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Study of acute toxicity of the biomass of *Cladophora* sp. and phycocyanin for *Daphnia magna*

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In the current economic conditions, an active search is conducted for cost-effective and well-balanced feeds and feed mixtures for fish farming. The development of functional feeds using biomass of wild algae is becoming a central focus in the fish feed production. Although these supplements offer a promising path to sustainable and environmentally friendly fish feeding, their impact on the ecosystem requires detailed research. The aim of the study was to determine the acute response of *Daphnia magna* to the influence of new feed products derived from the wild algae of the Baltic Sea, phycocyanin on its own, and their binary mixture. The study was conducted using the standard aquatic toxicology method. To establish the 48-hour LC_{50}, the probit analysis method was used. The results showed that *D. magna* is more sensitive to the effects of phycocyanin, with the following order of toxicity: phycocyanin > the mixture (1:1) > the biomass of *Cladophora* sp. The median lethal concentrations at 48 h were 100 mg/l, 920 mg/l, and 1720 mg/l, respectively. The obtained data allows to refer the tested compounds to low-hazard substances according to the Globally Harmonized System (GHS). The observed effects were compared with the effects of the mixture noticed in the toxic units (TU) model. In 48 hours, the mixture of the dry biomass of *Cladophora* sp. and phycocyanin at a ratio of 1:1 exhibited synergism. The obtained results will allow choosing the optimal ratio of the raw materials used in fish feeds without inflicting harm to the aquatic ecosystem and loss of their nutritional value.

Keywords: biomass of *Cladophora* sp., phycocyanin, toxicity

INTRODUCTION

Under the current economic conditions, the development of cost-effective and well-balanced feeds for fish nutrition is actively pursued. The use of novel feed additives of natural origin in fish feeding practices is one of topical issues for the fishing and food industries (Michalak et al., 2020)

The development of functional feeds on the base of wild algae biomass has emerged as

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a key point in the fish feed production. It is believed that in contrast to additives of chemical origin, feeds of natural origin are distinguished by their better environmental safety. Adding such natural additives to fish diets offers the advantage of the mitigation of the environmental risks associated with chemical additives. Nevertheless, recent studies emphasise the importance of the assessment of ecological implications of utilising natural feed additives derived from biomasses of wild algae in fish nutrition (Ahmad et al., 2022). While these additives offer a promising avenue for sustainable and environmentally friendly fish feeding practices, their impact on the ecosystem needs a detailed assessment. The use of unbalanced feeds can potentially induce metabolic disorders in the fish body, leading to a reduction in the energy content of the flesh and compromised fish resistance. In addition, the introduction of some substances into the environment can contribute to additional contamination, which in turn can exert adverse effects on the vital functions of fish and ecosystems. Therefore, the development of fish feeds should be accompanied with a toxicological assessment of their possible impacts on ecosystems.

Biotesting is currently considered an effective method for the evaluation of the potential hazards on ecosystems posed by chemical, physical, or biological factors. The use of *Daphnia magna Straus*, 1820 in toxicology studies dates back to 1944, when B. G. Anderson employed this crustacean to assess the toxicity of substances present in industrial effluents. The acute toxicity test protocols for *D. magna* were subsequently published in the 1960s (Persoone et al., 2009). Presently, the *D. magna* test is one of the most widely employed methods of assessing the toxicity of chemical compounds (Ahmed et al., 2023).

This test has several key advantages, including simplicity, short exposure time, sensitivity, and ease of culture maintenance. The simplicity of such testing is mainly related to the tiny size of *D. magna*. Despite midget size of *D. magna*, it is easily visible to the naked eye, which facilitates straightforward observation and quantification. Another significant benefit of chronic testing is the reproduction of *D. magna*, namely its ability to generate a relatively large number of generations during a short time (three generations during three weeks). Species of the genus *Daphnia* hold a crucial position in aquatic food chains, particularly concerning fish. Many countries mandate to test chemical compounds on various organisms before their production, release, or sale. *Daphnia* is recognised as a reference species for toxicological studies at international level (Olvera-Ramírez et al., 2010).

This genus belongs to the family Daphniidae, order Cladocera, class Branchiopoda, subclass Crustacea, and phylum Arthropoda. *Daphnia* is a typical inhabitant of lakes, puddles, and ponds and is thermophilic by nature. It is widespread in Europe, Asia, Africa, and North America, and has been extensively introduced into water bodies (Ebert, 2022). *D. magna* reproduces parthenogenetically under favourable conditions and rapidly attains a high level of its reproduction if environmental parameters are optimal. The maturation time of the species is six days at a temperature of 20 ± 2°C. Consequently, *D. magna* is readily available in most countries, and its environmental characteristics make it an effective and cost-efficient subject for cultivation.

Scientists have focused on the development of innovative functional compositions of fish feed from the biomass of wild algae *Cladophora* sp. and phycocyanin extracted from the biomass of wild cyanobacteria, as well as on the evaluation of their effect, in particular on *D. magna*.

Several studies explored the toxicity of phycocyanin for *D. magna*, revealing acute toxic effects when exposed to an aqueous extract of phycobiliproteins. In the study with aqueous phycobiliproteins extracts (phycoerythrin, phycocyanin, and allophycocyanin), which are taken from the crude extract obtained by cellular lysis of biomass *Pseudanabaena tenuis*, the average 48-hour LC_{50} was found to be 14.1 mg/l, indicating potential toxicity (Olvera-Ramírez et al., 2010). Moreover, phycocyanin can indirectly enter *D. magna* tissues from the intestine, with a bioavailability range of 21.6–32.8% (Lin et al., 2018).

It is reported that C-phycocyanin has many properties, such as anti-aggregant, hepatoprotective, antioxidant, anti-inflammatory, and cholesterol-lowering (Morya et al., 2023; Kannaujiya et al., 2023). A series of laboratory experiments were conducted to determine the antioxidant properties of C-phycocyanin compared to known compounds with antioxidant properties, the total content of flavonoids and antioxidant activity of the biomass of *Cladophora* sp. as the main components of the latest functional feeds (Agrawal et al., 2021; Hudz et al., 2022; Glinskaitė et al., 2023). However, the issue of the influence of these raw materials on ecosystems and their toxicological characteristics remains unclear. There are some reports connected to infections in freshwater fish and mussels caused by algae (*Chlorella* sp., *Chlorochytrium* sp., *Scenedesmus* sp., *Cladophora* sp.) (Hofbauer et al., 2021).

Despite the widespread application of *Cladophora* algae (Michalak et al., 2020), some studies addressed the impact of these algae on crustaceans as vital components of aquatic food chains.

While the utilisation of natural feed additives presents a favourable alternative (Singh et al., 2024), the intricacies associated with their impact on both fish health and the broader ecosystem need further investigation. This research is important for ensuring the sustainability and responsible implementation of algae-based fish feeds in the fishing and food industries.

The aim of our study was to evaluate the impact of the new feed products derived from the wild Baltic Sea algae, phycocyanin, and their mixtures on *D. magna* in laboratory conditions. The evaluation of the mixtures will allow clarifying the possible effects of synergism or antagonism between the components.

MATERIALS AND METHODS

The study was conducted according to the standard method of aquatic toxicology. The quality of the aquatic environment during the experiments adhered to the standardised requirements for biotesting with *D. magna*: oxygen saturation before the experiment reached 6.5 mg/l (whereas the requirement stipulates more than 6.0 mg/l), after the experiment 3.1 mg/l (the requirement being more than 2 mg/l); pH was within the range of 7.0–7.4 (in accordance with the requirements, pH should be in the range of 6.0–8.5), and the temperature was 18–20°C, namely, within the specified range of 20 ± 2°C.

Acute test on the reference toxicant

To assess the suitability of *D. magna* cultures for bioassays and their response to the reference toxicant, potassium dichromate ($K_2Cr_2O_7$) was used. Four solutions of potassium dichromate were prepared (0.50, 1.00, 1.5, 2.00 mg/l).

Acute toxicity test

Three acute toxicity tests were conducted: with *Cladophora* sp. biomass, phycocyanin, and a combination of *Cladophora* sp. biomass with phycocyanin at a ratio of 1:1 (Fig. 1).

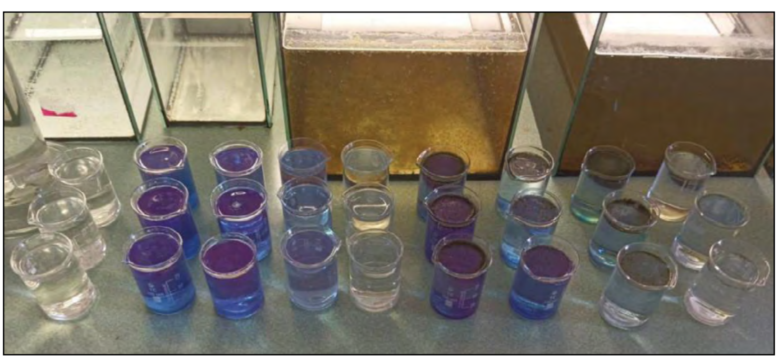

Fig. 1. Acute experiments on the tested products

D. magna at the age of less than 24 h old (neonates) from the third or fourth brood were used as test organisms. Before testing, the neonates underwent three 5-min rounds of washing using standard water. The test solutions were prepared using settled and aerated tap water for at least three days (for dechlorination and air saturation), which also served as a control. The experiment took place in a room free of toxic fumes or gases, in diffuse light, under conditions simulating natural day and night changes. The crustaceans were not fed during the experiment. The bioassay with *D. magna* was conducted in the measuring cups (V = 100 ml) at a temperature of 20 ± 2°C.

For each tested concentration, 30 organisms were used and divided into groups of ten individuals. Additional aeration of the aqueous solutions was not performed during the experiment as the maximum exposure of the test organisms did not exceed 96 h. The survival of the test organisms was visually assessed after 1, 6, 24, 48, 72, and 96 h of the exposure. The crustaceans that exhibited movement within the test tube for 15 s after gentle shaking were considered alive, while those that did not move were regarded dead. The LC_{50} values were calculated using graphs with a linear trend in mortality. Morbidity rates (%) were calculated by comparing the number of living *D. magna* in the control and experimental groups. These mortality percentages were transformed into statistical proportions, and graphs were plotted against the decimal logarithm of the concentrations of the test compounds.

The algorithm for the calculation of the average lethal concentration (LC_{50}). The percentage of the dead *D. magna* in the experiment compared to the control was expressed in conditional units – probits, and the concentration of the tested substance was expressed in decimal logarithms. The recalculation of the percentage of the dead *D. magna* into probits was carried out according to Finney's table (Finney et al., 1948). The average lethal concentration of the tested substance was found from the graph of the dependence of probits on the logarithms of concentrations (Microsoft Excel).

Data analysis and evaluation of the influence of joint toxicity

To determine the hazard category (class) of phycocyanin and the biomass of *Cladophora* sp. in the acute toxicity test on *D. magna*, we relied on the Globally Harmonized System of Classification and Labeling of Chemicals (GHS, 2006). The results were expressed as toxic units (TU) (Panouillères et al., 2007). The calculations of TU values for each component were based on the LC_{50} (mg/L) values obtained from the toxicity tests by the following formula:

$$TU = (1/LC_{50}) \times 100.$$

According to Personee et al. (Personee et al., 1993) and considering the values of TUs, there are following classes of hazard:

class I (TU = 0) 'no acute toxicity',
class II (0 < TU < 1) 'slightly toxic',
class III (1 < TU < 10) 'toxic',
class IV (11 < TU < 100) 'very toxic'.

To assess the combined effects of the binary mixture of phycocyanin and the dry biomass of *Cladophora* sp. on *D. magna* (Sprague, 1970; von der Ohe et al., 2013), the following formula was used:

$$TU = \frac{[A]}{LC_{50}[A]} + \frac{[B]}{LC_{50}[B]}$$

where [A] and [B] are the concentrations of compounds A and B connected with the LC_{50} of the mixture, and LC_{50}[A] (alone) (or LC_{50}[B] (alone)) is the LC_{50} of compound A (or B) when tested by itself.

If the sum of TUs equals 1.0, the toxicity is considered simply additive, i.e., the effects are as expected when each compound acts alone at its concentration. If the sum is less than 1, the compounds in the mixture interact antagonistically, resulting in an effect that is less than additive, where one component inhibits partially or fully the action of the other. Conversely, if the sum exceeds 1, the mixture demonstrates a synergistic effect, where the combined effect of two components is greater than additive interaction, indicating that one component potentiates the effects of the other (Wilbur et al., 2004).

RESULTS AND DISCUSSION

Acute test on the reference toxicant (potassium dichromate)

The LC_{50} at 48 h for the culture used in the experiments was determined to be 1.38 mg/l. The experimentally established response range of *D. magna*, within which the culture is considered suitable for testing, was 0.9–1.5 mg/l (Müller H. G., 1980). Therefore, the culture utilised in the experiments met the methodological requirements for biotesting (Fig. 2).

Mortality of *D. magna* under the influence of the biomass of *Cladophora* sp.

The acute toxicity of the biomass of *Cladophora* sp. was studied using 180 individuals of *D. magna* at the age of 24 h. We determined the toxicity of the biomass using its different concentrations in the aquatic environment: 10 g/l, 7.5 g/l, 5 g/l, 2.5 g/l, 0.5 g/l, 0 g/l (control). The mortality rate was calculated from the ratio of the dead individuals in the experiment to those in the control (Table 1).

For the period of 48 h, we calculated the value of the decimal logarithm of the concentration for probit 5 (Fig. 3). The lethal concentration by the antilogarithm was 1.72 g/l (1720 mg/l).

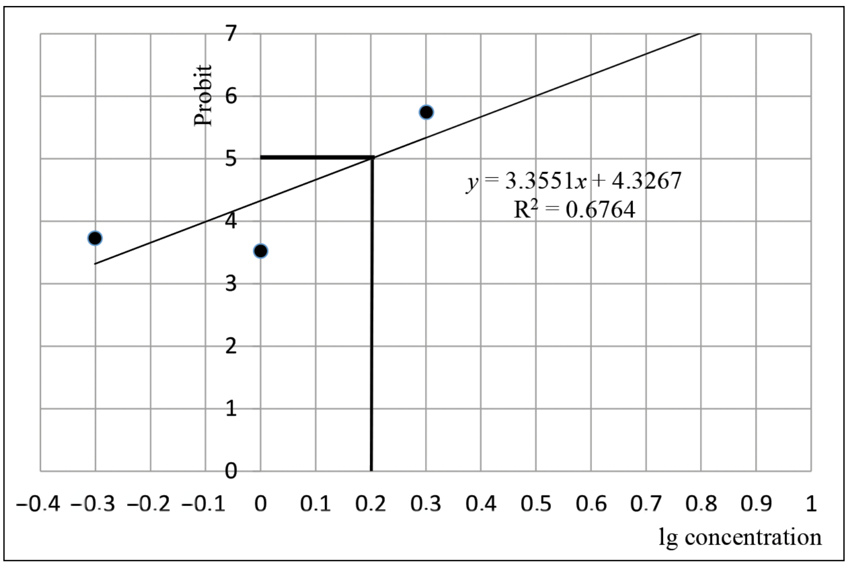

Fig. 2. A plot with a linear trend for the determinination of the LC_{50} at 48 h of the reference substance (potassium dichromate)

Table 1. **Average mortality of *D. magna* individuals relative to the control (%)**

Content, g/l	Duration of the experiment					
	1 hour	6 hours	24 hours	48 hours	72 hours	96 hours
0 (control)	0	0	0	**0**	0	0
10	0	37	100	**100**	100	100
7.5	0	20	100	**100**	100	100
5	0	23	87	**99.7**	100	100
2.5	0	3	20	**30**	77	100
0.5	0	0	10	**3**	0	12

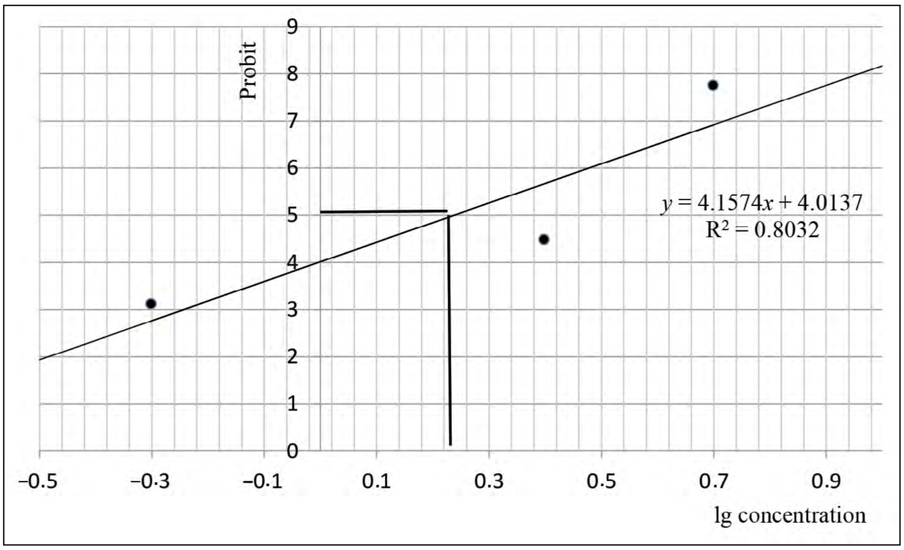

Fig. 3. A plot with a linear trend for the determination of LC_{50} 48 h of dry algae

It was determined that the concentration of 1.72 g/l of the studied dry algae is considered to be low-hazard toxic (GHS, 2006).

It should be taken into account that the suspended organic fraction, which includes *Cladophora* sp., is a key food source for many aquatic herbivore organisms, including zooplankton and fish. Thus, it can act as a source of pollution for water-filtering organisms. Supposedly, some pollutants can readily be adsorbed on the surface of algal cells, and, therefore, they can also be desorbed under certain conditions, for instance, in the digestive tract of some organisms (Long et al., 2019).

Mortality of *D. magna* under the influence of phycocyanin

We used 120 individuals of *D. magna* at the age of 24 h to study acute toxicity of phycocyanin. The toxicity of this substance was determined using its different concentrations in water (1 g/l, 0.5 g/l, 0.1 g/l, 0.05 g/l) compared to water.

Mortality of *D. magna* was calculated by the ratio of the dead individuals in the experiment to those in the control (water) (Table 2).

We observed mortality above 50% after 48 h of the experiment. For this period, we calculated the value of the decimal logarithm of the concentration for probit 5 (Fig. 4). Considering the LC_{50} at 48 h 0.10 g/L (100 mg/l), phycocyanin can be classified as a low-hazard substance according to the GHS (GHS, 2006).

It is clear that the tested substance becomes toxic after the beginning of its decay in water, i.e., two days after its release into the aquatic environment.

It should be noted that C-phycocyanin from spirulina, a protein of bright blue color, has recently become increasingly important. It is used

Table 2. **Average mortality of *D. magna* individuals relative to the control (%)**

Content, g/l	Duration of the experiment					
	1 hour	6 hours	24 hours	48 hours	72 hours	96 hours
0 (control)	0	0	0	**0**	0	0
1	0	0	0	**83**	100	100
0.5	0	0	0	**97**	100	100
0.1	0	0	0	**40**	57	63
0.05	0	0	0	**0**	13	17

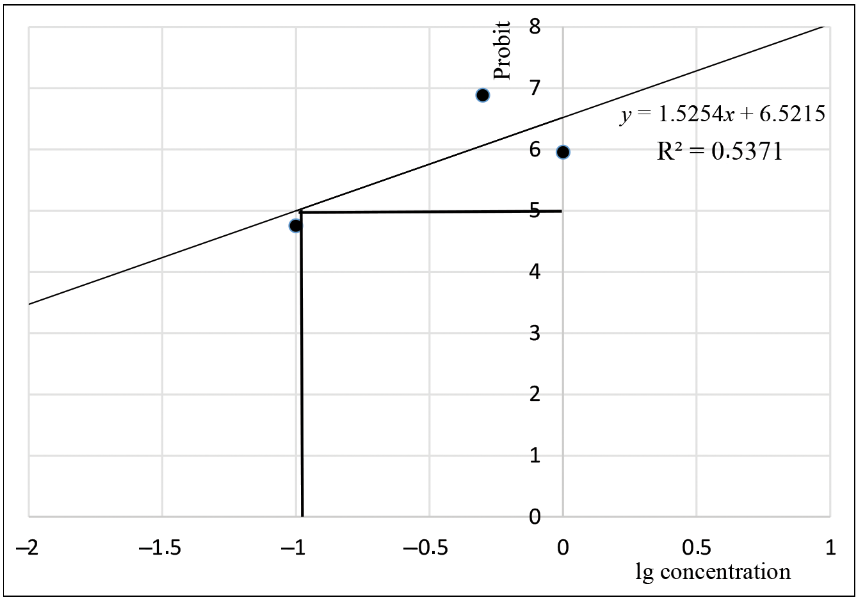

Fig. 4. A plot with linear trend for the determination of LC_{50} of phycocyanin

as a colorant for food products and for cosmetics. C-phycocyanin is reported to have many properties, such as antiplatelet, hepatoprotective, antioxidant, anti-inflammatory, and cholesterol-lowering (Kannaujiya et al., 2023; Morya et al., 2023). C-phycocyanin has weak antioxidant properties; moreover, pro-/antioxidant activities depend on its concentration (Hudz, 2022).

However, so far there are only few studies in the toxic effects of C-phycocyanin, and it is certain that further *in vivo* studies are needed to determine its low toxic potential for humans, animals, and the enviroment.

Mortality of *D. magna* under the influence of the biomass of *Cladophora* sp. and phycocyanin

We used 120 individuals of *D. magna* at the age of 24 h to estimate the acute toxicity of the mixture of phycocyanin and *Cladophora* sp. biomass at a ratio of 1:1. The total concentration of two components in water was 1 g/l, 0.5 g/l, 0.2 g/l, 0.1 g/l. Water was used as a control.

The ratio of the dead organisms in the experiment to those in the control was used to calculate their mortality (Table 3).

We observed mortality above 50% for 48 h of the experiment. For this period, we calculated the value of the decimal logarithm of the concentrations in probit 5 (Fig. 5). According to the antilogarithm, the lethal concentration (LC_{50} 48) was 0.92 g/L (920 mg/l). Therefore, the mixture belonged to low-hazard substances (GHS, 2006).

It is worth mentioning that the results of the experiment were reliable since there was no mortality in the control at the end of the experiment. In some cases, the mortality in the experimental tanks was more than 50%, which meets the requirements for biotesting.

Table 3. **Average mortality of *D. magna* individuals relative to the control (%)**

Concentration, g/l	Duration of the experiment					
	1 hour	6 hours	24 hours	48 hours	72 hours	96 hours
0 (control)	0	0	0	0	0	0
0.5 + 0.5	0	0	0	60	83	90
0.25 + 0.25	0	0	0	23	60	67
0.1 + 0.1	0	0	0	30	37	43
0.05 + 0.05	0	0	0	10	13	20

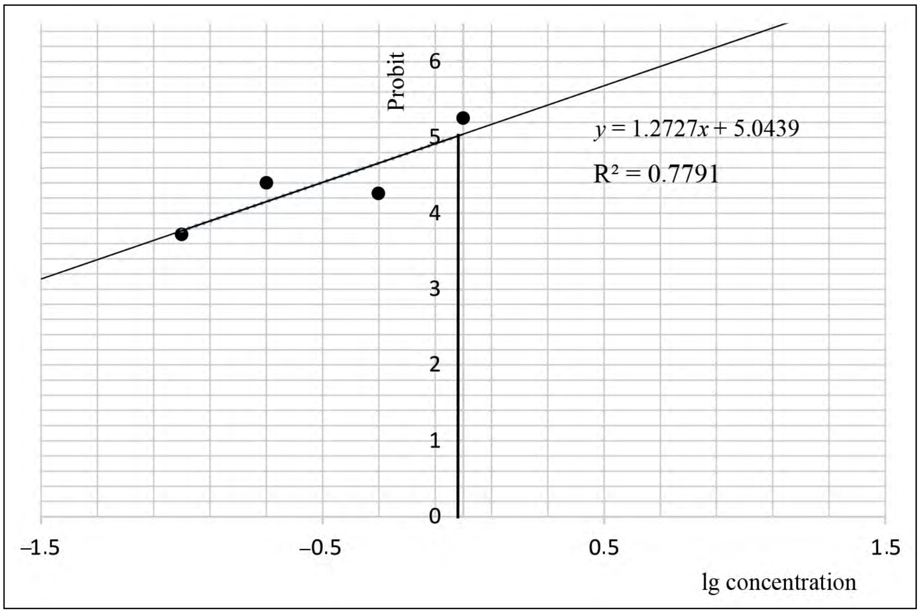

Fig. 5. A plot with linear trend for determining the LC_{50} of a mixture of phycocyanin and algae

Analysis of separate and combined effects of acute mixture exposure: the toxic unit approach

Since feed additives for fish could contain both dry algae biomass and phycocyanin, the assessment of the risk of this mixture requires considering their interactive effects. To understand these interactions, 48-hour LC_{50} values were determined both for the individual compounds and the binary mixture. Both dry algae biomass and phycocyanin, as well as their mixture, were classified under toxicity class III. The order of toxicity was as follows: phycocyanin > mixture > *Cladophora* sp. biomass (Table 4).

Based on the model of TU, the interaction between the mixture of the biomass of *Cladophora* sp. and phycocyanin at the ratio of 1:1 was greater than additive, namely, the mixture had a synergistic effect. According to the formula for combined effects of two components, we calculated a total TU for the mixture:

$$TU = \frac{460}{1720} + \frac{460}{100}.$$

Therefore, if the 48-hour LC_{50} value for each component was 460 mg/l (50% from 920 mg/l), then a calculated total TU of the mixture was 4.87. This total TU showed that the combined effect of the biomass of *Cladophora* sp. and phycocyanin is synergistic. This result is crucial for the assessment of the impact of these two components on aquatic ecosystems and other organisms as well.

Table 4. **The TU values of the biomass of *Cladophora* sp. and phycocyanin**

	***Cladophora* sp. biomass**	**phycocyanin**	**mixture**
LC_{50} (mg/l)	1720	100	920
TU	0.06	1.0	0.11

CONCLUSIONS

The results showed that *D. magna* is more sensitive to the effects of phycocyanin, with the following order of toxicity: phycocyanin > mixture (1:1) > biomass of *Cladophora* sp. The median lethal concentration values at 48 h were 100 mg/l, 920 mg/l, and 1720 mg/l, respectively. According to the LC_{50} value at 48 h, phycocyanin, mixture (1:1), and biomass of

Cladophora sp. can be classified as low-hazard substances.

The application of the concept of toxic units revealed that the mixture of phycocyanin and dry biomass of *Cladophora* sp. shows synergism against *D. magna*.

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CLADOPHORA SP. BIOMASĖS IR FIKOCIANINO ŪMAUS TOKSIŠKUMO *DAPHNIA MAGNA* TYRIMAS

Santrauka

Dabartiniu metu intensyviai ieškoma ekonomiškai efektyvių ir subalansuotų pašarų bei pašarų mišinių žuvininkystei. Pagrindinis dėmesys skiriamas žuvų funkcinių pašarų, naudojant laukinių dumblių biomasę, gamybai. Nors šie priedai atrodo patraukli tvarių ir aplinkai palankių žuvų pašarų galimybė, reikalingi išsamūs jų poveikio ekosistemai tyrimai. Tyrimo tikslas buvo nustatyti ūmų *Daphnia magna* atsaką į naujų pašarų prototipus, sukurtus iš Baltijos jūros laukinių dumblių *Cladophora* sp., fikocianino, išskirto iš melsvabakterijų, ar jų mišinio (1:1). Tyrimas atliktas naudojant standartinį vandens toksikologijos metodą. LC_{50} (vidutinės mirtinos koncentracijos) nustatyti 48 valandas buvo taikomas probito analizės metodas. Rezultatai rodo, kad *D. magna* yra jautresnė fikocianinui, nustatyta tokia toksiškumo seka: fikocianinas >> mišinys (1:1) > *Cladophora* sp. biomasė. Įvertintos vidutinės mirtinos koncentracijos reikšmės po 48 valandų: 100 mg/l, 920 mg/l ir 1720 mg/l atitinkamai. Gauti duomenys leidžia tirtas medžiagas priskirti mažo pavojingumo medžiagoms pagal Globaliai suderintą cheminių medžiagų klasifikavimo ir ženklinimo sistemą (GHS). Mišinio toksiškumas buvo įvertintas ir pagal toksinių vienetų (TU) modelį. Po 48 valandų nustatytas *Cladophora* sp. sausos biomasės ir fikocianino mišinio (1:1) sinerginis poveikis. Gauti tyrimo rezultatai padės pasirinkti optimalų žaliavų santykį žuvų pašaruose neprarandant jų maistinės vertės ir nekenkiant vandens ekosistemai.

Raktažodžiai: *Cladophora* sp. biomasė, fikocianinas, toksiškumas