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Ecotoxicological effects of glyphosate, 2,4 D and atrazine on freshwater systems: concentrations, risk assessment and establishment conditions of a tropical specie.

Juiz de Fora

2020

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Dissertação apresentada ao Programa de Pós-graduação em Biodiversidade e Conservação da Natureza, da Universidade Federal de Juiz de Fora como requisito parcial à obtenção do título de Mestre em Biodiversidade e Conservação da Natureza. Área de concentração: Processos ecológicos e Conservação da Natureza.

Orientadora: Profa. Dra. Raquel Fernandes Mendonça.

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À minha mãe, Cleuza, e tias, Creonice e Cremilda, por serem as principais responsáveis por todos os frutos da minha vida acadêmica.

“Em tudo há beleza, ela pode ser vista com os olhos e admirada com o coração”. (André Rodini)

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ABSTRACT

Pesticides are agents of global change, since they can be transported to environmental compartments, cause adverse effects on non-target species. The most worldwide used pesticide is glyphosate. Some studies have already shown adverse effects on aquatic species caused by glyphosate, which is triggering global discussions about its legislation and use. Brazil has up to date no prospect of reducing or banning the use of the three most used pesticides in the country, glyphosate, 2,4 D and atrazine. The knowledge on adverse effects of pesticides and other chemical contaminants in tropical species is still scarce, maybe because many do not have defined protocols for ecotoxicological test conditions. The purposes of the each chapter of this study were: (1) to investigate the global glyphosate concentrations in surface freshwaters, to compare the countries laws and to carry out environmental risk assessments; (2) to investigate the Brazilian concentrations of glyphosate, 2,4D and atrazine in surface freshwater, and to assess the potential environmental risks they represent; (3) to describe the steps and the challenges for culturing the tropical test species *Chironomus xanthus* as well as to discuss its current use in ecotoxicology. Information on glyphosate concentrations in surface freshwater is scarce and known values very irregular among the countries investigated, with 95% of the studied systems showing concentrations that represent a risk to aquatic species. Most countries evaluated did not have restrictive legislation for the glyphosate presence in water resources, resulting in the non-protection of aquatic organisms. There was an increase in the annual sales of 2,4D, atrazine and glyphosate between 2009 and 2018 in Brazil. Although most environment concentrations were below the limit allowed by Brazilian legislation, the observed concentrations represented a medium to high risk for ecosystems in 65%, 72% and 94% of the Brazilian states for 2,4 D, atrazine and glyphosate, respectively. The ecotoxicological effect of pesticides, as well as of other contaminants in freshwater sediments are very often performed with benthic macroinvertebrates. *Chironomus xanthus* has been increasingly used for the past of years. Therefore, conditions for *Chironomus xanthus* establishment, maintenance and operation in the laboratory are necessary, due to the absence of protocols for this species.

Keywords: Pesticide. Systematic review. Risk assessment. Cultivation. *Chironomus xanthus.*

RESUMO

Pesticidas são agentes de mudança global, uma vez que podem ser transportados para compartimentos ambientais e causar efeitos adversos em espécies não-alvo, incluindo o homem. O pesticida mais utilizado no mundo é o glifosato, e alguns estudos já demonstraram que ele pode causar efeitos adversos em espécies aquáticas, o que está desencadeando discussões globais sobre a sua legislação e uso. O Brasil é um dos países que não possui perspectivas de redução ou banimento do uso dos três pesticidas mais utilizados no país, glifosato, 2,4 D e atrazina. A avaliação dos efeitos adversos desses e de outros contaminantes químicos ainda são escassos em espécies tropicais, já que muitas não possuem protocolos definidos para condições de testes ecotoxicológicos. Com isso, os objetivos de cada capítulo do trabalho foram (1) investigar as concentrações mundiais de glifosato em águas doces superficiais, comparar as legislações dos países e realizar avaliações de risco ambiental. (2) investigar as concentrações de glifosato, 2,4D e atrazina em águas doces superficiais brasileiras e avaliar os potenciais riscos ambientais que eles representam. (3) descrever as etapas e os desafios para o cultivo da espécie teste tropical Chironomus xanthus, bem como discutir seu uso atual em ecotoxicologia. O registro das concentrações de glifosato em águas doces superficiais são escassos e irregulares entre os países investigados, e 95% dessas concentrações representam um risco às espécies aquáticas. A maioria dos países avaliados não possuía legislação restritiva para a presença do glifosato nos recursos hídricos, resultando na não proteção dos organismos aquáticos. Houve aumento nas vendas anuais de 2,4D, atrazina e glifosato entre 2009 e 2018 no Brasil. Embora a maioria das concentrações ambientais estivesse abaixo do limite permitido pela legislação brasileira, as concentrações observadas representaram um risco médio a alto para os ecossistemas em 65%, 72% e 94% dos estados brasileiros para 2,4 D, atrazina e glifosato, respectivamente. O efeito ecotoxicológico de pesticidas, bem como de outros contaminantes em sedimentos de água doce, é frequentemente realizado com macroinvertebrados bentônicos. A espécie *Chironomus xanthus* tem sido cada vez mais utilizada nos últimos anos. Portanto, são necessárias condições de estabelecimento, manutenção e operação de *Chironomus xanthus* em laboratório, devido à ausência de protocolos para essa espécie.

Palavras-chave: Pesticida. Revisão sistemática. Avaliação de Risco. Cultivo. *Chironomus xanthus.*

LIST OF FIGURES

Chapter 1 - Glyphosate concentrations in global freshwaters: are aquatic organisms at risk?

Chapter 2 - Three-bestseller pesticides in Brazil: freshwater concentrations and potential environmental risks

Chapter 3 - Chironomus xanthus (Diptera: Chironomidae) in ecotoxicology: laboratory cultures and tests

FINAL CONSIDERATIONS

LIST OF TABLES

SUMMARY

GENERAL INTRODUCTION

Synthetic chemicals has increased over the past years and it has outpaced the increase in other sources of environmental change, such as atmospheric CO_2 concentrations, eutrophication, global population increase, and biodiversity loss. Although concerns about the proliferation of synthetic chemicals - including pesticides - started the 1960s environmental movement, synthetic chemical pollution was not included in most analyzes of global change (Bernhardt et al., 2017).

Pesticides form a group of synthetic chemicals created to many pourposes such as to destroy, repel, or mitigate a pest in agricultural crops (EPA, 2020). The growing and indiscriminate use of synthetic pesticides is becoming a major global concern, due to their negative effects on terrestrial and freshwater ecosystems, and also on human health (Bhat et al., 2019).

Glyphosate is the most used pesticide worldwide and it can be transported to aquatic ecosystems by surface runoff and leaching, potentially affecting the aquatic environment (Yang et al., 2019). There is currently an intensive debate about glyphosate legislation in water, with several countries proposing to ban or reduce its use (BHAG, 2020), since many studies indicate negative effects of glyphosate on aquatic organisms and on human health (*e.g.* Villamar-Ayala et al., 2019, Ferreira-Júnior et al., 2017, Garza-Leon et al., 2017) . Goin in the oposite direction of these countries, Brazil has no prospect of reducing or banning glyphosate use (BHAG, 2020) and it is the second-largest exporter of agricultural products, depending largely on pesticides to keep this position.

The pesticides 2,4 D and atrazine are the second and third most used in Brazil, respectively (IBAMA, 2020) and, as well as glyphosate, 2,4 D and atrazine have a potential to cause adverse effects on aquatic ecosystems (*e.g.* Lozano et al., 2018, Basopo & Muzvidziwa, 2020). Therefore, knowing these pesticides concentrations in freshwaters and understanding the effect of the environmental concentrations on aquatic organisms are important and urgent issues to prevent ecosystem degradation and ultimately preserve human health.

The gap in ecotoxicological information is most remarkable in tropical aquatic ecosystems when compared to temperate ones (Ferreira et al., 2017). In line with this, there are very few official protocols for the use of tropical species in ecotoxicological

tests (Santos et al., 2018). It makes of ecotoxicological experiments involving tropical conditions a more complicated task. *Chironomus xanthus* Rempel, 1939 stands out on ecotoxicological studies at tropical regions. It is restricted to Brazil and Argentina, has great ecological and regional relevance (Janke et al., 2011). It is a good model organism to be used in ecotoxicological tests and to evaluate the quality of aquatic environments in tropical regions (Beguelli et al., 2018). However, many improvements are necessary in order to accomplish that. For instance, choose the best conditions for test and cultivate (e.g. temperature, photoperiod, larval instar) is essential to compare the results among the studies (Raimondo et al., 2009).

This study is composed of three chapters, with the following objectives: (1) to identify gaps by gathering worldwide glyphosate concentrations in surface freshwater through a systematic review, compare countries' legislations and to perform environmental risk assessments; (2) to investigate the Brazilian surface freshwater concentrations of glyphosate, 2,4D, and atrazine and evaluated the potential environmental risks they pose; and, finally, (3) to describe the steps and the challenges for culturing the tropical test species *Chironomus xanthus* as well as to discuss its current use in ecotoxicology.

1 CHAPTER 1: Glyphosate concentrations in global freshwaters: are aquatic organisms at risk?

(Chapter submit at the Environmental Science and Pollution Research, A2, FI: 3.056)

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Abstract: Glyphosate is the most used herbicide worldwide. Many studies have reported glyphosate risks to aquatic organisms of different trophic levels. Moreover, evidences suggests flaws in countries' legislation that may imply the non-protection of aquatic species exposed to glyphosate. Therefore, we aimed to investigate glyphosate concentrations in freshwater ecosystems worldwide based on a systematic literature review, to discuss the results considering each country's legislation, and to assess the relative tolerance and risk for aquatic species. Only articles providing in situ concentrations of glyphosate in freshwater systems were included in our study. In total, 73 articles met the inclusion criteria and were used in our analysis. The studies comprised freshwater ecosystems from 21 countries. Most countries evaluated (90%) did not have restrictive legislation for aquatic glyphosate concentrations, resulting in a potential non-protection of aquatic organisms. Glyphosate may pose a moderate to high risk in 95 % of the countries investigated, reaching a maximum concentration of 105 mg L^{-1}. Additionally, the risk analysis showed that glyphosate concentrations below 0.1 µg L^{-1} represent a low risk, whereas glyphosate concentrations above 1 µg L^{-1}, which is below the limit stablished by some countries' legislation, represent a high risk to aquatic organisms. Therefore, we strongly recommend a revision of the countries' legislation for glyphosate concentration in freshwater systems.

Keywords: non-target species, systematic review, relative tolerance, pesticide, legislation, risk assessment.

1.1.0 Introduction

Glyphosate dominates the global market of pesticides (Carretta et al., 2019; Gonzalez et al., 2019; Lupi et al., 2019), and its application in agriculture is continuously increasing (Hébert et al., 2018). The active ingredient glyphosate is an acid, has a low vapor pressure, which means that it is not likely to volatilize. Moreover, glyphosate has an intermediary sorption coefficient (Koc) and a low octanol/water coefficient (Kow), indicating intermediary mobility in the soil and weak biomagnification potential, respectively (Table 1.1; Battaglin et al., 2005). The relative potential to accumulate in soils is mainly related to glyphosate's capacity to bind to aluminum and iron oxides (Babić et al., 2005). However, when soil saturation is reached, glyphosate may be transported to water bodies by leaching (Lupi et al., 2019) since it also has a high solubility in water (169.07 g mol^{-1}; Table 1.1). The short half-life, based on specific environmental conditions (Table 1.1), implies that once in the water, glyphosate may be rapidly degraded into metabolites, such as aminomethylphosphonic acid (AMPA; Fernandes et al., 2019). However, the half-life must be analyzed with caution since it relies on several environmental conditions, such as temperature, pH, humidity, solar radiation, and microbial activity (Sviridov et al., 2015; Muskus et al., 2019). In the environment, glyphosate acts by inhibiting plant enzyme activities (e.g., 5-enolpyruvylshiquime-3 phosphate synthase), which may affect the synthesis of aromatic compounds, proteins and secondary compounds (Wang et al., 2016).

Table 1.1. Glyphosate physical-chemical properties. Data source: https://comptox.epa.gov/ (USEPA); https://sitem.herts.ac.uk/aeru/iupac/atoz.htm (IUPAC); http://npic.orst.edu/factsheets/archive/ (NPIC).

Physico-chemical properties	
CAS RN	1071-83-6
CAS name	N-(phosphonomethyl)glycine
Chemical formula	$C_3H_8NO_5P$
Valor pressure (mPa)	1.31×10^{-2}
Molecular weight (g mol^{-1})	169.07
Solubility in water (mg L^{-1}) - pH 7	157
Log Kow	Less than -3.2
Koc	300 – 20.100
DT_{50} water (days) - pH 7; 20ºC	9.9

CAS RN = CAS Registry number; DT_{50} = Half-life; Kow = Coefficient octanol/water; Koc = Sorption coefficient.

Previous studies showed the potential of glyphosate to harm primary producers (Perez et al., 2007; Felline et al., 2019) and even higher trophic levels, with the potential to disturb ecosystems as a whole (Villamar-Ayala et al., 2019). At the ecosystem level, a few studies have shown the potential of glyphosate to modify the aquatic trophic structure (Saxton et al., 2001; Perez et al., 2007; Hébert et al., 2018). However, an ecological risk assessment report concludes that glyphosate applied in aquatic environments does not impact aquatic invertebrates or fish (USEPA, 2015). Given the controversial effects of glyphosate and its occurrence in freshwater systems worldwide, it is essential to compare environmental concentrations - i.e. those concentrations obtained in situ in aquatic environments - with local legislations in order to identify the real potential risks to organisms. This type of study may support actions to prevent potentially adverse effects on human and environmental health (Daouk et al., 2013; Avigliano et al., 2015).

The use of glyphosate started in the 1970s, but a massive increase in its use was registered in the last decade, resulting in banning or reducing glyphosate in several countries (BHAG, 2019). Italy, Canada, Spain, Netherlands, and Portugal already reduced or banned glyphosate, while Italy, France, and Germany are processing glyphosate ban (BHAG, 2019; Klingelhöfer et al., 2021). Those decisions were made based on the uncertainty regarding glyphosate's adverse effects on the environment and human health. Even in some countries that are still using glyphosate (e.g. European countries), there are intensive discussions about

the maximum allowed values (MAV) in water and whether these values are ideal for preventing adverse effects on non-target species, including humans (Székács and Darvas, 2018). Although the legislation is meant to protect the non-target species, defining a MAV is a complex process in which many stakeholders are involved, such as public agencies, the scientific community, society, and economic sectors (Pozzetti and Gomes, 2018). In addition to legislation, enforcement of legislation attendance as well as establish of methods of use control are also important.

The intensive use of glyphosate may lead to a continuous input into water resources, although glyphosate's potential to bind to soils is considered high (Primost et al., 2017; Sasal et al., 2017). Aligned to the massive global use, the potential adverse effects on non-target species, the outdated regulations, and the lack of systematic information on glyphosate-contaminated areas turn glyphosate into an emergent risk for aquatic ecosystems (Maggi et al., 2020). Therefore, this study aims to i) analyze and discuss worldwide glyphosate concentrations in surface freshwaters (i.e., only aqueous concentrations) through a systematic review of the literature, ii) compare each country's glyphosate concentrations in surface freshwater with their national legislations, and iii) perform glyphosate relative tolerance and glyphosate environmental risk assessment for three aquatic species.

1.2.0 Methods

1.2.1 Systematic review of glyphosate concentration in freshwater

A systematic review was carried out according to the PRISMA methodology (Moher et al., 2009) by gathering peer-reviewed scientific articles about glyphosate concentrations in freshwater ecosystems on the platforms Web of Science, Scopus, and PubMed. The term "glyphosate" was used instead of general terms such as "pesticides" or "herbicides". The following search code was used: ((glyphosate) AND (river OR "water resource" OR riverside OR microbasin OR "hydrographic basin" OR "sub-basin" OR "watercourse" OR freshwater OR river basin OR stream OR tributary OR lake OR pond)). The search included scientific studies published until December 31st, 2019. Inclusion and exclusion of publications were done at two levels: i) title and abstract and ii) full text, according to the criteria below:

I. Only articles informing environmental concentrations of glyphosate in the water column

of natural freshwater systems were included in our dataset. Strictly experimental articles and those with no actual measurement of glyphosate concentrations in the environment were not included;

II. Ecotoxicological articles, development of quantification methods, or reviews were not considered. Moreover, articles that applied glyphosate in water for experimental purposes, describing glyphosate in soil, groundwater, or projections about glyphosate concentrations in the future were also not included. Finally, national reports or state-sponsored programs were not considered.

After the selection, the articles references were screened, and related literature matching our search criteria were included in our dataset (as "additional records"; Figure 1.1). The number of exclusions for each screening level is shown in Figure 1.1. In cases where the study location was not evident, the authors' affiliation was considered as the corresponding location. If specific coordinates of the sampling locations were not informed, the coordinates of the country or city specified in the study were considered. When a map was given with no coordinates, the coordinates were extracted by localizing the approximate location on Google Earth Pro.

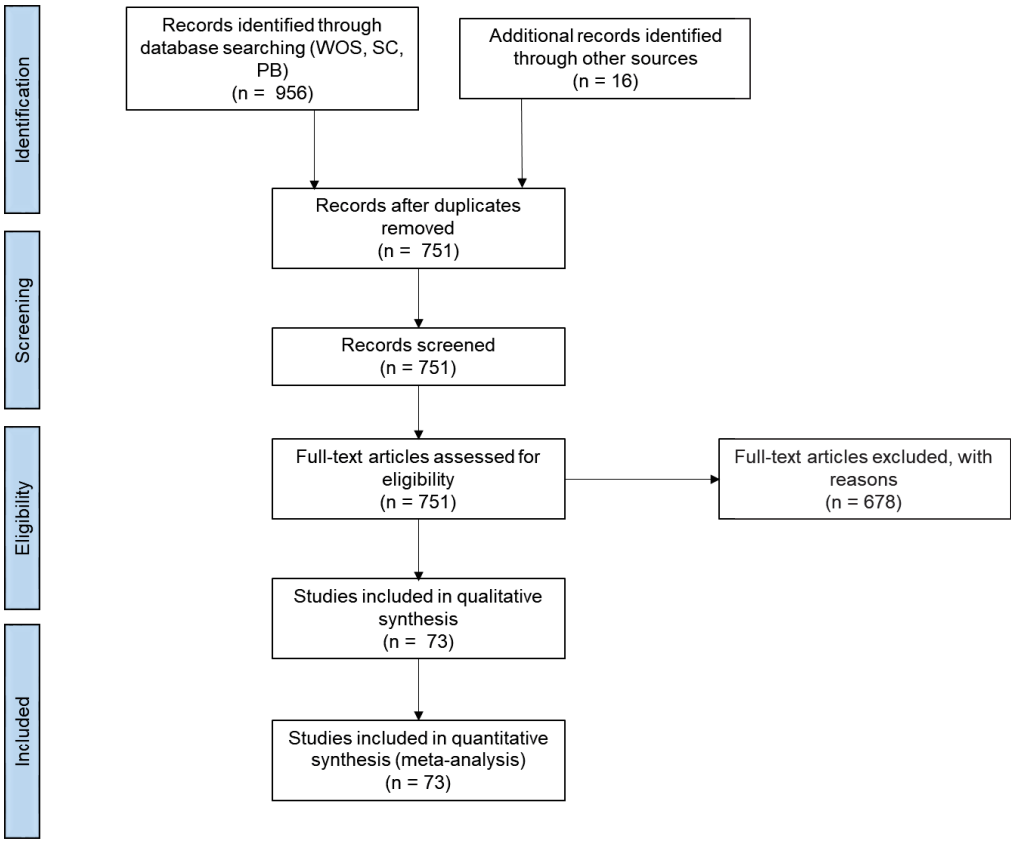

Figure 1.1: Systematic review diagram regarding glyphosate concentrations in surface freshwaters. Figure adapted from PRISMA methodology (Moher et al., 2009). Additional records identified through other sources refer to articles found in the reference lists of the articles obtained through the systematic review. WOS = Web of Science, SC = Scopus, PB = PubMed.

The environmental concentrations reported in the articles were compared to the MAV of glyphosate in surface freshwaters of each country. We were able to find MAV for aquatic biota protection of the following countries: Canada (CCME, 2012), Brazil (BRASIL, 2005), United States of America (USA) (USEPA, 2006), Argentina (Castro Berman et al., 2018), Sri Lanka (Gunarathna et al., 2018), Colombia (Ministry of Social Protection and Ministry of Environment, Housing and Territorial Development, 2007), Portugal (MA, 1998), Italy (Di Guardo et al., 2018), Switzerland, Netherlands, Germany, and France (Smit et al., 2014), Japan (Hamilton et al., 2003) and United Kingdom (WFD, 2010). The glyphosate MAV of drinking water was considered for Australia (Hamilton et al., 2003), Hungary, Spain, and Austria (EC, 1998) since surface freshwater legislation was

not found. We did not separate the environmental concentrations by water ecosystems (e.g., lake, river, etc.) because most of the studies did not refer to the type of aquatic system evaluated (see supplementary material Table S1.1).

1.2.2 Relative sensitivity to glyphosate

The Relative Tolerance approach (Trel) was used to assess the sensitivity of three test organisms to glyphosate the microalgae *Raphidocelis subcapitata* (Korshikov) F. Hindák, 1990, the microcustacean *Daphnia magna,* and the fish *Oncorhynchus mykiss* Walbaum, 1792, in which *D. magna* was used as a reference (Daam and Rico, 2016; Vilas-Boas et al., 2020). These organisms are commonly used to represent three trophic levels of aquatic ecosystems (primary producers, primary consumers, and secondary consumers), and the ecotoxicological data were available at the ECOTOX database (https://cfpub.epa.gov/ecotox/; 20th April 2020). The ECOTOX database is currently the largest single database containing information on aquatic and terrestrial species' sensitivity to chemical stressors. This database is also used in the most recent Ecological Risk Assessment of the United States (USEPA, 2015). Additionally, we checked the methodological quality of each article used. Trel was calculated by dividing the *R. subcapitata* and *O. mykiss* $LC(E)_{50}$ values by the mean $LC(E)_{50}$ values of *D. magna*. The geometric mean was calculated when more than one $LC(E)_{50}$ was reported for the species (see supplementary material Table S1.2). When the Trel value is equal to 1, it indicates tolerance to glyphosate equal to the reference species, whereas Trel values lower than 1 indicate greater sensitivity, and values greater than 1 indicate lower sensitivity.

1.2.3 Risk assessment

Risk quotients (RQs) were calculated considering the ratio between the measured environmental concentration and the predicted no-effect concentration (PNEC) (EC, 2003). The worst-case scenario approach was used, where the maximum environmental concentration (MEC) detected for each country was divided by the most sensitive PNEC value reported in the literature. We also considered 90th and 95th percentiles for comparing the results, which is a recommended method by drinking water risk assessment in the United States of America (USEPA, 2016). The MEC was calculated considering all the data reported in each country. The values below the limit of detection (LOD) or limit of

quantification (LOQ) were considered as LOD/2 or LOQ/2. The PNEC is calculated by dividing the $LC(E)_{50}$ or no-observed effect concentration (NOEC) values by the assessment factor (AF), which is applied according to the number of available ecotoxicological data (EC, 2003; Equation 1). The AF of 1000 is used when chronic studies are not available, 100 when one chronic test is available, 50 when two chronic tests are available, and 10 when one chronic test for each of the three trophic levels (represented by algae, microcrustacean, and fish) is available.

$$RQ = \frac{\text{MEC}}{(\text{NOEC or LC(E)50/AF})} \qquad (1)$$

Ecotoxicological studies of glyphosate active ingredient were selected from the ECOTOX database (https://cfpub.epa.gov/ecotox/; 27th January 2020). The PNEC was calculated from the NOEC reported by the microalgae *Scenedesmus obliquus* (Turpin) Kützing 1833 and *Oocystis lacustris* Chodat 1897 (Smebdol et al., 2017). Results from the three trophic levels were available, resulting in a PNEC value of 1 μg L^{-1}. The risk assessment calculation was also developed according to updated literature (Liu et al. 2015, Papadakis et al. 2015, Zhang et al. 2016, Carazo-Rojas et al. 2018, Iturburu et al., 2019). RQ lower than 0.1 was considered as a low environmental risk, between 0.1 and 1 as moderate risk, and greater than 1 as high risk (Iturburu et al., 2019). We also performed the RQ calculation using the MAV of each country as the MEC to evaluate whether the values are, indeed, protecting aquatic organisms based on the organisms selected for this study.

1.2.4 Graphical procedures

All graphics were produced using Excel 2013 and SigmaPlot 12.0. The maximum and minimum glyphosate concentrations reported for each country were used to create boxplot graphs. The maps of maximum glyphosate concentrations and risk assessment were made using ArcGIS version 10.6.1.

1.3.0 Results and Discussion

1.3.1 Systematic review of the literature and scientific production

Our systematic review returned 534 articles from Web of Science, 115 from Scopus, and 307 from PubMed. After removing the duplicates (i.e., first screening), 751 articles

remained. After applying the inclusion and exclusion criteria, 73 articles composed our dataset (Figure 1.1).

Our review indicated that the first scientific article reporting environmental glyphosate concentrations was published in 1998 (Figure 1.2A). Back in 1974, the United States Environmental Protection Agency (USEPA) registered the glyphosate use and, six years later, researchers started to investigate glyphosate concentrations in water matrices (Henderson et al., 2010). In the mid-1990s, the introduction of glyphosate-tolerant genetically modified crops probably facilitated the large-scale glyphosate application in agriculture (Székacs and Darvas, 2018). Indeed, an increase in glyphosate use from 1997 to 2016 was observed, peaking in 2000 (Székacs and Darvas, 2018). Despite this increase, the number of publications reporting glyphosate environmental concentrations in freshwater was relatively moderate from 1998 to 2016, with an average of 2 publications per year (Figure 1.2A). In 2017, the number of publications effectively increased, reaching 10 publications in 2017 and 11 in 2018. Studies investigating the adverse effects of glyphosate on freshwater species were also performed more often after 2016 (e.g., Pizarro et al., 2016; Felline et al., 2019; Sabio and García et al., 2020). The increasing number of publications in 2017 may be explained by the need to understand the risks posed by glyphosate to freshwater species. However, the number of publications decreased to 7 in 2019. Even though a decrease in the number of publications was observed, it is reasonable to expect an increase in the number of studies in the coming years due to recent advances in simplifying quantification methods. Currently, the most commonly used analytical method for determining glyphosate in water solution is the High-Performance Liquid Chromatography (HPLC). This method has higher recovery values and often requires derivatization, detectors for UV-vis and fluorescence compared to other methods. The gas chromatography technique also has good sensitivity, but it can become more complex due to the need to make glyphosate volatile (Melo et al., 2018). ELISA is another reliable method for determining glyphosate concentrations and it reaches lower detection limits compared to chromatography methods (Rubio et al., 2003).

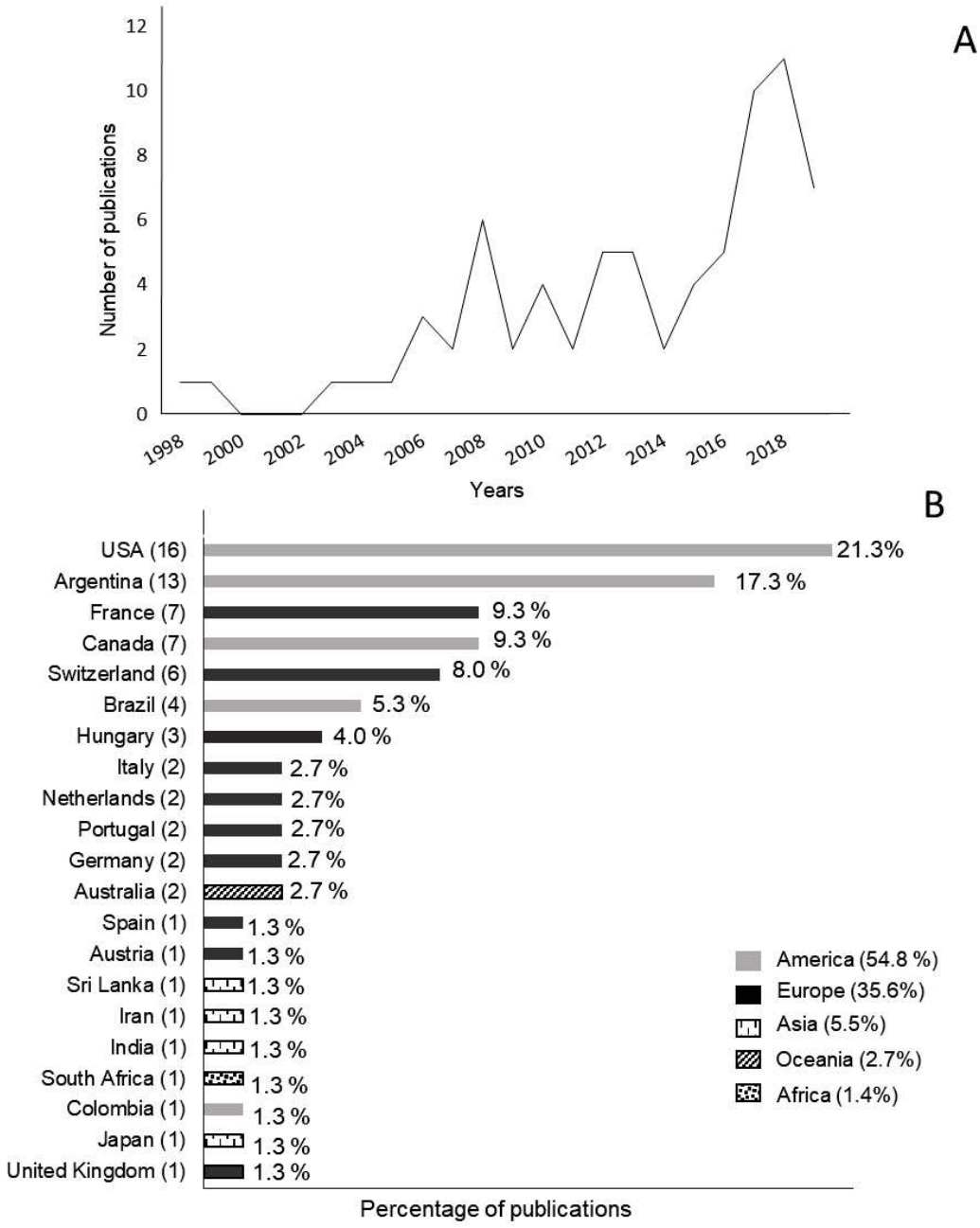

Figure 1.2: Number of glyphosate publications in surface freshwater over the years **(A)**. Percentage of publications by country and continent between 1980 e 2019 **(B).**

Our dataset was composed of glyphosate concentrations measured in surface freshwaters systems located in 21 countries. The top six countries with more publications were the USA (21.3 %), Argentina (17.3 %), France (9.3 %), Canada (9.3 %), Switzerland (8.0 %) and Brazil (5.3 %) (Figure 1.2B). The lack of published articles and the

concentrations reported by studies in only a few countries may be explained by the high costs and analytical methods' complexity to determine glyphosate concentration in environmental samples (Valle et al., 2019). The USA, Argentina, and Brazil are some of the largest users of glyphosate and resistant seeds globally (Richmond, 2018), which may explain the highest amount of studies in these countries. However, China and India are the primary producers and consumers of glyphosate in Asia, with China being the largest global producer (Richmond, 2018), and our review identified only two studies in India and no studies in China. In Africa, South Africa is a major glyphosate user (Richmond, 2018), and it is the only African country represented in this review, with only 1 article (Figure 1.2B and Figure 1.3).

Europe is the second continent with the most studies about glyphosate concentrations in surface waters (Figure 1.2B and Figure 1.3). Additionally, the European Union has fluctuated glyphosate use in recent years, probably due to regulatory issues (Richmond 2018; Table 1.2). Although glyphosate is still the most consumed herbicide worldwide, there has been a trend of reduction or even banning it due to its adverse effects on aquatic organisms (e.g, Garza-Leon et al., 2017; Ferreira et al., 2017) and the suspicion of carcinogenicity in humans (IARC, 2017).

Table 1.2. Maximum Allowed Value (MAV) of glyphosate in surface freshwater according to each country's legislation, attendance percentage (AP) with legislation, and glyphosate use prospects for each country.

	MAV (µg L^{-1})	AP (%)	Use	Reference
Argentina	280.0	94.4	A blanket ban is unlikely	Klingelhöfer et al. (2021)
Brazil	280.0	76.2	A blanket ban is unlikely	Klingelhöfer et al. (2021)
Canada	800.0	100.0	Restrict use	Klingelhöfer et al. (2021)
Colombia	100.0	0.0	N.A.	-
USA	700.0	100.0	A blanket ban is unlikely	BHAG*
Austria	0.1†	0.0	N.A.	-
France	28.0	70.6	Process of banning	BHAG*
Germany	0.1	50.0	Process of banning	BHAG*
Hungary	0.1†	0.0	N.A.	-
Italy	0.1	55.6	Process of banning	BHAG*
Netherlands	77.0	100.0	Prohibited use	BHAG*
Portugal	0.5	0.0	Prohibits in all public spaces	BHAG*
Spain	0.1†	50.0	Prohibited in some states	BHAG*
Switzerland	108.0	100.0	Prohibition denied	BHAG*
UK	196.0	100.0	Analysis process	BHAG*
India	700.0	100.0	N.A.	-
Japan	400.0	100.0	N.A.	-
Sri Lanka	700.0	100.0	Revoked the ban	BHAG*
Australia	10.0†	66.7	N.A.	-

*BHAG (https://www.bluehillhealthyecosystem.com/wp-content/uploads/2019/11/Where-is-Glyphosate-Banned-Baum-Hedlund-Aristei-Goldman.pdf)

N.A. Not available data.

† for drinking water.

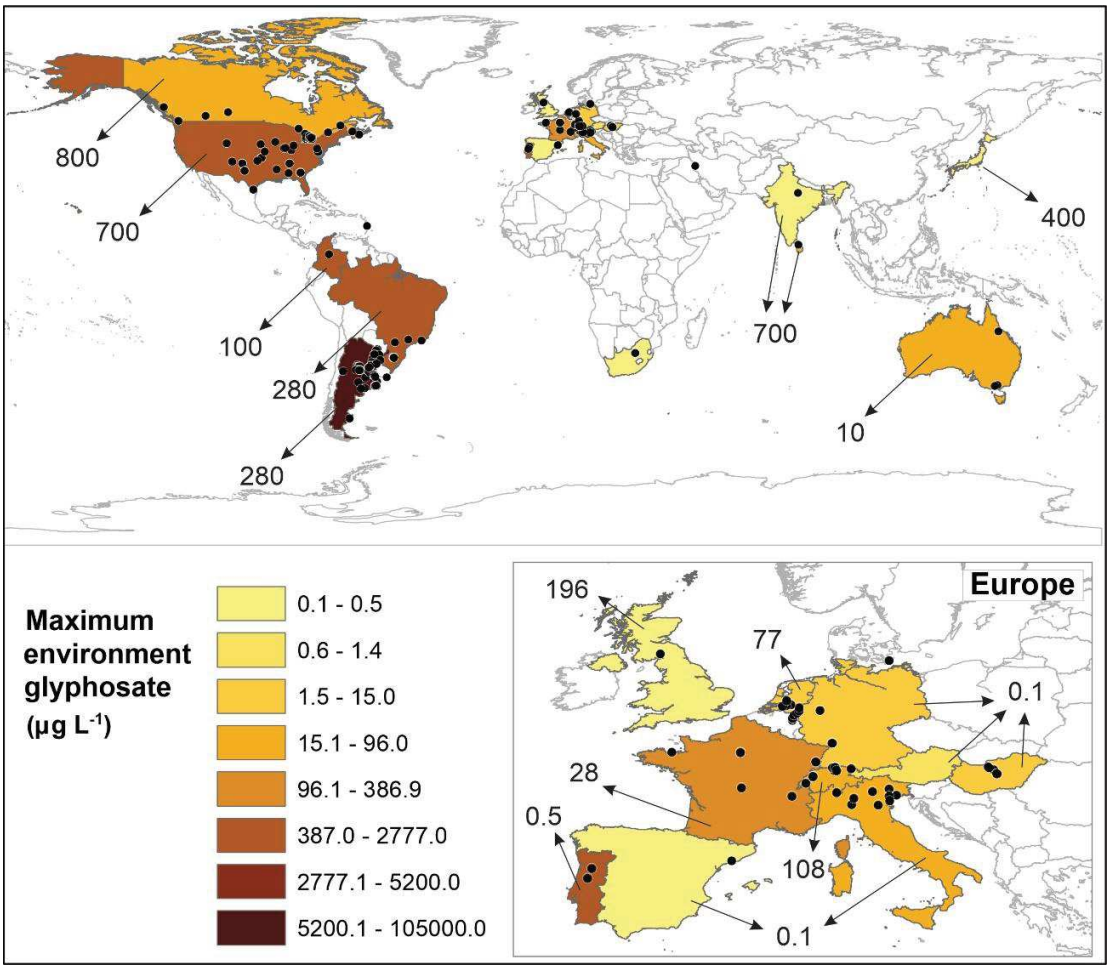

Figure 1.3: Maximum glyphosate concentration in freshwater systems. Values indicated by the arrows represent the Maximum Allowed Values (MAV, µg L^{-1}) for glyphosate in freshwater matrices. The black dots indicate the study sites. No data was found for the uncolored. countries.

1.3.2 Freshwater concentrations and national legislation worldwide

In our results, Argentina showed the highest glyphosate environmental concentration found (105000 µg L^{-1}; Sasl et al., 2017), followed by Colombia (2777 µg L^{-1}; Alza-Camacho et al., 2017) and Portugal (2460 µg L^{-1}; Silva et al., 2011) (Figure 1.3 and Figure 1.4). Argentina, as previously mentioned, is one of the largest users of glyphosate globally. It is noteworthy that this high concentration was found during the rainy season (Sasal et al., 2017), when glyphosate is usually carried out from the terrestrial ecosystems to surface waters by leaching (Byer et al., 2008; Daouk et al., 2013). Moreover, samples collected in aquatic systems near agricultural areas typically will represent higher

glyphosate concentrations (Montiel-Leon et al., 2019). The environmental characteristics, such as soil type, temperature, solar intensity, and microbial composition, also influence the glyphosate input into to water resources such as soil type, temperature, solar intensity, and microbial composition, which may explain the high variability found between countries regarding glyphosate concentrations. Additionally, the length of the period between glyphosate release in the environment and the sampling in the environment may also influence the glyphosate concentration to be obtained.

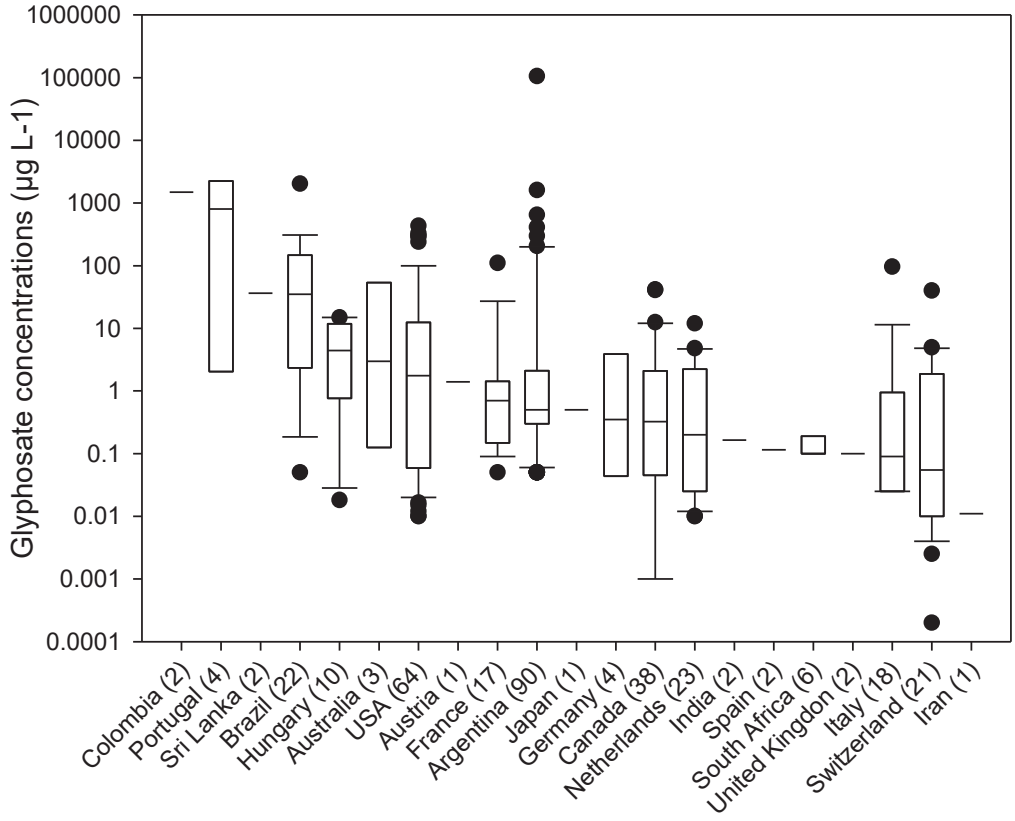

Figure 1.4: Glyphosate concentrations in surface freshwater matrices worldwide. The lines within the boxes indicate the median, the boxes delimit the 25th and 75th percentiles, and the whiskers delimit the 10^{th} and 90^{th} percentiles. Black dots represent outliers. The y-axis is represented on a logarithmic scale (Log_{10}), and the countries are ordered by decreasing median. Numbers in parentheses (x-axis) represent the number of glyphosate concentrations reported for each country.

The highest median value was found in Colombia (1489 μg L^{-1}) and the lowest in South Africa, the United Kingdom, Italy, Switzerland, and Iran (≤ 0.1 μg L^{-1}) (Figure 1.4). However, Colombia, South Africa, United Kingdom, and Iran had few samples (< 6 samples per country), and many of the studies in our dataset were performed in agricultural areas, which do not represent the overall freshwater systems within the countries. We, therefore, refrain from generating country - wide estimates based on our dataset. Instead, through our dataset, we show how large is the gap in knowledge about freshwater contamination by glyphosate, and we hope to call attention to urgently fill up this gap.

Although the comparison among different studies might be partly biased by the different methods used, we are confident that our analysis is not hampered since the liquid chromatographic method was primarily used (72.7%). In addition to liquid chromatography, ion chromatography (10.7 %), ELISA method (9.0 %), gas chromatography (4.6 %) and UV-Vis spectroscopy (3.0 %) were also used. Among the analyzed studies, the LOD ranged from 0.0002 μg L^{-1} to 200 μg L^{-1} and LOQ from 0.0007 μg L^{-1} to 530 μg L^{-1}. The lowest value of LOD and LOQ was found in Hanke et al., (2008) for Switzerland, in which the analytical method adopted was derivatization with 9-fluorenylmethylchloroformat (FMOC-Cl), solid-phase extraction (SPE), and liquid chromatography followed by electrospray tandem mass spectrometry (LC-ESI-MS / MS; see supplementary material Table S1.1). Noteworthy the possible interferes in the analytical methodology, since external substances may interfere in the final result, such as the presence of phosphate ion in the water sample (Zhu et al., 1999).

Among the 21 investigated countries, 19 have established legislation for MAV of glyphosate. We did not find MAV values set by Iran and South Africa. Canada, India, Sri Lanka, and the USA were the countries with less restrictive legislation, whereas European countries have presented the most restrictive legislation (Table 1.2). Countries that had the most restrictive MAV are also those trending to reduce/ban glyphosate use (Table 1.2). Brazil is the largest user of glyphosate, but, to the best of our knowledge, the number of existing studies in Brazil, according to our selection criteria, is relatively low. Moreover, Brazil does not present prospects for reducing/banning glyphosate use (Table 1.2). The dependence on agriculture may be identified as the main responsible for pesticide

application and production, which may be applicable to other countries that hold the economy through agriculture.

High median concentrations of glyphosate in freshwater were found in the USA, Brazil, and Argentina (Figure 1.4), countries with the least restrictive legislation compared to the others. These countries cultivate the largest shares of genetically engineered herbicide-tolerant crops globally, which improves the glyphosate use per hectare per year (Benbrook, 2016). Argentina mainly uses no-till practices, which consume more glyphosate than others cultivate practices (Okada et al., 2016). Soybean is the primary culture in the USA, Brazil, and Argentina (responsible for 82% of world production), and it is often the crop that receives the most glyphosate addition (Benbrook, 2016). Compared with other cultures (e.g., corn, sugar), soybean receives two more glyphosate applications in the cycle (up to 4.08 kg ha^{-1}), and leaves soil unprotected from erosion processes, which facilitates the entry of glyphosate in nearby watercourses (Okada et al., 2018). In addition to soybeans, glyphosate is also widely applied in corn and cotton crops in the USA (Benbrook, 2016), and corn and sugarcane in Brazil (IBGE, 2017).

Although most European countries showed environmental glyphosate concentrations in disagreement with the legislation, the MAV was about 1.5 to 8000 times more restrictive than American countries, such as Argentina, Canada, the USA, and Brazil (Figure 1.3). However, most of the studies investigated water resources near agricultural areas and, therefore, do not represent the entire area of the countries, but rather the most contaminated ones. Canada, the USA, Sri Lanka, India, the United Kingdom, Japan, Switzerland, and the Netherlands showed 100% of the freshwater samples with glyphosate concentrations below their legislation. Noteworthy that some of these countries present high MAV values, such as the USA with a MAV of 700 µg L^{-1}. Argentina had 94.4 % of compliance, Brazil presented 76.2 %, France 70.6 %, Italy 55.6 %, Germany and Spain 50 %. Colombia, Portugal, Austria, and Hungary showed 0% of legislation compliance (Table 1.2).

Creating legislation for surface water involves toxicological knowledge, the interests of non-governmental organizations, pesticide producers, farmers' associations, and political interests (Kudsk and Mathiassen, 2020). When legislation is based on "outdated

science" (i.e., when few toxicological data are available or/and analytical methods had higher limits of quantification), its review is crucial and urgent. Smaller uncertainty factors are conquered when ecotoxicological data are up-to-date (Umbuzeiro et al., 2010).

1.3.3 Aquatic risk assessment

The algae *R. subcapitata* and the fish *O. mykiss* were more sensitive to glyphosate than the microcrustacean *D. magna* (Figure 1.5, Table 1.3). Therefore, *D. magna* cannot be used to protect the species compared here. The assessment factor of 100, which corresponds to a Trel of 1, is applied to consider differences in sensitivity between species. Therefore, it resulted in a sufficient protection level concerning glyphosate for the standard test species represented here. The LE_{50} value used for *R. subcapitata* (15.4 mg L^{-1}; see supplementary material Table S1.2) was lower than the highest concentration recorded in Argentina (Figure 1.4). It means that the glyphosate concentrations in Argentinean freshwaters have the potential to affect primary producers and, consequently, may affect the entire food chain, harming other aquatic species and even the ecosystem services and functioning. Previous studies showed that Roundup®, a glyphosate-based product, has been shown to cause tissue damage and alterations in the activity of the antioxidant system of rainbow trout *Oncorhynchus mykiss* Walbaum, 1792 at 500 µg L^{-1} (Meshkini et al., 2018). Glyphosate concentrations of 1530 µg L^{-1} and 490 µg L^{-1} caused, respectively, late female emergence and rapid male emergence of *Chironomus xanthus* Rempel, 1939 (Ferreira et al., 2017). Another glyphosate-based product, Sulfosato Touchdown®, highly affected the survival, growth, reproduction, and intrinsic population growth rate of cladoceran microcrustaceans (*Daphnia magna* Straus, 1820 and *Ceriodaphnia dúbia* Richard, 1894) (Reno et al., 2018). Faena®, also a glyphosate-based product, caused a chronic effect (embryonic developmental phase and decreased fecundity) on the rotifer *Lecane papuana* Murray, 1913 and disruptive endocrine effects on cladocera *Alona guttata* Sars, 1862 at 700 µg L^{-1} (Garza-Leon et al., 2017).

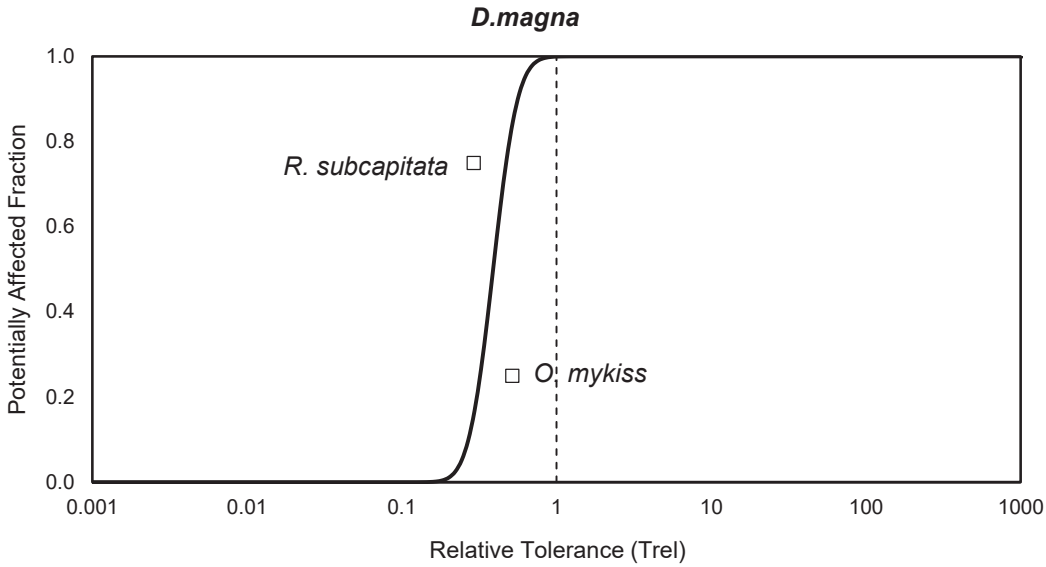

Figure 1.5: Relative tolerance values for *Raphidocelis subcapitata* and *Oncorhynchus mykiss* to glyphosate. The dashed line (Trel = 1) indicates the sensitivity of *D. magna*, where Trel <1 and Trel >1 indicate greater and lower sensitivity of species, respectively. The bold curved line indicates the Trel values distributed in a potentially affected fraction (PAF). The PAF represents the fraction of species exposed above the no-effect concentration (NOEC), being a measure that allows comparison in toxic stress between substances and areas.

Table 1.3. Number of relative tolerance (Trel) of *Raphidocelis subcapitata, Daphnia magna* and *Oncorhynchus mykiss* to glyphosate values separated by ranges. Trel < 1 indicates higher sensitivity and Trel > 1 indicates lower sensitivity.

Glyphosate	**<0.01**	**0.01-0.1**	**0.1-1**	**1-10**	**10-100**	**>100**	**<1**	**>1**
R. subcapitata	0	0	0	33.3	50	16.7	0	100
D.magna	0	0	0	12.5	50	37.5	0	100
O. mykiss	0	0	0	33.9	32.3	33.9	0	100

At the ecosystem level, glyphosate has been reported to favor the picocyanobacteria fraction of the phytoplankton communities and, therefore, eutrophication, due to the availability of phosphorus (P) in its molecule (Perez et al., 2007; Hébert et al., 2018). A microcosm study developed on Lake Erie (Canada) showed that glyphosate provides

nutrients (nitrogen - N and phosphorus - P) for tolerant phytoplankton species while killing the least tolerant ones (Saxton et al., 2001). Another microcosm study observed that the abundance and operational taxonomic units of dominant bacterias were affected by glyphosate (Sabio and García et al., 2020). A Roundup®-based mesocosm study performed in Argentina found the mortality of the periphyton community, which, consequently, favored the cyanobacteria group (Vera et al., 2010). This study also reported that glyphosate transforms clear environments into organic turbid, due to the growth of phytoplankton (Vera et al., 2010). Another mesocosm study testing glyphosate in both oligotrophic and eutrophic conditions showed that only one experimental application of glyphosate (2.4 mg L^{-1}) was enough to increase the P concentrations and, consequently, modify bacterioplankton and phytoplankton communities (Pizarro et al., 2016). Glyphosate also showed the potential to attract some species, such as Japanese quails (Ruuskanen et al., 2019).

All the countries investigated here presented a medium or high risk to the aquatic organisms when considering their MAV for the risk assessment (Figure 1.6B). Austria, Hungary, Spain, Italy, Germany, and Portugal showed a medium risk concerning their legislation values. However, Austria, Hungary, Spain, Italy, and Germany had their MAV at the limit to be considered as medium risk (0.1). The most significant risks of glyphosate MAV were found in the USA, Sri Lanka, Canada, and Argentina (see Figure 1.6B for other countries with high risk). The maximum, 95th and 90th glyphosate concentrations had the same result and 95 % of the countries (20 of the 21) showed a medium or high risk for the aquatic organisms (Figure 1.6A). Only environmental concentrations found in Iran presented a low risk, while the medium risk was found for India, Japan, South Africa, Spain, and the United Kingdom. The most significant risks of glyphosate in the environment were found in Argentina, the USA, Colombia, and Brazil (Figure 1.6A). For these countries, the RQs were greater than 100 or even 1000, a value that is assumed to have a serious potential to cause adverse effects to aquatic organisms (Giesy et al., 2000). In accordance with our analysis, a previous study has reported a high risk of glyphosate to the aquatic ecosystem in Argentina (Bonansea et al., 2018). Another study performed in Argentina estimated the mixture risk of pesticides and showed that glyphosate was largely responsible for the high ecological risk registered in the northern region of the country

(Iturburu et al., 2019). We noted that the countries with low risk were those with fewer studies, suggesting a possible bias in our interpretation due to the limited data available for the assessment. Therefore, the risk assessment may become more robust with more data on environmental concentrations in these countries. Additionaly, the risk assessment calculation applied generates an estimative of potential consequences. Noteworthy that it is not robust enough to demonstrate multiple stressors and mixture effects, for example. However, despite the limitations, this estimate was made in a more conservationist manner, precisely assuming these limitations, considering, for example, the most sensitive species that once affected may change community structures.

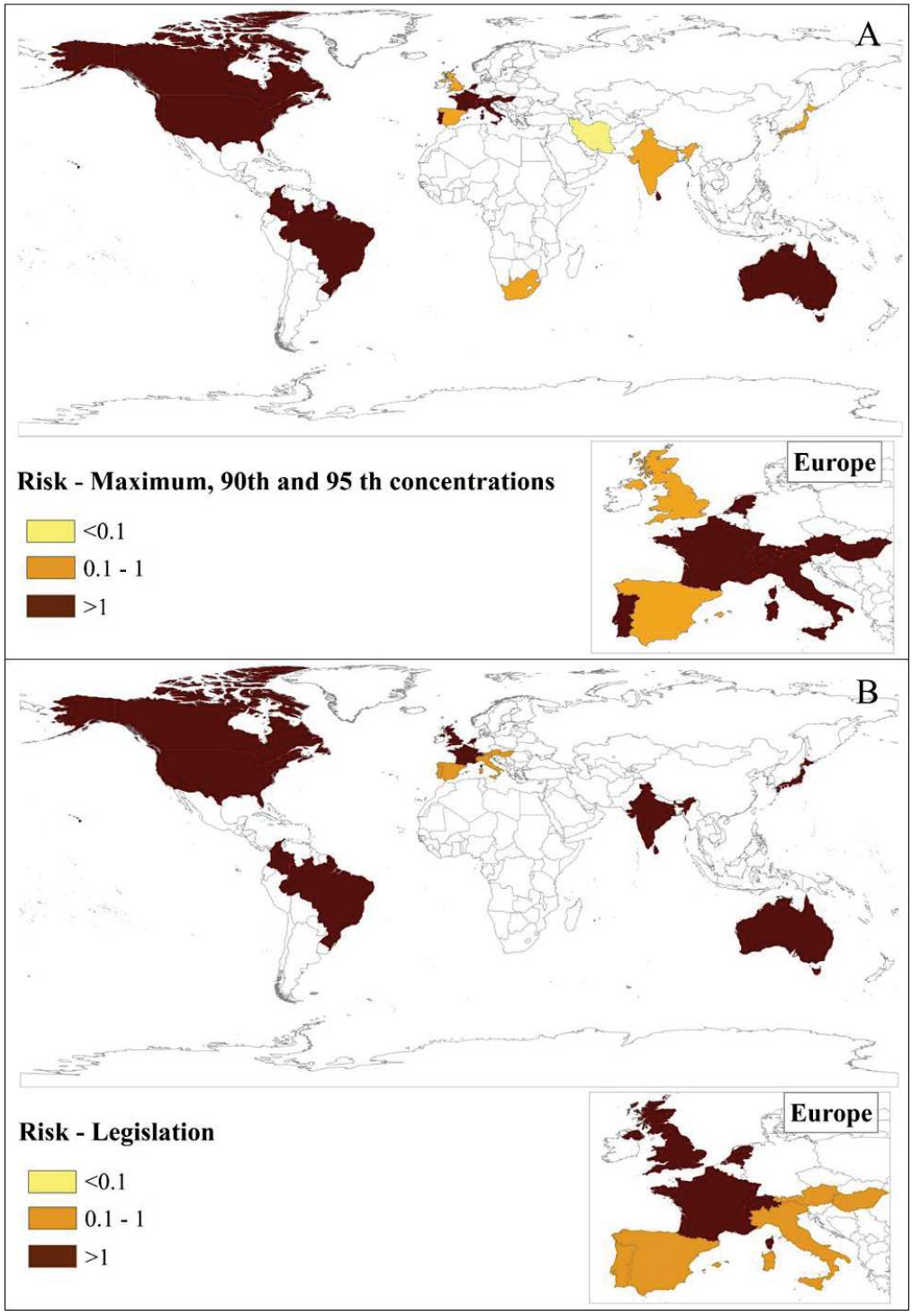

Figure 1.6: Risk assessment of glyphosate from the maximum, 90th and 95th environmental concentrations in surface waters globally **(A)** and the legislation of different countries **(B)**. The yellow countries indicate low risk (RQ < 0.1), the orange countries indicate medium risk (0.1≥ RQ > 1), whereas the dark red ones indicate a high risk (RQ > 1).

The risk assessment showed that glyphosate represents a medium-to-high risk for aquatic organisms in most evaluated countries, both in terms of legislation and environmental concentrations (maximum, 95th and 90th). Moreover, glyphosate may potentially affect entirely aquatic ecosystems by affecting non-target organisms, as shown here and by other studies (Perez et al., 2007; Vera et al., 2010).

According to our risk analysis, glyphosate concentrations below 0.1 μg L^{-1} represent a low risk to aquatic organisms, whereas above 1 μg L^{-1} poses a high risk. However, more studies are needed to improve our understanding of the remaining knowledge gaps, especially those related to endemic species' sensitivity, chronic effects, and mixture exposure.

1.3.4. Summary and future perspectives

Despite the increasing number of publications related to glyphosate concentrations in freshwater ecosystems, major scientific knowledge gaps remain. Nowadays, China is one of the largest glyphosate consumers, and we did not find any information on glyphosate concentrations in Chinese freshwater ecosystems. However, we do not contemplate national or state-sponsored programs, which may fill part of the gaps we identified. The higher glyphosate concentration was found in Argentina, and the country does not present prospects for reducing/banning glyphosate use, besides being one of the largest soybean producers in the world, as well as Brazil (Table 1.2; Klingelhöfer et al, 2021). Brazil and the USA, where glyphosate concentrations were also high, have less restrictive legislation and no prospect to reduce/ban glyphosate use in the near future. Therefore, we encourage more studies monitoring glyphosate concentrations in aquatic ecosystems, mainly in countries not represented here.

Although our focus was on surface freshwater (aqueous solution), glyphosate may also be found in other environmental matrices, such as suspended matter, soil, sediment, groundwater and even drinking water (Ronco et al, 2016; Gunarathna et al, 2018; Rendon-von et al, 2017). Therefore, we encourage other studies to better understand the glyphosate consequences in the environment in a more systemic view.

The MAV itself already represents a risk to the aquatic organisms, which means that even concentrations in agreement with the legislation may be harmful to aquatic organisms in many countries. Hence, we strongly suggest revisions of the glyphosate legislations in freshwater and monitoring their effectiveness. The MAV is a crucial tool for watershed management and should protect non-target species and guarantee water quality.

Acknowledgments

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2 CHAPTER 2: Three-bestseller pesticides in Brazil: freshwater concentrations and potential environmental risks

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Abstract: Agricultural production in Brazil is favored by weather conditions and by the large amount of available land. Therefore, Brazil currently is the second largest exporter of agricultural products globally. Pesticides are widely used in Brazilian crops due to their high efficiency, their low cost, and permissive legislation. However, pesticides tend to reach water resources threatening organisms and the water quality. Thereby, we aimed to review the surface freshwater concentrations of the three-bestseller pesticides in Brazil (glyphosate, 2,4D, and atrazine), and discuss the results with sales, legislation, toxicity and potential risks. For that, we performed a systematic review of quantitative studies of glyphosate, atrazine, and 2,4D in Brazilian freshwater and included monitoring data provided by the Brazilian Ministry of Health in our analysis. Finally, we calculated the risk assessment to the three pesticides. Only a few scientific studies reported concentrations of either of the three-bestseller pesticides in Brazilian freshwaters. Between 2009 and 2018, an increase in the sales of 2,4D, atrazine, and glyphosate was observed. It was not possible to evaluate the relation between concentrations and sales, due to limited number of studies, lack of standard criteria for sampling, individual environmental properties, and type of pesticide. Atrazine showed a higher toxicity compared to 2,4D and glyphosate. Regarding the environmental risks, 65%, 72%, and 94% of the Brazilian states had a medium to high risk to 2,4D, atrazine, and glyphosate, respectively. Finally, 80% of the Brazilian states evaluated showed a high environmental risk considering a mixture of the three pesticides. Although most of the environmental concentrations registered were below the allowed limits according to the Brazilian legislation, they are already enough to pose a high risk for the aquatic ecosystems. We, therefore, strongly recommend a revaluation of the maximum allowed values in the national surface freshwater Brazilian legislation.

Keywords: 2,4D, atrazine, glyphosate, legislation, systematic review.

2.1.0 Introduction

A huge amount of contaminants reach surface waters through different sources (Quadra et al., 2019), and water pollution has become one of the main environmental issues worldwide. Pesticides, which include an extensive amount of chemicals used for repelling and controlling plagues (*e.g.*, herbicides, insecticides, fungicides) are commonly found in aquatic ecosystems (Bonifacio and Hued, 2019). Studies indicate that pesticides use will enhance over the years to support the growing population, which will likely reach 9 billion people in 2050 (Verger and Boobis, 2013).

Brazil has favorable weather for extensive agricultural production, pushed by the huge demand for food both on national and global scale (Camargo et al., 2017). The country is, therefore, the second-largest exporter of agricultural products globally. That food demand, however, induces a massive use of herbicides in Brazil (IBAMA, 2020). More than 90% of the Brazilian farmers are reliant on pesticides (ANDV, 2009) and the country is the fifth-largest pesticide consumer worldwide (Worldatlas, 2018), representing ~20% of its global use (Albuquerque et al., 2016). Data from the Food and Agriculture Organization of the United Nations (2020) indicate that the pesticide use per cropland area in Brazil (5.94 kg/ha) is high when compared to the countries with the largest cropland area, i.e. 0.34 kg/ha in India, 2.54 kg/ha in the USA, and 0.62 kg/ha in Russia. Only China, with 13.07 kg/ha, uses more pesticides than Brazil. It is also worth mentioning that soy (32.2 Mha planted area), corn (15.8 Mha planted area) and sugar cane (10.1 Mha planted area) are the largest Brazilian crops (IBGE, 2017; Pignati et al., 2017), consuming on average 17.7L, 7.4L, and 4.8L pesticides per hectare, respectively (Pignati et al., 2017).

Data from 2009 to 2018 shows that the four-best-seller pesticides in Brazil are glyphosate, 2,4D, mineral oil, and atrazine (IBAMA, 2020). Those compounds are active ingredients, except for mineral oil, which is an adjuvant (MAPA, 2002) commonly used coupled with an active ingredient to improve its efficiency (Brazil, 2002). Glyphosate ranks first in Brazilian sales, but it is also the most used pesticide worldwide (Lupi et al., 2019; Okada et al., 2019; Villamar-Ayala et al., 2019), despite the proposed ban in Australia, France, India, Germany, Austria, and Spain (BHAG, 2020; https://www.baumhedlundlaw.com/). The relative high efficiency and low cost probably

explain the huge use of glyphosate globally (Green, 2012). Glyphosate in the environment is mostly degraded to aminomethylphosphonic acid (AMPA) (Fernandes et al., 2019), a process that depends on physical, chemical, and biological variables, such as temperature, pH, organic carbon availability, and microorganisms activity (Okada et al., 2019; Muskus et al., 2020). The glyphosate mode of action is mainly related to the inhibition of plant enzymes (5-enolpyruvylshiquime-3 phosphate synthase), which affects the production of aromatic compounds and, consequently, of proteins and secondary compounds (Wang et al., 2016). According to the Brazilian Health Surveillance Agency (ANVISA), glyphosate has a low toxicological level (four; Table 2.1). 2,4D is the second most used pesticide in Brazil, although it has a high toxicological level (one; ANVISA (2016); Table 2.1). 2,4D is also massively applied worldwide (Lozano et al., 2018) and acts by mimicking a natural auxin at a molecular level, overstimulating cell division and killing the plant (Song, 2014). Atrazine is the third one in the Brazilian rank of the most used pesticides (Figure 2.1). Atrazine has a moderate toxicological level (three; ANVISA (2016); Table 2.1) and the pesticide is banned in the European Union since 2003 (Montiel-León et al., 2019). Atrazine impairs the photosynthesis by blocking ATP, NADPH, and Hp production (Phyu et al., 2011).

Table 2.1. Physical-chemical properties of the three-bestseller pesticides in Brazil. Sources: https://comptox.epa.gov/ (US EPA); https://sitem.herts.ac.uk/aeru/iupac/atoz.htm (IUPAC); http://npic.orst.edu/factsheets/archive/ (NPIC); http://portal.anvisa.gov.br (ANVISA). K_{ow} = Coefficient octanol/ water; K_{oc} = Sorption coefficient.

Physical-chemical properties			
	2,4D	**Atrazine**	**Glyphosate**
CAS RN	94-75-7	1912-24-9	1071-83-6
Chemical formula	$C_8H_6C_{l2}O_3$	$C_8H_{14}ClN_5$	$C_3H_8NO_5P$
Molecular weight (g mol $^{-1}$)	221	215.68	169.07
Solubility in water (mg L^{-1}) - pH 7	44,558 ± 674	35	157
Log K_{ow}	0.177	2.7	Less than -3.2
K_{oc}	20 - 136	100	300 - 20,100
Bioaccumulation factor	68.8	18.9	0.893
Biodegradation Half-life (days)	3.55	4.92	4.47
Toxicological classification (ANVISA)	1	3	4

Previous studies have shown the ecotoxicological effects of glyphosate, 2,4D, and atrazine to aquatic organisms. Commercial formulas of glyphosate, for example, harmed algae photosynthesis (Felline et al., 2019), caused endocrine-disruptive effects on microcrustaceans (Garza-Leon et al., 2017), and changed the antioxidant system of fish (Meshkini et al., 2018). At the ecosystem level, that glyphosate induced picocyanobacteria growth in mesocosm experiments, with functional consequences to the whole system (Pizarro et al., 2016). Additionally, glyphosate adds phosphorus to the water resources potentially causing eutrophication (Hébert et al., 2019). 2,4D caused microalgae mortality (Lozano et al., 2018), reduced the survival and reproduction of microcrustaceans (Houssou et al., 2018), and affected chromosomal and DNA of fish (Ruiz de Arcaute et al., 2016). Furthermore, other investigation showed the potential of 2,4D to cause a misbalance on trophic chain levels (Villamar-Ayala et al., 2019). Atrazine also reduced photosynthesis in microalgae (Sun et al., 2019), changed diatom community composition (Wood et al., 2017), and bioaccumulated in fish muscle (Basopo and Muzvidziwa, 2020). Moreover, atrazine caused change to community composition and inhibited the production of periphyton (Herman et al., 1986).

The potential adverse effects described here and the massive use of glyphosate, 2,4D, and atrazine in Brazil, but also in other countries, makes it an urgent matter to understand the toxicity of these pesticides once in freshwater systems. With this study we aimed to investigate the surface freshwater concentrations of the three best-seller pesticides in Brazil, discuss the results with sales and legislation, as well as to perform an environmental risk assessment.

2.2.0 Methods

2.2.1 Systematic review

A systematic review was carried out using the PRISMA methodology (Moher et al., 2009). We searched for studies that quantified glyphosate, 2,4D, and atrazine in Brazilian surface freshwaters using the Scopus, Web of Science, PubMed, and Google Scholar platforms. The following search code was used: ((glyphosate OR atrazine OR "dichlorophenoxyacetic acid" OR 2,4D OR 2.4D OR 2.4-D) AND (lakes OR river OR “water resource” OR riverside OR microbasin OR “hydrographic basin” OR “sub-basin” OR “water

course" OR freshwater OR river basin OR "aquatic ecosystems") AND (Brazil)). The literature was also reviewed for each of the articles, and the ones that matched the criteria were also included in our analysis. The present review considers articles published between the 1st of January 2000 and 30th of April 2020.

The screening was performed by analyzing each entire content of the articles and gathering the concentrations of the three pesticides. Our exclusion criteria were: (1) articles that quantified the compounds in mixtures (*e.g.*, metabolites or other compounds); (2) articles that did not mention the limit of detection (LOD) and quantification (LOQ); (3) articles where LOD or LOQ were higher than the maximum allowed value according to Brazilian legislation number 357/2005 for class 2 and class 3 (Brazil, 2005); (4) articles that only reported pesticide concentrations in groundwater, drinking water, or seawater.

In Brazil, the legislation frames surface freshwaters into classes according to their main uses: special class and classes 1, 2, 3 and 4 (Brazil, 2005). Both classes special and 1 are more restrictive, since they are destined to human supply, aquatic life protection, irrigation and primary contact recreation. Most of the Brazilian rivers are classified as 2 and 3, which also is used for human supply after a more complex treatment, but also for irrigation, recreation, fishing and watering animals. Class 4 is mainly for navigation and landscape harmony (Brasil, 2005). Therefore, since most of the rivers are classified as 2 or 3, the maximum allowed values for those classes were considered in this study: 65 μg L^{-1} and 280 μg L^{-1} for glyphosate, 4 μg L^{-1} and 30 μg L^{-1} for 2,4D, and 2 μg L^{-1} for atrazine, respectively (Brazil, 2005).

All glyphosate, 2,4D, and atrazine concentrations were converted to a standard unit (μg L^{-1}) for the analysis (see supplementary material Table S1).

2.2.2 Government data

We also included in our analysis the concentrations of glyphosate, 2,4D, and atrazine in Brazilian surface freshwaters available at the Ministry of Health's "Open data" platform (http://www.dados.gov.br/) between 2014 and 2017. The data refer to the monitoring of water quality by water supply services in accordance with the Brazilian water quality standard for

human consumption (BRAZIL, 2017). In order to assure data quality, we excluded data that did not present LOQ and LOD values and values equal to 0. In the government data, glyphosate and 2,4D concentrations are analyzed in combination with AMPA and 2.4.5 T concentrations, respectively. Values below the LOQ was considered as LOQ/2 for statistical purposes, but only when the LOQ was above the maximum allowed value in Brazil (see supplementary material Table S2). The values in our dataset were grouped according to the Brazilian federal states in which the freshwater system is located. Distrito Federal, which is the smallest of the Brazilian federal units, was grouped with Goiás to ensure more even spatial distribution of data. The concentrations were compared to the maximum allowed values for the Brazilian surface freshwater (Brazil, 2005) and all the concentrations were standardized for $\mu g\ L^{-1}$.

2.2.3 Pesticide sales

National sales of glyphosate, 2,4D, and atrazine between 2009 and 2018 were taken from IBAMA (2020), including sales per Brazilian federal state. The database obtained was then compared to scientific and government publications. All analyses and graphs were produced in SigmaPlot (version 12.0).

2.2.4 Meta-analysis

We used the US EPA ECOTOX database (https://cfpub.epa.gov/ecotox/index.cfm; access on April 29th, 2020) to obtain ecotoxicological studies of the three herbicides. This database is currently the largest database on ecotoxicological studies of aquatic and terrestrial organisms. The database has some inclusion criteria to get reliable data, such as the use of peer-reviewed articles with well-described methods with valid species, chemical information, chemical concentrations, application of the observed effect and exposure time. We then compared the sensitivity of the standard test species *Raphidocelis subcapitata* Nygaard, 1987 (EC_{50} 1-5 h, growth inhibition, abundance), *Daphnia magna* Straus, 1820 (EC_{50} 1-2 h, immobility), and *Oncorhynchus mykiss* Walbaum, 1792 (LC_{50} 1-4 h, mortality) for each herbicide. These endpoints are usually in ecotoxicology studies when using standard species to evaluate the risk assessment (Rodriguez-Gil et al., 2018; Daam and Rico 2016). These organisms are the most used models in ecotoxicological testes to represent three

trophic levels. They also have complete data available at the US EPA ECOTOX database. The data reported by the US EPA ECOTOX database were verified by a thorough check of each manuscript in which the data was published in order to guarantee quality. The z–score was used here to standardize concentrations using the following equation:

$$z = \frac{x-\mu}{\sigma} \quad \text{(Equation 1)}$$

where x is the toxic concentration described in the studies with the standard test species, μ is the overall mean of all data (endpoint of all 3 different species) per herbicide, and σ is the standard deviation of all data for the three herbicides (See supplementary material Table S3, S4 and S5). This method is commonly used to compare different data (Gurevitch et al., 2018) and here, we followed the literature, where the z–score is also used as a meta-analytical approach to measuring differences in effect size in ecological and toxicological studies (Melvin and Wilson, 2013; Melvin and Leusch, 2016; Vilas-Boas et al., 2020). We first calculated the standardized and partial z–score values, standard deviation, and confidence interval, and then the sum of the squared deviations, using MS Excel. All graphs were produced in SigmaPlot (version 12.0).

2.2.5 Risk assessment

The risk assessment was performed according to updated literature with adaptations (Liu et al. 2015, Papadakis et al. 2015, Zhang et al. 2016, Carazo-Rojas et al. 2018, Iturburu et al., 2019). The 95% value of each pesticide in surface freshwater was calculated per Brazilian state. We used the 95% value instead of maximum environmental concentrations - which is commonly used to perform risk assessment - to avoid an overestimation. We thus divided the environmental concentration by the predicted no-effect concentration (PNEC) to estimate the environmental risk (Equation 2). The PNEC was calculated by dividing the no-observed effect concentration (NOEC) acquired from the US EPA ECOTOX database (May 13th 2020) by the assessment factor (AF; Equation 3).

$$Risk\ quotient\ (RQ) = \frac{Measured\ environment\ concentration}{Predicted\ no{-}effect\ concentration} \quad \text{(Equation 2)}$$

$$Predicted\ no-effect\ concentration\ (NOEC) = \frac{No-observed\ effect\ concentration}{Assessment\ fator}$$ (Equation 3)

The data from US EPA ECOTOX database were verified in the source articles to guarantee quality control and standardization to compare the risks and estimate the risk of the pesticide mixture. We tried to find the most sensitive reliable test available in the database for each pesticide. For 2,4D, the ecotoxicological test was conducted with *D. magna* (0.5 mg L^{-1}; Présing, 1981), for atrazine with *Poecilia reticulata* Peters, 1859 (0.013 mg L^{-1}; Shenoy, 2012) and for glyphosate with *Ceriodaphnia dubia* Richard, 1894 (0.01 mg L^{-1}; Huang et al., 2005). The NOEC results were divided by the AF to obtain the PNEC. The AF is applied to minimize the uncertainty from species to environmental toxicity extrapolation according to the availability of reliable data, in which higher values are applied to be conservative and protective (Carazo-Rojas et al., 2018). In our analysis, the AF of 50 was applied for 2,4D, while AF of 10 was applied for atrazine and glyphosate. Those AF are applied when long-term NOECs are available for two and three trophic levels, respectively (IHCP, 2003). We are aware about the uncertainties behind the risk assessment calculation, but it is indeed a good estimative of the risk based on measured environmental concentrations and reliable ecotoxicological tests, which is commonly used by studies worldwide (Liu et al., 2015, Papadakis et al., 2015, Zhang et al., 2016, Carazo-Rojas et al., 2018, Iturburu et al., 2019). The PNEC values were, thus, 1, 10, and 1.3 µg L^{-1} for glyphosate, 2,4D, and atrazine, respectively. Values higher than 1 were considered as high risk, values below 0.1 were considered low risk, and the values in-between were considered moderate risk (Liu et al., 2015, Papadakis et al., 2015, Zhang et al., 2016, Carazo-Rojas et al., 2018, Iturburu et al., 2019). A previous study had already assessed the environmental risk posed by the mixture of pesticides (Iturburu et al., 2019), and we applied that same methodology to our calculations. The map of glyphosate, 2,4D, atrazine, and mixture risk was elaborated using ArcGis version 10.3.1.

2.3.0 Results and discussion

2.3.1 Use of pesticides and academic production

A total of 113 articles were found, 34 from Scopus, 33 from Web of Science, 24 from Google Scholar, 19 from PubMed, and 3 from the references. After screening, only 44 were included in our analysis based on the inclusion and exclusion criteria (Figure 2.1). Among the three-bestseller pesticides, atrazine was the most studied (~ 73% of the articles), followed by 2,4D (~ 9%) and glyphosate (~ 9%). Two articles quantified both atrazine and glyphosate (4.5%), while other two atrazine and 2,4D (4.5%).

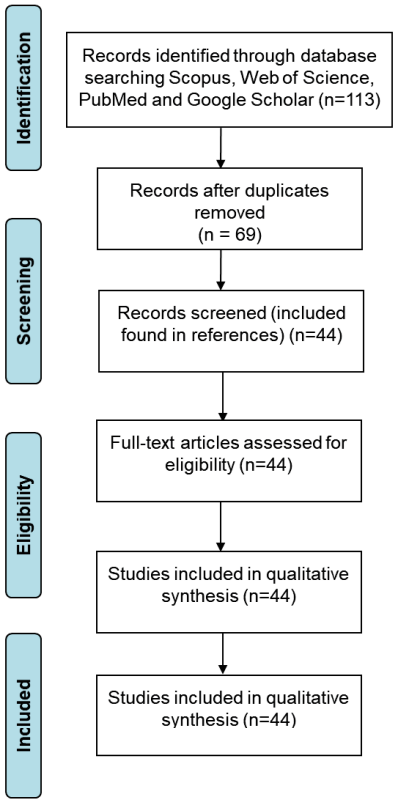

Figure 2.1. Flowchart of the systematic review of glyphosate, atrazine, and 2,4D studies in Brazilian surface freshwater. Adapted from PRISMA (Moher et al., 2009).

The pesticide sales in Brazil increased almost 85% from 2009 to 2018 (IBAMA, 2020), which is probably relate to a higher food demand (national and international) coupled with a greater transgenic grains consumption over the years, such as grains resistant to 2,4D and glyphosate (Fiocruz, 2019). Glyphosate sales increased by ~ 65% from 2009 to 2018, 2,4D by ~ 304% and atrazine by ~ 184% (Figure 2.2). Although glyphosate showed the lowest percentage growth of sales over ten years, it shows the highest accumulated sales

(more than 1.5 million tons), followed by 2,4D (~ 368 tons) and atrazine (~ 212 tons). The peak of sales for atrazine and glyphosate was in the last year evaluated (2018), while for 2,4D the peak was in 2017 (IBAMA, 2020, Figure 2.2).

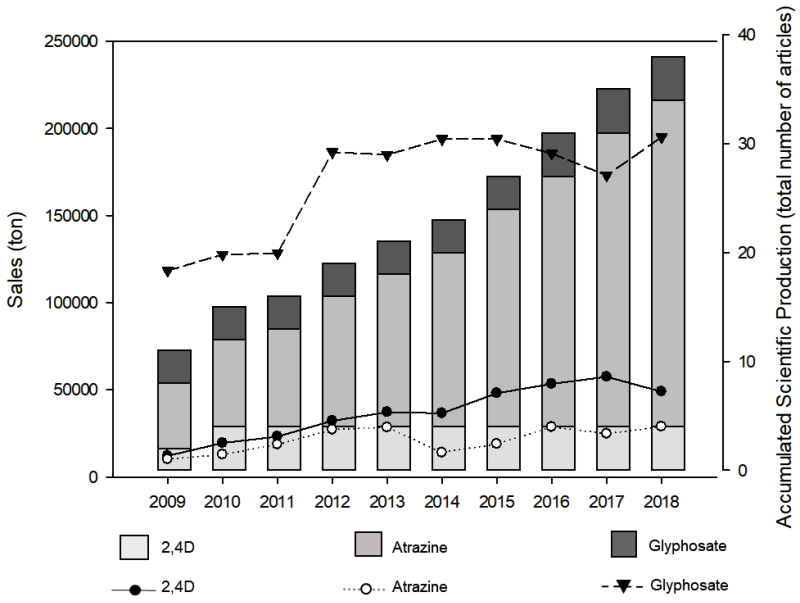

Figure 2.2. Annual sales and accumulated scientific production between 2009 and 2018. The marks and the lines represent the total annual sales for each of the herbicides (left y-axis). Bars represent the accumulated scientific production for each of the herbicides (right y-axis). Source of sales data: IBAMA, 2020 (www.ibama.gov.br).

Fluctuations in the commercialization of the herbicides over the years were observed, in which 2,4D and atrazine had two periods of decrease in sales, and glyphosate had four (Figure 2.2). From 2015 to 2017, glyphosate sales showed the largest decrease, which may be related to the increasing academic production and debate about possible risks, such as evidence regarding carcinogenic potential (IARC, 2017).

The three-bestseller pesticides were sold mainly in the Center-West region, which has become the largest agricultural area in Brazil in recent decades, particularly for grains (Bolfé et al., 2018). The South and Southeast regions were also important centers of consumption, which concentrate more than 70% of the total Brazilian agribusiness (Castro, 2013). The

Northeast and North regions presented the lowest pesticide sales over the years. Agriculture production is still limited in those regions due to several limitations, such as a lack of infrastructure and technology, and climate conditions mainly in the Northeast region (Castro, 2012, 2013).

The scientific production was more stable over time, without severe fluctuations. We expected to find more results regarding glyphosate due to its massive use, but atrazine was the most investigated in surface freshwater (n = 36), followed by glyphosate and 2,4D (n = 6). Therefore, there is a lack of information for glyphosate and 2,4D in surface freshwater, which are the first and second most sold pesticides in Brazil, respectively. The complexity of the glyphosate molecule may explain the results, which has high polarity and no chromophore, being necessary to apply derivatization reactions or to change its physical properties in order to quantify the compound in water using chromatography (Amarante Jr et al., 2012).

The number of publications was higher in the South (n = 16) and Southeast (n = 15) regions comparing to Center-West (n = 7), Northeast (n = 5) and North (n = 1). These results are in accordance with the sales data (IBAMA, 2020), except by Center-West publications. This spatial heterogeneity of scientific production in the country was already showed by previous studies, where fewer studies are normally conducted in the less-developed regions, such as the North and Northeast (Sidone et al., 2016). On the other hand, more universities and historically consolidated research institutes are located in the Southeast and South regions (Suzigan and Albuquerque, 2011), as well as a higher availability of human and financial resources (Albuquerque et al., 2002; Fundação de Amparo à Pesquisa do Estado de São Paulo, 2011).

Brazilian municipalities with the greatest increase in pesticide use coincide with the expansion of agricultural commodity areas, such as soy and sugar cane (IBGE, 2017; IPEA, 2020). Thus, the increase of pesticide use over the years in Brazil may be explained by the intensification of application in the large monocultures in the Southeast - the largest producer of sugarcane in the country - and in the South, as well as agricultural expansion in municipalities located on the Amazon edges, especially in Midwest and Northeast regions (IPEA, 2020).

In conclusion, we observed that the academic production is not following the increasing pesticide sales over time in Brazil. The high costs associated with the analytical methods and their complexity may limit the monitoring studies (Filizola et al., 2006; see supplementary material). The knowledge about the pesticides in freshwater resources is crucial, mainly considering the most sold ones, and, for that, more investment in this research area is need.

2.3.2 Environmental concentrations

Due to the low number of publications available regarding 2,4D, atrazine, and glyphosate in surface freshwater (n = 44), we included monitoring data provided by the Brazilian government in our analysis (Figure 2.3; See supplementary material Table S2). The sampling sites described in the literature were usually located at known contaminated areas and, therefore, the extrapolation of those environmental concentrations may represent an overestimation. Data from the government, on the other hand, is based on rivers that are used for human supply, which are expected to be less contaminated (Brasil, 2005). Therefore, the combination of these two approaches (government monitoring data + literature data) probably resulted in a more realistic estimation of the environmental concentrations.

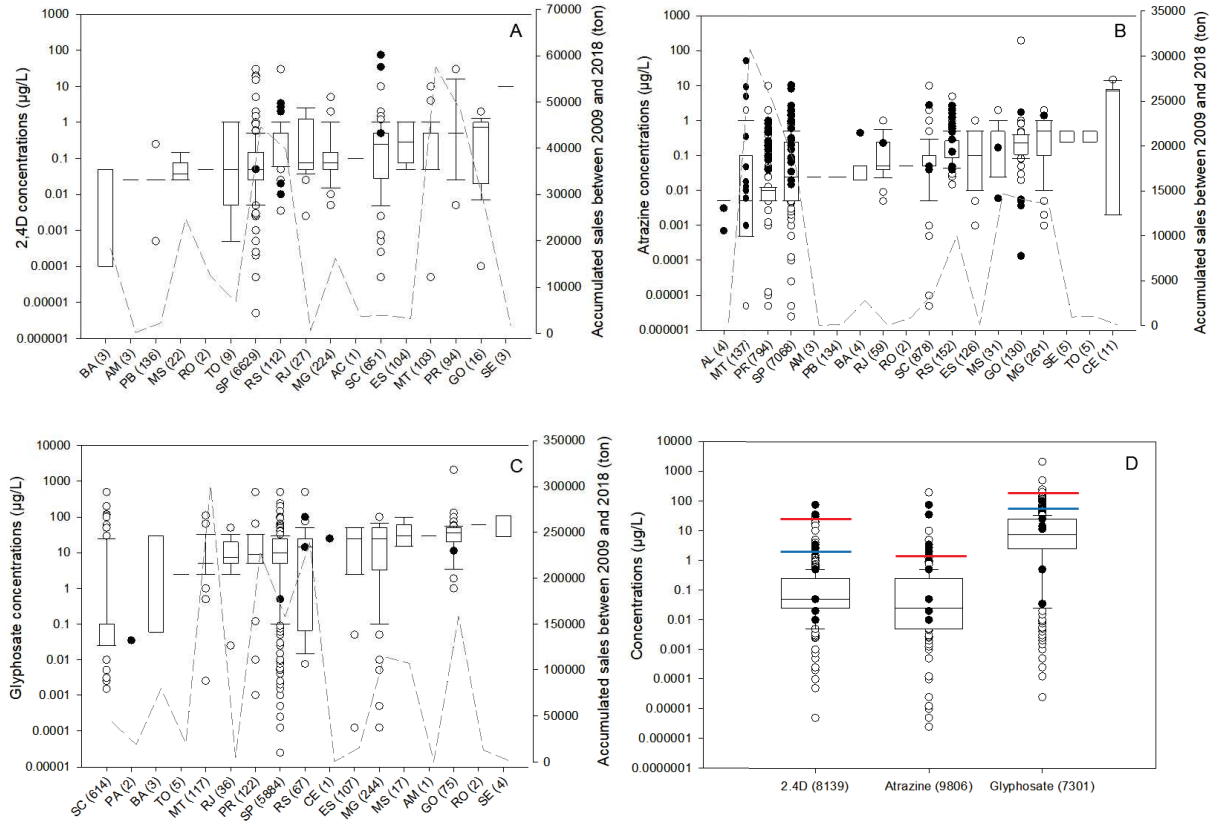

Figure 2.3. Pesticide concentrations in Brazilian surface freshwater and cumulative sales between 2009 and 2018. **A.** 2,4D concentrations and cumulative sales per Brazilian state. **B.** Atrazine concentrations and cumulative sales per Brazilian state. **C.** Glyphosate concentrations and cumulative sales per Brazilian state. **D.** 2,4D, atrazine, and glyphosate concentrations in Brazil. Boxplot corresponds to government data regarding pesticide concentrations in surface freshwater per Brazilian state and hollow dots correspond to outliers. Black dots correspond to literature data on pesticide concentrations in surface freshwater per Brazilian state. Dashed gray lines correspond to accumulated sales between 2009 and 2018 per Brazilian state. The box boundaries indicate the 25th and 75th percentiles; the line dividing the boxes represents the median; the error bars above and below the boxes indicate the 10th and 90th percentiles. Red and blue lines correspond to the maximum values allowed by Brazilian legislation for class 3 and 2, respectively (Brazil, 2005). The pesticide concentrations (y-axis) are on a logarithmic scale. The Brazilian states are ordered by ascendant median values. The numbers in parentheses represent the amount of data.

Brazilian states are: Acre (AC), Alagoas (AL), Amazonas (AM), Bahia (BA), Ceará (CE), Espírito Santo (ES), Goiás (GO), Mato Grosso (MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Pará (PA), Paraíba (PB), Paraná (PR), Rio de Janeiro (RJ), Rio Grande do Sul (RS), Santa Catarina (SC), São Paulo (SP), Sergipe (SE), and Tocantins (TO).

We did not find measured concentrations for the three-bestseller pesticides in the states of Amapá (AP), Maranhão (MA), Piauí (PI), Rio Grande do Norte (RN), and Roraima (RR). Sergipe state showed the highest median value of 2,4D (10 μg L^{-1}), but it was the 15th in accumulated sales (1647.23 ton). On the other hand, Bahia had the lowest median of 2,4D (0.0001 μg L^{-1}), but it was 7th in accumulated sales (18310.19 ton). The highest concentration found in surface freshwater was found in Santa Catarina state (74.5 μg L^{-1}), the 11th state in accumulated sales (3914.62 tons; Figure 2.3A). This concentration was found in small towns located in rural areas, usually related to agricultural zones (Pinheiro et al., 2010). The 2,4D concentrations in Brazil are similar to the ones found in other countries, such as Canada (1.68 μg L^{-1}; Metcalfe et al., 2019), Argentina (0.99 μg L^{-1}; Pérez et al., 2017), France (0.2 μg L^{-1}; Botta et al., 2012), and Spain (0.024 μg L^{-1}; Botta et al., 2012).

The highest median value of atrazine concentration was found in Ceará state (7 μg L^{-1}), which presented the lowest accumulated sales (16th; 59.59 tons). Alagoas had the lowest median value (0.004 μg L^{-1}) and was the 13th state in accumulated sales (361.61 tons; Figure 2.3B). Atrazine concentrations in Brazil were similar to those reported for other countries, such as Zimbabwe (6.15 μg L^{-1}; Basopo and Muzvidziwa et al., 2020), Argentina (1.4 μg L^{-1}; De Geronimo et al., 2014), Canada (0.66 μg L^{-1}; Montiel-León et al., 2019), and Germany (0.1 μg L^{-1}; Vonberg et al., 2014), but there reports are lower than the maximum registered in the country (195 μg L^{-1}; Figure 2.3B).

Considering glyphosate, the highest median value was found in Sergipe state (110 μg L^{-1}), which presented the lowest accumulated sales (15th; 1719.22 ton). The lowest median value was found in Santa Catarina, which showed one of the lowest sales of glyphosate (9th; 43887.58 ton). Reports of glyphosate in other countries showed higher values than the maximum reported in Brazil, such as Colombia (2777 μg L^{-1}; Alza-Camacho et al., 2016) and Argentina (10500 μg L^{-1}; Sasal et al., 2017), but other reports are lower, such as values registered in USA (9 μg L^{-1}; Kolpin et al., 2006), and France (0.8 μg L^{-1}; Slomber et al.,

2017). The Argentine economy is also highly dependent on agriculture, which is based on an intensive use of herbicides (Iturburu et al., 2019). Moreover, Brazil and Argentina are among the countries with largest glyphosate use worldwide (Richmond 2018).

The differences between the period of each data, systematic review (2000 – 2020), monitoring (2014 – 2017) and accumulated sales (2009 – 2018), probably favored the lack of correlation between pesticide concentrations and accumulated sales. Moreover, the highest median values was found for herbicides with a low number of samples and the higher the amount of data, the higher probability to dilute the median values. The monitoring data from government are derived from water supply sources, which, in theory, are less contaminated and may not represent contaminated areas like scientific studies. The lack of standard between contaminated or water supply sites and the number of samples per state may contribute to the divergences between the concentrations and pesticide sales by state, which was expected. The existence of a more robust monitoring program encompassing all states, as well as natural and contaminated areas, would be useful to a better understanding of pesticide environmental concentrations in Brazilian freshwater.

The 95th percentiles of 2,4D and atrazine were 1 µg L^{-1}, and the glyphosate 65 µg L^{-1} (Figure 2.3D). The maximum concentrations registered are quite higher comparing to 95th percentiles, which may be explained by the sampling season. Glyphosate had the highest median concentration in Brazilian surface freshwater (7.5 µg L^{-1}), followed by 2,4D (0.05 µg L^{-1}) and atrazine (0.03 µg L^{-1}; Figure 2.3D), and the exactly same order was observed for accumulated sales (Figure 2.2). However, the values registered for 2,4D and atrazine are similar, which may be explained by the higher half-life of atrazine in sediments (~5.0 days; Table 2.1), being susceptible to resuspension from the sediment to the water column.

Although herbicides are used in different crops, glyphosate is widely applied in soybeans, atrazine in corns, and 2,4D in sugar cane (Ackerman, 2007; Duke and Powles, 2008; Reis et al., 2008 a, b), which may help to explain the spatial national patterns found. Corn production is high in MT, MS, GO, MG, PR, and RS, while soybean in MT, GO, MS, PR, RS, MG, and BA (CONAB, 2019a) and sugarcane in the Southeast region, followed by the Center-West, Northeast, South, and North regions (CONAB, 2019b).

The weeds management in sugarcane culture is usually carried out throughout the plant's cycle. In rainy seasons, management is carried out in the post-emergence of weed plants; in the dry period, applications are made in the pre-emergency (Ismael, 2016). The sugar cane cultivation requires aerial pesticide spraying (Felisberto, 2015). Since aerial spraying causes the dispersion of pesticides in the environment, large areas can be contaminated (Fiocruz, 2018).

A common practice for Brazilian corn and soybean crops is the no-tillage system. Herbicide losses from the field due to surface runoff depend mainly on the occurrence of the next rainfall and the time interval between pesticide applications (Casara et al., 2012).

The herbicide application in the soybean crop, considered a summer crop (Rodrigues, 1993), usually occurs from the middle to the end of the cycle (Azevedo et al., 2016). There are some techniques that can be used aiming to assist the chemical control of integrated weeds management, such as the no-tillage cropping system, which is an effective soil management for weeds suppression trough the phytomass produced by cover plants (São Miguel et al., 2018).

About corn crops, there are some risks related to the pre-emergence weed control agents, since chemical management of weeds may carry pesticides to the environment, leading to soil and water contamination (PAS Campo, 2005). To reduce the probability of contamination it is necessary to minimize the pesticide use and, when not possible, find products that are not easily permeate into the soil and with low environmental half-life. Also, it is recommended the use of integrated pest management techniques (PAS Campo, 2005).

The herbicide sales and use are not the only factors accountable for the concentrations in the water, since many other environmental variables such as rainfall, soil retention capacity, slope, and other physical-chemical properties are also responsible for the runoff of pesticides to the aquatic ecosystems (Haith 1986; Dabrowski et al., 2002; Schriever and Liess 2007; Schriever et al., 2007). Comparing the K_{oc} (sorption coefficient) for each compound, glyphosate shows the higher adsorption potential while atrazine has a higher leaching potential (Table 2.1). Glyphosate is, however, much more used than 2,4D and atrazine in Brazil (Figure 2.1), which probably explain the higher concentrations in the water. Other properties, such as bioaccumulation and persistence potential, are similar for the three best-

seller pesticides (Table 2.1). Although these values may be considered low comparing to other organic pollutants, the high concentrations found in the environment make the compounds environmentally relevant and it is essential to control their application and residues in water resources.

2.3.3 Legislation

For 2,4D, the legislation compliance was 100% for most of the states, except Minas Gerais, Santa Catarina, Rio Grande do Sul, Sergipe, São Paulo, Mato Grosso, and Paraná (Table 2.2). Mato Grosso and Paraná states were also the largest 2,4D sellers in Brazil (Figure 2.3A).

Table 2.2. Percentage of samples that complied with the legislation for each Brazilian state according to water resource classes 2 and 3. For 2,4D: Class 2 = 4 µg L^{-1}; Class 3 = 30 µg L^{-1}. For atrazine: Class 2 and 3 = 2 µg L^{-1}. For glyphosate: Class 2 = 65 µg L^{-1}; Class 3 = 280 µg L^{-1} (Brasil, 2005). N.A.: Data not available.

Brazilian State	2,4D (%)		Atrazine (%)	Glyphosate (%)	
	Class 2	Class 3	Class 2 and Class 3	Class 2	Class 3
Acre	100	100	N.A.	N.A.	N.A.
Alagoas	N.A.	N.A.	100	100	100
Amazonas	100	100	100	100	100
Bahia	100	100	100	100	100
Ceará	N.A.	N.A.	36.4	100	100
Espírito Santo	100	100	100	100	100
Goiás	100	100	99.2	94.6	98.6
Mato Grosso	97.1	100	97.8	99.1	100
Mato Grosso do Sul	100	100	100	88.2	100
Minas Gerais	99.5	100	100	93.8	100
Para	N.A.	N.A.	N.A.	100	100
Paraíba	100	100	100	N.A.	N.A.
Paraná	90.4	100	99.8	99.2	99.2
Rio de Janeiro	100	100	100	100	100
Rio Grande do Sul	98.2	100	98	94	97
Rondônia	100	100	100	100	100
Santa Catarina	98.6	99.7	99.5	96.6	99.8
São Paulo	98.1	100	99.9	95	99.9
Sergipe	0	100	100	25	100
Tocantins	100	100	100	100	100

Regarding atrazine, Paraná, São Paulo, Santa Catarina, Goiás, Rio Grande do Sul, Mato Grosso, and Ceará did not comply 100% with the Brazilian legislation (Table 2.2). Mato Grosso was the state with the highest accumulated sales of atrazine in the country (Figure 2.3B) and, once again, one of the states with the lowest compliance with the

legislation. Although Rio Grande do Sul was not one of the biggest atrazine sellers, it showed the highest soybean production (IBGE, 2017), one of the main agricultural products in Brazil (Ortega et al., 2005), where atrazine is extensively applied (de Souza et al., 2020).

For glyphosate, Paraná, Mato Grosso, Santa Catarina, São Paulo, Goiás, Rio Grande do Sul, Minas Gerais, Mato Grosso do Sul, and Sergipe did not comply 100% with the legislation (Table 2.2), where Mato Grosso do Sul and Sergipe showed the lowest percentage of compliance. Although Mato Grosso do Sul was not among the largest sellers of glyphosate, the state is one of the largest sugar cane producers (IBGE, 2017), the second most important crop in Brazil, where glyphosate is largely applied (BHSA, 2016). Sergipe had only a few data, which may induce an overestimation of the concentrations found, as already commented previously.

Glyphosate and 2,4D were the herbicides with less legislation compliance considering class 2 (98.1%), while for class 3 atrazine showed less compliance (99.8%;Figure 2.3D). Although some concentrations are above the legal limit, there was generally no significant percentage of concentrations outside the standards stipulated by the Brazilian government. However, since the pesticides are intensively applied in Brazil and the concentrations found are comparable to others worldwide, this indicates that the maximum values allowed by the national legislation are high. Indeed, Europe seems to be more restrictive in terms of maximum allowed values of pesticides. For 2,4D, legislation in Canada, USA and Europe were not found, while for atrazine the maximum allowed values can be summarized as USA > Europe = Brazil > Canada and for glyphosate as Canada > USA > Brazil > Europe (Table 2.3). Therefore, although glyphosate legislation is more restrictive in Europe, for atrazine the Brazilian maximum allowed values are not in discrepancy comparing to other countries.

Table 2.3. Maximum values allowed for pesticides in surface freshwater in different countries (μg L^{-1}). ATZ = atrazine. GLY = glyphosate. N.A.: Data not available.

Pesticide	Brazil	Canada	European Union	USA
2,4D	4 (class 2); 30 (class 3) (Brazil, 2005)	N.A.	N.A.	N.A.
ATZ	2 (class 2 and 3; Brazil, 2005)	1.8 (CCME, 2012)	2 (EC, 2008)	10 (US EPA, 2006)
GLY	65 (class 2); 280 (class 3) (Brazil, 2005)	800 (CCME, 2012)	0.1 to 196 (Di Guardo & Finizio, 2018; WFD 2010)	700 S EPA, 2006)

2.3.4 Ecotoxicology and risk assessment

For *R. subcapitata*, 3 ecotoxicological data were selected from the US EPA ECOTOX database regarding 2,4D, 42 for atrazine, and 6 for glyphosate. For *D. magna*, 8 results were selected for 2,4D, 7 for atrazine and 9 for glyphosate. For *O. mykiss*, 8 results were selected for 2,4D, 18 for atrazine and 58 for glyphosate. Therefore, it was observed that *O. mykiss* was more studied considering the three-bestseller pesticides in Brazil, followed by *R. subcapitata* and *D. magna*. Considering the pesticides, glyphosate was more investigating regarding ecotoxicological studies, followed by atrazine and 2,4D, not directly related to the pattern of environmental concentrations, since atrazine was more studied. Besides that the ecotoxicological data consider studies developed worldwide, glyphosate raised concern over the years due to adverse effects related, as already discussed.

Atrazine was more toxic to all aquatic organisms evaluated (Figure 2.4). *R. subcapitata* and *D. magna* were more sensitive to 2,4D than glyphosate, while *O. mykiss* was more sensitive to glyphosate than 2,4D.

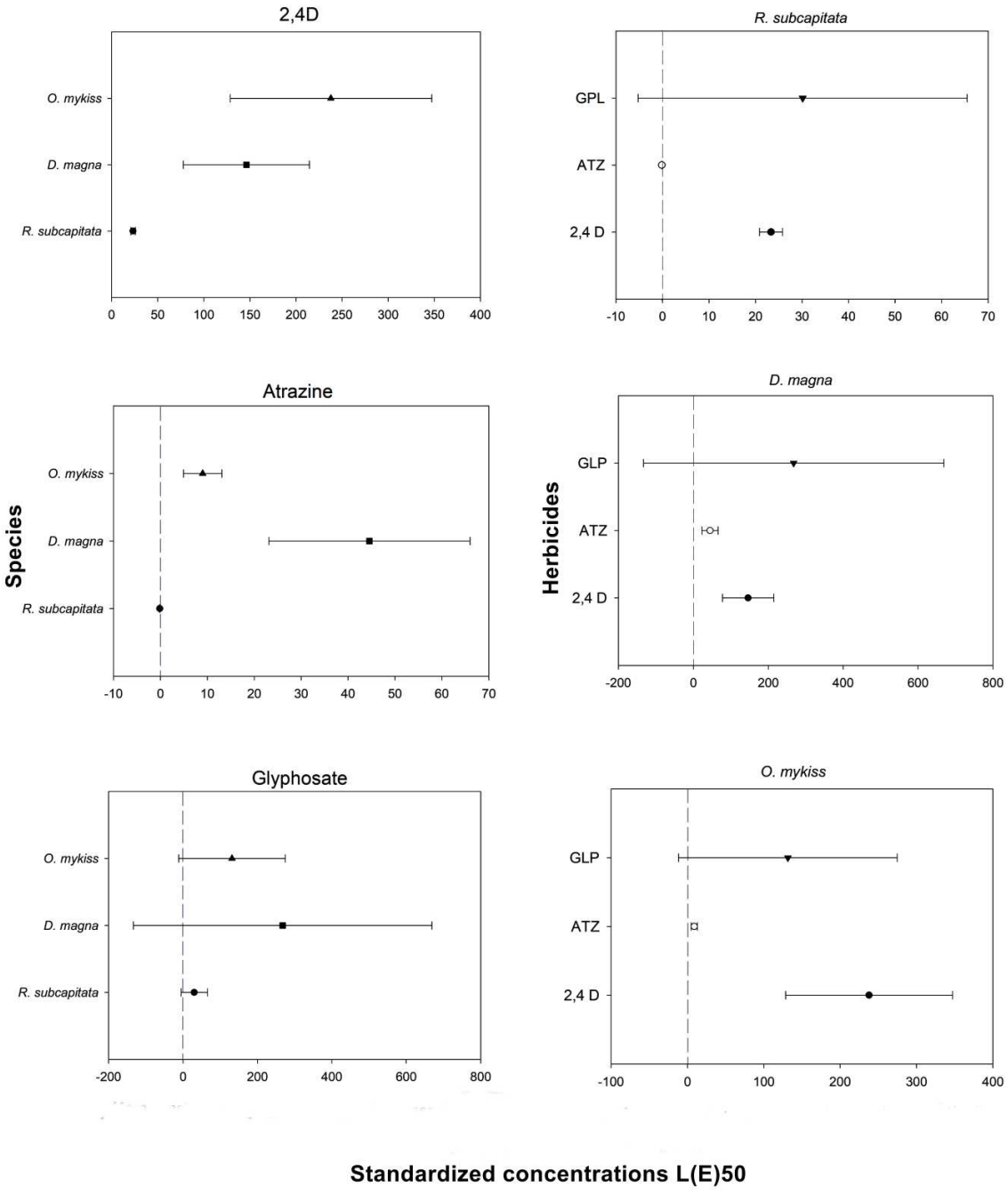

Figure 2.4. Average and confidence interval of the $L(E)_{50}$ z-score for standard test species and to 2,4D, atrazine (ATZ), and glyphosate (GLY). The numbers of sample $L(E)C_{50}$ values from the US EPA ECOTOX database (https://cfpub.epa.gov/ecotox/index.cfm; May 24th 2019) per herbicide were: 2,4D = 3, ATZ = 42, GLY = 6; 2,4D = 8, ATZ = 7, GLY =

9; 2,4D = 8, ATZ= 18, GLY = 58 for *R. subcapitata*, *D. magna*, *O. mykiss*, respectively. Overall effect size and confidence interval per herbicide and species: 37.65 (3.44, 71.86) for glyphosate, -0.13 (-0.16, -0.10) for atrazine, and 23.59 (21.14, 26.03) for 2,4D; -0.13 (-0.16; -0.10) for *R. subcapitata*, 54.20 (33.77, 74.63) for *D. magna*, and 9.42 (5.34, 13.49) for *O. mykiss*.

The risk assessment showed that 65% of the Brazilian states presented a medium or high risk considering 2,4D, but only concentrations from Paraná (PR) and Sergipe (SE) showed a high risk (Figure 2.5). Regarding atrazine concentrations, 72% of the states showed a medium or high risk, where Ceará (CE), Minas Gerais, and Rio Grande do Sul (RS) demonstrated a high risk (Figure 2.5). Glyphosate concentrations showed the highest risk, with 94% of the Brazilian states, which only Pará state (PA) did not represent a threat to the aquatic environment (Figure 2.5).

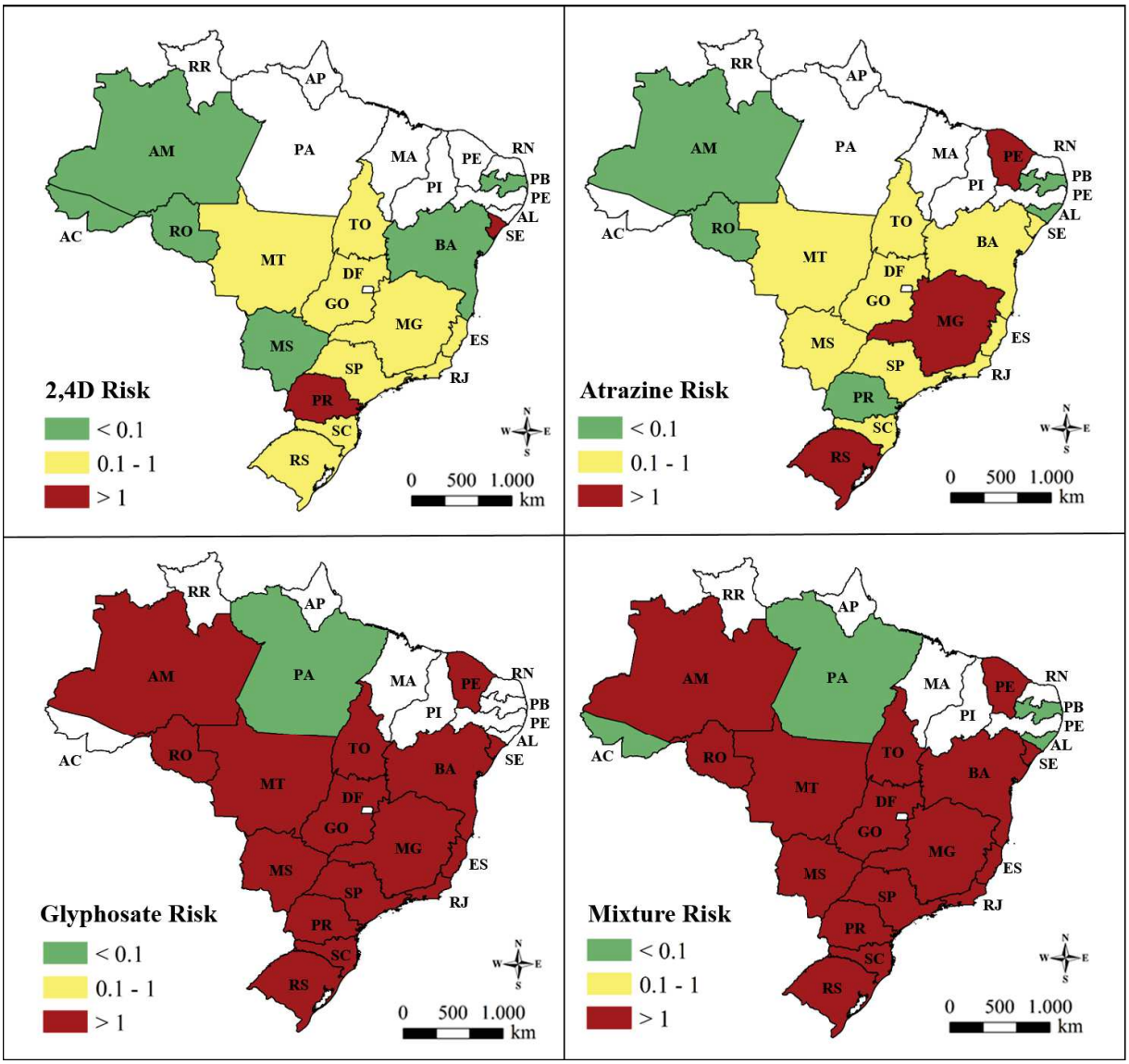

Figure 2.5. Environmental risk assessment of 2,4D, atrazine and glyphosate based on environmental concentrations found in Brazilian surface freshwaters. Hollow states had no data available. Brazilian states: Acre (AC), Alagoas (AL), Amapá (AP), Amazonas (AM), Bahia (BA), Ceará (CE), Espírito Santo (ES), Goiás (GO), Maranhão (MA), Mato Grosso (MT), Mato Grosso do Sul (MS), Minas Gerais (MG), Pará (PA), Paraíba (PB), Paraná (PR), Piauí (PI), Rio de Janeiro (RJ), Rio Grande do Norte (RN), Rio Grande do Sul (RS), Roraima (RR), Santa Catarina (SC), São Paulo (SP), Sergipe (SE), Tocantins (TO), Rondônia (RO) e Pernambuco (PE).

Higher risks were found in the South region for all pesticides (Figure 2.5), which also showed the highest accumulated sales, particularly in Paraná (PR) (Figure 2.3). However, Mato Grosso (MT), which also showed high accumulated sales, was not of main concern considering the risk analysis. The soybean productivity maps produced by Carvalho *et al.* (2016) and the risk assessment were similar, with a higher risk found in the Center-West and South regions coupled with higher productivity.

A previous study by Albuquerque et al. (2016) has already shown a high risk of 2,4D and atrazine in the Brazilian environment, but not with a nation-wide perspective, since the authors used only data from the literature, which is scarce in the country. The risk assessment considers environmental concentrations and toxicological effects of the pesticides on model species. The environmental concentrations of glyphosate were indeed higher than 2,4D and atrazine (Figure 2.3), and although the variation of the ecotoxicological effects was also higher, glyphosate showed relative high toxicity (Figure 2.4). Therefore, the glyphosate concentrations pose a higher threat to the aquatic environment compared to 2,4D and atrazine. Glyphosate may also contribute to eutrophication, since the phosphorus (P) presents in its the molecule may dissociated and be available to primary producers, being an additional threat to the environment (Table 2.1, Hébert et al., 2019).

The pesticides are commonly found in the environment as complex mixtures (Relyea, 2009). The mixture of 2,4D, atrazine and glyphosate would pose a high risk to 80% of the Brazilian states, and only the results from Acre (AC), Alagoas (AL), Pará (PA), and Paraíba (PB) showed a low risk. Important to mention that states with a low mixture risk did not have data for all the three-bestseller herbicides. The higher mixture risks found were mainly related to glyphosate concentrations, which were considerably higher than the other two pesticides. Relyea (2008) showed that a mixture of pesticides, including atrazine and 2,4D, would increase mortality in larval frogs by up to 99%. Another study found an additive effect of glyphosate and 2,4D on the phytoplankton community, but the glyphosate was the main responsible for the adverse effects found (Lozano et al., 2018). Considering the amount of chemicals present in the environment and other variables such as temperature, pH and nutrients, much more efforts to understand multiple stressors are needed.

Considering the PNEC based on our risk assessment, the Brazilian legislation for the three-bestseller pesticides would not protect the aquatic ecosystem. For 2,4D, the environmental concentrations below 1 μg L^{-1} would not pose a risk to the environment, while higher than 10 μg L^{-1} would pose a high risk. For atrazine, the environmental concentrations below 0.13 μg L^{-1} would not pose a risk to the aquatic ecosystem, while higher than 1.3 μg L^{-1} would pose a high risk. For glyphosate, the environmental legislation is even more disparate, since values below 0.1 μg L^{-1} would not pose a risk, but above 1 μg L^{-1} would already pose a high risk to the environment. The legislation that most closely matches the values that do not represent a risk to aquatic ecosystems, according to the data in this study, is the European one (Table 2.3). Thus, we argue that a review of the Brazilian freshwater legislation is necessary.

2.3.5 Perspectives

Given the continued use of pesticides in Brazil as well as the high concentrations and environmental risks found, alternatives must be developed to maintain a balance on the tripod of nature, society, and economy. In a country marked by agribusiness, stimulating agricultural production more environmental friendly is essential (Hobbs et al., 2008)

Regarding the export sector based on monocultures, a reassessment of the production methods based on intensive pesticides use must be conducted. The government and scientific community must work together establishing methodological standards for the monitoring of freshwater and improve the management of hydrographic basins, which would enable more precise and robust studies. The government should define taxes for large monocultures and distribute them to finance the monitoring of water resources, since they are the main responsible for pesticide contamination. Similarly, it is up to the government to invest in the development of less toxic compounds. The investment in foreign trade based on processed products instead of commodities only, would greatly contribute to increase the gross domestic product.

The family farming accounts for 23% of the total Brazilian agricultural area (IBGE, 2017) and more investment in this kind of agriculture is equally important (Assis, 2006). The incorporation of agroecological techniques, such as crop rotation, polyculture and the use of

biological control and natural methods as repellents, are among the viable ways that ensure the success of family farming (Hespanhol, 2008). By investing in family farming, the organic product costs would be reduced and, consequently, the consumption stimulated. Environmental education is essential in raising awareness among Brazilians about pesticide contamination in freshwater and food products, reinforcing conscious use and consumption.

This work offers a significant contribution to the understanding of the environmental issues related to glyphosate, atrazine, and 2,4D use in Brazil. However, it is important to emphasize the need for future work to assess impacts on other compartments besides surface freshwater, such as groundwater and drinking water, as well as trophic chain effects, bioaccumulation and biomagnification processes.

2.4.0 Conclusion

Although glyphosate is the most widely used herbicide in Brazil, the compound is not the main studied in Brazilian surface freshwater. Additionally, the sales of 2,4D, atrazine, and glyphosate increased between 2009 and 2018 in the country. In general, we did not find a correlation between the number of sales and herbicide concentrations by state, which is probably related to the small amount of data, lack of sampling standardization, local environmental traits (slope, temperature, soil type etc.), and the physical and chemical properties of each pesticide. The environmental concentrations showed a significant compliance percentage of the Brazilian legislation. However, the risk assessment showed that the maximum allowed values should be revised since they are not protecting the aquatic ecosystems.

Atrazine was the most toxic herbicide for the three species evaluated, representing three trophic levels in the aquatic ecosystem. However, glyphosate showed the highest environmental risk in Brazil, probably related to the high environmental concentrations. The mixture assessment showed a high risk in 80% of the states evaluated and should be of concern.

Acknowledgments

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3 CHAPTER 3: *Chironomus xanthus* (Diptera: Chironomidae) in ecotoxicology: laboratory cultures and tests

(Chapter in elaboration to be submit at the Aquatic Toxicology, A1, FI: 4.344)

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Abstract

Chironomus xanthus is a species restricted to tropical environments, easy to grow and to maintain in laboratory cultures. *C. xanthus* usually has many generations per year, which is an important criteria for selecting a test organism in ecotoxicology. This study aimed to: (1) review the methods for *C. xanthus* cultivate and its use in ecotoxicological tests, (2) establish a laboratory culture of *C. xanthus*, presenting the difficulties and discussing the ways to overcome them. The 4th instar larvae was the most used in acute studies, while the 1st instar larvae was the most used in chronic studies; 96 hours and 28 days were the most frequent durations in acute and chronic studies, respectively. The most common endpoints evaluated were organisms' survival and development, and most of the ecotoxicological studies using *C. xanthus* were performed in laboratory. Most of the tested contaminants were of the group of pesticides and these were also the ones that had the most adverse effects on organisms. Most mesocosms with environmental contaminated samples did not show adverse effects on *C. xanthus*. Chronic and field studies as well as those testing the effects of the mixture contaminants on *C. xanthus* were still deficient. Keeping the laboratory environment and equipment effectively sanitized was important for the success of the cultivate as well as maintain stabilized conditions of temperature, photoperiod, physical, chemical and biological water quality in cultures.

Keywords: Aquatic contaminants, ecotoxicological tests, macroinvertebrates cultivation.

3.1.0 Introduction

In the Chironomidae family, the genus *Chironomus* is the most diverse, encompassing about 2000 species in Europe, and 10000 only in Brazil (Callisto et al., 2017). Some species have hemoglobin in the hemolymph, allowing individuals to tolerate environments with low oxygen levels (Richardi et al., 2015), being used as bioindicators of environmental quality (Al-Shami et al., 2011; López et al., 2018). Their life cycles comprise four phases: ovigerous mass, larva (with five instars), pupa, and mosquito (Silva et al., 2019). The larva are aquatic sediment diggers and feed on detritus, which often expose them to chemicals via several uptake routes (Campagna et al., 2013).

Among the *Chironomus'* species, *Chironomus xanthus* Rempel, 1939 (considered synonymous of *Chironomus sancticaroli* Strixino & Strixino, 1981) stands out on ecotoxicological studies at tropical regions. It is restricted to Brazil and Argentina, has great ecological and regional relevance (Janke et al., 2011) and it is possible to culture and to maintain it in the laboratory, producing many new generations per year (Corbi et al., 2019), important criteria for selecting a test organism (Zagatto and Bertoletti, 2014). These characteristics make *C. xanthus* a good model organism to be used in ecotoxicological tests and to evaluate the quality of aquatic environments in tropical regions (Moreira-Santos et al., 2005; Beguelli et al., 2018). However, many gaps are to be filled before the establishment of robust protocols for the use of this species in ecotoxicology. For instance, choose the best conditions for test and cultivate (e.g. temperature, photoperiod, larval instar).

The start of ecotoxicological studies with *C. xanthus* occurred in 1985 (Strixino & Strixino, 1985) and has been increasing, reaching its peak in 2020 (Signorini-Souza et al., 2020; Morais et al., 2020; Macedo et al., 2020). This increase may be due to greater acceptance of this species as a suitable test organism in tropical environments (Dornfeld et al., 2019). Additionally, there is a recent interest in studying the occurrence of contaminants in aquatic environments, especially the emerging ones (i.e. contaminants, synthetic or natural, that have recently been introduced in the environment or with previously unrecognized negative effects on organisms; Wu et al., 2010).

Ecotoxicological tests with *C. xanthus* take into account the development of the organisms, laboratory and field conditions, routes of exposure and duration of the tests (Janke

et al. 2011; Morais et al., 2014; Rebecchi et al. 2014), but without standard protocols the toxicological data are usually based on different conditions. Standardizing the methodology is essential to reduce external variations and to compare the results among the studies (Raimondo et al., 2009). Therefore, this study aims to: (1) review the methods for *C. xanthus* cultivate and its use in ecotoxicological tests, (2) establish a laboratory culture of *C. xanthus*, presenting the difficulties and discussing the ways to overcome them.

3.2.0 Review of Ecotoxicological data

We found 110 articles being 34 from Web of Science, 40 from Scopus and 36 from Scielo. After applying the inclusion and exclusion criteria, we included 28 articles in the analysis (Figure 3.1; more details in supplementary material methods).

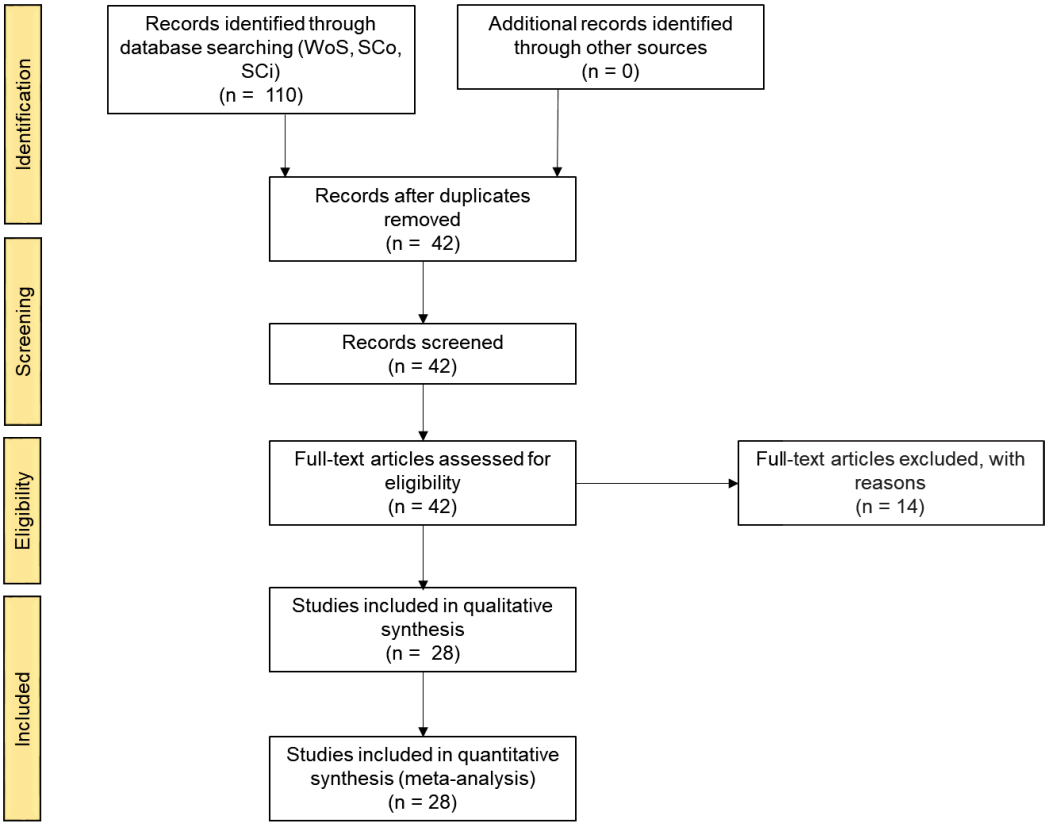

Figure 3.1. Systematic review diagram adapted from PRISMA (Moher et al., 2009).

3.2.1 Larval instars

The most used larval instars were 4th and 1st instar for acute and chronic tests, respectively (Table 3.1). The 4th instar is the most used in acute tests due to the organisms being bigger and, thus, easier to observe adverse effects on them during the test. Chronic tests need to assess the effects on part or all of the organism's life cycle, which is why the 1st instar is chosen. Most studies use sediment in the test vessels, since most of the organism's life cycle is benthic.

Table 3.1. Percentage of instar, duration and endpoints used for acute and chronic tests with *C. xanthus*. The reference numbers correspond to the ID provided in the supplementary material Table 3.1.

	Acute Test	Chronic Test	References
	Larval instar for initial test (%)		
Ovigerous mass	0.0	8.3	3
1st	11.1	50.0	8,9,12,15,16,24,28
2nd	14.8	25.0	2,7,14,23,26,27
3th	11.1	8.3	1,5,6,19
4th	63.0	8.3	1,3,4,5,6,8,9,10,11,12,13,14,17,18,20,21,22,25
	Test duration (%)		
24h	4.0	0.0	24
48h	32.0	0.0	4,11,12,13,15,16,24,25
96h	64.0	0.0	1,2,3,5,6,7,8,9,10,14,17,20,21,22,23,26
6 d	0.0	6.7	3
7 d	0.0	6.7	19
8 d	0.0	33.3	2,8,12,13,27
9 d	0.0	6.7	14
10 d	0.0	13.3	9,18
25 d	0.0	13.3	12,13
28 d	0.0	20.0	15,16,24
	Endpoint (%)		
Mortality	54.1	12.9	1,2,3,5,6,7,8,9,10,11,14,15,16,17,19,20,21,22,23,24,26,27,28
Enzymatic activity	21.6	16.1	3,6,8,9,11,12,13
Cellular changes	2.7	0.0	4,25
DNA damages	16.2	12.9	2,6,12,13,21,22,25
Histological biomarkers	2.7	0.0	8
Amount of lipid peroxidation	2.7	3.2	12
Growth	0.0	35.5	2,8,9,12,13,14,15,16,18,19,24,28
Emergence	0.0	12.9	9,12,13,15,16,18,24
Sex ratio	0.0	6.5	18,24

3.2.2 Test duration

Studies that analyzed the 4th instar generally performed the experiment within 96 h and they observed the survival of individuals (*e.g.* Printes et al., 2011, Novelli et al., 2011, Richardi et al., 2018; Table 3.1). This duration (96 h) is a limit to acute tests with this species, once a longer period will allow the larvae to develop for the next instar (Fonseca and Rocha, 2004), mischaracterizing the acute test. All acute test studies were carried out in very similar conditions (between 22 ºC and 29 ºC, photoperiod 12:12 hours) and most of them used artificial sediments as substrate in the test beakers.

Chronic tests with the 1st instar were performed in 8 (Richardi et al., 2018), 10 (Printes et al., 2011) and 28 days (Ferreira Junior et al., 2018; Table 3.1) at very similar conditions (between 22 ºC and 29 ºC, photoperiod 12:12 h). Ideally, chronic tests should analyze the effects over a large part of or the entire life cycle of the species and, for this reason, the most observed duration in the studies was 28 days. In addition, the tests duration also depends on the observed endpoint. For example, emergence and sex ratio need to be observed in the final cycle of the *Chironomus* (± 28 days).

3.2.3 Endpoints

Mortality and growth were the most used endpoints in acute and chronic tests, respectively (Table 3.1). These endpoints are also highly evaluated in other ecotoxicological studies (Sanchez et al., 2004, Pepin et al., 1991), and the methodological ease (in comparison to others endpoints methods) may explain their frequent use. However, other endpoints were already measured, such as enzymatic activity (Rebecchi et al., 2014; Richardi et al., 2018), DNA damages (Morais et al., 2014; Vicentini et al., 2017), emergence (Printes et al., 2011; Ferreira-Junior et al., 2017), sex ratio (Barbosa et al., 2019), cellular changes (Signorini-Souza et al., 2020), histological biomarkers (Richardi et al., 2018) and amount of lipid peroxidation (Morais et al., 2020). Emergence and sex ratio were parameters analyzed only in the tests with 28 days. In this period, low concentrations of a glyphosate-based herbicide caused delayed emergence of female, induced fast emergence of males and varied the sex ratio of *C. xanthus* (Ferreira-Júnior et al., 2017).

3.2.4 Laboratory and field studies

Most ecotoxicological studies were carried out in the laboratory (89%), while field experiments were used only few times (11%). Field studies can be more difficult, due to often complicated accessibility and difficulty to control the external variables, thus requiring more time and costing more (Ramasundaram et al., 2005). However, results from field experiments better represent the environmental conditions to which the organisms are subjected. The results of the tests made in laboratory and in the field with the same sediment/water could be different, as showed by Dornfeld et al. (2006), which reinforces that further field studies with this species are needed.

3.2.5 Contaminants and their effects on *C. xanthus*

Overall, studies have shown harmful effects of artificial chemicals on *C. xanthus* (Figure 3.2). Many showed deficiency in survival (Ferreira-Junior et al., 2017), growth (Morais et al., 2014), enzyme production (Rebecchi et al., 2014), emergence (Printes et al., 2011), sex ratio (Barbosa et al., 2019), and DNA damages (Vicentini et al., 2017). Then, this is another indication that *C. xanthus* it is a test organism suitable for carrying out ecotoxicological studies.

Among these studies, Ferreira-Junior et al. (2017) presented the largest LC50 (median lethal concentration; 251.5 mg L^{-1}). The authors evaluated the adverse effects of Roundup Original (glyphosate-based herbicide) on *C. xanthus.* Rebecchi et al. (2014) used the insecticide malathion and found the smallest LC50 (0.00251 mg L^{-1}). Both studies used pesticides as test substances, but toxicity varies inside the group and it is important to test different pesticide compounds on non-target species. The active chlorine decreases emergence in 22% of the population (Macedo et al., 2020) and the percentage of emerged *C. xanthus* was reduced by 33.3 and 45.8% by exposure to 0.0016 and 0.0032 mg L^{-1} of an insecticide (Ferreira-Junior et al., 2018).

All studies with heavy metals analyzed mortality and only one (Beguelli et al., 2018) analyzed morphological alterations, registering reduction in length and a higher occurrence of total damage on *C. xanthus.*

Two of the studies with flame retardants analyzed (96 h-test), used as endpoint the enzymatic activity, total protein concentration, DNA analysis, and cellular changes (Signorini-Souza et al., 2020; Palacio-Cortés et al., 2017). Only one analyzed, (28 days-test), development and emergence of larvae (Morais et al., 2019). They showed that flame retardants be caused a delayed larval development and decreased the number of emerging adults.

In general, studies that had no effect on *C. xanthus* carried out the experiment with environmental samples: sediment contaminated with heavy metals (Silvério et al., 2005), sludge contaminated with heavy metals (Sotero-Santo et al., 2007) and secondary effluent contaminated with disinfectant (da Costa et al., 2014). Only the study carried out with a pure nanomaterial - graphene oxide, did not show significant effects on *C. xanthus.*

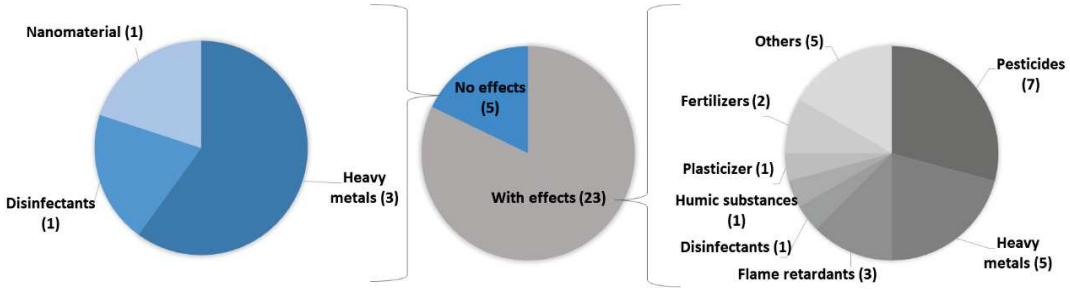

Figure 3.2. Number of studies that had adverse effects and no adverse effects on *C. xanthus.* Number in parenthesis represent the number of studies.

3.3.0 Review of laboratory cultures methods

We found 110 articles being 34 from Web of Science, 40 from Scopus and 36 from Scielo. After applying the inclusion and exclusion criteria, only 1 article was selected (Figure 3.3; Fonseca and Rocha, 2004; more details in supplementary material methods).

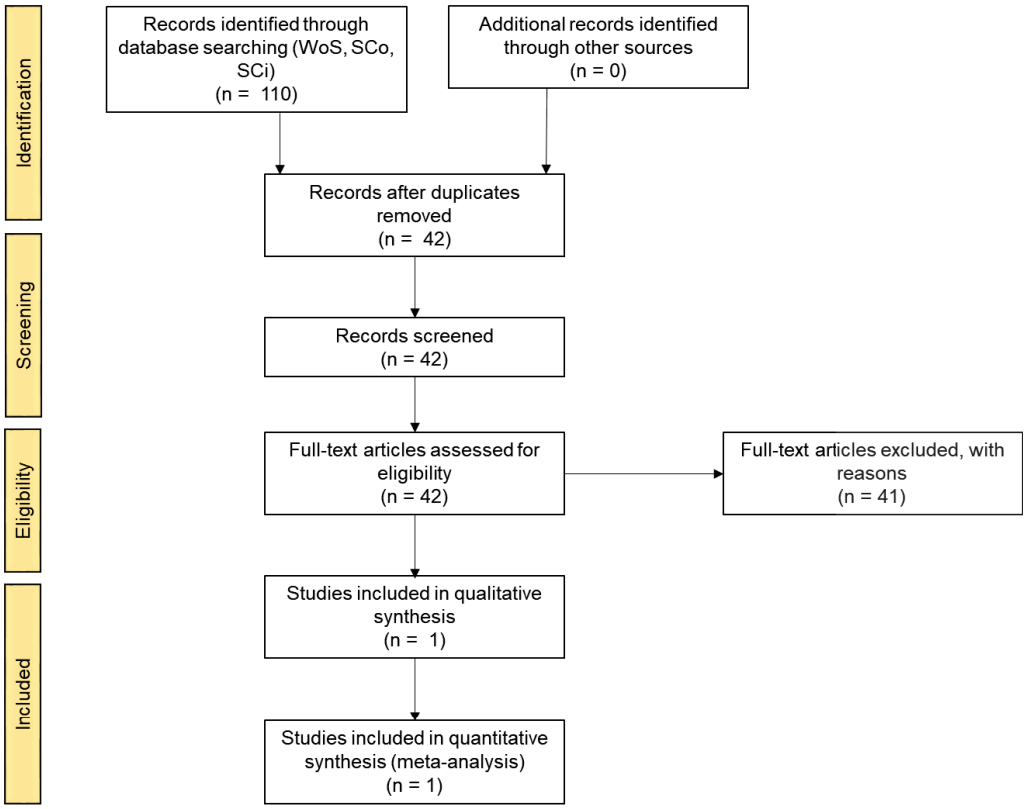

Figure 3.3. Systematic review diagram adapted from PRISMA (Moher et al., 2009).

Only Fonseca and Rocha (2004) describes the culture methods of *C. xanthus*, and 15% of the papers found have cited it. However, its study did not describe some difficulties and details (*e.g.* equipment clean, feeding, maintence) of *C. xanthus* establishment. This gap will be approach in the next topic. Some other studies cite another paper that describes a cultivation method for *C. decorus* as reference (Maier et al. 1990). The two methods are similar, using a tray or an aquarium filled with a sediment layer and water, covered with a screen with a mesh opening that prevents the mosquitoes exit while allowing them to copulate and to spawn. The major differences found are regarding the needs of each species such as photoperiod, temperature and water characteristics, among others.

3.4.0 Establishment of a new C. xanthus cultures: difficulties and pathways

Before starting a new culture of *C. xanthus*, it is necessary to prepare all the materials that are going to be used (Table 3.2) and prepare a place were the trays are going to be placed,

with controlled temperature and photoperiod. It is important that all the materials are exclusively used for the culture in order to avoid contamination.

Table 3.2. Materials needed to start one *C. xanthus* tray.

Item	Quantity
Sediment	1.5 kg
Culture water	4 liters
Tray	1 unity
Cage	1 unity
Fish food*	0.005g/ml stock solution (75 ml/week)
Air pump	1 unity
Algae solution*	10 ml
Tweezers	1 unity
Beakers	1 unity
Blender	1 unity

*In our cultures we have used Tetramin® fish food and Raphidocelis subcapitata algae cultures as food resource.

C. xanthus organisms were obtained from the Ecotoxicology Laboratory of the Center for Water Resources and Applied Ecology, at São Carlos School of Engineering, from the University of São Paulo (São Carlos, Brazil). Five ovigerous mass were brought to the Plankton Ecology Laboratory, at the University of Juiz de Fora (Juiz de Fora, Brazil) for the establishment of new cultures following the methods described by Fonseca and Rocha (2004). The ovigerous mass were kept in a 100 ml-becker and were fed with 10 ml of *Raphidocelis subcapitata* (Da costa et al., 2014; Barbosa et al., 2019). After 48 h, the hatched larvae (1st instar) were transferred for 7-liter plastic trays, containing 1/3 of sediment and 2/3 of water (about 4 liters). A cage was adapted in the top of the tray to avoid the entrance of unwanted organisms and to keep the new mosquitoes in contact for their mating and reproduction (Fonseca and Rocha, 2004). The cultivation water had a pH between 6.5 and 7.5, the room where the cultures were stablished had temperature ranging between 22 °C and 25 °C, with a photoperiod of 12h of light and 12h of darkness. The culturing trays were continuously aerated with air pumps (Figure 3.4). After 2 days of cultures establishment, the larvae were feed with 25 ml Tetramin® fish food solution (0.005 g/ml stock solution) three times a week. Water needs to be replenished frequently, until it reaches the initial volume of the tray

The first generation of new ovigerous masses was obtained 1 month after the establishment of the initial tray. After that, three masses from the main tray were removed and placed in a beaker filled with culture water and 10 ml of the *Raphidocelis subcapitata* algae solution. After hatching, the larvae of the 1st instar were placed in a tray as described above (300 organisms/tray). This procedure was repeated until the new culture had enough trays for a test (approximately 4 or 5 trays).

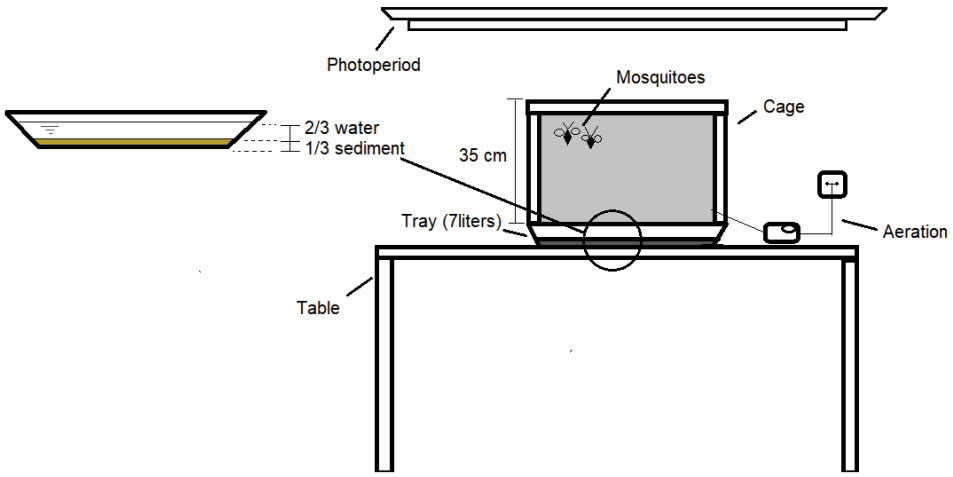

Figure 3.4. Schematics showing the necessary structure for *C. xanthus* establishment in laboratory cultures.

3.4.1 General care

When food solution or water are added to the tray, care must be taken to not revolve the sediment, stressing the larvae (Figure 3.5.A). When removing the cages to maintenance, it is important to tap the sides of the cage gently, so that mosquitoes fly upwards and do not leave the cage (Figure 3.5.B). Additionally, the pumps aeration must be soft, without revolving the sediment.

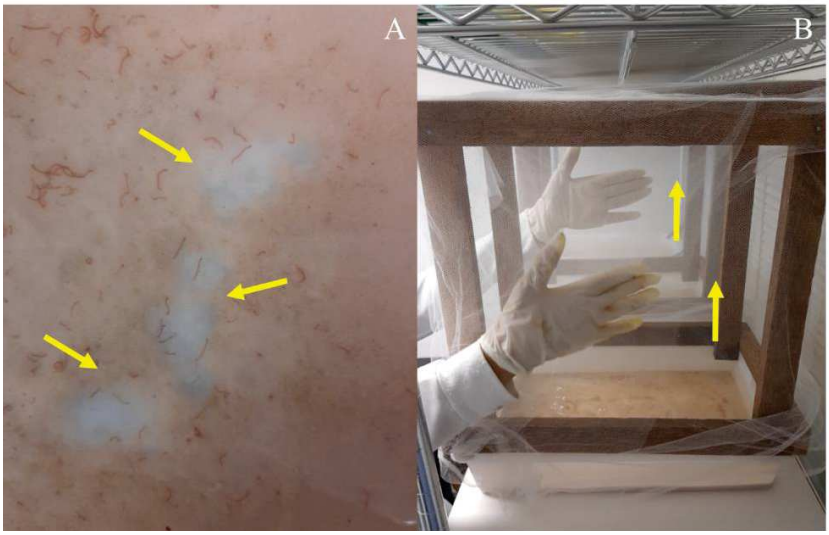

Figure 3.5. Maintenance details. **A.** Poor sand distribution caused by sediment overturnig during the feeding process. Arrows show spaces without sediment. **B.** Method to prevent mosquitoes from escaping the cage. Arrows show the direction in which the cages should be removed.

3.4.2 Feeding the organisms

As commented above, we have used Tetramin® solution to feeding the organisms. Most studies also used a solution of Tetramin® (Silva et al., 2019; Yamada et al., 2012; Sotero-Santo et al., 2017, Morais et al., 2020) or another fish food, such as Nutrafish® or Dog Chow® ration (Beguelli et al., 2018; Morais et al., 2014; Palacio-Cortes et al., 201), but information on methods for the preparation and stocking it are insufficient in the literature. We beat 2.5 g of Tetramin® solution and 500 ml of distilled water in a blender. After prepared, the stock solution must be stored in the refrigerator at 4°C to avoid the fermentation and it is important to remove the volume of solution that is going to be used 20 to 30 minutes before feeding the larvae or until it is at room temperature. Adding the cold solution may result in a delayed growth of the larval instars, due the drop of the culture's temperature (Strixino & Strixno, 1985).

3.4.3 Temperature of the culture room

Temperature is an important parameter for the development and survival of macroinvertebrate species in the laboratory (Strixino & Strixino, 1985). We have observed

this, when culture room was colder, consequently, life cycle was delayed, also delaying the start of tests. Therefore, any problem with controlling room temperature must be resolved quickly in order not to delay or accelerate *C. xanthus* development and survival. Our room temperature ranged between 22 °C and 25 °C and many studies maintain the culture in the same condition (see supplementary material Table S3.1), but it is important that each cultivation room has stabilized temperatures.

3.4.4 Hatching

In the beginning, the number of individuals hatching from the ovigerous mass in our laboratory culture ranged from 150 to 250 individuals. However, the study of Fonseca and Rocha (2004) reported that hatching around 500 to 600 individuals of the ovigerous mass. This difference was probably due to the manipulation during the collection of the mass on the trays and its transport to the beakers, or due to the amount of algae solution added, as many algae became entangled and prevented it from hatching completely. We used tweezers to collect the ovigerous mass very carefully, and the algae solution was added in the beaker only when the 1st instar larvae hatched. With these small precautions we verified an increase of survival larvae.

3.4.5 Cleaning the trays and the room

The culture and maintenance of the trays are extremely important and must be made with care to guarantee the larvae survival. The manipulation must be minimal, only to provide the essential, and all the materials used must be washed correctly (alkaline deterget, 1h-bath of 10-20% of HCl solution, and then a rinse with distilled water) to avoid contamination.

In one of our experiments we observed the existence of fungus in some of the trays, which in our case probably came from building wall where the experiment was performed. Therefore, in addition to the correct washing of materials and the tray, it is important that the culturing room be also cleaned constantly, including walls and, even the number of people who enter the place should be limited, requiring that everyone responsible for maintenance are equipped with the correct I.P.E (individual protection equipment) when entering the room (coat, gloves, and hair up). Taking these precautions, we make sure of the viability of the test organisms.

3.5.0 Future perspectives

To the best of our knowledge, this is the first study that shows solutions to the main difficulties that can be encountered during the cultivation, establishment and maintenance of *C. xanthus*. As well as test parameters and conditions most used in ecotoxicological studies with this species, and the contaminants effects assessed so far.

C. xanthus is suggested as a rising test organism for ecotoxicological studies, due to the recent increase in studies about this species, the lack of ecotoxicological data publications (only 28 studies) and the amount of contaminants present in the aquatic environment. For further ecotoxicological studies, we indicate the patters that we found in this study (cultivation conditions and maintenance, instar most used for test, duration test, endpoints). Additionally, we observed the need for more chronic and field studies, as well as effects of the mixture contaminants. This last effect is essential due to its proximity to environmental conditions.

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FINAL CONSIDERATIONS

We observed that most countries evaluated in this study did not have restrictive legislation for glyphosate in water resources, resulting in a potential non-protection of aquatic organisms. Because of this, and because 95% of the countries had glyphosate concentrations at moderate to high risk to aquatic organisms, we strongly recommend each country to review their legislation for glyphosate in freshwater systems.

Only a few studies assessed glyphosate concentrations in surface freshwater worldwide. Specifically, Brazil presented significant gaps in knowledge of the concentrations of glyphosate, 2,4 D, and atrazine in its freshwaters, also with only a few studies being found in the scientific literature. At the same time, there has been an increase in the sales of these three pesticides and most of the Brazilian states evaluated in this study showed a high environmental risk considering a mixture of the pesticides. Therefore, more assessments of pesticides concentrations in freshwaters and more studies evaluating their environmental impacts are strongly recommended.

Pesticides are part of an industrial system that still dominates the agricultural market worldwide. However, the scientific community has developed new ways to grow crops without the addition of external inputs such as pesticides and fertilizers. Agroecology may be an option to create more efficient and sustainable agricultural systems with minimal input of external chemicals to the environmental (Figure Final). Agroecology results in conserve and regenerate local agrobiodiversity, produces healthy food with internal inputs, restores local self-sufficiency, empowers farmers' organizations, offers more jobs per hectare and has more stability than industrial agriculture (Altieri and Toledo, 2011; Van der Plog et al., 2019).

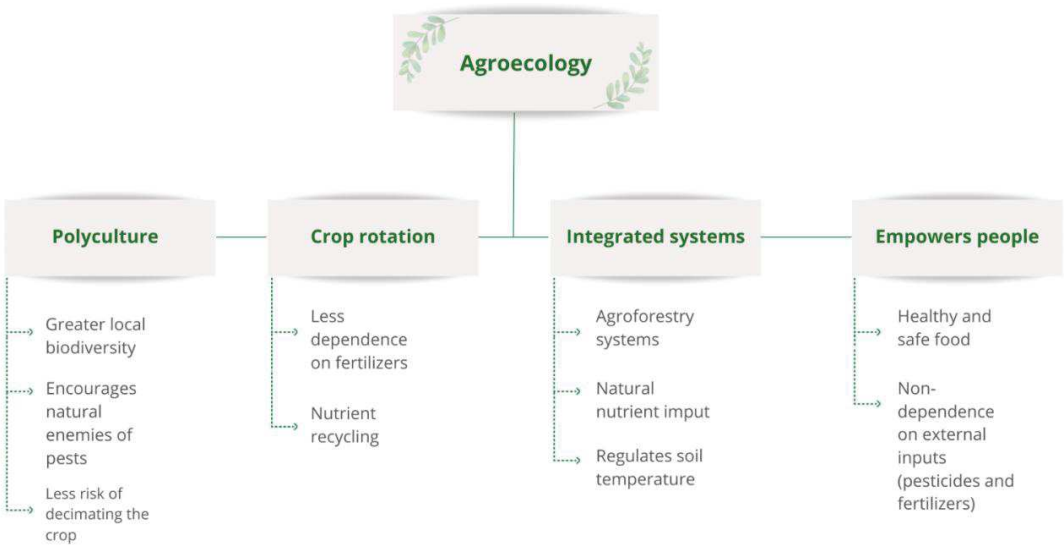

Figure Final. Agroecological practices in agricultural systems (Drexler, 2020; FAO, n.d.; Altieri & Nicholls, 2020).

Organic food are often referred as high-priced, but a study has shown that agro-ecological fairs have democratized this access with equal or even lower prices than conventional foods (Fantuzzi et al., 2017). Agroecological production established in opposition to the capitalist logic of production. Therefore, it is essential to break the myth that agroecological products have a higher price on the market, discouraging their acceptance by small producers and consumers. However, we, as consumers, we need to do our part by supporting as much as possible local farmers instead of corporate food chains and by avoiding food waste.

Clearly, the studies approaching the environmental effects of pesticides has been increasing in number and in scientific significance over the past years but such studies are still scarce and patchy, with less knowledge about tropical environments.

This study have drawn attention to a macroinvertebrate species that has a great potential as test organism in ecotoxicological studies in tropical systems - *Chironomus xanthus*. Ecotoxicological studies of *C. xanthus* has been increasing over the years, due to greater acceptance of this species as a suitable test organism. However, *C. xanthus* does not have detailed instructions published for its establishment conditions. To fill this gap, we provide instructions for establishing the *C. xanthus* cultivate and some ecotoxicological gaps

and directions to guide future studies with this species in tropical areas. We strongly recommended keeping the laboratory environment and equipment effectively sanitized as well as maintain stabilized conditions of temperature, photoperiod, physical, chemical and biological water quality in cultures.

Therefore, we suggest more ecotoxicological studies, mainly chronic and field studies, to verify potential adverse effects of contaminants on *C. xanthus* and to identify key concentrations (of tropical macroinvertebrates) that must be taken into account on environmental risk assessments in tropical countries.

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SUPPLEMENTARY MATERIAL

CHAPTER 1

Table S1.1

Author	Year	Method type	LOD	LOQ	Water resource	MiGC	AGC	MaGC
Okada et al.	2019	Liquid chromatography (LC) coupled with tandem mass spectrometry (MS/MS)	0.25	0.5	Creeks and rivers	< LOD	< LOD	< LOD
						2.0	0.6	3.0
Montiel-Léon et al.	2019	On-line SPE e UHPLC high-resolution mass spectrometry (HRMS), using a Q-Exactive Orbitrap mass spectrometer	0.002	N.A.	River and tributaries	0.0	0.0	3.0
Fernandes et al.	2019	Derivatization with FMOC-Cl. Ultra-high performance liquid chromatograph coupled to a tandem mass spectrometer.	3	10		< LOD	< LOD	< LOD
					River	3.0	56.5	110.0
						3.0	71.5	140.0
						10.0	20.0	30.0
						40.0	90.0	140.0
						140.0	155.0	170.0
						250.0	280.0	310.0
						90.0	200.0	310.0
Peruzzo et al.	2008	HPLC-UV detection, previous derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl)	40	100	Creeks	150	350	650
						70	220	410
						60	149.6	205
Carles et al.	2019	HPLC/FLD after a derivatization step	N.A.	0.1	Rivers	0.7	N.A.	0.4

Okada et al.	2018	Derivatization with FMOC-Cl. Waters® ACQUITY® UPLC and Waters® Micromass® Quattro Premier XE Mass Spectrometer (MS/MS) for detection	0.1	0.5	Creeks	0.1	0.4	8.2
Masiol et al.	2018	Derivatization with FMOC-Cl. High-performance liquid chromatography coupled with tandem mass spectrometry via an electrospray source (HPLC– ESI–MS/MS)	N.A.	0.05	Rivers and creeks	< LOQ	0.04	0.10
						< LOQ	N.A.	0.83
						< LOQ	0.40	1.40
						< LOQ	N.A.	1.30
						< LOQ	N.A.	0.51
						< LOQ	0.05	0.08
						< LOQ	N.A.	0.70
						< LOQ	N.A.	2.10
Gunarathna et al.	2018	Derivatization with FMOC-Cl. Liquid chromatography-mass spectrometry (LC-MS) with a mass selective detector.	0.01	0.1	Lake	28	36.3	45
Di Guardo & Finizio	2018	Derivatization with 9-fluorenylmethylchloroformate (FMOC-Cl), separation with high performance liquid chromatography (HPLC)	0.1	N.A.	Rivers and creeks	0.1	7.0	96
Castro Berman et al.	2018	Extracted by phosphate solution. The samples were analyse by HPLC-MS after derivatization with 9-fluorenylmethoxylcarbonyl chloride (FMOC-CL)	40	100	Lake	N.A.	N.A.	N.A.
						1.5	1.5	1.5
						N.A.	N.A.	N.A.
						< LOQ	< LOQ	< LOQ
						1.6	1.6	1.6
						2.2	2.2	2.2
						4.5	4.5	4.5
						1.6	1.6	1.6
						1.3	1.3	1.3

Bonansea et al.	2018	High-performance liquid chromatography coupled to mass spectrometry system (HPLC-ESI-MS), derivatized by FMOC–Cl solution	0.5	1	River	0.5	17.5	70
						0.5	35.2	125
						0.5	0.5	0.5
						0.5	0.5	0.5
						0.5	0.5	0.5
Bokony et al.	2018	Reversed-phase high-performance liquid chromatography coupled to tandem mass spectrometry - a derivatization with 9-fluorenylmethyl chloroformate	N.A.	N.A.	Lakes	2.4	8.4	15.0
						7.1	10.3	14.8
						6.5	8.8	10.9
Samargandhi et al.	2017	Gas-chromatography coupled with mass spectrophotometry	N.A.	N.A.	Source	< LOD	< LOD	0.011
Poiger et al.	2017	Derivatization with fluorenylmethyl chloroformate FMOC-Cl, combined with on-line solid phase extraction and LC-MS/MS detection.	N.A.	0.005	Creeks	< LOQ	0.1	40
Perez et al.	2017	Extract in the Ultra-high-performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS) system;Derivatized with FMOC-Cl	0.1	0.5	Creeks	< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						0.1	0.1	0.1
						0.1	0.1	0.1
						1.7	1.7	1.7
						0.1	0.1	0.1
						0.7	0.7	0.7
						0.5	0.5	0.5
Pérez et al.	2017	Extracted with borate buffer solution. Derivatized with FMOC-Cl. Analysed by UHPLC–MS/ MS	0.1	0.5	River	0.1	0.6	2.1
						0.0	0.5	1.1

Mottes et al.	2017	EPA-methods 507, 508, 610 and 625 (N.A.)	N.A.	0.01 to 0.2	River	0.05	N.A.	0.3
Mahler et al.	2017	Liquid chromatography tandem mass spectrometry LC-MS/MS.	0.2	N.A.	River	0.2	N.A.	20
						0.2	N.A.	50
						0.2	N.A.	4
						0.2	N.A.	10
Louch et al.	2017	Extraction and derivatized with FMOC-Cl. Samples analyzed by LC/MS-MS.	0.018	N.A.	River	0.0	N.A.	0.1
						0.0	N.A.	0.0
						0.1	N.A.	0.1
Bradley et al.	2017	N.A.	0.001	N.A.	River	0.0	2	10
Ronco et al.	2016	Extraction with K2HPO4. Derivatized with FMOC-Cl. Analyzed by HPLC-MS	0.3	N.A.	River	0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.4	0.5
						0.3	0.3	0.3
						0.3	0.4	0.4
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.4	0.5
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.4	0.5
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.5	0.6
						N.A.	0.3	0.3
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.3	0.3	0.3
						0.7	1.0	1.2

Lasier et al.	2016	Extracts by high-performance liquid chromatography using precolumn derivatization with FMOC-Cl	25	N.A.	River	< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
Desmet et al.	2016	N.A.	N.A.	N.A.	River	< 0.02	0.1	0.7
						< 0.02	0.1	0.3
						0.26	2.2	12.0
						< 0.05	0.4	3.2
						< 0.05	0.7	3.8
						0.05	1.3	4.6
						< 0.05	0.5	2.2
						< 0.05	0.1	0.8
						< 0.05	0.1	0.2
						< 0.05	0.3	4.8
						< 0.03	0.2	0.7
Battaglin et al.	2016	Sample extracts were analyzed on LC/MS; Derivatized with FMOC-Cl	0.5 - 1	N.A.	River	0.0	N.A.	2
Struger et. al.	2015	Ion chromatography electrospray ionization	0.01-5	N.A.	River	N.A.	0.0	0.2
						N.A.	N.A.	0.1

		triple quadruple mass spectrometry (IC/MS/MS)				N.A.	N.A.	0.0
						N.A.	0.0	3.4
						N.A.	0.0	0.7
						N.A.	0.0	0.7
						N.A.	0.1	2.4
						N.A.	0.1	10.3
						N.A.	0.1	1.4
						N.A.	0.0	0.7
						N.A.	0.0	0.3
						N.A.	0.0	0.1
						N.A.	0.2	41.9
						N.A.	N.A.	0.1
Douros et al.	2015	Competitive para-magnetic enzyme linked immunosorbent assay (ELISA) kit: Glyphosate Assay Kit (Product No. 500081);	N.A.	N.A.	River	0.0	N.A.	1.5
Avigliano & Schenone	2015	HPLC; Derivatized with FMOC-Cl, borate buffer and OPA-MPA solution (ortho-phthalaldehyde and 3mercaptopropionic acid).	N.A.	N.A.	River	N.A.	N.A.	1600
						N.A.	N.A.	200
						N.A.	N.A.	200
						N.A.	N.A.	200
						N.A.	N.A.	200
Armas et al.	2007	Glyphosate extraction in complexing resin. Analized by HPLC	0.01	1	River	< LOQ	< LOQ	< LOQ
Ramirez et al.	2014	HPLC; Derivatized with FMOC-Cl.	0.058	N.A.	Wetlands	0.2	2.7	59.9
Battaglin et al.	2014	Online SPE and liquid chromatography/tandem mass spectrometry (LC/MS/MS)	0.1	N.A.	Rivers, lakes, ditches and wetlands	N.A.	0.0	73.0
						N.A.	0.0	301.0
Nakashima et al.	2013	Conductivity detection-ion chromatography; extraction bay zirconia and titania	N.A.	1	River	N.A.	N.A.	< LOQ
Mortl et al.	2013	ELISA method (PN 500086)	0.05-0.12	N.A.	River and lake (together)	0.0	N.A.	1.0

Daouk et al.	2013	ULC-MS after their derivatization with 9-fluorenylmethyl chloroformate followed by solid-phase extraction.	N.A.	0.01	River and creeks	0.1	N.A.	5.0
Hanke et al.	2008	Derivatization with FMOC-Cl, SPE and LC-ESI-MS/MS.	0.0002	0.00007	River	0.0	N.A.	0.1
					Lake	0.0	N.A.	0.0
					Tributaries	0.0	N.A.	0.4
					Tributaries	0.1	0.1	0.1
Maillard et al.	2012	Derivatized with FMOC, solid-phase extracted and analyzed on an LC-MS-MS.	N.A.	0.1	Wetland	N.A.	8.7	110
Glozier et al.	2012	LC - IMS; Derivatized with FMOC-Cl; Extracted using hydrophilic–lipophilic balance	0.0004-0.001	N.A.	Rivers and creeks	0.0	0.0	0.3
						0.0	0.0	0.5
						0.0	0.0	0.3
						0.0	0.1	0.4
						0.0	0.0	0.0
						0.0	0.0	0.2
Coupe et al.	2012	Solid-phase extraction and analysis by HPLC/MS	0.02-0.1	N.A.		0.1	1.0	73.0
					Rivers	0.0	0.4	290.0
						0.2	69.5	430.0
						0.1	86.0	4.7
Botta et al.	2012	Two solid phase extractions and derivatized with FMOC-Cl. Detection by HPLC;	N.A.	N.A.	Rivers	N.A.	0.8	6.5
Silva et al.	2011	Spectrophotometer (USB 4000 UV-Vis) coupled via optical fibers to a liquid waveguide capillary cell (LWCC)	170	530	Springs and rivers	1600.0	2055.0	2460.0
Litz et al.	2011	Extraction and anlyzed by HPLC system with a fluorescence detector and two Knauer 64 as reagent pumps;	0.02	0.07	River	0.1	N.A.	5
Schriks et al.	2010	N.A.	N.A.	N.A.	River	N.A.	N.A.	1.2

Hanke et al.	2010	Derivatization with FMOC-Cl, followed by SPE of the derivatized and filtered sample and detection by LC-MS/MS	N.A.	0.02	River	0.0	N.A.	4.2
						0.0	N.A.	2.3
						0.0	N.A.	0.8
						0.0	N.A.	3.3
Boucherie et al.	2010	N.A.	N.A.	N.A.	River	N.A.	N.A.	0.7
Abrantes et al.	2010	HPLC (US EPA, 1999)	0.03	N.A.	Lake	1.0	2.5	5.2
Botta et al.	2009	Separated by HPLC and detected by fluorescence	N.A.	0.1	River	0.1	0.4	1.0
						0.1	0.1	0.2
						0.2	0.6	0.9
						1.3	1.5	1.6
Battaglin et al.	2009	Precolumn derivatization with FMOC-Cl, and deteccion by online solid-phase extraction followed by direct injection into a LC/MS.	0.02	N.A.	Vernal pools	0.0	52.6	328.0
						0.0	0.0	0.1
						0.0	1.1	12.0
						0.0	0.0	0.0
Struger et al.	2008	Ion chromatography, electrospray ionization, tandem mass spectrometry (IC/MS/MS)	0.01-0.02	N.A.	Creeks	N.A.	N.A.	40.8
Byer et al.	2008	ELISA test kits (Axys Glyphosate Method)	0.1	0.15	River	< LOD	2.1	12.0
						< LOD	0.2	0.9
						< LOD	0.2	0.7
Wan et al.	2006	Extract and determined for gas chromatographic analysis.	2	N.A.	River	2	4	9
Kolpin et al.	2006	Precolumn derivatization with FMOC-Cl by an automated online solid-phase extraction	0.1	N.A.	Streams	0.1	0.1	2

		and direct injection into a LC- MS						
Battaglin et al.	2005	Online Solid Phase Extraction and HPLC/MS	0.1	N.A.	Streams	0.1	0.2	9
Ramwell et al.	2004	Derivatization with FMOC-Cl and analysed by reverse-phase HPLC using a fluorescence detector.	0.1	N.A.	Streams	0.1	N.A.	0.1
Bauer et al.	1999	Coupling of IC with ES-MS without any derivatization	1	N.A.	River	< LOD	< LOD	< LOD
Skark et al.	1998	HPLC with post-column-derivatization and fluorescence detection	25	50	Surface water	0.0	N.A.	0.6
Santiago et al.	2018	Derivatised with FMOCCl and injected into HPLC/ESI-MS	0.5	1	Surface water	70	97.5	125
Adams et al.	2007	GC-MS method	N.A.	25	Stream water	< LOQ	< LOQ	< LOQ
Alza-Camacho et al.	2016	UV-Vis spectroscopy, according to AOAC method	40	50	Surface water	201	1088.6	2777
Freire et al.	2012	ion chromatography with suppressed conductivity detection	N.A.	N.A.	Stream water	0.0	44.4	2024.0
Pappas et al.	2008	HPLC with post-column derivitization and fluorescence detection	2	N.A.	Watershed	< LOD	N.A.	240.4
Sasal et al.	2017	ELISA	0.075	4	Surface water	0.1	N.A.	105000
Lutri et al.	2019	UHPLC MS/MS in EEA INTA Balcarce Laboratory (Argentina) with the methodology put forward by Aparicio et al. (2013).	0.1	N.A.	Surface water	< LOD	< LOD	< LOD
						0.2	0.2	0.2
						< LOD	< LOD	< LOD
						167.4	167.4	167.4
						0.5	0.5	0.5
						0.7	0.7	0.7

Horn et al.	2019	Enzyme-linked immunosorbent assays (ELISAs)	0.2	0.4	Surface water	< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	< LOD	< LOD
						< LOD	0.3	0.5
						< LOD	< LOD	< LOD
Medalie et al.	2019	USGS Organic Geochemistry Research Laboratory in Kansas using a LC/MS/MS method.	0.25	N.A.	Surface water	N.A.	0.1	0.3
						N.A.	0.0	0.1
						N.A.	0.2	0.6
						N.A.	0.1	0.3
						N.A.	0.2	0.6
Popp et al.	2008	Two-step solid-phase enrichment procedure. HPIC–ICP–DRC-MS.	42	N.A.	Receiving water downstream WWTPs	N.A.	N.A.	1.4
Huntscha et al.	2018	Derivatization with FMOC-Cl, online-enrichment, reversed-phase liquid chromatography, and tandem mass spectrometry.	N.A.	0.005	Lake	N.A.	0.2	1.4
Slomber et al.	2017	UPLC-MS/MS. Neutral and ionic compounds were extracted from the samples with solid-phase extraction	N.A.	0.05	River	0.05	N.A.	0.8
Pinto et al.	2018	UPLCTM-triple quadrupole mass spectrometer. Derivatization with FMOC-Cl and DLLME	0.35	1	Surface water	2.6	6.2	10.1
Székács et al.	2015	ELISA method	N.A.	N.A.	Surface water	0.12	N.A.	1.0
Davis et al.	2013	Extracted with dichloromethane. GCMS and LCMS.	0.01	N.A.	River and creek	N.A.	N.A.	54.0

Aparicio et al.	2013	Extract with potassium dihydrogen phosphate. Derivatized with 9-fluorenylmethylchloroformate (FMOC-CL). UPLC MS/MS.	0.1	0.5	Surface water	< LOD	N.A.	298.0
Ibánez et al.	2006	Acidified with HCl. Derivatized with FMOC-Cl. SPE–LC–ESI–MS/MS.	0.005	0.05	Surface water	0.03	0.1	0.2
Malone and Foster	2018	N.A.	N.A.	N.A.	Watershed	0.02	N.A.	0.1
You et al.	2003	ESI-CNLSD and coupled with capillary electrophoresis (CE)	200	N.A.	Lake	< LOD	< LOD	< LOD

All concentrations are in μgL^{-1}; MiGC – Minimum glyphosate concentration; AGC – Average glyphosate concentration; MaGC – Maximum glyphosate concentration; NA – Not available;

Table S1.2

Species	Type	LC50
Daphnia magna	Active ingredient	2.95
Daphnia magna	Active ingredient	5.3
Daphnia magna	Active ingredient	11.8
Daphnia magna	Active ingredient	12.4
Daphnia magna	Formulation	20
Daphnia magna	Formulation	21.88
Daphnia magna	Not coded	22
Daphnia magna	Active ingredient	61.72
Daphnia magna	Active ingredient	89
Daphnia magna	Active ingredient	95.96
Daphnia magna	Active ingredient	134
Daphnia magna	Formulation	146
Daphnia magna	Not coded	164.3
Daphnia magna	Active ingredient	199
Daphnia magna	Formulation	234
Daphnia magna	Active ingredient	2000
Oncorhynchus mykiss	Active ingredient	1.3
Oncorhynchus mykiss	Active ingredient	1.4
Oncorhynchus mykiss	Active ingredient	1.4

Oncorhynchus mykiss	Active ingredient	1.6
Oncorhynchus mykiss	Active ingredient	2.4
Oncorhynchus mykiss	Active ingredient	2.4
Oncorhynchus mykiss	Active ingredient	2.4
Oncorhynchus mykiss	Active ingredient	3.4
Oncorhynchus mykiss	Active ingredient	5.2
Oncorhynchus mykiss	Active ingredient	7.4
Oncorhynchus mykiss	Active ingredient	7.5
Oncorhynchus mykiss	Active ingredient	7.5
Oncorhynchus mykiss	Active ingredient	7.6
Oncorhynchus mykiss	Active ingredient	7.6
Oncorhynchus mykiss	Active ingredient	7.6
Oncorhynchus mykiss	Active ingredient	7.6
Oncorhynchus mykiss	Active ingredient	7.6
Oncorhynchus mykiss	Active ingredient	8.3
Oncorhynchus mykiss	Active ingredient	8.3
Oncorhynchus mykiss	Active ingredient	9
Oncorhynchus mykiss	Active ingredient	10
Oncorhynchus mykiss	Active ingredient	11
Oncorhynchus mykiss	Active ingredient	11
Oncorhynchus mykiss	Active ingredient	11
Oncorhynchus mykiss	Active ingredient	12
Oncorhynchus mykiss	Active ingredient	14
Oncorhynchus mykiss	Active ingredient	14
Oncorhynchus mykiss	Active ingredient	14
Oncorhynchus mykiss	Active ingredient	14
Oncorhynchus mykiss	Active ingredient	14
Oncorhynchus mykiss	Active ingredient	14
Oncorhynchus mykiss	Active ingredient	14
Oncorhynchus mykiss	Active ingredient	19
Oncorhynchus mykiss	Active ingredient	21
Oncorhynchus mykiss	Active ingredient	22
Oncorhynchus mykiss	Active ingredient	22
Oncorhynchus mykiss	Active ingredient	26
Oncorhynchus mykiss	Active ingredient	32
Oncorhynchus mykiss	Not coded	77.6
Oncorhynchus mykiss	Active ingredient	93
Oncorhynchus mykiss	Active ingredient	99
Oncorhynchus mykiss	Active ingredient	103
Oncorhynchus mykiss	Active ingredient	107

Oncorhynchus mykiss	Active ingredient	107
Oncorhynchus mykiss	Active ingredient	108
Oncorhynchus mykiss	Active ingredient	108
Oncorhynchus mykiss	Active ingredient	115
Oncorhynchus mykiss	Active ingredient	130
Oncorhynchus mykiss	Active ingredient	130
Oncorhynchus mykiss	Active ingredient	134
Oncorhynchus mykiss	Active ingredient	140
Oncorhynchus mykiss	Active ingredient	197
Oncorhynchus mykiss	Active ingredient	220
Oncorhynchus mykiss	Active ingredient	220
Oncorhynchus mykiss	Active ingredient	220
Oncorhynchus mykiss	Active ingredient	240
Oncorhynchus mykiss	Active ingredient	240
Oncorhynchus mykiss	Active ingredient	240
Oncorhynchus mykiss	Active ingredient	240
Oncorhynchus mykiss	Active ingredient	730
Oncorhynchus mykiss	Not coded	824
Oncorhynchus mykiss	Active ingredient	4290.8
Pseudokirchneriella subcapitata	Active ingredient	5.5554
Pseudokirchneriella subcapitata	Active ingredient	7.8
Pseudokirchneriella subcapitata	Active ingredient	12.54
Pseudokirchneriella subcapitata	Active ingredient	13.5
Pseudokirchneriella subcapitata	Active ingredient	14
Pseudokirchneriella subcapitata	Active ingredient	129

Species	**Geometric mean**
Oncorhynchus mykiss	27.31316986
Pseudokirchneriella subcapitata	15.38248492

Trel	**Number**	**Rank**	**Log10(Trel)**
0.518871107	1	0.25	-0.284940512
0.292222654	2	0.75	-0.53428612
		Average	-0.409613316
		SD	0.124672804

SSD		**Cd**	***D.magna***
Conc	**10Log(conc)**	**normdist**	
0.00001	-5	4.5057E-297	1
0.000011	-4.958607315	8.7653E-292	1
0.0000121	-4.91721463	1.5273E-286	1

0.00001331	-4.875821945	2.3838E-281	1
0.000014641	-4.834429259	3.3324E-276	1
1.61051E-05	-4.793036574	4.1727E-271	1
1.77156E-05	-4.751643889	4.68E-266	1
1.94872E-05	-4.710251204	4.7015E-261	1
2.14359E-05	-4.668858519	4.2306E-256	1
2.35795E-05	-4.627465834	3.4098E-251	1
2.59374E-05	-4.586073148	2.4616E-246	1
2.85312E-05	-4.544680463	1.5918E-241	1
3.13843E-05	-4.503287778	9.22E-237	1
3.45227E-05	-4.461895093	4.7834E-232	1
3.7975E-05	-4.420502408	2.2229E-227	1
4.17725E-05	-4.379109723	9.2528E-223	1
4.59497E-05	-4.337717037	3.4499E-218	1
5.05447E-05	-4.296324352	1.1521E-213	1
5.55992E-05	-4.254931667	3.4466E-209	1
6.11591E-05	-4.213538982	9.2352E-205	1
6.7275E-05	-4.172146297	2.2166E-200	1
7.40025E-05	-4.130753612	4.7654E-196	1
8.14027E-05	-4.089360927	9.1768E-192	1
8.9543E-05	-4.047968241	1.583E-187	1
9.84973E-05	-4.006575556	2.4459E-183	1
0.000108347	-3.965182871	3.3851E-179	1
0.000119182	-3.923790186	4.1967E-175	1
0.0001311	-3.882397501	4.6604E-171	1
0.00014421	-3.841004816	4.6359E-167	1
0.000158631	-3.79961213	4.1307E-163	1
0.000174494	-3.758219445	3.297E-159	1
0.000191943	-3.71682676	2.3572E-155	1
0.000211138	-3.675434075	1.5096E-151	1
0.000232252	-3.63404139	8.6606E-148	1
0.000255477	-3.592648705	4.4506E-144	1
0.000281024	-3.551256019	2.0487E-140	1
0.000309127	-3.509863334	8.4481E-137	1
0.000340039	-3.468470649	3.1206E-133	1
0.000374043	-3.427077964	1.0326E-129	1
0.000411448	-3.385685279	3.0606E-126	1
0.000452593	-3.344292594	8.1267E-123	1
0.000497852	-3.302899909	1.933E-119	1
0.000547637	-3.261507223	4.1187E-116	1

0.000602401	-3.220114538	7.8614E-113	1
0.000662641	-3.178721853	1.3442E-109	1
0.000728905	-3.137329168	2.059E-106	1
0.000801795	-3.095936483	2.8254E-103	1
0.000881975	-3.054543798	3.4731E-100	1
0.000970172	-3.013151112	3.82473E-97	1
0.00106719	-2.971758427	3.77324E-94	1
0.001173909	-2.930365742	3.33478E-91	1
0.001291299	-2.888973057	2.64036E-88	1
0.001420429	-2.847580372	1.87286E-85	1
0.001562472	-2.806187687	1.19014E-82	1
0.001718719	-2.764795001	6.77556E-80	1
0.001890591	-2.723402316	3.45582E-77	1
0.002079651	-2.682009631	1.57915E-74	1
0.002287616	-2.640616946	6.4649E-72	1
0.002516377	-2.599224261	2.37124E-69	1
0.002768015	-2.557831576	7.79235E-67	1
0.003044816	-2.516438891	2.29428E-64	1
0.003349298	-2.475046205	6.05225E-62	1
0.003684228	-2.43365352	1.43049E-59	1
0.004052651	-2.392260835	3.02941E-57	1
0.004457916	-2.35086815	5.74835E-55	1
0.004903707	-2.309475465	9.77346E-53	1
0.005394078	-2.26808278	1.48896E-50	1
0.005933486	-2.226690094	2.0326E-48	1
0.006526834	-2.185297409	2.4864E-46	1
0.007179518	-2.143904724	2.72549E-44	1
0.00789747	-2.102512039	2.67723E-42	1
0.008687217	-2.061119354	2.35671E-40	1
0.009555938	-2.019726669	1.85916E-38	1
0.010511532	-1.978333983	1.31442E-36	1
0.011562685	-1.936941298	8.32854E-35	1
0.012718954	-1.895548613	4.72975E-33	1
0.013990849	-1.854155928	2.40746E-31	1
0.015389934	-1.812763243	1.09837E-29	1
0.016928927	-1.771370558	4.49185E-28	1
0.01862182	-1.729977873	1.64669E-26	1
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4947698548	9.694403231	1	1
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CHAPTER 2

Supplementary material at chapter 2 can be found online at https://www.sciencedirect.com/science/article/abs/pii/S0048969720382875

CHAPTER 3

Methods

A systematic review was carried out according to the PRISMA methodology (Moher et al., 2009). We searched for studies that included laboratory cultures using *C. xanthus* on Scopus, Web of Science and Scielo platforms. The keywords chosen were ("*Chironomus xanthus*" OR "*Chironomus sancticaroli*"). Although being a broad combination, we were sure that we encompass as many ecotoxicological studies as possible. We added studies published until August 2020. Citations of the articles have been revised, and any work that contemplated ecotoxicological tests using *C. xanthus* has been included. The studies screening followed the analysis by: (i) title and abstract and (ii) full text.

Ecotoxicological tests

Only studies that analyzed an ecotoxicological test using *C. xanthus* were included. We excluded articles that made a physiological evaluation on *C. xanthus*, without testing a chemical compound. We also did not included articles that evaluated the best physiological and morphological conditions of *C. xanthus* to be used in the laboratory.

Laboratory cultures

We analyzed all articles which contained laboratory and field experiments using *C. xanthus*. We included only articles that described, with details, *C. xanthus* cultures establishment.

Table 3.1

Author	Country	Year	Type of study	Chemical substances	Test conditions	CL50/CE50
Janke et al.	Brazil	2011	Laboratory - Microcosmos	Calcium nitrate	22 ° C and 12 : 12 hours	N.M.
Morais et al.	Brazil	2014	Laboratory	Phenanthrene	25 ± 2 ° C and 12 : 12 hours	1.6 mg L-1
Rebecchi et al.	Brazil	2014	Laboratory	Pesticide malathion	26 ± 2 ° C and 12 : 12 hours	0.00251 mg L-1
Signorini-Souza et al.	Brazil	2020	Laboratory	BDE-17, BDE-47 e BDE-99	27 ± 2 ° C and 12 : 12 hours	N.M.
Yamada et al.	Brazil	2012	Laboratory - Microcosmos	Calcium nitrate	25 ± 1 ° C and 12 : 12 hours	N.M.
Vicentini et al.	Brazil	2017	Laboratory	Benzopyrene	23,1 ± 0,9 ° C ° C and 12 : 12 hours	0.00473 mg L-1

Sotero-Santo et al.	Brazil/Canada	2007	Laboratory - Microcosmos	Iron	25 ° C and 12 : 12 horas	4.09 mg L-1
Richardi et al.	Brazil	2018	Laboratory	Phenanthrene	25 ± 2 ° C and 12 : 12 hours	1.21 mg L-1
Printes et al.	Brazil	2011	Laboratory - Microcosmos	Pesticides	20 ± 2 ° C and 12 : 12 hours	N.M.
Novelli et al.	Brazil	2012	Laboratory	Pesticide	23 ± 2 ° C and 12 : 12 hours	0.00267 mg L-1
Moreira-Santos et al.	Brazil/Portugal/México	2005	Laboratory - Microcosmos	Pesticide	23° to 27°, natural light (external microcosm)	N.M.
Morais et al.	Brazil	2020	Laboratory	BBP (Benzyl butyl phthalate) - Plastic	25°C and 12:12 hours	N.M.
Morais et al.	Brazil	2019	Laboratory	Antimony	25°C and 12:12 hours	N.M.
Guimaraes-Souto et al.	Brazil	2018	Laboratory	Heavy metals	23° to 27° and 12:12h	N.M.
Ferreira-Junior et al.	Brazil/Portugal	2018	Laboratory	Pesticide	22 ± 1 °C and 12:12h	0.032 mg L-1

Ferreira-Junior et al.	Brazil/Portugal	2017	Laboratory	Pesticide	22 ± 1 °C and 12:12h	251.5 mg L-1
Dornfeld et al.	Brazil/Portugal	2019	Laboratory and in situ	Heavy metals	24 ± 1 °C and 12:12 h	0.3/0.7 mg L-1
Barbosa et al.	Brazil/Portugal	2019	Laboratory	Humic substances	23 °C ± 2 and 12:12h	N.M.
Beguelli et al.	Brazil/Spain	2018	In situ (field)	Heavy metals	In situ	N.M.
Campagna et al.	Brazil	2013	Laboratory	Heavy metals	25 ± 2 °C and 12h:12	1234.43/340.56 mg kg -1
Castro et al.	Brazil	2018	Laboratory	Nanomaterial	N.A	> 100 mg L -1
Colombo-Corbi et al.	Brazil	2017	Laboratory	Pesticide	N.A	N.M.
da Costa et al.	Brazil	2014	Laboratory	Disinfectants	23 °C ± 2 and 12:12h	N.M.
Macedo et al.	Brazil	2020	Laboratory	Disinfectants	22 ± 1 °C; 12/12h	14.64/1.02 mg L -1
Palacio-Cortés et al.	Brazil	2017	Laboratory	Flame Retardants	12:12 hours; 25±2 °C	N.M.
Silvério et al.	Brazil	2005	Microcosmos - Laboratory	Heavy metals	23°C +- 2°C and 12:12h	N.M.
Silva et al.	Brazil	2018	Microcosmos - Laboratory	Heavy metals	23 ± 2 °C photoperiod 12-12 h	N.M.
Strixino & Strixno	Brazil	1985	Laboratory	Temperature	For each treatment 12°C, 5°C, 20°C, 2°C, 25°C, 0°C, 35°C	N.M.

NA – Not available; NM – Not measured.

APPENDIX OF THESIS

The appendix shows another works that were published by Emília Brovini master's time:

- SILVA, I. R.; **BROVINI, E. M.;** PEREIRA, R. O.; GOMES, M. H. R. Influência da precipitação e do uso e ocupação do solo na qualidade da água da bacia do ribeirão Espírito Santo, Juiz de Fora/MG. Revista de Estudos Ambientais, v. 22, p. 35-51, 2020. DOI: 10.7867/1983-1501.2020v22n1p35-51
- **BROVINI, E. M.**; BROVINI, M M ; PEREIRA, R. O. ; GOMES, M. H. R. Caracterização preliminar da microbacia hidrográfica do ribeirão São João, em Mar de Espanha - Minas Gerais. Revista Brasileira de Meio Ambiente, v. 8, p. 71-91, 2020. DOI: 10.5281/zenodo.3970160
- **BROVINI, E. M.;** MOREIRA, G. S. V. S.; RESENDE, N. S.; CARDOSO, S. J.; PEREIRA, R. O.; GOMES, M. H. R. Avaliação temporal da quantidade e da qualidade físico-química e biológica das águas do ribeirão Espírito Santo. Principia: Caminhos da Iniciação Científica, v. 19, p. 1-11, 2019. DOI: 10.34019/2179-3700.2019.v19.31209