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# **Toxicity of Herbicides: Impact on Aquatic and Soil Biota and Human Health**

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Additional information is available at the end of the chapter

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## **1. Introduction**

During the last decades, the scientific community, including government and non-government organizations have increased their interest in detecting and controlling the environmental agents responsible for damages to the human health and sustainability of the ecosystems. This interest has been intensified by the frightening increase on the reports of the anthropogenic action on the environment responsible for damages to the ozone layer, accidental release of wastes and radioactive gases, as well as contamination by pesticides used in agriculture. However, the growth of the human population and of the activities associated with agriculture, industrialization and urbanization have contributed to the depredation of the biodiversity and genetic variability, resulting in the compromise of several species, including man [9].

After the industrial revolution, a great number of chemical substances have been released into the terrestrial and aquatic environments and in the atmosphere. These substances can be transported and transformed by different processes, whose transformation by-products can cause adverse effects on man, as well as damages to the terrestrial and aquatic ecosystems. Several studies have shown the presence of residues of several chemical substances in the air, water, soil, food and organisms in general [10].

Environmental pollution by genotoxic and mutagenic products affects the exposed organism and its future generations, this fact is observed both for animals, and in this case man is included, and for the other groups of organisms such as plants and microorganisms. In order to evaluate the consequences of the anthropogenic activities on the ecosystem it is necessary that the scientific community pays a special attention in the search for understanding the

modes of action of xenobiotics present in the ecosystem in the biota exposed. For this, extensive, detailed and ordered studies of the contaminants must be developed with the purpose of preventing the biological impairment, such as inductions of alterations in the genetic materials of the organisms [11].

Some studies have been performed in the attempt to evaluate the behaviour, transformations and effects of chemical agents, both in the environment and in the organisms. Toxicology establishes the limits of concentration or quantity of chemical substances acceptable in the environment by studies on the toxic effects of these substances in the organism and ecosystems [12].

Considering that the use of agrochemicals, such as herbicides, have caused a great environmental contamination, due to their widespread use, it has become indispensable to perform the assessment of the toxicity of these compounds.

### **1.1. The importance of herbicides**

Living beings are exposed to the action of numerous agents that are potentially toxic. These agents can be physical, chemical or biological and can provoke in the organisms physiological, biochemical, pathological effects and, in some cases, genetic effects [13]. A great variety of chemical substances with mutagenic potential, both natural and synthetic, have been investigated. Many of these substances are found in food, pharmaceutical drugs, pesticides and in complexes of domestic and industrial effluents. It is known that these compounds can cause detrimental inheritable changes in the genetic material, without these changes being expressed immediately [14]. Thus, several compounds dispersed in the environment can represent danger to human health, since they present a potential to induce mutations [15].

The production of food can occur both by agricultural activities and by livestock. The yield of food production is directly related with the relationship established between the species of interest for production and the other plant, animal, microbial and parasitic biological systems that compete for resources available in the environment [16]. Among the species that jeopardize the agricultural production there are the weeds that, when invade crops, can cause significant loss in the yield and quality of the harvest [17]. Therefore, in order to enhance the productivity and the quality of crops, the removal of weeds from agriculture becomes important.

Before the introduction of selective herbicides as an agricultural practice, the removal of weeds was accomplished manually in an extremely laborious form. Thus, the farmers sought other forms to control weeds, such as, integrating other weed control practices such as crop rotation, tillage and fallow systems [17].

The introduction of selective herbicides in the late 40's and the constant production of new herbicides in the following decades gave farmers a new tool in the control of weeds [17]. Therefore, the process of modernization of agriculture introduced, in the 60's, the use of new biological varieties considered more productive, but dependent on chemical fertilizers and intensive use of pesticides, in order to increase productivity. The use of these chemical agents resulted in the increase of productivity, but, on the other hand, brought adverse consequences,

since many are harmful substances for man and the environment. The world practice of using agrochemicals for long periods, often indiscriminate and abusive, has raised concerns among the public authorities and experts of public health and sustainability of natural resources [16].

Many agrochemicals are very toxic substances whose absorption in man are almost exclusively oral and can also occur by inhalation or dermally. As a consequence of the human exposure to pesticides, a series of disturbances can be observed, such as gastric, neurological and muscular [18].

Among the pesticides, the main agents of intoxication are the herbicides and insecticides. According to Vasilescu and Medvedovici [19], herbicides are defined as any substance, individually or in mixtures, whose function is to control, destroy, repel or mitigate the growth of weeds in a crop.

The use of herbicides, despite the fact that they are characterized as a highly effective tool in the control of weeds, has led to a change in the phytosociological composition of weeds and to a selection of biotypes resistant to herbicides, besides also causing impacts in the environment and human health. According to He et al. [20], herbicides are the most used chemical substances throughout the world. During the 90's, the global pesticide sales remained relatively constant, between 270 and 300 billions of US dollars, and 47% of this value corresponded to herbicides and 79% to insecticides. Since 2007, herbicides assumed the first place among the three major categories of pesticides (insecticides, fungicides/bactericides, herbicides) [21].

The use of herbicides to control weeds has been a common practice in global agriculture, mainly with the objective to increase agricultural production. However, when these chemicals are used in an uncontrolled manner, they can cause impacts on non-target organisms, especially on those that live in aquatic environments [22].

According to Chevreuil et al. [23], Kim and Feagley [24] and Abdel-Ramham et al. [25], most of the toxic effects of the herbicides on animals and plants were insufficiently investigated. As a consequence of the lack of information about the action of herbicides in the biological environment, these chemical agents can also represent a problem to human health [26, 27]. The impact of a pesticide in the environment depends on its dispersion mode and its concentration, as well as its own toxicity [28]. The mutagenic effects of the herbicides can result from several reactions with the organism, as a direct action of the compound on the nuclear DNA; incorporation in the DNA during cell replication; interference in the activity of the mitotic or meiotic division, resulting in incorrect division of the cell [29].

Some herbicides interfere directly in the cell division of plants, elongation and/or cell differentiation, causing disturbances in the functioning of the roots or vascular tissues [30]. In animals, herbicides can act in several tissues or organs and, sometimes, are associated with tumorigenic processes [31].

Jurado et al. [32] listed the general advantages and disadvantages of using herbicides. In this list, the authors cited as advantages: kill unwanted plants; help crops grow since it eliminates weeds that compete with crops for water, nutrients and sunlight; can be safely used in

plantations, while the manual or mechanical removal processes of weeds can cause damages to crops; can be used in geographically close crops; in most cases, only one application of the herbicide is sufficient to control the weeds, while the other methods must be constantly used; are easy to use; have fast action; are relatively inexpensive and are economically more viable than manual removal; non-selective herbicides can be used to eliminate vegetation cover in areas intended for the construction of residences and/or roads; to eradicate plants bearing diseases; and since some herbicides are biodegradable, they can become relatively inert after some time. The disadvantages listed by the authors are: some herbicides are not biodegradable and, thus, can persist in the environment for a long period of time; all herbicides are, at least, mildly toxic; can cause diseases and even accidental death (case of paraquat); can be carried into rivers by rainwater or be leached to groundwater polluting these environments; some herbicides can accumulate in the food chain and are toxic for animals, including man.

## 1.2. Herbicides classification

According to Moreland [33], herbicides are designated by common names approved by the Weed Science Society of America (WSSA) or by the British Standards Institution. Organic herbicides are classified according to their application method, chemical affinity, structural similarity, and by their mode of action [34]. In relation to the application methods, herbicides can be classified into two groups: soil application and foliar application. According to Jurado et al. [32], all the herbicides applied in the pre-planting (surface or incorporation) and pre-emergence (in crops, weeds or both) are classified as herbicides of soil application and those applied in the post-emergence are classified as foliar application.

Moreover, herbicides can be classified according to their mode of action. Following, it will be presented the classes of herbicides, according to their mode of action, based in the classification of Moreland [33] :

1. *chloroplast-associated reactions*: photo-induced electron transport and reaction coupled to phosphorylation occur in the chloroplast, any interference in these reactions inhibit the photosynthetic activity. Herbicides that inhibit the photo-chemically induced reactions are divided into the following classes:
  - a. electron transport inhibitors: electron transport is inhibited when one or more intermediary electron carriers are removed or inactivated or even when there is interference in the phosphorylation. Example: diuron, atrazine.
  - b. uncouplers: uncouplers dissociate the electron transport of the ATP formation through the dissipation of the energetic state of the thylakoid membrane, before the energy can be used to perform the high endergonic reaction of ADP phosphorylation. Example: perfluidone.
  - c. energy transference inhibitors: inhibition of energy transference inhibitors acts directly in the phosphorylation, as well as inhibitors of the electron transport, which inhibit both the electron flow and the formation of ATP in coupled systems. Example: 1,2,3-thiadiazol-phenylurea, nitrofen.

- d. inhibitory uncouplers: the term “inhibitory uncouplers” was used by Moreland [33] to indicate that the herbicides interfere in reactions affected by electron transport inhibitors and by uncouplers; These “inhibitory uncouplers” inhibit the basal transport, uncoupled and coupled of electrons. The herbicides classified in this group affect both the electron transport and the gradient of protons. Examples: acylanilides, dinitrophenols, imidazole, bromofenoxim.
  - e. electron acceptors: the compounds classified in this group are able to compete with some component of electron transport and consequently suffer reduction. Examples: diquat, paraquat.
  - f. inhibitors of the carotenoid synthesis: this class of herbicides acts to inhibit the synthesis of carotenoids, resulting in accumulation of precursors of carotenoid devoid colour (phytoene and phytofluene). The inhibition of carotenoid synthesis leads to the degradation of chlorophyll in the presence of light; degradation of 70s ribosomes; inhibition of the synthesis of proteins and loss of plastids. Examples: amitrole, dichlormate, SAN6706.
2. *mitochondrial electron transport and phosphorylation*: herbicides that interfere in the mitochondrial system are classified as:
    - a. electron transport inhibitors: defined as substances that have the ability to interrupt the electron flow in some point of the respiratory chain, acting in one of the complexes. Examples: diphenylether herbicides.
    - b. uncouplers: in appropriate concentrations, the classic uncouplers, that are weak lipophilic acids or bases, prevent the phosphorylation of ADP without interfering in the electron transport. Generally, any compound that promotes the dissipation of the energy generated by the electron transport, except for the production of ATP, can be considered as uncoupler. Example: isopropyl ester glyphosate.
    - c. energy transfer inhibitors: compounds of this group inhibit the phosphorylating electron transport, when the apparatus of energy conservation of the mitochondria is intact and the inhibition is circumvented by uncouplers. They combine with an intermediary in the coupling energy chain and, thus, block the phosphorylation sequence that leads to the ATP formation. No herbicide seems to act as an energy transfer inhibitor.
    - d. inhibitory uncouplers: most of the herbicides that interfere in the oxidative phosphorylation present a great variety of responses and are classified as uncoupling inhibitors. At low molar concentrations, herbicides fulfil almost all, if not all, of the requirements established for uncouplers, but at high concentrations they act as electron transport inhibitors. Herbicides that present this behaviour are the same classified as uncoupler inhibitors of the photoinduced reactions in the chloroplast. Example: perfluidone.
  3. *interactions with membrane*: herbicides can affect the structure and function of membranes directly or indirectly. When the herbicides disaggregate a membrane, they can influence directly the transport processes by interacting with the protein compounds, such as, ATPases and by altering the permeability by physicochemical interactions, or indirectly

by modulating the supply of ATP needed to energize the membrane. Interactions with the membrane can cause:

- a. compositional alterations: can modify or alter the composition of lipids in the membrane and can also act in the metabolism and synthesis of lipids. Examples: dinoben, chlorambem, perfluidone.
- b. effects in the permeability and integrity. Examples: paraquat, diquat, oryfluorfen, oryzalin.
4. *cell division*: herbicides may suppress cell division by interfering in the synthesis or active transport of precursors into the nucleus, which are necessary for the synthesis of DNA during interphase; modify the physical or chemical properties of the DNA or of their complexes; interfere in the formation and function of the spindle; and/or inhibits the formation of the cell wall. Several of the processes mentioned previously need energy and, therefore, interferences in the amount of energy caused by an herbicide could modulate the mitotic activity. The effects of the inhibitors of the cell division are dependent on the concentration and vary according to the species and the type of tissue. There is a relationship between cell division and cellular energy. In higher plants, cell division is prevented or suppressed in conditions in which the glycolysis or the oxidative phosphorylation is inhibited. Another form of the herbicide to alter cell division would be interacting with the microtubules, since these cellular structures are responsible for the orientation and movement of chromosomes during cell division. Examples of herbicides that interfere in cell division: N-phenylcarbamates, ioxynil, trifluralin.
5. *Synthesis of DNA, RNA and protein*: there are correlations between inhibition of RNA and protein synthesis and low concentration of ATP in tissues and these correlations suggest that interferences in the energy production, necessary to perform biosynthetic reactions, could be the mechanism by which the herbicides could express their effects. Moreover, they can inhibit the synthesis of DNA or RNA by altering the chromatin integrity and, in these cases, the synthesis of proteins is also affected. Examples: glyphosate, trifluralin.

The herbicides can still be classified according to the chemical affinity. Table 1 shows the chemical classes and examples of each class, according to Rao [34].

Class of the herbicide	Examples of herbicides
Acetamides	Acetochlor, alachlor, butachlor, dimethenamid, metolachlor, napropamide, pronamide, propachlor, propanil
Aliphatics	Chlorinated aliphatic acid (TCA), acrolein, dalapon
Arsenicals	Disodium methanearsonate (DSMA), monosodium methanearsonate (MSMA), cacodylic acid
Benzamides	Isoxaben
Benzoics	Dicamba
Benzothiadiazoles	Bentazon
Bipyridiliums	Diquat, paraquat
Carbamates	Asulam, desmedipham, phenmedipham

Class of the herbicide	Examples of herbicides
Cineoles	Cinmethylin
Cyclohexanediones (cyclohexenones)	Clethodim, cycloxdim, sethoxydim, tralkoxydim
Dinitroanilines	Benfenin, ethalfuralin, fluchloralin, pendimethalin, prodiamine, trifluralin
Diphenylethers	Acifluorfen, bifenox, fluoroglycofen, fomesafen, lactofen, oxyfluorfen
Imidazolidinones	Buthidazole
Imidazolinones	Imazapyr, imazaquin, imazethapyr, imazamethabenz
Imines	CGA-248757
Isoxazolidinones	Clomazone
Nitriles	Bromoxynil, dichlobenil, ioxynil
Oxadiazoles	Oxadiazon
Oxadiazolidines	Methazole
Phenols	Dinoseb
Phenoxyalkanoic acids	
Phenoxyacetics	2,4-D, MCPA, 2,4,5-T
Phenoxybutyrics	2,4-DB
Arylophenoxy propionics	Dichlorprop, diclofop, fenoxaprop, fluzafop-P, quizalofop-P
<i>N</i> -phenylphthalimides	Flumiclorac
Phenylpyridazines	Pyridate
Phenyl Triazinones (Aryl Triazinones)	Sulfentrazone
Phthalamates	Naptalam
Pyrazoliums	Difenzoquat
Pyridazinones	Norflurazon, pyrazon
Pyridinecarboxylic Acids	Clopyralid, picloram, triclopyr
Pyridines	Dithiopyr, thiazopyr
Pyridinones	Fluridone
Pyrimidinythio-benzoates (Benzoates)	Pyriothiobac
Quinolinecarboxylic acids	Quinclorac
Sulfonylureas	Bensulfuron, chlorimuron, chlorsulfuron, halosulfuron, metsulfuron, nicosulfuron, primisulfuron, prosulfuron, sulfometuron, thifensulfuron, triasulfuron, tribenuron
Tetrahydropyrimidinones	Yet to be commercialized
Thiocarbamates	Butylate, diallate, EPTC, molinate, pebulate, thiobencarb, triallate
Triazines	Ametryn, atrazine, cyanazine, hexazinone, prometryn, simazine
Triazinones	Metribuzin
Triazoles	Amitrole
Triazolopyrimidine Sulfonanilides	Flumetsulam
Uracils	Bromacil, terbacil, UCC-C4243
Ureas	Diuron, fluometuron, linuron, tebuthiuron,
Unclassified herbicides	Bensulide, ethofumesate, fosamine, glufosinate, glyphosate, tridiphane

**Table 1.** Classification of the herbicides according to the chemical affinity.



### 1.3. Aquatic and soil contamination due to the presence of herbicides

When a herbicide is used to control weeds, sometimes a majority of the compound ends up in the environment, whether it is in the soil, water, atmosphere or in the products harvested [17]. Due to the widespread use of these chemicals over the years, there has been an accumulation of these residues in the environment, which is causing alarming contaminations in the ecosystems [35] and negative damages to the biota. To Bolognesi and Merlo [3], the widespread use of herbicides has drawn the attention of researchers concerned with the risks that they can promote on the environment and human health, since they are chemicals considered contaminants commonly present in hydric resources and soils. According to the same authors, herbicides represent a high toxicity to target species but it can be also toxic, at different levels, to non-target species, such as human beings. Herbicides can cause deleterious effects on organisms and human health, both by their direct and indirect action [2]. Among the biological effects of these chemicals, it can be cited genetic damages, diverse physiological alterations and even death of the organisms exposed. Some herbicides, when at low concentrations, cannot cause immediate detectable effects in the organisms, but, in long term can reduce their lifespan longevity [4]. Herbicides can affect the organisms in different ways. As with other pesticides, the accumulation rate of these chemicals on biota depends on the type of the associated food chain, besides the physicochemical characteristics (chemical stability, solubility, photodecomposition, sorption in the soil) of the herbicide [5-6]. Thus, despite the existence of several toxicological studies carried out with herbicides, in different organisms, to quantify the impacts of these pollutants and know their mechanisms of action [7, 8, 2], there is a great need to expand even more the knowledge about the effects of different herbicides in aquatic and terrestrial ecosystems. Data obtained from *in situ*, *ex situ*, *in vivo* and *in vitro* tests, derived from experiments of simulation, occupational exposure or environmental contaminations, need to enhance so that it is possible to obtain even more consistent information about the action of these compounds.

According to Jurado et al. [32], when herbicides are applied in agricultural areas they can have different destinations, since being degraded by microorganisms or by non- biological means or even be transported by water, to areas distant from the application site. Thus, according to the same authors, the organisms can be then exposed to a great number of these xenobiotics as well as their metabolites.

The fate of the compound in the soil depends on the characteristics of the compound and the soil. The hydrogenionic properties of a compound in the soil determines its sorption characteristics, such as, acid herbicides in soils with normal pH are negatively charged and consequently are movable in most of the soils [17]. Some groups of pesticides are neutral in soils with normal pH but due to electronic dislocations in the molecules, they can bind to soil colloids by several forms [36].

According to Kudsk and Streiberg [17], during the last two decades, several studies have been completed to predict the behaviour of pesticides in the soil. Despite the numerous efforts to assess the effects of herbicides in the soil, there are conflicting data in the literature on the subject, where some studies show that the residues of pesticides can be sources of carbon and energy to microorganisms, and then are degraded and assimilated by them, while other reports

affirm that pesticides produce deleterious effects to the organisms and biochemical and enzymatic processes in the soil [37]. According to Hussain et al. [37], in general, the application of pesticides, and here it is also included herbicides, made long term, can cause a disturbance in the biochemical balance of the soil, which can reduce its fertility and productivity.

Once in the soil, herbicides can suffer alteration in their structure and composition, due to the action of physical, chemical and biological processes. This action on the herbicides is the one that will determine their activity and persistence in the soil. Some molecules, when incorporated into the soil, are reduced by volatilization and photo-decomposition. Once in the soil, herbicides can suffer the action of microorganisms, which, added to the high humidity and high temperature, can have their decomposition favoured [38]. If they are not absorbed by plants, they can become strongly adsorbed on the organic matter present in the colloidal fraction of the soil, be carried by rainwater and/or irrigation and even be leachate, thus reaching surface or groundwater [39].

The prediction of the availability of herbicides to plants has two purposes: 1. ensure that the herbicide reaches the roots in concentrations high enough to control weeds, without compromising the agricultural productivity; 2. predict if the compound is mobile in the soil to estimate how much of the herbicide can be leachate from the roots zone to groundwater [17].

The contamination of aquatic environments by herbicides has been characterized as a major world concern. This aquatic contamination is due to the use of these products in the control of aquatic plants, leachate and runoff of agricultural areas [40]. According to He et al. [20], it is a growing public concern about the amount of herbicides that have been introduced into the environment by leachate and runoff, not to mention that the contaminations of the aquatic environments generally occur by a mixture of these compounds and not by isolated substances.

Guzzella et al. [1] did a survey on the presence of herbicides in groundwater in a highly cultivated region of northern Italy. The researchers monitored for two years the presence of 5 active ingredients and 17 metabolites resulting from these compounds. The authors verified that atrazine, although banned in Italy since 1986, was the major contaminant of the groundwater of the sites studied, they also observed that the concentration of at least one of the compounds studied exceeded the maximum allowed concentration in 59% of the samples likely due in both cases to off-label herbicide use. This scenario could be, in long term, a serious problem for the quality of this water, which is used as drinking water.

Toccalino et al. [41] carried out a study to verify the potential of chemical mixtures existing in samples of groundwater used for public supply. In these samples, the most common organic contaminants were herbicides, disinfection by-products and solvents. The authors concluded that the combined concentrations of the contaminants can be a potential concern for more than half of the samples studied and that, even though the water destined to public supply pass through treatments to reduce contaminations and meet the legislations, it can still contain mixtures at worrying concentrations.

Saka [42] evaluated the toxicity of three herbicides (simetryn, mefenacet and thiobencarb) commonly used in rice planting in Japan, on the test organism *Silurana tropicalis* (tadpoles). The authors observed that the three herbicides, particularly thiobencarb, are toxic for tadpoles

(LD50 test), even for concentrations found in waters where the rice is cultivated. In a similar study carried out by Liu et al. [43], it was observed that the effect of the herbicide butachlor (most used herbicide in rice planting in Taiwan and Southeast Asia) on the organism *Fejervarya limnocharis* (alpine cricket frog) exposed to concentrations used in the field. In this study no effect on the growth of tadpoles of *F. limnocharis* was observed, but there was a negative action on survival, development and time of metamorphosis. The authors suggested that the herbicide butachlor can cause serious impacts on anurans that reproduce in rice fields, but this impact varies from species to species.

In a study conducted by Ventura et al. [8], it was observed that the herbicide atrazine has a genotoxic and mutagenic effect on the species *Oreochromis niloticus* (Nile tilapia). In this study, the authors observed that the herbicide can interfere in the genetic material of the organisms exposed, even at doses considered residual, which led the authors to suggest that residual doses of atrazine, resulting from leaching of soils of crops near water bodies, can interfere in a negative form in the stability of aquatic ecosystems.

Bouilly et al. [44] studied the impact of the herbicide diuron on *Crassostrea gigas* (Pacific oyster) and observed that the herbicide can cause irreversible damages to the genetic material of the organism studied. Moreover, the authors affirm that, due to the persistence of diuron in environments adjacent to its application site and that it is preferably used in spring, the pollution caused by its use causes negative impact in the aquatic organisms during the breeding season.

In general, when herbicides contaminate the aquatic ecosystem, they can cause deleterious effects on the organisms of this system. Thus, organisms that live in regions impacted by these substances, whose breeding period coincides with the application period of the herbicides, can suffer serious risks of development and survival of their offspring.

Hladik et al. [45] evaluated the presence of two herbicides (chloroacetamide and triazine), as well as their by-products, in drinking water samples of the Midwest region of the United States. The authors detected the presence of neutral chloroacetamide degradates in median concentrations (1 to 50 ng/L) of the water samples. Furthermore, they found that neither the original chloroacetamide herbicides nor their degradation products were efficiently removed by conventional water treatment processes (coagulation/flocculation, filtration, chlorination). According to Bannink [46], about 40% of the drinking water from Netherlands is derived from surface water. The Dutch water companies are facing problems with the water quality due to contamination by herbicides used to eliminate ruderal plants. These data serve as alerts for the presence of herbicides and their degradation products in drinking water, pointing out the need for the development of new treatment systems that could be more efficient to eliminate this class of contaminants.

According to Ying and Williams [40], organic herbicides, when in aquatic ecosystems, can be distributed in several compartments depending on their solubility in water. These compartments include water, aquatic organisms, suspended sediment and bottom sediment. The more hydrophilic the organic pesticide, the more it is transported to the aqueous phase, and the more hydrophobic a pesticide is, the more it will be associated to the organic carbon of the

suspended and bottom sediment [47]. The sorption of the herbicides in sediments in suspension can reduce the degradation rate of the herbicides in water, and the movement of the sediment in suspension can transport the pesticides from one place to another, entering into the tissue of organisms or settling on the bottom [40].

A study conducted by Jacomini et al. [48] evaluated the contamination of three matrices (water, sediment and bivalve molluscs) collected in rivers influenced by crops of sugar cane in São Paulo State-Brazil. In this study, the authors observed that the highest concentrations of residues of the herbicide ametrin were present in the sediment, showing the persistence of this compound in the sediments of rivers and its potential to mobilize between the compartments of the aquatic system, such as water and biota.

When the herbicides are dispersed in the water or sediments in suspension of the rivers, they can end up in other ecosystems such as estuaries. Duke et al. [49], when studying the effect of herbicides on mangroves of the Mackay region, found out that diuron, and even other herbicides, are potentially responsible for the mangrove dieback. According to the authors, the consequences for this death would be the impoverishment of the quality of the coastal water with an increase of the turbidity, nutrients and sediment deposition, problems in the fixation of seedlings and consequent erosion of the estuaries.

In a review conducted by Jones [50], the author highlights the contamination of marine environments by herbicides (such as diuron), discussing that the contamination of these environments can occur by transport of these substances of agricultural or non cultivated areas (roadsides, sports fields, train tracks), runoff by storms and tailwater irrigation release), pulverizations and accidental spills. These contaminations mean that the photochemical efficiency of intracellular symbiotic algae of the coral, in long term, may be compromised, leading to a loss in the symbiotic relationship of the coral with the algae and a consequent bleaching of corals. Still considering the marine ecosystem, Lewis et al. [51] verified that the runoff of pesticides from agricultural areas influence the health of the Great Barrier Reef in Australia and can disturb this sensitive ecosystem.

Considering the prior literature, it is likely possible that the effects of herbicides do not occur only at the places that they are applied but also in places distant from their application. Moreover, herbicides can induce alterations in non-target organisms, altering the survival and the equilibrium of the ecosystems, whether they are aquatic or terrestrial. Thus, much care must be taken when introducing these substances into the environment and more studies should be conducted in order to thoroughly understand the environmental consequences that herbicides can cause.

## **2. The effects of herbicides using different bioassays and test-organisms**

Many studies have evaluated the impact of different chemical classes of herbicides using different doses, organisms and bioassays, focusing on toxic, cytotoxic, genotoxic, mutagenic, embryotoxic, teratogenic, carcinogenic and estrogenic effects.

With respect to the toxicity, some herbicides pose major concerns when applied in regions close to water resources due to their highly toxic potential to many aquatic organisms [52].

Biological tests of toxicity and mutagenicity are, according to Moraes [53], indispensable for the evaluation of the reactions of living organisms to environmental pollution and also for the identification of the potential synergistic effects of several pollutants. The impact that toxic materials can promote in the integrity and function of DNA of several organisms has been investigated [54]. Several biomarkers have been used as tools for the detection of the toxic, genotoxic and mutagenic effects of pollution. Among them we can cite the presence of DNA adducts, chromosome aberrations, breaks in the DNA strands, micronuclei formation and other nuclear abnormalities, besides induction of cell death [55].

Most of the tests used to detect the mutagenic potential of chemical substances are based on the investigation of possible inductions of chromosome damages such as structural alterations, formation of micronuclei, sister chromatid exchanges, assessment of mutant genes or damages in the DNA, using different test organisms, such as bacteria, plants and animals, both *in vitro* and *in vivo* [56].

According to Veiga [57], it is possible to estimate the genotoxic, mutagenic, carcinogenic and teratogenic effects of agrochemicals by relatively simple methods. Several studies have been carried out by several researchers concerned with the harmful effects of pesticides in an attempt to verify their possible physiological [58, 59], mutagenic [7, 8, 60, 61, 62] and carcinogenic effects [63].

The interaction between different methods of evaluating the toxic, genotoxic and mutagenic potential provides a more global and comprehensive view of the effect of a chemical agent. For the monitoring of organisms exposed to chemical agents, the chromosome aberration test, micronucleus test and comet assay have been widely used [64]. A few studies also have shown the toxic effects of chemicals, by cell death processes, both necrotic and apoptotic [65].

According to Kristen [66], the dramatic expansion in the production of xenobiotic compounds by anthropogenic activities has compromised the environment by the introduction of millions of chemicals with toxic potential to biological systems.

Cytogenetic tests are adequate to identify the harmful effects of substances, in their several concentrations and different periods of exposure. These tests, generally performed with test organisms, are commonly applied in biomonitoring to the extent of pollution and in the evaluation of the combined effects of toxic and mutagenic substances on the organisms in the natural environment [53]. Micronuclei assays are efficient to assess the mutagenic activity of herbicides both in laboratorial and field assays [67]. The comet assay can be used to evaluate damages in proliferating cells or not, in *in vitro* or *in vivo* tests and can be applied with the purpose of genotoxicological analyses [68]. According to these same authors, these tests are considered one of the best tools to biomonitor several chemical compounds, including herbicides. According to Ribas et al. [69], the simplicity, reproducibility and rapidity of the comet test, associated to the ability of this assay in evaluating damages in the DNA, makes this technique highly applicable to environmental genotoxicology.

The toxic, cytotoxic, genotoxic, mutagenic, embryotoxic, teratogenic carcinogenic and estrogenic effects caused by herbicides on various organisms could be exemplified by studies as described below.

## 2.1. Atrazine

Atrazine is a triazinic herbicide, classified as moderately toxic of pre- and post-emergence, used for the control of weeds in crops of asparagus, corn, sorghum, sugarcane and pineapple [70]. According to Eldridge et al. [71], triazinic herbicides are among the most used pesticides in agriculture due to their ability to inhibit the photosynthesis of weeds in crops [16].

Triazine herbicides are extensively used in the United States to control grass, sedge and broadleaf weeds during the cultivation of maize, wheat, sorghum, sugarcane and conifers [72]. In Brazil, these herbicides are widely used on crops of sugarcane and maize. Due to the widespread use of triazine herbicides in the agriculture and, therefore, its high exposure potential for humans, the United States Environmental Protection Agency (USEPA) has conducted a special review on the published and non published data of several triazine herbicides [73]. According to Nwani et al. [22], the herbicide atrazine is widely used in crops worldwide. The dangers, both toxic and genotoxic of this herbicide have been revised; however, there is an urgent need for more detailed studies on the mode of action of this compound. Atrazine has been tested in several systems, but there are shortcomings in relation to certain tests performed and some evidences of the genotoxic effects, *in vivo*, still need to be confirmed [74].

Several studies using the test system *Aspergillus* have shown that atrazine is not mutagenic to these organisms [75, 76, 77], although it is considered mutagenic for other test systems such as *Drosophila melanogaster* [78, 79]. According to Ribas et al. [74], atrazine was responsible for a significant frequency of aneuploidies in *Neurospora crassa*, given by the chromosomal non-disjunction in *Aspergillus nidulans*, and by the induction of loss of sexual chromosomes in *Drosophila melanogaster*.

Sorghum plants treated with atrazine presented an increase in the number of their chromosomes, multinucleated cells, aneuploidy and polyploidy, and abnormalities in the mother cells of the pollen grain, which suggests that this herbicide interferes in the stability and also in the meiosis [80].

Popa et al. [70] observed that atrazine, when applied in high concentrations in maize seedlings, can induce chromosome breaks, visualized by the presence of single and paired chromosome fragments; a high frequency of chromatids and chromosome bridges; lagging chromosomes and presence of heteropolyploid or polyploid cells. Grant and Owens [81] showed that atrazine induced chromosome breaks (in mitosis and meiosis) in the species *Pisum sativum* and *Allium cepa*.

Hayes et al. [82] investigated the effect of the herbicide atrazine on wild leopard frogs (*Rana pipens*), in different regions of the United States. The authors observed that a great percentage of males exposed to the herbicide presented abnormalities in the gonads, such as development



retardation and hermaphroditism. This effect can, in long term, lead to a decline in the amphibian population of the sites contaminated with this herbicide.

According to Gammon et al. [83], some publications have reported a possible feminization of frogs, both in laboratorial assays and field studies. This effect is mainly due to the action of the enzyme aromatase; however, published research not shown the measures of this enzyme. Thus, there are doubts about the feminization theory, except for the studies that presented a great number of frogs with morphological alterations related to very high levels of atrazine.

Nwani et al. [22] evaluated the genotoxic and mutagenic effects of the herbicide Rasayanzine, whose active ingredient is atrazine, using the comet assay and micronucleus test, in erythrocytes and gill cells of the fish *Channa punctatus*. By the data analysis of the two cell types, significant effects for all the concentrations (4.24, 5.30 and 8.48 mg/L) and exposure periods tested (1, 3, 5, 7, 14, 21, 28 and 35 days) were observed. The highest damages were observed for the highest concentrations and exposure times, showing the genotoxic and mutagenic dose-response potential of atrazine for the aquatic organism. Furthermore, it was found that gills were more sensitive to the action of the herbicide, when compared to erythrocytes. From the results obtained, the authors suggested a careful and judicious use of the herbicide atrazine in order to protect the aquatic ecosystems and human population.

A study carried out by Çavas [84] compared the genotoxic effects of the active ingredient atrazine and its commercial formulation Gesaprim, in the concentrations of 5, 10 and 15 µg/L, by the comet assay and micronucleus test, in erythrocytes of the fish gibel carp (*Carassius auratus*). The results showed that there was a significant increase in the frequencies of the micronuclei and DNA strand breaks in the erythrocytes treated with all the concentrations of the commercial formulation of atrazine, showing the genotoxic and mutagenic potential of Gesaprim for this species of fish. While the commercial formulation presented a high genotoxic potential, the assays showed that the active ingredient atrazine is not genotoxic, suggesting that the adjuvants present in Gesaprim must be the responsible for the genotoxic effects observed in this species of fish. Despite the comparative analysis of the genotoxicity between the active ingredient and the commercial product has showed to be a very effective tool for the discovery of genotoxic environmental risks, it is not easy to determine the exact identity of the products used as adjuvants and of the agents of surface action of pesticides due to the existence of the patent protection system.

Atrazine has also been tested to evaluate the ability to induce cytogenetic damages in rodents. Meisner et al. [85] submitted rats to 20 ppm of atrazine (by water ingestion) and did not observe, after exposure to the herbicide, an increase in the number of chromosome aberrations. In a similar study, Roloff et al. [86] reported that there was no significant increase of chromosome aberrations in cells of rat bone marrow, when they were fed with 20 ppm of atrazine.

Wu et al. [87] assessed the embryotoxic and teratogenic effects of atrazine, at the doses of 25, 100 and 200 mg/Kg/day, in Sprague-Dawley rats. Prenatal exposure to the highest dose of the herbicide tested caused hypospadias in 10.23% of male newborn rats, and the lowest dose induced diverse embryotoxic damages in some individuals. According to Modic et al. [88], high doses of atrazine (50 or 200 mg/kg/day), administered daily in male Wistar rats at 60 days

of age, promoted alterations in the levels of several hormones in the serum of these individuals, observed by slight increases in the levels of androstenedione testosterone, estradiol, estrone, progesterone and corticosterone, quantified by radioimmunoassay.

To obtain more concise data on the genotoxicity of triazine herbicides, Tennant et al. [89] used the comet assay methodology, which showed to be highly sensitive for the detection of low rates of damages in the DNA. According to these authors, the comet assay showed that atrazine induced a small increase in the damages in the DNA in leukocytes of rats. Moreover, by the comet assay, Clements et al. [90] reported that atrazine induced a significant increase in the frequencies of damages in the DNA of erythrocytes of bullfrog tadpoles, noting the genotoxic potential of this herbicide for this species of amphibian, from the concentration of 4.8 mg/L.

Studies about the cytotoxicity, genotoxicity and mutagenicity of the atrazine herbicide (oral gavage - dose 400 mg/kg/day), carried out by Campos-Pereira et al. [91], have shown the induction of lipid peroxidation and liver damage, death of hepatocytes, and micronucleus formation in exposed Wistar rats. Tests performed by Ventura et al. [8] showed that the same triazine pesticide was able to induce significant DNA fragmentation when using the comet assay, and nuclear alterations and micronuclei using the micronucleus test in *Oreochromis niloticus* (Nile tilapia) erythrocytes exposed to different concentrations of atrazine (6.25, 12.5, 25 µg/L), thus corroborating the studies performed by Campos-Pereira et al. [91].

Ruiz and Marzin [92] assessed the genotoxic and mutagenic effects of the herbicide atrazine by two *in vitro* assays (*Salmonella* assay and SOS Chromotest), one to detect bacterial mutagenicity and the other to verify primary damages in the DNA. The assays were carried out both in the absence and in the presence of S9 fractions from rat liver homogenate (Sprague-Dawley). The authors found that the herbicide atrazine did not present genotoxic potential neither to the *in vitro* test with *Salmonella*/microsome nor by the SOS Chromotest, both in the absence and in the presence of the S9 fractions, when the strains were exposed to atrazine.

*In vitro* studies, performed with human lymphocytes, treated with 0.10 ppm of atrazine, detected a slight increase in the chromosome aberrations rates [85]. However, for concentrations below 0.001 ppm of this herbicide, chromosome aberrations were not detected [86] Lioi et al. [93] observed a small increase in the number of sister chromatid exchange but a great increase of chromosome aberrations in human lymphocytes exposed to atrazine. Meisner et al. [94] observed a significant increase in the frequency of chromosome breaks in human blood cells exposed to 1 ppm of the herbicide atrazine.

The genotoxicity of herbicides, such as atrazine, has also been evaluated by the comet assay by the use of human blood lymphocytes. According to Ribas et al. [69], blood cells treated with the herbicide atrazine, at concentrations of 50-200 µg/l, showed an extensive migration of DNA, mainly at concentrations of 100 and 200 µg/l.

In mammalian test systems, submitted to the action of the herbicide atrazine, most of the results seem to be negative, except for the results of Loprieno and Adler [95], who obtained a significant increase in the frequency of chromosome aberrations in bone marrow cells of rats, and the data obtained by Meisner et al. [94], who described an induction of chromosome aberrations in cultured human lymphocytes. While the results from bacteria and mammal test



systems are almost all negative, atrazine exhibits clear mutagenic effects in different plant test systems, by inducing chromosome aberrations in *Hordeum vulgare* and *Vicia faba* [96, 97], in *Zea mays* [98], in *Sorghum vulgare* [99] and in *Allium cepa* [62]; sister chromatid exchanges in maize [100]; and point mutation in maize [98].

Studies performed by Zeljezic et al. [101] had already reported that atrazine does not present genotoxicity or capacity to induce apoptosis or necrosis in human lymphocytes, while the treatment of these cells with the commercial formulation, Gesaprim, significantly increased the rates of damages in DNA, observed by the comet assay. Srivastava and Mishra [102] observed results that are in agreement with the findings of Zeljezic et al. [101] and Çavas [84], in which the exposure to different concentrations of Gesaprim inhibited the mitotic index and increased the frequencies of micronuclei and chromosome aberrations in somatic cells of *Allium cepa* and *Vicia faba*.

## 2.2. Atrazine and butachlor

Toxic effects of atrazine, alone or associated with the herbicide butachlor, for the freshwater species such as the green alga *Scenedesmus obliquus* and the cladoceran *Daphnia carinata*, were evaluated, showing values of 96 h-EC50 for *S. obliquus* (atrazine= 0.0147 mg/L and butachlor= 2.31 mg/L, and of 48h-LC50 for *D. carinata* (atrazine= 60.6 mg/L and butachlor= 3.40 mg/L) [20]. These results suggest that atrazine has a highly toxic potential for *S. obliquus* and slightly toxic for *D. carinata*, while butachlor exhibits a moderate toxic potential for both organisms. Now, the analysis of the mixture atrazine-butachlor allowed the authors to verify that the toxic effects were significantly antagonistic for *S. obliquus*, and that there was no significant synergism for *D. carinata* [20].

## 2.3. Atrazine, simazine and cyanazine

Simazine and cyanazine, as well as atrazine, are widely used as triazine herbicides of pre- and post-emergence weed control, whose residues have been carried to the source of drinking water of several agricultural communities. These compounds also present a potential risk to humans, mainly due to their presence in food [103]. Studies on the effect of atrazine, simazine and cyanazine performed by Kligerman et al. [104], found that there was not a significant increase in the sister chromatid exchanges and chromosome aberrations in cultured human lymphocytes exposed to these herbicides, up to the solubility limit in aqueous solution using 0.5% of dimethyl sulfoxide. However, Adler [105] observed that doses of 1500 and 2000 mg/Kg of atrazine, administered by oral gavage in rats, induced dominant lethal mutations and chromatin breaks in the bone marrow of these organisms.

Kligerman et al. [103] observed that the association of the herbicides atrazine, simazine and cyanazine did not induce micronuclei in polychromatic erythrocytes of bone marrow of female rats (C57B1/6) exposed by intraperitoneal injection, even when very high doses of these herbicides were administered (125, 250 and 500 mg/Kg of atrazine; 500, 1000 and 2000 mg/Kg of simazine; 100, 200 and 400 mg/kg of cyanazine), showing an absence of genotoxic potential of these compounds for the organism tested.

On the other hand, Hrelia et al. [106] showed that males and females of Sprague-Dawley rats exposed by oral gavage to doses of 56, 112 and 224 mg/kg of cyanazine, did not present significant increases in chromosome aberrations.

Taets et al. [107] evaluated the clastogenic potential of environmental concentrations of the triazine herbicides simazine (0.001 to 0.004  $\mu\text{g/mL}$ ), cyanazine (0.003 to 0.012  $\mu\text{g/mL}$ ) and atrazine (0.003 to 0.018  $\mu\text{g/mL}$ ), in Chinese Hamster Ovary (CHO) cells, using flow cytometry assay. The authors proved the clastogenic action for the herbicides atrazine and cyanazine, proven by the high indices of damages in the cells exposed to atrazine and by the significant frequencies of damages observed in the cells exposed to cyanazine.

#### 2.4. Terbutryn

The herbicide terbutryn is an s-triazine herbicide used pre- and post-emergence and widely used worldwide as an agent to control grass, sedge, and broadleaf weeds in vegetables, cereals and fruit trees. It is an herbicide persistent in the environment, which tends to dislocate by the flow of water and leachate [108].

An *in vitro* study performed by Moretti et al. [108] investigated the genotoxicity of the herbicide terbutryn, by analyzing the relationship between the cytogenetic damage, evaluated by the assays of SCE (sister chromatid exchanges) and MN (micronucleus), and the primary damage in the DNA, assessed by the comet assay, in leukocytes newly-isolated from peripheral human blood. The results showed that terbutryn did not produce significant increases of SCE or MN, both in the absence and in the presence of the metabolic activation system from rat liver (S9 fraction), although terbutryn has induced primary damages in the DNA in a more pronounced form in the absence of S9. The apparent lack of sensitivity of the assays of SCE and MN test for the genotoxicity of terbutryn, in comparison to the comet assay, can be attributed to the generation of specific types of damages, since the SCE and MN are determined in proliferative cells and are sensitive indicators of lesions that survive for, at least, one mitotic cycle, while the comet assay identifies repairable lesions in the DNA of on resting (G0) cells. According to these results, the authors suggest that terbutryn must be considered a genotoxic compound.

#### 2.5. 2,4-D (2,4-dichlorophenoxyacetic acid)

The 2,4-D (2,4-dichlorophenoxyacetic acid) is an herbicide from the group of the polychlorinated aromatic hydrocarbons that has been widely used throughout the world [109] since 1944, to control broadleaf weeds and woody plants [110]. Its action mimics the auxin of plants [111]. According to Martínez-Tabche et al. [112], this herbicide mimics the action of the hormone indole acetic acid, when used in small quantities but it is highly cytotoxic in high concentrations.

According to Ateeq et al. [113], the increase in the frequency of micronuclei and altered cells was significant, when erythrocytes of catfish (*Clarias batrachus*) were analyzed, after exposure to the herbicides 2,4-D and butachlor. There was a positive dose-response relationship in all exposures to the two herbicides and in all exposure periods tested.

Studies carried out by Suwalsky et al. [114] in nerve cells of *Caudiverbera caudiverbera* demonstrated the toxicity of the herbicide 2,4-D. The authors observed a reduction in the dose-dependent response to nerve stimulation in the simpact junction of the frog when they were exposed to this herbicide. This reduction is probably due to a mechanism of lipid perturbation and interference in the properties of the plasma membrane, such as protein conformation and/or interaction with protein receptors, which leads to an inhibition of the glandular chloride channel from the mucosal skin of this test organism.

According to Gómez et al. [115], the main and most common entrance route of 2,4-D in fish is through gills. This herbicide can cause several adverse symptoms to these organisms, such as bleeding, increased damage to the kidneys and renal functions, as well as hepatic degeneration.

Martínez-Tabche et al. [112] evaluated the toxicity of different concentrations of the herbicides 2,4-D and paraquat (0, 5, 75 and 150 mg/L), using several assays (acute lethality test, lipid peroxidation assay by quantification of MDA – Malondialdehyde – and comet assay) in rainbow trout (*Oncorhynchus mykiss*). For the acute lethality tests, it was observed a more evident toxic action for the organisms exposed to the treatment of 24 h with the herbicide paraquat, which presented high indices of mortality, analyzed by the values of  $LC_{50}$  ( $LC_{50}$  of paraquat = 0.084 mg/L;  $LC_{50}$  of 2,4-D = 362.38 mg/L). The authors also showed that individuals exposed to the two higher concentrations of both herbicides had apnoea and white spots in their scales. All concentrations of 2,4-D and paraquat induced a significant increase in the DNA damages and the amount of MDA in the gills exposed.

González et al. [116] proved the genotoxicity of 2,4-D due to a significant increase of SCE in CHO cells treated with the concentrations of 2 to 4 ug/mL of this herbicide. Madrigal-Bujaidar et al. [117] also showed the genotoxic potential of 2,4-D, due to a clastogenic effect of this herbicide at the doses of 100 and 200 mg/Kg, detected by a significant increase of SCE in bone marrow cells and germ cells of rats. Soloneski et al. [118] studied the genotoxic effects of different concentrations (0, 10, 25, 50 and 100 mg/mL) of the herbicide 2,4-D (2,4-dichlorophenoxyacetic) and its commercial derivative 2,4-D DMA (Dimethylamine 2,4-D salt), by the SCE assay and analyses of cell cycle progression and mitotic index human lymphocytes maintained in culture, in the presence (human whole blood - WBC) and absence (plasma leukocyte cultures - PLC) of erythrocytes. These compounds did not induce significant frequencies of SCE and only the concentration of 100 mg/mL of 2,4-D caused alterations in the progression of the cell cycle in PLC, while the different concentrations of 2,4-D and 2,4-D DMA induced a significant increase in the frequency of SCE and a significant delay in the cell proliferation rates in WBC. Moreover, both 2,4-D and 2,4-D DMA presented a dose-response inhibition of the mitotic activity in PLC and WBC. Based on these results, the authors concluded that the herbicide and its commercial derivative presented genotoxic potential, which was higher in the presence of human erythrocytes.

Morgan et al. [119] showed, by embryotoxicity and teratogenicity assays carried out with *Xenopus* (FETAX - frog embryo teratogenic assay – *Xenopus*), that high concentrations of 2,4-D, induce potentially more embryotoxic effects than teratogenic in frog embryos, demonstrated by the values of EC50 and LC50 of 245 mg/L and 254 mg/L, respectively, and by the Teratogenic Index of 1.04. Moreover, the same authors compared the teratogenic action of the

herbicide atrazine in relation to 2,4-D, showing that atrazine is potentially more teratogenic than 2,4-D, for frog embryos.

The estrogenic potential of 4 herbicides (triclopyr; 2,4-D; diquat dibromide and glyphosate), was evaluated by the *in vivo* de vitellogenin assay with rainbow trout. A significant estrogenic potential was shown for 2,4-D, since it induced a 93 fold increase in the levels of plasma vitellogenin of the fish treated with this herbicide during 7 days [120].

## 2.6. Glyphosate

Glyphosate is a non-selective organophosphorus, broad spectrum, post-emergence herbicide, widely used in agriculture, mainly to control grasses, sedges, and broadleaf weeds [121]. Its action occurs by the inhibition of the biosynthesis of aromatic amino acids [122]. Its main mode of action is by the inhibition of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), which is essential in plants for the synthesis of the referred amino acids. Since this enzyme is absent in animals, this herbicide should be relatively non toxic for these organisms [123]. There are many conflicting data on the toxicity of glyphosate and its commercial formulations.

According to Solomon and Thompson [124], environmental toxicology of glyphosate has been extensively reviewed by a series of international regulatory agencies. According to the authors, as glyphosate binds strongly with organic matter, it is considered immobile in soils and sediments. This binding also removes glyphosate from water, reducing efficiently, the exposure of aquatic organisms. As the acute exposures are most likely to occur, the measures of effect are the most adequate for the purpose of risk assessment. However, in general, the authors affirm that glyphosate presents a low potential of acute toxicity for wild animals, including mammals, birds, fish and aquatic invertebrates.

Williams et al. [125] carried out a critical review on the toxicity of the herbicide RoundUp™ and of its active ingredient glyphosate. The analysis of the toxicity data, carried out by pattern tests and evaluation criteria, indicated that there is no evidence that glyphosate causes direct damages in the DNA, both in assays performed *in vitro* and *in vivo*. The authors concluded that Roundup™ and its components do not represent a risk for the induction of inheritable/somatic mutations in humans. Furthermore, the authors assert that, by the studies performed, glyphosate is not carcinogenic or teratogenic, nor does it cause significant adverse effects in the reproduction, development or in the endocrine system of humans and other mammals and, therefore, does not represent a risk for the health of human beings.

A study on the impact of the herbicide glyphosate and its commercial formulation Roundup™, in three microorganisms of food interest (*Geotrichum candidum*, *Lactococcus lactis subsp. cremoris* and *Lactobacillus delbrueckii subsp. bulgaricus*), showed that Roundup™ has an inhibitory effect on the microbial growth and a microbiocide effect at concentrations lower than the recommended for agricultural use. It was also observed in this study that glyphosate did not induce significant toxic effects for the three microorganisms studied. These differences between the toxic actions resulted from Roundup™ and glyphosate could be explained by a possible amplified effect of the commercial formulation due to the presence, according to Cox

[126] of adjuvants, such as polyethoxylated tallowamine (POEA), used for a better stability and penetration of the chemical compound [127].

Relyea [128] assessed the toxic potential of environmentally relevant concentrations of glyphosate on three species of tadpoles (wood frog [*Rana sylvatica* or *syloaticus Lithobates*], leopard frog [*Rana pipiens pipiens* or L.], and American toad [*Bufo americanus* or *Anaxyrus americanus*]), by morphological analysis of individuals, before and after the application of the herbicide, showing that there is a significant induction of morphological alterations in the tadpoles of the three species. Specifically in the case of the wood frog and leopard frog, the exposure to the chemical compound has led to an evident alteration of the size of the tadpole tail, suggesting that the herbicide could be activating physiological mechanisms of development that are normally used as defence responses against predators. These results showed that glyphosate can have widespread and relevant effects on non target species, contradicting other studies, such as the one performed by Solomon and Thompson [124], who affirmed the inexistence or irrelevance of the toxicity of this compound on organisms and the environment.

Studies on the genotoxic potential of the active ingredient glyphosate, present in the commercial formulation Roundup, were performed on the roots of smooth hawkbeard (*Crepis capillaris* L.), in the concentrations of 0.05, 0.1, 0.5 and 1.0% of the active ingredient and for polychromatic erythrocytes (PCEs) of the bone marrow of C57BL rat, at doses inferior to half the LD<sub>50</sub> (1080 mg/Kg). In these studies the chromosome aberrations assay and micronucleus test were used, which showed that this chemical compound did not induce significant responses for any of the biological systems tested [129].

Martini et al. [123] studied the effects of the commercial formulation of glyphosate in the proliferation, survival and differentiation of the 3T3-L1 fibroblasts (a mammal cell line), by the cell viability test with Trypan, MTT test, enzymatic activity assay of caspase-3 and staining assay with annexin-V and propidium iodide. The results showed that glyphosate inhibits the cell proliferation and induces apoptosis in a dose-dependent way, besides decreasing significantly the ability of the fibroblasts to differentiate to adipocytes. These data suggest the occurrence of important cell damages mediated by the action of this herbicide, indicating that glyphosate presents a potential risk factor for human health and the environment.

Dallegrave et al. [130] evaluated the teratogenicity of the herbicide glyphosate, marketed in Brazil as Roundup (36% of glyphosate and 18% of the surfactant polyoxyethyleneamine), to females of Wistar rats. The females treated orally with three different doses of glyphosate (500, 750, 1000 mg/Kg) from the 6<sup>th</sup> to the 15<sup>th</sup> day of gestation. After performing caesarean sections on day 21 of gestation, the number of corpora lutea, implantations, live and dead foetuses and reabsorptions, as well as the external malformations and skeletal malformation were recorded and analyzed. It was observed a mortality rate of 50% of the females treated with the highest concentration of glyphosate; the authors verified that there was a dose-response relationship directly proportional to the increase in the number of skeletal alterations found. These results led the authors to conclude that the commercial formulation of glyphosate (Roundup) is toxic for females of Wistar rats and is able to induce a delay in the fetal skeletal development of this species. It is important to consider that the toxicity and teratogenicity observed can result from both the action of glyphosate as well as the surfactant present in the commercial formulation.

The oral administration of high doses of glyphosate (3500 mg/Kg) in Charles River COBS CD rats, between the 6<sup>th</sup> to the 19<sup>th</sup> day of pregnancy, and in rabbits, between the 6<sup>th</sup> to the 27<sup>th</sup> day of pregnancy, showed significant indices of maternal mortality for both species, as well as increase in the number of foetuses with reduced ossification of sternebrae [131], proving the toxicity and teratogenicity of this concentration of the herbicide for the organisms tested.

### 2.7. 2,4-D and glyphosate

Relyea [132] performed a study to observe the impact of two herbicides (glyphosate and 2,4-D) in the biodiversity of aquatic communities containing algae and more 25 species of animals. In this study the author observed that 2,4-D did not cause great impacts in the community and this is in agreement with previous studies that showed that this substance presents high LC-50 for several species. However, glyphosate had great impact in the community, causing a decrease of 22% of the species richness, while 2,4-D did not cause effects on this diversity. The authors also observed that neither of the two herbicides caused reduction in the periphyton biomass.

### 2.8. Diquat

Reglone is a bipyridylum herbicide, whose active ingredient is diquat (1,1'-ethylene -2,2'-ipyridyl dibromide), and of foliar application, used to eliminate weeds of different crops [133]. Reglone, in the concentrations tested (0.005, 0.01, 0.05 and 0.1% of the active ingredient for *Crepis capillaris* L.; 34.17 and 8.5 mg/Kg for mouse bone marrow polychromatic erythrocytes - PCEs), did not induce chromosome aberrations in any test system but promoted an increase in the frequency of micronuclei in both plant cells and PCEs [129], and thus is considered a potential mutagenic herbicide for these test organisms.

### 2.9. Pendimethalin

The herbicide Stomp 330, belongs to the dinitroanilines class, whose active ingredient is pendimethalin [N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine], it is applied as a systematic selective herbicide of the soil [133]. The responses of the two test systems for Stomp were very different: the concentrations tested (0.005, 0.1, 0.2 and 0.4% of the active ingredient for *Crepis capillaris* L.; 122.2, 244.5 and 489 mg/Kg for rats - PCEs) did not cause significant increases in the frequencies of chromosome aberrations in plant cells, but increased its incidence in rat cells, moreover, it induced an increase in the frequency of micronuclei in both test systems. This could be explained by the proven aneugenic effect of this herbicide, since all the concentrations tested produced C-mitoses in the assays with PCEs [129].

### 2.10. Paraquat

Paraquat (1,1'-dimethyl-4,4'-bipyridium dichloride) is a non-selective herbicide with fast action, widely used worldwide, mainly in the pre-harvest of cotton and potato and also to control a broad spectrum of weeds [134, 135, 136]. According to Tortorelli et al. [134], paraquat is able to modify the activity of several enzymes of fish, affecting the cardiac contraction and



opercular ventilation, effects that can alter the initial development of these organisms. According to Tomita et al. [137], paraquat causes oxidative stress in different species of fish by generating elevated levels of superoxide ion.

A study conducted by D'Souza et al. [138] evaluated the toxicity of the herbicide paraquat for germ cells of male Sprague-Dawley rats by dermal exposure to this chemical. The authors verified that paraquat, even at low doses, significantly reduced the amount of spermatozoa, increased the frequency of spermatozoa bearing abnormalities and the mortality rate of these germ cells, as well as affected the mobility of the spermatozoa of the individuals studied, showing that the herbicide is a cytotoxic and genotoxic agent for the germ cells of this organism.

Hanada [136], analyzing the karyotype of species of *Rana ornativentris*, after exposure for 6 hours to the herbicide paraquat at the concentrations of  $10^{-8}$  to  $10^{-6}$  M, showed that this compound is able to induce genotoxic effects in this organism. The author observed that paraquat promoted, in a dose-dependent manner, a significant increase in the quantity of chromosome breaks in leukocytes of this test organism, suggesting that this species of anuran is highly sensitive to the genotoxic action of the herbicide.

According to Bus et al. [139], the genotoxic action of paraquat may be associated with the transference of a single electron of reduced oxygen to paraquat, forming superoxide ions. The singlet oxygen can be formed from the superoxide ion and subsequently react with lipids to form hydroperoxides and fatty acids. According to Tanaka and Amano [140], lipid peroxidation is responsible for the origin of several chromosome aberrations. Bauer Dial and Dial [141] still affirm that the oxidative stress induced by paraquat may be related to the teratogenic action of this compound to embryos and tadpoles of anurans.

Speit et al. [142] evaluated the genotoxic potential of the herbicide paraquat in Chinese hamster V79 cells, by chromosome aberrations and comet assays. Using a modified protocol of the comet assay with the modified protein FPG (formamidopyrimidine-DNA glycosylase), a repair enzyme that specifically nicks the DNA at sites of 8-oxo-guanines and formamidopyrimidines, it was not possible to detect oxidative damages in the bases of DNA after treatment with paraquat. Now, when the cells were treated directly on the slides, after lysis (i.e., after the cell membrane barrier has been eliminated), a significant increase in the migration of DNA was observed, only after treatment with high concentrations of the herbicide. Thus, the authors verified that the herbicide induced chromosome aberrations but was not able to induce relevant DNA lesions to promote mutations in the gene HPRT in cultured V79 cells.

Ribas et al. [135] assessed the cytotoxic, genotoxic and mutagenic potentials of different concentrations of the herbicide paraquat (0, 1, 5, 25, 50, 100, 250, 500, 1000, 2000 and 4000  $\mu\text{g}/\text{mL}$ ), by the assays of SCE, chromosome aberrations and micronuclei, in lymphocytes maintained in culture. The results showed that paraquat is an agent that induces cytotoxicity for lymphocytes, since it promoted the reduction in the nuclear division rate in all the concentrations tested and a significant decrease in the cell proliferation rates, when the cells were exposed to the highest concentration of the herbicide. In relation to the genotoxicity, the herbicide induced a significant increase in the frequencies of SCE of the lymphocytes treated, whose damage was not modified by co-treatment with the metabolic activation (S9 fraction of

rat liver), but the data on the chromosome aberrations and micronuclei assays were not significant, which led the authors to conclude that paraquat is an inductor of primary damages in the DNA, although they have not shown that it has a clastogenic action.

A study performed by Hoffman and Eastin [143] evaluated the embryotoxic and teratogenic effects of two insecticides (lindane and toxaphene) and two herbicides (paraquat and 2,4,5-T), by external treatment of eggs of mallard duck (*Anas platyrhynchos*), using concentrations of field application. The authors showed that paraquat was the most significantly embryotoxic compound for this organism, independent of the type of vehicle in which the herbicide was associated, besides proving that paraquat impaired the growth of the organisms and was slightly teratogenic. The  $LC_{50}$  for this species was 1.5 Kg of the active ingredient/hectare in aqueous emulsion and 1 lb/acre in oil vehicles. When the organisms treated with paraquat were compared to the ones exposed to the herbicide 2,4,5-T, they presented little damages and it was observed few individuals bearing severe defects.

### 3. Harmful effects of herbicides on human health

The harmful effects of herbicides on human health are determined by several factors, such as the chemical class of those compounds, dose, time, and exposure route. Herbicides can be toxic to humans at high and lower doses [144]. The prolonged exposure can lead to a number of health effects, including the induction of diseases such as cancer and neurodegenerative [145, 146], reproductive and developmental changes [147] and respiratory effects [148].

Doll and Peto [149] estimated that 35% of all cases of cancer in the U.S. population originate from diet, and the herbicides present in foods are responsible. Estrogenicity assays made by Hernández et al. [150] show that organochlorine pesticides may act as endocrine disruption through more than one mechanism, including agonist or antagonist effects of different receptors. Chloro-s-triazine herbicides, pre-emergent pesticides used worldwide, have been generally considered as chemical compounds of low toxic potential for humans; however, there are many controversies on this issue. According to several international agencies, including the Environmental Protection Agency (EPA), Development for Environmental Assessment Center of the United States and IARC Monographs (International Agency for Research on Cancer), the herbicide atrazine, for example, was classified as a chemical agent probably carcinogenic to humans, although the basis for this conclusion is only evidenced in other animals [151, 152]. Due to the fact that atrazine induce mammary tumours in female Sprague-Dawley rats, the Peer Review Committee of the EPA Office of Pesticide Program (OPP) also concluded that atrazine should be considered in the Possibly Carcinogenic to Humans Group [153]. However, EPA [154] has classified this herbicide as a compound probably non carcinogenic to humans.

Some experimental studies have shown that exposure of humans to high doses of atrazine can result in an increased loss of body weight. However, a great number of epidemiological studies carried out with workers occupationally exposed to triazine herbicides indicate that these compounds do not have carcinogenic potential for these individuals. By analyses of different



studies, it was observed that, although the chloro-s-triazine herbicides interfere in the endocrine responses of different species of mammals, their potential impact on humans seem to be mainly related to reproduction and development and not with human carcinogenesis [155].

Gammon et al. [83] discussed the extensive list of epidemiological studies with the herbicide atrazine, which describes that the carcinogenic potential of this compound to humans is not conclusive, although some studies have indicated a relationship between a high risk of prostate cancer and exposure to the herbicide.

Mladinic et al. [156] evaluated the genotoxic and mutagenic effects of low concentrations of the herbicides glyphosate and terbuthylazine, considered safe and, therefore, considered possible to occur in occupational and residential exposures (ADI – Acceptable Daily Intake, REL – Residential Exposure Level, OEL – Occupational Exposure Level, and 1/100 and 1/16 LD<sub>50</sub> – Lethal Dose 50% - oral, rat), in human lymphocytes, with and without the use of metabolic activation (S9 fraction), by the FSH cytome assay, using pan-centromeric DNA probes to assess the content of micronuclei and other chromatinic instabilities. The authors verified that the frequencies of micronuclei, nuclear buds and nucleoplasmic bridges of cells treated with glyphosate slightly increased after the concentration of OEL 3.5 µg/mL, but no concentration induced an increase of the centromeric signals (C+) or DAPI (DAPI+). Now, the treatment with the herbicide terbuthylazine without metabolic activation showed a dose-response increase in the frequency of micronuclei of the lymphocytes exposed, and the significant data were from the concentration of 0.0008 µg/mL (REL) tested. The concentrations ADI (0.00058 µg/mL), REL (0.0008 µg/mL) and OEL (0.008 µg/mL) of terbuthylazine induced a significant occurrence of micronuclei hybridized with the centromeric probe (C+), regardless the presence or absence of S9, and of nuclear buds containing centromeric signals, only in the presence of S9. By the results obtained, it was suggested that the lowest concentrations of glyphosate do not have relevant harmful effects for the DNA molecule, while terbuthylazine presents a predominant aneugenic potential for the genetic material of human lymphocytes.

Terbuthylazine belongs to the chloro-s-triazine herbicides class, which inhibits the photosynthesis of weeds, by reaching the photosystem II. It is a chemical used for a variety of crops, such as maize, sugarcane, olive and pineapple [157]. Since the banishment of atrazine in European countries in 2006, terbuthylazine was recommended as its substitute. Due to the fact that the herbicide terbuthylazine is suspect of causing diseases in humans, such as non-Hodgkin lymphoma and lung cancer, Mladinic et al. [158] evaluated the effects of prolonged exposure (14 days) to low concentrations of this compound (0.58 ng/ml and 8 ng/ml) in human lymphocytes, using the comet assay and the comet-FISH assay (with the c-Myc and TP 53 genes). Treatment with terbuthylazine induced the migration of fragments of DNA in a significant manner, only for the highest concentration treated. The results showed an impairment of the structural integrity of c-Myc and TP 53, due to the prolonged exposure of human lymphocytes to terbuthylazine. The fact that several copies of TP53 were affected by the herbicide can indicate its ability to negatively interfere in the control of the cell cycle. However, the authors concluded that, for a more detailed assessment of the risk of cancer associated with exposure to terbuthylazine, it should be evaluated the impact of this pesticide on other housekeeping genes and markers.

Mladinic et al. [122] evaluated the genotoxic potential, by the comet assay and FISH, and oxidative damages, by the TBARS lipid peroxidation, of different concentrations of glyphosate (three similar to those observed in residential and occupational exposures and two related to  $LC_{50}$ ) in human lymphocytes. The comet assay showed that the concentration of 580  $\mu\text{g}/\text{mL}$  promoted a significant increase in the tail length, while the concentration of 92.8  $\mu\text{g}/\text{mL}$  caused an increase in the tail intensity, both in relation to the control test. With the addition of the S9 fraction, the tail length was significantly increased for all the concentrations tested. When the lymphocytes were exposed to the three highest concentrations without S9, there was an increase in the frequency of micronuclei, nuclear buds and nucleoplasmic bridges. The addition of a metabolic activation system only promoted a significant increase of the nuclear instabilities for the highest concentration tested. The values of TBARS significantly increased with the increase of the concentrations tested, regardless the presence or absence of the S9 fraction. Due to the fact that dose-dependent effects for all the assays used were not observed, the authors concluded that these concentrations of glyphosate are not relevant for human exposure, since they did not present a significant risk for human health.

According to Mladinic et al. [122], the increase in the number of crops genetically modified used in assays and diagnosis of resistance to glyphosate, may be related to the fact that these crops tolerate increasingly higher concentrations of the active ingredient necessary for an effective control of weeds, which results from the introduction of increasing amounts of glyphosate into the environment. Thus, some epidemiological studies have shown that human exposure to glyphosate present in the environment is correlated to the development of diseases such as the non-Hodgkin lymphoma [159, 160].

According to He et al. [161], paraquat, the second most widely used herbicide in the world, is able to selectively accumulate in human lungs by causing oxidative injury and fibrosis, leading several individuals to death. Chronic exposure to this herbicide is also associated with hepatic lesions, kidney failure and Parkinson's disease [162, 163].

Studies carried out by He et al. [161] evaluated the toxicity of paraquat on BEAS-2B normal cells (human bronchial epithelial cells), showing that it is dose-dependent and results in mitochondrial damages, oxidative stress, death of lung cells exposed, as well as production of cytokines, pro-fibrogenic growth factors and transformation of myofibroblasts. The authors also proved that administration of resveratrol, a polyphenolic phytoalexin naturally produced by several plants, to control bacteria and fungi, was able to inhibit the production of reactive oxygen species, inflammations and fibrotic reactions induced by paraquat, by the activation of the Nrf2 signaling (Nuclear Factor Erythroid-2), revealing a new molecular mechanism for the intervention against oxidative damages and pulmonary fibrosis resulted from the action of toxic chemical compounds.

The study on the influence of a complex mixture of herbicides (atrazine, 2,4-D, alachlor, ciazine and malathion) in workers occupationally exposed to them, was carried out using cytogenetic methods standardly established (chromosome aberrations and micronucleus assay) and the comet assay technique. This assay showed a significant increase in the DNA migration ( $P < 0.001$ ), suggesting that long-term exposure to the pesticides could cause damages in the genome of somatic cells and, therefore, would represent a potential risk to human health [164].

## 4. Conclusion

The authors present in this manuscript the bioassays and the test-systems most commonly used to evaluate the effects of herbicides and the test-organisms to best suit the assessments of herbicide effects. In these considerations, the authors attempted to present the most sensitive and efficient organisms capable of detecting environmental contamination resulting from the action of these chemical agents. Additionally, we present in this paper the need to carry out research aimed at more effective methods to prevent and/or reduce the deleterious effects of such compounds on the environment, the biota potentially exposed, and especially to human health.

In this study it was addressed several studies that used different methodologies, which evaluated the toxicity and action of herbicides on different non-target organisms, including human species. The table below summarizes the main researches addressed in the text.

Herbicide	Test-organism	Endpoint	Results	Tested concentrations	References
Atrazine	Erythrocytes of Nile tilapia ( <i>Oreochromis niloticus</i> )	micronucleus test; comet assay	increase in the DNA fragmentation; induction of micronuclei and nuclear abnormalities in all tested concentrations	6.25, 12.5, 25 µg/L	[8] Ventura et al., 2008
Atrazine	Wild leopard frogs ( <i>Rana pipiens</i> )	toxicity assay	induction of abnormalities in the gonads; developmental delay and hermaphroditism ( $\geq 0.1$ ppb)	0.01, 0.1, 0.4, 0.8, 1, 10, 25, 200 ppb	[82] Hayes et al., 2002
Atrazine	<i>Sorghum vulgare</i>	chromosome aberration assay	induction of multinucleated, aneuploid and polyploid cells; abnormalities in the mother cells of pollen grains; meiotic instability	2.7 Kg a.i./ha	[80] Liang et al., 1967
Atrazine	Human lymphocytes	chromosome aberration assay	increase in the chromosome aberrations frequency at 0.10 ppm	0.01, 1, 0.10 mg/ml	[85] Meisner et al., 1992
Atrazine	Human lymphocytes	chromosome aberration assay; SCE	increase in the frequency of chromosome aberrations; increase in the frequency of sister chromatid exchange in all tested concentrations	5, 8.5, 17, 51 µM	[93] Lioi et al., 1998
Atrazine	Human blood cells	chromosome aberration assay	Significant increase of chromosome breaks	1 ppm	[94] Meisner et al., 1993
Atrazine	Rat	chromosome aberration assay	there was no significant increase in the frequency of chromosome aberrations at 20 ppm	20 ppm	[85] Meisner et al., 1992
Atrazine	Bone marrow cells of rats	chromosome aberration assay	there was no significant increase in the frequency of chromosome aberrations	20 ppm	[86] Roloff et al., 1992
Atrazine	Human lymphocytes	chromosome aberration assay	induction of chromosome aberrations	0.0001 µg/mL	[94] Meisner et al., 1993

Herbicide	Test-organism	Endpoint	Results	Tested concentrations	References
Atrazine	Rat leukocytes	comet assay	increase in the damages in the DNA for 500 mg/Kg	125, 250, 500 mg/Kg	[89] Tennant et al., 2001
Atrazine	Erythrocytes of bullfrog tadpoles	comet assay	significant increase in the DNA damages, from the concentration of 4.8 mg/L	4.8, 19.75, 77, 308 mg/L	[90] Clements et al., 1997
Atrazine	Human lymphocytes	comet assay	significant increase in the DNA damages, mainly at the concentrations of 100 and 200 µg/L	50, 100, 200 µg/L	[69] Ribas et al., 1995
Atrazine	Hepatocytes of Wistar rats	lipid peroxidation assay; micronucleus test	increase in the rates of lipid peroxidation, hepatic damages, death of hepatocytes and induction of micronuclei.	400 ppm	[91] Campos-Pereira et al., 2012
Atrazine	Erythrocytes and gill cels of the fish <i>Channa punctatus</i>	micronucleus test; comet assay	induction of damages in the DNA and micronuclei, in the tested concentrations, in all the exposure periods (from 1 to 35 days), with more significant effects in the highest concentrations and exposure periods; higher sensitivity for gill cells	4.24, 5.30, 8.48 mg/L	[22] Nwani et al., 2011
Atrazine	Erythrocytes of the gibel carp fish ( <i>Carassius auratus</i> )	micronucleus test; comet assay	significant induction of DNA strand breaks and micronuclei, in all tested concentrations of the commercial product (Gesaprim), but there was not a induction of these genotoxic and mutagenic effects for the active ingredient.	5, 10, 15 µg/L	[84] Çavas, 2011
Atrazine	Human lymphocytes	comet assay	significant increase of damage in the DNA exposed to the commercial product Gesaprim, but there was no induction of genotoxicity for the active ingredient atrazine, for all tested concentrations.	0.047, 0.47, 4.7 ug/L	[101] Zeljezic et al., 2006
Atrazine	Somatic cells of <i>Allium cepa</i> and <i>Vicia faba</i>	chromosome aberration assay; micronucleus test	significant inhibition of the mitotic index, significant increase in the frequencies of micronuclei and chromosome aberrations of both test organisms, when exposed to the commercial product Gesaprim, but there was no induction of any significant effects when cells were exposed to the active ingredient atrazine, for all tested concentrations.	<i>A. cepa</i> : 15, 30, 60 mg/L; <i>V. faba</i> : 17.5, 35, 70 mg/L	[102] Srivastava and Mishra, 2009
Atrazine	<i>Salmonella</i> and hepatic cells of Sprague-Dawley rats	<i>Salmonella</i> assay and SOS Chromotest	there was no significant induction of genotoxic damages nor mutagenic	1 – 1000 µg/plate	[92] Ruiz and Marzin, 1997

Herbicide	Test-organism	Endpoint	Results	Tested concentrations	References
Atrazine	Sprague-Dawley rats	embryotoxic and teratogenic tests	induction of hypospadias in male newborns at 200 ppm and diverse embryotoxic damages at 25 ppm.	25, 100, 200 mg/kg/d	[87] Wu et al., 2007
Atrazine	Wistar rats	Radioimmunoassay	alterations in the levels of testosterone, androstenedione, estradiol, estrone, progesterone and corticosterone to 50 or 200 ppm for 60 days	50, 200 mg / kg / day	[88] Modic et al., 2004
Atrazine, Simazine and Cyanazine	Human lymphocytes	chromosome aberration assay and SCE	there was no significant increase of chromosome aberrations and sister chromatid exchanges	0.5, 5, 50 ppb	[104] Kligerman et al., 1993
Atrazine, Simazine and Cyanazine	Polychromatic erythrocytes of the bone marrow of female C57B1/6 rats	micronucleus test	there was no significant induction of micronuclei	0, 125, 250, 500 mg/kg	[103] Kligerman et al., 2000
Atrazine, Simazine and Cyanazine	Chinese Hamster Ovary – CHO – cells	flow cytometry assay	significant induction of chromosome damages by atrazine for the tested concentrations, proven clastogenic potential of cyanazine	0.003 µg/mL, 0.018 µg/mL (atrazine); 0.003 µg/mL, 0.012 µg/mL (cyanazine)	[107] Taets et al., 1998
Atrazine and Butachlor	Green alga <i>Scenedesmus obliquus</i> and cladoceran <i>Daphnia carinata</i>	acute toxicity assay	atrazine is highly toxic for <i>S. obliquus</i> and slightly toxic for <i>D. carinata</i> and butachlor is moderately toxic for both; the toxic effects of the mixture of the herbicides were significantly antagonistic for <i>S. obliquus</i> and there was no significant synergism for <i>D. carinata</i>	<i>S. obliquus</i> : 0, 0.5, 1, 2, 4, 8 mg/L (butachlor) and 0, 0.008, 0.016, 0.032, 0.064, 0.128 mg/L (atrazine) / <i>D. carinata</i> : 0, 1, 1.8, 3, 5, 8 mg/L (butachlor) and 0, 7.5, 15, 30, 60, 120 mg/L (atrazine)	[20] He et al., 2012
Butachlor	Alpine cricket frog ( <i>Fejervarya limnocharis</i> )	chromosome aberration assay	affected the survival, development and metamorphosis time of tadpoles in different concentrations; DNA damage (0.4-0.8 mg/L)	ranging from 0.025 to 3.2 mg/l	[43] Liu et al., 2011
Terbutryn	Human leukocytes	micronucleus test; comet assay; SCE	there was no significant induction of micronuclei and SCE; significant induction of DNA damages for all tested concentrations	0, 5, 10, 50, 100, 150 µg/mL	[108] Moretti et al., 2002
2,4-D	<i>Caudiverbera caudiverbera</i>	toxicity assay	dose-dependent reduction in the response of the simpatic junction to	0.01, 0.1, 1 mM	[114] Suwalsky et al., 1999

Herbicide	Test-organism	Endpoint	Results	Tested concentrations	References
			nerve stimulation due to inhibition of the glandular chloride channel in mucosa skin		
2,4-D	Gills of different species of fishes	toxicity assay	bleeding, renal increase, impairment of the renal functions and hepatic degeneration	400 mg/L	[115] Gómez et al., 1998
2,4-D	Chinese Hamster Ovary – CHO – cells	SCE	significant increase in the sister chromatid exchange at 2 and 4 µg/ml	2, 4, 6, 10 µg/mL	[116] González et al., 2005
2,4-D	Bone marrow and germ cells of rats	SCE	significant increase in the sister chromatid exchange at 100 and 200 ppm, for both cell types	50,100, 200 mg/kg	[117] Madrigal-Bujaidar et al., 2001
2,4-D	Frog <i>Xenopus</i>	FETAX - frog embryo teratogenic assay	significant induction of embryotoxic and teratogenic effects	245 mg/L	[119] Morgan et al., 1996
2,4-D and Butachlor	Erythrocytes of the catfish ( <i>Clarias batrachus</i> )	chromosome aberration assay; micronucleus test	significant increase in the frequency of micronuclei and altered cells in a dose-response manner for both herbicides	2,4-D: 25, 50, 75ppm; Butachlor: 1, 2, 2.5ppm	[113] Ateeq et al., 2002
2,4-D and Paraquat	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	acute lethality test, lipid peroxidation assay by quantification of MDA; comet assay	toxic action more evident for paraquat (high indices of mortality); apnea and white spots in the scales of individuals exposed to the 2 herbicides; increase in the rates of MDA and damages in the DNA after exposure to all concentrations of the tested herbicides	2,4-D: 316, 346, 389, 436, 489 mg/L; Paraquat: 0.055, 0.066, 0.083, 0.116, 0.133 mg/L	[112] Martínez-Tabche et al., 2004
2,4-D and 2,4-D DMA	Humanh lymphocytes and erythrocytes	SCE; analysis of the cell cycle progression and mitotic index	alterations in the cell cycle and induction of SCE for some concentrations only with more significant genotoxic effects for erythrocytes	10, 25, 50, 100 µg/mL	[118] Soloneski et al., 2007
2,4-D; Triclopyr; Diquat dibromide; glyphosate	Rainbow trout ( <i>Oncorhynchus mykiss</i> )	Vitellogenin estrogenic assay	significant increase in the levels of vitellogenin of the plasma of fishes exposed to 2,4-D	0.11, 1.64, 2.07, 1.25 mg/L	[120] Xie et al., 2005
Glyphosate	<i>Geotrichum candidum</i> , <i>Lactococcus lactis</i> subsp. <i>Cremoris</i> ; <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i>	microbial growth assay	inhibition of microbial growth by the commercial product Roundup; microbiocide effect at concentrations lower than the recommended by agricultural use for the commercial product Roundup; non induction of significant toxic effects for the three microorganisms by the active ingredient glyphosate	0.1, 1, 10, 100, 1000, 10000 ppm	[127] Clair et al., 2012
Glyphosate	Tadpoles of wood frog ( <i>Rana sylvatica</i> or <i>Sylvaticus lithobates</i> ),	acute toxicity assay	significant induction of morphological alterations of tadpoles of the three species; for the wood frogs and	0, 1, 2, or 3 mg acid equivalents [a.e.] /L of	[128] Relyea, 2012

Herbicide	Test-organism	Endpoint	Results	Tested concentrations	References
	leopard frog ( <i>Rana pipiens pipiens</i> or L), and American toad ( <i>Bufo americanus</i> or <i>Anaxyrus americanus</i> )		leopard frogs, exposure to glyphosate affected the size of the tail of tadpoles, for all tested concentrations	Roundup Original MAX	
Glyphosate	Roots from the smooth hawkbeard ( <i>Crepis capillaris</i> L); polychromatic erythrocytes of the bone marrow of C57BL rat	chromosome aberration assay; micronucleus assay	there was no induction of genotoxic and/or mutagenic effects for any of the species	<i>Crepis capillaris</i> : 0.05, 0.1, 0.5, 1 %; erythrocytes: doses inferior to half the LD <sub>50</sub> (1080 mg/Kg)	[129] Dimitrov et al., 2006
Glyphosate	Female Wistar rats	acute toxicity assay; teratogenicity assay	high mortality index of females treated with the highest concentration of the commercial product Roundup; increase in the dose-response of fetal skeletal alterations	500, 750, 1000 mg/kg	[130] Dallegrave et al., 2003
Glyphosate	Human lymphocytes	comet assay; FISH; lipid peroxidation assay – TBARS	significant increase in the DNA migration at 580 µg/mL; significant increase of the comet tail intensity at 92.8 µg/mL; greater lesion in the DNA in the presence of S9; increase in the frequency micronuclei, nuclear buds and nucleoplasmic bridges, without S9; significant increase of nuclear instabilities in the highest concentration tested with S9; significant dose-response increase of the levels of TBARS	0.5, 2.91, 3.5, 92.8, 580 µg/mL	[122] Mladinic et al., 2009
Glyphosate and 2,4-D	Algae and 25 species of aquatic animals	acute toxicity assay	there was no reduction in the biomass of periphyton by the 2 herbicides; there was no great impacts to the aquatic community by 2,4-D; high impact to the aquatic community by glyphosate by the significant decrease in the species richness	0, 1, 2, or 3 mg acid equivalents [a.e.] /L of Roundup Original MAX	[132] Relyea, 2005
Glyphosate and Terbutylazine	Human lymphocytes	cytome FISH	glyphosate caused an increase in the frequencies of micronuclei, nuclear buds and nucleoplasmic bridges of cells treated (3.5 µg/mL onward), but without induction of centromeric signals; terbutylazine induced an increase in the frequency of micronuclei hybridized with	0.5, 2.91, 3.50, 92.8, 580 µg/mL (glyphosate); 0,00058, 0,0008, 0,008, 25, 156,5 µg/mL (terbutylazine)	[156] Mladinic et al., 2009

Herbicide	Test-organism	Endpoint	Results	Tested concentrations	References
			centromeric probe and nuclear buds with centromeric signals in the presence of 59 (0.008 µg/mL onward)		
Terbuthylazine	Human lymphocytes	comet assay; comet assay-FISH	induction of the migration of fragments of DNA, significant only at the highest concentration; impairment of the structural integrity of c-Myc and TP 53 due to prolonged exposure to terbuthylazine	Terbuthylazine: 0.58 ng/ml, 8 ng/ml; carbofuran: 8 ng/ml, 21.6 ng/ml	[158] Mladinic et al., 2012
Paraquat	Several species of fishes	acute toxicity assay; enzyme activity assay	alteration in the activity of different enzymes; negative effects on cardiac contraction and opercular ventilation	0.1-2.0 mg/L	[134] Tortorelli et al., 1990
Paraquat	Several species of fishes	enzyme activity assay	induction of oxidative stress; increase in the levels of SOD	0.2-50 mM	[137] Tomita et al., 2007
Paraquat	Germ cells of Sprague-Dawley rats	cytotoxicity assay	reduction in the quantity of spermatozoa; increase in the mortality rates and abnormalities in spermatozoa for the higher concentrations	0, 6, 15, 30 mg/kg	[138] D'Souza et al., 2006
Paraquat	Leukocytes of <i>Rana ornativentris</i>	conventional cytogenetics assay	genotoxic effects, such as chromosome breaks	10 <sup>-6</sup> M	[136] Hanada, 2011
Paraquat	Human lymphocytes	chromosome aberration assay; micronucleus test; SCE	reduction in the cell division index; decrease in the cell proliferation rates; significant increase in the frequencies of SCE (50 µg/mL for 24h treatment; 4000 µg/mL for 2h treatment), significant increase in the MN frequencies (concentrations ≥ 25 µg/mL)	0, 1, 5, 25, 50, 250, 500, 1000, 2000, 4000 µg/mL	[135] Ribas et al., 1998
Paraquat	BEAS 2B normal cells (human bronchial epithelial cells)	cytotoxicity assay, oxidative stress assay	mitochondrial damage; oxidative stress; cell death; production of cytokines, pro-fibrogenic growth factors and transformation of myofibroblast	10 µM	He et al., 2012
Diuron	Pacific oyster ( <i>Crassostrea gigas</i> )	toxicity assay	irreversible damages to the genetic material, negative impacts in the reproduction of aquatic organisms	300 ng/L, 3 µg/L	[44] Bouilly et al., 2007
Diquat	Roots of smooth hawkbeard ( <i>Crepis capillaris</i> L.); polychromatic erythrocytes of the bone marrow of C57BL rat	chromosome aberration test; micronucleus test	there was no induction of chromosome aberrations for any test system; significant increase of the frequency of micronuclei for both test systems	<i>Crepis capillaris</i> : 0.005, 0.01, 0.05, 0.1%; erythrocytes: 8.5, 34.17 mg/Kg	[129] Dimitrov et al., 2006



Herbicide	Test-organism	Endpoint	Results	Tested concentrations	References
Pendimethalin	Roots of smooth hawkbeard ( <i>Crepis capillaris</i> L.); polychromatic erythrocytes of the bone marrow of C57BL rat	chromosome aberration test; micronucleus test	there was no significant increase in the frequencies of chromosome aberrations in plant cells, but an increase of their incidence in cells of rats; significant increase in the frequency of micronuclei for both test systems.	<i>Crepis capillaris</i> : 0.005, 0.1, 0.2, 0.4%; erythrocytes: 122.2, 244.5, 489 mg/Kg	[129] Dimitrov et al., 2006
Simetryn, mefenacet and thiobencarb	<i>Silurana tropicalis</i>	toxicity assay	toxic effects for tadpoles, more significant for thiobencarb	Thiobencarb: 6.85-2.92 mM	[42] Saka, 2010
Complex mixture of pesticides (atrazine, 2,4-D, alachlor, ciazine and malathion)	Workers exposed	chromosome aberration assay; micronucleus test; comet assay	significant increase in the migration of the DNA	Mixture of various concentrations of pesticides	[163] Garaj-Vrhovac and Zeljezic, 2002

**Table 2.** List of the main researches carried out with several bioindicators to evaluate the toxicity of herbicides.

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