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BEHAVIOR OF *COROPHIUM VOLUTATOR* (CRUSTACEA, AMPHIPODA) EXPOSED TO THE WATER-ACCOMMODATED FRACTION OF OIL IN WATER AND SEDIMENT

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Abstract—We investigated the short-term effects of the water accommodated fraction (WAF) of weathered Forties crude oil on the behavior of *Corophium volutator* in the Multispecies Freshwater Biomonitor® (MFB). When exposing *C. volutator* to 25 and 50% WAF in aqueous exposures, hyperactivity with an additional increase in ventilation was detected, whereas exposure to 100% WAF led to hypoactivity (narcosis). In a sediment exposure with 100% WAF, there was an increased tendency toward hyperactivity. In a pulse experiment, hyperactivity appeared at and after a 130-min exposure to 50% WAF in a majority of cases. Our experiments suggest that the behavior of *C. volutator* as measured in the MFB may be an appropriate parameter for coastal monitoring.

Keywords—Mud shrimp Oil Pulse pollution Locomotor activity

INTRODUCTION

Intertidal communities are highly vulnerable to oil spill incidents. This is a consequence of their location at the shoreline interface between water and land where floating oil is deposited by the waves [1]. The water-accommodated fraction (WAF) of oil is a combination of single-phase homogenous mixtures (water-soluble fractions) of hydrocarbons and dispersions of fine oil droplets in water [2]. It is this fraction that often represents the greatest risk to aquatic organisms.

The mud shrimp *Corophium volutator* is one of the most abundant organisms in estuarine mudflats of the North Atlantic, American, and European coasts, extending from western Norway to the Mediterranean and into the Black Sea and Azov Sea (http://www.marlin.ac.uk/species/Corophiumvolutator.htm). It can attain a size of approximately 10 mm and lives in self-constructed tubes in intertidal mudflats, salt-marsh pools, and brackish ditches (http://ip30.eti.uva.nl/bis/crustacea.php). It has the habit of swimming when in open water (http://www.marlin.ac.uk/species/Corophiumvolutator.htm) but generally shows low motility and burrows in the sediment most of the time [3]. *Corophium volutator* tolerates a wide range of salinity from near fully saline to almost freshwater and is locally abundant (http://ip30.eti.uva.nl/bis/crustacea.php). It has already been used in several marine bioassays to assess acute as well as chronic toxicity [4–8]. For behavior measurements with *C. volutator*, test parameters have been burrowing time, re-emergence from the sediment and activity prior to burrowing (exposure to WAF) [6], changes in swimming behavior, and locomotor and ventilatory activity (exposure to the pesticide Bioban® [Brenntag, Deerlijk, Belgium]) [7]. Behavior is considered to be a sensitive indicator for effects of contaminants [9].

Until now, only one behavioral study regarding WAF sediment exposure has been conducted [6]. The present study represents the first effort to investigate the effects of WAF (aqueous and in sediment) on behavior and survival of *C. volutator* using the Multispecies Freshwater Biomonitor® (MFB; LimCo International, Ibbenbüren, Germany), an online biomonitor that continuously and quantitatively records the behavior pattern of animals in both aqueous and sediment exposures.

The aims of the present study were to examine the suitability of the MFB for detecting effects of WAF (aqueous and in sediment) on *C. volutator*. The effects of several dilutions of WAF on the locomotor and ventilatory activity of *C. volutator* were to be assessed, as were any differences between behavior in aqueous and in sediment exposures. An additional objective was to examine the ability of *C. volutator* to recover from aqueous WAF exposure.

MATERIALS AND METHODS

Maintenance of test animals

Adult *C. volutator* and sediment were collected as described in Smith et al. [8] from an intertidal area of the Avon estuary near Aveton Gifford, South Devon, United Kingdom. Amphipods were separated from the sediment via sieving through a 500-μm sieve so that neonates passed through, while midsize individuals, which should be used for the tests, remained in the sieve. The animals were put into 5-L culture tanks holding field-collected and sieved (<300 μm) sediment as well as aerated and filtered seawater (25 ± 1‰). The tanks were maintained at 15 ± 1°C with a 12:12-h light:dark cycle. After an acclimation period of 7 d, sediment with embedded *C. volutator* was sieved again to extract the individuals. Size-matched specimens of medium size (~3–4 mm) were used for behavior measurements.

Preparation of WAFs and spiking of sediment

The WAFs were prepared using weathered Forties Blend crude oil consisting mainly of paraffines, naphthenes, and aromatics. They were prepared as described in Smith et al. [8]. For exposure preparations, 5-L Pyrex® bottles were used. Twenty-five milliliters of crude oil and 2,475 ml of 25‰ sea-

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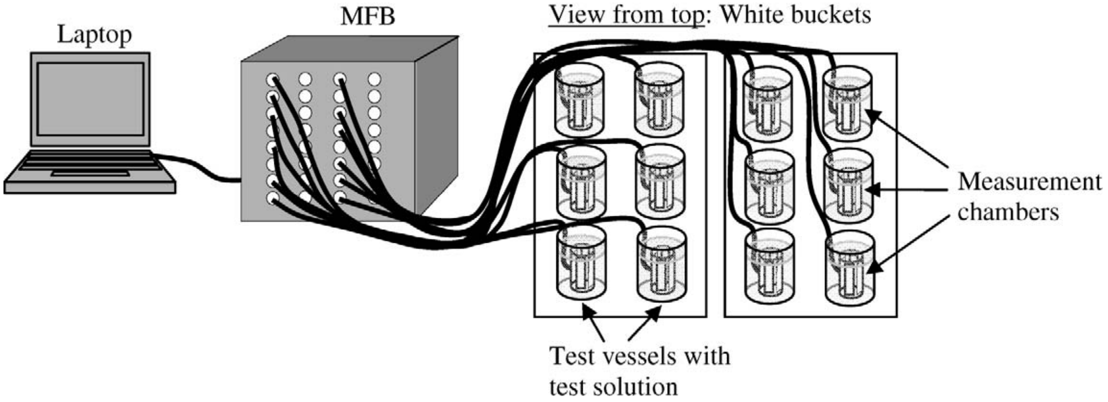

Fig. 1. Experimental setup for the behavior measurements with *Corophium volutator* in aqueous and sediment exposure. MFB = Multispecies Freshwater Biomonitor® (LimCo International, Ibbenbüren, Germany).

water were slowly vortex mixed in a temperature-controlled room (15 ± 1°C) for 24 h and then left to re-equilibrate for 1 h. Sampling was carried out by carefully siphoning off the water phase containing WAF by applying a gentle pressure of nitrogen to the top of the oil/seawater surface [8]. For the aqueous exposures, 100% WAF and dilutions of 25 and 50% WAF in 25‰ seawater were prepared.

For the sediment exposures, the sediment was spiked with 100% WAF as described in Smith et al. [8]. Portions of 160-ml-sieved sediment were placed in 500-ml-wide-neck glass bottles (Schott, Mainz, Germany), and an aliquot of 320 ml WAF (prepared with 25‰ seawater; see previous description) was added to each bottle. This mixture was shaken at 15°C for 3.5 h and 200 rpm on an orbital shaker. Afterward, the slurry from each bottle was transferred to 2-L Pyrex beakers. After allowing for 16 h of settlement, the supernatant was discarded [8]. Before the sediment was transferred to the measurement chambers, it was stirred for approximately 30 s with a spatula for final homogenization. Control treatments were prepared in the same way as the exposure treatments substituting 25‰ seawater for WAF.

Multispecies Freshwater Biomonitor

The MFB, an online biomonitor for continuously and quantitatively recording the behavior pattern of animals, consists of flow-through sensor chambers, a measuring unit, and a personal computer with specific software for data analysis [10]. The measuring principle in the sensor chamber is based on quadrupole impedance conversion. The behavioral signal from the animal is analyzed by a fast Fourier transformation, resulting in a histogram of different signal frequencies. Different frequency ranges can be attributed to different types of behavior, such as locomotion (summarized in frequency band 1: 0.5–2 Hz) and ventilation (summarized in frequency band 2: 2.5–8 Hz) [10]. The MFB nonoptical recording principle based on quadrupole impedance conversion allows for equal signal quality in water and sediment, as has already been shown in several studies [7,11,12]. It is presently the only automated biomonitoring system with this capability.

Experimental setup

Figure 1 shows the experimental setup for the behavior measurements. Experiments were conducted in 100-ml glass beakers filled with approximately 100 ml of test solution. One measuring chamber of the MFB was placed vertically in each beaker. The chambers used for *C. volutator* were 4 cm in length with a diameter of 1 cm, allowing for free movement of the individuals (length of *C. volutator*: ~3–4 mm). The chambers were sealed with a mesh lid at one end (mesh width 0.25 mm), the beaker was partially filled with test solution, and one amphipod was put into each chamber. After sealing the other end of the chamber, the beaker was completely filled with the test solution, and any remaining air bubbles in the chamber were removed.

Vessels for sediment exposure were also 100-ml glass beakers with one measuring chamber placed within each. The measurement chambers were half filled with sediment and half filled with water in order to give the animals the opportunity to swim in the water column and burrow in the sediment. The sediment was allowed to settle for approximately 1 h. The rest of the procedure was the same as for aqueous exposure.

Subsequently, the locomotor and ventilatory behavior of four to six individuals of *C. volutator* for each treatment was measured for a duration of 2 h in the acute aqueous (0, 25, 50, 100% WAF) and sediment exposures (0, 100% WAF). For acute aqueous exposures, three control treatments were conducted in total with each WAF exposure paired with a control in the system. For sediment exposures, the position of the animals in the chambers (in the water column or in the sediment) was noted several times during the exposure period of 2 h (minimum three times).

Moreover, in an additional stress and recovery pulse experiment, the behavior of six *C. volutator* was recorded for 2 h in 50% WAF and 25‰ seawater, respectively, with the previously described experimental setup for the aqueous exposure. After those 2 h, half the solution was removed and replaced with filtered 25‰ seawater. After an additional 1.5 h, all of the test solution was removed and replaced by seawater. After approximately 20 h, this pulse experiment was terminated. The animals were kept in a 12:12-h light:dark cycle (light from 8 AM to 8 PM). No food was added.

Data analysis

For each individual, mean locomotor (0.5–2 Hz, band 1) and ventilatory activity (>2 Hz, band 2) (% time spent on locomotion and ventilation, respectively) were calculated for the exposure time of 2 h. The behavior of four to six individuals of *C. volutator* was measured for each treatment. As the data of the three control treatments for aqueous exposures did not differ significantly, they were summarized for data analysis. For statistical evaluation, the data on time percentage of activity were arcsine transformed from proportional values. Non-

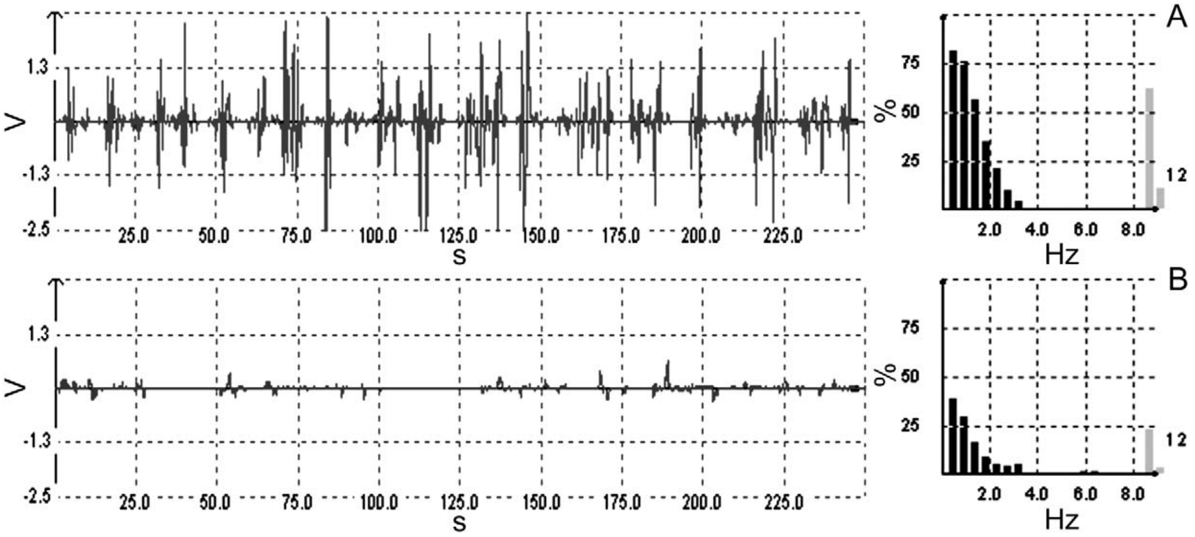

Fig. 2. Different behavior patterns of *Corophium volutator*. Left: Locomotor pattern (amplitude [V] vs time [s]). Right: Corresponding fast Fourier transformation histograms (locomotor activity in % of the time [250 s] vs frequency [Hz]) of *C. volutator* in uncontaminated water (**A**) and in sediment (**B**).

parametric methods were chosen because the data were only partially normally distributed (Shapiro–Wilk *W* test; JMP 4.0, SAS Systems, Cary, NC, USA). Differences between control and exposure treatments were analyzed for significance with a Wilcoxon two-group test (JMP 4.0) followed by a Bonferroni–Holm adjustment [13]. Behavioral data of the stress and recovery experiment were normalized to the reference data (ref, behavioral data of the 25‰ seawater treatment) for each data point throughout the whole exposure period ($f(x) = x/\mathrm{ref} \times 100$) to flatten out the normal circadian rhythm, while any circadian variation left in the curves could be interpreted as amplification of the rhythmicity due to exposure stress [14]. Afterward, the curve was split into increasing/decreasing and monotonous parts, and a spline run was performed with the data using a linear regression model (JMP 4.0).

RESULTS AND DISCUSSION

MFB signals for activity in sediment

Corophium volutator showed almost constant swimming activity in water, as reflected by continuous signals with high amplitude (Fig. 2A), and lower activity in sediment, as reflected by movement signals combined with pauses and a generally lower amplitude (Fig. 2B). In addition to locomotor activity, ventilation signals could be detected, as reflected by regular signals in the relevant frequency range (2.5–8 Hz) (Fig. 3A and C). The signals for locomotion were in the range of 0.5 to 2 Hz, whereas ventilation frequencies lay above 2 Hz (Figs. 2 and 3), similar to the description in Kirkpatrick et al. [7]. With increasing ventilation activity, the histogram changed from low to high frequencies (Fig. 3A).

Acute aqueous exposures to 25, 50, and 100% WAF

When exposed to 25 and 50% WAF, *C. volutator* displayed hyperactivity $p < 0.001$ (band 1, 25% WAF) and $p < 0.01$ (band 1, 50% WAF) compared to the control treatments. In the treatment with 100% WAF, the animals showed signs of narcosis and were lying on the bottom for most of the measurement time. In this treatment, variation in locomotor activity was high, three out of five animals showing only low locomotor activity (Fig. 4).

Hyperactivity (increased swimming activity) is a common symptom of toxic effects, indicating an avoidance/escape response [14]. This type of locomotor escape behavior has been described for *C. volutator* exposed to Bioban, [7] as well as for other organisms (e.g., *Gambusia holbrooki* [mosquitofish] exposed to acid mine drainage [14] and *Gammarus pulex* [freshwater shrimp] exposed to a simulated Cu-pulse [70 ppb] in situ [15]). Given the stressors (100% WAF, including oils and polycyclic aromatic hydrocarbons), narcosis would be a consistent response. This was suggested by seeing nonmoving amphipods lying on the sediment. Increased variability in behavior has been observed as a result of toxicity previously [16]. However, in the present study, significant behavioral responses occurred at much lower concentrations than those associated with narcosis (e.g., 25 vs 100% WAF). The fact that exposure to 100% WAF does not result in any significant difference to the control may be explained by the high variation in the behavior of the test animals. The differences could be clearly observed, as the amphipods were not using the whole chamber but only the lower area for swimming and were lying on the bottom most of the time. This increased variation in behavior is presumably due to the fact that some individuals were already affected by narcosis (i.e., less active), while others were not. Hyperactivity has been observed as a first sign of stress before. This behavioral response might present an attempt of the amphipods to escape the toxic area.

With 25 and 50% WAF, significant effects on ventilation occurred ($p = 0.003$ [band 2, 25% WAF] and $p = 0.007$ [band 2, 50% WAF]). A similar response could be observed for *C. volutator* exposed to high concentrations of Bioban [7] as well as for *G. holbrooki* and *Daphnia magna* (water flea) exposed to acid mine drainage [14]. An increase in ventilation might indicate an attempt by the animal to remove the toxins from the body surface [14].

Sediment 25‰ seawater and 100% WAF exposure

When exposed to sediment spiked with 100% WAF, *C. volutator* showed a tendency toward hyperactivity, although differences compared to the control were not significant (due to high interindividual variation) (Fig. 5). One of six animals

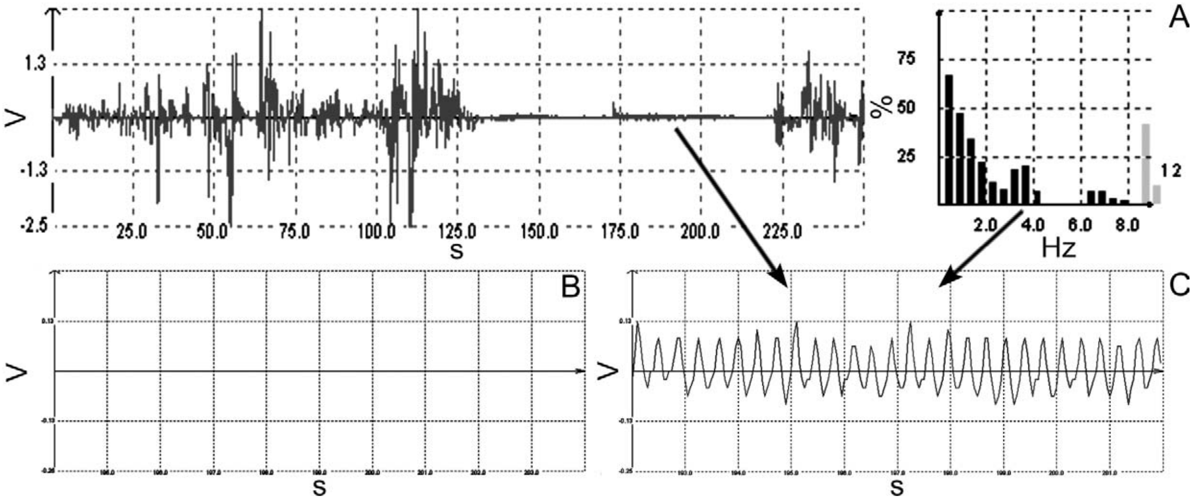

Fig. 3. Different behavior patterns of *Corophium volutator*. Left: Locomotor pattern (amplitude [V] vs time [s]). Right: Corresponding fast Fourier transformation histograms (locomotor activity in percent of the time [250 s] vs frequency [Hz]) of *C. volutator*. (**A**) Locomotion and ventilation signals in control water. (**B**) Signal of an empty chamber as baseline. (**C**) Enlarged view of ventilation signals.

in the WAF treatment did not burrow at all, while five others stayed in the sediment for a certain amount of time. Also, amphipods were swimming, as verified by visual observation and observation of the movement patterns, where swimming activity could be clearly distinguished from locomotor activity in sediment (Fig. 2A and B). In the control treatment, two of six animals frequently alternated between the water and sediment compartments, and four animals were constantly burrowing into the sediment.

When examining the burrowing behavior of *C. volutator* exposed to sediment spiked with Forties crude oil, *C. volutator* showed a greater tendency to avoid burrowing and to reemerge from the sediment [6]. In the present study, more animals in the 100% WAF exposure than in the control treatment spent part of the time swimming (six vs two out of six individuals). This finding supports the results of Scarlett et al. [6] but with differences in exposure conditions (pure oil in Scarlett et al.'s experiments [6] vs WAF in the present study). Moreover, there was less space to burrow in the measurement chambers in the present study than in the glass beakers in Scarlett et al. [6]. For more detailed sediment studies, it may be useful to give the amphipods more space to burrow (i.e., larger measurement chambers). To be able to distinguish better between behavioral signals in the sediment and in the water compartment in further studies, separate chambers stuck together should be used [7].

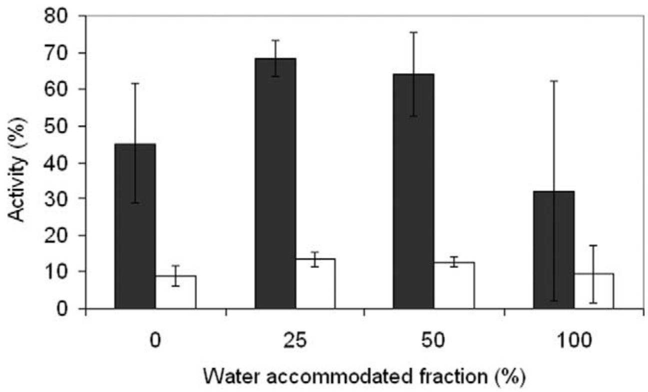

Fig. 4. Activity (%) of *Corophium volutator* in control treatments ($n = 4$–6 per treatment; summary of three control treatments: 14 individuals in total) and exposed to 25% ($n = 7$), 50% ($n = 6$), and 100% ($n = 5$) water-accommodated fraction in frequency bands 1 (0.5–2 Hz) (■: locomotion) and 2 (2.5–8 Hz) (□: ventilation) (mean ± standard deviation). Significant differences from control treatment: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

In a comparison of laboratory and in situ bioassays with *C. volutator*, the amphipods reacted more sensitively in in situ bioassays [17]. This may indicate that observed effects in the present study underestimate in situ effects.

No mortality occurred during any of the experiments of the present study. Also, in chronic studies with WAF (110 d), no significant effects on the survival of *C. volutator* were observed with 100% WAF [8]. These results demonstrate that behavioral responses may occur at lower concentrations (at 25 and 50% WAF) than effects to more traditional endpoints like malformations and mortality. This supports the higher sensitivity of behavioral endpoints.

Aqueous 50% WAF recovery exposure

In the stress and recovery pulse experiment with a 130-min pulse of 50% WAF (Fig. 6), *C. volutator* displayed a significant increase in locomotor activity (hyperactivity) compared to the control during the exposure (locomotor activity

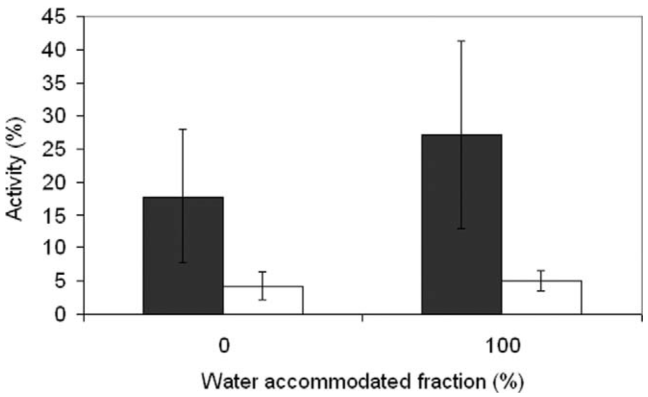

Fig. 5. Activity (%) of *Corophium volutator* in control sediment ($n = 6$) and in sediment spiked with 100% water-accommodated fraction ($n = 6$) in frequency bands 1 (0.5–2 Hz) (■: locomotion) and 2 (2.5–8 Hz) (□: ventilation) (mean ± standard deviation).

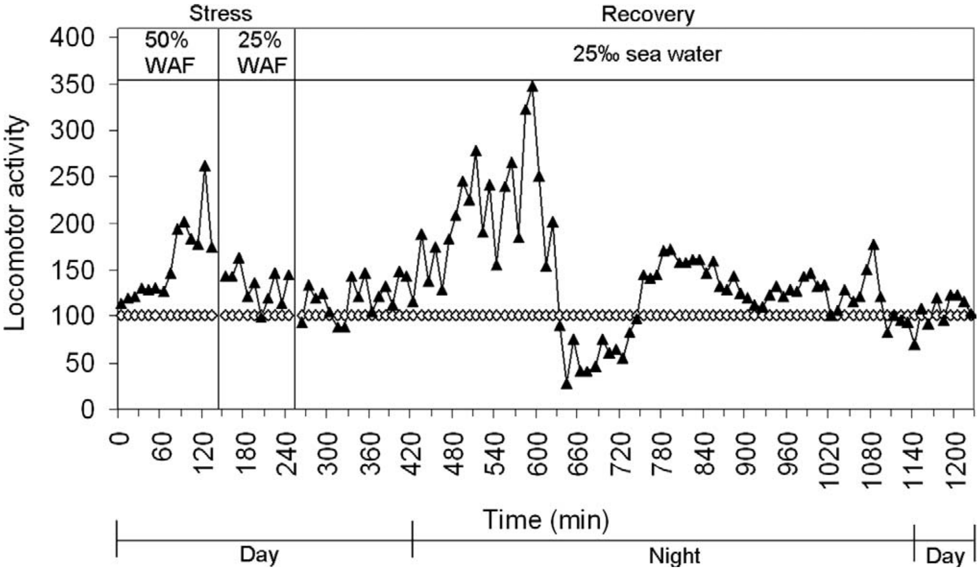

Fig. 6. Locomotor activity (%) of *Corophium volutator* in control treatment ($n = 6$) (◇: locomotion control) and exposed to 50% water-accommodated fraction (WAF) ($n = 6$) (▲: locomotion 50% WAF) (data normalized to control values, standard deviation [SD] range [control] = 9.31–26.44%; SD range [exposure] = 6.98–28.65%) in frequency band 1 (0.5–2 Hz) from 0 to 130 min, to 25% WAF from 150 to 240 min (data normalized to control values, SD range [control] = 21.55–26.29%; SD range [exposure] = 6.78–30.69%) and to control water from 260 to 1,220 min (data normalized to control values; SD range [control] = 12.05–30.20%; SD range [exposure] = 1.60–30.69%).

[normalized] = 97.684 + 0.979 min, $r^2 = 0.754$, $p < 0.001$). After exchanging half the solution with control water, the hyperactive locomotor activity decreased until the full exchange of solution after 240 min and remained near the control level afterward until 420 min. In the recovery period, three activity peaks could be observed. From 420 to 590 min, the locomotor activity increased significantly (locomotor activity [normalized] = −227.938 + 0.873 min, $r^2 = 0.528$, $p < 0.001$) and then decreased below control level (590–670 min, locomotor activity [normalized] = 2,438.827 − 3.655 min, $r^2 = 0.810$, $p < 0.001$). This was followed by a significant increase in activity (670–790 min, locomotor activity [normalized] = −761.004 + 1.179 min, $r^2 = 0.891$, $p < 0.001$), which decreased again afterward. A last small activity peak could be observed from 1,020 to 1,080 min (locomotor activity [normalized] = −1,038.828 + 1.112 min, $r^2 = 0.786$, $p = 0.008$). Afterward, the locomotor activity decreased to the control level and remained similar for the rest of the recovery time (1,110–1,220 min).

The results show that, if *C. volutator* was first exposed to 50% WAF (resulting in hyperactivity similar to the 2-h acute exposures), the subsequent 25% WAF exposure was sufficient to allow the activity to decrease to the control level (dissimilar to the 2-h acute exposures at this concentration level). This indicates that the effects of contaminants on the behavior may differ, depending on the previous exposure conditions. So in the environment, individuals of *C. volutator* that were previously exposed to a certain level of contaminants might be less sensitive than animals living in a noncontaminated environment. This may depend on the frequency of oil spill pulses and their respective concentration levels. The tendency toward higher activity of previously exposed amphipods compared to control animals suggests that effects of exposure to contaminants can continue even though the contaminant is no longer present in the environment. An increase in activity could also be observed for *Crangonyx pseudogracilis* (northern river crangonyctid) after exposure to a pulse of ammonium chloride [18]. In an in situ experiment at the Rhine River, *G. pulex* showed decreased activity due to an oil pollution peak [19]. This corresponds well with the narcotic effect of 100% WAF observed in our exposures with *C. volutator* and might have occurred in the pulse experiment if 100% WAF had been used.

It seems that *C. volutator* was able to recover from aqueous exposure to WAF approximately 18 h after the exposure period, but for a more detailed interpretation, further experiments with a longer recovery time and perhaps a second exposure period would be necessary. In a pulsed exposure experiment, carbamate insecticides were less toxic to *Chironomus riparius* (a midge larvae) larvae if recovery in clean water was permitted, but exposure to organophosphate insecticides proved to be equally toxic even after changes in the conditions [20]. In a pulsed exposures experiment with the freshwater amphipod *Hyalella azteca* using $CuSO_4$ and Na pentachlorophenol, recovery time had a significant effect on the mortality at secondary exposure [21]. If the animals were provided enough time between exposures, the amphipods were able to recover to a state similar to their original condition [21]. No data were available in the literature for pulsed exposure to oil or WAF.

According to Diamond et al. [22], pulsed exposure effects are dependent on the frequency, magnitude, and duration as well as the recovery period between pulses. They suggest that chronic water quality criteria and effluent permit limits may not be sufficient for protecting against such effects [22].

CONCLUSIONS

The MFB proved to be suitable for detecting behavioral effects of WAF on *C. volutator*. When comparing aqueous and sediment exposure, the effects of WAF on the locomotor activity of *C. volutator* were more pronounced in aqueous exposures than in sediment exposures. The higher sensitivity of aqueous exposure is partly outweighed by the lower environmental relevance because *C. volutator* spends most of the time in the sediment. This may also indicate that *C. volutator* can seek refuge in the sediment as a shelter from aqueous oil pollution spills and hence minimize toxic short-term effects. Clear differences between behavior in sediment and in water could be observed. The locomotor activity in sediment was lower than in water. *Corophium volutator* seemed to be able to recover from WAF exposure after approximately 18 h of recovery, but further and longer experiments are necessary to prove this conclusion.

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