CHAPTER 18

The utilizations of in vivo, in vitro and in silico tests in ecotoxicological studies: a narrative review

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ABSTRACT
With increasing demand for chemicals, the environment is increasingly exposed to toxic substances. To assess these exposures, ecotoxicological studies appear as an important tool. Tests for evaluations can be divided into *in vivo*, *in vitro* and *in silico*. The *in vivo* one differs from the others, as it uses living organisms in experimentation. The use of living organisms in research is an issue that has been discussed for a long time. There is a consensus among researchers on the importance of applying the 3Rs principles (reduction, substitution and refinement). Knowing this, the objective of the review is to evaluate the literature regarding the use of *in vivo* studies, emphasizing the relationship between model and non-model organisms for ecotoxicological studies, within the context of alternative tools such as *in vitro* and *in silico* assays. The review highlighted the increase in ecotoxicological studies in recent years and the importance of these studies for the assessment of environmental impacts. *Daphnia magna* is the most used invertebrate model organism in studies, while *Danio rerio* represents the most used vertebrate organism. The *in vitro* and *in silico* tests showed a large number of works carried out, which shows the importance of these tools, especially considering the application of the 3Rs. However, the work brought the importance of understanding which organism is being used in the study and how they vary within the different approaches.

Keywords: Ecotoxicology, 3Rs Principles, Model organism, Environmental impacts, Pollution.

1 INTRODUCTION

With the increase in urbanization and modifications promoted by the human population in recent years, environments are increasingly exposed to substances that may have toxic effects on organisms (Moraes, 2002; Jillella et al., 2021). According to Mucelin (2008), population growth and the consequent increase in activities, such as industry and agribusiness, generate environmental changes. Therefore, it is essential to evaluate environmental impacts through ecotoxicological studies, which are performed in order to identify potential damage to the environment (Almeida et al., 2017).

Ecotoxicological studies make it possible to identify the negative physiological and morphological effects of chemicals present in the air, water, soil and sediment, in contact with living beings (Walker, 2005; Silva et al., 2015). Besides allowing the evaluation of ecosystems already strongly affected, ecotoxicological tests are also useful to alert impacts in early stages that may have even more damaging effects in the long term (Zagatto, 2008).

For the development of ecotoxicological studies, tests with different methodological approaches can be applied, such as in vivo, in vitro and in silico tests. In *in vivo* tests, living organisms, usually model organisms, are used to conduct ecotoxicological studies. Model organisms are non-human individuals used in research that generate information that can be expanded and applied to other more complex organisms, such as humans (Leonelli & Ankeny, 2013). In addition, experimental models have some advantages , such as easy cultivation and the possibility of using a large number in the laboratory (Ankeny & Leonelli, 2011). According to US National Institute of Health (2018) examples of model organisms are the fruit fly *Drosophila melanogaster*, the plant *Arabidopsis thaliana* and the zebra fish *Danio rerio*.

Despite the widespread use of *in vivo* tests, the use of animals in scientific research is the subject of bioethical discussions (Silva et al., 2015). This debate is mainly brought by activists who criticize the use of animals in studies and ask for alternative methods to replace their use (Morales, 2008). In this regard, in order to reduce discomfort, pain and the number of animals used in scientific research, the 3Rs principle (reduction, replacement and refinement) was created (Cazarin et al., 2004). This program aims to promote greater applicability of *in vitro* (Petroianu, 1996; Cruz & Angelis, 2012) and *in silico* (Victal et al., 2014) studies by replacing animals with alternative tools. *In vitro* tests are those that use cells in culture instead of complex organisms (Quinn, 2014) and *in silico* tests are performed by computers through modeling and programs, allowing to identify potential threats that a given chemical substance can cause from its molecular structure (Victal et al., 2014).

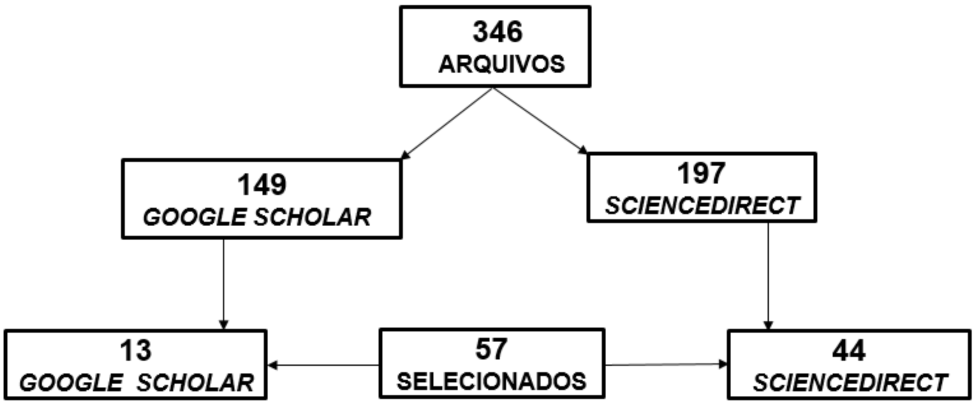
Therefore, knowing the diversity of existing tests, the objective of this study is to evaluate the literature regarding the use and importance of model and non-model organisms for ecotoxicological studies, within the context of alternative tools to the use of animals. Additionally, we evaluated the application of the 3Rs principles in ecotoxicological studies and identify the importance of these studies for environmental impact assessments.

2 METHODOLOGY

The present study is a narrative literature review that seeks to answer the following question, "Among the organisms used in ecotoxicological research, which ones are being used the most?" The databases used were Google Scholar and ScienceDirect. The methodology used in the review is similar to that of Ferrari (2015). The searches were done without delimitation of a specific period and were conducted between July 2021 to February 2022. The descriptors were used in the English language: model organisms, non model organisms, in vitro, in silico, in vivo and ecotoxicological, employed together with the Boolean operator AND (model organisms AND non-model organisms AND *in vitro* AND *in silico* AND *in vivo* AND ecotoxicological).

After the search, the titles and abstracts of the publications were read, and the review articles, reports, news, case reports, results of dissertations or theses, books and abstracts published in congress annals were excluded. Only original research articles in English were included, with at least one *in vivo*, *in vitro* or *in silico* ecotoxicological test in the materials and methods. The included articles were reviewed in full. To increase the scope of the review articles, we also used articles found in the references of the selected studies, which corroborate with the present study, but these were not included in the final number of selected articles, they were only used in the results and discussion topic. The dynamics of the selection process is described in Figure 1.

Figura 1. Flowchart of the selection of articles for the review.



Source: Authors (2022).

346 – archives
57 - selecteds

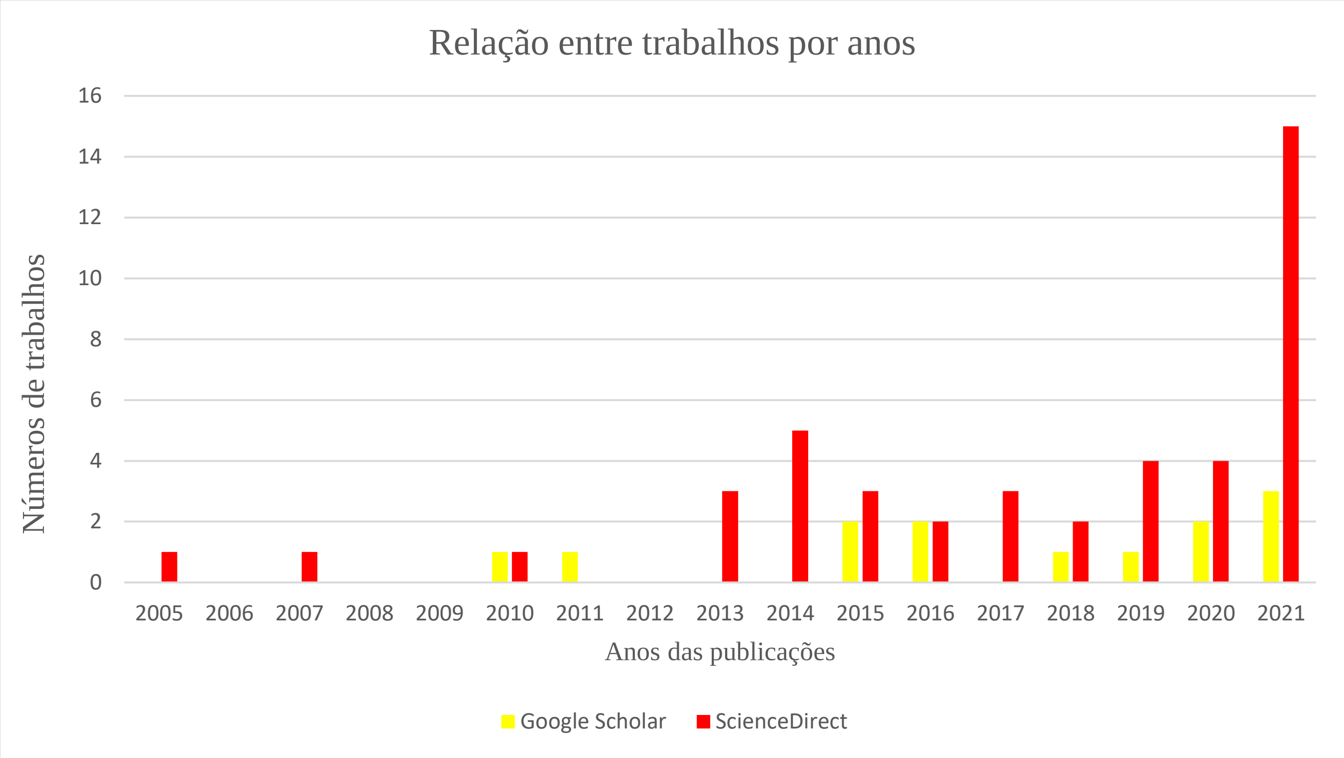
3 RESULTS AND DISCUSSION

3.1 GOOGLE SCHOLAR *VS* SCIENCEDIRECT

Through the initial searches to answer the question "Among the organisms used in ecotoxicological research, which are the most used?", using specific descriptors, 346 articles were found, 149 from Google Scholar and 197 from ScienceDirect. Among the 346 articles, after the inclusion process, 289 were excluded, leaving 57 articles, 13 from Google Scholar and 44 from ScienceDirect (Figure 1). The relatively low number of articles included from Google Scholar shows that although the database has a large number of files on the subject, many of them are review articles, books, dissertations, among others, which are listed within the exclusion factors of this evaluation. Moreover, it highlights the importance of searches in different platforms.

The ScienceDirect database showed a wider range of ecotoxicological articles according to the criteria used, and the 2 oldest articles (2005 and 2007) were found through this database. In Google Scholar the first selected articles were found from 2010 onwards (Figure 2). Both ScienceDirect and Google Scholar showed an increase in publications involving the evaluated theme and analysis criteria in recent years, highlighting the last three years, which corresponded to more than half (51%) of the selected articles and the year 2021, which presented 32% of the publications. This recent increase can be justified by the higher demand for industrial products (Miller et al., 2017) and applications of in silico studies (Rastogi et al., 2014; Gajewicz-Skretna et al., 2021a).

Figure 2. Timeline of the articles included in the evaluation.



Source: Authors (2022)

TRADUÇÃO:
Relação entre trabalhos por anos: relation among articles by years
Números de trabalhos: Number of articles
Anos das publicações: Years of Publication

3.2 FACTORS DRIVING TO THE EXPANSION OF ECOTOXICOLOGICAL STUDIES IN RECENT YEARS

The increase in ecotoxicological studies over the years is due to different factors, but we can highlight the inadequate disposal through agricultural and industrial activities in large cities, which threatens the environment (Seth et al., 2020). The products of this disposal, in contact with aquatic environments, can generate damage to the health of local biota and humans (Stinckens et al., 2016). Besides the aquatic environment, the terrestrial environment is also affected by the chemicals, as in the case of the antimicrobial Triclosan (TCS), 2-(2-4-dichlorophenoxy)-5-chlorophenol, which is widely used in the composition of personal care products (Zhu et al., 2018). Thus, ecotoxicological assessments are critical.

Studies have been conducted to evaluate the risk-benefit of industrial products (Jillella et al., 2021). According to REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), the body responsible for the protection of the environment and human health in the European Union, industries are required to make available existing information regarding their chemicals and, in cases of products without sufficient information, provide new data (Gubbels-Van Hal et al., 2005).

The agricultural system also causes concern with respect to ecotoxicology. In an epigenetic study, it was found that exposure of oysters (Crassostrea gigaspara) to the herbicide diuron causes increased methylation in the genetic material found in the digestive gland, which confirms the genotoxicity of this pesticide (Akcha, Barranger & Bachère, 2021). Furthermore, some pesticides have been reported to be toxic to birds (Zhang et al., 2015) and bees (Belsky et al., 2021). Certain pesticides are known to act as endocrine disruptors (EDs) (Legrand et al., 2016). EDs can cause problems during reproduction, growth, and development of organism exposed to them (Rodríguez et al., 2007).

As pharmaceutical products can cause great harm to the environment, there are several studies being conducted evaluating their ecotoxicity (Law et al., 2021; Minguez et al., 2014; Rastogi et al., 2014; Walker & Mceldowney, 2013). In aquatic ecosystems, these products promote various disturbances due to their chemical properties and because of transformation products (TPS) that are formed from biotic and abiotic actions (Rastogi et al., 2014). Due to the damage caused by TPSs, ecotoxicological studies have evaluated their potential harm (Law et al., 2021; Rastogi et al., 2014; Gutowski et al., 2015; Trawiński et al., 2021).

Currently, with the pandemic of COVID-19, some drugs have become eminent threats to the environment. Although the efficacy of azithromycin (AZT) and hydroxychloroquine (HCQ) are questioned (Jameleddine et al., 2020), both have become emerging drugs in terms of ecotoxicity due to their high use (Luz et al., 2021). This is due to the fact that the disposal of hospital waste is often not performed correctly, causing damage to the environment (Urban & Nakada, 2021). Another important factor is the incorrect disposal of pharmaceuticals into the sewage system (Minguez et al., 2014; Salgado et al 2021). As for AZT and HCQ, it has been reported that they can affect the aquatic environment, providing an increase in the energy expenditure of tadpoles (Physalaemus cuvieri), leading to adaptive physiological changes, which can cause problems in their reproduction and development in cases of prolonged exposures (Luz et al., 2021).

3.3 MODEL AND NON-MODEL ORGANISMS

Model organisms are characterized by their rapid life cycle (Legrand et al., 2016), well-characterized genetic material, and applicability of results that span other species, including humans (Howe et al., 2013; Martinez et al., 2018). In ecotoxicology, a well-known model organism is the zebra fish, *Danio rerio* (Prakash et al., 2021). This was evident during the present review, as it was the most commonly used organism in the experiments, along with the zooplankton species *Daphnia magna* (Table 1).

The zebra fish is the most commonly used vertebrate model organism in biomedical and ecotoxicological research (Prakash et al., 2021), and can be used from embryonic to adult stages (Stinckens et al., 2016). However, there is greater use of their embryonic stages, due to the fact that this stage of development represents a stage that is more sensitive to the damage caused by the toxicant (Strähle et al., 2012; Zhu et al., 2018; Prakash et al., 2021). It is worth pointing out that because the zebra fish is widely studied, there is a lot of information about its physiology, which makes it a model not only for *in vivo* studies, but also for *in silico* assessments (Walker & Mceldowney, 2013).

The use of mammals in research is a complex issue due to current legislation and the high financial cost of these studies (Siméon et al., 2020). Even for this reason, as shown in Table 1, the studies used in this review that used mammals, were performed with the application of *in vitro* assay or *in silico* assay. Thus, the zebra fish emerges as an alternative organism to using mammals, from systemic analyses, because it has about 70% of similar genes and neurophysiological circuits similar to humans (Howe et al., 2013; Martinez et al., 2018). Their characteristics, such as the high number of eggs per spawning and the fact that they exhibit rapid development (Goldsmith & Jobin, 2012) make them excellent model organisms..

Table 1. Quantitative number of organisms used in the ecotoxicological studies found by this review.

Living organisms	**Environmen**	**Exposed substance**	**Number of studies found**	**References**
Prokaryotes				
Vibrio fischeri (bacteria)	Aquatic	Organic UV filters; Diatrizoic acid; S-metolachlor.	4	(Law et al., 2021[1]); (Rastogi et al., 2014[2]); (Singh & Gupta, 2014[2]); (Gutowski et al., 2015[2])
Escherichia coli (bacteria)	Terrestrial	Diatrizoic acid	2	(Dom et al, 2010[1]); (Rastogi et al., 2014[2])
Salmonella Typhimurium (bacteria)	Terrestrial	Diatrizoic acid	1	(Rastogi et al., 2014[2])
Eukaryotes				
Danio rerio (fish)	Aquatic	2-Mercaptobenzothiazole; Aniline; Triclosan; Valproic acid; 4-methylbenzylidene camphor; 3,4,3′, 4′-tetrachloroazobenzene; Diclofenac; Ibuprofen; Levonorgestrel; Aldicarb Carbamate; Aldicarb Sulfoxide; Nano-Pd	10	(Wang et al., 2016[12]; Anila et al., 2021[1]; Küster & Altenburger, 2007[13]; Xiao et al., 2016[1]; Prakash et al., 2021[12]; Siméon et al., 2020[12]; Zhu et al., 2018[12]; Dom et al., 2010[12]; Stinckens et al., 2016[1]; Walker & Mceldowney, 2013[2])

Daphnia magna (crustacean)	Aquatic	Aniline; Organic UV filters; Azole fungicides; Sertraline; Clomipramine; Amitriptyline; Fluoxetine; Paroxetine; Mianserin; Citalopram; Venlafaxine; Fentanyl; Amitriptyline; Trazodone; Venlafaxine; Sodium dichromate; Chrysoidin; Benzoa-pyrene	10	(David et al., 2011[12]; Minguez et al., 2014[1]; Gottardi & Cedergreen, 2019[1]; Dom et al., 2010[12]; Law et al., 2021[2]; Gajewicz-Skretna et al., 2021b[2]; Trawiński et al., 2021[2]; Osawa et al., 2019[2]; Gajewicz-Skretna et al., 2021a[2]; Galimberti et al., 2020[2])
Pimephales promelas (fish)	Aquatic	Fentanyl; Amitriptyline; Trazodone; Venlafaxine; Pesticides; Cyclonite; Diethylstilbestrol; Fenanthrene; Perfluorooctane sulfonic acid; Perfluorinated compounds; 17α-ethinylestradiol	6	(Hala et al., 2015[12]; Wang, et al., 2016[12]; Trawiński et al., 2021[2]; Osawa et al., 2019[2]; Galimberti et al., 2020[2]; Ewald et al., 2020[2])
Oncorhynchus mykiss (fish)	Aquatic	3,4,3′, 4′-tetrachloroazobenzene; propanil; 3,4-dichloroaniline; Propranolol; Metoprolol; Atenolol; Formoterol; Terbutaline; Ranitidine; Imipramine; Diclofenaco; Ibuprofen; Levonorgestrel; S-metholachloro; Pesticidas; 17α-ethinylestradiol	6	(Alcaraz et al., 2021[12]; Xiao et al., 2016[3]; Stott et al., 2015[3]; Walker & Mceldowney, 2013[2]; Gutowski et al., 2015[2]; Galimberti et al., 2020[2])
Homo sapiens (human)	Terrestrial	Estrogen; Triphenyl phosphate	3	(Xiao et al., 2016[23]; Chan et al., 2019[3]; Wang et al., 2020[23])
Tetrahymena pyriformis (protozoan)	Aquatic	Various chemicals; Fentanyl; Amitriptyline; Trazodone; Venlafaxine	3	(Singh & Gupta, 2014[2]; Trawiński et al., 2021[2]; Osawa et al., 2019[2])
Raphidocelis subcapitata	Aquatic	Textile dyes; Pesticides; Polychlorinated biphenyls	3	(Jillella et al., 2021[2]; Galimberti et al., 2020[2]; Halm-Lemeille et al., 2014[12])

(microalgae)				
Oryzias latipestreinados (fish)	Aquatic		2	(Gajewicz-Skretna et al., 2021b[2]; Gajewicz-Skretna et al., 2021a[2])
Rattus norvegicus (rat)	Terrestrial	3,4,3′, 4′-tetrachloroazobenzene; propanil; 3,4 dichloroaniline; Sediment from Lake Sihwa - South Korea.	2	(Xiao et al., 2016[3]); (Cha et al., 2021[3])
Potamopyrgus antipodaru (mollusk)	Aquatic	Tributyltin; Cadmium	1	(Ruppert et al., 2017[1])
Tigriopus japonicus (crustacean)	Aquatic	Cadmium; Copper; Zinc	1	(Jeong et al., 2014[1])
Dunaliella tertiolecta (seaweed)	Aquatic	Polycyclic aromatic hydrocarbons; styrene oligomers; alkylphenols	1	(An et al.., 2021[12])
Isochrysis galbana (seaweed)	Aquatic	Polycyclic aromatic hydrocarbons; styrene oligomers; alkylphenols	1	(An et al., 2021[12])
Phaeodactylum tricornutum (seaweed)	Aquatic	Polycyclic aromatic hydrocarbons; styrene oligomers; alkylphenols	1	(An et al., 2021[12])
Piaractus mesopotamicus (fish)	Aquatic	Nano-TIO2	1	(Clemente et al., 2013[1])
Salmo trutta fario (fish)	Aquatic	Holtemme River in situ study	1	(Schmitz et al., 2021[1])

Physalaemus cuvieri (frog)	Terrestrial	Azithromycin; Hydroxychloroquine	1	(Luz et al., 2021[1])
Philodina acuticornis odiosa (rotifer)	Aquatic	Hydrogen peroxide; Sodium azide	1	(Olah et al., 2017[1])
Chironomus riparius (fly)	Terrestrial	Azole Fungicides	1	(Gottardi & Cedergreen, 2019[1])
Neomysis integer (shrimp)	Aquatic	Tebufenozide	1	(Wilde et al., 2013[123])
Melanogrammus aeglefinus (fish)	Aquatic	Petroleum	1	(Sørhus et al., 2021[1])
Micropterus salmoides (fish)	Aquatic	Tretinoin; Quercetin; Cyclosporine; Valproic acid; Copper sulfate; Methyl Methanesulfonate; Cobalt chloride, Acetaminophen, Atrazine, Formaldehyde	1	(Basili et al., 2018[12])
Colinus virginianus (bird)	Terrestrial	2,4-Dinitrotoluene	1	(Rawat et al., 2010[12])
Eurytemora affinis (crustacean)	Aquatic	Pyriproxyfen; Chlordecone	1	(Legrand et al., 2016[12])
Eisenia fetida (earthworm)	Terrestrial	AgNPs; Ag	1	(Novo et al., 2015[12])
Crassostrea gigas	Aquatic	Diuron Herbicide	1	(Akcha et al., 2021[13])

(mollusk)				
Gobiocypris rarus (fish)	Aquatic	Phenolic disinfection by-products. Aliphatic disinfection by-products	1	(Wang et al., 2021[1])
Apis mellifera (bee)	Terrestrial	Insecticide	1	(Belsky et al., 2021[1])
Pseudokirchneriella subcapitat (seaweed)	Aquatic	Aniline	1	(Dom et al., 2010[12])
Salmo salar (fish)	Aquatic	Diclofenac; Ibuprofen; Levonorgestrel	1	(Walker & Mceldowney, 2013[2])
Haliotis tuberculata (mollusk)	Aquatic	Biphenyl polychlorados; Sertraline; Clomipramine; Amitriptyline; Fluoxetine; Paroxetine; Mianserina; Citalopram; Venlafaxine	1	(Halm-Lemeille et al., 2014[23]); (Minguez et al., 2014[13])
Gasterosteus aculeatus (fish)	Aquatic	Ethinylestradiol; Trembolona	1	(Mintram et al., 2020[2])
Isochrysis galbana (seaweed)	Aquatic	ZnO; Ag; CeO; CuO ENPs	1	(Miller et al., 2017[23])
Aglais io (insect)	Terrestrial	Cry inseticida	1	(Baudrot et al., 2021[2])
Crassius auratus (fish)	Aquatic	Aldehydes; Phenols; Anilines; Alcohols	1	(Seth et al., 2020[3])
Poeciliopsis lucida (fish).	Aquatic	2,3,7,8-Tetrachlorodibenzo-p-dioxin; Polycyclic aromatic hydrocarbons	1	(Seth et al., 2020[3])

Xenopus tropicalis (frog)	Aquatic and terrestrial	Diclofenac; Ibuprofen; Levonorgestrel	1	(Walker & Mceldowney, 2013[2])
Daphnia pulex (crustacean)	Aquatic	Diclofenac; Ibuprofen; Levonorgestrel	1	(Walker & Mceldowney, 2013[2])
Anas platyrhynchos (bird)	Aquatic e terrestrial		1	(Zhang et al., 2015[2])
Nothura boraquira (bird)	Terrestrial		1	(Zhang et al., 2015[2])
Coturnix japônica (bird)	Terrestrial		1	(Zhang et al., 2015[2])
Scenedesmus obliguue (seaweed)	Aquatic		1	(Singh & Gupta, 2014[2])
Neocaridina davidi (shrimp)	Aquatic	Diacylhydrazine	1	(Chan et al., 2019[23])
Skeletonema costatum (microalgae)	Aquatic	Pesticides	1	(Yang et al., 2021[2])
Cyprinus carpio (fish)	Aquatic	S-metholachloro	1	(Gutowski et al., 2015[2])
Mus musculus (mouse)	Terrestrial	Saxitoxins	1	(RAMOS et al., 2018[3])
Lemna gibba (vegetable)	Aquatic	Pesticides	1	(Galimberti et al., 2020[2])
Chlorocebus sabaeus (monkey)	Terrestrial	Polycyclic Aromatic Hydrocarbons	1	(Bak et al., 2019[3])

Legend: Regarding the numbering of the references: [1] refers to *in vivo* studies; [2] are *in silico* studies; [3] are *in vitro* studies. Source: Authors (2022).

The other model organism widely used in ecotoxicological research is the zooplankton species *Daphnia magna* (Dom et al., 2010; Law et al., 2021; Gottardi & Cedergreen, 2019). This is an easy microcrustacean to maintain in laboratories because it is very small and has a short life cycle (Legrand et al., 2016). In addition, it presents high sensitivity to toxic products, which allows its use in ecotoxicological research (Gottardi & Cedergreen, 2019).

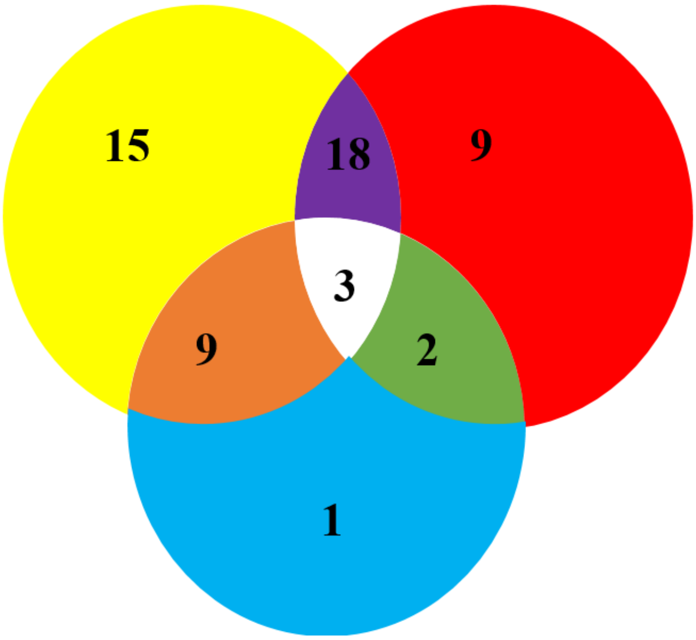
According to Table 1, D. *magna* is the most used invertebrate in ecotoxicological studies. In addition, this species is recommended as a model organism in genomic studies (Heckmann et al., 2008), and is considered a model organism in the evaluation of acute toxicity in aquatic environments (Cassotti et al., 2014). *Eisenia fetida* (earthworm), on the other hand, is classified as a model invertebrate in terrestrial environments, due to its importance in this ecosystem (Novo et al., 2015). However, its use appeared only once in Table 1, suggesting a greater focus on ecotoxicological research in aquatic environments and an apparent choice of non-model organisms in terrestrial environments.

Non-model organisms are those that do not match the characteristics of model organisms and are therefore used less in studies. However, the term does not mean that they are organisms that should not be used in research. One of the characteristics of non-model organisms is that they have their genetic material less understood compared to model organisms (Meyer et al., 2009; Rawat et al., 2010). However, advances in bioinformatics have been enabling a better understanding of the genetic material of these non-model organisms, which will enable a better use of them in studies (Vera et al., 2008; Rawat et al., 2010). One example is the case of the winter-rumped uru (*Colinus virginianus*), a non-model organism that had its material sequenced and exposed to 2,6-dinitrotoluene to perform an ecotoxicological evaluation. The result showed that the uru-do-norte has about 48% of similar genes to the model bird species (*Gallus gallus*), moreover, it showed some responses similar to those of the fatty fish on 2,6-dinitrotoluene exposure, which brings important systematic relationships of the uru-do-norte (Rawat et al., 2010). With this, the trend is that classic model organisms, such as *Daphnia magna* and *Danio rerio*, begin to share more space within ecotoxicological studies with other organisms with similar responses.

3.4 TYPES OF TESTING

There are different types of tests that can be conducted in toxicological research, highlighting *in vivo*, *in vitro* and *in silico* studies, which were quantified in the present review (Figure 3). The highest percentage of published papers involved *in silico* analyses, followed by *in vivo* analyses and lastly *in vitro* analyses. Of the 57 papers included in this review, only 1 (Stott et al., 2015) was entirely *in vitro*, suggesting the choice for integrated assessments (Gubbels-Van Hal et al., 2005).

Figure 3. Using the Ven Diagram to quantify the types of tests found from this review



Legend: Blue: *in vitro*; Red: *in vivo*; Yellow: *in silico*; Purple: *in silico* and *in vivo*; Green: *in vivo* and *in vitro*; Orange: *in silico* and *in vitro*; White: *in vitro, in silico* and *in vivo*
Source: Authors (2022)

3.4.1 *in vivo* assays

In vivo assays have the second largest number of studies (Figure 3). The *in vivo* assay has its use and application in many occasions in integrated analysis with *in silico* assay. The applications of *in vivo* tools in ecotoxicology seeks to analyze different "endpoints" from an established standard (Dom et al., 2010; Zhu et al., 2018; An et al., 2021), besides contributing to the expansion in the number of published works in this area. There are several organisms that can be used in these studies (Table 1), with the zebra fish being the most widely used. Through the zebra fish, one can assess, for example, mortality (Zhu et al., 2018), organ damage (Stinckens et al., 2016) and physiological processes (Xiao et al., 2016), as "endpoints" of the toxicological evaluation of substances.

The *in vivo* study is widely used in ecotoxicological research and is very important for conducting biomonitoring. With the current worrying scenario in aquatic ecosystems, the use of living organisms allows early identification of damage at the cellular level, from biomarkers, before it can become a major biological damage (Van der oos et al., 2003). One example is the use of micronucleus analysis in fish blood cells to assess the toxicity of a certain point in an aquatic environment (Schmitz et al., 2021).

The choice of living organisms in the experiment depends on the purpose of the study. In situations where it is desired to identify the damage caused by chronic exposure to local biota, organisms native to that region are used (Schmitz et al., 2021), but when the study aims to analyze the toxicity of a given substance, well-characterized model organisms are used, as in the case of *Daphnia magna* (Minguez et al., 2014) and *Danio rerio* (Prakash et al., 2021). Furthermore, one of the advantages of the *in vivo* method is the possibility of in situ study, which allows the use of organisms that are being directly exposed to the environment that is intended to be evaluated (Schmitz et al., 2021)

3.4.2 *in vitro* assays

Of the 57 articles included in this review, only 1 (Stott et al., 2015) was entirely *in vitro*. *In vitro* models are characterized by the possibility of evaluating cells from a controlled environment and isolated from the living organism (Halm-Lemeille et al., 2014), providing information at cellular and molecular levels (Binelli et al., 2009). Thus, *in vitro* studies enable the reduction of the number of animals that need to be killed in *in vivo* assays, constituting a relevant alternative for research.

Besides reducing the use of live organisms in research, *in vitro* studies contribute to the demands of ecotoxicological studies (Gubbels-Van Hal et al., 2005). Thus, in vitro assays play an important role not only ethically, but also as a way to promote chemical assessments. These assays are widely used in genotoxicity assessments (Binelli et al., 2009; Wang et al., 2020), such as in identifying methylations in DNA (Akcha et al., 2021) and in introducing reporter gene to evaluate harmful substances in monkey (Chlorocebus aethiops) cells and in humans. (Bak et al., 2019; Chan et al., 2019). In addition, they are fundamental in cytotoxicological studies, where through tools, such as the MTT ([3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] cell viability test) evaluate enzyme activities (Minguez et al., 2014; Halm-Lemeille et al., 2014).

3.4.3 in silico assays

Within ecotoxicology, *in silico* assays help in the identification of predictive responses, through computational and mathematical models (Dom et al., 2010). Due to obtaining results faster and at a lower cost compared to *in vivo* and *in vitro* assays, *in silico* assays have become the most widely used in ecotoxicological research, according to results obtained in the present review (Figure 3).

Applications of computational and mathematical tools allow simulation of chemical exposure to organisms (Baudrot et al., 2021). For example, PBPK (physiologically based pharmacokinetic models) mathematical modeling studies are able to simulate chemical exposure in zebra fish, which is a model organism often used in *in vivo* studies (Siméon et al., 2020). Thus, *in silico* studies allow the number of animals in research to be reduced, which also explains the increased use of this assay in ecotoxicological assessments (Figure 3).

One of the most widely used methodologies to predict toxicities of substances is Quantitative Structure-Activity Relationship (QSAR) (Zhu et al., 2018; Jillella et al., 2021; Law et al., 2021). Studies using QSAR primarily seek to develop and validate models for evaluating toxic products from the analysis of biological activity using mathematical forms (Singh & Gupta, 2014; Yang et al., 2021). One of the advantages of this method is that there are free QSAR software, such as ECOSAR, which makes this approach more accessible (DOM et al., 2010).

Genetics plays an important role in *in silico* studies, because with the advancement of bioinformatics, it has enabled a greater applicability of omics: genomics (Rawat et al., 2010) transcriptomics, proteomics and metabolomics (Wang et al., 2020). Often, omics studies integrate the use of *in silico* testing with *in vitro* (Wang et al., 2020) or *in vivo* (Alcaraz et al., 2021) testing, as their analyses are made from biomolecules and computational data (Schmitz et al., 2021). The application of omics science is only possible due to the availability of databases, such as GenBank (Novo et al., 2015) and National Center for Biotechnology Information Gene Expression Omnibus (NCBI) (Wang et al., 2016).

Omics analysis has enabled good ecotoxicological assessment (Schmitz et al., 2021). For example, joint analysis using transcripts, proteins, and metabolites has been shown to be effective in evaluating the pollutant triphenyl phosphate (TPP) in human cells (Wang et al., 2020). These assessments can be done separately as well, for example, several studies have been using only trascriptomic analysis in ecotoxicological assessments, such as in identifying genes responsible for the immune response of organisms exposed to heavy metals (Jeong et al., 2014) and in assessing genotoxicity in *Daphnia magna* (David et al., 2011).

3.5 3rs Principles

The 3Rs principles (reduction, replacement, and refinement) in the scientific environment emerged in 1959 with the publication of the book "The principles of humane experimental technique", with the purpose of reducing the number of animals and their discomfort in research (Russell & Burch, 1959). From then on, the 3Rs started to become present in research around the world, including being part of laws related to the use of animals in research (Astrogildo et al, 2018). For this reason, there is a need for and consequently, increased occurrence of alternative studies to *in vivo* ones, such as *in silico* and *in vitro* assays (Stinckens et al., 2016). In ecotoxicological studies, as shown in (Figure 3), the search for the alternative tests exceeded the number of *in vivo* studies. This shows that research does not always rely on studies with living organisms and that *in vitro* and *in silico* studies have been proving effective in ecotoxicological assessments.

In addition, the *in vivo* studies themselves are changing their methodologies, as in the case of studies with zebra fish, where most studies are being conducted in the embryonic stage (Küster & Altenburger, 2007; Zhu et al., 2018; Prakash et al., 2021;). Unlike the adult stage, the embryonic stage of these fish has no legislation preventing their use in studies conducted in Europe, so they are even used to replace animals in research (Strähle et al., 2012; Zhu et al., 2018; Prakash et al., 2021).

Regulatory agencies and research organizations seek the implementation of alternative tests not only for ethical reasons, but for technical reasons as well, since *in silico* testing, for example, presents as a faster and cheaper alternative (Gubbels-Van Hal et al., 2005). Furthermore, due to the increase in chemicals, there is a need to speed up their evaluation in the environment through vertebrate, invertebrate, *in vitro* or *in silico* experimental models. In this way computer-based approaches tend to increase further, as they are faster than those done on animals (Gajewicz-Skretna et al., 2021b).

4 CONCLUSION

The present review indicates that the invertebrate *Daphnia magna* and the vertebrate *Danio rerio* were the most commonly used organisms and thus a great importance in ecotoxicological assessments. In addition, model organisms collaborate to the creation of alternative methods, as in the case of *in silico* studies, which proved to have the highest rate of applications in ecotoxicological assessments. The results show that there is a concern for the application of the 3Rs principles in ecotoxicological studies. This can be seen from the analysis of the amount of each model applied, where there was a large amount of applications of *in vitro* and *in silico* studies.

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