

ECOLOGICAL IMPACT OF MICROPOLLUTANTS ON AQUATIC LIFE DETERMINED BY AN INNOVATIVE SUBLETHAL ENDPOINT *DAPHNIA MAGNA* HEARTBEAT RATE

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Abstract: In the last few decades growing attention has been paid to the occurrence and impact of micro-pollutants such as pharmaceuticals, personal care products, surfactants and other industrial additives on aquatic ecosystems. The effect on *Daphnia magna* of two pharmaceuticals (Na-diclofenac and paracetamol), a hormone (17 β -estradiol), a personal care product additive (triclosan), a psychoactive drug (nicotine) and of a pesticide (metazachlor) was determined at different concentrations (0.01–10,000 $\mu\text{g/L}$) during 24 and 48 h exposure. In order to test the sensitivity of this innovative heartbeat rate test the results were compared to those of the immobility and lethality tests, traditionally applied to *Daphnia*. The heartbeat rate as a toxicity endpoint for metazachlor, Na-diclofenac, β -estradiol and triclosane as well as for paracetamol showed significantly higher sensitivity than the immobility and lethality; the lowest observed effect concentrations were 0.05, 0.1, 0.1, 0.5 and 500 $\mu\text{g/L}$, respectively. Our study provides the evidence that the heartbeat rate of water flea is a more sensitive and ecologically more relevant endpoint than the conventionally applied immobilization and may become a novel model system for studying the effects of micro-pollutants on freshwater ecosystems.

Key words: ecotoxicity, *Daphnia magna*, heartbeat rate, immobilization, micro-pollutants, emerging contaminants

1. INTRODUCTION

There has been growing interest in emerging contaminants (ECs) since they proved to be potentially harmful to human health and water ecosystems (Kolpin et al., 2002; Daughton, 2004; Barnes et al., 2008; Loos et al., 2009). ECs are not new contaminants, they have been present in the environment for a long time, but their presence and significance are only now being recognised. They have potential secondary adverse effect on the ecosystem or human health, but many of them are currently not included in routine monitoring programmes (Boxall, 2012). Testing methods have not kept pace with scientific progress, the relevance and reliability of current methods is questionable and many of them do not meet animal welfare considerations (Gruiz et al., 2015). In Europe, the protection of animals used for experimental and scientific purposes is regulated by Directive

2010/63/EU (EU, 2010), which encourages the replacement of animals with non-animal systems or one animal species with a less highly developed one. There has been an extensive drive to have more relevant testing strategies accordingly, which also take account of animal welfare, including the 'Replacement, Reduction, and Refinement' ('3R') principles. Methods for testing ECs cannot be dealt separately in case of human and environmental concern, because ECs represent harmful effect both on human health and environment (Schiffelers et al., 2014).

Extensive research has been carried out to investigate the secondary adverse effects and the risks of micro-pollutants from the following sources: pharmaceuticals (non-steroidal anti-inflammatory drugs, steroids and hormones) detergents, cosmetics, industrial additives and agents, pesticides or nanomaterials. The potential influence of these emerging micro-pollutants on aquatic ecosystems is

currently not fully understood, they can be mutagenic, carcinogenic, reprotoxic or endocrine disrupting; furthermore they may have allergenic and sensitizing features (Focazio et al., 2008), similar to human exposures. Our knowledge is highly incomplete about the dose-response properties, the fate and the effects of these chemical substances toward non-target organisms at concentrations within ngL^{-1} to μgL^{-1} range in water matrices. The main problem is the lack of legislation and environmental quality criteria. Moreover they show unusual dose-response relationship, which means non-concentration-dependent or non-monotonic dose-response in many cases (Post et al., 2012; Lapworth et al., 2013).

The main sources of emerging contaminants are hospital effluents and communal wastewaters (Verlicchi et al., 2010; Deblonde et al., 2011; Jean et al., 2012; Bouissou-Schurtz et al., 2014). In fact, some of them cannot be completely eliminated from wastewaters by conventional water treatment technologies (Oppenheimer et al., 2007; Miége et al., 2008; Radjenovic et al., 2009). Because of the lack of effective water treatment technologies emerging pollutants may be present not only in industrial and municipal wastewaters or hospital effluents, but in natural surface and subsurface water matrices as well. Therefore, the probability of the environmental risk cannot be ruled out, and there is an urgent need for ecotoxicity assays applying sensitive and ecologically relevant measurement endpoints (Lishman et al., 2006; Jones et al., 2005; Castiglioni et al., 2006; Ternes & Joss 2006; Snyder et al., 2008; Bolong et al., 2009; Verlicchi et al., 2010). Table 1 summarises current literature data about surface water concentrations, EC_{50} and LC_{50} values of *Daphnia magna* lethality and immobilization tests conducted with micro-

pollutants of interest for this study.

Daphnia magna (water flea) is a crustacean widely applied in environmental toxicology for testing different water samples (Barata et al., 2008; Ohe et al., 2011). *Daphnia* have an open circulatory system consisting with a simple heart (Villegas-Navarro et al., 2003). Lethality and immobilization are the most commonly determined measurement endpoints (OECD 1996; OECD 2004; Barbosa et al., 2008; Mansour & Gad 2010). Acute and chronic assays applying this crustacean are among the most frequently used tests in aquatic toxicology using the conventional measurement endpoints, but some attempts have been also made to study sublethal, physiological effects. Some authors suggest morphological abnormalities, movement patterns, phototaxis index, feeding inhibition or heartbeat rate as an appropriate sublethal endpoint (Villegas-Navarro et al., 2003; Barata et al., 2008; Jeon et al., 2010; Leoni et al., 2008; Wu et al., 2008; Kovács et al., 2012). These measurement endpoints provide an alternative approach to assess the response of cladoceran to environmental micro-pollutants, but some of these characteristics are difficult to quantify.

The heartbeat rate test gives quantifiable results, however the available scientific literature contains scarce information about using heartbeat rate as endpoint for testing aquatic toxicity. Villegas-Navarro et al., (2003) studied the effects of four cardioactive drugs on the heart of *Daphnia magna*. They observed toxic effects in case of all drugs during a 48 h contact time experiment. They determined the following LC_{50} values: 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) studied the chronic effect of two β -blockers (propranolol, metoprolol) on *Daphnia*, measuring two different toxicity endpoints.

Table 1: $\text{EC}_{50}/\text{LC}_{50}$ values of *Daphnia magna* lethality and immobilization test and surface water concentrations for tested micro-pollutants

Substance	$\text{EC}_{50}/\text{LC}_{50}$ values of <i>Daphnia magna</i> immobilization / lethality test		Concentration in surface waters	
Metazachlor	22.3 mg/L (EC_{50} , 48h)	[FAO]	200 $\mu\text{g/L}$	[Kreuger 1998]
Na-diclofenac	48.5 mg/L (EC_{50} , 24h)	[Haap et al., 2008]	< 5 ng/L	[JDS-2 2008]
	56.5 mg/L (LC_{50} , 24h)	[Haap et al., 2008]		
	39.9 mg/L (LC_{50} , 48h)	[Ferrari et al., 2004]		
β -estradiol	3.67 mg/L (EC_{50} , 24h)	[Trenholm et al., 2006]	< 5 ng/L	[JDS-2, 2008]
	2.87 mg/L (EC_{50} , 48h)	[Brennan et al., 2006]		
	2.7 mg/L (LC_{50} , 48h)	[Hirano et al., 2004]		
Triclosan	0.390 mg/L (EC_{50} , 48h)	[Orvos et al., 2001]	66–184 ng/L	[Esteban et al., 2014]
Paracetamol	2.04 mg/L (EC_{50} , 24h)	[Kim et al., 2007]	2.24–289 ng/L	[Kim et al., 2012]
	30.1 mg/L (LC_{50} , 48h)	[Dave et al., 2012]		
Nicotine*	5.678 mg/L (EC_{50} , 24h)	[Martins et al., 2007]	6–80 ng/L	[Buerge et al., 2008]

* - Nicotine and its derivatives

The heartbeat rate proved to be the most sensitive toxicity endpoint. The above mentioned results suggest that the sublethal endpoints in case of *Daphnia magna* are sensitive at mg/L and µg/L concentration levels to the tested cardioactive and β-blocker substances.

Based on these studies we carried out the *D. magna* heartbeat rate test and immobilization and lethality tests for testing the effects of six micro-pollutants and for comparing this endpoint with the conventionally used immobility and lethality measurement endpoint described by the OECD 202 protocol. We aimed to prove that *Daphnia magna* heartbeat rate test may be a feasible bioassay to detect the toxic effect of various micro-pollutants at the level of µg/L and we expected to reach higher sensitivity compared to lethality and immobilisation. To our knowledge, this method has not been used yet for the ecotoxicity testing of aquatic micro-pollutants such as non-steroidal anti-inflammatory drugs (NSAID), psychoactive drugs, personal care products and pesticides.

2. MATERIALS AND METHODS

2.1. *Daphnia magna* test organisms

Daphnia magna was cultured in the laboratory in 5L volume beakers in a laboratory incubator (21.5±1°C; illumination: Juwel Aquarium, Day-Lite, 15W, 438 mm lamp, 560 Lumen, 6500 K, 16:8 h light:dark cycle). Test animals were fed every day with alga suspension cultivated in the laboratory containing *Scenedesmus obtusus*. The growth medium was boiled and cooled tap water. The electric conductivity value of the culture medium was kept between 250–500 mScm⁻¹ as recommended by Hebert et al., (2002). The dissolved O₂ concentration of the culture medium was more than 6mg/L.

2.2 Tested chemical substances

The model compounds (Na-diclofenac, paracetamol, 17β-estradiol, triclosan, metazachlor and nicotine) tested in this study were selected based on several criteria: high consumption and occurrence in the aquatic systems, suspected or proven unfavourable effect on the environment and human health and coverage of some characteristic classes of contaminants of emerging concern: pesticides, PPP additives, pharmaceuticals, etc.

The applied chemical substances were dissolved in the original culture medium in a series of six-member decimal dilution. The use of solvents

was not necessary as all of the substances are water soluble in the tested concentration. Since emerging micro-pollutants usually occur at µg/L concentrations in natural water samples, the concentration of the stock solutions were adjusted to have a maximum concentration of 10mg/L (nicotine, Na-diclofenac) and in some cases 5mg/L (triclosan, metazachlor, paracetamol) or 1mg/L (β-estradiol) due to the water solubility properties of the tested substances.

From stock solutions serial dilutions were prepared. The following freshly prepared test solutions were tested: Na-diclofenac and nicotine 10, 1, 0.1, 0.01, 0.001, and 0.0001 mg/L; in the case of triclosan, metazachlor and paracetamol 5, 0.5, 0.05, 0.005, 0.0005 and 0.00005 mg/L and in the case of β-estradiol 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001mg/L.

Paracetamol (CAS RN: 103-90-2, A5000-100G), nicotine (CAS RN: 68935-27-3, N9765-500MG) and β-estradiol (CAS RN: 50-28-2, E8875-250MG) were purchased from Sigma Aldrich; metazachlor (500 g/L) (CAS RN: 67129-08-2, Sultan 50SC) was purchased from Makhteshim-Agan Industries Ltd. Israel; Na-diclofenac (CAS RN: 15307-79-6) was purchased from CycloLab R&D Ltd., Budapest, Hungary. The test solutions has not been analysed, the evaluation of the results are based on nominal concentrations.

2.3. Experimental Procedures

2.3.1. Heartbeat rate test

Selection of the female animals: non-pregnant 10-day old *D. magna* individuals, not from first brood as Villegas-Navarro et al., (2003) recommended. The animals were not fed during the test. The electric conductivity of the test solutions was 250–500mScm⁻¹ recommended by Hebert et al. (2002), the dissolved O₂ concentration was more than 3 mg/L at the end of the test recommended by the OECD 202 Guideline. 10 animals were placed into 50 mL test solution in a 150 cm³ test vessel with the help of a special fabric spoon. As a control, 10 animals were placed into 50 mL of the culture medium in a 150 cm³ test vessel. The heartbeat rate of the animal was measured twice during the test, after 24 and 48 h contact times. Counting of the heartbeat rate was carried out under a stereomicroscope (NIKON SMZ800). The test animals were placed onto a single cavity microscope slide into a 50 µL droplet of the test solution, where the heartbeat rate of the test animals was measured one-by-one (individually), three times for 10 seconds.

2.3.2. Lethality and immobilisation

The effect of immobilization was determined for Na-diclofenac, paracetamol, 17 β -estradiol, triclosan, nicotine and metazachlor, using the OECD 202 acute immobilisation test. In addition to that the death of the animals was diagnosed with the heartbeat rate test on the same 10 animals included in the study.

2.4. Statistical evaluation and interpretation of results

The mean of the three measurements were expressed in heartbeat per minute (beats min⁻¹=bpm) and averaged for the 10 test animals. To determine if the applied concentration of the pollutants had a significant effect on the measured parameters, we performed analysis of variance (ANOVA) using StatSoft[®] Statistica 11[®].

The lowest observed effect concentration (LOEC) values were determined using Dunnet's ANOVA. The Dunnet test indicates whether the mean at any concentration of the measured data is lower (or higher) than the mean of the control. All *p* values less than 0.05 were considered statistically significant.

3. RESULTS AND DISCUSSION

3.1. Results

The data presented in figure 1 show the effect of the six investigated emerging pollutants (Na-diclofenac, paracetamol, 17 β -estradiol, triclosan, nicotine and metazachlor) on *D. magna* heartbeat rate. The comparative evaluation of the significant effects of different concentrations was carried out by one-way ANOVA. The following diagrams illustrate the effect of the applied emerging pollutants at different concentrations in case of 24 h and 48 h contact time.

In most cases the tested Na-diclofenac, β -estradiol and metazachlor concentrations showed significant effect compared to the control group (Fig. 1). We found that the inhibition effect of Na-diclofenac increased with the decreasing amount of chemical substance after 24 h exposure except at 10,000 mg/L concentration, where the detected inhibition was 14%. Na-diclofenac caused significantly lower heartbeat rate at all concentrations after 48 hours contact time than the control sample (inhibition=35–40%), (Table 2).

In case of animals exposed to 0.05–50 μ g/L paracetamol (acetaminophen) no significant effect on heartbeat rate was observed. The heartbeat rate

was significantly affected only in case of the highest experimental concentrations (500 and 5000 μ g/L) after 48 h exposure. Not only the lowering but increasing effect of this NSAID on heartbeat rate was learned at 500 and 5000 μ g/L concentrations following 24h contact time. After 48 h exposure paracetamol caused 22% and 43% inhibition at these concentrations compared to the control (Table 2). Different assumptions could be made about this observation: the effect of micro-pollutants, particularly drugs, does not show concentration-dependent response in case of the heartbeat rate. Secondly, there can be different phases and response types. For example, the heartbeat rate may increase due to an exaggerated response. The decreased heartbeat rate could be associated with inactivation and decreasing of the physiological activity.

A significant negative trend was observed in case of β -estradiol where low but significant inhibition of heartbeat rate was demonstrated. This effect was concentration dependent within 0.1–1000 μ g/L concentration range after 24 and 48 h contact time. At 0.01 μ g/L concentration the heartbeat rate increased by 11% by compared to the control after 24h exposure (Table 2). A slight decrease of the inhibition effect could be detected when comparing the 48h exposure to the 24h exposure. This phenomenon may be due to the adaptation in time.

Triclosan had the most severe toxic effect on *Daphnia magna*. This antibacterial agent applied in personal care products also demonstrated high toxicity which was not monotonic in the applied concentration range. However, the heartbeat rate test proved to be capable to detect the toxicity of triclosan at ppb (parts-per-billion) level (0.5 μ g/L). At 0.05 μ g/L and 0.5 μ g/L concentrations a slight decrease of the inhibition effect could be detected when comparing the 48 h exposure to the 24 h exposure. We could not detect any similar effect at higher concentrations (Table 2).

According to the bar diagrams (Fig. 1) the psychoactive drug nicotine caused significant decrease in heartbeat rate only at 10,000 μ g/L (24h) and 1000–10,000 μ g/L (48h) concentration levels, where 20% and 80% immobilisation effect was also detected, respectively.

Metazachlor showed concentration-dependent response of heartbeat rate in the chosen concentration range. This pesticide had 14% inhibition effect on the heart rate values at 5 μ g/L concentration after 48 h (Table 2.). Table 2 summarizes the results of the heartbeat rate, lethality and immobilisation tests. The data are given in inhibition percentage (H%) values.

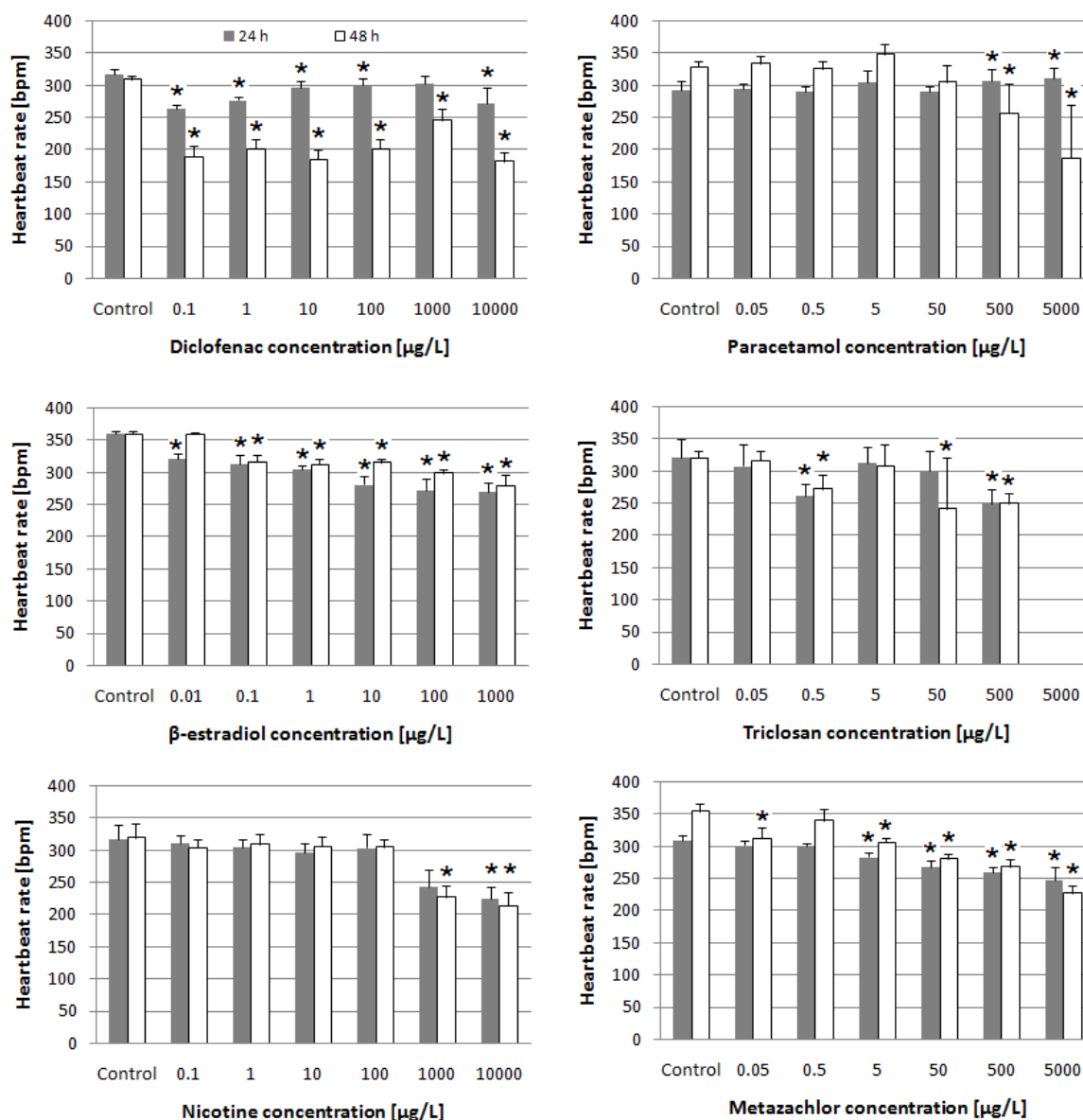


Figure 1. *Daphnia magna* heartbeat rate test results for the tested chemical substances in beat per minute units
 * Values represent significant decrease or increase of heartbeat rate compared to the heartbeat rate of the control group

Lethality was detected only in case of triclosan, and immobilisation was observed only in case of triclosan and nicotine. Where lethality occurred, the H% of heartbeat rate test was calculated only from the heartbeat rate of surviving animals. This way we separated the data of the lethal and sublethal measurement endpoints.

3.2. Discussion

As shown in table 2 data obtained in our study also indicate that the heartbeat rate as a measurement endpoint is significantly more sensitive than lethality and immobility. Although monotonic response of heartbeat rate was not

observed with exposure of all the tested chemical substances, statistical significance was found with all of the test compounds compared to the control group as shown in figure 1.

Lowest observed effect concentrations values calculated using Dunnet's ANOVA are presented in table 3 showing toxicity ranking. The new innovative toxicity endpoint, the heartbeat rate displays outstanding sensitivity to most of the tested substances compared to the immobilization test. The effect concentration values determined by immobilization test were orders of magnitude higher than measured by heartbeat rate test in the case of metazachlor, Na-diclofenac, β-estradiol and triclosan.

Table 2. Inhibition percentages of *Daphnia magna* heartbeat rate test for different chemical substances

Concentration [$\mu\text{g/L}$]	Na-diclofenac		Nicotine		β -estradiol	
	24 h	48 h	24 h	48 h	24 h	48 h
0.01	nm	nm	nm	nm	+11	0
0.1	-17	-39	-2	-5	-13	-12
1	-12	-35	-4	-3	-15	-13
10	-6	-40	-7	-4	-22	-12
100	-5	-34	-4	-5	-24	-17
1000	-4	-20	-24 (20)	-29 (20)	-25	-22
10,000	-14	-41	-30 (80)	-33 (80)	nm	nm

Concentration [$\mu\text{g/L}$]	Paracetamol		Triclosan		Metazachlor	
	24 h	48 h	24 h	48 h	24 h	48 h
0.05	+1	+2	-4	-1	-2	-12
0.5	-1	-1	-19	-5	-3	-4
5	+4	-6	-3 (60)	-4	-8	-14
50	-1	-7	-7 (90)	-25 (40)	-14	-21
500	+5	-22	-23 (100)	-22 (90)	-16	-24
5000	+5	-43	-100	-100	-20	-36

^a(): The numbers in parenthesis show the inhibition percentages of the *Daphnia magna* immobilization test.

^bnm: not measured

^cLethality was detected only in case of triclosan. For triclosan (24 h): 5000 $\mu\text{g/L}$: H%=100; 50 $\mu\text{g/L}$: H%=10; 5 $\mu\text{g/L}$: H=10%. Triclosan (48 h): 5000 $\mu\text{g/L}$: H%=100; 500 $\mu\text{g/L}$: H%=10; 50 $\mu\text{g/L}$: H%=50; 5 $\mu\text{g/L}$: H%=40; 0.5 $\mu\text{g/L}$: H%=10.

Table 3. Lowest observed effect concentrations of test substances on *Daphnia magna* determined by heartbeat rate and immobilization tests

Test substances	LOEC [$\mu\text{g/L}$] – 48 h	
	Heartbeat rate	Immobilization
Metazachlor	0.05	> 5000
Na-diclofenac	0.1	> 10,000
β -estradiol	0.1	> 1000
Triclosan	0.5	50
Paracetamol	500	>5000
Nicotine	1000	1000

The results obtained for diclofenac demonstrate the sensitivity and feasibility of the novel endpoint. For diclofenac Ferrari et al., (2003) reported 10days LOEC value *Ceriodaphnia dubia* at 2000 $\mu\text{g/L}$ applying reproduction inhibition which is much higher than the LOEC value obtained by our novel endpoint.

The lack of significant difference was observed in the case of nicotine between the lowest observed effect concentrations determined by heartbeat rate and immobility.

The current literature has only scarce data on the sublethal effect of the micro-pollutants tested in this study (Kim et al., 2012), which makes it difficult to compare and evaluate our results in the reflection of the findings of other researchers. However acute immobilisation effect data are available about some of the tested substances.

Quinn et al., (2011) indicated that standard

acute tests may not be appropriate to assess the potential toxicity of pharmaceuticals to animals, chronic data are needed. Numerous publications highlight the lack of chronic toxicity data (Boucard 2006, Fent et al., 2006). Results show that the conventionally applied and standardised immobilization test can detect the effect of Na-diclofenac only at a non-environmentally relevant mg/L concentration level (Quinn et al., 2011). The *D. magna* heartbeat rate test LOEC value (0.1 $\mu\text{g/L}$) is environmentally more relevant based on the Na-diclofenac concentration of treated waste water effluents (Vieno & Sillanpää 2014) and this LOEC value is closer to the environmental concentration of this pharmaceutical presented by the Liškaet al., (2008) (5 ng/L).

Brennan et al. (2006) determined the acute EC₅₀ (48 h) based on immobilisation, the EC₅₀ values were 2.87 mg/L for 17 β -estradiol. Trenholm In references list is et al., (2006) determined 3.67 mg/L for EC₅₀ in case of 17 β -estradiol. The *D. magna* heartbeat rate test resulted in 0.1 $\mu\text{g/L}$ for LOEC. For triclosan 0.39mg/L EC₅₀(48 h) was determined by acute immobilisation test (Orvos 2001), the heartbeat rate test resulted 0.5 $\mu\text{g/L}$ for LOEC. Kim et al., (2007) measured during an acute immobilisation test 2.04mg/L EC₅₀(24 h) for paracetamol, our study resulted in 500 $\mu\text{g/L}$ for LOEC using the heartbeat rate endpoint. LOEC determined by the heartbeat rate test is 0.05 $\mu\text{g/L}$ for metazachlor, acute immobilisation EC₅₀(48h) is

22.3mg/L according to the Food and Agriculture Organization of the United Nations.

Comparing the LOEC value determined by heartbeat rate endpoint with the concentration found in surface waters we can state that the concentrations of paracetamol and nicotine that affect heartbeat rate of the *Daphnia magna* were orders of magnitude greater than the levels reported in ambient water. But in the case of metazachlor, Na-diclofenac, β -estradiol and triclosan our results suggest that ecological impact of these are not negligible at the concentration found in the environment. Given the extensive use of these chemicals and loading to the aquatic ecosystem testing chronic effect of these test-substances may deserve further studies.

4. CONCLUSION

Development and application of sensitive and refined environmental toxicity methods for testing the impact of micro-pollutants on aquatic life may be one of the solutions addressing the water-quality problem facing the humanity nowadays. Toxicity tests were carried out for evaluation and comparison of the survival and immobility as endpoints applying Na-diclofenac, paracetamol, 17β -estradiol, triclosan, nicotine and metazachlor as model compounds. In addition special emphasis was put on a new endpoint, the heartbeat rate as a novelty.

Based on our results, it's obvious that the heartbeat rate of *Daphnia magna* can be the basis of toxicological studies and testing of aquatic micro-pollutants.

Heartbeat rate of *D. magna* responded to most of the tested micro-pollutants (except the nicotine) with a higher sensitivity than traditionally used lethality and immobility. In the case of metazachlor and triclosan the lowest observed effect concentrations determined by heartbeat rate test were comparable with the concentrations found in surface waters.

Our results suggest that this heartbeat rate acute toxicity test using *Daphnia magna* can be relevant in forecasting the ecological impact of micropollutants and may be used as an early warning system for monitoring of water quality.

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