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Research article

Application of biological early warning systems in wastewater treatment plants: Introducing a promising approach to monitor changing wastewater composition

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ABSTRACT

Wastewater treatment plants (WWTPs) are a major source of micropollutants to surface waters. Currently, their chemical or biological monitoring is realized by using grab or composite samples, which provides only snapshots of the current wastewater composition. Especially in WWTPs with industrial input, the wastewater composition can be highly variable and a continuous assessment would be advantageous, but very labor and cost intensive. A promising concept are automated real-time biological early warning systems (BEWS), where living organisms are constantly exposed to the water and an alarm is triggered if the organism’s responses exceed a harmful threshold of acute toxicity. Currently, BEWS are established for drinking water and surface water but are seldom applied to monitor wastewater. This study demonstrates that a battery of BEWS using algae (*Chlorella vulgaris* in the Algae Toximeter, bbe Moldaenke), water flea (*Daphnia magna* in the DaphTox II, bbe Moldaenke) and gammarids (*Gammarus pulex* in the Sensaguard, REMONDIS Aqua) can be adapted for wastewater surveillance. For continuous low-maintenance operation, a back-washable membrane filtration system is indispensable for adequate preparation of treated wastewater. Only minor deviations in the reaction of the organisms towards treated and filtered wastewater compared to surface waters were detected. After spiking treated wastewater with two concentrations of the model compounds diuron, chlorpyrifos methyl, and sertraline, the organisms in the different BEWS showed clear responses depending on the respective compound, concentration and mode of action. Immediate effects on photosynthetic activity of algae were detected for diuron exposure, and strong behavioral changes in water flea and gammarids after exposure to chlorpyrifos methyl or sertraline were observed, which triggered automated alarms. Different types of data analysis were applied to extract more information out of the specific behavioral traits, than only provided by the vendors algorithms. To investigate, whether behavioral movement changes can be linked to impact other endpoints, the effects on feeding activity of *G. pulex* were evaluated and results indicated significant differences between the exposures. Overall, these findings provide an important basis indicating that BEWS have the potential to act as alarm systems for pollution events in the wastewater sector.

1. Introduction

In the last decades, many regulations and methods have been put into force on wastewater discharge to limit introduction of various pollutants, to reach quality goals for rivers and to protect the ecosystem and human health (Ternes et al., 2004; Baumgarten et al., 2007).

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Currently, WWTPs are still considered as relevant point sources for the discharge of manmade substances of municipal or industrial origin into surface waters (Hofman and Teo, 2021). Micropollutants, defined as compounds occurring in low concentrations in aquatic ecosystems (ng/L to μg/L) but capable of eliciting effects on aquatic organisms, represent a large part of these pollutants (Schwarzenbach et al., 2006). Due to their persistence and polarity, a great variety of micropollutants cannot be fully eliminated by conventional WWTPs (Loos et al., 2009; Schäfer et al., 2011). Therefore, such compounds are still detectable in surface waters and can have a negative impact on organisms when entering the environment (Kienle et al., 2019).

Up to now, investigations of micropollutants in WWTPs are based on time-limited tests, using grab or composite water samples. Lab-based investigations consist of chemical analysis partly supplemented with ecotoxicological bioassays of various apical endpoints (Kienle, 2022). Biological early warning systems (BEWS), to date, are predominantly used to continuously monitor drinking and surface water quality (Mikol et al., 2007). They consist of an indicator organism that shows a reversible reaction to pollutant stress in the water, a measuring method that can register this response quantitatively, and a software that calculates an alarm from the generated data. Today, a broad variety of different aquatic BEWS is available for different applications and with test organisms of various trophic levels, such as bacteria, algae, invertebrates, and vertebrates (Bownik and Wlodkowic, 2021). These offer ecologically relevant, sensitive, fast and non-destructive parameters to monitor changing water composition (Gerhardt et al., 2007). So far, only few experiments on the suitability of BEWS for wastewater monitoring to detect potential micropollutant contamination have been conducted (Villa et al., 2018; Gerhardt, 2020). German authorities evaluated seven different continuous and semi-continuous BEWS for their application in wastewater (LAWA, 2000). Semi-continuous measurement methods, in which organisms are exposed to the tested water at intervals of a few minutes, performed well. The DF algae test (University of Regensburg), the Algae Toximeter (bbe Moldaenke) and the Regensburg luminescent bacteria test (University of Regensburg) were part of this group. The Daphnia Toximeter (bbe Moldaenke) was suggested by the authors as the best rated method for continuous measurement. The dynamic daphnia test (Elekton) and two mussel tests met the special requirements only to a very limited extent.

The role of continuous monitoring may not be crucial for households and hospitals as they have a relatively constant load, but for pesticides and pharmaceuticals, industrial wastewater can exhibit peak concentrations of compounds (up to 1000 times higher than background level) that may be overlooked as the production dynamics of different industrial companies vary significantly (Anliker et al., 2020a). BEWS could offer a new integrative approach to continuous wastewater quality monitoring that triggers a subsequent chemical analysis by limiting the investigation to the case of alarms.

The goal of the present study was threefold: (i) to investigate how the treated wastewater can be processed to ensure the functionality of the three BEWS over a period of several weeks (ii) to investigate if and how the organisms react to model toxicant peaks in wastewater (iii) to elaborate a statistical approach for the analysis of the behavioral data to identify potential adjustments to the system's internal alarm settings.

These questions were addressed in a series of one-week monitoring experiments utilizing three BEWS side-by-side with organisms of different trophic levels as well as three model substances spiked in cleaned wastewater. To link observed effects for the selected substances in BEWS to ecological parameters, feeding activity of gammarids was investigated in parallel.

2. Materials and methods

2.1. Chemicals and reagents

For the toxicant response tests, the substances diuron (≥98% purity), sertraline hydrochloride (≥98% purity) and chlorpyriphos methyl (analytical standard) were purchased from Sigma-Aldrich (Buchs, Switzerland). Two stock solutions with different concentrations were prepared for each substance with nanopure water. Concentrations of sertraline hydrochloride in this study refer to the active ingredient sertraline. Further information on the chemicals and their preparation is provided in Table A in section S1.1 in SM.

2.2. Test organisms

Chlorella vulgaris is a green algae that is often used as model organism in growth and photosynthesis inhibition assays and serves as an important food source for e.g. aquatic invertebrates (Hoek et al., 1995). The *C. vulgaris* culture for the experiments originated from a laboratory culture at Eawag (Dübendorf, Switzerland).

Daphnia magna as planktonic primary consumer, which feeds on phytoplankton and bacteria, is an important food source for organisms of higher trophic levels and has an important role in the field of ecotoxicology for acute and chronic toxicity assessment as well as behavioral evaluations (Ebert, 2005; Movahedian et al., 2005). For this study, *D. magna* was obtained from a laboratory breeding at Eawag, Switzerland.

Gammarus pulex is commonly represented in temperate streams and belongs to the class of omnivorous shredders, playing an important role in the decomposition of coarse organic matter (Verdonschot, 1990). *G. pulex* is increasingly used in ecotoxicological experiments and field tests including the assessment of feeding activity and behavior (Maltby et al., 2002). For our study, *G. pulex* were sampled from a small tributary of the Chriesbach River near Dübendorf, Switzerland (Latitude: 47.24277, Longitude: 8.37359). Additional information on handling cultivation of the organisms can be found in section S2.1 in SM.

2.3. Real-time biological early warning systems

The test organisms of different trophic levels were assessed in three BEWS (Table 1). An overview of the methods for the three BEWS is given below. Additional information can be found in section S3 in SM.

2.3.1. Monitoring of algae fluorescence inhibition in the Algae Toximeter

The semi-continuous measurements of fluorescence inhibition are based on a pulse amplitude modulation fluorometry (PAM fluorometry) (Noack and Walter, 1992). The measurement process starts by the determination of the concentration of the different algae classes in the wastewater alone and their activity, measured indirectly using the fluorescence activity of the algae. Then, *C. vulgaris* is added to the wastewater and the concentration and activity of the algae classes are again determined to evaluate a potential inhibition of algae photosynthetic activity by the wastewater. A further measurement, which includes only drinking water, is used as a reference value. This process is repeated every 30 min with a fresh algae suspension, wastewater and reference water. If the fluorescence inhibition exceeds a previously defined threshold, an alarm is triggered.

2.3.2. Behavioral monitoring of Daphnia magna in the DaphTox II

Behavioral tracking of *D. magna* in the DaphTox II is accomplished by using image analysis. A camera records changes in the location of individuals in test chambers from which the software creates single swimming lanes. Those serve as basis for calculating various behavioral parameters (Table 2) which result in the toxic index that determines the alarm (Lechelt et al., 2000). Changes in the parameters are recorded and evaluated in the toxic index in different ways: The "Hinkley detector" (ADH) (Moldaenke, 1998) is deployed for recognition of sudden changes within the measuring parameter in Table 2. The "Limit alarms" parameter checks if (upper or lower) parameter limits have been reached. With "Gradient alarms", the temporal course of the individual measurement values is observed.

Table 1
Selected Biological early warning systems used for monitoring wastewater.

System	Organisms	Species	Parameters	Vendor	Reference
Algaetox Toximeter	Algae	*Chlorella vulgaris*	Fluorescence	bbe Moldaenke	Noack and Walter (1992)
DaphTox II	Water flea	*Daphnia magna*	Swimming movements	bbe Moldaenke	Lechelt et al. (2000)
Sensaguard	Benthic invertebrates	*Gammarus pulex*	Swimming movements	REMONDIS Aqua	Voetz (2015)

Table 2
DaphtoxT II - Measured behavioral parameters that contribute to the calculation of the toxic index.

Parameter	Description
Number of active organisms	Describing immobilization and mortality
Average swimming distance (cm)	Describing the distance between individuals, social behavior
Average swimming height (cm)	Describing the vertical movement and calculated from the vertical location in the chamber
Average swimming speed (cm/s)	Describing how fast or slow the organisms are
Speed class index	Showing especially fast and slow individuals
Fractal dimensions	Classifies the momentary speed of all organisms

If the behavior deviates from normal behavior by a significant change in the parameters, an alarm response is generated. A flow through is achieved by an integrated pumping system, which directs the wastewater through the test chambers. For the experiments, ten juvenile *D. magna* (<72 h old) were introduced in each of the two chambers and exchanged every week.

2.3.3. Behavioral monitoring of Gammarus pulex in the Sensaguard

The Sensaguard (REMONDIS Aqua) measures the behavior of *G. pulex* individuals using impedance technique. The organisms are placed into eight cylindrical test chambers with net-carrying screw-lids. Equipped with four electrodes, a high-frequency alternating voltage is generated by one pair of electrodes. A second pair of electrodes measures the changes within the electromagnetic field induced by the movements of the organism in the sensor chamber (Voetz, 2015). The measured parameter is the intensity of the detected movements (amplitude), which is recorded continuously. The company-specific algorithm creates alarms by detecting differences between normal and deviant behavior in the average movement of the individuals. If the behavior and thus the measured activity values change, the short-term mean reacts faster than the long-term mean. Consequently, the difference between both mean values increases. If the behavior is constant, both mean values are equal and the difference is ideally 0. The differences of all chambers are added to the alarm sum. The alarm sum increases if a change in behavior is detected in several chambers at the same time. Eight adult male *G. pulex*, identified by the position of pre-copula pairs and bigger than 8 mm, were selected for experimentation. Individuals with visual infestation of acanthocephalan parasites were rejected (Bauer et al., 2005). Afterwards, one adult individual of *G. pulex* was introduced per test chamber, together with three leaf discs as a food source. All organisms were exchanged weekly.

2.4. Wastewater treatment plant and filtration module

All experiments were conducted at a pilot-scale WWTP (Eawag, Switzerland) that receives municipal wastewater and treats approximately 200 population equivalents (<72 m^3/d inflow). The mechanically treated wastewater is passing through a sedimentation stage, followed by denitrification and nitrification. Subsequently, it is collected in the secondary clarification tank (10.6 m^3).

Wastewater from the secondary clarifier was directed into a filtration tank, where a backflushable ultrafiltration membrane module (IPC 7, PVDF, pore size: 0.08 μm, BlueFootMembranes, Belgium) was placed. A constant permeate flux of 11.42 L/m^2h was provided from the membrane stack and directed into an aerated plexiglass container (permeate pool), where the permeate was conditioned to 17 °C. The treated and filtered wastewater was distributed continuously to the individual BEWS from the pool by pumping systems. Additional information on the membrane filter unit can be found in section 4.1 in SM.

2.5. Experimental setup

The first experiment served as negative control for the DaphTox II and the Sensaguard and was conducted with filtered lake water which was circulated through the BEWS for 6 days. For the Algae Toximeter no negative control was evaluated, as every measurement cycle includes reference water. For the second experiment, fluorescence and behavior of the test organisms in treated and filtered wastewater were assessed under flow-through conditions. This served as baseline for the comparison to the toxicant exposure experiments.

In the toxicant exposure experiments different substances were added to the filtered wastewater to simulate peak exposure concentrations for a limited amount of time. Three model substances, diuron, chlorpyrifos methyl and sertraline, representing herbicides, insecticides and pharmaceuticals, respectively, were selected because they are frequently found in surface and waste water. In addition, a broad range of information about their mode of action is available in the literature and they elicit effects in organisms in environmentally relevant concentrations. Two substance concentrations were considered: one based on effect concentration values from the literature for the test organisms and one concentration 0.5, 2, and 4 times higher, corresponding to diuron, chlorpyrifos methyl and sertraline (Table 3).

The set-up of the toxicant response experiments consisted of four phases: introduction of organisms (1 d), acclimation (2 d), exposure (2 × 10 h) and recovery phase (2 d) (Fig. 1). All phases were conducted under flow-through conditions, except of the exposure phase for the Sensaguard, where the water was circulated within the closed system to limit the necessary volume for the exposure experiments. For the exposure phase, a substance pool of 200 L was prepared with filtered wastewater and the respective toxicant. The stock solution (for preparation see S1.1 in SM) was added to the substance pool one hour before the exposure and mixing was ensured by an aquarium pump (CompactOn 5000, EHEIM, Deizisau, Germany). The pool was aerated (APS 300, Tetra, Pasadena, USA) and cooled (Ultra Titan 150, Hailea, China) to 17 °C.

2.6. Chemical analysis

Chemical analysis of diuron, sertraline and chlorpyrifos methyl was performed on an Agilent G6495C Triple Quadrupole (QQQ) Mass Spectrometer coupled to an UHPLC-System (Santa Clara, USA) for chromatographic separation. External standard calibration with

Table 3
Exposure concentrations for the evaluated toxicants in the present study and an overview on effect, environmental and WWTP effluent concentrations from the literature.

Toxicant	Species	Exposure concentration and duration (µg/L)		EC_{50}/LC_{50} (µg/L)	Literature	Environmental conc. (µg/L)	WWTP effuent conc. (µg/L)
		I.	II.				
Diuron	*C. vulgaris*	8.8 (10 h)	13.7 (10 h)	27.4 (20 min)	Podola and Melkonian (2005)	0.006	0.366
	D. magna			12,000 (24 h)	Baer (1991a)	Munz et al. (2017)	Köck-Schulmeyer et al. (2013)
	G. pulex			700 (24 h)[a]	Sanders (1969)		
Chlorpyrifos methyl	*C. vulgaris*	1.3 (10 h)	2.6 (10 h)	220 (>70 min)[b]	Bengtson Nash et al. (2005)	0.007	0.073
	D. magna			0.6 (48 h)	Schäfer et al. (2011)	Miclean et al. (2014)	Munz et al. (2017)
	G. pulex			0.4 (48 h)	Rubach et al. (2010)		
Sertraline	*C. vulgaris*	45.8 (10 h)	214 (10 h)	763.6[c] (96 h)	Johnson et al. (2007)	0.219	1.93
	D. magna			3100 (24 h)	Minagh et al. (2009)	Arnnok et al. (2017)	Mole and Brooks (2019)
	G. pulex			No data	–		

[a] For Gammarus lacustris.
[b] Inhibition concentration (IC_{10}).
[c] Growth inhibition.

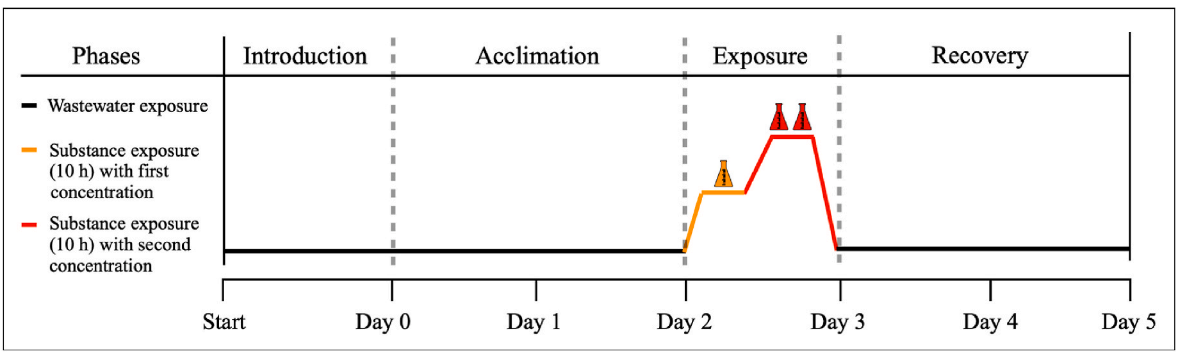

Fig. 1. Experimental set-up of toxicant response tests. Time-line of toxicant exposure. The color of the lines indicates exposure type (dark grey for filtered and treated wastewater, orange and red for wastewater with toxicant.

matching deuterated analytes (i.e. addition of the IS mixture to the sample directly before LC-MS/MS analysis) and calibration using a linear regression model were performed to determine the concentrations (ng/L) of the individual compounds by Agilent MassHunter Quantitative Analysis. Details of the chemical analysis are described in section S1.2 in SM.

2.7. Evaluation of Gammarus pulex feeding activity

Leaf discs of conditioned alder leaves (*Alnus glutinosa*) were prepared to evaluate the feeding activity of *G. pulex*. Each test chamber of the Sensaguard received three leaf discs for every one-week experiment. In addition, three control leaf discs were placed in an empty chamber alongside the other test chambers. Calculation of feeding rate for *G. pulex* was based on Maltby et al. (2002) and AFNOR standard (XP T 90-722-3, 2020) (equations (1) and (2)):

$$LDA_c = (LDA_1 \times BD) - LDA_2 \quad (1)$$

$$FR = \left(\frac{LDA_c}{W}\right) \bigg/ \text{D} \quad (2)$$

For the corrected leaf disc consumption (LDA_c), the leaf disc area before (LDA_1) and after (LDA_2) the experimental week was calculated by ImageJ. The average biological decay of the leaves (BD) was calculated by the mean difference in area between the control leaves before and after the experimental week. The feeding rate (FR) was calculated by the quotient of LDA_c and the dry weight of *G. pulex* in mg (W) divided by the total days of experimentation (D). More information on the evaluation of feeding activity can be found in section S2.2 in SM.

2.8. Statistics

All data were analyzed and visualized using the statistical software R, version 4.2.0 for Windows (R Core Team, 2022). In the case of DaphTox II and Algae Toximeter, the statistical data analysis was already integrated into the algorithms of the systems. Therefore, no additional statistical tests were carried out. Statistical analysis of the Sensaguard data focused on changes in behavior patterns for the eight individual organisms. A multivariate changepoint detection based on Pickering (2015) was applied to define time-points where a change in the mean of gammarids activity occurred during a one-week experiment. "Changepoints" were identified by using the "smop" package in R (Pickering, 2019). The amplitude values in the raw data of the Sensaguard (one per second) in the time-series were grouped in 5 min average intervals to reduce computational time. The individual detected changepoints were added to the average amplitude of the eight organisms, and a horizontal line representing the average amplitude of each segment was added. This allowed to display the differences in the average amplitude between the segments determined by the changepoints, which are marked in the figures by blue vertical lines. The changepoint analysis was accompanied by a simquant analysis, a linear random effects model, to assess the daily rhythms. In this model, hourly averages accounted as fixed effect, and the individuals' hourly behavior as random effects. For assessing the difference between hours, the package "emmeans" of R (Searle et al., 1980) was used, which allows to estimate marginal means and also yields adjusted p-values for the multiple comparisons, as well as visualisations. For comparison, the compact letter display was calculated by using the package "multcomp" (Bretz et al., 2016). To estimate prediction limits, the confidence bands were calculated via bootstrap. This calculation was based on seven weeks of baseline data during which *G. pulex* was exposed to treated wastewater.

The feeding rate of *G. pulex* was tested for normality and difference in the standard deviation. In the case of normal distribution and no significant difference in the standard deviation, parametric tests were applied. If this was not the case, a non-parametric test was carried out. As parametric test a one-way ANOVA followed by Dunn's multiple comparisons test was chosen and as non-parametric test a Kruskall Wallis test followed by Tukey's multiple comparison test. For more detailed information on the statistical analysis of daily rhythms and feeding activity see section S5.1 and S5.2 in SM.

3. Results and discussion

3.1. Municipal wastewater exposure induces no deviation from normal responses

In the first two experiments the BEWS were exposed to river water as negative control and filtered wastewater (Fig. 2). No alteration of photosynthesis activity was measured with *C. vulgaris* exposed to wastewater. In the DaphTox II, behavior of *D. magna* in wastewater was similar to the negative control (Fig. 2 A & C). In the Sensaguard, a circadian rhythm was visible in the average amplitude of *G. pulex* individuals in both experiments. The circadian rhythm is a 24 h autonomous biological clock (Zhao et al., 2020) in the organisms' average behavior. Typical of this is a fluctuating daily activity pattern, showing high activity at night (swimming) and low activity (ventilation) during the day. This seems to be the normal behavioral profile of the organisms (Janssens de Bisthoven et al., 2006). Fluctuations in circadian rhythm were present, and behavioral amplitudes were less pronounced in wastewater than in the negative control (Fig. 2 B & D). A decrease in behavioral activity in *G. pulex* upon exposure to WWTP effluent was also found by Love et al. (2020). The authors concluded that the presence of micropollutants and nutrients in wastewater may be related to this decrease. Most of the measured abiotic parameters, namely pH, dissolved oxygen and conductivity, remained stable during the conducted experiments (see Tables 4–5 in Excel-SI). Temperature in the Sensaguard fluctuated around ±2 °C due to the heating and cooling cycles of the thermostat.

Using a backwashable membrane unit proved to be a good solution to supply the BEWS with a largely germ- and particle-free wastewater matrix during 6 weeks. The application of ultrafiltration prevented undesirable effect like clogging of BEWS, which was predicted by Mikol et al. (2007). No mortality was observed in 100% filtered wastewater neither with daphnia nor with gammarids. In contrast, Gerhardt (2019) exposed *G. pulex* to 50% and 100% diluted WWTP effluent without filtration in an early warning system (Multispecies Freshwater Biomonitor®) and observed high mortality rates of 60% and 100% mortality after 4 days. The authors concluded that the presence of carbamates and high ammonia levels (>2 mg/L) in the treated unfiltrated wastewater might have been responsible for observed effects. By treating the wastewater using ultrafiltration, we were able to achieve our first goal, i.e., to ensure the operation of the BEWS during the entire experimental period of several weeks.

3.2. Diuron impairs algae photosynthesis and behavior of Gammarus pulex in wastewater

In the Algae Toximeter, photosynthetic activity of *C. vulgaris* was inhibited by 35% when exposed to 13.7 μg/L diuron in wastewater (Fig. 3A). Studies on herbicidal effects of substances (e.g., Ma et al., 2002a) often focus on growth inhibition of algae. However, strong correlations between effects on microalgae growth and reduced photosynthetic activity as measured by Pulse Amplitude Modulation (PAM) fluorometry has been demonstrated for several PSII inhibiting herbicides (Magnusson et al., 2008). In previous studies, the inhibition of effective quantum yield (ΔF/Fm') has been extensively applied for assessing the toxicity of herbicides in microalgae (Kienle et al., 2019; Schreiber et al., 2007; Bangston-Nash et al., 2005). A 20 min exposure of *C. vulgaris* to diuron on a biosensor, resulted in an EC50 value of 27 μg/L for photosynthesis inhibition (Podola and Melkonian, 2005), which is comparable to our results as the inhibition reached 35% when only half of the concentration was applied for 10 min. In the marine microalgae

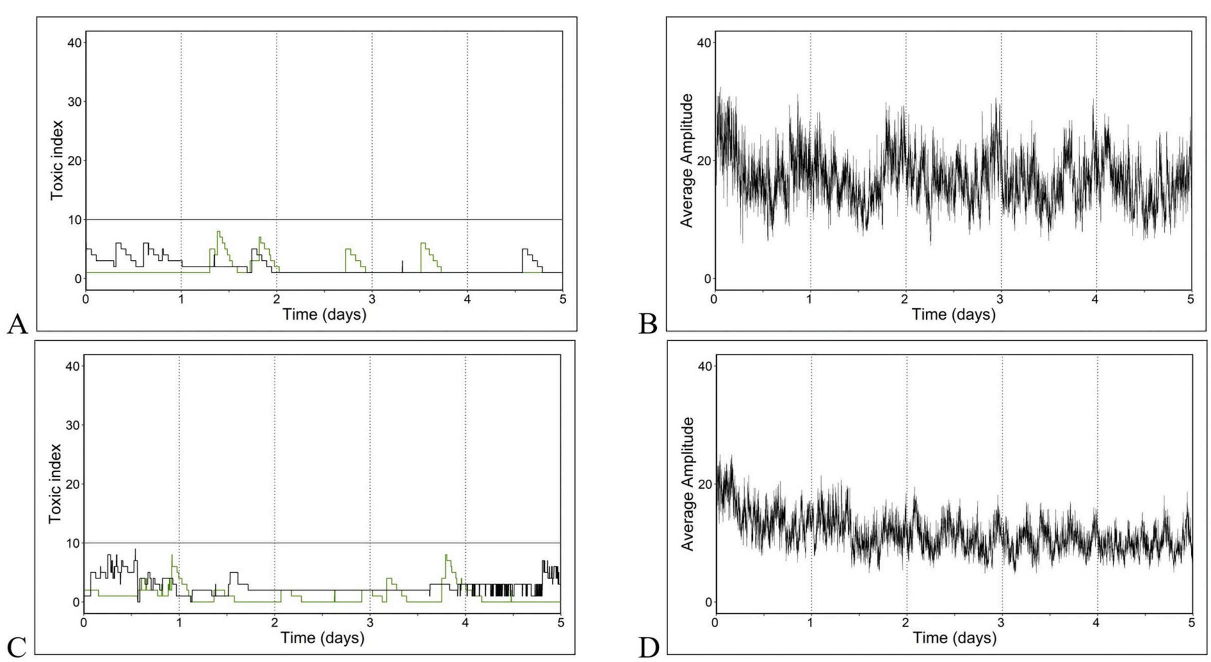

Fig. 2. Application of negative control (A–B) and wastewater (C–D) on the BEWS. A; C: DaphTox II - Toxic index of Daphnia magna: Black horizontal line indicates alarm threshold. Black and gray lines represent behavioral activity in the test chambers 1 and 2. B; D: Sensaguard – Average amplitude of Gammarus pulex: Black lines display the average behavioral activity of all 8 organisms in the test chambers.

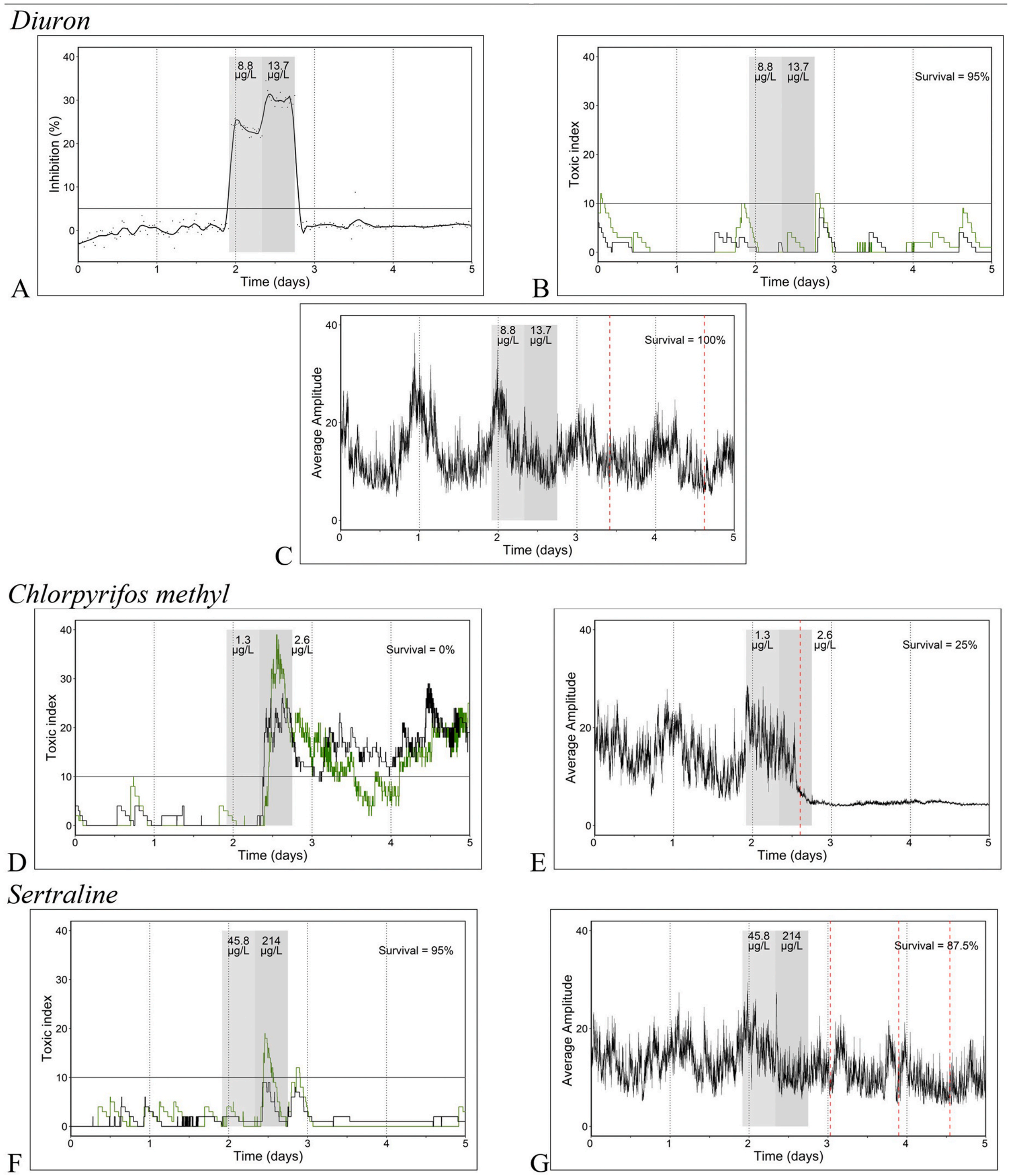

Fig. 3. Application of toxicants in BEWS. Experimental period as described in Fig. 1. A: Algae Toximeter - Measured inhibition (%) of photosynthesis activity of *C. vulgaris* during the exposure to diuron in treated wastewater. Horizontal black line indicates alarm threshold. Black dots with trend line indicate measured inhibition levels. B; D; F: DaphTox II - Measured toxic index of *D. magna*: Black horizontal line indicates alarm threshold. Black and gray lines represent behavioral activity in the test chambers 1 and 2. Survival rate of n = 20 after 6 days is depicted top-right. C; E; G: Sensaguard - Measured average amplitude of *G. pulex*: Black lines display the average behavioral activity of all 8 organisms. Red lines indicate detected alarms triggered by the system. Survival rate of n = 8 after 6 days is depicted top-right.

Rhodomonas salina, exposure to diuron resulted in a 24 h-EC_{50} value for PSII activity of 1.71 µg/L (Thomas et al., 2020), which is 10-fold lower than the values achieved with the Algae Toximeter using *C. vulgaris*. This does not necessarily indicate the insensitivity of the early warning system considering the difference in algae species and the substantially shorter exposure time of 10 min in the present study.

For *D. magna* exposed in the DaphTox II, the alarm threshold was exceeded twice by test chamber 1 during the experimental week with diuron (Fig. 3B). This occurred once during the acclimation phase, approximately 12 h after the introduction of the organisms into the system. This most likely can be assigned to the time the daphnia need to get used to the new environment until their normal behavior is established. A second exceedance occurred during the recovery phase, within 1 h after the end of the second exposure period to 13.7 µg/L. The cause of the second exceedance was a scattering of the measurement points for the parameter average height and an elevated average speed (Figure N in section S4.5 in SM). The increase in swimming speed, which is a typical large-scale swimming behavior and serves as an escape response of *D. magna* to pollutants in the water column (Uttieri et al., 2014), could be attributed to the presence of diuron. In earlier studies concentrations above 68 mg/L (24 h-EC_{50}), which is 500-fold higher than our exposure concentration, elicited lethal effects on *D. magna* (Baer, 1991). A similar scattering without an increased value in the toxic index was also visible during the change from the first to the second concentration of diuron. This change in behaviour might be attributed to a change in the water quality flowing through the system.

For *G. pulex* exposed in the Sensaguard, the circadian rhythm was particularly pronounced in the acclimation phase (Fig. 3C). After toxicant exposure, in the recovery phase, this pattern was clearly reduced but still visible. For other amphipod species, effect concentrations of 1.8 mg/L (48 h-LC_{50}, *G. fasciatus*) and 0.38 mg/L (48 h-LC_{50}, *G. lacustris)* diuron were reported (Sanders, 1969, 1970). While these data are based on lethal endpoints like immobility, it has to be considered that the BEWS provide insight into sub-lethal behavioral responses of the organism through movement monitoring. Accordingly, visible effects were observed in our study already at 10- to 100-fold lower diuron concentrations compared to the LC_{50} values reported in the literature. Higher sensitivity of behavioral biomonitoring has already been shown for *D. magna* (Bahrndorff et al., 2016). While the sensitivity of gammarids to diuron varies among species and tends to be higher than that of water flea, the effect on algae is higher than on crustaceans. This indicates that observing behavioral response of whole organisms may not be sufficient as an ecologically relevant measure for compounds such as PSII inhibiting herbicides, since the mode of action is mainly targeted on plants and does not induce stress responses of water fleas or gammarids in environmentally relevant concentrations.

3.3. Chlorpyrifos methyl concentrations caused acute mortality in both crustacean test organisms

In our study, exposure to chlorpyrifos methyl triggered an acute immobilization effect with constantly increasing inactivity in *D. magna* and *G. pulex* (Fig. 3D and E). The first concentrations step 1.3 (μg/L) evoked no behavioral alteration. The second concentration applied (2.6 μg/L) turned out to be acutely toxic to *D. magna*, since 50% of individuals were immobile 5 h after the beginning of the exposure (Fig. 3D). During the recovery phase, movements were limited to uncoordinated rotational motions at the bottom of the chamber. After the death of the organisms, there were some false effects recorded because the flow-through stream caused the dead organisms to float up during the recovery phase. Previous behavioral studies with *D. carinata* resulted in a 15-fold lower EC_{50} value of 0.13 μg/L after 24 h exposure to chlorpyrifos under laboratory conditions (Pablo et al., 2008). Zein et al. (2014) also studied short-term exposure (1.5 h) and showed by video tracking that 87.6 μg/L chlorpyrifos caused a change in swimming angle and a decrease in swimming distance in *D. pulex*. This is a 30-fold higher concentration than the one applied in our study, which also led to a slight decrease of swimming distance since the beginning of the exposure until mortality occurred after 5 h (See Figure N in S4.5 in SM). These findings indicate that minor concentrations of organophosphate insecticides may trigger sub-lethal neurotoxic effects that can alter swimming behavior of *D. magna*.

In the Sensaguard, a circadian rhythm was visible in the average amplitude during the acclimation phase (Fig. 3E). When exposure to 1.3 μg/L chlorpyrifos methyl started, the movement of *G. pulex* was in a normal range of average amplitude, but decreased rapidly after the concentration was increased to 2.6 μg/L. Six hours later, no behavioral activity was registered anymore, and an alarm was triggered. In another study, *G. pulex* was exposed to chlorpyrifos for 96 h, resulting in an LC_{50} value of 0.07μg/L (van Wijngaarden et al., 1993). Chlorpyrifos is considered more acutely toxic than chlorpyrifos methyl (Tomlin, 2009). Substances like chlorpyrifos and chlorpyrifos methyl interfere with different behavioral patterns, and there can be significant differences between organisms of different trophic levels in terms of their substance-specific sensitivity to pollutants (Marques et al., 2021). This is not only related to size differences, but also to diet type and natural habitat. While gammarids live benthically as detritus feeders and come into contact mainly with sediments, daphnia filter food particles from the water column (Maltby et al., 2002; Ebert, 2005). Previous studies on insecticides have shown that *G. pulex* is two to three orders of magnitude more sensitive to neonicotinoids than *D. magna*, and for organophosphates, *D. magna* is about a factor of six more sensitive than *G. pulex* (Ashauer et al., 2017). In our study, both organisms responded to chlorpyrifos methyl after similar exposure times.

3.4. Sertraline induces behavioral changes in Daphnia magna and Gammarus pulex during and after exposure

As expected for *C. vulgaris* no effects on photosynthesis activity could be detected with 45.8 and 214 μg/L sertraline. Yang et al. (2018) reported that exposure to 50 μg/L sertraline inhibited the growth of *C. vulgaris*, decreased the chlorophyll *a* (Chl-a) concentration and thus inhibited photosynthetic efficiency by 21% after 3 days of exposure. The short-term exposure (10 min) in our study could have been the reason why no effects occurred in the Algae Toximeter, suggesting that sertraline has only a long-term negative impact on algae.

The DaphTox II registered an exceedance of the alarm threshold during the 10 h exposure with 214 μg/L of sertraline. The toxic index went distinctly over the alarm threshold in test chamber 1 (Fig. 3F). Subsequently, the toxic index decreased and peaked again in the recovery phase after the exposure in the same chamber. In parallel, the toxic index in chamber 2 rose and fell, but remained below the alarm threshold. The peaks were caused by the alteration of several behavioral parameters displayed in Fig. 4. Additionally, a delay of the behavioral effects could be observed in comparison to other pollutants, possibly due to time for chemical uptake, distribution within daphnia and the time to apply a defense mechanism (Chevalier et al., 2015). This is consistent with the visible alarm that occurred after the exposure time and application of 214 μg/L sertraline in this experiment. The observation of the general behavioral response towards sertraline showed that *D. magna* returned to their normal condition after the alarms were triggered, which indicates that the organisms were able to recover from the exposure. Sertraline's low acute toxicity to *D. magna* (24 h-EC_{50} = 2770 mg/L) was already demonstrated by Minagh et al. (2009). On the other hand, studies investigating long-term effects on fecundity revealed that *D. magna* reproduction was altered by exposure to 30 μg/L for 14 days (Minguez et al., 2015). Our study observed sublethal effects on behavior of *D. magna* evoked by sertraline at concentrations below EC_{50} concentrations reported in the literature.

After the sertraline exposure, *G. pulex* indicated a visual disruption of the circadian rhythm in the Sensaguard (Fig. 3G). Compared to *D. magna*, *G. pulex* decreased their amplitude of movement during the second concentration and did not recover afterwards. The circadian rhythm was weak but still pronounced, as the exposure did not cause any acute mortality like chlorpyrifos. The changepoint analysis showed a decrease in behavior pattern over time (Fig. 5B). Other studies with sertraline showed that female *G. locusta* increased movement during a 48-day exposure to 1 μg/L (Neuparth et al., 2019). An increase in activity upon exposure to 1 μg/L for 21 days in crayfish (*Procambarus virginalis),* which moved greater distances, was revealed by Hossain et al. (2019). This would represent the opposite reaction to the one of the set up in the present study. However, as the study of Hossain et al. (2019) focused on chronic effects, it is possible that, upon exposure to higher concentrations during shorter periods, the behavioral response is more towards a physiological weakening.

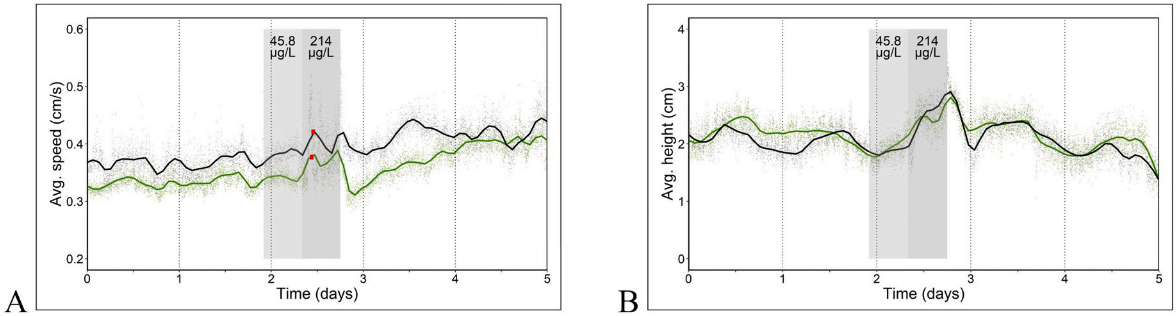

Fig. 4. DaphTox II - Calculated parameters during the experimental week with exposure of *Daphnia magna* to sertraline. Experimental period of 5 days is displayed. Light grey area indicates first concentration step (45.8 μg/L) and dark grey area indicates second concentration step (214 μg/L) of the toxicant. A: Average speed (cm/s) of D. magna. B: Average height (cm) of *D. magna*. Black and gray lines represent behavioral activity in the test chambers 1 and 2. Red dots represent sudden changes in behavior detected by Hinkley.

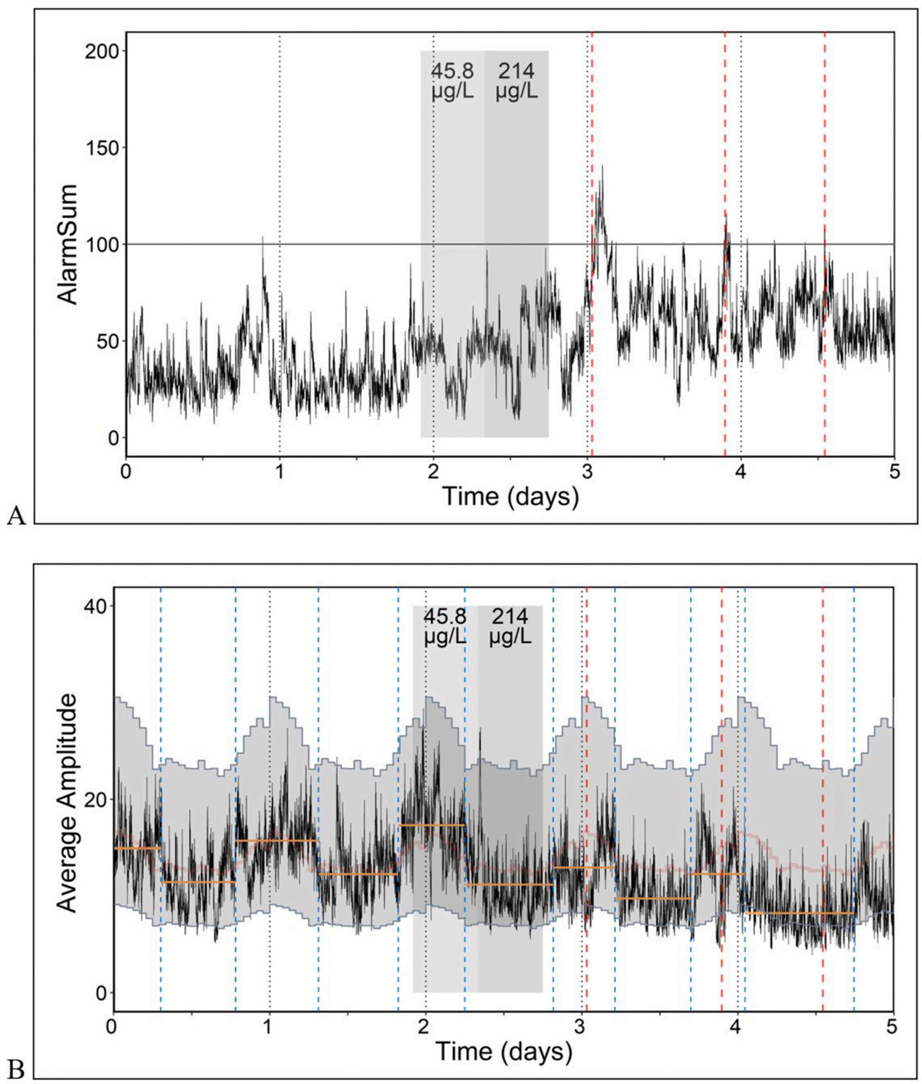

Fig. 5. Sensaguard – A) Alarm sum and B) average amplitude including changepoints and 95% confidence intervalls of *Gammarus pulex* behavior during the experimental week with sertraline exposure: Black horizontal line indicates alarm threshold. Blue dotted lines display the time-points detected in the multivariate changepoint analysis; yellow lines show the mean of each segment whose length is determined by the blue dotted lines. Vertical red lines show time points where alarms were triggered. Grey ribbons indicate model of 95% confidence intervals. Light grey area indicates first concentration step (45.8 μg/L) and dark grey area indicates second concentration step (214 μg/L) of the toxicant.

The experiments revealed differences between control water and treated wastewater as well as different responses to the model substances, which were chosen to represent different modes of action. This allowed us to answer our second research question ("if and how the organisms react to model toxicant peaks in wastewater"). In addition, differences in the sensitivity of the BEWS were observed depending on the compound tested.

3.5. Statistical methods to analyze alarm algorithms of behavioral data using sertraline as an example

3.5.1. Observing different behavioral parameters of D. magna

Sertraline's influence on *D. magna* became apparent by the alteration of swimming speed (Fig. 4A) which contributed to an increase in the toxic index above the alarm threshold (Fig. 3F). The parameter average height (Fig. 4B) showed a continuous increase already during the first concentration. The daphnia were located closer to the upper part of the chamber, near the water outflow, which indicates escape movement. The Hinkley detection registered a sudden change in swimming speed but not in height which has only little influence on the toxic index score system. Lopes et al. (2004) stated that an increase in swimming speed belongs to an escape response of *D. magna* to pollutants in the water column, which is also elicited by the presence of predators (Seely and Lutnesky, 1998). Based on these results, further experiments could be conducted to evaluate if the increased height should count as escape movement and receive a higher value in the creation of toxic points due to an earlier response to the toxic substance compared to other parameters. This could be done by a gradual increase of the concentration to gain a more defined insight in when the different parameters are reacting towards the toxicant.

With regard to our third goal ("to elaborate a statistical approach for the analysis of the behavioral data to identify potential adjustments to the system's internal alarm settings."), we found that the internal Hinkley detection method is already well established as a statistical evaluation. As individual alarm parameter values can be adjusted within this algorithm, there was no need for a further statistical approach to evaluate the behavior of *D. magna*.

3.5.2. Analysis of the movement patterns of G. pulex by examining raw amplitude pattern of the measuring chambers

The company specific alarm sum parameter, which fluctuated multiple times during exposure to the first concentration (45.8 μg/L) of sertraline, increased during exposure to the second concentration (214 μg/L) (Fig. 5A). After a steady increase, the value exceeded the alarm threshold and reached a level of 140 after the death of one individual. Overlooked by the alarm sum, a circadian rhythm was pointed out by the multivariate analysis in the average amplitude (Fig. 5B). The SMOP-Algorithm of the multivariate analysis detected no difference in the amount of changepoints before and after the exposure with sertraline. In the recovery phase, the night rhythm appeared shortened and the daytime activity seemed to last longer when looking at the length of the two last segments. The baseline data of the toxicity studies, which was used for modelling the circadian behavior, showed the tendency of larger activity at night and smaller activity in the afternoon indicated by the grey ribbons in Fig. 5B. The exposure to sertraline seems to have the effect of "flattening" the curvature, i.e., to suppress the circadian rhythm, although this is difficult to prove in a statistical manner. This corresponds to the multivariate changepoint analysis, where these patterns were clearly visible. Lopez-Mangas et al. (2023, unpublished), unpublished) experimented with river water and two toxicants in a similar setup. After creating a baseline, *G. pulex* was exposed to two doses of azoxystrobin and thiacloprid in a 7-day experiment. A general decrease of the circadian rhythm was observed over the baseline period and, after toxicants were applied, a clear disruption of this pattern was visible. The fact that changes in the circadian rhythm can act as an indicator of biological metabolic changes including daily metabolic rate and oxygen consumption makes it valuable for biomonitoring studies and for understanding the organism's behavior as recently shown by Yang et al. (2018).

Thus, for the Sensaguard, the introduction of the changepoint analysis to highlight the changes in the circadian rhythm and the use of prediction limits calculated from baseline data to visualise deviations from the gammarids normal behavior were two valuable statistical tools to analyze the raw data alongside its own alarm algorithm.

3.6. Feeding activity of G. pulex in the Sensaguard complementary to the toxicant response tests

The feeding activity was calculated for the individuals in the Sensaguard, and complemented the behavior measurements (Fig. 6). Behavior represents an integration of complex biochemical and physiological processes, and therefore endpoints like feeding activity may provide a sensitive indication of additional sublethal toxicant effects on the organisms used in the BEWS. The median of the feeding activity between individuals during exposure to wastewater only (baseline) was lower (median: 1.8 area/mg/d) compared to the negative control (median: 4.1 area/mg/d). This difference was, however, not significant, presumably due to the high variation of the negative control. A higher feeding rate in the negative control compared to the experiments with wastewater could be explained by the water matrix, which contains less nutrients than the wastewater. This was also indicated by another study where *G. pulex* was exposed to wastewater, which led to behavioral alterations but not to significant differences in feeding rate compared to control water (Love et al., 2020). For sertraline (median: 1 area/mg/d) and diuron (median: 2 area/mg/d) there was no big deviation from the baseline, but feeding activity of gammarids exposed to sertraline was significantly lower than feeding activity of gammarids from the negative control (ANOVA followed by Tukey's multiple comparisons test, p-value = 0.0258). The lowest feeding activity was measured in gammarids exposed to wastewater and chlorpyrifos methyl. However, it has to be considered that, in this treatment, just two individuals survived, which could be used to calculate the feeding rate. A reduction in feeding rates of *G. fossarum* of up to 70-90% compared to reference water under laboratory conditions was detected in previous studies investigating the impact of wastewater on detritivorous macroinvertebrates (Englert et al., 2013; Ganser et al., 2019). This is partially supported by the present experiment, which highlights that there might be a link between behavioral changes and feeding activity, and that changes in nutrient uptake might be accompanied by behavioral changes elicited by the exposure to substances like sertraline, but this has to be further investigated.

3.7. Relevance of BEWS for wastewater treatment plants and implications for practice

This study shows that the three selected BEWSion with a filtration module, are ready to be applied for wastewater monitoring. Municipal wastewater induced no deviation from normal behavior, but exposure to toxicants spiked to the wastewater simulating WWTPs with large industrial emissions clearly triggered alarms. The chemicals with different modes of action evoked different effects on the organisms in the BEWS, showing the value of using a battery of different BEWS, as recommended by Kramer and Botterweg (1991). However, dilution steps might be necessary in a real-world scenario with very high industrial input to avoid mortality of organisms, depending on the pollutant load. Such dilution should always be determined in pre-tests prior to the actual monitoring phase. BEWS' potential role in the WWTP environment could be complemented with the link to chemical monitoring. Anliker et al. (2020a) used 24-h composite samples from a time period of 3 months and non-target analysis to detect industrial peak emissions from regular production. They suggested monitoring with a high temporal resolution for extended time periods to identify critical contamination hotspots (Anliker et al., 2020b). Depending on production processes and product range, some companies produce the same products for years; others change their production from day to day. Accompanied by a tool which serves as a biological alarm system to indicate critical emission time periods would be a promising prioritization strategy to obtain the required chemical information much faster, without having to evaluate

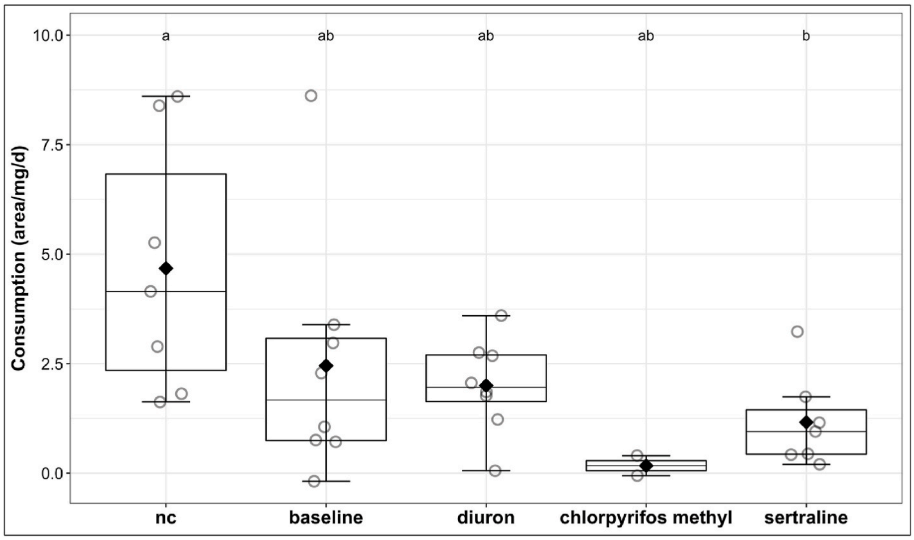

Fig. 6. Feeding activity of Gammarus pulex in the Sensaguard. Box plots describe the feeding activity by leaf disc feeding rate in area/mg/d in the single exposure experiments. Grey dots represent individual data points for each individual in the test chambers. The ends of the box are the upper and lower quartiles. The median is marked by a vertical line inside the box. Different letters indicate significant differences (ANOVA by Dunn's Multiple Comparison Test). Negative control is abbreviated with "nc". Note that for chlorpyrifos methyl only two organisms could be included.

a whole data set, and to trigger mitigation measures such as wastewater storage followed by extended treatment.

4. Conclusion

- The selected BEWS battery combined with ultrafiltration can be applied for wastewater surveillance with the aim to detect peak loads of pollutants.
- No strong differences between wastewater and control water were detected in the BEWS in terms of survival and behavior, which is an important prerequisite to be able to use these systems for continuous wastewater surveillance.
- BEWS reacted visibly on toxicant peak events and different behavioral effects could be observed during and after exposure, showing that they are capable of detecting peak loads of pollutants.
- The alarm algorithms of the BEWS proved to be reliable. However, by evaluating the individual and raw behavior patterns, additional relevant information could be gained, which can help further in increasing the sensitivity of the systems and enhance the understanding of specific behavioral responses.
- WWTPs with increased industrial input could benefit from such a continuous effect-based "non-target" monitoring method to quickly draw attention to critical inputs of pollutants, which could help operators to revise treatment strategies.

Credit author statement

Ali Kizgin: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Project administration, Danina Schmidt: Conceptualization, Validation, Formal analysis, Investigation, Visualization, Adriano Joss: Methodology, Software, Juliane Hollender: Conceptualization, Supervision, Writing – review & editing, Eberhard Morgenroth: Conceptualization, Supervision, Writing – review & editing, Cornelia Kienle: Conceptualization, Methodology, Supervision, Validation, Writing – review & editing, Project administration, Miriam Langer: Conceptualization, Methodology, Supervision, Validation, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

I have shared a link to my data in the file attach step.
Data Package for "Application of biological early warning systems in wastewater treatment plants: Introducing a promising approach to monitor changing wastewater composition"(Original data) (Mendeley Data)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2023.119001.

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