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Suitability of *Eucyclops serrulatus* (Fischer 1851) (Crustacea: Copepoda) for online biomonitoring of water quality using the new Microimpedance Sensor System

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ABSTRACT

Copepods are priority crustaceans for toxicity tests and higher tier environmental risk assessment, especially in marine ecosystems. *Eucyclops serrulatus* inhabit both epigean and stygal freshwater habitats; they represent optimal candidates for biomonitoring due to their wide range of habitats and geographical distribution, short life-cycle and easy laboratory culture. Their sensitivity towards acute exposures to copper, tributyltin (TBTH), bisphenol-A (BPA), carbamazepine (CBZ) and diclofenac (DFNA) was studied and their potential as sentinels in a novel online biomonitoring system was evaluated. Regarding the LC50s of BPA and TBTH, *E. serrulatus* adults were more sensitive than *D. magna* neonates, whereas the sensitivity towards Cu, diclofenac and carbamazepine was similar in both species. *E. serrulatus* was able to survive in the new Microimpedance Sensor System (MSS) for one week without food and showed constant activity when both light and flow stimuli were "on" during recording. In the MSS, they responded faster to a cooper pulse (0.5 mg/l) than *D. magna*, with a clear immediate increase in activity (escape behavior). However, they only slightly responded to nitrate pulses (50 and 100 mg/l). Furthermore, chronic exposures to nitrate led to significantly decreased activity in the copepods. The new MSS could be recommended to monitor groundwater and drinking water intakes with *E. serrulatus* as a new ecologically relevant and sensitive bio-indicator species.

KEYWORDS: *Eucyclops serrulatus*, online biomonitoring, groundwater, drinking water, toxicity, bio-indicator.

1. INTRODUCTION

Copepods play an important role in the earth' marine ecosystems. They inhabit benthic, interstitial, parasitic and free-floating habitats in high abundances and species richness, they account for a large part to the meiofauna biomass, and serve as a prey item for invertebrates, fish and mammals. They consume algae (incl. toxic algae) and detritus, hence serving as important "cleaners" and bio-indicators for ocean pollution [1]. Copepods are priority crustaceans for toxicity tests and higher tier environmental risk assessment due to their high abundances in a wide range of freshwater to marine ecosystems, and their diverse feeding habits in different life stages [2, 3].

Whereas copepods are frequently being used in eco-toxicity testing for saltwater, freshwater copepods have not yet received sufficient attention in test development in spite of their important role in lake ecosystems, where they can reach higher abundances than daphnids. Copepods have been proven to be more sensitive to insecticides and fungicides than daphnids [2]. Only a few studies on freshwater copepods (with cyclopids being the largest family with 800 species) have been reported at species level [2].

No tests on freshwater copepods are listed in the United States Environmental Protection Agency

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(US-EPA) and the Organisation for Economic Co-operation and Development (OECD) test guidelines [4]. Marus *et al*. [5] recently developed a test with *Cyclops vernalis* for survival and growth. Brown *et al*. [6] developed a test with *Bryocamptus zschokkei* in microwell plates with leaf discs as a food source. Cifoni *et al*. [3] proposed to perform (sub) chronic toxicity tests with *E. serrulatus* on nauplia (at 18 °C, 11 days) and adults (18 °C, 19 d), supporting that sensitivity towards pollutants is higher at 18 °C compared to 25 °C and younger stages being more sensitive than adults [7].

E. serrulatus (Fischer, 1851) is a frequent freshwater cyclopid, with females reaching a size of 1.7 mm and males up to 1.3 mm [8]. This species can be found around the world, except in Far East and East Asia [9]. It is primarily regarded as epigean hyperbenthic freshwater inhabitant, which also lives in transitional habitats, *i.e.* in the interstitial and in genuine groundwater habitats, where it has been found up to a depth of 1.5 m [7]. *E. serrulatus* has a wide ecological tolerance and reproduces in groundwater, if organically enriched [10]. This species represents an optimal candidate for biomonitoring due to a wide range of habitats and geographical distribution, short life-cycle and easy laboratory culture [11].

Continuous biological early warning systems (BEWS) using living organisms ranging from bacteria and algae, to daphnids, gammarids, mussels and even fish as indicators are based on both optical and non-optical recording technologies, some of them are being used for drinking water monitoring in waterworks throughout Europe on a voluntary basis, especially in those locations which are prone to accidental pollution (*e.g.* Rhine) or bio-terror attacks (*e.g.* Lake Constance) [12, 13, 14]. BEWS with whole organisms as bio-indicators are based on rapid and pollution-sensitive changes in behavior, survival and/or respiration as alarm indicators. Whereas daphnids are being used in online biomonitoring copepods are not. Due to their small size they might be even more sensitive than daphnids. Their longer life cycle, especially in groundwater species, allows to record chronic, long-term-effects of low-dose chemicals such as micro-pollutants (µg/l, ng/l range).

The aims of this study are (1) to provide background toxicity data for *E. serrulatus*, (2) to test the species' performance and sensitivity (pulses of Cu, nitrate) in the new Microimpedance Sensor System© (MSS), and (3) to evaluate their sensitivity to chronic nitrate exposure.

2. MATERIALS AND METHODS

2.1. Test species and culture

Copepods were cultivated in a 10 L glass aquarium in a thermostat at a constant temperature of 18 °C, in darkness with aerated stream water (Hockgraben, Konstanz: 47.66734 °N, 9.20123 °E) and powderized detritus from pre-conditioned alder leaves as both sediment and a food source. Water was renewed weekly.

2.2. Sensitivity assessment-acute toxicity

2.2.1. Test substances

Copper (CU (II) Cl: CAS 7447-39-4, Sigma Aldrich) was chosen as a representative of an essential metal trace element in biota with important catalytic functions in the cells [15]. Cu is being used in the construction industry (*e.g.* pipes) and as a biocide anti-fouling agent in shipping and agriculture industries [16]. Anthropogenic discharge of copper originates from smelters, mine waste emissions, fertilizers and biocide usages [17]. Whereas Cu is not very toxic towards human beings, it is highly toxic to aquatic organisms: LC50 values show that Gastropoda, Crustacea and Oligochaeta are sensitive, whereas Trichoptera and Odonata are more tolerant to copper with LC50 values ranging from as low as 5 µg/l (*Daphnia* sp. 48 h) to 64 mg/l (Trichoptera) [18]. Low concentrations of between 8-23 µg/l affected growth, behavior, reproduction and feeding in different invertebrates under chronic exposures [18]. One important mechanism of toxicity is that dissolved Cu affects the gill membranes of aquatic organisms, *e.g.* due to inhibition of ATPase [19]. Cu concentrations in surface water range from 0.2 to 20 µg/l with the world median of 3 µg/l in surface water [17, 20].

Bisphenol-A (BPA, 4,40-isopropylidinediphenol; C15H16O2; CAS 80-05-7, Alfa Aesar) was chosen as an important additive in plastic products with endocrine and cancerogenic effects on aquatic life. Concentrations of up to 0.49 µg/l were found in wastewater effluents [21, 22]. The substance is used as a stabilizer in diverse resins and plastic bottles used for human consumption. Due to its potential of being taken up orally *via* food items the limits for daily accepted intake have been lowered from 50 µg/kg body weight to 4 µg/kg body weight [23]. France has forbidden the use of BPA in food packaging since 2015. In surface water concentrations between 0.01-2.4 µg/l have been found and 6-30 µg/kg in the sediments. With the recent increasing awareness of plastic pollution in the oceans and rivers the debate on additives such as BPA, being released from (micro) plastics during degradation in aquatic environments, has received increasing attention.

Carbamazepine (CBZ, CAS 298-46-4, Sigma Aldrich) is a derivate functioning as antiepileptic and neuropsychological drug and serves as an anthropogenic marker in water bodies [24], where it has been found in concentrations up to 0.6 µg/l [25]. The acute toxicity towards aquatic invertebrate and fish has been reported as follows: LC50-96 h for *Daphnia magna*: 76.3 mg/l and *Oryzias latipes*: 35.4 mg/l [26]. *Chironomus riparius* (emergence) was affected at > 70 µg/kg in life-cycle exposures in spiked sediments [27].

Diclofenac (DFNA, Diclofenac sodium salt, item 70680 German Chemical Company) is a non-steroidal anti-inflammatory drug (NSAID), which is found in many water bodies due to its uncontrolled and increasing use as painkiller. As only 1/3 of the substance is being absorbed in human beings, great amounts of this chemical are released *via* wastewater treatment plants (WWTPs) into the aquatic environment. Due to its increasing environmental relevance it has been classified as a chemical of emerging concern and placed on the watch list of the European Union (EU). In tap water levels of about 10 ng/l were found, while in European surface water bodies levels range from 1-162 ng/l (UK) and 2.8-56 ng/l (Spain); a maximum value for WWTP-effluents has been reported at 0.99-1.3 µg/l [28].

Acute and chronic toxicity of diclofenac has been studied in fish (brown trout, zebrafish) and *G. fossarum* considering a series of endpoints such as mortality, juvenile/adult ratio and reproduction-related parameters [29]: the most sensitive parameter for gammarids was the juvenile/adult ratio with a lowest observed effect concentration (LOEC) of 2.6 mg/l compared to impairment of reproduction of *Daphnia magna* at LOEC = 6.25 mg/l. Brown trout were affected at 100 µg/l with slight decreases in body mass and survival after 14-25 d of exposure. Hatching of zebrafish eggs was delayed at 100 µg/l too. Juvenile *G. fossarum* had an LC50 (48 h) of 58 mg/l, whereas chronic exposures showed effects such as elevated mortality and decrease in egg-bearing females at 24 mg/l.

Tributyltin (TBTH, Tributyltinhydride, $C_{12}H_{28}Sn$, CAS 688-73-3) has been used as an anti-fouling biocide in ship paints, cages, fish nets as well as wood preservatives, disinfectants and biocides in cooling systems since 1960s [20, 30]. TBT is known to be toxic at very low concentration levels and able to accumulate in the food web [31]. Toxic effects such as mortality in mussels [32], and imposex in gastropods [33-36] have been reported. LC50 values reported in literature for TBT range from 0.015 ppb (*Tigriopus japonicas*) [37] to 36 ppb (*Tisbe biminiensis*) [38]. LOEC values range from 6 ng/l to 60 ng/l (*Psydodiaptomus marinus*) [39]. TBT has been forbidden internationally in 2008 [38].

2.3. Experimental setup

Stock solutions of all chemicals were prepared without solvents in bottled Volvic water (drinking water quality: pH 7.0; Calcium 12 mg/l, Chlorid 15 mg/l, Sodium 12 mg/l, Kalium 6 mg/l, Silizium 32 mg/l, Hydrogene Carbonate 74 mg/l, Magnesium 8 mg/l, Sulfate 9 mg/l). All experiments were performed in glass beakers, at 18 °C in a thermostat without illumination and aeration. The tests lasted for 4 days (acute) or 18 days (chronic nitrate test), with 3 recordings of survival and behavior in the MSS. Whereas copepods were not fed during acute tests, food (1 ml yeast suspension and 1 drop of living *Chilomonas paramecium* culture) was added on days 1, 6 and 12 during the chronic nitrate exposure test. The concentration levels tested are shown in Table 1.

Whereas in the acute tests five copepods were placed in each beaker, ten animals were used in the chronic nitrate test. Ten copepods (size: 0.9-1.2 mm) were placed in a 100 ml glass beaker filled with 50 ml solution and 0.5 ml fine-particulate sediment from the culture as a food source; 3 (chronic test) - 5 (acute tests) replicates were set up for each concentration level. Beakers were not aerated and covered with parafilm. Survival was counted (40x magn.) regularly and behavior was recorded several times in the MSS

Table 1. Test concentrations of the chemicals used in the acute toxicity experiments (96-h).

Cu (µg/l)	0	5	10	12.5	25	50	
BPA (µg/l)	0	10	100	500	1000	25000	50000
CBZ (mg/l)	0	25	50	75	100		
DFNA (mg/l)	0	5	10	20	50	80	100
TBT (µg/l)	0	0.0002	0.002	0.02	0.2		

Fig. 1. The Microimpedance Sensor System (MSS): Photograph: A. Stertzik. From left to right: reservoir with water (stained in pink for photography only), pump and electronic unit, chamber array set-up with 8 individual flow-through chambers and USB-camera on the top, laptop with specific software for data recording and analysis.

during the chronic nitrate experiment. Pulse experiments in the MSS (8 organisms) with a flow-through design were performed with copper and nitrate to simulate pollution pulses over 4 days. The chronic nitrate test was performed to mimic the actual problematics of chronically elevated nitrate levels in groundwater in Germany.

2.4. Performance of *Eucyclops serrulatus* in the MSS

The Microimpedance Sensor System (MSS) (Fig. 1) has recently been developed for recording small aquatic meiofauna and early life stages of aquatic invertebrates based on the technology of the Multispecies Freshwater Biomonitor© (MFB) [12, 13]. The new MSS consists of a chamber array made of polyoxamethylene (POM) based on Gerhardt [40] with 8 chambers (5 mm diameter and 5 mm depth) receiving individual flow-through from bottom to top *via* silicon tubes connected through a multichannel pump (Watson Marlow Model 040009-4A-005) with adjustable flow rates. The water is taken from a reservoir made of acrylglass (25 cm x 15 cm x 25 cm). Two pairs of stainless steel electrodes are mounted on the inner chamber walls with equal distances from each other (quadrupole impedance conversion technology) [12]. The chambers can be individually opened and closed on both sides with nylon net-bearing lids (mesh size: 0.1 mm). Within each chamber an LED is mounted for illumination, *e.g.* simulating a diurnal cycle or setting artificial light stimuli. On top of the chamber array a steel ring with a USB-camera is mounted to allow for additional visual observation of the animal within the chamber. The MSS is connected to the MFB-electronics which has been adapted to recording of small signals. In the software settings, new functions such as the settings of the pump (interval, flow rates), light stimuli (interval and duration), and new alarm algorithms (*e.g.* Double sigma detector, Jump detector) have been implemented for each channel individually.

2.4.1. Establishment of best monitoring conditions

Eight copepods (0.9-1.2 mm) were taken from the culture (18 °C) and placed individually in each chamber of the MSS. The flow rate was set to 1 ml/min. Experiments were performed at room temperature (20 °C) and lasted from 17:00 pm until 9:00 am of the following morning. Each experiment was run at least twice. During the experiments the MSS was covered with a black foil to prevent light from the surroundings. Recording periods lasted for 4 min., followed by intervals of 6 min each. The following settings were used in different binary combinations: light (L) and pump (P) on or off, either during recording or permanently.

2.4.2. Sensitivity of *E. serrulatus* in the MSS

Pulse tests (4 days' duration) with copper (0.5 mg/l) and nitrate (50 and 100 mg/l) were performed to establish the stress responses of the copepods to 2 different reference substances of relevance for groundwater pollution. Eight copepods were placed in the MSS and their normal activity was recorded in Volvic water for 24 h with the optimal settings as described above. Afterwards the toxic solution was added to the reservoir and slowly pumped individually through the chambers without any physical interference in the chambers or to the animals. It took 11 min. for the water from the reservoir to reach the chambers at a flow rate of 1 ml/min. The toxic pulse lasted for 24 h, followed thereafter by a recovery period in Volvic water for another 24 h. In the recovery period the toxic water in the reservoir was replaced by Volvic water without interfering the recording. During these 4 days no food was added as previous trials showed that the animals survive well during 4 days without adding food.

2.5. Statistical data analysis

All data were processed in Excel and SigmaPlot. LC50 values were calculated based on the Probit method. Comparisons of test parameters between different concentration levels and the controls were calculated with non-parametric tests such as repeated measurement (RM) analysis of variance (RM-ANOVA) on ranks or signed rank tests for time-dependent data.

3. RESULTS

3.1. Acute toxicity tests

Table 2 shows the results of the acute toxicity tests with *E. serrulatus*: TBTH being the most toxic, followed by Cu, Diclofenac, Carbamazepine and BPA.

3.2. Performance in the MSS

3.2.1. Establishment of best monitoring conditions

The highest activity with the least variance and outliers was achieved when both light and pump were on during the recording (Fig. 2). Both runs are very similar too (Mann Whitney U test: NS), *i.e.* reproducible results could be achieved with these settings (Fig. 3). All other settings were either more variable or achieved less activity of the copepods during the whole exposure period. In general, activity declined slightly throughout the exposure in all settings (Fig. 3). The setting with light and pump permanently switched on also showed similar and reproducible results with low variation, however at a lower general activity level. Previous experiments with 8 copepods exposed individually in well plates

Table 2. LC-50 (96 h) values for *E. serrulatus*.

96 h	LC50	Lower 95%-CI	Upper 95%-CI
Cu (µg/l)	14.3	12.4	17.0
BPA (µg/l)	793.2	501.2	1195.4
CBZ (mg/l)	54.6	42.7	67.2
DFNA (mg/l)	37.0	26.5	55.4
TBTH (µg/l)-72 h	0.28 (0.1 ng/l: 8 d)	-	-

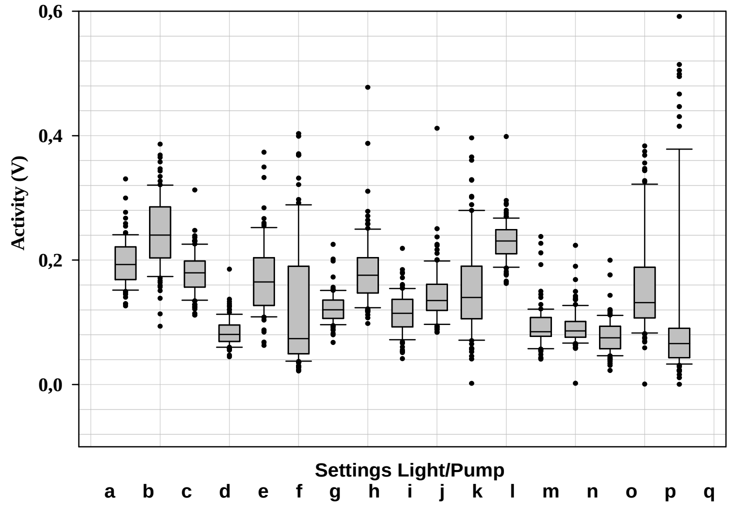

Fig. 2. Box plot of the locomotory activity of *E. serrulatus* (N: 8) over 16 h with different settings of light and flow stimuli. Grey boxes: median and 25% and 75% percentile; whiskers: variance; dots: outliers. Settings of light (L) and pump (P) stimuli: a, b: L+P: during recording on; c, d, e: L during recording on and P continuously on; f: L during recording on and P off; g, h: L continuously on and P during recording on; i, j: L+P continuously on; k: L continuously on and P off; l, m: L off and P during recording on; n, o: L off and P continuously on; p, q: L+P off.
The experiments were performed 1-3 times.

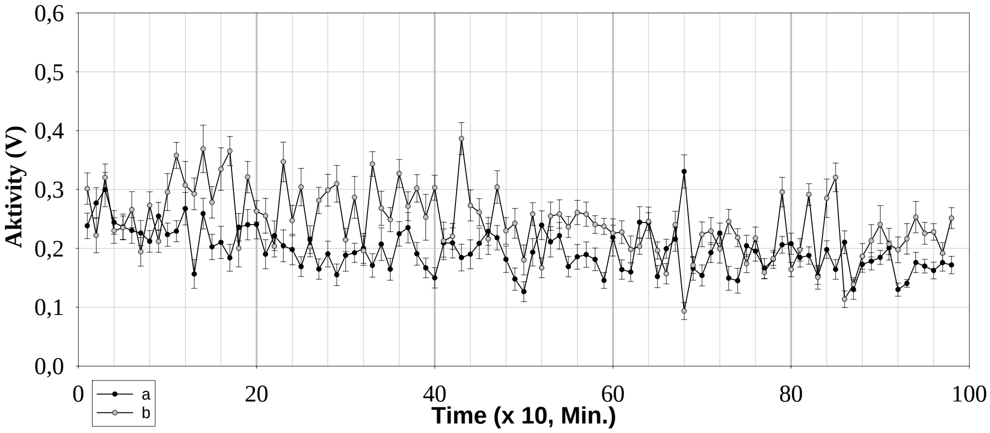

Fig. 3. Time course of locomotory activity of *E. serrulatus* (2 runs (a, b) with N: 8; means and sd) over 16 h with light and flow stimuli on during recording periods in the MSS.

in the dark, where the response to light stimuli with a torch (for 4 min, followed by an interval of 6 min.) was manually recorded over 400 min., showed that the animals can be activated by a light stimulus; however, their activity upon the light stimulus decreased and the response time to the onset of light increased from 10 to 30 sec. towards the end of the exposure, due to habituation after several days (Gerhardt, unpubl. data). The present automatic recordings in the MSS confirmed these visual observations and showed additionally that a combination of both light and pump stimuli in an interval setting keeps the activity of the copepods high for at least 16 hrs.

3.2.2. Sensitivity of *E. serrulatus* in the MSS

E. serrulatus reacted fast and in a reproducible manner to copper, with an immediate increase in

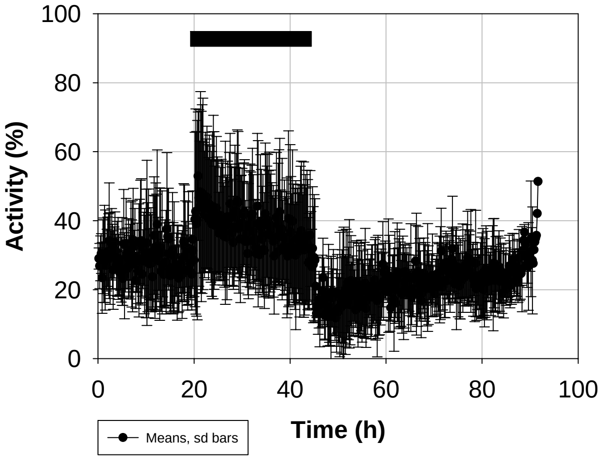

Fig. 4. Behavioral response of *E. serrulatus* in the MSS to copper (0.5 mg/l). Locomotory activity (%) of 24 organisms (means, sd), recorded over a total of 4 days. The black bar shows the start and duration of the Cu-pulse.

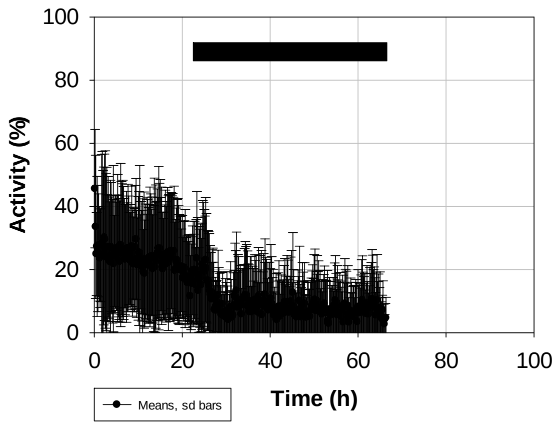

Fig. 5. Behavioral response of *Daphnia magna* in the MSS to copper (0.5 mg/l). Locomotory activity (%) of 8 organisms (means, sd), recorded over a total of 2.8 days without recovery period. The black bar shows the start and duration of Cu exposure.

spontaneous locomotory activity (escape behavior), which only decreased again in the recovery period with clean Volvic water (Fig. 4). All animals survived the pulse including the recovery period.

A similar test was performed with *Daphnia magna* (size 1.5 mm) in the MSS, showing a short and slight increase in locomotion after onset of copper dosing, followed by a decrease in activity and death of 43% of the daphnids until the end of the recordings in copper solution (Fig. 5).

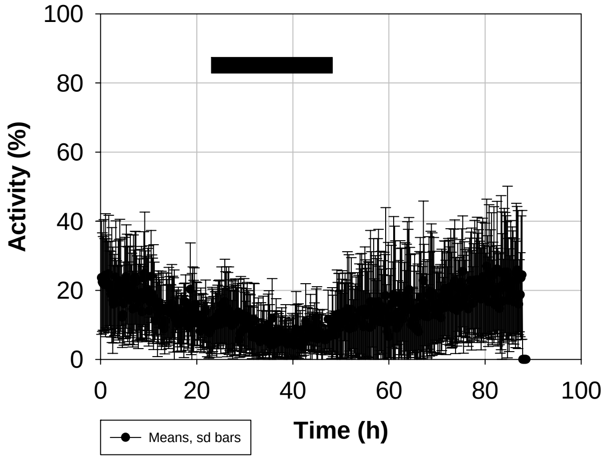

Fig. 6. Behavioral response of *E. serrulatus* in the MSS to nitrate (100 mg/l). Locomotory activity of 8 organisms (means, sd), recorded over a total of 3.6 days. The black bar shows the start and duration of the nitrate pulse.

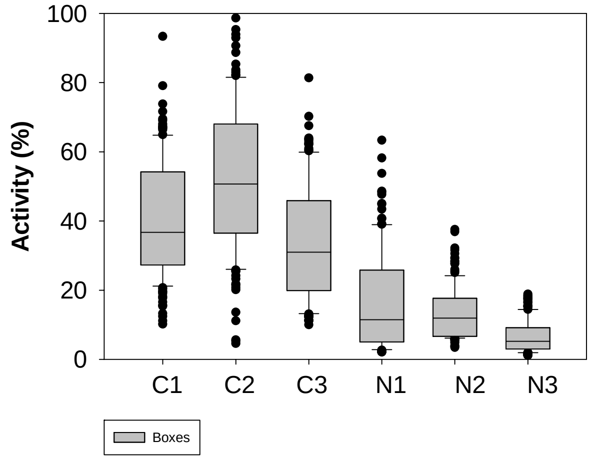

Fig. 7. Box plots of the locomotory activity of surviving *Eucyclops serrulatus* under chronic nitrate (50 mg/l) exposure (N: 8) in 3 subsequent experiments (1, 2, 3) compared to the resp. controls (C) over 18 days.

Both species can be used to record short-term copper pulses in the MSS.

Neither a pulse of 50 mg/l nor 100 mg/l nitrate had any severe effects on survival of *E. serrulatus*. All animals survived both tests, but slight differences in locomotory activity could be seen, such as (1) decrease in activity by ca. 10% during the nitrate pulse and (2) increase in activity and variability in the recovery period (Fig. 6).

3.3. Chronic nitrate exposures

Similarly to the acute nitrate pulses, the copepods responded to chronic nitrate exposures with reduced activity in 3 subsequent experiments (8 organisms) of 18 days' duration ($p < 0.001$, Signed Rank tests: control/exposure) (Fig. 7). In spite of regular feeding (every 6 days with both 1 ml yeast suspension and 1 drop of *Chilomonas* suspension) ca. 50 % of the animals died in the beakers in both control and nitrate exposures. This might have been due to the repeated handling stress of repeatedly taking the animals out of the beakers and placing them in the MSS for behavioral recording (3 times 2 h) and thereafter back again.

4. DISCUSSION

4.1. Sensitivity of *E. serrulatus*

Acute toxicity tests with different types of chemicals revealed new data on a new indicator species. Regarding copper, the LC50-48 h for neonates of *D. magna* are reported from 1.29 mg/l [41] to 8-51 mg/l (depending on hardness) [42]. *E. serrulatus* (adult!) showed an LC50-96 h of 14.3 mg/l, which is in the range of the literature data for daphnids. Moreover, it has to be taken into account that we tested adult copepods, while most literature data on *D. magna* report the testing of juveniles, which generally appear to be more sensitive compared to adult life stages. The LC50-48 h for BPA in neonates of *D. magna* was found at 10 mg/l [43] and the EC50-48 h for immobility at 12.9-19.8 mg/l depending on juvenile age [44]. Adults of *E. serrulatus* were more sensitive in the present study, with an LC50-96 h of 0.79 mg/l. Already at 100 µg/l BPA caused a 50% mortality in *E. serrulatus* after 7 days' exposure and locomotory activity increased on day 7 and 14 at this concentration level in a previous chronic toxicity test (Gerhardt, unpubl. data).

Carbamazepine and diclofenac were less toxic than copper and BPA to both species. The LC50 for neonates of *D. manga* were 76.3 mg/l (48 h) and 22.4 mg/l (96 h) [26] compared to the LC50-96 h for *E. serrulatus* of 54.6 mg/l in the present study. Also for diclofenac the LC50 value for *D. magna* neonates (22.4 mg/l, 48 h) [29] was comparable to that of *E. serrulatus* adults (37.0 mg/l, 96 h).

TBTH was found to be extremely toxic in both species: *E. serrulatus* adults had an LC50-96 h < 1 ng/l TBTH and in a previous study an LC50-48 h < 0.2 ng/l TBTH was found, compared to *G. fossarum* [45] and *Daphnia magna* neonates (LC50-48 h 1.67 µg/L TBTO, LC50-96 h 5.9 µg/l TBTCL [46]. At 1.1 µg/l a drastic increase in response time to a light stimulus was seen in the copepods already on day 1 and day 3; whereas on day 8 at > 0.15 µg/l TBTH response time increased significantly compared to the controls (Gerhardt, unpubl. data) (RM ANOVA on Ranks; control different from all other TBT-treatments ($p < 0.05$)). The fungicide Trihenyltinhydroxid was found to be highly toxic (LC50-96 h < 0.07 mg/l) to *Mesocyclops leuckarti* [2], and Triphenyltin acetate was toxic at < 0.0003 mg/l (LC50-96 h) to *Acanthocyclops venustus* [47]. Di Lorenzo *et al*. [48] reviewed toxicity studies on N-fertilizers and pesticides performed with freshwater copepods from 1948 to 2014, showing that insecticides and fungicides were more toxic than herbicides and that fertilizers are the least toxic in acute toxicity tests (mortality). The very few studies on hypogean copepods tend to reveal higher sensitivity of hypogean species compared to epigean species with regard to pesticides [48]. Moreover, they found juvenile stages to be more sensitive than adults to the herbicide Imazamox [7], which might be attributed to thinner exoskeleton, higher metabolic rates and less efficient detoxification systems in juveniles [49].

4.2. Performance of *Eucyclops serrulatus* as a bio-indicator in the MSS

E. serrulatus survived one week without food and could be cultivated easily with suspensions of yeast or *Chilomonas paramecium* over several weeks in Volvic water (Gerhardt unpubl. data). The culture is regarded at least as easy as that of daphnids. Cifoni *et al*. [3] developed a full life-cycle test with *E. serrulatus* and criteria for feeding and rearing this species. Kulkarni *et al*. [2] stressed the advantages of copepods in eco-toxicity testing. However, copepods are still not frequently used for freshwater toxicity evaluation and online biomonitoring.

E. serrulatus performed well in the new MSS, with reproducible locomotory activity under both light and flow stimuli during recording intervals. The copepods survived the standard stand-alone time for biomonitors of one week. During toxic pulse experiments with copper, both *D. manga* and *E. serrulatus* performed in the MSS; however, *E. serrulatus* showed a clear, immediate increase in activity upon Cu-dosing (escape behavior), whereas *D. manga* showed less clear behavioral responses, but increased mortality after continuous exposure. This indicates the potential of *E. serrulatus* as a rapid and sensitive behavioral bio-indicator in the MSS for early warning of changes in water quality. Such immediate increases in activity (hyperactivity, escape responses) to copper have been observed earlier in freshwater copepods [50], assuming that these responses might be “involuntary” reflexes with not necessarily positive ecological outcomes for the organisms, *e.g.* due to increased predation risk and high energetic costs.

However, not all chemicals induce behavioral early warning responses, *e.g.* nitrates. Whereas there was only a very slight trend towards a short increase in locomotion followed by a decrease in activity in *E. serrulatus* exposed to a nitrate pulse of 100 mg/l, a significant decrease in locomotion could be confirmed after a chronic exposure of the copepods to 50 mg/l nitrate (18 days).

The development of biological early warning systems dates back to the Sandoz accident in 1986; since then several online biomonitors based on physiological (*e.g.* respiration, bioluminescence of bacteria) or behavioral (*e.g.* immobility/mortality, locomotion, ventilation of aquatic invertebrates and fish) parameters have been developed, some of them have been implemented along large rivers, *e.g.* in Germany and the Netherlands [14]. As the Sandoz accident led to a temporary shortage of drinking water distribution in waterworks along the river Rhine, online biomonitoring systems found applications in waterworks using surface water as a source for drinking water. Storey *et al.* [51] and Bae and Park [52] reviewed technologies to monitor drinking water quality. The advantage of using whole organisms is the integration of mixture toxicity effects from a chemical cocktail including synergistic effects, which cannot be evaluated by the chemical analysis of single substances alone. As organisms and their intact populations represent the protection goal in water and nature conservation legislation, organisms represent the true sentinel for the evaluation and (continuous) surveillance of pollution. This insight leads to expanding fields of application of online biomonitoring, such as wastewater effluents and/or process control of selected purification steps [53].

Chemical online and inline sensors are meanwhile frequently used in both waterworks and wastewater treatment plants for real-time process control and detection of variations in selective parameters such as pH, conductivity, turbidity, chloride, nitrate and ammonium. Contrary to these simple sensors online biomonitors are more complex as they use living organisms, which need to be cultured and fed. Next to this, increased maintenance effort compared to chemical online sensors and the lack of trained staff often hampers their implementation. Recently new online biomonitors have been developed with less maintenance effort and longer stand-alone times, *e.g.* the MFB has successfully been applied for effluent control in a wastewater treatment plant with a stand-alone time of up to 3 weeks, using *Gammarus fossarum* as the bioindicator [53]. This stand-alone time is much longer than that reported for most other online biomonitors [54].

Moreover, the new MSS has the potential of application in groundwater and drinking water online biomonitoring with high sensitivity (enables the use of very small early life stages of aquatic organisms or small species such as copepods) and low maintenance (use of organisms with the ability to starve or feed on biofilm and fine organic matter from the raw water), both important criteria for the acceptance of online biomonitoring technology. The bio-indicator species is the “heart” of any online biomonitor system, and therefore systems with multi-species applications tend towards higher flexibility, wider application and higher sensitivity. *Eucyclops serrulatus* proved to be a sensitive and easy-to-handle new species to monitor, *e.g.* groundwater and drinking water intakes. Next to our setting of maintaining high locomotory activity *via* intervals of both light and flow stimuli other settings of the MSS are possible. In case of no stimuli *E. serrulatus* naturally settles after an acclimation period; this low activity might be considered as baseline and increasing activity due to escape responses regarded as alarms. However, in such a setting, chemicals causing decreases in activity (*e.g.* nitrate) cannot be detected. This shows that the choice of test species and the definitions of their early warning behavior have to be studied well and have to be adapted to the local monitoring needs before being applied in a biomonitor.

5. CONCLUSION

The freshwater copepod *E. serrulatus* proved to be highly sensitive to different toxic chemicals in acute exposures. Moreover, this species performed well in the Microimpedance Sensor System (MSS) and responded to pulses of both copper and nitrate and might be used in future online biomonitoring.

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CONFLICT OF INTEREST STATEMENT

There are no conflicts of interest.

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