A RAPID COMMUNICATION

Suppressed swimming activity in Zebrafish (Danio rerio) exposed to 1,4,5-oxadithiepane, a sulphur mustard degradation product*

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ABSTRACT

This rapid communication presents novel data on the ecotoxicity of the cyclic sulphur mustard degradation compound, 1,4,5-oxadithiepane. Groups of adult male Zebrafish (Danio rerio) were exposed to 1,4,5-oxadithiepane at 40.3 ± 2.9 μg L⁻¹, 133.3 ± 6.7 μg L⁻¹ and 1533.3 ± 266.7 μg L⁻¹, respectively. After 14 days of exposure, fitness and lethality were accessed and the swimming behaviour of the Zebrafish was measured by an automated video tracking system. The average swimming velocity was decreased significantly from 40.4 ± 2.5 mm s⁻¹ in the control group to 31.8 ± 1.6 mm s⁻¹ in the low dose. The low and high doses demonstrated suppressed average velocities, partly due to elevated immobile periods (rest time). Thus, at 40.3 ± 2.9 μg L⁻¹ and 1533.3 ± 266.7 μg L⁻¹ both parameters were significantly different from the control group. No adverse effects were found at the 1533.3 ± 266.7 μg L⁻¹ treatment level and no lethality, associated with the toxic properties of 1,4,5-oxadithiepane, was observed during the 14 days of exposure suggesting a 14 days No-Observed-Effect-Concentration above 1533.3 μg L⁻¹.

1. Introduction

In 1915, the first large-scale use of chemical warfare agents (CWAs) occurred in Ypres, Belgium, which started an extensive use of CWA during the First World War. More than one million people were struck by CWAs, resulting in 78,390 deaths (Gilchrist, 1928). During the Second World War, CWAs were not used but were still produced in large-scale. It has been reported that Germany produced and stockpiled 65,000 tonnes of CWAs (Gatsby, 1997). A large part of the German stockpile was disposed of by the Allied forces by dumping in the Baltic Sea. In the Bornholm Deep, the largest dumping area, 11,000 tonnes of CWAs were dumped. HELCOM (1994) reports that primarily sulphur mustard munition was dumped.

The environmental effects of these sea-dumped legacy compounds raise concern (Brewer & Nakayama, 2008; Sanderson, Fauser, Thomsen, & Sørensen, 2007). The ecotoxicity of these compounds is still poorly understood despite a growing list of scientific literature addressing this subject (e.g. Christensen et al., 2016). Especially, chronic, sub-chronic and acute studies are needed. The munitions were dumped at the sea bottom, and leakage of chemicals may pose a risk to marine animals, including the commercially important cod (Gadus morhua). Fish is an important group of organisms in ecosystems and presents a direct route of exposure to humans (Sanderson, Fauser, Thomsen, & Sørensen, 2008). Sulphur mustard degrades to different compounds in the marine environment. In the Baltic Sea, six different sulphur mustard degradation products have been detected (Söderström, 2014). One of the degradation products is the cyclic 1,4,5-oxadithiepane, and this compound was selected for this study for two reasons. Firstly, it has a high frequency of detection and occurs at the highest concentration of the six known sulphur mustard degradation products in sediment and pore water samples. Secondly, the compound has been characterised as toxic, according to the Globally Harmonized System of Classification and Labelling (GHS), with an EC₅₀ = 1700 μg L⁻¹ in the Microtox™ test (Christensen et al., 2016). This study presents ecotoxicity data for 1,4,5-oxadithiepane on fish. Groups of adult male Zebrafish were exposed to three different concentrations of 1,4,5-oxadithiepane for 14 days (sub-acute exposures), including a nominal environmental

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and behaviour measurements. Briefly, adult Zebrafish (Danio rerio) were purchased from Credo Fish (Aalborg, Denmark). The stock population, consisting of 300 male and female Zebrafish, was acclimatised and observed for health two weeks prior to experimentation. Three groups of adult male fish were exposed to 1,4,5-oxadithiepane at nominal concentrations of 19.3 μg L⁻¹, 193 μg L⁻¹ and 1930 μg L⁻¹. Concomitantly, a control group received only the carrier-solvent acetonitrile. The nominal concentration of acetonitrile was 26 μL L⁻¹ in all exposure tanks, and

relevant concentration of 19.3 μg L⁻¹ (Söderström, 2014). Subsequently, their swimming behaviour, representing an integrated response to the internal effects of the xenobiotic, was measured and analysed.

2. Materials & methods

2.1. Specimen and experimental design

The present study follows the experimental design described in (Baatrup & Henriksen, 2015) including fish handling

Figure 1. Two components in the spontaneous locomotor activity of the male Zebrafish (Danio rerio) exposed to 1,4,5-oxadithiepane at 40.3 μg L⁻¹ (n = 26), 133.3 μg L⁻¹ (n = 26), 1533.3 μg L⁻¹ (n = 27) and a solvent control (n = 27). (a) Average velocity (mm s⁻¹) of adult male Zebrafish during the 45-min recording (b) Mean maximum velocity (mm s⁻¹) of adult male Zebrafish during the 45-min recording. Note: Entries are presented as mean values ± SEM and the letters ‘a’ and ‘b’ indicate statistical significance.

Figure 2. Temporal average rest duration (s) of adult male Zebrafish (Danio rerio) during the 45-min recording. Notes: Average rest durations were categorised into 20 intervals of 135 s (0–2700 s) in the solvent control ■ (n = 26), low concentration group (40.3 μg L⁻¹) – (n = 26), the middle concentration group (133.3 μg L⁻¹) — (n = 27) and the high concentration group (1533.3 μg L⁻¹) — (n = 27). Error bars represent SEM.
hence, in accordance with OECD guidance document 23, did not exceed 100 μL L⁻¹ (Organisation for Economic Cooperation & Development [OECD], 2000). The actual concentrations of 1,4,5-oxadithiepane were determined using gas chromatography tandem mass spectrometry (GC-MS/MS) (Bełdowski et al., 2016) to 40.3 ± 2.9, 133.3 ± 6.7 and 1533.3 ± 266.7 μg L⁻¹. The lowest nominal concentration of 1,4,5-oxadithiepane is an environmental relevant concentration previously detected in pore water samples (Söderström, 2014).

A stock solution of 1,4,5-oxadithiepane (CAS: 3886-40-6; 99% purity, Envilytix A/S, Germany) was prepared at a concentration of 74.3 g L⁻¹ in acetonitrile and stored in darkness at −20 °C. Working solutions of the different treatment concentration were prepared at 7.4, 74.3 and 742.5 mg L⁻¹. Water to the exposure tanks was carried from a header tank at a flow rate of 25 L day⁻¹ via peristaltic pumps (Ole Dich Instrument Makers Aps, Hvidovre, Denmark). The header tank contained aerated, demineralised water at 27 °C, mixed with salinised (1.8 g NaCl L⁻¹) tap water (16:1) resulting in a conductivity of about 200 μS cm⁻¹. An outlet with a filter maintained the volume in the exposure tanks at 22 L, resulting in a continuous flow-through system. The 12 exposure tanks were (46 cm × 28 cm × 28 cm) seamless glass tanks. Oxygen saturation in the exposure tanks was maintained at 93%, fulfilling the recommendation of OECD 210 (OECD, 1992). Likewise, pH at 7.28 ± 0.02 is optimal for freshwater fish. Due to the large water exchange rate and the small number of fish in the exposure tanks, nitrous compounds were not measured. Two days before exposure, the water flow through the tanks was initiated. A programmable peristaltic pump (Ismatec ICP-N, IDEX Health and Science GmbH, Wertheim-Mondfeld, Germany) continually dosed the 1,4,5-oxadithiepane working solution to the inlet water using syringe needles. The flow rate of the working solutions was programmed to 45.1 μL min⁻¹ (65 mL day⁻¹) applicable to all treatments. To ensure the correct exposure conditions from day one, the exact amounts of 1,4,5-oxadithiepane and acetonitrile were pipetted to each exposure tank. Hereafter, the peristaltic pump dosing 1,4,5-oxadithiepane was started.

Behaviour trials were carried out with one presumed male from each of the four treatments. The four test males were divided between four test tanks measuring 28 × 21 × 13 cm (length × width × height) and containing 6 cm stock tank water (2.2 L) at a room temperature of 27 °C. The test tanks were placed on a sheet of glass 50 cm above diffusely lit white paper (79 lux). A GigE Vision colour progressive scan (non-interlaced) CCD camera (Leutron Vision, Switzerland) was mounted approximately 50 cm above the test tanks. The digital video signal from the camera consisted of a 1024 × 768-pixel image, giving a 0.27-mm spatial resolution of the visual field. When viewed from above by the camera, this arrangement resulted in clear silhouettes of the four fish. The behaviour measurements were controlled by the MOTIO vision system (Department of Bioscience, University of Aarhus, Denmark). During the 45-min recording, the images were captured at 16 frames per second (16 Hz). The male swimming behaviour was evaluated based on the following parameters: (1) total swimming distances

![Figure 3. Temporal average velocity (mm s⁻¹) of adult male Zebrafish (Danio rerio) during the 45-min recording. Note: Average velocities were categorised into 20 intervals of 135 s (0–2700 s) in the solvent control ▲, low concentration group (40.3 μg L⁻¹) —, middle concentration group (133.3 μg L⁻¹) —— and high concentration group (1533.3 μg L⁻¹) ———. Error bars represent SEM.](image-url)
(m), (2) maximum velocity averaged over one second intervals (mm s$^{-1}$), (3) average velocity (mm s$^{-1}$), (4) resting time (s), (5) number of stops (#), (6) turning rate per second (° s$^{-1}$) and (7) turning bias between left and right turns (° s$^{-1}$). Additionally, frequency distributions (20-intervals) of swimming velocities between 0 and 300 mm s$^{-1}$ were extracted from the data file, together with average swimming velocity for each 135 s during the 45 min (2700 s) recording. Likewise, frequency and temporal distributions of Turn Rate (number of turns in each interval from 0° to 180°), Turn Bias (numeration the number of turns ranging from $-180^\circ$ (right turns) to $+180^\circ$ (left turns)) and quiescent periods (rests) were calculated. After the 45-min behaviour measurement, the weight and length of the test fish were determined where after it was dissected for gender determination.

### 2.2. Statistical analyses

The three replicates were pooled as there were no statistical differences (ANOVA) in behaviour between groups. Differences in Fulton’s Condition Factor (calculated as: $100 \times \text{weight (g)} / \text{length (cm)}^3$) and behaviour were detected using ANOVA. None of the data complied with normality, and log transformations were therefore performed on all parameters except Turn Bias. A power transformation was performed on Turn Bias. All other parameters complied with the Shapiro–Wilks normality test ($p > 0.05$) after transformation except Rest time, Number of stops and Turn Bias which were analysed with the non-parametric Kruskal–Wallis test. All data complied with Levene’s mean test ($p > 0.05$) except Number of stops. Number of stops was similar between groups and will not be analysed any further (Kruskal–Wallis = 0.095). When ANOVA demonstrated differences between groups, the post-hoc Tukey Contrasts was carried out. If the Kruskal–Wallis test demonstrated a difference between groups, the post-hoc Mann Whitney U was carried out. The data analyses were carried out in R Commander Package 2.0–3 for Windows (R-Statistics, Bell Laboratories, Murray Hill, NJ, U.S.A.).

The various distributions were statistically examined by the $\chi^2$-test performed in Excel (2016, Microsoft Office). The statistical evaluation was performed by the command CHI2.FORD.RT of the $\chi^2$-value with 19 degrees of freedom (df). Data are presented as mean values ± SEM and a significance level of 0.05. Fitness (Fulton’s condition factor) and lethality were recorded at the end of the study.

### 3. Results & discussion

Only one fish died during the exposure period in the middle treatment group (133.3 ± 6.7 μg L$^{-1}$) whereas no fish died in the highest treatment group. Accordingly, it seems unlikely that the single death can be ascribed to toxicity of 1,4,5-oxadithiepane. All of the presumed male Zebrafish chosen for the experiment were after gender determination determined to be males (Supplementary Table 1). Fulton’s condition factor was identical across all treatment groups and the control group (Kruskal–Wallis, $p = 0.559$). This demonstrates an equal weight relative to length across all treatment groups and the control group, suggesting that 1,4,5-oxadithiepane at concentrations up to 1533.3 μg L$^{-1}$ over 14 days is unlikely to affect the growth or weight of adult male Zebrafish No-Observed-Effect-Concentration (NOEC > 1533.3 μg L$^{-1}$) (Supplementary Table 1). However, the exact age of each of the Zebrafish is unknown. It was found that the Fulton’s condition factor was significantly higher than the control, when Zebrafish were exposed to 3 ng L$^{-1}$ of the contraceptive hormone 17α-ethinylestradiol (EE2) (Baatrup & Henriksen, 2015) in a prolonged study from hatchling to adulthood. This is contrary to the present study which found identical Fulton’s condition factors (Kruskal-Wallis, $p = 0.559$).

In this behaviour study, the general trend is a decreased swimming activity in Zebrafish exposed to the environmentally realistic (low) concentration at 40.3 ± 2.9 μg L$^{-1}$ of 1,4,5-oxadithiepane. At the middle treatment group, the activity resembled the controls whereas the highest treatment group again displayed a decreased swimming activity. The average velocity statistical significantly decreased when Zebrafish were exposed to 40.3 ± 2.9 μg L$^{-1}$ (ANOVA, Tukey Contrast, $p < 0.05$) compared to the control, whereas the mean maximum swimming velocity decreased statistical significantly when Zebrafish were exposed to 40.3 ± 2.9 μg L$^{-1}$ compared to Zebrafish exposed to 133.3 ± 6.7 μg L$^{-1}$ (ANOVA, Tukey Contrast, $p < 0.05$) (Figure 1). The other components follow the above-mentioned general trend, but the differences were not statistically significant. All measured components of the Zebrafish swimming behaviour are presented in Table 1.

This study also examined frequency and temporal distributions during the 45-min recording. The distributions confirmed the general trend. The average rest duration in time intervals during the 45-min recording, shown in Figure 2, was highly altered. Zebrafish exposed to 40.3 ± 2.9 μg L$^{-1}$ and 1533.3 ± 266.7 μg L$^{-1}$ showed a higher number of stops during the recording period compared to the controls. A highly statistically significant elevated average rest duration in time intervals was found when Zebrafish exposed to 40.3 ± 2.9 μg L$^{-1}$ ($\chi^2 = 49.52$, df = 19, $p < 0.001$) and 1533.3 ± 266.7 μg L$^{-1}$ ($\chi^2 = 38.05$, df = 19, $p = 0.006$) compared to the control. This means that male Zebrafish exposed to 40.3 ± 2.9 μg L$^{-1}$ and 1533.3 ± 266.7 μg L$^{-1}$ have elevated rest periods over time than unexposed male Zebrafish. However, the average...
rest duration in rest intervals was statistically similar. Noteworthy, distributions of Turn Rates in intervals were statistically significantly altered at treatment 133.3 ± 6.7 μg L−1 compared to the control. Why so remains unknown. Turn Bias (representing the imbalance between right and left turns) was unaffected by the treatments.

The temporal average velocities during the measurement period were statistically significantly lowered velocities at treatment 40.3 ± 2.9 μg L−1 ($\chi^2 = 78.58$, df = 19, $p < 0.001$) and 1533.3 ± 266.7 μg L−1 ($\chi^2 = 53.18$, df = 19, $p < 0.001$) compared to the control group (Figure 3). Likewise, the duration of employed velocities in intervals were statistically significantly altered at treatment 40.3 ± 2.9 μg L−1 ($\chi^2 = 90.61$, df = 19, $p < 0.001$) and 1533.3 ± 266.7 μg L−1 ($\chi^2 = 53.15$, df = 19, $p < 0.001$) compared to the control group. This means that Zebrafish exposed to 1,4,5-oxadithiepane in the two above-mentioned concentrations employ different velocities during the 45-min recording.

The presented alterations in swimming behaviour should be seen in the context of the lacking dose–effect relationship resulting in a questionable causation of the treatment (Hill, 1965). To our knowledge, few studies report non-dose–effect relationships. However, one study notes, following oral exposure to the non-polar narcotic toluene, a non-dose-dependent increases in several endpoints of this study and the above-mentioned studies.

Table 1. Measured behavioral parameters in male zebrafish for the control group and each treatment.

| Actual 1,4,5-oxadithiepane concentration (N = 3 per concentration) | No. of adult males | 40.3 ± 2.9 μg L−1 | 133.3 ± 6.7 μg L−1 | 1533.3 ± 266.7 μg L−1 |
|---|---|---|---|---|
| 26 | 26 | 27 | 27 |
| Total path (m) | 97.9 ± 7.8 | 73.9 ± 4.8 | 91.8 ± 6.4 | 78.2 ± 5.8 |
| Mean max velocity (mm s−1) | 154.7 ± 9.2 | 134.9 ± 8.1* | 176.8 ± 12.2* | 168.1 ± 13.8 |
| Average velocity (mm s−1) | 40.0 ± 2.5 | 31.7 ± 1.5 | 37.3 ± 2.0 | 34.5 ± 2.1 |
| Turn rate (degrees s−1) | 502.7 ± 35.2 | 566.2 ± 28.4 | 482.1 ± 33.2 | 537.1 ± 24.9 |
| Turn bias (degrees s−1) | -0.7 ± 0.2 | 1.2 ± 0.4 | 1.5 ± 0.4 | 2.2 ± 0.8 |
| No. of stops | 2765 ± 457 | 3784 ± 389 | 2675 ± 395 | 3318 ± 388 |
| Total rest (s) | 3297.9 ± 65.2 | 4227.5 ± 50.2 | 297.9 ± 50.4 | 422.57 ± 68.3 |
| Total active time (s) | 2370.6 ± 65.2 | 2277.3 ± 50.2 | 2402.4 ± 50.4 | 2257.70 ± 68.3 |

Notes: Findings marked with bold are statistical significant from the control group and * indicates a significant difference between two groups. a negative value in turn bias indicates a turn preference to the left. The number of males shown are number of males undergoing statistical analysis. Others were omitted mainly due to damaged files.

Due to damaged files. Noteworthy, the present study finds behavioural alterations after exposure in the μg L−1 range of 1,4,5-oxadithiepane, whereas the above-mentioned studies all report their findings after exposure in the mg L−1 range. This might be due to a higher toxicity of 1,4,5-oxadithiepane, a higher accuracy of the automated tracking system (Baatrup, 2009) or the differences in exposure time from acute to sub-acute. McKim and colleagues (1987) described the baseline toxicity (narcotics) of fish acute toxicity syndromes (FATS) by altered body colouration, reduced startle response, hypoactive spontaneous locomotor activity and a rapid and shallow respiration (McKim et al., 1987), which are in agreement with the results of the present study as decreased swimming activity was found.

4. Conclusion

In summary, this study provides novel ecotoxicity data on 1,4,5-oxadithiepane. We found no toxicity on fitness and lethality after 14 days at 1533.3 μg L−1 flow-through exposure. Behavioural alterations when adult male Zebrafish...
was exposed to 1533.3 μg L⁻¹ of 1,4,5-oxadithiepane were observed. However, the causation of these requires further studies. The results obtained in this study assist in policy-making and risk assessment of CWA found in the Baltic Sea but can also be used at other known CWA-dumping sites. The alterations found may have implications for the health of the marine environment.

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Disclosure statement

The authors have no conflict of interest.

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