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Toxicity Assessment of 36 Herbicides to Green Algae: Effects of Mode of Action and Chemical Family

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Abstract: Aquatic ecosystems can suffer inadvertent contamination from widely used herbicides. This study delves into the relative toxicity of 36 herbicides on green algae, exploring 11 distinct modes of action and 25 chemical structure classes. Through a 72-h algal growth inhibition test, it was found that herbicides targeting acetolactate synthase (ALS), photosystem II (PSII inhibitors), microtubule assembly, very-long-chain fatty acid (VLCFA) synthesis, and lipid synthesis exhibited high toxicity, with 72-h EC50 (half-maximal effective concentration) values ranging from 0.003 mg/L to 24.6 mg/L. Other pesticide types showed moderate to low toxicity, with EC50 values ranging from 0.59 mg/L to 143 mg/L. Interestingly, herbicides sharing the same mode of action but differing in chemical composition displayed significantly varied toxicity. For instance, penoxsulam and pyribenzoxim, both ALS inhibitors, demonstrated distinct toxicity levels. Similarly, terbuthylazine and bentazone, both PSII inhibitors, also exhibited differing toxicities. Notably, herbicides approved for rice cultivation showed lower toxicity to green algae compared to those intended for terrestrial plants. These data offer valuable insights for assessing the potential risks posed by these chemicals to aquatic organisms. Additionally, to prevent or minimize herbicide residual effects, modern management practices were reviewed to offer practical guidance.

Keywords: herbicide; green algae; mode of action; chemical family; algal growth inhibition; EC50; ecotoxicity; risk assessment

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1. Introduction

Herbicides, utilized in agricultural practices to control weeds, often find their way into aquatic environments through surface runoff and atmospheric deposition [1]. This continuous influx not only disrupts agricultural ecosystems but also poses significant risks to aquatic life [2]. The widespread use of herbicides in agriculture, aimed at maximizing yields, compromises both aquatic and soil quality [1]. Soil contamination by herbicides can lead to groundwater pollution through leaching and affecting nearby rivers via surface runoffs [3]. Particularly in paddy fields, herbicide runoff presents a significant threat to aquatic flora, as toxic substances easily escape into surrounding water bodies [4]. Notably, the runoff of herbicides from paddy fields exceeds that from upland field pesticides [5]. Given their substantial impact on aquatic ecosystems, it is crucial to evaluate the adverse effects of herbicides on non-target organisms, especially algae, which occupy the base of the aquatic food chain [6]. Such assessments are vital for mitigating the detrimental effects of herbicide pollution on aquatic biodiversity.

Algae have been a focal point in numerous investigations due to their crucial role as primary producers in freshwater systems [7]. They play a pivotal role in maintaining the balance of aquatic ecosystems by serving as the foundational link in the trophic chain, generating organic matter and oxygen [7]. Algae, possessing characteristics akin to higher plants, are highly sensitive bio-indicators of water pollution [7]. Pollutants, particularly herbicides inhibiting photosynthesis, can significantly impact algal photosynthetic capacity and growth rates, detectable through water sample analyses and chemical assessments [8]. Generally, algae exhibit sensitivity to herbicides, and these chemicals have been observed to alter the species composition and community structure of benthic algal assemblages within natural aquatic environments [9]. Consequently, the impact of herbicides on non-target organisms, especially algae, is a critical concern in natural ecosystems. Green algae, in particular, have emerged as a standard species in conventional ecological effect assessments [10], further emphasizing their importance in understanding the ecological ramifications of herbicide exposure.

In recent decades, there has been a surge in studies aimed at elucidating the detrimental impacts of herbicides on algae. Many of these investigations have focused on assessing the toxicity of individual herbicides, such as glyphosate [11], metolachlor [12], and thiobencarb [13], among others. However, it is crucial to recognize that herbicides, each with unique chemical compositions and mechanisms, can exert varying levels of toxicity on algae. This variation stems from the intricate interplay of their chemical structures, properties, and familial characteristics, leading to diverse impacts on aquatic ecosystems. Herbicides are classified based on their mode of action and target specificity, encompassing mechanisms such as the inhibition, interruption, disruption, or mitigation of regular plant growth [14]. Understanding these diverse modes of action is essential for comprehensively assessing the potential risks posed by herbicides to algae and other aquatic organisms.

Previous studies have delved into the adverse effects of herbicide chemical compositions and mechanisms on algae. Ma et al. (2002) conducted green alga toxicity tests on 40 herbicides from 19 chemical structure classes and 11 modes of action, revealing that auxin herbicides exhibited the lowest acute toxicities to *Chlorella vulgaris*, while photosynthesis-inhibiting herbicides demonstrated the highest toxicity [15]. Similarly, Nagai et al. (2016) analyzed the toxicity of 20 herbicides on 5 periphytic algae, finding differential sensitivities across algal species to various herbicide classes [16]. Despite testing herbicides with diverse modes of action and chemical classes, most were formulated products or mixtures of formulated and technical products. Although toxicity discussions often focus on active compounds, the formulation of herbicides can potentially increase their toxicity to aquatic organisms, leading to inaccurate test results for active ingredients [17]. Obtaining a comprehensive range of active ingredients from herbicides with diverse chemical classes and mechanisms is challenging due to the predominance of formulated products in the market. Consequently, obtaining scientifically accurate results regarding the aquatic toxicity of herbicides becomes difficult when considering commercial formulations. Moreover, previous studies did not specify whether the tested herbicides were registered for use in specific countries by farmers. Our objective is to select herbicides registered for use in China, analyze their influence on green algae based on chemical classes and mechanisms, and provide scientifically sound results for regulatory agencies. This approach aims to enhance the scientific rigor and practical relevance of aquatic herbicide toxicity assessments.

In this study, 36 herbicides were selected and categorized into 11 groups based on their mode of action and into 25 groups according to their chemical properties. Our selection of herbicides covers a more comprehensive array of mechanisms and chemical categories compared to previous studies. Specifically, these 36 herbicides, all registered for use in China, were chosen to assess their toxic effects on green algae. The primary objectives of this study were to (i) investigate the impact of the mechanisms employed by these 36 herbicides on green algae; (ii) analyze the influence of the chemical families to which these herbicides belong on green algae; and (iii) compare the toxicity of herbicides used in paddy fields versus those used in upland fields on green algae. By addressing these objectives, this research aims to enhance the understanding of the ecological consequences of herbicide use in different agricultural settings and contribute to more informed regulatory decision making.

2. Materials and Methods

2.1. Herbicides

This study investigated the effects of thirty-six herbicides, as listed in Table 1. Following the classification of the Herbicide Resistance Action Committee (HRAC), these herbicides were grouped into eleven categories based on their mode of action and twenty-five categories based on their chemical properties, encompassing the primary action mechanisms and chemical families of herbicides. The selected herbicides are commonly utilized in agricultural practices across China. Specifically, among these herbicides, fourteen are approved for use in paddy fields and nineteen in dry farmland, and three are designated for exportation, as per registration data from the Institute for the Control of Agrochemicals, Ministry of Agriculture, China (ICAMA). Notably, active ingredients (a.i.) were utilized instead of commercial formulations, with the aim of isolating the effects of the neurotoxic molecules of the chemicals on mortality, excluding the potential influence of adjuvants added to commercial products.

Table 1. Classification of herbicides in groups with similar mode-of-action chemical characteristics according to the Herbicide Resistance Action Committee (HRAC) classification.

Herbicide	Technical Grade (a.i.) (%)	Chemical Group	Mode of Action	HRAC Groups
Cyhalofop-butyl	97.4	Aryloxyphenoxy-propionate	Inhibition of acetyl CoA carboxylase (ACCase)	A
Clethodim	37	Cyclohexanedione		
Rimsulfuron	99	Sulfonylurea	Inhibition of acetolactate synthase (ALS)	B
Prosulfuron	95	Sulfonylurea		
Flucarbazone-Na	95	Sulfonylurea		
Penoxsulam	98	Sulfonylurea		
Pyribenzoxim	95	Pyrithiobac-sodium		
Cloransulam-methyl	98	Triazolopyrimidine		
Florasulam	98	Triazolopyrimidine		
Phenmedipham	97.8	Phenyl-carbamate	Inhibition of photosynthesis at photosystem II (PSII inhibitor)	C_1
Terbuthylazine	95	Triazine		
Metamitron	98	Triazinone		
Metribuzin	97	Triazinone		
Amicarbazone	97	Triazolinone		
Flufenacet	98	Aryloxyphenoxy-propionate		
Bentazone	98	Benzothiadiazinone		
Bromoxynil	97	Nitrile		
Pyraflufen-ethyl	95	Phenylpyrazole	Inhibition of protoporphyrinogen oxidase (PPO)	E
Mesotrione	95	Triketone	Bleaching: inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD)	F_2
Isoxaflutole	96	Isoxazole		
Glyphosate	96	Glycines	Inhibition of 5-enol pyruvyl shikimic acid-3-phosphorus synthase (EPSP synthase inhibitor)	G_5
Glyphosate potassium salt	95	Glycines		
Glyphosate-isopropylammonium	95	Glycines		
Anilofos	97.3	Glycines		
Fluroxypyr-metyl	97	Pyridine	Microtubule assembly inhibition	K_1
Pendimethalin	98	Dinitroanilines		
Trifluralin	96	Dinitroanilines		

Table 1. *Cont.*

Herbicide	Technical Grade (a.i.) (%)	Chemical Group	Mode of Action	HRAC Groups
Metazachlor	98	Chloroacetamide	Inhibition of very-long-chain fatty acid (VLCFA inhibitor)	K_3
S-metolachlor	98	Chloroacetamide		
Prosulfocarb	98	Dithiocarbamate	Inhibition of lipid synthesis	N
Thiobencarb	97	Dithiocarbamate		
2,4-D butylate	98	Phenoxy-carboxylic-acid	Synthetic auxins	O
2,4-D isooctyl ester	96	Phenoxy-carboxylic-acid		
2-methyl-4-chloro-phenoxyacetic acid (MCPA)	95	Phenoxy-carboxylic-acid		
Dicamba	96.5	Benzoic acid		
Oxaziclomefone	97	Dithiocarbamate	Other	Z

2.2. Algal Strains

According to the Algal Growth Inhibition Test Guideline outlined in the Chinese Test Guideline on Environmental Safety Assessment for Chemical Pesticides [18] (Chinese National Standard, 2014), either the green algae *Selenastrum capricornutum* or *Scenedesmus obliquus* is employed as the test organism for algal growth inhibition tests in China. The toxicity data obtained from testing herbicides on these algae can be utilized for chemical pesticide registration applications in China and for further ecological risk assessments. In our study, both algae were sourced from the Institute of Hydrobiology, Chinese Academy of Sciences. To maintain stock cultures, the green algae were cultivated in 250 mL Erlenmeyer flasks containing 100 mL of liquid BG11 medium [19]. Algal strains were inoculated every 96 h, repeating the process two or three times to ensure exponential growth in the inoculum culture prior to testing. Before use in experiments, all glass vessels and culture media were sterilized at 121 °C for 30 min. Cultures were maintained in an illumination incubator at a temperature of 21 ± 1 °C, illuminated by cool-white fluorescent lights with a continuous light intensity of 5000 lux. These controlled conditions were essential for maintaining consistent and reliable algal cultures throughout the experiment.

2.3. Algal Growth Inhibition Test

A series of test solutions with varying concentrations of different herbicides (from 1 μg/L to 200 mg/L, depending on the specific herbicide) was prepared and sterilized at 121 °C for 30 min. Subsequently, 50 mL aliquots of the BG11 medium containing green algal cells were carefully transferred to 50 mL test solutions in Erlenmeyer flasks, achieving an initial cell density of approximately 50,000 cells/mL. These test solutions encompassed 5 to 7 concentrations, along with a control group. Additionally, when a solvent was utilized to solubilize the test substance, supplementary controls containing the solvent at the same concentration were included in the test. Each test concentration was replicated three times and incubated for 72 h at a constant temperature of 21 ± 1 °C, under continuous light intensity of 5000 lux, as prescribed by the Organisation for Economic Co-operation and Development (OECD) [20], the United States Environmental Protection Agency (USEPA) [21], and the Chinese National Standards (CNS) [18]. Aliquot samples were extracted from each of the triplicate flasks every 24 h. Chlorophyll fluorescence of the samples was quantified using a spectrophotometer (UV-2550, Shimadzu, Kyoto, Japan) with an excitation wavelength of 420 nm and an emission wavelength of 670 nm.

2.4. Data Processing and Statistical Analysis

Yield was determined by subtracting the starting biomass from the biomass at the end of the test for each individual vessel in both the control and treatment groups. Mean yield values were calculated for each test concentration and control, along with variance estimates. The percentage inhibition in yield (I_y) for each treatment replicate was then calculated using the following formula, in accordance with guidelines provided by the OECD [20], USEPA [21], and CNS [18]:

$$I_y = \frac{Y_c - Y_t}{Y_c} \times 100 \tag{1}$$

where I_y is the percent inhibition of yield (%), Y_c is the mean value for yield in the control group (cells/mL), and Y_t is value for yield for the treatment replicate (cells/mL).

Probit analysis was performed using SPSS® 16.0 to evaluate the toxicity of herbicides on green algae. The 72-h half-effect concentration (72 h-EC50), representing the herbicide concentration required to cause 50% inhibition of yield, along with its 95% confidence interval, were determined through a linear regression analysis of transformed herbicide concentrations as natural logarithm data versus percentage inhibition. Based on the obtained 72-h EC50 values, herbicides were categorized as having high toxicity (<0.3 mg/L), moderate toxicity (0.3 mg/L–3.0 mg/L), or low toxicity (>3.0 mg/L), following guidelines outlined in the CNS [18].

3. Results and Discussion

3.1. Mechanistic Influence on Algal Toxicity

Table 2 summarizes the acute toxicity of 36 herbicides to green algae, classified into 11 groups based on their mode of action. These modes include the inhibition of acetyl CoA carboxylase (ACCase), the inhibition of acetolactate synthase (ALS), the inhibition of photosynthesis at photosystem II (PSII inhibitor), the inhibition of protoporphyrinogen oxidase (PPO), the inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD), the inhibition of 5-enol pyruvyl shikimic acid-3-phosphorus synthase (EPSP synthase inhibitor), microtubule assembly inhibition, the inhibition of very-long-chain fatty acids (VLCFA inhibitor), and the inhibition of lipid synthesis, synthetic auxins, and others (Table 1). The mode of action significantly impacts green algae, with herbicides that inhibit cell division and target plant chloroplasts showing high toxicity to green algae. Approximately 50% of the total herbicides demonstrated moderate to high toxicity to green algae, with 72-h EC50 values ranging from 1.01×10^{-3} mg/L to 2.5 mg/L (Table 2). The mechanisms of action for these herbicides primarily involve ALS (e.g., prosulfuron, penoxsulam, cloransulam-methyl, and florasulam), PSII inhibition (e.g., terbuthylazine, metamitron, metribuzin, amicarbazone, and flufenacet), microtubule assembly inhibition (pendimethalin), VLCFA inhibition (metazachlor and S-metolachlor), and the inhibition of lipid synthesis (prosulfocarb and thiobencarb).

Table 2. Acute toxicity of 36 herbicides to green algae.

Herbicide	**Algal Species**	**72 h EC50 (mg a.i./L)**	**Confidence Interval**	**Toxicity Grade**
Cyhalofop-butyl	Scenedesmus obliquus	5.15	2.65–15.96	Low
Clethodim	Selenastrum capricornutum	2.50	2.0–3.2	Moderate
Rimsulfuron	Scenedesmus obliquus	0.71	0.57–0.85	Moderate
Prosulfuron	Selenastrum capricornutum	3.5×10^{-2}	2.7×10^{-2}–4.7×10^{-2}	High
Flucarbazone-Na	Selenastrum capricornutum	7.40	5.4–10	Low
Penoxsulam	Scenedesmus obliquus	0.18	0.16–0.22	High
Pyribenzoxim	Scenedesmus obliquus	24.60	—	Low
Cloransulam-methyl	Selenastrum capricornutum	3.0×10^{-3}	2×10^{-3}–4×10^{-3}	High
Florasulam	Scenedesmus obliquus	2.43×10^{-2}	2.13×10^{-2}–2.77×10^{-2}	High
Phenmedipham	Selenastrum capricornutum	7.20	6.45–8.23	Low

Table 2. *Cont.*

Herbicide	**Algal Species**	**72 h EC50 (mg a.i./L)**	**Confidence Interval**	**Toxicity Grade**
Terbuthylazine	Scenedesmus obliquus	4.4×10^{-3}	2.1×10^{-3}–9.4×10^{-3}	High
Metamitron	Scenedesmus obliquus	2.31	2.08–2.64	Moderate
Metribuzin	Selenastrum capricornutum	7.1×10^{-3}	6.3×10^{-3}–8.1×10^{-3}	High
Amicarbazone	Scenedesmus obliquus	0.49	0.36–0.65	Moderate
Flufenacet	Selenastrum capricornutum	4.1×10^{-3}	3.3×10^{-3}–5×10^{-3}	High
Bentazone	Scenedesmus obliquus	9.45	3.22–48.05	Low
Bromoxynil	Selenastrum capricornutum	4.40	3.8–5.1	Low
Pyraflufen-ethyl	Scenedesmus obliquus	8.22	5.3–12.7	Low
Mesotrione	Scenedesmus obliquus	18.60	9.9–34.7	Low
Isoxaflutole	Scenedesmus obliquus	3.34	3.19–3.49	Low
Glyphosate	Scenedesmus obliquus	29.11	25.55–33.22	Low
Glyphosate potassium salt	Scenedesmus obliquus	46.32	39.48–54.35	Low
Glyphosate-isopropylammonium	Scenedesmus obliquus	54.70	50.8–58.9	Low
Anilofos	Scenedesmus obliquus	0.59	0.41–0.85	Moderate
Fluroxypyr-metyl	Selenastrum capricornutum	>22	—	Low
Pendimethalin	Scenedesmus obliquus	8.94×10^{-3}	3.3×10^{-3}–5×10^{-3}	High
Trifluralin	Scenedesmus obliquus	5.14	4.26–6.62	Low
Metazachlor	Scenedesmus obliquus	1.01×10^{-3}	0.5×10^{-3}–1.8×10^{-3}	High
S-metolachlor	Scenedesmus obliquus	3.47×10^{-2}	2.88×10^{-2}–4.15×10^{-2}	High
Prosulfocarb	Scenedesmus obliquus	0.20	—	High
Thiobencarb	Selenastrum capricornutum	6.60×10^{-2}	3×10^{-2}–0.6	High
2,4-D butylate	Scenedesmus obliquus	86.09	43.03–192	Low
2,4-D isooctyl ester	Scenedesmus obliquus	4.78	3.67–6.39	Low
MCPA	Scenedesmus obliquus	143.00	129–159	Low
Dicamba	Scenedesmus obliquus	>96.5	—	Low
Oxaziclomefone	Selenastrum capricornutum	>30	—	Low

Numerous herbicides that target cell division and chloroplasts in plants exhibited high toxicity to green algae. The 72-hour EC50 values of ALS inhibitors ranged from 0.003 to 24.6 mg/L (Table 2 and Figure 1). Among the seven ALS inhibitor herbicides examined, four were classified as highly toxic, one as moderately toxic, and two as low toxicity. The high toxicity of ALS inhibitors primarily stems from their mode of action, which inhibits the acetolactate synthase enzyme, leading to the suppression of branched-chain amino acid biosynthesis (leucine, isoleucine, and valine), thus impeding cell division and causing weed mortality [15]. However, ALS inhibitors have a single target and may induce weed resistance if used continuously.

Similarly, the 72-hour EC50 values of PSII inhibitor herbicides ranged from 0.0041 to 9.54 mg/L. Among the eight PSII inhibitor herbicides tested, three were highly toxic, two were moderately toxic, and three had low toxicity. These PSII herbicides inhibit the photosynthetic pathway by binding to the protein complex site in the chloroplast thylakoid membrane [22]. Although the inhibition of photosynthesis could lead to slow plant starvation, rapid plant death is often attributed to the production of secondary toxic substances [23]. Due to excessive use, certain weeds have developed resistance to PSII herbicides based on this metabolic principle [24]. Previous studies have demonstrated that many ALS and PSII inhibitor herbicides exhibit high toxicity to green algae [15,16].

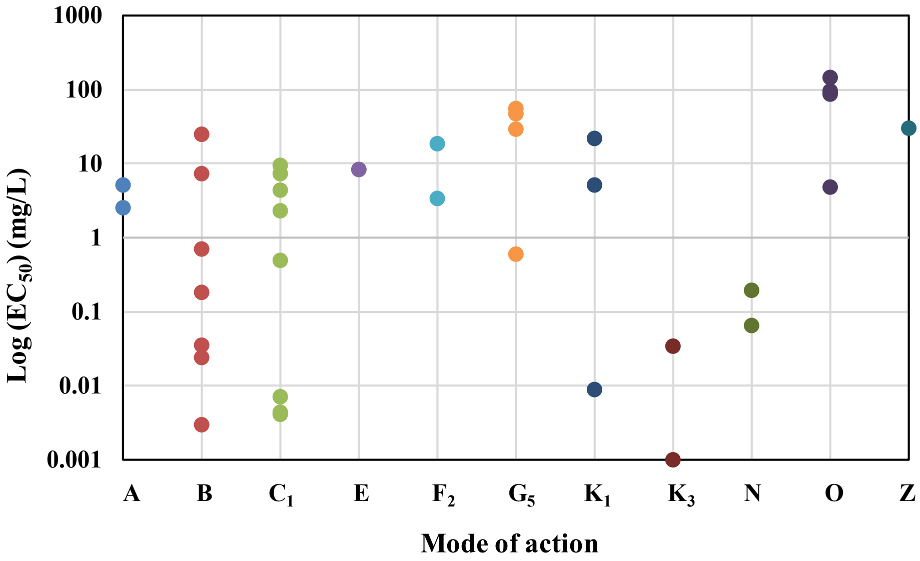

Figure 1. Toxicity of 11 herbicide groups to green algae: EC50 values (n = 36). The mode of action of the herbicide groups is identified by corresponding numbers detailed in Table 1.

Additionally, both VLCFA inhibitors and lipid synthesis inhibitors displayed high toxicity to green algae, with 72-hour EC50 values ranging from 0.001 to 0.195 mg/L. These herbicides, acting as shoot-growth inhibitors, are applied during soil preparation and effectively target emerging grass and broadleaf weeds [22]. Furthermore, one of the microtubule assembly inhibitor herbicides exhibited high toxicity, with a 72-h EC50 value of 0.0089 mg/L. Known as seedling root growth inhibitors, these herbicides impede cell division, ultimately halting root extension and growth [25]. Therefore, a comprehensive eco-toxicological assessment of the effects of these herbicide modes of action on algae is imperative.

In this study, nineteen herbicides demonstrated low toxicity to green algae, comprising approximately 50% of the total herbicides tested. The 72-h EC50 values for these nineteen herbicides ranged from 3.34×10^{-3} mg/L to 143 mg/L (Table 2 and Figure 1). The mechanisms of action for these herbicides primarily involved the inhibition of ACCase (cyhalofop-butyl), the inhibition of protoporphyrinogen oxidase (PPO) (pyraflufen-ethyl), the inhibition of 4-hydroxyphenyl-pyruvate-dioxygenase (4-HPPD) (mesotrione and isoxaflutole), the inhibition of 5-enol pyruvyl shikimic acid-3-phosphorus synthase (EPSP synthase inhibitor) (glyphosate, glyphosate potassium salt, and glyphosate-isopropylammonium), and the inhibition of synthetic auxins (2,4-D butylate, 2,4-D isooctyl ester, 2-methyl-4-chlorophenoxyacetic acid (MCPA), and dicamba).

The ACCase herbicide cyhalofop-butyl exhibited low toxicity, with a 72-hour EC50 value of 5.15 mg/L (Table 2 and Figure 1). These herbicides target and inhibit ACCase enzyme activity, commonly used for controlling grass during the cultivation of broadleaf crop varieties or crop rotation. In 2021, cyhalofop-butyl sales ranked second among herbicides usable for rice in the United States, owing to its high efficiency, low toxicity, and safety for subsequent crops [26].

Most EPSP synthase inhibitor herbicides demonstrated low toxicity to green algae, with 72-hour EC50 values ranging from 0.59 to 54.70 mg/L. These herbicides exert their action specifically on glycines, serving as nonspecific herbicides that inhibit amino acid synthesis [27]. Glyphosate, a prominent EPSP synthase inhibitor herbicide, is widely used worldwide due to its broad-spectrum efficacy and environmental and user-friendly characteristics [22].

Furthermore, the toxicity of PPO herbicides, 4-HPPD inhibitors, and synthetic auxins to green algae closely paralleled that of EPSP synthase inhibitor herbicides. PPO inhibitors disrupt cell membranes, categorized as cell membrane disruptors, with their site of action being the cell membrane. Meanwhile, 4-HPPD inhibitors act as pigment synthesis inhibitors, disrupting the green pigment chlorophyll and inhibiting pigment synthesis.

3.2. Influence of Chemical Class on Algal Toxicity

Thirty-six herbicides were categorized into 25 groups based on chemical classifications such as aryloxyphenoxy-propionate, cyclohexanedione, sulfonylurea, and others (Table 1). An analysis of the toxicity of these herbicides to green algae revealed that 19 herbicides exhibited low toxicity, accounting for 51% of the total, while 5 herbicides showed moderate toxicity, constituting 14%, and 12 herbicides displayed high toxicity, representing 35%. The toxicity levels varied significantly among the different herbicides tested on the single green algae. Notably, metazachlor in the chloroacetamide chemical group exhibited the highest acute toxicity, with a 72-h EC50 value of 1.01×10^{-3} mg/L. Conversely, 2-methyl-4-chlorophenoxyacetic acid (MCPA) in the phenoxy-carboxylic-acid chemical group demonstrated the lowest acute toxicity, with a 72-h EC50 of 143 mg/L.

The toxicity of green algae varied significantly due to the diverse chemical structures of herbicides. Herbicides with identical modes of action but differing chemical categories exhibited considerable variation in toxicity to green algae. For instance, among the ALS inhibitor herbicides, the triazolopyrimidine herbicides cloransulam-methyl and florasulam, as well as the sulfonylurea herbicides prosulfuron and penoxsulam, demonstrated high toxicity to green algae, with 72-h EC50 values ranging from 0.003 to 0.184 mg/L. In contrast, the sulfonylurea herbicide rimsulfuron exhibited moderate toxicity, while flucarbazone-Na and the pyrithiobac-sodium herbicide pyribenzoxim displayed low toxicity, with 72-h EC50 values exceeding 7 mg/L.

Similarly, among the PSII inhibitor herbicides, the aryloxyphenoxy-propionate herbicide flufenacet, the triazine herbicide terbuthylazine, and the triazinone herbicide metribuzin exhibited high toxicity to green algae, with 72-h EC50 values ranging from 0.0041 to 0.0071 mg/L. Conversely, the triazolinone herbicide amicarbazone and triazinone herbicide metamitron demonstrated moderate toxicity, while the nitrile herbicide bromoxynil, the benzothiadiazinone herbicide bentazone, and the phenyl-carbamate herbicide phenmedipham displayed low toxicity, with 72-h EC50 values exceeding 4 mg/L.

Moreover, herbicides within the same mode of action and chemical group exhibited varying toxicities against green algae. For instance, the sulfonylurea herbicides prosulfuron and penoxsulam were 211 and 40 times more toxic, respectively, than flucarbazone-Na to green algae. Additionally, herbicides in the sulfonylurea and triazolopyrimidine categories have prolonged residual times, increasing the risk of harm to subsequent crops and water resource pollution under natural conditions.

The dinitroanilines herbicide pendimethalin, the chloroacetamide herbicide metazachlor and S-metolachlor, and the dithiocarbamate herbicide prosulfocarb and thiobencarb exhibited similarly high toxicity to green algae, with 72-h EC50 values of 0.0089, 0.001, 0.003, 0.195, and 0.066 mg/L, respectively. In contrast, most of the other tested herbicides demonstrated low toxicity against green algae, with 72-h EC50 values exceeding 3 mg/L. These include aryloxyphenoxy-propionate herbicides (cyhalofop-butyl), phenylpyrazole herbicides (pyraflufen-ethyl), triketone herbicides (mesotrione), isoxazole herbicides (isoxaflutole), glycines herbicides (glyphosate, glyphosate potassium salt, and glyphosate-isopropylammonium), phenoxy-carboxylic-acid herbicides (2,4-D butylate, 2,4-D isooctyl ester, and MCPA), benzoic acid herbicides (dicamba), and dithiocarbamate (oxaziclomefone).

Some herbicides pose a risk of long-term environmental pollution due to various chemical properties such as volatility, water solubility, degradation velocity, and application method. Despite being low in toxicity to green algae, 2,4-D butylate can lead to serious crop poisoning incidents due to its high volatility [28]. This herbicide exhibits strong adhesion to plant surfaces and can volatilize into a gaseous state at temperatures as low as 15°C [28]. During pesticide spraying, larger droplets settle on the ground, while smaller droplets, less than 100 mm in diameter, can drift downwind for considerable distances [28]. As the mist containing 2,4-D butylate travels, moisture in the droplets evaporates rapidly, leaving behind a gaseous herbicide that can be carried further by the wind. It has been observed that under wind force scales of 3 or 4, the drift can extend up to nearly 100 m, and under wind force scales of 6 or 7, it can reach distances of 1500 to 2000 m [29]. Additionally, 2,4-D butylate may volatilize and drift downwind for a second time within 12 days of initial application [29]. In recent years, instances of frequent poisoning of dicotyledonous sensitive plants (e.g., cotton, wheat, corn, soybean, etc.) have been reported due to the drift of 2,4-D butylate. Consequently, the Chinese government ceased approving field trials and registration applications of 2,4-D butylate since 2016 [30].

Some herbicides pose a significant risk of water pollution due to their high water solubility. For instance, penoxsulam, which exhibits high toxicity to green algae, is exclusively permitted for use in rice cultivation in China and European countries [31]. With a solubility of 0.41 g/L at 19 °C, penoxsulam readily dissolves in water, exacerbating its potential to contaminate aquatic environments [31]. Given that paddy rice cultivation covers a substantial portion of agricultural land globally, including approximately one-fourth in China and more than half in Japan [32], the runoff of paddy herbicides into rivers and open aquatic systems through drainage channels is a significant concern. This runoff can range from a few percent to over fifty percent of the applied herbicide, exposing aquatic organisms, as well as people and animals relying on the polluted water, to harmful chemicals over prolonged periods [4]. Moreover, herbicides used in dry fields can also contribute to aquatic ecosystem harm [31]. In this study, several highly soluble herbicides permitted for use in upland fields exhibited high toxicity to green algae, including florasulam (solubility 6.36 g/L at 20 °C), metamitron (solubility 1.7 g/L at 25 °C), metribuzin (solubility 1.2 g/L at 20 °C), and amicarbazone (solubility 4.6 g/L at 20 °C). These herbicides, with their high water solubility, are prone to being washed and leached by rainwater, thereby contaminating surface and groundwater sources [33].

3.3. Registration Details of 36 Herbicides

As of 2023, all 36 herbicides included in the study had been registered in China, predominantly for use on crops such as rice, wheat, and corn. Six herbicides were registered for use on more than 10 different crops, while fourteen herbicides were registered for use on only 1 crop (refer to Table 3). Notably, due to their low toxicity to green algae, herbicides classified as glycines, triketone, and aryloxyphenoxy-propionate exhibited higher registration quantities compared to other types. Among the herbicides registered for use on rice, the top four formulations by registration quantity were glyphosate-isopropylammonium (328), glyphosate (305), mesotrione (294), and cyhalofop-butyl (203) (see Table 3). The most extensively registered herbicide was the dinitroanilines herbicide pendimenthalin, registered for use on 32 crops, including rice and cabbage, among others. Following closely is the glycines herbicide glyphosate, registered for use on 28 crops, such as rice and corn. Additionally, the chloroacetamide herbicide S-metolachlor was registered for use on 18 crops, including soybean and corn.

Table 3. Registration details of 36 herbicides for green algae in China.

Herbicide	Registration of Commercial Formulations	Registered Crops Species	Main Registered Crops
Cyhalofop-butyl	203	1	Rice
Clethodim	133	8	Soybean, oilseed rape, etc.
Rimsulfuron	35	5	Corn, potato, etc.
Prosulfuron	—	Export	—
Flucarbazone-Na	29	1	Wheat

Table 3. *Cont.*

Herbicide	Registration of Commercial Formulations	Registered Crops Species	Main Registered Crops
Penoxsulam	25	1	Rice
Pyribenzoxim	7	1	Rice
Cloransulam-methyl	5	1	Soybean
Florasulam	104	2	Wheat, tall fescue lawn
Phenmedipham	9	1	Sugar beet field
Terbuthylazine	—	Export	—
Metamitron	4	1	Sugar beet
Metribuzin	75	4	Soybean, corn, etc.
Amicarbazone	4	1	Corn
Flufenacet	7	1	Wheat
Bentazone	159	12	Rice, soybean
Bromoxynil	7	4	Wheat, sugarcane, etc.
Pyraflufen-ethyl	8	4	Wheat, cotton, etc.
Mesotrione	294	4	Rice, corn, etc.
Isoxaflutole	4	1	Corn field
Glyphosate	305	28	Rice, corn, etc.
Glyphosate potassium salt	26	7	Rice, rape field, etc.
Glyphosate-isopropylammonium	328	16	Rice, corn field
Anilofos	39	1	Rice
Fluroxypyr-metyl	76	10	Rice, wheat, etc.
Pendimethalin	188	32	Rice, Chinese cabbage, etc.
Trifluralin	77	8	Soybean, cotton, etc.
Metazachlor	3	1	Rape field
S-metolachlor	37	18	Soybean, corn, etc.
Prosulfocarb	—	Export	—
Thiobencarb	17	1	Rice
2,4-D butylate	157	8	Rice, wheat, etc.
2,4-D isooctyl ester	49	3	Soybean, wheat
MCPA	82	8	Rice, wheat, etc.
Dicamba	76	9	Wheat, corn, etc.
Oxaziclomefone	9	1	Rice

In China, rice cultivation accounts for the highest number of registered pesticide products, totaling 525 species and representing 26.49% of the total pesticide products. Notably, herbicide production reached 825,000 tons in 2023, constituting 63% of the total pesticide production, according to the Institute for the Control of Agrochemicals of China (ICAMA) [34]. Within the scope of this study, 14 herbicides registered for use on rice are suitable for paddy fields, including cyhalofop-butyl, penoxsulam, and anilofos, among others, while the remaining 19 herbicides are registered for terrestrial plants, with 3 herbicides intended for exportation (refer to Table 2). Interestingly, the herbicides registered for rice cultivation exhibit lower toxicity to green algae compared to those registered for terrestrial plants. Among the herbicides intended for use in paddy fields, 10 were classified as low toxicity (72 h EC50: 5.15 mg/L–143 mg/L), 1 as moderate toxicity (72 h EC50: 0.59 mg/L), and 3 as high toxicity (72 h EC50: 8.94×10^{-3} mg/L–0.184 mg/L). Conversely, among the herbicides designated for terrestrial plants, nine were categorized as exhibiting low toxicity (72 h EC50: 3.34 mg/L–96.5 mg/L), four as moderate toxicity (72 h EC50: 0.487 mg/L–2.5 mg/L), and six as high toxicity (72 h EC50: 1.01×10^{-3} mg/L–3.47×10^{-3} mg/L) to green algae.

In European Union countries, a total of 28 herbicides have been registered. Among these, 3 herbicides are approved for use in paddy rice, namely cyhalofop-butyl, penoxsulam, and glyphosate, while the remaining 25 are designated for terrestrial plants, according to the Food and Agriculture Organization of the United Nations (FAO) [35]. Notably, only penoxsulam exhibited high toxicity to green algae, with a 72 h EC50 value of 0.184 mg/L, whereas the other two herbicides demonstrated low toxicity, with 72 h EC50 values of 5.15 mg/L and 29.11 mg/L, respectively.

Similarly, the United States has registered 29 herbicides, with 5 authorized for use in paddy rice, namely cyhalofop-butyl, penoxsulam, bentazone, thiobencarb, and glyphosate, while the remaining 24 are intended for terrestrial plants, according to the USEPA [36]. Among these, penoxsulam and thiobencarb exhibited high toxicity against green algae, with 72 h EC50 values of 0.184 mg/L and 0.066 mg/L, respectively, whereas the other three demonstrated low toxicity, with 72 h EC50 values of 5.15 mg/L, 9.54 mg/L, and 29.11 mg/L, respectively.

3.4. Management Practices to Prevent or Minimize Herbicide Residual Effects

It is crucial to meticulously plan control strategies to mitigate the risk of herbicide carryover. This planning process should consider several factors, including the specific weed issues, available herbicide options (including formulations and persistence), soil characteristics, prevailing weather conditions, and crop rotation practices. Employing various management techniques such as selecting appropriate seeding dates, optimizing crop selection, and strategically placing fertilizers can promote the growth of robust competitive crops that outcompete weeds, thereby reducing the potential for herbicide carryover into subsequent growing seasons. Additionally, it is advisable to designate an untreated check area within the field to serve as a reference point for future comparisons. A comprehensive control plan aimed at minimizing or eliminating herbicide carryover should encompass the key components specified in the following subsections.

3.4.1. Choosing Herbicides with Minimal Residual Impact

Opting for an herbicide with minimal or no residual impact, tailored to your specific soil and weather conditions, can prevent future crop damage. Certain crops can withstand particular herbicide residues and can be replanted shortly after application, whereas others may remain susceptible to the herbicide's effects for a longer period. While some herbicides may dissipate after several half-lives and still pose a threat to certain crop varieties, others may persist longer but have milder effects on select crops [37]. Additionally, herbicides based on RNA interference (RNAi), a powerful tool built on natural regulatory mechanisms, have been increasingly utilized for crop protection against viruses, fungal pathogens, and insect pests [38].

Research indicates that applying herbicides early in the season when weeds are small not only reduces competition, enhancing crop yield, but also helps decrease the potential for carryover effects on subsequent crops [39]. It is advisable to apply herbicides at minimum rates to minimize the risk of carryover. Higher initial application rates result in the longer persistence of herbicide residues. Ensuring the precise, uniform application of herbicides is crucial to mitigate the risk of carryover [39]. Non-uniform application or inadequate incorporation may create localized "hot spots" with higher-than-recommended herbicide concentrations, leading to damage, particularly along field edges and corners or in strip patterns [40].

In conventional tillage systems, tillage helps distribute herbicide residues throughout the soil profile, accelerating microbial degradation and diluting residual concentrations [41]. Maintaining comprehensive field records, practicing crop rotation, and avoiding the consecutive use of herbicides from the same chemical group are effective strategies for minimizing carryover concerns [39]. Additionally, combining a non-residual herbicide with the lowest recommended rate of a residual herbicide in a tank mixture can further reduce carryover potential [42].

In instances where herbicide residue is detected or suspected, cultivating a tolerant crop capable of either storing or degrading the residue into non-toxic compounds is advisable. Soil composition also plays a significant role in herbicide breakdown processes [41].

3.4.2. Improving Herbicide Degradation through Various Techniques

Biostimulation: The term "biostimulation" refers to the practice of introducing electron acceptors, electron donors, or nutrients to stimulate existing microbial populations [43].

In essence, biostimulation involves providing adequate water, nutrients, and oxygen to the soil to enhance the activity of native microbial degraders or to facilitate co-metabolism [43]. This concept aims to amplify the natural degradation potential of contaminated soil by supplying amendments, nutrients, or other essential factors, and it has been applied to various types of pollutants. Incorporating organic matter, bioprocessed materials, or compost can naturally stimulate microbial activity in the soil and may be employed for soil remediation. Fresh bioprocessed materials are particularly rich in nitrogen, carbon, and other nutrients, making them conducive to microbial growth [43]. Research by Devi et al. (2022) demonstrated that the continual application of farmyard manure (FYM) to rice crops enhanced the degradation of butachlor, pretilachlor, and 2,4-D in the soil by promoting microbial activity [44].

Nutrient addition: Optimal microbial activity in soil often requires nutrient levels that are below the ideal concentration. Introducing necessary nutrients into such soils can stimulate microbial activity, facilitating the biodegradation of pollutants and offering a promising approach to enhancing bioremediation efforts in contaminated areas. Key nutrients such as carbon, nitrogen, and phosphorus play vital roles in stimulating microbes to produce essential enzymes for contaminant breakdown. Early studies, such as that by Rhine et al. (2003), demonstrated the positive impact of inorganic nutrient supplementation on the degradation of atrazine in soil [45]. Subsequently, researchers have increasingly explored the potential of microbial biostimulation through the manipulation of both organic and inorganic nutrient levels in soil. In certain instances, inducing inorganic nitrogen starvation has proven to be particularly effective in promoting degradation, as observed with atrazine and other heterocyclic compounds [46]. For instance, Qiu et al. (2009) found that the addition of phosphorus (P) resulted in the complete degradation of dichlobenil within 60 h, compared to a less than 40% degradation without P supplementation [47]. Fertilizer application not only supports the growth of tolerant plants, leading to increased herbicide uptake from the soil, but also fosters the proliferation of microflora, thereby enhancing the biological breakdown of herbicides. For example, phosphate supplementation has been shown to boost the microbial breakdown of phenoxy herbicides such as 2,4-D and MCPA [48].

Bioaugmentation: Bioaugmentation involves introducing specific microorganisms, whether indigenous or non-indigenous, with the aim of enhancing the biodegradation of a target compound or providing catabolic genes. Typically, this approach is combined with biostimulation [43]. Increasing the population of particular herbicide-degrading bacteria through artificial means can help address such challenges. Chen et al. (2011) reported that *Rhizopus oryzea*, a fungal isolate, showed potential for the bioremediation of alachlor from soil [49]. In experiments, the half-life values of alachlor in sterile and non-sterile soil incubated with *Rhizopus oryzea* were found to be 7.2 and 8.6 days, respectively [49].

3.4.3. Herbicide Deactivation to Mitigate Persistence and Minimize Adverse Impacts

Integration of organic matter: Herbicides can be rendered inactive through the incorporation of plant residues or organic matter into the soil. This process operates in two key ways. Initially, the application of FYM results in the adsorption of herbicide molecules onto the colloidal fraction, rendering them unavailable to crops and weeds. Subsequently, microbial populations thriving on organic matter commence decomposing herbicide residues at an accelerated pace, facilitated by the high moisture-holding capacity of organic matter in soils. In the study conducted by Kadian et al. (2008), the degradation of atrazine (25 ppm) in soil was investigated alongside various soil amendments, including a biogas slurry, mushroom spent compost, farmyard manure, and sodium citrate. The results indicated that the highest atrazine dissipation rate (34%) was observed in soil treated with biogas slurry. Interestingly, the synergistic effect of sodium citrate and farmyard manure initially exhibited a negative impact, but atrazine dissipation gradually increased after the first week, reaching a degradation rate of 32% after 21 days [50]. In another study, the impact of natural dissolved organic matter (DOM) on the toxicity of three herbicides (diuron, irgarol, and S-metolachlor) to two marine microalgae species was investigated [51]. The study highlighted the significant role of natural DOM in modulating herbicide toxicity to marine microalgae [51].

Employing non-phytotoxic oils, adjuvants, and surfactants: Non-phytotoxic oils, adjuvants, and surfactants not only enhance the effectiveness of weed control but also reduce residual hazards. Adjuvants alter specific physical properties of the spray solution, such as surface tension and wetting ability, which can affect how the solution moves within the soil. The addition of surfactants, particularly, can reduce water evaporation from the soil surface, thus contributing to a more effective application [52]. For instance, the introduction of olejan alongside trifluralin applications significantly boosted the herbicide degradation rate in both laboratory and pot-field experiments [53]. Cationic adjuvants may form neutral species by binding to certain anionic molecules in the soil, potentially dissolving the herbicide and reducing its mobility in the soil matrix. Surfactants, a crucial subset of adjuvants, serve as emulsifiers, wetting agents, and spreaders. Their inclusion can influence the degradation rate and distribution of herbicide residues in both soil and plants [53]. Typically, adjuvants are applied with herbicides at reduced doses, around 70–80% of the recommended amount, leading to lower herbicidal residues at harvest compared to treatments without adjuvants [53].

Utilizing adsorbents, protectants, and antidotes: These substances are applied to the soil, crop seeds, or transplanted plants to shield them from herbicide damage. Their mode of action typically involves the deactivation or adsorption of the herbicide, hindering its absorption and translocation by the crop. Activated charcoal, with its exceptionally large surface area, exhibits high adsorptive capacity and can be distributed or applied in narrow bands over seeds during planting [54]. Janaki et al. (2015) demonstrated that applying activated carbon at rates of 8 to 18 kg per hectare to tobacco along with imazaquin and chlorimuron reduced phytotoxicity and increased yields by two to four times [55]. Biochar application is another effective method for temporarily immobilizing herbicide residues in the soil, thereby allowing crops to avoid toxicity. The source material used for biochar production influences the sorption of herbicide residues [56]. Even small additions of biochar can significantly enhance the sorption of organic compounds in soils, as observed with diuron and pyrimethanil sorption [57]. Additionally, the adsorption of herbicide residues can be boosted by incorporating adsorbent materials such as activated charcoal and biochar [57]. While the widespread use of activated charcoal and biochar may not be economically feasible, its application in localized areas, such as spot treatments for chemical spills or in high-value crop production, may be justified [58].

Employing herbicide safeners: Herbicide safeners are a diverse group of synthetic chemicals designed to shield crop plants from damage caused by specific herbicides [59]. They are widely employed in commercial agriculture to enhance the selectivity of herbicides between crops and weed species. Safeners can be applied either as a mixture with the herbicide itself or as a treatment to crop seeds before planting [59]. Functioning as "bioregulators", they regulate the amount of herbicide reaching its intended target site in an active form [59]. The primary mechanism of action for safeners involves boosting the metabolic detoxification of herbicides within protected plants [59]. This process induces the activation of herbicide-detoxifying enzymes such as glutathione transferases (GSTs), cytochrome P-450 monooxygenases (Cyt P450s), esterases, and UDP-glucosyltransferases. At the molecular level, safeners are thought to activate or amplify the genes responsible for coding these enzymes, such as GSTs [59].

3.4.4. Exploring Natural Herbicides as Viable Alternatives

Allelopathy refers to a natural biological process where organisms release biochemicals that affect the growth, survival, and reproduction of other organisms [60]. These biochemicals, known as allelochemicals, can have either positive (beneficial) or negative (detrimental) effects on their target organisms. Allelochemicals offer potential alternatives to chemical herbicides in weed management. They fall into six classes: alkaloids, benzoxazinones, cinnamic acid derivatives, cyanogenic compounds, ethylene and other seed germination stimulants, and flavonoids [61]. These compounds have been isolated from various terrestrial and aquatic plant families. Unlike synthetic herbicides, allelochemicals do not have a universal mode of action or physiological target site [61].

Allelochemicals are found in various parts of plants, including leaves, flowers, fruits, stems, bark, roots, rhizomes, seeds, and pollen. They are released into the environment through processes such as volatilization, leaching, root exudation, and the decomposition of plant residues. During periods of stress, rainfall can cause the leaching of allelopathic substances from leaves, which then accumulate on the ground, inhibiting the growth and germination of crop plants [62]. This interference by allelochemicals not only affects neighboring plants but can also impact the interactions among different plant species. Certain chemicals may be released simultaneously, potentially exerting toxic effects in an additive or synergistic manner. Various crops, including beets, lupin, maize, wheat, oats, and barley, are known to exhibit allelopathic effects on other crops [62].

Allelopathic interactions often involve a combination of various compounds. Plant extracts with allelopathic properties can effectively manage weeds, as blends of allelopathic water extracts demonstrate greater efficacy compared to single-plant extracts [60]. Utilizing allelopathic extracts in conjunction with reduced herbicide doses, sometimes half the standard dose, can provide weed control comparable to that achieved with standard herbicide doses in several field crops. Employing lower herbicide doses may help mitigate the development of herbicide resistance in weed populations [62]. Thus, allelopathy presents an appealing and environmentally friendly alternative to pesticides in agricultural pest management [61]. Generally, allelochemicals exhibit stimulatory effects at low concentrations, while higher concentrations are inhibitory [61]. The concentration of allelochemicals in the producer plant may fluctuate over time and vary across different types of plant tissues.

Biotechnology's role in advancing allelopathy has garnered significant attention in recent times. Various crop species harbor distinct allelochemicals, each with unique potential for weed control [63]. Biotechnological methods enable the enhancement of genes responsible for allelochemical production, resulting in increased quantities of these compounds. Extensive research has been dedicated to mapping allelopathic Quantitative Trait Loci (QTLs) on chromosomes [63]. Additionally, some researchers propose transgenic approaches as effective tools, which involve introducing genes from high allelopathic genotypes into low or non-allelopathic genotypes [63]. Antisense knockout techniques and gene overexpression can alter both the quantity and quality of secondary metabolites in allelopathic plants [63].

4. Conclusions

This study delved into the impact of mode of actions and chemical classes on the toxicity of 36 herbicides against green algae. The findings underscored significant variations in herbicide toxicity across different modes of action. Herbicides that disrupt cell division (ALS inhibitors), target chloroplasts (PSII inhibitors), and inhibit root or shoot growth (microtubule assembly inhibitors, VLCFA inhibitors, and lipid synthesis inhibitors) exhibited high toxicity to green algae. Conversely, herbicides affecting ACCase enzyme activity (ACCase inhibitors), amino acid synthesis (EPSP synthase inhibitors), cell membranes (PPO inhibitors), and pigment synthesis (4-HPPD inhibitors) demonstrated lower toxicity levels. Notably, herbicides with identical modes of action but different chemical categories displayed considerable variability in toxicity to green algae. Among the ALS inhibitors, cloransulam-methyl, florasulam, prosulfuron, and penoxsulam exhibited higher toxicity compared to other ALS inhibitors like rimsulfuron, flucarbazone-Na, and pyribenzoxim. Similarly, within the PSII inhibitors, flufenacet, terbuthylazine, and metribuzin showed heightened toxicity relative to herbicides from different chemical families. Lastly, the study revealed that herbicides registered for rice cultivation exhibited lower toxicity to green algae compared to those registered for terrestrial plants. These findings shed light on the nuanced relationship between herbicide characteristics and their impact on aquatic ecosystems, emphasizing the importance of informed herbicide selection and environmental management practices.

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