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The Effects of Commercial Pesticide Formulations on the Function of In Vitro and In Vivo Assay Systems: A Comparative Analysis

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Abstract: Pesticides are commonly used in agriculture and are an important factor of food security for humankind. However, the overuse of pesticides can harm non-target organisms, and, thus, it is vital to comprehensively study their effects on the different metabolic pathways of living organisms. In the present study, enzyme-inhibition-based assays have been used to investigate the effects of commercial pesticide formulations on the key enzymes of the organisms, which catalyze a wide variety of metabolic reactions (protein catabolism, lactic acid fermentation, alcohol metabolism, the conduction of nerve impulses, etc.). Assay conditions have been optimized, and the limitations of the methods used in the study, which are related to the choice of the solvent for commercial pesticide formulations and optical effects occurring when commercial pesticide formulations are mixed with solutions of enzymes and substrates of assay systems, have been revealed. The effects of commercial pesticide formulations on simple chemoenzymatic assay systems (single-enzyme reactions) have been compared to their effects on complex multicomponent molecular systems (multi-enzyme reactions) and organisms (luminescent bacterium). The in vitro assay systems have shown higher sensitivity to pesticide exposure than the in vivo assay system. The sensitivity of the in vitro assay systems increases with the elongation of the chain of conjugated chemoenzymatic reactions. The effects exerted by commercial pesticide formulations with the same active ingredient but produced by different manufacturers on assay system functions have been found to differ from each other.

Keywords: enzyme inhibition-based assay; pesticides; bioluminescent assay; luminous bacteria; chemoenzymatic reactions

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1. Introduction

Currently used pesticides (CUPs) are chemicals that are widely used for controlling pests and vector-borne diseases; thus, they occur abundantly in the environment [1,2]. Pesticides are prepared as a variety of formulations and have diverse modes of action. Modern pesticide formulations do not have PBT (persistent, bioaccumulative, and toxic) properties, but some of them are non-specific and may cause damage to non-target species, including people and wildlife [3,4]. Pesticides, like any chemicals, can interact with each other and, thus, alter each other's resultant toxicity; therefore, their effects are not always predictable [5].

Practically every effect of a toxicant on the function of a living organism begins on a molecular level. Therefore, enzymes of bioindicators are often used as biomarkers of environmental pollution by various toxic compounds, including pesticides [6–8]. Pesticides are capable of inhibiting the activity of non-target enzymes such as cholinesterase enzymes (ChE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) [9–11]. The unwanted effects of pesticides on a molecular level are also exhibited as changes in the proportions of enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and a number of other biomarkers of oxidative stress [9,12]. The degree of change in the enzyme activity under the impact of toxicants in the organism is determined by the mode of action, duration of the contact, and resistance of a bioindicator species to different external toxic impacts [13,14]. Because of the quick response of enzymes as biochemical biomarkers to the presence of low concentrations of pollutants, they are regarded as useful tools of great toxicological relevance [9]. It is important that enzymes differ in their sensitivity to toxicants and show certain selectivity towards them.

These advantages of using enzymes in situ as pollution biomarkers served as prerequisites for creating in vitro enzyme-based assays. Under controlled laboratory conditions, such enzyme inhibition-based assays are used to detect toxicants in the tested medium [15–19]. This strategy is based on the specific inhibition of the enzymes by toxic substances, which is proportional to the amounts of toxicants in the sample, and it has been successfully used to develop procedures for water, air, soil, and food monitoring [20–23]. The in vitro enzyme-based assays have a great number of advantages, including the weaker effect of external factors on the experimental procedure and the higher sensitivity of the assays; the assays are rapid and simple to perform, and their results are reproducible; and their biological relevance is retained as well [14].

The purpose of the present study was to assess and compare the effects of commercial pesticide formulations on the in vitro and in vivo assay systems. Tests were performed with chemoenzymatic systems of varying complexity, including (1) single-enzyme reactions catalyzed by alcohol dehydrogenase (ADH), NAD(P)H:FMN-oxidoreductase (Red), lactate dehydrogenase (LDH), butyrylcholinesterase (BChE), alkaline phosphatase (ALP), and trypsin; and (2) multi-enzyme reactions catalyzed by the coupled system of luminescent bacteria NAD(P)H:FMN-oxidoreductase + luciferase (Red + Luc) and the three-enzyme systems of alcohol dehydrogenase + NADH:FMN-oxidoreductase + luciferase (ADH + Red + Luc) and lactate dehydrogenase + NADH:FMN-oxidoreductase + luciferase (LDH + Red + Luc). The effects of pesticides on the function of chemoenzymatic systems of varying complexity and the bioluminescence of the *Photobacterium phosphoreum* (*P. phosphoreum*) bacterial cell culture were studied and compared. Enzymes that were the most sensitive to the impacts of different classes of pesticides were identified; the effects of the pesticide active ingredients were distinguished from the effects of the inactive substances (formulants); and the potential specificity of the assay systems to pesticides was revealed. Commercial pesticides of different chemical classes (organophosphorus, pyrethroid, and neonicotinoid compounds) and having different target organisms (herbicides and insecticides) were tested in the current study.

2. Materials and Methods

2.1. Reagents and Pesticides

The following reagents were used: FMN (Serva, Heidelberg, Germany), NADH (Gerbu Biotechnik, Heidelberg, Germany), tetradecanal (Merck, Darmstadt, Germany), NAD (AppliChem, Darmstadt, Germany), Nα-Benzoyl-L-arginine ethyl ester (BAEE) (Sigma-Aldrich, St. Louis, MO, USA), 4-nitrophenyl phosphate disodium salt hexahydrate (Merck, Gillingham, Dorset, UK), HCl (SigmaTek, Khimki, Russia), S-BCh-I (Merck, Schaffhausen, Switzerland), 5.5′-Dithiobis(2-nitrobenzoic acid) (Sigma-Aldrich, Taufkirchen, Germany), pyruvate (Sigma-Aldrich, Tokyo, Japan), MgCl2 (Sigma-Aldrich, Petaling Jaya, Malaysia),

potassium-phosphate buffer pH 6.8–8.0, sodium-phosphate buffer pH 7.4, Clark and Lubs buffer pH 7.6, glycine NaOH pH 9.6, and 95% ethanol.

The study was performed using lyophilized LDH from rabbit muscle, 600 U/mg (Sigma-Aldrich, St. Louis, MO, USA); lyophilized ADH from baker's yeast, 300 U/mg (Sigma-Aldrich, St. Louis, MO, USA); lyophilized trypsin from porcine pancreas, 1300 BAEE U/mg (Sigma-Aldrich, St. Louis, MO, USA); lyophilized ALP from bovine intestinal mucosa 10 DEA U/mg (Merck, Gillingham, Dorset, UK); lyophilized BChE from equine serum, 900 U/mg (Sigma-Aldrich, St. Louis, MO, USA); Red from *Vibrio fischeri*, 0.15 U/mL (Institute of Biophysics, Siberian Branch of the Russian Academy of Sciences, Russia); and a lyophilized mixture of high-purity enzymes: 0.5 mg of Luc from recombinant *Escherichia coli* strain and 0.15 U of Red from *Vibrio fischeri* (Institute of Biophysics, Siberian Branch of the Russian Academy of Sciences, Russian Federation).

Bacterium *P. phosphoreum* 1889 was provided by the museum at the IBP SB RAS [24]. *P. phosphoreum* cells were grown for 24 h on solid medium for marine bacteria. The cells were suspended in the sodium-phosphate buffer pH 7.4.

Fifteen commercial pesticide formulations purchased in retail stores of Krasnoyarsk were used in analysis. The classification, properties, and manufacturers of these formulations are listed in Table 1. Pesticide solutions were prepared using distilled water, ethanol (95%), or acetonitrile 99.9% (PanReac AppliChem, Barcelona, Spain) as solvents.

Table 1. Classification of commercial pesticide formulations in accordance with the active ingredient and manufacturer.

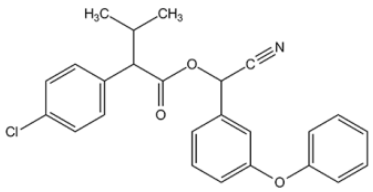
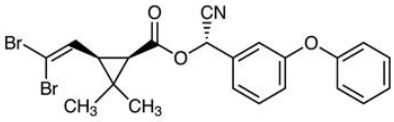
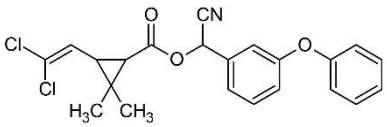
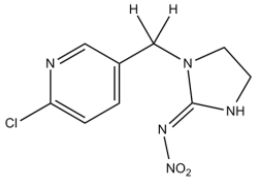
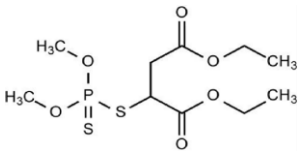
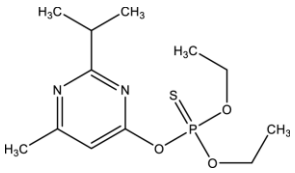
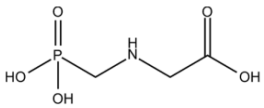
Pesticide Group	Active Ingredient	Commercial Pesticide Formulation	Form	Structural Formula	Manufacturer
Pyrethroids	Fenvalerate	Sempay	EC *		JSC "Avgust", Russia
		Fenaksin	Dust		"Agrovit" LLC, Russia
	Deltamethrin	Delcid	EC *		NVC "AgroVetZashita" LLC, Russia
	Cypermethrin	Inta-vir	ST **		"Garden Retail Service" LLC, Russia
		Briz	EC *		"Spetsbioservice" LLC, Russia
Neonicotinoids	Imidacloprid	Biotlin	WSC ***		JSC "Avgust", Russia
		Corado			ZPF Agrorus-Ryazan LLC, Russia
		Confidor Extra	WG ****		Bayer Garden, Germany

Table 1. *Cont.*

Pesticide Group	Active Ingredient	Commercial Pesticide Formulation	Form	Structural Formula	Manufacturer
OPs	Malathion	Aliot	EC *		JSC "Avgust", Russia
		Fufanon-Nova	Emulsion, oil in water		Firm "Gardener's Green Pharmacy" LLC, Russia
	Diazinon	Muravyed	EC *		JSC "Avgust", Russia
		Pochin	Granule		Firm "Gardener's Green Pharmacy" LLC, Russia
		Muravin	Bait (ready for use)		CJSC «TPK Technoexport», Russia
	Glyphosate	Liquidator	Water solution		CJSC «TPK Technoexport», Russia
		Tornado Extra			JSC "Avgust", Russia

Ops—organophosphorus pesticides. EC *—emulsifiable concentrate, ST **—water-soluble tablet, WSC ***—water-soluble concentrate, and WG ****—water-dispersible granule (according to classification in [25]).

Bioluminescence was measured using a Lumat LB 9507 bioluminometer (Berthold Technologies, Bad Wildbad, Germany) and a GloMax 20/20 luminometer (Promega Corporation, Madison, WI, USA). A Shimadzu UV-2600 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used to estimate the activities of single-enzyme reactions and to investigate the spectral properties of commercial pesticide formulations.

2.2. Effects of Commercial Pesticide Formulations on the Activities of Single-Enzyme Systems

Changes in enzyme activities in the presence and absence of analytes were measured using optical methods.

The activity of enzyme systems was determined from changes in the rate of conversion of specific substrates of the enzyme reaction, by measuring the absorbance of reaction mixture solutions or bioluminescence intensity in the control solution or in the solutions of the analyzed pesticide formulations.

The activities of single-enzyme reactions catalyzed by ADH, Red, and LDH were determined by changes in the solution absorbance at 340 nm.

The reaction mixture for the ADH-based enzyme reaction consisted of 0.75 U of ADH, 1495 µL of the potassium-phosphate buffer 0.05 M pH 7.85, 25 µL of 95% ethanol, and 40 µL of the 2.4 mM NAD solution. Control solutions were 500 µL of distilled water and 5 µL of acetonitrile.

The reaction mixture for the Red-based reaction comprised 6 mU of Red, 750 µL of the 0.05 M potassium-phosphate buffer pH 7.25, 10 µL of the 0.5 mM FMN solution, 100 µL of the 0.4 mM NADH solution, and 5–100 µL of the solvent (control) or the analyte solution.

The activity of the LDH-based enzyme reaction was estimated by using the reaction mixture of the following composition: 9 U of LDH, 850 µL of the 0.05 M potassium-phosphate buffer pH 8.0, 30 µL of the 69 mM pyruvate solution, 40 µL of the 3.25 mM NADH solution, and 50 µL of the solvent (control) or the analyte solution.

Changes in the activity of the single-enzyme reaction catalyzed by trypsin were estimated from changes in the solution absorbance at 253 nm. The reaction mixture contained 11.1 mU of trypsin, 490 µL of the 0.1 M Clark and Lubs buffer pH 7.6, 460 µL of the 0.5 mM BAEE solution, and 40 µL of 1 mM hydrochloric acid.

The activity of the single-enzyme reaction based on ALP was estimated in the reaction mixture of the following composition: 1.2 mU of the ALP solution, 988 µL of the glycine NaOH buffer pH 9.6 containing 0.5 mM $MgCl_2$, 30–50 µL of the analyte, and 8 µL of the 33 mM n-nitrophenyl phosphate solution. The absorbance of the solutions was measured at the 405-nm wavelength.

BChE activity was analyzed using Ellman's method [26]. The reaction mixture consisted of 70 mU of BChE, 800–850 µL of the 0.05 M potassium-phosphate buffer pH 8.0, 60 µL of 0.2 mM S-BCh-I, 60 µL of 0.2 mM 5,5′-dithiobis (2-nitrobenzoic acid), and 50–100 µL of the solvent (control) or the analyte solution. The absorbance of the solutions was measured at the 412-nm wavelength.

The effects of the solvents and commercial pesticide formulations on the activity of the enzymes in the single-enzyme reactions were estimated as the relative activity using the formula $A = (A_{treat} / A_{contr}) \cdot 100\%$, where A_{treat} and A_{contr} are the enzyme activity in the presence of the analyte and in the control solution, respectively.

2.3. The Effects of Commercial Pesticide Formulations on the Activity of Multi-Enzyme Systems

The activities of the Red + Luc coupled-enzyme system and the ADH + Red + Luc and LDH + Red + Luc three-enzyme systems were estimated from the change in the luminescence intensity in the presence of the analyzed sample relative to the control.

The reaction mixture for analyzing the luminescence intensity of the Red + Luc coupled-enzyme system contained 290 µL of the 0.05 M potassium-phosphate buffer pH 6.8; 50 µL of the 0.0025% tetradecanal solution; 5 µL of the Red + Luc mixture, preliminarily diluted in 5 mL of the buffer solution; 100 µL of the 0.4 mM NADH solution; 50 µL of the 0.5 mM FMN solution; and 10–200 µL of the solvent (control) or the analyte solution.

The reaction mixture for analyzing the luminescence intensity of the ADH + Red + Luc three-enzyme system contained 350 µL of the 0.05 M potassium-phosphate buffer pH 6.9; 5 µL of 0.5 mg/mL ADH; 5 µL of the Red + Luc solution, preliminarily diluted in 5 mL of the buffer solution; 50 µL of the 0.0025% tetradecanal solution; 100 µL of the 0.4 mM NAD solution; 10 µL of the 0.5 mM FMN solution; 5 µL of 95% ethanol; and 10–100 µL of the solvent (control) or the analyte solution.

An analysis of the activity of the LDH + Red + Luc three-enzyme system was performed using the reaction mixture consisting of 300 µL of the 0.05 M potassium-phosphate buffer pH 7.1; 5 µL of 0.5 mg/mL LDH; 10 µL of the Red + Luc solution, preliminarily diluted in 5 mL of the buffer solution; 10 µL of the 15 mM lactate solution; 100 µL of the 0.5 mM NAD solution; 50 µL of the 0.0025% tetradecanal solution; 10 µL of the 0.5 mM FMN solution; and 10–50 µL of the solvent (control) or the analyte solution.

The strength of the effect of the solvents and commercial pesticide formulations on the multi-enzyme reactions was determined from the residual luminescence intensity calculated using the formula $I = (I_{treat} / I_{contr})\ 100\%$, where I_{treat} and I_{contr} are the average values of luminescence intensity in the presence of the analyzed and control samples, respectively.

The inhibitory effect of a commercial pesticide formulation on the enzyme activity and the bioluminescence intensity of a multi-enzyme system was quantified using the parameter IC_{50}, which is the concentration of the active ingredient decreased the activity of the enzyme system by 50%.

2.4. The Effects of Commercial Pesticide Formulations on Bioluminescence of the Assay System Based on P. Phosphoreum Luminescent Bacterium

The effects of commercial pesticide formulations on the luminescent bacterium were estimated by changes in the bacterial luminescence intensity in the presence of the analyte relative to the control value. The control value was the intensity of the luminescence of bacterial cells in the presence of the solvent. Bacterial suspension (450 µL) and 5 µL of ethanol or 50 µL of distilled water, used as solvents for commercial pesticide formulations, were placed into a luminometer cuvette, and luminescence intensity (I_{contr}) was measured after 1 min. Another 450-µL sample of bacterial suspension and 5 or 50 µL of the solution

of a commercial pesticide formulation (depending on the solvent used) were placed into a luminometer cuvette, and luminescence intensity (I_{treat}) was measured after 1 min. The residual luminescence intensity was calculated using the formula $I = (I_{treat} / I_{contr}) \cdot 100\%$.

The effect of a commercial pesticide formulation on the *P. phosphoreum*-based assay system was estimated using parameter EC_{50}, and the concentration of the active ingredient decreased the intensity of bioluminescence of the bacterium by 50%.

2.5. Statistical Analysis

The results were statistically processed using the EXCEL software package (Microsoft, Redmond, Washington, DC, USA). Each data point was the result of at least five measurements. The means and standard deviations were calculated for the maximum luminescence intensity (I_{treat}, I_{contr}) and enzyme activity (A_{treat} and A_{contr}).

3. Results

3.1. Physico-Chemical Characterization of Commercial Pesticide Formulations

Enzyme system research requires a considerable amount of additional work. During the initial phase, it is important to choose the solvent that will be capable of producing a homogenous solution of the tested toxicant without exerting any significant effects on the enzyme assay systems. The solvents used in the present study were distilled water, ethanol, and acetonitrile. Acetonitrile and ethanol are the best solvents for most of the pesticides (Table 2). Of the 15 commercial formulations analyzed in the present study, only three (Confidor Extra, Liquidator, and Tornado Extra) were soluble in water. None of the solvents used was able to produce homogenous solutions of such commercial formulations as Fenaksin, Inta-vir, Fufanon-Nova, Pochin, and Muravin; these formulations formed suspended particles, which later settled to form sediment or emulsions. The representatives of pyrethroids (Sempay, Delcid, and Briz) and neonicotinoids (except Confidor Extra) dissolved equally well in ethanol and acetonitrile. Ethanol was the best solvent for most of the OPs (Aliot, Muravyed, and Tornado Extra).

Table 2. The solubility of the commercial pesticide formulations used in the study in water, 95% ethanol, and acetonitrile.

Commercial Pesticide Formulation	Active Ingredient	Distilled Water	Alcohol	Acetonitrile
Sempay	Fenvalerate	−	+	+
Fenaksin		−	−	−
Delcid	Deltamethrin	−	+	+
Inta-vir	Cypermethrin	−	−	−
Briz		−	+	+
Biotlin	Imidacloprid	−	+	+
Corado		−	+	+
Confidor Extra		+	−	−
Aliot	Malathion	−	+	−
Fufanon-Nova		−	−	−
Muravyed	Diazinon	−	+	+
Pochin		−	−	−
Muravin		−	−	−
Liquidator	Glyphosate	+	−	−
Tornado Extra		+	+	−

"+" The commercial pesticide formulation was soluble in the used solvent. "−" The commercial pesticide formulation was insoluble in the used solvent.

Then, we investigated the effects of the solvents on the activity of enzyme assay systems and the intensity of bioluminescence of the luminescent bacterium. For most of the enzyme assay systems, the maximal amount of distilled water that did not result in the pronounced inhibition of the enzymatic reaction was 10–15% of the total reaction mixture volume. For the ADH-based single-enzyme system and for the Red + Luc coupled-enzyme

system, the acceptable amount of water could reach 25% of the total reaction mixture volume. Ethanol and acetonitrile considerably inhibited the enzymatic reactions. For most of the enzyme assay systems, the maximal amount of ethanol in the reaction mixture was determined at 1–2%; higher ethanol concentrations, but not exceeding 5%, could be used for LDH, ALP, and BChE. Similar results were obtained for acetonitrile: because of the strong inhibitory effect of this solvent on enzymatic reactions, its concentration in the reaction mixture must be no more than 5%.

In the in vivo study, ethanol and distilled water were used as solvents of pesticide formulations; the largest amounts of solvents contained in the reaction mixture, which insignificantly inhibited *P. phosphoreum* luminescence, were 1 and 10%, respectively. Acetonitrile produced a considerable inhibitory effect on bacterial bioluminescence, even at a low concentration in the solution (below 1%).

Then, we analyzed the spectral characteristics of the commercial pesticide formulations. That stage was needed for the accurate interpretation of results, as activities of the enzyme assay systems were determined using optical methods. The absorbance peaks of most of the pesticide solutions were in the short wavelength range, about 200–300 nm (Figure 1).

For most of the assay systems, changes in the absorbance of the solutions are recorded in the longer wavelength region (340 nm for ADH, LDH, and Red; 405 and 412 nm for ALP and BChE, respectively). Thus, the study did not show any significant effect of the optical characteristics of the commercial pesticide formulations on the parameters of the analyzed enzyme assay systems except for the trypsin-based assay system.

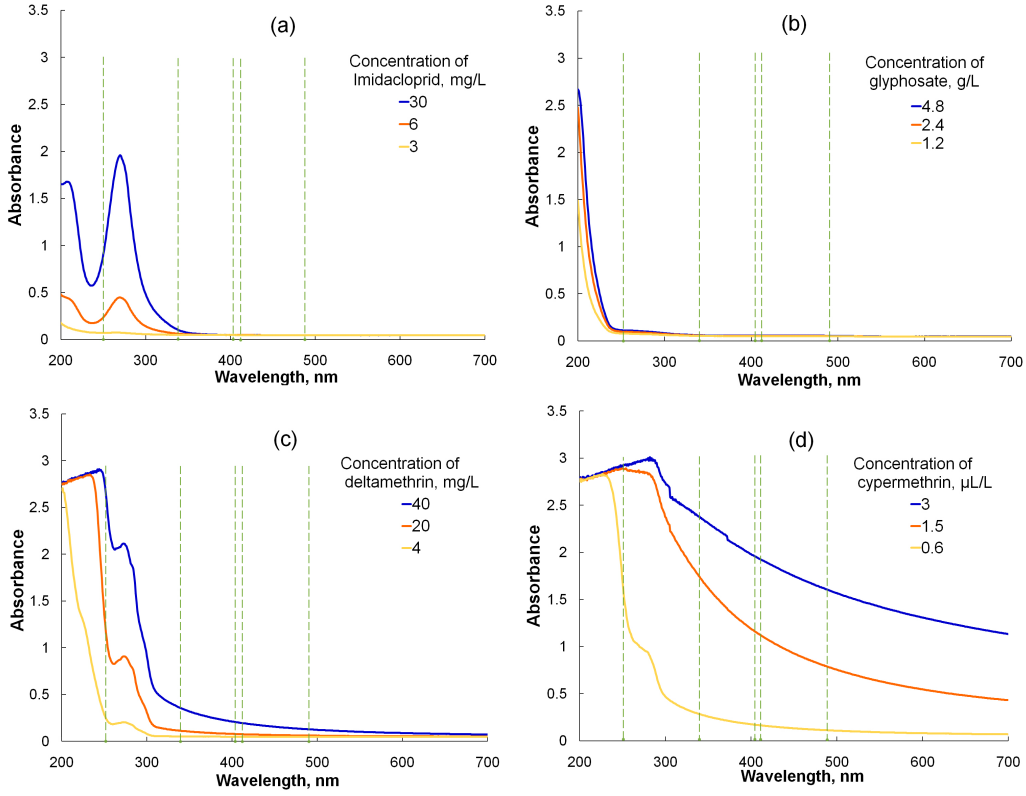


Figure 1. Absorption spectra of commercial pesticide formulations as dependent on concentrations of their active ingredients: (**a**) Confidor Extra water solution (active ingredient—imidacloprid), (**b**) Liquidator water solution (active ingredient—glyphosate), (**c**) Delcid ethanol solution (active ingredient—deltamethrin), and (**d**) Briz acetonitrile solution (active ingredient—cypermethrin). Vertical dotted lines indicate the absorbance of the pesticide solutions at the wavelengths used in the chemoenzymatic analysis.

If the addition of the pesticide solution to the reaction mixtures of the enzyme assay systems resulted in the interaction of the components followed by the production of suspended particles; turbidity; or color change in the solution, the procedure of sample preparation was adjusted. For example, the pesticide solution was additionally diluted with distilled water or sequentially diluted with acetonitrile/ethanol and water, thus minimizing the unwanted optical effects for a number of formulations (Figure 1a–c). However, changes in the sample preparation did not always result in an improvement. For instance, the rate of enzymatic reaction catalyzed by trypsin is measured at a 253-nm wavelength, where the Briz insecticide has the peak absorbance (Figure 1d). Because of the limitations of the optical methods, we were unable to estimate the effects of such pesticides on enzyme activity.

3.2. Estimating Sensitivity of Assay Systems to Commercial Pesticide Formulations

3.2.1. The Effect of Pesticide Formulations on the Activity of Single-Enzyme Assay Systems

Single-enzyme reactions are simple assay systems: their response indicates the presence of the inhibitors to which the enzymes are specific. Enzyme inhibition-based assay was used to assess the effects of commercial pesticide formulations on the single-enzyme reactions catalyzed by trypsin, LDH, ADH, BChE, ALP, and Red. The concentration dependencies of the assay system activities were obtained using three groups of CUPs (organophosphorus, pyrethroid, and neonicotinoid compounds); IC_{50} values were determined.

The enzyme systems showed selective sensitivity towards the commercial pesticide formulations of the groups of CUPs studied in this work. The Biotlin and Confidor Extra neonicotinoids and the Delcid pyrethroid had the strongest inhibitory effects, but the sensitivity of the assay systems to these formulations differed by several orders of magnitude. For example, the 50% decrease in the activities of LDH and Red was observed in the presence of 1 and 14.9 mg/L of the Confidor formulation, whereas for BChE, that parameter was higher by four orders of magnitude: IC_{50} was 80 g/L (Table 3).

All the assay systems exhibited sensitivity to glyphosate, the active ingredient of the Tornado Extra and Liquidator commercial formulations. The strongest effect was achieved with the ADH-based assay system: IC_{50} values were 2.1 mg/L and 1.5 mg/L, respectively. Figure 2 demonstrates differences in the effects of the Tornado Extra commercial formulation on the activities of single-enzyme assay systems. The IC_{50} values for hydrolase BChE and oxidoreductase Red were 2.4 and 5.0 mg/L, while for the two other hydrolases—ALP and trypsin—IC_{50} values were 100 and 1000 times higher, respectively.

Of the three dehydrogenases (ADH, LDH, and Red) used in this study as assay systems, the LDH-based assay system showed high sensitivity to most pesticides: the exposure of the LDH-based assay system to five pesticides out of ten resulted in IC_{50} values that were lower than for the two other dehydrogenases. At the same time, exposure to cypermethrin (Briz), imidacloprid (Corado), and glyphosate (Liquidator) resulted in lower IC_{50} values for the ADH-based assay system.

Table 3. IC_{50} (mg/L) and EC_{50} (mg/L) values determined from the effects of commercial pesticide formulations on in vivo and in vitro assay systems.

Active Ingredient	Commercial Pesticide Formulation	In Vitro Assay Systems									In Vivo Assay System	
		Single-Enzyme Assay Systems					Multi-Enzyme Assay Systems				P. Phos-phoreum	MRL RUS mg/kg
		Trypsin	ALP	BChE	LDH	ADH	Red	Red + Luc	ADH + Red + Luc	LDH + Red + Luc		
Fenvalerate	Sempay	*	*	–	0.2	*	*	0.0014	0.0006	0.0007	*	0.02–0.1
Deltamethrin	Delcid	–	–	0.76	6.2	16.7	146	39.5	12.7	11.5	*	0.01–0.3
Cypermethrin	Briz	*	–	30930	150	100	300	5	3	1	*	0.01–2.0
Imidacloprid	Biotlin	*	*	200	–	0.17	0.09	0.003	0.006	0.01	2000	0.1–1.0
	Corado	*	*	–	180	0.08	–	0.07	0.04	0.04	500	
	Confidor Extra	–	*	80,000	1	49.9	14.9	34.4	47.8	1.9	110	
Malathion	Aliot	*	*	4	30	*	*	0.1	0.05	0.014	*	0.05–1.0
Diazinon	Muravyed	*	*	20	0.05	0.2	*	0.009	0.01	0.005	*	0.1–0.5
Glyphosate	Liquidator	5400	600	1000	6000	1.5	9.0	1.11	1.4	1.1	400	0.1–5.0
	Tornado Extra	2400	220	2.4	52	2.1	5.0	1.8	2.0	3.3	400	

"*" The parameter could not be determined because of physico-chemical properties of the formulation or interaction of the formulation with the reaction mixture components. "–" No inhibitory effect of the formulation was detected in the tested concentration range.

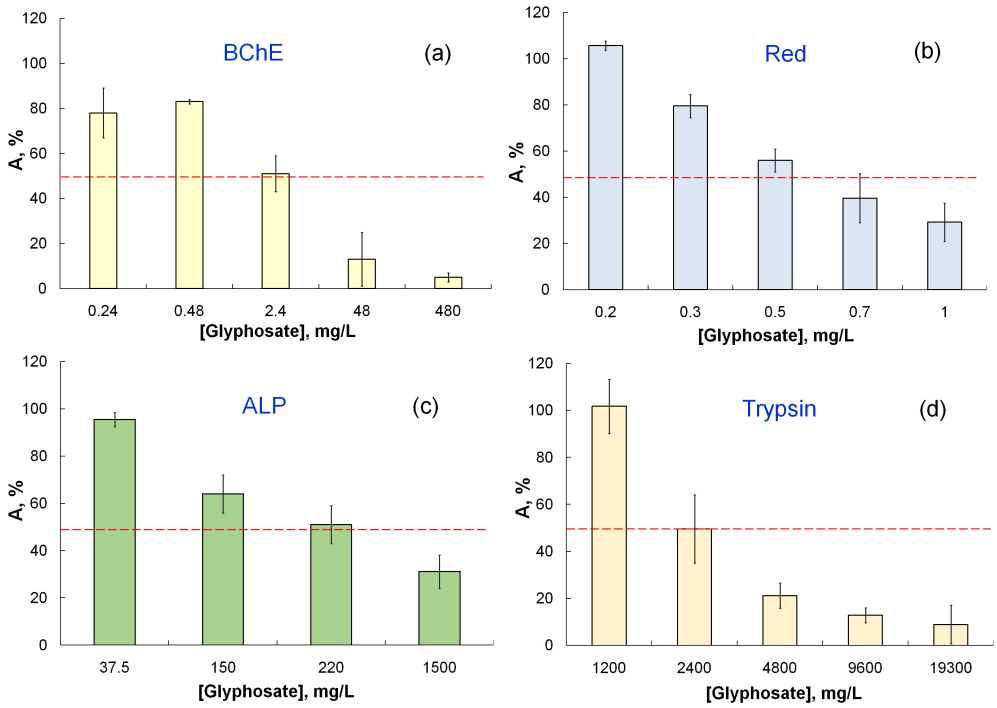


Figure 2. The effect of the Tornado Extra commercial pesticide formulation (with glyphosate as the active ingredient) on single-enzyme assay systems: (**a**) BChE, (**b**) Red, (**c**) ALP, and (**d**) trypsin.

The analysis of the results showed the certain specificity of the enzyme systems to pesticide formulations. For example, ADH was effectively inhibited by neonicotinoids: for imidacloprid, IC_{50} values below MAC were recorded in treatments with two of the three commercial formulations containing this pesticide (Table 3). LDH activity was chiefly inhibited by pyrethroids. The assay systems with trypsin and ALP were inhibited by high concentrations of the Ops—glyphosate-based herbicides Liquidator and Tornado Extra. The effects of other pesticides were not determined because of the limitations of the optical methods used in this study, which allow for the presence of only low concentrations of pesticides.

A comparison of the effects of commercial pesticide formulations containing the same active ingredient but produced by different manufacturers showed differences in the strength of the inhibition of the assay systems by these formulations. The imidacloprid neonicotinoid had a strong inhibitory effect on the activity of BChE, but the IC_{50} value obtained for imidacloprid in the Biotlin formulation was 400 times lower than for the Confidor Extra formulation (Table 3). As the active ingredient of Corado, imidacloprid did not exert any detectable effect in the concentration range between 0.38 and 38 g/L (Figure 3). Similar results were obtained for the organophosphorus pesticide glyphosate. The IC_{50} values for glyphosate as the active ingredient of Tornado Extra and Liquidator, inhibiting the BChE activity, differed by a factor of 417 (Table 3).

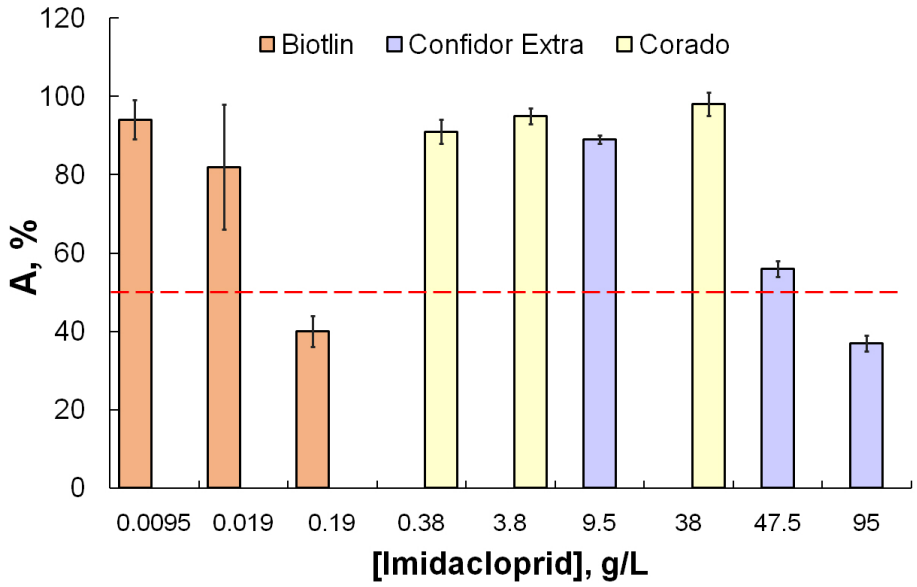


Figure 3. BChE activity vs. concentration of imidacloprid as the active ingredient of the commercial pesticide formulations Biotlin, Corado, and Confidor Extra.

3.2.2. The Effect of Pesticide Formulations on Multi-Enzyme Systems

Multi-enzyme reactions are models of metabolic pathways in the organism, and they potentially more fully reflect the biological effects of xenobiotics. Therefore, our next step was to investigate the toxic effect of the commercial pesticide formulations on the complex molecular systems (multi-enzyme reactions) and compare it with the pesticide effect on the simple assay systems (single-enzyme reactions).

We obtained concentration dependencies of pesticide effects on the coupled and three-enzyme reactions: Red + Luc, ADH + Red + Luc, and LDH + Red + Luc. All assay systems showed high sensitivity to a number of commercial pesticide formulations. The coupled Red + Luc enzyme system was sensitive to such formulations as Sempay, Biotlin, Corado, Aliot, Muravyed, Tornado, and Liquidator, at concentrations equal to or below MRLs of their active ingredients in food (Table 3). The three-enzyme LDH + Red + Luc system exhibited the highest sensitivity to organophosphorus compounds—commercial formulations Aliot (malathion) and Muravyed (diazinon): the IC_{50} values for malathion and diazinon were 0.014 and 0.005 mg/L, respectively.

The sensitivity of the assay systems to toxic substances became considerably higher as the length of the chain of conjugated enzyme reactions was increased from the single-enzyme to three-enzyme reactions. For instance, the sensitivity of the three-enzyme LDH + Red + Luc and ADH + Red + Luc systems exposed to imidacloprid (the active ingredient of the Corado formulation) at a concentration of 0.05 mg/L was 20 times higher than the sensitivity of the single-enzyme LDH system (Figure 4). The IC_{50} value for diazinon (the active ingredient of Muravyed) was 0.05 mg/L for the single-enzyme LDH system and 0.005 mg/L for the three-enzyme LDH + Red + Luc system (Table 3). The three-enzyme ADH + Red + Luc system showed the highest sensitivity to the effects of most of the tested commercial formulations. The neonicotinoid pesticide imidacloprid produced the strongest inhibitory effect on this assay system: the luminescence intensity of the three-enzyme ADH + Red + Luc system was reduced by a factor of two when exposed to the Biotlin and Corado formulations at concentrations that were several orders of magnitude below the MRL of imidacloprid in food.

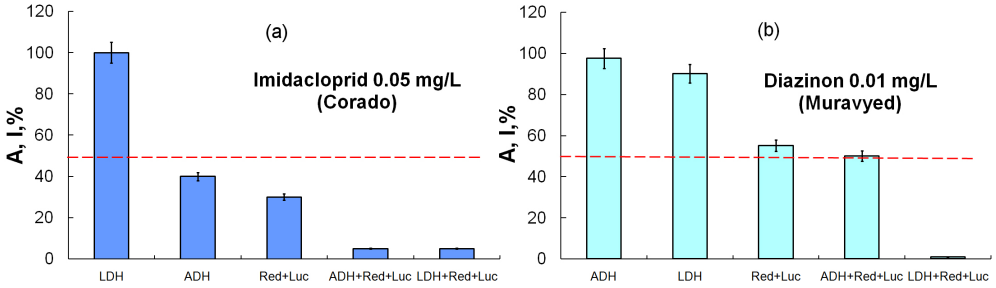


Figure 4. Comparison of the effects produced by the active ingredients of pesticide formulations on enzyme assay systems with different lengths of the enzyme conjugation chain. (**a**) 0.05 mg/L of imidacloprid in Corado; (**b**) 0.01 mg/L of diazinon in Muravyed.

The change in the sensitivity of the single-enzyme, coupled-enzyme, and three-enzyme systems was the most pronounced in the treatments with glyphosate-based herbicides. All the assay systems demonstrated sensitivity to glyphosate as the active ingredient of the Tornado and Liquidator formulations. The activity of Red was 50% inhibited in the presence of 5.0 and 9.0 mg/L of the formulations, respectively. However, of all tested enzyme systems, the three-enzyme reactions exhibited the highest sensitivity to organophosphorus compounds. For the LDH + Red + Luc-based assay system, the IC_{50} value for glyphosate (Liquidator) was 1.1 mg/L, which corresponded to the MRL of this pesticide in fruit and vegetables.

Of the pyrethroid compounds, the Sempay pesticide formulation (with fenvalerate as the active ingredient) had the most pronounced effect on the assay systems. The sensitivity to fenvalerate (Sempay) decreased as follows: ADH + Red + Luc > LDH + Red + Luc > Red + Luc > LDH. For the enzyme systems based on ADH, Red, trypsin, and ALP, the IC_{50} values for fenvalerate could not be determined because of interactions with the components of the reaction mixture. The exposure to the Delcid formulation (deltamethrin) clearly demonstrated the selective sensitivity of assay systems to toxic compounds: the IC_{50} values for deltamethrin varied between 0.76 and 146.2 mg/L, and the sensitivity of the assay systems to the inhibitory effect of the formulation increased as follows: Red < Red + Luc < ADH < ADH + Red + Luc < LDH + Red + Luc < LDH < BChE. At the same time, this commercial formulation did not produce any pronounced effect on the activity of ALP within the tested concentration range and exerted an insignificant stimulatory effect on trypsin activity.

3.3. Comparison of the Effects of Commercial Pesticide Formulations on In Vivo and In Vitro Assay Systems

The *P. phosphoreum* luminescent bacterium was used as an in vivo assay system to study the effects of commercial pesticide formulations on the intensity of its luminescence. *P. phosphoreum* culture was found to be sensitive to the effects of commercial pesticide formulations of the CUPs studied. Neonicotinoids and OPs produced the strongest effects on the assay system. For the imidacloprid neonicotinoid (Confidor Extra) and glyphosate (an organophosphorus compound (Liquidator)), the EC_{50} values were 110 mg/L and 400 mg/L, respectively (Table 3). For commercial pesticide formulations based on pyrethroids, we managed to determine only EC_{20} values—pesticide concentrations causing a 20% decrease in bacterium luminescence intensity. For deltamethrin and fenvalerate, the EC_{20} values were 400 mg/L and 500 mg/L, considerably exceeding their MRLs in food. The EC_{50} values for these pesticides could not be determined because their increased concentrations in the reaction mixture caused the pesticides to interact with the reaction mixture components.

Like in the in vitro experiments, pesticide formulations based on the same active ingredient but produced by different manufacturers had dissimilar effects on the bacterium luminescence intensity. All three neonicotinoid pesticide formulations based on

imidacloprid considerably inhibited bacterial luminescence. However, the 50% residual luminescence was observed in the presence of 110 mg/L of imidacloprid in Confidor Extra, 500 mg/L of imidacloprid in Corado, and 2000 mg/L of imidacloprid in Biotlin. No difference was observed between *P. phosphoreum* sensitivity to the glyphosate-based Tornado Extra and Liquidator formulations.

We compared the effects exerted by pesticides on the intensity of bacterial luminescence and activity of enzyme assay systems. In some treatments, commercial pesticide formulations produced similar effects on the in vitro and in vivo assay systems. For instance, the Tornado Extra commercial formulation had a strong inhibitory effect on both single-enzyme systems with ALP and BChE and on the coupled-enzyme Red + Luc and three-enzyme ADH + Red + Luc systems; that pesticide considerably inhibited bacterial luminescence as well. For some other formulations, the results differed substantially. For instance, the Corado formulation inhibited the intensity of *P. phosphoreum* bioluminescence but did not produce any inhibitory effect on BChE activity, whatever pesticide concentration was used. However, that was the exception rather than the rule.

Most pesticide formulations had stronger inhibitory effects on the in vitro assay systems than on the luminescent bacterium. That was particularly evident in treatments with bioluminescent multi-enzyme assay systems. For example, 50% inhibition of bacterial luminescence was observed in the presence of 400 mg/L of glyphosate (the active ingredient of the Liquidator commercial formulation) and 2000 mg/L of imidacloprid (Biotlin). However, for the bioluminescent Red + Luc enzyme assay system, the IC_{50} values of the same formulations were 1.11 and 0.003 mg/L. Sensitivity to the glyphosate-based Tornado Extra formulation differed by a factor of more than 200 (Figure 5). Thus, although both in vitro and in vivo assays measured the same parameter (bioluminescence intensity), the results of the two assay types did not always correlate with each other.

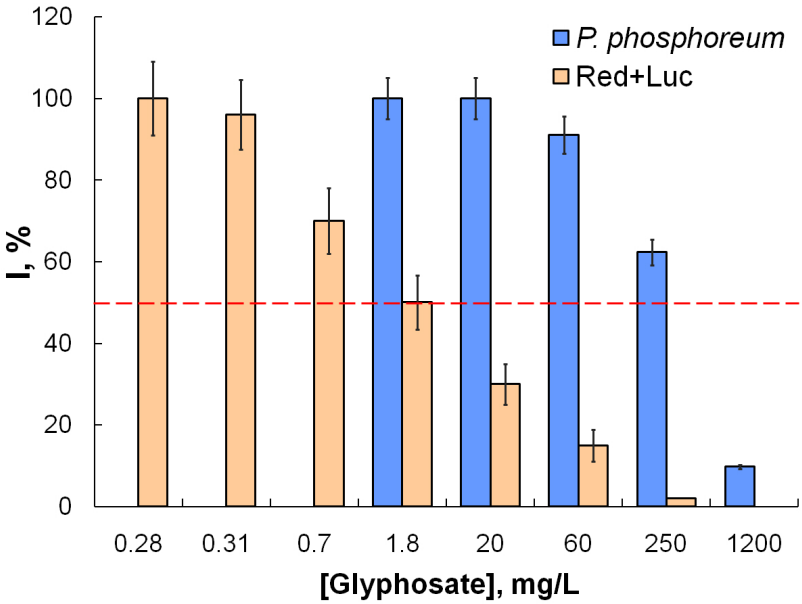


Figure 5. The residual luminescence intensity of luminous bacterium *P. phosphoreum* and the Red + Luc coupled-enzyme system exposed to commercial pesticide formulation Tornado Extra (glyphosate as the active ingredient).

4. Discussion

Pesticide residues have been proven to be able to produce cytotoxic effects on humans [27]. In addition to the disruption of the cell layer integrity, these effects include changes in the activity of enzymes; the induction or reduction in amino acid, lactate, and urea levels [28]; and adverse effects on the rates of other metabolic pathways [29,30], which, at least partly, accounts for the high toxicity of pesticides for non-target species.

Enzymes play an essential part in the activation and detoxification of xenobiotics, and biotransformation leading to the formation of metabolites that have different and, sometimes, more toxic and bioaccumulative properties than the initial pesticides [31,32]. One of the most extensively discussed indirect impacts of pesticides on enzymes is the generation of reactive oxygen species (ROS). Pesticide residues and transformation products are capable of disrupting the balance of oxidative processes in the organism, causing the accumulation of oxidation products with high reactivity. Research shows that oxidative stress is a key factor in the mode of action of imidacloprid at low doses [33]. The interaction of the generated ROS with cell macromolecules can induce DNA damage, oxidative modifications of protein and lipid molecules, and mitochondrial dysfunction and may eventually result in cell necrosis or apoptosis [34,35]. Thus, pesticide-caused oxidative stress, which stimulates ROS generation, may result in biochemical, cellular, and physiological changes in the affected organism. This interaction of consecutive molecular events with subclinical and clinical manifestations is supported by the adverse outcome pathway (AOP) framework [36].

As primary changes induced by environmentally relevant concentrations of pesticide formulations occur at the molecular level, changes in the activities of enzymes serving as biomarkers of oxidative stress such as CAT, SOD, GPx, GST, etc., are widely used to detect contamination by pesticide residues [37].

In the present study, we used enzyme inhibition-based assay to compare the effects of different pesticide classes on enzymes in assay systems of increasing complexity: from simple single-enzyme reactions to multi-enzyme reactions and organisms. Such experiments need to be thoroughly prepared, and certain complications may arise during the preliminary work. Many commercial pesticide formulations are water-insoluble and, being mixed with water, form dispersions, emulsions, or suspensions, which results in turbidity and sediment formation during the experiment. An important stage in research using ecotoxicology methods is choosing a proper solvent, which both effectively dissolves pesticide formulations and produces the minimal inhibitory effect on enzyme assay systems.

The careful choice of the solvent is not easy, although it is often given too little emphasis. All currently used pesticides consist of the active ingredient and inactive substances added to it. Formulants or co-formulants are the ingredients that are added to enhance pesticide efficiency and stability, prolong pesticide shelf life, and delay pesticide degradation in the environment [38–41]. Therefore, pesticides with the same active ingredient may be marketed in the form of granules, solutions, emulsions, etc. [25,42], which differ in the method of application, effect, and toxicity [43]. Thus, the solubility of commercial pesticide formulations is by no means always determined by the solubility of their active ingredients.

During the preliminary phase, we selected solvents (water, ethanol, and acetonitrile) to prepare solutions of commercial pesticide formulations that would be suitable for analysis by chemoenzymatic methods. In some instances, we failed to achieve the intended effect because of the interactions of solvents with components of enzyme systems. In addition, certain limitations are imposed by optical methods of the analysis of enzyme activity. For example, we did not manage to determine the inhibitory effect of malathion, as the active ingredient of the Aliot pesticide formulation, and diazinon (Muravyed) on the assay systems based on trypsin, ALP, and Red. The interactions of the pesticides with reaction mixture components resulted in the coloring of the solution and the formation of suspension, which caused a critical increase in the absorbance and prevented accurate analysis. A pyrethroid formulation (Sempay) produced similar optical effects, and the IC_{50} value was only determined for the LDH-based assay system. With some of the pesticides, these difficulties were overcome by additionally diluting the initial pesticide solution. That action, however, reduced pesticide concentration in the reaction mixture, and, hence, no inhibitory effect was detected.

The results obtained using enzyme inhibition-based assays demonstrate that different classes of enzymes, namely, hydrolases (ALP, BChE, and trypsin) and oxidoreductases (LDH, ADH, Red, and Luc), differ enormously in their sensitivity to exposure to commercial

pesticide formulations. The experiment with the selected commercial pesticide formulations showed certain specificity of assay systems towards pesticide active ingredients. For instance, pyrethroids considerably inhibited the LDH-based assay system, and the imidacloprid neonicotinoid, as the active ingredient of two commercial pesticides at concentrations corresponding to their MRLs in food, inhibited ADH. Multi-enzyme assay systems were significantly inhibited by commercial pesticides representing all three groups studied in this work: Sempay (with fenvalerate as the active ingredient), Biotlin (imidacloprid), and Muravyed (diazinon).

The current study showed that the increase in the length of enzyme conjugation chain in assay systems resulted in the more pronounced inhibitory effect of pesticides. The difference between the sensitivity of the enzymes in simple (single-enzyme) and complex (multi-enzyme) assay systems reached four orders of magnitude. The coupled Red + Luc enzyme system of the luminescent bacterium exhibited sensitivity to most of the commercial pesticide formulations at concentrations equal to and below their MRLs in food. The three-enzyme ADH + Red + Luc and LDH + Red + Luc systems were the most sensitive to pesticide exposure. Some of the enzymes remained specific to definite pesticides in both single-enzyme and multi-enzyme systems. For example, Confidor Extra considerably inhibited both the LDH-based assay system and the three-enzyme LDH + Red + Luc system. Such results could be caused both by disruption of the structural and functional properties of a single enzyme as a component of the multi-enzyme system exposed to pesticides, which led to changes in the rate of the entire reaction, and by the effect of the pesticide on interactions between enzymes in the conjugation chain. Biological systems do have rigid organization, and the inactivation of one of the metabolic pathway components may lead to unpredictable consequences and the dysfunction of the whole organism [44]. These data combined suggest that multi-enzyme systems are promising tools for monitoring pesticide residues in the environment. The use of conjugation chains of enzymatic reactions in designing biosensors increases the detection limit and specificity of biosensors [45].

An interesting finding of this study is that the change from the molecular-level assay system to the organism-level assay system does not necessarily entail an increase in the sensitivity to pesticide exposure. In the present study, we estimated the effects of pesticide formulations using an alternative analysis procedure, based on the in vivo bacterial bioluminescence assay, which had been proven effective in ecotoxicology monitoring [46–48]. As toxicants induce the disruption of cellular metabolism and, hence, a quick decrease in light emission, the in vivo method based on *P. phosphoreum* is a simple and convenient screening assay [46,49]. However, the results of the present study considered as a whole demonstrate that the sensitivity of in vitro and in vivo assay systems to exposure to commercial pesticide formulations increased as follows: the assay based on *P. phosphoreum* < single-enzyme reactions < multi-enzyme reactions. We suppose that the reason for the low sensitivity of *P. phosphoreum* to the pesticides compared to the sensitivity of the enzyme assays is that the cells have a protection system, e.g., a tough cell wall [50]. This is partly indicative of the higher efficacy of enzyme reactions as the basis for biosensors.

The prospects of enzymes as bioselective elements of various chemosensors are confirmed by comparing the obtained data on the sensitivity of enzymatic bioassays and other tests. In particular, in the study of pesticide toxicity on the early-life stages of *Pacific oyster*, the EC_{50} value for the neoncotinoid imidacloprid exceeded 200 mg/L [51]. To determine the glyphosate-based herbicide toxicity, a variety of bioassays based on microorganisms are used [52]. Thus, using a biotest based on the green microalgae *Chlorella kessleri*, the inhibitory effect of the commercial pesticide ATANOR (glyphosate as the active ingredient) was established, and EC_{50} was 55.62 mg/L [53]. An acute response test was used to establish the effect of exposure to the pyrethroid pesticide cypermentrin on earthworms, resulting in an LC_{50} (the concentration that kills 50% of a test animals) value of 86.04 mg/kg [54]. Thus, the in vitro and in vivo bioassay systems proposed in our work generally have similar,

or in some cases higher, sensitivity to the active ingredients of pesticide formulations Since bioassays on living organisms presented in the literature differ significantly in their sensitivity to pesticides and their results depend on many factors [14], the reproducibility of bioassays based on chemoenzymatic reactions can also be considered as their advantage.

This study also demonstrated differences in the effects of commercial pesticide formulations containing the same active ingredient but made by different manufacturers, on in vivo and in vitro assay systems. Manufacturers often do not disclose the data on the detailed composition of formulants, whose amounts sometimes reach 90% of the total formulation, and, thus, information on ecotoxicity of the entire product remains unavailable to consumers and researchers [38,55,56]. Formulants are classified as inert ingredients and, as a rule, are not tested for toxicity as separate components, which creates a false sense of security. Some of the formulants, however, are more toxic than the active ingredient, as confirmed by a number of convincing studies [57–59]. A study by Takács et al. [60] reported that the commercial formulation based on neonicotinoid clothianidin was 46.5 times more toxic for *Daphnia magna* than the active ingredient alone. The results demonstrating that commercial pesticide formulations were more toxic for the test organisms than active ingredients alone were also obtained in bacterial cytotoxicity bioassay, an algal photosynthesis bioassay [61], and the zebrafish embryo toxicity test [43]. A study of two test models of aquatic macrophytes showed the difference in the species sensitivity in exposures to active ingredients alone and commercial pesticide formulations [62]. A number of studies relate the more pronounced effects of pesticide formulations on non-target organisms to their effect on enzymes and cytotoxicity [63,64].

In the present study, commercial pesticide formulations similar in their mode of action, targets, and active ingredient but produced by different manufacturers were found to either inhibit assay systems significantly or exert no effect on them whatever pesticide concentration was taken. The experiment with the single-enzyme BChE system was a vivid example: the inhibitory effects of imidacloprid as the active ingredient of Biotlin and Confidor Extra differed by a factor of 400. Similarly, the sensitivity of the *P. phosphoreum* luminescent bacterium to the imidacloprid neonicotinoid decreased as follows: Confidor Extra > Corado > Biotlin. The effects differed both between the forms of the same formulation, such as WSC and WG (Biotlin and Confidor Extra), and between different formulations, such as Liquidator and Tornado Extra, having the same form—a water solution. These results provide evidence confirming that formulants, whose composition and concentration differ by manufacturer, make a considerable contribution to the toxic effect of pesticides. These inert substances may be able to increase the inhibitory effects of the active ingredients of pesticides or even function as inhibitors of enzyme activity.

5. Conclusions

The study investigated the effects of a number of commercial pesticide formulations representing different chemical classes (organophosphorus, pyrethroid, and neonicotinoid compounds) and having different targets (herbicides and insecticides) on the activity of single-enzyme, coupled-enzyme, and three-enzyme assay systems and the assay system based on the *P. phosphoreum* luminescent bacterium. The assay systems differed in their sensitivity to exposure to commercial pesticide formulations, which increased as follows: the assay system based on *P. phosphoreum* < single-enzyme reactions < multi-enzyme reactions. The assay systems based on the enzymes catalyzing a wide variety of metabolic reactions sometimes respond even to insignificant concentrations of commercial pesticide formulations and can serve as a model for estimating the potential effects of pesticides at a molecular level.

An important finding was that assay systems showed varying sensitivity to commercial pesticide formulations with the same active ingredient but produced by different manufacturers, which suggested a considerable contribution of formulants to the combined effect of the commercial pesticides on the assay systems tested in this study. Thus, studies of the effects of commercial pesticide formulations on assay systems should take into

account not only the active ingredients but also formulants, to underestimate the negative side effects.

A comparison of the sensitivities of the in vitro and in vivo assay systems to pesticide exposure suggests the considerable potential of enzyme-based assay systems as chemosensors for the toxicological assessment of commercial pesticide formulations. Moreover, their sensitivity can be enhanced by elongating the chain of conjugated enzymatic reactions. As the tested chemoenzymatic systems differ in their sensitivity to pesticide exposure, they can be integrated into a complex enzyme assay, including biomarker enzymes with different specificities to pesticides, which can be used to detect pesticides.

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