Chapter 7

Lethal and Sublethal Effects of Pesticides on Aquatic Organisms: The Case of a Freshwater Shrimp Exposure to Roundup®

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Additional information is available at the end of the chapter

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1. Introduction

In the last few decades, rapid human population growth with its concomitant astronomical increase in urbanisation, industrialisation and technology has had its toll on natural resources of the world. Climate change, acid rain, nutrient enrichment of aquatic environments, pollution by pesticides, metals, and synthesised toxic substances on local, regional and global scales are the result of such anthropogenic disturbances. Recent events, as witnessed the world over such as large scale mortality of wildlife (e.g. sea mammals, birds), increasing menace to human health (e.g. cancerous cells, chronic respiratory disease, damage to organs such as brain, lung, heart, liver, kidneys) and algal bloom in many water bodies are all effects of the anthropogenic perturbations of the biosphere. The biosphere is part of the earth that supports life. It comprises of the lithosphere, hydrosphere and atmosphere. The hydrosphere is the total mass of water on planet Earth, which includes oceans, lakes, streams, groundwaters and glaciers. Saline water account for 97.5 % while freshwater accounts for 2.5 %. The bulk of freshwater, 68.7 %, is stored in ice and permanent snow cover, while 29.9 % exists as groundwater. Only 0.26 % is found in lakes, river systems and reservoirs [1]. However, among all the components of hydrosphere, freshwater ecosystems are the most vulnerable to pollution due to anthropogenic stresses [2-3]. Agricultural, industrial and domestic activities are the major sources of this pollution [4]. These activities use more than one-third of the Earth's accessible freshwater resources and have contaminated water with numerous synthetic and geogenic compounds [4]. For instance, about 300 billion kilograms of synthetic compounds used in industrial, consumer and agricultural products find their way into natural freshwater systems every year

[5]. Ten percent of the globally accessible runoff is used, generating a stream of wastewater, which flows or seeps into groundwater, rivers, lakes, or the oceans [5].

The use of agrochemicals is necessary to control pests and increase yields in order to produce adequate food for the global population, estimated at 6.8 billion in 2009 [5], and recently reported to have reached 7 billion [6]. Underdeveloped countries, where 1.02 billion people (15 %) are undernourished and 1.3 billion people (19 %) live on an inadequate diet [5], need an adequate food supply. However, the agricultural sector's annual application of over 140 billion kilograms of fertilizers and large amounts of pesticides creates massive sources of diffuse pollution of freshwater systems [4]. The presence of these toxic chemicals in both aquatic and terrestrial ecosystems has become an important issue globally. Growing research-based evidence shows that pesticides, metals and many industrial chemicals interfere with the health and normal functioning of the endocrine systems of a wide range of organisms, including humans [7-9]. It is believed that effects of these chemicals on the normal functioning of the endocrine system are responsible for a number of developmental anomalies in a wide range of species, from invertebrates to higher mammals [10-13].

2. Pesticides

2.1. Pesticide pollution in ecosystems

Pesticides are substances or mixture of substances designed to control, repel, mitigate, kill or regulate the growth of undesirable biological organisms. These undesirable biological organisms (pests) do not only compete with humans for food, transmit diseases and destroy property, but are generally nuisance. Pests include insects, plant pathogens, weeds, molluscs, fish, birds, mammals, nematodes and microorganisms such as bacteria and viruses. Pesticides may be classified as being biological or synthetic. Biological pesticides are derived from natural sources such as extracts from plants (e.g. pyrethrin insecticide from chrysanthemum plants and azadriachtin from neem trees). Majority of pesticides are synthetic as they are made through industrial processes. A pesticide may also be classified as broad-spectrum when used to control a wide range of species or as narrow-spectrum when used to control a small group of species. However, the most common classification of pesticides is based on the type of pest they are used to control. These include insecticides (control insects), herbicides (control weeds) and fungicides (control fungi). Pesticides are used in agriculture to maintain high production efficiency and there is a constant demand for stable crop production to support the growing human population. Therefore, use of pesticides is expected to increase in the near future [14]. However, their use is an environmental hazard and can affect non-targeted organisms, other than the targeted pests [15].

Pesticide pollution affects both aquatic and soil ecosystems. Factors that promote pesticide pollution include drainage patterns, properties of the pesticide, rainfall, microbial activity, treatment surface and rate of application. Pesticides are able to move from one ecosystem to another through processes such as transfer (mobility) and transformation (degradation). Transfer may occur through surface runoff, vapourization to atmosphere, sorption (adsorp-

tion/desorption), plant uptake or soil water fluxes. Transformation occurs through chemical, microbial and photo-degradation [16]. A risk to a water body by a particular pesticide is dictated by the unique properties of the pesticide. For example, half-life, mobility and solubility are three properties of pesticides which determine their specific effects.

Although pesticides are used on a local scale, their effects are ubiquitous and can be felt regionally and globally [17]. They are transported into aquatic systems through processes such as direct applications, surface runoffs, spray drifts, agricultural returns and groundwater intrusions; either as single chemicals or complex mixtures [18]. The transportation of pesticides to their final destination in the aquatic ecosystem may result in adverse health effects on the organisms found there. All members that form the different communities of an ecosystem, from the smallest invertebrates to birds and humans, are affected by pesticides. Most toxic pesticides in urban and agricultural settings are responsible for the deaths of many birds, fish and zooplanktons that fish depend on for food [19]. It has been reported that pesticides contaminate many breeding sites of amphibians and that some of them may persist in the environment for a very long time even at lower concentrations. Some effects of pesticides only become highlighted after long term exposure. For example, the survival patterns for early green frogs and late wood frogs are affected only after 24 days of exposure to atrazine [17].

2.2. Herbicides: weed control pesticides

Weeds are plants that grow in places people do not wish them to grow because they compete with "beneficial and desirable" plant species. Until the last century, much of the energy used in farming went into removing weeds to provide suitable conditions for efficient cropping. However, during the industrial revolution, more people moved to work in factories, thus creating a shortage of labour on farms and it became necessary to develop more efficient ways to control weeds [5]. Herbicides are chemical substances used to suppress or kill unwanted vegetation (weeds). They are only one of the many types of pesticides that include insecticides, fungicides, rodenticides and nematocides [5]. Herbicides may be classified based on the time of application: pre-plant herbicides are applied to the soil before the crop is planted; pre-emergence herbicides are applied to the soil after the crop is planted, but before the crop or weeds emerge; post-emergence herbicides are applied to both crop and weeds after they have germinated and emerged from the soil. Herbicides may also be classified by the way they kill or suppress plants. These include hormone inhibitors, cell division inhibitors, photosynthesis inhibitors, pigment synthesis inhibitors, lipid synthesis (cell membrane) inhibitors, or cell metabolism (e.g. amino acid biosynthesis) inhibitors [20].

All herbicide products have chemical properties that influence their ability to suppress growth or kill plants. While some of these properties are inherent in the chemical nature of the herbicides, others are added to enhance their efficacy. The following are some chemical properties of herbicides that influence their use:

- *Chemical structure*: The biologically active portion of a herbicide product is the *active ingredient*. It is the fundamental molecular composition and configuration of the herbicide.

The physical and chemical properties of a herbicide can also determine the method of application and use.

- *Water solubility and polarity*: Herbicides that are produced as salts dissolve quite well in water and are usually formulated to be applied in water, while non-polar herbicide sources are not. Water is the main substance used to disperse (spray) herbicides, and hence the water solubility of a herbicide influences the type of product that is formulated, how it is applied and the movement of the herbicide in the soil profile.
- *Volatility*: Herbicides with a high vapour pressure volatilise easily, while those with a low vapour pressure are relatively non-volatile. The volatility of a herbicide can determine the mode of action and the herbicide's fate in the environment.
- *Formulations*: Commercial herbicide products contain an active ingredient and "inert" ingredients. An "inert" ingredient could be a carrier that is used to dilute and disperse the herbicide (e.g. water, oil, certain types of clay, vermiculite, plant residues, starch polymers, certain dry fertilizers) or an adjuvant (e.g. activator, additive, dispersing agent, emulsifier, spreader, sticker, surfactant, thickener, wetting agent) that enhances the herbicide's performance, handling, or application [20]. In recent years, carriers and adjuvants have been implicated in adding to the toxicity of the active ingredients, and in some cases, have been even more toxic than the active ingredient alone [20].

Before herbicide products are registered for use, the registration authorities require experimental information on their toxicology, biology, chemistry, and biochemical degradation in addition to their effect on air and water quality, soil microorganisms, and wildlife. Although commercial herbicide products contain several different ingredients, toxicity tests are usually conducted only on the active ingredient, which is the component of the product believed to actually affect the target organism [20]. The criteria for assessing the possible effects of herbicides on the safety of humans, animals and the environment are the herbicide's toxicity (including carcinogenicity, mutagenicity, endocrine disruption, reproduction and developmental abnormalities), biomagnification, and persistence in the environment ([20].

Given the scarcity of water resources in South Africa, aquatic herbicides are of special interest. The potential of an aquatic herbicide to adversely affect aquatic organisms depends on its inherent toxicity to the specific organism and the organism's exposure to the compound in terms of concentration and duration [21]. The inherent toxicity of the pesticide, which is due to its mode of action, is a specific relationship between the organism and the chemical, whereas factors such as application rates and techniques, chemical and physical properties of the pesticide, and environmental conditions at the time of application can make exposures highly variable.

Herbicides now lead all other pesticide groups in terms of amount produced, total acreage treated, and total value from sale. Over the past decades, public awareness of the worldwide increase in the use of herbicides and their adverse effects on aquatic ecosystems has been growing [22]. Herbicides may reach water bodies directly by overhead spray of aquatic weeds, or indirectly through processes such as agricultural runoff, spray drift and leaching. Potential problems associated with herbicide-use include injury to non-target vegetation, injury to crops,

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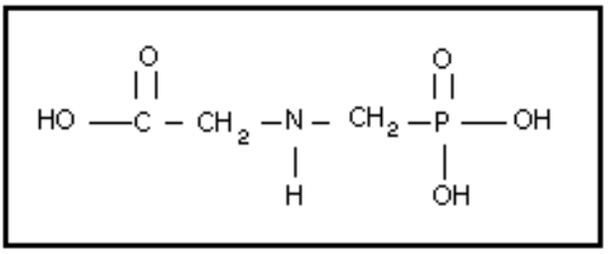

Figure 1. Molecular structure of N-(Phosphonomethyl) glycine

residue in soil or water, toxicity to non-target organisms, and concerns for human health and safety [20]. Herbicides can decrease environmental water quality and ecosystem functioning by reducing species diversity, changing community structure, modifying food chains, altering patterns of energy flow and nutrient recycling, and reducing resilience of ecosystems, among others [22].

3. Roundup®

3.1. Glyphosate-based herbicides

Glyphosate-based herbicides are the world's leading post-emergent, organophosphonate systemic, broad-spectrum and non-selective herbicides for the control of annual and perennial weeds [22]. Roundup® is the major glyphosate-based herbicide in which glyphosate (the active ingredient) is formulated as isopropylamine (IPA) salt, polyoxyethylene amine (POEA) (a surfactant), and water. Other formulations (e.g. Rodeo®) contain the IPA salt of glyphosate without POEA, and in some countries are primarily used for controlling aquatic weeds [23-24]. Other trade names of glyphosate-based herbicides include Roundup®, Roundup Ultra®, Roundup Pro®, Accord®, Honcho®, Pondmaster®, Protocol®, Rascal®, Expedite®, Ranger®, Bronco®, Campain®, Landmaster®, Fallow Master® and Aquamaster® by Monsanto; Glyphomax®, Glypro® and Rodeo® by Dow Agrosciences; Glyphosate herbicide by Du Pont; Silhouette® by Cenex/Land O'Lakes; Rattler® by Helena; MirageR® by Platte; JuryR® by Riverside/Terra; and Touchdown® by Zeneca [25].

Glyphosate is an aminophosphonic analogue of the natural amino acid glycine [22].The International Union of Pure and Applied Chemistry's (IUPAC) name for glyphosate is N-(phosphonomethyl) glycine and the Chemical Abstracts Service (CAS) registry number is 1071-83-6. The glyphosate molecule has several dissociable hydrogens, especially the first hydrogen of the phosphate group (Figure 1). Thus, a typical glyphosate molecule is an acid, and is often referred to as the technical grade glyphosate.

Technical-grade glyphosate has a relatively low solubility in water (12 g/L at 25° C and 60 g/L at 100° C), and is insoluble in other solvents because of strong intermolecular hydrogen bonds that stabilise the crystal lattice [26]. For this reason, commercial herbicide formulations contain glyphosate in the form of salt, which has much higher solubility but still maintains the

herbicidal properties of the parent compound [22]. Formulations of glyphosate in salt form include monoammonium salt, diammonium salt, isopropylamine salt, potassium salt, sodium salt, and trimethylsulfonium or trimesium salt. Of these, the isopropylamine, sodium, and monoammonium salt forms are commonly used in formulated herbicide products [27].

The isopropylamine salt is the most commonly used in commercialised formulated products (e.g. Roundup®). The physical and chemical properties of glyphosate acid and two of its salt forms are listed in Table 1. The concentration of glyphosate is commonly expressed as mg a.i./L (active ingredient/Litre) or mg a.e./L (acid equivalents/Litre) [22]. Acid equivalent is the theoretical percent yield of parent acid from a pesticide active ingredient, which has been formulated as a derivative (usually esters, salts or amines) [28].

Active ingredient	Form	Vapour pressure	Henry's constant	Molecular weight	Solubility in water	Log K_{ow}	K_{oc}
Glyphosate acid	Odourless, white solid	1.31 x 10^{-2} mPa (25° C); 1.84 x 10^{-7} mmHg (45° C)	4.08 x 10^{-19} atm·m^3/mol	169.07 g/mol	pH 1.9: 10,500 mg/L; pH 7: 157,000 mg/L	< -3.2	300 - 20,100
Glyphosate Isopropylamine salt	Odourless, white solid	2.1 x 10^{-3} mPa (25° C); 1.58 x 10^{-8} mmHg (25° C)	6.27 x 10^{-27} atm·m^3/mol	228.19 g/mol	pH 4.1: 786 ,000 mg/L	-3.9 or -5.4	300 - 20,100
Glyphosate ammonium salt	Odourless, white solid	9 x 10^{-3} mPa (25° C); 6.75 x 10^{-8} mmHg (25° C)	1.5 x 10^{-13} atm·m^3/mol	186.11 g/mol	pH 3.2: 144,000 mg/L	-3.7 or -5.3	300 - 20,100

Table 1. Physical and chemical properties of glyphosate acid, glyphosate isopropylamine salt, and glyphosate ammonium salt [27]

3.2. Mode of action of glyphosate

As a systemic herbicide, glyphosate is readily translocated through the phloem to all parts of the plant. Glyphosate molecules are absorbed from the leaf surface into plant cells where they are symplastically translocated to the meristems of growing plants [22]. Glyphosate's phytotoxic symptoms usually start gradually, becoming visible within two to four days in most annual weeds, but may not occur until after seven days in most perennial weeds. Physical phytotoxic symptoms include progress from gradual wilting and chlorosis, to complete browning, total deterioration and finally, death [22]. The primary mode of action of glyphosate

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is confined to the shikimate pathway aromatic amino acid biosynthesis, a pathway that links primary and secondary metabolism.

Shikimate (shikimic acid) is an important biochemical intermediary in plants and microorganisms, such as bacteria and fungi. It is a precursor for the aromatic amino acids phenylalanine, tryptophan and tyrosine. Other precursors of the shikimate pathway are indole, indole derivatives (e.g. indole acetic acid), tannins, flavonoids, lignin, many alkaloids, and other aromatic metabolites. The biosynthesis of these essential substances is promoted by the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), the target enzyme of glyphosate (Figure 2). This enzyme is one of the seven enzymes that catalyse a series of reactions, which begins with the reaction between shikimate-3-phosphate (S3P) and phosphoenolpyruvate (PEP). The shikimate pathway accounts for about 35 % of the plant mass in dry weight and therefore any interference in the pathway is highly detrimental to the plant. Glyphosate inhibits the activity of EPSPS, preventing the production of chorismate, the last common precursor in the biosynthesis of numerous aromatic compounds in bacteria, fungi and plants. This causes a deficiency in the production of the essential substances needed by the organisms to survive and propagate [22, 29]. The pathway is absent in animals, which may account for the low toxicity of glyphosate to animals.

However, acute effects in animals, following intraperitoneal administration of high glyphosate doses suggest altered mitochondrial activity, possibly due to uncoupling of oxidative phosphorylation during cellular respiration [26]. In summary, glyphosate ultimately interrupts various biochemical processes, including nucleic acid synthesis, protein synthesis, photosynthesis and respiration, which are essential life processes of living things.

3.3. Environmental fate of glyphosate

Glyphosate has a strong soil adsorption capacity, which limits its movement in the environment. The average half-life of glyphosate in soil is two months, but can range from weeks to years [25]. The presence of glyphosate in water systems may be due to runoff from vegetation surfaces, spray drift, and intentional or unintentional direct overspray, with an average half-life of two to ten weeks [25]. Glyphosate is susceptible to chemical and photo-degradation, although microbial degradation is the primary dissipation mechanism in soils. The rate of degradation in water is generally slower than in most soils because of fewer microorganisms in water than in soils [30]. When glyphosate degrades, it produces aminomethylphosphonic acid (AMPA) and carbon dioxide [31], both of which reduce pH when dissolved in water. However, pH is known to affect the stability of glyphosate in water. For instance, glyphosate did not undergo hydrolysis in buffered solution with a pH of 3, 6, or 9 at 35° C, while insignificant photodegradation has been recorded under natural light in a pH 5, 7, and 9 buffered solutions [27]. In natural water systems, glyphosate dissipates through degradation, dilution, and adsorption on organic substances, inorganic clays and the sediment (the major sink for glyphosate in water bodies) [25, 30]. With its long half-life and its ability to cause the death of organisms in aquatic systems, it is recommended that glyphosate should be used as an aquatic herbicide to treat only one-third to half a water body at any one time [25].

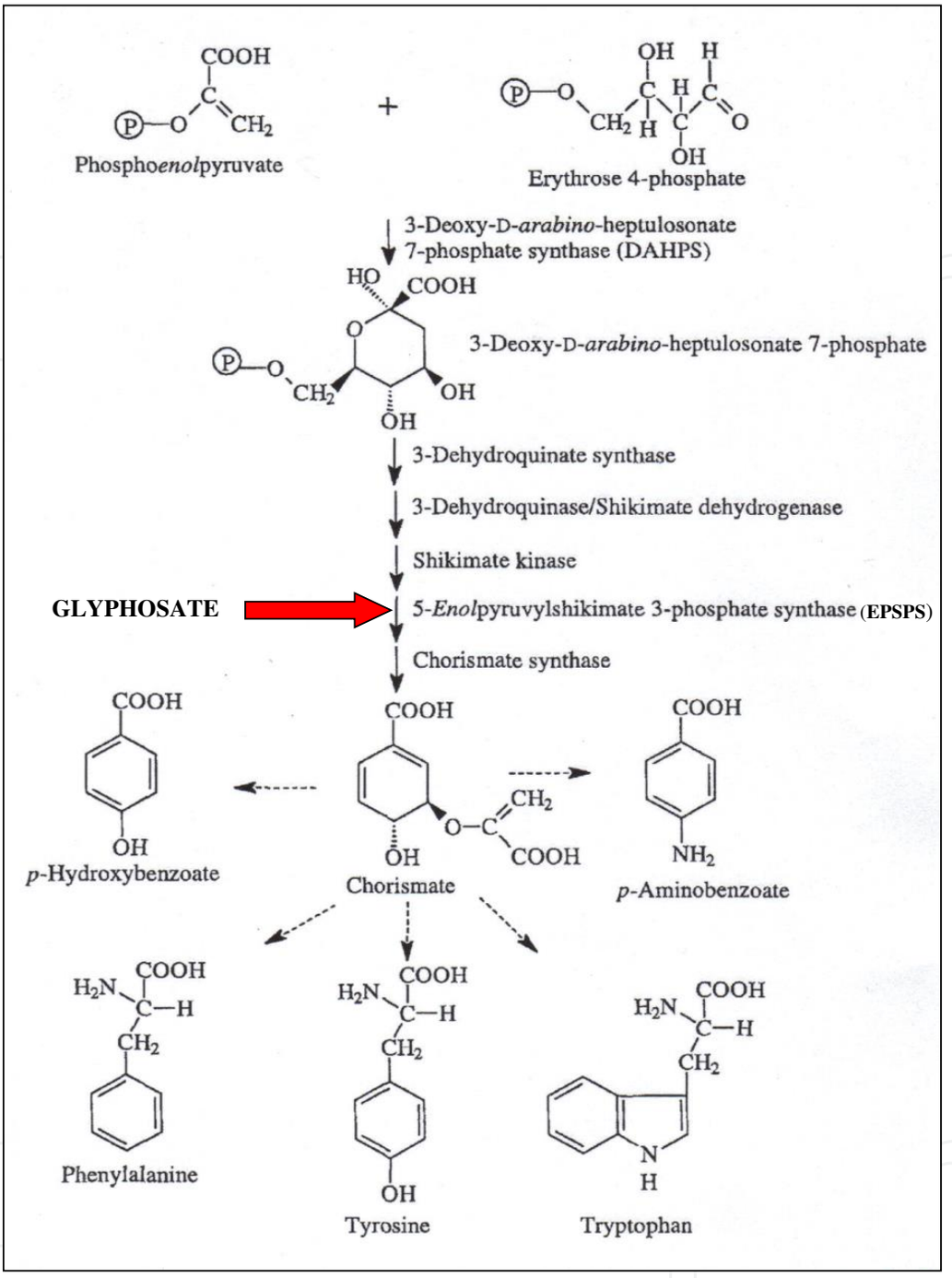

Figure 2. Glyphosate mode of action in plants with red arrow pointing to the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase (modified from [32])

3.4. Toxicology of glyphosate

Ecotoxicologists are greatly concerned about the exposure of non-target aquatic organisms to glyphosate formulations because of its high water solubility and the extensive use of glyphosate-based herbicides in the environment, especially in shallow water systems [23]. The surfactant polyoxyethylene amine (POEA) is thought to be responsible for the relatively high

toxicity of Roundup® to several freshwater invertebrates and fishes, although isopropylamine (IPA) salt of glyphosate also contributes its share [23, 33]. Technical grade glyphosate is slightly to very slightly toxic, with reported LC50 values of greater than 55 mg/L and a 21 d NOEC value of 100 mg/L.

Conversely, formulations of glyphosate are moderately to very slightly toxic with 2 d EC50 values of 5.3-5600 mg/L and 21 d MATC values of 1.4-4.9 mg/L reported [26]. The LC50 values also determine which glyphosate formulation can be applied in aquatic systems. For example, Touchdown 4-LC® and Bronco® have low LC50s for aquatic species (<13 mg/L), and are not registered for aquatic use, while Rodeo® has relatively high LC50s (>900 mg/L) for aquatic species and is permitted for use in aquatic systems. In the same manner, Roundup® is not registered for use in aquatic systems in the United States because its 96-hour LC50 for *Daphnia* is 25.5 mg/L, while that of glyphosate alone is 962 mg/L [25].

3.5. Effects of glyphosate-based herbicides on aquatic animals

Glyphosate-based herbicides are used globally to control both aquatic and terrestrial weeds. In recent years, its use has increased tremendously and is likely to impact on non-target organisms in the environment. Even though it is generally regarded as having a low potential for contaminating surface waters due to its perceived rapid dissipation and strong adsorption to soils and sediments, it has been detected in surface waters long after being used to kill aquatic weeds [34]. In fact, its mode of action was designed to affect only plants [29], but various studies in recent years have reported adverse impact on non-target animals [23, 33, 35]. These impacts could be lethal or sublethal. Lethal effects are mainly mortality and immobility endpoint measures. However, there are several endpoint measures that can be used to assess sublethal effects. At the 'physical' level, measures of survival, growth, morphological changes, and behavioural changes exposed animals are used as endpoint indicators. Measures of reproductive performance that are often used to assess sublethal response include sexual maturity, time to first brood release, time required for egg development, fecundity, gonad histopathology, and alterations in reproductive characteristics. Biochemical measures used as possible endpoints to assess exposed animals include metabolic disruption, steroid metabolism, vitellogenin induction, lipid peroxidation, acetylcholinesterase activity, cytochrome P450enzymes and blood glucose levels.

4. Exposure effects

4.1. Classification of exposure effects

The effect caused by exposure to chemicals can be classified according to different exposure time (short-term or long-term) and exposure type (lethal or sublethal). Short-term exposure time is usually defined as not more than 96 h, while long-term exposure time defined as being more than 96 h (Table 2). There are different possibilities of effect to expect when animals are exposed to chemicals. Lethal exposure to stress can possibly cause a biological system to respond in short-term or long-term. Similarly, biological systems can experience sublethal

responses to a stressor in short-term or long-term (Table 3). Lethal exposure will often result in mortality (i.e. immobility, decolouration and degeneration), whereas sublethal exposure normally results in a cellular, molecular or biochemical level response including growth (length, weight and moulting), reproduction (embryo and gonads) and biochemical (acetylcholinesterase and lipid peroxidation) (Table 3).

Exposure classification	Effect classification	Description
Exposure time	Short-term	≤ 96 h
	Long-term	≥ 96 h
Exposure type	Lethal	Mortality measure as endpoint
	Sublethal	Cellular/molecular/biochemical/physiological level measure as endpoint

Table 2. Exposure-effect classification of chemicals

Effect classification	Description
Short-term lethal	≤ 96 h and mortality measure as endpoint
Short-term sublethal	≤ 96 h and cellular/molecular/biochemical/physiological level measure as endpoint
Long-term lethal	≥ 96 h and mortality measure as endpoint
Long-term sublethal	≥ 96 h and cellular/molecular/biochemical/physiological level measure as endpoint

Table 3. Different possibilities of effect of animals exposed to chemicals

4.2. Effects of lethal exposure

Mortality is the most common endpoint measure when organisms are exposed to a lethal dose, although immobility is also considered as lethal effect of exposure. In [36] the relevance of using mortality and immobility as endpoints to reflect the toxicity of the organophosphorous insecticide chlorpyrifos in fourteen different freshwater arthropods was evaluated. Using dose response models and species sensitivity distributions (SSDs), they compared the differences in response dynamics during 96 h of exposure with these two endpoints across the different species. Their study suggests that freshwater arthropods vary less in their immobility response than in their mortality response. They suggested immobility as the relevant endpoint for SSDs and ERA (environmental risk assessment) because they found it was a more sensitive endpoint than mortality, with less variability across the tested species. Generally, effect concentrations for immobility and mortality will converge to the same value with time, but this does not occur with the same speed for all species [36]. However, a good match between effective (immobility) and lethal (mortality) concentrations can exist right from the start of a toxicity test where LC50/EC50 ratios equal one, approximately. For some species, the differences between LC50 and EC50 can remain relatively constant within the 96 h of testing. Furthermore, the extent to which LC50 and EC50 values differ for certain time points is species specific [36]. For example,

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exposure concentrations may not induce any significant incipient mortality in a particular species, but will induce immobility at very low concentrations in another species. This is due to differences in toxicokinetics and/or toxicodynamics between the species. For instance, differences in toxicokinetics may enable one species to decrease or regulate uptake and eliminate the test chemical, or detoxify it quickly, thereby significantly delaying incipient mortality. Toxicodynamic differences, such as differences in the interaction of the stressor and target enzyme, or in the ability to compensate or repair damage, may cause different species to respond differently to the test chemicals [36].

Mortality was also used as an endpoint response measure by [37] when they studied the acute mortality of adults and sub-lethal embryo responses of *Palaemonetes pugio* to endosulfan. Their findings suggest that the insecticide endosulfan may preferentially affect male grass shrimp, and exposed female grass shrimp may produce embryos with delayed hatching times. They suggested that the size difference between male and female grass shrimp might be the cause as mortality decreases by 25 % with a corresponding increase in size of 1 mm.

Some studies have reported correlation between lethal and sublethal effects. In [38], the correlation between 96 h mortality and 24 h acetylcholinesterase inhibition in three life stages of the grass shrimp (*Palaemonetes pugio*) after exposure to organophosphate pesticides was investigated. They found a strong positive relationship ($R^2 = 0.962$) between the ratio of the lowest observed effect concentration and 20 % effect concentration (LOEC/EC20). Therefore, they concluded that sublethal endpoints could be used as a predictor of 96 h mortality for the life stages of *P. pugio*.

4.3. Growth measures used as sub-lethal responses to exposure

Body weight and length are two direct measures of growth that may be used in the assessment of sub-lethal effects on arthropods. Simple dry weight can be determined by drying sampled animals at an average temperature of 60º C, and a mean drying time of 48 hours to constant weight [39]. However, for many invertebrates, ash-free dry weight (AFDW) is often used as the appropriate weight measurement because the method reduces any inaccuracies that might be introduced by inorganic constituents in the animal's body. Inorganic components may arise from processes such as the development of skeletal components, or from feeding (the ingestion of sediment) [39]. In small-sized crustaceans, such as caridean shrimps and mysids, the removal of ash from the dry weight measurement is unnecessary since it would have a negligible effect on the accuracy of the measurement [40]. Separate determinations should be made for male and female crustaceans because they might be different sizes [39]

Different body length dimensions of shrimp can be measured to determine growth. These may include the distance from the base of the eye-stalks to the tip of the telson or to the tip of the exopod; or from the tip of the carapace to the tip of the exopod along the midline of the body [39]. Sometimes, it is difficult to measure preserved animals because of the body curvature that results from the fixation process. Relaxing the animal and then determining length as the sum of a series of relatively straight-line measurements prior to fixation may reduce inaccuracy. Animals may be anaesthetized in soda water to relax them prior to length measurements [41-42].

Reduced growth may not be a particularly sensitive endpoint, but it is the most common response to sub-lethal exposure to toxicants [39]. Reduced growth is connected to reproductive success since the size of female crustaceans is directly related to fecundity [43]. The age of test animals and the toxicant concentration are related to the effect of toxicant exposure on growth. In general, young crustaceans are more sensitive than adults to toxicant exposure. However, effects of toxicants on juvenile survival do not always lead to reductions in population growth rate since survivors may compensate for the lost individuals by increasing their own reproduction [44]. Similarly, effects at the individual level may sometimes run opposite to the population level effects. This shows complex relationship between toxicant effects on individual performance versus population dynamics. [44] evaluated the effects of nonylphenol on two life-history traits (i.e. juvenile survival and fecundity) of the parthenogenetic springtail, *Folsomia candida,* in relation to population growth rate. They reported that the presence of nonylphenol stimulated fecundity and the body-growth rate of test organisms, but did not affect population growth rate. The authors found that the effect of the test chemical on fecundity was the main contributor of the observed effect on growth rate. However, since relative sensitivity of fecundity (elasticity) was very low, large changes in fecundity resulted in a minimal effect on population growth rate. Conversely, juvenile survival had higher elasticity, but was not affected by nonylphenol, and hence did not contribute to effects on population growth rate. The study by [44] revealed that increase in body size and fecundity after exposure to chemicals does not necessarily translate into increase in population growth rate. Their study also shows that effects of chemicals on individual life-history traits are attenuated at the population level and that population growth rate is an appropriate endpoint for ecotoxicological studies.

Moulting is an important physiological process in arthropods because it allows them to grow [45-46]. It is regulated by the interaction of moult stimulating hormones (MSHs, generally referred to as ecdysteroids), and nervous system secretions produced in the cephalothorax, and with moult-inhibiting hormones (MIHs) produced in the eyestalks [39]. In higher crustaceans such as the Malacostraca, paired cephalic endocrine organs called Y-organs (absent in lower crustaceans such as Entomostraca) secrete three different ecdysteroids, namely ecdysone (E), 25-deoxyecdysone (25dE), and 3-dehydroecdysone (3DE). Usually these organs produce either E + 25dE, or E + 3DE. Activities of the Y-organ are regulated mainly by the MIH, an inhibitory neuropeptide secreted from the X-organ-sinus gland complex [45-46]. Since hormones regulate moulting in crustaceans, moulting is a clear indicator of the adverse effects of endocrine disrupting chemicals, which include many pesticides. Hormonal regulation of moulting in crustaceans makes the process vulnerable to the adverse effect of endocrine disrupting chemicals (EDCs), including many pesticides [39]. Furthermore, since substantial growth in crustaceans can only occur as a result of moulting, any disruption in the moulting process could affect growth. Therefore, estimation of moulting frequency may be a useful endpoint.

Moult stage is a useful technique for measuring growth [39]. If moult stages are classified based on duration of different stages under normal laboratory conditions, then the environmental effects on relative duration of stages can be evaluated, using the moult-stage technique [47].

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However, moult-cycle chronology is a prerequisite for the use of moult staging in growth studies. The moult-stage technique was used to determine the main moult stages for juveniles and young adults of *Mysis mixta* and *Neomysis integer* under different temperature conditions and feeding. The technique was also used in the field to determine the moult cycle duration of *Mysis mixta* [47].

4.4. Reproductive measures used as sublethal responses to exposure

Embryotoxicity and gonad histopathology are two main reproductive measures used as sublethal responses to exposure. Embryo development time (or incubation period) in caridean shrimps is measured as the number of days between the first appearance of embryos in the brood pouch and the first release of neonates. In uncontaminated systems, incubation period is related to environmental temperature, salinity and an interaction between the two factors. However, the effect of most contaminants is to lengthen hatching time. In many embryotoxicity studies, either gravid females are placed in exposure containers, or fertilized eggs are removed from the female and placed in exposure containers where they develop to hatching. In [48], both gravid maternal and isolated embryos of *Daphnia magna* were exposed to the agricultural fungicide fenarimol to evaluate embryo development and susceptibility to the anti-ecdysteroidal properties of the fungicide. They reported that exposure of either gravid maternal animals or isolated embryos to the test toxicant resulted in embryo abnormalities which ranged from early partial developmental arrest to incomplete development of antennae and shell spines. They found that such developmental abnormalities were linked to suppressed ecdysone levels in the embryos and that the abnormalities could be prevented by co-exposure to 20-hydroxyecdysone. The results also showed how environmental anti-ecdysteroids, such as fenarimol, in many agro-chemicals disrupt the normal development of crustacean embryos.

Effective embryotoxicity investigations are based on identification of specific developmental features during embryogenesis and the susceptibility of such features to chemical exposure. Embryonic development of *C. nilotica* under laboratory conditions was investigated by [49] and identified stages in embryonic development which could be used as quantifiable experimental endpoints in toxicity tests. The author identified and described seven potential developmental stages that could be used in toxicity tests to study exposure-response relationships to stressors.

Histopathology is a technique that combines knowledge and experience of fundamental animal anatomy, physiology, endocrinology, pathology, and toxicology. It can enhance relevant biological information in sublethal exposure tests by allowing proper and more specific hazard identification, such as the organs targeted by toxic substances and mechanisms of action in aquatic ecotoxicological studies [50]. Histopathology is relevant to an ecological assessment of toxicants because it can detect critical adverse biological effects (e.g. reproductive abnormality) and is more sensitive than the classical toxicological testing, since histological effects are visible at lower exposure concentrations than they are at toxicological endpoints, such as mortality or behavioural changes [50]. The use of small crustaceans in practical histopathology makes it possible to embed the animals *in situ* for a quick overview of various relevant organs, making screening fast and comprehensive [50].

4.5. Biochemical measures used as sub-lethal responses to exposure

Acetylcholinesterase activity (AChE) and lipid peroxidation (LPx) are two biochemical measures often used as to assess sublethal responses to exposure. The main physiological function of the enzyme AChE is to hydrolyze acetylcholine (ACh), a neurotransmitter of cholinergic synapses during transduction of nerve impulses. Inhibition of AChE prevents the hydrolysis of ACh in nerve synapses and neuromuscular junctions, causing accumulation of excess ACh at these sites. This results in over-excitation of the synaptic and muscular tissues, which may lead to abnormal behaviours such as hyperactivity, asphyxia and death. AChE activity is therefore regarded as a good biomarker to detect a range of toxic compounds in aquatic animals, including insecticides, herbicides, surfactants and metals [51-52]. In a study to evaluate AChE activity in the oyster *Crassostrea corteziensis*, [53] exposed the organisms to the pesticide dichlorvos. The results of their study revealed that AChE activity was 65 % lower in oysters exposed to the pesticides than in control animals. Based on this outcome, they suggested using AChE activity in oysters as early biomarkers of effects and exposure to pesticides in aquatic environments. Similar observations and suggestions were made when the mosquito fish *Gambusia affinis* was exposed to the pesticide chlorpyrifos [54]. Although AChE is used as a classical biomarker in biomonitoring studies with regard to the exposure of a number of organophosphate and carbamate pesticides, recent studies have shown the existence of sublethal effects of glyphosate-based compounds on biomarkers of neurotoxicity including AChE [33, 55-56]

Lipid peroxidation is a recognised mechanism of cellular injury in plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds which include reactive carbonyl compounds. Polyunsaturated fatty acid peroxides decompose to produce malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), and the measurement of MDA and HAE is used as an indicator of lipid peroxidation. Whether cells and tissues are susceptible to oxidative stress when exposed to pesticides reflects the balance between oxidative stress and the anti-oxidant defence capability. Since free radicals and hydroperoxides are potentially harmful, toxicants that stimulate lipid peroxidation and/or weaken anti-oxidant defence capability may cause or increase cellular susceptibility to oxidative damage. Animals exposed to pesticides may have their anti-oxidant defence capabilities directly or indirectly modified, rendering them susceptible to oxidative stress. Oxidative damage of cells and tissues of animals exposed to pesticides may be the result of insufficient anti-oxidant potential [57]. Developing biomarkers of oxidative stress as a pollution-mediated mechanism of toxicity requires knowledge of how anti-oxidant biochemical systems and target molecules are influenced by test toxicants [58].

Different toxicants may produce different anti-oxidant/pro-oxidant responses in organisms, depending on whether the organism can produce reactive oxygen species and anti-oxidant enzymes to detoxify them. Changes in juveniles of the freshwater crustacea *Daphnia magna* anti-oxidative processes in were assessed by [58] after exposure to paraquat and endosulfan in a 48 h sublethal toxicity test. They evaluated lipid peroxidation and activities of key anti-oxidant enzymes including catalase, superoxide dismutase, glutathione peroxidase and glutathione S-transferases. They found that increased lipid peroxidation produced low anti-

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oxidant enzyme activity for endosulfan, while decreased lipid peroxidation enhanced levels of anti-oxidant enzyme activities for paraquat. In [59], the authors suggested that glyphosate exposure and metabolism in the liver of animals can lead to excessive production of MDA and oxidative stress through unregulated generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, hydroxyl radical, peroxyl radicals and singlet oxygen. Excessive ROS in turn can be detrimental to cell structure through oxidative damage of lipids, proteins or DNA, and altered regulation of gene functions critical for development, differentiation, and aging.

5. Lethal and sublethal effects of *Caridina nilotica* exposure to Roundup®

5.1. *Caridina nilotica*: a decapod freshwater shrimp

The exposure of non-target aquatic organisms to glyphosate-based herbicides is of great concern because of the high water solubility of glyphosate and its extensive use in the environment. Thus, it is important to investigate the effects of these bioactive chemicals on aquatic organisms. *Caridina nilotica* (Decapoda: Atyidae) (Figure 3) is the most common of four indigenous freshwater caridean species found in South Africa. It has been used in ecotoxicological studies in South Africa since the early 1990s. Roundups® was selected as a representative of glyphosate-based herbicides by the virtue of it being the most popular and widely used herbicide in South Africa and most parts of the world [60-61]. In this section of this chapter, summary of findings of some exposure tests are given to demonstrate lethal and sublethal effects of Roundup® to *C. nilotica* at different biological scales. Mortality was the lethal effect investigated, whereas the sublethal effects studied were growth, acetylcholinesterase activity and lipid peroxidation. The tests were all aimed at demonstrating the use of *C. nilotica* as an early detection sensor system of pesticides pollution in South African aquatic ecosystems. Comprehensive reports of these tests are reported in [62-65].

5.2. Short-term lethal tests — Mortality

The toxicity of the herbicide Roundup® was assessed using three different life stages of *C. nilotica*. Neonate (<7 days post hatching (dph)), juvenile (>7 dph and <20 dph) and adult (>40 dph) shrimps were exposed to varying concentrations of the herbicide in 48 and 96 h short-term lethal tests in order to determine the most sensitive life-stage. Mortality was calculated at the end of each test period. Based on this, Roundup® 48 h and 96 h LC50 (median lethal concentration) values and the associated 95 % confidence limits were calculated for *C. nilotica* using the probit method. The results showed neonates to be more sensitive to Roundup® than both juveniles and adults. The estimated 96 h LC50 of neonates is much lower than the field application rate, though the application's impact will depend on the dilution rate of the applied concentration in the environment. This study shows that low levels of the Roundup® may adversely affect *C. nilotica* health and survival. Thus, the herbicide should be carefully managed to minimize any negative impact on non-target freshwater organisms.

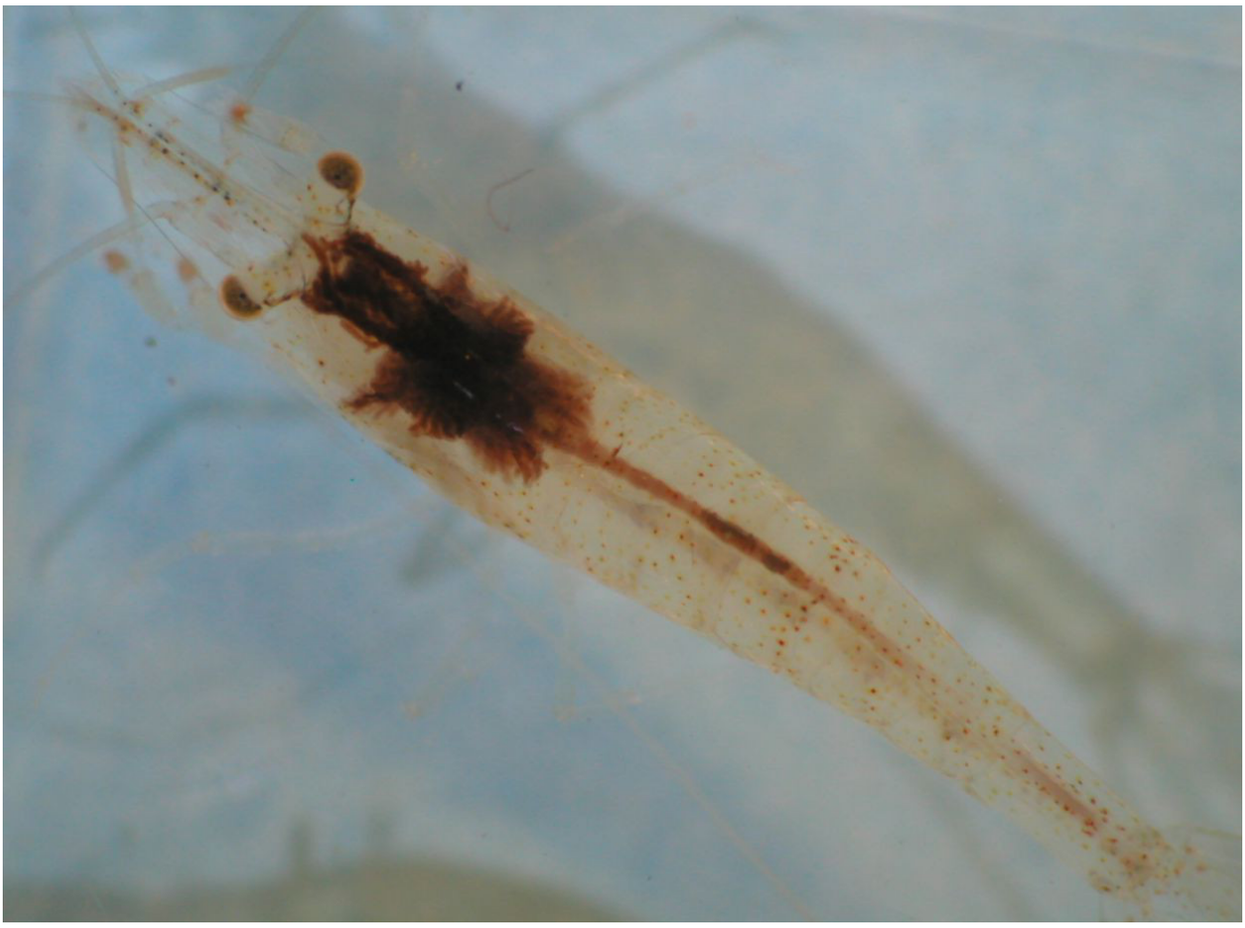

Figure 3. Adult *Caridina nilotica*

5.3. Long-term sublethal tests — Growth

The possible use of growth measures in *C. nilotica* as biomarkers of Roundup® pollution as part of aquatic life in South Africa were evaluated. Using static-renewal methods in a 25-d growth toxicity test, 40 dph shrimps were exposed to different sublethal Roundup® concentrations. Shrimp total lengths and wet weights were measured every fifth day. These data were used to determine the shrimp's growth performance and feed utilization in terms of percent weight gain (PWG), percent length gain (PLG), specific growth rate (SGR), condition factor (CF), feed intake (FI), feed conversion ratio (FCR) and feed conversion efficiency (FCE). Moulting was observed for 14 d and the data used to determine the daily moult rate for each concentration. Results of growth performance and food utilization indices showed that growth was significantly impaired in all exposed groups compared to control. Moulting frequency was also higher in all exposed groups than in control. Although all the tested growth measures proved to be possible biomarkers of Roundup® pollution, moulting frequency gives a clearer indication of the sublethal effects of Roundup® toxicity to *C nilotica*.

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5.4. Short-term and long-term sublethal tests — Biochemical

The use of *C. nilotica* whole-body acetylcholinesterase (AChE) activity and lipid peroxidation as potential biomarkers of Roundup® pollution of aquatic ecosystems was investigated. Forty days post hatch (dph) shrimps were exposed to different concentrations of Roundup® in a 96 h short-term sublethal test and a 21 d long-term sublethal test. Shrimp whole-body AChE activities were determined at the end of the exposure periods by spectrophotometric assay of sample extract. Final AChE activities were expressed as nmol/min/mg proteins. Shrimp whole-body LPx was estimated by thiobarbituric acid reactive species (TBARS) assay, performed by a malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA) measured spectrophotometrically. Final MDA concentrations were expressed as nmol MDA produced/mg protein. The results showed that AChE activity was concentration-dependent, with percent activity levels decreasing monotonically from control to the highest concentration. Conversely, LPx was significantly lower in control than in shrimps exposed to different Roundup® concentrations, increasing monotonically. The study provides ecotoxicological basis for the possible use of AChE activity and LPx in *C. nilotica* as possible biomarkers for monitoring effects of Roundup® pollution in freshwater systems.

6. Concluding remarks

In this chapter, the effects of rapid human population growth on aquatic ecosystems have been discussed. These effects are seen in such phenomena as climate change, nutrient enrichment of aquatic environments, and pollution by all types of chemicals including pesticides on local, regional and global scales. These anthropogenic disturbances adversely impact the normal functioning of organisms and are responsible for a number of developmental anomalies in a wide range of species; from invertebrates to higher mammals. It is expected that the use of pesticides, especially herbicides, will continue to increase and eventually becoming environmental hazard to non-target organisms at different biological scale levels unless proactive measures are taken. The case study, i.e. lethal and sublethal exposures of *C. nilotica* to varying environmentally relevant concentrations of Roundup®, showed that *C. nilotica* can be used as early detection system to assess glyphosate-based herbicides pollution effects on aquatic ecosystems.

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