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Pillar[5]arenes as Modulators for the Glyphosate and 2,4-D Herbicidal Activity: The Effect of Self-Assembly on Phyto- and Ecotoxicity

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Abstract

The widespread use of herbicides in agriculture results in their accumulation in the environment, which has a negative impact on non-target biota. One way to reduce environmental risks while maintaining the effectiveness of plant protection products is to apply supramolecular chemistry principles to agricultural practices. Although pillar[n]arenes are used in the production of sensors and antidotes for pesticides, their influence on the herbicidal properties and ecotoxicity of herbicides toward aquatic organisms and higher plants has hardly been studied. The effect of pillar[5]arenes on the herbicidal activity of 2,4-dichlorophenoxyacetic acid (**2,4-D**) and glyphosate (**Glyp**), as well as the ecotoxicity of the resulting binary systems toward *Ceriodaphnia affinis* and *Paramecium caudatum*, was assessed for the first time. The association constants of pillar[5]arenes with **Glyp** ($\log K_a$ = 3.92–4.06) were an order of magnitude higher than the corresponding values for **2,4-D** ($\log K_a$ = 2.66–3.06) with the stoichiometry of 1:1. The formation of stable associates (143–177 nm) with negative zeta potential values (from −20.9 to −7.8 mV) was demonstrated for the pillar[5]arene/herbicide systems. Low phytotoxicity of pillar[5]arenes against *Chlorella vulgaris* was shown. The addition of pillar[5]arenes to **2,4-D** reduced the wheat (*Triticum aestivum* L.) germination index by 4.5-fold compared to the pure herbicide. Forming associates between decamethoxypillar[5]arene and **Glyp** increased the LC_{10} by more than twofold compared to the individual herbicide against *Paramecium caudatum* and *Ceriodaphnia affinis*. It was demonstrated that combining pillar[5]arenes with **Glyp** can reduce ecotoxicity while partially preserving or selectively modifying phytotoxicity. The results obtained in this study are encouraging for the development of materials and supramolecular systems that could boost agricultural efficiency while reducing its environmental impact.

Keywords: pillar[5]arene; self-assembly; glyphosate; 2,4-D; herbicidal activity; ecotoxicity

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1. Introduction

The rapidly growing population and climate change across the planet are giving rise to challenges for modern agriculture, including soil degradation, reduction in agricultural

land, unsustainable use of natural resources, and excessive application of agrochemicals, among others [1–3]. According to data from the United Nations, among the 17 Sustainable Development Goals, the eradication of poverty and hunger is identified as the top priority [4]. It has been estimated that, in order to ensure food security by 2050, agricultural output must increase by 60% compared with 2005 levels [5]. However, maintaining current levels of traditional agricultural practices will lead to the additional degradation of 16 million square kilometers of farmland, accompanied by a 12–14% decline in its productivity [6]. The development of novel fertilizers, the search for innovative plant protection methods, the breeding of resistant crop varieties, and the optimization of agronomic practices constitute the primary approaches to enhancing agricultural efficiency [7,8]. Pesticides serve as a tool for protecting crops and regulating their growth, with herbicides accounting for 40% of all pesticides currently in use [9,10]. However, the majority of these compounds have limited water solubility and require the use of adjuvants (organic solvents, surfactants, stabilizers), which increases environmental risks [11–14].

The widespread application of herbicides, particularly glyphosate (**Glyp**) and 2,4-dichlorophenoxyacetic acid (**2,4-D**), leads to their accumulation in soils as well as surface and groundwater, with demonstrated toxic effects on non-target biota [12,15–17]. **Glyp** is one of the most widely used herbicides (700,000 tons per year [18,19]). Glyphosate-based herbicides have adverse effects on a broad range of terrestrial and aquatic organisms due to their relatively high solubility in water (12 g/L at 25 °C) [20]. The global use of **2,4-D** is estimated to be 150,000 tons per year [21]. Studies have shown that the effects of **2,4-D** include changes in growth, pigment composition, antioxidant enzyme activity, and cellular ultrastructure. Additionally, **2,4-D** has been shown to induce cyanotoxin production in phytoplankton and cyanobacteria [15,16]. Despite the low solubility of **2,4-D** (approximately 0.54 g/L) and its ester form (the most widely used form in commercial herbicides due to more efficient penetration into weeds) [22], **Glyp** and **2,4-D** can alter the structure of phytoplankton and periphyton communities in freshwater microcosms, reducing chlorophyll content and algal abundance [15]. Moreover, extensive data have also been reported regarding the combined effects of **Glyp** and **2,4-D** on microalgae, periphyton, and terrestrial plants, including dose-dependent growth inhibition, changes in species composition, oxidative stress, and morphological damage [12,15,16,23,24]. Given these considerations, there is a growing demand for the development of innovative approaches that reduce environmental risk while maintaining the effectiveness of herbicides, marking this as a vital field within agriculture.

The integration of developments in supramolecular chemistry into agricultural practice was considered a promising strategy for improving the effectiveness of existing pesticides while simultaneously decreasing the quantities applied to soil, thereby sustaining or enhancing crop productivity [25–27]. To date, the literature has demonstrated the use of macrocyclic compounds as nanopesticides, pesticide sensors, pesticide antidotes, and as materials that enhance pesticide efficacy while preserving pesticidal properties and reducing ecotoxicity [28–33]. Pillar[n]arenes, first reported in 2008 [34], have demonstrated considerable promise as a macrocyclic platform for agriculture, which is attributed to their ease of functionalization, one-step synthesis, low toxicity of various derivatives, and propensity for host–guest complexation [35–37]. Specifically, pillar[5]arenes have been shown to form stable complexes with benquitrione, leading to an expansion of the herbicide's spreading area on hydrophobic leaf surfaces [38], as well as to bind paraquat and diquat, with a concomitant reduction in reactive oxygen species formation, thereby restricting their harmful impact on cultivated plants [39,40]. Haibing Li and co-workers reported the construction of an organic porous polymer derived from a decasubstituted pillar[5]arene bearing sulfur-containing spacers, which enabled highly efficient and selective

adsorption of methylparathion [41]. Our research team has shown that the genotoxicity of glyphosate-based formulations can be mitigated via competitive binding of pillar[5]arenes with biomacromolecules [42]. However, the literature lacks data on the influence of complexation between pillar[n]arenes and herbicides on herbicidal properties, particularly the effects on target plant species, as well as on ecotoxicity, specifically the effects on non-target biota, including aquatic organisms and higher plants.

In this work, decamethoxypillar[5]arene and monohydroxypillar[5]arene (as the most synthetically accessible representatives of the pillar[5]arene family [29,34–36]) were used to modulate the herbicidal activity of **Glyp** and **2,4-D** against the microalga *C. vulgaris*, common wheat (*Triticum aestivum* L.), and garden radish (*Raphanus sativus* L.), alongside the ecotoxicity of the resulting herbicide-containing systems against *P. caudatum* and *C. affinis* (Figure 1). The findings presented herein offer considerable promise for the application of pillar[5]arene derivatives and supramolecular systems based on them in improving both the efficacy and safety of contemporary agricultural practices.

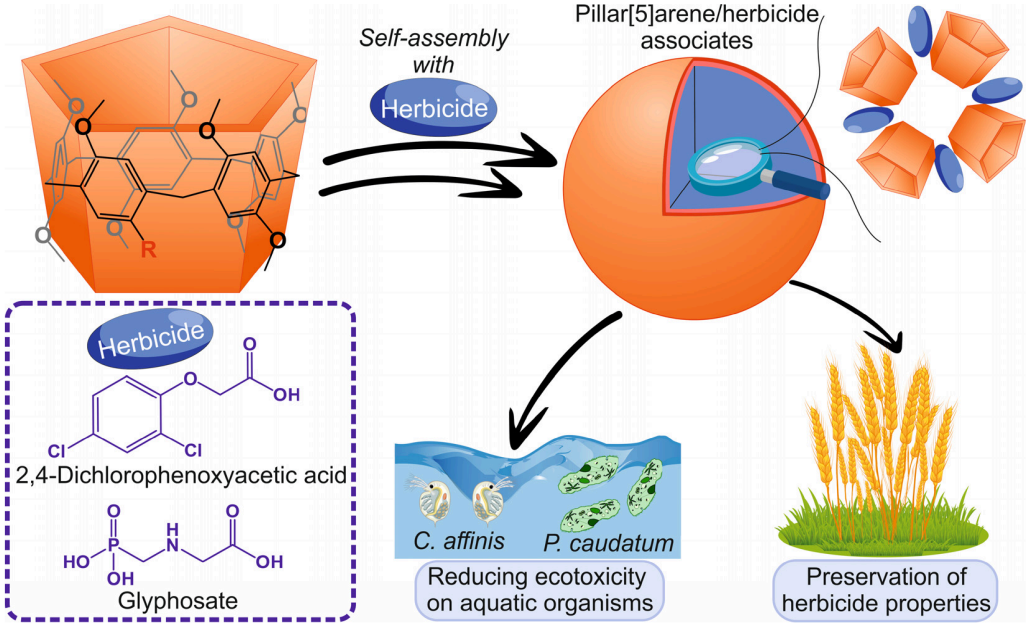

Figure 1. Sketch image of the design of the pillar[5]arene/herbicide systems.

2. Materials and Methods

2.1. General Experimental Procedures

^{1}H and ^{13}C NMR spectra were recorded on the Bruker Avance-400 spectrometer (Bruker Corp., Billerica, MA, USA) (^{13}C{^{1}H} 100 MHz, ^{1}H 400 MHz). Chemical shifts were referenced to the signals of residual protons of deuterated solvent (DMSO-d_6). The compound's concentration was equal to 3–5% *w*/*w* for all measurements. The FTIR ATR spectra were recorded on the Spectrum 400 FT-IR spectrometer (Perkin–Elmer, Seer Green, Llantrisant, UK) with the Diamond KRS-5 attenuated total internal reflectance attachment (resolution 0.5 cm^{-1}, accumulation of 64 scans, recording time 16 s in the wavelength range of 400–4000 cm^{-1}). Elemental analysis was performed on the Perkin–Elmer 2400 Series II instruments (Perkin–Elmer, Waltham, MA, USA). Melting points were determined using the Boetius microscope, having a micro-heating table (VEB Kombinat Nagema, Radebeul, Germany). All chemicals were purchased from Acros (Fair Lawn, NJ, USA). Organic solvents were purified in accordance with standard procedures. All the aqueous solutions were prepared using the Millipore-Q (Merck Group, Burlington, MA, USA) deionized water (>18.0 MW cm at 25 °C).

2.2. General Procedures for the Synthesis of Compounds **1** *and* **2**

Initial decamethoxypillar[5]arene **1** was obtained from commercially available 1,4-dimethoxybenzene using the literary method [43]. Further removal of one methoxy protection led to monohydroxypillar[5]arene **2** [44].

Decamethoxypillar[5]arene (pillar[5]arene **1**). Mp: 249 °C. ^{1}H NMR ($CDCl_3$, 400 MHz, 298 K) δ_H (ppm): 3.72 (s, 30H, -OCH_3), 3.76 (s, 10H, -CH_2-), 6.80 (s, 10H, ArH).

4,8,14,18,23,26,28,31,32-Nonamethoxy-35-hydroxypillar[5]arene (pillar[5]arene **2**). Mp: 203 °C. ^{1}H NMR ($CDCl_3$, 400 MHz, 298 K) δ_H (ppm): 6.90, 6.83, 6.76, 6.74, 6.72, 6.68, 6.65, 6.62, 6.60 (s, 10H, Ar<u>H</u>), 3.81, 3.78, 3.77, 3.75, 3.74, 3.71, 3.65, 3.63, 3.62, 3.59, 3.54 (s, 37H, -C<u>H_2</u>– and -OC<u>H_3</u>).

2.3. Fluorescence Spectroscopy

Fluorescence spectra were recorded on the Fluorolog 3 luminescence spectrometer (Horiba Jobin Yvon, Longjumeau, France). The excitation wavelength was set to 290 nm. The emission scan range was 310–450 nm. Excitation and emission slits were 4 nm for pillar[5]arene **1** and its mixtures with **2,4-D** and **Glyp**. Excitation and emission slits were set to 3 nm for pillar[5]arene **2** and its mixtures with **2,4-D** and **Glyp**. Quartz cuvettes with an optical path length of 10 mm were used. The cuvette was placed at the front-face position to avoid the inner-filter effect. Fluorescence spectra were recorded at 298 K in an H_2O + 5% DMSO system and automatically corrected using the Fluorescence program. In the initial fluorescence experiments, 300 μL of pillar[5]arenes **1** or **2** (100 μM) was added to 300 μL of the herbicide solution (1 mM), and the mixture was diluted to a final volume of 3 mL. In the fluorescence titration experiments, aliquots of 1 mM solution of **2,4-D** or **Glyp** (30, 150, 300, 450, 600, 900, 1200, 1500, 1800, 2100, 2400, 2700 μL) were added to 30 μL of a 1 mM pillar[5]arene solution and diluted to a final volume of 3 mL. The fluorescence spectra of the solutions were then recorded. The stability constants of associates were calculated by Bindfit [45]. Three independent experiments were carried out for each series.

2.4. Dynamic Light Scattering (DLS)

The distribution of particles by number, volume, intensity, and the polydispersity index was determined by dynamic light scattering (DLS) on a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK) in quartz cuvettes. The instrument is equipped with a 4 mW He-Ne laser (633 nm). Measurements were performed at a detection angle of 173°. The error in determining the particle size is less than 2%. The results were processed by the DTS program (Dispersion Technology Software 4.20). An H_2O + 5% DMSO system was used to prepare solutions for studying the association of pillar[5]arenes **1** and **2**. During the experiment, the concentrations of macrocycles were 10 μM. Binary systems (pillar[5]arene with herbicide) were prepared similarly to those studied by fluorescence spectroscopy. The particle sizes were measured 1 h after mixing. Measurements were carried out after 72 h to evaluate kinetic stability. Three independent experiments were carried out for each series.

2.5. Zeta Potentials

Zeta (ζ) potentials were determined by electrophoretic light scattering (ELS) on a Zetasizer Nano ZS from Malvern Instruments (Worcestershire, UK). Samples were prepared for the DLS measurements and were transferred with the syringe into the disposable folded capillary cell for measurement. The zeta potentials were measured using the Malvern M3-PALS method and averaged from three measurements.

2.6. Transmission Electron Microscopy (TEM)

TEM measurements were made at the Interdisciplinary Center for Analytical Microscopy of Kazan Federal University. Analysis of samples was carried out using a Hitachi HT7700 Exalens transmission electron microscope (Tokyo, Japan) with an Oxford Instruments X-Maxn 80T EDS detector working in STEM mode. Samples of the pillar[5]arenes (10 μM) and a binary system, the pillar[5]arenes (10 μM) with herbicides (10 μM), were prepared similarly to those studied by the DLS method. A H_2O + 5% DMSO system was used as a solvent. The solution (10 μL) was placed on a carbon-coated 3 mm copper grid and dried at room temperature using a special holder for microanalysis. After drying, the grid was placed in the transmission electron microscope and analyzed at an accelerating voltage of 80 kV.

2.7. Evaluation of the Herbicidal Activity of Compounds on a Microalgae Culture in 96-Well Microtiter Plates

An algologically pure culture of the unicellular green alga *Chlorella vulgaris* was used as the test object. The microalgae were cultivated in Tamiya medium (macronutrients (g/L): KNO_3—5, $MgSO_4 \times 7H_2O$—2.5, $KH_2PO_4 \times 3H_2O$—1.25, ferric citrate—0.003; micronutrients: solution A (g/L) (H_3BO_3—2.86; $MnCl_2 \times 4H_2O$—1.81; $ZnSO_4 \times 5H_2O$—0.222) and solution B (g/L) (MoO_3—0.018; NH_4VO_3—0.023, dissolved with heating) [46]. Outside the experimental period, the alga was maintained in an active state by culturing it in 5% Tamiya medium. One day prior to the biotesting experiment, the alga was subcultured into 50% Tamiya medium and cultivated under constant illumination at a temperature of 25 ± 3 °C with stirring at 100 rpm for 24 h. The optical density of the 24 h culture was measured using a Multiskan FC microplate photometer (Thermo Fisher Scientific (Shanghai) Instruments Co., Ltd., PuDong, Shanghai, China) at a wavelength of 620 nm after intensive shaking for 15 s prior to the measurement. A culture with an optical density of 0.6–0.8 arbitrary units (a.u.) was used as the inoculum for the experiment.

The experiment was conducted in the wells of a 96-well microtiter plate following recommendations with modifications [47]. During the experiment, optical density was measured using a microplate photometer at a wavelength of 620 nm after intensive shaking for 15 s prior to each measurement. The obtained data were normalized to the initial optical density values and the negative control (DMSO or water); the percentage of inhibition was then calculated according to the established recommendations.

2.8. Phytotoxicity of Pillar[5]arenes **1** *and* **2**

Phytotoxicity assessment was performed using a contact method on common agricultural plants: common wheat (*Triticum aestivum* L. cv. "Chernobrovaya") and garden radish (*Raphanus sativus* L. cv. "Zhara"). The test was conducted in Petri dishes (90 mm diameter). A filter paper disk served as the artificial substrate. The disks were moistened with 5 mL of the respective test solution. Ten seeds were placed in each dish. The dishes were placed in a plant growth chamber (VeFarm Clima Pro, Agroaspect Plus LLC, Yekaterinburg, Russia) set to a 16/8 h light/dark photoperiod. After 72 h, the seed germination rate (%) was determined, and the length of the main root was measured. Filter paper disks moistened with a dimethyl sulfoxide (DMSO) solution were used as a control for the tested compounds, as well as distilled water, which served as a negative control for the DMSO sample. Based on the collected data, seed germination (%), root elongation inhibition/stimulation relative to the control (%), and the germination index (GI, %) were calculated using the formulas described by Selim et al. [48].

Germination rate, %:

$$\frac{\text{Number of germinated seeds (treatment)}}{\text{Number of germinated seeds (control)}} \times 100$$

Root length relative to control, %:

$$\frac{\text{Mean root length (treatment), mm}}{\text{Mean root length (control), mm}} \times 100$$

GI, %:

$$\frac{\text{Germination rate, \%} \times \text{Root length relative to control, \%}}{100}$$

2.9. Ecotoxicity Characterization of the Tested Compounds

Ecotoxicity assessment was performed on a range of aquatic organisms: the protozoan *Paramecium caudatum* and the crustacean *Ceriodaphnia affinis*. The maximum working concentration for toxicity evaluation was 0.2 g/L. Subsequently, a series of dilutions was prepared down to the maximum non-toxic concentration. The tests were conducted according to a previously described method [49]. To determine the toxicity of the studied samples, the LC_{10} value was calculated for each sample for *P. caudatum* and *C. affinis*.

2.10. Statistical Processing

All experiments were performed in at least two independent series, with a minimum of three replicates per series. A statistical analysis was performed using the R-4.5.2 package. A test for normal distribution was carried out using the Shapiro–Wilk test. This was followed by a one-way analysis of variance (ANOVA) and post hoc multiple comparisons. Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Molecular Binding and Self-Assembly of Pillar[5]arenes with Herbicides

Our research group has previously examined complexation between various herbicides (glyphosate, paraquat, pyridate, 3-(methylphosphinico)propionic acid, and glufosinate-ammonium) and pillar[5]arenes functionalized with amino acid and betaine groups [42,50]. We demonstrated that competitive binding of these pillar[5]arene derivatives to biomacromolecules suppresses **Glyp** interaction with them [42]. The ability of pillar[5]arenes containing *L*-tryptophan residues to bind paraquat, pyridate, 3-(methylphosphinico)propionic acid, and glufosinate-ammonium was demonstrated in another study [50]. In the present study, we proposed to evaluate the effect of molecular binding of pillar[5]arenes **1** and **2** with **Glyp** and **2,4-D** on herbicidal properties, as well as the ecotoxicity of the resulting pillar[5]arene/herbicide systems. The choice of macrocycles **1** and **2** was justified by the relative simplicity of their synthesis and the good yields achieved in the reactions. The structures of the macrocyclic compounds and herbicides used in the study are presented in Figure 2.

The interaction of macrocycles **1** and **2** with the herbicides was initially investigated by fluorescence spectroscopy. This technique was chosen because of its high sensitivity, which allows for the detection of minor changes in the microenvironment of the compounds studied. The binding of pillar[5]arenes with **Glyp** and **2,4-D** was studied in a binary solvent system (H_2O + 5% DMSO) using tenfold molar excesses of the herbicides (Figure 3). It was found that the addition of herbicides to solutions of the macrocycles resulted in fluorescence quenching of the macrocycle regardless of the herbicide's nature. Upon the addition of **Glyp** to compound **1**, a fluorescence quenching of the macrocycle by more

than half was observed, whereas the addition of **2,4-D** led to a decrease in fluorescence intensity of approximately 1.5-fold (Figure 3A,B). Similarly, fluorescence quenching of the macrocycle was observed in the case of pillar[5]arene **2** (Figure 3C,D).

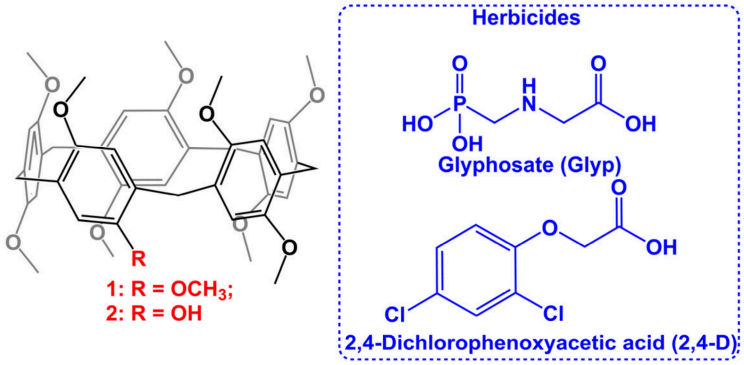

Figure 2. Structures of pillar[5]arenes **1** and **2** and herbicides **Glyp** and **2,4-D**.

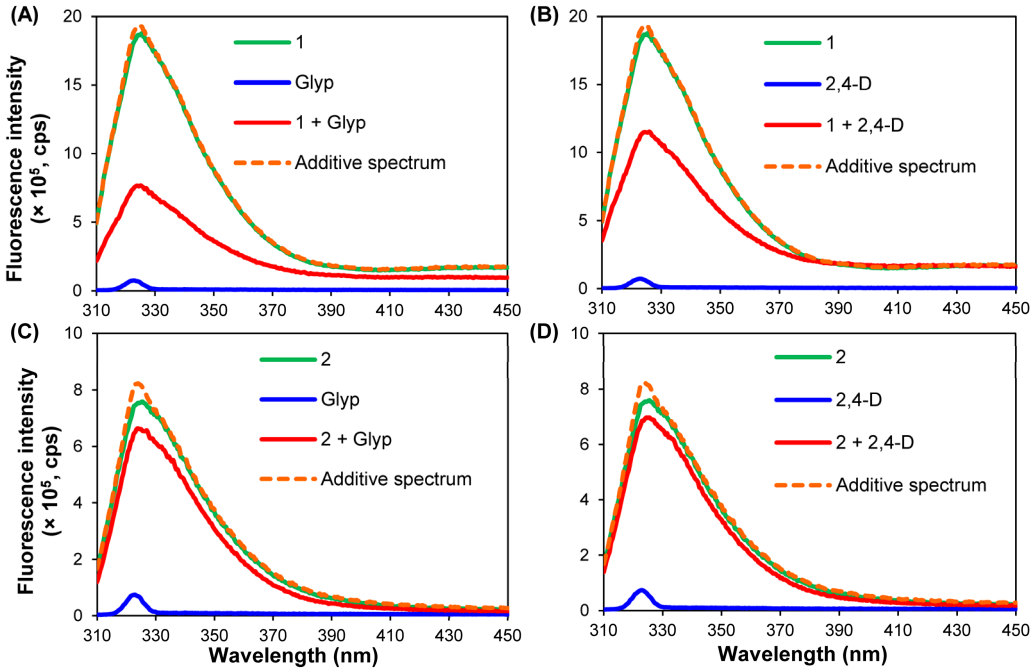

Figure 3. Fluorescence spectra (H_2O + 5% DMSO, 298 K, excitation wavelength 290 nm) of pillar[5]arene **1** (10 μM) with (**A**) **Glyp** (100 μM) and (**B**) **2,4-D** (100 μM), and pillar[5]arene **2** (10 μM) with (**C**) **Glyp** (100 μM) and (**D**) **2,4-D** (100 μM). The additive spectrum is the theoretical sum of the pillar[5]arene and herbicide spectra.

To quantify the interaction, fluorescence titration experiments were conducted (Figures 4A and S1–S3), wherein the macrocycle concentration was maintained constant. The resulting data were analyzed using Bindfit v0.5 software [45,51]. The stoichiometry of the associates was determined to be 1:1, which was also confirmed by titration data processed using pillar[5]arene:herbicide ratios of 1:2 and 2:1. However, in these cases, the association constants were determined with a large error (Figures S4–S7). The logarithms of the association constants are presented in Table 1. **Glyp** binding appears to be more efficient, as indicated by the higher association constant values observed with pillar[5]arenes. In contrast, the binding strength with **2,4-D** was an order of magnitude lower.

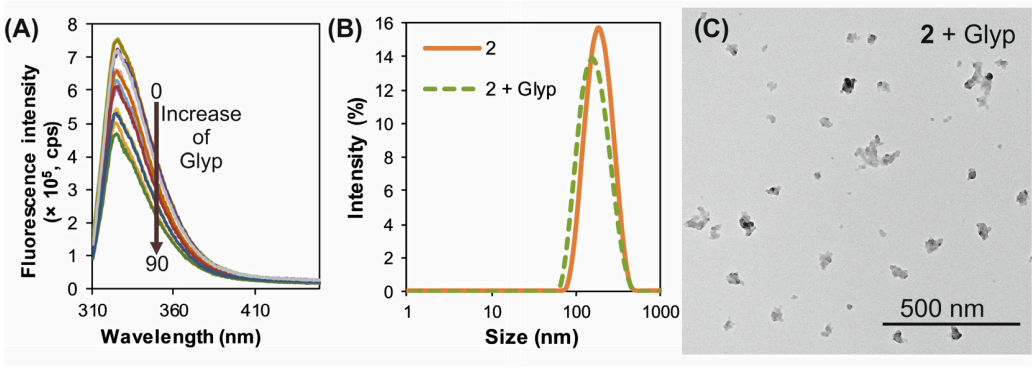

Figure 4. (**A**) Fluorescence spectra (H_2O + 5% DMSO, 298 K, excitation wavelength 290 nm) of **2** (10 μM) with various concentrations of **Glyp** (from 0- to 90-fold excess). Arrow means hypochromic effect with increasing concentration of **Glyp**; (**B**) size distribution of particles by intensity for **2** (10 μM, solid line) and **2** with **Glyp** (1:1 ratio, dashed line) in H_2O + 5% DMSO (298 K); (**C**) TEM image of associates formed by **2** with **Glyp** (1:1 ratio).

Table 1. Values of logK_a of pillar[5]arenes with herbicides determined by fluorescence titration in H_2O + 5% DMSO system.

System	1 + 2,4-D	1 + Glyp	2 + 2,4-D	2 + Glyp
logK_a	3.06 ± 0.32	3.92 ± 0.34	2.66 ± 0.36	4.06 ± 0.36

NMR spectroscopy is another convenient approach for binding investigation. Solutions of pillar[5]arenes **1** and **2** were prepared in DMSO-d_6 due to the limited solubility of the macrocycles in the H_2O + 5% DMSO system at 10 mM. The interaction of pillararenes with herbicides (10 mM) was studied at a 1:1 ratio. Unfortunately, it was found that **Glyp** had low solubility at 10 mM in DMSO, making further analysis of the proton spectra of mixtures of compounds **1** and **2** with **Glyp** unfeasible. Analysis of the ^{1}H NMR spectra of macrocycles **1** and **2** in the presence of **2,4-D** showed no changes in the chemical shifts in the pillar[5]arenes or the herbicide (Figures S8 and S9). This suggests that the herbicide molecule is not included in the macrocyclic cavity. Thus, based on fluorescence and NMR spectroscopy data, we hypothesize that the formation of ensembles in which the entropy factor plays a decisive role in stabilizing the resulting supramolecular systems is possible [52].

The next stage of the study involved investigating the association between pillar[5]arenes **1** and **2** with **Glyp** and **2,4-D** by dynamic and electrophoretic light scattering (Figures S10–S29). Initially, the association of individual macrocycles **1** and **2** was studied in a binary solvent system (H_2O + 5% DMSO) at 10 μM (Table 2), which was used for the fluorescence studies. It was shown that compound **1** formed polydisperse systems (Figure S12) characterized by a polydispersity index (PDI) exceeding 0.25 and submicron-sized associates. In contrast, macrocycle **2** formed stable associates with a mean diameter of approximately 181 nm and a PDI of 0.08 (Figure S13). This behavior is likely due to the presence of a hydroxyl group in its structure, which provides additional stabilization of self-assembled nanoparticles through hydrogen bonding [53,54]. The association of compounds **1** and **2** with the herbicides was studied under the same conditions at 10 μM for each pillar[5]arene (Table 2) and a macrocycle-to-herbicide ratio of 1:1 (Figures S14–S21). The addition of herbicides, regardless of their nature, to compound **1** led to the stabilization of the systems, resulting in the formation of nanometer-sized particles (143–153 nm) with a PDI ranging from 0.18 to 0.20 (Figures S1 and S2). The zeta potential value of the macrocycle **1**/herbicide systems was negative, which can be attributed to the presence of carboxyl and/or phosphonic moieties in the **Glyp** and **2,4-D** molecules. Association studies of

pillar[5]arene **2** with herbicides demonstrated that the size of the associates formed remains largely unchanged compared to that of macrocycle **2** alone. Notably, the addition of **2,4-D** to macrocycle **2** resulted in particles with an average diameter of 145 ± 6 nm (Figure S16), whereas the **2** + **Glyp** systems produced associates measuring 177 ± 2 nm (Figure S20). The polydispersity indices of the systems increased only slightly upon herbicide addition to pillar[5]arene **2** (PDI = 0.11–0.12). The resulting systems also exhibited negative zeta potential values. For the macrocycle/**2,4-D** systems, the ζ-potential value was roughly two times lower than that for the pillar[5]arene/**Glyp** systems, as **Glyp** contains both carboxyl and phosphonic groups, which may explain its higher absolute zeta potential value. The stability of the systems derived from pillar[5]arenes **1** and **2** with herbicides was evaluated after the solutions had been stored for three days (Table S1, Figures S22–S29). Systems based on macrocycle **2** exhibited high sedimentation stability, with the hydrodynamic characteristics of the colloidal systems remaining essentially unchanged (Figures S26–S29). In contrast, solutions of **1** + **Glyp** and **1** + **2,4-D** showed increased polydispersity indices (0.29–0.34) alongside only minor fluctuations in particle size (151–169 nm) (Figures S22–S25).

Table 2. Hydrodynamic diameters (d, nm), polydispersity indices (PDI), and zeta potential (ζ, mV) of pillar[5]arenes **1** and **2** (10 μM) and their mixtures (1:1) with herbicides (H_2O + 5% DMSO, 298 K) by intensity. Data are presented as mean ± SD, n = 3.

System	d, nm	PDI	ζ, mV
2,4-D	340 ± 75	0.84 ± 0.09	N/D [1]
Glyp	259 ± 61	0.66 ± 0.01	N/D
1	2632 ± 133	0.69 ± 0.09	2.0 ± 0.3
1 + **2,4-D**	143 ± 2	0.18 ± 0.01	−(9.4 ± 1.3)
1 + **Glyp**	153 ± 9	0.20 ± 0.01	−(20.9 ± 1.2)
2	181 ± 2	0.08 ± 0.03	−(6.0 ± 0.1)
2 + **2,4-D**	145 ± 6	0.12 ± 0.02	−(7.8 ± 0.2)
2 + **Glyp**	177 ± 2	0.11 ± 0.02	−(20.8 ± 1.2)

[1] N/D—no determination.

Transmission electron microscopy (TEM) was used to characterize the associate morphology for pillar[5]arenes **1** and **2** with **Glyp** and **2,4-D**. All systems formed spherical particles with average diameters of 50–100 nm, which then coalesced into larger associates (Figures 3C and S1–S3). TEM data confirmed that the particle sizes were consistent with those measured by DLS.

Thus, fluorescence and NMR spectroscopy, DLS, and TEM revealed that pillar[5]arenes **1** and **2** effectively bind **Glyp** and **2,4-D**, forming stable supramolecular associates with hydrodynamic diameters of 143–177 nm. ^{1}H NMR spectroscopy of the pillar[5]arene/herbicide systems showed that the herbicide molecule did not enter the macrocyclic cavity and remained outside of it.

*3.2. Evaluation of the Herbicidal Activity of Pillar[5]arenes and Their Systems with **Glyp** and **2,4-D***

To date, most publications in this field have focused on the formation of host–guest complexes between pillar[n]arenes and herbicides [55–57], with paraquat and its derivatives being the most widely studied. At the same time, data on the herbicidal activity of such complexes, the potential for targeted herbicide delivery, and their sustained release remain limited [41]. One of the aims of this study was to evaluate the herbicidal properties and ecotoxicity of **Glyp**, **2,4-D**, and pillar[5]arene/herbicide systems. The herbicidal activity was assessed using plants at different organizational levels, including lower plants (microalgae) and higher plants from various classes. Accordingly, the phytotoxicity of pil-

lar[5]arenes **1** and **2** and pillar[5]arene/herbicide systems was studied using the microalga *Chlorella vulgaris*, common wheat (*Triticum aestivum* L.), and garden radish (*Raphanus sativus* L.). These species exhibit specific herbicide responses due to differences in their structure and physiology, including growth inhibition and reduced seed germination [58].

It is well known that **Glyp** and **2,4-D** inhibit the growth and development of radish and wheat in a dose-dependent manner [59,60]. Higher concentrations are employed in vegetation and field experiments, a practice attributed to the adsorption of herbicides by soil and their degradation by soil microbial communities. For example, the recommended working concentration for **Glyp** when applied as the commercial formulation Tornado 500 (JSC August Crop Protection, Russian Federation) is 5 g/L, whereas for **2,4-D** in the commercial formulation Ballerina (JSC August Crop Protection, Russian Federation) it does not exceed 2 g/L. One of the objectives of this study was to assess whether the concentration of the herbicide could be reduced while maintaining its activity through association with macrocycles. For this reason, a concentration of 0.2 g/L was selected for further experiments. This was the minimum concentration at which herbicidal properties were previously observed for both **Glyp** and **2,4-D** in eluate experiments [61,62]. Also, the reasons for choosing a concentration lower than the concentration of the working solution included the possibility of a quantitative change in the test function at sublethal concentrations and the dilution factor in natural environments.

3.2.1. Herbicidal activity of Pillar[5]arenes and Their Systems with **Glyp** and **2,4-D** Toward *Chlorella vulgaris*

Glyp has been reported to inhibit the growth of *C. vulgaris* at concentrations exceeding 5 mg/L [63–65]. In contrast, the effects of **2,4-D** on microalgae have been addressed in far fewer studies. In particular, the growth of *C. vulgaris* has been inhibited, and the synthesis of both protein and pigment has been suppressed at **2,4-D** concentrations exceeding 90 μM (~20 mg/L) [66,67].

Decamethoxypillar[5]arene **1** was shown to stimulate the growth and development of the algae *C. vulgaris* (−13%), whereas pillar[5]arene **2** displayed weak inhibitory activity (5%) toward *C. vulgaris* (Figure 5). Notably, the phytotoxicity of **2,4-D** toward *C. vulgaris* was slightly increased when the herbicide was bound with macrocycles **1** and **2** compared to free **2,4-D**. These findings are consistent with the fluorescence titration results, which yielded nearly identical association constants for macrocycles **1** and **2** with **2,4-D** within the range of 2.66–3.06 (Table 1). The binding of **Glyp** with pillar[5]arenes **1** and **2** resulted in dramatically different effects. The **1** + **Glyp** system was found to stimulate microalgae growth (Figure 5A). In contrast, the phytotoxicity of **Glyp** was increased two-fold when bound by macrocycle **2** compared to free **Glyp** (Figure 5B).

3.2.2. Herbicidal Activity of Pillar[5]arenes and Their Systems with **Glyp** and **2,4-D** Toward Common Wheat (*T. aestivum*) and Garden Radish (*R. sativus*)

According to the literature, both **2,4-D** and **Glyp** exhibit phytotoxic properties against radish [61,68]. It has also been reported that **2,4-D** is used to control wild radish in wheat cultivation. Even comparatively low concentrations (1 μM) of **2,4-D** are known to elicit oxidative stress, membrane damage, reduced pigment content, and impaired growth parameters at the seedling stage and in laboratory models [62,69–71]. **Glyp**, by contrast, is essentially non-phytotoxic to resistant crops when applied at the recommended rate. However, sublethal doses have been shown to reduce both the height and yield of susceptible wheat cultivars [72], including the variety used in this study.

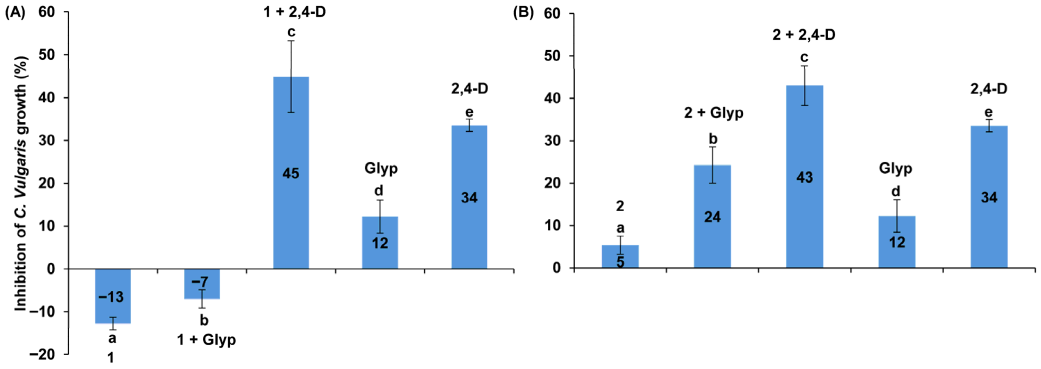

Figure 5. Phytotoxicity of (**A**) pillar[5]arene **1** and (**B**) pillar[5]arene **2** and their systems with **Glyp** and **2,4-D** toward microalga *C. vulgaris* ($C_{pillar[5]arene}$ = $C_{herbicide}$ = 0.2 g/L). Different letters (a–e) indicate statistically significant differences between treatments ($p < 0.05$). Data are presented as mean ± SD, n = 5.

This study evaluated the phytotoxicity of **2,4-D** and **Glyp** (Table 3) against both garden radish (*R. sativus*) and common wheat (*T. aestivum*). The presence of phytotoxic activity against both monocotyledonous and dicotyledonous plants was necessary to assess the effect of pillar[5]arene–herbicide interactions on their biological activity. Radish exhibited greater sensitivity to **2,4-D** (Table 3), a finding consistent with the distinct mechanisms of action of the two herbicides. As a synthetic auxin, **2,4-D** saturates the auxin signaling pathway, leading to aberrant growth, increased ethylene and abscisic acid levels, oxidative stress, cell wall rupture, and ultimately cell death—effects that are predominantly observed in dicotyledonous species [73,74]. **Glyp**, in contrast, acts by inhibiting EPSPS (a key enzyme in the shikimate pathway), thereby blocking the biosynthesis of aromatic amino acids and impairing both protein synthesis and secondary metabolite production across all plant species, regardless of whether they are monocots or dicots [75–77].

Table 3. Phytotoxicity of pillar[5]arenes **1** and **2** and their systems with **2,4-D** and **Glyp** toward common wheat (*T. aestivum*) and garden radish (*R. sativus*) ($C_{pillar[5]arene}$ = $C_{herbicide}$ = 0.2 g/L). Different letters (a–d) indicate statistically significant differences between treatments ($p < 0.05$). Data are presented as mean ± SD, n = 15.

System	Wheat			Garden Radish		
	Germination Rate, %	Average Root Length, % of Control	GI, %	Germination Rate, %	Average Root Length, % of Control	GI, %
2,4-D *	57 ± 3 ^{a}	15 ± 1 ^{a}	9	15 ± 1 ^{a}	14 ± 1 ^{a}	2
Glyp *	68 ± 10 ^{a}	6 ± 1 ^{b}	4	81 ± 17 ^{b}	30 ± 5 ^{b}	24
DMSO **	104 ± 6 ^{b}	24 ± 9 ^{a}	24	77 ± 22 ^{b}	72 ± 13 ^{c}	55
1 *	89 ± 11 ^{b}	16 ± 10 ^{a}	14	69 ± 12 ^{b}	56 ± 14 ^{d}	39
1 + **2,4-D** *	50 ± 14 ^{a}	4 ± 1 ^{b}	2	19 ± 7 ^{a}	25 ± 13 ^{b}	5
1 + **Glyp** *	50 ± 6 ^{a}	5 ± 2 ^{b}	2	77 ± 2 ^{b}	54 ± 10 ^{d}	42
2 *	89 ± 11 ^{b}	14 ± 3 ^{a}	13	85 ± 24 ^{b}	26 ± 3 ^{b}	22
2 + **2,4-D** *	64 ± 12 ^{a}	4 ± 1 ^{b}	2	19 ± 6 ^{a}	16 ± 8 ^{ab}	3
2 + **Glyp** *	79 ± 17 ^{ab}	4 ± 1 ^{b}	3	69 ± 12 ^{b}	39 ± 14 ^{bd}	27

* means a DMSO sample was used as a control. ** means a water sample was used as a control.

Next, the phytotoxicity of macrocycles **1** and **2** was evaluated against wheat and radish, both with and without herbicides. It is important to note that the original solvent, dimethyl sulfoxide (DMSO), had a negative impact on the growth and development of both wheat and radish seedlings. In wheat, DMSO reduced root length and the germination index (GI) by 76%. The inhibitory effect was less pronounced in radishes, with root length and GI decreasing by 28% and 45%, respectively. Nevertheless, DMSO also negatively affected

radish germination. Therefore, all parameters for the experimental samples were calculated relative to the DMSO control to evaluate the effects of the tested compounds exclusively. A slight inhibition of wheat seed germination rate (89%) was observed for pillar[5]arenes **1** and **2**, regardless of the macrocycle structure (Table 3). The wheat root length was reduced by 84% for macrocycle **1** and by 86% for compound **2**. The wheat germination index (GI) was found to be 13–14% for both macrocycles. Systems consisting of pillar[5]arenes **1** and **2** in combination with herbicides exhibited divergent effects on phytotoxicity toward wheat. The **1** + **2,4-D** and **1** + **Glyp** systems, in particular, resulted in reductions in root length of 1–11% and decreases in germination rate of 7–18%. Macrocycle **2**/herbicide systems reduced wheat root length by 2–11% (Table 3). Thus, adding pillar[5]arenes **1** and **2** to **2,4-D** decreased GI approximately 4.5-fold (from 9% to 2%) in wheat, whereas **Glyp** systems showed almost no effect on germination.

For garden radish, pillar[5]arene **1** inhibited seed germination (69%), while macrocycle **2** showed lower phytotoxicity (85%) versus control (Table 3). The root length decreased by 74% (compound **1**) and 44% (compound **2**) compared to the control. The radish germination indices for macrocycle **1** (39%) and **2** (22%) were higher than wheat GI values, indicating pillar[5]arene selectivity toward monocots. **2,4-D**-based systems containing both macrocycles did not show significant enhancement of herbicidal activity against radish. The GI values of pure **Glyp** (24%) and the **2** + **Glyp** system (27%) were similar. However, the **1** + **Glyp** system showed an almost twofold increase in GI to 42%, indicating reduced phytotoxicity through the formation of macrocycle–herbicide associates.

Thus, the modulation of the herbicidal properties of **2,4-D** and **Glyp** by pillar[5]arenes was studied using model plants (*C. vulgaris*, *T. aestivum*, *R. sativus*). The low phytotoxicity of pillar[5]arenes **1** and **2** was demonstrated against the microalga *C. vulgaris*. The GI of wheat was found to be 13–14% for both macrocycles. Meanwhile, the germination indices for radish were higher, at 22–39%. A synergistic effect was found for the pillar[5]arene/**2,4-D** systems, resulting in increased phytotoxicity compared to **2,4-D** itself. A similar pattern was observed in the case of the **2** + **Glyp** system, where phytotoxicity towards *C. vulgaris* increased. Assessment of macrocycle–herbicide interactions revealed an approximate fourfold decrease in wheat GI upon combining pillar[5]arenes **1** and **2** with **2,4-D**, whereas binding with **Glyp** showed negligible impact on the germination index. No significant effect of macrocycles on **2,4-D** phytotoxicity was evident in radishes. However, the addition of pillar[5]arene **1** to **Glyp** resulted in a GI that was almost twice that of the free herbicide.

*3.3. Ecotoxicity of Pillar[5]arenes and Their Systems with **Glyp** and **2,4-D** Toward P. caudatum and C. affinis*

A key requirement of host molecules designed to bind pesticides is low toxicity, so as to minimize their harmful impact on non-target microbiota when released into water and soil. In this regard, the final stage of the study involved assessing the ecotoxicity of pillar[5]arenes **1** and **2** and their systems with herbicides against the aquatic organisms *C. affinis* and *P. caudatum* (Table 4, Figures 6 and 7). *P. caudatum* and *C. affinis* are widely used in ecotoxicological analyses due to their sensitivity to pollutants and their key role in aquatic ecosystems. *P. caudatum* is sensitive to the effects of heavy metals and organic herbicides [78–80]. *C. affinis* is a standard, highly sensitive test species used to evaluate the toxicity of various environmental contaminants, including pesticides [81,82]. Using these organisms allows the evaluation of the effects of herbicides and herbicide-containing systems on aquatic and terrestrial ecosystems at multiple biological levels.

Pillar[5]arenes **1** and **2** showed ecotoxicity against *C. affinis* exceeding that of **Glyp** and **2,4-D** at 0.2 g/L (Figure 6). There was no significant change in ecotoxicity for **2,4-D** systems with macrocycles **1** and **2**. However, **Glyp**-based systems were found to reduce ecotoxicity compared to the free components. The marked decrease in ecotoxicity for **Glyp** likely

reflects the higher association constants of pillar[5]arenes **1** and **2** with **Glyp** determined by fluorescence titration (Table 1).

Table 4. Ecotoxicity of pillar[5]arenes **1** and **2** and their systems with **2,4-D** and **Glyp** toward the aquatic organisms *C. affinis* and *P. caudatum*.

System	Ecotoxicity (LC_{10}, g/L)	
	C. affinis	*P. caudatum*
2,4-D	0.082	0.158
Glyp	0.087	0.116
1	0.045	0.176
1 + **2,4-D**	0.045	>0.200
1 + **Glyp**	**>0.200**	**>0.200**
2	0.102	0.120
2 + **2,4-D**	0.060	**0.200**
2 + **Glyp**	**0.145**	0.116

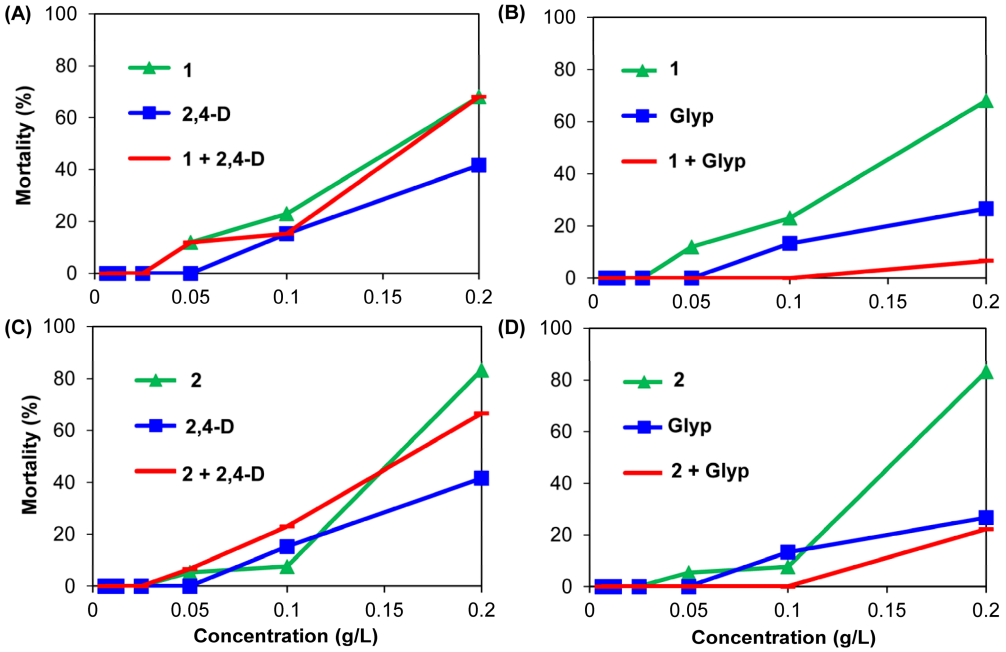

Figure 6. Ecotoxicity toward the aquatic organism *C. affinis* of pillar[5]arene **1** and its systems with (**A**) **2,4-D** and (**B**) **Glyp**; pillar[5]arene **2** and its systems with (**C**) **2,4-D** and (**D**) **Glyp**.

The ecotoxicity of pillar[5]arenes **1** and **2** at a concentration of 0.2 g/L was either lower than or not statistically different from that of **Glyp** toward *P. caudatum* (Figure 7). Compared to **2,4-D**, macrocycle **2** at 0.2 g/L caused a higher mortality rate in *P. caudatum* (26%) than the unformulated herbicide (15%). No modulation of herbicide toxicity by the macrocycles was detected for systems combining pillar[5]arenes **1** and **2** with **2,4-D**. In contrast, the **1** + **Glyp** system exhibited a marked reduction in ecotoxicity toward *P. caudatum* at 0.2 g/L, decreasing to 4% compared to 27% for **Glyp** alone.

Toxicity data from serial dilutions were used to determine LC_{10} (concentration causing < 10% mortality) for aquatic organisms (Table 4). The **1** + **Glyp** system showed an increase in LC_{10} of more than twofold for both species versus **Glyp** alone, indicating reduced toxicity. Similarly, the **2** + **Glyp** system decreased toxicity nearly twofold toward *C. affinis* relative to free **Glyp**. The greater reduction in ecotoxicity for **Glyp** systems with macrocycles **1** and **2** is probably due to their higher association constants with the herbicide.

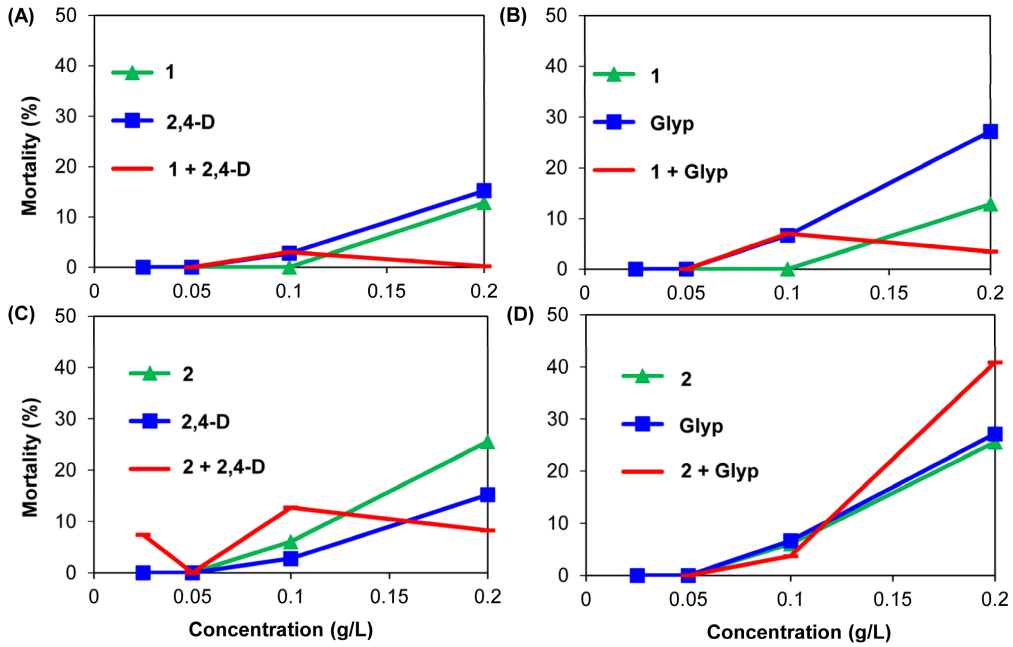

Figure 7. Ecotoxicity toward the aquatic organism *P. caudatum* of pillar[5]arene **1** and its systems with (**A**) **2,4-D** and (**B**) **Glyp**; pillar[5]arene **2** and its systems with (**C**) **2,4-D** and (**D**) **Glyp**.

3.4. General Assessment of Changes in Herbicidal Properties and Ecotoxicity of Pillar[5]arene Systems with **2,4-*D*** *and* ***Glyp***

The overall effect of pillar[5]arenes **1** and **2** and their herbicide-containing systems was assessed, given that they exhibited distinct herbicidal and ecotoxic properties. The ratio of the value of the macrocycle–herbicide system to the value of the same parameter for the pure herbicide was calculated for each parameter. This calculation allowed us to determine the effect of the herbicide binding to pillar[5]arene on changes in parameters (increases or decreases), eliminating the need for units of measurement and enabling comparisons between different parameters. A score was calculated by summing the parameters that positively characterize the associates and subtracting the parameters that negatively characterize them. A weighting system was not used. This was partly because seven parameters characterized herbicidal properties in this summary assessment (three for each plant species and one for microalgae inhibition), while only two parameters assessed ecotoxicity. This allowed for a greater overall weighting of herbicidal properties. A summary table (Table 5) was compiled showing parameter changes relative to the parent herbicide (**2,4-D** or **Glyp**), and the difference between ecotoxicity and herbicidal parameters was determined. Thus, the **1** + **Glyp** system was found to exhibit the greatest positive effect. A reduction in ecotoxicity by a factor of 2.6–3.4 was demonstrated, along with enhanced herbicidal properties, which were most pronounced against the monocotyledonous plant common wheat. Furthermore, the association of both macrocycles with **Glyp** was more effective than their association with **2,4-D**.

However, it is important to note that this score should be viewed as a descriptive, comparative index, rather than a validated biological indicator. Future studies will further refine the proposed evaluation system, including determining weighting coefficients for each parameter and incorporating phytotoxicity data for the tested compounds against model weed species such as *Arabidopsis thaliana*.

Table 5. Summary table of changes in herbicidal activity and ecotoxicity of pillar[5]arene–herbicide systems versus the parent herbicides (**2,4-D** and **Glyp**).

Parameter	Optimal Value	1 + 2,4-D	1 + Glyp	2 + 2,4-D	2 + Glyp
Herbicidal activity assessment against algae					
Toxicity, %	maximum	1.3	5.0	1.3	2.0
Herbicidal activity against wheat					
Germination rate, %	minimum	0.9	0.7	1.1	1.2
Average root length, % of control	minimum	1.0	0.8	1.0	0.7
GI, %		0.9	0.6	1.1	0.8
Herbicidal activity against radish					
Germination rate, %	minimum	1.3	1.0	1.3	0.9
Average root length, % of control	minimum	1.8	1.8	1.1	1.3
GI, %		2.2	1.7	1.4	1.1
Ecotoxicity					
C. affinis	maximum	0.5	3.4	0.7	1.7
P. caudatum	maximum	1.4	2.6	1.3	1.0
Overall score					
All parameters		−4.7	0.0 *	−3.8	−1.3
Score for wheat		0.5	4.5 *	0.0	2.0
Score for radish		−3.3	1.5 *	−1.8	−0.6

* The variant with the highest total score when compared separately for radish and wheat is highlighted in green.

4. Conclusions

This study presents the first assessment of the effect of pillar[5]arenes on the herbicidal activity of 2,4-dichlorophenoxyacetic acid (**2,4-D**) and glyphosate (**Glyp**) on *C. vulgaris*, *T. aestivum*, and *R. sativus*, as well as an evaluation of the ecotoxicity of the resulting pillar[5]arene/herbicide systems toward *C. affinis* and *P. caudatum*. The interaction of pillar[5]arenes with **2,4-D** and **Glyp** was investigated using fluorescence and NMR spectroscopy, DLS, and TEM. The logarithms of the association constants for the macrocycle/herbicide systems were found to range from 2.66 to 4.06, and the stoichiometry was determined to be 1:1. The higher association constants indicated that **Glyp** binding was more efficient. NMR spectroscopy showed the absence of classical inclusion complexes between the herbicide molecules and the pillar[5]arenes. Decamethoxypillar[5]arene formed polydisperse colloidal systems, whereas monohydroxypillar[5]arene formed stable self-associates (d = 181 ± 2, PDI = 0.08 ± 0.03). This behavior was probably due to the presence of a hydroxyl group in its structure, which provided additional stabilization of the self-assembled nanoparticles through hydrogen bonding. The studied pillar[5]arenes were shown to form stable associates with **2,4-D** and **Glyp**, with mean particle diameters ranging from 143 to 177 nm and polydispersity indices of 0.11–0.20. The systems based on monohydroxypillar[5]arene and the herbicides demonstrated high sedimentation stability over three days. An evaluation of the herbicidal activity revealed that the pillar[5]arenes themselves exhibited low phytotoxicity toward the microalga *C. vulgaris*. The germination index for wheat was determined to be 13–14% for both macrocycles, while the corresponding values for radish were higher, ranging from 22 to 39%. A synergistic effect was observed for the pillar[5]arene/**2,4-D** systems, resulting in increased phytotoxicity against *C. vulgaris* compared to the herbicide alone. A similar pattern was also demonstrated for the system

formed by monohydroxypillar[5]arene with **Glyp** toward the microalga. The germination index for wheat decreased 4.5-fold with the addition of pillar[5]arenes to **2,4-D**. However, the formation of associates with **Glyp** had virtually no effect on the germination index. In the case of radish, no effect of the macrocycles on the phytotoxicity of **2,4-D** was observed, whereas the addition of decamethoxypillar[5]arene to **Glyp** led to an approximately twofold increase in the germination index compared to the herbicide alone. The formation of associates between decamethoxypillar[5]arene and **Glyp** was found to increase the LC_{10} value by more than twofold compared to the individual herbicide against *P. caudatum* and *C. affinis*. This increase was likely due to the higher association constants of the macrocycle with **Glyp**. Thus, the association of pillar[5]arenes with **Glyp** made it possible to reduce the ecotoxicity of the herbicide while partially preserving its phytotoxic effects toward wheat under the applied laboratory screening conditions. This study is an important step toward evaluating how pillar[5]arenes affect the herbicidal properties and ecotoxicity of herbicides. Furthermore, the presented findings open up prospects for the development of environmentally safer formulations of plant protection products based on pillar[5]arenes.

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Abbreviations

The following abbreviations are used in this manuscript:

Glyp	Glyphosate
2,4-D	2,4-dichlorophenoxyacetic acid
DMSO	Dimethyl sulfoxide
NMR	Nuclear magnetic resonance
TEM	Transmission electron microscopy
PDI	Polydispersity index
DLS	Dynamic light scattering
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GI	Germination index
LC_{10}	The concentration of a pesticide that causes the death of 10% of the test organism population within a specified exposure time

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