

UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS NATURAIS E EXATAS
PROGRAMA DE PÓS-GRADUAÇÃO EM BIODIVERSIDADE ANIMAL

Victor Hugo dos Santos Mollmann

**DIVERSIDADE E CONSERVAÇÃO DE *AEGLA* LEACH, 1820
(CRUSTACEA, ANOMURA) EM ÁREA PRIORITÁRIA PARA
PRESERVAÇÃO**

Victor Hugo dos Santos Mollmann

**DIVERSIDADE E CONSERVAÇÃO DE *AEGLA LEACH, 1820* (CRUSTACEA,
ANOMURA) EM ÁREA PRIORITÁRIA PARA PRESERVAÇÃO**

Dissertação de Mestrado apresentado ao Programa de Pós-Graduação em Biodiversidade Animal da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do título de **Mestre em Ciências Biológicas**

Orientador: Prof^ª. Dr^ª. Marlise Ladvocat Bartholomei-Santos
Coorientador: Prof. Dr. Sandro Santos

Santa Maria, RS
2022

Ficha gerada com os dados fornecidos pelo autor

Mollmann, Victor Hugo dos Santos
Diversidade e conservação de *Aegla* Leach, 1820 (Crustacea, Anomura) em área prioritária para preservação / Victor Hugo dos Santos Mollmann. - 2022.
128 p.; 30 cm

Orientador: Marlise Ladvocat Bartholomei-Santos
Coorientador: Sandro Santos
Dissertação (Mestrado) – Universidade Federal de Santa Maria, Centro de Ciências Naturais e Exatas, Programa de Pós-graduação em Biodiversidade Animal, Santa Maria, RS, 2022.

1. Limnologia 2. Unidades de Conservação 3. *Aegla* 4. Complexos de espécies crípticas I. Mollmann, Victor Hugo dos Santos II. Bartholomei-Santos, Marlise Ladvocat III. Santos, Santos IV. Diversidade e conservação de *Aegla* Leach, 1820 (Crustacea, Anomura) em área prioritária para preservação



Victor Hugo dos Santos Mollmann

**DIVERSIDADE E CONSERVAÇÃO DE *AEGLA LEACH, 1820* (CRUSTACEA,
ANOMURA) EM ÁREA PRIORITÁRIA PARA PRESERVAÇÃO**

Dissertação de Mestrado apresentado ao Programa de Pós-Graduação em Biodiversidade Animal da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do título de **Mestre em Ciências Biológicas**.

Aprovado em 16 de agosto de 2022:

Marlise Ladvocat Bartholomei-Santos, Dr^a. (UFSM)
(Presidente/Orientador)

Sérgio Luiz de Siqueira Bueno, Dr. (USP)

Gustavo Monteiro Teixeira, Dr. (UEL)

AGRADECIMENTOS

Antes de tudo, a mim, pela dedicação na execução desse trabalho, por perseverar em meio aos problemas, por achar luz quando tudo parecia sombrio – por nunca pensar em desistir.

Aos meus pais, Rosa e Hugo, e à minha vó Galdina, pela ética e valores passados. Além de todo o suporte estrutural e financeiro, em especial, agradeço-lhes por me apresentar à minha primeira e eterna “namorada” – os estudos.

À minha orientadora, Marlise Ladvocat Bartholomei-Santos, por me guiar na execução desse trabalho, me apresentando as eglas, a genética e suas possibilidades. Pela confiança e liberdade concedida na realização desse trabalho, mostrando-me como ensinar pode ser leve e tranquilo.

Ao meu coorientador, Sandro Santos, principalmente por ter me encantado com todos os detalhes do mundo das eglas, me fazendo acreditar ainda mais na enorme relevância que esse trabalho traz. Bem como nos ensinamentos das técnicas de campo – é debaixo das pedras maiores que tem as eglas maiores!

À professora Georgina Bond-Buckup (in memoriam) pelo delineamento desse estudo, desde os sítios de coleta à valiosas dicas sobre as hospedagens na região. Sem ela, esse trabalho não seria possível. Não pude conhecê-la pessoalmente, infelizmente, mas posso crer que seu legado estará sempre presente pelo resto da minha vida.

À minha querida amiga e parceira de pesquisa Graci, por seu acolhimento e coração enorme. Também, pela valiosa ajuda nos trabalhos de campo, que foram fundamentais para atingir nossos objetivos.

Aos meus amigos e colegas do laboratório de diversidade genética e de carcinologia da UFSM, em especial à Gislaine pela ajuda na parte metodológica e pelas longas conversas sobre nossas ideias envolvendo trabalhos com Aegla.

À Amanda, pelo incrível suporte nesses últimos e decisivos meses do mestrado.

Aos meus amigos de Tuparendi, há anos compartilhando risadas, cervejas e uma parceria enorme. Em especial, ao Alex, pela grandiosa ajuda na parte computacional.

Aos meus amigos e colegas biólogos presentes desde o tempo da graduação, que de alguma maneira me apoiaram e incentivaram no decurso do mestrado.

Às administrações das Unidades de Conservação onde o estudo foi realizado, por nos viabilizar as coletas dentro das unidades. Da mesma forma, ao Eduardo Pezzuto, pela permissão de coletar dentro de sua RPPN.

À Universidade Federal de Santa Maria - UFSM, o Programa de Pós-graduação em Biodiversidade Animal – PPGBA, e seus professores pela qualidade do ensino oferecido.

À banca, Dr. Sérgio Bueno e Dr. Gustavo Teixeira, pelo aceite e tempo utilizado nas sugestões para melhoria do presente estudo.

À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES pela concessão de bolsa durante o período de pesquisa e ao Conselho Nacional do Desenvolvimento Científico e Tecnológico – CNPq, pelo financiamento dos custosos trabalhos de campo e o material necessário para ele.

Muito obrigado!

*"O segredo é não correr atrás das borboletas.
É cuidar bem do jardim para que elas venham
até você".*

Mario Quintana

RESUMO

DIVERSIDADE E CONSERVAÇÃO DE *AEGLA* LEACH, 1820 (CRUSTACEA, ANOMURA) EM ÁREA PRIORITÁRIA PARA PRESERVAÇÃO

AUTOR: Victor Hugo dos Santos Mollmann
ORIENTADORA: Marlise Ladvoat Bartholomei-Santos
COORIENTADOR: Sandro Santos

Crustáceos dulcícolas do gênero *Aegla* Leach, 1820, são componentes importantes da fauna de rios, riachos, lagos e cavernas de diversos países da América do Sul, de onde são endêmicos. Atualmente o gênero é representado por 93 espécies descritas, porém, estima-se que este número possa ser muito maior, visto os complexos crípticos descobertos nas espécies de *Aegla paulensis*, *Aegla longirostri* e *Aegla uruguayana*. Complexos de espécies crípticas são um problema taxonômico presente em diversos taxa, principalmente aqueles com conservação morfológica, fazendo com que se subestime a real diversidade de espécies, trazendo implicações para a conservação e manejo da biodiversidade. No Brasil, a região sul concentra a maior parte da diversidade de espécies de *Aegla*, e estudos prévios demonstraram que as ecorregiões do Alto Uruguai, Tramandaí-Mampituba e Laguna dos Patos formam uma grande área prioritária para conservação de eglídeos, devido ao alto número de espécies e diversidade filogenética, elevado endemismo, e ameaças ao habitat aquático. E justamente essa ameaça ao ambiente aquático serve como background para o capítulo I, no qual avaliamos a preservação de ambientes de água doce dentro dessas áreas prioritárias, onde se localizam quatro grandes Unidades de Conservação (UC). A qualidade da água foi avaliada em 33 pontos de coleta distribuídos dentro e fora das UCs, através de análises físico-químicas, microbiológicas e detecção de pesticidas e compostos farmacêuticos, com a finalidade de observar fatores que ameaçam a biodiversidade aquática. A qualidade da água não diferiu significativamente entre locais protegidos e não protegidos, e indicou clara poluição, com evidências de influxo de esgoto, mesmo em áreas bem conservadas. Encontramos 19 pesticidas e cinco fármacos nas amostras de água da região analisada. Todos os pontos amostrados apresentaram ao menos um composto pesticida e fármaco, com destaque para o 2,4-D, presente em 91% destes. As diferentes fontes de dados nos permitiram observar que UCs não são suficientes para mitigar impactos antrópicos, e medidas de preservação e manejo devem levar em conta toda a bacia hidrográfica, visto que os rios e riachos extrapolam os limites das UCs. Os resultados servem de contexto para a avaliação da influência dos impactos antropogênicos na biodiversidade aquática, em especial, as espécies do gênero *Aegla*, visto que, a degradação de ambientes aquáticos é um dos principais fatores responsáveis pelo grande número de espécies do gênero que estão ameaçadas de extinção, e medidas preventivas para preservação do ambiente aquático se tornam essenciais para proteger a biodiversidade. No capítulo II, acessamos a diversidade e distribuição de *Aegla* na área prioritária especificada anteriormente, também testando a hipótese de existência de complexos crípticos entre as espécies da região. Para isso, utilizamos métodos de delimitação de espécies aplicados a sequências do gene mitocondrial *COI*, juntamente com análises filogenéticas e morfológicas. Observamos 22 novas espécies potenciais, divididas em três complexos crípticos: sete espécies putativas em *Aegla jarai*, três em *Aegla franciscana* e uma em *Aegla camargoi*, além de impressionantes 11 outras espécies putativas não incluídas em nenhum agrupamento, evidenciando que a real diversidade do grupo ainda é muito desconhecida. Nossos resultados indicam que a região de estudo tem a maior riqueza de espécies de *Aegla*, o que a torna ainda mais prioritária para a conservação do gênero e preservação de seus habitats. Destaca-se também a taxonomia integrativa como o melhor meio para detectar novas espécies em grupos com morfologia conservada.

Palavras-chave: Água doce. Poluição. Pesticidas. Unidades de Conservação. *COI*. Complexos crípticos. Taxonomia integrativa.

ABSTRACT

DIVERSITY AND CONSERVATION OF *AEGLA* LEACH 1820, (CRUSTACEA, ANOMURA) IN A PRIORITY AREA FOR PRESERVATION

AUTHOR: Victor Hugo dos Santos Mollmann
ADVISOR: Marlise Ladvocat Bartholomei-Santos
CO-ADVISOR: Sandro Santos

Freshwater crustaceans of the genus *Aegla* Leach, 1820 are important components of the fauna of rivers, streams, lakes, and caves of several countries in South America, where they are endemic. Currently, the genus is represented by 93 described species, but it is estimated that this number could be much higher, given the cryptic complexes discovered in *Aegla paulensis*, *Aegla longirostri*, and *Aegla uruguayana*. Cryptic species complexes are a taxonomic problem present in several taxa, especially those with morphological conservation, underestimating the real diversity of species, bringing implications in conservation and management of the biodiversity. In Brazil, the southern region concentrates most of the species' diversity of *Aegla*, and previous studies have shown that the ecoregions of Upper Uruguay, Tramandaí-Mampituba, and Laguna dos Patos Basin compose a large priority area for eglid conservation, due to the high number of species and phylogenetic diversity, high endemism, and threats to the aquatic habitat. And precisely this threat to the aquatic environment serves as the background for Chapter I, in which we evaluate the preservation of freshwater environments within these priority areas, where four large Protected Areas (PA) are located. Water quality was assessed in 33 sampling sites distributed inside and outside the PAs, through physicochemical and microbiological analysis and detection of pesticides and pharmaceutical compounds, to observe factors that threaten aquatic biodiversity. Water quality did not differ significantly between protected and unprotected sites, but indicated clear pollution, with evidence of sewage inflow, even in well-conserved areas. We found 19 pesticides and five pharmaceuticals in water samples from the analyzed region. All the sampled sites presented at least one pesticide and one pharmaceutical compound, especially 2,4-D, present in 91% of them. The different data sources allowed us to observe that PAs are not enough to mitigate anthropic pressures, and preservation and management measures must take into account the entire watershed since rivers and streams go beyond the limits of PAs. The results provide a context for evaluating the influence of anthropogenic impacts on aquatic biodiversity, especially species of the genus *Aegla*, since the degradation of aquatic environments is one of the main factors responsible for a large number of species of the genus that are threatened with extinction, and preventive measures for the conservation of the aquatic environment become essential to protect biodiversity. In Chapter II, we accessed the diversity and distribution of *Aegla* in the priority region specified above, also testing the hypothesis of the existence of cryptic complexes among species in the region. For this purpose, we used species delimitation methods applied to mitochondrial *COI* gene sequences, together with phylogenetic and morphological analyses. We observed 22 potential new species, divided into three cryptic complex clusters: seven putative species in *Aegla jarai*, three in *Aegla franciscana*, and one in *Aegla camargoi*, plus an impressive 11 other putative species not included in any cluster, evidencing that the real diversity of the group is still largely unknown. Our results indicate that the study region has the highest species richness of *Aegla*, which makes it an even higher priority for the conservation of the genus and preservation of its habitat. Also, integrative taxonomy is the best way to detect new species in groups with conserved morphology.

Keywords: Freshwater. Pollution. Pesticides. Protected Areas. *COI*. Cryptic complexes. Integrative taxonomy.

LISTA DE FIGURAS

CAPÍTULO I

- FIGURE 1 – Sampling sites (1 to 33) grouped into six areas, represented by symbols. The protected areas (PAs) are delimited by shades.....52
- FIGURE 2 – Comparisons of physicochemical and biological parameters from streams sampled in the various study areas: (a) temperature, (b) dissolved oxygen, (c) conductivity, (d) pH, (e) *Escherichia coli* and (f) total coliforms in the water samples from the six regions analysed. 1 = São Joaquim National Park (SJNP) and Private Natural Heritage Reserve Portal das Nascentes (PNHR); 2 = region outside SJNP; 3 = Aparados da Serra National Park (ASNP) and Serra Geral National Park (SGNP); 4 = region outside ASNP and SGNP; 5 = São Francisco de Paula National Forest (SFPNF); 6 = region outside SFPNF. Statistically significant differences ($p < 0.05$) between samples indicated by t-tests and analyses of variance with Tukey post hoc tests are represented by upper-case letters. Lower-case letters represent statistically non-significant comparisons. Asterisks (*) represent significant differences ($p < 0.05$) in comparisons between protected areas (PAs). Within the boxplots, the horizontal lines represent medians, the white circles show means and the boxes and bars indicate the quartiles. Black circles represent outliers. CFU = colony-forming unit.....53
- FIGURE 3 – Comparisons of pesticide classes among the various study areas (for area codes, see Fig. 2): (a) herbicides, (b) insecticides and (c) fungicides. Statistically significant differences ($p < 0.05$) between samples indicated by t-tests and analyses of variance with Tukey post-hoc tests are represented by upper-case letters. Lower-case letters represent statistically non-significant comparisons. Asterisks (*) represent significant differences ($p < 0.05$) in comparisons between areas within PAs. Within the boxplots, the horizontal lines represent medians, the white circles show means and the boxes and bars indicate the quartiles. Black circles represent outliers. PA = protected area.....54
- FIGURE 4 – (a) Comparisons of the caffeine concentrations between protected areas (PAs) and unprotected (outside) areas. (b) Comparisons of the caffeine concentrations between the various study areas (for area codes, see Fig. 2). Statistically

significant differences ($p < 0.05$) between samples indicated by t-tests and analyses of variance with Tukey post hoc tests are represented by upper-case letters. Lower-case letters represent statistically non-significant comparisons. Within the boxplots, the horizontal lines represent medians, the white circles show means and the boxes and bars indicate the quartiles. Black circles represent outliers.....55

FIGURE S1 – Comparison of physicochemical and biological parameters in protected (PAs) and unprotected (outside) areas using t-tests and Mann-Whitney tests. Significant differences between samples ($p < 0.05$) are represented by capital letters. In the boxplot, the horizontal line represents the median, the white circle shows the mean, boxes and bars indicate the quartiles, and black circles represent the outliers.....56

FIGURE S2 – Contribution of each pesticide compound in water at each sampling site. Values are the averages of concentrations recorded in 2020 and 2021. (H) herbicide; (I) insecticide; (F) fungicide.....56

FIGURE S3 – Average contribution (%) of each pesticide class in water at each sampling site. (H) herbicide; (I) insecticide; (F) fungicide.....57

FIGURE S4 – Comparison of pesticide classes in protected (PAs) and unprotected (outside) areas using t-tests and Mann-Whitney tests. Significant differences ($p < 0.05$) between samples are represented by capital letters. Within the boxplot, the horizontal line represents the median and the empty circle shows the mean. Within the boxplot, the horizontal line represents the median, the white circle shows the mean, boxes and bars indicate the quartiles. Black circles represent the outliers.....57

CAPÍTULO II

FIGURE 1 – **(a)** Location of the 33 sampling sites (red dots) along the watersheds of the study area, represented by the different colors, and protected areas (green shades). **(b)** Haplotype network colored following its respective watershed, showing the connectivity of the 96 *COI* haplotypes of *Aegla* for the study region, with size

proportional to haplotype frequency. Black circles represent missing/not sampled haplotypes.....81

FIGURE 2 – Bayesian tree depicting phylogenetic relationships and cryptic diversity in the genus *Aegla* based on *COI* mtDNA sequences from this study and from GenBank (GB). Sequences from GB relevant to delimitate species are highlighted in red. Numbers above branches represent Bayesian posterior probabilities. Posterior probabilities <0.75 are not shown. The colors in the branches represent the clades following Pérez-Losada et al. (2004). The sampling site column (SS) with colored boxes represent sites with sympatric occurrences. Molecular entities delimited by GMYC, bPTP and ASAP algorithms are also shown, with black bars representing GB delimitations not relevant for the study. Red and orange bars in bPTP delimitation represent groupings of phylogenetic distant sequences (for example, P209, P308 and P216 sequences were grouped).....87

LISTA DE TABELAS

CAPÍTULO I

- TABLE S1 – Average and standard deviation of physicochemical and biological parameters in water samples from streams inside and outside Protected Areas in Southern Brazil. Sampling sites are grouped into six areas, corresponding to Protected Areas (São Joaquim National Park - SJNP, Aparados da Serra National Park - ASNP, Serra Geral National Park - SGNP, São Francisco de Paula National Forest – SFPNF, Private Natural Heritage Reserve Portal das Nascentes – PNHR) and outside areas (E).....58
- TABLE S2 – Pesticides and pharmaceuticals analyzed in water samples from 33 sites in southern Brazil and their respective Limit of detection (LOD) and Limit of quantification (LOQ). All concentrations are in $\mu\text{g/L}$60
- TABLE S3 – Average concentrations ($\mu\text{g/L}$) and standard deviation of three classes of pesticides in water samples from streams inside and outside Protected Areas in Southern Brazil. Sampling sites are grouped into six areas, corresponding to Protected Areas (São Joaquim National Park - SJNP, Aparados da Serra National Park - ASNP, Serra Geral National Park - SGNP, São Francisco de Paula National Forest – SFPNF, Private Natural Heritage Reserve Portal das Nascentes – PNHR) and outside areas (E).....67
- TABLE S4 – Pesticides detected in water samples from streams inside and outside Protected Areas in southern Brazil, collected in November 2020 and November 2021. All concentrations are in $\mu\text{g/L}$. Sampling sites are grouped into six areas, corresponding to Protected Areas (São Joaquim National Park - SJNP, Aparados da Serra National Park - ASNP, Serra Geral National Park - SGNP, São Francisco de Paula National Forest – SFPNF, Private Natural Heritage Reserve Portal das Nascentes – PNHR) and outside areas (E).....69
- TABLE S5 – Mean concentrations ($\mu\text{g/L}$) and standard deviation of the pharmaceuticals recorded in water samples from streams inside and outside Protected Areas in Southern Brazil. Sampling sites are grouped into six areas, corresponding to Protected Areas (São Joaquim National Park - SJNP, Aparados da Serra National

Park - ASNP, Serra Geral National Park - SGNP, São Francisco de Paula National Forest – SFPNF, Private Natural Heritage Reserve Portal das Nascentes – PNHR) and outside areas (E). AI = anti-inflammatory, V = Vermifuge, AB = antibiotic, S = sulfonamide.....73

CAPÍTULO II

TABLE S1 – Specifications of each of the 33 sampling sites selected for the study and the species recorded in each through different sources. Site number in bold represent sampling sites within Protected Areas. For spatial localization, see Figure 1. The asterisk (*) refers to the phylogenetic clades established by Pérez-Losada et al. (2004) for the whole *Aegla* genus. The dash (-) represents sites where no specimens were collected.....108

TABLE S2 – Performance of the models tested in the phylogenetic analysis, ranked according to Stepping Stone (SS) sampling and Bayes Factor (BF) values. All BF values were calculated against the coalescent model with strict molecular clock (considered model 1 in the calculations), according to Kass & Raftery (1995). Positive BF values indicate support for model 1.....111

SUMÁRIO

1	INTRODUÇÃO	16
2	OBJETIVOS	22
2.1	OBJETIVOS ESPECÍFICOS	22
3	CAPÍTULO I - TERRESTRIAL PROTECTED AREAS DO NOT FULLY SHIELD THEIR STREAMS FROM EXOGENOUS STRESSORS	23
	Summary	24
	Introduction	25
	Methods	26
	Results	30
	Discussion	34
	References	41
	Figures and Tables	52
4	CAPÍTULO II - HIDDEN DIVERSITY OF <i>AEGLA</i> (CRUSTACEA: DECAPODA: ANOMURA) IN A PRIORITY AREA FOR CONSERVATION.....	75
	Abstract	76
	Introduction	78
	Methods	80
	Results	85
	Discussion	88
	References	95
5	DISCUSSÃO GERAL.....	112
	REFERÊNCIAS BIBLIOGRÁFICAS	121

1 INTRODUÇÃO

Unidades de Conservação (UCs) ou Áreas Protegidas (PAs) são a espinha dorsal da conservação da biodiversidade em todo o mundo, desempenhando papel fundamental na preservação de diversos taxa (GELDMAN et al., 2014), principalmente aqueles que se encontram sob alguma categoria de ameaça (LIU et al., 2001), visto seu desempenho na mitigação de distúrbios antrópicos (BEATTY et al., 2014), como a conversão do uso do solo, fragmentação, perda de habitat, contaminação por pesticidas, caça e desmatamento (GRIGNOLIO et al., 2011; FENG et al., 2021; YANG et al., 2021). Em 2022, a rede de PAs cobre cerca de 16.6% da superfície terrestre e de águas continentais (UNEP-WCMC & IUCN, 2021), quase alcançando o objetivo de 17% de cobertura estabelecido no *Aichi Biodiversity Targets* para a década de 2011-2020, durante a Convenção sobre a Diversidade Biológica em 2010 (<https://www.cbd.int/>). No âmbito de proteção à biodiversidade, a maior parte das UCs mundiais são caracterizadas pela preservação de espécies terrestres e carismáticas, com proteção incidental à biodiversidade aquática (DUDLEY & STOLTON, 2003), apesar desta abrigar cerca de 10% de toda a diversidade. Tal valor aumenta de magnitude ao considerarmos que águas continentais cobrem apenas 0,8% da superfície terrestre (ACREMAN et al., 2020). Embora haja diversos casos positivos de proteção a ambientes aquáticos dentro de áreas protegidas delineadas exclusivamente pelo ambiente terrestre (ACREMAN et al., 2020), a biodiversidade de ecossistemas de água doce continua em declínio. Baseado no índice utilizado pelo Fundo Mundial para a Natureza (*World Wildlife Fund* - WWF) desde 1970, as populações de ambientes aquáticos de água doce sofreram uma redução de 83% em seu tamanho, mais que o dobro em comparação com espécies marinhas e terrestres (WWF, 2018).

No Brasil, as UCs ocupam o equivalente a 18.8% (1,600,827.34 km²) do território terrestre, abrangendo também águas continentais, distribuídas em 2.647 UCs de diferentes categorias de manejo. Conforme especificado no Sistema Nacional de Unidades de Conservação (SNUC) estabelecido pela Lei nº 9.985 (BRASIL, 2000), são 12 categorias de UCs, agrupadas em unidades de proteção integral, cujo uso é mais rígido, e unidades de uso sustentável, que permitem uso de parte dos recursos naturais (BRASIL/MMA, 2022). Entretanto, a maior parte das áreas protegidas se encontra na Amazônia (28.6% em 357 UCs). O bioma da Mata Atlântica, um dos mais ameaçados do mundo, apresenta apenas 11.33% de sua área protegida, entretanto, tem o maior número de UCs em comparação aos outros biomas brasileiros, totalizando 1.589, caracterizados em sua maioria pela pequena extensão e por serem reservas particulares do patrimônio natural ou de uso sustentável. Surpreendentemente, mais de

70% das UCs nesse bioma não possuem Plano de Manejo nem Conselho Gestor registrados no Painel de Unidades de Conservação Brasileiras (BRASIL/MMA, 2022), o que pode ocasionar diminuição na sua efetividade, visto que o Plano de Manejo possui papel fundamental em diversos aspectos que gerem a proteção à biodiversidade nas UCs (ACREMAN et al., 2020). Além disso, investimento, infraestrutura e pesquisas científicas são fatores que mais beneficiam a efetividade de UCs em reduzir o desmatamento (FENG et al., 2021).

Diferentemente da classificação das UCs atribuídas pelo SNUC para o Brasil, a *International Union for Nature Conservation* (IUCN) estabelece sete diferentes categorias de UCs, conforme objetivo e restrição de uso. As categorias vão desde a Ia e Ib, estritamente delineadas para proteção integral da biodiversidade, permitindo apenas uso científico e de monitoramento, até a categoria VI, que permite uso sustentável dos recursos naturais, bem como visitação. A maior parte das UCs no Brasil se enquadra na categoria IV (1,048 UCs, 32%), sendo áreas de relevante interesse ecológico ou reservas particulares do patrimônio, seguido de UCs de categoria II (480, 15%) como parques nacionais (UNEP-WCMC, 2022).

O presente estudo inclui cinco UCs, que juntas cobrem cerca de 13% (817,53 km²) da região de estudo (ver Figura 1, Capítulo I). O Parque Nacional de São Joaquim (497 km²), o Parque Nacional de Aparados da Serra (131 km²) e o Parque Nacional da Serra Geral (173 km²) são enquadrados na categoria II da IUCN, cujo principal objetivo é manter o ecossistema preservado, enquanto permite usos como a visitação. A Floresta Nacional de São Francisco de Paula (16 km²) enquadra-se na categoria VI, que permite uso sustentável em parte de sua área. Por fim, a Reserva Particular do Patrimônio Natural Portal das Nascentes (0,16 km²) pertence a categoria IV, designada para proteger habitat de Mata Atlântica em zona de amortecimento do Parque Nacional de São Joaquim. Contrariando a perspectiva nacional relatada anteriormente, todas estas UCs mencionadas possuem Plano de Manejo e Conselho Gestor.

Diversos estudos apontam a necessidade de considerar ambientes aquáticos no delineamento e manejo de áreas protegidas, o que aumenta a eficiência na proteção da biodiversidade em comparação com áreas protegidas que consideram ambientes aquáticos apenas posteriormente (HERMOSO et al., 2016; DORJI et al., 2020). Geralmente, a maior parte das UCs aloca menos recursos e ações destinadas à conservação da água doce do que em outras atividades, e em alguns casos, absolutamente nenhum recurso é voltado à proteção e manejo do ecossistema aquático em UCs do EUA (THIEME et al., 2012). Porém, são necessários mais pesquisas e conhecimento acerca da biodiversidade aquática, com a finalidade de contribuir com a conservação destes locais. Em relação a isto, a maior parte dos estudos em ambientes aquáticos dentro de UCs considera apenas vertebrados, majoritariamente peixes (ACREMAN

et al., 2020). Poucos são aqueles que incluem dados sobre invertebrados (MANCINI et al., 2005; SNYDER et al., 2013; NG et al., 2015). Essa desatenção à fauna invertebrada nas UCs deve-se a diversos motivos, sendo um dos principais fatores a falta de sensibilização sobre sua importância e ecologia (D'AMEN et al., 2013). Nesse contexto, Barros et al. (2020) identificaram diversas lacunas no conhecimento sobre a fauna invertebrada de água doce no Brasil, principalmente devido a vieses geográficos, restringindo o uso das espécies de invertebrados como bioindicadoras. Entretanto, é necessário reconhecer que pesquisas sobre a biodiversidade em áreas extensas, como o caso dos biomas brasileiros, são exaustivas e dificilmente viáveis (D'AMEN et al., 2013). Porém, assim que disponíveis, dados sobre a biodiversidade de invertebrados devem ser utilizados na priorização de áreas protegidas (BROOKS et al., 2006), bem como em assistência ao manejo dos recursos hídricos abrangidos por estas. Nesse sentido, a comunidade de macroinvertebrados é componente fundamental em ecossistemas aquáticos devido, principalmente, a seu papel no fluxo de nutrientes orgânicos (MANCINI et al., 2005), e informações sobre sua diversidade devem servir como referência no processo de entendimento de ambientes dulcícolas em áreas de proteção.

Um invertebrado notável de ecossistemas aquáticos são os crustáceos do gênero *Aegla* Leach, 1820, são os únicos anomuros que habitam exclusivamente águas continentais, restringindo-se a cursos d'água de pequena magnitude e com bom aspecto físico-químico, além de ambientes cavernícolas e lacustres (BOND-BUCKUP & BUCKUP, 1994). Eglídeos apresentam papéis essenciais em ecossistemas aquáticos, atuando no fluxo de matéria orgânica através da fragmentação de detritos foliares recobrando o bento de rios e riachos (COGO & SANTOS, 2013; COGO et al., 2014), agindo também como predadores de larvas de insetos aquáticos, como Diptera, Ephemeroptera, Coleoptera e Trichoptera (BUENO & BOND-BUCKUP, 2004). Apesar da dieta onívora, nota-se a preferência da espécie por material vegetal que, dentre outros fatores, são de maior abundância em riachos neotropicais e são de fácil obtenção pelos eglídeos. Ainda sobre sua ecologia trófica, há relatos que *Aegla* faz parte da dieta de diversos taxa, como aves, rãs, peixes e répteis, como *Caiman latirostris* (BURNS, 1972; MELO, 1990). Então, macroconsumidores, como as espécies do gênero *Aegla*, atuam como espécie-chave em ambientes aquáticos (COGO et al., 2014), reiterando a importância da conservação do táxon.

Até então, foram descritas 93 espécies de *Aegla* (MARÇAL et al., 2021; WoRMS, 2022), distribuídas em bacias hidrográficas no Chile, Argentina, Bolívia, Paraguai, Uruguai e Brasil (BOND-BUCKUP & SANTOS, 2007). Porém, estima-se que o número seja maior, dada a descoberta de complexos crípticos em algumas espécies do gênero, como em *Aegla paulensis*

(MORAES et al., 2016), *Aegla longirostri* (CRIVELLARO et al., 2018) e *Aegla uruguayana* (ZIMMERMANN et al., 2021). Essa cripticidade deve-se, entre outros fatores, à morfologia geral do gênero ser muito conservada, limitando a quantidade de caracteres disponíveis para o uso na identificação dos indivíduos (BARTHOLOMEI-SANTOS et al., 2020; ZIMMERMANN et al., 2021). Desta forma, a utilização de marcadores genéticos e métodos como DNA *Barcoding* (HEBERT et al., 2003) se tornam ferramentas poderosas para auxiliar na definição das espécies de eglídeos. Da mesma forma, dados genéticos implementados em metodologias para delimitação de espécies, tais como *Yule-Coalescent Model* (GMYC) (PONS et al., 2006), *Automatic Barcode Gap Discovery* (ABGD) (PUILLANDRE et al., 2012), *Poisson Tree Process* (PTP) (ZHANG et al., 2013), *Bayesian Phylogenetics and Phylogeography* (BPP) (YANG & RANNALA, 2014), *Bayes Factor Delimitation* (BFD) (LEACHÉ et al., 2014) e *Assemble Species by Automatic Partitioning* (ASAP) (PUILLANDRE et al., 2020), auxiliam na formulação de hipóteses para a distinção entre espécies próximas, servindo como base para futuras descrições de novas espécies e identificação de complexos crípticos.

Pesquisas recentes acerca do status de conservação de eglídeos nos mostram que quase 70% das espécies do gênero estão ameaçadas de extinção (BUENO et al., 2017; MORAES et al., 2017; SANTOS et al., 2017; BOOS et al., 2020). Características como distribuição restrita, alta taxa de endemismo e a dependência de ambientes aquáticos com alto teor de oxigênio dissolvido, tornam os eglídeos vulneráveis à poluição e destruição de habitats, levando ao declínio populacional, observado nos últimos anos (MAIA et al., 2013; BUENO et al., 2014). Efluxo de esgoto doméstico e industrial nos cursos d'água, bem como supressão da mata ciliar e assoreamento são os principais danos antrópicos, limitando a disponibilidade de micro-habitats no ambiente, e influenciando negativamente a abundância e distribuição desses organismos (TREVISAN et al., 2009). Práticas silviculturais de espécies exóticas, como *Pinus* sp. e *Eucalyptus* sp., juntamente com o uso inadequado de pesticidas para controle de espécies consideradas pragas nessas áreas de plantio, ocasionam a degradação da qualidade das águas nas bacias hidrográficas, por consequência do escoamento superficial e lixiviação, contribuindo para a deterioração de nichos aquáticos (BOND-BUCKUP & SANTOS, 2007; QUEIROZ et al., 2010).

No Brasil, a região sul concentra a maioria das espécies do gênero *Aegla* (SANTOS et al., 2017; BOOS et al., 2020) especialmente nas ecorregiões da Laguna dos Patos, Alto Uruguai e Tramandaí-Mampituba, que abrangem os estados do Rio Grande do Sul e Santa Catarina. Essas áreas são consideradas prioritárias para a conservação das espécies de *Aegla*, devido à

alta riqueza de espécies e diversidade filogenética, elevado endemismo, e ameaças ao habitat aquático (PÉREZ-LOSADA et al., 2009, GONÇALVES et al., 2018). Uma das potenciais ameaças aos eglídeos na região decorre do perfil produtivo, onde a produção agrícola se dá em conjunto com práticas que utilizam pesticidas pré- ou pós-plantio. Em 2005, 56% dos municípios do estado de Santa Catarina relataram contaminação do solo por pesticidas, sendo o estado com a maior proporção de contaminação no Brasil (PINHEIRO & ROSA, 2008). Em 2019, o governo do estado do Rio Grande do Sul proibiu o uso de 2,4-D, um herbicida comumente utilizado em lavouras de soja e trigo até o final daquele ano, devido à alta proporção do composto encontrado em áreas sem produção agrícola, e reforçou o controle e a vigilância acerca da aplicação deste herbicida (RIO GRANDE DO SUL, 2019). A preocupação aumenta devido ao fator de que os compostos agroquímicos aplicados irregularmente nas culturas podem alcançar rios e córregos através do processo de lixiviação, causando a contaminação do ambiente de água doce, conforme exposto anteriormente (RICO et al., 2021). Em conjunto, há a preocupação crescente com a contaminação de recursos hídricos por compostos farmacêuticos, que geralmente atingem estes através da descarga de águas residuais humanas (AUS DER BEEK et al., 2016; BRADLEY et al., 2020), pois produtos farmacêuticos de uso humano e veterinário são excretados principalmente na urina, e podem atuar como um indicador de poluição. A região do estudo ainda é reconhecida pela produção animal, cujos efeitos também podem influenciar negativamente a qualidade da água, morfologia dos canais, alteração na vegetação ripária, compactação do solo e até influenciar a população de macroinvertebrados em rios e córregos (BELZKY et al., 1999; RANGANATH, et al., 2009). A influência destes contaminantes, assim como o uso da terra em relação à preservação dos recursos hídricos, é crucial para compreender os riscos para o ecossistema aquático, já que eles ameaçam espécies não-alvo, como peixes, anfíbios e invertebrados aquáticos (NOWELL et al., 2009; NOWELL et al., 2014; BATTAGLIN et al., 2016; LINK et al., 2022).

Dados estes fatores, compreender a diversidade das espécies de *Aegla* nessa região prioritária é de fundamental interesse, tanto do ponto de vista filogenético – reconhecer as relações entre as espécies da área, sua distribuição, identificar complexos crípticos e potenciais novas espécies, quanto conservacionista – utilizar dados de distribuição dos eglídeos e qualidade do habitat para avaliar ameaças aos rios e sua biodiversidade, tanto dentro de UCs quanto fora destas, a fim de gerar evidências para auxiliar no manejo e preservação da biota aquática de maneira geral. Com isso, essa dissertação está estruturada em dois capítulos: o primeiro caracteriza o habitat aquático onde foram coletados indivíduos de *Aegla*, servindo como background acerca das ameaças às espécies aquáticas e fornecendo subsídios para futuras

medidas de preservação. O segundo aborda diretamente as espécies de *Aegla* ocorrentes na região e evidencia as relações filogenéticas entre estas, com geração de hipóteses de novas espécies e a implicação deste conhecimento no manejo e preservação do gênero.

2 OBJETIVOS

O presente estudo visou avaliar se as Unidades de Conservação (UCs) são efetivas na proteção das espécies de eglídeos em uma região prioritária para preservação.

2.1 OBJETIVOS ESPECÍFICOS

Capítulo I:

- a) Comparar os padrões de qualidade físico-química e microbiológica da água dos riachos amostrados dentro e fora de UCs, em uma área prioritária para conservação de eglídeos;
- b) Identificar contaminações por herbicidas, inseticidas, fungicidas e medicamentos;
- c) Compreender o papel das UCs terrestres na mitigação de impactos antrópicos sobre riachos.

Capítulo II:

- a) Usar a taxonomia integrativa para identificar as espécies de *Aegla* ocorrentes em uma área prioritária para conservação, comparando os dados dentro e fora de UCs;
- b) Identificar possíveis complexos crípticos de espécies e novas unidades taxonômicas através de métodos filogenéticos e filogeográficos.

3 CAPÍTULO I - TERRESTRIAL PROTECTED AREAS DO NOT FULLY SHIELD THEIR STREAMS FROM EXOGENOUS STRESSORS

Formatado conforme especificação da revista. Publicado na revista *Environmental Conservation*, p. 1-10 (2022). DOI:10.1017/S0376892922000261.

Submetido em 13 de abril de 2022. Aceito em 28 de junho de 2022.

Terrestrial protected areas do not fully shield their streams from exogenous stressors

Victor Hugo dos Santos Mollmann¹, Sandro Santos¹, Gracieli Fernandes², Emerson Contreira Mossolin³, Marcelo Marchet Dalosto⁴, Sônia Maria Vaz Sanches Cardoso⁵, Osmar Damian Prestes⁵, Renato Zanella⁵, Marlise Ladvoocat Bartholomei-Santos¹

¹ Programa de Pós-Graduação em Biodiversidade Animal, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil;

² Programa de Pós-Graduação em Ciência do Solo, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil;

³ Departamento de Ciências Biológicas, Universidade Federal de Catalão (UFCAT), Catalão, GO, Brazil;

⁴ Instituto Federal de Educação, Ciência e Tecnologia Farroupilha, Campus Alegrete, RS, Brazil;

⁵ Programa de Pós-Graduação em Química, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil.

Author for correspondence:

Prof. Dr^a Marlise L. Bartholomei-Santos

E-mail: marliselbs@gmail.com

Summary

Protected areas (PAs) represent a powerful refuge for maintaining and safeguarding biodiversity. Generally, PAs are delineated to protect terrestrial taxa, providing incidental protection to the aquatic ecosystem within their borders. Here, we compare water quality within protected and non-protected areas in southern Brazil, encompassing remnants of the Atlantic Forest biome, to assess whether PAs serve as a buffer from external pressures for aquatic ecosystems within their boundaries. In addition to physicochemical and microbiological water parameters, we analyzed 147 pesticide and 31 pharmaceutical compounds in water samples from 33 sites within, and outside PAs. The water quality did not differ between PAs and non-PAs but indicated clear pollution from sewage discharges. We found 19 pesticides and five pharmaceuticals in streams within the study area. We detected pesticides in all sampling sites, with the herbicide 2,4-D present in 91% of them. Our data show that PAs are not enough to mitigate impacts from the catchments, and the running water that reaches their domains already shows signs of anthropogenic interference, which may affect aquatic biodiversity. Protection and management measures require consideration of the whole watershed to protect freshwater habitat and biota.

Keywords: aquatic pollution; contaminating pharmaceuticals; pesticide's impact; physicochemical parameters; wastewater discharge; water quality.

Introduction

Freshwater environments are habitat to at least 10% of the global biodiversity, corresponding to approximately 126,000 species (Dudgeon et al. 2006; Strayer & Dudgeon 2010). This biodiversity is even more remarkable given that fresh waters cover only 0.8% of the Earth's surface (Acreman et al. 2020). Notwithstanding, threats to fresh waters have become more intense in recent decades, making this environment one of the most threatened in the world, with a sharp decline in its biodiversity (Dudgeon et al. 2006; Vörösmarty et al. 2010; Reid et al. 2019). Pollution and wastewater discharge are major drivers of biodiversity loss and, consequently, great efforts are necessary to understand the negative effects of these factors on the aquatic community and its conservation (Barros et al. 2020; Reid et al. 2019). Harmful effects also reach humans who are strictly dependent on inland waters, from the more basic needs such as water and food supply to more complex uses such as industry. Hence, conserving freshwater environments is of utmost urgency.

Inland aquatic ecosystems could benefit from protected areas (PAs), which are crucial to biodiversity conservation globally (Abell et al. 2007; Gaston et al. 2008). PAs can minimize anthropogenic pressures, like land conversion, fragmentation, habitat loss, hunting, and logging (Grignolio et al. 2011; Feng et al. 2021; Yang et al. 2021). However, the effectiveness of PAs in protecting aquatic biodiversity has been questioned and the PAs' potential to protect fresh water needs strengthening (Hermoso et al. 2016; Acreman et al. 2020). In addition, there is a lack of consideration by policymakers for freshwater ecosystems in PAs' design and management, providing only incidental protection to rivers, creeks, streams, and wetlands within their borders (Herbert et al. 2010; Thieme et al. 2012; Quenta-Herrera et al. 2022).

Recent studies had appointed pesticides, pharmaceuticals, and personal care products contamination inside PAs, raising global concern about the influence of these compounds even in highly preserved regions (Elliot & VanderMeulen, 2017; Bradley et al. 2021). For example, Bradley et al. (2020) recorded 80 different bioactive compounds in freshwater samples from five PAs in the USA. Sources of threats to water systems in PAs may be diverse, such as visitation (Weissinger et al. 2018), agriculture, and land conversion in surrounding areas (Battaglin et al. 2016; Silva et al. 2021), which directly or indirectly influence the watershed. Therefore, accessing the risks to streams inside PAs is a key point to improve PA effectiveness in protecting freshwater biota.

Here, we compared freshwater quality in streams inside and outside PAs designed to protect the last remnants of the Atlantic Forest in Southern Brazil and, consequently, consider whether freshwater organisms could benefit from better water quality inside these PAs. This biome is one of the world's foremost biodiversity hotspots, with high species richness and endemism levels, but it is also severely threatened (Ribeiro et al. 2009; Mittermeier et al. 2011). For this purpose, physicochemical and biological parameters, as well as pesticides and pharmaceutical contamination in water were analyzed.

Methods

Study area

Our study was carried out in and around four large protected areas located in the southern region of the Atlantic Forest, comprising the São Joaquim National Park (SJNP, 496.72 km²), Aparados da Serra National Park (ASNP, 131.48 km²), Serra Geral National Park (SGNP, 173.02 km²), and São Francisco de Paula National Forest (SFPNF, 16.16 km²). We also

included a Private Natural Heritage Reserve (PNHR Portal das Nascentes, 0.16 km²) since it is a conservation unit near to SJNP (ICMBio/SISBIO lic. N°73877-1). The whole study area lies within the Brazilian Atlantic Forest biome, with different types of phytophysiognomy but mainly *Araucaria* seasonal evergreen forests and highland prairies (Oliveira-Filho et al. 2013).

We selected 33 sampling sites in rivers and streams, 10 within and 23 outside PAs; the latter being 1.3-49.5 km distant from the nearest PA (Fig. 1). Six streams inside the PAs originate within their boundaries (sites 1, 4, 5, 6, 23, and 24), and four streams have their sources outside the conservation units (sites 22, 25, 26, and 30). In each site, surface water samples were taken in November 2020 and November 2021. In total, 30 different water bodies comprise the study, characterized by low-order streams, with a predominantly rocky substrate and depths not exceeding 50 cm at the margins. The riparian vegetation varies among sites, both in composition, given the different phytophysiognomies of the study area and in conservation level and size. Based on the distribution and distance of the sites to the PAs, we divided the study region into six different areas (see Table S1 and Fig. 1): areas 1 (SJNP and PNHR), 3 (SGNP and ASNP), and 5 (SFPNF) correspond to PAs, and areas 2, 4 and 6 correspond to their respective outside regions. We grouped SJNP and PNHR into one area, as well as SGNP and ASNP, since they are adjacent.

Physicochemical and biological parameters

The temperature of the water (°C), dissolved oxygen concentration (mg/L), conductivity (µS/cm), and pH were measured three times at each site, at different points along the stream (2-3 m between them), using a Multiparameter Meter HI 9829 (Hanna Instruments, UK), and the data averaged. Due to technical problems in the equipment at some sites, some measurements could not be taken; when only one measure was obtained, it was used without averaging, and

if no measure could be taken (as for dissolved oxygen at site 8), the site parameter was excluded from statistical analysis.

The biological parameters – presence of *Escherichia coli* and total coliforms – were obtained using the Colipaper Alfakit® (Alfakit Eireli, Brazil) dip slide, following the steps specified by the manufacturer.

Pesticide and pharmaceutical determination

Approximately 500 mL of water were bottled in a dark glass container and stored in thermal boxes with ice. The detection and quantification of 147 pesticides and 31 pharmaceuticals (Table S2) were performed using solid-phase extraction (SPE) and ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). For SPE, 500 mL of water sample were filtered on polytetrafluoroethylene (PTFE) membrane (47 mm and 0.45 µm porosity, from Agilent Technologies, Santa Clara, USA), and the selected volume was percolated through Oasis® (Waters Corp., USA) Hydrophilic-Lipophilic Balance (HLB) 60 mg cartridges at a flow rate of 2-5 mL/min (Donato et al. 2012).

Chromatographic analyses were performed with a Waters (USA) UHPLC-MS/MS system equipped with: an Acquity UPLC™ binary pump; Xevo TQ™ MS/MS triple quadrupole detector; autosampler; column temperature controller, and MassLynx V4.1 software for system control and data acquisition. In addition, an Acquity UPLC ethylene bridged hybrid (BEH) C18 analytical column (50×2.1 mm, particle size 1.7 µm), kept at 40 °C, was used for chromatographic separation. The mobile phase consisted of (A) water containing 2% (v/v) methanol, and (B) methanol, both with 0.1% (v/v) formic acid and 5 mmol/L ammonium formate. In the gradient elution program, the percentage of the organic phase (B) was: 0 min, 5%; 7.75 min, 100%, maintained until 8.50 min; and 8.51 min, 5%, remaining constant until 10 min. The flow rate was constant at 0.225 mL/min with an injection volume of 10 µL. The

quadrupole mass spectrometer operates in selected reaction monitoring (SRM) mode using two transitions, one for quantification and one for identification. The UHPLC-MS/MS conditions were: electrospray ionization (ESI) in positive and negative modes, capillary voltage 2 kV, desolvation gas temperature 500 °C, desolvation gas flow (nitrogen) 600 L/h, spray flow 80 L/h, collision gas flow (argon) 0.15 mL/min, and source temperature 150 °C (Donato et al. 2017). Despite the same method being implemented to detect and determine pharmaceuticals, there were subtle variations, following Oliveira et al. (2019).

Data analysis

Data on physicochemical, biological, pesticides, and pharmaceutical parameters were first analyzed for normality and homogeneity using the Shapiro-Wilk, Bartlett, and Levene tests (when data showed non-normal distribution). Comparisons between the dependent variables (physicochemical, biological, agrochemical and pharmaceutical) and the independent variables (PAs and non-protected sites) were performed using Student's *t*-test and Mann-Whitney U-test. Analysis of Variance (ANOVA) was used to highlight differences in the data among the six areas, combined with a Tukey post-hoc test when values showed normal distribution. When the data were non-normal, we used a Kruskal-Wallis test along with a Student-Newman-Keuls (SNK) post-hoc test. A *p* value of 0.05 was used as the significance threshold. The analyses were performed using R 4.04 (R Core Team 2021) implemented in Rstudio 1.4 and BioStat 5.0 (Ayres et al. 2007).

In some cases, pesticides and pharmaceuticals were detected but not quantified since their values were higher than the Limit of Detection (LOD) but lower than the Limit of Quantification (LOQ), which means that the compound is present but cannot be accurately quantified. In such cases, we used the average of LOD and LOQ values (see Table S2), in order to have quantifiable values to use in statistical analysis. Otherwise, we would have to use only

presence/absence data, losing information. Because of this, at some sites, the concentrations are lower than the LOQ.

Results

Physicochemical and biological parameters of the streams

The mean values obtained for the physicochemical parameters at 33 sampling sites are summarized in Table S1, as well as the counts of *E. coli* and total coliforms CFUs. At least one pesticide and one pharmaceutical were detected at each sampling site.

When comparing the physicochemical water parameters from sampling sites within PAs with those outside the PAs, temperature ($t = 1.859$) and conductivity ($U = 50.5$) showed statistically significant differences, while dissolved oxygen ($t = 0.934$) and pH ($U = 96$) did not differ (Fig. S1). Biological parameters (*E. coli* and total coliforms) showed no difference between protected and outside areas ($U = 98$; $t = -1.235$, respectively).

Streams inside the SJNP and PNHR (area 1) had lower temperatures ($t = -4.407$) and conductivity ($U = 3.5$) compared to values obtained for outside streams (area 2). No differences between the two areas were observed in dissolved oxygen ($t = -1.295$), pH ($U = 15$), *E. coli* ($t = -0.576$), or total coliforms ($t = 0.026$) (Fig. 2a-f).

Also, no differences were observed for physicochemical (Fig. 2a-d) or biological parameters (Fig. 2e-f), between areas 3 (ASNP and SGNP) and 4 (outside these PAs). Dissolved oxygen levels were significantly lower in the SFPNF stream (area 5) than those outside (area 6) ($t = 2.196$; Fig 2b). There was also a difference in the pH of these two areas, but, although statistically significant, this was small (mean 7.63 for area 5 and 7.34 ± 0.16 for area 6; $t = -2.239$; Fig 2d). Temperature and conductivity showed no statistically significant difference

between areas 5 and 6 ($t = 0.219$ and 0.820 , respectively; Fig. 2a, c). The indicator of biological contamination *E. coli* also presented significantly higher values within area 5 in relation to outside streams ($t = -2.052$; Fig. 2e). The mean value of colony-forming units within the PA (area 5) was 1520 CFU/100 mL, while outside the mean was 368 ± 142 CFU/100 mL (about four times lower). Total coliform counts showed no significant differences ($t = -1.143$; Fig. 2f), despite the high level in area 5 (3160 CFU/100 mL).

Comparing the results among the three PAs (areas 1, 3, and 5), four physicochemical parameters, besides the biological ones, exhibited significant variations. We observed these differences in temperature ($F = 7.123$) between areas 1 and 3 (SJNP and SGNP + ASNP; $Q = 4.68$) and 1 and 5 (SJNP and SFPNF; $Q = 4.33$), but areas 3 and 5 did not differ ($Q = 0.73$) (Fig 2a).

The dissolved oxygen ($F = 6.725$) concentration values in the water for areas 1 and 3 were similar (mean of 7.53 ± 0.67 mg/L O₂ for SJNP and 7.35 ± 0.25 mg/L O₂ for SGNP and ASNP; $Q = 0.776$). In contrast, area 5 had significantly lower dissolved oxygen levels (mean 6.08 mg/L of O₂) than areas 1 ($Q = 4.963$) and 3 ($Q = 4.514$) (Fig 2b). The pH values differed significantly ($F = 5.478$) only between areas 1 and 3 ($Q = 4.1245$), where mean pH values of 7.58 ± 0.23 and 6.87 ± 0.47 were observed, respectively. There were no significant pH differences between areas 1 and 5 ($Q = 0.223$) or between areas 3 and 5 ($Q = 3.538$) (Fig 2d). There were also no significant differences in the electrical conductivity values among the PAs ($H = 5.119$) (Fig 2c).

Significant differences in the values of the biological indicator *E. coli* ($F = 5.132$) occurred between areas 1 and 5 ($Q = 4.1030$), and between areas 3 and 5 ($Q = 4.222$) (Fig. 2 e, f). The variation between these sites is due to the high *E. coli* count in the samples from area 5, averaging 1520 CFU/100 mL. On the other hand, the mean values for this parameter in areas 1

and 3 were 160 ± 134 and 168 ± 177 CFU/100 mL, respectively ($Q = 0.031$). The values of total coliforms did not vary significantly among the PAs ($H = 1.648$).

Pesticides

We detected 19 out of 147 (13%) compounds assessed, being seven herbicides, eight insecticides, and four fungicides (Fig. S2). Pesticides were detected in all sampled sites (Fig. S3, Table S3). In nine out of 33 sites (about 27%), we found only one type of pesticide, which was herbicide, in most cases. In 20 sites (61%), two classes of substances were detected simultaneously, herbicides and insecticides, except for site 14, where herbicides and fungicides were present. At four sites (12%), all three classes were present (Table S3).

Among the herbicides, 2,4-dichlorophenoxyacetic acid (2,4-D) was present in 91% of the water samples collected (30 out of 33). Another widely used herbicide, diuron, appeared in 27% (9 of 33) of the streams sampled, all located in areas 1 and 2, followed by atrazine, present in three sites (9%) (Fig. S2). Herbicide concentrations ranged from zero to 0.121 $\mu\text{g/L}$. The highest average herbicide concentration was detected at site 31 (area 6), where the compound metribuzin contributed to the high value in the November 2021 sample (Table S4). Near this sampling point, site 32 had the second-highest concentration of herbicides (area 4), showing an average value of 0.110 $\mu\text{g/L}$, with 2,4-D, atrazine, and saflufenacil having the greatest preponderances. The highest herbicide concentration within the PAs was detected at site 1 (area 1), averaging 0.0725 $\mu\text{g/L}$. Herbicides were not detected in only two sites, one outside a PA (area 4) and one within a PA (area 3).

Insecticides were detected in fewer sites than herbicides, although they were present in approximately 76% of them (Table S3, Fig. S3). The insecticide ethoprophos was identified in 61% of the water samples (21 of 30), present in all six areas. Chlorantraniprole was recorded in six sampled sites (17%). The insecticide compound detected in the highest concentration was

imidacloprid, at site 18 (0.529 $\mu\text{g/L}$, area 4), and the mean concentration at this site was the highest observed (0.271 $\mu\text{g/L}$). We did not detect insecticides in eight sites, which were both inside (site 1, area 1; site 27, area 4) and outside (sites 3, 9, 10, 11, and 14, area 2; site 33, area 6) PAs.

Mean fungicide concentrations ranged from zero to 0.046 $\mu\text{g/L}$, being detected in only five sites (13, 14, and 15, area 2; site 26, area 3; site 31, area 5) (Table S3, Fig. S3). Pyrimethanil and carbendazim were responsible for a great portion of detected fungicides (Table S4, Fig. S2). The other identified compounds were detected in only one or two sites throughout the study area, contributing less than 5% to the total concentration observed.

We did not record statistically significant differences in the mean concentrations of the three classes of pesticides between streams inside and outside PAs ($U = 111, 109.5$ and 105.5 for herbicides, insecticides and fungicides, respectively) (Fig. S4). However, the mean concentrations observed were always lower inside (herbicides 0.0411 ± 0.02 $\mu\text{g/L}$; insecticides 0.0078 ± 0.006 $\mu\text{g/L}$; fungicides 0.0007 ± 0.002 $\mu\text{g/L}$) than outside PAs (herbicides: 0.0460 ± 0.03 $\mu\text{g/L}$; insecticides: 0.0201 ± 0.05 $\mu\text{g/L}$; fungicides: 0.0033 ± 0.01 $\mu\text{g/L}$).

Mean concentrations of herbicides within PAs were significantly higher in streams from area 1 than area 3 ($t = 2.198$). Other comparisons among areas showed no statistically significant differences in mean concentrations of the three pesticide classes (Fig. 3a-c). Data on the concentrations of each compound class are found in Table S3. The compounds recorded in the two sampling campaigns can be seen in Table S4.

Pharmaceuticals

We recorded five out of 31 (16%) pharmaceutical compounds tested for in the study area: caffeine, diclofenac (anti-inflammatory), albendazole (vermifuge), trimethoprim (antibiotic), and sulfadoxine (sulfonamides) (Table S5).

Caffeine was detected in all 33 sampling sites, the mean concentration ranging from 0.0033 µg/L at sites 4 (area 1), 13 (area 2), 21 (area 4), 22, 23, 24 (area 3), and 33 (area 6) to 0.0585 µg/L in site 19 (area 4) (Table S5). Diclofenac and trimethoprim were detected at only one site each (site 15, area 4, and site 26, area 3, respectively). Sulfadoxine was detected at sites 8 (area 2) and 33 (area 6). The vermifuge albendazole was found at sites 20, 21 (area 4), and 22 (area 3) (Table S5).

Statistical tests were performed for caffeine since the other drugs were not detected at most sites. We did not observe statistically significant differences in caffeine between PA and non-PA sites ($t = 1.163$) (Fig. 4a). However, significant differences in mean caffeine levels were recorded between streams in each PA and outside them: areas 3 and 4 (area 3 = 0.0067 µg/L, area 4 = 0.0266 µg/L; $U = 4.5$) and between areas 5 and 6 (area 5 = 0.0225 µg/L, area 6 = 0.0111 µg/L; $t = -2.403$). The remaining inter-area comparisons performed were not statistically significant (Fig. 4b).

Discussion

We found that the water quality of streams inside the PAs mirrored the exogenous anthropogenic inputs and expressed the condition of the streams in the region as a whole. Outliers in these parameters represent possible sources of pollution. However, we are aware that our results comprise only a particular time fraction since the analyzed physicochemical parameters and contaminants vary in function of rainfalls, pesticide application time, and many other factors. In this sense, temporal data are required (e.g., different seasons) to circumvent these factors and fortify assumptions.

In general, the physicochemical water quality parameters in our study were not harmful to freshwater biodiversity. According to the Environmental National Council (CONAMA Brazil) resolution 357/2005 and the aquatic life criteria established by the United States Environmental Protection Agency (US EPA 1986), levels of dissolved oxygen should be higher than 6 or 5 mg/L, respectively, and pH should range between 6.5-9.0 or 6.5-8.0, respectively. Only sites 18 (area 4) and 30 (area 5) deserve more attention. Both showed patterns of decreasing DO concentration that could result in hypoxic conditions and negatively affect aquatic organisms, forcing them to shift their breathing patterns or reduce their activity levels. This, in turn, could retard the development or cause reproductive impairments, such as increased embryo mortality and defects (Cox 2003). It should be noted, however, that re-aeration from the atmosphere is the main factor contributing to the oxygenation of low order streams, being affected by temperature, water depth and velocity, wind, and waterfalls or dams (Cox 2003; Franklin 2014). Site 30 is a shallow and narrow, low order stream with slow water flow, which could be responsible for its low dissolved oxygen levels. Moreover, site 18 also presented pH measures lower than 6.5, along with site 24 (area 5). Future monitoring actions at these sites should be carried out to obtain more data and confirm if this phenomenon persists or if it is due to seasonal variations in the watercourse.

The presence of *E. coli* and thermotolerant coliforms indicates contamination by fecal waste since these bacteria come from the fecal material of warm-blooded animals. The study area includes different land use types, including cattle raising, which could contaminate the watercourses. Coliform bacteria as a whole are naturally present in the environment and most of them are not harmful, but when present in water, they may be a strong indicator of the presence of *E. coli* and other pathogens correlated with infectious diseases (Noble et al. 2003). In general, streams inside the PAs do not seem protected from biological contaminants. There is no standard for biological contamination in freshwater for protection of aquatic life, but *E.*

E. coli and total coliforms should be absent from water intended for public supply, as stipulated by the Brazilian decree 1.469/00 (Brasil, 2001) and by the U.S. EPA (2017). All PAs had average counts of *E. coli* and total coliforms higher than the recommended threshold for excellent water quality for human bathing and primary contact, that is, higher than 200 *E. coli* and 200 total coliforms per 100 mL of water (CONAMA resolutions 274/00 and 357/05). The biological contamination possibly originates upstream of the PAs, worsening downstream water quality inside PAs. An integrative approach should be implemented, with constant monitoring, focusing on preserving springs with pristine waters and protecting riparian vegetation in the entire catchment area (Pontes et al. 2019). Indeed, the lack of a watershed management approach is one of the main factors that may weaken the conservation of fresh waters in PAs (Acreman et al. 2020).

Contrary to what was observed in other PAs, area 5 (SFPNF) showed a worsening of the dissolved oxygen concentration and pH level, and *E. coli* and total coliform counts, compared to the streams outside this PA (area 6). SFPNF is classified as a PA of category VI by International Union for Conservation of Nature (IUCN) (Borrini-Feyerabend et al. 2017), being a PA with sustainable use of natural resources that supports types of human interference that may influence its running waters. Also, the SFPNF is considerably smaller than the other PAs sampled in this study, except the NHPR (in area 1), so the land use in its surroundings could influence its watercourses more intensely. NHPR is in IUCN category IV, which aims to protect particular species or habitats, but this small PA is close to SJNP (both in area 1) and could benefit from this proximity. In other way, national parks such as SJNP (area 1), ASNP, and SGNP (area 3) are in IUCN category II. This kind of PA does not support sustainable use of the natural resources, and restricts human interference to activities such as tourism. This could be a key point to understand the differences among PAs.

Large amounts of pesticides and pharmaceutical contaminants reach waterways through different sources, including spray drift from adjacent areas, drainage, runoff, precipitation and wastewater discharge (Park et al. 2017; Quadra et al. 2019; Bradley et al. 2020), and water contamination has thus become one of the major environmental issues worldwide (Brovini et al. 2021). Pesticides are commonly found in aquatic ecosystems (Bonifacio & Hued 2019; Fernandes et al. 2019), even in PAs with wide swaths of riparian forest (Rocha et al. 2020; Bradley et al. 2020). Riparian forests play a key role in conserving water resources, as they act as ‘protective shields’ of waterways against contamination by chemical compounds by reducing the runoff connectivity (Rasmussen et al. 2011; Rocha et al. 2020). The transfer of pesticides is maximized when the vegetation only sparsely covers the soil (Rheinheimer et al. 2020). Therefore, one could expect no (or at least significantly reduced) influence of pesticides in waters from pristine and conserved regions. However, we found no differences between streams inside and outside PAs; even the streams originating inside the PAs had pesticides detected in their water samples. These data indicate that the forest cover may not be controlling residues’ entry into river waters since both are contaminated by pesticide pollutants. Also, current water conservation practices have been insufficient to limit the transfers of water, sediment, and pesticides from crops to river systems.

In addition to the presence of vegetation, several other factors can influence the transfer of pesticides to water bodies, such as the frequency and intensity of rainfall events, the application period of pesticides (Lefrancq et al. 2017; Yorlano et al. 2022), the terrain relief and slope (González 2007), and the mobility/solubility of the molecules involved (Andrade et al. 2021). Herbicides were the most frequently detected class of pesticides in our study areas. Generally, herbicides are more soluble than insecticides and fungicides and, therefore, comprise a large fraction of the pesticides frequently detected in surface water monitoring studies (Schreiner et al. 2016; Bighiu et al. 2020) since the runoff rate of compounds with higher solubility tends to

be greater (Chen et al. 2019). 2,4-D, detected in almost all sampling sites, is one of the most used herbicides worldwide (Maggi et al. 2019) and the second most used pesticide in Brazil, despite its high toxicity (Moraes 2019), which has led to its prohibition in some countries (Pan 2021). As for atrazine, it is the most frequently detected herbicide in Brazilian surface waters (Caldas et al. 2019) and several other studies report the massive presence of this compound or its degradate hydroxyatrazine in surface waters of the USA (Bradley et al. 2017; Bradley et al. 2020), Argentina (Iturburu et al. 2019; Pérez et al. 2021), Iran (Almasi et al. 2020), and even in Europe (Slaby et al. 2022) where atrazine was banned almost 20 years ago. However, in aquatic environments, atrazine tends to be adsorbed and immobilized by sediments due to high lipophilicity and poor water solubility (Qu et al. 2017), which may explain its low detection frequency at our sampling sites. Among the harmful effects of atrazine on aquatic life are reducing primary production in freshwater communities by inhibiting photosynthesis, showing strong toxicity toward aquatic plants and adverse reproductive consequences in amphibians and other wildlife (He et al. 2019; US EPA 2017).

As the presence of pesticides in the surface water is a matter of global concern, environmental legislation worldwide establishes maximum allowable limits, but the regulations vary greatly from one country to another (Li & Fantke 2022). In both sampling campaigns and for all sampling sites, the concentrations of pesticides detected were below the maximum limits permitted by current Brazilian legislation (Ministry of Health, Brazil 2021). However, it should be noted that many pesticides already prohibited in the European Union and other countries are still allowed in Brazil and that the maximum limits allowed are high, which can compromise human and animal health. Moreover, while there is a limit to the total amount of pesticides that can be present in water in the European Union, this is not the case in Brazil. The main problem is that the determination of maximum limits per active ingredient (and not for the total amount

of pesticides) facilitates the formation of pesticide cocktails, with implications that are little studied (Moraes 2019).

Environmental contamination by pharmaceutical compounds is of increasing concern since they might negatively affect human, animal and ecosystem health (Ebele, Abdallah & Harrad; Rico et al. 2021; Wilkinson et al. 2022). Continuous exposure of the freshwater biota to pharmaceutical residues in the environment may adversely affect the organisms (Frederic & Yves 2014; Ondarza et al. 2019), leading to potential biomagnification and bioaccumulation processes, mainly on primary consumers of the aquatic ecosystems (Lagesson et al. 2016; Świacka et al. 2022).

Of the compounds detected in our study, caffeine was present in all sampled streams. This compound is a natural alkaloid that acts as a stimulant on the central nervous system (Mandel 2002). It is considered an emerging contaminant because there is no standard or legislation to regulate its discharge or monitoring despite potential environmental risks (Li et al. 2020). Caffeine is largely consumed worldwide through caffeinated beverages, food, and medicines, and since high concentrations of this substance have been found in the aquatic environment, it has been considered an indicator of anthropogenic inputs of pharmaceutical compounds in freshwater environments (Li et al. 2020; Wilkinson et al. 2022). Caffeine has been detected in rivers and streams on every continent, including Antarctica, raising concern about its effect on aquatic communities (Wilkinson et al. 2022). Despite the widespread contamination, there is no established maximum limit for this substance in aquatic ecosystems. A significant correlation between caffeine and coliform bacteria was observed, evidencing that this compound may indicate recent fecal contamination (Daneshvar et al. 2012). Considering the simultaneous record of caffeine and coliforms in the sampled sites, there is evidently sewage inflow, potentially recent, in the streams of the sampled region. Although present, the

concentration recorded is considered low when compared to other studies (Wilkinson et al. 2022).

The detection of the anti-inflammatory drug diclofenac at site 15 may be related to its veterinary use in treating mastitis and acute infections, among other disorders in cattle (Bastos 2017). Relationships between environmentally relevant concentrations of diclofenac in water with oxidative stress and enzyme inhibition in the fish species *Danio rerio* and *Astyanax altiparanae* have been found (Bio & Nunes, 2020; Muñoz-Peñuela et al. 2022). Also, the interaction of caffeine with diclofenac may inhibit acetylcholinesterase activity in muscle tissue (Muñoz-Peñuela et al. 2022). Although the concentration found at site 15 is low, it serves as a warning of potential contamination by this substance and potential problems for aquatic biota. The ecotoxicological effect of the vermifuge albendazole on aquatic organisms, found in three relatively close sites, is unknown.

Among water pollutants, antibiotics have received the most attention in recent studies, given their effects on aquatic ecosystem populations and human health implications (Gothwal & Shashidhar 2014). We detected trimethoprim in a water sample from a PA, but at a many-times lower concentration than that suggested to avoid complications to aquatic biota (Fick et al. 2010). In some sites, we detected sulfadoxine, which has been related to wastewater treatment plants (Čelić et al. 2019).

Although the contaminants found were detected in low concentrations in our study area, the main concern emerging from our data relates to the cumulative or synergistic effects among several pesticide and pharmaceutical compounds on the aquatic biota, both inside and outside PAs. Even in low concentrations, a single application of combined pesticides/pharmaceuticals may have drastic effects on several taxonomic groups (Relyiea 2009; Bradley et al. 2019). Future studies are needed to elucidate the possible relationship of the pesticides found with land

use and seasonality. Also, research on the potential effects of these compounds on taxa occurring in low-order streams could enhance our comprehension of the risks to aquatic biota.

The biodiversity in Atlantic Forest streams may not be effectively protected within the sampled terrestrial PAs. Our findings corroborate the idea that contamination by biological, pesticide and pharmaceutical pollutants affects the whole catchment area and influences the waters inside PAs, which are supposed to be safe from such anthropogenic threats. Terrestrial PAs, such as national parks, are a very effective way of preserving terrestrial biodiversity (Gray et al. 2016; Koskimäki et al. 2021), but there is a need for PAs to be designed to better protect the freshwater biota through the protection of springs and catchments as a whole.

Supplementary material. For supplementary material accompanying this paper, visit <https://www.cambridge.org/core/journals/environmental-conservation>.

Acknowledgements. We are thankful to Prof. Dr. Danilo Rheinheimer dos Santos for sharing some lab equipment with us and to Prof. Dr. Georgina Bond-Buckup (*in memoriam*) for helping delineate the project. We also thank two anonymous reviewers and N. Polunin for their helpful suggestions.

Financial support. We thank CAPES foundation (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the fellowship to VHSM and CNPq for the productivity grant to SS (process 311142/2014-1).

Conflict of interest. The authors declare none.

Ethical standards. None.

References

Abell R, Allan JD, Lehner B (2007) Unlocking the potential of protected areas for freshwaters.

Biological Conservation 134: 48-63.

Acreman M, Hughes KA, Arthington AH, Tickner D, Duenas MA (2020) Protected areas and freshwater biodiversity: a novel systematic review distils eight lessons for effective conservation.

Conservation Letters 13: e12684.

- Almasi H, Takdastan A, Jaafarzadeh N, Babaei AA, Birgani YT, Cheraghian B, Saki A et al. (2020) Spatial distribution, ecological and health risk assessment and source identification of atrazine in Shadegan international wetland, Iran. *Marine Pollution Bulletin* 160: 111569.
- Andrade VS, Gutierrez MF, Regaldo L, Paira AR, Repetti MR, Gagneten AM (2021) Influence of rainfall and seasonal crop practices on nutrient and pesticide runoff from soybean dominated agricultural areas in Pampean streams, Argentina. *Science of The Total Environment* 788: 147676.
- Ayres M, Júnior MA, Ayres DL, Santos AA (2007) BIOESTAT – Aplicações estatísticas nas áreas das ciências bio-médicas. Ong Mamiraua. Belém, PA.
- Barros G, Brito MTS, Peluso LM, Faria E, Izzo TJ, Teixido AL (2020) Biased research generates large gaps on invertebrate biota knowledge in Brazilian freshwater ecosystems. *Perspectives in Ecology and Conservation* 18: 190-196.
- Bastos MC (2017). Study of the environmental contamination of human and veterinary medicines in the south Brazil. [Doctoral dissertation, University of Santa Maria, Brazil]. [www document]. URL <http://repositorio.ufsm.br/handle/1/22378>.
- Battaglin WA, Smalling KL, Anderson C, Calhoun D, Chestnut T, Muths E (2016) Potential interactions among disease, pesticides, water quality and adjacent land cover in amphibian habitats in the United States. *Science of the Total Environment* 566-567: 320-33.
- Bighiu MA, Höss S, Traunspurger W, Kahlert M, Goedkoop W (2020) Limited effects of pesticides on stream macroinvertebrates, biofilm nematodes, and algae in intensive agricultural landscapes in Sweden. *Water Resources* 174: 115640.
- Bio S, Nunes B (2020) Acute effects of diclofenac on zebrafish: Indications of oxidative effects and damages at environmentally realistic levels of exposure. *Environmental Toxicology and Pharmacology* 78: 103394.

- Bonifacio AF, Hued AC (2019) Single and joint effects of chronic exposure to chlorpyrifos and glyphosate based pesticides on structural biomarkers in *Cnesterodon decemmaculatus*. *Chemosphere* 236: 124311.
- Borrini-Feyerabend G, N Dudley, T Jaeger, B Lassen, N Pathak Broome, A Phillips, T Sandwith (2017) Governança de Áreas Protegidas: da compreensão à ação. Série Diretrizes para melhores Práticas para Áreas Protegidas, No. 20, Gland, Suíça: UICN. xvi: 124 pp.
- Bradley PM, Battaglin WA, Clark JM, Henning F, Hladik ML, Iwanowicz LR, Journey CA et al. (2017) Widespread occurrence and potential for biodegradation of bioactive contaminants in Congaree National Park, USA. *Environmental Toxicology and Chemistry* 36: 3045-3056.
- Bradley PM, Journey CA, Berninger JP, Button DT, Clark JM, Corsi SR, DeCicco LA et al. (2019) Mixed-chemical exposure and predicted effects potential in wadeable southeastern USA streams. *Science of The Total Environment* 655: 70-83.
- Bradley PM, Romanok KM, Duncan JR, Battaglin WA, Clark JM, Hladik ML, Huffman BJ et al. (2020) Exposure and potential effects of pesticides and pharmaceuticals in protected streams of the US National park Service southeast region. *Science of the Total Environment* 704: 135431.
- Bradley PM, Kulp MA, Huffman BJ, Romanok KM, Smalling KL, Breitmeyer SE, Clark JM (2021) Reconnaissance of cumulative risk of pesticides and pharmaceuticals in Great Smoky Mountains National Park streams. *Science of the Total Environment* 781: 146711.
- Brasil, Fundação Nacional de Saúde (2001) Portaria nº 1.469/2000, de 29 de dezembro de 2000: aprova o controle e vigilância da qualidade da água para consumo humano e seu padrão de potabilidade. Fundação Nacional de Saúde: 32 p.
- Brovini EM, Deus BCT, Vilas-Boas JA, Quadra GR, Carvalho L, Mendonça RF, Pereira RO et al. (2021) Three-bestseller pesticides in Brazil: freshwater concentrations and potential environmental risks. *Science of the Total Environment* 771: 144754.

- Caldas SS, Arias JLO, Rombaldi C, Mello LL, Cerqueira MBR, Martins AF, Primel EG (2019) Occurrence of pesticides and PPCPs in surface and drinking water in southern Brazil: data on 4-Year monitoring. *Journal of the Brazilian Chemical Society* 30: 71-80.
- Čelić M, Gros M, Farré M, Barceló D, Petrovic M (2019) Pharmaceuticals as chemical markers of wastewater contamination in the vulnerable area of the Ebro Delta (Spain). *Science of the Total Environment* 652: 952–963.
- Chen C, Guo W, Ngo HH (2019) Pesticides in stormwater runoff—a mini review. *Frontiers of Environmental Science & Engineering* 13: 1–72.
- CONAMA - Conselho Nacional de Meio Ambiente (2000) Resolução CONAMA n° 274, de 29 de novembro de 2000. [www document] URL http://pnqa.ana.gov.br/Publicacao/Resolu%C3%A7%C3%A3o_Conama_274_Balneabilidade.pdf.
- CONAMA - Conselho Nacional de Meio Ambiente (2005) Resolução n° 357 de 17 de março de 2005. [www document] URL https://www.icmbio.gov.br/cepsul/images/stories/legislacao/Resolucao/2005/res_conama_357_2005_classificacao_corpos_agua_rtfda_altrd_res_393_2007_397_2008_410_2009_430_2011.pdf.
- Cox BA (2003) A review of dissolved oxygen modelling techniques for lowland rivers. *The Science of the Total Environment* 314–316: 303–334.
- Daneshvar A, Aboulfadl K, Viglino L, Broséus R, Sauvé S, Madoux-Humery AS, Weyhenmeyer GA (2012) Evaluating pharmaceuticals and caffeine as indicators of fecal contamination in drinking water sources of the Greater Montreal region. *Chemosphere* 88: 131–139.
- Donato FF, Bandeira NMG, Santos GC, Prestes OD, Adaime MB, Zanella R (2017) Evaluation of the rotating disk sorptive extraction (RDSE) technique with polymeric sorbent for multiresidue

- determination of pesticides in water by ultra-high-performance liquid chromatography-tandem mass spectrometry. *Journal of Chromatography* 1516: 54-63.
- Donato FF, Kemmerich M, Facco JF, Friggi CA, Prestes OD, Adaime MB, Zanella R (2012) Simultaneous determination of pesticides and antibiotic residues at trace levels in water samples by SPE and LC-MS/MS. *Brazilian Journal of Analytical Chemistry* 7: 331-340.
- Dudgeon D, Arthington AH, Gessner MO, Kawabata Z-I, Knowler DJ, Lévêque C, Naiman RJ et al. (2006) Freshwater Biodiversity: importance, threats, status and conservation challenges. *Biological Reviews* 81: 163-182.
- Ebelle AJ, Abdallah MAE, Harrad S (2017) Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. *Emerging Contaminants* 3 (1): 1-16.
- Elliot SM, VanderMeulen DD (2017) A regional assessment of chemicals of concern in surface waters of four Midwestern United States national parks. *Science of the Total Environment* 579: 1726-1735.
- Feng C, Cao M, Wang W, Wang H, Liu F, Zhang L, Du J (2021) Which management measures lead to better performance of China's protected areas in reducing forest loss? *Science of the Total Environment* 764: 142895.
- Fernandes G, Aparicio V, Bastos MC, Geronimo E, Labanowski J, Prestes OD, Zanella R et al. (2019) Indiscriminate use of glyphosate impregnates river epilithic biofilms in southern Brazil. *Science of the Total Environment* 651: 1377-1387.
- Fick J, Lindberg RH, Tysklind M, Larsson DGJ (2010) Predicted critical environmental concentrations for 500 pharmaceuticals. *Regulatory Toxicology and Pharmacology* 58: 516-523.
- Franklin PA (2014) Dissolved oxygen criteria for freshwater fish in New Zealand: a revised approach. *New Zealand Journal of Marine and Freshwater Research* 48(1): 112-126.

- Frederic O, Yves P (2014) Pharmaceuticals in hospital wastewater: their ecotoxicity and contribution to the environmental hazard of the effluent. *Chemosphere* 115: 31e39.
- Gaston KJ, Jackson SF, Cantú-Salazar L, Cruz-Piñón G (2008) The Ecological Performance of Protected Areas. *Annual Review of Ecology, Evolution, and Systematics* 39: 93–113.
- González S (2007) Contaminación difusa de las aguas. INIA Tierra adentro. [www document]. URL <https://biblioteca.inia.cl/handle/123456789/6373>.
- Gothwal R, Shashidhar T (2014) Antibiotic Pollution in the Environment: A Review. *Clean – Soil, Air, Water* 42(9999): 1–11.
- Gray CL, Hill SLL, Newbold T, Hudson LN, Börger L, Contu S, Hoskins AJ et al. (2016) Local biodiversity is higher inside than outside terrestrial protected areas worldwide. *Nature Communications* 7: 12306.
- Grignolio S, Merli E, Bongi P, Ciuti S, Apollonio M (2011) Effects of hunting with hounds on a non-target species living on the edge of a protected area. *Biological Conservation* 641-649.
- He J, He H, Yan Z, Gao F, Zheng X, Fan J, Wang Y (2019). Comparative analysis of freshwater species sensitivity distributions and ecotoxicity for priority pesticides: Implications for water quality criteria. *Ecotoxicology and Environmental Safety* 176: 119-124.
- Herbert ME, McIntyre PB, Doran PJ, Allan JD, Abell R (2010) Terrestrial reserve networks do not adequately represent aquatic ecosystems. *Conservation Biology* 24(4): 1002–1011.
- Hermoso V, Abell R, Linke S, Boon P (2016) The role of protected areas for freshwater biodiversity conservation: challenges and opportunities in a rapidly changing world. *Aquatic Conservation: Marine and Freshwater Ecosystems* 26: 3–11.
- Iturburu FG, Calderon G, Amé MV, Menone ML (2019) Ecological Risk Assessment (ERA) of pesticides from freshwater ecosystems in the Pampas region of Argentina: Legacy and current use chemicals contribution. *Science of the Total Environment* 691: 476-482.

- Koskimäki T, Eklund J, Moulatlet GM, Tuomisto H (2021) Impact of individual protected areas on deforestation and carbon emissions in Acre, Brazil. *Environmental Conservation* 48: 217–224.
- Lagesson A, Fahlman J, Brodin T, Fick J, Jonsson M, Byström P, Klaminder J (2016) Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web - Insights from a field experiment. *Science of the Total Environment* 568: 208-215.
- Lefrancq M, Jadas-Hécart A, La Jeunesse I, Landry D, Payraudeau S (2017) High frequency monitoring of pesticides in runoff water to improve understanding of their transport and environmental impacts. *Science of The Total Environment* 587–588: 75–86.
- Li Z, Fantke P (2022) Toward harmonizing global pesticide regulations for surface freshwaters in support of protecting human health. *Journal of Environmental Management* 301: 113909.
- Li S, Wen J, He B, Hu X, Liu J (2020) Occurrence of caffeine in the freshwater environment: Implications for ecopharmaco vigilance. *Environmental Pollution* 263: 114371.
- Maggi F, Tang FHM, Cecilia D, Mcbratney A (2019) Pest-Chemgrids, global gridded maps of the top 20 crop specific pesticide application rates from 2015 to 2025. *Scientific Data* 6: 170.
- Mandel HG (2002) Update on caffeine consumption, disposition and action. *Food and Chemical Toxicology* 40: 1231–1234.
- Ministry of Health, Brazil (2021) Portaria GM/MS N° 888/2021. [www document] URL <https://www.in.gov.br/en/web/dou/-/portaria-gm/ms-n-888-de-4-de-maio-de-2021-318461562>.
- Mittermeier RA, Turner WR, Larsen FW, Brooks TM, Gascon C (2011) Global Biodiversity Conservation: The Critical Role of Hotspots. In: *Biodiversity Hotspots*, eds. FE Zachos and JC Habel, pp. 1-22. Springer-Verlag Berlin Heidelberg.
- Moraes RF (2019) Agrotóxicos no Brasil: padrões de uso, política da regulação e prevenção da captura regulatória. *Textos para Discussão* 2506: 1-76. Brasília: Instituto de Pesquisa Econômica Aplicada (IPEA).

- Muñoz-Peñuela M, Moreira RG, Gomes ADO, Tolussi CE, Branco GS, Pinheiro JPS, Zampieri RA (2022) Neurotoxic, biotransformation, oxidative stress and genotoxic effects in *Astyanax altiparanae* (Teleostei, Characiformes) males exposed to environmentally relevant concentrations of diclofenac and/or caffeine. *Environmental Toxicology and Pharmacology* 91: 103821.
- Noble RT, Moore DF, Leecaster MK, McGee CD, Weisberg SB (2003) Comparison of total coliform, fecal coliform, and enterococcus bacterial indicator response for ocean recreational water quality testing. *Water Research* 37: 1637–1643.
- Oliveira JA, Izeppi LJP, Loose RF, Muenchen DK, Prestes OD, Zanella R (2019) A multiclass method for the determination of pharmaceuticals in drinking water by solid phase extraction and ultra-high performance liquid chromatography-tandem mass spectrometry. *Analytical Methods* 11: 2333-2340.
- Oliveira-Filho AT, Budke JC, Jarenkov JA, Eisenlohr PV, Neves DRM (2013) Delving into the variations in tree species composition and richness across South American subtropical Atlantic and Pampean forests. *Journal of Plant Ecology* 8(3): 242–260.
- Ondarza PM, Haddad SP, Avigliano E, Miglioranza, KSB, Brooks BW (2019) Pharmaceuticals, illicit drugs and their metabolites in fish from Argentina: implications for protected areas influenced by urbanization. *Science of the Total Environment* 649: 1029e1037.
- PAN. Pesticide Action Network (2021) [www document]. URL <http://pan-international.org/pan-international-consolidated-list-of-banned-pesticides/>.
- Park YK, Oh JA, You AS, Park SJ, Jo YM, Lee JB, Kim CS (2017) Acute toxicity of pesticides to carp (*Cyprinus carpio*) and changes of toxicity to the exposure time. *Korean Journal of Pesticide Science* 21(4) 453–492.
- Pérez DJ, Iturburu FG, Calderon G, Oyesqui LAE, Gerónimo E, Aparicio VC. Ecological risk assessment of current-use pesticides and biocides in soils, sediments and surface water of a mixed land-use basin of the Pampas region, Argentina. *Chemosphere* 263: 128061.

- Pontes PRM, Cavalcante RBL, Sahoo PK, Júnior ROS, Silva MS, Dall’Agnol R, Siqueira JO (2019) The role of protected and deforested areas in the hydrological processes of Itacaiúnas River Basin, eastern Amazonia. *Journal of Environmental Management* 235: 489–499.
- Qu M, Li H, Li N, Liu G, Zhao J, Hua Y, Zhu D (2017) Distribution of atrazine and its phytoremediation by submerged macrophytes in lake sediments. *Chemosphere* 168:1515–1522.
- Quadra GR, Teixeira JRPVA, Barros N, Roland F, Amado AM (2019) Water pollution: one of the main Limnology challenges in the Anthropocene. *Acta Limnologica Braziliensia* 31: 203.
- Quenta-Herrera E, Crespo-Pérez V, Mark BG, Gonzales AL, Kulonen A (2022) Mountain freshwater ecosystems and protected areas in the tropical Andes: insights and gaps for climate change adaptation. *Environmental Conservation* 49: 17–26.
- R Core Team (2021) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
- Rasmussen JJ, Baattrup-Pedersen A, Wiberg-Larsena P, McKnight US, Kronvang B (2011) Buffer strip width and agricultural pesticide contamination in Danish lowland streams: implications for stream and riparian management. *Ecological engineering* 37:1990–1997.
- Reid AJ, Carlson AK, Creed IF, Eliason EJ, Gell PA, Johnson PTJ, Kidd KA et al. (2019) Emerging threats and persistent conservation challenges for freshwater biodiversity. *Biological Reviews* 94: 849-873.
- Relyea RA (2009) A Cocktail of Contaminants: How mixtures of pesticides at low concentrations Affect Aquatic Communities. *Oecologia* 159: 363-376.
- Rheinheimer DS, Lima JAMC, Vargas JPR, Bastos MC, Santos MAS, Mondamert L, Labanoeski J (2020) Pesticide Bioaccumulation in Epilithic Biofilms as Biomarkers of agricultural activities in a representative watershed. *Environmental Monitoring and Assessment* 192(6): 381.

- Ribeiro MC, Metzger JP, Martensen AC, Ponzoni FJ, Hirota MM (2009) The Brazilian Atlantic Forest: How much is left, and how is the remaining forest distributed? Implications for conservation. *Biological Conservation* 142: 1141–1153.
- Rico A, Oliveira R, Nunes GSS, Rizzi C, Villa S, López-Heras I, Vighi M et al (2021) Pharmaceuticals and other urban contaminants threaten Amazonian freshwater ecosystems. *Environment International* 155: 106702.
- Rocha MC, Santos MB, Zanella R, Prestes OD, Gonçalves AS, Schuch AP (2020) Preserved riparian forest protects endangered forest-specialists amphibian species against the genotoxic impact of sunlight and agrochemicals. *Biological Conservation* 249: 108746.
- Schreiner VC, Szöcs E, Bhowmik AK, Vijver MG, Schäfer RB (2016) Pesticide mixtures in streams of several European countries and the USA. *Science of The Total Environment* 573: 680–689.
- Silva IA, Dupas FA, Costa CW, Medeiros GOR, Souza AR (2021) Spatiotemporal changes in land cover land use in protected areas of Serra da Mantiqueira, Southeastern Brazil. *Environmental Challenges* 4: 10019.
- Slaby S, Cor FL, Dufour V, Auger L, Pasquini L, Cardoso O, Curtet L et al. (2022) Distribution of pesticides and some of their transformation products in a small lentic waterbody: Fish, water, and sediment contamination in an agricultural watershed. *Environmental Pollution* 292: 118403
- Strayer DL, Dudgeon D (2010) Freshwater biodiversity conservation: recent progress and future challenges. *Journal of the North American Benthological Society* 29(1): 344-358.
- Swiacka K, Maculewicz J, Kowalska D, Caban M, Smolarz K, Swiezak J (2022) Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – Current state of knowledge. *Journal of Hazardous Materials* 424: 127350.
- Thieme ML, Rudolph J, Higgins J, Takats JA (2012) Protected areas and freshwater conservation: A survey of protected area managers in the Tennessee and Cumberland River Basins, USA. *Journal of Environmental Management* 109: 189-199.

Tong STY, Cheng W (2002) Modeling the relationship between land use and surface water quality. *Journal of Environmental Management* 66: 377-393.

U.S. EPA (Environmental Protection Agency) (1986) Quality criteria for water. EPA-440/5-86-001. Office of Water Regulations and Standards, Washington, DC.

U.S. EPA (Environmental Protection Agency) (2017) Causal Analysis/Diagnosis Decision Information System (CADDIS) <https://www.epa.gov/caddis-vol2>. *Volume 2. Sources, Stressors, Responses*. Office of Research and Development, Washington, DC. [www document] URL <https://www.epa.gov/caddis-vol2>.

Vörösmarty C, McIntyre P, Gessner M, Dudgeon D, Prusevich A, Green P, Gildden S et al. (2010) Global threats to human water security and river biodiversity. *Nature* 467: 555–561.

Weissinger RH, Blackwell BR, Keteles K, Battaglin WA, Bradley PM (2018) Bioactive contaminants of emerging concern in National Park waters of the northern Colorado Plateau, USA. *Science of the Total Environment* 636: 910–918.

Wilkinson JL, Boxall ABA, Kolpin DW, Leung KMY, Lai RWS, Galbán-Malagón C, Adell AD et al. (2022) Pharmaceutical pollution of the world's rivers. *PNAS* 119(8): e2113947119.

Yang H, Viña A, Winkler JA, Chung MG, Huang Q, Dou Y, McShea WJ (2021) A global assessment of the impact of individual protected areas on preventing forest loss. *Science of the Total Environment* 777:145995.

Yorlano MF, Demetrio PM, Rimoldi F (2022) Riparian strips as attenuation zones for the toxicity of pesticides in agricultural surface runoff: Relative influence of herbaceous vegetation and terrain slope on toxicity attenuation of 2,4-D. *Science of The Total Environment* 807: 150655.

Figures and Tables

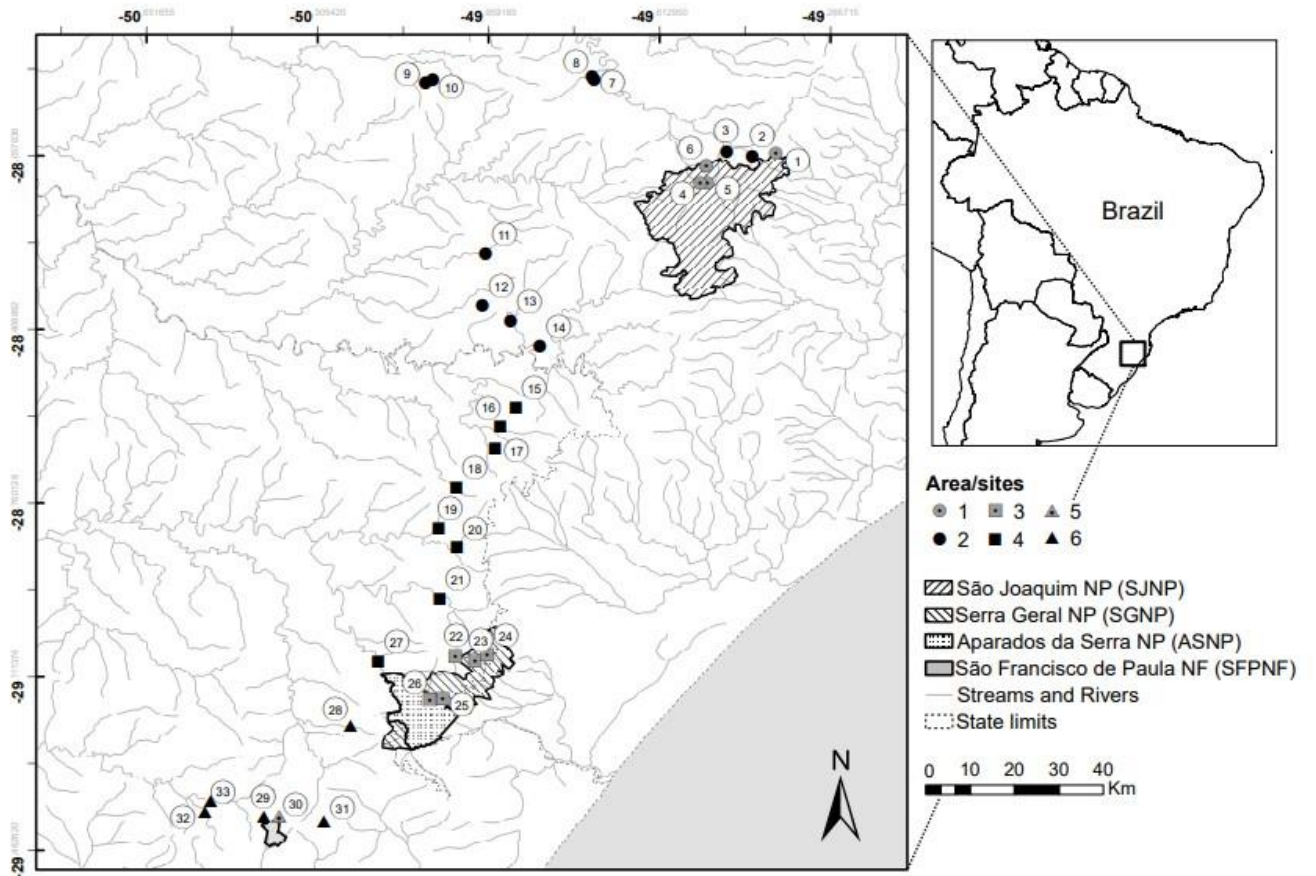


Fig. 1. Sampling sites (1 to 33) grouped into six areas, represented by symbols. The protected areas (PAs) are delimited by shades.

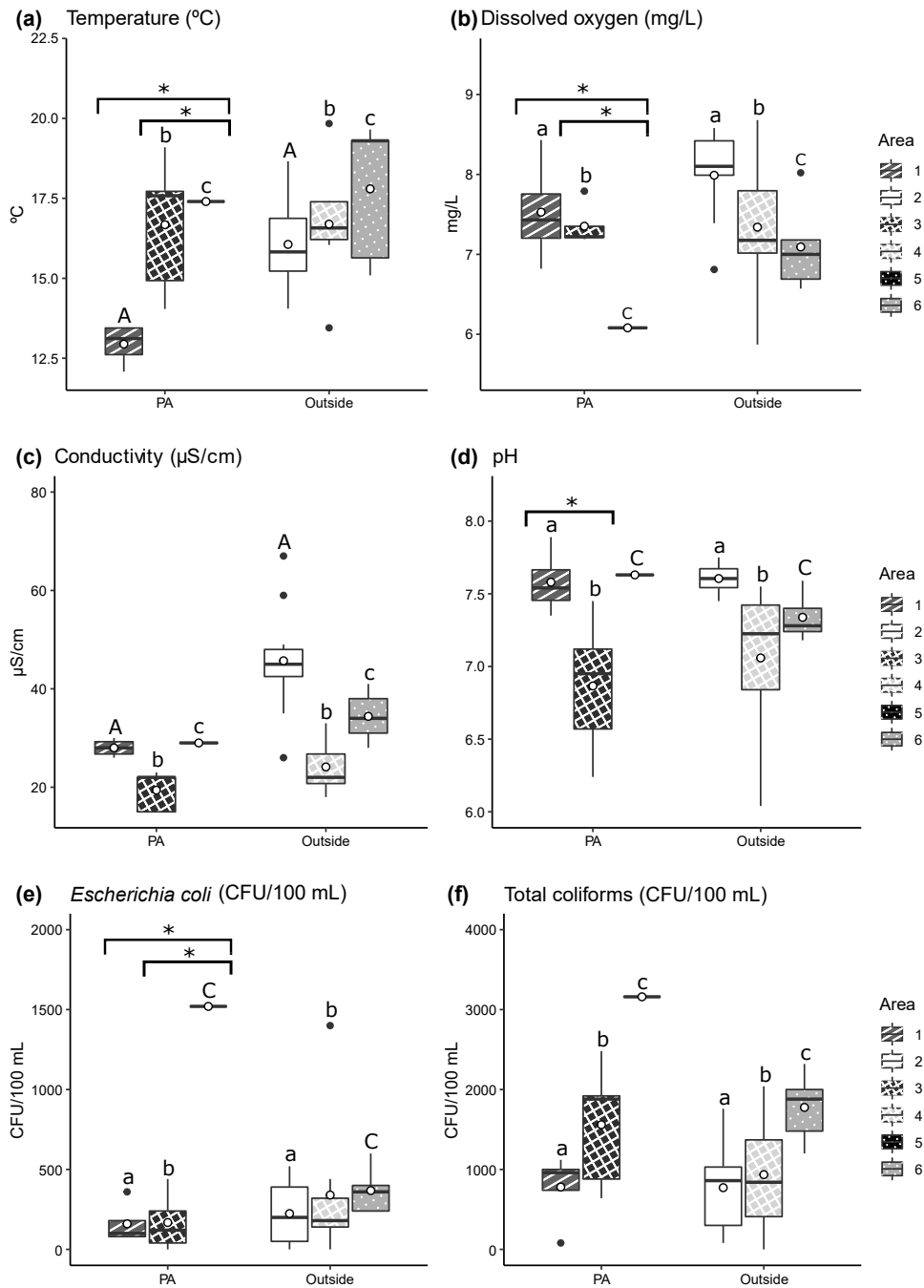


Fig. 2. Comparisons of physicochemical and biological parameters from streams sampled in the various study areas: (a) temperature, (b) dissolved oxygen, (c) conductivity, (d) pH, (e) *Escherichia coli* and (f) total coliforms in the water samples from the six regions analysed. 1 = São Joaquim National Park (SJNP) and Private Natural Heritage Reserve Portal das Nascentes (PNHR); 2 = region outside SJNP; 3 = Aparados da Serra National Park (ASNP) and Serra Geral National Park (SGNP); 4 = region outside ASNP and SGNP; 5 = São Francisco de Paula National Forest (SFPNF); 6 = region outside SFPNF. Statistically significant differences ($p < 0.05$) between samples indicated by t-tests and analyses of variance with Tukey post hoc tests are represented by upper-case letters. Lower-case letters represent statistically non-significant comparisons. Asterisks (*) represent significant differences ($p < 0.05$) in comparisons between protected areas (PAs). Within the boxplots, the horizontal lines represent medians, the white circles show means and the boxes and bars indicate the quartiles. Black circles represent outliers. CFU = colony-forming unit.

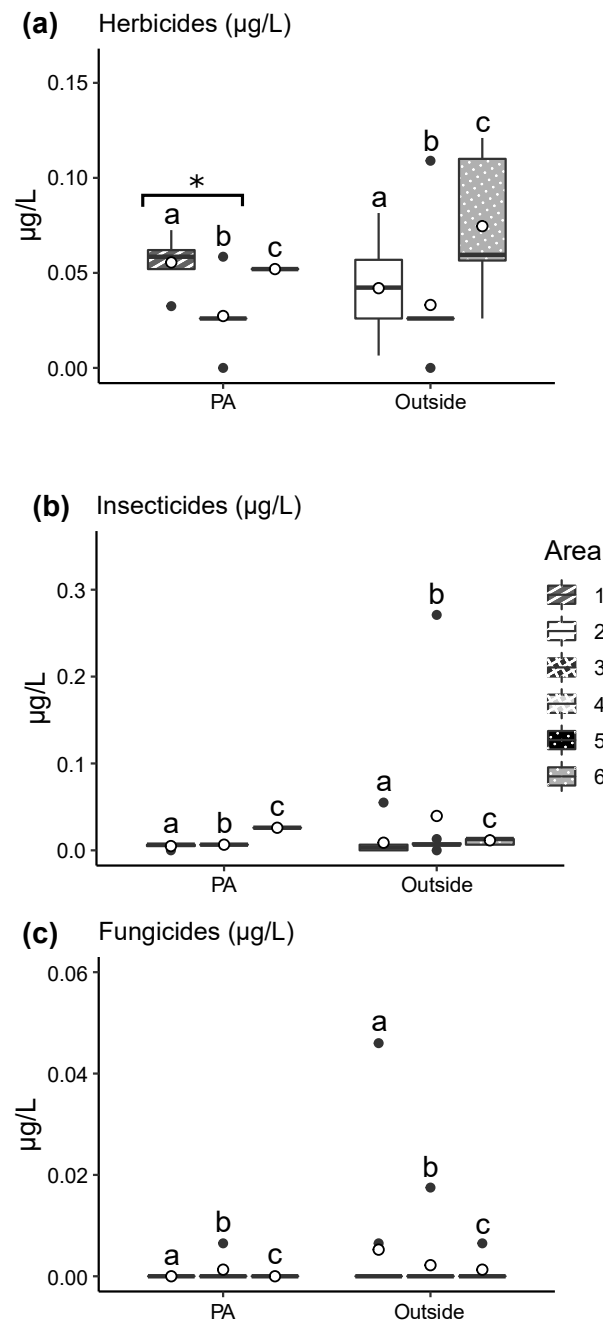


Fig. 3. Comparisons of pesticide classes among the various study areas (for area codes, see Fig. 2): (a) herbicides, (b) insecticides and (c) fungicides. Statistically significant differences ($p < 0.05$) between samples indicated by t-tests and analyses of variance with Tukey post-hoc tests are represented by upper-case letters. Lower-case letters represent statistically non-significant comparisons. Asterisks (*) represent significant differences ($p < 0.05$) in comparisons between areas within PAs. Within the boxplots, the horizontal lines represent medians, the white circles show means and the boxes and bars indicate the quartiles. Black circles represent outliers. PA = protected area.

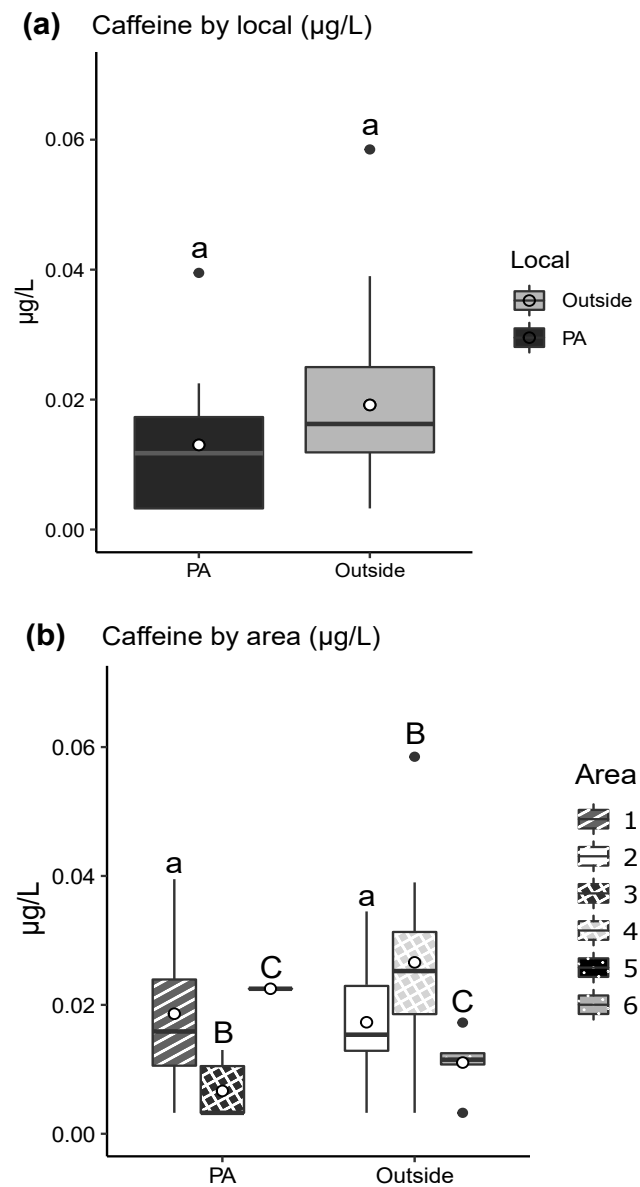


Fig. 4. (a) Comparisons of the caffeine concentrations between protected areas (PAs) and unprotected (outside) areas. (b) Comparisons of the caffeine concentrations between the various study areas (for area codes, see Fig. 2). Statistically significant differences ($p < 0.05$) between samples indicated by t-tests and analyses of variance with Tukey post hoc tests are represented by upper-case letters. Lower-case letters represent statistically non-significant comparisons. Within the boxplots, the horizontal lines represent medians, the white circles show means and the boxes and bars indicate the quartiles. Black circles represent outliers.

Supporting Information

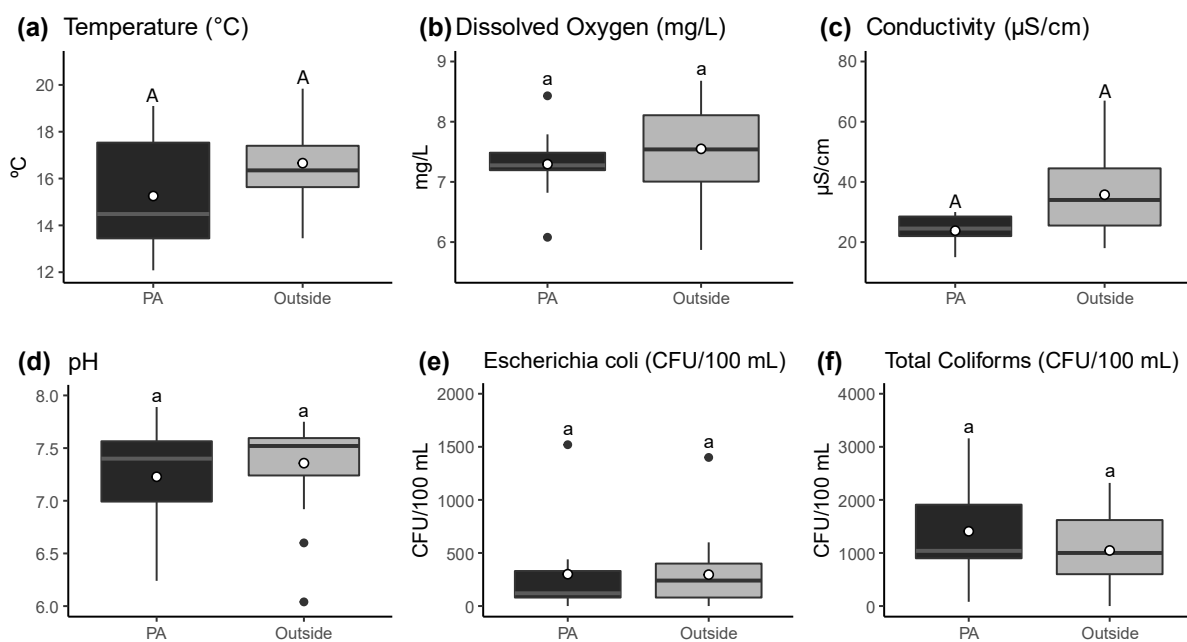


Fig. S1. Comparison of physicochemical and biological parameters in protected (PAs) and unprotected (outside) areas using t -tests and Mann-Whitney tests. Significant differences between samples ($p < 0.05$) are represented by capital letters. In the boxplot, the horizontal line represents the median, the white circle shows the mean, boxes and bars indicate the quartiles, and black circles represent the outliers.

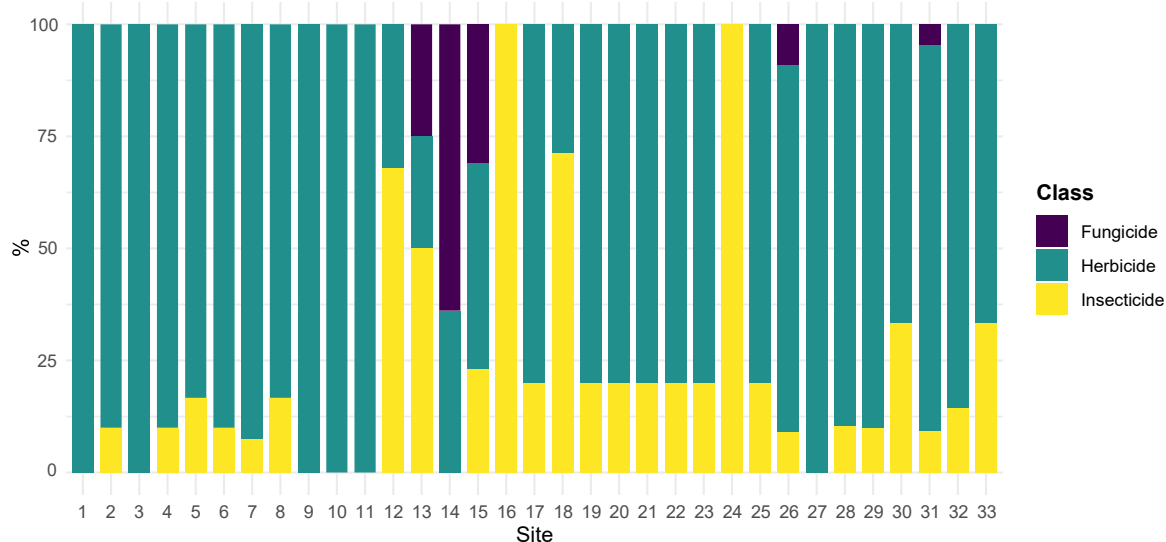


Fig. S2. Contribution of each pesticide compound in water at each sampling site. Values are the averages of concentrations recorded in 2020 and 2021. (H) herbicide; (I) insecticide; (F) fungicide.

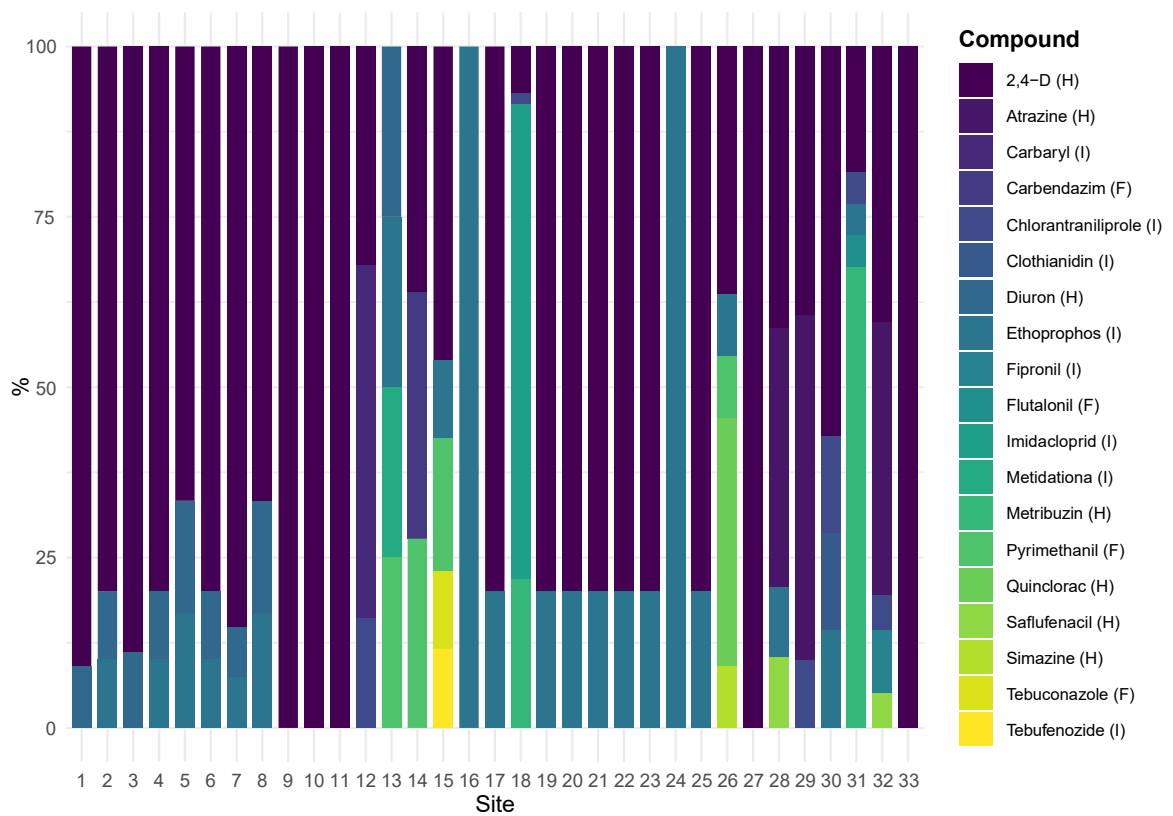


Fig. S3. Average contribution (%) of each pesticide class in water at each sampling site. (H) herbicide; (I) insecticide; (F) fungicide.

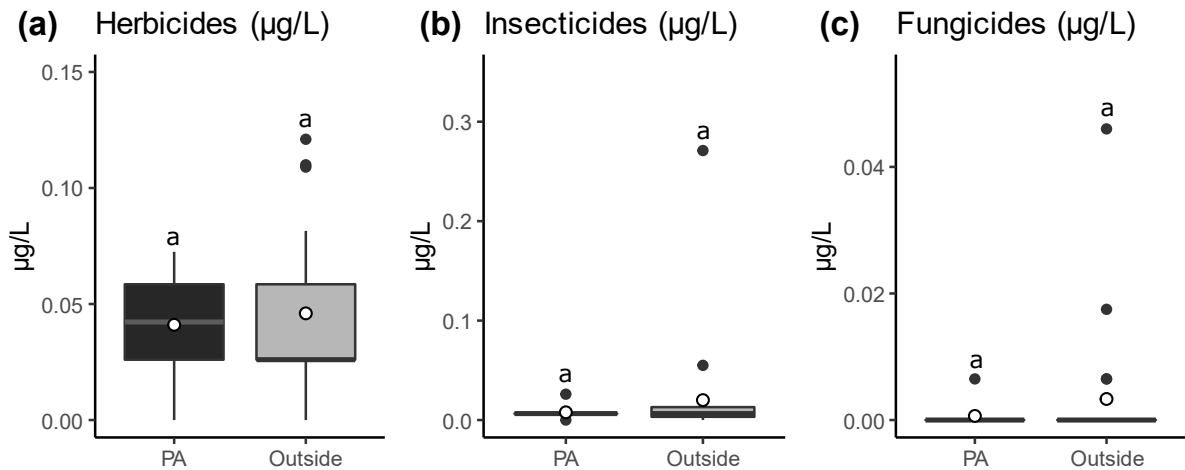


Fig. S4. Comparison of pesticide classes in protected (PAs) and unprotected (outside) areas using *t*-tests and Mann-Whitney tests. Significant differences ($p < 0.05$) between samples are represented by capital letters. Within the boxplot, the horizontal line represents the median and the empty circle shows the mean. Within the boxplot, the horizontal line represents the median, the white circle shows the mean, boxes and bars indicate the quartiles. Black circles represent the outliers.

Table S1. Average and standard deviation of physicochemical and biological parameters in water samples from streams inside and outside Protected Areas in Southern Brazil. Sampling sites are grouped into six areas, corresponding to Protected Areas (Parque Nacional de São Joaquim - SJNP, Parque Nacional de Aparados da Serra - ASNP, Parque Nacional da Serra Geral - SGPN, Floresta Nacional de São Francisco de Paula – SFPNF, Private Natural Heritage Reserve – PNHR) and outside areas (E).

Site	Area	Latitude (S)	Longitude (W)	Stream	Temp. (°C)	OD (mg/L)	Cond. (µS/cm)	pH	E. coli (UFC/100 mL)	Total Coliforms (UFC/100 mL)
01	1 (SJNP)	28° 3'12.30"S	49°22'40.04"W	Corvo Branco	12.79 ± 1.0	8.43 ± 1.3	26 ± 2.4	7.35 ± 0.2	80 *	80 *
02	2 (E)	28° 3'29.24"S	49°25'32.63"W	Bispo	14.85 ± 2.3	7.99 ± 0.9	35 ± 0.7	7.60 ± 0.5	440 ± 509	1040 ± 226
03	2 (E)	28° 2'56.53"S	49°28'38.58"W	Cachimbo	15.09 ± 2.3	8.10 ± 0.6	45 ± 1.1	7.65 ± 0.3	360 ± 396	1000 ± 735
04	1 (SJNP)	28° 6'41.50"S	49°30'56.21"W	Urubici tributary	12.08 ± 1.1	6.82 ± 2.2	27 ± 1.2	7.59 ± 0.3	360 ± 283	960 ± 792
05	1 (SJNP)	28° 6'47.01"S	49°31'50.77"W	Urubici tributary	13.46 *	7.33 *	29 *	7.49 *	80 *	1120 *
06	1 (SJNP)	28° 4'36.74"S	49°31'17.60"W	Urubici tributary	13.44 ± 0.9	7.53 ± 0.8	30 ± 2.5	7.89 ± 0.3	120 ± 170	960 ± 566
07	2 (E)	27°54'9.46"S	49°44'45.52"W	Lagoa Preta	15.63 ± 0.4	7.39 ± 1.4	45 ± 7.1	7.61 ± 0.2	80 ± 0	920 ± 57
08	2 (E)	27°53'54.73"S	49°44'59.98"W	Tigre	15.64 *	-	42 *	7.45 *	0 *	80 *
09	2 (E)	27°54'33.99"S	50° 5'14.32"W	Caveira tributary	14.05 ± 2	6.81 *	49 ± 2.8	7.75 ± 1.3	0	120 ± 57
10	2 (E)	27°54'13.36"S	50° 4'21.49"W	Caveirinha	16.50 ± 1.4	8.58 *	59 ± 3.5	7.70 ± 0.9	520 ± 735	800 ± 339
11	2 (E)	28°15'22.05"S	49°57'56.34"W	Antoninha	17.00 ± 0.5	8.43 *	45 ± 3.9	7.68 ± 0.4	40 ± 57	160 ± 113
12	2 (E)	28°21'37.46"S	49°58'17.07"W	São Mateus tributary	16.01 ± 1.7	8.11 *	26 ± 3.2	7.55 ± 0.3	80 ± 113	1120 ± 556
13	2 (E)	28°23'27.02"S	49°54'51.28"W	Invernadinha	18.66 ± 0.3	8.42 ± 0.1	44 ± 5.7	7.54 ± 0.3	400 ± 566	720 ± 1018
14	2 (E)	28°26'32.93"S	49°51'18.28"W	Sete Arroio	17.16 ± 0.5	8.07 ± 0.5	67 ± 18.1	7.52 ± 0	320 ± 113	1760 ± 1358
15	4 (E)	28°33'58.81"S	49°54'13.68"W	Lajeadozinho	16.04 ± 0.3	7.69 ± 0.2	25 ± 0.9	7.52 ± 0	80 ± 113	480 ± 0
16	4 (E)	28°36'21.35"S	49°56'7.27"W	Marco	19.84 ± 0.3	7.32 ± 0.1	21 ± 6.1	7.21 ± 0.3	200 ± 283	1200 ± 792
17	4 (E)	28°38'54.91"S	49°56'46.85"W	Silveira tributary	17.39 ± 1.9	7.02 ± 0.8	18 ± 0.7	6.60 *	280 ± 396	800 ± 792

18	4 (E)	28°43'38.22"S	50° 1'28.34"W	Manuel Leão	16.27 ± 0.7	5.87 ± 0.2	20 ± 3.7	6.04 *	1400 ± 1980	2040 ± 2885
19	4 (E)	28°48'39.99"S	50° 3'37.13"W	das Antas tributary	13.45 ± 2.5	8.68 ± 0.1	33 ± 12.4	7.55 *	160 ± 226	200 ± 283
20	4 (E)	28°51'1.46"S	50° 1'25.28"W	das Antas	16.80 ± 1.9	8.11 ± 0.3	22 ± 4.9	7.24 *	0 *	0 *
21	4 (E)	28°57'11.32"S	50° 3'30.63"W	Reserva tributary	16.35 ± 0.2	7.00 ± 0.4	22 ± 8	6.92 *	160 *	880 *
22	3 (ASNP and SGNP)	29° 4'8.90"S	50° 1'35.08"W	Reserva	17.72 ± 1.8	7.22 ± 0.7	22 ± 6.8	6.95 *	0	640 ± 0
23	3 (ASNP and SGNP)	29° 4'43.01"S	49°59'12.38"W	Pedras	19.10 ± 0.4	7.35 ± 0.1	15 ± 5.7	6.57 *	40 ± 57	1920 ± 113
24	3 (ASNP and SGNP)	29° 4'1.98"S	49°57'45.61"W	Pedras tributary	17.58 ± 0.7	7.79 ± 1	15 ± 5.2	6.24 *	440 ± 622	880 ± 226
25	3 (ASNP and SGNP)	29° 9'25.95"S	50° 3'6.02"W	Perdizes	14.04 ± 1.6	7.21 ± 0.7	22 ± 6.1	7.45 ± 0.5	240 ± 113	1880 ± 57
26	3 (ASNP and SGNP)	29° 9'28.43"S	50° 4'44.80"W	Perdizes	14.93 ± 2.3	7.19 ± 0.1	23 ± 5.9	7.12 ± 0.4	120 ± 57	2480 ± 566
27	4 (E)	29° 4'50.17"S	50°10'56.51"W	Camisa	17.41 ± 2	7.03 ± 0.4	32 ± 10.3	7.39 ± 0.1	440 ± 622	1880 ± 509
28	6 (E)	29°12'36.37"S	50°14'20.61"W	Baio Branco	15.09 ± 1.6	7.00 ± 0.1	28 ± 9.9	7.40 ± 0.4	360 ± 509	1880 ± 2546
29	6 (E)	29°23'38.00"S	50°24'51.95"W	Rolantinho	15.64 ± 2.4	7.18 ± 0.2	41 ± 20.7	7.59 ± 0.2	240 ± 113	1200 ± 679
30	5 (SFPNF)	29°24'43.40"S	50°22'58.70"W	Rolantinho tributary	17.40 ± 1.9	6.08 ± 0.5	29 ± 14.4	7.63 ± 0.1	1520 ± 1471	3160 ± 3111
31	6 (E)	29°24'20.74"S	50°17'34.39"W	Forqueta	19.31 ± 1.2	8.02 ± 0	38 ± 14.5	7.24 ± 0	600 ± 735	2000 ± 1358
32	6 (E)	29°21'49.24"S	50°31'20.56"W	Caí	19.65 ± 1.9	6.69 ± 0	31 ± 5.7	7.28 ± 0.2	240 ± 339	2320 ± 339
33	6 (E)	29°23'3.95"S	50°32'2.75"W	Caí tributary	19.30 ± 2.1	6.57 ± 0.2	34 ± 17.3	7.18 ± 0.2	400 ± 566	1480 ± 735

Table S2. Pesticides and pharmaceuticals analyzed in water samples from 33 sites and their respective Limit of detection (LOD) and Limit of quantification (LOQ). All concentrations are in $\mu\text{g/L}$.

Pesticide	Analyzed in 2020	Analyzed in 2021	Limit of detection (LOD $\mu\text{g/L}$)	Limit of quantification (LOQ $\mu\text{g/L}$)
2,4-D	✓	✓	0.024	0.080
3-hidroxi-carbofuran	✓	✓	0.012	0.040
Acetamiprid	✓	✓	0.006	0.020
Aldicarb		✓	0.012	0.040
Ametrine	✓	✓	0.006	0.020
Atrazine	✓	✓	0.006	0.020
Azaconazole	✓	✓	0.006	0.020
Azamethiphos	✓	✓	0.006	0.020
Azinphos-ethyl	✓	✓	0.012	0.040
Azinphos-methyl	✓	✓	0.006	0.020
Azoxystrobin	✓	✓	0.006	0.020
Bentazon	✓	✓	0.024	0.080
Bitertanol	✓	✓	0.012	0.040
Boscalid	✓	✓	0.006	0.020
Bromuconazole	✓	✓	0.012	0.040
Buprofezin	✓	✓	0.006	0.020
Carbaryl	✓	✓	0.006	0.020
Carbendazim	✓	✓	0.024	0.080
Carbofuran	✓	✓	0.006	0.020
Carboxin		✓	0.006	0.020
Chloramphenicol	✓		0.012	0.040
Chlorantraniliprole	✓	✓	0.006	0.020
Chlorfenvinphos	✓	✓	0.006	0.020
Chlorimuron-ethyl	✓	✓	0.012	0.040
Chlorpropham		✓	0.120	0.400

Chlorpyrifos ethyl	✓	✓	0.006	0.020
Chlorpyrifos methyl	✓	✓	0.012	0.040
Clomazone		✓	0.006	0.020
Clothianidin	✓	✓	0.006	0.020
Cyanazine	✓	✓	0.006	0.020
Cyazofamid	✓	✓	0.006	0.020
Cymoxanil	✓	✓	0.006	0.020
Cyproconazole		✓	0.012	0.040
Demeton-S-methyl-sulfon	✓	✓	0.006	0.020
Diazinon		✓	0.006	0.020
Dichlorvos	✓	✓	0.024	0.080
Diclosulam	✓	✓	0.024	0.080
Dicrotophos	✓	✓	0.006	0.020
Difenoconazole	✓	✓	0.006	0.020
Diflubenzuron	✓	✓	0.012	0.040
Dimethoate	✓	✓	0.006	0.020
Dimoxystrobin	✓	✓	0.006	0.020
Diniconazole	✓	✓	0.006	0.020
Diuron		✓	0.006	0.020
Epoxiconazole	✓	✓	0.006	0.020
Ethiofencarb sulfone	✓		0.024	0.080
Ethoprophos		✓	0.006	0.020
Ethoxysulfuron	✓	✓	0.006	0.020
Fenamidone	✓	✓	0.006	0.020
Fenamiphos		✓	0.006	0.020
Fenarimol	✓	✓	0.012	0.040
Fenazaquin	✓		0.006	0.020
Fenehexamida	✓		0.006	0.020
Fenhexamid		✓	0.012	0.040

Fenoxycarb	✓	✓	0.024	0.080
Fenpropathrin	✓	✓	0.006	0.020
Fenpropimorph	✓	✓	0.006	0.020
Fenthion	✓	✓	0.006	0.020
Fipronil	✓	✓	0.006	0.020
Fluquinconazole	✓	✓	0.006	0.020
Fluroxypyr	✓	✓	0.024	0.080
Flusilazole	✓	✓	0.006	0.020
Flutolanil	✓	✓	0.006	0.020
Flutriafol	✓	✓	0.006	0.020
Furathiocarb	✓		0.006	0.020
Imazalil	✓	✓	0.006	0.020
Imazamox	✓	✓	0.006	0.020
Imazapic	✓	✓	0.006	0.020
Imazapyr		✓	0.024	0.080
Imazaquin	✓	✓	0.006	0.020
Imazethapyr	✓	✓	0.006	0.020
Imidacloprid	✓	✓	0.012	0.040
Iprovalicarb	✓	✓	0.006	0.020
Kresoxim-Methyl	✓	✓	0.006	0.020
Linuron	✓	✓	0.006	0.020
Malathion	✓	✓	0.006	0.020
Mecarbam	✓	✓	0.006	0.020
Mephospholam	✓	✓	0.006	0.020
Mepronil	✓	✓	0.006	0.020
Metalaxyl	✓	✓	0.006	0.020
Metconazole	✓	✓	0.006	0.020
Methamidophos		✓	0.060	0.200
Methiocarb	✓	✓	0.006	0.020

Methiocarb Sulfone	✓	✓	0.006	0.020
Methomyl	✓	✓	0.060	0.200
Metidationa	✓	✓	0.006	0.020
Metribuzin	✓	✓	0.006	0.020
Metsulfuron-methyl	✓	✓	0.006	0.020
Mevinphos	✓	✓	0.006	0.020
Molinate	✓	✓	0.012	0.040
Monocrotophos	✓	✓	0.006	0.020
Monolinuron	✓	✓	0.006	0.020
Myclobutanil	✓	✓	0.006	0.020
Nicossulfurum	✓	✓	0.006	0.020
Nuarimol	✓	✓	0.006	0.020
Oxadixyl	✓	✓	0.024	0.080
Oxamyl	✓	✓	0.120	0.400
Paraoxon-ethyl	✓	✓	0.006	0.020
Parathion methyl	✓	✓	0.006	0.020
Penconazole	✓	✓	0.006	0.020
Pencycuron	✓	✓	0.006	0.020
Penoxsulam	✓	✓	0.012	0.040
Phorate		✓	0.024	0.080
Phosalone	✓	✓	0.012	0.040
Phosmet	✓	✓	0.006	0.020
Picloram		✓		
Picoxystrobin	✓	✓	0.006	0.020
Piperonyl butoxide		✓	0.006	0.020
Piridafentiona	✓	✓	0.006	0.020
Pirimicarb	✓	✓	0.006	0.020
Pirimiphos -ethyl	✓	✓	0.006	0.020
Pirimiphos-methyl	✓	✓	0.006	0.020

Prochloraz	✓	✓	0.006	0.020
Profenofos	✓	✓	0.006	0.020
Prometryn	✓	✓	0.006	0.020
Propanil	✓	✓	0.006	0.020
Propiconazole	✓	✓	0.006	0.020
Propoxur	✓	✓	0.006	0.020
Pyraclostrobin	✓	✓	0.006	0.020
Pyrazophos	✓	✓	0.006	0.020
Pyrimethanil	✓	✓	0.006	0.020
Quinalphos	✓	✓	0.006	0.020
Quinclorac	✓	✓	0.024	0.080
Quinoxifen	✓	✓	0.006	0.020
Quizalofop-P-ethyl	✓		0.012	0.040
Saflufenacil	✓	✓	0.006	0.020
Simazine	✓	✓	0.006	0.020
Tebuconazole	✓	✓	0.006	0.020
Tebufenozide	✓	✓	0.006	0.020
Terbufos	✓	✓	0.024	0.080
Tetraconazole	✓	✓	0.006	0.020
Thiabendazole	✓	✓	0.012	0.040
Thiacloprid	✓	✓	0.006	0.020
Thiamethoxam	✓	✓	0.060	0.200
Thiobencarb	✓	✓	0.006	0.020
Thiodicarb	✓	✓	0.006	0.020
Tolclofos-methyl	✓	✓	0.006	0.020
Tolyfluanid	✓	✓	0.006	0.020
Triadimefon	✓	✓	0.006	0.020
Triadimenol	✓	✓	0.012	0.040
Triazofos	✓	✓	0.006	0.020
Trichlorfon	✓		0.060	0.200

Tricyclazole	✓	✓	0.006	0.020
Trifloxystrobin	✓	✓	0.006	0.020
Trifloxysulfuron	✓	✓	0.006	0.020
Triflumuron		✓	0.006	0.020
Vamidothion	✓	✓	0.006	0.020

Pharmaceutical	Analyzed in 2020	Analyzed in 2021	Limit of detection (LOD µg/L)	Limit of quantification (LOQ µg/L)
Albendazole	✓	✓	0.003	0.010
Albendazole sulfone	✓	✓	0.003	0.010
Albendazole-2-amine	✓	✓	0.006	0.020
Albendazole-sulfoxide	✓	✓	0.003	0.010
Androstenedione	✓	✓	0.003	0.010
Atenolol	✓	✓	0.003	0.010
Caffeine	✓	✓	0.003	0.010
Carbamazepine	✓	✓	0.003	0.010
Chloramphenicol	✓	✓	0.003	0.010
Chlortetracycline	✓	✓	0.012	0.040
Ciprofloxacin		✓	0.003	0.010
Clindamycin	✓	✓	0.003	0.010
Diclofenac	✓	✓	0.003	0.010
Doxycycline	✓	✓	0.012	0.040
Ivermectin	✓	✓	0.006	0.020
Lincomycin	✓	✓	0.003	0.010
Mebendazole	✓	✓	0.003	0.010
Metronidazole	✓	✓	0.012	0.040
Nalidixic acid	✓	✓	0.003	0.010
Norfloxacin	✓	✓	0.006	0.020
Ofloxacin	✓	✓	0.003	0.010
Paracetamol	✓	✓	0.012	0.040

Progesterone	✓	✓	0.003	0.010
Propranolol	✓	✓	0.003	0.010
Sulfadiazine	✓	✓	0.030	0.100
Sulfadoxine	✓	✓	0.003	0.010
Sulfamethoxazole	✓	✓	0.003	0.010
Testosterone	✓	✓	0.003	0.010
Tetracycline	✓		0.003	0.010
Thiabendazole	✓	✓	0.003	0.010
Trimethoprim	✓	✓	0.003	0.010

Table S3: Average concentrations ($\mu\text{g/L}$) and standard deviation of three classes of pesticides in water samples from streams inside and outside Protected Areas in Southern Brazil. Sampling sites are grouped into six areas, corresponding to Protected Areas (Parque Nacional de São Joaquim - SJNP, Parque Nacional de Aparados da Serra - ASNP, Parque Nacional da Serra Geral - SGPN, Floresta Nacional de São Francisco de Paula – SFPNF, Private Natural Heritage Reserve – PNHR) and outside areas (E).

Site	Area	City	Stream	Herbicides ($\mu\text{g/L}$)	Insecticides ($\mu\text{g/L}$)	Fungicides ($\mu\text{g/L}$)
01	1 (SJNP)	Urubici	Corvo Branco	0.0725 ± 0.0106		
02	2 (E)	Urubici	Bispo	0.0585 ± 0.0092	0.0065 ± 0.0092	
03	2 (E)	Urubici	Cachimbo	0.0585 ± 0.0092		
04	1 (SJNP)	Urubici	Urubici tributary	0.0585 ± 0.0092	0.0065 ± 0.0092	
05	1 (SJNP)	Urubici	Urubici tributary	0.0325 ± 0.0460	0.0065 ± 0.0092	
06	1 (SJNP)	Urubici	Urubici tributary	0.0585 ± 0.0092	0.0065 ± 0.0092	
07	2 (E)	Rio Rufino	Lagoa Preta	0.0815 ± 0.0417	0.0065 ± 0.0092	
08	2 (E)	Rio Rufino	Tigre	0.0325 ± 0.0460	0.0065 ± 0.0092	
09	2 (E)	Paínel	Caveira tributary	0.052 ± 0		
10	2 (E)	Paínel	Caveirinha	0.052 ± 0		
11	2 (E)	São Joaquim	Antoninha	0.026 ± 0.0368		
12	2 (E)	São Joaquim	São Mateus tributary	0.026 ± 0.0368	0.055 ± 0.0594	
13	2 (E)	São Joaquim	Invernadinha	0.0065 ± 0.0092	0.013 ± 0.0184	0.0065 ± 0.0092
14	2 (E)	São Joaquim	Sete Arroio	0.026 ± 0.0368		0.046 ± 0.0467
15	4 (E)	São José dos Ausentes	Lajeadoinho	0.026 ± 0.0368	0.013 ± 0	0.0175 ± 0.0247

16	4 (E)	São José dos Ausentes	Marco		0.0065 ± 0.0092	
17	4 (E)	São José dos Ausentes	Silveira tributary	0.026 ± 0.0368	0.0065 ± 0.0092	
18	4 (E)	São José dos Ausentes	Manuel Leão	0.109 ± 0.1174	0.271 ± 0.3649	
19	4 (E)	São José dos Ausentes	das Antas tributary	0.026 ± 0.0368	0.0065 ± 0.0092	
20	4 (E)	São José dos Ausentes	das Antas	0.026 ± 0.0368	0.0065 ± 0.0092	
21	4 (E)	Cambará do Sul	da Reserva tributary	0.026 ± 0.0368	0.0065 ± 0.0092	
22	3 (ASNP e SGPN)	Cambará do Sul	da Reserva	0.026 ± 0.0368	0.0065 ± 0.0092	
23	3 (ASNP e SGPN)	Cambará do Sul	das Pedras	0.026 ± 0.0368	0.0065 ± 0.0092	
24	3 (ASNP e SGPN)	Cambará do Sul	das Pedras tributary		0.0065 ± 0.0092	
25	3 (ASNP e SGPN)	Cambará do Sul	Perdizes	0.026 ± 0.0368	0.0065 ± 0.0092	
26	3 (ASNP e SGPN)	Cambará do Sul	Perdizes	0.0585 ± 0.0827	0.0065 ± 0.0092	0.0065 ± 0.0092
27	4 (E)	Cambará do Sul	Camisa	0.026 ± 0.0368		
28	6 (E)	São Francisco de Paula	Baio Branco	0.0565 ± 0.0799	0.0065 ± 0.0092	
29	6 (E)	São Francisco de Paula	Rolantinho	0.0595 ± 0.0106	0.0065 ± 0.0092	
30	5 (SFPNF)	São Francisco de Paula	Rolantinho tributary	0.052 ± 0.0368	0.026 ± 0.0092	
31	6 (E)	São Francisco de Paula	Forqueta	0.121 ± 0.0976	0.013 ± 0	0.0065 ± 0.0092
32	6 (E)	São Francisco de Paula	Caí	0.11 ± 0.0453	0.0185 ± 0.0262	
33	6 (E)	São Francisco de Paula	Caí tributary	0.026 ± 0.0368	0.013 ± 0.0092	

Table S4. Pesticides detected in water samples from streams inside and outside Protected Areas in southern Brazil, collected in November 2020 and November 2021. All concentrations are in µg/L. Sampling sites are grouped into six areas, corresponding to Protected Areas (Parque Nacional de São Joaquim - SJNP, Parque Nacional de Aparados da Serra - ASNP, Parque Nacional da Serra Geral - SGPN, Floresta Nacional de São Francisco de Paula – SFPNF, Private Natural Heritage Reserve – PNHR) and outside areas (E).

Site	Area	2,4 D		Atrazine		Carbendazim		Carbaryl		Chlorantraniiprole		Clothianidin		Diuron		Ethoprophos		Fipronil		Flutalonil		Imidacloprid		
		2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	2020	2021	
1	1 (RPPN)	0.08	0.052												0.013									
2	2 (E)	0.052	0.052												0.013		0.013							
3	2 (E)	0.052	0.052												0.013									
4	1 (SJNP)	0.052	0.052												0.013		0.013							
5	1 (SJNP)		0.052												0.013		0.013							
6	1 (SJNP)	0.052	0.052												0.013		0.013							
7	2 (E)	0.052	0.098												0.013		0.013							
8	2 (E)		0.052												0.013		0.013							
9	2 (E)	0.052	0.052																					
10	2 (E)	0.052	0.052																					
11	2 (E)	0.052																						
12	2 (E)	0.052							0.084	0.013	0.013													
13	2 (E)														0.013		0.013							
14	2 (E)	0.052				0.052																		
15	4 (E)	0.052															0.013							
16	4 (E)																0.013							
17	4 (E)	0.052															0.013							

2	2 (E)					
3	2 (E)					
4	1 (SJNP)					
5	1 (SJNP)					
6	1 (SJNP)					
7	2 (E)					
8	2 (E)					
9	2 (E)					
10	2 (E)					
11	2 (E)					
12	2 (E)					
13	2 (E)	0.013		0.013		
14	2 (E)		0.027	0.013		
15	4 (E)		0.022		0.013	0.013
16	4 (E)					
17	4 (E)					
18	4 (E)	0.14	0.026			
19	4 (E)					
20	4 (E)					
21	4 (E)					
22	3 (ASNP e SGNP)					
23	3 (ASNP e SGNP)					

24	3 (ASNP e SGNP)				
25	3 (ASNP e SGNP)				
26	3 (ASNP e SGNP)	0.013	0.052		0.013
27	4 (E)				
28	6 (E)			0.013	
29	6 (E)				
30	5 (SFPNF)				
31	6 (E)	0.19			
32	6 (E)			0.013	
33	6 (E)				

Table S5: Mean concentrations ($\mu\text{g/L}$) and standard deviation of the pharmaceuticals recorded in water samples from streams inside and outside Protected Areas in Southern Brazil. Sampling sites are grouped into six areas, corresponding to Protected Areas (Parque Nacional de São Joaquim - SJNP, Parque Nacional de Aparados da Serra - ASNP, Parque Nacional da Serra Geral - SGP, Floresta Nacional de São Francisco de Paula – SFPNF, Private Natural Heritage Reserve – PNHR) and outside areas (E). AI = anti-inflammatory, V = Vermifuge, AB = antibiotic, S = sulfonamide.

Site	Area	Caffeine ($\mu\text{g/L}$)	Diclofenac (AI) ($\mu\text{g/L}$)	Albendazole (V) ($\mu\text{g/L}$)	Trimethoprim (AB) ($\mu\text{g/L}$)	Sulfadoxine (S) ($\mu\text{g/L}$)
01	1 (RPPN)	0.0395 \pm 0.0361				
02	2 (E)	0.0250 \pm 0.0028				
03	2 (E)	0.0163 \pm 0.0138				
04	1 (SJNP)	0.0033 \pm 0.0046				
05	1 (SJNP)	0.0130 \pm 0.0184				
06	1 (SJNP)	0.0188 \pm 0.0173				
07	2 (E)	0.0345 \pm 0.0035				
08	2 (E)	0.0145 \pm 0.0205				0.0100
09	2 (E)	0.0168 \pm 0.0145				
10	2 (E)	0.0118 \pm 0.0074				
11	2 (E)	0.0133 \pm 0.0095				
12	2 (E)	0.0128 \pm 0.0088				
13	2 (E)	0.0033 \pm 0.0046				
14	2 (E)	0.0250 \pm 0.0028				
15	4 (E)	0.0208 \pm 0.0202	0.0100			
16	4 (E)	0.0120 \pm 0.0170				

17	4 (E)	0.0288 ± 0.0315	
18	4 (E)	0.0390 ± 0.0156	
19	4 (E)	0.0585 ± 0.0530	
20	4 (E)	0.0275 ± 0.0092	0.017
21	4 (E)	0.0033 ± 0.0046	0.011
22	3 (ASNP e SGNP)	0.0033 ± 0.0046	0.013
23	3 (ASNP e SGNP)	0.0033 ± 0.0046	
24	3 (ASNP e SGNP)	0.0033 ± 0.0046	
25	3 (ASNP e SGNP)	0.0105 ± 0.0148	
26	3 (APNP e SGNP)	0.0130 ± 0.184	0.00650
27	4 (E)	0.0230 ± 0.0325	
28	6 (E)	0.0108 ± 0.0060	
29	6 (E)	0.0125 ± 0.0177	
30	5 (SFPNF)	0.0225 ± 0.0078	
31	6 (E)	0.0173 ± 0.0152	
32	6 (E)	0.0115 ± 0.0163	
33	6 (E)	0.0033 ± 0.0046	0.0065

4 CAPÍTULO II - HIDDEN DIVERSITY OF *AEGLA* (CRUSTACEA: DECAPODA: ANOMURA) IN A PRIORITY AREA FOR CONSERVATION

Formatado conforme normas do periódico *Biodiversity and Conservation*, para o qual foi submetido no dia 14/09/2022.

Hidden diversity of *Aegla* (Crustacea: Decapoda: Anomura) in a priority area for conservation

Victor Hugo dos Santos Mollmann¹ (ORCID: 0000-0002-7490-7962), Marlise Ladvoocat Bartholomei-Santos¹ (ORCID: 0000-0002-4837-8219), Gracieli Fernandes² (ORCID: 0000-0002-7264-1785), Gislaine Puli¹, Emerson Contreira Mossolin³ (ORCID: 0000-0002-0569-9135), Marcelo Marchet Dalosto⁴, and Sandro Santos¹ (ORCID: 0000-0001-9305-1154)

¹ Programa de Pós-Graduação em Biodiversidade Animal, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil;

² Programa de Pós-Graduação em Ciência do Solo, Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brazil;

³ Departamento de Ciências Biológicas, Universidade Federal de Catalão (UFCAT), Catalão, GO, Brazil;

⁴ Instituto Federal de Educação, Ciência e Tecnologia Farroupilha, Campus Alegrete, RS, Brazil;

Correspondence:

Sandro Santos, Programa de Pós-Graduação em Biodiversidade Animal, Departamento de Ecologia e Evolução, Universidade Federal de Santa Maria (UFSM), 97105-900, Av. Roraima, Santa Maria, RS, Brazil

E-mail: sandro.santos@ufsm.br

Abstract

Aegla is a genus of freshwater anomurans encompassing 93 described species occurring in rivers, streams, and caverns in septentrional South America; Brazil harbors the greatest diversity of species, mainly in its southern region, and cryptic species were already reported in the country. Prior studies identified that the Upper Uruguay, Tramandaí-Mampituba, and Laguna dos Patos freshwater ecoregions encompasses a large priority area for *Aegla* conservation, due to high species richness, phylogenetic

diversity, elevated endemism, and threats to habitat. Here, we assessed *Aegla* richness and distribution in this priority area and tested the hypothesis of crypticism in the species of the region. Sampling sites were distributed along the study region specified above, inside and in the surroundings of five protected areas (PAs), covering the major distribution range of several species described for southern Brazil. We applied three species delimitation methods to *COI* mitochondrial gene data, coupled with phylogenetic and morphological analyses. By integrating species delimitation based on the *COI* data and traditional taxonomy, we identified a high proportion of hidden diversity among *Aegla* in our study area and retrieved nine known species besides impressive 22 new ones. Of these, 11 are possible new taxonomic units that have not fitted any known species, and 11 are divided into three cryptic groups among *Aegla jarai* complex, *Aegla franciscana* complex, and *Aegla camargoi* complex. Most of these species have a narrow distribution and were sampled only in one or two sites, consistent with the pattern observed for some species of the group, making them highly vulnerable to habitat degradation. Despite the real richness of the genus *Aegla* still being largely unacknowledged, the high diversity of aeglid species revealed here makes our study area the richest known region regarding the number of *Aegla* species within its distribution across South America, turning this area even more worthy for conservation efforts.

Keywords Cryptic species complex, Freshwater, Atlantic Forest biodiversity, DNA *barcoding*.

Introduction

The underestimation of species diversity is a well-known taxonomic problem for several taxa like birds and terrestrial or aquatic vertebrates (Cadenillas and Elía 2021; Chang et al. 2011; Jaramillo et al. 2020; Martins et al. 2015; Torres-Carvajal and Terán 2021; Walker, Lyra and Haddad 2018), and a vast array of invertebrates (Adamowicz and Purvis 2003; Cicconardi et al. 2013; Sabatelli et al. 2021). The iconic publication of Mora et al. (2011) predicted *c.* 9,920,000 terrestrial and marine animal species on earth; by that time, 11% of these had been described (1,124,516). Nowadays, the number of species records should be higher but still far from expected. In addition, the knowledge concerning species diversity suffers from the increasing extinction risks in many groups, powered by anthropogenic interference and climate change (Dawson et al. 2011; Stork et al. 2009). In this sense, knowing to protect is a cliché with more meaning than never, as unveiling the earth's biodiversity is a prerequisite for recognizing how many species are threatened with extinction (Costello 2015). The same author highlights that freshwater habitats have the most significant proportion of extinct and endangered species, and habitat loss is the main driver.

The increase in species descriptions in the recent decades (more than 18,000 per year, on average) (Costello et al. 2013) may have a link with the advances in molecular technology. Nowadays, genetic tools play a key role in unveiling hidden diversity and delimitating species, in collaboration with the traditional taxonomy based on morphology – resulting in what is called the integrative approach. These tools are beneficial for taxa comprising cryptic complexes – groups of species with indistinguishable morphologies but genetically divergent and reproductively isolated from each other, often incorrectly recognized as a single species (Bickford et al. 2007; Crivellaro et al. 2018). Cryptic complexes are frequently associated with taxonomy relied only on morphological characters, leading to molecular paraphyly in several groups (Funk and Omland 2003; Lefébure et al. 2006). The use of molecular data has boosted the detection rate of cryptic species since the 1990s, and in 2015, around 90% of the papers on the subject used genetic markers (Struck and Cerca 2019; Struck et al. 2018). Several tools for delineating species boundaries based on genetic data have been developed and are widely used. Some of these methods include the general mixed Yule-Coalescent model (GMYC) (Pons et al. 2006),

automatic barcode gap discovery (ABGD) (Puillandre et al. 2012), Poisson tree process (PTP) (Zhang et al. 2013), Bayesian Phylogenetics and Phylogeography (BPP) (Yang and Rannala 2014), Bayes Factor Delimitation (BFD) (Leaché et al. 2014), assemble species by automatic partitioning (ASAP) (Puillandre et al. 2020), and others, which successfully provided preliminary taxonomic hypotheses for further species discoveries. Nevertheless, it is essential to note that the use of molecular data does not discard the use of phenotypic characters, and a combination of both methods is usually the best way to get stronger assumptions regarding crypticism, especially in morphologically conserved groups (Li et al. 2019; Noguerales et al. 2018).

One group that suffers from unsolved taxonomy and hidden diversity is the freshwater crustaceans of the genus *Aegla* (Aeglidae). Reports of paraphyly and cryptic complexes (Pérez-Losada et al. 2004), as in *Aegla paulensis* (Moraes et al. 2016), *Aegla longirostri* (Crivellaro et al. 2018), and *Aegla uruguayana* (Zimmermann et al. 2021), make the actual 93 described species (Marçal et al. 2021; WoRMS 2022) a small part of the real diversity of the group. This hidden crypticity found in the genus is due, among other factors, to the highly conserved morphology limiting the use of phenotypic characters in the taxonomic identification (Bartholomei-Santos et al. 2019; Zimmermann et al. 2021). Thus, using molecular markers in phylogenetic and taxonomic studies becomes a powerful tool to assist in identifying and delimitating aeglid species.

The genus *Aegla* is distributed in watersheds of Argentina, Bolivia, Chile, Paraguay, Uruguay, and Brazil (Santos et al. 2017); the latter concentrates the greatest diversity of species, mainly in its southern region. Almost 70% of the species of *Aegla* are categorized under some level of extinction risk, according to the criteria established by the International Union for Conservation of Nature (IUCN) (Boos et al. 2020; Bueno et al. 2016; Santos et al. 2017). Characteristics such as restricted distribution, high endemism rate, and dependence on aquatic environments with high oxygenation rates make aeglids vulnerable to pollution and habitat loss, leading to the population declines observed in recent years (Bond-Buckup and Buckup 1994; Bueno et al. 2014; Maia et al. 2013).

Previous studies identified Upper Uruguay, Tramandaí-Mampituba, and Laguna dos Patos freshwater ecoregions (Abell et al. 2008) as a large priority area for *Aegla* conservation, due to high

species richness, phylogenetic diversity, elevated endemism, and threats to the habitat (Gonçalves et al. 2018; Pérez-Losada et al. 2009). Also, some species found within those ecoregions, such as *Aegla franciscana* and *Aegla jarai*, were recovered as paraphyletic in the molecular phylogeny of the genus (Pérez-Losada et al. 2004), and may represent cryptic species complexes, which leads to severe implications for the genus' conservation. Hence, it is essential to understand the diversity and threats to these crustaceans, to the posterior establishment of preservation efforts and policies. Using the *COI* mitochondrial gene for DNA *barcoding*, phylogenetic and phylogeographic approaches, and supplemented by morphological characters, we investigated the diversity and distribution of *Aegla* species in this priority region. Also, we tested the hypothesis of crypticism among the species in the area.

Methods

Study area

Our study area comprises *c.* 9,000 km² in the core of the Atlantic Forest Biosphere Reserve (MMA/IBAMA, 2003) in the southern region of Brazil (Fig. 1), within the freshwater ecoregions of Upper Uruguay, Laguna dos Patos, and Tramandaí-Mampituba. The region encompasses the Uruguay and Atlantic South Hydrographic regions and their respective sub basins: Canoas (sites 1 to 10), Pelotas (sites 11 to 14), Apuaê-Inhandava (sites 15 to 18), Taquari-Antas (sites 19 to 22, 27 and 28), Mampituba (sites 23 to 26), Sinos (sites 29 and 30), Tramandaí (site 31), and Caí (sites 32 and 33) rivers watersheds. The phytophysiognomy of the biome encompasses mainly *Araucaria* evergreen seasonal forests and highland prairies (Oliveira-Filho et al. 2013). The annual mean temperature ranges from 12 to 14 °C, with the annual rainfall averaging between 1600 and 2200 mm. These characteristics fit the area into the subtropical climate with temperate summer (Cfb) according to Köppen's climate classification as detailed in Alvares et al. (2014). Within the study region, there are four large Protected Areas (PAs) [São Joaquim National Park (SJNP), Aparados da Serra National Park (ASNP), Serra Geral National Park (SGNP), and São Francisco de Paula National Forest (SFPNF)] and one Private Natural Heritage Reserve (PNHR Portal das Nascentes) adjacent to SJNP.

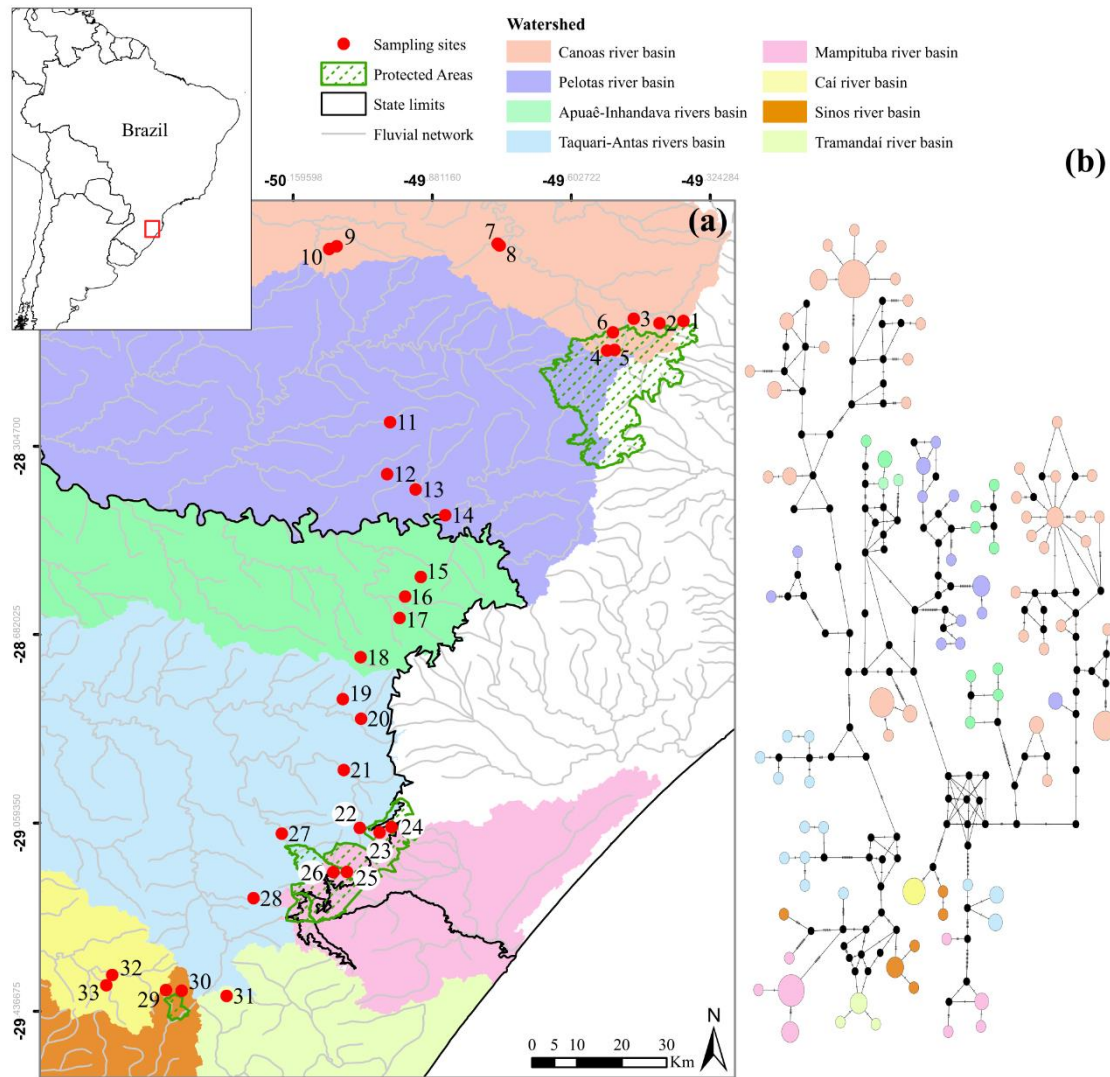


Fig. 1 (a) Location of the 33 sampling sites (red dots) along the watersheds of the study area, represented by the different colors, and protected areas (green shades). **(b)** Haplotype network colored following its respective watershed, showing the connectivity of the 96 *COI* haplotypes of *Aegla* for the study region, with size proportional to haplotype frequency. Black circles represent missing/not sampled haplotypes.

Sampling and morphological identification

Based on the distribution of the *Aegla* species (Bond-Buckup and Buckup, 1994; Santos et al. 2017) and the assumption that the study region includes priority areas for *Aegla* conservation (Gonçalves et al. 2018), we selected 33 sampling sites in rivers and streams, of which ten are located inside PAs (Fig. 1 and Table S1) (ICMBio/SISBIO lic. N°73877-1). The altitude of sampling points ranged from 791 to

1451 meters. In total, we sampled 30 different water bodies, comprising the surroundings of type localities of some of the *Aegla* species occurring in the region (*Aegla brevipalma*, *Aegla leachi*, and *Aegla oblata*) as well as locations never sampled before. These streams are of small magnitudes, with a predominantly rocky substrate. The riparian vegetation at each of these sites varies both in composition, given the different phytophysiognomies of the study area, and in conservation level and width of the vegetation patch. Specimens were collected in three sampling periods: February/2020, November/2020, and November/2021 using nets or manual search performed by removing rocks from the substrate. Collected individuals were preserved in ethyl alcohol and later taken to the Laboratory of Carcinology at the Federal University of Santa Maria (UFSM), Brazil.

Individuals were morphologically identified based on Bond-Buckup and Buckup (1994) and Santos et al. (2012), following variations in the shape and ornaments of the chela, rostral shape, size and shape of the subrostral process, and shape of the anterior angle of the second epimeron, using stereoscopic microscopy. The external morphology of *Aegla* species tends to be conserved, complicating the identification process, and demanding a certain level of expertise and experience. For this reason, information on the species distribution was a crucial factor when necessary. Vouchers were deposited at the Crustacean Collection of the Department of Ecology and Evolution, UFSM, Brazil.

DNA extraction, PCR amplification, and Sequencing

Gill tissue fragments from each specimen were used for DNA extraction, using Chelex® 100 chelating resin (Bio-Rad), following the manufacturer's instructions. A fragment of the mitochondrial *Cytochrome C Oxidase Subunit I (COI)* gene was amplified using the primers LCOI-f and COIA2-r (Xu et al. 2009). Polymerase chain reaction (PCR) was set up in a total volume of 15 µL containing about 30 ng of DNA template, 1.5 mM MgCl₂, 200 µM each dNTP, 0.4 µM each primer, DMSO 1X, 1U of *Taq* DNA polymerase, and 1X *Taq* buffer. Cycling conditions were initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 seconds, 47 °C for 45 seconds, and 72 °C for 1 minute, with a final extension at 72 °C for 5 minutes. PCR products were checked through agarose gel electrophoresis, purified, and sequenced in both directions. Sequences were BLASTed against GenBank

(GB, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to safeguard from contamination and aligned using the Muscle algorithm (Edgar 2004) implemented in Mega X (Kumar et al. 2018). The same program was used to translate the *COI* gene fragment into amino acids to detect the presence of stop codons, indicative of pseudogene amplification, using the mitochondrial genetic code for invertebrates.

Phylogenetic analyses

To investigate the positioning of our sampled specimens in relation to the *Aegla* phylogeny proposed by Pérez-Losada et al. (2004), as well as the possible presence of paraphyly and help the species identification, we inferred a phylogenetic tree using Bayesian Inference (BI). In addition to our 151 sequences, we included 60 *COI* gene sequences of *Aegla* available in GB for most species of Brazilian clades as defined by Pérez-Losada et al. (2004), as well as two sequences of *Aegla abtao* and *Aegla riolimayana* (both Chilean species) as outgroups. The resulting dataset comprised 213 sequences in a 559 bp-alignment. First, we selected the best nucleotide substitution model for our data using jModelTest 2.1.10 (Darriba et al. 2012), which was used in the following analyses. Second, in order to select the best clock model (strict or lognormal relaxed clock) and tree prior (coalescent or Yule process) to use in the phylogenetic inference, we calculated the marginal likelihood values of the four possible combinations between clock model and tree prior were estimated using stepping-stone sampling (Xie et al. 2011) and Monte Carlo Markov Chain (MCMC) with 10 million generations and 50 path-steps in Beast 1.8 (Drummond et al. 2012). Competing models were ranked after applying the Bayes Factor (BF) approach ($2\ln Bf$) (Kass and Raftery 1995); BF values greater than ten were indicative of strong support in distinguishing between models (Grummer et al. 2014).

Third, using the previously selected models, we performed a Bayesian analysis using the MCMC method in Beast 1.8 (Drummond et al. 2012), with 30 million chains and sampling every 3000 generations. Posterior probabilities were calculated with a burn-in of 3 million states and checked for convergence using Tracer 1.7.1 (Rambaut et al. 2014). Finally, all the results were visualized in FigTree 1.4.4 (Rambaut 2014).

In order to visualize the distribution of the *COI* haplotypes along the river basins, we constructed a haplotype network. Using DnaSP 6.12 (Rozas et al. 2017), we calculated the number of different haplotypes present in the 151 sequences obtained, and inferred the network using PopART (Leigh and Bryant 2015), with the Median Joining method (Bandelt et al. 1999).

Species delimitation

We used three different species delimitation methods applied to the *COI* sequence dataset. The first, General Mixed Yule Coalescent (GMYC) (Pons et al. 2006), uses a phylogenetic tree as input to fit the speciation and coalescent processes based on the differences in branching rates at the level of species and populations. GMYC method was carried out in the package “splits” in R v4.1.3 (R core team, 2022), using the ultra-metric tree previously obtained in BEAST, with the default parameters and a single threshold that specifies the transition time between- to within-species branching. The second method is the Bayesian Poisson Tree Process (bPTP), which also uses a phylogenetic tree as input to estimate branching event, but differently from GMYC, it uses a Bayesian framework to support the delimited species (Reid and Carsten 2012). Analyses with bPTP were run in the web interface (<https://species.h-its.org/ptp/>) (Zhang et al. 2013). The third method is the recent Assemble Species by Automatic Partitioning (ASAP) (Puillandre et al. 2020). It implements a hierarchical clustering algorithm based on pairwise genetic distances, identifying the “barcode gap”, and proposes species partitions based on a new scoring system with no biological prior of intraspecific diversity. We used the online version of this program (<https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html>) with Kimura (K80) as substitution model and transition/transversion rate of 2.73 (calculated using MEGA 11 (Tamura et al. 2021)). These delimitation methods are widely used for single gene analysis, each one having its advantages and shortcomings. Due to this assumption, authors often propose the use and comparison of different methods (Puillandre et al. 2020).

Results

Morphological analyses

Of the 33 sampling sites, we found *Aegla* specimens in 27 of them during the three sampling campaigns. The identification based on the morphology culminated in twelve known species: *A. brevipalma*, *Aegla camargoi*, *A. franciscana*, *Aegla inconspicua*, *Aegla itacolomiensis*, *A. jarai*, *A. leachi*, *Aegla leptodactyla*, *A. oblata*, *Aegla plana*, *Aegla serrana*, and *Aegla spinosa*. Of these, the identities of *A. itacolomiensis* and *A. plana* were not confirmed by comparison with *COI* sequences from GB. Specimens whose morphological identification was not possible were referred to as *Aegla* sp. (Table S1).

Phylogenetic analyses

Our entire dataset resulted in 213 *COI* sequences, including samples from GB, 559 bp unambiguous aligned. The number of parsimony informative sites was 157. The best fit molecular clock and tree prior model were strict and coalescent, respectively (Table S2). The phylogenetic tree recovered using these parameters and the GTR + I + G substitution model is shown in Figure 2. The phylogenetic analysis positioned the species of the study region in two distinct clades, C and E, as specified in Pérez-Losada et al. (2004). The division between the two clades was evident, with populations from sites 1 to 17 belonging to clade C, while populations from sites 19 to 33 belong to clade E (no specimens were found in site 18). The only exception was the population of site 9, which fit into both clade C and E since in this site we have *A. brevipalma*, *A. oblata* (Clade C), and *A. leachi* (Clade E) (Santos et al. 2012). Site 9 is the type-locality of *A. leachi*, and site 10 is the type locality of *A. oblata* (Santos et al. 2012).

We recovered 96 haplotypes for the study region (Fig. 1b), most of which were located in the Canoas River watershed, where most sampling sites were. By visualizing the network, we can notice the division between the basins that distinguish clades C (sites 1 to 17) and E (sites 19 to 33), in coherence with the generated phylogeny (Fig. 2). The exceptions, such as the haplotypes obtained for samples from Caí

and Sinos river basins, were placed near the haplotypes of the Canoas and Apuaê-Inhandava river basins, despite being geographically distant from them.

Species delimitation and integrative taxonomy

The GMYC analysis based on the *COI* dataset produced 99 ML entities (including already described species and outgroups). The bPTP analysis indicated the presence of 97 putative species. Meanwhile, ASAP suggested 82 potential species (Fig. 2). Since ASAP showed to be more conservative than the other two methods, we opted to couple its results to the phylogenetics and morphological analyses when delimiting species in order to not overestimate the diversity in the study area.

From the 82 groups delimited by ASAP, 26 are putative new species (or MOTUs) for the region. Using the integrative approach, based on the ASAP delimitation, the phylogenetic grouping, and morphology, we identified 22 new species, by bringing some monophyletic entities together (Fig. 2). Moreover, based on the morphological resemblance to known species, we identified three cryptic complexes: *A. jarai*, *A. franciscana*, and *A. camargoi*, comprising seven, three and one putative new species, respectively (excluding the species *sensu stricto*), totaling 11 species. The remaining 11 possible new MOTUs neither grouped with known species nor could be morphologically identified. According to our phylogeny, 14 new MOTUs belong to clade C and 8 to clade E of Pérez-Losada et al. (2004).

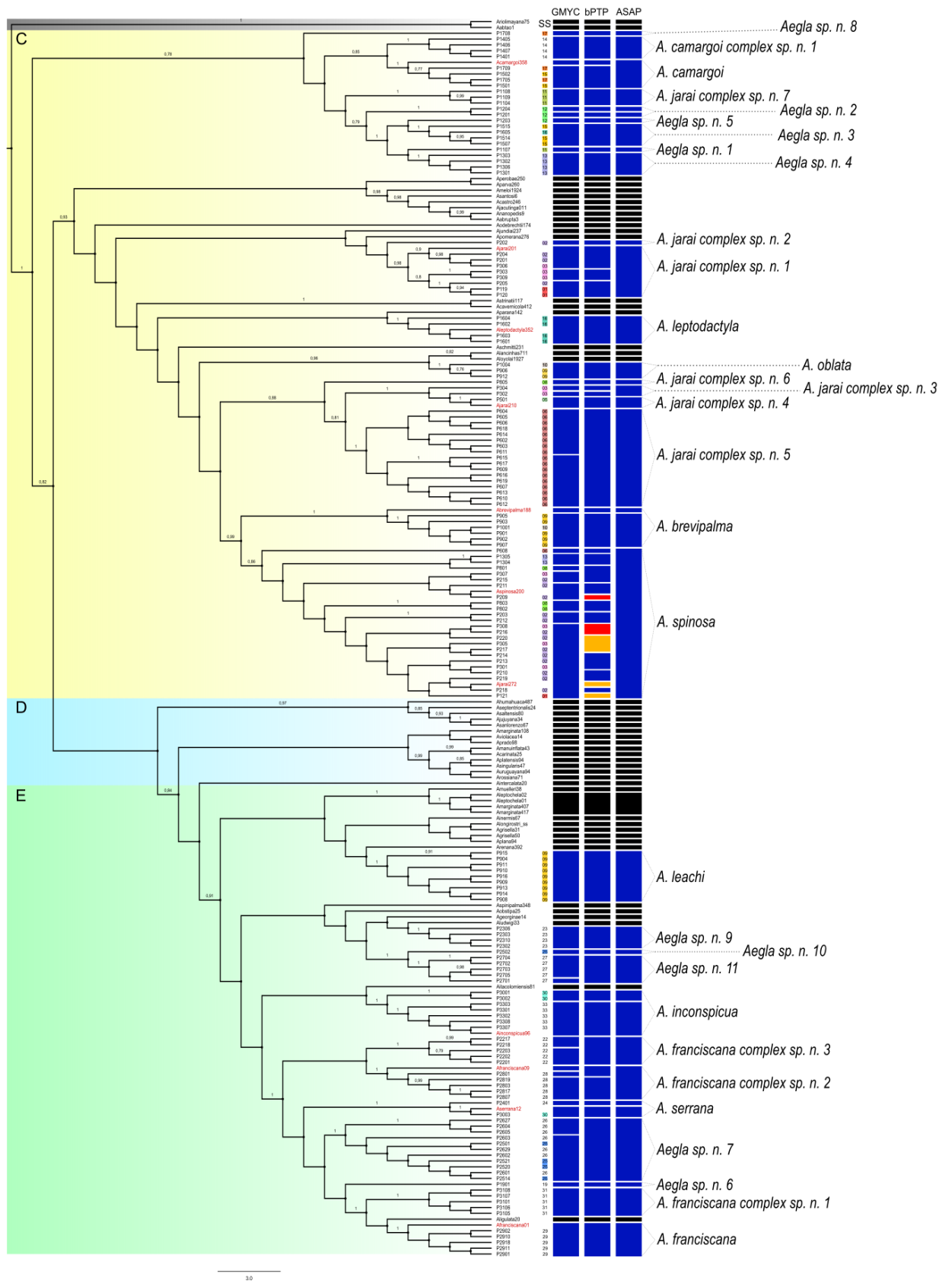


Fig. 2 Bayesian tree depicting phylogenetic relationships and cryptic diversity in the genus *Aegla* based on *COI* mtDNA sequences from this study and from GenBank (GB). Sequences from GB relevant to

delimitate species are highlighted in red. Numbers above branches represent Bayesian posterior probabilities. Posterior probabilities <0.75 are not shown. The colors in the branches represent the clades following Pérez-Losada et al. (2004). The sampling site column with colored boxes represent sites with sympatric occurrences. Molecular entities delimited by GMYC, bPTP and ASAP algorithms are also shown, with black bars representing GB delimitations not relevant for the study. Red and orange bars in bPTP delimitation represent groupings of phylogenetic distant sequences (for example, P209, P308 and P216 sequences were grouped).

Putative new species were found in the Canoas river basin (six species, all from the *A. jarai* complex), Pelotas river basin (six species, being one from the *A. jarai* complex and one from the *A. camargoi* complex), Apuaê-Inhandava river basin (two species), Taquari-Antas river basin (four species, with two belonging to the *A. franciscana* complex), Mampituba (three species) and Tramandaí river basin (one species from *A. franciscana* complex) (see Table S1). We did not record new MOTUs for the Sinos and Caí river basins.

Six of the new putative species (27%) were found only within PAs (of these, two are from the *A. jarai* complex, one from the *A. franciscana* complex, and three others), while 15 (68%) were found only in sites outside PAs. One putative specie (*Aegla jarai* complex sp. n. 1) was registered in both areas. Based on the integrative species delimitation, we found sympatric occurrences in 15 sites (55%), considering already described species and new ones, with three sites (2, 3, and 9) having three species and 12 sites (1, 6, 8, 10, 11, 12, 13, 15, 16, 17, 25 and 30) with two species.

Discussion

We identified a significant proportion of hidden diversity within the sampled in a priority area for conservation in Brazil, revealing that the diversity of the genus is greatly underestimated. Our results highlight the need to use integrative taxonomy to study taxa that are not easily identified by morphological characters only, allowing us to recognize their hidden diversity properly, an essential

knowledge to access the taxa conservation status and propose adequate conservation measures. *Aegla* is a highly speciose genus, with 93 currently known species (Marçal et al. 2021; WoRMS 2022) and many others to be described, some of them belonging to cryptic species complexes (Crivellaro et al. 2018; Zimmermann et al. 2018; Zimmermann et al. 2021). We confirmed the presence of cryptic complexes in *A. franciscana* and *A. jarai*, and we also revealed the existence of cryptic diversity in *A. camargoi*, summing 11 cryptic species. Surprisingly, 11 additional potential species were identified. These findings open the way for future studies describing the new species and solving problems of paraphyletic groups within clades C and E, increasing the resolution of the phylogenetic relationships among the species of the genus and improving our understanding of its evolutionary history.

A molecular phylogeny of the genus *Aegla*, using both nuclear and mitochondrial genes, recovered *A. jarai* as paraphyletic and closely related to *A. spinosa* (Pérez-Losada et al. 2004). We also recovered *A. jarai* as a paraphyletic group in our *COI*-based phylogeny, and our integrative approach to delimiting species suggests the presence of at least seven species within the *A. jarai* complex. The *COI* sequences available in GB are from specimens of *A. jarai* collected elsewhere than the type-locality, and our study area did not include the type-locality of this species; hence, we considered that all the putative new species are *A. jarai sensu lato*, belonging to the *A. jarai* species complex. It is worth noting that one sequence of *A. jarai* from GB is grouped within a clade including several of our sequences and a sequence from *A. spinosa* (also from GB). Due to the morphological identification of our specimens as *A. spinosa*, we opted to identify that clade as *A. spinosa*. Both *A. spinosa* and *A. brevipalma* are closely related to *A. jarai* in our study, and the phylogeny by Pérez-Losada et al. (2004) (*A. brevipalma* is identified as *Aegla* n. sp. 3 in this study since it was not yet described in 2004). Moreover, these three species exhibit some resemblance, so we could consider that they all belong to the *A. jarai* complex.

Aegla franciscana, recovered as paraphyletic by Pérez-Losada et al. (2004), was also confirmed as a species complex, comprising at least four species, including *A. franciscana sensu stricto*. We considered the specimens collected at site 29 as being *sensu stricto* since this site is near the type-locality of *A. franciscana*. It is interesting to note that the *A. jarai* and *A. franciscana* complexes are not monophyletic. For the *A. jarai* complex, besides a clade including the new species 3, 4, 5 and 6 from the *A. jarai*

complex along with *A. brevipalma* and *A. spinosa*, there is another clade including *A. jarai* n. sp. 1 and 2, along with *Aegla pomerana* and *Aegla Jundiai*, and plus other with *A. jarai* n. sp. 7 along with the new species *Aegla* sp. n. 1 to 5 (Fig. 2). Convergent evolution might be responsible for the morphological similarities between these non-monophyletic taxa.

In the case of *A. franciscana*, however, beyond the possibility of convergent evolution among the morphologically similar species of the complex, we cannot rule out a second hypothesis: the monophyletic clade ((((((*A. franciscana* + *Aegla ligulata*) + *A. franciscana* complex n. sp. 1) + *Aegla* n. sp. 6) + *Aegla* n. sp. 7) + *A. serrana*) (*A. franciscana* complex n. sp. 2 + *A. franciscana* complex n. sp. 3)) may correspond to only one species complex (see Fig. 2). The overall similarity among the species in this clade and their distributions areas agree with this idea. Recently diverged species may not have accumulated substantial morphological differences yet (Bickford et al. 2007).

Aegla camargoi was also revealed to be a possible complex including at least two species. *COI* sequences from the specimens morphologically identified as *A. camargoi* were recovered as two reciprocally monophyletic groups. While the species delimitation method ASAP suggested two putative species, GMYC and bPTP suggested three, separating the sequence retrieved from GB as a different entity. Recent speciation with no morphological differentiation is the most parsimonious explanation for this complex.

Although we have opted to be conservative and not oversplit species, by integrating traditional and molecular taxonomy, we cannot disregard the possibility that *A. serrana*, *A. inconspicua*, *A. spinosa*, and *A. brevipalma* might encompass more than one species, as suggested by the delimitation methods. More studies are necessary to investigate if these species also bear hidden diversity.

Integrative taxonomy reflects the best currently available framework for systematic studies, addressing different perspectives in the search for the best identification of new species and their description (Pante et al. 2015; Wei et al. 2022). In addition, information about the species distribution along the watersheds was also useful and should always be considered. The genus *Aegla* is sometimes problematic, with limited morphological characters and low taxonomic variation between species (Bond-Buckup and Buckup 1994), which could mislead identifications that rely solely on morphology,

overlooking interspecific differences. That is one of the reasons for the discrepancy between the number of species described and the potential number of species (Crivellaro et al. 2018). In our study, we had several specimens for which we could not surely identify the morphospecies, as the diagnostic characters did not wholly match those described for the species. The genetic data were extremely helpful in circumventing this situation to reveal the diversity hidden within the subtle morphological differences.

These different sources of evidence (morphological and genetic) are complementary to each other and, whenever possible, should be coupled in identifying and describing *Aegla* species. Furthermore, there is a need for new diagnostic characters for the identification and description of *Aegla* species (Crivellaro et al. 2018). Even with several tools used to access the diversity in the region, particular care should be taken regarding the consideration of the MOTUs as true species, that is, if they are reproductively isolated (Fiser et al. 2018). Despite that, they represent important evolutionary units with high potential to be confirmed as being new species with further investigation.

Based on the *Aegla* records for the study region (Bond-Buckup and Buckup 1994; Santos et al. 2010; Santos et al. 2012, 2017), the predominance of species from clades C and E was expected. We can note that the division between the Apuaê-Inhandava and Taquari-Antas rivers' basins is the breakpoint of these two clades for the region, with species from clade C dispersed in Apuaê-Inhandava and northern catchments (Pelotas and Canoas rivers' basins). In contrast, while species from clade E are dispersed through Taquari-Antas and southern and eastern catchments (Caí, Sinos, Mampituba, and Tramandaí river basins). Looking on a larger scale, species from clade C were found in the Uruguay River Hydrographic Region and species from clade E occurred in the South Atlantic Hydrographic Region, except *A. leachi* (clade E).

Our study area has a particular topology, with mountain ranges forming clear divisions between watersheds. Mountainous landscapes represent geographic barriers, with elevations forming habitat patches and breaking connectivity between different regions (Bertuzzo et al. 2016), acting as drivers of diversification in many taxa (Boschmann and Condamine 2022; Vences et al. 2009). In fact, aeglid richness positively correlates with altitude (Tumini et al. 2016), and the study region holds one of the highest aeglid diversity in Brazil (Pérez-Losada et al. 2009). Due to habitat isolation created by the

geomorphology, ancestral populations were probably split, favoring speciation and resulting in the high number of putative species observed. Many other factors can drive speciation events. In *Aegla*, factors such as low dispersion capability (Ayres-Peres et al. 2011), direct development, parental care, and habitat specialization should act as dispersion constraints (Baumart et al. 2015; López-Greco et al. 2004). These biological factors, together with geographic isolation, reduce the gene flow between populations and increase speciation rates (Loretán et al. 2020).

Sympatry between species of *Aegla* is commonly reported, with cases already recorded for the study region (Bond-Buckup and Buckup 1994; Castro-Souza and Bond-Buckup 2004). Using the integrative approach, we identified sympatric species in 55% of the sampled sites. Species from the *A. jarai* complex were involved in 40% of these cases. It is worth noting that sympatry did not occur between closely related species according to our phylogeny, except in site 2, where three species coexist (*A. spinosa*, *A. jarai* complex sp. 2, *A. jarai* complex sp. 3), with two of them being sister species. The high proportion of sympatry among aeglid species highlights the importance of morphological and molecular identification of several specimens when studying populations in order to avoid underestimation of richness or incorrect inferences due to species misidentification.

One of the main implications of this study is for the conservation of aeglids, a subject of urgent interest due to the high levels of endemism and increasing anthropic pressure on the freshwater habitat. The putative species unveiled by our analyses could reduce the geographical range of the known species for the region and have consequences for their management and conservation. Consider the case of *A. jarai*, which is distributed throughout the basins of the Canoas, Pelotas, and Itajaí-Açu rivers and was classified as Least Concern (LC) (Santos et al. 2017). The discovery that this species actually encompasses a complex may change the limits of the known geographic distribution of *A. jarai sensu stricto* and consequently impact its conservation status. On the other way, the new putative species *Aegla* n. sp 1 to 11 were found in only one site, except *Aegla* sp. n. 3 and 7 (found in two sampling sites), which makes these potential species, with a narrow preliminary distribution, almost automatically considered threatened following the guidelines established by IUCN to classify species extinction risks. New assessments should be conducted in the region to verify the range of these putative species and

confirm their narrow distribution. Also, an analysis to assess these populations' conservation status is of the utmost importance.

Among the nine already described species we found in our study area, considering those morphologically identified and confirmed by molecular analysis (*A. brevipalma*, *A. camargoi*, *A. franciscana*, *A. inconspicua*, *A. leachi*, *A. leptodactyla*, *A. oblata*, *A. serrana*, and *A. spinosa*), seven are under some level of threat (Critically Endangered, Endangered or Vulnerable – Santos et al. 2017). Of these threatened species, the most worrisome case is *A. brevipalma*, which is critically endangered, given the ongoing threats to the quality of its habitat – few sites in less than ten km² in the Canoas river basin (Santos et al. 2012). However, this study's records of *A. brevipalma* represent new occurrence sites for the region and may now be considered in future monitoring programs and studies. The two exceptions are *A. franciscana* and *A. spinosa*, classified as LC, but this classification may change for *A. franciscana*, given its cryptic diversity.

Overall, habitat degradation threatens almost every population of *Aegla*, and it could increase even more the number of endangered species in the genus, which is already extremely high (c. 70%, Santos et al. 2017). The outflow of domestic and industrial sewage into waterways, as well as suppression of riparian forests and siltation, are the main anthropogenic drivers of freshwater habitat degradation, limiting the availability of microhabitats in the environment and negatively influencing the abundance and distribution of these organisms (Bueno et al. 2016; Trevisan et al. 2009). Also, silvicultural practices of exotic species, such as *Pinus* sp. and *Eucalyptus* sp., along with the inappropriate use of pesticides, cause the degradation of water quality in the watershed as a result of surface runoff and lixiviation, contributing to the deterioration of aquatic niches (Chen et al. 2019; Queiroz et al. 2010; Rasmussen et al. 2011). In fact, a recent survey detected the presence of 19 different types of pesticides and five pharmaceuticals in streams in the study area, such as 2,4-dichlorophenoxy-acetic acid and atrazine, even in pristine protected areas (Mollmann et al. 2022). The harmful effect of pesticides in *A. longirostri* has been investigated, with an increase in oxidative stress and high levels of protein and lipid damage in tissues (Cerezer et al. 2020), highlighting the risks of exposure to these environmental contaminants.

Almost one-third of the new putative species of *Aegla* were found in streams inside terrestrial PAs, which are considered one of the greatest strongholds to protect biodiversity from different sources of threats (Geldman et al. 2014), such as the ones mentioned before. Although many PAs are designed to preserve terrestrial environments/taxa, providing only incidental protection to the water courses present within their borders (Dudley and Stolton 2003), they may offer adequate protection to the freshwater biota in some cases (Acreman et al. 2020). However, the streams inside PAs where we sampled the aeglids are not completely buffered from external chemical and biological contaminants (Mollmann et al. 2022), so species within terrestrial PAs still require attention. Ideally, the design of PAs should consider the freshwater habitats to protect their biota effectively. The high diversity of aeglid species unveiled makes our study area the richest known region regarding the number of *Aegla* species within its distribution area, making this region even more priority for conservation efforts. Our results reinforce the importance of the Upper Uruguay, Laguna dos Patos, and Tramandaí-Mampituba ecoregions for *Aegla* conservation. The sampling effort revealed new occurrence records for the genus in several sites, amplifying the knowledge of the distribution area of several species in the region. Finally, this article brings to light new cases of cryptic diversity within *Aegla*, which are continuously increasing with the use of molecular tools. New studies, expanding the sampling area, including specimens from type-localities of already described species and a set of nuclear and mitochondrial genes, will be carried out to confirm the taxonomic status of the new putative species and help in their future description. After this, the conservation status of the group should be reassessed, allowing the proposal of effective protection measures for this group of ecologically and evolutionarily unique anomuran crustaceans.

Supplementary information The online version contains supplementary material available at ...

Acknowledgments We are thankful to Prof. Dr. Georgina Bond-Buckup (*in memoriam*) for helping to delineate the project, to the administration of the PAs for permitting us to perform the research in the Parks, and Eduardo Pezzuto for letting us collect in his Private Natural Heritage Reserve Portal das Nascentes. We also thank Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for the sampling license.

Author contributions Conceptualization and developing methods: MLB-S, SS. Conducting the research: VHSM, MLB-S, SS, GF, ECM, MMD. Data analysis: VHSM, MLB-S, SS, GP. Data interpretation, preparation of figures and tables: VHSM, MLB-S, SS, GP. Original draft preparation: VHSM, MLB-S, SS. Writing, review and editing: VHSM, MLB-S, SS, GF, ECM, MMD, GP.

Funding This work was supported by CAPES foundation (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the fellowship to VHdSM and CNPq for the productivity grant to SS (process 311142/2014-1).

Data availability DNA sequence data are available at GenBank under accession numbers ***

Declarations

Conflict of interest The authors declare no conflict of interest.

References

Abell R, Thieme ML, Revenga C, Bryer M, Kottelat M, Bogutskaya N, Coad B, Mandrak N, Balderas SC, Bussing W, Stiassny MLJ, Skelton P, Allen GR, Unmack P, Naseka A, Ng R, Sindorf N, Robertson J, Armijo E, Higgins JV, Heibel TJ, Wikramanayake E, Olson D, López HL, Reis RE, Lundberg JG, Pérez MHS, Petry P (2008) Freshwater Ecoregions of the World: A New Map of Biogeographic Units for Freshwater Biodiversity Conservation. *BioScience* 58(5):403–414. <https://doi.org/10.1641/B580507>

- Acreman M, Hughes KA, Arthington AH, Tickner D, Duenas MA (2020) Protected areas and freshwater biodiversity: a novel systematic review distils eight lessons for effective conservation. *Conserv Lett* 13:e12684. <https://doi.org/10.1111/conl.12684>
- Adamowicz SJ, Purvis A (2005) How many branchiopod crustacean species are there? Quantifying the components of underestimation. *Global Ecol Biogeogr* 14:455-468. <https://doi.org/10.1111/j.1466-822X.2005.00164.x>
- Alvares CA, Stape JS, Sentelhas PC, Gonçalves JLM, Sparovek G (2014) Köppen's climate classification map for Brazil. *Meteorol Z* 22(6):711–728. <https://doi.org/10.1127/0941-2948/2013/0507>
- Ayres-Peres L, Coutinho C, Baumart JS, Gonçalves AS, Araujo PB, and Santos S (2011) Radio-telemetry techniques in the study of displacement of freshwater anomurans. *Nauplius* 19:41–54.
- Bandelt H, Forster P, and Röhl A (1999) Median-joining networks for inferring intraspecific phylogenies. *Mol Biol Evol* 16(1): 37–48. <https://doi.org/10.1093/oxfordjournals.molbev.a026036>
- Bartholomei-Santos ML, Santos S, Zimmermann BL, Pérez-Losada M, Crandall KA (2019) Evolutionary history and phylogenetic relationships of Aeglidae. In Santos S. and Bueno SLS (eds) *Aeglidae: Life history and conservation status of unique freshwater Anomuran decapods*, CRC Press, Boca Raton, pp 1-27.
- Baumart JS, Dalosto MM, Gonçalves AS, Palaoro AV, Santos S (2015) How to deal with a bad neighbor? Strategies of sympatric freshwater decapods (Crustacea) for coexistence. *Hydrobiologia* 762:29–39. <https://doi.org/10.1007/s10750-015-2331-0>

- Bertuzzo E, Carrara F, Mari L, Altermatt F, Rodriguez-Iturbe I, Rinaldo A (2016) Geomorphic controls on elevational gradients of species richness. *PNAS* 113(7):1737-1742. <https://doi.org/10.1073/pnas.1518922113>
- Bickford D, Lohman DJ, Sodhi NS, Ng PK, Meier R, Winker K, Ingram KK, Das I (2007) Cryptic species as a window on diversity and conservation. *Trends Ecol Evol* 22:148–155. <https://doi.org/10.1016/j.tree.2006.11.004>
- Bond-Buckup G, Buckup L (1994) A família Aeglidae (Crustacea, Decapoda, Anomura). *Arquivos de Zoologia* 32(4): 159–346. <https://doi.org/10.11606/issn.2176-7793.v32i4p159-346>
- Boos H, Salge PG, Pinheiro AA (2019) Conservation Status and Threats of Aeglidae: Beyond the Assessment. In Santos S. and Bueno SLS (eds) *Aeglidae: Life history and conservation status of unique freshwater Anomuran decapods*, CRC Press, Boca Raton, pp 233-251.
- Boschman LM, Condamine FL (2022) Mountain radiations are not only rapid and recent: Ancient diversification of South American frog and lizard families related to Paleogene Andean orogeny and Cenozoic climate variations. *Global Planet Change* 208:103704. <https://doi.org/10.1016/j.gloplacha.2021.103704>
- Bueno SLS, Santos S, da Rocha SS, Gomes KM, Mossolin EC, Mantelatto FL (2016) Avaliação do estado de conservação dos eglídeos (Decapoda: Aeglidae). In: Pinheiro M, Boss H. *Livro Vermelho dos Crustáceos do Brasil: Avaliação 2010–2014*. Porto Alegre: Sociedade Brasileira de Carcinologia–SBC, pp 35–63.
- Bueno SLS, Shimiz RM, Moraes JCB (2016) A Remarkable Anomuran: The taxon *Aegla* Leach, 1820. Taxonomic remarks, distribution, biology, diversity and conservation. In: Kawai T. and Cumberlidge N. (eds.), *A Global Overview of the Conservation of Freshwater Decapod Crustaceans*. Cham, Switzerland: Springer International Publishing AG, pp 23-64.

- Bueno SLS, Takano BF, Cohen FPA, Moraes JCB, Chiquetto-Machado PI, Vieira LCM, Shimizu RM (2014) Fluctuations in the Population Size of the Highly Endemic *Aegla Perobae* (Decapoda: Anomura: Aeglidae) Caused by a Disturbance Event. *J Crustacean Biol* 34 (2):165–173. <https://doi.org/10.1163/1937240X-00002224>
- Cadenillas R, D'Elía G (2021) The number of species of degus (genus *Octodon*) is currently underestimated: An appraisal of species limits and their phylogenetic relationships (Rodentia: Hystricomorpha: Octodontidae). *Mol Phylogenet Evol* 159:107111. <https://doi.org/10.1016/j.ympev.2021.107111>
- Castro-Souza T, Bond-Buckup G (2004) O nicho trófico de duas espécies simpátricas de *Aegla* (Crustacea, Aeglidae) no tributário da bacia do Rio Pelotas, Rio Grande do Sul, Brasil. *Rev Bras Zool* 21(4):805–813. <https://doi.org/10.1590/S0101-81752004000400014>
- Cerezer C, Marins AT, Cerezer FO, Severo ES, Leitemperger JW, Bandeira NMG, Zanella R, Loro VL, Santos S (2020) Influence of pesticides and abiotic conditions on biochemical biomarkers in *Aegla aff. longirostri* (Crustacea, Anomura): Implications for conservation. *Ecotox Environ Safe* 203:110982. <https://doi.org/10.1016/j.ecoenv.2020.110982>
- Chang SW, Oshida T, Endo H, Nguyen ST, Dang CN, Nguyen DX, Jiang X, Li Z-J, Lin L-K (2011) Ancient hybridization and underestimated species diversity in Asian striped squirrels (genus *Tamiops*): inference from paternal, maternal and biparental markers. *J Zool* 285:128-138. <https://doi.org/10.1111/j.1469-7998.2011.00822.x>
- Chen C, Guo W, Ngo HH (2019) Pesticides in stormwater runoff – a mini review. *Front Env Sci Eng* 13:1–72. <https://doi.org/10.1007/s11783-019-1150-3>
- Cicconardi F, Fanciulli PP, Emerson BC (2013) Collembola, the biological species concept and the underestimation of global species richness. *Mol Ecol* 22:5382-5396. <https://doi.org/10.1111/mec.12472>

- Costello MJ (2015) Biodiversity: The Known, Unknown, and Rates of Extinction. *Curr Biol* 25:R362–R383. <https://doi.org/10.1016/j.cub.2015.03.051>
- Costello MJ, May RM, Stork NE (2013) Can We Name Earth's Species Before They Go Extinct? *Science* 339:413–416. <https://doi.org/10.1126/science.1230318>
- Crivellaro MS, Zimmermann BL, Bartholomei-Santos ML, Crandall KA, Pérez-Losada M, Bond-Buckup G, Santos S (2018) Looks can be deceiving: species delimitation reveals hidden diversity in the freshwater crab *Aegla longirostri* (Decapoda: Anomura). *Zool J Linn Soc Lond*, 182(1):24–37. <https://doi.org/10.1093/zoolinnean/zlx030>
- Darriba D, Taboada GL, Doallo R, Posada D (2012) jModelTest 2: More models, new heuristics and parallel computing. *Nat Methods* 9:772. <https://doi.org/10.1038/nmeth.2109>
- Dawson, TPP, Jackson ST, House JI, Prentice IC, Mac GM (2011) Beyond Predictions: Biodiversity Conservation in a Changing Climate. *Science* 332:53–58. <https://doi.org/10.1126/science.1200303>
- Drummond AJ, Suchard MA, Xie D, Rambaut A (2012) Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Mol Biol Evol* 29:1969–1973. <https://doi.org/10.1093/molbev/mss075>
- Dudley N, Stolton S (2003) Running Pure: The Importance of Forest Protected Areas to Drinking Water. *World Bank/WWF Alliance for Forest Conservation and Sustainable Use*. World Bank. <https://openknowledge.worldbank.org/handle/10986/15006>
- Edgar RC (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797. <https://doi.org/10.1093/nar/gkh340>
- Fiser C, Robinson C T, Malard F (2018). Cryptic species as a window into the paradigm shift of the species concept. *Mol Ecol* 27, 613–635. <https://doi.org/10.1111/mec.14486>

- Funk DJ, Omland KE (2003) Species-Level Paraphyly and Polyphyly: Frequency, Causes, and Consequences, with Insights from Animal Mitochondrial DNA. *Annu Rev Ecol Evol S* 34(1):397-423. <https://doi.org/10.1146/annurev.ecolsys.34.011802.132421>
- Geldmann J, Barnes M, Coad L, Craigie ID, Hockings M, Burgess ND (2013) Effectiveness of terrestrial protected areas in reducing habitat loss and population declines. *Biol Conserv* 161:230–238. <http://dx.doi.org/10.1016/j.biocon.2013.02.018>
- Gonçalves AS, Costa GC, Bond-Buckup G, Bartholomei-Santos ML, Santos S (2018) Priority areas for conservation within four freshwater ecoregions in South America: A scale perspective based on freshwater crabs (Anomura, Aeglidae). *Aquat Conserv* 28:1077–1088. <https://doi.org/10.1002/aqc.2965>
- Grummer JA, Bryson RW, Reeder TW (2014) Species delimitation using Bayes factors: Simulations and application to the *Sceloporus scalaris* species group (Squamata: Phrynosomatidae). *Syst Biol* 63:119–133. <https://doi.org/10.1093/sysbio/syt069>
- Jaramillo AF, Riva IDL, Guayasamin JM, Chaparro JC, Gagliardi-Urrutia G, Gutiérrez RC, Brcko I, Vilà C, Castroviejo-Fisher S (2020) Vastly underestimated species richness of Amazonian salamanders (Plethodontidae: *Bolitoglossa*) and implications about plethodontid diversification. *Mol Phylogenet Evol* 149:106841. <https://doi.org/10.1016/j.ympev.2020.106841>
- Kass RE, Raftery AE (1995) Bayes Factors. *J Am Stat Assoc* 90(430):773-795. <https://doi.org/10.2307/2291091>
- Kumar S, Stecher G, Li M, Knyaz C, Tamura K (2018) MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Mol Biol Evol* 35:1547-1549. <https://doi.org/10.1093/molbev/msy096>

- Leaché AD, Fujita MK, Minin VN, Bouckaert RR (2014) Species delimitation using genome-wide SNP data. *Syst Biol* 63(4):534–542. <https://doi.org/10.1093/sysbio/syu018>
- Lefébure T, Douady CJ, Gouy M, Gibert J (2006) Relationship between morphological taxonomy and molecular divergence within Crustacea: proposal of a molecular threshold to help species delimitation. *Mol Phylogenet Evol* 40:435–447. <https://doi.org/10.1016/j.ympev.2006.03.014>
- Leigh JW, Bryant D (2015) PopART: Full-feature software for haplotype network construction. *Methods Ecol Evol* 6(9):1110–1116. <https://doi.org/10.1111/2041-210X.12410>
- Li H, Kong L, Wang K, Zhang S, Motokawa M, Wu Y, Wang W, Li Y (2019) Molecular phylogeographic analyses and species delimitations reveal that *Leopoldamys edwardsi* (Rodentia: Muridae) is a species complex. *Integr Zool* 14:494–505. <https://doi.org/10.1111/1749-4877.12378>
- López-Greco LS, Viau V, Lavolpe M, Bond-Buckup G, Rodriguez EM (2004) Juvenile hatching and maternal care in *Aegla uruguayana* (Anomura, Aeglidae). *J Crustacean Biol* 24:309–313. <https://doi.org/10.1651/C-2441>
- Loretán G, Rueda EC, Cabrera JM, Pérez-Losada M, Collins PA, Giri F (2020) Geographical isolation and restricted gene flow drive speciation of *Aegla singularis* (Decapoda: Anomura: Aeglidae) in southern South America. *Biol J Linn Soc* 129:177–189. <https://doi.org/10.1093/biolinnean/blz148>
- Maia KP, Bueno SLS, Trajano E (2013) Ecologia populacional e conservação de eglídeos (Crustacea: Decapoda: Aeglidae) em cavernas da área cárstica do Alto Ribeira, em São Paulo. *Revista da Biologia* 10:40–45. <https://doi.org/10.7594/revbio.10.02.07>
- Marçal IC, Páez FP, Silva PF, Souza-Shibatta L, Teixeira GM (2021) Cryptic diversity among populations of *Aegla* Leach, 1820 (Decapoda: Anomura: Aeglidae) from Tibagi Riverbasin,

Paraná state, Brazil, with descriptions of three new species. *J Nat Hist* 55: 2145–2171.

<https://doi.org/10.1080/00222933.2021.1984599>

Martins FC, Cox SC, Irestedt M, Prÿs-Jones RP, Day JJ (2020) A comprehensive molecular phylogeny of Afrotropical white-eyes (Aves: Zosteropidae) highlights prior underestimation of mainland diversity and complex colonisation history. *Mol Phylogenet Evol* 149:106843.

<https://doi.org/10.1016/j.ympev.2020.106843>

MMA/IBAMA – Plano de Manejo do Parque Nacional de Aparados da Serra e Serra Geral (2003).

Available at: https://www.gov.br/icmbio/pt-br/assuntos/biodiversidade/unidade-de-conservacao/unidades-de-biomas/mata-atlantica/lista-de-ucs/parna-da-serra-geral/arquivos/DCOM_pm_Parna_de_Aparados_da_Serra_Encarte_1.pdf

Mollmann VHS, Santos S, Fernandes G, Mossolin EC, Dalosto MM, Cardoso SMVS, Prestes OD, Zanella R, Bartholomei-Santos ML (2022) Terrestrial protected areas do not fully shield their streams from exogenous stressors. *Environ Conserv* 1–10.

<https://doi.org/10.1017/S0376892922000261>

Mora C, Tittensor DP, Adl S, Simpson AGB, Worm B (2011) How Many Species Are There on Earth and in the Ocean? *PLoS Biol* 9(8):e1001127. <https://doi.org/10.1371/journal.pbio.1001127>

Moraes JC, Terossi M, Buranelli RC, Tavares M, Mantelatto FL, Bueno SLS (2016) Morphological and molecular data reveal the cryptic diversity among populations of *Aegla paulensis* (Decapoda, Anomura, Aeglididae), with descriptions of four new species and comments on dispersal routes and conservation status. *Zootaxa* 4193:1–48. <https://doi.org/10.11646/zootaxa.4193.1.1>

Noguerales V, Cordero PJ, Ortego J (2018) Integrating genomic and phenotypic data to evaluate alternative phylogenetic and species delimitation hypotheses in a recent evolutionary radiation of grasshoppers. *Mol Ecol* 27:1229–1244. <https://doi.org/10.1111/mec.14504>

- Oliveira-Filho AT, Budke JC, Jarenkov JA, Eisenlohr PV, Neves DRM (2013) Delving into the variations in tree species composition and richness across South American subtropical Atlantic and Pampean forests. *J Plant Ecol* 8:242–260. <https://doi.org/10.1093/jpe/rtt058>
- Pante E, Schoelinck C, Puillandre N (2015) From Integrative Taxonomy to Species Description: One Step Beyond. *Syst Biol* 64(1):152–160. <https://doi.org/10.1093/sysbio/syu083>
- Pérez-Losada M, Bond-Buckup G, Jara CG, Crandall KA (2004) Molecular systematics and biogeography of the southern South American freshwater “crabs” *Aegla* (Decapoda: Anomura: Aegliidae) using multiple heuristic tree search approaches. *Syst Biol* 53:767–780. <https://doi.org/10.1080/10635150490522331>
- Pérez-Losada M, Bond-Buckup G, Jara CG, Crandall KA (2009) Conservation assessment of Southern South American freshwater ecoregions on the basis of the distribution and genetic diversity of crabs from the genus *Aegla*. *Conserv Biol* 23:692–702. <https://doi.org/10.1111/j.1523-1739.2008.01161.x>
- Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, Hazell S, Kamoun S, Sumlin WD, Vogler AP (2006) Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Syst Biol* 55:595–609. <https://doi.org/10.1080/10635150600852011>
- Puillandre N, Lambert A, Brouillet S, Achaz G (2012) ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Mol Ecol* 21:1864–1877. <https://doi.org/10.1111/j.1365-294X.2011.05239.x>
- Puillandre N, Brouillet S, Achaz G (2020) ASAP: assemble species by automatic partitioning. *Mol Ecol Resour* 21:609–620. <https://doi.org/10.1111/1755-0998.13281>
- Queiroz GMP, Silva MR, Bianco RJF, Pinheiro A, Kaufmann V (2011). Transporte de Glifosato pelo escoamento superficial e por lixiviação em um solo agrícola. *Revista Química Nova* 34:190-195.

- R Core Team (2022). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL <https://www.R-project.org/>.
- Rambaut A (2014) Figtree v.1.4.2. Software. Institute of Evolutionary Biology, University Edinburgh. Available at: <http://tree.bio.ed.ac.uk/software/figtree/>
- Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA (2018) Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol* 67:901. <https://doi.org/10.1093/sysbio/syy032>
- Rasmussen JJ, Baattrup-Pedersen A, Wiberg-Larsena P, McKnight US, Kronvang B (2011) Buffer strip width and agricultural pesticide contamination in Danish lowland streams: implications for stream and riparian management. *Ecol Eng* 37: 1990–1997. <https://doi.org/10.1016/j.ecoleng.2011.08.016>
- Reid NM, Carstens BC (2012) Phylogenetic estimation error can decrease the accuracy of species delimitation: A Bayesian implementation of the general mixed Yule-coalescent model. *BMC Evol Biol* 12(1):196. <https://doi.org/10.1186/1471-2148-12-196>
- Rozas J, Ferrer-Mata A, Sánchez-DelBarrio JC, Guirao-Rico S, Librado P, Ramos-Onsins SE, Sánchez-Gracia A (2017) DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Mol Biol Evol* 34(12):3299–3302. <https://doi.org/10.1093/molbev/msx248>
- Sabatelli S, Ruspantini P, Cardoli P, Audisio P (2021) Underestimated diversity: Cryptic species and phylogenetic relationships in the subgenus *Cobalius* (Coleoptera: Hydraenidae) from marine rockpools. *Mol Phylogenet Evol* 163: 107243. <https://doi.org/10.1016/j.ympev.2021.107243>
- Santos S, Bond-Buckup G, Buckup L, Pérez-Losada M, Finley M, Crandall KA (2012) Three new species of *Aegla* (Anomura) freshwater crabs from the upper Uruguay river hydrographic basin in Brazil. *J Crustacean Biol* 32(4):529-540. <https://doi.org/10.1163/193724012X635935>

- Santos S, Bond-Buckup G, Gonçalves AS, Bartholomei-Santos ML, Buckup L, Jara CG (2017) Diversity and conservation status of *Aegla* spp. (Anomura, Aeglidae): An update. *Nauplius* 25:e2017011. <https://doi.org/10.1590/2358-2936e2017011>
- Santos S, Bond-Buckup G, Pérez-Losada M, Jara CG, Crandall KA, Buckup L (2010) New records and description of a new species of Aeglidae (Crustacea: Anomura) from river basins in Southern Brazil. *Nauplius* 18(1):79-86.
- Stork NE, Coddington JA, Colwell RK, Chazdon RL, Dick CW, Peres CA, Sloan S, Willis K (2009) Vulnerability and Resilience of Tropical Forest Species to Land-Use Change. *Conserv Biol* 23:1438-1447. <https://doi.org/10.1111/j.1523-1739.2009.01335.x>
- Struck TH, Cerca J (2019) Cryptic Species and Their Evolutionary Significance. In eLS, John Wiley and Sons, Ltd. <https://doi.org/10.1002/9780470015902.a0028292>
- Struck TH, Feder JL, Bendiksby M, Birkeland S, Cerca J, Gusarov VI, Sonja Kistenich, Larsson K-H, Liow LH, Nowak MD, Stedje B, Bachmann L, Dimitrov D (2018) Finding Evolutionary Processes Hidden in Cryptic Species. *Trends Ecol Evol* 33(3):153-163. <https://doi.org/10.1016/j.tree.2017.11.007>
- Tamura K, Stecher G, Kumar S (2021) MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Mol Biol Evol* 38:3022-3027. <https://doi.org/10.1093/molbev/msab120>
- Torres-Carvajal O, Terán C (2021) Molecular phylogeny of Neotropical Parrot Snakes (Serpentes: Colubrinae: *Leptophis*) supports underestimated species richness. *Mol Phylogenet Evol* 164:107267. <https://doi.org/10.1016/j.ympev.2021.107267>
- Trevisan A, Hepp LU, Santos S (2009) Abundância e distribuição de Aeglidae (Crustacea: Anomura) em função do uso da terra na bacia hidrográfica do Rio Jacutinga, Rio Grande do Sul, Brasil. *Zoologia* 26(3):419–426. <https://doi.org/10.1590/S1984-46702009000300006>

- Tumini G, Giri F, Williner V, Collins PA (2016) The importance of biogeographical history and extant environmental conditions as drivers of freshwater decapod distribution in southern South America. *Freshwater Biol* 61:715–728. <https://doi.org/10.1111/fwb.12742>
- Vences M, Wollenberg KC, Vieites DR, Lees DC (2009) Madagascar as a model region of species diversification. *Trends Ecol Evol* 24(8):456-465. <https://doi.org/10.1016/j.tree.2009.03.011>
- Walker M, Lyra ML, Haddad CFB (2018) Phylogenetic relationships and cryptic species diversity in the Brazilian eggbrooding tree frog, genus *Fritziana* Mello-Leitão 1937 (Anura: Hemiphractidae). *Mol Phylogenet Evol* 123:59–72. <https://doi.org/10.1016/j.ympev.2018.02.012>
- Wei C, Sangster G, Olsson U, Rasmussen PC, Svensson L, Yao C, Carey GJ, Leader PJ, Zhang R, Chen G, Song G, Lei F, Wilcove DS, Alström P, Liu Y (2022) Cryptic species in a colorful genus: Integrative taxonomy of the bush robins (Aves, Muscicapidae, *Tarsiger*) suggests two overlooked species. *Mol Phylogenet Evol* 175:107580. <https://doi.org/10.1016/j.ympev.2022.107580>
- WoRMS Editorial Board (2022) World Register of Marine Species. Available from <https://www.marinespecies.org> at VLIZ.
- Xie W, Lewis PO, Fan Y, Kuo L, Chen MH (2011) Improving marginal likelihood estimation for Bayesian phylogenetic model selection. *Syst Biol* 60:150–160. <https://doi.org/10.1093/sysbio/syq085>
- Xu J, Pérez-Losada M, Jara CG, Crandall KA (2009) Pleistocene glaciation leaves deep signature on the freshwater crab *Aegla alacalufi* in Chilean Patagonia. *Mol Ecol* 18:904– 918. <https://doi.org/10.1111/j.1365-294X.2008.04070.x>
- Yang Z, and Rannala B (2014) Unguided species delimitation using DNA sequence data from multiple loci. *Mol Biol Evol* 31:3125–3135. <https://doi.org/10.1093/molbev/msu279>

Zhang J, Kaplil P, Pavlidis P, Stamatakis A (2013) A general species delimitation method with applications to phylogenetic placements. *Bioinformatics* 29(22):2869–2876.

<https://doi.org/10.1093/bioinformatics/btt499>

Zimmermann BL, Crivellaro MS, Hauschild CB, Bartholomei-Santos ML, Crandall KA, Pérez-Losada M, Giri F, Collins P, Santos S (2018) Phylogeography reveals unexpectedly low genetic diversity in a widely distributed species: The case of the freshwater crab *Aegla platensis* (Decapoda: Anomura). *Biol J Linn Soc* 123:578–592.

<https://doi.org/10.1093/biolinnean/blx166>

Zimmermann BL, Buzatto I, Santos S, Giri F, Mello FT, Crandall KA, Pérez-Losada M, Bartholomei-Santos ML (2021) Entangled Aegliidae (Decapoda, Anomura): Additional evidence for cryptic species. *Zool Scr* 50:473–484. <https://doi.org/10.1111/zsc.12483>

Table S1 – Specifications of each of the 33 sampling sites selected for the study and the species recorded in each through different sources. Site number in bold represent sampling sites within Protected Areas. For spatial localization, see Figure 1. The asterisk (*) refers to the phylogenetic clades established by Pérez-Losada et al. (2004) for the whole *Aegla* genus. The dash (-) represents sites where no specimens were registered.

Site	Latitude (S)	Longitude (W)	Altitude (m)	Stream	River Basin	Morphological identification	Integrative taxonomy	Phylogenetic clade*
01	28°3'12.30"S	49°22'40.04"W	1031	Corvo Branco	Canoas	<i>A. jarai</i>	<i>A. spinosa</i> and <i>A. jarai</i> complex <i>sp. n. 1</i>	C
02	28°3'29.24"S	49°25'32.63"W	960	Bispo	Canoas	<i>A. jarai</i> and <i>A. spinosa</i>	<i>A. spinosa</i> , <i>A. jarai</i> complex <i>sp. n. 1</i> and <i>A. jarai</i> complex <i>sp. n. 2</i>	C
03	28°2'56.53"S	49°28'38.58"W	966	Cachimbo	Canoas	<i>A. jarai</i> and <i>A. spinosa</i>	<i>A. spinosa</i> , <i>A. jarai</i> complex <i>sp. n. 1</i> and <i>A. jarai</i> complex <i>sp. n. 3</i>	C
04	28°6'41.50"S	49°30'56.21"W	1451	Urubici tributary	Canoas	-	-	-
05	28°6'47.01"S	49°31'50.77"W	1440	Urubici tributary	Canoas	<i>A. leachi</i>	<i>A. jarai</i> complex <i>sp. n. 4</i>	C
06	28°4'36.74"S	49°31'7.60"W	1377	Urubici tributary	Canoas	<i>A. jarai</i> and <i>Aegla sp.</i>	<i>A. spinosa</i> and <i>A. jarai</i> complex <i>sp. n. 5</i>	C
07	27°54'9.46"S	49°44'45.52"W	865	Lagoa Preta	Canoas	-	-	-
08	27°53'54.73"S	49°44'59.98"W	868	Tigre	Canoas	<i>A. jarai</i> and <i>A. spinosa</i>	<i>A. spinosa</i> and <i>A. jarai</i> complex <i>sp. n. 6</i>	C
09	27°54'33.99"S	50° 5'14.32"W	1125	Caveira tributary	Canoas	<i>A. brevipalma</i> , <i>A. leachi</i> and <i>A. jarai</i>	<i>A. brevipalma</i> , <i>A. leachi</i> and <i>A. oblata</i>	C and E
10	27°54'13.36"S	50° 4'21.49"W	1139	Caveirinha	Canoas	<i>A. jarai</i> and <i>A. leachi</i>	<i>A. brevipalma</i> and <i>A. oblata</i>	C
11	28°15'22.05"S	49°57'56.34"W	1163	Antoninha	Pelotas	<i>A. jarai</i> and <i>A. oblata</i>	<i>Aegla sp. n. 1</i> and <i>A. jarai</i> complex <i>sp. n. 7</i>	C

12	28°21'37.46"S	49°58'17.07"W	1243	São Mateus tributary	Pelotas	<i>A. jarai</i> and <i>A. leachi</i>	<i>Aegla</i> sp. n. 2 and <i>Aegla</i> sp. n. 5	C
13	28°23'27.02"S	49°54'51.28"W	1050	Invernadinha	Pelotas	<i>A. spinosa</i>	<i>A. spinosa</i> and <i>Aegla</i> sp. n. 4	
14	28°26'32.93"S	49°51'18.28"W	946	Sete Arroio	Pelotas	<i>A. camargoi</i>	<i>A. camargoi</i> complex sp. n. 1	C
15	28°33'58.81"S	49°54'13.68"W	1290	Lajeadozinho	Apuaê-Inhandava	<i>A. camargoi</i>	<i>A. camargoi</i> and <i>Aegla</i> sp. n. 3	C
16	28°36'21.35"S	49°56'7.27"W	1151	Marco	Apuaê-Inhandava	<i>A. leptodactyla</i>	<i>A. leptodactyla</i> and sp. n. 3	C
17	28°38'54.91"S	49°56'46.85"W	1176	Silveira tributary	Apuaê-Inhandava	<i>A. camargoi</i> and <i>A. oblata</i>	<i>A. camargoi</i> and <i>Aegla</i> sp. n. 8	C
18	28°43'38.22"S	50° 1'28.34"W	1188	Manuel Leão	Apuaê-Inhandava	-	-	-
19	28°48'39.99"S	50° 3'37.13"W	1006	das Antas tributary	Taquari-Antas	<i>A. plana</i>	<i>Aegla</i> sp. n. 6	E
20	28°51'1.46"S	50° 1'25.28"W	1004	das Antas	Taquari-Antas	-	-	-
21	28°57'11.32"S	50° 3'30.63"W	862	Reserva tributary	Taquari-Antas	-	-	-
22	29°4'8.90"S	50° 1'35.08"W	1004	Reserva	Taquari-Antas	<i>A. itacolomiensis</i>	<i>A. franciscana</i> complex sp. n. 3	E
23	29°4'43.01"S	49°59'12.38"W	1008	Pedras	Mampituba	<i>A. serrana</i>	<i>Aegla</i> sp. n. 9	E
24	29°4'1.98"S	49°57'45.61"W	1026	Pedras tributary	Mampituba	<i>A. serrana</i>	<i>A. serrana</i>	E
25	29°9'25.95"S	50° 3'6.02"W	927	Perdizes	Mampituba	<i>A. itacolomiensis</i>	<i>Aegla</i> sp. n. 7 and <i>Aegla</i> sp. n. 10	E
26	29°9'28.43"S	50° 4'44.80"W	921	Perdizes	Mampituba	<i>A. itacolomiensis</i>	<i>Aegla</i> sp. n. 7	E
27	29°4'50.17"S	50°10'56.51"W	894	Camisa	Taquari-Antas	<i>A. itacolomiensis</i>	<i>Aegla</i> sp. n. 11	E
28	29°12'36.37"S	50°14'20.61"W	926	Baio Branco	Taquari-Antas	<i>A. franciscana</i>	<i>A. franciscana</i> complex sp. n. 2	E
29	29°23'38.00"S	50°24'51.95"W	864	Rolantinho	dos Sinos	<i>A. franciscana</i>	<i>A. franciscana</i>	E
30	29°24'43.40"S	50°22'58.70"W	919	Rolantinho tributary	dos Sinos	<i>A. inconspicua</i>	<i>A. inconspicua</i> and <i>A. serrana</i>	E

31	29°24'20.74"S	50°17'34.39"W	877	Forqueta	Tramandaí	<i>A. rossiana</i>	<i>A. franciscana</i> <i>complex sp. n. 1</i>	E
32	29°21'49.24"S	50°31'20.56"W	791	Caí	Caí	-		E
33	29°23'3.95"S	50°32'2.75"W	812	Caí tributary	Caí	<i>A. franciscana</i>	<i>A. inconspicua</i>	E

Table S2 - Performance of the models tested in the phylogenetic analysis, ranked according to Stepping Stone (SS) sampling and Bayes Factor (BF) values. All BF values were calculated against the coalescent model with strict molecular clock (considered model 1 in the calculations), according to Kass & Raftery (1995). Positive BF values indicate support for model 1.

	SS	$2\ln\text{BF}$	Rank
Model 1 (Coalescent Strict)	-8671.54		1
Model 2 (Coalescent Relaxed)	-8913.17	483.27	2
Model 3 (Yule Relaxed)	-8959.93	576.77	3
Model 4 (Yule Strict)	-8971.55	600.03	4

5 DISCUSSÃO GERAL

À medida que a expansão das áreas urbanas e agrícolas cresce, maior é a pressão humana sobre os corpos de água, potencialmente interferindo na conservação da biodiversidade aquática. Nesse sentido, as UCs são fundamentais para evitar a degradação de habitat e perda da biota. Estudos prévios identificaram algumas áreas prioritárias para a conservação de crustáceos de água doce no sul do Brasil, que abrangem remanescentes do bioma Mata Atlântica, uma região altamente impactada por distúrbios antropogênicos, e que localizam-se próximas a algumas UCs (PÉREZ-LOSADA et al., 2009; GONÇALVES et al., 2018). Comparando conjuntos de parâmetros de quatro fontes diferentes - físico-químicos, biológicos, pesticidas e fármacos – dentro e em torno de quatro UCs terrestres, observamos que a qualidade da água dos rios e riachos dentro das UCs reflete as pressões externas e expressa a “saúde” dos corpos d’água na região como um todo. *Outliers* visualizados nos resultados do Capítulo I representam possíveis focos pontuais de poluição recente. A seguir, discutimos brevemente sobre cada um dos parâmetros de qualidade da água analisados.

Todos os pontos de amostragem apresentaram temperatura média da água abaixo de 20°C, que pode ser considerada adequada para a área de estudo no final da primavera, quando as amostragens foram realizadas. Entretanto, não há um padrão considerado “normal” para a temperatura da água, uma vez que esta depende não apenas de aspectos locais como altitude e temperatura do ar, mas também é influenciada pelas atividades humanas. Retirada de água dos rios, descargas industriais e domésticas e remoção de matas ciliares são exemplos de atividades que aumentam a temperatura da água; por outro lado, a liberação de água fria de um reservatório termicamente estratificado pode diminuir a temperatura da água de rios e riachos (EUA/EPA, 2017).

Como o oxigênio é essencial para o processo respiratório, sua concentração dissolvida na água não é apenas uma medida da saúde do rio ou riacho, mas também um fator limitante para a maioria dos organismos (FRANKLIN, 2014). Os fatores que influenciam a concentração de oxigênio na água incluem a produção fotossintética de oxigênio por algas e outras vegetações aquáticas, a introdução de oxigênio de tributários e a aeração da atmosfera; esta última, por sua vez, é afetada pela temperatura, profundidade e velocidade da água, ventos e presença de cachoeiras ou represas (COX, 2003; FRANKLIN, 2014). A maioria dos cursos d’água analisados apresentou concentrações acima de 6 mg/L de O₂ dissolvido, que é a concentração mínima para a água ser considerada de boa qualidade, segundo a resolução 357/2005 do Conselho Nacional de Meio Ambiente (BRASIL, 2005), para classificação de corpos d’água na

Classe 1, e de acordo com os critérios de vida aquática estabelecidos pela Agência de Proteção Ambiental dos Estados Unidos (EUA/EPA, 1986). Porém, os pontos de coleta 18 e 30 merecem atenção. Ambos mostraram valores de oxigênio dissolvido limítrofes, o que poderia indicar o início de processos de eutrofização, resultar em condições hipóxicas e afetar os organismos aquáticos. Baixos teores de oxigênio dissolvido podem forçar os animais a mudar seus padrões respiratórios ou diminuir seu nível de atividade, o que, por sua vez, poderia retardar o desenvolvimento e/ou causar deficiências reprodutivas, tais como aumento da mortalidade e defeitos embrionários (COX, 2003).

A condutividade é um importante parâmetro físico da água, e quanto maior o valor (em micro Siemens por centímetro) maior a concentração de íons inorgânicos na água, relacionando-os a potenciais poluidores (HORNE & GOLDMAN, 1994; BAIRD & BRIDGEWATER, 2017). Todos os pontos de coleta apresentaram valores considerados baixos, variando de 15 $\mu\text{S}/\text{cm}$ a 67 $\mu\text{S}/\text{cm}$. Embora aceitável, a faixa de valores de condutividade que encontramos é muito maior do que aquela encontrada para córregos no Parque Estadual da Serra do Mar (12,2 a 21,7 $\mu\text{S}/\text{cm}$), UC na Mata Atlântica localizada no estado de São Paulo (KUHLMANN et al., 2014). As diferenças entre os estudos podem ser devidas a diferentes estruturas de vegetação ripária nas UCs analisadas, já que a variação da condutividade elétrica e do oxigênio dissolvido na água está relacionada à estrutura ripária em locais com presença de agricultura (DE SOUZA et al., 2013). Os impactos da condutividade elétrica sobre os seres vivos geralmente estão relacionados à presença de íons presentes em compostos como sais e metais pesados, os quais levam aos altos valores de condutividade (OERTLI & PARRIS, 2019). Em riachos centrais dos Montes Apalaches, nos Estados Unidos, foram identificadas grandes perdas nas populações de macroinvertebrados, algas e bactérias quando a condutividade excedeu 150 $\mu\text{S}/\text{cm}$, mesmo com baixos níveis de impacto de mineração (SIMONIN et al., 2021). Em condições experimentais, a sobrevivência embrionária do anuro *Hyla versicolor* foi negativamente correlacionada à condutividade durante o desenvolvimento (BRAND et al., 2010).

A concentração de íons de hidrogênio, ou pH, afeta a maioria dos processos químicos e biológicos na água, atuando como um dos mais importantes fatores ambientais limitando a distribuição das espécies em habitats aquáticos. Diferentes espécies desenvolvem-se dentro de diferentes faixas de pH, mas a extensão ideal para a maioria dos organismos aquáticos situa-se entre pH 6,5 - 8 (EUA/EPA, 2017). O pH flutuante ou constante fora desta faixa estressa muitas espécies fisiologicamente e pode interferir nas taxas de reprodução e crescimento, levar a doenças ou morte, resultando eventualmente na redução da diversidade biológica nos cursos

d'água. Nos rios, o pH é afetado principalmente pela geologia da fonte de água e por contaminantes químicos (MACKIE, 2004; EUA/EPA, 2017). No Brasil, os critérios de qualidade da água para o pH em água doce sugerem uma faixa de 6,0 a 9,0 (BRASIL, 2005). Um dos parâmetros mais básicos de qualidade da água, o pH não diferiu significativamente entre os riachos amostrados, estando dentro da amplitude aceitável e, portanto, não representando uma potencial ameaça à vida aquática na área de estudo. Entretanto, vale a pena mencionar a área 3, que compreende dois grandes Parques Nacionais (Aparados da Serra e Serra Geral), onde registramos valores próximos ao limite mínimo (6,0) para este parâmetro.

Considerando-se em conjunto os parâmetros físico-químicos avaliados, nota-se que, de forma geral, todos os pontos amostrados apresentam-se dentro de limites estabelecidos como apropriados para a qualidade da água. Os riachos no interior de UCs refletiram as condições externas, não apresentando qualidade físico-química superior ou inferior aos riachos externos.

Quanto aos parâmetros microbiológicos, identificamos a presença massiva de coliformes fecais e *Escherichia coli* em todos os pontos amostrados, com exceção de um ponto. Bactérias do grupo coliforme estão naturalmente presentes no ambiente e a maioria delas não é prejudicial, mas quando presentes na água destinada ao consumo podem indicar a presença de *E. coli* e outros patógenos que podem acarretar doenças infecciosas (NOBLE et al. 2003) e, por sua vez, indicam a contaminação por resíduos fecais. A baixa proteção fornecida pelas UCs aos seus cursos internos de água contra contaminantes biológicos parece ser comum no bioma da Mata Atlântica, como encontrado por outros estudos (SIMÕES, 2008; KUHLMANN et al., 2014; AVIGLIANO & SCHENONE et al., 2015). Apesar de manter grande parte da cobertura vegetal ripária e com um alto grau de conservação, a contaminação por coliformes dos riachos no interior de UCs possivelmente tem origem fora dos limites da UC e, portanto, quando o fluxo de água atinge seus domínios, ele ainda carrega a influência de áreas externas (PONTES et al., 2019). A presença significativa de bactérias do grupo coliforme é uma das várias consequências de alterações no uso e cobertura do solo e da crescente urbanização (TONG & CHENG, 2002; SNELDER et al, 2022), que permitem que a contaminação chegue aos corpos d'água. Para minimizar esses efeitos, uma abordagem integrativa precisa ser implementada, com monitoramento constante, foco na preservação das nascentes, e na restauração da vegetação ribeirinha em toda a bacia hidrográfica (PONTES, 2019).

Em relação aos pesticidas, tais compostos foram encontrados em todos os 33 pontos de coleta, mesmo em locais com boa preservação e longe de influência antrópica. Dos 19 diferentes compostos registrados destacam-se o 2,4-D, diurom, atrazina, etoprofós, pirimetanil. Observamos menores concentrações de herbicidas, inseticidas e fungicidas dentro das UCs,

porém, sem diferenças estatisticamente significativas entre os locais protegidos e suas áreas adjacentes, indicando que a cobertura florestal não está sendo suficientemente eficiente no controle da entrada de resíduos de pesticidas na água dos rios. Estas substâncias podem chegar à água superficial por diferentes caminhos, incluindo a deriva eólica por pulverização nas áreas adjacentes, drenagem, escoamento, ou com a precipitação (ANDRADE et al., 2021), efeito potencializado pela remoção ou redução da vegetação ripária, bem como transformação do uso e cobertura da terra, que facilitam a chegada desses compostos até a rede fluvial (RHEINHEIMER et al., 2020). Com a presença de vegetação, devido à porosidade relativamente alta da zona radicular, a capacidade de infiltração e a retenção de sedimentos e água de escoamento é aumentada (PROSSER et al., 2020), reduzindo assim a contaminação de cursos d'água. A presença desses compostos indica que as práticas atuais de conservação da água são insuficientes para limitar as transferências de água, sedimentos e pesticidas das plantações para os sistemas fluviais. Ao monitorar a presença de pesticidas em córregos do Parque Estadual Turvo - (Rio Grande do Sul, Brasil), Da Rocha et al. (2020) detectaram 2,7 vezes mais tipos de compostos em locais próximos a cultivos agrícolas em comparação com locais preservados, inseridos completamente dentro do Parque. Entretanto, mesmo com a proteção proporcionada pela floresta, ainda foram detectados pesticidas na água dentro da UC.

A contaminação ambiental por compostos farmacêuticos emergiu como mais uma preocupação nos últimos anos, podendo ter efeitos negativos na saúde humana, animal e nos ecossistemas (WILKINSON et al., 2022). Dos compostos detectados em nosso estudo, destacamos a cafeína, que estava presente em todos os rios e riachos amostrados. Este composto é um alcaloide natural que atua como estimulante no sistema nervoso central (MANDEL, 2002); é considerado um contaminante emergente porque não há nenhuma norma ou legislação que regule sua descarga ou monitoramento apesar de seus riscos potenciais ao meio ambiente (MARASCO JUNIOR et al., 2019; LI et al., 2020). A cafeína é amplamente consumida em todo o mundo, em bebidas, alimentos e medicamentos, e como foram encontradas altas concentrações desta substância no ambiente aquático em diversos estudos, ela tem sido considerada um indicador de influência antropogênica em ambientes de água doce (FERREIRA, 2005; LI et al., 2020). Wilkinson et al. (2022) identificaram o composto em rios e riachos em todos os continentes, incluindo a Antártica, sendo encontrado em mais de 50% dos locais de amostragem em todo o mundo. Na América do Sul, os registros de cafeína ocorreram em quase 70% dos locais de amostragem, levantando a preocupação sobre a fonte de efluxo deste composto, bem como seu efeito sobre a comunidade aquática. Apesar da presença massiva de cafeína no mundo inteiro, não há limite máximo estabelecido para o

composto nos ecossistemas aquáticos. Foi observada uma correlação significativa entre presença de cafeína e de bactérias do grupo coliformes, evidenciando que este composto pode servir como indicador de contaminação fecal recente (DANESHVAR et al., 2012). Considerando o registro simultâneo de cafeína e coliformes (incluindo aqui *E. coli*) nos locais amostrados, pode-se afirmar que há um influxo de esgoto, sendo potencialmente recente, nos riachos da região analisada.

Apesar das baixas doses detectadas em riachos dentro e fora das UCs, tanto de produtos farmacêuticos quanto de agroquímicos, não podemos desconsiderar que possa haver um efeito sinérgico entre diferentes substâncias químicas presentes na água em pequenas doses. A exposição contínua da biota de água doce a resíduos farmacêuticos e pesticidas pode afetar negativamente os organismos, levando a processos de biomagnificação e bioacúmulo, principalmente em consumidores primários (FREDERIC & YVES 2014; LAGESSON et al. 2016; ONDARZA et al. 2019; ŚWIACKA et al. 2022). Em resumo, nossos resultados indicam que a biodiversidade nos córregos da Mata Atlântica pode não estar efetivamente protegida, pois de forma geral os riachos dentro das UCs terrestres amostradas não estão protegidos contra contaminações biológicas, pesticidas e compostos farmacêuticos. Mesmo considerando que nossos resultados são um retrato do momento da amostragem da água, uma vez que os parâmetros físico-químicos analisados e os contaminantes variam em função das chuvas, do período de aplicação de pesticidas e muitos outros fatores, os dados indicam que a contaminação por poluentes (biológicos, agroquímicos e farmacêuticos) afeta toda a bacia hidrográfica e influencia as águas dentro de áreas protegidas, um local supostamente seguro de pressões antrópicas. Este fato não diminui a importância das UCs terrestres, como os parques nacionais, que são uma forma muito eficaz de preservar a biodiversidade (GRAY et al., 2016). Ao invés disso, aponta para a necessidade de UCs destinadas a proteger a biota de água doce, que não estão presentes no Brasil.

A principal implicação dos resultados relacionados ao Capítulo I desse estudo é acerca da conservação da biota aquática, em especial, das espécies de *Aegla* presentes na área amostrada. Eglídeos têm a característica de habitar ambientes aquáticos com boa qualidade, principalmente com alta taxa de oxigênio dissolvido, dado sua alta taxa metabólica comparada a outros crustáceos dulcícolas (DALOSTO & SANTOS et al., 2011). Considerando-se os parâmetros físico-químicos, os pontos de amostragem selecionados são “adequados” ao gênero. Entretanto, observam-se as pressões antrópicas, como a elevada presença de coliformes e a contaminação por pesticidas e fármacos. Os efeitos da contaminação biológica sobre eglídeos não são conhecidos, mas sabe-se que compostos pesticidas podem trazer sérias consequências

aos indivíduos de *Aegla*, como o aumento do estresse oxidativo e altos níveis de danos às proteínas e lipídeos nos tecidos (CEREZER et al., 2020). Cabe a estudos futuros avaliar os limites ambientais aceitáveis dos parâmetros de qualidade da água para as espécies de *Aegla*, tornando-se imprescindíveis no processo de monitoramento das suas populações.

A região de estudo é caracterizada pela crescente expansão de atividades agrícolas, principalmente com o cultivo de batatas e maçãs, além da prática da silvicultura, que empregam o amplo uso de pesticidas (SANTOS et al., 2012). Entretanto, é necessário refletir acerca do uso sustentável desses compostos, e se perguntar até que ponto tais práticas são seguras tanto para a biodiversidade, quanto para a saúde humana. Em um estudo recente, Panis et al. (2022) revelaram uma correlação entre a contaminação de água por pesticidas em 127 municípios do estado do Paraná com casos de câncer durante o período de 2014 a 2017. Nesse sentido, é imprescindível a presença da comunidade científica nas tomadas de decisões, principalmente aquelas relacionadas às regulações de compostos como herbicidas, inseticidas e fungicidas, como estabelecimento de seus limites máximos permitidos, banimentos e permissões.

Os resultados do capítulo II evidenciam a surpreendente diversidade de espécies encontradas na região de estudo. Foi identificada a presença de três complexos de espécies crípticas, *A. jarai*, *A. franciscana* e *A. camargoi*, com sete, três e uma espécies putativas, respectivamente, excluindo-se as espécies *stricto sensu*, em cada complexo. Além disso, outras 11 potenciais espécies foram identificadas. Entre as implicações deste estudo está a de que as espécies crípticas reveladas por nossas análises poderiam reduzir o alcance geográfico das espécies conhecidas para a região e ter consequências para o manejo e conservação das mesmas. Considere o caso de *A. jarai*, que está distribuída pelas bacias dos rios Canoas, Pelotas e Itajaí-Açu, e está classificada na categoria Pouco Preocupante (*Least Concern* - LC), de acordo com os critérios da IUCN (SANTOS et al., 2017). A descoberta que esta espécie engloba um complexo críptico pode mudar os limites da distribuição geográfica conhecida de *A. jarai sensu stricto*, e conseqüentemente, ter implicações para o seu status de conservação. O mesmo pode acontecer com as espécies de *A. franciscana* e *A. camargoi*, que também possuem cripticidade. Todas as novas espécies putativas não pertencentes a complexos crípticos (*Aegla* sp. n. 1 a 11) foram registradas em apenas um ponto ou dois sítios de coleta, evidenciando preliminarmente a distribuição restrita dessas populações, e fazendo com que emergjam preocupações em relação ao risco de extinção destas potenciais espécies. Novas avaliações devem ser realizadas na região para ampliar a área de busca e confirmar sua distribuição.

Considerando as espécies de *Aegla* presentes nos riachos dentro das UCs, será que poderíamos considera-las mais protegidas contra ameaças em comparação às espécies que não

ocorrem dentro dessas áreas? Nossos dados indicam que, ao menos nas UCs analisadas, os eglídeos estão expostos a contaminantes biológicos, pesticidas e compostos farmacêuticos tanto quanto os que vivem em áreas externas às UCs. As áreas protegidas são consideradas os melhores redutos para proteger a biodiversidade de diferentes fontes de ameaças (GELDMAN et al., 2014), tais como conversão do uso do solo, fragmentação, perda de habitat. Entretanto, uma grande proporção das UCs é delineada para preservar ambientes terrestres ou taxa, fornecendo apenas proteção acidental aos cursos d'água presentes dentro de seus limites (DUDLEY & STOLTON, 2003). E visto os dados referentes ao capítulo I, observa-se que UCs podem não ser suficientes para servir como método de proteção efetivo à biodiversidade aquática. Em projeções baseadas em modelagem ecológica a partir da distribuição conhecida das espécies de *Aegla*, para o período presente e para cenários futuros de alterações climáticas, as UCs apresentaram pouca efetividade para a conservação do grupo (PULI, 2019). Na nossa região de estudo, as UCs cobrem cerca de 13% da área total, e apesar disso, abrigam um terço do total de espécies registradas. Considerando todas as espécies identificadas no estudo, registramos sete espécies somente dentro de UCs (uma descrita e seis putativas) e 21 espécies somente em sítios externos (seis descritas e 15 putativas). Três espécies – *A. spinosa*, *A. inconspicua* e *A. jarai* complexo sp. n. 1 – ocorrem em ambos os locais (ver Tabela S1, capítulo II).

O acesso a essa diversidade oculta no gênero *Aegla* para a região de estudo foi facilitado pelo uso de métodos moleculares, principalmente das ferramentas de delimitação de espécies GMYC, bPTP e ASAP. O quebra-cabeça de unificar estes métodos de delimitação, a filogenia e a morfologia para delinear as espécies putativas foi bastante desafiador, pois foram encontradas divergências em alguns grupos, principalmente por causa da incongruência entre os números de entidades delimitadas pelos três métodos. Isso acontece devido à metodologia que cada um emprega para definir os limites das espécies. O GMYC tende a dividir mais as espécies em relação ao bPTP e ABGD (PUILLANDRE et al., 2012; FUJISAWA & BARRACLOUGH, 2013), enquanto este último é mais conservador (PENTINSAARI et al., 2017). O ASAP é muito semelhante ao método ABGD; ambos os métodos são baseados em distâncias genéticas pareadas, porém o ASAP usa as distâncias para construir uma lista de partições de grupos (sequências de DNA), ranqueadas por um escore (PUILLANDRE et al., 2012). Quando estas incongruências aconteceram, a morfologia foi fundamental para resolver as divergências, apesar da baixa variação morfológica em *Aegla*. Um caso em particular é o que denominamos como complexo *A. jarai*, o qual agrupa várias espécies morfológicamente identificadas como *A. jarai*, mas que não se apresentaram monofiléticas na filogenia baseada

no gene *COI*; além disso, os métodos de delimitação de espécies empregados identificaram entre sete e 11 possíveis espécies no complexo. A taxonomia integrativa reflete a melhor prática atualmente disponível para estudos sistemáticos, abordando diferentes perspectivas na busca da identificação de novas espécies e sua descrição (PANTE et al., 2015; WEI et al., 2022). Por outro lado, considerar apenas os dados genéticos na taxonomia molecular não é um procedimento seguro, podendo levar à superestimação da diversidade, já que o número de espécies delimitadas pode variar de acordo com o método empregado e pode não corresponder à realidade. Além disso, o conhecimento sobre a distribuição das espécies ao longo das drenagens também foi útil para auxiliar na identificação morfológica e deve ser considerado juntamente com as outras informações.

Como já mencionado, a morfologia no gênero *Aegla* é às vezes problemática, com limitação de caracteres morfológicos úteis para a taxonomia, seja em função da baixa variação interespecífica ou da grande variação intraespecífica e consequente sobreposição de caracteres entre espécies (BOND-BUCKUP & BUCKUP, 1994), o que dificulta a identificação baseada apenas na morfologia. Essa é uma das razões da discrepância entre o número de espécies descritas e o número potencial de espécies (CRIVELLARO et al., 2018). Neste estudo, houve casos de dúvidas durante a identificação morfológica da espécie, uma vez que os caracteres diagnósticos não correspondiam exatamente à descrição da espécie. Neste sentido, os dados genéticos foram extremamente úteis para evidenciar a diferença entre espécies e revelar a diversidade escondida nas sutis diferenças morfológicas do gênero.

Considerando-se os nossos resultados em conjunto com dados de outros estudos (por exemplo, CRIVELLARO et al., 2018; ZIMMERMANN et al., 2018; ZIMMERMANN et al., 2021), percebe-se que a diversidade de eglídeos é ainda muito maior e mais surpreendente do que conhecemos atualmente. Reunindo todos esses dados, o número de espécies de eglídeos aproxima-se de 150, o que abre inúmeras perspectivas de estudos sobre esse gênero peculiar tão especioso. Além disso, a região de estudo, já previamente identificada como apresentando alta riqueza e endemismo de espécies de *Aegla*, revela-se como a região com o maior número de espécies ocorrentes do gênero em toda a sua área de distribuição no sul da América do Sul, tornando-se ainda mais prioritária para esforços de conservação. Porém, apenas identificar novas espécies e revelar a presença de complexos crípticos não é suficiente nem para descrever a biodiversidade nem para proteger os eglídeos contra os fatores que os ameaçam de extinção. Assim, o próximo passo neste estudo será a utilização de mais marcadores moleculares para confirmar as prováveis espécies novas, seguido pela descrição dessas espécies e acesso aos seus

status de conservação. Desta forma, será possível propor medidas de proteção e manejo das espécies de *Aegla* e do habitat aquático.

REFERÊNCIAS BIBLIOGRÁFICAS

ACREMAN, Michael *et al.* Protected areas and freshwater biodiversity: a novel systematic review distills eight lessons for effective conservation. **Conservation Letters**, v. 13, p. e12684, 2020.

ANDRADE, Victoria S. *et al.* Influence of rainfall and seasonal crop practices on nutrient and pesticide runoff from soybean dominated agricultural areas in Pampean streams, Argentina. **Science of The Total Environment**, v. 788, p. 147676, 2021.

AUS DER BEEK, Tim *et al.* Pharmaceuticals in the environment - Global occurrences and perspectives. **Environmental Toxicology and Chemistry**, v. 35, p. 823–835, 2016.

AVIGLIANO, Esteban & SCHENONE, Nahuel E. Human health risk assessment and environmental distribution of trace elements, glyphosate, fecal coliform and total coliform in Atlantic Rainforest mountain rivers (South America). **Microchemical Journal**, v. 122, p. 149-158, 2015.

BAIRD, Rodger & BRIDGEWATER, Laura. **Standard methods for the examination of water and wastewater**. 23 ed. Washington, D.C. American Public Health Association, 2017.

BARROS, Graciele *et al.* Biased research generates large gaps on invertebrate biota knowledge in Brazilian freshwater ecosystems. **Perspectives in ecology and conservation**, v. 18, p. 190-196, 2020.

BARTHOLOMEI-SANTOS, Marlise L. *et al.* Evolutionary history and phylogenetic relationships of Aeglidae. *In*: SANTOS, Sandro & BUENO, Sérgio L. S. **Aeglidae: Life history and conservation status of unique freshwater Anomuran decapods**: CRC Press, 2020. p. 1–27.

BATTAGLIN, William A. *et al.* Potential interactions among disease, pesticides, water quality and adjacent land cover in amphibian habitats in the United States. **Science of the Total Environment**, v. 566–567, p. 320–332, 2016.

BEATTY, William *et al.* The role of protected area wetlands in waterfowl habitat conservation: Implications for protected area network design. **Biological Conservation**, v. 176, p. 144-152, 2014.

BELSKY, Ana J. *et al.* Survey of Livestock Influences on Stream and Riparian Ecosystems in the Western United States. **Journal of Soil and Water Conservation**, v. 54, p. 419-431, 1999.

BOND-BUCKUP, Giorgina & BUCKUP, Ludwig. A família Aeglidae (Crustacea, Decapoda, Anomura). **Arquivos de Zoologia**, v. 32, p. 159-346, 1994.

BOND-BUCKUP, Giorgina & SANTOS, Sandro. 2007. Crustáceos anomuros de águas continentais: Diversidade e aspectos biológicos. **Ciência e ambiente**, v. 35, p. 47-54, 2007.

BOOS, Harry *et al.* Conservation Status and Threats of Aeglidae: Beyond the Assessment. *In*: SANTOS, Sandro & BUENO, Sérgio L. S. **Aeglidae: Life history and conservation status of unique freshwater Anomuran decapods**: CRC Press, 2020. p. 233–251

BRADLEY, Paul M. *et al.* Exposure and potential effects of pesticides and pharmaceuticals in protected streams of the US National park Service southeast region. **Science of the Total Environment**, v. 704, p. 135431, 2020.

BRAND, Adrienne B. *et al.* Lethal and sublethal effects of embryonic and larval exposure of *Hyla versicolor* to stormwater pond sediments. **Archives of Environmental Contamination and Toxicology**, v. 58, p. 325–331, 2010.

BRASIL. Conselho Nacional do Meio Ambiente – CONAMA. **Resolução CONAMA N° 357**. CONAMA, 17 de março de 2005. Assunto: classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, bem como estabelece as condições e padrões de lançamento de efluentes, e dá outras providências. Disponível em: https://www.icmbio.gov.br/cepsul/images/stories/legislacao/Resolucao/2005/res_conama_357_2005_classificacao_corpos_agua_rtfcd_a_altrd_res_393_2007_397_2008_410_2009_430_2011.pdf. Acesso em: jul. 2022.

BRASIL. **Lei N° 9.985, de 18 de julho de 2000**. Assunto: Institui o Sistema Nacional de Unidades de Conservação da Natureza e dá outras providências. Brasília: Presidência da República, 2000. Disponível em http://www.planalto.gov.br/ccivil_03/leis/19985.htm. Acesso em: jul. 2022.

BRASIL. Ministério do Meio Ambiente. Departamento de Áreas Protegidas. **Painel de Unidades de Conservação Brasileiras**. Disponível em: <https://app.powerbi.com/view?r=eyJrIjoiMGNmMGY3NGMtNWZlOC00ZmRmLWExZWItNTNiNDhkZDg0MmY4IiwidCI6IjM5NTdhMzY3LTZkMzgtNGMxZi1hNGJhLTMzZThmM2M1NTBlNyJ9&pageName=ReportSection0a112a2a9e0cf52a827>. Acesso em: jul. 2022.

BROOKS, Thomas *et al.* Global biodiversity conservation priorities. **Science**, v. 313, p. 58–61, 2006.

BUENO, Abel. A. P. & BOND-BUCKUP, Giorgina. Natural Diet of *Aegla platensis* Schmitt and *Aegla ligulata* Bond-Buckup & Buckup (Crustacea, Decapoda, Aeglidae) from Brazil. **Acta Limnologica Brasiliensia**, v. 16, n. 2, p. 115-127, 2004.

BUENO, Sérgio L. S. *et al.* A new species of stygobitic aeglid from lentic subterranean waters in southeastern Brazil, with an unusual morphological trait: short pleopods in adult males. **Nauplius**, v. 25, p. 1-18, 2017.

BUENO, Sérgio. L. S. *et al.* Fluctuations in the Population Size of the Highly Endemic *Aegla Perobae* (Decapoda: Anomura: Aeglidae) Caused by a Disturbance Event. **Journal of Crustacean Biology**, v. 34, n. 2, p. 165–173, 2014.

BURNS, James W. 1972. The distribution and life history of South American freshwater crabs (*Aegla*) and their role in trout streams and lakes. **Transactions of the American Fisheries Society**, v. 101, p. 595-607, 1972.

CEREZER, Cristina M. *et al.* Influence of pesticides and abiotic conditions on biochemical biomarkers in *Aegla aff. longirostri* (crustacea, anomura): Implications for conservation. **Ecotoxicology and Environmental Safety**, v. 203, p. 110982, 2020.

COGO, Gláucia B. & SANTOS, Sandro. The role of aeglids in shredding organic matter in neotropical streams. **Journal of Crustacean Biology**, v. 33, n. 4, p. 519–526, 2013.

COGO, Gláucia G *et al.* The effect of the macroconsumer *Aegla longirostri* (Crustacea, Decapoda) on the invertebrate community in a subtropical stream. **Acta Limnologica Brasiliensia**, v. 26, p. 143-156, 2014.

COX, B. A. A review of dissolved oxygen modelling techniques for lowland rivers. **The Science of the Total Environment**, v. 314–316, p. 303–334, 2003.

CRIVELLARO, Marcelo S. *et al.* Looks can be deceiving: species delimitation reveals hidden diversity in the freshwater crab *Aegla longirostri* (Decapoda: Anomura). **Zoological Journal of the Linnean Society**, v. 182, p. 24–37, 2018.

D'AMEN, Manuela *et al.* Protected areas and insect conservation: questioning the effectiveness of Natura 2000 network for saproxylic beetles in Italy. **Animal Conservation**, v. 16, p. 370-378, 2013.

DALOSTO, Marcelo & SANTOS, Sandro. Differences in oxygen consumption and diel activity as adaptations related to microhabitat in Neotropical freshwater decapods (Crustacea). **Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology**, v. 160, p. 461-466, 2011.

DANESHVAR, Atlasi *et al.* Evaluating pharmaceuticals and caffeine as indicators of fecal contamination in drinking water sources of the Greater Montreal region. **Chemosphere**, v. 88, p. 131–139, 2012.

DORJI, Tshering *et al.* Freshwater conservation planning in the context of nature needs half and protected area dynamism in Bhutan. **Biological Conservation**, v. 251, p. 108785, 2020.

DUDLEY, Nigel & STOLTON, Sue. **Running Pure: The importance of forest protected areas to drinking water**. World bank/WWF Alliance. 112 p. 2003.

EUA. Environmental Protection Agency, EPA. **Causal Analysis/Diagnosis Decision Information System (CADDIS)**. Office of Research and Development, Washington, DC, 2017. Disponível em: <https://www.epa.gov/caddis-vol2>. Acesso em: mar. 2022.

EUA. Environmental Protection Agency, EPA. **Quality criteria for water**. EPA-440/5-86-001. Office of Water Regulations and Standards, Washington, DC, 1986. Acesso em: mar. 2022.

FENG, Chunting *et al.* Which management measures lead to better performance of China's protected areas in reducing forest loss? **Science of the Total Environment**, v. 764, p. 142895, 2021.

FERREIRA, Aldo *et al.* Caffeine as an environmental indicator for assessing urban aquatic ecosystems. **Cad. Saúde Pública**, v. 21, n. 6, 2005.

FRANKLIN, Paul A. Dissolved oxygen criteria for freshwater fish in New Zealand: a revised approach. **New Zealand Journal of Marine and Freshwater Research**, v. 48, n. 1, p. 112-126, 2014.

FREDERIC, Orias & YVES, Perrodin. Pharmaceuticals in hospital wastewater: their ecotoxicity and contribution to the environmental hazard of the effluent. **Chemosphere**, v. 115, p. 31-39, 2014.

FUJISAWA, Tomochika & BARRACLOUGH, Timothy G. Delimiting Species Using Single locus Data and the Generalized Mixed Yule Coalescent (GMYC) approach: A revised method and evaluation on simulated datasets. **Systematic Biology**, v. 62, p. 707–724, 2013.

GELDMAN, Jonas *et al.* Effectiveness of terrestrial protected areas in reducing habitat loss and population declines. **Biological Conservation**, v. 161: p. 230-238, 2013.

GONÇALVES, Alberto S. *et al.* Priority areas for conservation within four freshwater ecoregions in South America: A scale perspective based on freshwater crabs (Anomura, Aeglidae). **Aquatic Conservation: Marine and Freshwater Ecosystems**, v. 28, p. 1077–1088, 2018.

GRAY, Claudia L. *et al.* Local biodiversity is higher inside than outside terrestrial protected areas worldwide. **Nature Communications**, v. 7, p. 12306, 2016.

GRIGNOLIO, Stefano *et al.* Effects of hunting with hounds on a non-target species living on the edge of a protected area. **Biological Conservation**, v. 144, p. 641-649, 2011.

HERBERT, Paul D. N. *et al.* Biological identifications through DNA barcodes. **Proceedings of the Royal Society B: Biological Sciences**, v. 1512, p. 313-21, 2003.

HERMOSO, Virgilio *et al.* The role of protected areas for freshwater biodiversity conservation: challenges and opportunities in a rapidly changing world. **Aquatic Conservation: Marine and Freshwater Ecosystems**, v. 26, p. 3–11, 2016.

HORNE, Alexander J. & GOLDMAN, Charles R. (1994). **Limnology**. New York: McGraw-Hill, 1994.

KUHLMANN, Mônica L. *et al.* Effects of human activities on rivers located in protected areas of the Atlantic Forest. **Acta Limnologica Brasiliensia**, v. 26, n. 1, p. 60-72, 2014.

LAGESSON, Annelie *et al.* Bioaccumulation of five pharmaceuticals at multiple trophic levels in an aquatic food web - Insights from a field experiment. **Science of the Total Environment**, v. 568, p. 208-215, 2016.

LEACHÉ, Adam D. *et al.* Species delimitation using genome-wide SNP data. **Systematic Biology**, v. 63, n. 4, p. 534–542, 2014.

LI, Shulan *et al.* Occurrence of caffeine in the freshwater environment: Implications for ecopharmacovigilance. **Environmental Pollution**, v. 263, p. 114371, 2020.

LINK, Moritz *et al.* Pesticide effects on macroinvertebrates and leaf litter decomposition in areas with traditional agriculture. **Science of the Total Environment**, v. 828, p. 154549, 2022.

LIU, Jianguo *et al.* Ecological degradation in protected areas: the case of Wolong Nature Reserve for Giant Pandas. **Science**, v. 292, p. 98–101, 2001.

MACKIE, Gerald L. **Applied Aquatic Ecosystem Concepts**. Dubuque: Kendall/Hunt Publishing, 2004.

MAIA, Kate *et al.* Ecologia populacional e conservação de eglídeos (Crustacea: Decapoda: Aeglidae) em cavernas da área cárstica do Alto Ribeira, em São Paulo. **Revista da Biologia**, v. 10, p. 40–45, 2013.

MANCINI, Laura *et al.* Biological quality of running waters in protected areas: The influence of size and land use. **Biodiversity & Conservation**, v. 14, n. 2, p. 351–364, 2005.

MANDEL, H. G. Update on caffeine consumption, disposition and action. **Food and Chemical Toxicology**, v. 40, p. 1231–1234, 2002.

MARASCO JÚNIOR, César A. *et al.* Occurrence of caffeine in wastewater and sewage and applied techniques for analysis: a review. **Eclética Química Journal**, v. 44, n. 4, p. 11–26, 2019.

MARÇAL, Ingrid C. *et al.* Cryptic diversity among populations of *Aegla* Leach, 1820 (Decapoda: Anomura: Aeglidae) from Tibagi Riverbasin, Paraná state, Brazil, with descriptions of three new species. **Journal of Natural History**, v. 55, p. 2145–2171, 2021.

MELO, Maria T. Q. **Dieta de *Caiman latirostris* (Daudin, 1802) na estação ecológica do Taim, RS**. 1990. 63 f. Dissertação de mestrado – Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, 2004.

MORAES, Juliana C. B. *et al.* Morphological and molecular data reveal the cryptic diversity among populations of *Aegla paulensis* (Decapoda, Anomura, Aeglidae), with descriptions of four new species and comments on dispersal routes and conservation status. **Zootaxa**, v. 4193, p. 1–48, 2016.

MORAES, Juliana C. B. *et al.* Taxonomic review of *Aegla marginata* Bond Buckup and Buckup, 1994 (Decapoda, Anomura, Aeglidae) with description of a new species. **Zootaxa**, v. 4323, p. 519–533, 2017.

NG, Daniel J. J. *et al.* Conservation challenges and action for the critically endangered Singapore freshwater crab *Johora singaporensis*. **Oryx**, v. 49(2), p. 345–351, 2015.

NOBLE, Rachel T. *et al.* Comparison of total coliform, fecal coliform, and enterococcus bacterial indicator response for ocean recreational water quality testing. **Water Research**, v. 37, p. 1637–1643, 2003.

NOWELL, Lisa H. *et al.* Pesticide toxicity index - A tool for assessing potential toxicity of pesticide mixtures to freshwater aquatic organisms. **Science of the Total Environment**, v. 476, p. 144–157, 2014.

NOWELL, Lisa H. *et al.* Regression models for explaining and predicting concentrations of organochlorine pesticides in fish from streams in the United States. **Environmental Toxicology and Chemistry**, v. 28, p. 1346–1358, 2009.

OERTLI, Beat & PARRIS, Kirsten M. Review: Toward management of urban ponds for freshwater biodiversity. **Ecosphere**, v. 10, n. 7, p. e02810, 2019.

ONDARZA, Paola M. *et al.* Pharmaceuticals, illicit drugs and their metabolites in fish from Argentina: implications for protected areas influenced by urbanization. **Science of the Total Environment**, v. 649, p. 1029e1037, 2019.

PANIS, Carolina *et al.* Widespread pesticide contamination of drinking water and impact on cancer risk in Brazil. **Environment International**, v. 165, p. 107321, 2022.

PANTE, Eric *et al.* From Integrative Taxonomy to Species Description: One Step Beyond. **Systematic Biology**, v. 64, n. 1, p. 152–160, 2015.

PENTINSAARI, Mikko *et al.* Algorithmic single-locus species delimitation: Effects of sampling effort, variation and nonmonophyly in four methods and 1870 species of beetles. **Molecular Ecology Resources**, v. 17, p. 393–404, 2017.

PÉREZ-LOSADA, Marcos *et al.* Conservation assessment of Southern South American freshwater ecoregions on the basis of the distribution and genetic diversity of crabs from the genus *Aegla*. **Conservation Biology**, v. 23, p. 692–702, 2009.

PÉREZ-LOSADA, Marcos *et al.* Molecular systematics and biogeography of the southern South American freshwater “crabs” *Aegla* (Decapoda: Anomura: Aeglidae) using multiple heuristic tree search approaches. **Systematic Biology**, v. 53, p. 767–780, 2004.

PINHEIRO, Adilson & ROSA, Fabiana C. Classificação dos pesticidas usados na bacia hidrográfica do Itajaí (SC) ao risco de degradação dos recursos hídricos. **Pesticidas: Revista de ecotoxicologia e meio ambiente**, v. 18, p. 45-58, 2009.

PONS, Joan *et al.* Sequence-based species delimitation for the DNA taxonomy of undescribed insects. **Systematic Biology**, v. 55, p. 595–609, 2006.

PONTES, Paulo R. M. *et al.* The role of protected and deforested areas in the hydrological processes of Itacaiúnas River Basin, eastern Amazonia. **Journal of Environmental Management**, v. 235, p. 489–499, 2019.

PROSSER, Ryan S. *et al.* A review of the effectiveness of vegetated buffers to mitigate pesticide and nutrient transport into surface waters from agricultural areas. **Journal of Environmental Management**, v. 261, p. 110210, 2020.

PULLANDRE, Nicolas *et al.* ABGD, Automatic Barcode Gap Discovery for primary species delimitation. **Molecular Ecology**, v. 21, p. 1864–1877, 2012.

PULLANDRE, Nicolas *et al.* ASAP: assemble species by automatic partitioning. **Molecular Ecology Resources**, v. 21, p. 609–620, 2020.

PULI, Gislaine. **Filogeografia e modelagem de nicho ecológico de *Aegla leach* 1820 (Crustacea, Anomura): um olhar sobre o passado e projeções para o futuro**. 2019. Dissertação (Mestrado em Biodiversidade Animal) – Universidade Federal de Santa Maria, Santa Maria, RS, 2019.

QUEIROZ, Gabriela *et al.* Transporte de Glifosato pelo escoamento superficial e por lixiviação em um solo agrícola. **Revista Química Nova**, v. 34, p. 190-195, 2011.

RANGANATH, Sheila C. *et al.* Livestock exclusion influences on riparian vegetation, channel morphology, and benthic macroinvertebrate assemblages. **Journal of Soil and Water Conservation**, v. 64, n. 1, p. 33-42, 2009.

RHEINHEIMER, Danilo S. *et al.* Pesticide Bioaccumulation in Epilithic Biofilms as Biomarkers of agricultural activities in a representative watershed. **Environmental Monitoring and Assessment**, v. 192, n. 6, p. 381, 2020.

RICO, Andreu *et al.* Pharmaceuticals and other urban contaminants threaten Amazonian freshwater ecosystems. **Environment International**, v. 155, p. 106702, 2021.

RIO GRANDE DO SUL. Secretaria da agricultura, pecuária e desenvolvimento rural. **Instrução Normativa SEAPDR n° 06/2019**. Porto Alegre, RS: Gabinete do Secretário, 2019. Assunto: Estabelece o cadastro de aplicadores de produtos agrotóxicos hormonais, regulamenta sua aplicação e dá outras providências. Disponível em: <https://www.agricultura.rs.gov.br/upload/arquivos/201907/05160456-instrucao-normativa-seapdr.pdf>. Acesso em: fev. 2022.

ROCHA, Marcelo C. *et al.* Preserved riparian forest protects endangered forest-specialists amphibian species against the genotoxic impact of sunlight and agrochemicals. **Biological Conservation**, v. 249, p. 108746, 2020.

SANTOS, Sandro *et al.* Diversity and conservation status of *Aegla* spp. (Anomura, Aeglidae): An update. **Nauplius**, v. 25, p. e2017011, 2017.

SANTOS, Sandro *et al.* Three new species of *Aegla* (Anomura) freshwater crabs from the upper Uruguay river hydrographic basin in Brazil. **Journal Of Crustacean Biology**, v. 32, n. 4, p. 529-540, 2012.

SIMÕES, Fabiano S. *et al.* Water quality index as a simple indicator of aquaculture effects on aquatic bodies. **Ecological Indicators**, v. 8, p. 476–484, 2008.

SIMONIN, Marie *et al.* Consistent declines in aquatic biodiversity across diverse domains of life in rivers impacted by surface coal mining. **Ecological Applications**, v. 31, n. 6, p. e02389, 2021.

SNELDER, Ton H. *et al.* Attribution of river water-quality trends to agricultural land use and climate variability in New Zealand. **Marine and Freshwater Research**, v. 73, p. 1–19, 2022.

SNYDER, Marcia N. *et al.* Landscape-scale disturbance and protected areas: Long-term dynamics of populations of the shrimp, *Macrobrachium olfersi* in lowland Neotropical streams, Costa Rica. **Journal of Tropical Ecology**, 29(1), p. 81–85, 2013.

SOUZA, Andréa L. T. *et al.* Influence of riparian vegetation and forest structure on the water quality of rural low-order streams in SE Brazil. **Forest Ecology and Management**, v. 298, p. 12–18, 2013.

SWIACKA, Klaudia *et al.* Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – Current state of knowledge. **Journal of Hazardous Materials**, v. 424, p. 127350, 2022.

THIEME, Michele L. *et al.* Protected areas and freshwater conservation: A survey of protected area managers in the Tennessee and Cumberland River Basins, USA. **Journal of Environmental Management**, v. 109, p. 189-199, 2012.

TONG, Susana T. Y. & CHENG, Wenli. Modeling the relationship between land use and surface water quality. **Journal of Environmental Management**, v. 66, p. 377-393, 2002.

TREVISAN, André *et al.* Abundância e distribuição de Aeglidae (Crustacea: Anomura) em função do uso da terra na bacia hidrográfica do Rio Jacutinga, Rio Grande do Sul, Brasil. **Zoologia**, v. 26, n. 3, p. 419–426, 2009.

UNEP-WCMC & IUCN. **Protected Planet Report 2020**. 2021. Disponível em: <https://livereport.protectedplanet.net/chapter-3>. Acesso em: jun. 2022.

UNEP-WCMC. **Protected Area Profile for Brazil from the World Database on Protected Areas**. 2022. Disponível em: www.protectedplanet.net. Acesso em: jun. 2022.

WEI, Chentao *et al.* Cryptic species in a colorful genus: Integrative taxonomy of the bush robins (Aves, Muscicapidae, *Tarsiger*) suggests two overlooked species. **Molecular Phylogenetics and Evolution**, v. 175, p. 107580, 2022.

WILKINSON, John L. *et al.* Pharmaceutical pollution of the world's rivers. **PNAS**, v. 119, n. 8, e2113947119, 2022.

WWF. **Living planet report (2018) risk and resilience in a new era**. Gland, Switzerland: WWF International, 2018. Disponível em: <https://www.worldwildlife.org/pages/living-planet-report-2018>.

YANG, Hongbo *et al.* A global assessment of the impact of individual protected areas on preventing forest loss. **Science of the Total Environment**, v. 777, p. 145995, 2021.

YANG, Ziheng & RANNALA, Bruce. Unguided species delimitation using DNA sequence data from multiple loci. **Molecular Biology and Evolution**, v. 31, p. 3125–3135, 2014.

ZHANG, Jiajie *et al.* A general species delimitation method with applications to phylogenetic placements. **Bioinformatics**, v. 29, n. 22, p. 2869–2876, 2013.

ZIMMERMANN, Bianca L. *et al.* Phylogeography reveals unexpectedly low genetic diversity in a widely distributed species: The case of the freshwater crab *Aegla platensis* (Decapoda: Anomura). **Biological Journal of the Linnean Society**, v. 123, p. 578–592, 2018.

ZIMMERMANN, Bianca. L. *et al.* Entangled Aeglidae (Decapoda, Anomura): Additional evidence for cryptic species. **Zoologica Scripta**, v. 50, p. 473–484, 2021.