Chapter 15

Relationship Between Biomarkers and Pesticide Exposure in Fishes: A Review

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Human population growth and sustainability

In the modern world, small livelihood-based farms that grow multiple crops have been mostly replaced by large, agricultural conglomerates, in which food is grown in monocultures. This shift has allowed for the expansion of suburbs and new cities, but it has also made these places isolated, dirty, degraded, abandoned, and depersonalized. Owing in no small part to pesticide use, one of the legacies of the twentieth century is the potential for severe anthropogenic ecological damage. It has been recognized since the 1970s that byproducts and waste from technical-industrial development degrade the biosphere and threaten to irretrievably poison the environment to which we, as humans, also belong [1].

The term "sustainable" means that an activity can be continued and repeated indefinitely and predictably into the future. It is concerning that, in large part, human activities are logically unsustainable: global human population cannot continue to increase in size forever. We cannot continue to take fish out of sea faster than their populations can recover if we want to have fish to eat in the future. We cannot continue to develop crops in forests if soil quantity and quality deteriorates and water supplies become eutrophic or toxic. We cannot continue to use the same pesticides if an increasing number of pests and pathogens become resistant to them. We cannot maintain nature's diversity if we continue to cause species extinction [2].

The source of many environmental problems, not to say of all, is simply our current level of rapid human population growth [Figure 1]. More people means more demand for energy, more consumption of non-renewable resources such as oil and minerals, more pressure on the renewable ones like forests and fisheries, more need for crops and food production, and so on. Surely, this cannot continue sustainably [2]. In addition to—or as a consequence of—this population growth, environmental pollution is also increasing.

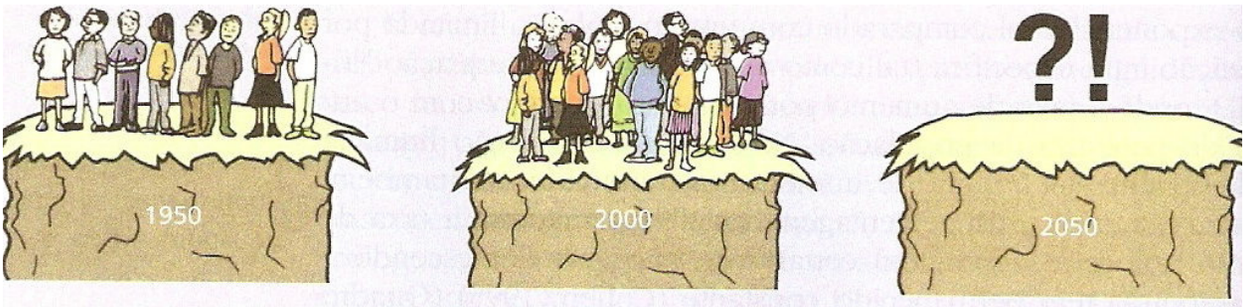

Figure 1. Representation of human population growth – before agricultural revolution [18th century] human population took around 1000 years to double in size. Currently, total population has been doubling in the last decade. Source: [2]

While world population [more than seven billion people] has doubled in the last decade, the demand for water supply increased by a factor of six according to United Nations data for the year 2000. Water demand grows faster than the population, mainly due to the introduction of more hygienic habits globally and the omnipresent desire to increase the productivity of food and industrialized products, the latter of which are generally also intended to maximize agricultural production. However, this increase in per-capita consumption exacerbates the imbalance between the distribution of water on Earth and its centers of human population density. Thus, the cheapest and most viable way to supply a growing world population is to learn how to use the available water in a more efficient manner [3].

1.2. Water sustainability as a resource

Water, when taken on the whole, can be considered an abundant resource on the planet. Total reserves sum up around of 1,265,000 trillion m^3, distributed among solid [ice], liquid [rivers, lakes, oceans, water tables] and gaseous [atmosphere] phases [Figure 2].

However, out of the proportions shown in Figure 2, the relatively small parcel of water present in freshwater courses stands out. These waterways constitute the main source of water supply and are also the most common recipients of sewage discharge. Total world water demand is only around 11% of the mean discharge of rivers. Of this, 70% is used in agricultural activities, 20% in industrial activities and 10% goes to domestic and municipal use [3]. Therefore the actual crisis is not in terms of global water scarcity, but in its heterogeneous distribution. This is aggravated by the disorderly growth of local demand and, above all, by the fact that water degradation has reached unprecedented levels, not just in urban areas, but it rural areas as well [3].

In the same manner, water scarcity is not an issue that is exclusive to arid and semi-arid regions. Many areas with abundant water supplies, although insufficient to attend excessively high demands, have also experienced conflicts regarding water use and suffered consumption restrictions that have affected economic development and quality of life [4]. In addition, there has been severe extraction pressure on many aquifers to the point where many have been degraded nearly to extinction, especially near big cities or metropolises.

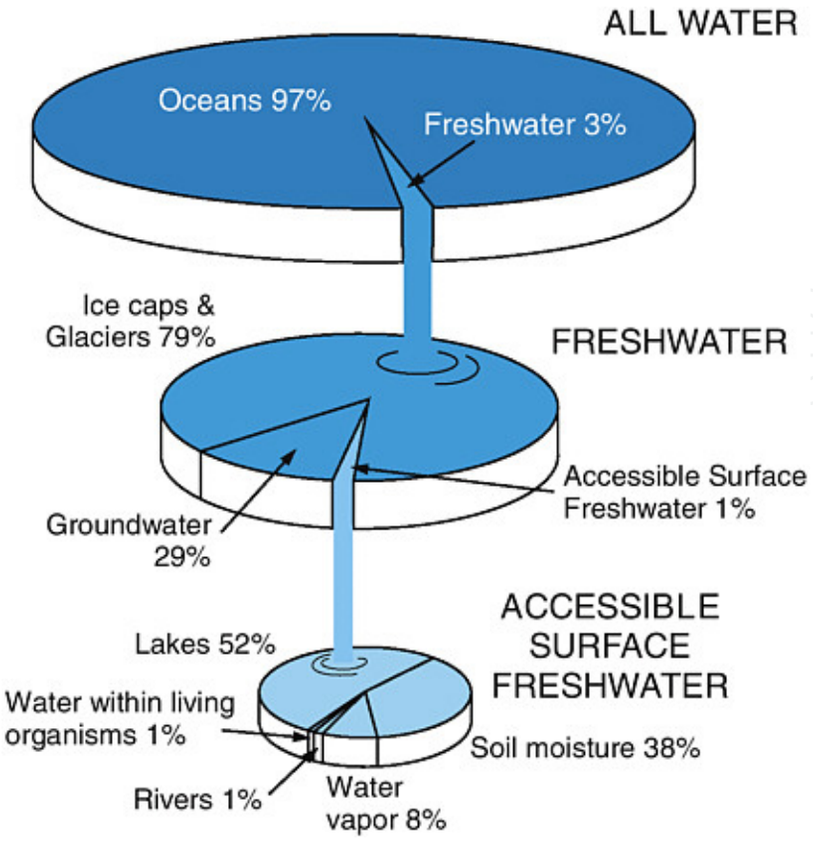

Figure 2. Distribution of the world's water. Source: Courtesy "Earth Update" CD-ROM, Rice University and the Houston Museum of Natural Science; used with permission.

The physical expansion of cities toward wellsprings has been causing serious damage, often focing their relocation.

The demand for a clean and safe water supply for human consumption, agriculture, and recreational purposes has been rising rapidly in the last few years. Water has become a limiting factor for agricultural, urban and industrial development. Recipient waterways, such as lakes, rivers and coastal areas receive great amounts of industrial, agricultural and urban waste directly via inputs and conveyance as well as indirectly through atmospheric deposition of aerial emissions. A complex mixture of toxic substances with an increasing number of contaminants has been deposited in these waters, posing a threat to aquatic ecosystems as much as to the health and well-being of human populations [5].

Regrettably, the waterways that are becoming contaminated are otherwise especially valuable resources. Unfortunately, contamination is very easy, but decontamination is often very costly and in some cases impossible to achieve [2]. In contrast with waste disposed in terrestrial environments that has more-or-less local effects, toxic waste in aquatic environments can be easily transported by currents and dispersed over large areas. Toxic chemicals in water, even in low amounts, can be concentrated to lethal levels by filtering aquatic organisms and top predators [6]. Pesticides, herbicides, oil waste and leakages, heavy metals [such as mercury, zinc and lead], detergents, and industrial waste can harm and kill organisms that live in or use this contaminated water. The potential risks of

contamination in aquatic biota and humans can to be evaluated through biomonitoring programs. The relevance of these studies rises with the growth of urban, industrial, and agricultural activities around water sources [*i.e.*, rivers, lakes and reservoirs] associated with frequently inadequate or insufficient water treatment. This is reflected in attempts to establish rules concerning the safety of water supplies in reservoirs and possible risks to environmental and human exposure [7]. These kinds of studies fits in a discipline called ecotoxicology.

2. Ecotoxicology

The term ecotoxicology was introduced by Truhaut in 1969 and was derived from the root words ecology and toxicology. The introduction of this term reflected a growing interest in the effects of chemicals in other, non-human species. Truhaut identified a field of study that was interested in the harmful effects of these substances within the concept of ecology. Ecotoxicology can be defined as *the study of the harmful effects of chemicals on ecosystems, including effects on individuals as well as consequences in populations and higher levels of organization* [8].

Despite the definition above, many of the first works in ecotoxicology had little to do with ecology or ecotoxicology. At the time, principal importance was placed on the detection and determination of pollutants in animal and plant samples, although the analytical results could sometimes be related to the effect on populations and communities. Analytical techniques such as chromatography, thin layer chromatography and atomic absorption facilitated the detection of very low concentrations of chemicals in living organisms, but establishing the biological significance of their presence or the organismal response to a specific dose of these substances remained difficult [8].

A substance is considered a pollutant when it is detected in levels above those that would normally occur in a particular environment. This immediately brings up the question "what level is considered to be normal?" For most synthetic organic chemicals, such as pesticides, the answer is simple: no detectable level is "normal," because these substances do not exist in the environment until they are introduced by humans. On the other hand, substances such as metals, sulfur dioxide, nitrogen oxides, polycyclic aromatic hydrocarbons [PAHs] and methyl mercury naturally occur and their presence in the environment pre-dates humans. Naturally, there is variation in the concentration of these compounds across different sites and through time. This complicates the assessment of what is normal [8].

There is a conventional distinction between the definitions of the terms "pollutant" and "contaminant": a pollutant is a substance that causes real environmental damage, while the term "contaminant" does not necessarily imply that the chemical is harmful. Still, it is difficult to deal with this distinction. First, there is the general toxicological principle that toxicity is related to dose. In this way, a pollutant can fit the description of pollutant in one situation [at high doses] but not in others [low concentrations]. Second, there is no general agreement on what constitutes environmental damage. Some scientists consider deleterious biochemical changes in organisms to be an environmental damage; others apply the term to

population decrease. Third, the effects of the levels of chemicals measured in living organisms – or in their environment – are frequently unknown, even though the term pollutant is applied to them. This subject becomes even more complicated due to the possibility of toxicity increasing when organisms are exposed to a suite of environmental chemicals; such synergistic effects may result in chemicals having more a deleterious influence on organisms when in a mixture than they would otherwise in isolation [8].

Determining whether a contaminant is a pollutant also depends on its concentration in the environment, on the organisms to be considered, and on the possible damages of the contaminant to the organism. Thus, a compound can fit the description of a pollutant for one organism but not for another [8]. In order to minimize these problems of terminology, the term "pollutant" is used for environmental chemicals that exceed normal levels and cause damage. And *environmental damage* includes biochemical and physiological changes that adversely affect individual organisms, birth, growth and mortality rates[8], and reproduction.

An exciting aspect of ecotoxicology is that it represents an approach that extends from molecules to ecosystems, from genes to physiology [8]. This is further explored in the discussion of response levels in biomarkers.

3. Pesticides

When pesticides began to be developed on an industrial scale, manufacturers were not very concerned about the specificity of their products. These chemicals could damage anything, so long as they did not harm the crop, human beings, or their animals. A good example of this was when P. H. Müller received the Nobel Prize in Physiology or Medicine in 1948 "for his discovery of the high efficiency of Dichloride-Diphenil-Trichlorethane [DDT] as a contact poison against several arthropods" [9]. This insecticide was widely used after World War II to exterminate mosquitoes that caused malaria and typhus. It is cheap and very effective in the short run, but in the long run it is harmful to human health and possibly carcinogenic. It also interferes with animal life, leading to, for example, higher mortality in birds. In less than two decades DDT was banned in many countries, and today its use is forbidden almost worldwide.

The study of the impacts caused by pesticides gained attention from 1979 on, inspired by discoveries of pollution by nematicides in aquifers of many north-American states. Following this, many other cases of pesticide contamination of soil, water resources, animals and, more critically, human beings were diagnosed in the temperate regions, but little investigation was carried in tropical regions [10].

Many chemicals used to kill plagues have become important environmental pollutants. These pesticides are pulverized or released above plague areas, but only a small amount reaches the target, with most of it falling over resident crops or bare soil. Therefore, such pesticides are used in excessive quantities. This occurs especially with herbicides because they are cheaper than insecticides and fungicides [2]. The real problem emerges when the

pesticide is toxic to species other than the target ones and, in particular, when they are transported outside the areas where they were applied and persist in the environment longer than expected.

Agricultural production is currently highly dependent on the use of pesticides, and the abandonment or reduction in their use would lead to a decrease in production, a rise in production costs, higher consumer prices, and, in some places, hunger and malnutrition[11].

The sale of pesticides involves billions of dollars a year. Current transgenic technology has increased the commercialization of certain kinds of pesticides, which can be deliberately used on resistant crops. With many of these substances reaching the environment, especially aquatic ecosystems where they are most concentrated, it is natural to think that some could be accumulating in individuals that compose food chains [12]. Due to their biological activity and to the huge quantity in which they are spread annually in the environment, pesticides can harm human health and the environment; for example, the induction of DNA damage can lead to adverse reproductive reactions, cancer and many other chronic diseases [13]. Recent evidence of negative effects of herbicides in amphibians, reptiles, fish and many other organisms continue to elucidate the fact that we are still discovering the extent to which populations can be affected by the current use of pesticides [14–18].

Therefore, the goal of this chapter is to discuss the methodologies and results of experiments and field surveys that analyze the effects of pesticides, primarily on aquatic communities and especially in fish.

4. Biomarkers

In ecotoxicology, there are many levels of response that can be evaluated.

The presence of a pesticide or other xenobiotic compounds in a portion of the aquatic environment does not, by itself, indicate a deleterious effect. Connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects. The evaluation of these adverse effects—particularly if it is based upon only one level of response—can be affected by the ability of various pollutants [and their derivatives] to mutually affect toxicity, or even to act synergistically [19].

Deleterious effects on populations are often difficult to detect in feral organisms because many of these effects tend to be made manifest only after longer periods of time. When the effect finally becomes clear, the destructive process may have gone beyond the point where it could be reversed by remedial actions or risk reduction. In these sceneries the importance of early-warning signals, or biomarkers, that detect adverse biological responses towards anthropogenic environmental toxins become critical. A biomarker is any biological response to a chemical agent present in the environment that can be measured in the organism [or in its cells], in its metabolic products [urine, feces], or in hair, feathers, etc., that is indicative of some deviation in the standard pattern found in non affected organisms [20].

Pollutant stress generally triggers a cascade of biological responses, each of which may, in theory serve as a biomarker [21]. In the established reference response levels [22][Figure 3],

biomarkers evaluate the precocious responses to pollutants. The responses in higher hierarchical levels are late response measures, frequently when the entire environment is already impacted. Biomarkers are important because they give us much more information on the biological effects of a certain pollutant than simply its quantification. Moreover, by the use of multiple biomarkers important information can be obtained. Biomarkers can be used after trophic, environmental, or occupational exposure, to elucidate the relation of cause-effect and dose-effect in health risk assessment, and in clinical diagnoses and for monitoring purposes [19].

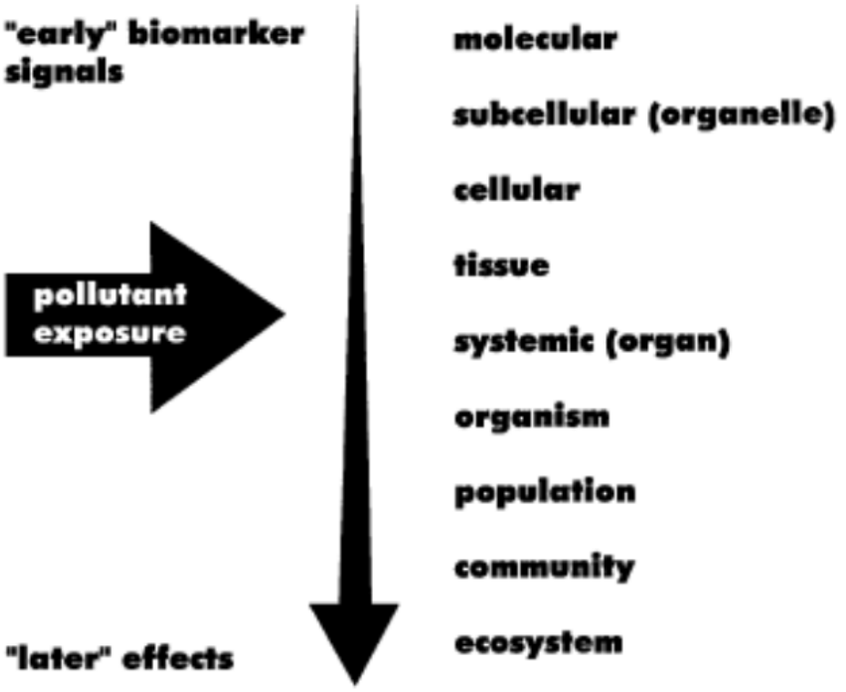

Figure 3. Schematic representation of the sequential order of responses to pollutant stress within a biological system. Modified from [22].

In developing a better understanding of the toxicity of contaminants, two kinds of studies can be carried out: *bioassays*, which are laboratory experiments, or *biomonitoring*, with direct field surveys. Although bioassays generate complementary data, it is important to note that experimental conditions do not always entirely reflect the natural environment [23].

On the other hand, we should not confound the term *biomarker* with *bioindicator*. A bioindicator is defined as an organism whose presence or absence, behavior, or some other characteristic gives information on the environmental conditions of its habitat. Fish species have attracted considerable interest as bioindicators in studies assessing the biological and biochemical responses to environmental contaminants. Fish can be found virtually everywhere in the aquatic environment and they play a major ecological role in the aquatic food-web because of their function as carriers of energy from lower to higher trophic levels [24]. Well like mammals, fish can suffer bioaccumulation, and have the advantage as bioindicators because they can respond to mutagenic agents in low concentrations by

activating the P450 cytochrome enzymatic system, a system of monoxigenase enzymes with a heme group and different specificities per substrate. These enzymes play a fundamental role in the metabolism of xenobiotic substances and of endogen compounds [25].

Genetic, biochemical, and histopathological biomarkers are among the most common biomarkers in ecotoxicological studies with fish.

4.1. Genetic biomarkers

Genetic biomarkers evaluate the most precocious level of response: at the molecular level. DNA is a molecule that contains all the necessary information for the survival and perpetuation of an organism [26]. The exposure of an organism to genotoxic substances can lead to a sequence of events [27] that affect higher levels of response. *Genetic ecotoxicology* can be defined as the study of pollutant-induced changes in the genetic material of biota in nature and has two components: first, initially, the genotoxicity of pollutants, such as structural alterations in the DNA, and second, consequently, the procession and expression of DNA damage in mutant gene products, resulting in long-term heritable effects, such as changes in gene frequency within exposed populations, mutational events, etc. [28].

Many biomarkers have been used as tools for exposure detection and for the evaluation of the effects of genotoxic pollution. These biomarkers consist of tests such as the evaluation of chromosomal abnormalities, DNA adducts and breaks, the measurement of micronucleus frequency and other chromosomal anomalies, and the Comet Assay [29]. Here we will discuss surveys that used the Piscine Micronucleus Test [in conjunction with nuclear morphological alterations] and the Comet Assay.

Among many mutagenicity assays, piscine micronucleus and nuclear morphological alterations test [Figure 4] has been applied successfully because it is simple, safe, sensitive and it does not depend on the karyotypic characteristic of the study animal [30]. This last point is important because most fish have a relatively large number of small chromosomes, which are hard to visualize [31]. When fish erythrocytes are used there is also no excessive time consumption or animal suffering. Thus the micronucleus test in fish erythrocytes has been shown to be a promising technique in the investigation of environmentally-caused mutagenesis [32].

Micronuclei are small cytoplasmic chromatin masses present outside of the main nucleus of the cells that can originate from a chromosomal break as well as from a dysfunction in the mitotic spindle apparatus [33]. They are whole or partial chromosomes that were incorporated inside the nucleus of the daughter cell during cellular division and appear with a small dark round structure identical in appearance to the cellular nucleus [29]. Although there is a measurable basal level of spontaneous formation of micronuclei in most fish species [32], broad scale exposure to environmentally-relevant levels of clastogenic compounds in the laboratory [29,34,35] has been shown to elevate the frequency of micronuclei.

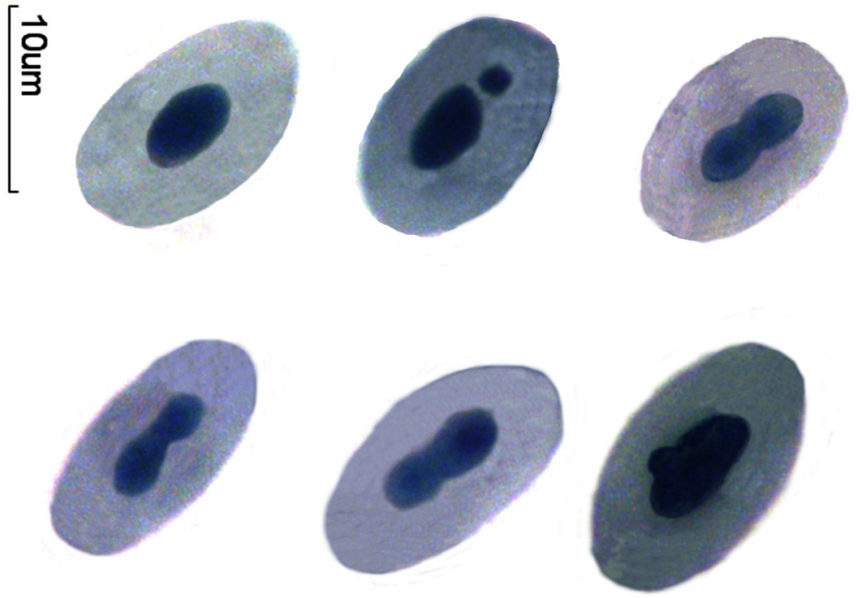

Figure 4. Photos of a micronucleus and nuclear morphological alterations found in erythrocytes of neotropical fish *Astyanax altiparanae*. The first cell represents a normal nucleus, the second cell has a micronucleus and all other cells show nuclear morphological alterations.

In addition to the presence of micronuclei, nuclear morphological alterations can also occur, such as when the nucleus does not show a regular oval shape, but has a projection or an invagination of chromatin. In reference [36], they showed that these alterations are induced by well known genotoxic compounds, even when the micronucleus has not been formed. It is believed that these nuclear anomalies are due to problems with the nuclear lamina, because this structure confers the regular oval shape and stability on the nucleus [37].

Tests that directly evaluate breaks in DNA strands or chain alterations followed by DNA damage are commonly used to analyze the genotoxic impact in aquatic animals [38]. The Single Cell Gel Electrophoresis [SCGE], or Comet Assay, was firstly applied in ecotoxicology fifteen years ago, and has become one of the most popular tests for the detection of strand breaks in aquatic animals under *in vitro*, *in vivo* and *in situ* exposure [39].

The Comet Assay is a rapid, quantitative technique in which visual evidence of DNA damage in karyotic cells can be measured [Figure 5]. It is based on the quantification of denaturized DNA fragments that migrate out of the cell nucleus during electrophoresis. This method has been broadly used in many areas, including biomonitoring, genotoxicity, ecological monitoring, and also as a tool for DNA damage research or reparation in many kinds of cells in response to a variety of DNA-damaging agents [40].

There are many advantages to the Comet Assay: [a] genotoxic damage is detected at the individual cell level; [b] most eukaryotic organisms can be used in the Comet Assay; [c] a small number of cells is required; [d] it is usually easier to perform and more sensitive than other methods for the evaluation of strand breaks; [e] DNA strand breaks form quickly after a genotoxic exposure, so the essay provides an early evaluation of biota's response [38].

The Comet Assay is usually done with erythrocytes because they are easily obtained through non-destructive methods and do not require the additional cellular isolation step. However, other tissues have also been tested for the genotoxic effects of contaminants because genotoxic effects can be tissue-specific [41].

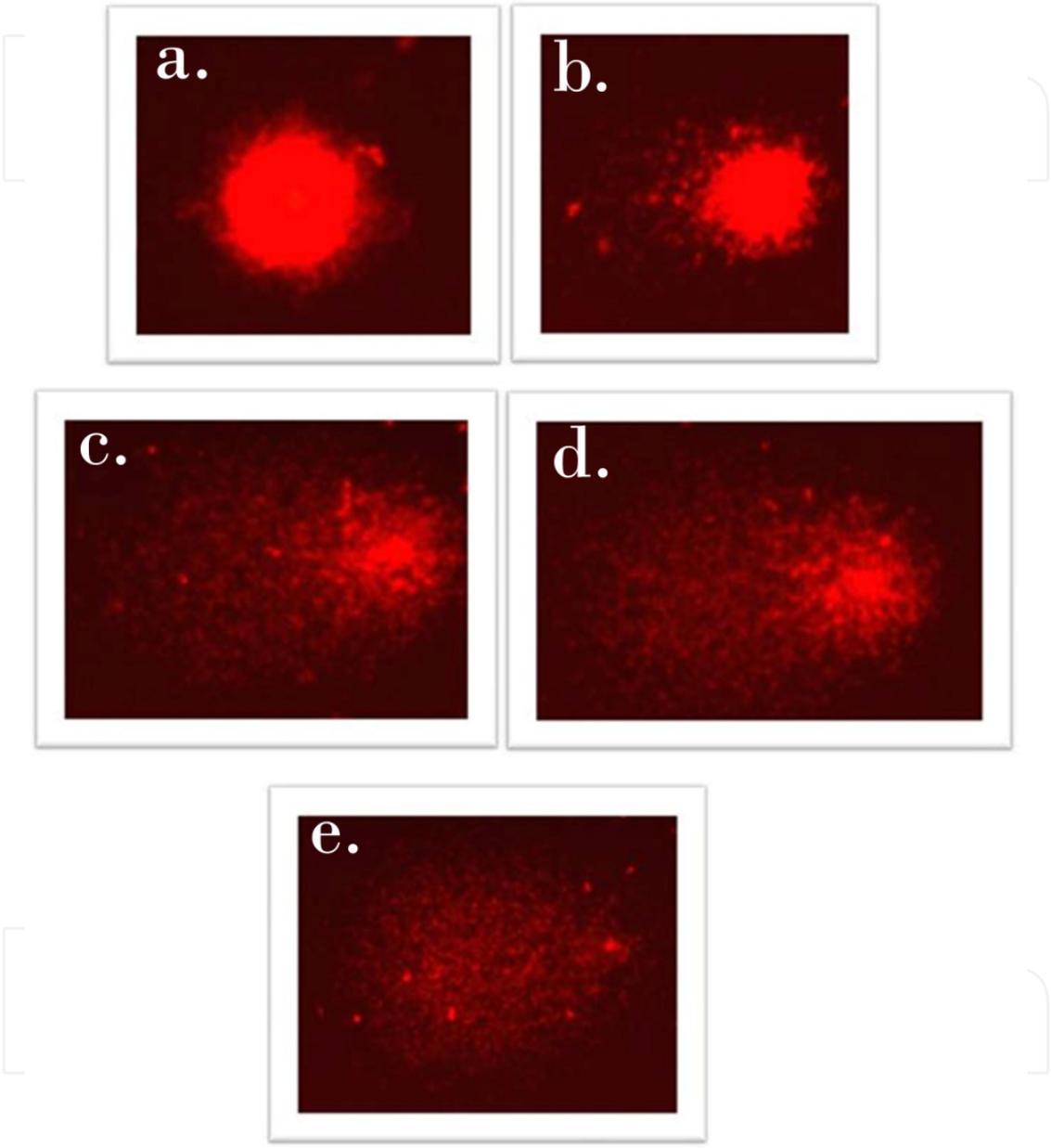

Figure 5. Pictures of five different damage rates in the Comet Essay through an immersion lens. a. zero damage; b. damage one; c. damage two; d. damage three; e. damage four [possibly in apoptosis]. Source: the author [2009].

4.2. Biochemical biomarkers

According to the Central Dogma of Molecular Biology [DNA→ mRNA→protein], DNA is indirectly responsible for protein production [26]. Therefore, DNA alterations can lead to

damage in proteins [and the resulting enzymes]. These alterations can be quantified through biochemical biomarkers.

The most sensitive biomarkers are usually changes in the level and activity of biotransformation enzymes. Biotransformation is the conversion, catalyzed by enzymes, of a xenobiotic compound into a more water-soluble form, facilitating its excretion [19]. The enzymes responsible for biotransformation reactions are found throughout an organism [blood, kidneys, lungs, skin, nervous tissue, small intestine, and liver], but the liver is undoubtedly the organ in which they are most concentrated [42]. The biochemical biomarkers to be highlighted in this chapter are the activities of GST [Glutathione S-transferase], CAT [Catalase], lipoperoxidation [LPO], and Acetylcholinesterase [AchE].

The enzyme Glutathione S-transferase [GST] belongs to phase II of metabolism, and is responsible for the conjugation of electrophilic components or those that come from phase I with GST. The conjugation reaction started by GST is important to cells because it acts in the hydrolysis of lipophilic substances, which can then be excreted as inert substances in the organism. This super family of enzymes occurs in prokaryotes, plants, mollusks, crustaceans, insects, amphibians, reptiles, fish, and mammals [19].

Catalases are intra-cellular enzymes located in the peroxisomes that facilitate the removal of hydrogen peroxide, which is transformed into molecular oxygen and water [43]. Catalases are also cited as detoxication enzymes on some substrates, such as phenols, alcohols, formic acid and formaldehyde [44,45].

The lipid peroxidation or oxidation of polyunsaturated fatty acids is a regular physiological process that is important in cellular maturation [46–48] and lipid mobilization [49,50]. Some classes of contaminants, however, can have detrimental effects on this process [51,52] and can lead to damage in cellular function [52,53] and malfunction of cellular membranes and essential organelles, in addition to potentially affecting transporting processes, metabolites and ion gradient maintenance, and receptor-mediated signals transduction [54], with subsequent structural modification of the lipoproteinic complexes in cellular membranes [55,56].

Lipoperoxidation has been used successfully as a xenobiotic-induced oxidative stress measurement in organisms exposed *in vivo* to myriad chemicals such as metals [iron, cadmium, mercury and lead], paraquat, malathion, deltamethrin, and glyphosate [19,57–61].

The term cholinesterase [AChE] usually refers to the sum of the activities of pseudo-cholinesterase, or butirilcholinesterase, and acetylcholinesterase, or real cholinesterase, both of which are present in muscles [62]. The measurement of the AChE activity is often used to diagnose the exposure to anticholinesterasic toxins in fish, and can be considered one of the most ancient biomarkers [62,63]. Some authors [19] indicate that fish exposed to pesticides can show a reduction in acetylcholinesterase activity that is proportional to concentration and exposure time. The enzymatic measurement of cholinesterase allow the detection of sub-lethal toxicological effects, mainly of organophosphate compounds and carbamates, even without the presence of clinical symptoms.

4.3. Histophatological biomarkers

We can also observe damage in higher, cellular and tissue, response levels, which are detected through histopathological techniques. Morphological techniques such as light microscopy have been used in toxicology because they allow an evaluation of the possible effects of xenobiotics on target organs and tissues. According to [64], the effects in cell and tissue structure are important parameters to be considered in the evaluation of the potential toxicity of contaminants in living organisms.

Some authors [65] report that, through morphology, it is possible to reveal the most-affected target organs as well as to detect an organism's sensitivity to the toxicity level of the compounds to which it was exposed. Histopathology also permits the differentiation of injuries promulgated by disease from those caused by environmental factors, such as the exposure to pollutants [66].

The advantage of histopathology as a biomarker lies in its use at intermediate levels of biological organization. Histological changes appear as a medium-term response to sub-lethal stressors, and histology provides a rapid method for detecting effects from xenobiotic compounds, especially chronic ones, in various tissues and organs [67]. For example, fish exposure to chemical contaminants is likely to induce a number of lesions in different organs [68,69]. Gills [70], kidneys [71,72], liver [73,74] and skin [75] are suitable organs for histological examination in order to determine the effect of pollution.

The article [67] propose an index of histopathological tests for any given organ, which leads to standardized quantification and allows legitimate comparison between different studies and, with restrictions, between different organs. This tool leads to a better understanding of the significance of histological findings after exposure to contamination.

Certain organs are the primary markers for aquatic pollution. For example, gills and skin have large surfaces that are in direct and permanent contact with potential irritants, and both have mucous cells. The liver plays a key role in metabolism and subsequent excretion of xenobiotics and is also the site of vitellogene production. The kidneys are very important for maintenance of a stable internal environment with respect to water and salt, excretion, and partially for the metabolism of xenobiotics [67].

5. Relationship between biomarkers and pesticide exposure in fishes

Every week, new articles are published showing the detrimental effects of many pesticides. These effects can be seen at all response levels: molecular, cellular, histological, individual, or even at higher ecological levels such as population, community, or ecosystem.

It is important to evaluate the effect of pesticides at lower response levels for the purposes of early damage detection, before they affect higher levels and decimate an entire community or ecosystem. Hence, we will focus on lower level responses at the molecular, cellular and histological level.

Mutagenic chemicals have a high probability of inducing carcinogenic effects in various fish species. A majority of these chemicals have been found to cause tumors at specific or multiple sites in fish [76]. Herbicides and pesticides comprise a large group of mutagenic chemicals, but information on herbicidal genotoxicity is lacking. Penthachlorophenol [PCP] and 2,4-dichlorophenoxyacetic acid [2,4-D] are chlorinated phenols widely used in agriculture. Chlorinated phenols in general are noted for exhibiting strong biological effects. For example, 2,4-dinitrophenol decouples oxidative phosphorylation; intervening in the oxidative pathways of metabolism. A clinical manifestation of this effect is the very rapid onset of rigor mortis in victims of pentachlorophenol poisoning [77]. Another study with humans revealed a significant increase in chromosomal abnormalities observed in the lymphocytes of workers exposed to PCP, leading to possible carcinogenic effects [78]. [79] surveyed the mutagenic effect of these two pesticides [PCP and 2,4-D] in the fish *Channa punctatus*. Using the Piscine Micronucleus Test [PMT], which evaluates the rate of permanent DNA damage, they observed that an increase in the dose and exposure time to both pesticides increased the rate of mutation in fish erythrocytes. In this work, they concluded that PCB was more toxic than 2,4-D in terms of Micronucleus induction.

The same fish species was used to evaluate the acute genotoxic effect of the insecticide Endosulfan [80]. Endosulfan is one of the most abundant organochlorine pesticides in the global atmosphere and is capable of undergoing long range transport to remote locations such as the Arctic [81]. Using the Comet Assay with liver and gill tissue, the authors observed a dose-dependent response; that is the higher the dose, the higher the rate of DNA breakdown in *C. punctatus*. In this case, there is evidence that gill cells are more sensitive than hepatic ones.

We reported on the case of an accidental spill of about 8000 liters of endosulfan in Paraıba do Sul River in the state of Rio de Janeiro State [Brazil], in November 2008 [7]. In this study, we analyzed the fish *Pimelodus maculatus* before [dry season] and after [rainy season] the spill in two affected drinking water reservoirs [Ilha dos Pombos and Santa Cecília] and in one that was unaffected [Santa Branca] These reservoirs are destined for human water provision. Fish from the Ilha dos Pombos reservoir [rainy season] that had been affected by the endosulfan spill showed several histopathological alterations in the gills and liver. Gill alteration index was similar in the fish from the three reservoirs in the dry season, but increased in the affected reservoirs during the rainy season, probably due to the endosulfan spill that occurred two months before this sampling. Figures 6 and 7 show the alterations found in the Paraíba do Sul River fishes. With regard to biochemical biomarkers, Cholinesterase activity in axial muscle was higher in *P. maculatus* from Ilha dos Pombos [33X] and Santa Branca reservoirs [11X] during the rainy season sampling after the endosulfan spill. Although we do not have normal values for acetylcholinesterase activity in this fish species, based on studies with other fish species the activity in the muscle showed a decrease in the dry season in all the reservoirs. Several pollutants such as organophosphates, carbamates [82], metals [83], hydrocarbons, and endosulfan [84] can decrease cholinesterase activity through inhibition or reduced expression, although an increase in activity has also been reported for fish muscle [85].

A similar study to [7] was also carried out in polluted and unpolluted areas in Estuarine Lakes at Santa Catarina Coast in southern Brazil, using the bioindicator fish *Geophagus brasiliensis* [86]. Estuaries are important sinks of pollutants derived from anthropogenic activities. The lakes in Santa Catarina are of great economic importance to the surrounding areas, enabling cultivation of rice crops and pig farming by various irrigation and drainage channels, as well as providing fish and shrimp to support ~10,000 artisanal fishermen. The results showed that both studied lakes are impacted by potential genotoxic substances. Severe lesions in the livers of *G. brasiliensis* were also observed. The inhibition of acetylcholinesterase activity suggested the presence of pesticides or metals in the study sites. The presence of large areas of rice crops around Santa Marta Lake [one of the studied lakes] may provide an explanation for the occurrence of substances with neurotoxic potential in the lakes. Three pesticides widely used on rice crops in southern Brazil [Clomazone, Quinclorac and Metsulfuron-methyl] have been shown to inhibit AChE activity in another species of fish, *Rhamdia quelen* [87]. The inhibition of AChE activity in fish can have adverse consequences for the animal itself, mainly by affecting its swimming ability and therefore its ability to find food and escape from predators [88].

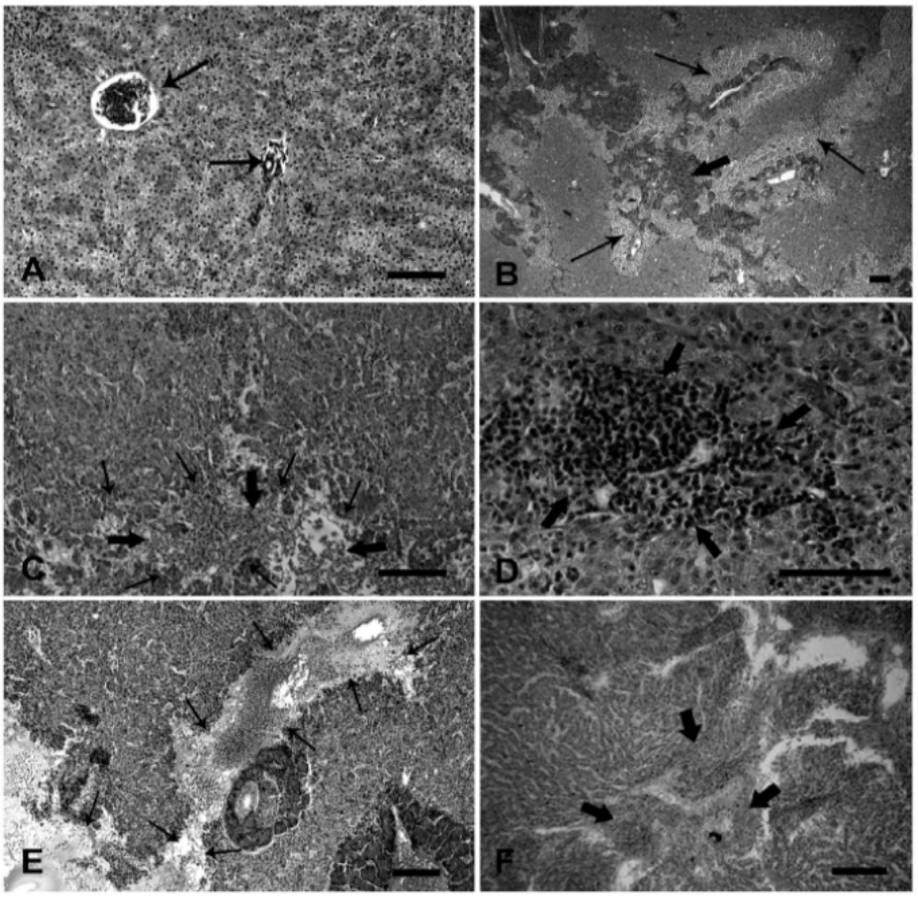

Figure 6. Histopathological findings from the liver of *Pimelodus maculatus* in Paraıba do Sul River. [A] Normal tissue. Arrows show vessels. [B] The presence of pancreatic tissue [large arrow] and the high incidence of melanomacrophage centers [small arrows]. [C] Occurrence of differentiated tissue [small arrows]. [D] Arrows show leukocyte infiltration. [E] Large necrosis area [arrows]. [F] Arrows show a large differentiated area of tissue. Scale bar=100 mm. Font: [7]

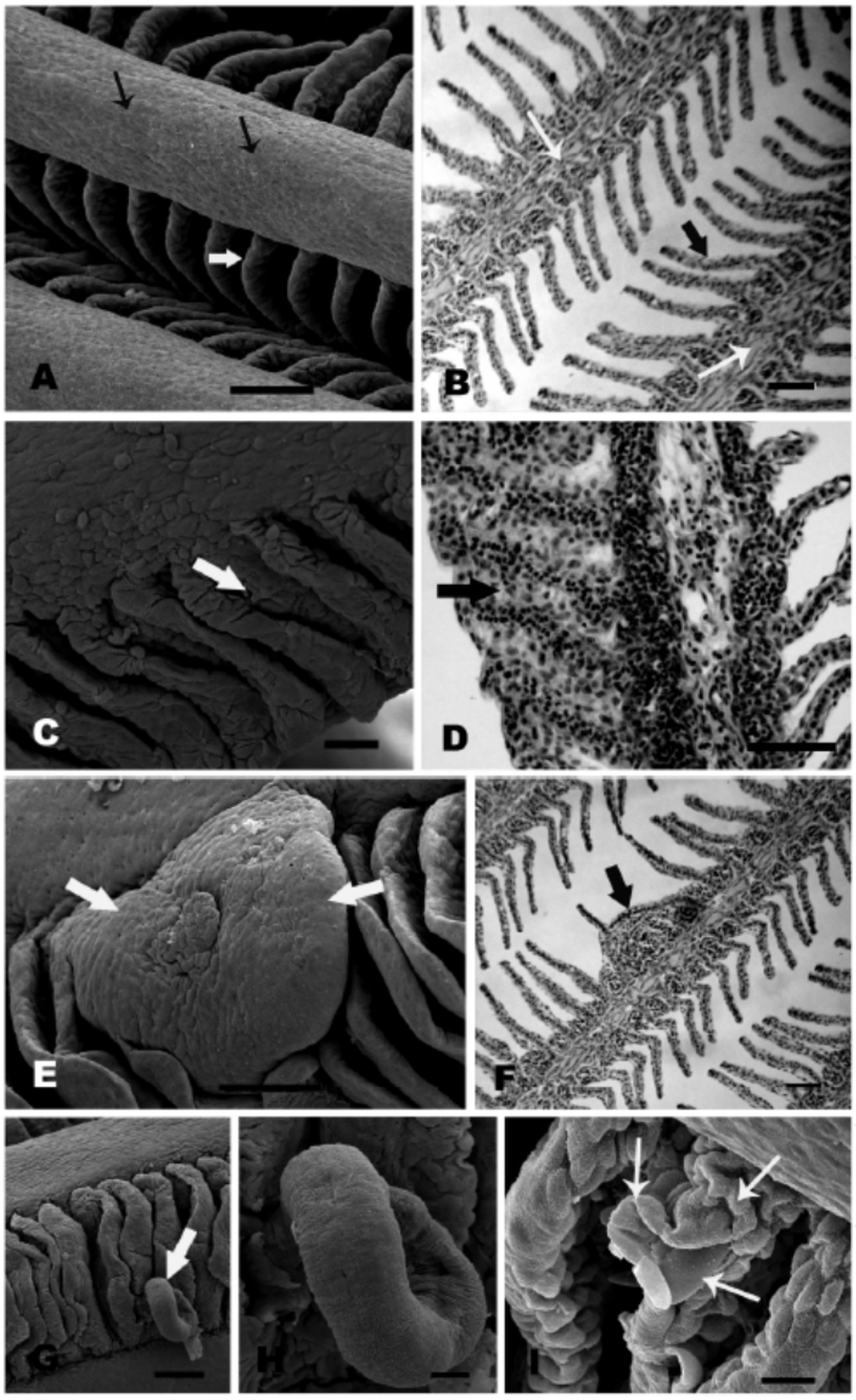

Figure 7. Gills of *Pimelodus maculatus* from Paraíba do Sul River. A and B: Normal aspect of gills showing primary [small arrows] and secondary lamellas [large arrows]. Scale bar = 50 and 100μm respectively. C and D: arrows: fusion among secondary lamellas. Scale bar = 20 and 50μm respectively. E and F: neoplasia. Scale bar = 20 and 100μm. G and H: arrow= ectoparasite. Scale bar = 50 and 10μm. I: Observe the epithelial cells alterations [arrows]. Scale bar = 10μm. Font: [7].

Another field study compared two areas in southern California with records of chlorinated hydrocarbon [DDTs and PCBs] contamination to one less contaminated site. The frequency of micronuclei in circulating erythrocytes of two sea fishes was much higher in the contaminated areas. The DNA damage rate was up to four times lower in the uncontaminated site [89]. Organochlorine compounds such as in the DDT family, used as pesticides in agriculture, and polychlorinated biphenyls or PCB, which are important industrial chemicals and are used as non-flammable oils in many commercial products, are extremely persistent and difficult to degrade. Despite the fact that these compounds have been forbidden in many developed countries and their worldwide production and use have drastically decreased in recent years [90], at present they are widespread and have become ubiquitous contaminants of natural systems. PCBs are currently the most abundant chlorinated aromatic contaminants in the environment.

It was not until after DDT use had become widespread that the impacts of pesticides started to gain world's attention and an environmental revolution began. This happened in 1962, with the release of the famous book Silent Spring, by Rachel Carson [91]. She described the process know as biomagnification, through which DDT and other organochlorine insecticides become more concentrated in higher levels of the food chain, being detected in the breast milk of women around the world and in the fatty tissues of Eskimos, inhabitants of isolated lands in Arctic. DDT is responsible for making bird's egg shells thinner, particularly in birds of prey; this compound nearly drove the Peregrine falcon to extinction. DDT blocks calcium absorption, which makes the eggs easily broken and interrupts incubation, consequently undermining reproduction.

Currently, the pesticides with the highest sales rates worldwide are those based on glyphosate. Their sales have risen 20% a year, mainly due to the advent of biotechnology, which has provided plants that are resistant to this herbicide. Described by the manufacturers as pesticides low in toxicity and with good environmental compatibility, the glyphosate-based herbicides can seem like a silver bullet to those dealing with unwanted vegetation. However, there is public interest in the ecological, safety, and health concerns that may arise through the use of products from transgenic harvests [92].

There is some literature on the undesirable effects of glyphosate. Laboratory studies have detected adverse effects in every toxicological test category: medium-term toxicity [salivary gland lesions], long-term toxicity [inflammations of the mucous membranes of the stomach], genetic damage [human blood cells], reproductive effects [reduction in the number of spermatozoa in mice; higher frequency of abnormal spermatozoa in rabbits], and carcinogenicity [higher frequency of liver tumors in male mice and thyroid cancer in female mice] [93].

The author [94] cites many positive results for the mutagenicity of glyphosate for a variety of test systems [e.g. *Salmonella typhimurium* – reverse mutation test, *Drosophila melanogaster* - induced sex-related lethal recessive mutations, and chromosomal aberrations in *Allium cepa* and cultures of human lymphocytes].

The most popular commercial product based on glyphosate is Roundup®. Its active ingredient is the 48% acid equivalent of the isopropylamine salt of N-[phosphonomethyl] glycine [$C_3H_8NO_5P$; Monsanto Agricultural Co, St. Louis, MO, USA]. Roundup is a broad-spectrum, nonselective, postemergent herbicide that is used to kill unwanted plants in a wide variety of agricultural, lawn and garden, aquatic, and forestry situations. Despite its long and extensive use, the ecotoxicological data for Roundup are scarce.

A study by [95] evaluated the genotoxic potential of Roundup® in blood cells of the European eel [*Anguilla anguilla*]. In a bioassay, they subjected the fish to realistic exposure concentrations of 58 and 116 μg/L for 1-3 days, and also addressed the possible association with oxidative stress. Comet and erythrocytes' nuclear abnormalities assays were used as genotoxic end points, reflecting different types of genetic damage. The authors showed higher rates of DNA damage in the contaminated fish than in the control group after 3 days of exposure [the same result was obtained in the Piscine MicronucleusTest]. The biochemical markers were assessed through enzymatic [catalase, glutathione-S-transferase, glutathione peroxidase and glutathione reductase] and non-enzymatic [total glutathione content] antioxidants, as well as by lipid peroxidation [LPO] measurements. Antioxidant defenses were unresponsive to Roundup. LPO levels increased only for the high concentration after the first day of exposure, indicating that oxidative stress in blood caused by this agrochemical was not severe. Overall results suggested that both DNA damaging effects induced by Roundup are not directly related with an increased pro-oxidant state.

Another study [96] showed different results. These authors evaluated the effects of Roundup Transorb® [RDT] on the Neotropical fish *Prochilodus lineatus*. Juvenile fish were acutely exposed [6, 24 and 96 h] to 1 mg/L of RDT, 5 mg/L of RDT, or only water [control]. They performed antioxidant analysis in the liver and acetylcholinesterase [AChE] determination in brain and muscle. After 6 h of exposure fish showed a transient reduction in superoxide dismutase and catalase activity. RDT also inhibited glutathione-S-transferase after 6 and 24 h of exposure. The reduction in these enzymes is probably related to the occurrence of lipid peroxidation [LPO] in fish exposed to the herbicide for 6 h. LPO returned to control levels after 24 and 96 h exposure to RDT, when fish showed an increased activity of glutathione peroxidase. The content of reduced glutathione also increased after 96 h exposure. Thus, after 24 and 96 h the antioxidant defenses were apparently enough to combat ROS, preventing the occurrence of oxidative damage. The exposure to RDT for 96 h led to an inhibition of AChE in brain and muscle at rates, which may not be considered a life-threatening situation.

The contradictory results of these studies warrant closer inspection. First, the concentration used in [96] was up to 86 times higher than the one used by [95]. Some studies show that biomarker responses are dose-dependent [19]. Second, the sensitivity of the fish must be taken into account. Not all fish have the same response to the same contaminant. The exposure time to the contaminant is also a factor that can be responsible for the differences in the results. Finally, the products used had different commercial names and different surfactants in their composition. Virtually all pesticides have other ingredients other than the *active* one, which actually has the exterminating action. Such ingredients are mistakenly

called *inert*. Their purpose is to facilitate the use of the product or to make it more efficient. Usually the inert compounds are not identified in the pesticide's label. In the case of glyphosate-based products, many "inert" ingredients were identified [93]. Differences in the test-organisms' responses to glyphosate and to Roundup, its commercial formula, can be attributed to the toxicity of different compounds and surfactants in the commercial formula. Research has revealed that Roundup can be up to 30 times more toxic to fish than the pure glyphosate, due to the so-called *inert* compounds in the formula [94].

Some studies report pathological damage in fish exposed to glyphosate. The author [97] exposed *Oreochromis niloticus* to sub-lethal concentrations [5 and 15 mg/L] of Roundup for 3 months, and the organs exhibited varying degrees of histopathological change. In the gills, filament cell proliferation, lamellar cell hyperplasia, lamellar fusion, epithelial lifting, and aneurysm were observed. In the liver, vacuolation of hepatocytes and nuclear pyknosis occurred. Kidney lesions consisted of dilation of Bowman's space and accumulation of hyaline droplets in the tubular epithelial cells. The results indicated that long-term exposure to glyphosate at sub-lethal concentrations had adverse effects stemming from histopathological and biochemical alterations in the fish. [98] has exposed *Cyprinus carpio* to immersion in Roundup [205 mg of glyphosate/L and 410 mg of glyphosate/L] in concentrations of 40 to 20-fold lower than those used in practice. Electron microscopy revealed that Roundup caused appearance of myelin-like structures in carp hepatocytes, swelling of mitochondria and disappearance of the internal mitochondrial membrane at both exposure doses. In this case, both studies, even though with different concentrations and species, confirmed that glyphosate can cause damages to fish tissues.

A study with the neotropical fish *Corydoras paleatus* contaminated with 3.20 μg/L glyphosate [6.67 μg/L Roundup®] showed that this pesticide might have genotoxic effects even at very small concentrations [99]. In this work, we performed PMT and Comet Assays with blood and liver cells, after the fish had been exposed to herbicide for 3, 6 and 9 d. A similar study [100], evaluated the sublethal effects of Roundup on the fish *Astyanax sp*. for 4 days. They tested two concentrations of Roundup: 3 μL/L and 6 μL/L. The PMT outcome was that only the highest dose showed any difference in response compared to the control group. Both works used the same commercial product, tested similar doses, and had similar responses, even though they were conducted with different fish.

The study [101] observed that Roundup® could affect cellular function [e.g., DNA] and that Roundup® and several glyphosate-based products interfered with cell-cycle regulation. In this work, the dose-response curves of the formulation products indicated a threshold for cell cycle induction even at very small concentrations, in agreement with other studies cited above. Failure in the cell cycle checkpoints leads to genomic instability and subsequent development of cancers from the affected cell [102,103]. Several lines of evidence have shown the highly conserved molecular basis of the cell cycle, from simple unicellular eucaryotes such as yeast to complex metazoans such as fishes or humans [104].

As discussed in the first pages of this chapter, a substance is considered harmful when it is detected in the environment at a higher concentration than it would *normally* occur. But

what is the normal level for each substance? For many synthetic organic chemicals, such as pesticides, the answer is quite simple – no detectable level is normal because these compounds do not exist in the nature unless they are introduced by humans [8]. However, considering the current worldwide dependence on pesticides, it is impossible to avoid their entering natural environments, reaching animals, contaminating our food supplies and drinking water, etc. For this reason, countries try to establish a maximum tolerance limit for each pesticide in each component of the environment. One of the lowest limits is the one established by the European Union legislation, which is 0.10 μg/L [or 0.0001 mg/L] for all pesticides [individually] in water designated for human consumption [105]. Many studies have shown that this limit is safe [106]. In Brazil, Ministry of Health law 518 establishes the limits of some agrochemicals in drinking water, such as atrazine [0.002 mg/L or 2 μg/L], 2,4 D [0.03 mg/L], DDT [0.002 mg/L], Endosulfan [0.02 mg/L] and glyphosate [0.5 mg/L][107].

In 1974, the US Congress passed the Safe Drinking Water Act. This law requires the US Environmental Protection Agency [EPA] to determine the level of contaminants in drinking water at which no adverse health effects are likely to occur. These non-enforceable health goals, based solely on possible health risks and exposure over a lifetime with an adequate factor of safety, are called maximum contaminant level goals [MCLG]. Maximum contaminant levels [MCLs] are set as close to the health goals as possible, considering costs, benefits, and the ability of public water systems to detect and remove contaminants using suitable treatment technologies. The MCLG for glyphosate is 0.7 mg/L, or 700 ppb. EPA has set an enforceable MCL regulation for glyphosate at 0.7 mg/L, or 700 ppb. The MCLG for 2,4-D is 0.07 mg/L, or 70 ppb. For atrazine, the MCLG is 0.003mg/L; and for PCBs [Polychlorinated biphenyls] the MCLG is zero, and the MCL is 0.0005mg/L [108].

Canada has the Guidelines for Canadian Drinking Water Quality, which are intended to protect freshwater and marine life from anthropogenic stressors such as chemical inputs or changes in physical components. In this, the Maximum Acceptable Concentration [MAC] for atrazine and its metabolites is 0.005 mg/L; for 2,4-D is 0.1 mg/L, and for glyphosate the MAC is 0.28 mg/L [109].

6. Conclusions

In this chapter, we make explanations about some pesticides, and the effects of these on fishes, in field or laboratory assays. In addition to the pesticides cited above, many others are spread daily in the environment. However, little is known about the individual or synergistic effects that these products may have at the various levels of biological systems [in the short or long run]. Thus, many efforts have been made to explore to the deleterious effects of pesticides on non-target species, but there is still a lot to be done. These efforts are of great importance in understanding the impacts of pesticides in organisms and in the environment as well as in establishing of safe limits on the use of these products in the environment.

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