# Toxicological evaluation of feed contaminated with mycotoxins using a luminescent microorganism: *Photobacterium phosphoreum*

*O. Orobchenko*, O. Kurbatska, A. Paliy and A. Palii*

## Abstract

The possibility of using luminescent microorganisms *Photobacterium phosphoreum* (strain IMB B-7071; Sq3) for rapid toxicological evaluation of feed contaminated with mycotoxins was examined based on the reduction of the luminescence intensity. Under the conditions of this study, feed with Ochratoxin A mycotoxin content at the level of maximum residue limits (MRL 0.05 mg/kg) is characterized as non-toxic, while for T2-mycotoxin, deoxynivalenol, fumonisins and aflatoxin B1 (MRL 0.1, 1.0, 5.0, and 0.01 mg/kg, respectively) is characterized as toxic, and for zearalenone (MRL 1.0 mg/kg) as highly toxic. This indicates the need for further research to study the toxicological characteristics of mycotoxins in the body of laboratory and productive animals, possibly with further revision of the maximum allowable levels of relevant contaminants in feed in Ukraine.

**Key words:** *bioluminescence; feed; mycotoxins; toxicity; Photobacterium phosphoreum*

## Introduction

Obtaining quality livestock products is not possible without scientifically sound, complete feeding of productive animals. The quality and safety of the feed used, and the level of their contamination by abiotic and biotic factors are important (Gosling et al., 2021; Orobchenko et al., 2022).

Mycotoxins are secondary metabolites of microscopic moulds, which mainly

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belong to the genera *Aspergillus, Penicillium*, and *Fusarium*. Mycotoxin contamination of feed raw materials and feed for farm animals can occur both in the field and during storage (Pleadin et al., 2012; Logrieco et al., 2021). It has been estimated that about 25% of the world's crops may be contaminated with mycotoxins. Many mycotoxins can be present in feed along with moulds, increasing their damaging effects on animals (Streit et al., 2012; Kępińska-Pacelik and Biel, 2021).

The level of mycotoxins in feed can vary significantly depending on the raw materials used for their production and the geographical location of farms (climatic conditions), which create the preconditions for establishing acceptable levels of mycotoxins in feed, in particular, five mycotoxins (aflatoxins (AFB1), deoxynivalenol (DON), zearalenone (ZON), fumonisins (FBs) and ochratoxin A (OTA)) fall under the action of EU legislation (regulation or recommendation) (Guerre, 2016; Pleadin et al., 2020). In Ukraine today, six mycotoxins are subject to testing in feeds and feed materials: AFB1, DON, ZON, OTA, FBs and T-2 toxin) (On approval of the List of maximum permissible levels…, 2017).

Many high-tech laboratory test methods are used to ensure animal feed does not contain excess mycotoxins, the most common of which are high performance liquid chromatography with UV detector (Curticapean et al., 2011), liquid chromatography with mass spectrometry (Tsiplakou et al., 2014), thin layer chromatography (Liang et al., 1996), enzyme linked immunosorbent assay (Beyene et al., 2019) and others. They allow for high accuracy detection and quantification of mycotoxins, and are also used for screening and monitoring residual mycotoxins in feed materials and finished feeds. However, they are quite expensive, difficult to perform, time-consuming, and most importantly do not respond to the question of how dangerous (toxic) the feed with a certain content of mycotoxins may be for an animal.

To address this issue in the practice of veterinary medicine, the bioassay method is used on models of different levels of organisation: target and laboratory animals, insects, crustaceans, ciliates, bacteria, cell cultures, etc. (Jeong et al., 2005; GOST, 2014; Gerssen et al., 2019). Bioassay on laboratory animals is the most illustrative model for determining the toxicity of feed, though the global scientific community tends to minimise the use of living organisms in experiments with the implementation of the 3R principle (Replace, Reduce, Refine) (Gorzalczany and Rodriguez Basso, 2021), so the development of alternative tests to determine toxicity is relevant today.

Particularly promising is the use of photobiosensors, which are already widely used to monitor the state of the environment and ecosystems (Senko et al., 2019). Moreover, bioassays with the use of live bioluminescent bacteria come to the fore, which differ from others in that the intensity of their luminescence is measured as a parameter of vital activity (Ismailov and Aleskerova, 2015; Efremenko et al., 2016; Li et al., 2020).

There are 12 species of luminescent bacteria, classified into four genera: *Vibrio, Photobacterium, Shewanella, Xenorharbdus*. Most members of this group are marine species, including both wild and symbiotic forms (Burtseva et al., 2020). However, despite the fairly wide range of toxicants and compounds that have been studied for bacterial photoluminescence, these microorganisms have not been commonly used to determine feed toxicity, especially with mycotoxin contamination.

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

## Materials and Methods

The study was conducted in the toxicological monitoring laboratory of the National Scientific Centre "Institute of Experimental and Clinical Veterinary Medicine" (Kharkiv).

The studies used mycotoxins that are included on the List of maximum allowable levels of unwanted substances in raw materials and feed for animals (On approval of the List of maximum permissible levels of undesirable substances in feed and feed raw materials for animals of the Ministry of Agrarian Policy of Ukraine; Order, List dated March 19, 2012, No. 131 as amended on October 11, 2017 Order No. 550), in the form of standard samples (SS), namely: T2-toxin (Sigma-Aldrich, 33947), ZON (Sigma-Aldrich, CRM46916), DON (Sigma-Aldrich, CRM46911), OTA (Sigma-Aldrich, CRM46912), FB (Sigma-Aldrich, 32606) and AFB1 (Sigma-Aldrich, CRM44647).

Under the study conditions, corn grits without toxic properties and free of these mycotoxins were used as the "matrix". The absence of toxic properties of feed used as the "matrix" was determined using the standard method (DSTU 3570-97. Feed grain, grain by-products, compound feeds. Methods for the determination of toxicity: determination of toxicity using the infusoria (*Colpoda Steinii*). The study of mycotoxins in the "matrix" was performed using thin layer chromatography (Kaminska et al. 2019): the content of mycotoxins was below the limit of quantitation of the method. Toxicants were added to the matrix at different concentrations (five levels prepared by ethanol dilution, depending on the maximum residue limits (MRL)) (Table 1).

A portion of the control and experimental "matrices" weighing 10.0 g was added to glass vials, to the experimental samples the appropriate amount of SS mycotoxins was added and 96% ethanol in a volume of 20.0 $cm^3$ was added and extracted, left for 24 hours, then centrifuged at 1.5–2.0 x 1000 rpm for 10 minutes. After that the supernatant was collected and added in a volume of 0.02 $cm^3$ to culture fluid in a volume of 1.0 $cm^3$ (previously prepared and introduced into the luminometer cuvette).

As a test culture, we used lyophilised culture *Photobacterium phosphoreum* (strain IMB B-7071; Sq3) (*P. phosphoreum*), obtained from the Depository of Microorganisms of the Institute of Microbiology and Virology named

**Table 1.** Mycotoxins that were studied for the intensity of the luminescence of luminescent bacteria *P. phosphoreum*

| Mycotoxin | Investigated levels (doses), mg/kg of feed | Maximum residue limits, mg/kg of feed* |
|---|---|---|
| T2-toxin | 0.01; 0.05; 0.1; 0.5; 1.0 | 0.1 |
| ZON | 0.1; 0.2; 1.0; 5.0; 10.0 | 1.0 |
| DON | 0.05; 0.1; 0.5; 1.0; 2.0 | 1.0 |
| OTA | 0.005; 0.01; 0.05; 0.1; 0.5 | 0.05 |
| FB | 0.5; 1.0; 5.0; 10.0; 15.0 | 5.0 |
| AFB1 | 0.001; 0.005; 0.01; 0.05; 0.1 | 0.01 |

Note * According to (On approval of the List of maximum permissible levels..., 2017)

after D.K. Zabolotny of the National Academy of Sciences of Ukraine (Kyiv). *P. phosphoreum* are gram-negative polymorphic asporogenic rods with a size of 0.8–1.0 × 1.2–1.4 μm.

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: sodium chloride (NaCl), 2.5–2.7 wt.%; potassium phosphate disubstituted ($K_2HPO_4$), 1.4–1.6 wt.%; ammonium phosphate disubstituted $(NH_4)_2HPO_4$, 0.04–0.06 wt.%; magnesium sulphate heptahydrate ($MgSO_4 \bullet 7H_2O$), 0.01–0.02 wt.%, 0.4–0.6 wt.%; peptone, 0.4–0.6 wt.%; yeast extract, 0.4–0.6 wt.%; glycerin, 0.2–0.4 wt.%; chalk ($CaCO_3$). 0.01–0.03 wt.%; and the rest distilled water (up to 10 $cm^3$) for 22.0±2.0 hours (Declaratory patent of Ukraine for a utility model № 143070 IPC (51) C12N 1/20; applicant and patent holder National Research Center "Institute of Experimental and Clinical Veterinary Medicine"; stated 21.01.2020 (u 2020 00341); publ. 10.07.2020, 13/2020. 4). Before the start of the study, the intensity of bacterial luminescence in the "working" suspension was determined to be in the range exceeding the background value of the device by 25–150 times. To obtain reliable results, the intensity of the glow must exceed the background indicator of the device by 25–250 times.

Luminous intensity of *P. phosphoreum* was examined on an EMILITE luminometer 1003 A (BioChemMac, Russian Federation). During testing, the exposure time was noted and changes in luminous intensity were recorded on the instrument after 20–25 minutes. Measurements were performed in pairs of control-experiment. The spectral range of the luminometer is 350–950 nm. To obtain reliable values, we examined six replicates of control and experimental samples.

To quantify the degree of toxicity of the sample relative to the effect of different levels of mycotoxins on the luminescence of bacteria *P. phosphoreum*, we used the toxicity index (T) as a dimensionless value equal to the ratio (formula 1):

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

where:

$I_0$ 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 conclusion of the toxicity of mycotoxins for the bacterium *P. phosphoreum* was made on the basis of the degree of inhibition of their luminescence under the influence of various doses, *i.e.,* the decrease in the intensity of bioluminescence is proportional to the toxic effect of the mycotoxin.

The toxicity index T reflects the concentration of mycotoxin that causes cessation of biosensor luminescence (*P. phosphoreum*) at a fixed exposure time of the test sample. As the upper limit indicator, a 50% decrease in the luminescence intensity of bacteria compared to the control was determined, which is displayed by a toxicity index of 50. This allows classifying samples with a toxicity index of 50 and higher as "highly toxic". The lower limit of the toxicity index is 20, which means that the luminescence of bacteria is reduced by 20% compared to the control and allows samples with a toxicity index of 20 or less to be classified as "non-toxic". All T values between 20 and 50 allow samples to be classified as "toxic" in which, if properly diluted, toxicity can be reduced.

*Statistical analysis.* The results were processed by variation statistics using the analysis of variance software package

**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 |

(ANOVA) StatPlus 5 (6.7.0.3) (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).

## Results

The effect of mycotoxin $T_2$ on the luminescence of *P. phosphoreum* depending on concentration is shown in Fig. 1. Under conditions of application of feed extracts with different levels of mycotoxin $T_2$ to the test culture *P. phosphoreum* at the 5th minute after application, glow suppression was observed at all levels: for 0.01 mg/kg of feed – not reliable (by 5.4%); for 0.05 mg/kg of feed – by 18.0% ($P$<0.05); for 0.1 mg/kg – by 31.9% ($P$<0.05); for 0.5 mg/kg – by 39.6% ($P$<0.05) and for 1.0 mg/kg of feed, the intensity of the glow decreased by 44.0% ($P$<0.05) relative to the control.

At the 10th minute of the experiment, an increase in luminescence was observed at the level of mycotoxin $T_2$ in feed 0.01 mg/kg (not reliable 0.8%), while at 0.05, 0.1, 0.5 and 1.0 mg/kg of feed luminous ($P$<0.05) by 11.6 %, 28.8%, 49.5% and 45.1%, respectively, relative to the control.

At the 15th minute of the experiment, an increase in luminescence was observed at $T_2$ mycotoxin levels of 0.01 and 0.05 mg/kg of feed by 33.0% and 16.0%, respectively ($P$<0.05), while at 0.1, 0.5 and 1.0 mg/kg, the luminous intensity decreased ($P$<0.05) by 17.5%, 34.6% and 39.4%, respectively, relative to the control.

A similar pattern was observed at the 20th minute of the experiment: increased glow at levels of mycotoxin $T_2$ of 0.01 and 0.05 mg/kg of feed by 37.2% and 20.8%, respectively ($P$<0.05), while at 0.1, 0.5 and 1.0 mg/kg luminous intensity decreased ($P$<0.05) by 15.7%, 37.2% and 43.6%, respectively, relative to the control.

At the 25th minute after the introduction of extracts from feed, it was found that at levels of mycotoxin $T_2$ of 0.01 and 0.05 mg/kg of feed *P. phosphoreum* luminous intensity increased by 38.8% and 20.7%, respectively ($P$<0.05), and at the levels of 0.1, 0.5 and 1.0 mg/kg of feed luminous intensity decreased ($P$<0.05) by 28.5%, 42.7% and 55.5%, respectively, relative to the control (Fig. 1).

The same trend was observed in the last study period (30 minutes): increase in luminous intensity ($P$<0.05) at the level of mycotoxin $T_2$ of 0.01 and 0.05 mg/kg of feed by 33.3% and 10.8% and decrease in luminous intensity ($P$<0.05) at levels 0.1, 0.5 and 1.0 mg/kg of feed by 33.0%, 46.2% and 63.7%, respectively, relative to the control (Fig. 1).

The percentage of reduction in the intensity of the luminous *P. phosphoreum* corresponded to the toxicity index, allowing for toxicological evaluation of feed with different levels of mycotoxin $T_2$. Thus, for the content of mycotoxin 0.01 and 0.05 mg/kg of feed, the toxicity index at the 20th – 25th minutes (recommended period of registration of fluorescence) was negative and averaged -38.0 and -20.8. At a concentration of 0.1 mg/kg of feed (MRL indicator) T was 22.1; for 0.5 mg/kg of feed it was 40.0; and for 1.0 mg/kg of feed, the average toxicity index was 49.6. The obtained data suggest that feeds with a mycotoxin $T_2$ content less than 0.01

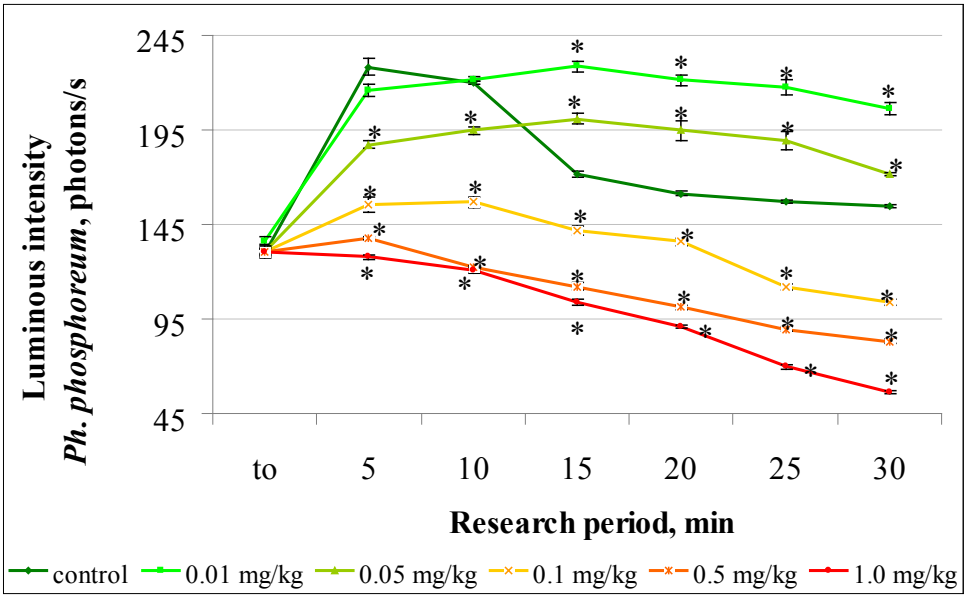


**Figure 1.** Dynamics of luminous intensity of *P. phosphoreum* under conditions of introduction of different doses of $T_2$ mycotoxin into feed (M±m; *n*=6, * – *P*<0.05 – relative to control).

and up to 0.05 mg/kg of feed are non-toxic (T less than 20), and at a content from 0.1 to 1.0 mg/kg inclusive, the feeds are toxic (T from 20 to 50).

The effect of mycotoxin ZON on the luminescence of *P. phosphoreum* depending on concentration is shown in Fig. 2. Under the conditions of application of feed extracts with different levels of mycotoxin ZON to the test culture *P. phosphoreum* at the 5th minute after application, we observed the suppression of luminescence at all levels (*P*<0.05): for 0.1 mg/kg of feed by 10.1%; for 0.2 mg/kg of feed by 33.7%; for 1.0 mg/kg by 40.2%; for 5.0 mg/kg by 33.7% and for 10.0 mg/kg of feed, the intensity of luminosity decreased by 55.0% relative to the control.

At the 10th minute of the experiment, there was an increase in luminescence at the level of ZON in feed 0.1 mg/kg (*P*<0.05) by 41.0%, and at the level of 0.2 mg/kg no reliable deviations in luminous intensity were observed (although it decreased by 11.4%), while for 1.0, 5.0 and 10.0 mg/kg of feed, the luminous intensity decreased (*P*<0.05) by 24.8%, 30.5% and 49.5%, respectively, relative to the control.

A similar dynamic was observed at the 15th minute of the experiment: at the level of ZON in the feed 0.1 mg/kg an increase in the luminescence *P. phosphoreum* was 13.3% (*P*<0.05), while at the levels of 0.2, 1.0, 5.0 and 10.0 mg/kg of feed the luminescence intensity decreased (*P*<0.05) by 32.6%, 26.5%, 51.0% and 61.2%, respectively, relative to the control.

At the 20th minute after administration of feed extracts at the level of ZON in the feed 0.1 mg/kg no reliable differences from the control were observed, and at the levels of mycotoxin 0.2, 1.0, 5.0 and 10.0 mg/kg luminous intensity decreased (*P*<0.05) by 43.2%, 52.3%, 73.9% and 79.5%, respectively, relative to the control.

At the 25th minute of the experiment at the level of ZON in the feed of 0.1 mg/kg we also did not observe reliable

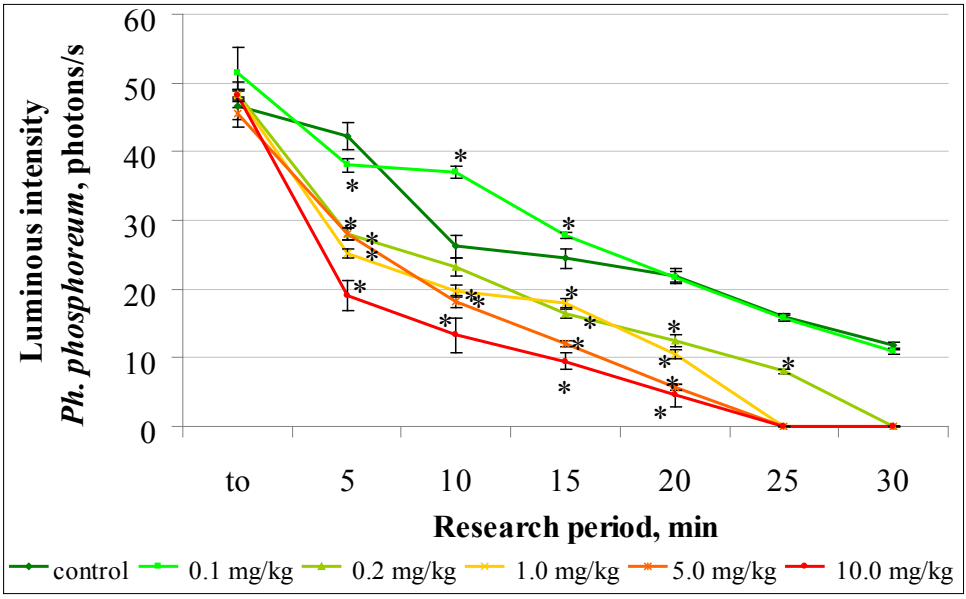


**Figure 2.** Dynamics of luminous intensity *P. phosphoreum* under conditions of administration in feed different doses of ZON (M±m; *n*=6, * – *P*<0.05 – relative to control).

differences in the intensity of the luminescence of *P. phosphoreum*. At 0.2 mg/kg mycotoxin in feed, the reliable luminous suppression was 50.0%, and at the levels of 1.0, 5.0 and 10.0 mg/kg at this period of time we recorded complete luminous suppression.

In the final period of the study, at the level of 0.1 mg/kg mycotoxin of feed, no reliable differences in the *P. phosphoreum* luminous intensity were observed, while at levels of ZON of 0.2, 1.0, 5.0 and 10.0 mg/kg we recorded complete luminous suppression (Fig. 2).

The percentage of the reduction in the *P. phosphoreum* luminous intensity corresponded to the toxicity index, which allowed for toxicological evaluation of feed with different levels of mycotoxin ZON. Thus, at a mycotoxin content of 0.1 mg/kg of feed, the toxicity index at 20–25$^{th}$ minutes (recommended registration time of fluorescence) was 1.4; for the content of 0.2 mg/kg of feed T was 46.6; for 1.0 mg/kg (MRL indicator) it was 76.2; for 5.0 mg/kg of feed, 87.0, and for 10.0 mg/kg of feed the average toxicity index was 89.8. The data obtained suggest that feeds with a mycotoxin ZON content of less than 0.1 mg/kg inclusive are non-toxic (T less than 20), for a content of 0.2 mg/kg of feed are toxic (T from 20 to 50), and for a content from 1.0 to 10.0 mg/kg inclusive are highly toxic (T is more than 50).

The effect of mycotoxin DON on the luminescence of *P. phosphoreum* depending on concentration is shown in Fig. 3. Under the conditions of administration of feed extracts with different levels of DON into the test culture *P. phosphoreum* at the 5$^{th}$ minute after application, we observed luminous suppression at all levels (*P*<0.05): for 0.05 mg/ kg of feed by 17.7%; for 0.1 mg/kg of feed by 36.9%; for 0.5 mg/kg by 61.3%; for 1.0 mg/kg by 63.3% and for 2.0 mg/kg of feed the luminescence intensity decreased by 65.2% relative to the control.

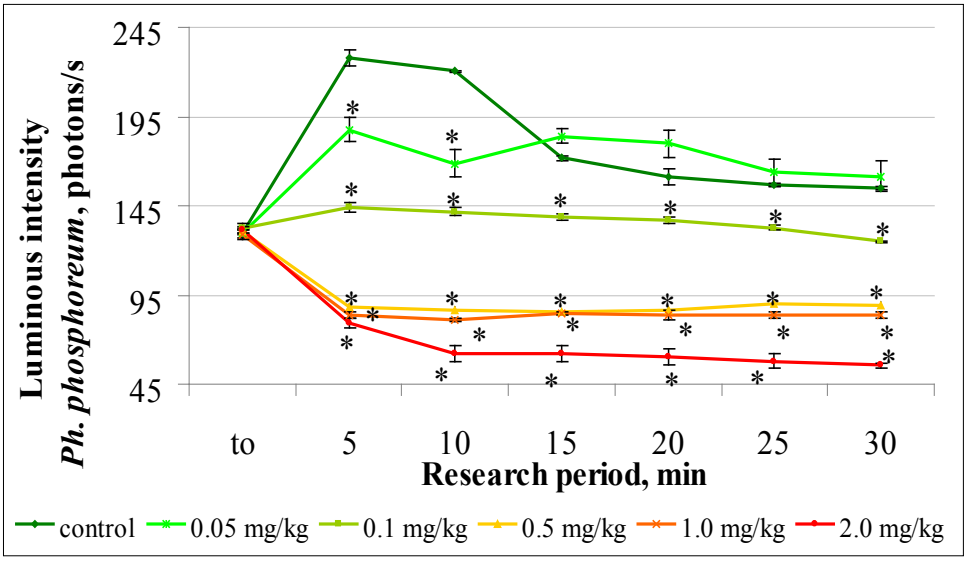


**Figure 3**. Dynamics of luminous intensity of *P. phosphoreum* under conditions of administration into the feed of different doses of DON (M±m; *n*=6, * – *P*<0.05 – relative to control).

At the 10th minute of the experiment, a similar picture was observed: for 0.05 mg/kg DON in the feed, the luminous intensity decreased by 23.5%; for 0.1 mg/kg of feed by 35.6%; for 0.5 mg/kg by 60.6%; for 1.0 mg/kg by 63.1% and for 2.0 mg/kg of feed the luminous intensity decreased by 71.6% relative to the control.

At the 15th minute of the experiment at the DON level of 0.05 mg/kg of feed there was an unreliable increase in luminosity (7.1%), while at mycotoxin levels of 0.1, 0.5, 1.0 and 2.0 mg/kg luminous intensity decreased (*P*<0.05) by 19.2%, 50.4%, 50.7% and 63.9%, respectively, relative to the control.

At the 20th minute of the experiment at the DON level of 0.05 mg/kg of feed, an unreliable increase in the luminous intensity of *P. phosphoreum* (11.5%) was observed, while at mycotoxin levels of 0.1, 0.5, 1.0 and 2.0 mg/kg, the luminescence intensity decreased (*P*<0.05) by 15.2%, 46.5%, 48.2% and 62.5%, respectively, relative to the control.

At the 25th minute of the experiment at the DON level of 0.05 mg/kg of feed we observed an unreliable increase in the *P. phosphoreum* luminous intensity (4.3%), while at mycotoxin levels of 0.1, 0.5, 1.0 and 2.0 mg/kg the luminescence intensity decreased (*P*<0.05) by 15.3%, 42.6%, 46.6% and 63.0%, respectively, relative to the control (Fig. 3).

The dynamics of luminescence was similar at the 30th minute of the experiment: at the DON level of 0.05 mg/kg of feed there was an unreliable increase in the *P. phosphoreum* luminous intensity (4.4%), while at mycotoxin levels of 0.1, 0.5, 1.0 and 2.0 mg/kg, the luminous intensity decreased (*P*<0.05) by 19.4%, 42.3%, 45.9% and 64.1%, respectively, relative to the control.

The percentage of reduction in the *P. phosphoreum* luminous intensity corresponded to the toxicity index, allowing for toxicological evaluation of feed with different levels of the mycotoxin DON. Thus, at a mycotoxin content of 0.05 mg/kg of feed, the toxicity index at

the 20th – 25th minutes (recommended time of registration of fluorescence) was negative and averaged -7.9; at the content of 0.1 mg/kg of feed T was 15.3; for 0.5 mg/kg it was 44.6; for 1.0 mg/kg of feed (MRL) it was 47.4, and for 2.0 mg/kg of feed the average toxicity index was 62.8. The obtained data suggest that feeds with a DON mycotoxin content from less than 0.05 to 0.1 mg/kg inclusive are non-toxic (T less than 20), with a content of 0.5 to 1.0 mg/kg of feed are toxic (T from 20 to 50), and with a content of 2.0 mg/kg inclusive the feed are highly toxic (T higher than 50).

The effect of mycotoxin OTA on the luminescence of *P. phosphoreum* depending on concentration is shown in Fig. 4. Under conditions of administration of feed extracts with different levels of mycotoxin OTA into the test culture *P. phosphoreum*, at the 5th minute after administration, a slight increase in the intensity of the luminescence was observed for 0.005 mg/kg of feed, while at the other levels luminous suppression was observed ($P$<0.05): at 0.01 mg/kg of feed by 5.7%; for 0.05 mg/kg by 11.5%; for 0.1 mg/kg by 12.7% and for 0.5 mg/kg of feed by 43.0% relative to the control.

At the 10th minute of the experiment at the level of OTA of 0.005 mg/kg of feed we observed a slight luminous suppression, while at other levels we observed a reliable suppression of luminescence ($P$<0.05): for 0.01 mg/kg of feed by 6.4%; for 0.05 mg/kg by 10.2%; for 0.1 mg/kg by 23.4% and for 0.5 mg/kg of feed by 44.5% relative to the control.

On the 15th minute of the experiment at mycotoxin levels in feed of 0.005, 0.01 and 0.05 mg/kg we recorded a reliable increase in the *P. phosphoreum* luminous intensity ($P$<0.05) by 25.9%, 18.0% and 5.8%, respectively, while at the levels of 0.1 and 0.5 mg/kg of feed, on the contrary, the decrease was by 8.6% and 39.7% ($P$<0.05) relative to the control.

The dynamics of the luminous intensity at the 20th minute of the experiment was

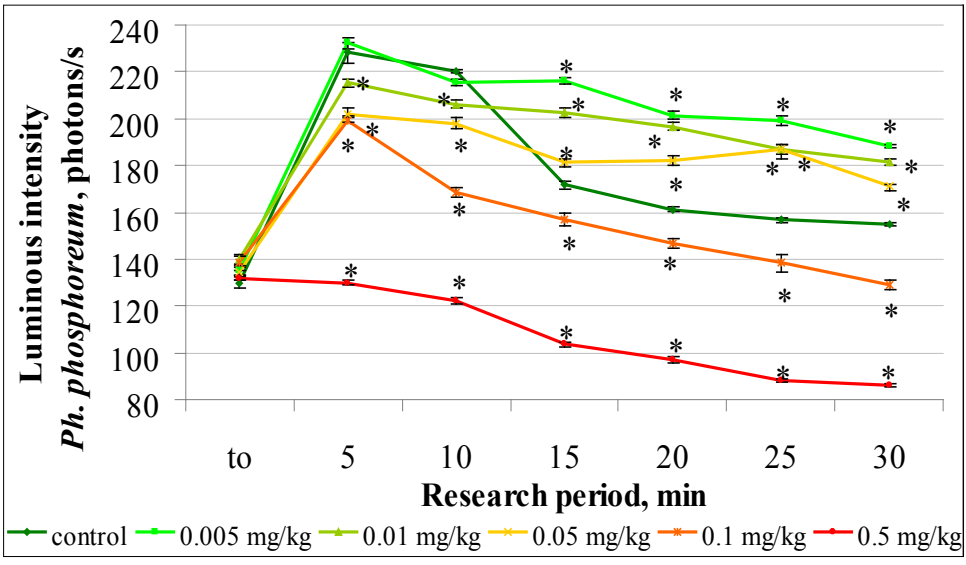


**Figure 4.** Dynamics of *P. phosphoreum* luminous intensity under conditions of administration of different doses of OTA into the feed (M±m; $n$=6, * – $P$<0.05 – relative to control).

similar: at OTA levels in feed of 0.005, 0.01 and 0.05 mg/kg, a reliable increase ($P$<0.05) in the *P. phosphoreum* luminous intensity was recorded by 25.0%, 22.0% and 12.9%, respectively, while at the levels of 0.1 and 0.5 mg/kg of feed, on the contrary, the decrease was by 9.0% and 39.8% ($P$<0.05) relative to the control.

At the 25$^{th}$ minute of the experiment at OTA levels in feed of 0.005, 0.01 and 0.05 mg/kg, we recorded a reliable increase ($P$<0.05) in the *P. phosphoreum* luminous intensity by 27.1%, 19.3% and 19.1%, respectively, while at the levels of 0.1 and 0.5 mg/kg of feed, on the contrary, the decrease was by 11.8% and 43.7% ($P$<0.05) relative to the control.

In the final period of the study, at the levels of OTA in feed of 0.005, 0.01 and 0.05 mg/kg we recorded a reliable increase in *P. phosphoreum* luminous intensity ($P$<0.05) by 21.8%, 17.1% and 10.7%, respectively, while at the levels of 0.1 and 0.5 mg/kg of feed, on the contrary, the decrease was by 16.6% and 44.4% ($P$<0.05) relative to the control (Fig. 4).

The percentage of the reduction in the *P. phosphoreum* luminous intensity corresponded to the toxicity index, allowing for toxicological evaluation of feed containing different levels of the mycotoxin OTA. Thus, at the content of mycotoxin of 0.005, 0.01 and 0.05 (MRL) mg/kg in feed, the toxicity index at the 20$^{th}$–25$^{th}$ minutes (recommended time for fluorescence registration) was negative and averaged -26.0, -20.7 and -16.0; and at a content of 0.1 and 0.5 mg/kg of feed, the average toxicity index was 10.4 and 41.8. The obtained data suggest that feeds with a content of mycotoxin OTA from less than 0.005 to 0.1 mg/kg inclusive are non-toxic (T less than 20), and with a content of 0.05 mg/kg are toxic (T from 20 to 50).

The effect of mycotoxin FB on the *P. phosphoreum* luminescence depending on concentration is shown in Fig. 5. Under conditions of administration of feed extracts with different levels of mycotoxin FB to the test culture *P. phosphoreum* on the 5$^{th}$ minute after administration at 0.5–10.0 mg/kg of feed, we observed an increase in the luminous intensity, and at 0.5, 1.0 and 5.0 mg/kg reliable ($P$<0.05) (by 50.6%, 18.6% and 16.2%), and at the level of 10.0 mg/kg not probable (1.6%), while at the mycotoxin level of 15.0 mg/kg the luminous intensity was 11.7% lower than the control ($P$<0.05).

At the 10$^{th}$ minute of the experiment at a mycotoxin level of 0.5 mg/kg of feed, *P. phosphoreum* luminous intensity increased by 44.3% ($P$<0.05), at the levels of 1.0 and 5.0 mg/kg unreliable deviations from control were registered, while at the levels of 10.0 and 15.0 mg/kg of feed the luminous intensity was 18.3% and 35.3% lower than the control, respectively ($P$<0.05).

At the 15$^{th}$ minute of the experiment at a mycotoxin level of 0.5 mg/kg of feed, an increase in the *P. phosphoreum* luminous intensity by 35.1% ($P$<0.05) was observed, at the level of 1.0 mg/kg unreliable deviations from control were registered, while at the levels of 5.0, 10.0 and 15.0 mg/kg of feed the luminescence intensity was 13.2%, 41.6%, and 49.0% lower than the control, respectively ($P$<0.05) (Fig. 5).

At the 20$^{th}$ minute of the experiment at a mycotoxin level of 0.5 mg/kg of feed, an increase in the *P. phosphoreum* luminous intensity by 23.9% ($P$<0.05) was recorded, and at the levels of 1.0, 5.0, 10.0 and 15.0 mg/kg of feed the luminous intensity was 14.9%, 18.0%, 47.8% and 54.3% lower than the control, respectively ($P$<0.05).

At the 25$^{th}$ minute of the experiment at the level of mycotoxin of 0.5 mg/kg of feed, no reliable deviations were observed in the intensity of the *P. phosphoreum* luminescence, while at the levels of 1.0, 5.0, 10.0 and 15.0 mg/kg of feed the luminescence intensity was 20.5%, 22.7%, 52.2% and 56.8% lower than the control, respectively ($P$<0.05).

A similar pattern was recorded in the last period of the study: at the level of FB of 0.5 mg/kg of feed no reliable

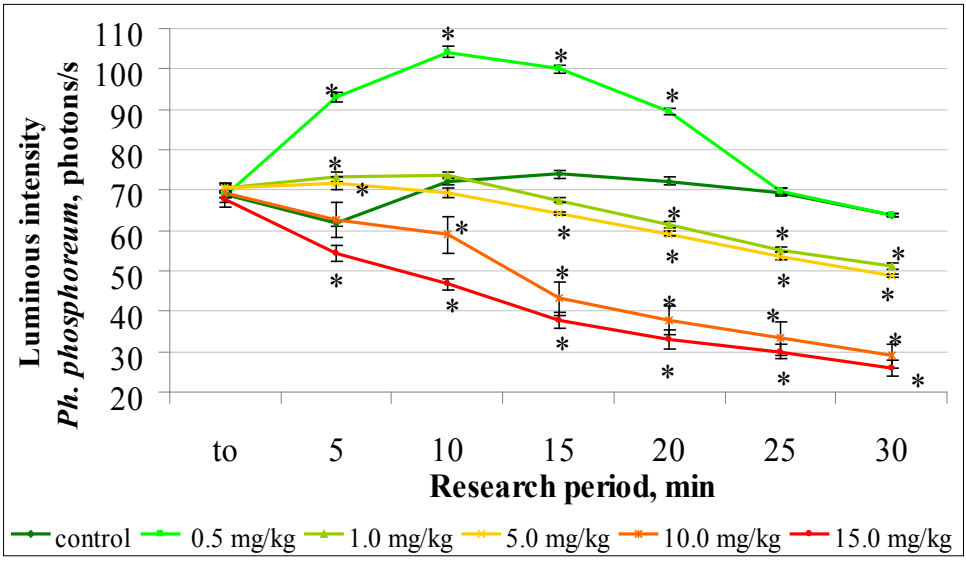


**Figure 5.** Dynamics of the *P. phosphoreum* luminous intensity under conditions of administration into the feed of different doses of FB (M±m; $n$=6, * – $P$<0.05 – relative to the control).

deviations were observed in the intensity of *P. phosphoreum* luminescence, and at the levels of 1.0, 5.0, 10.0 and 15.0 mg/kg of feed the luminescence intensity was 19.6%, 23.5%, 54.5% and 59.6% lower than the control, respectively ($P$<0.05) (Fig. 5).

The percentage of reduction in the intensity of the *P. phosphoreum* luminous intensity corresponded to the toxicity index, allowing for toxicological evaluation of feed with different levels of mycotoxin FB. Thus, at a mycotoxin content of 0.5 mg/kg of feed, the toxicity index on the 20$^{th}$ – 25$^{th}$ minutes (recommended period of registration of fluorescence indicators) was negative and averaged -12.1; and for the content of 1.0, 5.0 (MRL), 10.0 and 15.0 mg/kg of feed, the average toxicity index was 17.7, 20.3, 50.0 and 55.6, respectively. The data suggest that feeds with a content of mycotoxin FB from less than 0.5 to 1.0 mg/kg inclusive are non-toxic (T less than 20), with a content of 5.0 to 10.0 mg/kg are toxic (T 20 to 50) and 15.0 mg/kg are very toxic (T is more than 50).

The effect of mycotoxin AFB1 on *P. phosphoreum* luminescence depending on concentration is shown in Fig. 6. Under conditions of administration of feed extracts with different levels of mycotoxin AFB1 to the test culture *P. phosphoreum* in 5 min after administration at 0.001 and 0.005 mg/kg of feed, we observed an increase in the intensity of its fluorescence ($P$<0.05) by 24.2% and 21.0%, while at levels of mycotoxin of 0.01, 0.05 and 0.1 mg/kg luminous intensity was 19.3%, 48.2% and 46.4% lower than the control, respectively ($P$<0.05).

At the 10$^{th}$ minute of the experiment at AFB1 levels of 0.001 and 0.005 mg/kg of feed, an increase in *P. phosphoreum* luminous intensity ($P$<0.05) by 62.2% and 21.6% was recorded, while at mycotoxin levels of 0.01, 0.05 and 0.1 mg/kg the luminescence intensity was 24.9%, 49.1% and 56.9% lower than the control, respectively ($P$<0.05) (Fig. 6).

At the 15$^{th}$ minute of the experiment at the levels of mycotoxin of 0.001 and 0.005 mg/kg of feed, we observed an increase

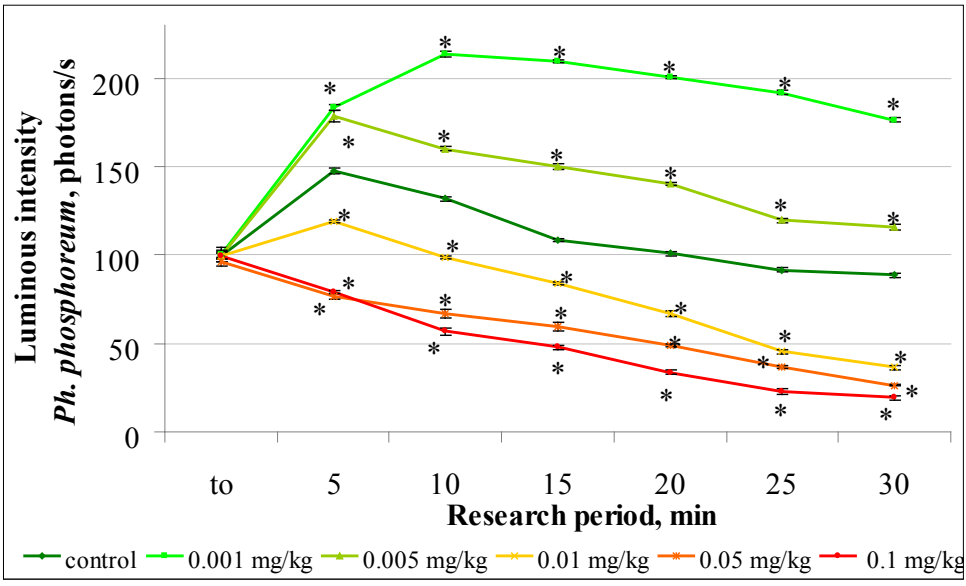


**Figure 6.** Dynamics of the *P. phosphoreum* luminous intensity under conditions of introduction into the feed of different doses of AFB1 (M±m; *n*=6, * - *P*<0.05 – relative to control).

in the *P. phosphoreum* luminous intensity (*P*<0.05) by 92.4% and 38.2%, while at mycotoxin levels of 0.01, 0.05 and 0.1 mg/kg the luminous intensity was 22.8%, 45.3% and 56.1% lower than the control, respectively (*P*<0.05).

At the 20$^{th}$ minute of the experiment at mycotoxin levels of 0.001 and 0.005 mg/kg of feed, we observed an increase in the *P. phosphoreum* luminous intensity (*P*<0.05) by 99.0% and 39.2%, while at mycotoxin levels of 0.01, 0.05 and 0.1 mg/kg the luminous intensity was 33.7%, 51.4% and 66.7% lower than the control, respectively (*P*<0.05).

At the 25$^{th}$ minute of the experiment at AFB1 levels of 0.001 and 0.005 mg/kg of feed, an increase in the *P. phosphoreum* luminous intensity (*P*<0.05) by 109.8% and 30.9% was recorded, while at mycotoxin levels of 0.01, 0.05 and 0.1 mg/kg the luminous intensity was 50.3%, 59.8% and 75.1% lower than the control, respectively (*P*<0.05).

A similar pattern was recorded in the last period of the study: at levels of AFB1 of 0.001 and 0.005 mg/kg of feed, an increase in the *P. phosphoreum* luminous intensity (*P*<0.05) by 99.4% and 30.8% was recorded, while at levels of mycotoxin of 0.01, 0.05 and 0.1 mg/kg the luminous intensity was 58.8%, 70.1% and 78.2% lower than the control, respectively (*P*<0.05) (Fig. 6).

The percentage of reduction in the *P. phosphoreum* luminous intensity corresponded to the toxicity index, allowing for toxicological evaluation of feed with different levels of mycotoxin AFB1. Thus, for the content of mycotoxin of 0.001 and 0.005 mg/kg of feed, the toxicity index in the 20$^{th}$–25$^{th}$ minutes (recommended time of registration of fluorescence) was negative and averaged -104.4 and 35.1; and at a content of 0.01 (MRL), 0.05 and 0.1 mg/kg of feed, the average toxicity index was 42.0, 55.6 and 70.9, respectively.

The obtained data suggest that feeds with a content of mycotoxin AFB1 from less than 0.001 to 0.005 mg/kg inclusive

are non-toxic (T less than 20), with a content of 0.01 mg/kg are toxic (T from 20 to 50) and higher than 0.05 mg/kg are highly toxic (T is more than 50).

## Discussion

The solution of current problems of production of high-quality livestock products lies in an integrated approach taking into account technological and veterinary-sanitary measures (Alders et al., 2021; Paliy et al., 2021). To this end, a number of modern technologies and veterinary drugs have been developed and introduced into production, taking into account regional and epizootological features (Oosting et al., 2014; Paliy et al., 2018; O'Mara et al., 2021). At the same time, the assessment of the quality and safety of animal feed at the current level becomes particularly relevant (Kuiper et al., 2015; Giraldo et al., 2019).

It should be noted that in the scientific literature, there is little data on the effect of mycotoxins isolated directly from feed on the luminescence of *Photobacterium phosphoreum*.

Yates and Porter (1982) were among the first researchers to discover the possibility of using the bacterial bioluminescence of *Photobacterium phosphoreum* for toxicological analysis for mycotoxins. They studied rubratoxin B, ZON, penicillic acid, citrinin, OTA, PR-toxin, AFB1, and patulin. The effect of the above mycotoxins on bioluminescence was determined after 5, 10, 15 and 20 minutes of incubation with bacterial suspensions. The concentration of rubratoxin B required to reduce the luminous intensity by 50% (half-maximal inhibitory concentration ($IC_{50}$) increased over time, while the concentration of citrinin, penicillic acid, patulin and PR-toxin decreased over time. The concentrations of ZON, AFB1, and OTA required for $IC_{50}$ over time became very low (Yates and Porter, 1982). Based on these studies, the first bioluminescent sensor system for the analysis of ecosystem pollution (Microtox®) was developed, but it was not adapted for toxicological evaluation of animal feed.

The effect of T2-mycotoxin on *P. phosphoreum* luminescence was established (Katsev et al., 1999), namely, suppression of *P. phosphoreum* luminescence after 10 minutes of incubation of bacteria at the level of 50% at a toxin concentration of 12 μg/mL of test material, and the effect of mycotoxins AFB1 in the amount of 10 μg/mL and DON of 20 μg/mL on the luminescence of photobacteria was studied by Sarter et al. (2008) (however, using *Vibrio fischeri* as the test culture). The results indicated an inhibition of luminescence by AFB1 and enhancement by DON.

The inhibitory effect of various mycotoxins (AFB1, DON, ZON, T2-toxin and ochratoxin) on the luminescence of another test culture of photobacteria (*Aliivibrio fischeri*) was detected at the concentration of toxins 1–20 μg/mL. The tested bacterium was most sensitive to AFB1, to a lesser extent (but with pronounced inhibition of luminescence) relative to ZON, while other mycotoxins did not affect the fluorescence intensity of *A. fischeri* (Krifaton et al., 2010). This is consistent with our data regarding AFB1 and ZON and contrary to the results obtained for DON, T2-toxin and OTA, as they showed a pronounced inhibition of *P. phosphoreum* luminescence in the presence of their high content in feed.

The toxicity of fusaric acid mycotoxin produced by *Fusarium proliferatum* in banana fruit, was evaluated using a strain *Vibrio qinghaiensis* sp. Q67 by the inhibition of luminescence. Luminescent bacterial bioevaluation has been shown to be effective for the monitoring *F. proliferatum* fusaric acid (Li et al., 2012).

Strong correlations ($R^2$>0.98) were found between mycotoxin concentrations ($FB_1$, DON, ZON, OTA, patulin and

citrinin) and *V. qinghaiensis* sp. Q67 luminous intensity. In addition, *Fusarium proliferatum* ($IC_{50}$=17.49%) showed greater luminescence inhibition than *Fusarium semitectum* ($IC_{50}$=92.56%) or *Fusarium oxysporum* ($IC_{50}$=28.61%), which corresponded to the determined higher content of $FB_1$, $FB_2$ and DON measured by high performance liquid chromatography-tandem mass spectrometry (Jian et al., 2017). This indicates the possibility of photobacteria use for toxicological evaluation of feed contaminated with mycotoxins.

New strategies for detecting the presence of mycotoxins in the natural environment were presented by Efremenco et al. (2021) and described by García (2021). The authors compared the characteristics of rapid quantitative analysis of various mycotoxins (DON, OTA, patulin, sterigmatocystin and ZON) using acetyl-, butyrylcholinesterases and photobacterial strains of luminescent cells. The best bioindicators in terms of sensitivity and working range (μg/mL) were: *Photobacterium* spp. 17 for analysis for DON (0.8–89) and patulin (0.2–32); *Photobacterium* spp. 9.2 for analysis for OTA (0.4–72) and ZON (0.2–32).

It should be noted that the working concentrations of mycotoxins in the above literature sources were mainly on environmental objects (soil, water, etc.) and food products and were significantly higher than the content of mycotoxins in feed. Calculating the approximate concentration of mycotoxins in the final extract from feed samples in the present study, we get the following: for T2-toxin, the range was 0.005–0.5 μg/mL, for AFB1 0.0005–0.05 μg/mL, for DON 0.025–1.0 μg/mL, for ZON 0.05–5.0 μg/mL, for FBs 0.25–7.5 μg/mL and for OTA 0.0025–0.25 μg/mL.

Feed toxicity was confirmed for T2-toxin from 0.05 μg/mL, for AFB1 0.005 μg/mL, for DON 0.25 μg/mL, for ZON 0.1 μg/mL, for FBs 2.5 μg/mL and for OTA from 0.025 μg/mL. The concentrations of mycotoxins that cause inhibition of luminescence are not significant at all, which confirms the results obtained by Yates and Porter (1982). Comparing our results with those of Katsev et al. (1999) and Sarter et al. (2008), it was found that *P. phosphoreum* occurs at much lower concentrations of mycotoxins. The closest to the results obtained here are the data of Krifaton et al. (2010) (inhibition of luminescence at concentrations of 1–20 μg/mL), Efremenco et al. (2021) and García (2021) (depending on the mycotoxin, the inhibitory concentration is 0.2–89 μg/mL).

The results of this study confirm the possibility of using luminescent bacteria for the analysis of mycotoxin contamination of feed, which is a relevant and popular rapid test of laboratory diagnosis.

## Conclusions

Mycotoxins exhibit a strong inhibitory effect on the luminescence of *P. phosphoreum.* Feed toxicity using the bioluminescence effect was confirmed for T2-toxin at concentrations in the final feed extract from 0.05 μg/mL, for AFB1 0.005 μg/mL, for DON 0.25 μg/mL, for ZON 0.1 μg/mL, for FBs 2.5 μg/mL and for OTA from 0.025 μg/mL, which corresponded to their content in the feed of 0.1; 0.01; 1.0; 0.2; 5.0 and 0.05 mg/kg, respectively.

The luminescence of *Photobacterium phosphoreum* can be used for rapid (up to 30 minutes) toxicological evaluation of feed contaminated with mycotoxins, 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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# Toksikološka procjena hrane za životinje kontaminirane mikotoksinima uporabom luminiscentnih mikroorganizama *Photobacterium phosphoreum*

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Na bazi redukcije intenziteta luminiscencije uspostavljena je mogućnost uporabe luminiscentnih mikroorganizama *Photobacterium phosphoreum* (soj IMB B-7071; Sq3) za izražavanje (do 30 minuta) toksikološke procjene hrane za životinje kontaminirane mikotoksinima. Međutim, ako je za okratoksin A u uvjetima ispitivanja hrane za životinje s udjelom mikotoksina pri razini najvećih dopuštenih količina rezidua (MRL) (0,05 mg/kg) hrana za životinje okarakterizirana kao netoksična, tada je za T2-mikotoksin, deoksinivalenol, fumonizin i aflatoksin B1 pri MRL (0,1; 1,0; 5,0, odnosno 0,01 mg/kg) hrana za životinje okarakterizirana kao toksična, a za zearalenon (MRL 1,0 mg/kg) – kao visoko toksična, što ukazuje na potrebu dodatnog istraživanja da se ispitaju toksikološke karakteristike mikotoksina u tijelu laboratorijskih i produktivnih životinja, moguće s dodatnom revizijom (naniže) najvećih dopuštenih količina relevantnih kontaminanata u hrani za životinje u Ukrajini.

**Ključne riječi**: *bioluminiscencija, hrana za životinje, mikotoksini, toksičnost, Photobacterium phosphoreum*