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Federal University of Santa Catarina (UFSC) and
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Ecological mixture risk assessment between polystyrene nanoplastic and ivermectin
seeking an approach at various trophic levels:
Bioassays, modeling, and regulatory aspects.

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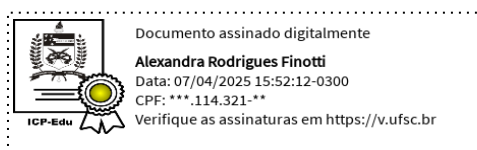
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Trust in an uncertain, indeed improbable, future worth living may seem foolish, but there is no way of avoiding the option because there is nowhere to stand outside of the alternative of either consenting to or refusing the challenge as it is addressed to each of us. (Stengers, 2013)]

ABSTRACT

Increasing amounts of pollutants are constantly being generated and disposed in the natural environment even after wastewater treatment plants. To assess its effects on the biota closer to the effects occurring on the environment, the present work aims to understand the effects of environmentally relevant concentrations of the polystyrene nanoplastic (PSNP), ivermectin (IVM), and their mixture (PSNP-IVM) across different trophic levels through bioassays and modeling. This thesis is comprised of three chapters, 1. Characterization of the involved pollutants and Toxicological assessment; 2. Mixture Risk Assessment, and 3. Regulatory approach. The characterization evaluated the stability in suspension of PSNP and IVM thus providing information on the possible mobility of the particles on the water column, affecting then the bioassays and generating data on the toxicity using organisms from different trophic levels such as producers, primary consumers, and decomposers, with further Species Sensitivity Distribution curve. Then, a Mixture Risk Assessment model was developed to help policy makers to apply the Mixture Assessment Factor (MAF) and correct regulatory thresholds. The last chapter provides a new framework to the regulatory practices have been helping each other towards a better understanding of toxicological models, their respective mixture pollutant evaluation and, policy implementation with a focus on the global north-south relations. PSNP was not toxic in environmentally relevant concentrations to none of the tested species, while IVM was toxic to *D. magna* and *E. crypticus*. The mixture showed no pattern between species; however the SSD provided an HC5 of 29.49 ppt of IVM for aquatic organisms and 36.87 for terrestrial organisms. With the model from chapter 2 providing a strong scientific base for the application of MAF on the correction of regulatory thresholds, the discussion on chapter 3 points towards a greater integration between all stakeholders though the GORIES framework which stands for: Global south-centered, Open access data, Regionalization, Integration, and Energy Saving principle. Therefore, this work helps bridge the gap between academia and the regulatory bodies and shows a possible way forward. When all interested parts get involved it is possible to profit from all forms of knowledge and provide greater environmental protection.

Keywords: Regulatory Toxicology. Ecological risk assessment. Mixture toxicity. Species Sensitivity Distribution curve. Polystyrene nanoplastic. Ivermectin.

RÉSUMÉ

Des quantités croissantes de polluants sont constamment générées et déposées dans l'environnement naturel, même après les usines de traitement des eaux usées. Afin d'évaluer ses effets sur le biote plus près des effets qui se produisent dans l'environnement, les présents travaux visent à comprendre les effets des concentrations pertinentes pour l'environnement de polystyrène nanoplastique (PSNP), d'ivermectine (IVM) et de leur mélange (PSNP-IVM) à différents niveaux trophiques grâce à des essais biologiques et à la modélisation. Cette thèse se compose de trois chapitres, 1. Caractérisation des polluants en cause et évaluation toxicologique ; 2. Mélange de l'évaluation des risques et 3. Approche réglementaire. La caractérisation a évalué la stabilité en suspension du PSNP et de la VM, fournissant des informations sur la mobilité possible des particules dans la colonne d'eau, affectant les essais biologiques et générant des données sur la toxicité utilisant des organismes de différents niveaux trophiques, tels que les producteurs, les consommateurs primaires et les décomposeurs, avec une courbe ultérieure de la distribution de la sensibilité des espèces. Ensuite, un modèle d'évaluation des risques pour les mélanges a été mis au point pour aider les décideurs à appliquer le facteur d'évaluation des mélanges (FEM) et à corriger les limites réglementaires. Le dernier chapitre fournit un nouveau cadre pour les pratiques réglementaires qui se sont aidées mutuellement à mieux comprendre les modèles toxicologiques, leur évaluation respective des mélanges de polluants et la mise en œuvre de politiques axées sur les relations nord-sud mondiales. Le PSNP n'était pas toxique à des concentrations pertinentes pour l'environnement pour aucune des espèces testées, tandis que l'IVM était toxique pour *D. magna* et *E. crypticus*. Le mélange n'a pas montré de modèle entre les espèces ; toutefois, la DSS a fourni un HC5 de 29,49 ppt IVM pour les organismes aquatiques et de 36,87 ppt pour les organismes terrestres. Le modèle du chapitre 2 fournissant une base scientifique solide pour l'application du CRG dans la correction des limites réglementaires, la discussion au chapitre 3 indique une plus grande intégration entre toutes les parties prenantes grâce au cadre GLORIES, qui signifie : Centré sur le Sud global, les données en libre accès, la régionalisation, l'intégration et le principe d'économie d'énergie. Par conséquent, ce travail aide à réduire la distance entre l'academie et les organismes de réglementation et montre une voie possible pour aller de l'avant. Lorsque toutes les parties prenantes s'impliquent, il est possible de bénéficier de toutes les formes de connaissances et d'assurer une meilleure protection de l'environnement.

Mots-clés: Toxicologie réglementaire. Évaluation des risques écologiques. Concentrations pertinentes pour l'environnement. Toxicité des mélanges. Courbe de distribution de la sensibilité des espèces. Micro/nanoplastique de polystyrène. Ivermectine.

RESUMO EXPANDIDO

Introdução

Quantidades crescentes de poluentes são constantemente geradas e descartadas no ambiente natural, mesmo após estações de tratamento de esgoto. Estes poluentes são chamados de poluentes emergentes quando não existem limites regulatórios que os mantenham em níveis seguros para o meio ambiente ou para os seres humanos. Portanto, a regulação se apresenta enquanto critério de definição para os novos poluentes que podem causar danos ambientais. Dentre estes poluentes encontram-se os nanoplásticos que são amplamente observados na natureza principalmente através da degradação do plástico de uso comum pela ação de diversos fatores ambientais como o atrito, radiação UV, pH ou biodegradação. Outro poluente que apresentou um aumento no uso durante o período da pandemia foi o medicamento ivermectina, um antiparasitário que foi utilizado como medicamento para a prevenção da COVID-19, fato que nunca foi comprovado em escala real. Estes dois componentes uma vez no meio ambiente podem causar impactos em conjunto tanto entre si quanto junto a outros poluentes ambientais oriundos do despejo de efluentes tratados ou não.

Objetivos

Para avaliar seus efeitos na biota mais próximos dos efeitos que ocorrem no meio ambiente, o presente trabalho tem como objetivo entender os efeitos de concentrações ambientalmente relevantes do poliestireno nanoplástico (PSNP), ivermectina (IVM) e sua mistura (PSNP-IVM) em diferentes níveis tróficos por meio de bioensaios. O segundo objetivo desta tese é desenvolver uma ferramenta de avaliação de risco para a inclusão de fatores de avaliação de misturas para que as regulamentações possam ser mais próximas aos efeitos observados na natureza e, portanto, a proteção ambiental seja baseada em condições mais próximas das reais. Quanto ao último objetivo desta tese, tem-se o desenvolvimento de uma ferramenta para auxiliar a gestão da informação em agências reguladoras quanto à utilização de fatores de atualização de legislação, especialmente focados na atualização das regulamentações quanto a mistura de poluentes.

Metodologia

Esta tese é composta por três capítulos, 1. Caracterização dos poluentes envolvidos e avaliação toxicológica; 2. Avaliação de risco de mistura e 3. Abordagem regulatória. A caracterização avaliou a estabilidade em suspensão do PSNP e do IVM, fornecendo informações sobre a possível mobilidade das partículas na coluna d'água, afetando os bioensaios e gerando dados sobre a toxicidade usando organismos de diferentes níveis tróficos, como produtores, consumidores primários e decompositores, com posterior curva de Distribuição de Sensibilidade das Espécies. Em seguida, foi desenvolvida uma ferramenta de Avaliação de Risco de Mistura para ajudar os formuladores de políticas a aplicarem o Fator de Avaliação de Mistura (FAM) e corrigir os limites regulatórios. Esta ferramenta consiste na

inclusão de diferentes métodos que isoladamente avaliam os caminhos bioquímicos para a obtenção do efeito (*Adverse Outcome Pathways* – AOP) e a avaliação do peso da evidência de cada possível mistura avaliada (*Weight of Evidence* – WoE). O último capítulo fornece um novo arcabouço para as práticas regulatórias que vêm se ajudando mutuamente a uma melhor compreensão dos modelos toxicológicos, sua respectiva avaliação de misturas poluentes e implementação de políticas com foco nas relações globais norte-sul. Tendo em vista que a geração de dados através de monitoramento é frequentemente gerada e fornecida por países do norte global, este método permite que países do sul global cujo monitoramento pode ser menos extenso através da utilização e inclusão de informações encontradas em artigos ou bases de dados internacionais de caráter aberto. Desta maneira é possível que sejam economizados recursos por parte dos países do sul global porém sem reduzir a proteção ambiental.

Resultados e discussão

O PSNP não foi tóxico em concentrações ambientalmente relevantes para nenhuma das espécies testadas, enquanto o IVM foi tóxico para *D. magna* e *E. crypticus*. A mistura não apresentou padrão entre as espécies; no entanto, o SSD forneceu um HC5 de 29,49 ppt de IVM para organismos aquáticos e 36,87 para organismos terrestres. Isto indica que as concentrações encontradas no meio ambiente podem apresentar riscos para os organismos mais sensíveis. Em caso nos quais estes organismos desempenhem um serviço ecossistêmico relevante, os efeitos em cadeia podem ser maiores afetando os níveis tróficos superiores ou inferiores. A ferramenta do capítulo de avaliação de risco (capítulo 2) apresentou uma nova possibilidade para a inclusão de dados obtidos na literatura para a atualização de valores regulatórios. O método apresenta um caminho que utiliza as abordagens de AOP e WoE como reforços para avaliadores de risco que não possuem recursos para os bioensaios em laboratórios próprios. A utilização de dados disponíveis online e de maneira aberta é imprescindível para a disseminação do conhecimento e para o aumento na proteção ambiental subsequente. Com o modelo do capítulo 2 fornecendo uma forte base científica para a aplicação do MAF na correção de limites regulatórios, a discussão no capítulo 3 aponta para uma maior integração entre todas as partes interessadas por meio da estrutura GLORIES, que significa em inglês: Centrado no sul global, dados de acesso aberto, regionalização, integração e princípio de economia de energia. Portanto, este trabalho ajuda a preencher a lacuna entre a academia e os órgãos reguladores e mostra um possível caminho a seguir. Quando todas as partes interessadas se envolvem, é possível se beneficiar com todas as formas de conhecimento e proporcionar maior proteção ambiental.

Considerações finais

A presente tese reflete sobre os possíveis impactos ambientais negativos da ausência de regulamentação ambiental para poluentes específicos, denominados poluentes emergentes. Desta maneira, foi possível observar nos resultados dos bioensaios que concentrações ambientais de ambos os poluentes, nanoplastico de poliestireno e ivermectina, podem causar efeitos negativos isoladamente e em

mistura. Desta maneira, a ferramenta de atualização da legislação apresenta indicativos de como agências reguladoras poderiam atualizar seus sistemas regulatórios, bem como a utilização de ferramentas disponíveis de maneira aberta para que seja aumentada a proteção ambiental oriunda da regulamentação. Enquanto para o último capítulo, foi possível observar que as agências reguladoras poderão fazer uso de uma nova ferramenta regulatória na qual será possível utilizar dados oriundos de fontes abertas para aumentar a proteção ambiental.

Palavras-chave: Toxicologia regulatória. Avaliação de risco ecológico. Concentrações ambientalmente relevantes. Toxicidade da mistura. Curva de distribuição de sensibilidade da espécie. Micro/nanoplástico de poliestireno. Ivermectina.

1 ABBREVIATIONS

ANT – Antagonism	NP – Nanoplastics
AOP – Adverse Outcome Pathways	OECD – Organisation for Economic Co-operation and Development
BBM – Bold Basal Media	OTH – Others
BBM - Bold Basal Medium CPCC – Canadian Phycological Culture Center	PDI – Polydispersity Index
DMSO – Dimethylsulfoxide	PE – Polyethylene
EC ₁₀ – Effective concentration that causes an effect on 10% of the organisms	PEC – Predicted Environmental Concentration
EC ₅₀ – Effective concentration that causes an effect on 50% of the organisms	PET – Polyethylene terephthalate
EcotoQ – Quebec Environmental Toxicology Research Center	PP – Polypropylene
Eexp – Expected Effect	PPGEA – Graduate Program on Environmental Engineering
Eobs – Observed Effect	PS – Polystyrene
EU – European Union	PSNP – Polystyrene nanoplastics
GRIL – Interuniversity Research Group in Limnology	PVC – Vinyl Polychloride
HC5 – Hazardous Concentration to 5% of the species	RAC – Regulatory Accepted Concentration
HD – Hydrodynamic Diameter	RO – Reverse Osmosis RreSSa – Research Group on Resource Recovery in Sanitation Systems
ISO – International Organization for Standardization	SSD – Species Sensitivity Distribution
IVM – Ivermectin	SYN – Synergism
LABTOX – Environmental Toxicology Laboratory	TEM – Transmission Electron Microscopy
LC ₅₀ – Lethal Concentration for 50% of the organisms	TI – Toxicity Index
LDPE – Low-density polyethylene	TOXEN – Environmental Toxicology Research Group
MAF – Mixture Assessment Factor	TU – Toxic Units
MoA – Mode of Action	UFSC – Universidade Federal de Santa Catarina
MP – Microplastics	UQAM – Université du Québec à Montréal
MRA – Mixture Risk Assessment	UV – Ultraviolet
NAM – New Approach Methodology	WFD – Water Framework Directive
NOAEL – No Observed Adverse Effect Level	WHO – World Health Organization
NOEL – No Observed Effect Level	WoE – Weight of Evidence
	ZP – Zeta Potential

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3 THESIS CONTEXT

Increasing complexity in environmental pollution demands that scientists, industry, and policymakers take action to reduce the human pressure on natural habitats. This thesis elaborates on the risk assessment of two important pollution sources: plastics, using nanoplastics as representatives, and contaminants of emerging concern, with ivermectin as the representative. Both compounds were analyzed using an interdisciplinary evaluation connecting socioeconomic and environmental discussions, using characterization, bioassays, modeling, and regulatory perspectives.

Thesis development started at the beginning of the COVID-19 pandemic in June 2020 at the Graduate Program on Environmental Engineering (PPGEA) at Universidade Federal de Santa Catarina (UFSC) and in September 2020 in Environmental Sciences at the Université du Québec à Montréal (UQAM). The required courses for a cotutelle Ph.D. were already completed at UFSC and UQAM. The in-person activities of the cotutelle at UQAM started on May 2022 and finished with the return to Brazil in May 2024.

Integrating four laboratories, this thesis was advised in Brazil by Professor Maria Elisa Magri (Research Group on Resource Recovery in Sanitation Systems – RRESSA/UFSC), co-advised by Professor William Gerson Matias (Environmental Toxicology Laboratory – LABTOX/UFSC). In Canada, this thesis was advised by Professor Philippe Juneau (Department of biological sciences - GRIL, EcotoQ, TOXEN/UQAM) and co-advised by Professor David Dewez (Department of Chemistry - NANOQAM/UQAM). Given the multi-disciplinary aspect of the thesis the integration of different areas was of great relevance to the better completion of the thesis.

4 THESIS INTRODUCTION

Environmental assessments are usually done on a single compound basis, while the environment is increasing complexity when you consider that multiple compounds are discharged simultaneously (KORTENKAMP et al., 2007). This complexity for the understanding and modeling these compounds is partially due to the natural compounds present in the environment such as organic matter, humic acids that vary regionally and seasonally. Effluent characteristics could also vary significantly depending on the source, if it is either industrial or domestic effluent. Some of the relevant pollutants to understand environmental contamination are Polystyrene nanoplastics (PSNP) and ivermectin (IVM). The first is found at high concentrations and is ubiquitous in the environment and the last has high environmental impacts on the biota even when found in small concentrations in orders such as $\mu\text{g L}^{-1}$ or ng L^{-1} (SANDERSON et al., 2007a; SIEGFRIED et al., 2017). PSNP is obtained mainly from the degradation of macroplastics in the natural environment and IVM is widely used on livestock, being mostly found on soil, sediments and water (SANDERSON et al., 2007a; SIEGFRIED et al., 2017).

Ivermectin is an antiparasitic medication primarily utilized in livestock to safeguard against both internal and external parasites. This includes protection from nematodes affecting the gastrointestinal and respiratory systems, as well as infestations by lice, flies, and ticks (SANDERSON et al., 2007a). IVM is the highest selling antiparasitic in North America for animal use, with a regular dose of $0.2 \text{ mg kg}_{\text{bodyweight}}^{-1}$ (BOXALL et al., 2004). The main excretion route of IVM is through the feces, leading up to a potential impact on soil biota, mainly having an insecticide

effect on *Coleoptera sp.* and *Scarabaeidae sp.* raising concerns on its accumulation on the soil, reducing the productivity (SANDERSON et al., 2007a). The concentrations of IVM found in wastewater were measured up to 0.9794 mg L⁻¹ (INARMAL; MOODLEY, 2023) while in water ranged from 0.1 to 1 µg L⁻¹ (SANDERSON et al., 2007b).

IVM consumption by humans as an antiparasitic treatment is typically done at doses ranging from 150 to 200 µg L.d⁻¹ (FAILLOC-ROJAS et al., 2023). However, during the COVID-19 pandemic, misinformation circulated regarding its effectiveness in preventing the viral infection following a successful *in vitro* study (CALY et al., 2020), even though *in vivo* assays did not present conclusive data (ZAHEER et al., 2021). The use and even administration by doctors was observed at the first symptom as an 'early treatment' for COVID-19 (USFDA, 2021). Although WHO discouraged the use of IVM, its usage was continuous throughout the pandemic in Latin America (OLIVEIRA-FILHO et al., 2021; WHO, 2022). Widespread use by humans can displace this concern from rural livestock production areas to urban centers, where it could interact with other contaminants since the half-life of IVM in water can vary from <6h to 2.9 days and up to 15 days in the sediment (GWENZI et al., 2022) and over 100 days in marine environments. Thus, indicating that under certain conditions, IVM could be considered persistent (PRASSE; LÖFFLER; TERNES, 2009). The concentrations of IVM found in wastewater were measured up to 0.9794 mg L⁻¹ (INARMAL; MOODLEY, 2023) while in water ranged from 0.1 to 1 µg L⁻¹ (SANDERSON et al., 2007b).

Effects observed on the biota were the most significant for *Daphnia magna* with an LC₅₀ ranging from 1.2 to 25 ng/L, and No Observed Effect Level (NOEL) (SANDERSON et al., 2007a). Liebig et al. 2010 compiled the effects on the algae *Pseudokirchneriella subcapitata* (EC₅₀_{72h, growth rate} >4 µg/L), the fish *Oncorhynchus mykiss* (LC₅₀_{96h} = 3 µg/L) and the fish *Salmo salar* (LC₅₀_{96h} = 17 µg/L) (LIEBIG et al., 2010). The concentration of IVM in water is estimated to be on average 2 ng/L in areas nearby its contamination due to an increased affinity of this compound to the soil (Koc > 12.600) thus, most of its concentration remains in the soil matrix (SANDERSON et al., 2007a). Therefore, Sanderson et al. state that IVM was considered not to pose risk to aquatic biota, but raised concern over the impacts on soil and sediment organisms (SANDERSON et al., 2007a).

However, the concentrations for EC₅₀ found by Garric et al. (2007) started the range of concentrations at 1.2 ng/L, thus indicating that some risk may still be present even though were not considered initially. Briefly, IVM is discharged in the environment either through wastewater treatment plants final effluent, from non-treated wastewater discharge in bodies of water (INARMAL; MOODLEY, 2023), or from the surface runoff carrying the IVM present in livestock feces from the rural areas to the rivers and lakes in the region (SANDERSON et al., 2007a). With a Koc of that magnitude, an interaction between IVM and other pollutants with greater hydrophobicity could be expected (KOELMANS et al., 2016; ZICCARDI et al., 2016). The main pollutant that is going to be used for this interaction is considered to be ubiquitous in the environment, present in all biomes around the globe even without

human population, the pollutant is the polystyrene nanoplastic (PSNP) (KOELMANS et al., 2016; ZICCARDI et al., 2016).

PSNP can be manufactured in nanoscale e.g., for the cosmetics industry as an abrasive, however it is mostly present in the environment as a product of the degradation of macroplastics (GEYER; JAMBECK; LAW, 2017). This fragmentation can occur due to the effects of UV radiation, water agitation, chemical degradation, salinity and biodegradation (GIGAULT et al., 2018). These plastics in the environment are present mainly due to an incorrect disposal, observed through a recycling rate of only 9% of all the amount of plastic material produced worldwide (GEYER; JAMBECK; LAW, 2017). Those discarded in landfills find a favorable environment to the fragmentation of micro and nanoplastics debris, resulting from degradation in the acidic environment of landfills (DRIS et al., 2015; GEYER; JAMBECK; LAW, 2017; GIGAULT et al., 2018; JIANG et al., 2018; RIST et al., 2017). The degradation and fragmentation of macroplastics can lead to a greater dispersion of the materials since the smaller particles are lighter and can pass through small orifices. When summed with the increase on the ratio surface area / volume of the size reduction of the plastic particles, the chances of micro and nanoplastics interacting with environmental pollutants are increased significantly (KOELMANS et al., 2016; ZICCARDI et al., 2016).

Due to the widespread use of PSNP, its effects represent an essential material for understanding environmental plastic toxicity in low concentrations since the concentrations that can cause harm to 5% of the species (HC5) range from 0.02

to 2.0 $\mu\text{g L}^{-1}$ (ADAM; VON WYL; NOWACK, 2021; BESSELING et al., 2019; IWASAKI et al., 2023).

According to its density, the plastic material can be deposited in sediments, such as vinyl polychloride (PVC), or transported superficially through river waters to sea currents such as low density polyethylene (LDPE), polypropylene (PP) and polystyrene (PS) (MORÉT-FERGUSON et al., 2010). The transport and destination of micro and nanoplastic particles is difficult to monitor due to the variation in type, morphology and size of these materials. To identify the destination of PS spherical particles at milli, micro and nanometric scales in aquatic environments, Besseling et al. (2017) simulated the transport of particles from the river system to the marine environment. The result showed that nano and millimetric particles are more likely to accumulate in sediment along the river, while microparticles are able to reach the marine environment. However, Gigault et al. (2016) showed evidence of the presence of NP in ocean systems due to the accelerated physical-chemical degradation of millimetric scale plastics for nanometric scale, since the conditions of salinity, UV radiation, mechanical friction and biodegradation are adequate for this process.

The high surface area/volume ratio of eroded nanoplastics combined with the hydrophobic characteristics of micro and nanoplastics favor their use as transport vehicles of other emerging pollutants present in the environment via sorption and desorption processes. Such processes facilitate the dispersion, ingestion and possible penetration of emerging pollutants into the cells (BESSELING et al., 2014; BOUWMEESTER; HOLLMAN; PETERS, 2015; HIRAI et al., 2011; WAGNER;

LAMBERT, 2018). The main characteristics altering for the degree of sorption and desorption of an emerging pollutant in nanoplastics are (TEUTEN et al., 2009; WAGNER; LAMBERT, 2018):

- Chemical composition: Whether or not there is a chemical affinity between the nanoplastic and the referred pollutant;
- Crystallinity level: Plastic particles are not crystalline, but the greater the irregularity in the structure the greater the possible interaction with other compounds in the media, allowing the adhesion of pollutants;
- Particle size: The higher the ratio between the surface area and the volume of each particle, the greater the carrier potential;
- Surface properties: Surface coating or functionalization with or without the presence of loads may facilitate or hinder the role of a polluting vehicle.

The process of sorption and desorption increases the probability of interaction between compounds present in low concentrations and aquatic biota such as ingestion (TEUTEN et al., 2009). In this sense, aquatic organisms can ingest microplastics (MP) and nanoplastics (NP) particles of various sizes and shapes, including granules, fragments and fibers (BESSELING et al., 2014; JEMEC et al., 2016; RIST et al., 2017). After ingestion, these remain in the intestines of organisms, reducing their feeding capacity and consequently negatively affecting their development. A thorough assessment of the potential toxicological effects of these

pollutants on organisms is hampered because the composition of plastic is basically hydrocarbons. Thus, there are methodological limitations for the identification and quantification of ingested plastic, since the presence of micro and nanoplastics can be confused with the food itself in the body and in the sediment or water with organic matter and other hydrophobic compounds present in terrestrial and aqueous environments. Thus, the presence of plastics in various environments can cause deleterious effects to the biota due to the ingestion of plastics or their adhesion in gills including MP and NP (GREGORY, 2009).

PS is the most important polymer derived from styrene, being widely used for its low cost, ease of processing and good mechanical properties (CANEVAROLO JR., 2006). The main PS composite products are eyeglass frames, plastic cups, plastic egg trays, packaging and insulation in construction (expanded PS) (PLASTICSEUROPE; EPRO, 2017). Due to its wide use, the degradation of this polymer represents an important contribution to PSNP. Its amorphous structure confers a more irregular characteristic of its surface, allowing the physical adhesion of other hydrophobic compounds. Its density varies between 0.96 and 1.05 g/cm³, being close to that of water, which facilitates its transport through the water flow (WAGNER; LAMBERT, 2018). Despite the density indicating fluctuation, it is important to highlight the role that both biofilm and watercourses turbulence provide for the arrival of these materials in any water column (COLE et al., 2013).

The difference in chemical affinity, that is, the hydrophobicity of PSNP hinders the interaction with water. However, the high ratio between surface area/volume of its particles promotes stabilization of PSNP in aqueous media. Metric

limits for defining plastic debris as being micro or nanoplastics have been recently reported by Da Costa et al. (2016), which classified MP as particles with a diameter of less than 5 mm and NP with a diameter below 1 μm . Similarly, Gigault et al. (2018) determined that NP is the isolated particles or set of particles with dimensions between 1 nm and 1 μm .

Some studies aim to find the environmentally relevant concentrations of micro and nanoplastics, but the methodological and technical limitations for the collection, sampling and identification of materials in micro and nanometric scales are still barriers to be overcome. Variations in concentrations are verified due to the greater or lesser presence of plastic pollution in the region. The concentrations of environmental relevance for the NPPS were summarized in Table 1.

Table 1 – Review of environmental concentrations of polyethylene (PE), polystyrene (PS), polypropylene (PP), and polyethylene terephthalate (PET).

Sample type	Local	Polymer found	Concentrations	References
Freshwater	Meuse River, Netherlands Rhine River, Netherlands	PE, PS , PP	0,14 mg/m ³ or 9,7 MP/m ³ 0,56 mg/m ³ or 56 MP/m ³	Urgert (2015)
Freshwater	20 rivers and lakes from Wuhuan, China	PET, PP, PE, polyamide and PS	From 1.660 $\pm$ 639 to 8.925 $\pm$ 1.591 particles/m ³	Wang et al. (2017)
Sediments	Sarikum Lake, Turkey	PS	MP from 1 to 5 mm: 0,005-0,024 particles/g MP smaller than 1mm: 0,027-0,049 particles/g	Visne e Bat (2016)
Sediments	Rhine-Main area, Germany	PE, PP and PS	1 g/kg or 4.000 particles/kg	Klein, Worch and Knepper (2015)

Sample type	Local	Polymer found	Concentrations	References
WWTP effluent	Chicago metropolitan area of northeastern Illinois and northwestern Indiana and Central Illinois, United States	PP, PE and PS	1.338.757 particles/day	McCormick et al. (2016)
WWTP effluent	China	No specification	Mass concentrations: Plastic: [influent] – [effluent] MPs: 26.23 – 1.75 µg/L NPs: 11.28 – 0.71 µg/L	Zhang et al. (2023)

Source: Adapted from Wan et al. (2018).

Environmental analyses showed important variations amongst nanoplastics regarding their morphology, size and their surface chemistry (biofilm, organic matter, for example) (JIANG et al., 2018; LYNCH et al., 2014). These modifications occur mainly due to the plastics origins and fate in the environment. In the natural environment, the degradation of larger plastic particles occurs through UV radiation, salinity of the waters, animal ingestion and handling of plastic materials, friction with solid particles, biodegradation and changes in temperature, to list a few (FOTOPOULOU; KARAPANAGIOTI, 2015). The shape of these particles is irregular with some concave structures that help to increase the surface area and thus retain potentially more of what is present in the media: organic matter or other pollutants (FOTOPOULOU; KARAPANAGIOTI, 2015).

As a means of obtaining the behavior of different pollutants that could be interacting with each other, several methods in environmental toxicology were adapted or developed, such as the A in the presence of B approach. This approach aims to investigate the behavior of compound A in the presence of a fixed concentration of compound B. The next step to understand the adverse effects on

biota is the application of risk assessment methods, which are well known and have a high confidence. Although the last statement is true for single chemicals, the greater the diversity of pollutants, the lower the predictability of their effects as a mixture due to their spatial, temporal variation and effects on the more diverse organisms of the potentially affected biota (ARIS; SHAMSUDDIN; PRAVEENA, 2014; DIETRICH et al., 2010).

One way to fill the gaps left by pollutant mixtures is to apply environmental risk assessment models for the mixture of various pollutants, called Mixture Risk Assessment (MRA) (DRAKVIK et al., 2020a; KIENZLER et al., 2016). Simplification is often used through the segregation of pollutants in similar action groups so that a model can be used for each group and then a model for pollutants of different actions to then finalize the modeling of the complex mixture (DRAKVIK et al., 2020a). A relatively new approach to understand the toxicity is the Adverse Outcome Pathways (AOP) framework, which works based on the simplification of the natural processes of adverse outcomes in several key events that are measurable and well defined (ANKLEY et al., 2010; VILLENEUVE et al., 2014). Therefore, the importance of representing complex biochemical processes in a simpler, easily comprehensible and linked in a chain of events is crucial for helping professionals outside academia.

From this scenario emerges the need for a mixture risk assessment model employing simple and comprehensive methods that regulators could apply. Models that could implement calculations that would consider the impact of multiple compounds could create a structure in which the environment is protected based on the measure of its global exposure and not on a single compound basis. For that

reason, regulation started becoming more important at the same pace that new compounds are added to the environment, and new concepts emerged, such as the exposure load, emerged (WILLEY et al., 2021).

One model that is being applied worldwide and has shown promising evaluations is the Species Sensitivity Distribution (SSD) curve. After acquiring toxicity endpoints from the literature, it is possible to plot a distribution curve of the values, through which the Hazardous Concentration to 5% of the species (HC5) can be calculated for each compound, thus providing a safe concentration for most organisms. Several countries use SSD as a part of their regulatory framework on at least one stage of the evaluation. The SSD method was developed to be used for single compounds only; however, it is important to merge the results of the SSD curves into one analysis for more compounds to better understand the behavior in the environment. This fact highlights the importance of integrating calculation methods to the understanding of mixture toxicity on a regulatory level, a gap in regulation that is yet to be fulfilled. Few countries have started discussing the inclusion of mixture toxicity as evaluating factors for the water quality in a more direct way. What is currently done is usually the use of whole-mixture toxicity testing using the effluents that are being discharged into the environment. Somehow the toxicity is being tested, however, once discharged the environment, different effluents will interact with each other causing interaction between pollutants that would not be considered co-occurring.

The practical use for the risk assessments developed in academia is to help chemical developers in industries and regulatory agencies professionals to make

decisions with increased confidence and reduced damage to the environment. One problem observed in this dynamic is the need in academia to produce great amounts of data that are not always being used by other stakeholders. Regulatory agencies on the other hand are overloaded with work and cannot always take the time to make decisions based on a literature review that could take months, when the action that must be taken in chemical contamination is happening in a matter of days.

In that regard a better connection between academia and regulatory agencies is necessary to better direct the time and energy spent producing such high-quality information. Therefore, the gap between academia and regulatory practices could be reduced by developing methods that would use the data generated and provide update numbers to each regulation. This data could be provided by academia, while the application of the methods could be validated and applied by regulatory agencies.

This work aims to integrate three aspects of the mixture risk assessment field: bioassays, modelling, and regulatory aspects through the generation of bioassays data, modelling including the generating data, and discussing how could the regulatory framework be adapted to accommodate for the increasing number of new compounds being discharged in the environment. The following chapters elaborate on those three aspects and help promote a greater integration between generated data and applied knowledge.

5 OBJECTIVE

5.1 MAIN OBJECTIVE

Perform an ecological mixture risk assessment across different trophic levels using polystyrene nanoplastics and ivermectin as case study, providing a comprehensible understanding of the process from compounds applied to model development and its application in regulatory scenarios.

5.1.1 Specific objectives

1. Identify the effects caused by polystyrene nanoplastic and ivermectin at environmentally relevant concentrations (chronic level) and concentrations higher than the environmental concentrations (acute level) on the test organisms;
2. Create a calculation method for updating regulatory thresholds based on toxicity data from the literature using the mixture toxicity of an emerging contaminant and a regulated contaminant;
3. Propose a framework method for a greater application of ecological mixture risk assessment in the environmental regulatory agencies through an action plan, understanding the socioeconomic applications of ecological risk assessment.

5.2 HYPOTHESIS

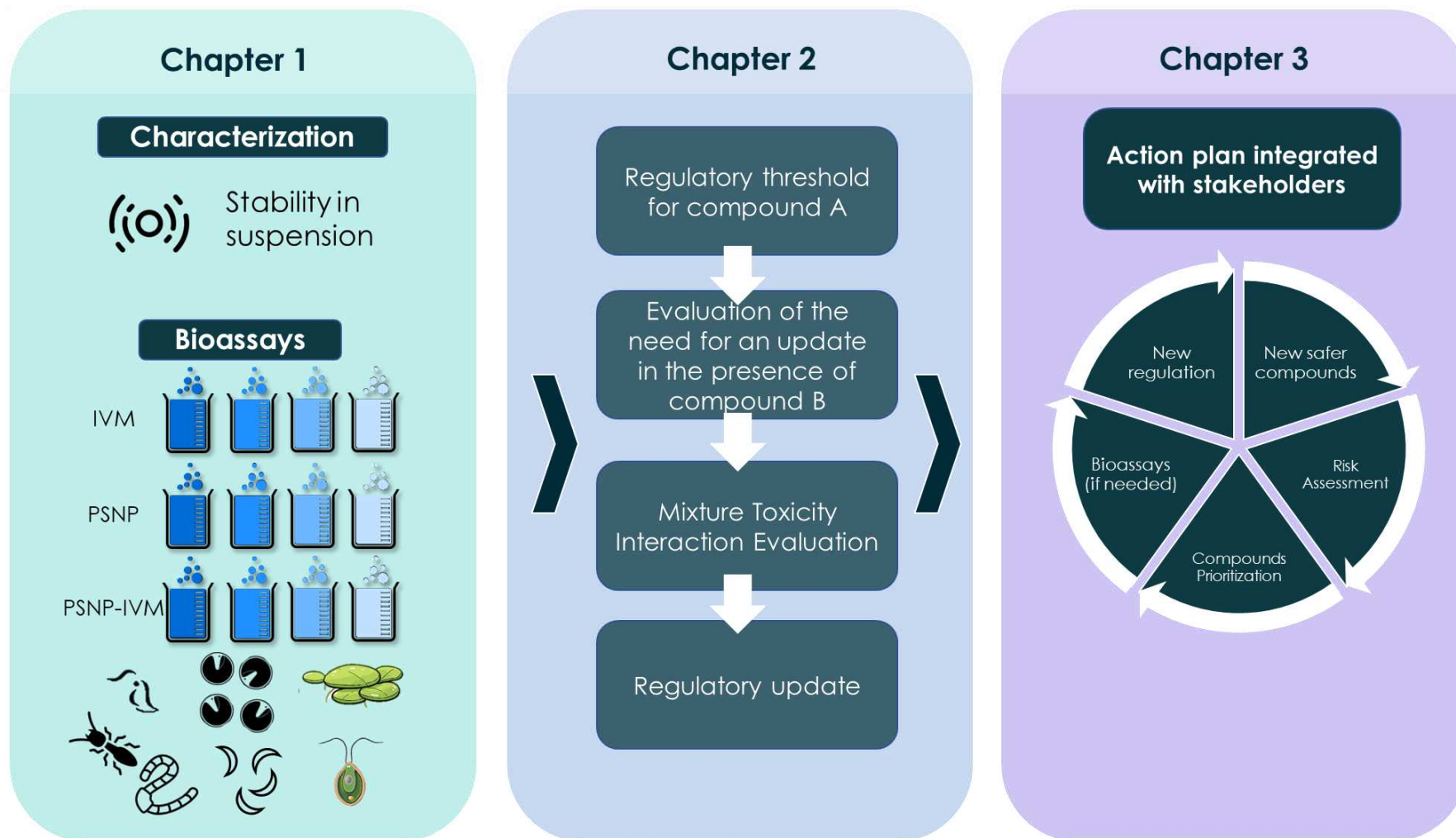
1. The ivermectin will adhere to the polystyrene nanoplastics surface causing the mixture between ivermectin and polystyrene nanoplastics to promote a

greater impact (synergism) than the expected additive effect for the isolated compounds due to the probable trojan horse effect;

2. The proposed framework and inherent calculations based on the species sensitivity distribution curve, adjusted for specific toxicological interactions identified in the literature; will update the water quality threshold and thus increase environmental protection when needed;

3. A structured framework integrating ecological risk assessment with socioeconomic analysis will enhance the precision on the updating values for water quality thresholds by environmental regulatory agencies, leading to more effective management of ecological risks associated with chemical mixtures, due to a greater integration between academia, industry, regulatory bodies, and social aspects.

6 THESIS SCHEME



7 CHAPTER 1 – CHARACTERIZATION AND BIOASSAYS

7.1 INTRODUCTION

Environmental pollution occurs in an increasingly complex and diffuse way. The presence of several pollutants in the environment presents a complexification of the environmental assessment of its effects on the biota. (KORTENKAMP et al., 2007). Some of these pollutants can be present in various environmental compartments due to their ubiquitous use or incorrect disposal (BESSELING et al., 2017). Polystyrene nanoplastics (PSNP) and ivermectin (IVM) are among the pollutants with high environmental relevance. The first is found at high concentrations in the environment in places with high plastic pollution due to the degradation of macroplastics, and the last has high environmental impacts on the biota even when found in small concentrations in orders such as $\mu\text{g L}^{-1}$ or ng L^{-1} from which the main route towards the environment is through its use on livestock (SANDERSON et al., 2007; SIEGFRIED et al., 2017).

Ivermectin is an antiparasitic drug most often used on livestock, protecting the animals from internal and external parasites such as nematodes on the gastroenteric and respiratory tracts, lice, flies, and ticks (SANDERSON et al., 2007a). IVM is the highest-selling antiparasitic in North America for animal use, with a regular dose of $0.2 \text{ mg kg of body weight}^{-1}$ (BOXALL et al., 2004). The main excretion route of IVM is through the feces, leading up to a potential impact on soil biota, mainly having an insecticide effect on *Coleoptera sp.* and *Scarabaeidae sp.*, raising concerns about its accumulation on the soil due to reduced productivity (SANDERSON et al., 2007a).

The half-life of IVM in the soil ranges from 11.5 days in clay soil to 15.5 days in sandy soil (GWENZI et al., 2022). More than 90% of the administered concentration of the active parent drug can remain in the feces of treated livestock (e.g., 39% in cattle, 43% in swine, and 69% in sheep) (VOKŘÁL et al., 2019). Even though IVM is an antiparasitic drug, Vokral et al. (2019) found a 20% reduction in the root growth of the white mustard (*Sinapis alba* L.) at the concentration of 50 nM, demonstrating the possible impact on non-target species.

Humans can also consume IVM as an antiparasitic, with an average of 150 to 200 $\mu\text{g kg}^{-1}$ (FAILOC-ROJAS et al., 2023). Furthermore, during the COVID-19 pandemic, some authors advanced a possible efficiency to protect against viral infection after a successful *in vitro* test. (CALY et al., 2020), even though *in vivo* assays did not present conclusive data (OLIVEIRA-FILHO et al., 2021; ZAHEER et al., 2021).. The use and even administration by doctors was observed at the first symptom as an 'early treatment' for COVID-19 (USFDA, 2021). Although WHO discouraged the use of IVM, its usage was continuous throughout the pandemic in Latin America (OLIVEIRA-FILHO et al., 2021; WHO, 2022). Widespread use by humans can displace this concern from rural livestock production areas to urban centers, where it could interact with other contaminants since the half-life of IVM in water can vary from <6h to 2.9 days and up to 15 days in the sediment (GWENZI et al., 2022) and over 100 days in marine environments. Thus indicating that under certain conditions, IVM could be considered persistent (PRASSE; LÖFFLER; TERNES, 2009). The concentrations of IVM found in wastewater were measured up

until 0.9794 mg L⁻¹ (INARMAL; MOODLEY, 2023) while in water ranged from 0.1 to 1 µg L⁻¹ (SANDERSON et al., 2007b).

Since plastics are ubiquitous in the natural environment, nanoplastics are mostly present as either primary plastics, which were produced on the nanometric scale, or as a consequence of the degradation of macroplastics (GEYER; JAMBECK; LAW, 2017). This fragmentation can occur due to UV radiation, water agitation, chemical degradation, salinity, and biodegradation (GIGAULT et al., 2018). These plastics in the environment are present mainly due to incorrect disposal, leading to a recycling rate of only 9% of all the plastic material produced worldwide (GEYER; JAMBECK; LAW, 2017). Those discarded in landfills find a favorable environment for the fragmentation of micro and nanoplastics debris resulting from degradation in the acidic environment of landfills (DRIS et al., 2015; GEYER; JAMBECK; LAW, 2017; GIGAULT et al., 2018; JIANG et al., 2018; RIST et al., 2017).

The high surface area/volume ratio of eroded nanoplastics and the hydrophobic characteristics of micro and nanoplastics favor their use as transport vehicles of other emerging pollutants present in the environment, such as IVM, via sorption and desorption processes. These processes facilitate the dispersion, ingestion, and possible penetration of emerging pollutants into the cells (BESSELING et al., 2014; BOUWMEESTER; HOLLMAN; PETERS, 2015; HIRAI et al., 2011; WAGNER; LAMBERT, 2018). Due to its widespread use, the effects of PSNP represent an essential material for understanding environmental plastic toxicity in low concentrations since the concentrations that can cause harm to 5% of

the species (HC5) range from 0.02 to 2.0 $\mu\text{g L}^{-1}$ (ADAM; VON WYL; NOWACK, 2021; BESSELING et al., 2019; IWASAKI et al., 2023).

This work aims to investigate the ecological effects of PSNP, IVM, and a mixture between them (PSNP-IVM) by i) performing bioassays with the non-target species *Chlamydomonas reinhardtii*, *Microcystis aeruginosa*, *Lemna minor*, *Daphnia magna*, *Folsomia candida* and *Enchytraeus crypticus*; and ii) generate a Species Sensitivity Distribution (SSD) curve for the IVM with the tested species and perform a risk assessment by complementing the curve with literature data.

7.2 METHODOLOGY

7.2.1 Materials

The contaminants used in this research were IVM obtained at $\geq 98\%$ purity (Sigma-Aldrich, CAS 70288-86-7), and PSNP previously synthesized by Vicentini et al. in which paper further details about the synthesis process are described. The water used throughout the research is Reverse Osmosis (RO) water, with a conductivity of $0.1 \mu\text{S cm}^{-1}$. Ethanol (purity $\geq 95\%$) (Sigma-Aldrich, CAS 64-17-5) was used to increase IVM solubility on the aquatic assays, while DMSO (purity $\geq 99\%$) (Sigma-Aldrich, CAS 67-68-5) was used for the terrestrial organisms.

7.2.2 Stability of the PSNP in suspension

Stability in suspension measures the properties associated with a solid in a given aqueous solution to maintain itself in suspension. Zeta Potential (ZP),

Hydrodynamic Diameter (HD), and Polydispersity Index (PDI) are the parameters chosen to be measured in this work since they give a good perspective on the stability (VICENTINI et al., 2019). All the stability parameters, ZP, HD, and PDI, were measured using the Nanobrook Haven 90Plus PALS equipment. The isolated compounds and their mixture on every liquid media of testing (ISO, M4, CHU, and BBM) had their stability quantified. The concentrations that reached the limits of detection were determined experimentally. However, priority was given to the lower concentrations to reduce error due to interference on the equipment. Stability in suspension values were measured for solutions at the same concentrations applied during the acute toxicity test in *Daphnia magna*. The concentrations were kept constant for the PSNP at 66.03 mg L⁻¹, and each increment added 1.97 ng L⁻¹ of IVM; all alteration promoted by the presence of IVM on stability is summarized in the Supplementary material (S1).

ZP evaluates the stability using the electrophoretic mobility method, which states that the greater the absolute value of the ZP in mV, the greater the stability (MURDOCK et al., 2007). Murdock et al. (2007) also state that a sample is considered stable if the ZP is greater than 30 mV in absolute values. The ED value is the dimension of the suspended particles obtained through Dynamic Light Scattering, which measures the scattering of light through a certain angle and provides information about the movement of the particles (VICENTINI et al., 2019). The PDI indicates the agglomeration of the particles in suspension, meaning that values equal to or smaller than 0.3 (dimensionless) are monodisperse. Monodispersity is a property that indicates a low level of agglomeration and, thus, a

narrow range of diameter values (JIANG; OBERDÖRSTER; BISWAS, 2009; KÜLKAMP et al., 2009). A higher agglomeration can mean a higher tendency of the particles to leave the water column and accumulate on the sediment, thus giving an important exposure aspect to the contaminant.

The results were statistically treated using the one-way ANOVA analysis for variance between each parameter; normality was tested through D'Agostino & Pearson normality tests in the GraphPad Prism 6 software ®. This software has the homoscedasticity test as a built-in function of the ANOVA testing, thus being standardized.

7.2.3 Bioassays

All bioassays performed in this study were compiled in Table 2 with their respective endpoints and concise methodology. All IVM dilutions were solubilized in ethanol to create a stock solution of 1 mg L^{-1} , and all tests' control treatments with ethanol were performed.

Table 2 - Test organism and their respective trophic levels, environmental compartments where they can be found, type of bioassay, endpoint, and brief methodology.

Test organisms	Trophic level	Environmental compartment	Bioassay	Endpoint	Method
<i>Daphnia magna</i>	Primary consumer	Freshwater	Acute	EC50	The immobilization of the Daphnids will be measured after the neonates (<24h) are exposed to the pollutants for 48h (OECD, 2004).
			Chronic	NOEC LOEC	The growth, reproduction, and longevity of the organisms will be measured after exposure to the pollutants for 21 days, comparing the control treatment with the exposed ones (OECD, 2012).
<i>Lemna minor</i>	Primary producer	Freshwater	Growth inhibition	EC20 NOEC	After a 7-day exposure to the pollutants, the growth inhibition will be measured by the alterations in the frond numbers (OECD, 2006).
<i>Folsomia candida</i>	Decomposer	Soil	Reproduction	EC50	Exposition of the organisms to different concentrations for 28 days and measured the number of juveniles generated, compared with the control treatments (ISO, 2014).
<i>Enchytraeus crypticus</i>	Decomposer	Soil	Chronic	EC50	Exposition of the organisms to different concentrations for 21 days and measured the number of juveniles generated, compared with the control treatments (ISSO, 2004).
<i>Microcystis aeruginosa</i> <i>Chlamydomonas reinhardtii</i>	Primary producers	Freshwater	Growth inhibition	EC50 NOEC	Growth inhibition is stated in the OECD guidelines for testing algae. The test takes 72 hours, and the results performed by counting the cells and the effect of the different concentrations in inhibiting the phytoplankton growth (OECD, 1984)

7.2.3.1 *Daphnia magna*

The *Daphnia magna* organisms were cultivated in an artificial M4 medium according to the ISO 6341 protocols (ISO, 2012). Individual batches were kept in 2 L beakers with a 16h:8h light/dark photoperiod at 20 ± 2 °C, and the *D. magna* were fed with the algae *Scenedesmus subspicatus* (6×10^5 cells mL⁻¹), cultured in CHU medium (Chu, 1942). The acute toxicity tests followed the OECD Test Guidelines No. 202 (OECD, 2004), in which ten neonates per container were exposed to the pollutants for 48 hours. The applied concentrations were as follows: IVM (10.00, 12.50, 15.00, 17.50, and 20.00 ng L⁻¹), PSNP (31.25, 62.50, 125.00, 250.00, and 500.00 mg L⁻¹), and their mixture (all treatments had a constant PSNP concentration of 63.00 mg L⁻¹ and the IVM was varied as 0.00, 1.91, 3.82, 5.72, and 7.63.00 ng L⁻¹).

The chronic toxicity test was performed following Test Guideline No. 211 (OECD, 2012), in which ten neonates were exposed to the contaminants, each in a 50mL beaker with fresh media renewed three times a week to maintain the contaminants concentration constant throughout the experiment. The applied concentrations were as follows IVM (10.00, 12.50, 15.00, 17.50, and 20.00 ng L⁻¹), PSNP (0.053, 0.53, 5.30, 53.00, and 530.00 mg L⁻¹), and their mixture (all treatments had a constant PSNP concentration of 5.30 µg L⁻¹ and the IVM was varied as 0,0001, 0,001, 0,01, and 0,1 ng L⁻¹). The parameters of size and longevity were measured

after 21 days of exposure, while reproduction was measured during every media renewal.

7.2.3.2 *Lemna minor*

The stock organisms were obtained from cultures held at the Ecotoxicology of Aquatic Microorganisms Laboratory (UQAM, Montreal, Canada) in a growth chamber and at a constant temperature of 24.0 ± 1.0 °C, at $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and a 14h/10h light/dark photoperiod and transferred every week to keep them at the exponential growth phase using Bold Basal Medium (BBM) as culture media. The growth inhibition test with *Lemna minor* followed the guidelines of OECD No. 221 (OECD, 2006a). The exposed organisms composed ten fronds in total and were exposed for seven days in 250 mL Erlenmeyer flasks with 100 mL culture media in triplicate. At the end of the test, the following parameters were analyzed: frond number, root length, and surface area. The applied concentrations were as follows: IVM (0.10, 1.00, 10.00, 100.00, and 1 000.00 $\mu\text{g L}^{-1}$), PSNP (0.10, 1.00, 10.00, 100.00, and 1 000.00 mg L^{-1}), and their mixture (all treatments had a constant PSNP concentration of 10.00 mg L^{-1} and the IVM was varied as 0.10, 1.00, 10.00, 100.00, and 1 000.00 $\mu\text{g L}^{-1}$). The average growth was measured by using the same equation used for the microorganisms but changing X_i and X_j for each parameter evaluated and i and j for seven days (KITAMURA et al., 2023; LALAU et al., 2014).

7.2.3.3 *Folsomia candida*

The collembolans *F. candida* were cultivated in laboratory conditions according to ISO 11267 (ISO, 2014) in a substrate comprised of plaster of Paris (powdered), distilled water, and activated charcoal (powder) in a proportion of 10:6:1 (w:w:w), respectively. They were fed granulated dry yeast (*Saccharomyces cerevisiae*) twice a week when the substrate moisture was adjusted with distilled water. The tests were performed using Tropical Artificial Soil (TAS), a standard substrate recommended for terrestrial ecotoxicological assays in tropical and subtropical regions (GARCIA et al., 2004), composed of 75% fine sand, 20% kaolin clay and 10% coconut fiber.

Chronic toxicity tests evaluated the reproduction of the organisms based on the ISO 11267 guidelines (ISO, 2014), in which ten organisms aged between 10 and 12 days were used in each replicate and exposed to a serial dilution of all tested compounds for 28 days. For PSNP, a stock solution was prepared with distilled water. IVM was diluted in dimethylsulfoxide (DMSO) before its application in soil samples. An additional DMSO control test was performed to evaluate the possible effect of the solvent. Individuals were fed at the beginning of the test and after 14 days, and the soil moisture was adjusted weekly. The applied concentrations were as follows: IVM (1.00, 2.00, 4.00, 8.00, and 16.00 mg kg⁻¹), PSNP (0.10 mg kg⁻¹), and their mixture (all treatments had a constant PSNP concentration of 0.10 mg kg⁻¹ and the IVM was varied as 1.00, 2.00, 4.00, 8.00, and 16.00 mg kg⁻¹). At the end of the test, the content of each replica was submerged in water, and a few drops of

black ink were added to the water to produce a dark background that facilitated the visualization of the surviving adults and juveniles. All replicates were photographed in high resolution through a digital camera, and the images were assessed using the ImageJ® software to count the number of juveniles generated during the assay.

7.2.3.4 *Enchytraeus crypticus*

The *E. crypticus* individuals were also exposed to the spiked TAS to perform chronic assays according to ISO 16387 (ISO, 2004). Ten organisms with similar length and visible clitellum were placed in 200 mL glass containers with 30 g of moist soil spiked with tested compounds or only distilled water (control soil). A control treatment containing DMSO was also prepared. The applied concentrations were as follows: IVM (1.00, 2.00, 4.00, 8.00, and 16.00 mg kg⁻¹), PSNP (0.10 mg kg⁻¹), and their mixture (all treatments had a constant PSNP concentration of 0.10 mg kg⁻¹ and the IVM was varied as 1.00, 2.00, 4.00, 8.00, and 16.00 mg kg⁻¹). Feeding was performed weekly using oat flour. Following the methodology of Bandeira et al. (2021), eight replicates were performed for the control and four replicates for treatments containing ivermectin. After 21 days of exposure, enchytraeids were fixed in 70% ethanol and stained with five drops of Bengal rose solution (1% in ethanol), and the juveniles produced were counted using a stereo microscope.

7.2.3.5 *Chlamydomonas reinhardtii* (CPCC5) and *Microcystis aeruginosa*
(CPCC632)

The stock organisms were obtained from Canadian Phycological Culture Center (CPCC, Waterloo) and kept in a growth chamber at constant temperature of 24.0 ± 1.0 °C, at $100 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and a 14h/10h light/dark photoperiod with gentle daily manual agitation and transferred every three days to keep them in their exponential growth phase using Bold Basal Medium (BBM) with added vitamin B12 as culture media. The growth inhibition was assessed after 72h exposition to the contaminants following the OECD Test Guidelines No. 201 (OECD, 1987). The test organisms were kept in 150 mL Erlenmeyer flasks with a total of 60 mL of culture media, and added IVM (0.10, 1.00, 10.00, 100.00, and 1 000.00 $\mu\text{g L}^{-1}$), PSNP (1.00, 25.00, 50.00, 75.00, and 100.00 mg L^{-1}), and their mixture (all treatments had a constant PSNP concentration of 0.10 mg L^{-1} and the IVM was varied as 0.10, 1.00, 10.00, 100.00, and 1 000.00 $\mu\text{g L}^{-1}$). The cell density was measured at the beginning (with an initial concentration of 5×10^5 cells) and the end of the assays using a Multisizer 3 Coulter Counter particle analyzer (Beckman Coulter Inc., USA). The growth rate was determined as the OECD establishes $\mu_{i-j} = [\ln(X_j) - \ln(X_i)] / (t_j - t_i)$, where μ is the average specific growth rate, X is the cell concentration, t is time in hours, and i and j are the initial and final test times, resulting in 72h.

7.2.4 Mixture Risk Assessment

Based on the environmental scenario in which there was a rapid increase in consumption of IVM during the COVID-19 pandemic (GWENZI et al., 2022; OLIVEIRA-FILHO et al., 2021), the methodology of the mixture assays for both acute and chronic tests applied the “A in the presence of B” mixture toxicity approach (GESTEL, 2012). The application of the method consists of keeping constant the concentration of the first pollutant while varying the concentration of the second and understanding the effects caused by the second compound on global toxicity. This approach explains the real effects of the sudden increase in IVM concentration in the environment. All tested concentrations are specified in Table 2.

The Abbott method was used to evaluate the toxicological interactions by calculating the ratio between the expected effect (E_{exp}) and the observed effect (E_{obs}) (GIBSON et al., 2019). The expected effect is calculated based on the effect of the isolated compounds (compound A and compound B), thus $E_{Exp} = A + B - \left(\frac{A*B}{100}\right)$. The ratio between E_{obs}/E_{exp} is established as the Toxicity Index (TI) will determine if the effect was antagonism ($TI < 0.7$), additivity ($0.7 < TI < 1.3$), or synergism ($TI > 1.3$) as proposed by Chen et al. (2014).

The toxic units (TU) methodology was applied to cases where it was possible to calculate the EC50 for the IVM. TUs are used to define the mixture concentrations as a parametrization of the toxicity of both compounds to its EC50 of the same organism and effect that was tested in cases in which the EC50 for IVM was found (FOUCQUIER; GUEDJ, 2015; RODEA-PALOMARES et al., 2015). The applied

concentrations are shown in Table 4, followed by their respective concentrations in mg L⁻¹ and ng L⁻¹ and the respective TU sum for each treatment.

7.2.5 Species sensitivity distribution (SSD) curve

The SSD curve was elaborated using primary (bioassays done in this work) and secondary data. (data obtained from the literature). Since the toxicity profile of IVM is not completely understood, two criteria were established to filter the data to compose the SSD curve: i) the endpoint chosen for the curve was the EC50, and ii) the effect must be of the acute exposure, either mortality, immobility, or growth rate. This selection aims to identify if non-target species are at risk of exposure to IVM. The tool used to generate the curve was the Species Sensitivity Distribution Generator recommended by the USEPA (USEPA, 2005). The calculations use Probit modeling.

Recently, a microplastics SSD curve was elaborated, calculating an HC5 between 0.02 and 2.0 µg L⁻¹ varying based on the size of the particle and the morphology (either a sphere/fragment or fiber) (Iwasaki et al., 2023) was similar to those found with different methodologies (Adam et al., 2021; Besseling et al., 2019), even with micro and nanoplastics varying significantly between experiments in size, shape, chemical composition, additives, or degradation level. Their unit endpoints often differ from mg L⁻¹ to particles L⁻¹, and the concentrations at which the effects are observed are usually too high to have environmental significance. Thus, this study will elaborate only on an IVM SSD.

7.3 RESULTS

7.3.1 Stability in suspension

Table 3 summarizes the results by the PSNP isolated samples and the highest IVM concentration tested for *D. magna*. The same concentration was used for all media to provide a more insightful behavior comparison of PSNP in the presence of IVM.

Table 3 – Zeta Potential (ZP), Effective Diameter (ED), and Polydispersity Index (PDI) values for PSNP, IVM, and their mixture (PSNP-IVM) in all tested media. RO: reverse osmosis water; BBMI: Bold Basal Medium used for Lemna minor; BBM m: Bold Basal Medium used for the microorganisms.

Media	Samples	pH	ZP	ED	PDI
		--	mV	nm	nm
RO	PSNP	7.02	-62.00 ± 1.24	130.66 ± 3.31	0.213 ± 0.005
	PSNP-IVM (1.50)	7.02	-33.47 ± 0.7	134.05 ± 1.17	0.203 ± 0.004
ISO	PSNP	7.03	-43.78 ± 0.36	135.65 ± 1.28	0.290 ± 0.153
	PSNP-IVM (1.50)	7.03	-35.64 ± 2.57	167.63 ± 3.91	0.286 ± 0.017
M4	PSNP	7.00	-44.9 ± 0.69	137.93 ± 2.73	0.203 ± 0.008
	PSNP-IVM (1.50)	7.00	-46.38 ± 0.95	132.22 ± 1.23	0.220 ± 0.023
BBMI	PSNP	6.80	-58.03 ± 1.16	169.95 ± 1.08	0.229 ± 0.01
	PSNP-IVM (1.50)	6.80	-61.86 ± 0.67	170.48 ± 3.25	0.236 ± 0.004
BBMm	PSNP	6.80	-53.64 ± 0.88	242.03 ± 7.09	0.257 ± 0.047
	PSNP-IVM (1.50)	6.80	-65.79 ± 1.54	194.69 ± 3.48	0.282 ± 0.029

The PZ measurement of both PSNP and mixture resulted in high particle stability in all measured media, at pH corrected for the values applied on the tests: 7.0 for reverse osmosis water (RO) M4, and ISO; and 6.80 for Bold Basal Medium used on the Lemna minor tests (BBMI) and Bold Basal Medium used on the tests with the microorganisms (BBMm) (Table 3). According to one-way variance analysis

(ANOVA), there was a statistically significant difference ($p < 0.05$) between the ZP data when compared in pairs. Thus, it was possible to notice a reduction in the stability of PSNP and IVM in RO water compared to culture media (Table 3). Such behavior was expected, given that the ionic strength of the culture medium posed by the salts with different polarities in solution may reduce the stability of the suspended compounds (MURDOCK et al., 2007). Despite this reduction, PSNP particles, both isolated and in the mixture, continued to be significantly stable ($ZP > 30.0$ mV). However, the stability of BBMm showed an increase that could be explained by being the only medium with biotin in its composition. Biotin is a neutral polymer, and the stability in suspension tends to increase in the presence of such compounds (BROOKS, 1973; KWON; KWON; HONG, 2008). Regarding the dispersion of particles, the PDI showed no statistical difference between its measurements. All measurements show that the samples are monodisperse ($PDI < 0.3$), so the suspended particles do not present tendencies to the formation of agglomerates.

7.3.2 Bioassays

*7.3.2.1 Effects on *Daphnia magna**

The results for the same PSNP sample with *D. magna* are previously generated, giving an EC50 of 133.04 ± 17.42 mg L⁻¹ ($n = 5$) (VAZ, 2020). The isolated IVM provided an EC50 of 7.63 ± 1.09 ng L⁻¹ ($n = 4$). The value for IVM corroborates with those in the literature and the PSNP with the toxicity observed for other plastics produced in the same lab (SANDERSON et al., 2007a; VICENTINI et

al., 2019). The PSNP-IVM mixture evaluation using the Abbott method showed an antagonistic effect in the mixtures M1 and M2. At the same time, the higher the IVM concentration additivity was, the effect mostly observed on the immobility of *D. magna*. For further information, the graphs are presented in appendix A.

Chronic effects on *D. magna* showed little to no effect on PSNP and PSNP-IVM. However, significant ($p < 0.05$) reductions in reproduction, size, and longevity were observed at the IVM concentration of 0.1 ng L⁻¹ (Table 4), meaning that the lowest IVM NOEC is 0.001 ng L⁻¹ for the parameter size. However, even with the small effect, evaluating the toxicological interactions between the pollutants compared to the isolated forms was possible.

As for the toxicological interaction, it was possible to observe (Table 5) that when comparing the isolated compounds to their mixture, the Abbott method indicated a tendency for synergism on reproduction and antagonism on size and survival, except at the highest mixture concentration (M5), in which synergism was observed for longevity. This means that, in general, the presence of PSNP attenuated the effects of IVM on size and longevity and increased its effect on reproduction.

7.3.2.2 Effects on *Lemna minor*

The effects of the pollutants on *L. minor* are shown in Tables 4 and 5. The surface area showed no significant ($p > 0.05$) effect caused by PSNP. However, the effects from the exposition of 100 and 1,000 µg L⁻¹ of IVM showed significant ($p >$

0.05) effects, increasing the surface area. When exposed to PSNP-IVM, the surface area was reduced significantly ($p > 0.05$) compared to both controls. PSNP increased the root length of the same organisms with the exposition at 100 mg L⁻¹, while both IVM and PSNP-IVM had no significant ($p < 0.05$) effect on root length. The Abbott method (Table 4) indicated overall antagonism for all concentrations applied, meaning that both PSNP and IVM in mixtures reduced their isolated effects on both surface area and root length for *L. minor*. For further information, the graphs are presented in appendix B.

7.3.2.3 Effects on *Microcystis aeruginosa* and *Chlamydomonas reinhardtii*

PSNP induced adverse effects on *M. aeruginosa* for all samples; however, only for PSNP the EC₅₀ (Table 3) was possible to calculate, generating an EC₅₀ of 43.49 ± 7.85 mg L⁻¹. The growth rate was slightly reduced in presence of 0.10 and 1.00 µg L⁻¹ IVM, but the concentration of 1,000 µg L⁻¹ increased the average growth rate by 22%. The effects of PSNP on *C. reinhardtii* (Table 4) showed a PSNP EC₅₀ of 13.45 ± 3.45 mg L⁻¹, while it was not possible to calculate the EC₅₀ for neither the PSNP nor PSNP-IVM. PSNP-IVM provided an increase in the average growth rate of 32%. For further information, the graphs are presented in appendix C.

The mixture effect can be categorized between synergism for *M. aeruginosa* and additivity for *C. reinhardtii*, with a tendency for synergism with the increase of IVM concentration for both species (Table 5).

7.3.2.4 Effects on *Folsomia candida* and *Enchytraeus crypticus*

The IVM toxicity results showed an EC50 of 5.66 ± 1.64 mg/kg for *F. candida* and 47.98 ± 8.61 mg/kg for *E. crypticus*. The mixture PSNP-IVM applied to *F. candida* demonstrated antagonism in the first concentration applied and additivity when the IVM concentration was increased, meaning that this mixture is dose-dependent for this organism, with a tendency to increase the effects the bigger the IVM concentrations. As for *E. crypticus*, the first mixture concentration presented synergistic effects, while the increase in IVM concentration provided additivity as the main effect. The RI had low variation between the additive mixtures, meaning the increase in IVM concentration did not alter their toxicological interaction. For further information, the graphs are presented in appendix D and E.

The results of all bioassays are presented in table 4 below, but also on appendix F in which all comparisons are performed in terms of LOEC and NOEC to help understand the difference in magnitude from one species to another. By presenting the results in comparable endpoints it is easier to draw conclusions.

Table 4 – Bioassays compiled results for all the test organisms using isolated ivermectin (IVM) and polystyrene nanoplastics (PSNP).

Test organisms	Bioassay	Endpoint	IVM results	PSNP results
<i>Daphnia magna</i>	Acute	EC50	7.63 ± 1.09 ng L ⁻¹	133.04 ± 17.42 mg L ⁻¹
<i>Daphnia magna</i>	Chronic	NOEC LOEC	LOEC Reproduction: 0.01 ng L ⁻¹ NOEC Size: 0.001 ng L ⁻¹ LOEC Longevity: 0.1 ng L ⁻¹	NOEC Reproduction: 0.053 µg L ⁻¹ NOEC Size: 0.053 µg L ⁻¹ NOEC Longevity: 530 µg L ⁻¹
<i>Lemna minor</i>	Growth inhibition	NOEC	Surface area: 10 µg L ⁻¹ Root length: 1 mg L ⁻¹	Surface area: 100 mg L ⁻¹ Root length: 10 mg L ⁻¹
<i>Folsomia candida</i>	Reproduction	EC50	5.66 ± 1.64 mg kg ⁻¹	-
<i>Enchytraeus crypticus</i>	Reproduction	EC50	47.98 ± 8.61 mg kg ⁻¹	-
<i>Microcystis aeruginosa</i>	Growth inhibition	EC50 NOEC	NOEC: 100 ng L ⁻¹	EC50: 43.49 ± 7.85 µg L ⁻¹
<i>Chlamydomonas reinhardtii</i>	Growth inhibition	EC50 NOEC	NOEC: 1 µg L ⁻¹	EC50: 13.45 ± 3.45 mg L ⁻¹

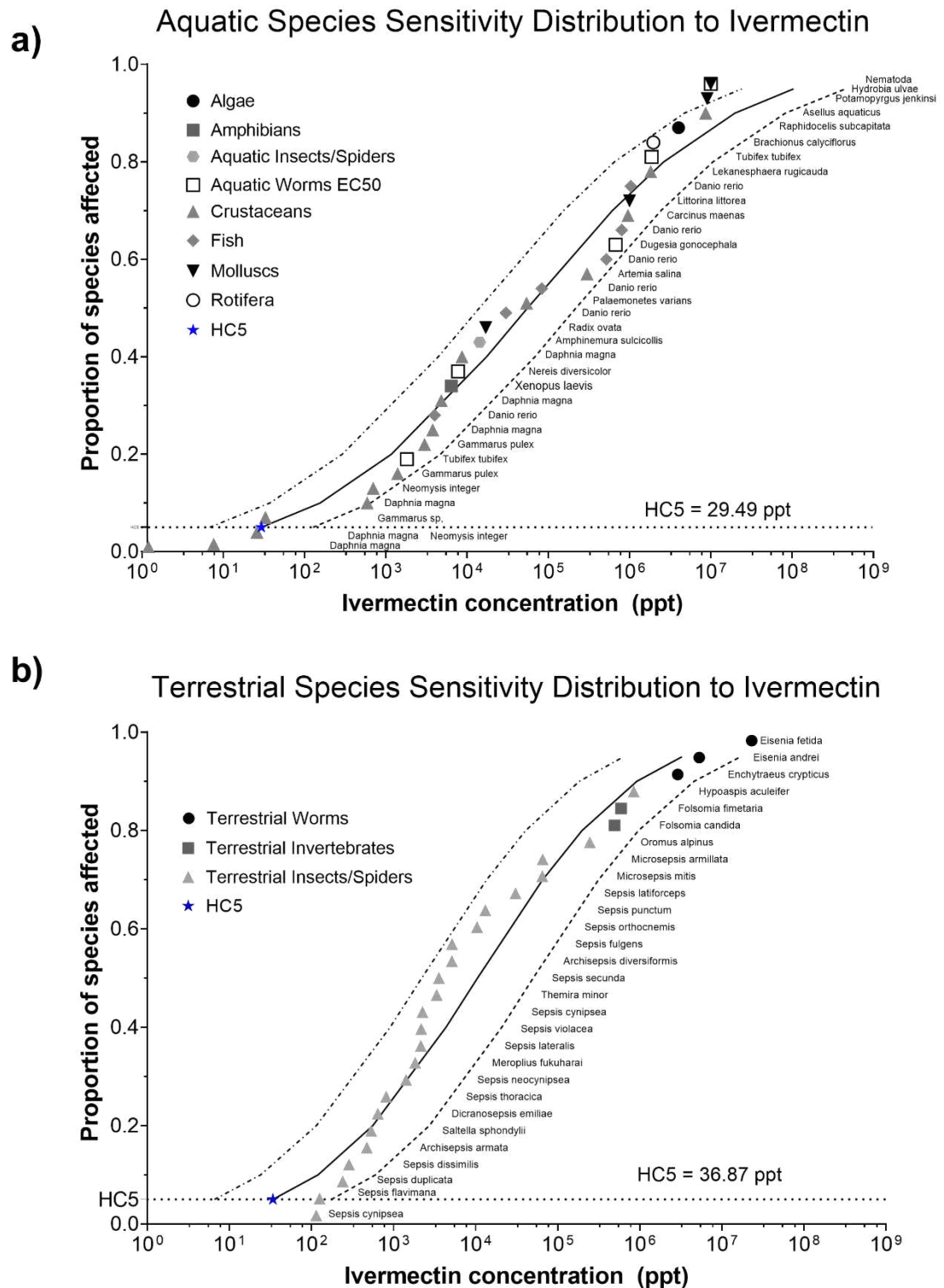
Table 5 – Compiled bioassays for the mixture between ivermectin (IVM) and polystyrene nanoplastics (PSNP) for the organisms *Daphnia magna* and *Lemna minor*.

Test organisms	Samples	PSNP concentration	IVM concentration	Endpoint	Ratio of Inhibition (RI)	Interaction	Endpoint	Ratio of Inhibition (RI)	Interaction	Endpoint	Ratio of Inhibition (RI)	Interaction
		mg L ⁻¹	ng L ⁻¹									
<i>Daphnia magna</i> (acute)	M1	63,00	0,00	Immobility	0,46	ANT	-	-	-	-	-	-
	M2	63,00	1,91		0,66	ANT		-	-		-	-
	M3	63,00	3,82		0,89	ADD		-	-		-	-
	M4	63,00	5,72		0,94	ADD		-	-		-	-
	M5	63,00	7,63		0,99	ADD		-	-		-	-
<i>Daphnia magna</i> (chronic)		mg L ⁻¹	ng L ⁻¹									
	M1	5.30	0,00	Reproduction	2,27	SYN	Size	0,63	ANT	Longevity	0,69	ANT
	M2	5.30	0,01		1,99	SYN		0,58	ANT		0,55	ANT
	M3	5.30	0,10		1,54	SYN		0,61	ANT		0,65	ANT
	M4	5.30	1,00		3,20	SYN		N/A	N/A		1,25	SYN
<i>Lemna minor</i>		µg L ⁻¹	ng L ⁻¹									
	M1	5,30	0,10	Surface area	0,43	ANT	Root length	0,49	ANT	-	-	-
	M2	5,30	1,00		0,37	ANT		0,53	ANT		-	-
	M3	5,30	10,00		0,42	ANT		0,49	ANT		-	-
	M4	5,30	100,00		0,47	ANT		0,47	ANT		-	-
	M5	5,30	1000,00		0,42	ANT		0,47	ANT		-	-

7.3.3 Species sensitivity distribution curve

The main parameter extracted from an SSD (Figure 1) is the Hazardous Concentration 5 (HC5), which is the concentration that is hazardous to 5% of the species, was 0.001 $\mu\text{g L}^{-1}$. The list of the species comprised on the analysis, their references, and trophic level are in the Supplementary material.

Figure 1 - Species Sensitivity Distribution curve for ivermectin. HC5 value was obtained as 29.49 ppt for aquatic species and 36.87 ppt for terrestrial species.



7.4 DISCUSSION

The higher the suspension stability, the greater the permanence of the solid in the water column, indicating a higher probability of interaction between PSNP and other pollutants, as well as aquatic organisms. The presence of IVM in the solution significantly altered the stability of PSNP (Table 3) in RO, ISO, and BBMm; however, it was not enough to make any samples unstable. This behavior has been described by Ma *et al.* (2016), who observed microplastic sedimentation in the presence of other pollutants, while nanoplastics tended to remain in suspension. The similarities suggest that the particles of PSNP-IVM will remain longer stable in the water column than the MP particles, evidencing the importance of studies with NP in addition to those already performed with MP.

Although there were ions in the medium, the Effective Diameter values were equivalent to those calculated by the TEM technique in a previous work (VAZ, 2020). The ED measurements presented significant statistical differences ($p < 0.05$) in ISO, M4, and BBMm. This behavior was also observed by Fadare *et al.* (2019), who justified neutralizing the nanoplastic surface with the medium ions. Thus, using the PDI as a parameter, it was not possible to affirm whether there was any complexation of IVM on the surface due to the lack of significant alteration in this diameter. This does not mean that this interaction did not occur. Still, it indicates that it was not significant enough to be detected through this measure, verified by FTIR measures in which no chemical interaction was observed. In short, PSNP particles are stable in solution and maintain their nanometric dimension, even in the presence of IVM.

The acute IVM toxicity of *D. magna* established in the present work was similar to that found in the literature with an EC50 ranging from 1.2 to 25 ng L⁻¹ and No Observed Effect Level (NOEL) of 10 ng L⁻¹ (SANDERSON et al., 2007a). The concentration of 1 µg L⁻¹ of IVM increased the surface area of *L. minor*, possibly due to the increased concentration of ions on the media formed by the solubilization of IVM. The other aquatic organisms had no effect caused by IVM, corroborating the results presented by Sanderson *et al.* (2007a) that IVM was considered not to pose a risk to aquatic biota but raised concern over the impacts on soil and sediment organisms. This behavior of causing no impacts in aquatic organisms but significant effects on soil organisms was observed as IVM was toxic for the soil organisms *F. candida* and *E. crypticus*, providing values for EC50 of 5.66 ± 1.64 mg/kg and 47.98 ± 8.61 mg/kg, respectively. Limited data exists on the environmental concentrations for IVM. However, the EC10 values for the same species were 1.90 ± 1.35 mg/kg and 16.95 ± 7.85 mg/kg, indicating that species near livestock activities might be at a higher risk of observing some effect.

PSNP only had a significant effect ($p < 0.05$) on the acute toxicity of *D. magna* and increased root length on *L. minor*, both in high concentrations of microplastics, far from that can be found in the environment. This corroborates what is said in the literature that the acute effects of nanoplastics can only be observed in high concentrations (FERREIRA; VENÂNCIO; OLIVEIRA, 2023). However, when in a mixture with additives on the plastic, such as plasticizers, flame retardants, coloring, or other pollutants in the media this toxicity could be altered (BANDEIRA et al., 2021; FADARE et al., 2019; FERREIRA; VENÂNCIO; OLIVEIRA, 2023). Nanoplastics

have already been studied as vectors for other pollutants. (KOELMANS et al., 2016; ZICCARDI et al., 2016) Thus, IVM was used as a mixture compound in this study. The intensity of the antagonistic effect was similar between all treatments meaning that the antagonistic effect showed no correlation with the increase in IVM concentration.

Regarding the mixture effects, there was no clear tendency between all organisms. This means that for these organisms presented, the mixture PSNP-IVM is synergistic for the reproduction of *D. magna*, for the growth rate of *M. aeruginosa*, and in the concentration M1 for the reproduction of *E. crypticus*. The mixture was antagonistic for the surface area and root length of *L. minor* and the size and longevity of *D. magna*. Additivity was observed on concentrations M3, M4, and M5 for immobility, concentrations M2, M3, M4, and M5 for the reproduction of *Folsomia candida*, M2, M3, M4, and M5 for the reproduction of *Enchytraeus crypticus*, and the growth rate of *C. reinhardtii*. When observed together, the chronic mixture effects on *Daphnia magna* might indicate that when exposed to the mixture, the organisms shifted their biomass allocation towards an increased number of offspring (COORS; HAMMERS-WIRTZ; RATTE, 2004) to the detriment of their size and longevity.

The SSD curve showed that 29.49 ppt of IVM could cause harm to 5% of the aquatic species, while the HC5 for the terrestrial organisms was 36.87 ppt, thus meaning that even such low concentrations could already have some negative effects since environmental IVM concentrations ranged from 0.1 to 1 $\mu\text{g L}^{-1}$ (SANDERSON et al., 2007b). When considering the mixture with other pollutants in

different environmental compartments, its toxicity could be altered, as shown in this work, in ways that are difficult to predict, especially based on the nature of the effects.

From the main groups of organisms described in the SSD curve some can be highlighted as important takeaways. The crustaceans are present alongside the whole curve with a bigger concentration on the lower part of the graph meaning that these organisms tend to be more sensitive to ivermectin than other organisms while algae and mollusks stayed mostly on the top part of the graph meaning that our organisms that are more resistant to the ivermectin. As for the terrestrial graph we can observe that terrestrial insects/spiders are present all throughout the curve and terrestrial worms tend to be more resistant to ivermectin than some species of insects and spiders the terrestrial invertebrates also presented a more

IVM has its effects associated with its binding to glutamate-gated chloride ion channels of invertebrate muscle and microfilaria nerve cells (EL-SABER BATIHA et al., 2020), leading to an increase in the permeability of the cell to chloride ions, generating a hyperpolarization of the cell and subsequent paralysis and death of the parasite (EL-SABER BATIHA et al., 2020). The IVM could also act as an agonist for the neurotransmitter gamma-aminobutyric acid (GABA), meaning vertebrates could have GABA-mediated nervous system processes affected. (EL-SABER BATIHA et al., 2020). Furthermore, plants also use GABA as a defense substance and signaling molecule in different processes, meaning that IVM could have had an impact on plants even though it was not developed for this purpose (GUO et al., 2023).

7.5 FINAL CONSIDERATIONS

Mixture toxicity has become increasingly relevant due to the number of chemicals being released into the environment yearly. This work helps to understand the possible effects of the mixture of PSNP and IVM to demonstrate possible scenarios in the natural environment. It can collaborate with the system to regulate emerging contaminants. Suspension stability showed that the PSNP is likely to stay in the water column due to its high stability, and the presence of IVM or the media ions mostly altered its stability but not significantly enough, preserving its stable characteristics and thus increasing its bioavailability.

Isolated PSNP caused an effect only in concentrations far from those observed in natural water, soil, and sediments, meaning that its true concerns regarding its impacts are physical interaction and possible interactions with other compounds. IVM showed little to no effects on the non-target organisms and significant negative effects on *D. magna*, *Enchytraeus crypticus*, and *Folsomia candida*. Thus, this evaluation corroborated the existing data and showed that concerns should be raised over environmental safety in aquatic regions with wastewater discharge or soils close to livestock production.

The mixture PSNP-IVM is synergistic for the reproduction of *D. magna*, for the growth rate of *M. aeruginosa*, antagonistic for the surface area and root length of *L. minor*, and for the size and longevity of *D. magna*. Additivity was mostly observed for *Daphnia magna* immobility, for the reproduction of *Folsomia candida*, for the reproduction of *Enchytraeus crypticus*, and for the growth rate of *C. reinhardtii*. The

SSD curve helped understand the possible impact on the environment that these concentrations might have, providing an HC5 of 29.49 ppt of IVM for aquatic organisms and 36.87 for terrestrial organisms, denoting its impact in the environment with low concentrations.

This paper provides further information on PSNP and IVM toxicity isolated and in mixture, highlighting the need to monitor and assess the levels of these compounds in the environment. Environmental safety concerns should be addressed by regulatory bodies to evaluate and help predict the consequences of the consumption of pharmaceuticals as it changes over,

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8 CHAPTER 2 - MODELING

8.1 INTRODUCTION

Environmental pollution is becoming more complex daily by creating several new compounds that might be discharged into the environment after use. For that reason, regulation started becoming more important at the same pace that new compounds are added to the environment, and new concepts, such as the exposure load, emerged (WILLEY et al., 2021). Willey et al. (2021) defined the exposure load as “the number of compounds that are exceeding a defined concentration threshold,” and it was applied to the context of human exposure; however, by definition, this concept could be extrapolated to environmental exposure as well.

Usually in aquatic ecosystems, organisms are exposed to the toxic effects of several compounds, sometimes in concentrations only one order of magnitude less than the respective acute toxicity effects for the organisms (WEITERE et al., 2021). Chemical pollution, habitat degradation, nutrients, and invasive species usually impact riverine organisms. Thus, the environmental stress could be higher than when considering only chemical pollution (BIRK et al., 2020). For that reason, to increase good management practices from regulatory bodies, it is important to characterize the region where regulation occurs by understanding land use and occupation, type of soil, and water origin to achieve good environmental conditions (FAGGIANO et al., 2010).

Implementation of regulations such as the Water Framework Directive (WFD), the European Union (EU) water quality regulation, demands an integration between modeling-based and monitoring-based approaches to achieve water quality

standards (YANG; WANG, 2010). Modeling can help understand complex problems as a group of interlinked variables and parameters that provide an adequate representation given its simplifications and constraints, such as the existence of emerging compounds that have not yet been regulated. Environmental modeling often works with estimations and simplifications of natural phenomena. Mixture toxicity assessments are some of the simplifications that can be performed while maintaining representative of the real processes (RODEA-PALOMARES et al., 2015). However, it is common for biochemical models to be of higher complexity due to the complex nature of the processes.

One of the used approach to understanding toxicity is the Adverse Outcome Pathways (AOP) framework, which works based on the simplification of the natural processes of adverse outcomes in several key events that are measurable and well-defined (ANKLEY et al., 2010; VILLENEUVE et al., 2014). This method can work well with a mixture of risk assessments due to the possibility of using the AOP wiki, an online database constantly receiving new AOPs. For each new process and event, the possible interactions between them are highlighted in the database, making it simpler for risk assessors to make connections between several different pollutants since the professionals doing these analyses are not often biologists (VILLENEUVE et al., 2014).

One model that is being applied worldwide and has shown promising evaluations is the Species Sensitivity Distribution (SSD) curve. After acquiring toxicity endpoints from the literature, it is possible to plot a distribution curve of the values, through which the Hazardous Concentration to 5% of the species (HC5) can

be calculated for each compound, thus providing a safe concentration for most organisms. Several countries use SSD as a part of their regulatory framework on at least one stage of the evaluation such as Argentina (GABAY et al., 2018), Australia (WARNE et al., 2018), Brazil (CHAM et al., 2017), Canada (Health Canada, 2023), China (YAN et al., 2023), Japan (NAGAI, 2021), Netherlands (ZAJDLIK, 2016), New Zealand (WARNE et al., 2018), South Africa (ZAJDLIK, 2016), South Korea (ZAJDLIK, 2016), and the United States (ZAJDLIK, 2016). The SSD method was developed to be used for single compounds only; however, it is important to merge the results of the SSD curves into one analysis for more compounds to better understand the behavior in the environment.

Mixture toxicity is the branch of environmental toxicology concerned with understanding the effects of two or more compounds on the organisms (RODEA-PALOMARES et al., 2015). With mixture evaluations, it is possible to identify if a given mixture is causing the expected sum of effects of the single compounds (additivity), more effect than the expected sum (synergism), or less effect than the expected sum (antagonism) (RODEA-PALOMARES et al., 2015). This sum could be defined by two models, either Concentration Addition or Independent Action, depending on the behavior of the isolated compounds and their mode of action (RODEA-PALOMARES et al., 2015). Some mixture evaluation methods have been developed to take the theoretical knowledge onwards and apply it in a real scenario by providing calculations and a scientific basis for policymakers. As an example, the Mixture Assessment Factor (MAF) was developed.

The MAF method encompasses a group of calculations to provide a value with which it is possible to correct a regulatory threshold and is currently proposed as a stronger method (DE ZWART; POSTHUMA, 2005; POSTHUMA et al., 2019; PRICE; JUNGHANS, 2023). However, it is still an intermediary stage to the point where it will be possible to use more robust models to accommodate environmental changes and reproduce environmental and biochemical conditions to better understand toxic effects. Some calculations for the MAF include a risk quotient for a given compound i , which is the ratio between the predicted environmental concentration and the predicted no-effect concentration for compound i .

The empirical findings indicate that a MAF value of 10 is sufficient for protecting the environment for >70% of the mixtures encountered in monitoring studies conducted in the aquatic freshwater, the MAF of 20 was sufficient for 95% of the samples (TREU et al., 2024). The data indicate that a MAF of $n/2$ is necessary for highly complex mixtures with more than 30 compounds (TREU et al., 2024).

Thus, this work proposes a new framework model to be used by regulatory bodies for the risk assessment of mixtures between regulated and unregulated compounds (contaminants or emerging concerns). This framework will provide a mixture assessment factor (MAF) for updating current water quality regulatory thresholds and indicate, when applicable, the creation of a new regulatory threshold for unregulated compounds. Therefore, the framework can run on different compounds, changing the variables to the ones relating to a given chemical, mainly working with parameters and variables of the environment, such as the SSD and AOP.

8.2 METHODOLOGY

8.2.1 framework Paper selection

The paper selection process for the creation of the framework employs a systematic approach to find and analyze relevant papers using the Scopus, Google Scholar, PubMed, and Springer databases. The review began by using the keywords “water quality standard”, “mixture risk assessment”, “regulatory threshold updating”, “weight of evidence”, “adverse outcome pathways”, and “mixture assessment factor”. The period of 20 years was evaluated, ranging from 2004 to 2024, of papers published in English. To better understand the current scenario and provide an updated contribution we used as inclusion criteria papers that proposed frameworks, methodologies, and procedures; and defined as exclusion criteria any papers that would not focus on aquatic environments regulation as to specify the media in which this analysis is performed.

Evaluation criteria were established for the selected papers, and they are:

- **Similarity:** The papers were assessed for their similarities, particularly focusing on recurring ideas that may appear globally.
- **Applicability:** Preference was given to papers that presented straightforward and direct concepts, as these were deemed more applicable when compiling potential reference models for the proposed framework.

- **Relevance:** Papers that are highly cited or originate from established organizations were regarded as highly relevant due to their significant influence. Organizations such as the World Health Organization (WHO), the United Nations (UN), and the European Commission were identified as particularly relevant sources.
- **Scope:** Papers addressing methodologies for evaluating mixture toxicity in water were considered critically important, whereas those discussing the water quality of individual compounds were categorized as of moderate importance.
- **Reach:** The extent of the methodologies in terms of geographical coverage was also evaluated, with papers on watershed management practices being classified as highly important, given that the proposed framework is intended to operate at a local level.

As a result of this analysis, we propose a new framework for updating regulatory threshold for water quality to be used mainly by regulators but could also be used by the civil society by giving a Birdseye view to better understand the process, by knowing in which parts of the processes they could act, and what stakeholders to contact when needed. It is important to highlight that any validation processes with regulators are out of the scope of this work, further works will validate the applicability and scalability.

8.2.2 WEIGHT OF EVIDENCE (WoE)

The toxicological data used as input for the framework model were chosen using the Scopus, Google Scholar, PubMed, ECOTOX EPA®, and Springer databases. These papers were done by searching for the target contaminants for the framework. Further details on the workings of the framework are stated in the Results section since they are a product of this paper. Categorization and evaluation of those papers was done by using the WoE approach.

The WoE can be defined as a method to compile different evidence to answer a single question by classifying the response data on their relevance through an analysis of significance for said question. (EVANS et al., 2016; HARDY et al., 2017; NORDBERG et al., 2007). Depending on which information is necessary, the papers could have a different classification for the risk assessment based on exposition, routes of exposure, duration, and animal models (NORDBERG et al., 2007).

The evaluation process will provide the updated value for the regulation threshold through a series of calculations. Firstly, there will be research for all the papers containing the mixture between compounds A and B. The next step would be to summarize all the toxicological interactions observed in each paper, separate them in which endpoints are relevant for the analysis, and segregate them into synergistic, antagonistic, or additive. Usually, for regulatory purposes, the intended endpoints are related to mortality, immobility, or growth rate, thus these are the endpoints chosen for this work.

For the WoE evaluation, the interactions will be separated into two categories: SYN for synergism and OTH for other interactions, mainly antagonism

and additivity. Then, the equation applied to guide the evaluation forward will be equation X. If $WoE > 1$, there is sound evidence that synergism is the main effect observed; if $WoE < 1$, then antagonism or additivity are the main effects observed. Thus, the regulatory threshold is not updated; thus, no evaluation is needed.

Equation 1 – Adapted Weight of Evidence (WoE) applied equations

a) $WoE = \ln\left(\frac{SYN}{OTH}\right)$

b) If $OTH = 0$, then $WoE = \ln(SYN)$

c) If $SYN = 1$, then $WoE = 1$

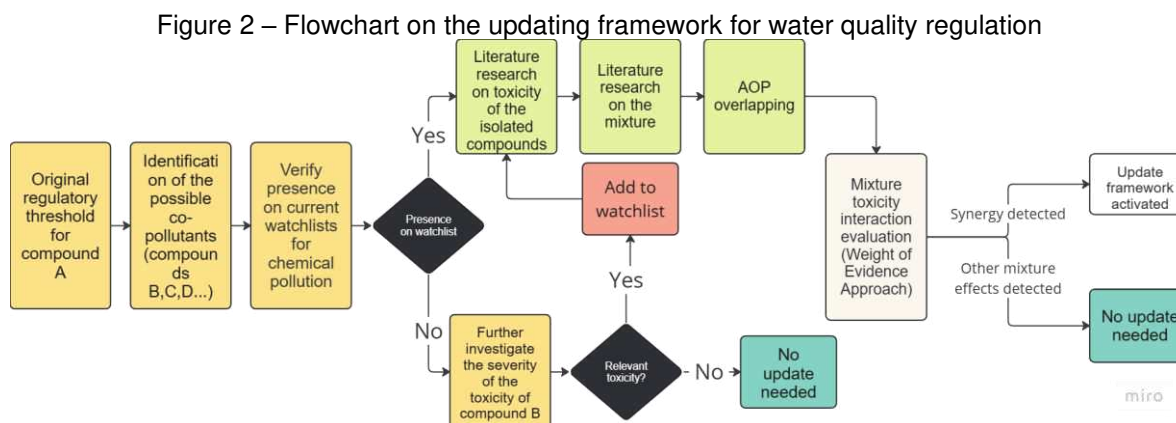
8.3 SPECIES SENSITIVITY DISTRIBUTION CURVE

Another method that was applied to this research is the Species Sensitivity Distribution (SSD) curve (BOECKMAN; LAYTON, 2017) as a means of including ecological data on the evaluation process. After acquiring the acute toxicity results and other results from the literature it is possible to plot an SSD through which the Hazardous Concentration to 5% of the species (HC5) were calculated for each compound. SSD analysis helps understand how a given contaminant could affect different taxa, and by listing the organism, it is possible to understand if a given file is more sensitive to the given chemical.

Input data for the SSD curve was provided by literature research and databases such as the ECOTOX ® from EPA. This database contains toxicological information on thousands of chemicals and their effects on biota, classified by endpoint, organism, and compound. Consulting such reliable sources gives the SSD greater confidence in its defined concentrations.

8.4 RESULTS

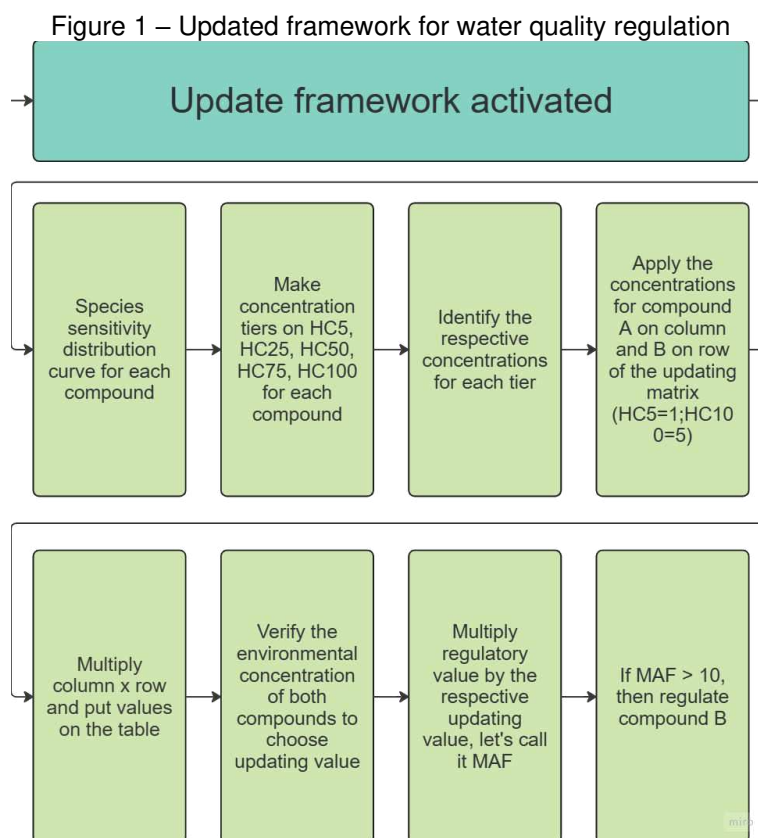
Thus, the following framework, represented in Figure 2, was elaborated on and further described on the following topics.



8.4.1 Updating framework for regulatory bodies

This framework aims to update regulatory thresholds for contaminants in water quality standards due to their possible interaction with other compounds in the media that are not yet regulated, such as contaminants of emerging concern. The Figure 3 is a flowchart of the framework and its possible paths beginning with the discovery of possible mixture toxicity effects in the literature of the original regulatory threshold for a given compound (here called compound A) and its co-pollutant (here called compounds B, C, D, etc.). The need for updating compound A will be determined by the observed toxicity in the mixture, with compound B being higher

than expected for the individual compounds (synergism); the first paper will provide a direction to which further investigation is needed.



The next step would be to check if the co-pollutant compound B is listed on the current local watchlists of chemicals in surface water; if no list is established in the country, the process should consult pre-defined lists such as the Surface Water Watchlist of the European Commission (GOMEZ CORTES et al., 2022). If compound B is not on the watchlist, then a further investigation on the severity of the toxicity must be conducted, given that the toxicity is irrelevant. For example, by only causing effects in high concentrations that are not environmentally relevant, then no update is needed. However, the compound must be added to the watchlist if the effects are relevant in environmental concentrations.

When compound B is already present on the watchlist, further literature research must be done on compound A + compound B mixture, and we propose that this analysis is done through Adverse Outcome Pathways. This approach describes the toxicity of chemicals through a series of connected events that start with the molecular initiating event, followed by key events that will generate an adverse outcome whether on the organism, population, or community level (VILLENEUVE et al., 2014).

The main advantage of AOP is that it is non-species-specific, meaning that the molecular initiating events and subsequent adverse outcomes are provided on a biomolecular basis of any processes that can be measured (either by intensity, duration, frequency, or any other relevant variable) (VILLENEUVE et al., 2014). We propose that compound A and compound B be consulted on the AOP Wiki (<https://aopwiki.org/>), and if they have any overlapping key events, then it is an indication that the mixture evaluating process should be stricter in regard to possible effects.

8.5 MIXTURE TOXICITY EVALUATION

The mixture toxicity evaluation process will provide the MAF with which the regulatory thresholds will be updated. For that, the regulatory values being assessed should be multiplied by one/MAF thus reducing the limit to a more acceptable level.

8.5.1 Species Sensitivity Distribution

When synergism is detected, the next step is to evaluate the magnitude of this impact for both compounds by elaborating two SSD curves with as many organisms as possible from different trophic levels for both compounds. The first step would be to verify the existence of published papers with an SSD curve for the desired compound; in case it is non-existent, it is possible to use as input the data found in databases for ecotoxicology such as the ECOTOX database (US EPA), the Norman Network (EU). For compounds such as micro and nanoplastics from which reliable and consistent data is difficult to find standardized, it is possible to create SSD curves using the SCCWRP Microplastics Tools and Data Repositories (<https://microplastics.sccwrp.org/>). This database provides quick and reliable toxicity values for different plastics and converts the different concentration units so it is possible to easily perform calculations.

In these platforms, it is possible to select different endpoints to build an SSD curve that is suitable to what is being regulated, meaning that depending on the local practice, it is possible to use NOEC, LOEC, EC50, EC20, or any other endpoint that is relevant to the given regulatory updating. The most common method for the elaboration of the SSD is the USEPA method using their spreadsheets; however, it is possible to make the curve using different methods such as R, Python, or other programming languages. This framework proposes using the USEPA SSD Toolbox based on its current widespread usage ([Species Sensitivity Distribution \(SSD\) Toolbox | US EPA](#)).

With the curve, regulators should identify the respective hazardous concentrations that cause significant damage to 5, 25, 50, 75, and 100% of the species. The percentage is provided on the Y-axis, and the X-axis projection of the SSD curve will provide the concentrations.

8.5.1.1 Risk Matrix

The next step in this framework is the use of an adapted risk weighting matrix from the World Health Organization (WHO) in which each HCx value for each compound will be attributed to a risk index and will be placed: compound A on the left side of the matrix and compound B on the top part of the matrix (Table 6). Then, the current environmental concentration of both compounds should be assessed either through monitoring in cases in which the structures are held in place, and data is available or through literature research for the respective regulatory area, studies from the same country, province, state, or region that have identified and quantified the compounds.

Table 6 – Risk matrix with the MAF values calculated.

		Risk Index (RI)	Compound B				
			HC100	HC75	HC50	HC25	HC5
Compound A			3	2,5	2	1,5	1
	HC100	3	9	7,5	6	4,5	3
	HC75	2,5	7,5	6,25	5	3,75	2,5
	HC50	2	6	5	4	3	2
	HC25	1,5	4,5	3,75	3	2,25	1,5
	HC5	1	3	2,5	2	1,5	1
Risk level			Re-evaluation	Very high	High	Medium	Low
MAF = RIa X RIb X WoE			>9	9 ≥ MAF ≥ 7.5	7.4 ≥ MAF ≥ 5.0	4.9 ≥ MAF ≥ 2.5	2.4 ≥ MAF ≥ 1

Subsequently, regulators should identify which concentration tier the current environmental concentration is found in based on the SSD curve. This means that the environmental concentration will provide an HCx with $x > 100$ and based on the x value; they should follow the indication below for each tier and their respective Risk Index:

- **Safe:** when $x \leq 5$ and $RI = 1.0$;
- **Concerning:** when $6 \leq x \leq 25$ and $RI = 1.5$;
- **Slightly harmful:** when $26 \leq x \leq 50$ and $RI = 2.0$;
- **Harmful:** when $51 \leq x \leq 75$ and $RI = 2.5$;
- **Very harmful:** when $76 \leq x \leq 100$ and $RI = 3.0$.

After selecting which tier best describes each compound, the combination between both should be evaluated using the Mixture Assessment Factor (MAF) extracted from Equation 2 below. The equation also considers that the greater the number of papers reporting synergism between the compounds, the greater the protection since the evidence is stronger for synergism.

Equation 2 – MAF calculation consisting of the product between the Risk Index from compounds A and B and the Weight of Evidence (WoE).

$$MAF = RI_A * RI_B * WoE$$

If the environmental concentrations for both compounds are high enough to cause effect to 100% of the species, that means that action needs to be taken to

restore safety. Therefore, the framework incorporates a safety metric, such that an $MAF > 9$ necessitates the formulation of new regulations for the emerging compound, suggesting to regulators that special attention is needed to compensate for the environmental damage being provoked.

8.6 APPLICATION OF THE FRAMERORK

Aiming to be more practical, the authors chose to apply this framework to existing data from previous work, providing a contaminant of emerging concern, ivermectin. Ivermectin is an antiparasitic drug that is widely used for humans and livestock (ZAHEER et al., 2021). During the pandemic, there was a great increase in the consumption of ivermectin due to claims of avoiding infection from the COVID-19 virus. Thus, using this data, it is possible to update the regulation value for a given compound. A good candidate for this example is glyphosate, since it is a highly studied compound and is regulated by different countries. The premise was that we are updating a regulation for the Brazilian freshwater quality threshold in a Class I body of water (most strict), and this threshold is $65 \mu\text{g L}^{-1}$.

Firstly, we identify compound A (glyphosate) and compound B (ivermectin) and the presence of ivermectin on watchlists; currently, ivermectin is not a part of the regular watchlists, leading to "Further investigate the severity of the mixture toxicity." After further investigation, ivermectin causes relevant effects on key species for the environment, such as *Daphnia magna*, in concentrations up to 0.01 ng L^{-1} (GARRIC et al., 2007). Thus, it was considered relevant toxicity, and it is indicated that such a compound has been added to the watchlists.

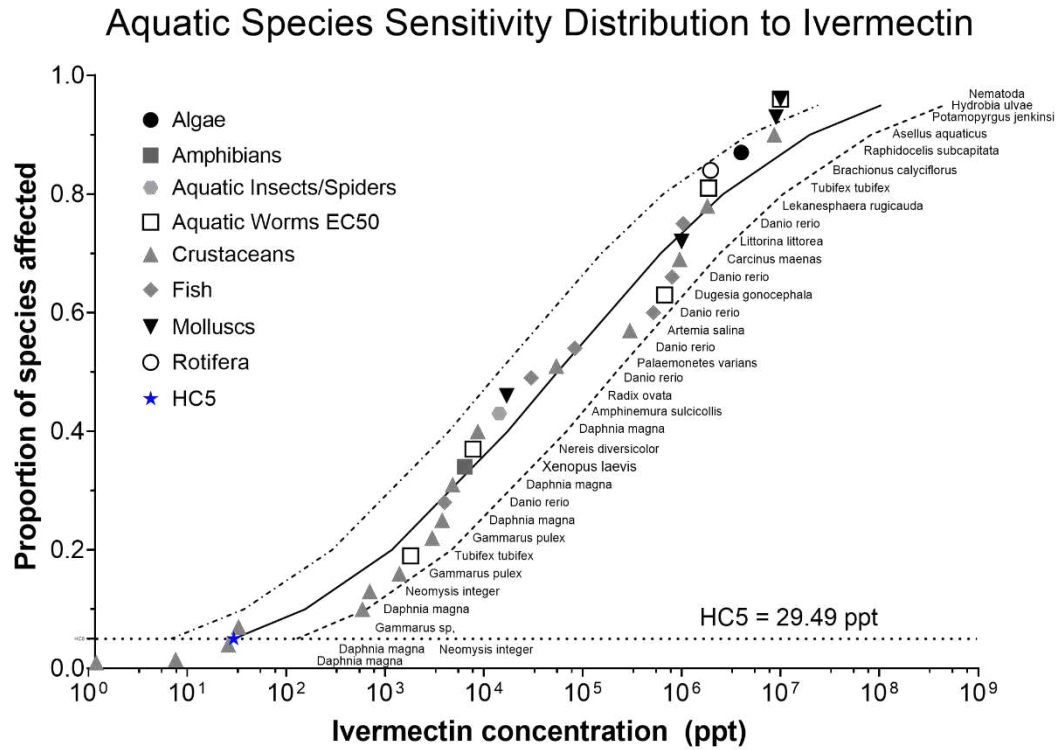
The next step is to verify the literature if there is any evidence of a mixture between these two compounds. Thus, it was used as search prompts for the terms “glyphosate” + “ivermectin” + “toxicity” and researched on the platforms Google Scholar, Pubmed, ECOTOX®, and Scopus, and we found one paper on this mixture and the effect is synergistic in this case the equation 1c which gives a WoE value of 1. This implies that the WoE will not contribute to the MAF value. Continuing the evaluation steps, the AOP should be consulted on the AOP wiki, and no AOPs were found for glyphosate or ivermectin. Since synergy was detected, this triggered the start of the mixture toxicity interaction evaluation.

This phase of the evaluation starts with the SSD curve, and since there are SSD curves, one for each compound in the example, the glyphosate curve will be extracted from FAN et al., (2022). As for the ivermectin, the SSD present on Chapter 1 was used for the concentrations, and both the respective values for the HCx are presented in Table 7 with an estimation from the plotted curve and the original graphs are displayed on Figure 2 and Figure 3.I

Table 7 – Hazardous concentrations for the respective percentage of species affected of 5, 25, 50, 75, and 100 obtained through the SSD curve method for glyphosate, with data extracted from Fan et al. (2022), and ivermectin with data extracted from Vaz et al. (2024).

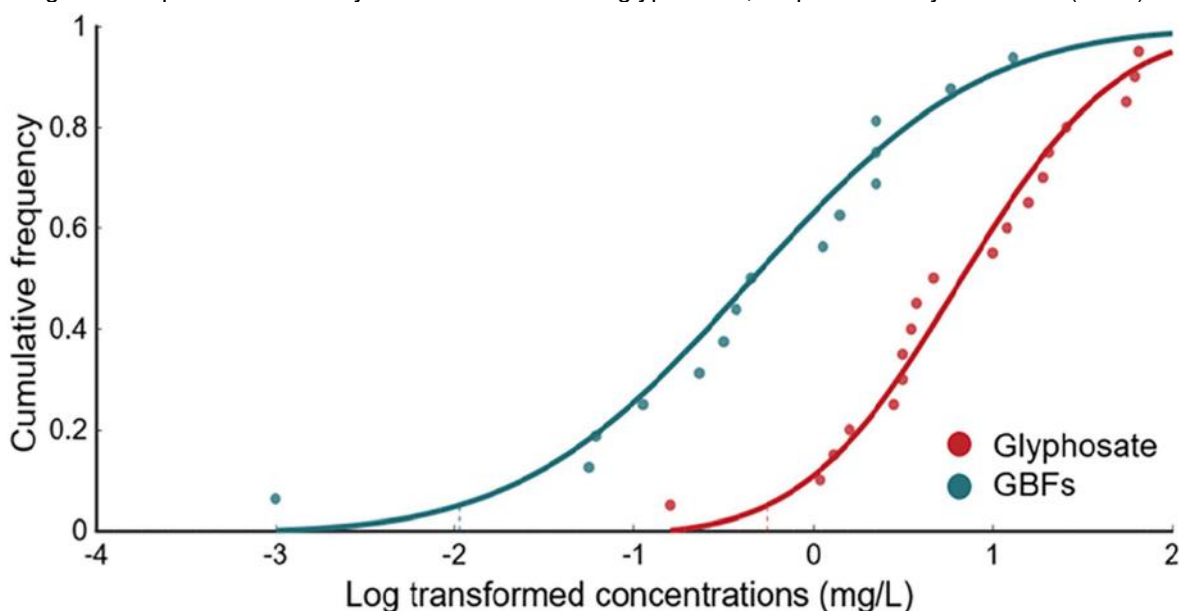
<i>Hazardous concentration</i>	<i>Glyphosate</i>	<i>Ivermectin</i>
	mg L ⁻¹	µg L ⁻¹
<i>HC5</i>	8.31	0.001
<i>HC25</i>	35.48	1.100
<i>HC50</i>	89.13	10.250
<i>HC75</i>	223.87	100.450

Figure 2 - Aquatic Species Sensitivity Distribution curve to ivermectin from Chapter 1



Source: the author.

Figure 3 - Species Sensitivity Distribution curve to glyphosate, as produced by Fan et al. (2022)



Source: Fan et al. (2022).

With the concentrations defined by the literature papers aforementioned, we should provide either a estimated Predicted Environmental Concentration (PEC) or quantified data measured on surface waters in the region where the regulation applies. For glyphosate, the quantified value in the environment varies from 6.1 to 0.97 mg L⁻¹ (LIMA et al., 2023) which categorizes the use of HC5 as the evaluation tier. As for ivermectin, it ranged from 0.1 to 1 µg L⁻¹ (SANDERSON et al., 2007b) and for this evaluation, the bigger concentration will be chosen since it will provide greater protection. Thus, the HC5 tier is chosen for ivermectin. This step is done by consulting available data in the literature and classifying the percentage of species possibly affected by each pollutant.

Since both compounds are on the HC5 range, it is possible to extract from the risk matrix (table 7) the risk index value of 1 for *RIa* and *RIb*, and since the MAF

value is comprised of the product $RIa * Rib * WoE$, then $MAF = 1 * 1 * 1$, thus $MAF = 1$. Meaning that for the mixture between glyphosate and ivermectin, the MAF would not change the regulatory threshold value since the mixture between compounds has not proven itself to be hazardous in the environmental concentrations found.

8.7 FRAMEWORK LIMITATIONS

It should be noted that although it is necessary to model the mixtures to help fill in the knowledge gaps, there are some important confounding factors, such as the interaction between the compounds and the media. The presence of bodily fluids in any given organism can alter a molecule and reduce its bioavailability considerably (HEYS et al., 2016). Thus, the use of non-target species to assess the toxicity is important as we cannot yet fully understand the dynamic of the pollutants in the media. Cleuvers (2004) proved that the interaction between ibuprofen and acetylsalicylic acid showed significative effects on *Daphnia magna*, a non-target species for the pharmaceutical compounds.

As a limitation in terms of compounds for the model, macroplastics, microplastics, and nanoplastics are not possible to be included yet, due to the difficulty in measuring and homogenizing the plastics data for regulation. Another aspect that could not be covered in this framework model was the seasonal variation that could generate different pollutants that could affect different taxonomic groups, shifting risk assessment from one season to another (BECKERS et al., 2018). It is possible to segregate the compounds by season or constant emission. In cases in

which the compound is constant, it would be of greater importance its regulation (BECKERS et al., 2018).

Another limitation for the framework is that it was developed for an evaluation based on two compounds being analyzed per cycle, therefore it could only be applied for two compounds each time. This could be incremented in future applications for this model elaborating on how to include multiple compounds at once or discussing them as groups and regulating as groups, a practice that is well known in regulatory agencies.

The validation of the framework is a next step that can be performed in collaboration with regulatory agencies in different countries, specially those that do not have a great network of monitoring sites , to test the efficacy or the feasibility of the proposed calculations.

8.8 FINAL CONSIDERATIONS

The framework model proposed in this paper was developed with the intention of being used by regulatory bodies to help facilitate the updating of water quality thresholds. Integration between biological variables (SSD) and biochemical analysis (AOP) provides strength to the evaluation. During the demonstration, it was proven that not every case needs an update; however, after the evaluation, it is possible to suppose that this mixture between these two compounds is not a pressure on the ecosystem.

Modeling can help predict compounds of interest and the most dangerous mixtures. Therefore, the need for bioassays is reduced; the SSD provided in this

paper is an example. It is possible to evaluate toxicity without greater animal usage and providing data that are safe and reliable. The perspectives for the future are the reduction of animal usage on bioassays and increased confidence in the data generated by modeling.

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9 CHAPTER 3 – REGULATORY ASPECTS

9.1 INTRODUCTION

Regulatory practices often are responsible for maintaining environmental concentrations of hazardous chemicals of concern below the threshold of significant effect, usually concentrations at which no effect was observed such as NOAEL, NOAEC, and Benchmark dose (KORTENKAMP; FAUST, 2018). These policy thresholds regarding new tests for new compounds or effluents from specific activities are established by performing literature research on the subject to perform a data-based and robust decision on the limits for each particular compound (DRAKVIK et al., 2020a; KORTENKAMP; FAUST, 2018). Different effects and species tested can be standardized such as soil and water organisms (ISO, 2012; OECD, 2004, 2006a, 2006b, 2012) for environmental concentrations and *in vitro* testing for human exposure, to provide the ideal concentrations based on actual effects observed such as disturbances in the standardized endpoints (BEEN et al., 2021).

Occurring in all environmental compartments, chemicals can have several different co-occurring exposure routes to both humans and the ecosystem, therefore all relevant chemicals should be evaluated regarding their possible adverse effects on the biota. The main issue is the number of chemicals being generated and their complexity when in a mixture of naturally occurring and other pollutants in the environment, especially in regions where environmental monitoring of chemicals is not performed regularly due to lack of resources (QUANG et al., 2021). Mixtures of

chemicals present in the environment are a threat to seven out of the 17 UN sustainability development goals such as 3) good health and well-being, 6) clear water and sanitation, 11) sustainable cities and communities, 12) responsible consumption and production, 13) climate action, 14) life below water, and 15) life on land.

A shift in approach has been noted to consider the effect of the mixture itself in the regulatory assessment due to possible interactions between the compounds of each mixture because of possible synergistic or antagonistic effects (KORTENKAMP; FAUST, 2018; LIU; SAYES, 2024). Although mixture toxicity has been researched for a few decades, it has already shown that the concentrations of isolated compounds do not represent fairly what may be occurring in the natural environment. Thus, the importance of connecting what is being produced in academia with the regulatory practices is indisputable as a means of connecting the knowledge produced by both ends, academia, and regulatory bodies, and optimizing their outcome by saving efforts for what has already been produced (DRAKVIK et al., 2020a).

Different methods have been applied to evaluate different mixtures in risk assessment aiming to increase confidence in the safety values provided by legislation. It is possible to demonstrate the importance of environmental regulation frameworks that can protect the environment but also allow the reference values to be updated after new information has been acquired by applying different computational methods, consulting the known literature, or via publications of new toxicological data. Despite substantial development, regulatory agencies are still

skeptical about embracing new technologies to update their accepted environmental concentrations, sometimes called regulatory accepted concentrations (RACs) (SAUER et al., 2017). A few challenges delay mixture risk assessment from being fully applied such as the novelty factor; overlapping regulatory silos (KORTENKAMP; FAUST, 2018); data gaps in risk/toxicity/Mode of Action (MoA) of specific compounds and mixtures (LIU; SAYES, 2024; SAUER et al., 2017), different knowledge-producing levels on exposure, impacts and governance in different regions of the world (BRACK et al., 2022); and a comprehensive assessment of the uncertainties associated with mixtures and the communication of these uncertainties with policy-makers.

Data gaps on mixture toxicity of pollutants exist due to the exponential and almost infinite number of mixtures possible and the lack of continuous and reliable monitoring, especially in global south countries in which data are not often readily available (BOPP et al., 2019; QUANG et al., 2021). Given that time and resources are limited, the mixture risk assessment should be more utilized as a trustworthy methodology to evaluate the possible effects of pollutants. The problem arises from this approach as the amount of data required as inputs for the model increases with its complexity (BOPP et al., 2019; DRAKVIK et al., 2020a). Data-driven solutions point toward a strategy including all involved stakeholders: academia, regulatory agencies, and industry (BRACK et al., 2022; LANGAN et al., 2024). It could be possible to manage the risks by regulating and prioritizing mixtures of concern and then modeling and testing them to update regulations (FAUST et al., 2019).

Around the world, different approaches are implemented for chemical regulation, but overlapping competence is standard in most countries (KORTENKAMP; FAUST, 2018). Different organizations often regulate human health, environmental quality, agriculture, and mining, and if integrated, their efforts could provide a holistic understanding of the effects observed in humans and the environment (KORTENKAMP; FAUST, 2018). However, regulation tends to lack procedural promptness to include New Approach Methodologies (NAMs) that are often elaborated by academics through scientific publications (LANGAN et al., 2024).

Due to the extensive effort, it would take to model all compounds and their respective combinations, the current work aims to bridge the gap between mixture toxicity modeling techniques and current watershed/regional water management practices through predictive indirect methods and effluent toxicity evaluation. This paper will first present the entirety of the proposed framework and further elaborate on each route separately.

10 METHODOLOGY

This paper proposes a framework to help bridge the gap between academia, industry, regulatory agencies, and civil society, by providing a comprehensible and easy to follow guideline to promote integration and a more efficient knowledge-flux

between all stakeholders. The frameworks were developed by literature review and were performed using the methodology described below.

To understand the current scenario, the keywords researched on SCOPUS, Web of Science, and Google Scholar were “regulatory toxicology”, “water quality thresholds”, “watershed management”, “environmental impact study”, and “effluent toxicity”. The period evaluated was comprised of the last 20 years of data, thus from 2004 until 2024. The inclusion criteria were papers, guidelines, and official regulatory documents referring to water management methodologies, toxicity evaluation methods, and environmental management practices. Exclusion criteria were papers, guidelines, and methodologies concerning soil and air quality since the focus of this work is aquatic ecosystems regulation.

Evaluation criteria were established for the selected papers, and they are:

- **Similarity:** The papers were evaluated for their similarity between each other, focusing on those ideas that might be repeated worldwide;
- **Applicability:** Papers with simpler and direct ideas were considered more applicable and thus were given preference when listing the possible reference models for the presented framework;
- **Relevance:** Highly cited papers or guidelines from consolidated organizations were considered of high relevance due to their impact. Organizations such as the World Health Organization (WHO), United Nations (UN), or the European Commission were some of the deemed “high relevance”.

- **Scope:** Papers concerning methodologies for mixture toxicity evaluation in water were deemed of high importance, while papers that discussed water quality of single compounds were classified as medium importance;
- **Reach:** The broadness of the methodologies in terms of area were also considered and papers discussing watershed management practices were considered of high importance, since the proposed framework aims to work locally.

As a result of this analysis, we propose a new framework for updating regulatory threshold for water quality to be used mainly by regulators but could also be used by the civil society by giving a Birdseye view to better understand the process, by knowing in which parts of the processes they could act, and what stakeholders to contact when needed. It is important to highlight that any validation processes with regulators are out of the scope of this work, further works will validate applicability and scalability.

11 RESULTS AND DISCUSSION

11.1 PROPOSED FRAMEWORK

Criteria were established to guide the process and insure that it would be useful for regulators, civil society, and industries. The main principles of the

framework give its name as GLORIES: i) Global south-centered; ii) Open access data generated on the watershed as a means for environmental protection; iii) Regionalization of the information to the watershed level since surface water contamination occurs usually through treated/untreated effluent discharge; iv) Integration between different forms of knowledge produced in academia, industry, regulatory bodies, and civil society; and v) “Energy Saving principle” meaning that groups and discussions established on the local scale could be used as “polinizers” for the framework.

11.1.1 Principles

11.1.1.1 Global south-centered

Due to extensive monitoring, the global North has a bigger database from which it is possible to elaborate trustworthy models and thus sound regulation (QUANG et al., 2021). However, the reduced size of monitoring data on the global south means that using the same models applied for developed countries (with extensive data) on developing countries (with less data available) might lack robustness. Global north and south have different environmental characteristics such as temperature, humidity, rainfall, climate, biomes, land occupation, and use. While the efforts towards harmonization are intense, there are limits to any model, especially when input data are scarce.

Creating a global south-centered framework means that the focus is to generate the necessary data for safe and reliable regulation while guiding local governments on how to use them. Recognizing the knowledge generated on the

global south as trustworthy and applicable is also one of the ways of altering the flow of knowledge, fully including local stakeholders means trusting local information.

11.1.1.2 Open access

Data availability is crucial to cheaper methodologies since paying for input data to any proposed environmental model would not be necessary. Governmental information on environmental quality if generated with public funds should already return to society as open data. However, private organizations that generate and manage wastewater must present its characteristics such as physicochemical parameters and toxicity results to regulatory agencies. Thus, these data are usually not open access. Data from environmental studies are also available when consulting the regulatory agencies. However, it is not always open access and needing to use these data usually demands extra steps, such as providing a reasoning to the use and evaluation of the provided data. As examples of this suggestion, there is the NORMAN database system which is a “network of reference laboratories, research centers, and related organizations for monitoring of emerging environmental substances” that compiles monitoring data and facilitates their reach (BRACK; DULIO; SLOBODNIK, 2012). NORMAN provides environmental monitoring data for indoor air quality, surface water quality, food and safety, that are available as open source information.

It is comprehensible that sensitive data should be preserved from the public. However, accessibility to the environmental quality of surface water could be simplified. Therefore, organizing data that has already been generated periodically

in an open database could help civil society and regulatory agencies as to what are the information demands of the general public after access is established. A given community, when given access to effluent toxicity data, could learn that their water has a high pollutant concentration and demand extra care from their local regulatory agency. This principle gives civil society more power, giving them information on the workings of the industries in the region, thus gaining communities' trust while helping academia and regulatory agencies have more precise environmental protection.

11.1.1.3 Regionalization

Regulating using published papers to verify the toxicity of a given compound is a method that favors environmental protection while having a scientific basis. However, biota and environmental conditions vary regionally, and regulation is being elaborated using standardized organisms, such as *Daphnia magna*, from temperate climates. Those organisms might not reflect with the necessary precision the local pollution dynamics of the several global south countries. Thus, local regulatory agencies could benefit from the local data generated either from the presence of local species or the effluent characteristics. After creating an open database from the previous principle, consultation on information at the watershed level might be available. Perception of what pollutants are present in the watershed could help predict the effects on the local biota.

11.1.1.4 Integration

Academia, industry, regulatory bodies, and civil society all benefit from exchanging information on environmental protection, either from industry and civil society consuming resources at a sustainable pace for longer or regulation and academy promoting guided and informed decisions (POSTHUMA et al., 2016). When information is kept between paywalls or literal walls in the case of environmental studies that are only accessible *in situ*, it is not only cost-ineffective but also a waste of time and effort that was put into generating information and using it less optimally (EVANS et al., 2016). The benefits from integration could come from the conversations between stakeholders that instead of trying to find the responsible for a given problem would work together on finding possible solutions.

11.1.1.5 Energy Saving Principle

This framework recognizes all the efforts being made towards a safer environment by different organizations and, therefore, tries to include them in the process, thus, saving the energy of creating this methodology from the start. The energy for the framework would be saved using methods already in place such as Adverse Outcome Pathways (AOPs) and organizations such as the watershed management committees.

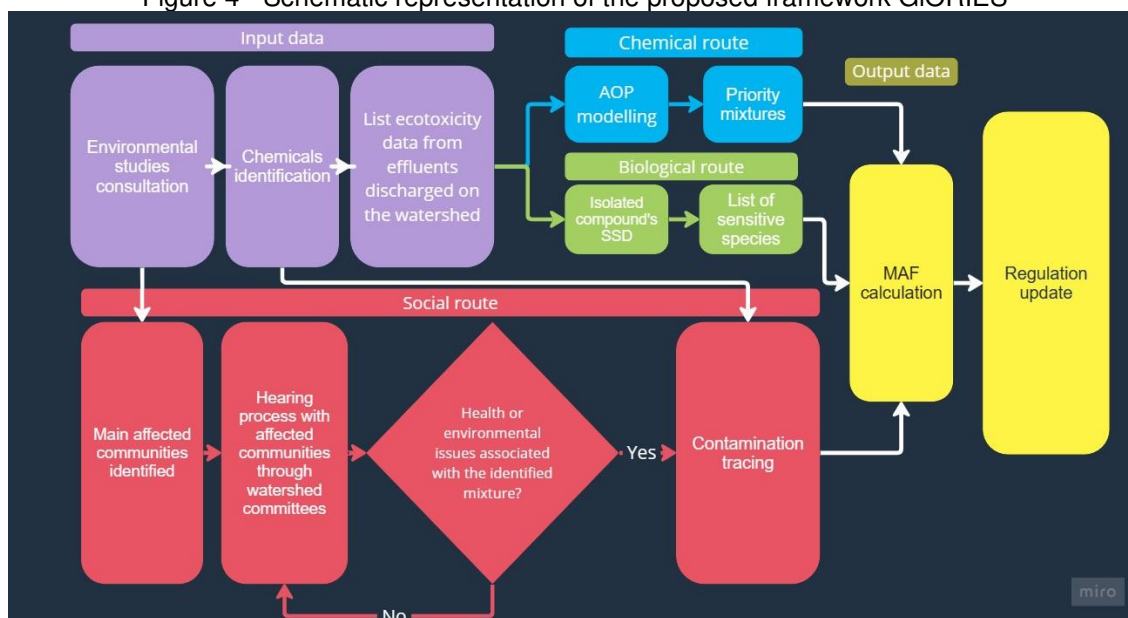
The watershed management committees are in different countries worldwide and usually include all relevant stakeholders regarding water use in quantity and quality. Pairing with an important organization that shares bonds with local communities, the framework would be implemented through workshops to

empower local agents to become pollinators of the presented ideas. Thus, it would be necessary to form a scientific council that would be integrated into each committee and generate a way to implement the framework for the local reality.

11.1.2 Framework Graphical Representation

In Figure 4, the authors propose a new approach to updating regulations regarding chemical mixtures, which are divided into three main routes: chemical, biological, and social. This classification can help policymakers by highlighting essential steps in the process, such as including all affected parties in the analysis and remembering how other species are affected by the chemicals in the water. The method could also be extended to include climate change as it is flexible enough to accommodate stressors other than chemical ones. Three routes are proposed for that: chemical, biological, and social.

Figure 4 - Schematic representation of the proposed framework GIORIES



11.1.3 Scale

To simplify the analysis, the proposed framework uses the watershed as the management scale as it is the geographical boundary in which the surface water carries most of its pollutants. By separating each watershed as management hotspots, it is easier to engage the population as some countries already have as surface water management practices a watershed committee comprised of the affected parties: regulation, industries, and civil society (PIHA; DULIO; HANKE GEORG, 2010). Therefore, it is simpler to use the network created locally and cultivate the trust of the people involved aiming a greater success rate of implementation for the proposed framework.

It may be essential to highlight that this framework aims to include a mixture of factors into surface water regulatory thresholds; factors related to groundwater, drinking water, and soil quality are not considered in this preliminary proposition.

11.1.4 Consulting of preexisting studies and reports

Regulatory agencies can demand environmental studies during environmental licensing for different industries, businesses, and land uses (PEREIRA et al., 2018; RODRIGUES et al., 2019). The studies aim to guide these agencies on the expected impacts and the environmental liabilities that might be brought to the region (PEREIRA et al., 2018; RODRIGUES et al., 2019). Depending on the size and relevance of the proposed activity, the influence area can be a few kilometers from the site, include the whole watershed, or can have much larger influence areas such as ports.

Usually, third-party companies are hired for the elaboration of these studies and the regulatory agencies evaluate the quality and verify if all the essential topics are covered (RODRIGUES et al., 2019). After being approved, the document has already been revised by different professionals with different backgrounds, which enriches the document and increases its confidence. Therefore, these studies contain detailed information on different aspects of the influence area including fauna, flora, geomorphology, pluviometry, fluviometry, biomes, land use and occupation, and the presence of traditional communities such as autochthones, indigenous or quilombolas (afro-Brazilian rural communities) that usually are more at risk and perceive contamination first due to their close ties to the water resources as a food source (RODRIGUES et al., 2019).

Avoiding contamination at its source is usually done by demanding the industries perform effluent toxicity tests with the whole effluent and the results are

not above a certain threshold (RODRIGUES et al., 2019). These assays are performed with more than one species, usually in at least two trophic levels (the most used are algae or marine bacteria and daphnids). However, the results of such tests are not publicly available, meaning that there is a specific frequency in which whole mixture toxicity is being assessed, and the results are usually only used by lawmakers to attest to the accordance with the law.

We propose a consultation of all the environmental studies performed on the analyzed watershed through environmental agencies, including implementation or construction studies and the consultation of the whole mixture toxicity tests coupled with a list of all the source chemicals used in the process. This could mean that with a database large enough to perform modeling techniques such as Adverse Outcome Pathways (AOP), the toxicity could be more easily understood, and highly toxic chemicals could be reduced from the source.

The two data sources proposed in the section do not demand the implementation of monitoring techniques or expensive equipment. This methodology is being proposed so that countries can easily apply them without an extensive monitoring network but already using generated data. The challenge of this crucial step is to enforce companies to make public their data on toxicity. Though it could increase the population's trust in the regulatory agency, the companies could reduce their participation due to an unwillingness to cooperate.

11.1.5 Chemical route

After creating a database with the whole mixture toxicity data coupled with the list of all the compounds used during the process, it could be easier to understand which compounds contribute mainly to the observed toxicity. The input data for the database should be accessible to policymakers and the public, following the FAIR (Findability, Accessibility, Interoperability, and Reuse) principles of open data (WILKINSON et al., 2016). With open data, new research could merge from evaluating the mixture toxicity on a chemical level by modeling with other software such as Quantitative Structure-Activity Relationship (QSAR) (ROY; KAR; DAS, 2015), thus promoting a regulatory gain and a scientific increase in knowledge about mixture toxicity.

The mixture toxicity assays are either the compounds approach or the whole mixture approach. The first means that each compound present in the mixture is tested isolated and then in the mixture, and the latter means that the effluent is tested as a whole. The toxicity is the primary outcome of the analysis, not necessarily the chemical interaction between the parts of the mixture. Regardless of complexity, usually, the toxicity is caused mainly by ten compounds (POSTHUMA et al., 2016), which could mean that a priority list of compounds from each watershed could be elaborated. This list should also be integrated with other watchlists for emerging contaminants such as the European watchlist.

With a finite number of compounds to be evaluated and validated by the watchlist of the region, it is possible to try and model their biochemical interactions on the species present in the watershed with methods such as AOP (KNAPEN et

al., 2018). AOP is a modeling technique comprising a mechanistic approach to toxicity, in which the toxic pathway is separated into different vital events that could be compared with each other since they are non-species specific (VILLENEUVE et al., 2014). That means that a part of the mixture toxicity could be understood by using AOP to extrapolate some toxic effects amongst species from the closest phylogenetic group possible (VILLENEUVE et al., 2014). This would mean that if an AOP is developed for *Daphnia magna* but on the watershed only *Daphnia pulex* was identified, a simplification could be made that these organisms have enough biological similarity so that the *Daphnia magna* AOP could be regionalized to *Daphnia pulex*.

11.1.6 Biological route

Consulting the environmental studies for the watershed being analyzed, it is possible to highlight the organisms susceptible to being harmed by chemical pollution and those that could be endangered species. Elaborating a list of the species present in the watershed could help policymakers prioritize certain species during the modeling step of the framework (SALA et al., 2012). Using the list could also help prevent further damage if after some research it is discovered that some species are susceptible to the chemicals used on the watershed before any modeling application, the use could be reduced, or the effluent treatment could increase its efficiency.

Discovering the sensibility of the species present through literature research is usually how biological data are included in regulations for surface water. Data on

single compound toxicity is widely used, mainly when extracted from databases such as ECOTOX from the USEPA. However, to increase confidence in environmental protection, regulatory bodies could start applying methods such as the Species Sensitivity Distribution (SSD) that are more robust and include several species in the analysis (SALA et al., 2012).

SSD consists of listing as many toxicity values as possible for single compounds and ordering them from the most sensitive to the less sensitive species in a given endpoint, usually the LC50 for mortality or EC50 for immobility (SALA et al., 2012). From this graph, a safe concentration for 95% of the species (HC5) can be extracted, thus simplifying the species-by-species analysis. Including as many species as possible increases the confidence of the method, however, it can be challenging to find information on several species in the same compound. Sala et al. (2012) demonstrate that there is no statistically significant difference between SSDs of 9 to 10 species and 92 species, which means that for regulatory purposes, ten species could suffice to start the evaluation.

Therefore, this framework proposes elaborating an SSD curve for the prioritized compounds, and using the prioritized species, it is an effort to regionalize the SSD to each watershed. In cases in which data on the toxicity of the chemicals for those species is sparse, an SSD for another chemical with a similar mode of action could be applied using the same hypothesis as Sala et al. (2012). The authors state that if the mode of action is similar between chemicals, toxicological effects are comparable, thus the slope of the curve could be used as an approximation, in which the classes of organisms could be similarly placed.

Another proposition would be to use SSDs for different classes of chemicals instead of the isolated compounds, meaning that with data from the standard species alone, it could be possible to develop an SSD (SALA et al., 2012). Those values could serve as a guideline to policymakers on a low-data scenario since data on the toxicity of organisms such as daphnids, algae, or trout are usually available (SALA et al., 2012). Regulatory bodies demand this toxicity information and thus are more widely accessible.

11.1.7 Social route

Amongst the main studies and documents elaborated at the watershed level, the watershed management plans are some of the most important, since they could be consulted to profit from the network of stakeholders that these plans contain. Part of these documents list the main land uses and industries present in the region and their impacts on the water resources. Usually, watershed management plans consult the local communities to ensure that all involved parties are heard (BERA; BANIK, 2019).

Civil society could play a key role in reporting the impacts of chemical pollution to the authorities, through actions such as recording and documenting the environmental alterations e.g. a fish population decline, water properties visibly changing such as color and turbidity, or reporting cases of new diseases caused by pollution. The latter should also be consulted with data from whichever departments of the region are responsible for tracking epidemiological data, be it the health

surveillance department, environmental monitoring agency, or any department that would gather toxicity data from the compounds used in the watershed.

Consulting with traditional local communities such as autochthones, indigenous groups, or traditional communities such as quilombolas, is a crucial step in reducing the distance between all stakeholders and the people whose livelihoods might be significantly affected by chemical pollution. Including these communities at the elaboration stage could reduce the damage caused by environmental racism, which is a recent definition for the centuries-old problem of marginalized communities experiencing more significant environmental impacts than their non-marginalized counterparts.

Integrating the watch list of chemicals and their possible adverse effect using AOP could help health departments and hospitals keep track of health records and relate the data with water quality. Thus, all the routes proposed are interconnected and would help each other in reinforcing protection.

11.1.8 Regulation updating

Integration between routes is the primary key of the proposed framework since it is already known that one of the problems in environmental regulation is how distant regulatory bodies can be from each other given that there are several possible exposure routes for humans and biota (EVANS et al., 2016). Those regulatory silos (EVANS et al., 2016) are one of the challenges in creating an environmental regulation that could be easily updated and thus realistically protect the environment.

Alternatives to further include mixtures in the regulation have already been discussed and one of the most promising ones is the Mixture Assessment Factor (MAF) (DRAKVIK et al., 2020b). This methodology consists of creating a factor to adjust current regulations to natural environment scenarios that include several compounds co-occurring in the same body of water. The proposed factor could be used as a transitory stage from the current moment to the future. We could better integrate modeling with regulatory practices and further protect the environment with more accurate data on the physical- and biochemical processes.

Drakvik et al. (2020) stated that the MAF could be based on different aspects considered essential for the region. It could be based on synergism already observed in the literature, for example, it was observed that diclofenac and sulfamethoxazole have a synergistic effect and the authors calculated a mixture toxicity index of 3.29 for the effect of these compounds on mortality of *Eusemia fetida* (DRZYMAŁA; KALKA, 2023). Thus, if the regulators chose this parameter as their MAF, the regulatory thresholds for both these compounds could be corrected 3.29 times to reduce this effect.

11.2 CONNECTIONS BETWEEN ACADEMIA REGULATORY BODIES AND INDUSTRY

Greater integration between all stakeholders is needed to implement the proposed framework efficiently, given that it applies new concepts and methodologies relevant to academia, regulatory bodies, and industry. Therefore, to increase the likelihood of the application's success, we propose the creation of a

workgroup for guidance and feedback on how to use the proposed methodology and how it could be improved. This workgroup would be comprised of scientists, policymakers, and industry professionals to better convey the knowledge to each stakeholder.

First, contact could be made through the watershed committees or similar structures since they are the groups responsible for including all relevant stakeholders regarding surface water quality. Profiting from previous efforts makes it easier to implement any new proposed methodology. The primary method to diffuse the framework to interested parties would be through elaborating courses, seminars, and workshops promoted by interdisciplinary groups of experts from different organizations and scientists. Groups such as ICLEI already tackle bridging this gap by promoting activities with policymakers e.g. workshops on contaminants of emerging concern and river-specific pollutants.

Workshops would promote a greater understanding of mixture toxicity and associated risks and explain how the proposed framework could be integrated into the current workflow by teaching how to use the New Approach Methodologies integrated in this paper. It is essential to highlight a feedback section to include all stakeholders' opinions on the implementation stage.

11.3 FRAMEWOK LIMITATIONS

The proposed framework has some limitations and they are as follows.

Currently, the framework cannot include in its evaluation pollutants without defined regulatory thresholds such as macro, micro or nanoplastics. Therefore, other

pollutants without a water quality standard will also not be able to be included. The solution to this question would be to rely in other water quality threshold definitions to first define a threshold and then update it with the method hereby presented.

12 CONCLUSION

New emerging scenarios such as chemical pollution and climate change are demanding greater adaptations from all sectors for environmental protection, especially in the global south. To address these issues, this paper creates a framework that integrates a few different methodologies that converge to promote environmental quality standards closer to actual environmental conditions since no single methodology can fully encompass all subjects into regulation.

The innovation associated with this framework proposition is related to its integration between different sectors like the biological, social, and environmental routes. These were considered the most relevant factors for environmental regulation due to their relevance on the regulatory steps. The biological and chemical routes are connected to a greater extent due to their high conceptual connection, especially in environmental toxicity data. This highlights that countries that do not have access to extensive research could use data generated by other sources and still have a safer environment with a data-based analysis.

However, most frameworks do not include the social route even though it is an essential aspect of the success rate of any policy. Including the participation of civil society and marginalized communities in the conversation could also increase

the sense of belonging and importance to otherwise forgotten communities and thus start a positive feedback loop with more engagement and knowledge from every part involved.

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14 THESIS CONCLUSION

Mixture toxicity has gained significant attention due to the increasing release of various chemicals into the environment each year. This study aimed to elucidate the potential effects of the combination of polystyrene nanoparticles (PSNP) and ivermectin (IVM), thereby simulating possible environmental scenarios. The findings could assist regulatory bodies in managing emerging contaminants.

The stability of PSNP suspensions indicates that these particles are likely to remain suspended in the water column, attributed to their high stability (>30mV). While the presence of IVM or media ions influenced this stability, the alterations were not significant enough to compromise the stability of PSNPs, thereby enhancing their bioavailability.

Isolated PSNPs demonstrated effects only at concentrations significantly removed from those typically found in natural water, soil, and sediments. This suggests that the primary concerns regarding PSNPs involve their physical interactions and potential synergistic effects with other compounds. In contrast, IVM exhibited minimal impact on non-target organisms but caused notable negative effects on *Daphnia magna*, *Enchytraeus crypticus*, and *Folsomia candida*. Consequently, this evaluation reinforces existing data and emphasizes the need for heightened environmental safety measures in aquatic environments receiving wastewater discharges or in soils adjacent to livestock operations.

The combination of PSNP and IVM revealed synergistic effects on the reproduction of *Daphnia magna*, as well as on the growth rate of *Microcystis aeruginosa*. Conversely, antagonistic interactions were observed concerning the

surface area and root length of *Lemna minor*, along with the size and longevity of *Daphnia magna*. Additive effects were primarily noted regarding immobility in *Daphnia magna*, reproduction in *Folsomia candida*, reproduction in *Enchytraeus crypticus*, and growth rate in *Chlamydomonas reinhardtii*.

The species sensitivity distribution (SSD) curve elucidated potential environmental impacts associated with these concentrations, yielding a hazardous concentration for 5% (HC5) of 29.49 parts per trillion (ppt) for aquatic organisms and 36.87 ppt for terrestrial organisms, underscoring the environmental ramifications even at low concentrations. This section of the thesis contributes valuable insights into the toxicity profiles of PSNPs and IVM, both individually and in combination, highlighting an urgent need for monitoring and assessing these compounds within environmental contexts.

Regulatory agencies must address environmental safety concerns to evaluate and predict the consequences arising from pharmaceutical consumption as it evolves. The model proposed is designed for use by regulatory bodies to facilitate updates to water quality thresholds. By integrating biological variables (SSD) with biochemical analyses (AOP), this model enhances evaluation robustness.

The demonstration indicated that not all cases need updates; however, it is reasonable to conclude that this specific mixture does not cause pressure on ecosystems. The SSD presented here exemplifies a method to assess toxicity while minimizing animal usage, yielding safe and reliable data. Future prospects include further reduction in animal utilization for bioassays and increased confidence in data generated through modeling approaches.

Emerging challenges such as chemical pollution and climate change are reshaping environmental protection efforts, particularly in developing regions. This thesis establishes a novel framework to tackle these issues by integrating diverse methodologies that converge to enhance environmental quality standards reflective of actual conditions, recognizing that no single methodology can adequately encompass all regulatory subjects. The proposed framework delineates three primary routes—biological, social, and environmental—aimed at categorizing significant factors pertinent to environmental regulation into broader domains. The biological and chemical routes are closely interconnected due to their strong association with environmental toxicity data. However, incorporating a social dimension is crucial as it facilitates engagement with civil society and marginalized communities in discussions surrounding these critical issues.

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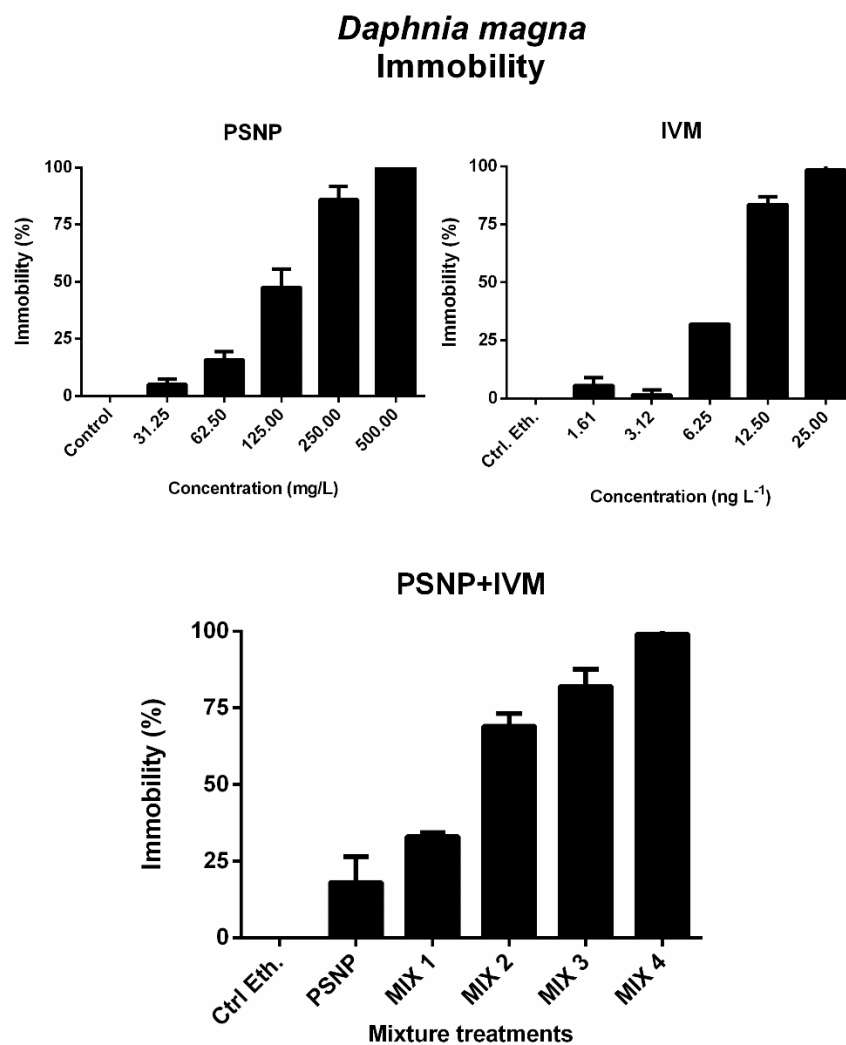
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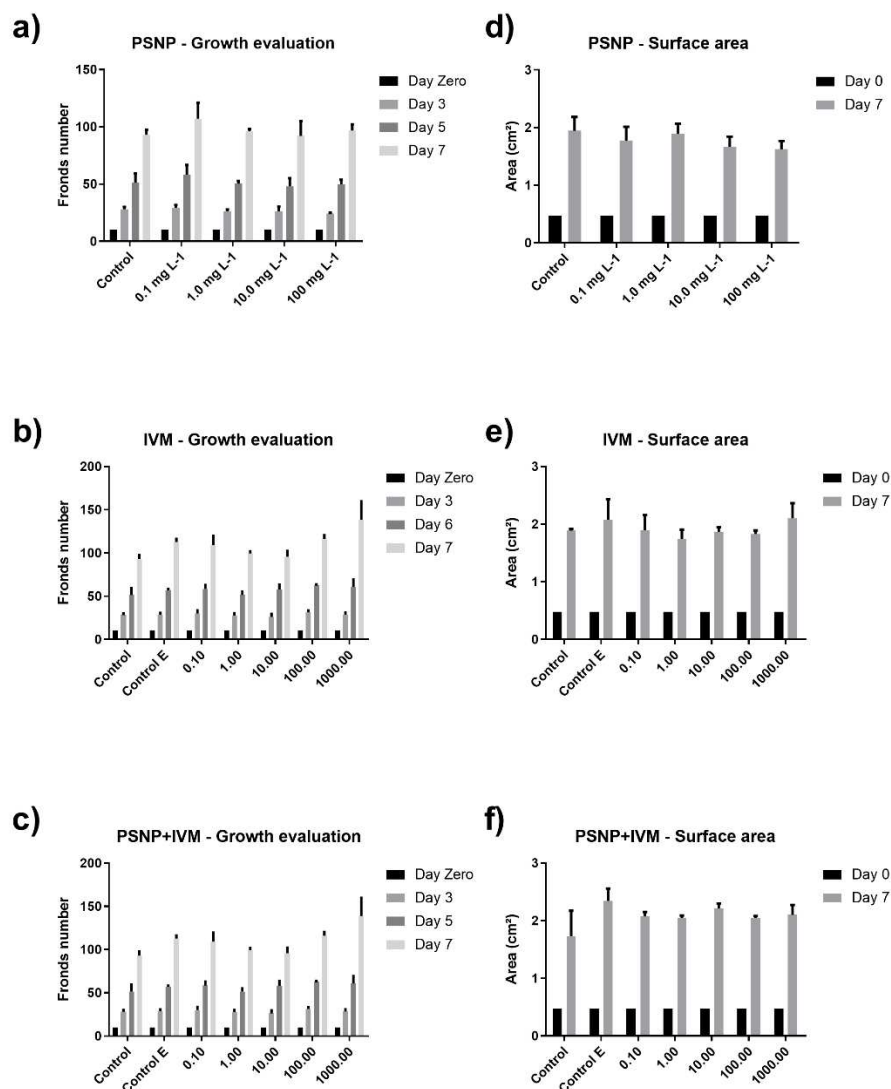
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16 APPENDIX]

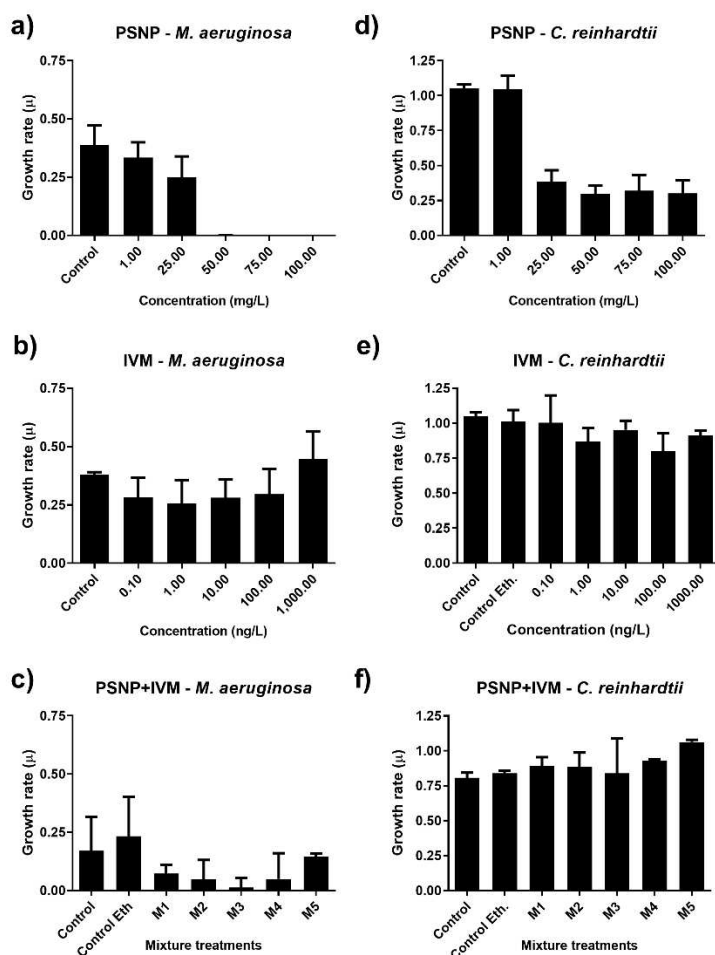
APPENDIX A – *Daphnia magna* immobility results for polystyrene nanoplastics (PSNP) and ivermectin (IVM). The data provides the endpoints EC₅₀ and LOEC. The EC₅₀ of PSNP is 133.04 mg L⁻¹ and the EC₅₀ for IVM is 7.63 ng L⁻¹. The LOEC of PSNP as 31.25 mg L⁻¹, and the LOEC of IVM 1.61 ng L⁻¹.



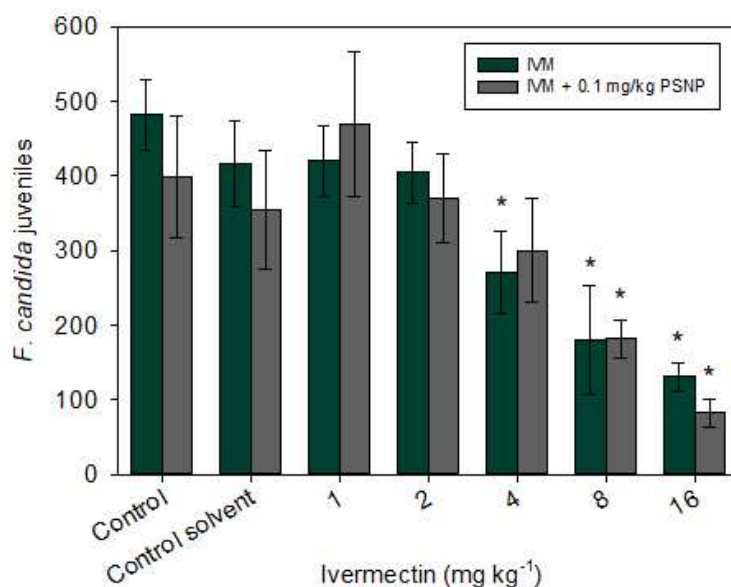
APPENDIX B – *Lemna minor* growth rate bioassay for polystyrene nanoplastics (PSNP) and ivermectin (IVM). The data provides the endpoint NOEC. The NOEC of PSNP is 100 mg L⁻¹ for surface area and 10 mg L⁻¹ for root length. As for IVM the NOEC of 10 ug L⁻¹ for surface area and 1 mg L⁻¹ for root length.



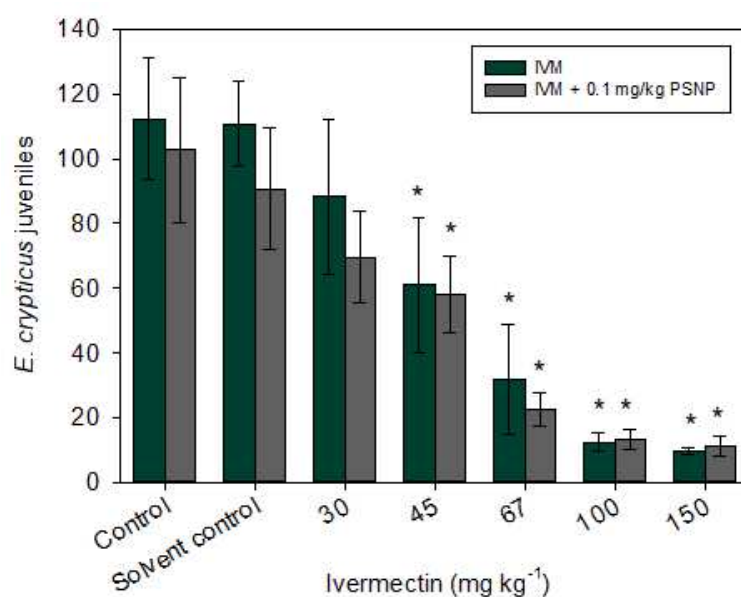
APPENDIX C – *Microcystis aeruginosa* and *Chlamydomonas reinhardtii* growth rate bioassays. The tests provided the endpoints EC50, LOEC, and NOEC. The *M. aeruginosa* EC50 for PSNP is 43.49 $\mu\text{g L}^{-1}$ and it was not possible to calculate the EC50 for IVM, thus the LOEC for IVM was 0.10 ng L^{-1} . was the NOEC for IVM is 100 ng L^{-1} . As for *C. reinhardtii*, the EC50 of PSNP was 13.45 mg L^{-1} and it was not possible to calculate the EC50 for IVM, thus the NOEC is 1 $\mu\text{g L}^{-1}$. The IVM NOEC was 0.10 ng L^{-1} .



APPENDIX D – Mean number ($\pm$ standard deviation; $n = 5$) of *Folsomia candida* juveniles found after 28 days of exposure to increasing concentrations of ivermectin (IVM, green bars) or their mixtures with polystyrene nanoplastic (IVM + PSNP, gray bars) in a tropical artificial soil (TAS). Asterisk (*) indicate significant reduction on the number of juveniles compared to the respective control. The NOEC was defined as 1 mg kg^{-1} .



APPENDIX E - Mean number ($\pm$ standard deviation; $n = 4$) of *Enchytraeus crypticus* juveniles found after 21 days of exposure to increasing concentrations of ivermectin (IVM, green bars) or their mixtures with polystyrene nanoplastic (IVM + PSNP, gray bars) in a tropical artificial soil (TAS). Asterisk (*) indicate significant reduction on the number of juveniles compared to the respective control. The LOEC was defined as 30 mg kg⁻¹.



APPENDIX F – Compiled bioassays data results in terms of NOEC or LOEC

for comparison purposes.

Test organisms	Bioassay	Endpoint	PSNP Results	IVM Results
<i>Daphnia magna</i>	Acute	LOEC	31.25 mg L ⁻¹	1.61 ng L ⁻¹
<i>Daphnia magna</i>	Chronic	NOEC LOEC	NOEC Reproduction: 0.053 µg L ⁻¹ NOEC Size: 0.053 µg L ⁻¹ NOEC Longevity: 530 µg L ⁻¹	LOEC Reproduction: 0.01 ng L ⁻¹ NOEC Size: 0.001 ng L ⁻¹ LOEC Longevity: 0.1 ng L ⁻¹
<i>Lemna minor</i>	Growth inhibition	NOEC	100 mg L ⁻¹	10 µg L ⁻¹
<i>Folsomia candida</i>	Reproduction	NOEC		1 mg kg ⁻¹
<i>Enchytraeus crypticus</i>	Chronic	NOEC		30 mg kg ⁻¹
<i>Microcystis aeruginosa</i>	Growth inhibition	LOEC	1 mg L ⁻¹	0.10 ng L ⁻¹
<i>Chlamydomonas reinhardtii</i>	Growth inhibition	NOEC	1 mg L ⁻¹	0.10 ng L ⁻¹