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# Establishment of Chronic Toxicity Testing with *Cloeon Dipterum* including Transcriptomics-based Molecular Profiling

Kirsten Germing[1], Maria Vogt[1], Lena Kosak[1] and Sebastian Eilebrecht[1]

[1]Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg, Germany
E-mail contact: kirsten.germing@ime.fraunhofer.de

## 1. Introduction

Aquatic larvae of the so-called EPT-taxa are frequently exposed to multiple contaminants and are shown to be highly sensitive. For the risk assessment, often standardized test systems with *Daphnia* sp. are used but can cause an underestimation of the risk since *Daphnia* are less sensitive than EPT-taxa in many cases. In Europe we are still lacking standardized test systems with more sensitive species, particular for chronic tests. We adapted a test system for chronic toxicity tests with the lentic mayfly species *Cloeon dipterum* developed by Gaiac (2021) [1] and included transcriptomics-based molecular profiling. However, the application of transcriptomics with field-collected test organisms can be challenging due to high genetic variation and the influence of various environmental factors in field populations. Nevertheless, within this study we successfully developed an optimized procedure for short-term exposure of relevant developmental stages of *C. dipterum* larvae.

## 2. Materials and Methods

Two chronic toxicity tests with field-collected larvae of *C. dipterum* in the early development stage L3 (based on Cianciara (1976) [2]) were performed (Figure 1A). The insecticide Fipronil was used as test item in environmentally relevant concentrations (nominal: 0.037 – 0.60 µg/L). The tests were performed in glass beakers filled with 500 mL test solution under conditions of standing water (Figure 1B). During the tests, the larvae were fed with diatoms of *Navicula pelliculosa* grown on small tiles and pieces of carrots. The exposure was semi-static and the test solutions and food were replaced twice a week. At each media renewal the development of the larvae was determined. The development stages based on Cianciara (1976) [2] were renamed with numbers to create metric data for further statistical evaluation (L3 = 0; L4 = 1; L5 = 2; …). As endpoints the larval development throughout the test, emergence, mortality of the larvae and the wing size of imagines were examined. In each test the concentration of Fipronil was determined by LC-MS/MS.

A long-term exposure test over 38 days was conducted until all individuals had emerged or died. Emerged female individuals were sampled for further transcriptomic analysis. The statistical evaluation was based on geometric mean concentrations. Based on the results of the long-term exposure test the test design of a test with short-term exposure over 7 days was adapted. In this test the transcriptomic analysis was performed with the larvae. For this, at test end three larvae per replicate were immediately frozen before RNA was extracted for transcriptomic analysis. Statistical evaluation presented in this abstract was based on nominal concentrations. Extracted RNA samples were subjected to library preparation before RNA sequencing on an Illumina NovaSeq system. Sequence reads were mapped to the *Cloeon dipterum* reference genome assembly CLODIP2 using STAR [3]. Differential gene expression analysis was performed using DESeq2 [4].

## 3. Results and Discussion

### 3.1. Long term exposure over 38 days

In the 38 day test the mortality in the control was 10 % at test end. Based on validity criteria of established test protocols, e. g. 30 % control mortality in OECD 233 [5], the test is considered to be valid. In the treatments a mortality between 25 % (Concentration 0.041 µg/L) and 75 % (Concentration 0.563 µg/L) was examined. Based on geometric mean concentrations an LC50 of 0.181 µg/L was determined for the endpoint mortality. For the sublethal endpoint larval development statistical evalutations were performed for several time points. After 7 days the mean larval stage in the control was 3.45 (Figure 1C). In the treatments, the mean larval stage ranged from 3.21 (Concentration 0.037 µg/L) to 2.00 (Concentration 0.60 µg/L). An EC50 of 0.70 mg/L was determined.

For this test transcriptomics revealed no significantly differentially expressed genes when comparing treatments and control. This may have been due to two main reasons: upon ermergence, the emerged imagines had no contact with the test item until test end and the imago is a final stage for mayflies, in which

several physiological processes are altered or stopped. Thus, we concluded that potentially the larval stage may be more appropriate and sensitive for transcriptome analysis. Based on these results, we decided to repeat the test with an adapted test design.

### 3.2. Short-term exposure over 7 days

The short-term exposure test was conducted for 7 days. At test end, the mortality in the control was 0 %. Thus the test is considered to be valid. In the treatments a mortality between 10 % (Concentration 0.075 and 0.30 µg/L) and 20 % (Concentration 0.037 and 0.15 µg/L) was examined. No $LC_x$ could be determined. After 7 days the mean larval stage in the control was 3.33. In the treatments, the mean larval stage ranged from 3.23 (Concentration 0.037 µg/L) to 2.44 (Concentration 0.15 µg/L). For the endpoint larval development an EC10 of 0.029 mg/L (nominal) was determined.

Indeed, with this test setup, significantly differentially expressed genes (DEGs) were detected in the larvae using transcriptomics (Figure 1D). We observed a concentration-dependent increase in the numbers of DEGs as well as strong positive correllation for their expression changes when comparing the different exposure concentrations.

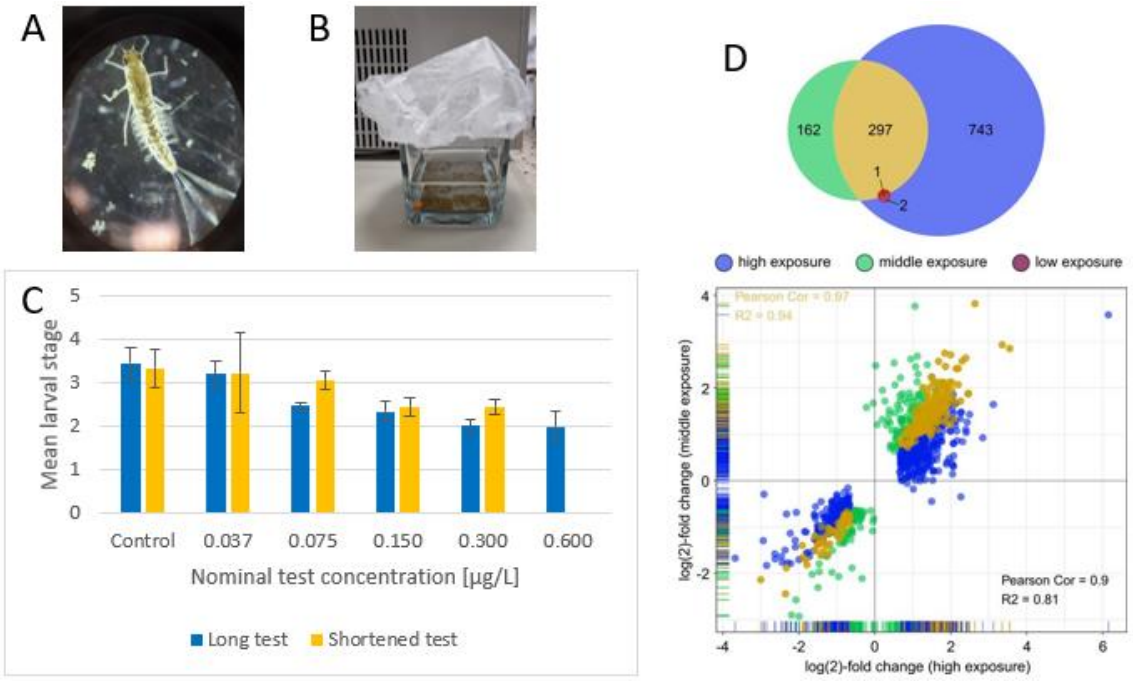


***Figure 1: A:* C. dipterum *larvae in stage N1. B: Test vessel with test solution and food tiles and carrots. C: Mean larval stage per treatment after 7 days in the long-term exposure and short-term exposure test. Larval development was based on Cianciara (1976) (L3 = 0, L4 = 1, L5 = 2, …) D: Differential gene expression in the short-term exposure test.***

## 4. Conclusions

At Fraunhofer IME a test system for chronic toxicity tests with the lentic mayfly species *C. dipterum* was successfully established. Larval development was pointed out as appropriate sensitive sublethal endpoint. Both tests with short-term and long-term exposure revealed a reproducible effect on the larval development. Furthermore, transcriptomics were successfully applied to wild-catches of this model organism observing concentration-dependent gene expression changes, which after successful functional annotation may enable the differentiation of molecular modes of action of toxicity in mayfly. Overall, within this study comparable effects in the larval development and transcriptomic data were observed indicating that these methods can be applied in future screening of *C. dipterum* as representative for EPT-taxa.

## 5. References


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