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Use of Locomotor Activity of Rainbow Trout Juveniles in Identifying Sublethal Concentrations of Landfill Leachate

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Abstract**—**Landfill waste is a common problem as it has an economic and environmental impact even if it is closed. Landfill waste contains a high density of various persistent compounds such as heavy metals, organic and inorganic materials. As persistent compounds are slowly-degradable or even non-degradable in the environment, they often produce sublethal or even lethal effects on aquatic organisms. The aims of the present study were to estimate sublethal effects of the Kairiai landfill (WGS: 55°55‘46.74“, 23°23‘28.4“) leachate on the locomotor activity of rainbow trout *Oncorhynchus mykiss* juveniles using the original system package developed in our laboratory for automated monitoring, recording and analysis of aquatic organisms' activity, and to determine patterns of fish behavioral response to sublethal effects of leachate. Four different concentrations of leachate were chosen: 0.125; 0.25; 0.5 and 1.0 mL/L (0.0025; 0.005; 0.01 and 0.002 as part of 96-hour LC50, respectively). Locomotor activity was measured after 5, 10 and 30 minutes of exposure during 1-minute test-periods of each fish (7 fish per treatment). The threshold-effect-concentration amounted to 0.18 mL/L (0.0036 parts of 96-hour LC50). This concentration was found to be even 2.8-fold lower than the concentration generally assumed to be "safe" for fish. At higher concentrations, the landfill leachate solution elicited behavioral response of test fish to sublethal levels of pollutants. The ability of the rainbow trout to detect and avoid contaminants occurred after 5 minutes of exposure. The intensity of locomotor activity reached a peak within 10 minutes, evidently decreasing after 30 minutes. This could be explained by the physiological and biochemical adaptation of fish to altered environmental conditions. It has been established that the locomotor activity of juvenile trout depends on leachate concentration and exposure duration. Modeling of these parameters showed that the activity of juveniles increased at higher leachate concentrations, but slightly decreased with the increasing exposure duration. Experiment results confirm that the behavior of rainbow trout juveniles is a sensitive and rapid biomarker that can be used in combination with the system for fish behavior monitoring, registration and analysis to determine sublethal concentrations of pollutants in ambient water. Further research should be focused on software improvement aimed to include more parameters of aquatic organisms' behavior and to investigate the most rapid and appropriate behavioral responses in different species. In practice, this study could be the basis for the development and creation of biological early-warning systems (BEWS).

Keywords**—**Fish behavior biomarker, landfill leachate, locomotor activity, rainbow trout juveniles, sublethal effects.

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I. Introduction

LANDFILL sites are associated with a lot of adverse effects on the environment [1]-[5]. Landfill leachate is a liquid material formed when landfill waste decomposes. When entering ambient waters, landfill leachate may affect water quality because it changes the chemical composition of water and that of bottom sediments [6]. Moreover, leachate can disrupt the biological and nutritional balance between organisms or cause various long-term unpredictable changes in populations of the aquatic ecosystem [7].

Landfill waste contains various persistent compounds such as heavy metals, organic and inorganic materials, that are slowly degradable or even non-degradable, and. therefore, remain in the natural environment for a long-time [8], [9]. Heavy metals (i.e. copper, chrome, nickel, zinc, cadmium, mercury) that are found in leachate at higher concentrations are particularly toxic in their soluble forms that are able to oxygenate cells (e.g. hexavalent chrome), damage gill cells (e.g. zinc), disrupt the process of osmosis (e.g. mercury) leading to the depletion of inner organ cells [10]. In addition, the accumulation of metals in tissues of internal organs of the aquatic biota may cause various metabolic alterations and undesirable changes, and consequently, severe effects and health hazards [11]. Investigations into leachate toxicity and its effect on fish behavior showed that heavy metals may affect cells of fish gills and consequently, disorganize their behavior [12], [13]. Moreover, numerous studies have demonstrated that copper, cadmium, lead, and zinc are common metals that cause olfactory toxicity in fish leading to decreased sense of smell. Changes in the olfactory organs reduce the ability of fish to respond rapidly to pollutants [14]-[16]. Leachate metals may be bound by suspended organic matter that could reduce metal toxicity in water. In some cases, basic, unstable, acidic, and nonpolar organics could be the major toxicants in leachate [17]. Therefore, toxicity of landfill leachate depends on what has been deposited in the landfill for years and on leachate composition. The great diversity of effects that landfill leachate exerts on organisms has aroused a greater interest of scientists encouraging them to undertake investigations into toxicity effects of complex effluents on aquatic biota [17]-[19].

Individual pollutants in water usually do not exceed the maximum-allowable-concentration (MAC). However, due to the existing synergistic or antagonistic effects of toxicants, the combination of different chemicals produces a stronger effect on aquatic organisms than individual toxicants [20], [21].

Therefore, it is essential to determine the level of landfill leachate toxicity as a mixture of contaminants outgoing from closed landfills [22].

Toxicity assessment of complex effluents using only physicochemical measurements does not provide accurate and full information on the possible synergistic or antagonistic effects that landfill leachate may have on aquatic biota. Therefore, in order to assess water toxicity, it is necessary to carry out biological tests. Bioassays involve the use of the most sensitive aquatic species and their test-responses. Test-objects should be easy to culture and maintain under laboratory conditions, their behavioral parameters should be easy to record and be responsive not only to acute toxicity levels, but also to sublethal concentrations [22], [23].

Behavior of certain fish species is a highly sensitive biomarker of environmental water contamination [12], [24]-[29]. Fish locomotor behavior is defined as a variety of fish movements and their ability to move from one place to another [30]. Furthermore, movement may also involve "undirected' orientation or 'directed" locomotor response to a gradient of natural stimuli such as temperature, light, salinity, chemicals (i.e. pheromones, food odor) and also chemical toxicants in water [30]. Parameters of locomotion indicating fish movements such as mobility and swimming intensity can be easily recorded [30]. They are ecologically-significant responses to low sublethal concentrations of contaminants, allowing fish to detect (*detection response*) and avoid (*avoidance response*) contaminated sites [31], [32]. Integrated rapid behavioral responses observed when conducting a complex test in a short-period of time allow determining sublethal effects of contaminants or evaluating the level of water toxicity as well as calculating the expected effective and non-effective concentrations of certain chemicals [29], [33].

Unfortunately, biological tests designed to assess water quality based on fish behavior are still not standardized by the International Organization of Standards (ISO). However, behavioral responses of fish are sometimes reported to be 100 [34], [35] or even 1000 times [36] more sensitive than acute toxicity tests and, therefore, are highly recommendable compared to standardized biological tests. The American Society and Testing Materials (ASTM) has developed the guide for fish behavioral response measurement during standard laboratory toxicity tests, which, however, contains only some general information on methods for qualitative and quantitative fish behavior assessment [37]-[39]. Unfortunately, standardization processes in Europe are still in the stage of development.

New and more advanced systems and methods for monitoring, registration and analysis of fish behavioral responses have been developed over the last decades [30], [31], [40]-[46]. The novelty of the current study lies in the application of the original system package developed for the automatic monitoring, recording and analysis of behavioral response patterns in aquatic organisms. The system software is designed to convert the video record of fish behavior into a digital form, which facilitates further data processing and analysis. In practice, digitized data analysis can be useful for modeling with several different parameters such as fish response to stimuli and environmental factors.

For the experiments, we chose the rainbow trout (*Oncorhynchus mykiss,* Walbaum, 1792) that is very sensitive to aquatic pollutants, especially in early stages of its development [25], [26], [28]. The sensitivity of a test *species* is usually compared with that of a *standard species* (e.g. *rainbow trout*) [47], [48].

The aim of the present study was to experimentally evaluate the effect of the Kairiai landfill leachate at sublethal concentrations on the rainbow trout behavior based on the locomotor activity analysis (1); to determine the threshold-effect-concentration (TEC) of leachate for test fish (2); to compare the data obtained with the results of acute toxicity tests (3); to determine behavioral response dependence on leachate concentration and exposure duration (4); to establish patterns of fish behavioral response to sublethal effects of leachate (5).

II. Materials and Methods

The tests were conducted on artificially bred (Meškerinė hatchery, Svenčionys District, Lithuania) 1-2 month old rainbow trout juveniles. The average length of test fish was 6.45 ± 0.63 mm and the total weight was 3.90 ± 0.38 g (mean ± S.E.M., n = 28, respectively).

The Kairiai landfill leachate (Šiauliai District, Lithuania, WGS: 55°55'46.74 "23°23'28.4") was used as a toxicant. Leachate samples were collected in July, 2014. Physicochemical parameters of the investigated leachate were as follows: anions: Cl^- (1 889), SO_4^{2-} (60.4), HCO_3^- (1,930), CO_3^{2-} (8.71), NO_2^- (2.89), NO_3^- (<0.050); cations: Ca^{2+} (1,196), Mg^{2+} (582), K^+ (90.9), Na^+ (103), NH_4^+ (100) mg/L, respectively; heavy metals: Cu^{2+} (2), Zn^{2+} (76), Ni^{2+} (100), Cr^{6+} (620), Pb^{2+} (3), Hg^{2+} (0.2) µg/L, respectively; pH = 8.45, electrical conductivity 9,450 µS/cm at 25 °C, permanganate index 234 mg O/L, total dissolved solids 5,963 mg/L. The physicochemical parameters of leachate were determined using standardized procedures [49]-[56].

TABLE I
The Ratios of Leachate Dilution

Dilution ratio	mL/L	Fraction of 96-hour LC50
1:8000	0.125	0.0025
1:4000	0.25	0.005
1:2000	0.5	0.01
1:1000	1.0	0.02

Deep-well water was used as dilution water. The main physicochemical characteristics of the water were as follows: hardness 271-296 mg/L (as $CaCO_3$), alkalinity 190-210 mg/L (as $CaCO_3$), pH 7.9 to 8.1, dissolved oxygen content was 8-10 mg/L and dissolved organic carbon (DOC) concentrations were below the detection limit of the device (<0.3 mg/L).

Four different sublethal concentrations of leachate were chosen based on the median-lethal-concentration (96-hour LC50) to rainbow trout juveniles of 50 (27.52 – 90.84) mL/L, which had been derived from acute toxicity tests performed under the same laboratory conditions [57]. Leachate dilution ratios are presented in Table I.

Each test fish was placed into a five liter experimental white fish tank (210 mm x 310 mm x 160 mm), filled with 2 liters of deep-well water cooled at a temperature of 12-13°C. Aeration was provided through an inner chamber, in which the fish was separated from the air-stone aerator by a stainless steel baffle (mesh size 1.5 mm). In the course of the test, the temperature of the cooled water was maintained constant. Before the beginning of the experiment, the fish were allowed 30 minutes to adapt to test conditions. Seven fish individuals were analyzed per concentration.

During the experiment, the locomotor activity of rainbow trout juveniles in control (clean water) and treated (with leachate) solution was recorded after 5, 10 and 30 minutes of exposure with a digital video camera (SONY Handycam® HDR-CX305E). The tracked video data were further processed using the original software (Fish tank monitor).

The locomotor activity was measured in ratios of coordinate values (X, Y) of object movements and expressed in relative value units of delta K (ΔK), which were calculated using the software. Higher ΔK values indicate an increased intensity of fish movements.

The sensitivity of behavioral responses was evaluated by determining the threshold-effect-concentration (TEC), which was calculated as the geometric mean of the lowest-observed-effect-concentration (LOEC) and the no-observed-effect-concentration (NOEC) [58].

The results were compared with the concentration that is theoretically defined as "safe" for fish [59] and is equal to 0.01 (1/100) as a fraction of 96-hour LC50 [the ratio between the maximum-acceptable-toxicant-concentration (MAC) and the median lethal concentration (LC50)], i.e. the concentration which does not cause any sublethal or lethal effects in fish.

The statistical analysis of the results was performed using the software STATISTICA 10 (StatSoft Inc., Tulsa, Oklahoma, USA). Significant differences between samples were tested using the parametric one-way analysis of the variance test (parametric one-way ANOVA, post-hoc Dunnett's test, $p < 0.05$).

TABLE II
LOCOMOTOR ACTIVITY (ΔK) OF RAINBOW TROUT JUVENILES AT DIFFERENT CONCENTRATIONS OF LANDFILL LEACHATE AND VARYING DURATION OF EXPOSURE

Leachate concentration (mL/L)	Exposure time (min)			
	Control	5	10	30
0.125	18.4±2.0 (201)	14.9±1.6 (197)	13.1±0.6* (199)	17.4±1.6 (196)
0.25	10.1±1.3 (204)	41.0±5.3* (195)	40.9±4.3* (204)	34.2±4.9* (198)
0.5	13.6±1.8 (206)	54.1±4.8* (232)	106.4±8.6* (220)	63.3±4.9* (216)
1.0	13.1±1.3 (265)	55.1±4.1* (284)	101.6±6.0* (260)	70.7±4.5* (259)

Note: Coordinate values (X, Y) were calculated in relative value units of ΔK (mean ± S.E.M., number of measurements (n) is given in brackets), (n – 7 fish per treatment); *Asterisks (*) indicate significant differences compared to control fish group (parametric one way ANOVA, post hoc Dunnett's test, $p < 0.05$).

III. RESULTS

The study into the locomotor activity of rainbow trout juveniles revealed number fish behavioral patterns. At the lowest leachate concentration (0.125 mL/L) the test fish demonstrated no relevant behavioral response, while at higher test concentrations, the intensity of locomotor activity reached a significant level and increased with the rising concentration of leachate. The data obtained are presented in Table II.

The lowest tested leachate concentration that did not elicit any relevant behavioral response in rainbow trout juveniles (Fig. 1) was 0.125 mL/L (0.0025 as a part 96-hour LC50). In general, the mean values (± S.E.M) of the rainbow trout locomotor activity showed no significant differences between treatments ($p > 0.05$), except after 10 minutes of exposure, when swimming intensity of fish juveniles decreased 1.4-fold compared with the control level. The intensity of locomotor activity of fish recorded after 5 minutes (14.9 ± 1.6 ΔK) and 30 minutes (17.4 ± 1.6 ΔK) of exposure was close to the control level. At this leachate concentration only one statistically significant difference was obtained, which could be explained by individual differences in the behavior of fish that had been randomly selected for the experiment from the stock. Generally, there was no evident change in fish locomotor activity observed compared to the control fish group. This concentration was defined as a no-observed-effect concentration (NOEC) ($p = 0.063$).

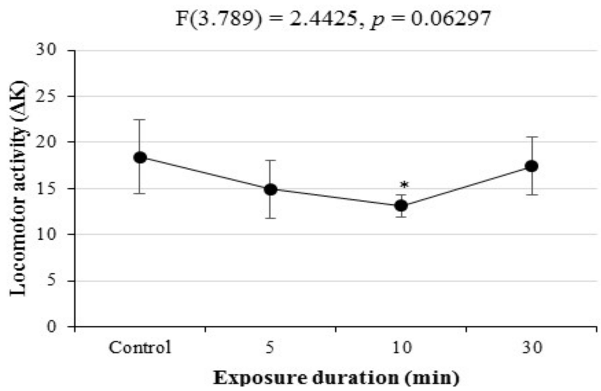

Fig. 1 Changes in locomotor activity (mean ± confidence intervals of 95%) of rainbow trout juveniles after 5, 10 and 30-minute exposure to the 0.125 mL/L (0.0025 as a fraction of 96-hour LC50) concentration of leachate; Asterisks (*) denote significant differences compared to the control fish group ($p < 0.05$)

During the test, part of rainbow trout juveniles was more responsive, and another one – less, so the mean values of the locomotor activity of the tested fish were not at zero levels. In fact, our original software (Fish tank monitor) can track any hardly conspicuous movements of fish from the recorded video data.

At the concentration of 0.25 mL/L (0.005 as a part 96-hour LC50), the intensity of locomotor activity reached a significant level and kept increasing throughout the exposure duration (Fig. 2). The comparison of behavioral response with the control fish group showed a relevant increase (4.6-fold) after 5 minutes (41.0 ± 5.3 ΔK) of exposure. Higher mean values were obtained after 10 and 30 minute (40.9 ± 4.3 ΔK and 34.2 ± 4.9 ΔK, respectively) exposure duration. Increased intensity of locomotor activity was considered to be a response of rainbow trout juveniles to altered environmental conditions and stimuli. In this case, fish response to the sublethal concentration of leachate was observed. This concentration was defined as the lowest-observed-effect-concentration (LOEC) ($p < 0.001$).

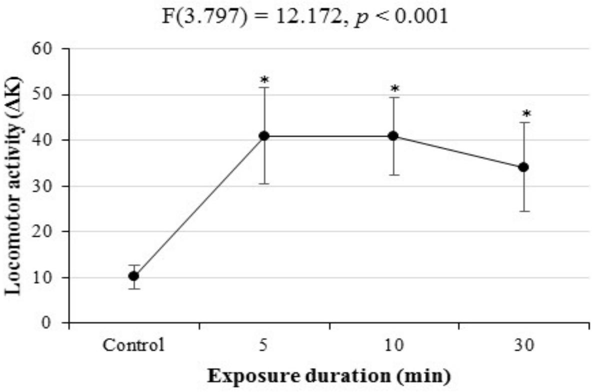

Fig. 2 Changes in locomotor activity (mean ± confidence intervals of 95%) of rainbow trout juveniles after 5, 10 and 30 minute exposure to the 0.25 mL/L (0.005 as a fraction of 96-hour LC50) concentration of leachate; Asterisks (*) denote significant differences compared to the control fish group ($p < 0.05$)

Exposure to the highest leachate concentrations of 0.5 and 1.0 mL/L (0.01 and 0.02 as a fraction of 96-hour LC50, respectively) revealed its sublethal effects on the behavior of rainbow trout juveniles (Figs. 3 and 4). Compared to the activity of the control fish, the locomotor activity of the test fish was increased, after 10 minutes of exposure reaching maximum values at both concentrations: 106.4 ± 8.6 ΔK (increased 7.8-fold) at 0.5 mL/L and 101.6 ± 6.0 ΔK (increased even 7.8-fold) at 1.0 mL/L at 1.0 mL/L, respectively. The locomotion of juveniles observed after 30 minutes of exposure decreased evidently, but remained significantly higher than the control results: at the concentration of 0.5 mL/L (0.01 96-hour LC50) the locomotor activity decreased to 63.3 ± 4.9 ΔK (4.7-fold higher than control) and at 1.0 mL/L (0.02 96-hour LC50) dropped to 70.7 ± 4.5 ΔK (5.4-fold higher than control).

The recorded differences in locomotion intensity could be explained by the mechanism of fish behavioral response to the effect of pollutants. The avoidance response and locomotor behavior are grouped under the same category of behavioral responses and are closely related [24], [60], [61]. Substances such as heavy metals, organic and inorganic materials that are found in landfill leachate irritate chemosensory (smell and taste) receptors of fish evoking avoidance response and increased locomotor activity, i.e. under these circumstances, stressed fish tend to bypass the contaminated area thus reducing exposure to it and avoiding stress stimuli [14]-[16]. The response to stress is considered to be an adaptive mechanism that allows fish to cope with real or perceived stress in order to maintain its normal or homeostatic state [62]. Fish adaptation to stress stimuli (e.g. sublethal concentrations) by biochemical and physiological self-regulation mechanisms allows fish to survive in altered conditions of the aquatic environment [63].

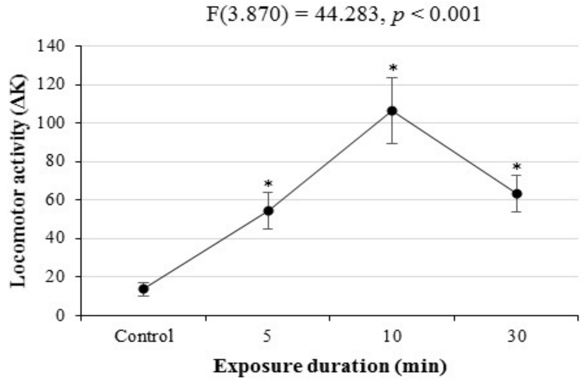

Fig. 3 Changes in locomotor activity (mean ± confidence intervals of 95%) of rainbow trout juveniles after 5, 10 and 30-minute exposure to the 0.5 mL/L (0.01 as a fraction of 96-hour LC50) concentration of leachate; Asterisks (*) denote significant differences compared to the control fish group ($p < 0.05$)

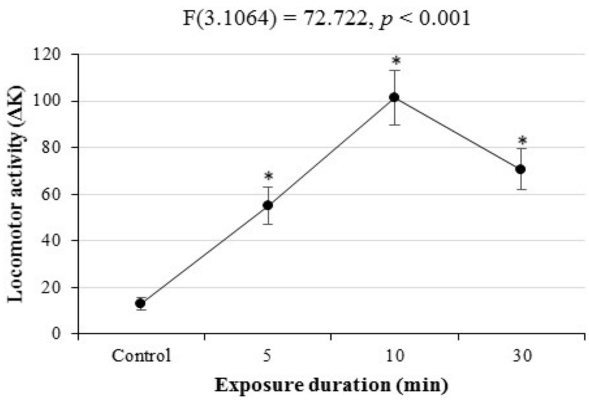

Fig. 4 Changes in locomotor activity (mean ± confidence intervals of 95%) of rainbow trout juveniles after 5, 10 and 30 minute exposure to the 1.0 mL/L (0.02 as a fraction of 96-hour LC50) concentration of leachate; Asterisks (*) indicate significant differences compared to the control fish group ($p < 0.05$)

Sensitivity of each endpoint of behavioral parameters should be compared with standard toxicological data, for example, the median lethal concentration [33], [60], [61], [64]. The concentration of pollutants theoretically defined as "safe" for fish as in [59] amounted to 0.01 (1%) part of 96-hour LC50. In this case, the "safe" concentration of landfill leachate should be 0.5 mL/L, when the established 96-hour LC50 is 50 mL/L. The estimated threshold-effect-concentration (TEC) of leachate is 0.18 mL/L, or it is equal to 0.0036 part of 96-hour LC50. The obtained TEC is 2.8-fold lower than the assumed "safe" concentration.

The test variables such as exposure duration, landfill leachate concentration and the locomotor activity in rainbow trout juveniles were used in combination for the creation of the 3D model. Results of the current study show that with increasing concentrations of leachate (0.5 and 1.0 mL/L, respectively) the locomotor activity of rainbow trout juveniles heightened showing significantly greater values than those of the control fish groups at higher concentrations but slightly decreased values with increasing exposure duration (Fig. 5). At the lowest tested concentration (0.125 mL/L), the effect of landfill leachate on locomotor activity was not exposure duration dependent. In general, the modeling of results showed a significant correlation between leachate concentration and exposure duration ($p < 0.001$).

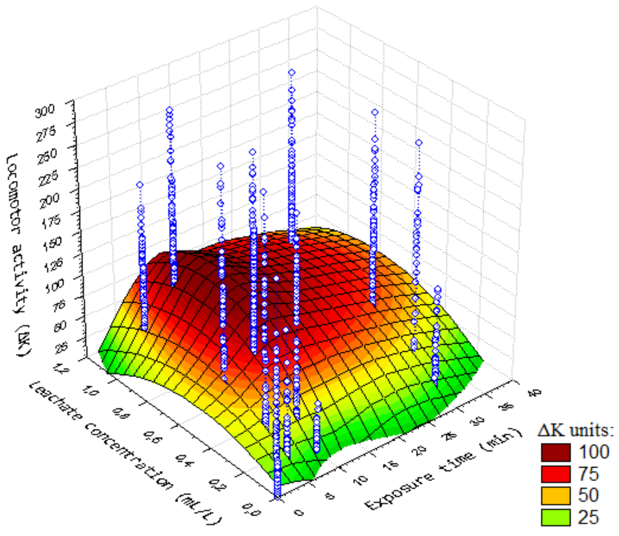

Fig. 5 Locomotor activity of *Oncorhynchus mykiss* juveniles after exposure to different landfill leachate concentrations, (n = 3,536)

IV. Discussion

The results obtained from the experiment confirm that fish behavior is quite a sensitive biomarker in the assessment of sublethal concentrations in aquatic environments. However, the bioassay using fish behavior as a biomarker has not been accepted yet as a standard by the international organization of standardization (ISO), although the American Society for Testing and Material (ASTM) has been working in this field and has developed a few quantitative and qualitative evaluation methods for fish behavior assessment. Besides, this organization was founded as the world leader in developing high standards for biological toxicity tests. Fish sensitivity to chemical contaminants may vary depending on environmental conditions in which fish live. As a rule, standardized toxicity tests are carried out to assess concentrations of pollutants that are hazardous or lethal to fish. In contrast, acute and chronic biological tests do not reveal toxic effects on fish at very low concentrations of contaminants. Therefore, fish behavior allows identifying sublethal concentrations of pollutants. Moreover, the lack of information on the range of effects produced by pollutants and on biological processes in fish may affect the distribution of fish populations in waters. In this case, it is necessary to review the threshold limit values set for chemical substances in the aquatic environment.

Our study confirms that fish behavior is a useful and appropriate biomarker in the assessment of water toxicity and even in setting new guidelines for the aquatic biota protection. The follow-up studies showed that relatively low concentrations of pollutants can be also environmentally-significant in biological tests. Toxicity investigations that are based only on the parameters traditionally used in standardized tests (death rate, reproduction, etc.) and that do not include fish behavioral parameters are not comprehensive and, therefore, debatable.

Biological early-warning systems (BEWS) have been developed based on the behavioral response of organisms making it possible to detect a wide range of contaminants and, thus, ensuring efficient water quality monitoring and management. However, an individual behavioral variation and a large amount of data non-linearity are inappropriate to BEWS [65]-[69]. To ensure fast processing and proper interpretation of data, it is also necessary to improve software. Thus, more detailed studies are needed for the application of fish behavior in practice. For this purpose, this study will be further extended to software improvement aimed at making it possible to include more behavioral parameters and to search for the most rapid and appropriate behavioral response of different aquatic species that may be suitable for BEWS.

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