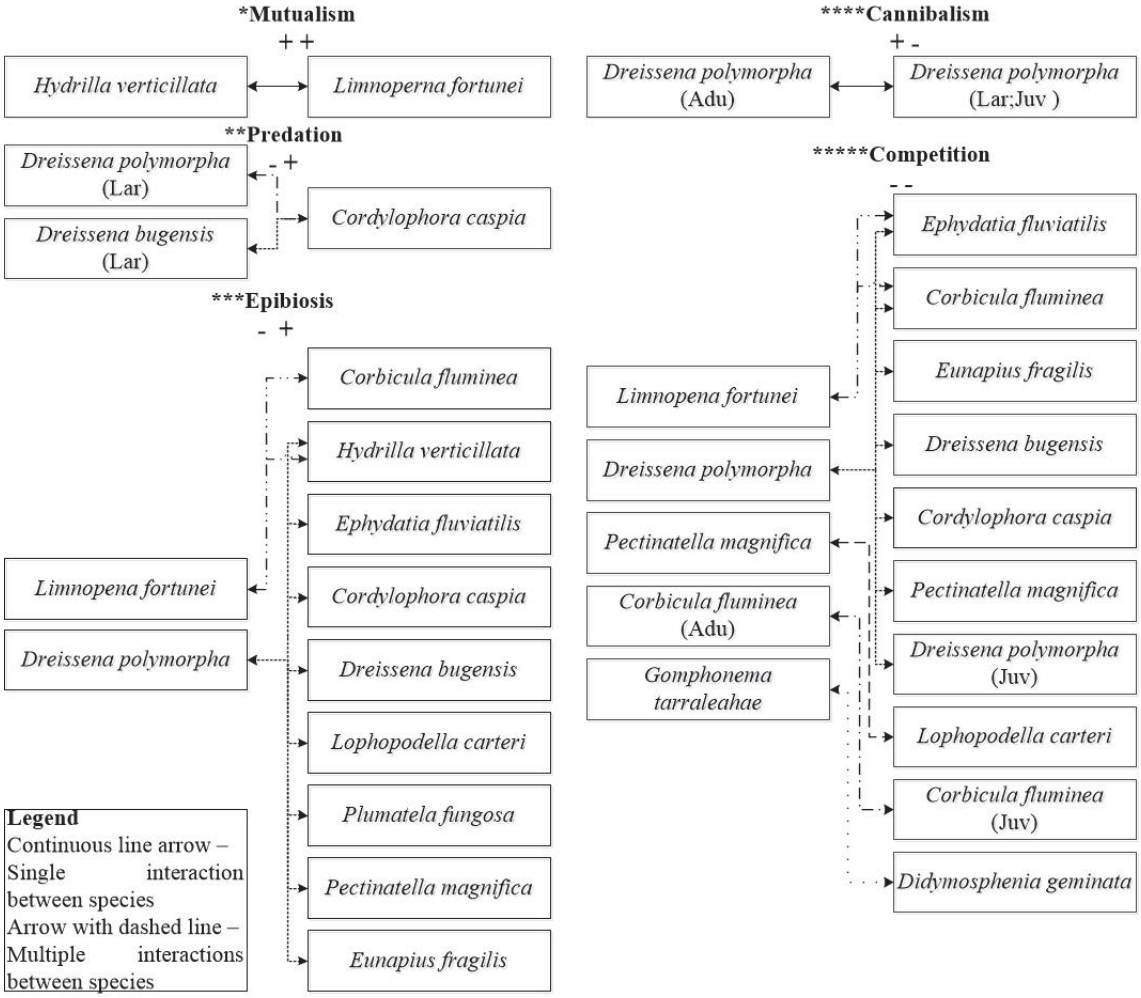

Fig 4 Schematic representation of ecological interactions and thei effects between fouling invasive aquatic species in limic environments. *Mutualism: Sousa et al. (2009a); **Predation: Olenin and Leppäkoski (1999), Pucherelli et al. (2016); ***Epibiosis:, Ricciardi and Reiswig (1994), Ricciardi (1996), Lauer et al. (1999a), Cummings and Graf (2010), Aldridge et al. (2014), Borges (2014), Michelan et al. (2014), ****Cannibalism: Wood (2013); *****Competition: Lewandowski (1982), Ricciardi and Reiswig (1994), Ricciardi (1996), Molloy et al. (1997), Wormer (2009), Cummings and Graf (2010), Ravizza (2015)

Table 6. Number and frequency of reports on ecological interactions between freshwater invasive fouling species described in bibliography

Ecological Interaction	Nº reports	Frequency %	Reference
Competition	28	50,00	(Ernandes-Silva et al. 2016; Karatayev et al. 2010; Wormer 2009)

Ecological Interaction	Nº reports	Frequency %	Reference
Epibiosis	17	30,35	(Lauer et al. 1999b; Ricciardi 1996)
Predation	3	05,35	(Molloy et al. 1997; Olenin and Leppäkoski 1999; Pucherelli et al. 2016)
Mutualism	8	14,28	(Michelan et al. 2014; Sousa et al. 2009a)
Cannibalism	2	3,7	(Lewandowski 1982; Maclsaac et al. 1991a)
TOTAL	58	100	

3.4 Discussion

Dreissenids were the group most strongly correlated to the ecological impacts caused in limnic ecosystems, as corroborated by authors such as Griffiths et al. (1991); Padilla (1997); Daunys et al. (2006); Dölle and Kurzmann (2020); Griffiths et al. (1991); Karatayev et al. (2007); Padilla (1997); Wawrzyniak-Wydrowska et al. (2019); Dölle and Kurzmann (2020). Dreissenid reproduction coincides with the warmest periods of the year (spring and summer) (Ackerman et al. 1994; Nichols 1996) and, as they are filtering organisms, the relationship between the abundance of these organisms and food (phytoplankton) is clear, which depends on the presence of dissolved nutrients (mainly phosphorus) in water. Several studies show that invasive organisms are related to the preference or need of these species to live in homogeneous environments, usually associated with significant loss of biodiversity, and high genetic and taxonomic similarity (Olden 2006; Olden et al. 2004; Ricciardi 2001; Ricciardi and MacIsaac 2011b). Therefore, it is not uncommon for biological invasions and local rarefaction or extinction of native species to occur in parallel (Olden 2006; Olden et al. 2004; Ricciardi and MacIsaac 2011b; Simberloff 2009). The predation and competition pressures exerted by invasive species play an essential role in this process (García-Ramos and Rodríguez 2002; Rodriguez 2006). Environmental changes caused by fouling invasive aquatic species may also benefit and facilitate the entry of other non-fouling invasive species, contributing to intensify the process of habitat disturbance (Ricciardi 2001; Rodriguez 2006; Simberloff 2009; Simberloff and Von Holle 1999).

Freshwater fouling invasive species are concentrated in Mollusca, Cnidaria, Bryozoa, Porifera, Bacillariophyta, and Magnoliophyta phyla. Limnic molluscs were the most

representative group. Problems related to them have been reported in practically all continents, such as in South America (*L. fortunei* and *C. fluminea*) (Darrigran 2002; Mansur and Garces 1988a; Uliano-Silva et al. 2013); North America (*D. polymorpha, D. bugensis* and *C. fluminea)* (Armero and Halcón 2001; Pucherelli et al. 2018); Europe (*D. polymorpha, D. bugensis* and *C. fluminea)* (Armero and Halcón 2001; Pucherelli et al. 2018), and Oceania (*C. fluminea*) (Whitton et al. 2009). North America and Europe are the continents with the largest number of studies assessing the ecological interactions related to invasive aquatic fouling species. The highest frequency of reported cases occurred in natural lakes and the lowest in reservoirs of hydroelectric power plants located in North America (Ricciardi 2001; Wong and Gerstenberger 2015), South America (Borges 2014), and Oceania (Ravizza 2015; Whitton et al. 2009).

Ecological interactions can be related to several impacts and also to the degree of severity of impacts caused by bioinvasive fouling. The increased frequency of introduction of invasive species ends up making ecosystems more susceptible to invasions and requiring ever greater financial and operational resources to mitigate the associated impacts (Ricciardi et al. 2013; Ricciardi and MacIsaac 2011a). The impacts caused by invasive organisms are also intrinsically related to the abundance of individuals in a given area and the possibility of synergy between different species (Simberloff and Von Holle 1999). The economic and ecological impacts caused, individually or jointly, by *L. fortunei*, *C. caspia,* and *C. fluminea* in South America are well documented (Darrigran 2002; Mansur and Garces 1988b; Uliano-Silva et al. 2013). At high densities these organisms obstruct the hydraulic systems and heat exchangers in hydroelectric plants, which can reduce the efficiency of energy production (Aldridge and Muller 2001; Belz et al. 2016; Borges 2013; Borges 2014). Interactions between these species can accelerate trophic changes and compromise the availability of food for native species (Belz et al. 2010; Darrigran 2002; Darrigran et al. 2007; Uliano-Silva et al. 2013). In North America, operational problems in hydraulic systems of power plants and water supply caused by interactions between species such as *D. polymorpha*, *D. bugensis*, *C. fluminea*, *L. carteri* and *E. fragilis* were reported by Lauer et al. (1999a); Armero and Halcón (2001); Lauer et al. (1999a); Ricciardi (2001); Pucherelli et al. (2018). These species also cause ecological impacts, such as competition for resources with native

species (Lauer and McComish 2001). In Europe, *D. polymorpha*, *D. bugensis* and *C. fluminea* cause obstruction of hydraulic systems (Armero and Halcón 2001; Pucherelli et al. 2018), clogging of cooling filters, plates and condensers (Armero and Halcón 2001) and pipes of power and water treatment plants (Folino-Rorem and Indelicato 2005; Lipsey Jr and Chimney 1978; Pucherelli et al. 2018). In Oceania, it was found that *D. geminata* e *G. tarraleahae* can cause the obstruction of hydraulic systems, in addition to competing for substrates, light and nutrients with native species (Ravizza 2015; Whitton et al. 2009). Seven of the freshwater invasive fouling species reported in our study (*D. bugensis*, *D. polymorpha*, *Corbicula* sp., *L. fortunei*, *C. caspia*, *Plumatella* sp., and *P. magnifica*) were also identified by Nakano and Strayer (2014) causing economic impacts, mainly related to biofouling in water treatment plant hydraulic systems and hydroelectric plants.

The 13 fouling invasive aquatic species reported here identified share biological and especially reproductive characteristics that facilitate the bioinvasion process, such as rapid growth rates, early sexual maturity, existence of one or more successful breeding strategies, dispersal and opportunistic behaviour. Such characteristics are present in *D. bugensis* (Wong and Cross 2015)*, D. polymorpha* (Karatayev et al. 2010; Nichols 1996; Sprung 1991), *C. fluminea* (Byrne and McMahon 1994; Darrigran 2002; McMahon 1982)*, L. fortunei* (Boltovskoy 2015; Boltovskoy and Correa 2015; Boltovskoy et al. 2015; Darrigran 2002), *C. caspia* (Folino-Rorem 2000; Folino-Rorem et al. 2006; Nakano and Strayer 2014), *E. fluviatilis* (Manconi et al. 2008; Manconi and Pronzato 2008), *E. fragilis* (Braekman and Daloze 1986; Williams et al. 1985), *P. magnifica* (Gontar 2012), *L. carteri* (Ricciardi and Reiswig 1994; Wood 1989), and *P. fungosa* (Wood and Okamura 2005); Magnoliophyta: *H. verticillate* (Michelan et al. 2014); Bacillariophyta: *G. tarraleahae* (Andrewartha et al. 2007; Ravizza 2015) and *D. geminata* (Erickson and Shearer 2006; Shearer and Erickson 2006).

Some of these same biological characteristics are facilitators of ecological

relationships involving invasive fouling species, causing these species to relate through predation at various moments of the life cycle, (Burlakova et al. 2012; Nakano and Strayer 2014), epibiosis (Lauer and Spacie 2004; Wahl 2008), ecological niche competition, substrate, food or territory (Burlakova et al. 2014; Garton et al. 2013; Lancioni and Gaino 2007). The individual action of invasive species can also lead to changes that favour other invasions (Michelan et al. 2014; Ricciardi 1996; Ricciardi 2001; Simberloff 2013; Simberloff and Von Holle 1999).

Interactions with direct negative effects (competition) and indirect positive effects (epibiosis, cannibalism and predation) reached similar proportions in studies by different authors. Often, these interactions involved species with some proximity to their geographical origin or evolutionary history. In some cases, such as in the study by Ricciardi (2001) with *D. bugensis*, *D. polymorpha*, *C. caspia* and *L. carteri*, conducted in the Great Lakes region, indicated that positive one-way interactions, such as herbivory, predation and parasitism, are more common and relevant than purely negative ones. Other studies have demonstrated the predominance of negative or neutral interactions among invasive species (Jackson 2015; Kuebbing and Nuñez 2015).

Mutualism is considered a positive and direct interaction. The species involved in mutualistic relations described include the golden mussel *L. fortunei,* invader in South America (Darrigran and Pastorino 1995) and the macrophyte *H. verticillata* invader in several continents like Europe, South America, North America, Africa and Australia (Cook and Lüönd 1982; Madeira et al. 2007). Both species are known to cause impacts on the environment and also in hydraulic systems for water uptake and hydroelectric power plants (Michelan et al. 2014; Sousa 2011).

Facilitating interactions between freshwater fouling invasive species can have direct consequences on the bioinvasion process itself, as ecosystems become more easily

invaded as the number of invasions increases (Ricciardi 2001). Macrophyte *H. verticillata* achieves high biomass (Sousa et al. 2009b) and ends up providing substrate for colonization by *L. fortunei* (Michelan et al. 2014). However, there is still little experimental research demonstrating the role of interspecific interactions as a success factor for invasive species in freshwater environments, especially in the case of more complex relationships. For example, *C. caspia* has a filamentous structure responsible for its adhesion and encrustation in different substrates. These filaments serve as a preferred substrate for *D. bugensis* and *D. polymorpha* settlement (Folino-Rorem et al. 2006; Pucherelli et al. 2016). As the later life forms of these dreissenids have a preference for more solid surfaces, they are detached from the filaments produced by *C. caspia*, migrating to adjacent substrates (Ackerman et al. 1994; Folino-Rorem et al. 2006). *Cordylophora caspia* is a predator (Folino-Rorem 2000; Folino-Rorem et al. 2006) and initially benefits from this relationship by preying on dreissenid mussel larvae (Moreteau and Khalanski 1994; Pucherelli et al. 2016) and also using their shells as substrate (Folino-Rorem et al. 2006; Olenin and Leppäkoski 1999). However, if the number of dreissenids increases, they compete with *C. caspia* for substrate, which can cause the death of the invading hydrozoan (Pucherelli et al. 2016). As postulated by Lasiak and Barnard (1995), dreissenid mussel larval settlement patterns may be repeated by other bivalve species that have veliger larvae. Therefore, it is possible that this relationship also occurs with other invading freshwater bivalves.

Epibiosis is present in 11 of 13 invasive species and involves the Mollusca, Porifera, Bryozoa, Magnoliophyta, and Cnidaria phyla. *Dreissena polymorpha* as the most representative species in the epibiosis relationship among freshwater fouling invasive species. It has been reported that this species may serve as a substrate for *D. bugensis* (Aldridge et al. 2014; Lauer et al. 1999a), *E. fluviatilis* (Gaino 2005; Ricciardi 1996;

Ricciardi 2005), *E. fragilis* (Molloy et al. 1997; Ricciardi 2005), *P. magnifica* (Ricciardi and Reiswig 1994), *P. fungosa* (Ricciardi and Reiswig 1994), *L. carteri* (Cummings and Graf 2010; Lauer et al. 1999b), and *C. caspia* (Pucherelli et al. 2016). Bryozoans have been reported in several studies relating to *D. polymorpha*, mainly reducing the growth rates of the dreissenid and harming its development (Cummings and Graf 2010; Molloy et al. 1997; Ricciardi and Reiswig 1994). Bryozoan colonies can reduce the surfaces for the mussel larvae settlement. Moreover, they grow directly on the bivalve shells, causing difficulty in breathing and feeding to them. It is known that *L. carteri* can inhibit mussels in several ways, the three main ones are described by Lauer et al. (1999b): 1) bryozoans can sort and select food particles through their lateral cilia (Okamura and Doolan 1993), which can physically displace mussel larvae during its settlement and feeding process; 2) the dense cover formed by bryozoans on the substrate limits the availability of substrates for mussel larvae settlement; 3) bryozoans have coelomic fluid, which is a defence mechanism that causes neurotoxic effects and eventually repels bivalves (Collins et al. 1966; Tenney and Woolcott 1964).

Cannibalism was recorded among individuals of *D. polymorpha.* This relationship is a significant mechanism of population dynamics and maintenance (Lewandowski 1982; Sprung 1989). Adult bivalves can consume a wide range of zooplanktonic organisms up to 400 µm in length, including its veligers (Shevtsova et al. 1986). According to Maclsaac et al. (1991b), intraspecific predation by adult zebra mussels has been identified as an important cause of mortality. The author concludes that *D. polymorpha* veligers may suffer mortality rates of up to 70% due to cannibalism by adult mussels (> 20 mm).

Competition between freshwater invasive fouling species was one of the most frequently described forms of interactions by researchers. Several authors reported the occurrence of *D. polymorpha* competing with other species of Mollusca (Karatayev et al.

2015; Molloy et al. 1997; Vaate et al. 2010), Porifera (Gaino 2005; Ricciardi and Reiswig 1994; Wood 2013), Cnidaria (Molloy et al. 1997), and Bryozoa (Molloy et al. 1997; Nakano and Strayer 2014). There were also reports of intraspecific competition for space, substrate and food among *D. polymorpha* juveniles (Lewandowski 1982; Molloy et al. 1997). *Eunapius fragilis* and *E. fluviatilis* share and compete with *D. polymorpha* by the same substrate types (Cummings and Graf 2010; Gaino 2005; Lancioni and Gaino 2007; Ricciardi et al. 1995). Competition between these species begins when the sponge develops over the mussel valves, may causing them to occlude, obstructing the siphons, reducing movement to capture food and interrupt breathing, which may lead to molluscs to death (Gaino 2005; Lauer and Spacie 2004; Ricciardi et al. 1995). Sponges adhere firmly to the substrate, are very efficient in capturing food, have easy to occupy new ecological niches and use such characteristics to facilitate their dispersion (Ricciardi 1996). When associated with mussels, these species increase their chances of spreading in new environments (Lauer and Spacie 2004; Ricciardi 1996), since porifers have minimal capacity for locomotion. *Dreissena polymorpha* and *D. bugensis* have a high degree of niche overlap (Stoeckmann 2003), consequently can compete for substrate and food (Diggins 2001; Karatayev et al. 2014; Molloy et al. 1997; Nalepa et al. 2010). *Dreissena bugensis* has some advantages over *D. polymorpha*, such as gonadal maturation and spawning over a wider temperature range (Orlova et al. 2004); Higher tolerance to low dissolved oxygen concentrations (Baldwin et al. 2002); higher efficiency in food assimilation and lower respiratory rate (Stoeckmann and Garton 1997). In contrast, *D. polymorpha* presents earlier reproduction, which allows individuals to produce larger numbers of offspring throughout their lives, increasing their ability to spread geographically and to conquer new habitats, which makes this species a very efficient bioinvader (Mills et al. 1996; Stoeckmann 2003).

3.5 Conclusion

Problems related to invasive species are usually addressed in isolation according to the most significant impacts caused by each species. However, when the invaded environments are studied holistically it is observed that interactions between fouling aquatic invasive species are potentially critical in defining the associated environmental and economic implications, and this is a problem that is spreading rapidly all over the world. Interactions among invasive fouling species can cause significant changes in native communities, increase the pressure of invasive propagules, and financial losses. The interactions among fouling invaders are dynamic, quite variable in their form and degrees of associated impacts. *Dreissena polymorpha* is the species with the highest frequency of interactions with other invasive fouling species, presenting ecological effects that can be directly negative (competition) or indirectly positive (predation and epibiosis). Competition is the most common form of interaction between invasive fouling species. Predation, epibiosis and cannibalism can occur concomitantly with other interactions. Not infrequently, fouling invasive species play the role of facilitating the colonization process of other invaders, potentiating the impacts of bionvasions. Positive ecological interactions, even though less common, eventually connect and facilitate the process of colonization, establishment and spread of invasive aquatic species.

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4 CAPÍTULO II - ECOLOGICAL INTERACTIONS BETWEEN INVASIVE AND NATIVE FOULING SPECIES IN THE RESERVOIR OF A HYDROELECTRIC PLANT

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Abstract
In this study we investigate the main ecological interactions between fouling aquatic organisms (both invasive and native) present in the reservoir of the Governador José Richa hydroelectric plant, located in southern Brazil, and to identify the most suitable period for the interruption of machinery operation for cleaning and maintenance of the hydraulic systems of this plant. A total of 32 experimental plates were fixed to a metallic structure positioned close to the plant's water intake. Three invasive fouling species were

identified in our samples (*Limnoperna fortunei* [Mollusca], *Cordylophora caspia*, and *Hydra* sp. [Cnidaria]) and six native taxa belonging to the phyla Protozoa, Ciliophora, Amoebozoa, and Arthropoda. Spring and summer were the seasons with the highest fouling rates, as well as densities of fouling organisms. The highest levels of diversity were recorded during the colder seasons. Several interactions between the organisms were identified, such as mutualism, commensalism, competition, epibiosis, cannibalism and predation. The data obtained suggest that, from the biological point of view, the most suitable period for machine shutdown destined for the removal of biological fouling in the hydraulic systems of the studied plant is between the end of spring and the beginning of summer.

Keywords: Ecological succession; facilitation; freshwater environment; biofouling; seasonality.

4.1 Introduction

Fouling invasive aquatic organisms have caused severe economic impacts around the world (Callow 1993; Morton 1977; Nakano and Strayer 2014; Pimentel et al. 2001; Ricciardi and MacIsaac 2008; Simberloff and Vitule 2014; Simberloff and Von Holle 1999). There have been records of clogging of pipes in water collection and treatment stations (Melo and Bott 1997; Pu et al. 2009; Rajagopal and van der Velde 2012). Industries such as pulp and paper mills are also frequently impacted by fouling organisms, as these sectors depend directly on the capture of water from natural bodies to carry out their production processes (Coetser and Cloete 2005; Rajagopal and van der Velde 2012). In these cases, the damage caused by fouling organisms is associated with both increased corrosion rates and clogging of hydraulic structures (Characklis 1981; Farhat et al. 2019; Martin et al. 2016; Melo and Bott 1997; Pu et al. 2009; Singh et al. 2020). However, the most widely affected sector has been hydroelectric power generation (Armour et al. 1993; Boltovskoy 2017; Boltovskoy and Correa 2015; Boltovskoy et al. 2006; Cataldo 2001; Jernelöv 2017; MacIsaac 1996; Mansur et al. 2016; Portella et al. 2009).

In hydroelectric projects affected by fouling, the frequency of interventions aimed at cleaning and maintaining hydraulic structures increases, with a consequent increase in operating costs (Booy et al. 2017; Flemming and Cloete 2010; Flemming 2011; Grohmann 2008b; Nelson 2019). Each machine shutdown implies the need for specialized labor to carry out cleaning activities, in addition to the interruption of energy production during the entire cleaning period. Frota et al. (2014), based on the value of the energy priced in the Brazilian spot market, concluded that the lost profits for the energy not generated in a single day of maintenance of a 44 MW Francis turbine would be US$ 180,000. The associated economic impact is even greater when considering that machine shutdown for maintenance and cleaning are always greater than 24 h (Flemming 2002; Grohmann 2008a; Pucherelli et al. 2018). Therefore, identifying the most suitable periods for machine shutdowns for maintenance and cleaning would allow the optimization of efforts and costs in the mid- and long term, given that it would allow for maintenance efforts to be more effective and concentrated in time, which in turn would minimize control efforts in the intervening months (Boltovskoy et al. 2015b; Frota et al. 2014; Grohmann 2008c; Latombe et al. 2017; Oreska and Aldridge 2011; Venkatesan and Murthy 2008).

In South America, three fouling invasive aquatic species have been reported to cause significant operational and economic losses to companies in the hydroelectric sector: the Asian clam, *Corbicula fluminea*, the cnidarian *Cordylophora caspia*, and the golden mussel *Limnoperna fortunei*. *Corbicula fluminea* obstructs heat exchangers of plants due to the accumulation of their shells (Karatayev et al. 2005; Ludwig et al. 2014; Mansur et al. 2016). *Cordylophora caspia* affects hydraulic pipes, filters and protective grids (Danrigran and De Drago 2000; Nakano and Strayer 2014; Portella et al. 2009; Sylvester et al. 2011). Finally, *L. fortunei* causes damage to the protection grids (Darrigran 2002), filters, generators, floodgates (Boltovskoy et al. 2006), pipes, and heat exchangers (Cataldo et al. 2003; Darrigran and Damborenea 2011; Uliano-Silva et al. 2013).

In addition to the economic impacts, fouling aquatic organisms have generated several negative impacts on the ecology of communities in freshwater environments, interfering mainly in the trophic chain of native species (David et al. 2017; Emery-Butcher et al. 2020). There are relatively well-documented cases of competition between these organisms and incumbent species for food and/or space, as well as shifts of nutrient cycling, among other environmental disturbances (David et al. 2017; Ricciardi and MacIsaac 2011). Invasive species often interact with each other and often act as “facilitators” (a term that refers to those species that have direct positive effects on other species) (Hacker and Gaines 1997). This interaction can favor the increase in density or biomass of at least one of the involved species, which can facilitate the bioinvasion process (Ricciardi and MacIsaac 2000; Rodriguez 2006; Silknetter et al. 2019; Velde and Rajagopal 2006). Thus, this type of interaction would contribute to the increase in the number of species introduced into aquatic environments (Simberloff and Von Holle 1999). The impacts of facilitating species are more frequent when they favor or provide a limiting resource, increasing the complexity of the habitat, functionally replacing a native species or competing for resources and ecological niche (Kéfi et al. 2016; Ricciardi 2001; Ricciardi et al. 1996; Simberloff 2006; Simberloff and Von Holle 1999). The present study

aimed to investigate the main ecological interactions between the invasive fouling aquatic species and the native species present in the reservoir of a hydroelectric plant located in the southern region of Brazil and to evaluate the most suitable period for machine shutdown for cleaning and maintenance of the hydraulic systems of this plant.

4.2 Methods

4.2.1 Study Design

The experiment was carried out between September 2018 and December 2019, at the HPP Governador José Richa reservoir (25°32'34.99"S, 53° 29'45.82"W), located in the Lower Iguaçu River section, in the border between the municipalities of Capitão Leônidas Marques and Nova Prata do Iguaçu, state of Paraná, Brazil. Samples were obtained using polystyrene experimental settlement plates (15 x 15 cm), fixed with nylon clamps to a metallic structure of 0.58 m². This structure was positioned one meter below the surface and was fixed by ropes to the log boom located near the dam of the HPP. For five seasons (Spring/2018: October, November and December; Summer /2019: January, February and March; Autumn/2019: April, May and June; Winter/2019: July, August and September; Spring /2019: October, November and December), a total of 32 experimental plates were sequentially installed, six plates (two replicas/month) in each season, and two more test plates that were installed in the first month (September 2018) and removed only in the last month (December 2019). Each month, two experimental plates were removed, so that at the end of the season all six seasonal plates had been removed (two plates/month). At the beginning of the next season, the procedure was repeated (Figure *5*).

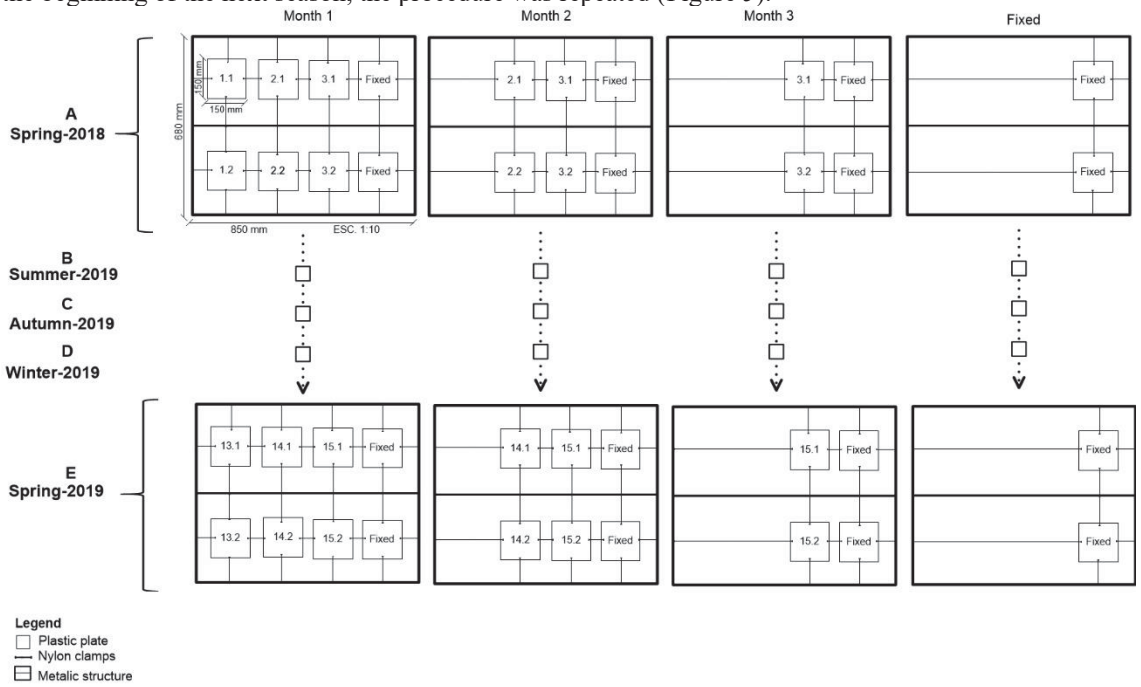


Figure 5 Schematic representation of the supporting structure of the experimental settlement plates and the methodological procedures adopted: at the beginning of each season, the structure had eight experimental plates; at the end of each month, two of them were removed for analysis of fouling and two remaining plates ("fixed") were removed after 15 months of monitoring; A) Sping-2018, B) Summer-2019; C) Autumn-2019; D) Winter-2019 and E) Springer -2019.

The experimental settlement plates removed from the floating structures were packed in plastic bags. The biological material was fixed in 6% formaldehyde and preserved in 70% ethanol. The automated hourly measurement of the reservoir water temperature during the 15 months of the study was carried out and the data made available by Companhia Paranaense de Energia.

4.3 Analysis of experimental plates

In the laboratory, all experimental settlement plates were photographed, both with a digital camera (Nikon, Coolpix B500 Brindes, Japan) and in a stereoscopic microscope (Zeiss, Stereo Discovery.V8 Crisp, Germany), in order to organize an image database for visual analysis the fouling process. The images were analyzed using the ImageJ software (version 1.52a) (Schneider et al. 2012) to identify the recruitment pattern and the degree of occupation of the experimental plates. The calculation of the coverage area in each experimental plate was performed using the Watershed plugin, a tool that calculates based on the intensity or grey level of the pixels present (Papadopulos et al. 2007). The values were then transformed into the percentage of the fouled area in relation to the total area of the plate. After measuring the area occupied by fouling organisms, macroscopic individuals (> 1 cm) were removed from the experimental plates, with the aid of surgical instruments, fixed in 4% formaldehyde buffer, preserved in 70% alcohol, identified to the lowest possible taxonomic level, and quantified. The mollusks were also weighed to obtain the wet total weight (meat and shell), on a scale (Bell Engineering, S2202h – 2,200 g, Italy), making it possible to calculate the relative biomass (g/cm²).

For the identification and quantification of microscopic organisms (<1 cm), two methodological procedures were established. When the experimental settlement plates had a fouling area greater than 30% (as observed in **Erro! Fonte de referência não encontrada.** A), a subsample of fouling organisms was obtained using a standardized grid containing 225 squares (1 x 1 cm), of which 20 (8.8% of the total area). The selection of the squares was carried out randomly over the entire plate, including non-fouled surfaces (Figure 6 B), following the methodology proposed by Borges (2013). After this procedure, the biological material embedded in these selected cells was removed with the aid of a scalpel, stained with cane rose; and identified using a stereoscopic microscope (Pinto-Coelho 2004). Alternatively, if the fouling area covered 30% or less of the experimental settlement plates, they were scrapped, the material stained with a cane rose and analyzed in a stereomicroscope. The relative density of each taxon on each plate was directly calculated for Amoebidae, Daphniidae, Calocalanidae, Chironomidae, Vaginicolidae, Centropyxidae, and Mytilidae. However, obtaining reliable counts for Cnidaria is made dificult by the frequent presence of fragmented individuals in the samples, mainly in the case of Cordylophoridae. Therefore, the analysis of Cordylophoridae and Hydridae was done qualitatively, considering the number of hydrorrhizae (filaments) or hydrae present in each sample. The recruitment patterns, as well as interactions between the groups found, were based on direct observations under a stereoscopic microscope. The observational data were supplemented with bibliographic information about the reproductive biology, behavior, and ecological relationships between individuals invading limnic environments, as well as between invasive and native species.

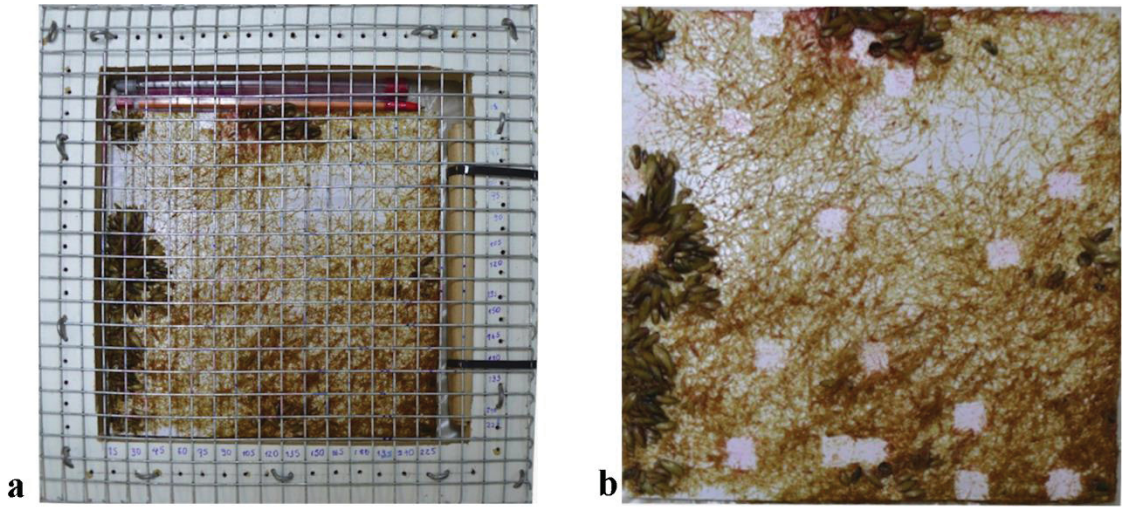


Figure 6 Auxiliary structure for analyzing recruitment patterns of fouling and native invasive aquatic groups, (A) Grid with 225 squares (1 x 1 cm) showing the pattern of visualization of the encrustation on the experimental settlement plate, below the grid, (B) Experimental settlement plate after taking samples in the 20 random squares

4.4 Data analysis

To characterize the diversity of organisms over the sampling period, the Shannon-Weaner diversity index was used (Pielou 1966). Normality and homogeneity of the variances of density, biomass, diversity and temperature data were analyzed by the Levene and Cochran tests; the homoscedasticity was analyzed using the residuals of variance. The Kruskal-Wallis analysis was used to determine the significant differences between the variables tested, always at the 95% confidence level. Spearman's correlation coefficient was applied to assess the degree of association between dependent variables (coverage area, density, biomass, diversity) and water temperature (independent variable). The analyses were performed using the software Statistica 10.0 (StatSoft®).

4.5 Results

4.5.1 Environmental conditions

The highest average water temperatures in the reservoir were recorded in January 2019 and the lowest in August 2019. When temperatures are compared across seasons, they were similar ($p > 0.05$) in spring (2018 and 2019) and in autumn (2019), but higher in summer and lower in winter (Figure 7).

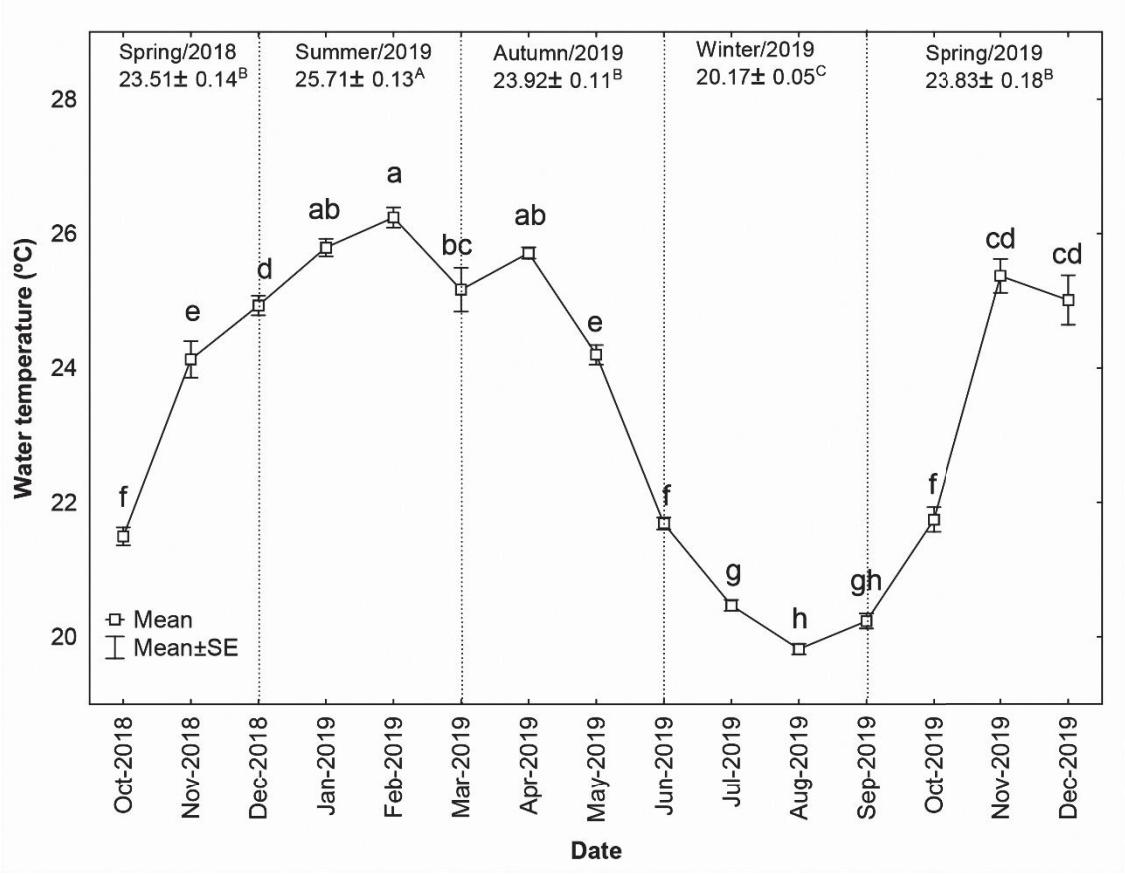


Figure 7 Temporal variation (average values and standard error) of the water temperature in the reservoir of the HPP José Richa, from October 2018 to December 2019. Similar capital letters indicate statistical similarities between the seasons and the lower letters between the months of the year

4.5.2 Identification of the recorded taxa

Macro and microinvertebrate organisms present in the analyzed samples were identified, and three of them (*Limnoperna fortunei*, *Cordylophora caspia* and *Hydra* sp.) are considered fouling invasive species belonging to the phyla Mollusca and Cnidaria (Table 7).

Table 7 Taxonomic classification of families identified in the experimental settlement plates in the reservoir of the HPP Gov. José Richa. Taxa that could not be identified at the species level are indicated as “NI”.

Phylum	Class	Order	Family	Species	Category
Protozoa	Lobosa	Amoebida	Amoebidae	NI*	Native
Arthropoda	Branchiopoda	Cladocera	Daphniidae	NI	Native
Arthropoda	Maxillopoda	Calanoida	Calocalanidae	NI	Native
Arthropoda	Insecta	Diptera	Chironomidae	NI	Native
Ciliophora	Oligohymenophorea	Sessilida	Vaginicolidae	NI	Native
Amoebozoa	Tubulinea	Tubulunida	Centropyxidae	NI	Native
Mollusca	Bivalvia	Mytilida	Mytilidae	*Limnoperna fortunei*	Invasive
Cnidaria	Hydrozoa	Anthoathecata	Cordylophoridae	*Cordylophora caspia*	Invasive
Cnidaria	Hydrozoa	Anthoathecata	Hydridae	*Hydra* sp.	Invasive

4.5.3 Recruitment pattern of identified taxa

The plates kept submerged for 15 consecutive months recorded, on average, at the end of this period, 79.3% of their area occupied by colonizing organisms. In relation to the seasons, the highest occupations involved plates obtained during the spring of 2018, which showed 56.0% of their occupied area at the end of three months. The values quantified in these periods were significantly higher than those observed in other seasons (Figure 8 A). The average density of organisms present in the test experimental settlement plates was higher in the spring, 2018 and the summer, 2019 Figure 8 B). The highest rates of seasonal diversity were recorded in the winter and autumn of 2019, with lower diversities observed on the test experimental settlement plates kept submerged for 15 months and in the spring of 2019 (Figure 8 C). The period considered most critical regarding the presence of fouling invasive organisms took place between late spring and early summer. In the following seasons, the relative density of these organisms was significantly reduced. Mainly in the specimens kept submerged for 15 months, the density was close to the values found in early summer, with the difference that in this case there were practically only one encrusted species (*L. fortunei*) (Figure 8 D).

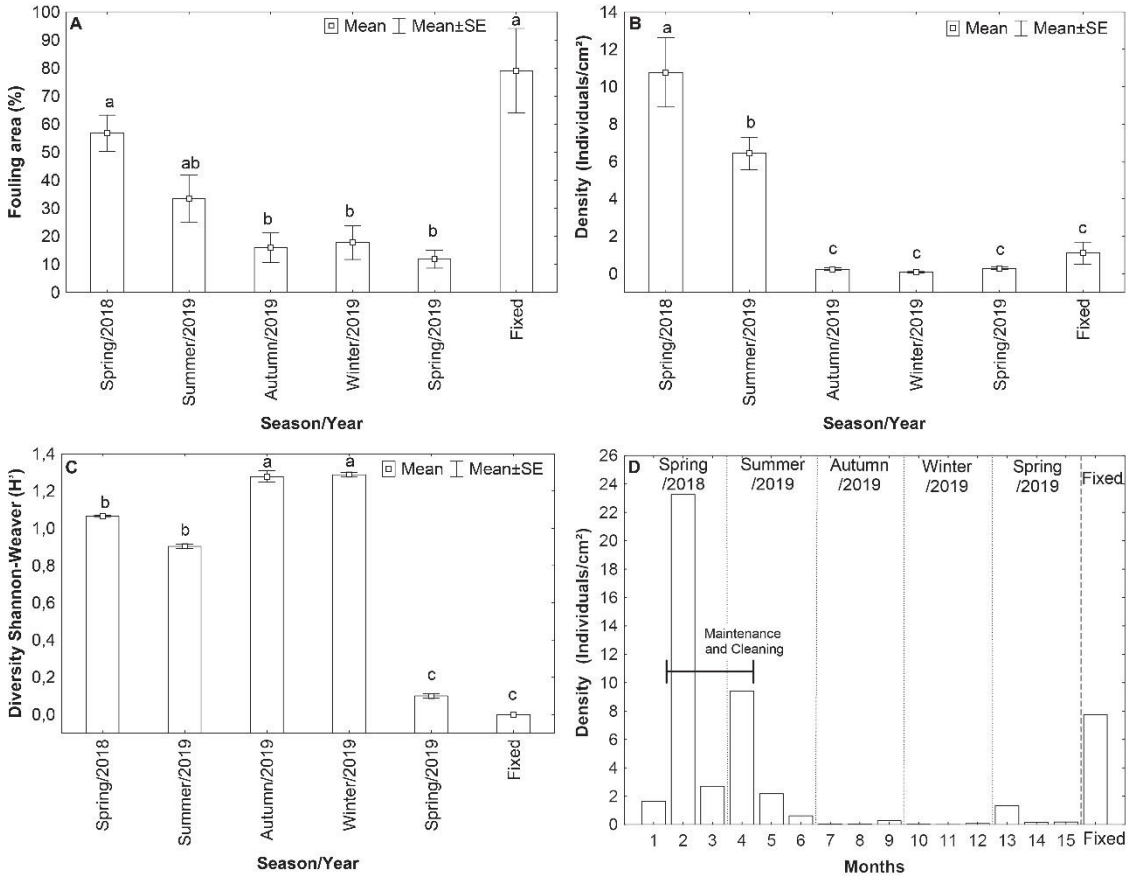
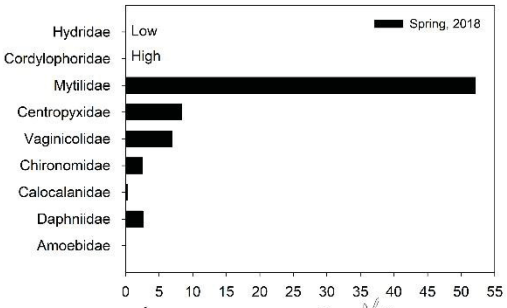
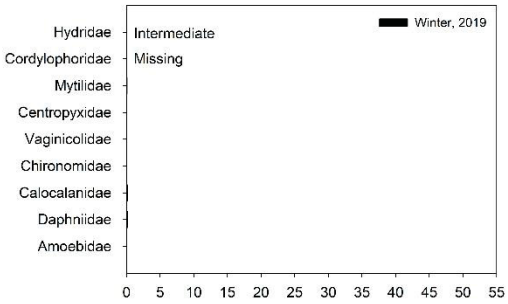
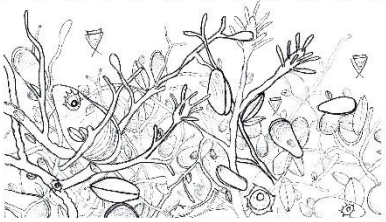


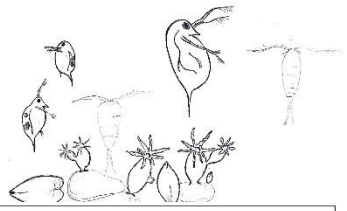
Figure 8 A) The area occupied by fouling and native organisms. B) Seasonal variation in the general density of identified organisms. C) Seasonal variation of the Shannon-Weaver diversity index. D) Monthly density and the recommended period for the maintenance and cleaning of the hydraulic installations (Month: 2- November; 3-December and 4- January) of the Governador José Richa hydroelectric power plant

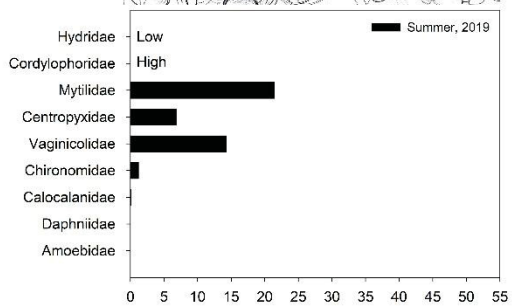
The average density of organisms was 10.4 individuals/cm² during the spring 2018; 6.3 in the summer 2019; 0.3 in the spring 2019; 0.2 in the autumn 2019 and 0.1 in the winter 2019. *Limnoperna fortunei* was the predominant invasive organism throughout the analyzed period. The average density of *L. fortunei* was higher in the spring, 2018 and the summer, 2019. This species also completely dominated the experimental settlement plates kept submerged for 15 consecutive months (Figure 9). The largest biomass of *L. fortunei* was recorded in the experimental settlement plates kept submerged for 15 months in the reservoir. The highest seasonal biomasses were recorded in winter (Table 8). There was a significant ($p<0.05$) but low correlation ($r = -0.39$) between the biomass of *L. fortunei* and water temperature.

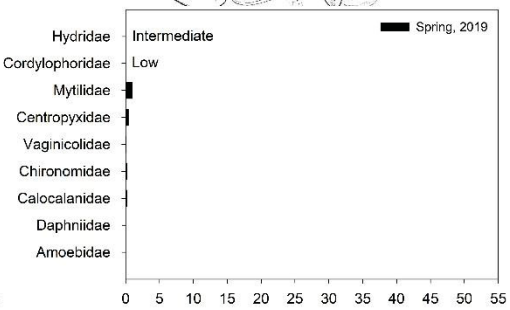

Spring, 2018
Hydridae
Cordylophoridae
Mytilidae
Centropyxidae
Vaginicolidae
Chironomidae
Calocalanidae
Daphniidae
Amoebidae
Low
High
0 5 10 15 20 25 30 35 40 45 50 55

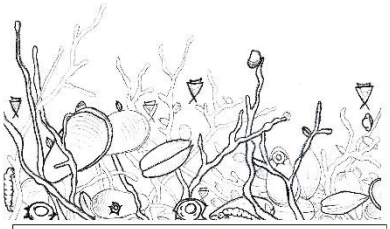

Winter, 2019
Hydridae
Cordylophoridae
Mytilidae
Centropyxidae
Vaginicolidae
Chironomidae
Calocalanidae
Daphniidae
Amoebidae
Intermediate
Missing
0 5 10 15 20 25 30 35 40 45 50 55

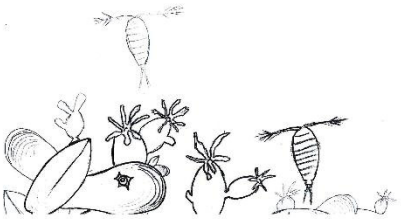


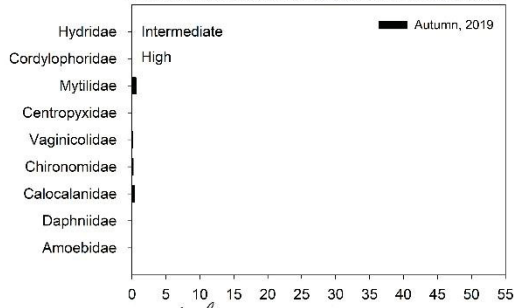


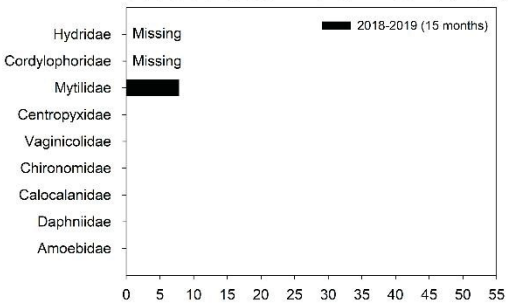

Summer, 2019
Hydridae
Cordylophoridae
Mytilidae
Centropyxidae
Vaginicolidae
Chironomidae
Calocalanidae
Daphniidae
Amoebidae
Low
High
0 5 10 15 20 25 30 35 40 45 50 55

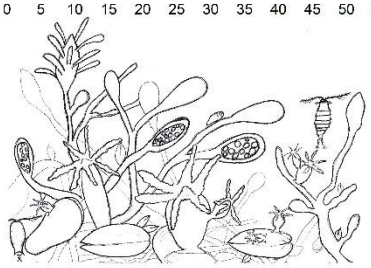

Spring, 2019
Hydridae
Cordylophoridae
Mytilidae
Centropyxidae
Vaginicolidae
Chironomidae
Calocalanidae
Daphniidae
Amoebidae
Intermediate
Low
0 5 10 15 20 25 30 35 40 45 50 55

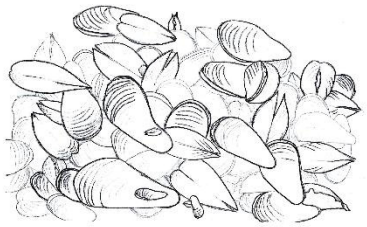


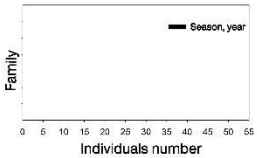



Autumn, 2019
Hydridae
Cordylophoridae
Mytilidae
Centropyxidae
Vaginicolidae
Chironomidae
Calocalanidae
Daphniidae
Amoebidae
Intermediate
High
0 5 10 15 20 25 30 35 40 45 50 55


2018-2019 (15 months)
Hydridae
Cordylophoridae
Mytilidae
Centropyxidae
Vaginicolidae
Chironomidae
Calocalanidae
Daphniidae
Amoebidae
Missing
Missing
0 5 10 15 20 25 30 35 40 45 50 55






Season, year
Family
0 5 10 15 20 25 30 35 40 45 50 55
Individuals number

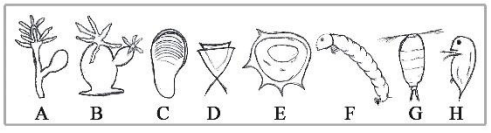

A
B
C
D
E
F
G
H

Figure 9 Schematic representation of the ecological succession observed in the experimental settlement plates in the reservoir of the Governador José Richa HPP, indicating the groups of dominant organisms throughout the seasons: Spring 2018; Summer 2019; Autumn 2019; Winter 2019; Spring 2019; The experimental settlement plates kept submerged for 15 months. The values presented graphically represent the average density of the organisms. A) Cordylophoridae; B) Hydridae; C) Mytilidae; D) Vaginicolidae; E) Centropyxidae; F) Chironomidae; G) Calocalanidae and H) Daphniidae

Table 8 Variation of the average biomass of *Limnoperna fortunei* in the experimental settlement plates kept in the reservoir of Governador José Richa HPP.

Season/Year	The average number of specimens	Biomass (mg/cm²) of *Limnoperna fortunei*	
		Mean	SE
Spring, 2018	30	0.9^{abc}	0.13
Summer, 2019	78	0.5^{c}	0.04
Autumn, 2019	46	0.5^{c}	0.07
Winter, 2019	40	1.1^{a}	0.12
Spring, 2019	40	0.9^{abc}	0.14
2018-2019 (15 months)	175	293.8^{a}	87.50

4.5.4 Ecological interactions between the families of the identified organisms

The ecological interactions between the main groups identified in the experimental settlement plates were classified as positive and direct (mutualism), positive and indirect (commensalism), negative and direct (competition), negative and indirect (epibiosis, cannibalism and predation) (Figure 10). Mutualism was observed between adult and juvenile individuals of the family Mytilidae, as well as between individuals of the families Cordylophoridae and Mytilidae. Commensalism occurred between Mytilidae and insect larvae of the Chironomidae family. Competition for food and space was observed among fouling invaders (Cordylophoridae, Mytilidae and Hydridae), especially when the space available for fouling on the substrate was more limited. It was observed among invasive and native organisms (Daphniidae, Calocalanidae, Chironomidae, Vaginicolidae and Centropyxidae). Cannibalism was identified only in adult individuals of Mytilidae, which can feed on larvae of the same species. Epibiosis was characterized by the use of organisms from certain groups, such as basibionts. For example, individuals from Hydridae were used as substrate by Cordylophoridae, Mytilidae and Centropyxidae. Cordylophoridae, on the other hand, were recorded hosting Hydridae, Centropyxidae and Mytilidae. Among the organisms involved in epibiosis, the relationship between Cordylophoridae and Mytilidae was the most evident and recorded on several occasions. The predation occurred with Hydridae preying on Chironomidae, Daphniidae and Calocalanidae. Cordylophoridae can feed on larvae of Mytilidae, as well as individuals from the family Daphniidae and Calocalanidae in the juvenile and adult phases. Mytilidae can prey on protozoa including Vaginicolidae and Centropyxidae, and microcrustaceans in the juvenile stage like Daphniidae and Calocalanidae.

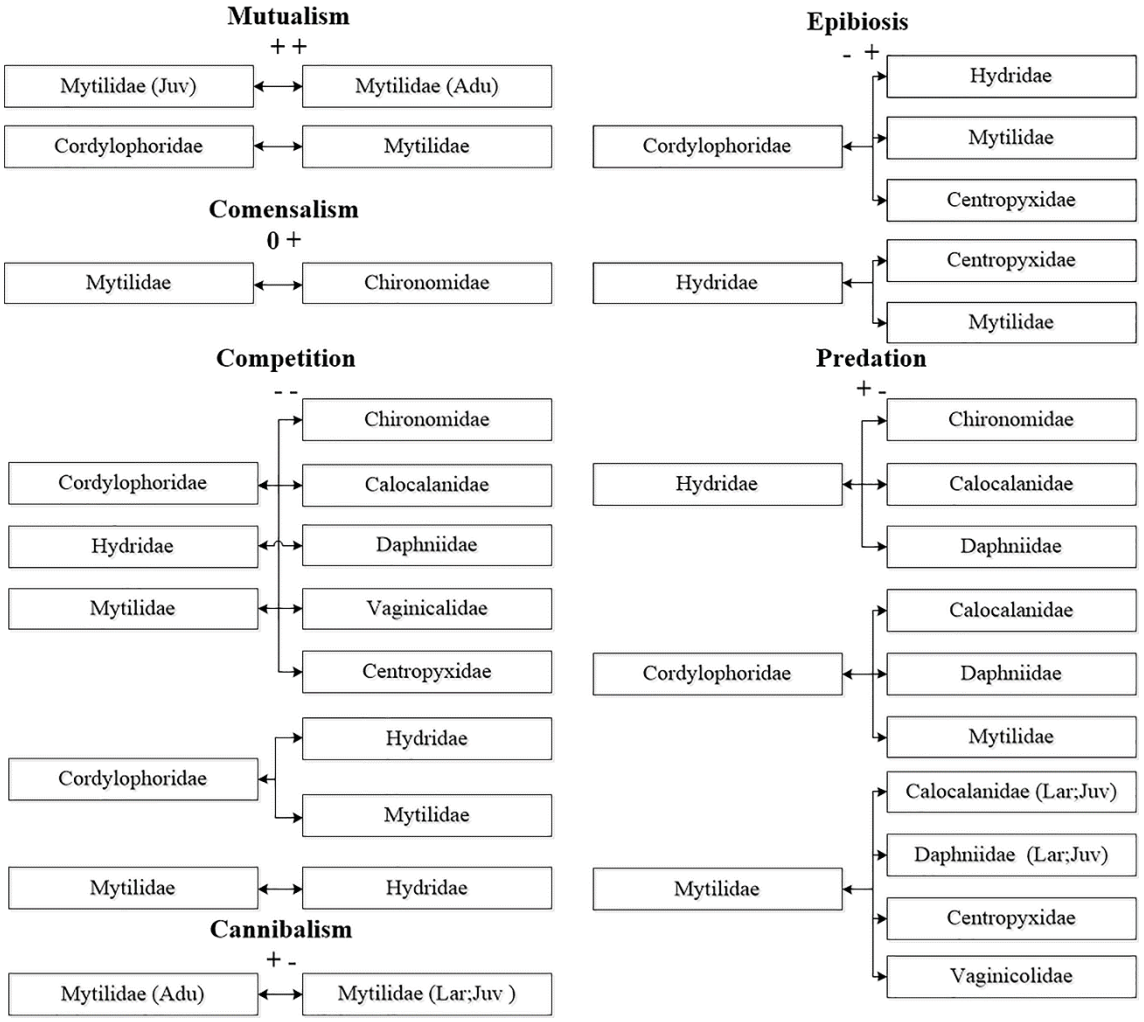


Figure 10 Schematic representation of the ecological interactions recorded between the families of the fouling and native invader groups present in the experimental settlement plates positioned in the reservoir of the Governador José Richa HPP.

4.6 Discussion

In subtropical aquatic environments, higher temperatures tend to have positive effects on the rates of fouling and colonization of aquatic communities (Lansac-Tôha et al. 2019; Rodrigues et al. 2015), especially on the density of micro and macroinvertebrates (Belz et al. 2010; Borges 2014; Borges et al. 2017). Temperature interferes with reproductive cycles, the supply of recruits and the trophic conditions of aquatic environments (Chen et al. 2019; Cifuentes et al. 2010; McPherson et al. 1984; Mieczan and Rudyk-Leuska 2019; Patil and Anil 2005). In the present study, however, in addition to the water temperature not having a significant direct influence on the composition or density of most of the identified communities, in the only case in which this correlation existed (concerning the *L. fortunei* biomass) it was negative and of only low degree (r = -0.39). On the other hand, the highest rates of fouling and density seasonal of organisms in the experimental settlement plates were recorded precisely during spring and summer. The density of organisms presents on the plates installed during spring 2018 and spring 2019 did not follow the same pattern of fouling or maximum achieved densities. This seasonal and interannual variation underscores the high degree of complexity of fouling processes by invasive aquatic organisms, which has also been reported in other studies (Berntsson and Jonsson 2003; Dehmordi et al. 2011; Fortunato et al. 2017; Underwood and Anderson 1994). It is possible to postulate that other environmental factors, which were not individually monitored here, but which were observed in other studies, such as availability of nutrients and food, rainfall patterns, incidence of sunlight (Fernandez and Navarrete 2015; Masi et al. 2016; Melo and Bott 1997; Navarrete et al. 2019; Pamplin et al. 2006), or related to the physical characteristics

of the reservoir, such as hydrodynamics or its depth (Albano and Obenat 2019), contributed to the obtained results.

Another noteworthy fact is that both the density and the area occupied in the experimental plates by invading organisms were systematically superior to those related to native fauna organisms. It is known that the typically opportunistic behavior of invading organisms is closely associated with their biological characteristics (such as high growth rates, the existence of successful reproduction strategies and ease of dispersal) and, especially, reproductive (early sexual maturity and high fertility), which facilitate the establishment process during bioinvasion. Such characteristics are present in *C. caspia* (Folino-Rorem 2000; Folino-Rorem et al. 2006; Nakano and Strayer 2014), *Hydra* sp. (Deserti et al. 2017; Hobmayer et al. 2012; Rodrigues et al. 2016) and *L. fortunei* (Boltovskoy 2015; Boltovskoy and Correa 2015; Boltovskoy et al. 2015a; Darrigran 2002).

The settlement periods of the studied groups were well defined, mainly for *L. fortunei*, which occurred in all monitored seasons. This observation coincides with the result of a study developed by Damborenea and Penchaszadeh (2006), which concluded that *L. fortunei* could produce sperm and oocytes continuously throughout the year. Boltovskoy and Correa (2015), showed that in about 80% of the time series studied in South American reservoirs, researchers had identified an density of at least 10 larvae/m³ of golden mussel in the analyzed samples. In the present study, the highest occurrence of *L. fortunei* settlement occurred in spring, 2018 and summer, 2019, coinciding with the reproduction peaks already reported in several other studies (Boltovskoy et al. 2009; Canzi et al. 2005; Darrigran and Damborenea 2006; Magara et al. 2001).

The biomass of *L. fortunei* was higher in those experimental settlement plates maintained throughout the study period. On these plates, the individuals present were predominantly adults. In adulthood, *L. fortunei* generally shows greater adaptation to ecological ranges and to stressful environmental factors (Liu et al. 2020). The smallest golden mussel biomass was observed in autumn, 2019. This period was characterized by intermediate temperatures, which tend to limit the growth rates of individuals and interfere with the quality and availability of food (Boltovskoy and Correa 2015; Boltovskoy et al. 2009; Nakano et al. 2011; Oliveira et al. 2015).

Over the course of ecological succession, the supporting organisms are the first to establish themselves (microorganisms, filamentous algae, diatoms, protozoa and others) (Martín-Rodríguez et al. 2015; Silknetter et al. 2019), followed by macroinvertebrates (mollusks, insects and cnidarians) (Abarzua and Jakubowski 1995). *Cordylophora caspia* and *L. fortunei* were predominant in this process. The cnidarian has hydrorrhizae, a filamentous structure responsible for adhesion and encrustation on different substrates. These filaments end up serving as the preferred substrate for the settlement of *L. fortunei* larvae, which use them to adhere, protect themselves from local hydrodynamics and develop during the first stages of life. Similar behavior has also been reported between *C. caspia* and *Dreissena polymorpha* in North America (Ackerman et al. 1994; Folino-Rorem et al. 2006; Pucherelli et al. 2016).

The trophic position and the capacity that each group has to occupy the substrate establish changes in the structuring of these communities over time. Some individuals end up dominating the substrates and making them more homogeneous (Olden 2006; Olden et al. 2004; Ricciardi and MacIsaac 2011). In the present case, the highest levels of diversity occurred in a period of lower rates of fouling of the experimental plates. As *L. fortunei* was the dominant species in almost all seasons, when it was less abundant, there was a greater opportunity for the colonization of the experimental plates by other organisms, which increased the diversity indexes. Spaccesi and Rodrigues (2012) also recorded lower rates of diversity in substrates dominated by *L. fortunei* in the La Plata River. The authors also report that diversity was also lower in periods of low temperatures (autumn and winter).

Experimental studies carried out in South American reservoirs that assess ecological interactions, including fouling and native invasive groups, are still relatively limited (Boltovskoy and Correa 2015; Nakano et al. 2011). In the present case, it was possible to identify interactions with positive effects (mutualism and commensalism), indirect negative effects (predation, cannibalism and epibiosis) and purely negative effects (competition) substrate used for the settlement can be a limiting resource in certain environments and, when mutualistic interactions occurred, in the present case, they involved the sharing of substrates by *L. fortunei* and *C. caspia*. Portella et al. (2009) evaluated fouling in the same reservoir of HPP José Richa and reported the presence of these two invaders in coexistence, potentiating the operational and economic impacts to the plant. According to Simberloff and Von Holle (1999) and Gallardo and Aldridge (2018), mutualistic relationships between freshwater invaders are poorly studied in the literature. However,

understanding the role of each group involved in mutualism is of great importance, given that the effects of both species individually, in addition to their ecological interactions, often lead to severe negative economic impacts (Green et al. 2011; Rolla et al. 2019; Wegner et al. 2019). This mutualistic relationship can also contribute to the entry and spread of secondary invaders, in addition to raising the propagule pressure at different levels of the ecosystem (Green et al. 2011; Ricciardi 2001; Ricciardi 2015; Ricciardi and Reiswig 1994). Invaders also modify the habitats of native species and contribute to making these environments more homogeneous (Olden 2006; Olden et al. 2004; Ricciardi and MacIsaac 2011). For example, *L. fortunei* may favor an increase in the number of macroinvertebrates in the Chironomidae family. This happens due to the accumulation of shells of the golden mussel clusters, which end up increasing the physical complexity of the substrate (Burlakova et al. 2012; Gutiérrez et al. 2003), as well as enriching these environments with the increase of stool bio-deposits and pseudofeces (Darrigran et al. 1998; Sardiña et al. 2008). This enrichment causes changes in the density of benthic fauna, as well as leading to the biological imbalance of these environments (Darrigran and Damborenea 2011; Karatayev et al. 2007). Burlakova et al. (2012) evaluated the composition of communities in locations with the presence and absence of two ecosystem engineers: *L. fortunei* and *D. polymorpha*. Where the species were present, the authors observed an increase in the richness and density of native macroinvertebrates (Burlakova et al. 2012). However, over time, native communities tend to become more homogeneous (Olden 2006; Olden et al. 2004; Ricciardi 2001; Ricciardi and MacIsaac 2011; Stewart et al. 1998).

Among interactions with a negative effect, the competition was the predominant one, both in the present study and in other cases studied (Jackson ; Kuebbing and Nuñez 2015). This relationship can happen for several reasons, including competition for territories, substrates, food or ecological niche (Jones et al. 2012). *Limnoperna fortunei* and *C. caspia*, compete for substrate, especially when they are scarce. These organisms are fixed in common structures, which makes them close at different times or stages of life during the bioinvasion process. Competition for substrates is also reported between *C. caspia* and *D. polymorpha* (Folino-Rorem et al. 2006; Pucherelli et al. 2016).

Cannibalism was recorded among adults and larvae of *L. fortunei*. Adult mollusks can feed on particles ranging in size from 4 to 1,000 µm in length, which includes their own larvae (Molina et al. 2015). In studies with *D. polymorpha*, cannibalism was responsible for mortality rates of up to 70% (Maclsaac et al. 1991). In epibiosis, *C. caspia* and *Hydra* sp. can serve as a substrate for other groups, such as *L. fortunei* and individuals of the family Centropyxidae. The relationship between *C. caspia* and *L. fortunei* is the most evident and involves several stages during the fouling process. However, it is more noticeable when *L. fortunei* is in its settlement phase, when they prefer to settle in places protected from turbulent currents (Cataldo and Boltovskoy 1999; Sylvester et al. 2007).

Simultaneously with epibiosis, the facilitation relationship can occur between these same invaders. The colonies of *C. caspia* have a three-dimensional structure, which initially favors the settlement of *L. fortunei*. In the second moment of this relationship, the mollusks develop a preference for harder substrates and to continue their development they start to colonize these hard substrates originally occupied by *C. caspia*, compromising the survival of the organisms of this species. Epibiosis between zebra mussels and other different groups of fouling invaders is well studied, especially the sponges *Ephidatia fluviatilis* (Gaino 2005; Ricciardi 2005; Ricciardi et al. 1996), *Eunapius fragilis* (Molloy et al. 1997; Ricciardi 2005), with the bryozoans *Pectinatella magnifica*, *Plumatella fungosa* (Ricciardi and Reiswig 1994), *Lophopodella carteri* (Cummings and Graf 2010; Lauer et al. 1999) and also with the cnidarian *C. caspia* (Pucherelli et al. 2016).

Predation has also been described for several groups, for example, between *C. caspia* and larvae of *L. fortunei*. This cnidarian can also use juvenile and adult golden mussel shells as substrate (Folino-Rorem et al. 2006; Olenin and Leppäkoski 1999). *Limnoperna fortunei* can prey on juvenile micro crustaceans, such as Daphniidae and Calocalanidae, as well as protozoa Vaginicolidae and Centropyxidae. Micro crustaceans are among the favorite foods of the golden mussel, as they have a larger size and even greater biomass compared to phytoplankton (Molina et al. 2015).

Based on the density values quantified throughout the present study, the recruitment pattern, the ecological succession and the interactions observed between the dominant groups at the HPP José Richa; we recommend that maintenance and cleaning of hydraulic structures to remove biofouling should be carried out during spring or early summer. This is the most critical period in terms of recruitment of young forms of fouling organisms and, in particular, *L. fortunei*, which is the species that most causes problems with the clogging of filters, grids and pipes. In autumn and winter, fouling rates naturally fall by up to 80%, reducing the operational and economic risks of the machine shutdowns and cleaning. This conclusion is also supported by other studies, which reported decreased fouling levels and speed during colder seasons,

which would be related to the heterothermia of invertebrates and their consequent dependence on temperature for regulating metabolic processes (Poloczanska et al. 2010; Pörtner 2002; Berntsson and Jonsson 2003; Dehmordi et al. 2011; Fortunato et al. 2017; Underwood and Anderson 1994). Therefore, by promoting the shutdown of machines and cleaning of hydraulic systems in the most critical period of this process, there would be less time for the establishment of colonies during the peak of spring and clean structures when the beginning of peak summer colonization, ensuring operating conditions.

4.7 Acknowledgments

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4.8 Compliance and ethical standards

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4.9 Author Contributions

Conceptualization, A.B., R.L., and A.O., and.; methodology and field collection, A.B., A.O., R.L., and A.H., and O.N.; software, A.O. and A.B.; writing - preparing the manuscript, A.B., A.O., R.L., and A.H.; writing – review and editing, A.B., A.O., A.H., R.L., and M.P.; supervision, A.O., M.P. and T.Z.

4.10 Availability of data and material

Raw data used in our analyses is available from the authors upon request.

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5 CAPÍTULO III- AN EXPERIMENTAL ASSESSMENT OF THE DISTRIBUTION OF ENVIRONMENTAL DNA ALONG THE WATER COLUMN

An experimental assessment of the distribution of environmental DNA along the water column

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Freshwater; eDNA, Free DNA; DNA distribution; biodiversity monitoring.

Abstract

The study of environmental DNA (eDNA) is increasingly becoming a valuable tool to survey and monitor aquatic communities. However, there are important gaps in our understanding of the dynamics governing the distribution of DNA under natural conditions. In this report we carry out controlled experiments to assess the extent and timing of eDNA distribution along the water column. A sample of known DNA concentration was placed at the bottom of a 5-m high tube (20 cm in diameter and total volume of 160 L), and water samples were obtained at different depths over an 8 h-period. The presence of the target DNA was assessed by qPCR analysis. This sampling protocol allowed for assessing the timescale for the diffusion of DNA while minimizing the influence of turbulence. We demonstrate that, after a time-period of as little as 30 min, the eDNA had spread across the entire container. The implications of these results for eDNA sampling protocols in the field are discussed.

5.1 Introduction

Environmental DNA (eDNA) is a method based on the detection of trace genetic material shed from organisms into their surroundings (Barnes and Turner 2016). Environmental DNA is composed by a range of particles, such as free DNA, organelles, cells, tissue fragments and metabolic waste (Turner et al. 2014; Wilcox et al. 2015). When suspended in an aquatic environment, this material can be sampled together with the water, extracted, and detected through molecular biology techniques (Ficetola et al. 2008). Surveillance and monitoring of aquatic species through eDNA is widely applied

with advantages over traditional methods. This strategy, for example, can detect single or multiple species in one environmental sample (Harper et al. 2018), and the results can quantify relative biomass (Pilliod et al. 2014; Takahara et al. 2012). The main advantages of this approach are the shorter time requirements, increased cost-effectiveness, increased taxonomic resolution and non-invasive sampling (Thomsen et al. 2012; Hunter et al. 2015; Eiler et al. 2018). Several studies applied this method for aquatic organisms, such as fish (Miya et al. 2015), mussels and snails (Goldberg et al. 2013; Marshall and Stepien 2019), jellyfish (Minamoto et al. 2017), sharks (Bakker et al. 2017), amphibians (Pope et al. 2020) and arthropods (Toju and Baba 2018).

Although DNA is a powerful technique, it is far from being standardized, as several methods are applied to capture and analyze field samples (Hinlo et al. 2017). Water samples, methods consist mostly of sampling the water column or the sediment (Buxton et al. 2017; Katano et al. 2017; Wittwer et al. 2018). However, studies rarely sample more than one depth, and when they do, the only parameter to compare them is detection rates or biodiversity (Yamamoto et al. 2016; Andruszkiewicz et al. 2017). Spread of eDNA horizontally was recently explored by studying flow from rivers (Sansom and Sassoubre 2017; Pont et al. 2018; Jo et al. 2019; Villacorta-Rath et al. 2020) but the vertical distribution is still fairly explored from a functional perspective. Vertical zoning is the structuring of communities through layers of species and communities across depths, which can potentially change dramatically in a matter of meters (Chappuis et al. 2014). eDNA concentration, composition and spatial distribution is then expected to vary as communities change through depth due to vertical zoning. While some studies conclude that there is a negligible impact on the detection and composition (Eichmiller et al. 2014; Currier et al. 2018; Cordier et al. 2019; Harper et al. 2020; Lafferty et al. 2020) others report differences (Moyer et al. 2014; Hänfling et al. 2016; Yamamoto et al. 2016;

Andruszkiewicz et al. 2017; Minamoto et al. 2017; Lacoursière-Roussel et al. 2018; Uthicke et al. 2018; Cordier et al. 2019; Murakami et al. 2019; Jeunen et al. 2020; Kuehne et al. 2020; Lor et al. 2020; Sigsgaard et al. 2020; Zhang et al. 2020). These studies, however, vary significantly between the water body architecture, water composition, depth sampled, sampling strategy, target organism, detecting technique, extraction protocol and molecular marker. This leads to an inconsistent pattern that can be interpreted as detection relies more on the organism's biology than depth (Minamoto et al. 2017). However, these studies do not consider how DNA moves through the column as both (a) the sources of biological material are still in the water body, releasing particles, at the same time that the particles that are still in the water are being degraded and moved horizontally, and (b) they do not consider time as one of their variables, only depth.

Water bodies are complex systems with varied hydraulic dynamics. Studying the vertical aspects of DNA in a natural system is a difficult task due to many factors acting in the water column at once (Jane et al. 2015). Flow, hyporheic exchanges, streambeds, surface-subsurface exchange, sediment and colloidal interactions are some of these factors that contribute to this complexity (Shogren et al. 2016; Shogren et al. 2019). Controlling these variables in a field experiment to understand how they affect the vertical dynamics of DNA is not logistically viable, so they must be studied individually in a controlled environment.

In this study, we aim to understand how free DNA behaves in a controlled water column. To this end, we built a 5-m high and 20-cm diameter PVC tube, injected DNA at the bottom and monitored how it spread through the water column for 8 hours. Understanding how eDNA behaves in the water column is important to interpret species distribution in a water body and improve sampling strategies. A controlled environment

is ideal for this because one can introduce variables as our understanding of these dynamics improves.

5.2 Materials and Methods

5.2.1 Experimental setup

We build an experimental apparatus to emulate the water column of lentic, freshwater conditions using a 5-m high polyvinyl chloride (PVC) pipe (20 cm in diameter). We placed chromatographic septa at six depths (*i.e.* 0, 1, 2, 3, 4, and 5 m) to allow water sampling by the external side of the cylinder by using a medical sterile 1-mL syringes and thus minimizing the generation of turbulence in the water column inside the cylinder. Prior to each experiment, the entire apparatus was decontaminated in a two-step process. First, we used a dichloroisocyanurate solution (0.06 g L^{-1}) to thoroughly wash the pipe. We then rinsed away the chlorine with previously treated DNA-free water. This treatment consisted of decontaminating the water with a 10% sodium hypochlorite solution (0.2 mL L^{-1}), followed by chlorine neutralization using a 50% sodium thiosulfate solution (0.1 mL L^{-1}). The second step was repeated three times in order to ensure that there was no leftover chlorine in the system, coupled with a colorimetric method to detect chlorine after each washing (Zall et al. 1956).

We generated a test solution of eDNA by amplifying a ~100 bp fragment of the COI gene117from a genomic sample of the golden mussel Limnoperna fortunei (Mytilidae), which is an organism commonly known for its biofouling impacts on hydraulic systems (Darrigran and Damborenea 2011; Boltovskoy 2015; Boltovskoy 2017). Each assay was run in a 25 μL final volume reaction, with concentrations: 100 μM each primer, 0.25 mM dNTP mix, 1 U Platinum Taq DNA Polymerase, 1 X Platinum Taq buffer and 2 mM $MgCl_2$; Thermocycling conditions followed: 1 min at 95 °C for initial denaturation, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 30 sec.

and at 70 °C for 30 sec. To obtain a high DNA concentration for the stock test solution, we carried several independent PCRs and the resulting products were pooled, quantified using Qubit 4 fluorometer, and frozen at -80 °C. Immediately before the beginning of the experiments, the DNA solution thawed at room temperature, and each experiment used a 1 mL aliquot (2000 ng of target DNA).

Each experiment began by filling the entire apparatus with DNA-free water up to 5 m, followed by a 15 min period to allow for the water movement to subside. At this point, 1 mL water samples were then collected from each depth using disposable, DNA-free syringes to serve as negative controls. The experimental solution aliquot was then injected at the base of the pipe (5 m depth), and immediately, 1 mL samples were collected from all depths using disposable syringes. Sampling was then repeated at 30 min, 1, 2, 4 and 8 hours after the injection. The entire experiment was run in triplicate. Water samples were then stored at 2 mL decontaminated microtubes, and frozen at -20 °C until analysis.

5.2.2 eDNA amplification and quantification

Each water sample from the experimental apparatus was processed for DNA extraction using a Solid Phase Reversible Immobilization (SPRI) protocol (DeAngelis et al. 1995). First, 1 mL of collected sample was incubated with a final concentration of 12.5% weight/volume PEG-8000, 0.7 M NaCl and 0.02 mg/mL carboxylated magnetic beads at room temperature for 10 min to condense DNA and bind it onto the magnetic beads. Samples were then magnetized using a neodymium rare earth permanent magnets (NEB), and the supernatant was carefully removed using a micropipette. Samples were dried at room temperature, eluted into 100 µL of TE Buffer and gently mixed. After unbinding DNA from the magnetic beads, samples were magnetized again and the supernatant containing DNA was removed and stored in individual vials.

After extraction, samples were quantified using qPCR with a hydrolysis probe (TaqMan) targeting the 100 bp fragment previously amplified COI fragment (Pie et al. 2017). Each assay was run in a 10 µL final volume, with concentrations as follows: 75 µM each primer, 25 µM probe and 1 X QuantiNova Probe PCR Kit (Qiagen). Each sample was run in triplicate, with 3 µL of extract being used in each reaction. Cycling conditions were: 2 min at 95 °C for enzyme activation, followed by 50 cycles of denaturation at 95 °C for 5 s, and combined annealing and extension at 60 °C for 5 sec. Assay was run in RotorGeneQ 5plex+HRM (Qiagen). For quantification, a standard curve was built by running a six-order serial dilution of the stock solution previously quantified using Qubit, also performed in triplicate. Each run was analyzed using RotorGeneQ Series Software (Qiagen), with Quantification analysis. Threshold was calculated with automatic option, with a 0.35 upper bound limit, and quantification was done with slope correct mode.

5.2.3 Analyses

We used two approaches to assess the vertical distribution of DNA over time. First, we tested the relationship between depth and concentration using linear regressions for each experimental period and determined the time until this relationship became nonsignificant (i.e. DNA concentrations were homogeneous between depths) as an indication of non-homogeneous distribution of DNA across the apparatus. Second, we fit cubic smoothing splines to each dataset (degrees of freedom = 4). Given that the final concentrations are unlikely to become precisely equal due to measurement error, we compared the observed data to re-sampled splines in which concentrations and depths were randomly shuffled (N=1000 pseudo-replicates). This procedure allowed for the generation of a visual expectation of the expected variation in concentration estimates given the inherent variability of the environmental setup used in our study. All analyses were carried out using R 4.0.2 (R Core Team 2020).

5.3 Results

The vertical distribution of experimental DNA at different time periods is shown in Figure 1. There was a significant relationship between depths and Ct immediately after the beginning of the experiments ($t = 2.99$, $p = 0.008$) and after 30 min ($t = 5.36$, $p = 6.32e\text{-}05$), but that relationship became non-significant after 1 h ($p = 0.48 - 0.98$) (Figure 11). This difference was accompanied by an increase in the DNA concentration across all depths in a manner consistent with the homogenization of DNA concentration throughout the entire apparatus. These results were consistent with the comparison between the splines fit to the observed data and those obtained from shuffled samples. The only two time periods that were outside the simulated data were immediately after and 30 min after the beginning of the experiments. Interestingly, in the latter, the DNA distribution was midway between the state at t=0 and the complete homogenization found at the end of the experiments, with higher-than-expected concentrations up to 3 m from the origin of the DNA.

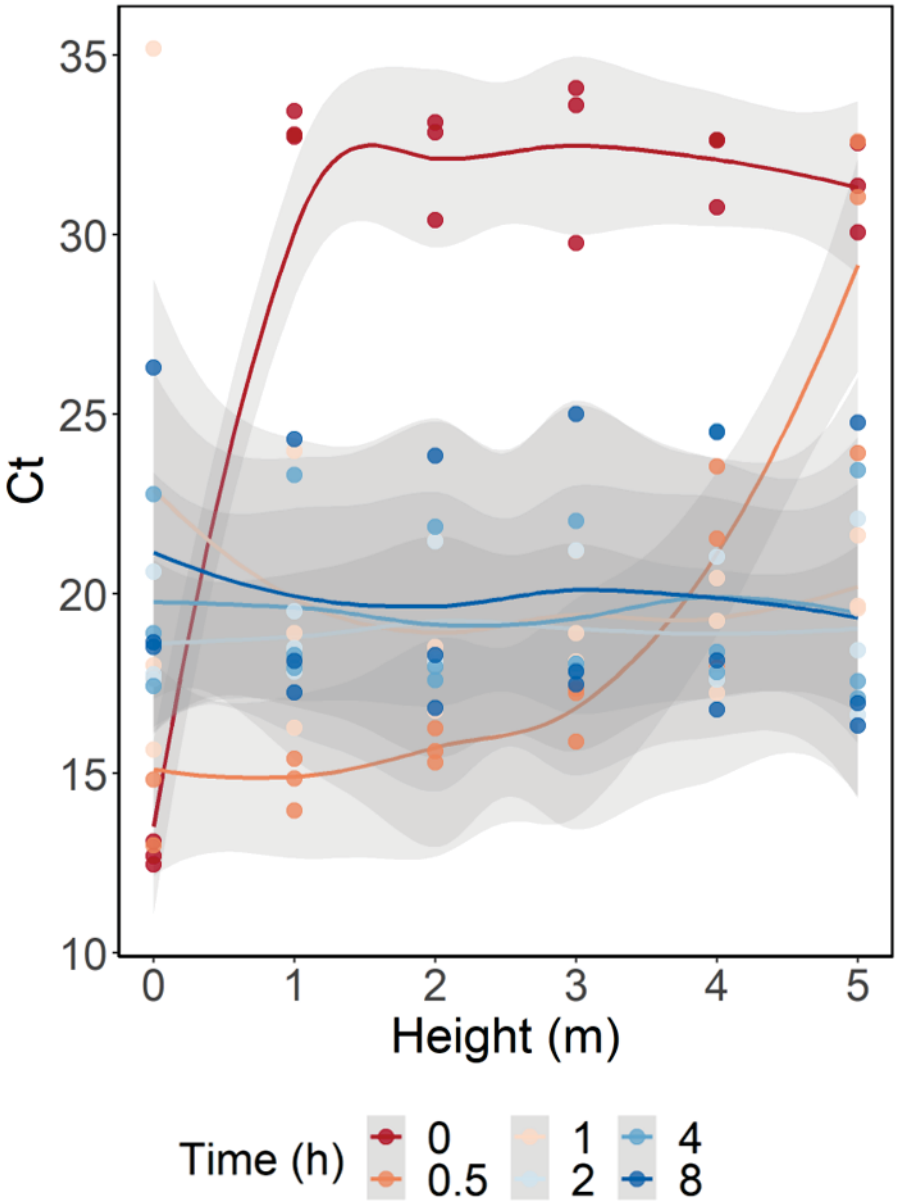

Figure 11 Variation in threshold cycle (Ct) in our experiments from immediately after the addition of DNA (t = 0 h) to eight hours later. Colored lines show cubic splines across the three replicates of each experimental group (see legend). Gray lines indicate 1000 similar splines with datasets in which concentration and depth data were randomly shuffled.

5.4 Discussion

As DNA studies become increasingly used to monitor different components of the aquatic biota, it is crucial to understand the factors determining the distribution of DNA in the water column. In our study, we demonstrate that the diffusion of DNA along the water column takes place rapidly, in the time scale of minutes, even in the absence of

turbulence. This result is important given that, under field conditions, the water currents would tend to accelerate the homogenization process. Thus, there does not seem to be "an optimal location" to obtain water samples for DNA analyses in a lentic system, as DNA tends to not accumulate in a specific part of the water column. These results are intriguing, given that previous studies suggested a differential accumulation of DNA on either the surface (e.g. (Murakami et al. 2019) or the bottom (e.g. (Moyer et al. 2014), or even near the layer that the organism lives (e.g. (Minamoto et al. 2017).

It is important to note that, although we used free DNA molecules in our experiment, DNA is not a monodisperse phase in nature (Turner et al. 2014; Wilcox et al. 2015). It is composed of particles ranging from single DNA molecules to tissue fragments (e.g. between 0.2 and 180 μm, but mostly between 1-10 μm (Turner et al. 2014)). Particle size composition also plays an important role in how studies comparing different depths report due to how they interact with filter pore size. Although this distribution range seems to be constant between close-related taxa (such as fish, (Barnes et al. 2020)), it seems to vary between different taxa (such as water fleas) (Moushomi et al. 2019). This distribution also changes with time, as bigger particles tend to break down into smaller particles (Murakami et al. 2019). As the sampling and processing methods (volume used, filtration technique, time between sampling and water composition measurements) in these comparative studies are not standardized, it is expected that particle size distribution will play a major role in the results. The behavior of different particle sizes on the water column is unknown. This is a potential source of bias on the sampling, as the captured DNA can differ significantly from true DNA source amount on a determined sampling point, because of pore size and volume configuration, and this error can vary between sampling points.

We also expect that the solubility of these different particles influences how they behave in the water column. While most of the DNA particles tend to have a hydrophilic nature, some are hydrophobic. When considering colloidal particles in the water, DNA particles can bind to it and behave differently from how they would if they were suspended, mostly due to weight changes. This can lead to accumulation in certain parts of the water column, or changing speed of diffusion (Cai et al. 2006a; Cai et al. 2006b). When bound particles are too dense, it can also promote deposition and accumulation of eDNA in the substrate (Zhai et al. 2019). Size of suspended particles also influences this dynamic, as finer substrates tend to capture more DNA due to smaller pores (Shogren et al. 2016). This can lead to an effect of accidentally re-suspending trapped DNA into the water column while sampling, which can cause a sampling bias where capturing water near the bottom is actually capturing the substrate (Turner 2004; Turner et al. 2014). Hydrogeomorphic features of the system being studied should be assessed in order to evaluate slopes (which influence depth variations) and adsorption sites (which can sequester eDNA) (Fremier et al. 2019).

It is also important to emphasize that our results only pertain to a specific aspect of DNA distribution, namely the vertical diffusion process over time in the absence of water currents. The movement of water in lotic conditions might provide qualitatively different conditions, given that water velocity varies with depth. For instance, under laminar flow, water near the surface might include DNA from farther upstream than those near the bottom (Curtis et al. 2020). However, as water speed becomes faster, the onset of turbulent flow might lead to homogenization of bottom and top water layers (Mächler et al. 2020). Water flow and stratification are also important factors that can create different degrading zones in the water column (Curtis et al. 2020). Liquid flow is known to degrade eDNA due to mechanical forces (Levy et al. 1999). When hyporheic exchanges (water

from the main river flow being exchanged with water kept in porous substrates) are considered, we would expect it to create less intense flow zones. These islands could potentially serve as less degrading spaces, where it would be more advantageous to sample near porous substrates both due to sequester of DNA and due to irreversible sorption to bed sediment (Foppen et al. 2013). Little is known about the dynamic of these spaces regarding eDNA particles and their distribution. In another scenario, when there's permanent water column stratification (such as in the sea), depth becomes an important sampling factor (Jeunen et al. 2020). It is unknown if DNA can pass these barriers (i.e. if convection is enough to break these barriers and homogenize DNA). It's also unknown if there are clines through the same zones, causing in-between convection to cycle the water and homogenize the water in each water break.

While our results show the behavior of a monodisperse phase of DNA particles in a relatively small water column, it highlights how this system would behave without interference. With so many factors acting at once in a complex water body system, it is important to break down its components and understand how they behave separately, so we can build a better model that can be incorporated in realistic field conditions. Understanding the interplay between turbulence, colloidal particles and DNA transport is a particularly important frontier of eDNA research.

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6 CAPÍTULO IV- EFFECTS OF ABIOTIC VARIABLES ON DNA DEGRADATION IN A LIMNIC ENVIRONMENT

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Abstract

Molecular techniques are increasingly used in the assessment and monitoring of invasive fouling species. Our aim was to assess the environmental factors responsible for the degradation and persistence of environmental DNA (eDNA) over time in an environment that is not fully controlled. In this study, this was achieved by measuring the effects of these factors on the eDNA persistence of *Limnoperna fortunei* and *Cordylophora* sp. under limnic conditions. After a pilot experiment to determine DNA degradation in the field, the experimental phase began at two hydroelectric power plant, in Paraná, Brazil, with two experiments, EI, which was made with bottles contained DNA extract of *L. fortunei* and EII, which bottles contained water from the reservoir itself, with eDNA of both species. In both experiments, temperature, luminosity, turbidity and transparency were monitored and DNA concentration was measured by qPCR. Sampling units consisted of two sets of sterile glass tubes for two treatments, one with transparent tubes and one with tubes covered with black tape, to prevent the influence of sunlight. The units were arranged in triplicate and attached to a guide rope held vertically in the water column where they were distributed at 0.0; 0.3; 0.6; 1.5; 3.0; 4.3, 7.0; and 10.0 m depths and remained submerged for 24, 72, 168 and 264 hours, respectively. To monitor DNA degradation during transport (T0), we used initial controls with known DNA concentrations that were stored and frozen at -20 ºC until analysis. We concluded that the presence of eDNA could still be detected after 12 days under different environmental conditions, but the degradation process of the molecule was clearly accentuated in the first 24 hours. The rapid degradation of eDNA in aquatic habitats allowed monitoring of species practically in real time, as the DNA identified was the result of a recent release.

Keywords: eDNA, invasive fouling species, monitoring, qPCR, turbidity

6.1 Introduction

The rapid proliferation of molecular techniques, particularly those based on the use of genetic markers and polymerase chain reaction (PCR), has revolutionized several areas of science and contributed to knowledge on the identification, detection and monitoring of biodiversity in limnetic environments (Ficetola et al., 2008; Green and Field, 2012; Pilgrim et al., 2011; Zhou et al., 2009). These techniques include identification or detection of species using environmental DNA (eDNA) (Kasai et al., 2020; Pilliod et al., 2013), which is released into the environment by organisms in various forms, e.g. cells, faeces, scales, mucus, skin, and gametes (Barnes and Turner, 2016). After genomic material is released into the environment, its fragments can remain in the water for days or even weeks, depending on the environmental conditions to which the molecules are exposed (Barnes et al., 2014; Dejean et al., 2011). Therefore, traces of this eDNA can be collected, identified and quantified in aquatic environments through water sampling, following a procedure that includes filtration (Goldberg et al., 2011; Thomsen et al., 2012; Yamanaka and Minamoto, 2016), preservation (Jerde et al., 2011), extraction and amplification of the DNA contained in the samples (Goldberg et al., 2011; Sanches and Schreier, 2020).

Molecular techniques are increasingly and widely used in the assessment and monitoring of invasive fouling species that cause operational and economic impacts at energy production facilities (Boltovskoy and Correa, 2015; Boltovskoy et al., 2006; Karatayev et al., 2007; Nakano and Strayer, 2014; Pie et al., 2006; Pucherelli et al., 2016; Tokumon and Boltovskoy 2018). In addition to detecting species in the environment, in principle it is also possible to assess their relative biomass (Doi et al., 2017; Takahara et al., 2012). These techniques open up new and efficient possibilities for early detection and mitigation of impacts caused by invading fouling organisms in reservoirs and hydroelectric power plants (HPPs) (Pie et al., 2017). However, if the results are to be reliably associated with the environmental processes that influence them, adequate knowledge of the factors that determine the degradation of DNA in the environment is essential (Jerde et al., 2011; Yamanaka and Minamoto, 2016). This association can be challenging and this is considered one of the most important limitations for detecting species with eDNA techniques (Strickler et al., 2015). Samples exposed to factors that accelerate degradation can compromise results and make environmental monitoring impossible, especially if the target species is at low density (Bailey et al., 2007) as in the

case of the initial bioinvasion at a particular site (Strickler et al., 2015). Studies have shown the effects of abiotic factors on the degradation of DNA molecules, including high temperatures (Collins et al., 2018; Strickler et al., 2015; Tsuji et al., 2018), ultraviolet radiation (Pilliod et al., 2014) and an acidic pH (Collins et al., 2018; Torti et al., 2018).

Although the use of eDNA has been shown to be accurate, there are still gaps in the many protocols available. The persistence and integrity of the DNA molecule exposed to abiotic factors *in situ* are important variables for quantitative assessment of environmental processes, as they affect the integrity of eDNA (Jo et al. (2020); Pilliod et al. (2013); Tsuji et al. (2018); Wilcox et al. (2020)). As Sansom and Sassoubre (2017) point out, using these realistic systems provides a more reliable biomonitoring tool compared to testing under perfectly controlled conditions. Therefore, we proposed to assess the environmental factors responsible for the degradation and persistence of eDNA over time in an environment that is not fully controlled. To this end, we measured the effects of environmental factors on the eDNA persistence of the two invasive species *Limnoperna fortunei* (Of Muller 1774) and *Cordylophora* sp. (Pallas, 1771) in a limnic environment. Our goal is to test eDNA degradation in three scenarios: (i) with a model sample simulating the presence of a species with a high DNA release; (ii) with a model of species with an intermediate DNA release; and (III) with a model of species releasing a low DNA concentration.

6.2 Material and methods

6.2.1 Pilot experiment

A pilot experiment was conducted with a DNA extract from *Limnoperna fortunei* to ensure that the experimental design used (time period and depth) was sufficient to estimate the rate of degradation of samples containing eDNA from invasive species *in situ*. In this experiment, we used samples with a DNA extract that did not contaminate the environment, as the invasive organism is already present in the study area.

6.2.1.1 DNA extraction from Limnoperna fortunei

Individuals of *L. fortunei* from the reservoir of HHP Governador José Richa (-25.142, -48.869), state of Paraná, Brazil, were maintained under laboratory conditions [temperature (25.0°C ± 1.0°C) and constant aeration (6.5 ± 1.5 mg/O_2/L)] to obtain tissue samples for complete DNA extraction. For this purpose, the samples were digested in a

buffer solution with the enzyme proteinase K and purified using the SPRI (Solid Phase Reversible Immobilization) procedure with magnetic beads (microspheres surrounded by magnetite and carboxyl), that bind to DNA (carboxyl-DNA binding). After this procedure, we quantified the extracts using the Qubit® 4.0 Fluorometer (Thermo Fisher Scientific®, Brazil) and the Broad Range (BR) Fluorometry Kit. The DNA fragments present in the samples were analyzed qualitatively by electrophoresis in a 0.6% agarose gel. The results were visualized under ultraviolet radiation on an E-Gel Imager (UV Light base, Thermo Fisher Scientific). The extracts that showed good quality and no vertical resistance in the gel were quantified by quantitative real-time PCR (qPCR) using hydrolysis probes (TaqMan) with a Rotor - Gene Q (Qiagen, Germany). The extracts were then added in a single flask and diluted with ultrapure water, creating the working solution. The sequences of the primers and probes for TaqMan assays that we used to amplify the DNA of *L. fortunei* (Pie et al., 2017).

6.2.2 Experiment I (Scenarios i)

6.2.2.1 Experimental design I

The experiment was conducted in the reservoir of the HPP Governador Pedro Viriato Parigot de Souza. The experimental apparatus consisted of a guide rope (nylon, 8-mm thick and 12-m long), the ends of which were attached to a buoy, and a ballast that held the guide rope in a vertical position in the water column. Sets of sterile 2.0 mL glass vials with crimp caps were attached along the rope with plastic clips. Each set consisted of one vial wrapped with insulating tape (opaque treatment) and another without tape (transparent treatment). We placed both types of vials at each depth to evaluate the effect of light penetration. Immediately before the start of the experiment, 2 mL of the working solution containing DNA from *L. fortunei* was injected into the flasks (0.9 mL of extract and 698.2 mL of high purity H_2O) with an average initial concentration of 1.6 (ng/μL). The sets were arranged in triplicate and distributed at 0 (at the air/water interface); 0.3; 0.6; 1.5; 3.0; 4.3, 7.0 and 10.0 m depth. We placed a total of twelve experimental units in the reservoir and submerged them. Three were taken after 24 hours, three after 72 hours, three after 168 hours and the last three after 264 hours. The samples were transported in a polystyrene box with ice. In addition to baseline control samples with known DNA concentration that were conditioned and frozen at -20 ºC until analysis (T0), we used

control bottles that were monitored during transport of the samples to check for DNA degradation during transport.

For the turbidity analysis, we took water samples at the same experimental depths using a Van Dorn bottle. Using a turbidimeter AT 2K (Alfakit, Brazil) and a Secchi disc, we measured turbidity and transparency, respectively, at each sampling time (24, 72, 168 and 264 h). We used data loggers (HOBO®, USA) to continuously record water temperature and light intensity at each depth and at the surface of the reservoir (with data loggers directly attached to a float) and in the thermal boxes used to transport the samples. In the laboratory, we fractionated and froze (-20 °C) the samples to later quantify the DNA concentration by qPCR. The quantification test was performed in a final volume of 10 µL [QuantiNova Probe PCR MasterMix® 1X; primer (specific for *L. fortunei*); qPCR probe; BSA (bovine serum albumin); ultrapure water and sample] with a Rotor-Gene Q (Qiagen®, Germany).

6.2.3 Experiment II (Scenarios ii and iii)

6.2.3.1 Experimental design II

We collected water samples containing eDNA of *Limnoperna fortunei* (with an average initial concentration of 0.015 ng/µL) and *Cordylophora* sp. (with an initial concentration of 0.000048 ng/µL) from HPP Governador José Richa reservoir, and filled them into 200-mL sterile plastic bottles. The experimental apparatus consisted of a guide rope (nylon, 8 mm thick and 12 m long), the ends of which were attached to a buoy and a ballast. Along the rope, sets of sterile plastic bottles with screw caps were attached with plastic clips. Each set, arranged in triplicate, consisted of one bottle of amber material (opaque treatment) and one bottle of transparent material (transparent treatment), distributed at the same depth and over the same period of time as described in experimental design I. The bottles were then placed in the reservoir. Twelve experimental units were placed in the reservoir and removed three by three at the end of each period. The collected samples were transported in a polystyrene box with ice. We also used control bottles with known initial DNA concentration (T0). These control samples were immediately filtered and the filters were placed in an Eppendorf tube containing 99% ethanol and frozen at -20 ºC until analysis.

We used data loggers (HOBO®, USA) to continuously record water temperature and light intensity at each depth and also at the surface of the reservoir (from data loggers

attached directly to the float) and in the thermal boxes used during sample transport. Transparency and turbidity measurements followed the same methodology as in experimental design I. Samples were filtered immediately after collection using a vacuum pump and a cellulose nitrate membrane (Unifil, Germany) with a diameter of 47 mm and a pore size of 0.45 µm. The filters were kept in an Eppendorf tube containing 99% ethanol and stored at -20 °C until analysis. In the laboratory, we extracted the samples according to the SPRI (Solid Phase Reversible Immobilization) protocol for further quantification of DNA concentration by qPCR. The quantification test was performed in a final volume of 10 µL [QuantiNova Probe PCR MasterMix® 1X; primer specific for *L. fortunei* and *Cordylophora* sp. (forward AGGACGCAGTATCTTGACTGTG, reverse GTCGCCCCCAACTAAACTACCAAA and FAM/BHQ1 probe CACTCGCCACTTAATTGGTGGATAGT (Agostinis, 2016)) qPCR, BSA (Bovine Serum Albumin), ultrapure water and sample] using the Rotor-Gene Q (Qiagen®, Germany).

6.3 Data analysis

We analyzed the data independently for the three data sets, the DNA extract of *L. fortunei* (scenarios i), the eDNA of *L. fortunei* (scenarios ii) and the eDNA of *Cordylophora* sp. (scenarios iii). The normality of the variances of the DNA concentrations was tested with the Shapiro-Wilk test and the homoscedasticity with the Fligner-Killeen test. Both tests were performed using the one-way tests V.2.6. package (Dag et al., 2018) in the software R V4.2 (R Core, 2020). As the DNA concentration data for the three datasets did not show a normal distribution, Kruskal-Wallis analysis was used. The effect size of each variable was compared with eta^2 using the package 'rstatix' V0.7.0 (Kassambara, 2020). We determined whether differences between time, which was considered a categorical variable, and treatments (transparent and opaque) were significant in the R software. To determine which groups differed significantly, we used the post-hoc Wilcox test in R.

To determine which variables best explain DNA degradation, a selection of generalized linear models based on the Bayesian Information Criterion (BIC) was run for each dataset (DNA extract of L. fortunei, the eDNA of *L. fortunei* and the eDNA of *Cordylophora* sp. as responsible variables) using the 'performance' V.0.8.0 R package (Lüdecke et al., 2021). We started with a model that accounted for all variables and

excluded non-significant variables. In addition, we included a null model with only one intercept. In total, we obtained 17 models, including the null model, the different abiotic variables, and possible interactions between them, as shown in Table S 1.

6.4 Results

6.4.1 Environmental conditions

The parameters evaluated to characterize the environment studied included transparency (at the surface), turbidity, luminosity, and water temperature (at different depths). In experiment I, with the DNA extract of *L. fortunei*, the transparency of the water, as methodically applied to the surface layer of the water body, showed homogeneous values, with greater transparency at 2.1 m. Turbidity, in turn, was classified as low, reaching higher values between 1.5 and 4.3 m, with an average of 4.5 NTU. In experiment II, with eDNA from *L. fortunei* and *Cordylophora* sp., turbidity ranged between 3.0 and 4.3 m, with an average of 4.4 NTU, and the highest transparency was 6.9 m. In experiment I, the temperature in the surface layer remained relatively stable at around 22 ºC. From 7 m, it decreased significantly, reaching an average of 16.4 °C (Figure12a). The average luminosity reached 13,283 lux (31.8%) at 0 m depth and stabilized at about 694.5 lux (1.7%) from 4.3 m (Figure12b). In the experiment II, the luminosity reached 9.5 lux and stabilized from 7 m with an average value of 2.1 lux. The temperature showed the same pattern as in experiment 1, with the highest value near the water surface at 27 ºC.

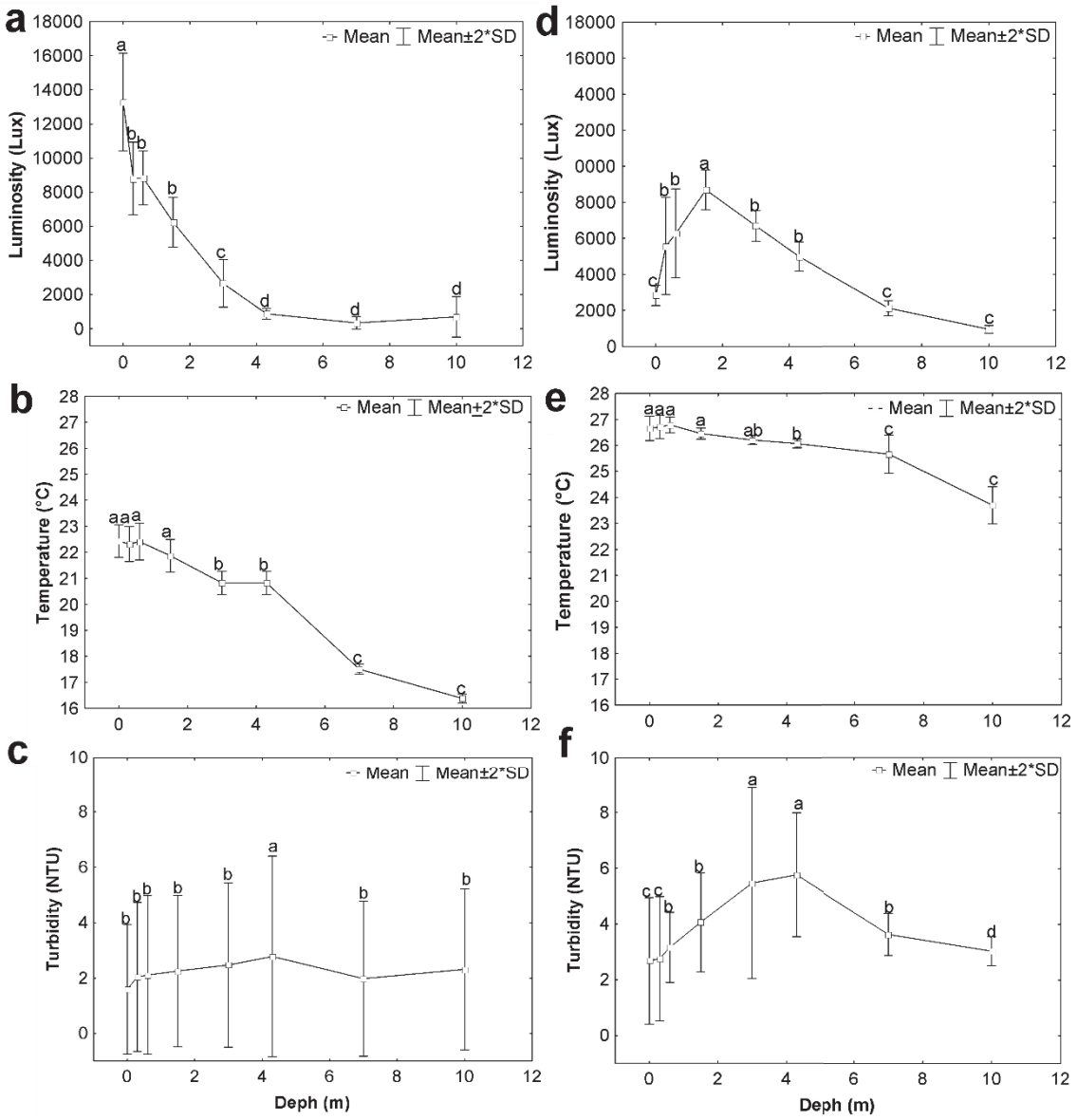

Figure12. Results of experiments from experiment I conducted at the HPP Parigot de Souza reservoir, using DNA extract samples of *Limnoperna fortunei,* a) mean values of luminosity, b) temperature and c) turbidity; experiment II conducted at the HPP Jose Richa, using eDNA samples *of Limnoperna fortunei* and *Cordylophora* sp., mean values of d) luminosity, e) temperature and f) turbidity,

6.4.2 DNA Concentration Analyses

6.4.3 Experiment I DNA extract

The effects of sampling period and DNA concentration were considered significant ($p < 0.001$) and with a large effect ($eta^2 = 0.23$). The results show that DNA concentration decreases in the first 24 hours and is significantly different from the other periods. After 24 hours, the DNA concentration have a low but significant increasement (Table 9), remaining low and constant in the other time periods (Figure 13). DNA was still detected at low concentrations (0.15 ng/µL) 264 hours after the start of the experiment. The opaque

and transparent treatments had no effect on the degradation of the samples ($p > 0.05$). In the model selection analysis, the best model is the one with the lowest BIC and the highest weight (wBIC) that retains time and depth, as highlighted in bold in Table S 1.

Table 9. Wilcoxon test for different experimental periods (0, 25, 75, 168 and 264 hours) in relation to the concentration of *Limnoperna fortunei* DNA extract samples. Significant values are highlighted in bold ($p < 0.05$).

	Period (hours)				
	0	24	72	168	264
0	-				
24	**0.00031**	-	-		
72	**0.00031**	**5.40 E-13**	-		
168	**0.00031**	**5.00 E-09**	0.65421	-	
264	**0.00031**	**3.60 -08**	0.73884	0.57244	-

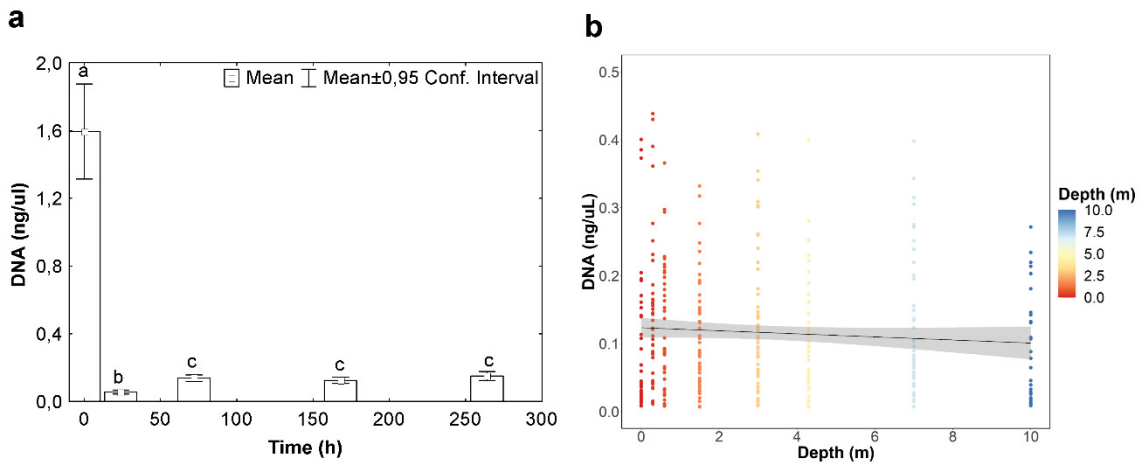

Figure 13 a) Concentration of DNA molecules (µg/µL) in the extract samples of *Limnoperna fortunei* depending on the experimental period as a categorical variable; b) Relationship between DNA concentration (ng /µL), depth.

6.4.4 Experiment II: Environmental DNA

There was also a large and significant effect for *L. fortunei* ($eta^2 = 0.53$, $p < 0.001$) and *Cordylophora* sp. ($eta^2 = 0.32$, $p < 0.001$) in relation to sampling period and DNA concentration (Table 10). The results show that the DNA concentration decreases in the first 24 hours for *Cordylophora* sp., while this decrease is less pronounced for *L. fortunei*. In *Cordylophora* sp. the DNA concentration after 24 hours is close to zero in all time periods, while in *L. fortunei* there is an increase in DNA concentration in the first 24 h, after which DNA tends to stabilize (Figure 14).

Table 10 Result of the Wilcoxon test for different temperatures. The values on the lower diagonal are from *Cordylophora* sp. and on the upper diagonal from *Limnoperna fortunei*. Significant values are highlighted in bold ($p < 0.05$). Only the time of 264 h was not significantly different from zero for *Cordylophora* sp.

	Period (Hours)				
	0	24	72	168	264
0	-	**0.00047**	**1.10E-10**	**1.10E-10**	**7.80E-09**
24	**8.50E-08**	-	**< 2e-16**	**< 2e-16**	**1.30E-15**
72	**2.90E-07**	**3.30E-09**	-	**9.40E-10**	**1.00E-06**
168	**1.30E-06**	**5.40E-05**	**0.022**	-	**0.01272**
264	**1.30E-06**	0.131	**5.00E-08**	**2.80E-06**	-

There was a small but significant effect of treatment type on DNA concentration for *L. fortunei* ($eta^2 = 0.02$, $p = 0.003$) and a moderate effect for *Cordylophora* sp. ($eta^2 = 0.12$, $p < 0.001$). Although turbidity was significant for *Cordylophora* sp., we can conclude that environmental DNA degradation occurs mainly in the first 24 hours, independent of other factors. The opaque and transparent treatments had no effect on the degradation of the samples ($p > 0.05$). In the model selection analysis, the best model was the interaction of time and turbidity for *Cordylophora* sp. and only time for *L. fortunei*.

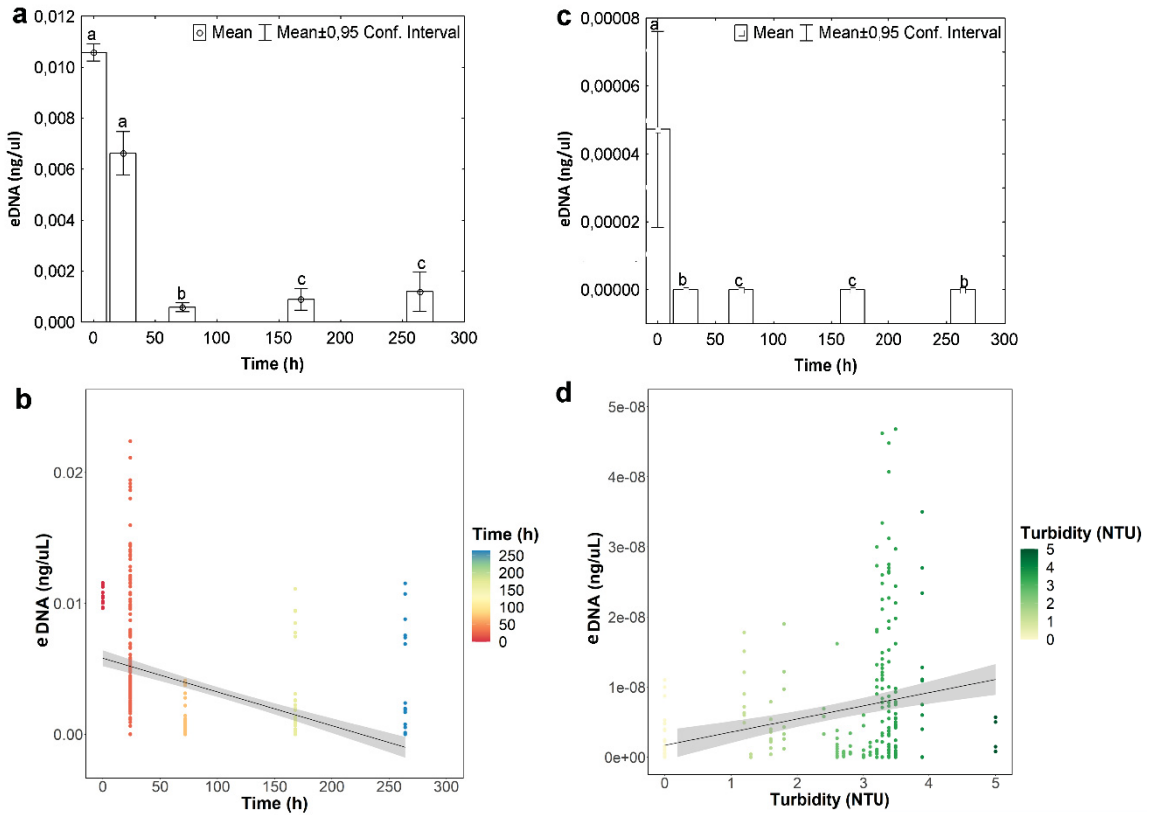

Figure 14) a) eDNA concentration as a function of experimental period (0, 24, 75, 168 and 264 hours) as categorical variable; b) scatter plot of eDNA concentration data with time (h) as continuous variable for *Limnoperna fortunei*. c) eDNA concentration per period (0, 24, 75, 168 and 264 hours) as categorical variable; and d) scatter plot of eDNA concentration data with turbidity for *Cordylophora* sp.

6.5 Discussion

We investigated the effects of abiotic conditions on DNA degradation in different types of DNA samples in a non-controlled environment. We hypothesized three scenarios to understand the degradation process of DNA samples. The first scenario uses as a model sample simulating the presence of a species with a high release of DNA concentration (samples with *L. fortunei* DNA extract, initial average concentration of 1.6 ng/µL). The second scenario uses as a model a species with an intermediate release of DNA (samples with *L. fortunei* eDNA, initial average concentration of 0.015 ng/µL). And the third scenario uses a species releasing a low concentration of DNA (*Cordylophora* sp. eDNA samples, initial average concentration of 0.000048 ng/µL).

Our results show a strong effect of DNA degradation in samples under the influence of environmental variables, with DNA degraded about 16 times faster in samples with DNA extracts than in samples with environmental DNA from *L. fortunei*. The abiotic factors that most accelerate DNA degradation are time plus depth for the *L. fortunei* DNA extract, time, and turbidity interacting with time for *Cordylophora* sp. eDNA samples e just time for eDNA samples of *L. fortunei*.

Many abiotic factors in water samples can affect the detection of eDNA (Stewart, 2019). In a non-controlled environment, as in our study, the abiotic factors are correlated and interact with each other. In our study, the depth and turbidity associated with time influenced the degradation of DNA molecules in the limnetic environment depending of species and experiment. As showed in the analysis of environmental data, depth, turbidity, and temperature vary together and our statistical analysis kept the parameter with higher explanatory power in the model, but all this variable may influenced the DNA degradation in a synergistic way (Barnes et al., 2014; Caza-Allard et al., 2021). Apart from depth and turbidity parameters, the water flow rate (Hauger et al., 2020; Song et al., 2017), temperature (Robson et al., 2016; Strickler et al., 2015), pH (Torti et al., 2015) and climatic changes such as heavy rainfall or severe drought (King et al., 2022) can also influence DNA degradation. Climate shapes certain characteristics of watercourses, such that heavy rainfall creates an eDNA dilution effect (Buxton et al., 2017; Harper et al., 2019) and increasing concentrations of suspended particles (Deiner et al., 2017; Stoeckle

et al., 2017; Wang et al., 2021), while drought can create scenarios with a low probability of detection, due to low water levels and other associated factors (Deiner et al., 2016; Sales et al., 2019).

In the eDNA samples from experiment II, there was an increase in DNA concentration in the first 24 hours, in contrast to experiment I, with the DNA extract. This DNA peak can be explained by the degradation of tissues and cell organelles leading to an increased release of intracellular DNA into the environment (Mauvisseau et al., 2021). However, this dynamic is complex, as other factors, both abiotic (such as pH) and biotic (microbial and enzymatic activity), can even synergistically influence the process of DNA release and degradation (Caza-Allard et al., 2021).

Other studies have already shown that the degradation of eDNA is very rapid and can even take place within minutes in aquatic environments (Sassoubre et al., 2016; Yamanaka and Minamoto, 2016). This process is triggered by hydrolysis, oxidation and microbial activity, among others (Lindahl, 1993; Torti et al., 2015). This rapid degradation of eDNA in aquatic communities is a very interesting finding, as the short persistence of DNA allows monitoring of organisms present in the environment in virtually real time (Seymour et al., 2018; Stefanni et al., 2022).

The DNA degradation in our study were higher in the eDNA samples of *L. fortunei* than in the samples containing *Cordylophora* sp. Studies with species from very different taxonomic groups also showed different degradation rates, as in the study by Andruszkiewicz Allan et al. (2021), who reported lower concentration in samples from jellyfish, followed by fish and shrimp. These differences may be related to physiological conditions (age or life stage) and density (Pilliod et al., 2013) which ultimately influence the state of eDNA produced and released by the organisms and thus on the concentration (Caza-Allard et al., 2021). In the study by Sansom and Sassoubre (2017) it was described that most of the eDNA released by the bivalve *Lampsilis siliquoidea* is due to the excretion of faeces or tissue cells from the body cavity. The latter is not the case in the cnidarian *Cordylophora*, which has a body cavity with little fluid and a chitinous periderm (Slobodkin and Bossert, 2010), suggesting that the anatomy of the organism may favour or hinder the excretion of eDNA into the environment. Material containing eDNA from cnidarians can be collected by the presence of larvae in the sexual reproductive phase and menonts (dormant stage) in the resting phase (Agostinis, 2016).

It is also important to assess the biomass of the organisms studied. In another study conducted by our group (Bertão et al., 2021) at the José Richa HPP, a higher density of *L. fortunei* was found compared to cnidarians, which may indicate a positive correlation found in relation to the higher concentration and DNA of *L. fortunei* found in this study. We have studied the effects of exposure to light at depths of up to 10 m on samples stored in opaque and transparent containers, as there is no consensus on the effects of light on DNA degradation. One example is that some studies have found no effects of UV light on sample integrity (Andruszkiewicz et al., 2017; Machler et al., 2018; Merkes et al., 2014) in contrast to Pilliod et al. (2014), who reported greater DNA degradation in samples exposed to UV light. Such seemingly inconsistent results highlight the need for additional studies under different conditions to better investigate the effects of UV light on DNA integrity (Eichmiller et al., 2016; Pilliod et al., 2013; Strickler et al., 2015). Here we were able to show that the opaque and transparent bottles showed no significant difference ($p > 0.05$) and the luminosity had no effect on the degradation rate of the samples. In the eDNA experiment, the samples were placed on the cables near the safety signal buoys of the reservoir, which might have caused shading at the first experimental depths (0, 0.3 and 0.6 m). However, this bias does not seem to have had a significant effect on the decomposition rates, as they showed a similar pattern to the extract samples placed on the cables away from the signal buoys in experiment I.

6.6 Conclusion

Our results showed that in both samples containing extract and eDNA of *L. fortunei* and samples containing eDNA of *Cordylophora* sp., qPCR of aquatic samples could detect the presence of DNA after exposure to different environmental conditions for 12 days. However, the degradation process of the molecule is much more pronounced in the first 24 hours. Because of the faster degradation in the first hours, immediate filtration, preservation and storage of the water samples, preferably on site, is critical for accurate DNA measurement. This rapid degradation of eDNA in aquatic communities is promising, as the short residence time of eDNA in the environment allows monitoring of species in virtually real time.

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Table S 1. Models used to determine which variations best explain DNA degradation. The best model (lower Schwarz Bayesian Information Criterion - BIC, Broadly Applicable Bayesian Information Criterion - higher wBIC) included turbidity with time interaction factor (in hours) for *Cordylophora* sp., for *Limnoperna fortunei* (extract) included depth with interaction factor or time (in hours), and unique time (in hours) for *Limnoperna fortunei* (eDNA). The values of the two best models are highlighted in bold.

	***Cordylophora* sp.**		***Limnoperna fortunei***			
	(eDNA)		**(eDNA)**		**(Extrat)**	
Model	**BIC**	**wBIC**	**BIC**	**wBIC**	**BIC**	**wBIC**
[DNA] ~ 1	-7159.72	< 0.001	-2862.32	< 0.001	-61.92	< 0.001
[DNA] ~ Brightness + Depth + Turbidity + Temperature + Time + Treatment	-8836.51	< 0.001	-2806.78	< 0.001	-627.39	0.006
[DNA] ~ Depth + Turbidity + Luminosity + Time + Treatment	-8840.82	< 0.001	-2812.07	< 0.001	-627.58	0.006
[DNA] ~ Depth + Turbidity + Temperature + Time	-8844.63	0.003	-2779.34	< 0.001	-627.08	0.005
[DNA] ~ Turbidity	-8842.7	0.001	-2685.71	< 0.001	-613.68	< 0.001
[DNA] ~ Temperature	-8260.7	< 0.001	-2804.97	< 0.001	-340.24	< 0.001
[DNA] ~ Time	-7806.56	< 0.001	**-2944.13**	**1**	-56.85	< 0.001

[DNA] ~ Depth	-8819.34	< 0.001	-2678.16	< 0.001	-614.94	< 0.001
[DNA] ~ Depth + Time	-8833.06	< 0.001	-2786.96	< 0.001	**-636.53**	**0.545**
[DNA] ~ Turbidity + Temperature	-8837.15	< 0.001	-2669.18	< 0.001	-607.86	< 0.001
[DNA] ~ Turbidity + Time	-8848.51	0.019	-2778.54	< 0.001	-635.42	0.312
[DNA] ~ Temperature + Time	-8255.66	< 0.001	-2897.27	< 0.001	-404.16	< 0.001
[DNA] ~ Depth + Turbidity	-8838.09	< 0.001	-2698.59	< 0.001	-609.47	< 0.001
[DNA] ~ Depth + Turbidity + Time	-8845.61	0.005	-2779.81	< 0.001	-632.54	0.074
[DNA] ~ Turbidity + Temperature + Time	-8843.53	0.002	-2770.54	< 0.001	-630.85	0.032
[DNA] ~ Turbidity * Treatment	-8834.35	< 0.001	-2670.25	< 0.001	-608.22	< 0.001
[DNA] ~ Temperature * Time	-8390.93	< 0.001	-2891.71	< 0.001	-464.16	< 0.001
[DNA] ~ Turbidity * Time	**-8856.35**	**0.97**	-2772.56	< 0.001	-629.97	0.020

6.8 Contributions

Conceptualization: AB, RL, PM and AO; methodology and field collection: AB, AO, RL, and AH; F, S, V and O, S, M, N; molecular analysis: PV; software: CDH and AB; writing—preparing the manuscript: AB, AO, RL, and AH, CDH; writing—review and editing: AB, AO, AH, RL, GGC, CDH and MP; supervision: AO, AH, MP, GGC and TZ.

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6.11 Conflict of interest

The authors declare that they have no conflict of interest.

7 CAPÍTULO V- ESPÉCIES INVASORAS INCRUSTANTES límnicas: Interações ecológicas, desafios empregados na detecção e amostragem por métodos moleculares

ESPÉCIES INVASORAS INCRUSTANTES LÍMNICAS

Interações ecológicas, desafios empregados na detecção e amostragem por métodos moleculares

Por Ana Paula da Silva Bertão

QUEM SÃO?

Os organismos invasores incrustantes límnicos (que vivem em ambientes como rios, lagos e reservatórios) são aqueles que estão fora de sua área de distribuição natural; que se reproduzem sem controle; que possuem estruturas de fixação que facilitam a sua adesão a diferentes substratos; e que, embora muito pequenos, costumam formar colônias numerosas e causar grandes impactos econômicos e ambientais. Um exemplo desses organismos é o mexilhão-dourado (*Limnoperna fortunei*) e o hidrozoário *Cordylophora* sp. Além de impactos ambientais, essas duas espécies causam grandes problemas para as instalações hidráulicas de usinas hidrelétricas de energia da América do Sul.

Essas espécies interagem entre si?

Sim, em diversas situações. Os organismos que vivem juntos em um mesmo ecossistema, na maioria das vezes, dependem, pelos mais variados motivos, uns dos outros. Para isso estabelecem diversas formas de interação ecológica. Assim, estas interações podem acontecer entre os indivíduos de uma mesma espécie (interações intraespecíficas) ou entre indivíduos de diferentes espécies (interações interespecíficas). Elas podem ser classificadas como harmônicas ou desarmônicas, dependendo, respectivamente, das consequências positivas ou negativas que provocam aos organismos/espécies envolvidos.

Nos estudos de interação avaliados na tese de doutorado de Ana Paula da Silva Bertão, realizada junto ao curso de Pós-Graduação em Zootecnia, da Universidade Federal do Paraná, foram identificados os principais organismos aquáticos invasores incrustantes e também os nativos presentes nos ambientes límnicos, em especial no reservatório da Usina Hidrelétrica Gov. José Richa. As interações identificadas ao longo do trabalho foram classificadas segundo seus efeitos positivos e diretos (mutualismo), positivos e indiretos (comensalismo), negativos e diretos (competição), negativos e indiretos (epibiose, canibalismo e predação). Como apresentado no quadro 1.

Quadro 1. Interações ecológicas entre organismos invasores, nativos e seus efeitos

Interações ecológicas	Efeitos			
	Organismo 1		Organismo 2	
Mutualismo [1]	*Limnoperna fortunei*	+	Cordylophora sp.	+
Competição[2]	*Cordylophora* sp.	-	*Hydra* sp.	-
Epibiose[3]	*Hydra* sp.	+	*Limnoperna fortunei*	-
Canibalismo[4]	*Limnoperna fortunei*	+	*Limnoperna fortunei*	-
Predação[5]	*Cordylophora* sp.	+	*Limnoperna fortunei*	-
Comensalismo[6]	*Limnoperna fortunei*		Chironomidae	+

1-Interação intraespecífica e harmônica ocorre quando diferentes espécies se interagem para aumentar suas chances de sobrevivência. Ambas ofertam e recebem benefícios (que pode ser um serviço ou um produto que seu parceiro não pode conseguir sozinho).

2- Interações que podem ocorrer de forma intraespecífica ou interespecífica. A competição pode acontecer por meio de uma disputa por território, alimento, nicho ecológico e até mesmo por parceiros sexuais.

3- É um tipo de interação interespecífica, geralmente o basibionte se adere a um substrato a afim de se estabelecer, crescer ou, em alguns casos, reproduzir - se. Já o epibionte se assenta sobre o basibionte durante o início da fase séssil do seu ciclo biológico.

4- Nesse tipo de interação, os indivíduos podem se alimentar de outros organismos da mesma espécie. Essa relação acaba contribuindo para eliminação de larvas e juvenis menos aptos, além de poder ter um efeito sobre o número de Indivíduos na população.

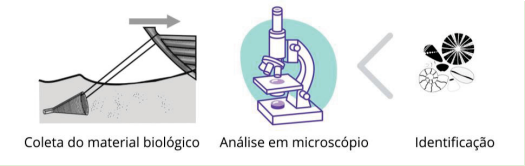
5- É uma relação que se caracteriza como interespecífica e desarmônica. Os organismos obtêm seu alimento a partir da captura de organismos.

6- Relação ecológica em que duas espécies estão associadas, mas, enquanto uma delas é significativa, e claramente beneficiada pela associação, a outra não tem nenhum ganho ou prejuízo. Como o exemplo das colônias de mexilhão-dourado que acabam formando um ambiente propicio para o desenvolvimento de larvas de insetos que se alimentam das pseudofezes e fezes dos mexilhões.

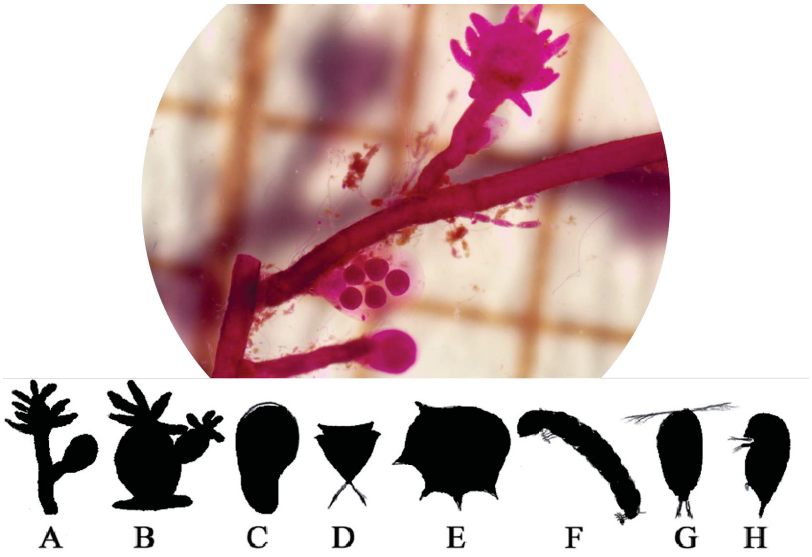
Métodos de coleta de amostras

Método convencional

Os métodos convencionais de coleta de amostras de aquáticas sempre foram utilizados pela ciência através de procedimentos tradicionais. Normalmente esse método utiliza-se redes de pesca (peixes, crustáceos e outros), armadilhas como covo (crustáceos) e redes de plâncton (plâncton) com o intuito de capturar o organismo e transportá-lo até o laboratório para a identificação por microscópio ou através da análise das características morfológicas baseadas em chaves taxonômicas.



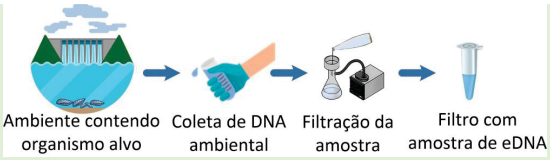
Para a identificação e quantificação de organismos aquáticos em estágios microscópicos, como, moluscos (larvas), peixes (ovos e larvas) e crustáceos (larvas e adultos), por exemplo, os organismos - já mortos - são analisados em microscópio estereoscópico. Esta técnica demanda tempo, técnicos especializados e os resultados nem sempre são precisos quanto deveriam ser.



Organismos incrustantes invasores e nativos identificados no reservatório da usina hidrelétrica de energia (UHE) José Richa: A e B) Cnidários; C) Molusco; D) Ciliophora; E) Amoebozoa; F, G e H) Arthropoda. Imagem em close (destacado em corante rosa de bengala), um hidrante de Cordylophora sp. representado por gonóforos (estrutura responsável pela reprodução).

Métodos moleculares – DNA ambiental

O DNA ambiental (eDNA) é uma mistura complexa de DNA genômico oriundo de organismos inteiros ou partes deles, presentes em amostras ambientais. Essas amostras podem ser, por exemplo, de solo, água ou sedimento. Métodos baseados em eDNA têm ganhado espaço nas pesquisas e no monitoramento de espécies. A coleta é realizada com amostragem de água, filtração, conservação do filtro contendo a amostra.



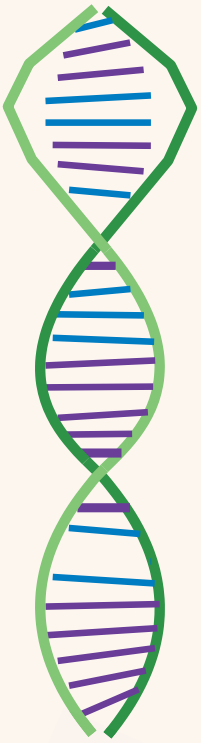
A amostra deve ser fixada em álcool e armazenada em gelo até seguir para o laboratório para realização de análises moleculares futuras. Esta técnica tem avançado muito e contribuído para diversas pesquisas utilizando monitoramento de organismos aquáticos e terrestres.

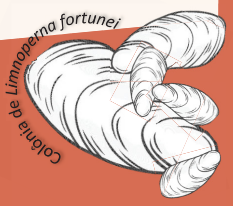
Detecção de espécies invasoras incrustantes

Um dos grandes problemas relacionados a bioinvasão de espécies incrustantes é detectar sua introdução em um ambiente de forma precoce. Visto que identificar moluscos em especial nos estagios iniciantes de vida (larvas planctônicas) é muito dificil. E diversas vezes utilizar métodos convensionais de identificação é quase impossível, pois as larvas são muito parececidas com microcrustáceos nativos, como com outras espécies invasoras (*Corbicula fluminea*).

É um grande desafio definir pontos estratégicos e manter o monitoramento ativo para viabilizar a detecção de espécies introduzidas antes que as populações atinjam tamanho significativo. E estabelecer medidas para conter os indivíduos em certa área onde ocorre invasão para evitar que haja dispersão ou proteger outras áreas da invasão pelas espécies incrustantes. Dentre as adversidades, utilizar métodos de gestão para o controle de espécies aquáticas invasoras incrustantes são particularmente desafiadores devido às grandes limitações na detecção, quantificação e identificação precoce.

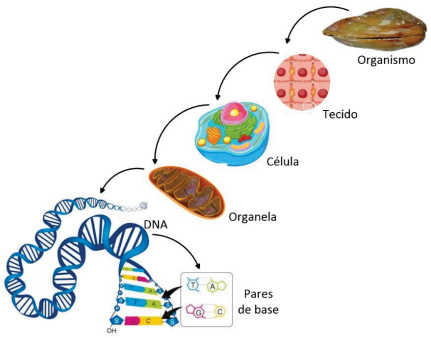
O uso de metodologias moleculares como técnicas baseadas em eDNA tornou- se uma das formas mais inovadoras e precisas de monitoramento de espécies em diferentes ecossistemas. A degradação do DNA presente nas amostras coletadas é um dos grandes obstáculos ainda a serem superados, visto que a não integridade da molecula dificulta a análise, gerando resultados falsos negativos.





Degradação da molécula de DNA

Sabe-se que a degradação de materiais biológicos inicia-se tão logo ele é liberado, começando pelos fragmentos de tecidos, depois por células inteiras, organelas (por exemplo, mitocôndrias) e, por último, ocorre a degradação da própria molécula de DNA. Essa degradação é acelerada por microrganismos, por enzimas exógenas, por reações químicas espontâneas e por vários fatores físicos, como temperatura e incidência luminosa (radiação ultra violeta). Esses fatores podem começar a atuar tão logo ocorra a liberação do DNA, mas também após a coleta das amostras, caso não sejam adotados procedimentos metodológicos adequados para fixação das mesmas.



O eDNA está em constante processo de degradação e é preciso compreender como os fatores naturais o afetam após sua liberação, bem como frear este processo, após a coleta de amostras. Caso contrário, os resultados a serem obtidos em laboratório serão imprecisos. Por isso, é importante que as amostras sejam filtradas e fixadas ainda em campo. Mas, apesar dos desafios metodológicos ainda existentes, as ferramentas genéticas moleculares vieram para ficar e revolucionarão, de modo irreversível, a forma como monitoramos os ambientes, comunidades e identificamos os organismos. Em um futuro não muito distante, por exemplo, coletar (e muitas vezes, sacrificar) organismos, principalmente aqueles raros ou em risco de extinção, apenas com a justificativa de poder estudá-los, não será mais algo normal, se isso pode ser feito apenas analisando as pistas de DNA que eles deixam no ambiente.

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8 CONSIDERAÇÕES FINAIS

Nesta tese, identificamos as principais espécies invasoras incrustantes de ambientes límnicos e suas interações, além de termos investigado os desafios para a detecção precoce dessas espécies. Globalmente, foram identificadas 13 espécies límnicas invasoras incrustantes que interagem, de diversas forma. A competição foi o tipo de interação ecológica mais frequentemente relatado entre espécies invasoras de água doce na literatura, seguida por epibiose, predação, mutualismo e canibalismo. Estas interações têm implicações nos processos de invasão biológica, pois elas afetam diretamente a dispersão e o estabelecimento dessas espécies em seus habitats. Tais espécies possuem grande potencial de causar impactos ecológicos entre as espécies nativas e econômicos em diversos setores como de abastecimento de água, produção de energia hidrelétrica, pesca, aquicultura e outras. Neste trabalho identificamos como uma importante lacuna experimentos que avaliam sobre as interações ecológicas de espécies incrustantes invasoras no ambiente límnico.

Neste estudo foi realizado a avaliação de interações ecológicas entre três espécies límnicas invasoras incrustantes (*L. fortunei*, *Cordylophora* sp., e *Hydra* sp.) e seis táxons nativos pertencentes aos filos Protozoa, Ciliophora, Amoebozoa e Arthropoda em um reservatório de uma UHE. Entre as interações mais relatadas, a competição foi a mais prevalente, essa relação pode acontecer por vários motivos, incluindo competição por territórios, substratos e alimentos. Os organismos invasores incrustantes mais relatados, foram *L. fortunei* e *Cordylophora* sp. que competem por substrato, principalmente quando esses são escassos, o que os tornam próximos em diferentes momentos ou fases da vida durante o processo de bioinvasão. Dentre os períodos avaliados (primavera, verão, outono e inverno) descobrimos que entre o final da primavera e início do verão é o momento mais adequado para realizar remoção dessas incrustações nos sistemas hidráulicos, pois, após este período as incrustações diminuem naturalmente, reduzindo os riscos operacionais e econômicos das paradas e limpeza das máquinas. Isso pode garantir menores gastos econômicos pelas empresas de UHE.

Uma forma de minimizar esses impactos é realizando a identificação precoce dessas espécies envolvendo algumas abordagens utilizando amostras de DNA. Recomendamos que amostragens de coleta de água para avaliar o DNA dessas espécies devem ser realizadas próximas a coluna de água, visto que essa molécula tende a se movimentar do fundo para a superfície. Além da distribuição na coluna de água, outros fatores influenciam a detecção das espécies, como a degradação da molécula de DNA no ambiente. Desta forma, também descobrimos que fatores abióticos como a turbidez e o tempo (24h) podem influenciar na persistência do DNA no ambiente. Concluímos, assim, que a rápida degradação do eDNA em habitats aquáticos proporciona o monitoramento das espécies praticamente em tempo real, o que permite uma avaliação acurada da presença dessas espécies em um determinado local.

Acreditamos termos contribuído para o avanço no entendimento desse tema tão importante devido aos seus imensos impactos negativos biológicos e econômicos. Detectar precocemente a presença de tais espécies é fundamental para evitar maiores danos, e aqui demonstramos maneiras efetivas, rápidas e economicamente viáveis para isso. Entendemos que para um efetivo plano de redução de impactos ambientais devemos espalhar o conhecimento produzido para além dos muros das universidades.

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