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Research article
Ερευνητικό άρθρο

Toxicological evaluation of feed contaminated with herbicides using luminescent microorganisms *Photobacterium Phosphoreum*

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ABSTRACT: *Photobacterium phosphoreum* (strain IMB B-7071; Sq3), a luminous microbe, can be used to undertake a quick toxicity assessment (up to 30 minutes) of herbicide-contaminated feed. However, when the herbicides Greenfort Horse (chisalofop-p-ethyl) and Astralid (clopyralid) were evaluated at the maximum residue limit (MRL) levels (0.04 and 2.0 mg/kg), the feed was deemed non-toxic. While for the herbicides Skat (chisalofop-p-tefuryl), Agroshield Super (glyphosate potash), Greenfort Extra (metolachlor+terbutazine) and Greenfort NK 40 (nicosulfuron) MRL (0.04; 1.0; 0.1 and 0.2 mg/kg) feed is classified as toxic for Soteira (imazamox+imazapyr), Greenfort Premium (2,4-D 2-ethylhexyl ether+florazulam) and Astanes (acetochlor) (MRL 0.05; 0.05 and 0.03 mg/kg) – classified as extremely toxic, indicating the need for further studies on the toxicological properties of the herbicide in laboratory and farm animals and possible further (downward) feed revision the maximum allowable levels of relevant pollutants.

Keyword: animal feed; bioluminescence measurements; herbicides; *Photobacterium phosphoreum*; toxicity tests.

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INTRODUCTION

Herbicides for weed management are a common feature of modern industrial agriculture, accounting for 47.5% of total pesticide use. However, this technique offers considerable environmental and health concerns in global agricultural systems (Sharma et al., 2019; Zaller, 2020; Clapp, 2021). For example, glyphosate alone accounts for 33% of the pesticides used in Europe. Glyphosate is used annually to one-third of annual crop systems and half of perennial tree crops (Antier et al., 2020).

German researchers discovered that leftover pesticides can pollute the air and have been found in insects from Germany's protected areas (Brühl et al., 2021; Kruse-Plaß et al., 2021). Additionally, herbicide residues have been detected in grass samples from public places in some areas of Northern Italy with intensive crop cultivation (Linhart et al., 2019; Linhart et al., 2021). Herbicides, including atrazine, linuron, and pendimethalin, have been found in the food that wild birds feed their young (Haroune et al., 2015). Similarly, mesosulfuron-methyl, fluroxypyr, and florasulam have been detected in the organs of gray partridge, indicating that these substances are ingested by birds through food or water (Millot et al., 2015). This poses a potential threat to semi-free-range hunting farms, which are rapidly developing (Pepko et al., 2022). Herbicides, such as glyphosate, 2-methyl-4-chlorophenoacetic acid, and metholachlor, can enter the human body through food and water (Cech et al., 2022).

Although herbicides are primarily employed to eliminate weeds, their active ingredients (AIs) can have both direct and indirect effects on non-target organisms. These consequences include deadly and sublethal effects on honey bees (Motta et al., 2018; Cullen et al., 2019; Straw et al., 2021), earthworms (Gaupp-Berghausen et al., 2015; Zaller et al., 2021), and birds (Gill et al., 2018). Furthermore, herbicides have been linked to negative impacts on human health, including the risk of cancer, embryotoxicity, and acute toxicity (Mesnage et al., 2021; Rani et al., 2021).

To address this issue, Europe has formulated the Farm-to-Fork Strategy, which aims to reduce overall pesticide use and risks by 50% by 2030 (EC COM/2020/381, 2020) and provides for the establishment of maximum permissible levels of pesticides in feed for farm animals (Cech et al., 2022).

In order to prevent feeding animals with excessive amounts of herbicides, many highly technical laboratory methods are used for their screening and monitoring in feed raw materials and finished feeds, of which the most commonly used are liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/MS) (Penagos-Tabares et al, 2023), ELISA (Zhao et al., 2018), zwitterionic hydrophilic interaction liquid chromatography (ZIC-HILIC) (Heydebreck, 2021), and others. However, they are quite expensive, difficult to perform, time-consuming, and most importantly, they do not provide an answer to the question of how dangerous (toxic) the feed with a certain content of a particular herbicide is for the animal.

The degree of feed hazard is currently determined by bioassay on models of different levels of organization: target and laboratory animals, insects, crustaceans, infusoria, bacteria, cell cultures, etc. (Jeong et al., 2005; GOST, 2014; Gerssen et al., 2019), but the most indicative model for determining feed toxicity is the bioassay on laboratory animals.

At the same time, the global scientific community is working on developing alternative toxicity tests to reduce the use of living organisms in experiments (the three Rs: Replace, Reduce, and Refine) (Gorzalczany and Rodriguez Basso, 2021). The use of photobiosensors shows great promise. Biotests using live bioluminescent bacteria are particularly noteworthy, as the intensity of their luminescence can be measured as a parameter of vital activity (Ismailov and Aleskerova, 2015; Efremenko et al., 2016; Li et al., 2020). This is especially true given our previous studies, which have established the possibility of toxicological assessment of feed contaminated with mycotoxins using luminescent microorganisms such as *Photobacterium phosphoreum* (Orobchenko et al., 2023).

Our work aimed to provide a toxicological evaluation of feeds with different levels of herbicides based on the study of their effects on the luminescence of *Photobacterium phosphoreum*.

MATERIALS AND METHODS

The place of the experiment

The research was conducted in the laboratory of toxicological monitoring of the National Scientific Center «Institute of Experimental and Clinical Veterinary Medicine» (Kharkiv, Ukraine).

Experimental herbicides and feed

Herbicides were used in the form of commercial drugs of different groups registered over the past 5

years and authorized for use in Ukraine, respectively, namely Soteira, Greenfort Premium, Greenfort Hors, Skat, Agroshield Super, Greenfort Extra, Astanes Acetochlor, Astralid, Greenfort NK 40 (Table 1).

Under the research conditions, compound feed that is non-toxic and does not contain the above-mentioned herbicides is used as the "matrix". Characteristics of the compound feed: complete compound feed PK 62 for calves aged 1-6 months, manufactured in accordance with state national standard of Ukraine (SNSU) 8530:2015 "Compound feeds for cattle. Technical conditions" with the following composition: wheat - 15%, barley - 15%, corn - 30%, protein-vitamin mineral supplement - 40%. The absence of toxicity in the feed used as "substrate" was determined according to standard methods (SNSU 3570-97). Feed grains, grain by-products, and compound feeds. Method for determining toxicity: use infusoria *Colpoda Steinii*) to determine toxicity. A gas chromatography-mass spectrometer (Shimadzu GS-17A MS-QP5050A, Japan) was used to detect the herbicide in the "matrix": the content of the active ingredient in the herbicide was lower than the quantification limit of the method. Different concentrations of toxins were added to the matrix (five concentrations were prepared by dilution with ethanol, depending on the maximum residue limit (MRL)) (Table 1).

Experiment design

Glass vials were filled with a portion of the control and experimental "matrices" weighing 10.0 g. The experimental samples were treated with the appropriate amount of herbicides and 96° ethanol in a volume of 20.0 cm^3, which was added and left to extract for 24 hours. The supernatant was then collected and added in a volume of 0.02 cm^3 to culture fluid that had been previously prepared and introduced into the luminometer cuvette in a volume of 1.0 cm^3.

We employed the lyophilized culture of *Photobacterium phosphoreum* (strain IMB B-7071; Sq3) (*Ph. Phosphoreum*) are gram-negative polymorphic asporogenic rods with a size of 0.8-1.0 × 1.2-1.4 μm. The test culture obtained from the Depository of Microorganisms of the Institute of Microbiology and Virology named after D.K. Zabolotny of the National Academy of Sciences of Ukraine (Kyiv).

The experiment involved photobacteria cultivation in tubes on a liquid and dense nutritional medium (pH 6.8-7.2) with sodium chloride added at a temperature of 27.0±1.0°C in thermostat. Cultivation of photobacteria during the experiment was carried out in a thermostat under aerobic conditions at a temperature of (27.0±1.0) °C in tubes on a liquid and dense nutrient medium (pH 6.8-7.2), containing, wt. %: sodium chloride (NaCl) – 2.5-2.7, potassium phosphate disubstituted (K_2HPO_4) – 1.4-1.6, ammonium phosphate disubstituted $(NH_4)_2HPO_4$) – 0.04-0.06, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) – 0.01-0.02, peptone – 0.4-0.6, yeast extract – 0.4-0.6, glycerin – 0.2-0.4, chalk ($CaCO_3$) – 0.01-0.03, all the rest distilled water (up to 1.0 dm^3) for (22.0±2.0) hours (Declaratory patent of Ukraine for a utility model № 143070). Prior to the commencement of the research, the luminescence intensity of the bacteria in the "working" suspension was measured. It was found to be in the range of 25–150 times greater than the device's background value (in order to produce data that can be trusted, the luminescence intensity needs to surpass the background indicator of the device by 25–250 times).

*Ph. phosphoreum*s luminosity was measured using an EMILITE luminometer (1003 A) (BioChemMac, russian federation). After 20 to 25 minutes of testing, the exposure duration was measured and the instrument's changes in luminous intensity were recorded. Measurements were carried out in control experiment pairs. The luminometer's spectral range is 350–950 nm. We looked at six duplicates of the control and experimental samples in order to get reliable values.

Interpretation of results

The toxicity index (T), a dimensionless number equal to the ratio (formula 1) (Kurbatska and Orobchenko, 2021), was used to measure the sample's level of toxicity in relation to the impact of various herbicide concentrations on the luminescence of the bacteria *Ph. phosphoreum*.

$$T = \frac{I_0 - I}{I_0} \times 100 \text{ , where} \quad (1)$$

I_0 and I – respectively, the intensity of the control and experiment,

100 – conversion factor.

The data were interpreted at the three threshold levels of the toxicity index (Table 2).

The degree to which different doses of herbicides suppress the luminescence of *Ph. phosphoreum* bacteria is used to determine how harmful the chemicals are to these bacteria. That is, the herbicide's harmful effects are directly correlated with a drop in bioluminescence intensity.

Table 1. Herbicides that were studied for the intensity of the luminescence of luminescent bacteria *Ph. Phosphoreum*

Herbicide (active substances) (a.s.) and brief description	Investigated levels (doses), mg/kg of feed	Maximum residue limits*, mg/kg of feed
Soteira (imazamox+imazapyr). Preparative form: solution concentrate. Chemical class: imidazolinones. Active ingredient concentration: 33+15 g/l. Purpose: herbicide for the destruction of cereal and dicotyledonous weeds in sunflower crops (hybrids and varieties resistant to imidazolinones)	0.01; 0.025; 0.05; 0.1 and 0.25	0.05** (imazamox)
Greenfort premium (2,4-D 2-ethylhexyl ether + florasulam). Preparative form: Suspo-emulsion. Chemical class: triazolepyrilidines. Active ingredient concentration: 452.42+6.25 g/l. Purpose: systemic herbicide for the protection of grain crops and corn against annual and some perennial dicotyledonous weeds	0.01; 0.025; 0.05; 0.1 and 0.25	0.05** (florasulam)
Greenfort chors (chisalophop-p-ethyl). Preparative form: emulsifiable concentrate. Chemical class: aryloxyphenoxypropionic acids. Active ingredient concentration: 125 g/l. Purpose: systemic post-emergence herbicide for the protection of rapeseed, sunflower and soybean against cereal weeds	0.008; 0.02; 0.04; 0.08 and 2.0	0.04
Skat (chisalophop-p-tephuryl). Preparative form: emulsion concentrate. Chemical class: aryloxyphenoxypropionates. Active ingredient concentration: 40 g/l. Purpose: for the destruction of annual and perennial cereal weeds in crops of sugar beet, rapeseed, sunflower, soybean and other broadleaf crops	0.008; 0.02; 0.04; 0.08 and 2.0	0.04
Agroshield super (potassium salt of glyphosate). Preparative form: solution concentrate. Chemical class: glycine derivatives. Active ingredient concentration: 676 g/l. Purpose: for processing winter wheat, spring wheat, soybeans, sunflower	0.1; 0.5; 1.0; 2.5 and 5.0	1.00
Greenfort extra (metholachlor+terbutylazine). Preparative form: suspension concentrate. Chemical class: triazine chloroacetanimides. Active ingredient concentration: 312.5+187.5 g/l. Purpose: soil herbicide of combined action for the protection of sunflower and corn against annual cereal and annual dicotyledonous weeds	0.02; 0.05; 0.1; 0.2 and 0.5	0.1** (metholachlor)
Astanes (acetochlor). Preparative form: emulsion concentrate. Chemical class: chloroacetanilides. Active ingredient concentration: 900 g/l. Purpose: effective pre-emergence herbicide of selective action, destroys germinating annual cereal weeds and some dicotyledonous weeds before the crop rows close	0.006; 0.015; 0.03; 0.06 and 0.15	0.03
Astralid (clopyralid). Preparative form: solution concentrate. Chemical class: pyridine derivatives. Active ingredient concentration: 300 g/l. Purpose: treatment of sugar beets against annual cereal dicotyledonous weeds	0.4; 1.0; 2.0; 4.0 and 10.0	2.00
Greenfort NK 40 (nicosulfuron). Preparative form: suspension concentrate. Chemical class: sulfonylureas. Active ingredient concentration: 40 g/l. Purpose: systemic post-emergence herbicide for the protection of corn against annual and perennial cereals and the most common dicotyledonous weeds	0.04; 0.1; 0.2; 0.4 and 1.0	0.20

Note * According to (https://eur-lex.europa.eu/); ** - MRL active substances.

Table 2. Classification of the substance toxicity by the T value

Toxicity index level	T value	Conclusion on the toxicity level
1	less than 20	non-toxic sample
2	from 20 to 50	toxic sample
3	more than 50	highly toxic sample

The concentration of herbicide that causes the biosensor luminescence (*Ph. phosphoreum* bacterium) to be quenched at a fixed exposure period of the test sample is reflected in the toxicity index "T". A 50% reduction in the luminescence intensity of the bacteria relative to the control was found to be the top limit indicator. This is represented by a toxicity index of 50, and it enables the classification of substances with a toxicity index of 50 and higher as "highly toxic". We can designate samples with a toxicity index of 20 or less as "non-toxic" since the lower limit of the toxicity index is 20, which indicates that the luminescence of bacteria is 20% lower than the control. Samples are categorized as "toxic" for all "T" values between 20 and 50; however, toxicity can be decreased if the samples are diluted appropriately.

Statistical analysis

To obtain reliable results, the study for each pesticide was conducted in 6 replicates. The results were processed by variation statistics using the analysis of variance software package (ANOVA) (one-way analysis of variance, M±m) StatPlus 7 (7.6.5.0) (AnalystSoft Inc., USA). The reliability of the obtained results was evaluated by Fisher's criterion at a reliability level of 95.0% (P<0.05) (StatPlus for Windows, 2021).

RESULTS

Figure 1 illustrates the impact of Soteira herbicide on *Ph. phosphoreum* luminescence based on concentration. By determining the percentage decline in *Ph. phosphoreum* luminescence intensity in relation to the toxicity index (T), we were able to perform a toxicological evaluation of feed containing varying concentrations of Soteira herbicide. After 25 and 30 minutes of the experiment, the toxicity index was consistently 100 at all herbicide levels, showing high feed toxicity in the presence of the drug at doses ranging from 0.01 to 0.25 mg/kg.

Figure 2 depicts how the concentration of Greenfort Premium herbicide affects *Ph. phosphoreum* luminescence. By calculating the percentage decrease in *Ph. phosphoreum* luminescence intensity in relation to the toxicity index (T), we were able to perform a toxicological evaluation of feed con-

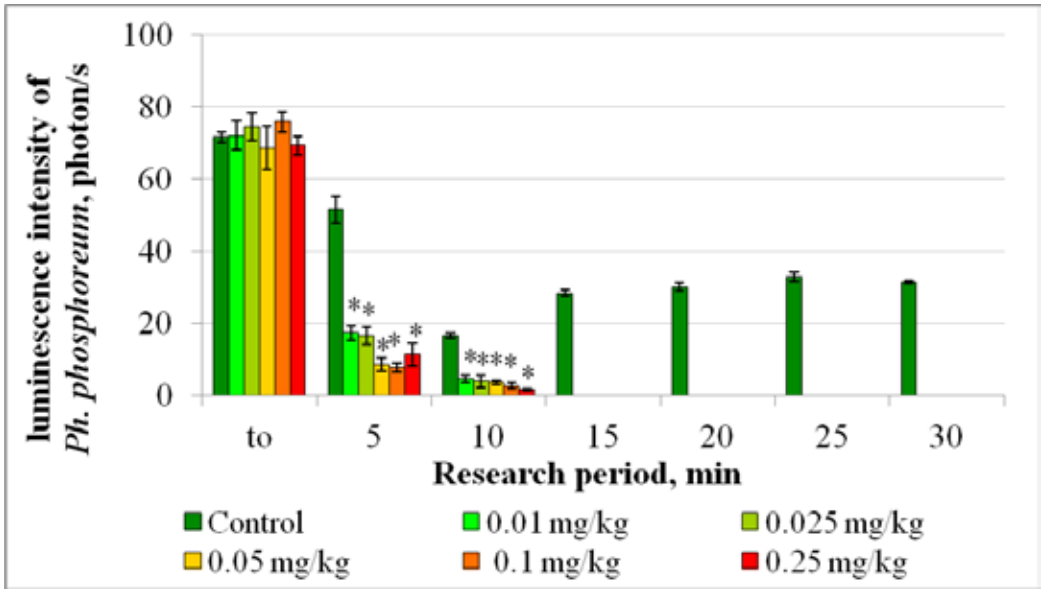

Figure 1. Dynamics of *Ph. phosphoreum* luminescence intensity when different doses of Soteira herbicide (a.s. imazamox+imazapyr) were added to the feed (M±m, n=6, *- p<0.05 - relative to control).

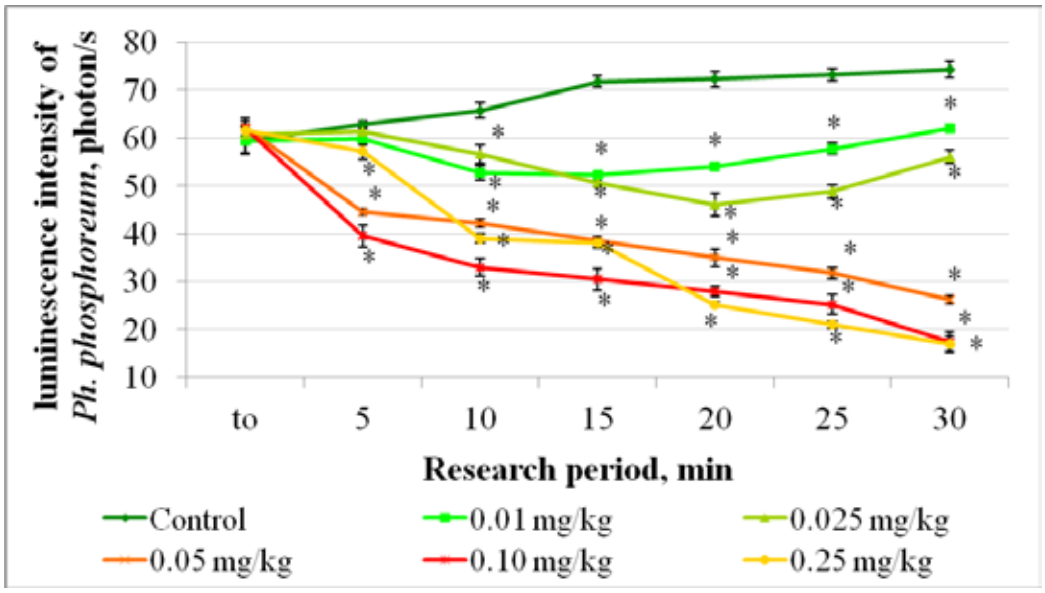

Figure 2. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of feeding different doses of Greenfort Premium herbicide (a.s. 2,4-D 2-ethylhexyl ether+florasulam) (M±m, n=6, *- p<0.05 - relative to control).

taining varying concentrations of Greenfort Premium herbicide. The toxicity index, therefore, averaged 18.9 at a drug content of 0.01 mg/kg of feed for (20–25) minutes (the recommended period for recording fluorescence indicators); 29.0 at a content of 0.025 mg/kg of feed; 60.7 at 0.05 mg/kg of feed (MRL indicator); 71.0 at 0.10 mg/kg of feed; and 74.2 at a content of 0.25 mg/kg of feed. According to the data, feeds containing less than 0.01 mg/kg of Greenfort Premium herbicide are considered non-toxic (toxicity index less than 20), feeds containing 0.025 mg/kg are considered toxic (toxicity index between 20 and 50), and feeds containing 0.05 to 0.25 mg/kg are considered highly toxic (toxicity index greater than 50).

Figure 3 illustrates how the concentration of the herbicide Greenfort Horse affects *Ph. phosphoreum* luminescence. The toxicity index (T) and the % decline in *Ph. phosphoreum* luminescence intensity allowed for a toxicological evaluation of feed containing varying concentrations of Greenfort Horse herbicide. The toxicity index therefore averaged 13.9 at a drug content of 0.008 mg/kg of feed for (20–25 minutes), which is the recommended period for recording fluorescence indicators; 14.9 at a content of 0.02 mg/kg of feed; 15.8 at 0.04 mg/kg of feed (MRL indicator); 52.1 at 0.08 mg/kg of feed; and 54.2 at 0.2 mg/kg of feed. According to the statistics, feeds containing less than 0.008 to 0.04 mg/kg of feed, inclusive, are considered non-toxic (toxicity index less than 20). Conversely, feeds containing 0.08 to 0.2 mg/kg of feed are considered very toxic (toxicity index greater than 50).

Figure 4 illustrates the effect of the herbicide Skat on *Ph. phosphoreum* luminescence. The toxicity index (T) and the percentage decline in *Ph. phosphoreum* luminescence intensity allowed us to do a toxicological evaluation of feed containing varying concentrations of Skat herbicide. The toxicity index was thus negative and averaged -3.0 at a drug content of 0.008 mg/kg feed for (20–25) min (the recommended period for recording fluorescence indicators); 12.6 at a content of 0.02 mg/kg feed; 28.3 at 0.04 mg/kg feed (MRL indicator); 40.6 at 0.08 mg/kg feed; and 67.3 at a content of 0.2 mg/kg feed. As follows, feeds containing less than 0.008 to 0.02 mg/kg herbicide of feed inclusively are considered non-toxic (toxicity index less than 20), while feeds containing 0.04 to 0.08 mg/kg are considered hazardous (toxicity index between 20 and 50). Feeds that contain 0.2 mg/kg herbicide Skat are extremely hazardous (toxicity index greater than 50).

Figure 5 illustrates how the concentration of the herbicide Agroshield Super affects *Ph. phosphoreum* luminescence. The toxicity index (T) and the % decline in *Ph. phosphoreum* luminescence intensity allowed us to do a toxicological evaluation of feed

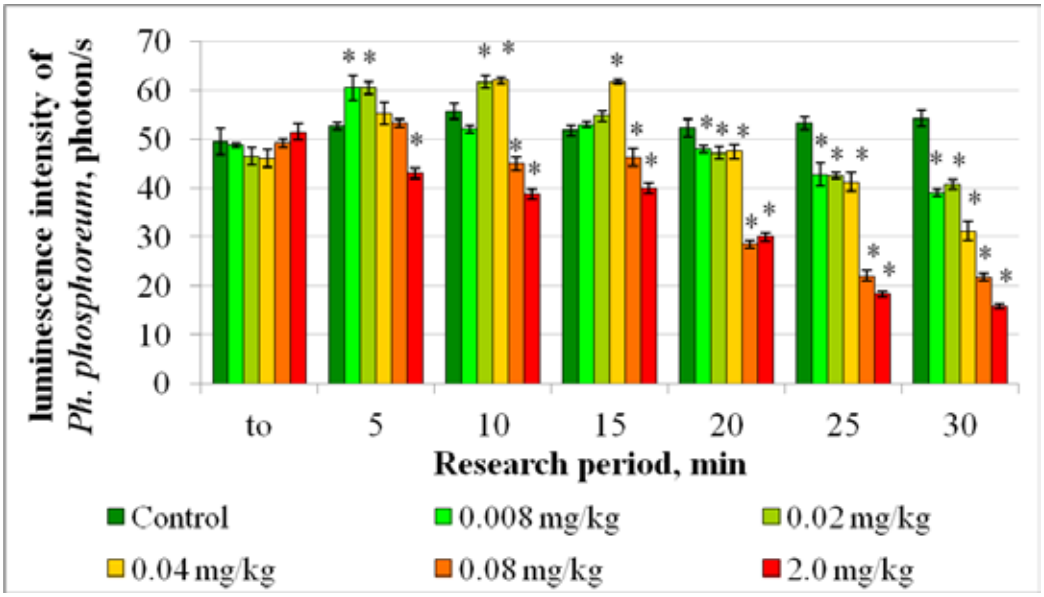

Figure 3. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of feeding different doses of Greenfort Horse herbicide (a.s. chisalofop-p-ethyl) (M±m, n=6, *- p<0.05 - relative to control).

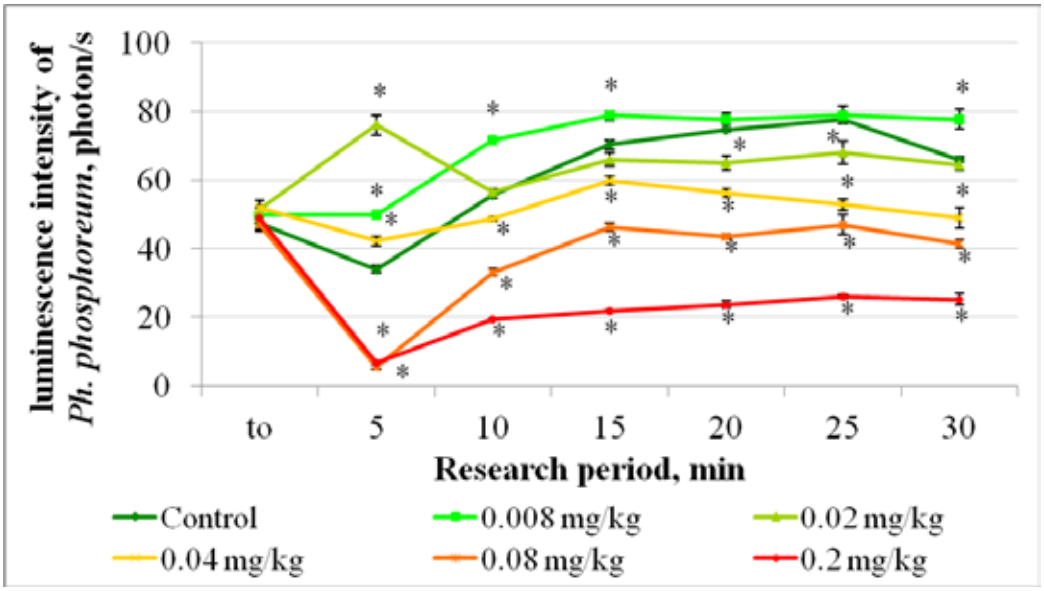

Figure 4. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of feeding different doses of the herbicide Skat (a.s. chisalofop-p-tefuryl) (M±m, n=6, *- p<0.05 - relative to control).

containing varying concentrations of the herbicide Agroshield Super. The toxicity index, therefore, averaged 16.5 at a drug content of 0.1 mg/kg of feed for 20–25 minutes (the recommended period for recording fluorescence indicators); 18.3 at a content of 0.5 mg/kg of feed; 40.8 at 1.0 mg/kg of feed (MRL indicator); 61.2 at 2.50 mg/kg of feed; and 71.0 at 5.0 mg/kg of feed. According to the data, feeds containing less than 0.1 to 0.5 mg/kg of Agroshield Super herbicide, inclusively, are considered non-toxic (toxicity index less than 20), feeds containing 1.0 mg/kg are considered toxic (toxicity index between 20 and

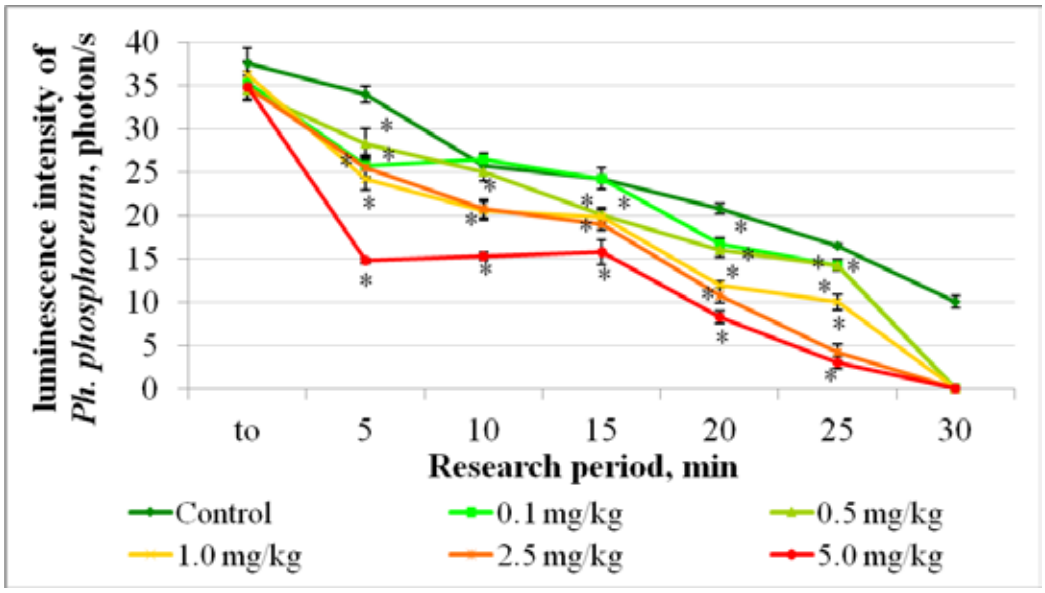

Figure 5. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of feeding different doses of the herbicide Agroshield Super (a.s. potassium salt of glyphosate) (M±m, n=6, *- p<0.05 - relative to the control).

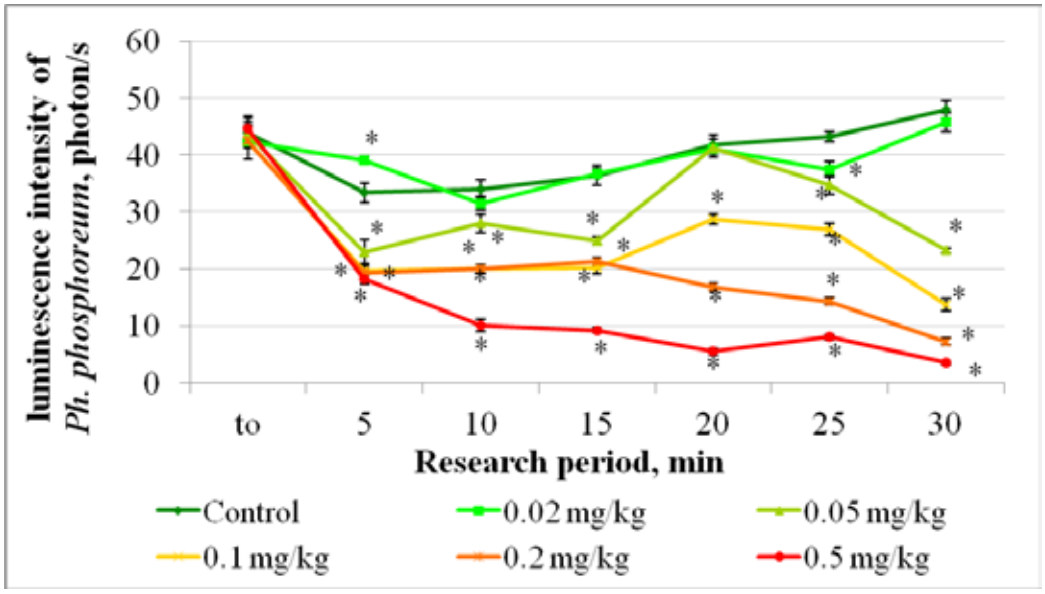

Figure 6. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of feeding different doses of Greenfort Extra herbicide (a.s. metholachlor+terbutylazine) (M±m, n=6, *- p<0.05 - relative to control).

50), and feeds containing 2.5 mg/kg are considered highly toxic (toxicity index greater than 50).

Figure 6 illustrates the effect of Greenfort Extra herbicide, based on concentration, on *Ph. phosphoreum* luminescence. The toxicity index (T) and the % decline in *Ph. phosphoreum* luminescence intensity allowed for a toxicological evaluation of feed treated with varying concentrations of Greenfort Extra herbicide. The toxicity index therefore averaged 7.6 at a drug content of 0.02 mg/kg of feed for (20–25) minutes (the recommended period for recording fluorescence indicators); 10.4 at a content of 0.05 mg/kg of feed; 34.4 at 0.1 mg/kg of feed (MRL indicator);

63.5 at 0.20 mg/kg of feed; and 84.2 at 0.5 mg/kg of feed. According to the data, feeds containing less than 0.02 to 0.05 mg/kg of Greenfort Extra herbicide, inclusively, are considered non-toxic (toxicity index less than 20), 0.1 mg/kg of feed is considered toxic (toxicity index between 20 and 50), and 0.2 mg/kg of feed is considered highly toxic (toxicity index more than 50).

Figure 7 depicts how the concentration of the herbicide Astanes affects *Ph. phosphoreum* luminescence. By calculating the percentage decrease in *Ph. phosphoreum* luminescence intensity in relation to the toxicity index (T), we were able to perform a toxicological evaluation of feed containing varying concentrations of Astanes herbicide. The toxicity index was thus negative and averaged -12.8 at a drug content of 0.006 mg/kg of feed for (20–25) min (the recommended period for recording fluorescence indicators); at a content of 0.015 mg/kg of feed, it was 25.3; at 0.03 mg/kg of feed (MRL indicator), it was 59.9; at 0.06 mg/kg of feed, it was 60.3; and at 0.15 mg/kg of feed, it was 67.9. According to the data, feeds containing less than 0.006 mg/kg of Astanes herbicide are considered non-toxic (toxicity index less than 20), while feeds containing more than 0.015 mg/kg of Astanes herbicide are considered hazardous (toxicity index greater than 20 to 50). They are extremely hazardous, with a concentration of 0.03 mg/kg of feed (toxicity index greater than 50).

Figure 8 depicts how the concentration of the herbicide Astralid affects *Ph. phosphoreum* luminescence. By calculating the percentage decrease in *Ph. phosphoreum* luminescence intensity in relation to the toxicity index (T), we were able to perform a toxicological evaluation of feed containing varying concentrations of Astralid herbicide. The toxicity index was thus negative and averaged -10.4 at a drug content of 0.4 mg/kg feed for (20–25) min (the recommended time for recording fluorescence indicators); 8.5 at a content of 1.0 mg/kg feed; 19.9 at 2.0 mg/kg feed (MRL indicator); 60.3 at 4.0 mg/kg feed; and 71.0 at 10.0 mg/kg feed. Astralid herbicide-containing feeds with less than 0.4 and up to 2.0 mg/kg of feed inclusively are not toxic (toxicity index less than 20), but feeds with 4.0 mg/kg and above are highly toxic (toxicity index more than 50). This is indicated by the results collected.

Figure 9 illustrates how the concentration of the herbicide Greenfort NK 40 affects *Ph. phosphoreum* luminescence. The toxicity index (T) and the percentage decline in *Ph. phosphoreum* luminescence intensity allowed us to do a toxicological evaluation of feed containing varying concentrations of Greenfort NK 40 herbicide. The toxicity index averaged 10.8 at a drug content of 0.04 mg/kg of feed for (20–25) minutes (the recommended time for recording fluorescence indicators); 19.4 at a content of 0.1 mg/kg of feed; 35.2 at 0.2 mg/kg of feed (MRL

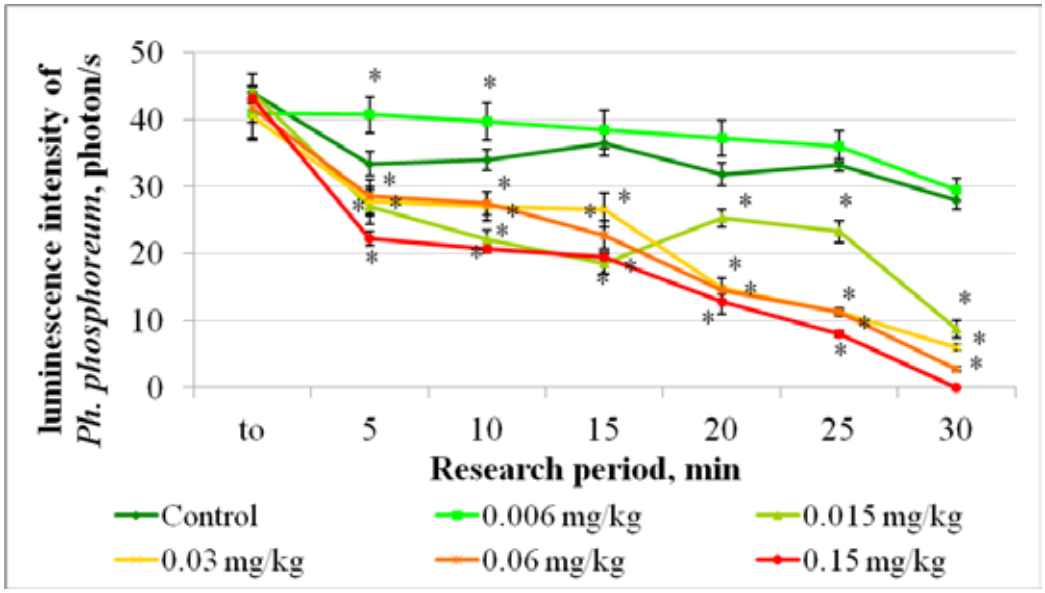

Figure 7. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of different doses of Astanes herbicide (s.a. acetochlor) (M±m, n=6, *- p<0.05 - relative to control).

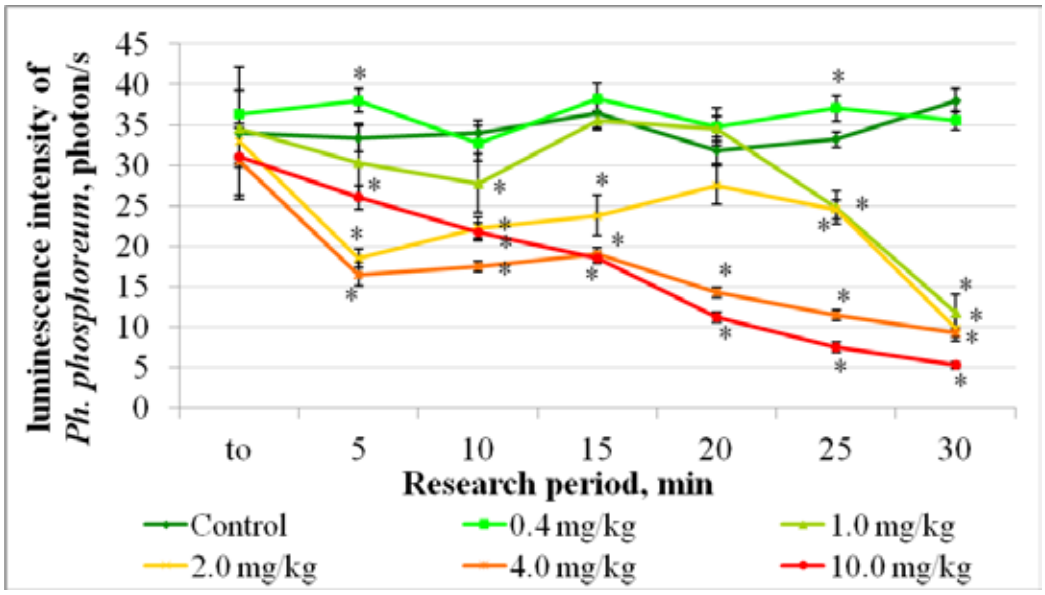

Figure 8. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of feeding different doses of Astralid herbicide (a.s. clopyralid) (M±m, n=6, *- p<0.05 - relative to control).

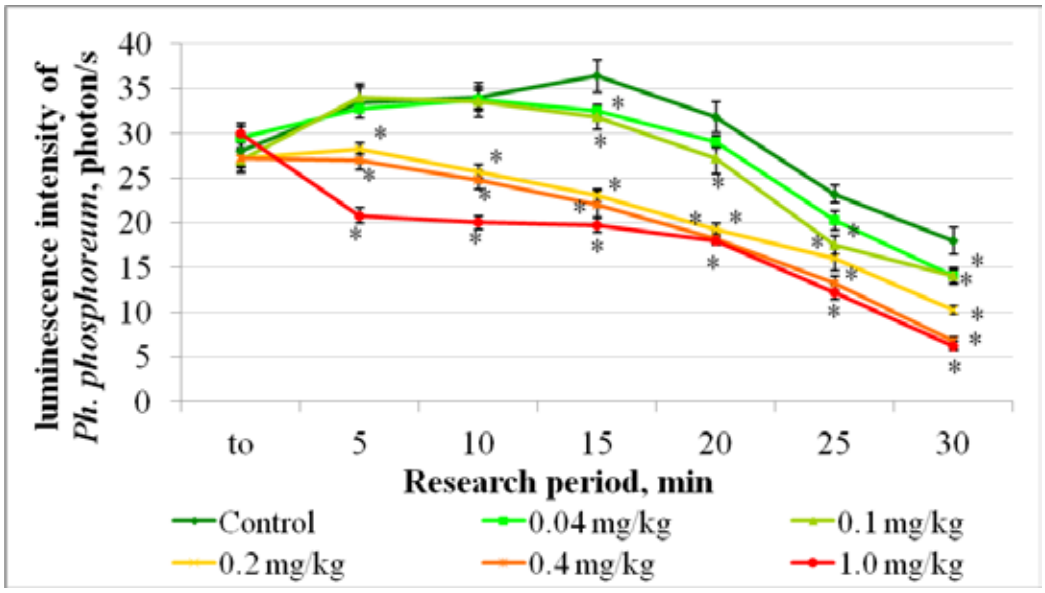

Figure 9. The dynamics of *Ph. phosphoreum* luminescence intensity under the conditions of feeding different doses of Greenfort NK 40 herbicide (a.s. nicosulfuron) (M±m, n=6, *- p<0.05 - relative to control).

indicator); 42.7 at 0.4 mg/kg of feed; and 45.3 at 1.0 mg/kg of feed. As follows, feeds containing less than 0.04 and up to 0.1 mg of Greenfort NK 40 herbicide/kg of feed inclusively are considered non-toxic (toxicity index < 20), while feeds containing 0.2 mg/kg inclusively and above are considered harmful (toxicity index 20–50).

DISCUSSION

Bioluminescent bacteria are increasingly commonly utilized to investigate the toxicology of environmental items. Jonkers et al. (2020) suggested a biosensor based on *Bacillus WT* and *E. coli FhuAT* with a detection limit of 0.043-324.0 μg/L to detect antibiotics in water. *Ph. leiognathi* was used to develop

a biosensor capable of detecting mercury at a limit of 9.87 μg/L. Borisover et al. (2019) employed *E. coli mutants* to detect chlorine at a limit of 1.0 μg/L. Dieudonne et al. (2020) used *E. coli* as a biosensor for detecting arsenite, with a detection limit of 39.6 μg/L.

The most widely used biotest is Microtox (Strategic Diagnostics, Inc, Germany, USA) (based on *Ph. phosphoreum*, strain NRRL-B-11177, sometimes also called *Vibrio fischerii*, strain NRRL-B-11177), which was developed first and is widely used in laboratory and field studies to control the quality of industrial and natural waters in several countries, to determine the degree of toxicity of chemical compounds and pharmaceuticals being developed (Johnson, 2013; Kunz et al., 2017).

A biosensor tests ToxAlert 100® (Merck, Germany) and LUMIStox 300 (HACH LANGE, Germany) were developed, based on the inhibition of bioluminescence in lyophilized *V. fisheri*. They are used for the analysis of soils, groundwater, wastewater and sludge. The BioTox™ system, based on *Aliivibrio fischeri* and *Ph. phosphoreum*, is also used for the toxicological monitoring of aquatic environments. In addition, commercial production of test systems has been realized: «Mitatox» (USA), «Vitotox» (GENAUR Molecular Products, Belgium) based on *Vibrio fischeri* and «Mutatox» based on *Aliivibrio fischeri* (Hurtado-Gallego et al., 2022; Li et al., 2022; Huang et al., 2023; Li et al., 2024).

Regarding the impact of herbicides on the luminescence of photobacteria, *Aliivibrio fischeri* was used to detect terbuthrin in water, with a detection limit of 81.0 μg/L (Vermeirssen et al., 2018).

It should be noted that not all herbicides have been studied for their effect on the luminescence of photobacteria: no data on the effect of florasulam, chisalofop-p-ethyl, and chisalofop-p-tefuryl have been found in the scientific literature.

In our experiment, the concentrations of the herbicide Soteira (by AS imazamox) in the final extract were 0.005, 0.0125, 0.025, 0.05, and 0.125 mg/l (corresponding to the levels in the feed - 0.01, 0.025, 0.05, 0.1, and 0.25 mg/kg), while complete suppression of luminescence at all studied levels was observed within 15 minutes. Olkova (2022) on luminescent bacteria revealed a high degree of toxicity in waters with concentrations of imazamox 0.01-3.0 mg/l. Also, the high degree of toxicity of this herbicide can be explained by the presence of the second component (imazapyr), which probably potentiates the effect of the main AS.

Vurm et al. (2021) determined the effect of glyphosate on the luminescence of *Aliivibrio fischeri*: EC_{50} was 0.811 μg/ml. In our studies, the EC_{50} of the herbicide Agroshield Super (AS potassium salt of glyphosate) was approximately between 1.0 and 2.5 mg/kg of feed (percentage of inhibition 40.8 and 61.2%, respectively), which in terms of the final concentration in the extract averaged 0.875 μg/ml, i.e. our data are consistent with the literature.

The concentrations of the herbicide Greenfort Extra (according to AS metolachlor) in the final tested extract were 0.01, 0.025, 0.05, 0.1, and 0.25 mg/l (corresponding to the levels in the feed - 0.02, 0.05, 0.1; 0.2 and 0.5 mg/kg), while the EC_{50} was approximately between 0.1 and 0.2 mg/kg of feed (percentage of inhibition 34.4 and 63.5 %, respectively), which in terms of the final concentration in the extract averaged 0.075 mg/l, which was consistent with the data of Osano et al. (2002) who reported the EC_{50} relative to *Vibrio fischeri* at the herbicide level of 0.006-4.9 mg/l.

The herbicide Astanes (AS acetochlor) at feed levels of 0.006, 0.015, 0.03, 0.06 and 0.15 mg/kg in the final extracts had concentrations of 0.003, 0.0075, 0.015, 0.03 and 0.075 μg/ml, respectively, with the percentage of inhibition exceeding the 50% mark already at a concentration of 0.015 μg/ml, which contradicted the data obtained by Souissi. et al. (2013) (EC_{50} was greater than 0.2 μg/mL).

The concentrations of the herbicide Astralid (AS clopyralid) in the final extract tested were 0.2, 0.5, 1.0, 2.0 and 5.0 mg/L (corresponding to the levels in the feed - 0.4, 1.0, 2.0, 4.0 and 10.0 mg/kg), with the percentage of inhibition exceeding 50% at a concentration of 2.0 μg/ml, which was slightly lower than the data obtained by Vurm et al. (2021) (EC_{50} 5.07 mg/L).

The herbicide Greenfort NK 40 (AS nicosulfuron) at levels in the feed of 0.04, 0.1, 0.2, 0.4 and 1.0 mg/kg in the final extracts had concentrations of 0.02, 0.05, 0.1, 0.2 and 0.5 μg/ml, respectively, while the percentage of inhibition at the studied levels did not exceed 50% (the maximum percentage of inhibition of the luminescence of *Ph. phosphoreum* 45.3 was observed at a concentration in the final extract of 0.5 μg/ml), which was slightly higher than the results obtained by Joly et al. (2013) (EC_{50} was 0.1678±0.0218 μg/mL).

At the same time, some herbicides in low doses stimulated the luminescence of *Ph. phosphoreum*, in particular, Skat (chisalofop-p-tetufuryl), Astanes and Astralid (clopyralid), which can be explained by the phenomenon of hormesis (stimulation of any organism's system by external influences that are not strong enough to manifest harmful factors) (Belz and Cedergreen 2010; Baran et al., 2019).

The results of our research confirm the possibility of luminescent bacteria use for the analysis of contaminated with herbicides feed, which is a relevant and popular rapid test of laboratory diagnosis.

CONCLUSIONS

The influence of different levels of herbicides on the intensity of *Ph. phosphoreum* luminescence was studied and the toxicological assessment of feeds by the percentage of decrease in luminescence intensity was given: Soteira (imazamox+imazapyr) completely suppressed the luminescence of *Ph. phosphoreum* at all studied levels of herbicide in feed, Greenfort premium (2,4-D 2-ethylhexyl ether+florasulam) on average - by 18.9-74.2%, Greenfort Horse (chisalofop-p-ethyl) - by 13,9-54.2%, Skat (chisalofop-p-tefuryl) - by 12.6-67.3%, Agroshield Super (potassium salt of glyphosate) - by 16.5-71.0%, Greenfort Extra (metholachlor+terbutylazine) - by 7.6-84.2%, Astanes (acetochlor) - by 25.3-67.9%, Astralid (clopyralid) - by 8.5-71.0%, Greenfort NK 40 (nicosulfuron) - by 10.8-45.3%, which allowed to assess feed with the content of herbicides Greenfort Premium less than 0.01 mg/kg, Greenfort Horse less than 0.008-0.04 mg/kg, Skat less than 0.008-0.02 mg/kg, Agroshield Super less than 0.1-0.5 mg/kg, Greenfort Extra less than 0.02-0.05 mg/kg, Astanes less than 0.006 mg/kg, Astralid less than 0.4-2.0 mg/kg, Greenfort NK 40 less than 0.04-0.1 mg/kg as non-toxic; with the content of Greenfort Premium 0.025 mg/kg, Skat 0.04-0.08 mg/kg, Agroshield Super from 1.0 mg/kg, Greenfort Extra from 0.1 mg/kg, Astanes 0.015 mg/kg, Greenfort NK 40 from 0.2 mg/kg as toxic and with the content of Soteira 0.01-0, 25 mg/kg, Greenfort Premium 0.05-0.25 mg/kg, Greenfort Horse 0.08-0.2 mg/kg, Skat from 0.2 mg/kg, Agroshield Super from 2.5 mg/kg, Greenfort Extra from 0.2 mg/kg, Astanes from 0.03 mg/kg, Astralid 4.0 mg/kg and above as highly toxic feeds.

The luminescence of *Photobacterium phosphoreum* can be used for rapid (up to 30 minutes) toxicological evaluation of feed contaminated with herbicides, which will avoid economic damage from the shortage of agricultural products due to poisoning and ensure the safety of the resulting products for humans.

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CONFLICT OF INTEREST

The authors declare that they have no potential conflict of interest with respect to the authorship and/or publication of this article.

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