

ECOTOXICOLOGICAL METHODS FOR MONITORING THE EFFECTS OF MICROPOLLUTANTS IN WATERS

C.2-Developments in site investigation and monitoring

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ABSTRACT

Three different aquatic testorganisms: *Daphnia magna* (water flea), *Lemna minor* (common duckweed) and *Heterocypris incongruens* were applied to measure the effect of three emerging pollutants. A pharmaceutical (Na-diclofenac), a pesticide (metazachlor) and a psychoactive compound (nicotine) were tested in different concentrations. Innovative toxicology endpoints were used to reach higher sensitivity. In case of *D. magna* the heartbeat rate was determined, in case of *L. minor* the total chlorophyll content and in case of *H. incongruens* the average velocity and the total distance of the movement. Results show, that these innovative toxicity endpoints are more sensitive, than the commonly applied endpoints. The moving behaviour and heart rate of crustaceans were the most sensitive endpoints, with clear effects observed even at the µg/L level of some pollutants (diclofenac, β-estradiol, nicotine, triclosane) revealing the sublethal stresses caused by exposure to these emerging pollutants.

INTRODUCTION

In recent years there has been growing concern about emerging pollutants found in treated and untreated waste-waters, surface and subsurface waters because of their potential environmental and health risk (Kolpin et al., 2002; Daughton et al., 2004). Emerging contaminants can be pharmaceuticals and personal care products, pesticides, disinfection by-products, industrial additives and by-products etc. in the aquatic environment. These emerging substances are suspected of having secondary adverse effects, such as mutagenicity, carcinogenicity, reprotoxicity, and endocrine disrupting, immune-disrupting and allergizing effects (Focazio et al., 2008; 2008; Bolong et al., 2009; Loos et al., 2009). While more effective analytical methods are being developed for chemical monitoring of these contaminants, biological methods providing more complex results and capable of predicting and measuring chronic effects are still not available (Barnes et al., 2008). Prior to conducting appropriate and feasible environmental risk assessments of emerging micropollutants, ecotoxicological methods with more sensitive end-points need to be developed.

In connection with this topic and in line with the 3R-concept "Reduction, Refinement and Replacement of Animals Toxicity Testing" (DIRECTIVE 2010/63/EU) our research activity aimed at developing innovative, sensitive environmental toxicology tests applying sublethal endpoints. Historically, toxicological endpoints of standardized animal tests included survival, growth and reproduction. In this paper we examined the effects of emerging contaminants focusing on common endpoints of various testorganisms. We have also assessed the physiological responses (respiration, moving behaviour, heart rate) using novel procedures. To detect physiological and behavioural responses of animal testorganisms over a wide range of pollutant concentrations digital microscope camera observation and a computer aided evaluation system were used.

We have done comparative assessment of the newly developed and commonly used endpoints of environmental toxicology tests. The problem-specific integrated methodology included: alga tests with three unicellular alga species (*Pseudokirchneriella*, *Scenedesmus*, *Chlorella*), *Tetrahymena pyriformis* (protozoon) reproduction and respiration test, *Lemna minor* reproduction inhibition test with the

determination of chlorophyll content, *Heterocypris incongruens* (freshwater ostracode) lethality and movement test, and the *Daphnia magna* immobility and heart rate test.

This paper reports the results of the three most sensitive environmental toxicology tests, namely the *Daphnia magna* heartbeat rate test, the *Lemna minor* reproduction inhibition test with the determination of chlorophyll content and the *Heterocypris incongruens* movement test.

Daphnia magna is a crustacea widely used for environmental toxicology evaluation of different chemical substances in water samples (Ohe et al., 2011). This crustacean is an accepted testorganism and indicator for environmental impacts. One of the commonly used toxicology endpoints is the reproduction (OECD, 1998; Pieters et al., 2005). Lethality and immobilization are also commonly determined (Assmuth and Penttila (1995); OECD, 1996; OECD, 2004; Brennan et al. (2006;); Lee et al (2009)).

Villegas-Navarro et al. (2003) examined the effects of four cardio active drugs on the heart of *D. magna*. Toxic effect was observable in case of all drugs during a 48 h contact time experiment. The following LC₅₀ values were detected: 2.03 mg/L for ouabain, 7.04 mg/L for verapamil, 32.45 mg/L for metaproterenol and 76.21 mg/L for metoprolol. Dzialowski et al. (2006) tested the chronic effect of propranolol and metoprolol using different toxicity endpoints with *Daphnoids*. They found that the heartbeat rate is the most sensitive endpoint. Based on their results we would like to prove the feasibility of *Daphnia magna* heartbeat rate test for the characterization of the effect of emerging pollutants.

To our knowledge, the potential hazardous effect of emerging pollutants on *Lemna* species has not been thoroughly investigated yet. Brain et al. (2004) studied the effect of eight pharmaceuticals (carbamazepine, levofloxacin, sertraline, atorvastatin, acetaminophen, caffeine, sulfamethoxazole, and trimethoprim) on *L. gibba*. They observed clear concentration-effect between the pharmaceutical concentration and the toxic effect. Cedergreen et al. (2007) and Gorzerino et al. (2009) tested the toxicity of various pesticides on *L. minor*, but they did not test any other types of emerging micropollutants such as pharmaceuticals, industrial additives and agents or personal care products. According to these studies, *L. minor* is a promising testorganism for the evaluation of emerging micropollutants.

The *Heterocypris incongruens* is a freshwater ostracod, its body is covered with calcareous shell. Their reproduction can occur both by fertilized and virgin eggs. *H. incongruens* is a bottom-dwelling animal and feeds on mostly algae and small aquatic organisms. The first application of *Heterocypris incongruens* as an environmental toxicology testorganism was published by Hubálek et al. (2007). *H. incongruens* can be sensitive to water dissolved and sediment-bound contaminants, this way the total toxicity of the tested medium can be measured. The commonly used toxicity endpoints are lethality and immobilization.

MATERIALS AND METHODS

Materials

Na-Diclofenac is a nonsteroidal anti-inflammatory drug (NSAID) (CYCLOLAB R&D Ltd., CAS: 15307-79-6).

Nicotine is a parasympathomimetic alkaloid found in the nightshade family of plants (*Solanaceae*). It is a nicotinic acetylcholine receptor agonist found in the roots and leaves of the plants (Sigma Aldrich, N3876 SIGMA, ≥99% (GC), liquid, CAS: 54-11-5) (<http://enfo.agt.bme.hu>).

Metazachlor is a residual herbicide for controlling broad leaved weeds and annual grasses. It is a synthetic compound and a member of the chloroacetamide chemical family. It is applied directly to the soil or by aerial spray (SULTAN 50 SC (500 g/L), Makhteshim Agan Hungary Zrt., CAS: 67129-08-2) (www.agchemaccess.com).

Experimental – test species and test procedures

***Daphnia magna* immobilisation test**

The test was carried out based on the OECD 202 *Daphnia sp.* Acute Immobilisation Test Protocol.

***Daphnia magna* heart rate test**

Testorganism

We applied a culture of *Daphnia magna*, which was maintained in our laboratory in a 5 litres volume beaker in a 21.5±1 °C thermostat with 16:8 h light: dark cycle. The adults were approx. 10 days old female individuals, fed every second day with alga suspension of *Scenedesmus subspicatus*. Boiled and cooled, aerated tap water was used as growth medium, of <500 mS cm⁻¹ electric conductivity value (Hart et al., 1999).

Test solutions

The testing substances were dissolved in the original culturing medium in a series of six members decimal dilution. We conducted the experiment with the following testsolutions: nicotine, metazachlor and Na-diclofenac at 10; 1; 0.1; 0.01; 0.001; 0.0001 mg/L concentration.

Experimental procedure

The experiment was carried out based on the work of Villegas-Navarro et al. (2003) and Dzialowski et al. (2006) with minor modifications. The appropriate testorganisms in 200 µL tested chemical solution were placed onto a single cavity microscope slide with the help of a special fabric spoon, and then the heartbeat rate of each testanimal was measured three times for 10 seconds. The measurement was carried out with NIKON SMZ800 stereomicroscope. After the measurement 10 testanimals were placed into 50 mL of each testsolution and into a control container with the culturing medium. After 24 and 48 hours the heartbeat rate of the testanimals was counted again as explained above.

Evaluation and interpretation of results

The mean of the three measured heartbeat values were calculated before and after exposure, and averaged for the ten testanimals using Microsoft Office Excel program. The mean heartbeat rate of the samples was plotted on a bar diagram in increasing order of the concentration of the tested chemical substance. In case of all samples standard deviation was calculated for the ten individuals.

***Lemna minor* reproduction inhibition test**

Testorganism

A culture of laboratory maintained *Lemna minor* was used in the experiments. The testorganisms were cultured in a 20x30x7 cm glass container kept in a 21.5±1 °C thermostat with 16:8 h light: dark cycle (illumination: Juwel Aquarium, Day-Lite, 15W, 438 mm lamp, 560 Lumen, 6500 K). For the test healthy, two-leaf *L. minor* individuals were used, cultivated in Hoagland's nutrient medium explained below:

Hoagland's nutrient medium (Missouri Botanical Garden) (www.mobot.org):

MgSO ₄ ·7H ₂ O (24,6 g/100ml):	1,0 ml/l
Ca(NO ₃) ₂ ·4H ₂ O (23,6 g/100ml):	2,3 ml/l
KH ₂ PO ₄ (13,6 g/100ml):	0,5 ml/l
KNO ₃ (10,1 g/100ml):	2,5 ml/l
Microelement solution:	0,5 ml/l
Fe-EDTA solution:	20,0 ml/l

Microelement solution:

H ₃ BO ₃	2,86 g/l
MnCl ₂ ·4H ₂ O	1,82 g/l
Na ₂ MoO ₄ ·2H ₂ O	0,09 g/l
CuSO ₄ ·5H ₂ O	0,09 g/l
ZnSO ₄ ·7H ₂ O	0,22 g/l

Fe-EDTA solution:

FeCl ₃ ·6H ₂ O	0,121g/250ml
EDTA	0,375g/250

Test solutions

We applied testing substances dissolved in the original Hoagland's nutrient medium in a series of five members decimal or twofold dilution. The experiments were carried out with the following testsolutions: nicotine at 10; 1; 0.1; 0.01; 0.001; 0.0001 mg/L concentration, metazachlor at 100; 10; 1; 0.1; 0.01 mg/L concentration and Na-diclofenac at 50; 25; 12.5; 6.25, 3.125 mg/L concentration.

Experimental procedure

On the first day 10 healthy and two-leaf *L. minor* individuals were placed into 50 mL of each dilution member of the testsolutions. The experiment was carried out with three parallels in 150 cm³ beakers. Hoagland's nutrient medium was applied as control. The beakers were covered with a translucent plastic film to avoid evaporation and concentration of the testsolutions during the experiment. The assembled test systems (beakers) were incubated in a 21,5 ± 1 °C thermostat for 7 days under the following light conditions: 16:8 h light: dark cycle (illumination: Juwel Aquarium, Day-Lite, 15W, 438 mm lamp, 560 Lumen, 6500 K).

On the seventh day *L. minor* individuals were removed from the test-solutions, then dried with filter paper. The wet weight of *L. minor* biomass was determined in case of each sample. The dried biomass was placed into ground-necked test tubes containing 5 mL of 96% ethanol. After 24 hours the optical density of the samples was determined spectrophotometrically (Sanyo SP55 UV/VIS spectrophotometer) at 470, 649 and 664 nm wavelength values.

Evaluation and interpretation of results

From the measured optical density values the total chlorophyll content was determined using the following formula (Lichtenthaler, 1987):

$$C_{a+b} = \frac{5,24 \cdot A_{664} + 22,24 \cdot A_{649}}{\text{sample}}, \text{ in which}$$

C_{a+b}: total chlorophyll content of the sample (mg/sample)

A₆₆₄: absorbance values at 664 nm wavelength

A₆₄₉: absorbance values at 649 nm wavelength

From the total chlorophyll values an inhibition percentage was calculated as compared to the values of the control sample using the following formula:

$$H\% = \frac{C - S}{C} \times 100, \text{ in which}$$

H%: inhibition percentage

C: total chlorophyll content values of the control sample

S: total chlorophyll content values of the sample

***Heterocypris incongruens* immobilization test**

The test was carried out based on OSTRACODTOXKIT F.

***Heterocypris incongruens* movement test**

Testorganism

We applied a culture of *Heterocypris incongruens*, which was maintained in our laboratory in a 0,5 liter volume beaker kept in a 21.5 ± 1 °C thermostate with 16:8 h light: dark cycle. The test adults were approx. 10 days old female individuals, fed every second day with alga suspension (a mixture of *Chlorella vulgaris*, *Pseudokirchneriella subcapitata* and *Scenedesmus subspicatus* species). Standard water (0.6 g $MgSO_4$; 0.96 g $NaHCO_3$; 0.04g KCl; 0.6 g $CaSO_4 \cdot 2 H_2O$; in 10 L distilled water; pH=6,4) was used as growth medium.

Test solutions

We applied testing substances dissolved in distilled water in a series of four members dilution. The experiments were carried out with the following test-solutions: nicotine in 10; 1; 0.1; 0.01 mg/L concentration, metazachlor and Na-diclofenac in 50; 5; 0.5; 0.05 mg/L concentration.

Experimental procedure

3 testorganisms were placed into a 2 mL volume (h=15 mm; d=12 mm) glass container and the movement of the testorganisms was registered by a QImaging 5.0 Micropublisher RTV digital microscope camera of SMZ800 Nikon stereo microscope and Image-Pro Plus 7.0 software. The video record contained 150 images. The test containers were illuminated from the bottom. The movement of the selected coloured points was followed by the software. From several options (eg.: acceleration, angular velocity) the average speed and total travelled distance were the basis of comparison.

For the initial (t=0 min) measurements test-animals were placed into 1 mL of the culturing medium, which was also used as the control solution, then video records were made at t=0 min and after a 30 minutes contact time in the test-solution. After that the test-animals were put into a thermostate (21.5 ± 1 °C) for 24 and 48 hours in 10 mL of the test-solutions with the same composition used for the 30 minutes contact time test. Distilled water was also used as control test-solution.

RESULTS

Innovative toxicity endpoints were applied to investigate the harmful effect of three emerging pollutants (metazachlor, Na-diclofenac and nicotine). In case of *D. magna* the heartbeat rate was determined, in case of *L. minor* the total chlorophyll content and in case of *H. incongruens* the average velocity and the total distance of the movement.

***Daphnia magna* immobilisation test**

No immobilisation effect was observed in case of metazachlor and diclofenac. Nicotine had immobilisation effect on *D. magna*, 10 mg/L concentration immobilised 80% of the testorganism, 1 mg/L concentration immobilised 20% of the testorganism.

***Daphnia magna* heartbeat rate test**

In the case of nicotine after 10 minutes contact time a significant attenuation could be observed already at 1 mg/L concentration. There was no significant change after 24 and 48 h (Fig. 1). The pesticide, metazachlor did not show considerable inhibitory effect after 10 minutes, but after 24h and 48 h a significant attenuation of the heartbeat rate was observed even at 0.1 mg/L concentration level (Fig. 2). Na-diclofenac caused the most irregular concentration-effect relationship. Significant toxic effect appeared in all of the samples, but only after 48 h contact time (Fig. 3). But this significant inhibition could be also observed at very low concentration (0.1 ppb) level. These phenomena draw the attention to the importance of testing with different contact time to study the nature of the inhibition effect of a particular contaminant (eg.: the irregular concentration-effect relationship or the property of the testorganism to become accustomed to the toxic materials).

None of the tested chemical substances produced the usual sigmoid concentration-relationship curve. The results of *Daphnia* heart rates test proved that this method with this new endpoint can be a promising tool to fulfill the increasing need for more sensitive ecotoxicity test methods.

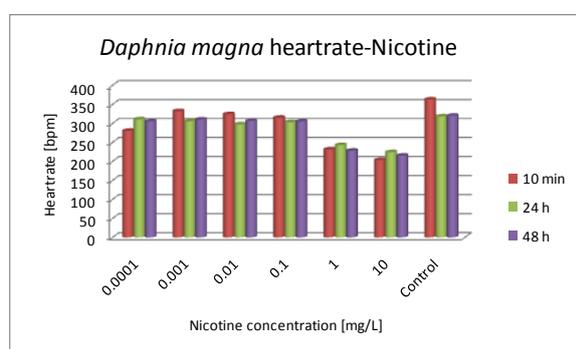


Fig. 1. The attenuation effect of nicotine on *Daphnia magna* heartrate

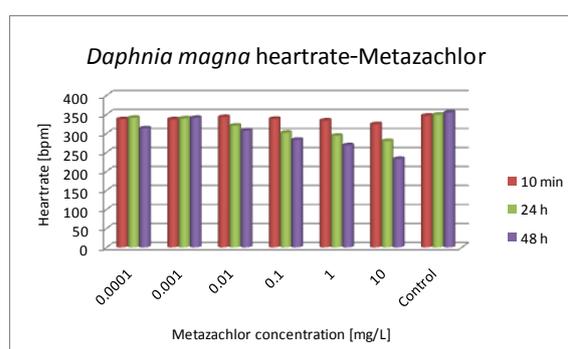


Fig. 2. The attenuation effect of metazachlor on *Daphnia magna* heartrate

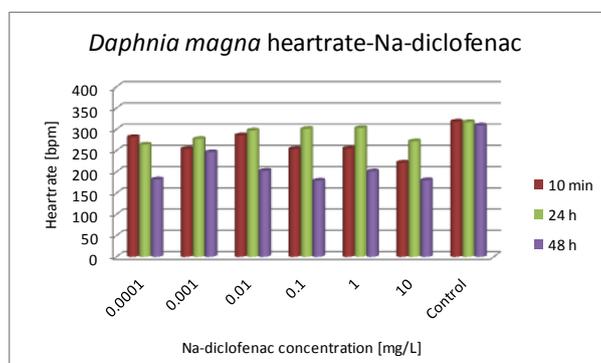


Fig. 3. The attenuation effect of Na-diclofenac on *Daphnia magna* heartrate

***Lemna minor* reproduction inhibition test**

The results of the duckweed toxicity tests performed on emerging pollutants solutions (nicotine, metazachlor and Na-diclofenac) are presented in the following figures (Fig 4–6).

Na-diclofenac had only a slight inhibitory effect (H%=15) only at 25 and 50 mg/L concentrations (Fig. 6). Metazachlor, which is a pesticide showed the expected strongest inhibitory effect of the tested chemical substance. The inhibition percentages were almost the same value from 0.1 to 100 mg/L concentrations (approx. 60%). Metazachlor in a 10 mg/L concentration resulted 46% inhibition. Based on the measured H% values we can conclude, that metazachlor is hardly toxic to *L. minor* (Fig. 5). Nicotine caused 84% inhibition at 10 mg/L concentration, but showed an irregular concentration-response relationship at other concentration (Fig. 4). For the investigation of this phenomenon and the mode of action further researches with more dilute solutions are needed.

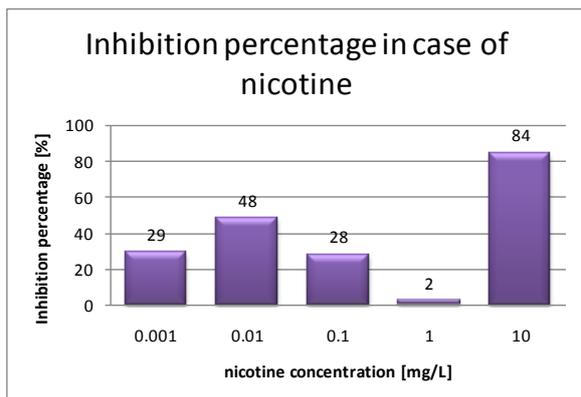


Fig. 4. Inhibition effect of nicotine on *Lemna minor* growth

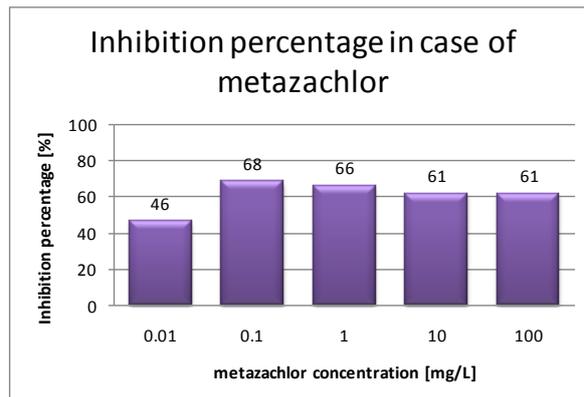


Fig. 5. Inhibition effect of metazachlor on *Lemna minor* growth

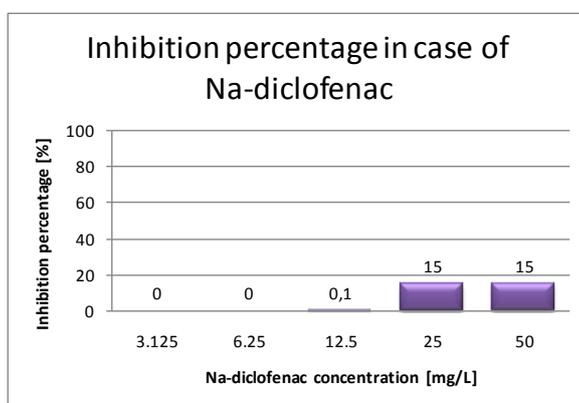


Fig. 6. Inhibition effect of Na-diclofenac on *Lemna minor* growth

***Heterocypris incongruens* immobilisation test**

By OSTRACODTOXKIT F™ CHRONIC Test the immobilisation effect (EC₅₀ values) of nicotine, metazachlor and diclofenac was determined. The EC₅₀ was 0.257 mg/L for nicotine, 10.000 mg/L for metazachlor and 12.227 mg/L for diclofenac.

***Heterocypris incongruens* movement test**

The results of the toxicity tests carried out by the freshwater ostracods performed on emerging pollutants solutions (nicotine, metazachlor and Na-diclofenac) are presented in the following figures (Fig 7–9).

The results show, that the average velocity of the test organisms decreased in time independent from the concentration, otherwise the inhibitory effect of nicotine at different concentrations is well observable. After 30 minutes and 24 hours contact time more intense movement is observable in some cases (0.4 and 0.08 mg/L concentrations) thanks to the toxic effect of the tested material. In this case the testorganism reacts with a heightened life-phenomenon for the increased amount of the toxic material. (Further experiments are needed to find out exactly how these parameters are related to each other.) Then, after a 48 hours exposure the movement of the test-animals is negligible in the 10 mg/L nicotine solution and as the nicotine concentration increases, the average velocity decreases depending on the concentration.

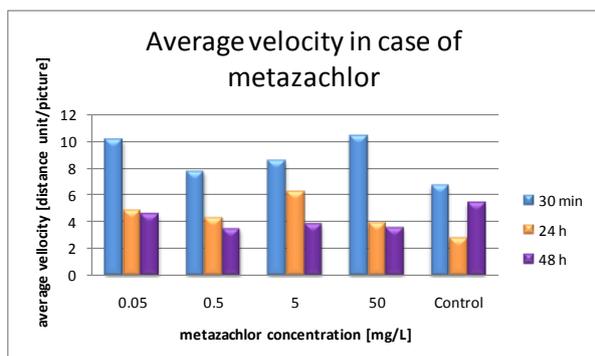


Fig. 7. The effect of metazachlor on *Heterocypris incongruens* movement velocity

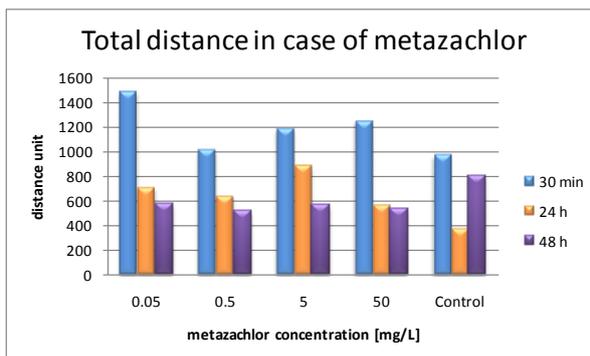


Fig. 8. The effect of metazachlor on *Heterocypris incongruens* movement distance

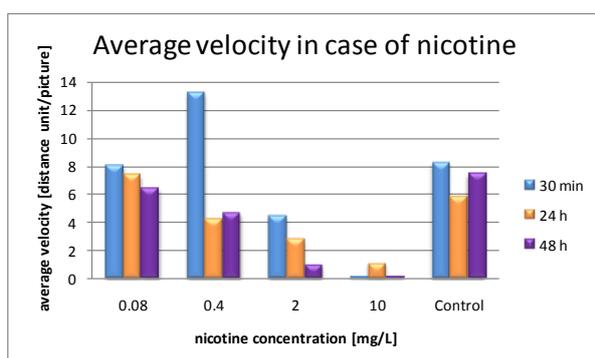


Fig. 9. The effect of nicotine on *Heterocypris incongruens* movement velocity

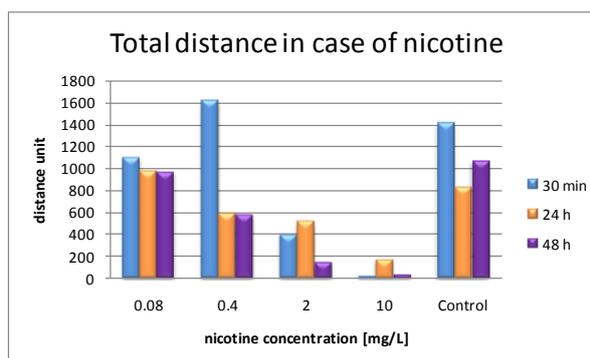


Fig. 10. The effect of nicotine on *Heterocypris incongruens* movement distance

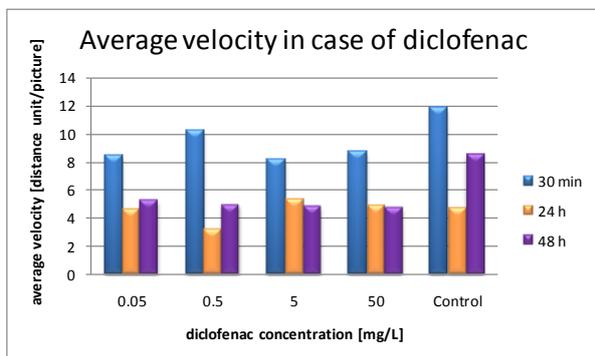


Fig. 11. The effect of diclofenac on *Heterocypris incongruens* movement velocity

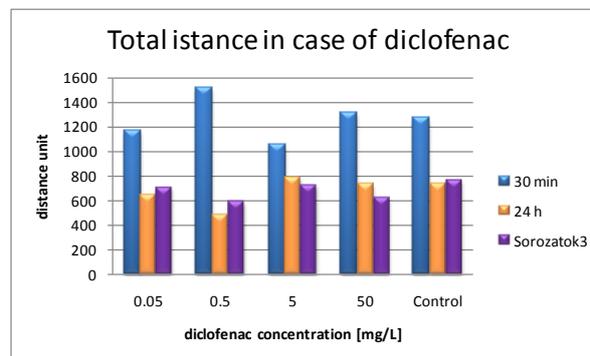


Fig. 12. The effect of diclofenac on *Heterocypris incongruens* movement distance

After 30 minutes of contact time even the 0,4 mg/L nicotine solution caused greater mobility compared to the control group, however 24 hours later the inhibitory effect in this concentration was observable (Fig. 9). Similar observations can be done by examining the total distance values (Fig. 10).

Table 1. Inhibition percentage calculated from the measured average velocities and EC₅₀ values for nicotine

Inhibition %	10	2	0,4	0,08	EC ₅₀
24 h	81	51	26	27	2.485 mg/L
48 h	97	87	37	14	1.004 mg/L

In case of metazachlor concentration-dependent effect on the average velocity was observable neither after 30 minutes, nor after 24 hours, however after 48 hours contact time severe toxic effect of the pesticide was observable, which phenomenon shows the feasibility of the method for the testing of emerging micropollutants (Fig. 7). Already 0.05 mg/L concentration of metazachlor resulted in 33% inhibition of the total distance (Fig. 8). In case of diclofenac the effect on the total distance and the average velocity differs after 48 hours. The total distance does not show smaller value in the presence of the tested chemical (Fig. 12), but the metazachlor has 45% inhibitory effect on the average velocity at 0.05 mg/L concentration (Fig. 11).

Further developments are needed in the future in connection with the evaluation possibilities: the new movement characteristics features for the better understanding of the accelerated movement phenomena.

DISCUSSION

Comparative assessment of environmental toxicology tests was carried out to find new, more sensitive endpoints for testing the toxic effect of emerging micropollutants found in freshwater ecosystems. The three most sensitive environmental toxicology tests and their results are presented here, namely the *Daphnia magna* heartbeat rate test, the *Lemna minor* reproduction inhibition test with the determination of chlorophyll content and the *Heterocypris incongruens* movement test.

In the *H. incongruens* immobilisation test the EC₅₀ values were 0.26 mg/L for nicotine, 10.00 mg/L for metazachlor and 12.23 mg/L for diclofenac. Comparing this immobilisation test with the *H. incongruens* movement test more intense movement was observable at 0.08 mg/L nicotine concentration after 30 minutes and 24 hours contact time. Already 0.05 mg/L concentration of metazachlor resulted in 33% inhibition of the total distance and 45% inhibitory effect on the average velocity.

In the *D. magna* immobilisation test no immobilisation effect was observed in case of metazachlor and diclofenac within the 0.0001-10 mg/L concentration range. Nicotine had immobilisation effect on *D. magna*, 10 mg/L concentration immobilised 80% of the testorganism, 1 mg/L concentration immobilised 20% of the testorganisms. Comparing this immobilisation test with the *D. magna* heartbeat rate test in the case of nicotine a significant attenuation could be observed already at 1 mg/L concentration after 10 minutes contact time. The pesticide, metazachlor showed considerable inhibitory effect after 24h and 48 hours even at 0.1 mg/L concentration level. Na-diclofenac caused the most irregular concentration-effect relationship. Significant toxic effect appeared in all of the samples, but only after 48 h contact time. This significant inhibition could be also observed at very low concentration (0.1 mg/L) level. None of the tested chemical substances produced the usual sigmoid concentration-relationship curve. The results of *D. magna* heartbeat test and *H. incongruens* movement test proved that these methods with this new endpoints can be a promising tool to fulfill the increasing need for more sensitive ecotoxicity test methods.

These newly developed procedures with sub-lethal endpoints offered the possibility for easy and quick estimation of environmental toxicity. Furthermore an important conclusion of the research is that there is no universal toxicity test to detect the effect of all types of chemical substances; reliable risk assessment of micropollutants requires a battery of bioassays.

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