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RESEARCH ARTICLE

How Does Experimental Design Modify the Result of *Daphnia magna* Heartbeat Rate Test? — Analyses of Factors Affecting the Sensitivity of the Test System

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Abstract

Development of an unconventional test method involves usually the comparison of biological responses under a variety of test conditions. The quality of these biological methods relies on an appropriate experimental design. The Daphnia magna heartbeat rate as a physiological endpoint for assessing aquatic pollution has been of minor interest so far; nonetheless, this could be an early and sensitive indicator of the harmful effect of micropollutants. Our aim was to set up the optimal experimental design of the heartbeat rate test. The studied factors were the composition of the test medium, the age of the test organism, and the exposure time, at triclosan concentrations between 0.2–2000 µg/L. According to the evaluation of test results the optimal test condition for the heartbeat rate test assumes tap water as test medium, 10-day-old test organisms and 48 h exposure time.

Keywords

Daphnia magna, heartbeat rate, micropollutants, triclosan

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1 Introduction

In recent years new and more sensitive analytical methods and environmental ecotoxicity tests are being developed to study the fate and transport of pharmaceuticals and personal care products (PPCP) as well as to detect the secondary adverse effects of PPCPs at environmentally relevant concentrations in order to investigate their adverse effects on non-target species in aquatic ecosystems even at trace levels [1-7].

The cladocerans *Daphnia spp.* have been established as useful test systems in environmental toxicology but usually the classical endpoints (immobilization and lethality) are applied for impact assessment of toxic substances [8-11]. Despite the fact that the *Daphnia magna* heartbeat rate has proved to be a promising sublethal toxicity endpoint in the case of cardioactive drugs, this method has been primarily applied in pharmacology studies so far in an infinitesimal number of publications [12-14]. Studies applying the *D. magna* heartbeat rate endpoint to investigate the environmental effects of chemical substances and environmental samples were targeting concentrations that cannot be considered environmentally relevant [15-17].

Our research aimed at developing a simple, reliable, and cost effective method for measuring heartbeat rate based on previous findings of existing literature. The *D. magna* heartbeat rate test described by Fekete-Kertész et al. [18] was established for studying the physiological effect of micropollutants (Na-diclofenac, 17β-estradiol, paracetamol, triclosan and metazachlor) on freshwater ecosystems. A detailed comparison of the *D. magna* heartbeat rate test methods is shown in Table 1 summarizing the applications of this unconventional endpoint from the past three decades. The heartbeat rate test gives quantifiable results of the effect of micropollutants at environmentally relevant concentrations, however, the available scientific literature contains scarce information about using this endpoint for especially ecotoxicological purposes.

According to our previous results [18] and data from current literature (Table 1), the *D. magna* heartbeat rate could be an early and sensitive indicator of the harmful effects of micropollutants, therefore it deserves further investigations to set up the optimal experimental design.

Table 1 Application of *D. magna* heartbeat rate in current literature

Test methodology	Method of heartbeat rate determination	Test organism	Test substance	Reference
3 hours of adaptation period before determination of the heartbeat rate in a 1 cm^3 volume silicon-rubber bottomed plexiglass chamber filled with test solution. Medium: water from Lake Balaton; 35 min of light adaptation period prior to each measurement. After 3 hours of adaptation period the water from Lake Balaton is replaced by sample solution. Measurements every 30 min for 6 hours.	Registration of the heartbeat rate with an opto-electric instrument: the *D. magna* specimen is trans-illuminated with a Carl Zeiss profile projector, the density change of the heart tissue causes a change of light intensity. This change is synchronous with the contraction and proportional to the heart beat amplitude.	*D. magna* 3.2−3.6 mm size. older than 4th instars	dikonirt: 250 and 500 mg/L	[15]
The heartbeat rate is measured in an anodized aluminium chamber (test medium: 1:14 diluted sea-water after 1 hour of acclimatization.	Registration of the heartbeat rate with a digital image processing software at 15°C based on pixel intensity rhythmic variation.	*D. magna* 2.5±0.2 mm size. female animals	Assessing the effect of anoxia on the heartbeat rate and heart contraction	[24]
Determination of the heartbeat rate of 5 test animals/sample on a microscope slide in one droplet of sample after 0, 30, 60 and 90 sec exposure time.	Registration of the heartbeat rate under light microscope. No further details.	no data	chinetrin: 0.01−0.5 ml/l; Surface water samples from River Tisza, Lake Balaton and River Séd.	[16]
Test animals are incubated individually in a 50 mL volume container for 2 hours at room temperature in the test medium, then test medium is removed and the heartbeat rate is determined in a droplet of test medium.	Digital recording of the heart contractions under inverse microscope (250 frames/sec), then determination of the heartbeat rate by further data processing.	*D. magna* age: 10 days	ouabain: 0.0006–5.85 mg/L verapamil: 0.0455−45.46 mg/L metaproterenol: 0.021−21.13 mg/L metoprolol: 0.027−26.74 mg/L	[12]
Testing in a cooled (10−11°C) chamber in 50 µL test medium, which is natural surface water or artificial salt solution.	Registration of the heartbeat rate under phase-contrast microscope oculometrically for 3×15 sec.	*D. pulex* 1−2 mm size	caffeine: 194.2−1942 mg/L isoproteronol: 2.11−211 mg/L adrenaline: 18.32 mg/L propranolol: 25.93 mg/L carbachol: 18.27 mg/L	[13]
Test animals are incubated individually in a 30 mL volume container for 30 min at room temperature in the test medium, then the test animal is placed into a droplet of test medium on a single cavity microscope slide. Medium: reconstituted hard water.	Digital recording of the heart contractions under inverse microscope for 15 sec, then heart rate was calculated from the digital video clips by replaying the video frame by frame and counting the number of heart beats in a 5-sec span using Image Pro software.	*D. magna* age: 4 days	propanolol: 0.8; 1.6; 3.2 mg/L metoprolol: 32; 64; 128 mg/L	[14]
D. magna were tethered to a squirrel hair and the test animals were allowed to acclimate at least 45 minutes to the tether prior to testing in 100 mL hard reconstituted water. Pre-exposure baseline rates of heartbeat rate were obtained for 30 minutes prior to adding nanoparticles. Nanoparticles were then added to the vessel with a pipette and the animal was recorded for 1 hour in every 15 minutes.	Digital recording of the heart contractions under a phase-contrast microscope for 8.7 sec (250 frame/sec).	*D. magna*	nano-C_{60}: 0.26 mg/L $C_{60}H_xC_{70}H_x$: 0.26 mg/L nTiO_2: 2 mg/L	[5]
D. magna were placed on concave microscope slides coated with petroleum jelly and kept in 200 µL of distilled water. Heart rate was measured twice 2 minute intervals between 2–4 and 10–12 minutes after administering experimental compounds.	Registration of the heartbeat rate under a phase-contrast microscope oculometrically.	*D. magna*	curcumin: 1.35 and 2.67 µM diphenhydramine: 1.09 µM	[17]

For further investigations triclosan was selected in this study as a model compound since the heartbeat rate test presented outstanding sensitivity (LOEC = 0.5 µg/L) to this selected micropollutant [18]. Our decision was supported by the endocrine disrupting potential [19] and the inhibitory potential of *Daphnia* HR96 receptor which is a promiscuous endo- and xenobiotic nuclear receptor involved in acclimation to toxicants [20].

Our aim in this work was to set up the optimal experimental conditions for the *D. magna* heartbeat rate test by analysing the factors affecting the sensitivity of the test system in the specific case of triclosan. Regarding the standard lethality and immobilization test methods Samel et al. [21] determined the optimal test medium and experimental circumstances, but similar efforts targeting the heartbeat rate test are not known in the current literature.

2 Materials and methods

2.1 *D. magna* test organism

A colony of *D. magna* cultured in the laboratory was used in a series of experiments. The test animals were cultured in 5 L beakers in a 21.5±1ºC thermostatic chamber with 16:8 h light: dark cycle (illumination: Juwel Aquarium, Day-Lite, 15 W, 438 mm lamp, 560 Lumen, 6500 K). Adult (10 days old) and young (3 days old) female animals were used for the test, fed every two days by an alga suspension cultivated in the laboratory containing *Scenedesmus obtusiusculus*. For maintaining *D. magna* aged, dechlorinated tap water and OECD M7 medium were used. The electric conductivity value of the media was presumably less than 500 mS cm^{-1} [22]. To check the sensitivity of the *D. magna* culture acute toxicity tests were performed with potassium dichromate ($K_2Cr_2O_7$) as reference toxicant at about every six months interval. Sensitivity of *D. magna* culture to $K_2Cr_2O_7$ ranged within the limits (EC_{50}, 24 h = 0.6–2.1 mg/L) set by guideline OECD 202 [23].

2.2 Tested chemical substance and quantification

Triclosan was purchased from Sigma-Aldrich (CAS Number: 3380-34-5; 72779-5G-F ≥97.0% HPLC; pka = 7.9; log Kow = 4.76; water solubility at 20°C = 10 mg/L). Saturated triclosan solution was prepared by adding excess solute to MilliQ® ultrapure water. To reach maximum dissolution the solution was stirred in sealed bottle at room temperature overnight. The use of organic solvents was not necessary because triclosan is water soluble in the tested concentration. The saturated solution was centrifuged at 8000 rpm and the undissolved crystal-free supernatant was used as stock solution. Triclosan concentration of the saturated stock solution was determined by High Performance Liquid Chromatography (Merck LaChrom Hitachi HPLC instrument with UV-Vis absorbance detector, Phenomenex® Kinetex 2.6 µm XB-C18 150x4.6 mm Column, eluent: 60:40 acetonitrile:water with 0.1 M cc. H_3PO_4, column temperature: 22°C, flow rate: 1 mL/min, injection volume: 10 µL, 282 nm detector wavelength). Effect of triclosan was tested at a series of five-member decimal dilution. The tested concentration range covered the environmentally relevant triclosan concentrations determined in surface waters. Serial dilutions were prepared from the stock solution with determined concentration using the applied culturing media (dechlorinated tap water or OECD M7 medium) in order to freshly prepare the test solutions of the following nominal concentrations: 0.2, 2, 20, 200 and 2000 µg/L. The saturated stock solution was stored in a dark refrigerator at 4ºC and proved to be stable throughout the series of experiments.

2.3 Experimental procedure

In order to find the optimal experimental conditions for the *D. magna* heartbeat rate test several factors were taken into consideration i.e. the composition of the medium, the age of the test organism, and the exposure time. The factors and their levels are summarized in Table 2.

Table 2 The investigated factors and their levels

Categorical variable	Levels of the investigated factor	
Test medium	Tap water	OECD M7 medium
Age of the test organism	3 days	10 days
Exposure time	24 h	48 h

Selection of the female animals: non-pregnant 3-day old and 10-day old *D. magna* individuals, not from the first brood as recommended by Villegas-Navarro et al. [12]. The animals were not fed during the test, the electric conductivity of the test solutions was 250−500 mS cm^{-1}, the dissolved O_2 concentration was more than 3 mg/L at the end of the test as recommended by the OECD 202 Guideline [23]. As test medium, M7 medium was used recommended by OECD 202 Guideline. Dechlorinated tap water was also applied as test medium. However, the composition of tap water is not identical in different laboratories, it can be an appropriate test medium based on the same consideration as using good quality surface waters from different sites for ecotoxicological testing. 10 animals of appropriate age were placed into 50 mL test solution in 150 mL test vessels with the help of a special fabric spoon. As a control, the original culturing media were used. The heartbeat rate of the animals was measured twice during the test, after 24 and 48 h exposure times. Counting of the heartbeat rate was carried out under a stereomicroscope (NIKON SMZ800, 63-times magnification). However, the heartbeat rate could be examined with a simple light microscope, its pointed light source affects (accelerates) the heartbeat rate and the inhibition caused by the chemical substances could not be detected, while thanks to the dim light of a stereo microscope, this phenomenon can be avoided. 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. The measured heartbeat rate of an individual was considered valid if the animal did not spent more time on the microscope slide than 45 seconds in order to avoid prolonged exposure to room temperature compared to the temperature of the medium in the thermostated test vessels. ossible error or variability associated with manual counting of the *Daphnia* heartbeat rate was addressed and eliminated through collection of data by visual inspection of heartbeats of the same test animal by five different individuals and repeating this practice on multiple animals till consistency in counting heartbeat rates was accomplished by the experimenter. Variability in heartbeat rate due to disturbances or stress from physical handling of the test organisms proved to be insignificant and might be considered as uniform across the test groups.

3 Results and discussion

The result of our experiments was a series of datasets, each containing heartbeat rates for a certain level of the investigated three factors at five different triclosan concentrations. As the whole set of experiments was repeated twice, this yielded a total of 16 datasets with altogether approx. 800–1000 heartbeat rates each coming from three successive measurement of an individual. The proper way of processing these data would have been to construct an appropriate ANOVA model and search for significant effects and interactions. However, there were various obstacles for this straightforward solution. To mention a few: (1) As some of the test organisms inevitably perished in our experiments their heartbeats were treated as missing data and so the amount of data for each experimental setup was not the same (if all test organisms perished their heartbeats were taken as zero). There are various ways of handling imbalance, e.g. adding cell averages as raw data, removing randomly selected raw data, or using a statistical model which takes into account this type of imbalance [25], but it certainly cannot be neglected. (2) There were certain limits of randomization in the experimental design. The test organisms were not incubated individually but in sets of ten which was very convenient from the experimental point of view but resulted in heartbeat rates that were not independent from each other. Experiments conducted with 48 hour exposition times were actually the continuation of the 24 hour exposition time experiments, so those results were not independent from each other. And finally, due to the large number of samples the complete temporal segregation of treatments could not be prevented. Disregarding these limits of randomization could yield to fallacy as *p* values would be unduly low and thus significances unduly high. Constructing a proper statistical model which considers all these limits would be an elegant solution, but it would be quite sophisticated and it is outside the scope of this paper. Instead, a simpler approach was followed, which produced satisfactory results.

First, heartbeat rates for each vessel were averaged. This balanced the design and by lowering the degrees of freedom to a more appropriate level it compensated for the limit of randomization introduced by not incubating the test organisms separately (which the authors believe had the highest influence on the outcome of the analysis).

Next, relative inhibition rates were calculated at each experimental condition for each successive triclosan concentration. For example, the relative inhibition at 20 μg/L triclosan concentration was calculated by comparing the average heartbeat rates at 20 μg/L to those at 2 μg/L for each experimental setup and each vessel in parallel. The resulting dataset of 80 averaged heartbeat rates and relative inhibitions (the highest administered concentration of triclosan yielded full inhibition in all cases and thus those results were omitted from further analysis) was split by the five remaining triclosan concentrations (control, 0.2, 2, 20, 200 μg/L) into 5 subsets and then the 16 entries (two parallels for each of the 8 experimental conditions) in each of the 5 data subsets were ranked separately by decreasing relative inhibitions. Finally, the relative inhibition ranks gained at each triclosan concentration were summed for each experimental condition (Sum of ranks, SOR).

Table 3 shows that most tested experimental conditions performed roughly equally, but one of them seemed to perform substantially better than the rest.

Table 3 Sum of ranks (SOR) and EC_{50} values [μg/L] calculated for each experimental condition

Medium	Age	Time	**SOR**	**EC_{50}**	**LCI[a]**	**UCI[b]**
Water	10 day	48 hour	53	35.2	6.55	276
Water	3 day	24 hour	75	303	207	466
Water	10 day	24 hour	81	327	200	587
M7	3 day	48 hour	86	334	243	480
M7	3 day	24 hour	91	329	247	450
M7	10 day	24 hour	92	340	259	453
M7	10 day	48 hour	96	379	310	469
Water	3 day	48 hour	106	369	315	437

[a]LCI: Lower Confidence Interval of EC_{50}

[b]UCI: Upper Confidence Interval of EC_{50}

[c]EC_{50} values were derived with OriginLab 8.0 software applying Dose Response Function fitting: y = A1 + (A2–A1)/(1 + 10^((LOGx0–x)*p))

To determine whether this difference was significant, the sum of ranks was calculated for each parallel of each experimental condition, resulting in two sums of ranks for each experimental condition. Kruskal-Wallis test on the ranks themselves produced a *p*-value of 0.076 (H0: the performance of the 8 experimental conditions is the same, H1: at least one of the experimental conditions performs differently than the rest) indicating that the experimental condition that scored the best might indeed be better than the others. The averaged inhibition percentage values of the

Table 4 Inhibition percentage values of the *D. magna* heartbeat rate test under different experimental conditions

	Inhibition percentage [%]							
Exposure	24 h				48 h			
Medium	tap water		M7 medium		tap water		M7 medium	
Age	3 days	10 days	3 days	10 days	3 days	10 days	3 days	10 days
0.2 µg/L	8	18	5	6	13	23	11	12
2 µg/L	7	18	12	16	8	28	15	14
20 µg/L	14	26	13	14	12	42	18	24
200 µg/L	31	29	22	19	5	48	24	9
2000 µg/L	100	100	100	100	100	100	100	100

two parallels in the case of the eight different experimental setups are summarised in Table 4. However decimal dilution series are not optimal for determining EC_{50} values, an attempt was made to compare the sensitivity of the different experimental conditions with the help of effective concentration values. The effective concentration values are summarised in Table 3. It has to be noted that the EC_{50} values are in line with the SOR values (correlation coefficient r=0.903, p=0.02, carried out by Pearson correlation using Dell Statistica 13® data analysis software system), furthermore the lowest ranked experimental setup (10-day old cladoceran in tap water incubated for 48 h) is characterized by the lowest heartbeat rate EC_{50} value in the case of 10-day old cladoceran in tap water incubated for 48 h.

Box&Whisker diagrams of the best (tap water, 10-day-old daphnids, 48 h), the worst (tap water, 3-day-old daphnids, 48 h) and two moderately performing experimental setups (M7, 3-day-old daphnids, 48 h; M7, 3-day-old daphnids, 24 h) (Fig. 1) clearly show that the cladoceran heartbeat rate confirmed a sensitive concentration-dependent response to triclosan. The highest decrease in heartbeat rate was observed in the case of 10-day old cladoceran in tap water incubated for 48 h. The lowest exposure concentration of triclosan (0.2 µg/L) resulted in significantly lower heartbeat rate compared to control, demonstrating that the assessed sublethal endpoint is a sensitive endpoint and thus it is suitable as a potential early stress indicator of the exposure to triclosan. This finding is of paramount importance because sublethal effects of pollutants on *D. magna* may lead to population decline and consequently may generate alteration of other aquatic biota populations. The results showed that daphnids of various ages respond differently to the toxicant in different media and also at different exposure times. However, the current study did not aim to investigate the mechanism underlying the mode of action of triclosan concerning the different sensitivity of 3-day-old and 10-day-old test animals.

In aquatic ecosystems, triclosan possesses the potential to cause adverse effects on large number of species. Considering the effect of triclosan there is a strong evidence that aquatic organisms such as algae, invertebrates and certain types of fish are much more sensitive to triclosan than mammals. This widely applied antibacterial agent used as additive in a variety of consumer products is highly toxic to algae and exhibits developmental as well as reproductive adverse effects to fish [26-28].

Huebner et al. [29] examined age-related vulnerability of *Daphnia magna* to UV-B radiation. The age-related relationships between UV-B dosage and reproduction, and the effects of duration of PRR (photorecovery radiation) on survival and fecundity were determined. Their results demonstrated the need to consider age when examining the effects of UV-B on zooplankton and the need to monitor responses over a sufficient length of time, which was the case in our experiments as well. Contrary to assumptions, Klein [30] demonstrated, that juvenile daphnids of the species *D. magna* Straus exhibited a very varied sensitivity towards toxic chemicals between the ages of 0–24 h, which can be a possible reason why the experimental setup with 10-day-old daphnids performed better.

The toxicity of triclosan is often associated with the formation of reactive oxygen species (ROS) and the crucial mode of action is primarily attributed to increasing ROS causing DNA damage [31, 32]. In the case of 10-day-old daphnids the more severe effect of triclosan may be attributed to the increasing stress due to accumulated free radical damage [33] compared to 3-day-old daphnids with shorter lifespan before administering triclosan.

Peng et al. [34] also demonstrated correlation between triclosan exposure and oxidative stress. They found that triclosan enhanced the activity of glutathione S-transferase and decreased the superoxide dismutase activity, which may point to the damage in the cell membranes, confirming that triclosan caused oxidative stress.

4 Conclusion

Due to the subtle effects of PPCP chemicals measured by conventional ecotoxicity methods at environmentally relevant concentrations their effects are underestimated and there is a high demand for new, more sensitive environmental ecotoxicity tests. Since the results and the sensitivity of ecotoxicity tests may be influenced by various factors, the development of an unconventional test method involves usually the comparison of biological responses under a variety of test conditions.

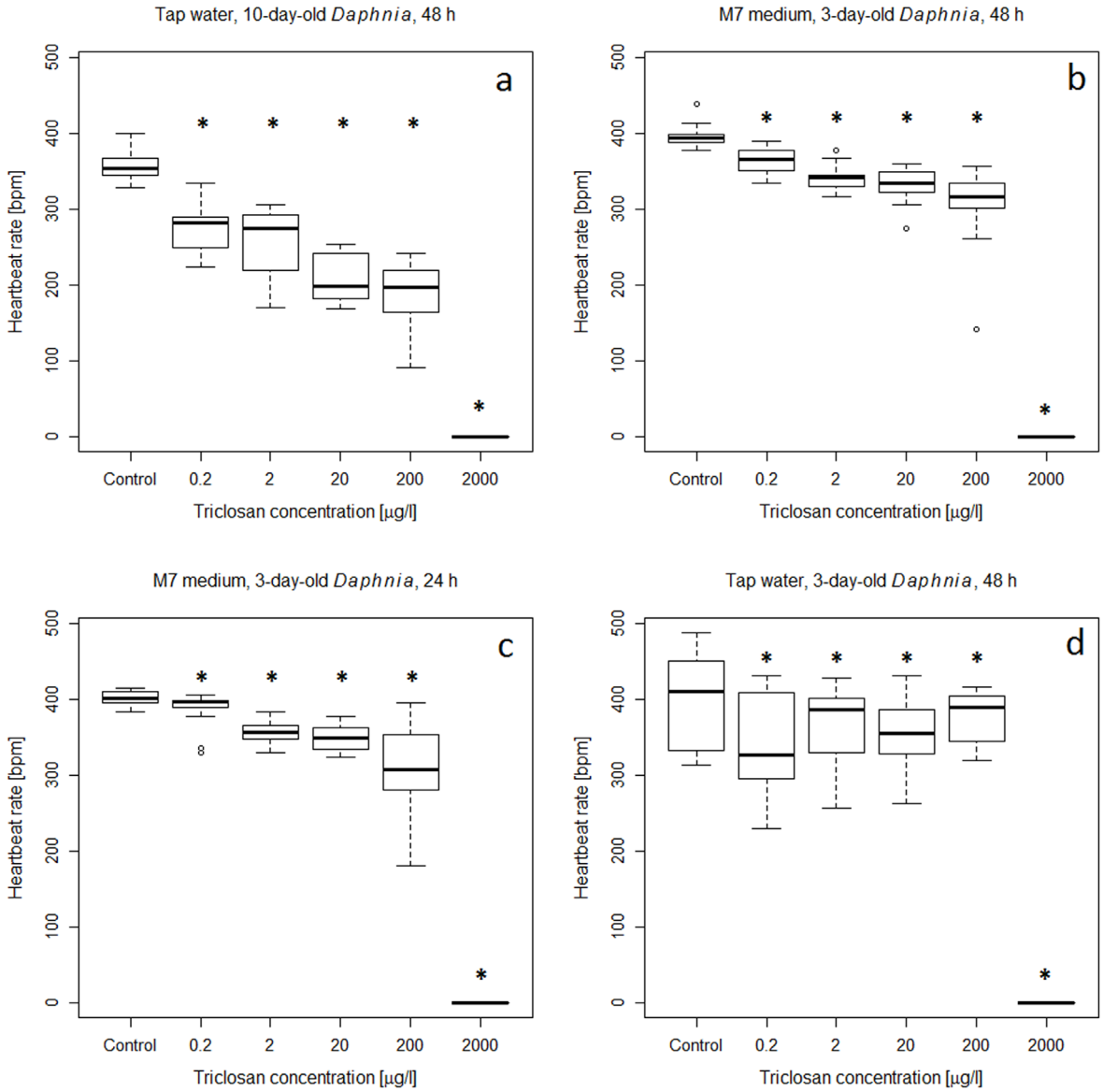

Fig. 1 Box&Whisker diagrams for selected experimental setups. On the diagrams bold horizontal lines represent the 25 and 75% percentile values; whiskers: minimum and maximum of the dataset; circles: outliers. Significant inhibition compared to control is marked by asterisk (*).

In line with the current efforts in the field of ecotoxicology assessment of aquatic micropollutants our aim was to establish a simple, reliable and cost effective heartbeat rate determination method by setting up its optimal experimental conditions. The sensitivity of the *D. magna* heartbeat rate test was compared in altogether 16 set of experiments with 8 different experimental setups (factors were the quality of the test medium, the age of the test organism, and the exposure time) at five different triclosan concentrations.

The 10-day-old daphnids cultivated for 48 h in tap water showed the most expressed response (highest inhibition percentages, lowest EC_{50} value) amongst all the experimental setups. The applied method provided a user-friendly and useful tool for optimizing the sensitivity of the heartbeat rate test (HBRT) for small sublethal effects, and the approach may be employed also to other ecotoxicity test systems.

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