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Original Article

Ecotoxicological effects of commercial herbicides on the reproductive system of aquatic arthropod *Limnocoris submontandoni* (Hemiptera: Naucoridae)

Efeitos ecotoxicológicos de herbicidas comerciais no sistema reprodutivo do artrópode aquático *Limnocoris submontandoni* (Hemiptera: Naucoridae)

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Abstract

Worldwide, conventional agriculture makes extensive use of pesticides. Although the effects of herbicides are relatively well known in terms of environmental impacts on non-target organisms, there is very little scientific evidence regarding the impacts of herbicide residues on aquatic arthropods from tropical conservation areas. This study evaluates for the first time the toxicity of the herbicides ametryn, atrazine, and clomazone on the aquatic insect *Limnocoris submontandoni* (Hemiptera: Naucoridae). The lethal concentration (LC_{50}) of herbicides was evaluated for these insects, as well as the effect of the herbicides on the insects' tissues and testicles. The estimated LC_{50} was 1012.41, 192.42, and 46.09 mg/L for clomazone, atrazine, and ametryn, respectively. Spermatocyte and spermatid changes were observed under the effect of atrazine, and effects on spermatogenesis were observed for some concentrations of clomazone, with apparent recovery after a short time. Our results provide useful information on the effects of herbicide residues in aquatic systems. This information can help minimize the risk of long-term reproductive effects in non-target species that have been previously overlooked in ecotoxicology studies.

Keywords: ametryn, atrazine, clomazone, *Limnocoris*, Naucoridae.

Resumo

Em todo o mundo, a agricultura convencional faz uso extensivo de pesticidas. Embora os efeitos dos herbicidas sejam relativamente bem conhecidos em termos de impactos ambientais em organismos não-alvo, há pouca evidência científica sobre os impactos de resíduos de herbicidas em artrópodes aquáticos de áreas de conservação tropicais. Este estudo avalia pela primeira vez a toxicidade dos herbicidas ametryn, atrazine e clomazone sobre o inseto aquático *Limnocoris submontandoni* (Hemiptera: Naucoridae). A concentração letal (LC_{50}) de herbicidas foi avaliada para esses insetos, bem como o efeito dos herbicidas nos tecidos e testículos dos insetos. A LC_{50} estimada foi de 1012,41, 192,42 e 46,09 mg/L para clomazone, atrazine e ametryn, respectivamente. Alterações nos espermatócitos e espermátides foram observadas sob o efeito de atrazine, e efeitos na espermatogênese foram observados para algumas concentrações de clomazone, com aparente recuperação após um curto período de tempo. Nossos resultados fornecem informações úteis sobre os efeitos de resíduos de herbicidas em sistemas aquáticos. Essas informações podem ajudar a minimizar o risco de efeitos reprodutivos de longo prazo em espécies não-alvo que foram negligenciadas anteriormente em estudos de ecotoxicologia.

Palavras-chave: ametryn, atrazine, clomazone, *Limnocoris*, Naucoridae.

1. Introduction

Pesticides are divided into different categories, depending upon their target. Some of these categories include herbicides, insecticides, fungicides, rodenticides, molluscicides, nematicides, and plant growth regulators (Rani and Shanker, 2018). These pesticides are applied at conventional farming as pest control to avoiding economic losses. Thus, the use of pesticides is important in avoiding economic losses and guaranteeing production of food,

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fiber, and biofuels. The intensive use of pesticides can allow toxins to disturb natural flora, fauna and aquatic life, but by complying with the recommendations found in integrated pest or weed management protocols, the risks can be reduced (Ferreira et al., 2019; Renaud et al., 2018; Shuman-Goodier and Propper, 2016).

However, in many places the overuse of herbicides is carried out, due to the lack of knowledge of these protocols, which carries a risk of pollution of aquatic systems (Dellamatrice and Monteiro, 2014).

The destination and transport of pesticides are influenced by several processes. Herbicides enter the water via drift, runoff, leaching through the soil, or direct application to surface water in some cases, such as for weed control (Breckels and Kilgour, 2018; Ippolito and Fait, 2019; Yang et al., 2019). Aquatic plant death induced by herbicide application can lead to lethal anoxic environments for all aquatic communities (Mueller et al., 2006). Also, effects on non-target aquatic organisms may be a threat to aquatic food webs, such as the genus Limnocoris (Dehnert et al., 2019; Fiorino et al., 2018; Papoulias et al., 2014).

The Naucoridae has approximately 400 valid species, divided into 40 genera and five subfamilies (Cheirochelinae, Cryphocricinae, Laccocorinae, Limnocorinae e Naucorinae). *Limnocoris* is one of the most diverse genera of the Naucoridae, endemic to the Americas with 71 valid species (Nieser and Ruf, 2001). They are often apex predators in the benthic microhabitat (Sites and Willig, 1991). *Limnocoris submontandoni* is an example of this species found in the Jequitinhonha River basin, Brazil. It is an endemic species of the Brazilian Atlantic Forest (La Rivers, 1974; Nieser and Ruf, 2001; Pelli et al., 2006; Souza et al., 2006; Ribeiro et al., 2009; Rodrigues, 2018). This species is registered only for the southeast of Brazil, in the states of Minas Gerais, Rio de Janeiro, and São Paulo. The lack of studies with this species for many ecological issues place to risk its occurrence. Thus, our study may be useful to contribute in terms of information that awakening curiosity for future research with *L. submontandoni.*

Atrazine (6-chloro-N2-ethyll-N4-isopropyl-1,3,5-triazine-2,4-diamine), ametryn, (N2-ethyl-N4-isopropyl-6-methylthio-1,3,5-triazine-2,4-diamine) and clomazone, 2-(2-chlorobenzyl)-4,4-dimethyl-1,2-oxazolidin-3-one) are three herbicides applied to weed control in sugar cane culture (Elbashir and Aboul-Enein, 2015). The application is pre and post-emergence of weeds, acting as inhibitors of photosystem II and in the synthesis of carotenoids (Mohammadi et al., 2009). However, due to physico-chemical properties, such as relatively high-water solubility, low sorption coefficient, or long half-life, they present a high risk of leaching in surface and groundwater (Sandoval-Carrasco et al., 2013; Alencar et al., 2020).

One of the world's most widely used herbicides is glyphosate (Annett et al., 2014; Cattani et al., 2014; Wang et al., 2016). There are several studies that investigate the impact of glyphosate on non-target organisms (Harayashiki et al., 2013; Lopes et al., 2014; Sánchez et al., 2017). However, there is a gap of studies regarding the impacts of other widely used herbicides, such as atrazine and clomazone, on predators' insects, hemipterans, and tropical species (Cao et al., 2018; Freitas et al., 2017). We hypothesized that the pollution of herbicides led to effects in the morphology of the reproductive systems of *L. submontandoni*. Thus, knowledge about the effects of these herbicides on the morphology of reproductive systems of aquatic arthropods such as *L. submontandoni* may be useful in understanding possible impacts on non-target aquatic organisms and promote the development of strategies that minimize water contamination in order to conserve local biodiversity. In this study, we aimed to evaluate the toxicity of commercial herbicides on the reproductive system of *L. submontandoni* using a simulate situation of formulated product enter an aquatic system would be by direct application to the surface of the water.

2. Materials and Methods

2.1. Insects

Limnocoris submontandoni adults used in the experiment were obtained from a conservation unit mosaic known as the Biribiri State Park in the state of Minas Gerais, Brazil (18° 11′ 09.3″ S 043° 36′ 54.9″ W) (Rylands and Brandon, 2005; Medeiros, 2006). Insects were collected by hand netting, with 0.5 mm mesh, which is typically used to capture specimens associated with vegetation and benthic substrate. A total of 90 individuals and water from the collection site were collected and placed inside plastic pots and taken to the Laboratory of Integrated Management of Weeds, Department of Agronomy at the Universidade Federal dos Vales do Jequitinhonha e Mucuri, Diamantina-MG, Brazil. The specimens were identified as *L. submontandoni* following identification keys (La Rivers, 1974; Nieser and Ruf, 2001). *L. submontandoni* adults were released in each plastic tray (40 × 25 × 7 cm) with two liters water from the collection site and acclimated to the laboratory for 24 hours with an individual aeration system. The additional alimentation was not performed, since we aim to represent a similar situation in the field. Thus, alimentation by *L. submontandoni* were done from possible foods to bring in the water from the collection site.

2.2. Herbicides

The following commercial herbicides were used in the experiments: atrazine (Atrazine Nortox 500®, concentrated suspension at 500 g active ingredient (a.i.)/L, NORTOX S/A Brasil, Arapongas, Paraná, Brazil); clomazone (Gamit 360®, encapsulation suspension at 360 g active ingredient (a.i.)/L, FMC Quimica do Brasil Ltda, Campinas, São Paulo, Brazil); and ametryn (Metrimex 500®, concentrated suspension at 500 g active ingredient (a.i.)/L, Oxon Brasil Defensivos Agrícolas Ltda Brasil, São Paulo, SP – Brazil). The propose to use the commercial product because additive components of the formulation can protect the herbicide molecules in the soil until it reaches the watercourse (Carboneras et al., 2020). Some additives can even enhance the effect of the herbicide by allowing more excellent absorption by non-target organisms (Santos et al., 2005).

2.3. Concentration-mortality bioassays

Herbicides (atrazine, clomazone, ametryn) was diluted in 1000 mL of distilled water to obtain a stock solution. Five different concentrations each of atrazine (30; 250; 467; 937; 1950 mg/L) and clomazone (162; 666; 1008; 1350;1750 mg/L), and six concentrations of ametryn (45; 87.5; 175; 350; 700;1400 mg/L), were then prepared and used to assess herbicide toxicity and determine relevant toxicological endpoints. Four replicates of five insects each were used for each concentration tested following a completely random design. Each experimental unit was composed of a plastic tray (40 × 25 × 7 cm) with an individual aeration system. Each tray was filled with two liters of water from the collection site and two milliliters of each concentration was applied using a precision single channel mechanical micropipette with variable volume (brand DIGIPET, made in Curitiba, state of Parana, Brazil). Water from collection site was used as a control. The number of dead adults was assessed after 24 h, and then daily for four days. Insects were considered dead if they did not respond to prodding with fine-haired paintbrush.

2.4. Histological structure bioassays

A total of five male adults of *L. submontandoni* were subjected to histological analysis. Taking into account that sublethal effects are as important as lethal effects for the development of *L. submontandoni,* the concentrations used were 45 mg/L for ametryn, 125 mg/L for atrazine, and 162 mg/L for clomazone. Water from collection site was used as a control. After 96 hours, the insects were fixed by immersion in 10% formaldehyde solution for 72 hours in order to preserve organs and tissues. After fixation, the specimens were transferred to a 70% ethyl alcohol solution. To ensure fixation of the histological material, longitudinal cuts were made on one side of the insect. After fixation, the material was dehydrated with a series of 1 h immersions in each of four increasing concentrations of ethyl alcohol solution (70%, 80%, 90%, and 100%).

After dehydration, the insects were mounted in resin, and the samples were cast in molds and placed in a greenhouse. After resin polymerization, the samples were placed in small blocks of wood to make 3 µm thick cuts in a LEICA 2055 MULTICUT microtome. The sections were fixed on slides and stained with toluidine blue. The material was examined and photographed using an OLYMPUS BX 41 photomicroscope.

2.5. Statistical analyses

Lethal concentrations (LC_{50}) of herbicides to *L. submontandoni* were calculated using the Trimmed Spearman–Karber method (Hamilton et al., 1977). A descriptive analysis was performed for histological structure bioassays.

3. Results

3.1. Concentration-mortality

Based on the LC_{50} measures obtained from these concentration–mortality bioassays, ametryn was more toxic than atrazine and clomazone at lower concentrations. The LC_{50} estimates of herbicides showed a concentration of 46.09 mg/L for ametryn, 192.42 mg/L for atrazine, and 1012.41 mg/ L for clomazone.

3.2. Histological structure

Germ cells at different stages of development were observed in the testicles of most groups analyzed (Figure 1).

Each testis is composed of one or more long, helicoidal tubule whose wall is formed by flat-core cells. Within these tubules are the germ cells. In order to describe the histological changes observed in this study, a brief description of the main morphological aspects of the testis of *L. submontandoni* follows, as observed in the control group. In the center of the testis are located the spermatogonia. These are the smallest cells of the spermatogenic lineage and form small groups with spherical nuclei and very evident nucleoli (Figure 1A). Spermatocytes are easily recognized owing to their large size. They are the largest cells of the spermatogenic lineage (Figure 1B). Large areas of cytoplasm surrounding the nucleus contain structures with very low affinity for 1% Toluidine Blue, allowing a clear view of the nucleus. Between the nucleus and the cell membrane are areas of intense dye affinity, characterizing the formation of the acrossromatic vacuole typical of spermatoids. During spermatocyte maturation, the poorly stained structures migrate toward the cell poles (Figures 1A, B, C).

Ametryn caused degradation on the internal structures of *L. submontandoni*, thus it was not possible to evaluate spermatogenic structures. However, we were able to observe visible effects on the tissues of insects submitted to atrazine and clomazone at concentrations below LC_{50}. Results are illustrated in Figures 2 and 3 for atrazine and clomazone, respectively.

An examination of histological sections indicated that treatment with atrazine caused degeneration to the spermatocytes (Figure 2A), and especially to the spermatids (Figure 2B).

An examination of the tissues of *L. submontandoni* submitted to clomazone revealed signs of degeneration in spermatids (SPTI) (Figure 3A, B). However, the health of spermatogonia (Figure 3A, B) and spermatocytes (Figure 3B) indicate that spermatogenesis should normalize; and the high concentration of sperm cells (Figure 3C) demonstrates that exposure to clomazone did not alter sperm production.

4. Discussion

This study examines effects of herbicide exposure that can potentially have long-term impacts on the health of the species without direct mortality. Morphological changes to the reproductive system are negative effects to non-target species that should be taken into account when evaluating the effects of herbicides. Ametryn is used mainly in sugarcane crops. Residues of this herbicide have been detected into water bodies adjacent to the sugarcane areas in concentrations of 10–300 mg L^{-1} and 0-3 mg L^{-1} by Kennedy et al. (2012) and by Lewis et al. (2009) respectively. The impact of this herbicide is not new in the

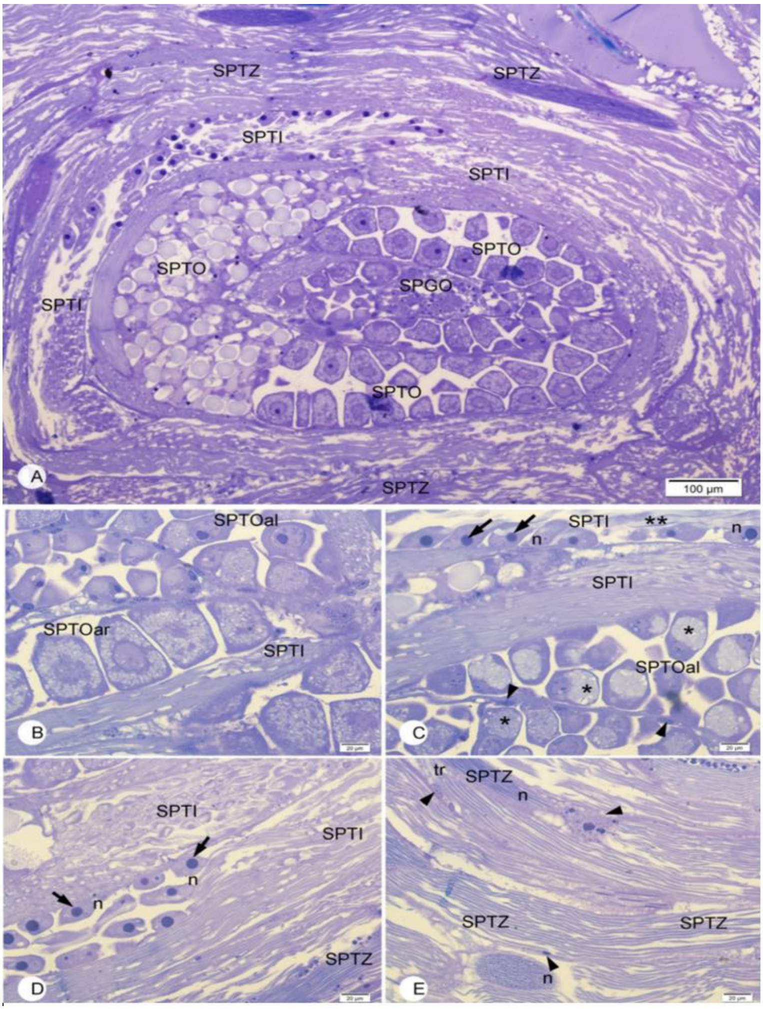

Figure 1. Photomicrographs of the control group of *Limnocoris submontandoni*, (A) helical distribution of the seminiferous tubule with germ cells, spermatogonia (SPGO), spermatocytes (SPTO), spermatids (SPTI), sperms (SPTZ), (B) spermatocytes (SPTO) and spermatids (SPTI) in different degrees of maturation, (C) spermatocytes (SPTO) with slightly colored areas (*) displaced to one of the cell poles, spermatids (SPTI) with different degrees of maturation, acrosome vesicle (arrows), elongation of slightly colored structures (**), elongated cells forming the tubular wall (arrowhead), core (n), (D) Spermatids (SPTI) in different degrees of maturation, spermatozoa (SPTZ) forming compact bundles and very elongated nucleus, nucleus (n) and acrosome vesicles (arrows) of some spermatoids and (E) Compact sperm bundles (SPTZ), sperm nuclei (n), tubular wall cells (arrowhead) and tracheole (tr). Dye: Toluidine Blue.

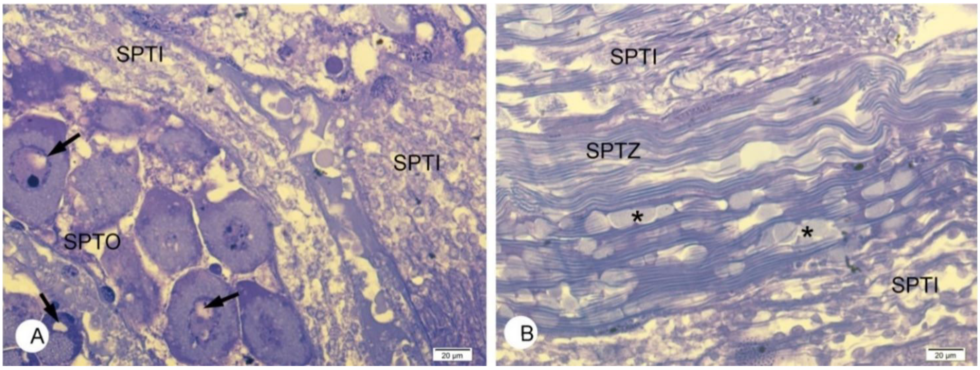

Figure 2. *Limnocoris submontandoni* testis with atrazine 125 mg/L, (A) Hyalinization of cytoplasm, nuclear vacuoles (arrows) in spermatocyte (SPTO) and spermatid degeneration (SPTI), (B) spermatid degeneration (SPTI) altered morphology and large number of cellular remains (*) observed in the outer layers next to the sperms. Dye: Toluidine Blue.

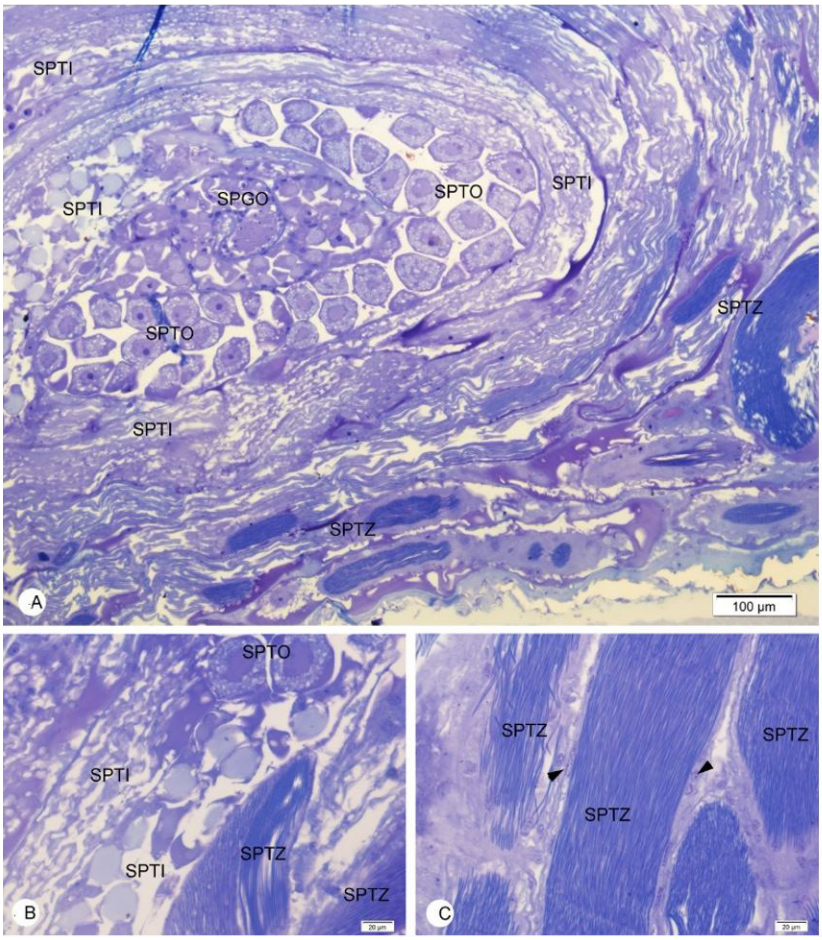

Figure 3. *Limnocoris submontandoni* testis with clomazone 162 mg / L, (A) Apparent enlargement of the testicular interstice and degeneration of some spermatoids (SPTI), with normal concentrations of spermatozoa (SPTZ) in the outer regions, (B) spermatocytes (SPTO) with normal appearance and sperm (SPTZ) with slight alteration of spermatids (SPTI), C) profusion of sperm (SPTZ) with a high degree of compaction, indicating high rates of spermatogenesis and integrity of tubular cells (arrowhead). Dye: Toluidine Blue.

aquatic system. However, its effect on *L. submontandoni* is shown the first time in our results.

We highlighted that our simulation situation uses a dose highest than related by published studies of rivers contaminated by herbicides but provide a useful information to determine risk for *L. submotandoni* in case of spray drift onto the surface of the water or when users clean the sprayers and throws the remains of products in water ways. In these cases, there is a highest the likelihood of exposure to the formulated product. Besides that, studies have shown that the toxicity manifested by herbicides to aquatic organisms is largely due to the surfactant in the mixture evidencing an importance in ecotoxicology studies the use of formulated herbicides (Folmar et al., 1979; Mann and Bidwell, 1999; Edginton et al., 2004).

The negative effects of ametryn on *L. submontandoni* may relate to its capacity to enter the body of aquatic organisms due to its high octanol-water partition coefficient (Kow) (Daam et al., 2019; He et al., 2013). A significant correlation between Kow and LC_{50} values have been previously reported (He et al., 2013). The degradation of internal structures of *L. submontandoni* even at a low dose of ametryn highlights its high capacity to affect non-target organisms. We suggest that new studies exploring the sublethal effects of ametryn on *L. submontandoni* are needed.

The herbicide atrazine was toxic to individuals tested, with an LC_{50} of 192.42 mg/L. The lowest concentrations tested caused disturbances in the permatogenic tissues, in addition to causing degeneration in spermatids. Although many sperm cells are still produced, their quality is likely to be altered due to the extensive changes observed at earlier stages of development. Because of this, the herbicide may affect the next generations of *L. submontandoni*. Atrazine is one of the most dangerous herbicides to the environment (Székács et al., 2015); it has a high soil persistence of 60 to 100 days, and a high manufacturer-recommended dosage averaging 6.5 L/ha. Atrazine is leachable due to its high Kow (275). Thus, strategies for reducing the risks of this herbicide to aquatic environments need to be formulated.

Clomazone is widely used to control annual broadleaf weeds and grasses. Its residues have been detected in fish liver and muscle samples, raising concerns over its potential adverse effects (Menezes et al., 2011; Lazartigues et al., 2013). Our results show negative effects of clomazone to *L. submontandoni*, even in low doses. Clomazone may inhibit several metabolic pathways, including the citric acid cycle.

In conclusion, our investigation is the first report on herbicide toxicity to the reproductive system of *L. submontandoni*. Our results bring attention to the necessity of considering the impacts of herbicides on non-target organisms that have not been extensively studied, particularly in aquatic toxicology research.

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