Current Topics in
Toxicology
Vol. 16, 2020

Original Article

In situ online biomonitoring of groundwater quality using freshwater amphipods exposed to organic fertilizer and rainfall events

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ABSTRACT

Nitrate levels originating from agricultural fields can reach groundwater concentrations far above the allowed threshold values. In this context we studied the effects of pig manure applied as fertilizer on a field with crop during several simulated rain events. The effects on groundwater crustaceans were monitored continuously using the Multispecies Freshwater Biomonitor© (MFB) adapted to groundwater conditions and for application in the field. *Gammarus fossarum* (Koch, 1835) and *Niphargopsis casparyi* (Pratz, 1866) served as indicator species in the biomonitor. Pig manure was applied on the test field, once spiked with sulfamethoxazole. Three rainfall events of different intensity and duration were simulated directly after the fertilization and the responses of the animals were quantitatively recorded in the online biomonitor for 24 h to 7 days. *G. fossarum* responded within 24 h with decreasing spontaneous locomotor activity to elevated Nitrate levels being washed out from the surrounding soils. *N. casparyi* responded in two experiments of 7 days' duration with decreasing activity to elevated Nitrate levels in the 1st experiment and in the 2nd experiment to increased Nitrite and sulfamethoxazole levels showing decreased activity and 62.5% mortality. The three field experiments showed (1) successful operation of the online biomonitor MFB *in situ*, (2) *G. fossarum* sensitivity to Nitrate, (3) groundwater crustaceans' sensitivity to Nitrate, Nitrite and sulfamethoxazole. We recommend the use of subterraneous crustacean species for online biomonitoring of groundwater quality in agricultural areas as an effective measure to assess excess nutrients and pollutants aiming at subsequent reduction and compliance to the European groundwater directive.

KEYWORDS: groundwater, online biomonitor, *Niphargopsis casparyi*, *Gammarus fossarum*, Nitrate, antibiotics.

INTRODUCTION

Groundwater represents the largest freshwater reservoir in the world (up to 97%) and offers essential provisional ecosystem services for, *e.g.* human consumption, irrigation, cooling, storage, buffer of surface water levels and water purification. Safeguarding a minimum water level for running water ecosystems even in drought periods groundwater sometimes contributes up to 90% of the stream water, showing the importance of groundwater quality for surface water quality. On the other hand, surface water quality is important for groundwater quality as surface water contributes to the enrichment of groundwater reservoirs. In spite of this significance, only very few research has been conducted so far, regarding the effects of toxins, nutrients and climate change in groundwater [1, 2].

Agricultural practices affect river ecosystems, but studies on water quality of subterraneous communities are rare; however, such knowledge is crucial for

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sustainable use of agricultural and freshwater resources [3]. The groundwater chemical status is threatened by pollution, land use and irrigation. Therefore, identification and monitoring of protected areas is urgently needed for both surface and groundwater bodies with regard to drinking water, bathing water, endangered areas for water-related habitats and species as well as threatened areas according to the Nitrate guideline, with exceeding threshold values. As groundwater is a major resource for drinking water in Germany, threshold values for Nitrate (50 mg/L) have been aligned with the German drinking water directive (98/83/EG). However, some countries have stronger regulations, *e.g*. Nitrate levels in Switzerland may only reach 25 mg/L in groundwater bodies. In 2018, the European Court doomed Germany for a violation of the European Nitrate Guideline (91/676/ EG), as 28% of the monitoring sites exceeded the Nitrate threshold until 2014 [4]. The recent EU fertilizer regulation (EU 2019/1009) aims to harmonize, regulate and improve the standards and use of fertilizers in the EU in order to reduce pollution with nutrients and toxins.

Climate change might cause more droughts leading to decreased surface and groundwater levels and higher pollutant and nutrient concentrations. In order to understand the estimated changes in both agriculture and water sectors caused by climate change, it is important to perform continuous long-term monitoring at the groundwater sites [5].

The Water Framework Directive, WFD (2000/60/EG) requires both a good chemical and ecological status of surface water bodies including a deterioration ban. The European Groundwater Directive, GWD (2006/118/EG), on the legal basis of the WFD, demands a good chemical and quantitative status and sets quality standards for Nitrate, some biocides, pesticides and other chemicals. Emerging organic compounds, such as pharmaceuticals have not yet been considered. Moreover, there is no mandatory watch list of substances of emerging concern [6]. The Environmental Quality Standard Directive, EQS (2008/105/EG) requires that freshwater pollution with dangerous priority substances should be prevented, and pollution with other priority substances should be reduced. Regarding groundwater ecosystems a 1st attempt to summarize data on pharmaceuticals in groundwater leading towards a groundwater watch list has recently been published [6]. The authors found 31 pharmaceutical substances in groundwater bodies in several European countries, including antibiotics such as tetracyclines and sulfamethoxazole. Even though the levels in processed drinking water are about 1000x below the minimum acute therapeutic doses (MTD) and below analytical detection limits of 20 ng/l, chronic toxicity towards aquatic organisms, resistance formation, life-time exposure to humans and toxicity of transformation products are regarded of high concern [6, 7]. As health effects of lifetime exposure are still unknown, the widespread use of antibiotics for both human and veterinary treatment, especially for growth promotion and disease prophylaxis in intensive farming needs to be reduced.

Next to the provisional ecosystem services which are threatened by both pollution and climate change, also the groundwater ecosystem itself including its still unknown biodiversity and sensitivity towards pollution and climate change is at risk. Groundwater habitats have very specific abiotic and biotic characteristics, such as lack of light, constant temperature, low flow, small populations combined with slow reproduction, very few dominant invertebrate species and simplified food webs, long life cycles etc.; all these factors might make groundwater invertebrates more sensitive to pollution compared to their freshwater relatives [2, 8]. Ecotoxicological studies with groundwater species are very scarce and mainly focus on acute exposures to metals under laboratory conditions (69% of the available data) and a few macro- or microcrustacean species [2].

In this study we investigated for the first time the effects of the combination of fertilization and storm water events on a crop field in a realistic scenario on groundwater crustaceans *in situ*.

MATERIALS AND METHODS

Experimental setup and irrigation

The experimental field (48° 02’ 41.4’’N; 7°47’ 36.0’’E) represented a crop field (30 x 30 m^2) with high groundwater levels (ca. 1.5 m). Different groundwater level gauges were installed as well as a basic solar energy station (180-250 Wp) including 4 storage batteries (12V, 2 x 90 Ah, 2 x 100 Ah) and different types of sensor probes and autosamplers

(Fig. 1). The MFB-based Groundwater Biomonitor was operated during 3 experiments with *Gammarus fossarum* (24 h), and *Niphargopsis casparyi* (2 x 7 days), using 8 animals for real-time online biomonitoring of the groundwater quality during the experimental treatments. The artificial rainfall with groundwater from a nearby pipe outside the experimental field took place simultaneously at 12 sprinkling sites equally distributed in the experimental field with overlapping sprinkling range.

In the 1st experiment (24 h) the whole setup was tested and the flow direction of the water through the soil and groundwater horizons was mapped by labelling the irrigation water with Deuterium. The effects of the irrigation on 8 specimens of *G. fossarum* were continuously monitored in the Groundwater Biomonitor. A multiprobe recorded concentrations of oxygen, temperature and Nitrate. After placement of the animals in the biomonitor test chambers in the groundwater pipe in ca. 1.5 m depth, the irrigation (with 12 sprinklers) started and simulated a rainfall event of 42.16 mm rain with an intensity of 28.11 mm/h. This event lasted for 1.5 h. After 24 h a 2nd rainfall event was simulated for 3 h (amount 43.55 mm, intensity: 14.52 mm/h), supported by natural rainfall.

In the 2nd experiment (7 days) the effects of simulated rainfall and fertilization (pig manure) were studied on 8 specimens of *N. casparyi*, previously collected in a groundwater pipe near Neuenburg (47° 48' 45.1'' N; 7°32' 50.5'' E), also situated in the Rhine valley. The animals were placed in the biomonitor 16 h before the start of the irrigation experiments and their behavior and survival monitored continuously for 7 days during and after the experiment. The pig manure (580 L) was distributed equally in aliquots of 8 L on 72 sites (1.5 m x 3 m) in a core section of the experimental area (18 x 18 m^2: in a 90° angle to the groundwater flow direction). After application a rainfall event of 30 min (amount: 11.18 mm; intensity: 28.36 mm/h) was simulated followed by a subsequent irrigation for 2 h (amount: 9.05 mm; intensity: 4.52 mm/h). Daily water samples were analyzed for Nitrate and Nitrite by ion chromatography (930 compact IC Flex, detection limit: 0.5 mg/L).

In the 3rd experiment (7 days) the effects of simulated rainfall, fertilization and antibiotics (4 g Sulfamethoxazole added to 800 L aged pig manure) were studied. 580 L of this mixture was applied on the experimental core field as described above and the effects on survival and behavior of 8 specimens of *N. casparyi* monitored in the online biomonitor. The artificial rainfall event (amount: 10.63, intensity: 21.26 mm/h) was performed through 11 sprinkler units for 30 minutes. The antibiotics were analyzed with high-performance liquid chromatography-mass spectrometry,

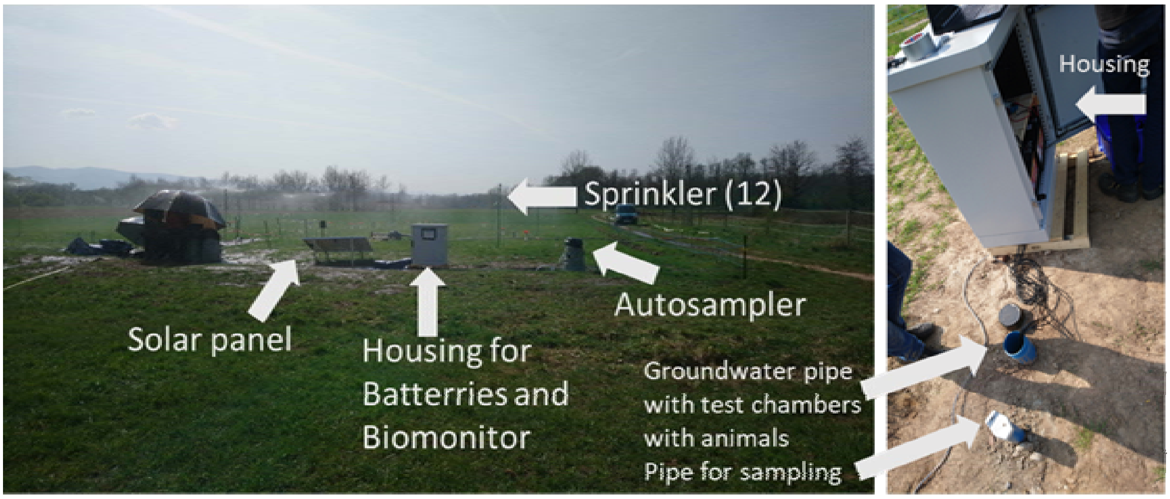

Fig. 1. Experimental field area with 12 sprinkler units for the simulation of rainfall events, housing for the biomonitor and batteries, next to the solar panel for the operation of the Groundwater Biomonitor, automatic water samplers and the groundwater pipe containing the biomonitor test chambers (right side) (Photos by N. Badouin).

HPLC-MS/MS (HPLC-Agilent 1200, Tandem MS API 4500 von AB Sciex) after solid phase extraction with Strata X columns (Phenomenex). The following columns were used to separate doxycycline and sulfamethoxazole: Kintetex and MZ-Aqua Perfect C 18, respectively.

Indicator species

Gammarus fossarum (Koch 1835)

The surface water amphipod *G. fossarum* is widely distributed in clean, forested and morphologically intact headwater streams in the Northern Hemisphere, where it can occur in high numbers and biomass, thus representing a keystone species in the aquatic invertebrate community. As omnivorous animals they have a broad food spectrum; however, they are also important leaf decomposers, and serve as prey for large invertebrates, fish and waterfowl [9]. The amphipods were kept in a laboratory culture in a thermostat (darkness, 12.5 °C, filtered water from Lake Constance of drinking water quality, leached alder leaves as food source).

Niphargopsis casparyi (Pratz 1866)

As a subsurface relative of *G. fossarum* the amphipod *N. casparyi* inhabits groundwater habitats in the Rhine Valley in the neighborhood of Neuenburg (*e.g.* 47°48' 45.1''N; 7°32'50.5''E). The animals were sampled a day before the field experiment and kept meanwhile in a thermostat box at 10 °C in groundwater from the sampling site.

Multispecies Freshwater Biomonitor©, MFB

The Multispecies Freshwater Biomonitor (MFB) records quantitatively in real-time the survival and behavior (*e.g*. locomotion, ventilation, inactivity) of aquatic animal species of a size of 3 mm onwards. The animals are placed individually in the flow through test chambers sealed on both ends with nylon mesh containing screw lids (mesh size 0.5 mm). On the inner walls of the cylindrical test chambers two pairs of steel electrodes are mounted, one pair is generating a high frequency alternating current, which does not affect the animals [10], while the 2nd pair of electrodes, placed in an angle of 90° to the 1st electrode pair is recording the changes in the electrical field caused by the animal's movements [11]. Different types of movements generate different signal frequencies, thus different behavioral fingerprints. Pollution pulses cause changes in the behavioral pattern of the organisms, which lead to an alarm in the software, if certain threshold values are surpassed [12]. In the BMBF GroundCare project the system was adapted to groundwater crustaceans, such as *Niphargopsis casparyi* by performing different laboratory toxicity tests to develop the optimal software settings and test chamber size for the groundwater indicator species [13-15]. Moreover, the *in-situ* operation of this new Groundwater Biomonitor with battery-buffered solar energy supply was validated.

RESULTS

Rainfall event with *G. fossarum* (24 h)

The first 4 recordings showed high and variable activity due to the immediate exposure of the gammarids in the groundwater pipe and the start of the sprinklers. During the spray irrigation phase the activity of the gammarids was normally high (30-40%) and apart from a short increase in activity at the end of the sprinkling the activity remained high for 2 more hours before it drastically declined to values below 20% after ca. 4 hours since the start of the experiment and an alarm (behavioral alarm: yellow) was given. The activity remained low (10-20%) until the end of the experiment (24 h), in spite of a 2nd spray irrigation (Fig. 2a). At the end all animals survived, but 4 organisms were very passive, *i.e.* did not react to a physical stimulus with the pipette. The values for Nitrate (multiprobe exposed in the center of the experimental field) showed initially levels above 100 mg/L, which increased rapidly during the spray irrigation up to a maximum of 300 mg/L after 5-7 hours (Fig. 2b). During this rain event Nitrate, was washed out of the soil in the groundwater horizon and provoked an immediate response of the gammarids. In the course of the experiment Nitrate levels decreased down to 100 mg/L again, but increased slightly to 200 mg/L due to natural rainfall in the crop field and remained high until the 2nd artificial spray irrigation, where Nitrate levels fell back to 100 mg/L (Fig. 2c). Moreover, local Doxycycline background levels in the groundwater were 0.044 mg/L at the start of the experiment, groundwater temperature 9 °C and oxygen saturation around 60%.

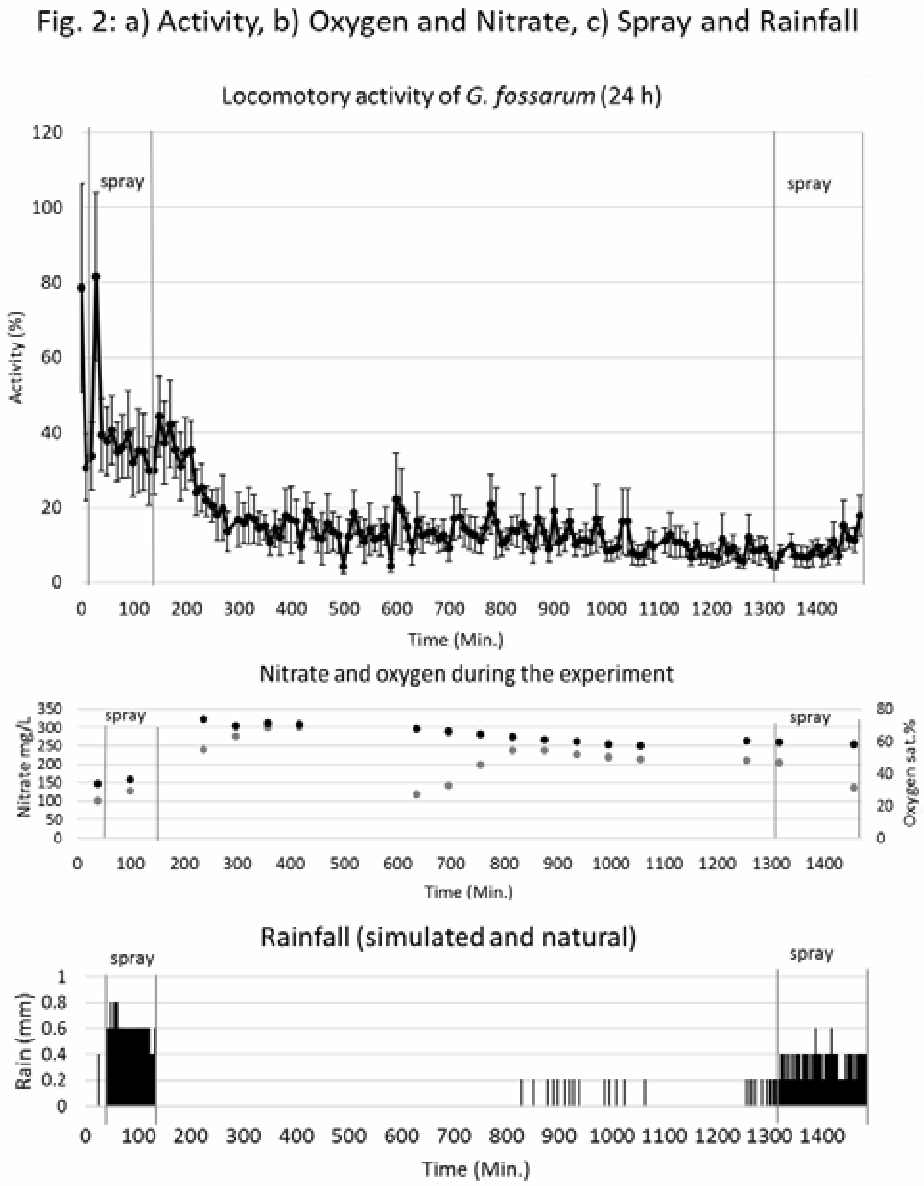

Fig. 2. Spontaneous locomotor activity of *Gammarus fossarum* (2a, top), recorded as means and SD bars, from 8 organisms over 24 h. Spray periods mark the artificial rainfall simulation by sprinkling from a hydrant (1: 1,5 h, 2: 3 h). Monitoring of oxygen saturation (black dots) and Nitrate (grey dots) (2b, middle), recorded periodically with a multiprobe. Natural and simulated rainfall (2c, bottom) during the experiment.

Rainfall events with *Niphargopsis casparyi* (7 d)

The groundwater organisms were exposed in the Groundwater Biomonitor 16 hours prior to the manure application and subsequent spray irrigation on the test field (2nd field experiment).

All 8 animals survived during the whole exposure period in the groundwater pipe and the pig manure application on the field. Prior to the fertilization followed by the subsequent spray irrigation (0,5 h) the activity of the animals was low, increased during the sprinkling and remained high during the following day, indicating an avoidance response (Fig. 3a). Thereafter, the activity of the organisms decreased drastically provoking a behavioral alarm, which coincided with the increase of Nitrate levels (78.89-94.12 mg/L) (Fig. 3b). Nitrite levels were low. During the following

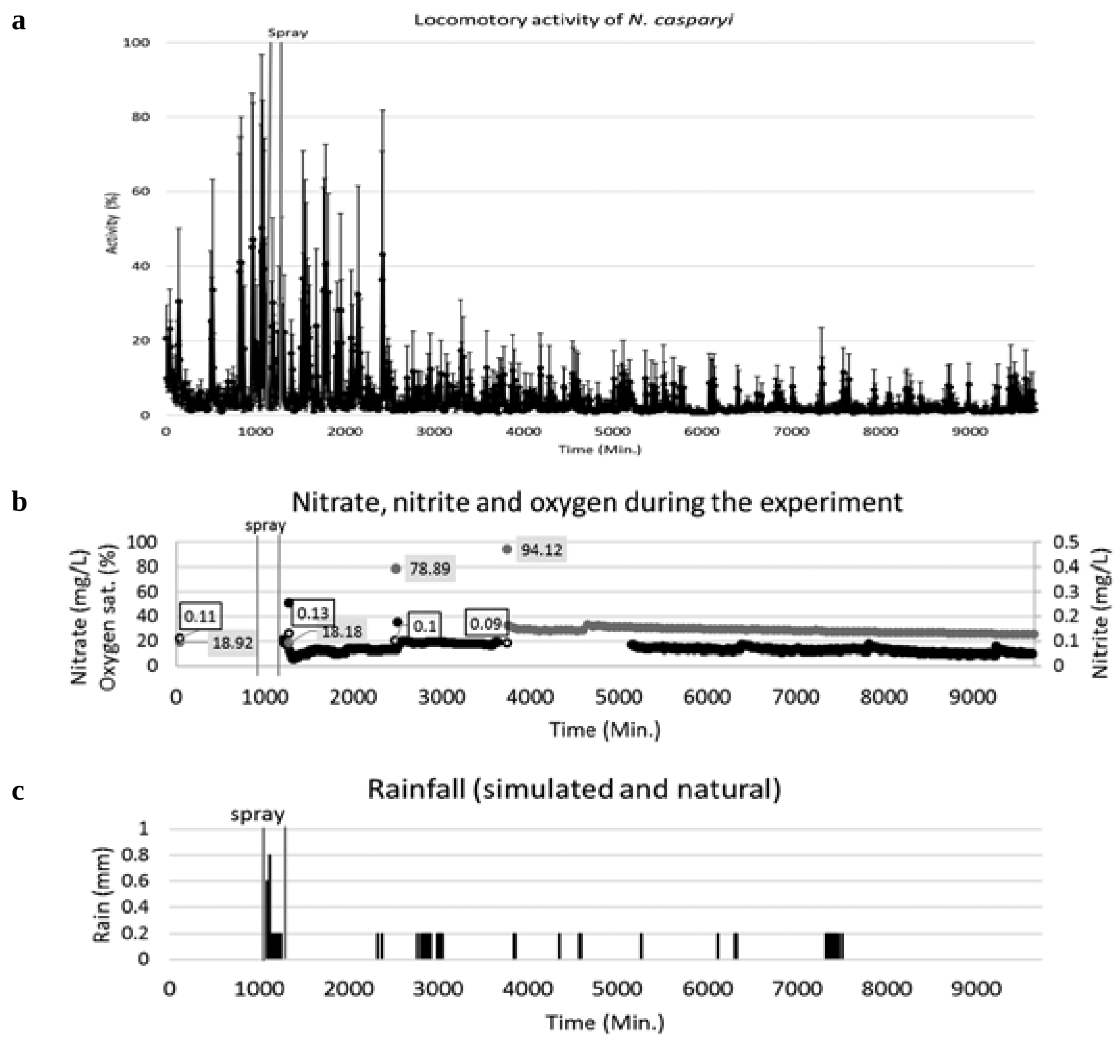

Fig. 3. Spontaneous locomotor activity of *Niphargopsis casparyi* (3a, top), recorded as means and SD bars, from 8 organisms over 7 days. Spray periods mark the artificial rainfall simulation by sprinkling (0.5 h) from a hydrant after application of pig manure on the test field. Monitoring of oxygen saturation (black dots/line) and Nitrate (grey dots/line) (3b, middle), recorded periodically with a multiprobe. Nitrite values (0.09 - 0.13 mg/L) and Nitrate values (18.18 - 84.12 mg/L) were additionally measured by ion chromatography. Natural and simulated rainfall (3c, bottom) during the experiment.

days of the experiment the activity of the organisms remained low (below 20%). In this experiment oxygen saturation was low (< 20% saturation) apart from some higher values during the spray irrigation and natural rainfall, which occurred sporadically in negligible amounts (Fig. 3c). The Nitrate levels of the multiprobe in a neighboring groundwater pipe showed low values (ca. 20 mg/L) for the second half of the experiment (Fig. 3b). Groundwater temperature was 10 °C, and the local background Doxycycline level was 0.025 mg/L at the beginning of the field experiment.

In another field experiment (Fig. 4a-c), performed two weeks after the previous one, we applied pig manure spiked with the antibiotic Sulfamethoxazole (3.6 mg/L) to increase the stress on the test organisms. During the overnight recordings before the application *N. casparyi* showed some elevated activity peaks during the night, similar to the previous experiment (Fig. 3a). During the spray irrigation (0.5 h) after the application of the pig manure fortified with the antibiotics the activity of the groundwater crustaceans increased for a few hours, then fell drastically below 10% provoking a behavioral

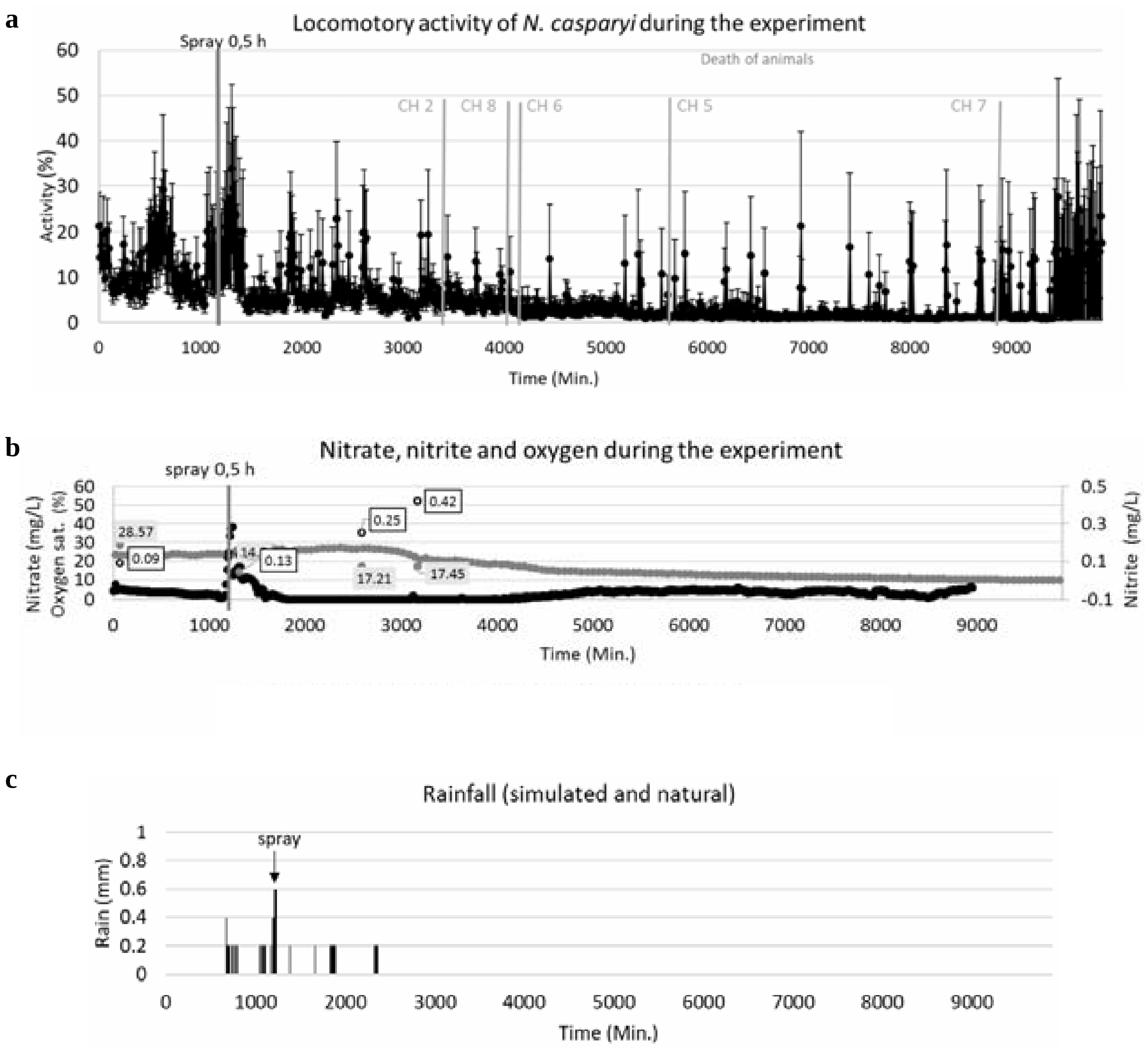

Fig. 4. Spontaneous locomotor activity of *Niphargopsis casparyi* (4a, top), recorded as means and SD bars, from 8 organisms over 7 days. Spray periods mark the artificial rainfall simulation by sprinkling (0.5 h) from a hydrant after application of pig manure on the test field. Monitoring of oxygen saturation (black dots/line) and Nitrate (grey dots/line) (4b, middle), recorded periodically with a multiprobe. Nitrate levels (17.21 - 28.57 mg/L) and Nitrite levels (0.09 - 0.42 mg/L) were additionally measured with ion chromatography. Natural and simulated rainfall (4c, bottom) during the experiment.

alarm. During the following 2 days the activity remained low between 10-20% and some animals died from day 2 onwards. 50% mortality was reached at day 4, revealing the acute toxicity of the applied mixture of pig manure and the antibiotics. At the end of the experiment the sulfamethoxazole level decreased down to 0.07 mg/L, showing degradation. Natural background levels of Doxycycline in the groundwater also decreased from 0.063 mg/L at start down to 0.020 mg/L at the end. High activity of the animals during the spray phase was correlated with high oxygen saturation, which otherwise was generally low as in the previous experiment (Fig. 4b). Whereas Nitrate levels were lower than in the previous experiment, Nitrite values reached toxic concentrations of up to 0.42 mg/L at the time when the first animal died. Until the end of the experiment 5 of 8 organisms died after this Nitrite pulse. Similar results were found in a laboratory study with the freshwater amphipod *Echinogammarus toletanus* exposed to 0.5, 5 and 10 mg/L Nitrite-N for periods of 1, 8, 24 and 48 h revealing detrimental effects of Nitrite on survival and delayed mortality after the exposure [16].

DISCUSSION

For the 1st time, real-time online biomonitoring of groundwater quality was performed with groundwater species *in situ*, using the new Groundwater Biomonitor, based on the Multispecies Freshwater Biomonitor (MFB) [11, 12]. Online biomonitoring of both toxic pulses and chronic exposure to pollutants is essential for (1) risk assessment and evaluation of groundwater quality and (2) control where drinking water is prepared from surface-water-enriched groundwater bodies. Moreover, groundwater used for irrigation in human food production needs real-time monitoring of its toxic potential as well. The rapid responses of the groundwater crustaceans to the pollutants showed that they are appropriate groundwater test animals for long-term online groundwater biomonitoring.

Oxygen levels are generally lower in groundwater compared to surface water, especially in streams. Therefore, a test species for groundwater biomonitoring needs to tolerate low oxygen levels. *N. casparyi* survived low oxygen saturation levels (< 20%), whereas *G. fossarum* needs more than 50% oxygen saturation ([17]: 5.3 mg/L oxygen, 15 °C, threshold for survival and rheotactic activity of *G. fossarum*). In wastewater, *G. fossarum* survived for one week at 30% oxygen saturation [18]. *G. limnaeus* tolerated 3 mg/L oxygen at 6.4 °C (resp. 25% saturation) with 80% survival for more than 4 days, while 1 mg/L oxygen at 20 °C (resp. 11% saturation) was extremely toxic within 3 hours for *G. pulex* [19]. *G. pulex* appeared to be 5-fold more sensitive towards oxygen depletion than *Asellus aquaticus* [20].

Nitrogen values in both surface and groundwater bodies have reached alarming levels due to intensive farming and fertilization with manure in agriculture. In the 1st field experiment *G. fossarum* responded rapidly to Nitrate levels up to 300 mg/L ($N0_3$-N = 67.5 mg/L) with decreased fitness within 24 h. These values correspond roughly to literature data. Acute LC_{50}-48h values for different invertebrates revealed *E. toletanus* and *Echinogammarus echinosetosus* to be more sensitive towards NO_3 compared to *Daphnia magna* (*E. t.*: 180 mg/L; *E.e.*: 107 mg/L; *D. magna* neonates: 465 mg/L), whereby *Cheumatopsyche pettiti* and *Hydropsyche occidentalis* were the most sensitive species studied (6.7, resp. 4.5 mg/L) [21]. In chronic exposures to Ammonium or Nitrate, *G. pulex* proved to be more sensitive compared to other invertebrate species [22]. Increasing Nitrate levels caused a shift in the Gammarus/Asellus ratio towards Asellus in field surveys [23].

NH_3 toxicity threshold in *G. pulex* was 4.3 mg/L (adults) resp. 6.2 mg/L (juv.) [20]. NH_3-N concentrations of > 0.14 mg/L negatively affected feeding activities of *Echinogammarus* (*Eulimnogammarus*) *toletanus* [24]. The LC_{50}-96 h of Nitrite-N was 2.59 mg/L for *E. echinosetosus* and 2.09 mg/L for *E. toletanus* [25]. Moreover, increasing chloride had an ameliorating effect on Nitrite toxicity, as both ions compete for the receptors of the chloride cells in the gills [26]. No literature data are available for groundwater crustaceans; however, our 2 field experiments show that Nitrate levels around 78 to 94 mg/L and Nitrite levels up to 0.42 mg/L may affect their behavior negatively, showing a higher sensitivity compared to surface water amphipods. As elevated nitrogen levels affected the studied freshwater species it might be concluded that this also influences the hyporheic community in long terms. Kibichii *et al.* [3] found in a study of the invertebrate communities at several hyporheic sites in Ireland that especially Crustacea and EPT-taxa (Ephemeroptera-Plecoptera-Trichoptera) densities and species richness was reduced at higher Nitrate levels in agricultural catchments.

Antibacterials are commonly used in intensive farming, hence can be found in pig manure fertilizers, too. About 1/3 of the pharmaceuticals concentration in surface waters reaches groundwater horizons, showing both their toxic potential as well as their resistance potential in drinking water [27]. However, the amount of antibiotics used in veterinary practice and the percentage being excreted unaltered varies from substance to substance, *e.g.* only 2% of Doxycycline consumed by farm animals is excreted unaltered whereas 33% of Sulfamethoxazole is excreted unaltered, *i.e.* is available in the environment [27]. However, a recent study showed that 15-30% Sulfamethoxazole and 70% Doxycycline are excreted [28]. The drinking water threshold levels in Australia are set at 10 µg/L for Doxycycline and 35 µg/L for Sulfamethoxazole [28]. The chronic lowest observed effect concentration (LOEC) for effects on reproduction in *Daphnia magna* was 3.1 mg/L Sulfamethoxazole [29]. However, another study found *Ceriodaphnia dubia* to be negatively affected in their reproduction within 7 days at levels as low as 0.2 mg/L Sulfamethoxazole [30]. Crane *et al*. [31] also found *C. dubia* to be affected in their reproduction by 0.25 mg/L Sulfamethoxazole, whereas cyanobacteria were much more sensitive (0.059 mg/L). In our 3rd field experiment 3.6 mg/L Sulfamethoxazole was applied in the manure on the soil and distributed into the groundwater by the artificial rainfall event. In the pipe where the Groundwater Biomonitor was exposed about 40% of the spray water and 6% of the manure from the sites of application reached the groundwater according to the Deuterium marker experiment (1st experiment). This means that about 0.0036 mg/L Sulfamethoxazole might theoretically have reached the animals in the Groundwater Biomonitor. After 7 days' exposure 0.07 mg/L Sulfamethoxazole was measured in the pipe next to the biomonitor. Even though the actual levels in the exposure pipe of the groundwater biomonitor might be as low as this estimation the crustaceans showed drastic effects not only on behavior but also on survival. More than 50% mortality was recorded at the end of the 7 days' exposure, showing that the combination of Nitrite and Sulfamethoxazole was acutely toxic, supported by the abovementioned data from laboratory experiments in the literature.

Based on a predicted environmental concentration (PEC) of 10 µg/L Sulfamethoxazole a predicted no effect concentration (PNEC) for aquatic life was estimated at 0.59 µg/L [32]; these data have been derived from bacteria, algae and aquatic plant tests only. According to the estimation from our field experiment, this value appears sufficiently protective for groundwater crustacean species. Moreover, Sulfamethoxazole may act synergistically with other antibiotics, such as Doxycycline in this study. A mixture of 10 antibiotics was estimated as not toxic anymore if an application factor of 10 was applied to the PNEC values for the single antibiotics based on the concentration addition approach for mixture toxicity [32].

Doxycycline is a frequently used broad spectrum tetracycline antibiotic to treat respiratory and intestinal diseases in farm animals. In our field experiments background levels up to 0.063 mg/L were recorded. The values were much lower than reported toxic thresholds in the literature. In the Microtox assay the LC_{50} values for *Vibrio fisheri* were 19.8 mg/L (5 Min.) and 5.2 mg/L (15 Min. exposure) [33]. The LC_{50}-48 h for *D. magna* was 156.14 mg/L, the LC_{50}-96 h for zebrafish was > 50 mg/L, similar to biochemical effects detected in fish cell-lines (EROD: 51.57 mg/L). The most sensitive tested species was *Chlorella vulgaris* with an LC_{50}-48h of 15.2 mg/L. If a risk factor of 100 is applied, the estimated PNEC threshold level would be 0.15 mg/L Doxycycline, which is higher than the background values that were recorded in the present field study [34].

CONCLUSIONS

The new Groundwater Biomonitor based on the Multispecies Freshwater Biomonitor can be operated with stygobiont amphipods *in situ*. Groundwater crustaceans are appropriate biomonitor species as they are key species in groundwater ecosystems and tolerate low oxygen levels.

Groundwater crustaceans are sensitive biomonitor species as they react sensitively to Nitrate, Nitrite and Sulfamethoxazole at environmentally realistic concentrations.

ACKNOWLEDGEMENTS

The *Niphargopsis casparyi* was taxonomically determined by Cene Fiser, Univ. of Ljubljana, Slovenia. Andreas Tiehm, Astrid Thoma, Anna-Lena Schneider and Charlotte Schäfer (TZW) are greatly acknowledged for the chemical analyses of Nitrate, Nitrite and the antibiotics during the field experiments. Britta Kattenstroth (University of Freiburg) is acknowledged for the hard work of setting up the experimental field and its equipment and performing the artificial rainfall and manure spray experiments *in situ*. The Helmholtz Zentrum, München is acknowledged for offering the WTW multiprobe used by the University of Freiburg. The study was co-financed by the German Federal

Ministry of Education and Research (BMBF) as part of the funding measure “Regional Water Resource Management for Sustainable Protection of Waters in Germany” (ReWaM) on the project Groundcare 2.0 (funding code 033W037H).

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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