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

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Introduction

- EPT-taxa (Ephemeroptera, Plecoptera, Trichoptera) were shown to be highly sensitive against various environmental pollutants.
- Until now, EPT taxa are not represented in current regulatory frameworks and the literature data is sparse, especially for chronic testing.

Two-step approach

1. Adaptation of a test system with *Cloeon dipterum* developed by Gaiac (2021) [1]

- Covering the life span of young larvae until emergence
- Larval development and emergence as sublethal endpoints

2. Transcriptomics-based molecular profiling after 7 days exposure

- Covering the most sensitive life stages
- Testing for chronic toxicity effects after short exposure

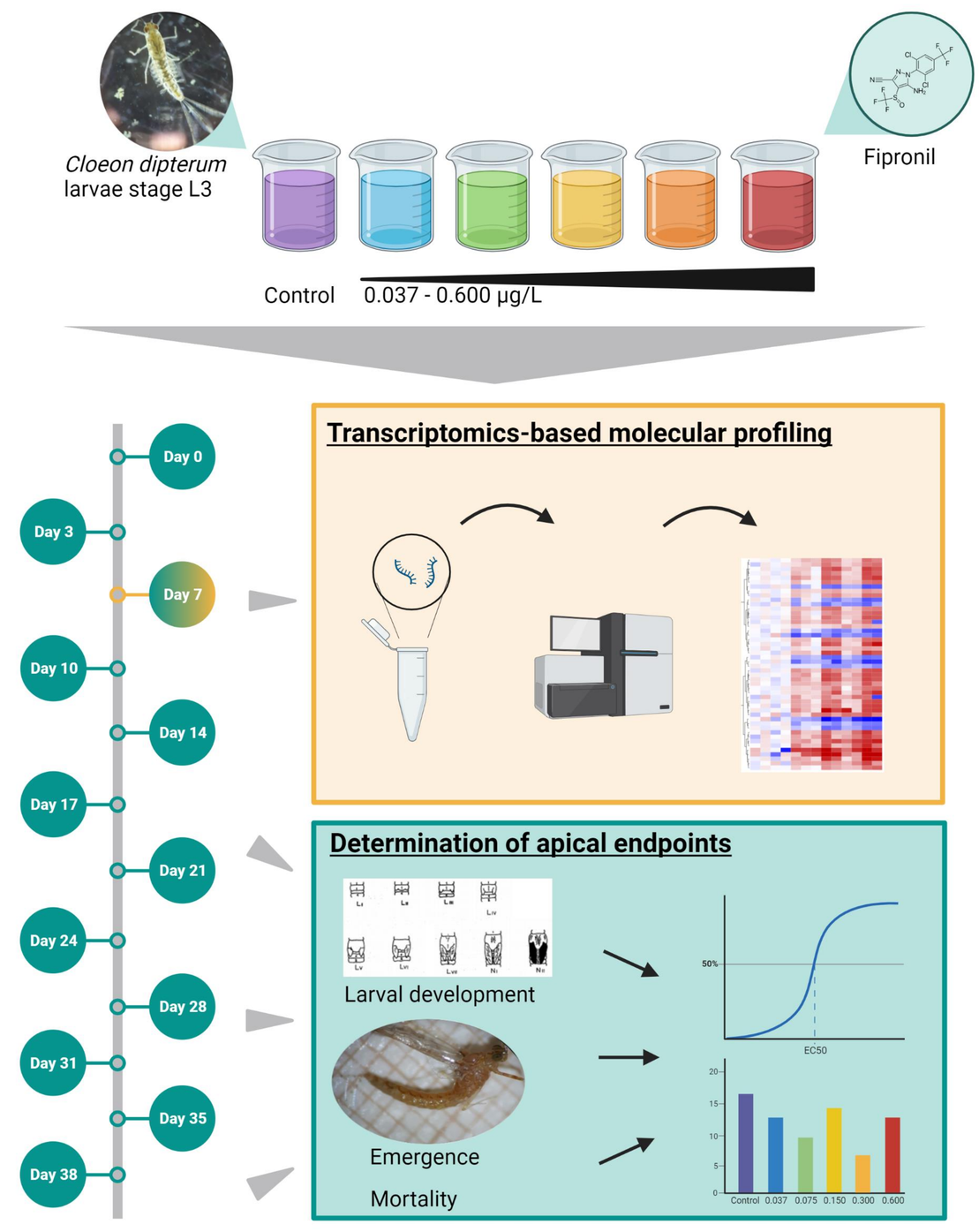

Figure 1: Methods: Chronic toxicity testing with *C. dipterum*: Field-collected young larvae in the larval stage L3 (based on Cianciara (1976) [2]) were introduced to a control and five test concentrations containing 0.037 – 0.600 µg/L Fipronil in Elendt M4 medium [3]. For each treatment, 4 replicates containing 5 larvae each were prepared. The test design was semi-static with two media renewals per week. Larvae were fed with diatoms of *Navicula pelliculosa* and carrots. The apical endpoints larval development, emergence and mortality were determined twice per week. RNA-extraction was performed after 7 days: 3 larvae per replicate were used for extraction. (Created with BioRender.com)

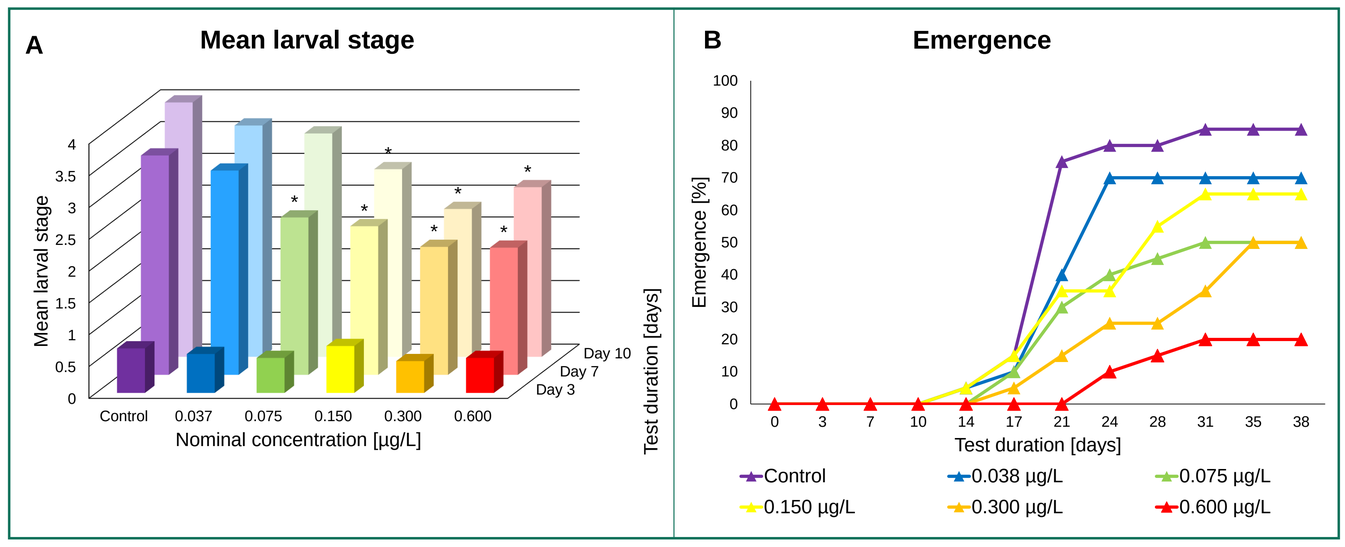

Figure 2: A: Mean larval stage (based on classification by Cianciara (1976): L3 = 0, L4 = 1, L5 = 2, …) per treatment after 3, 7 and 10 days in the chronic toxicity test with a total test duration of 38 days. A significant reduction in larval development compared to the control is marked (*). **B:** Emergence per treatment in the chronic toxicity test with *C. dipterum* with a total test duration of 38 days.

Table 1: Overview of EC_x (Effect concentrations) and NOEC (No-observed-effect-concentration) based on nominal Fipronil concentrations determined for several endpoints in the chronic toxicity test with *C. dipterum* with a total test duration of 38 days.

Endpoint	EC_{10}	EC_{50}	NOEC
	[µg/L] (95 % confidence limit)		[µg/L]
Larval development after 7 days	n. d.	0.768 (0.457 – 2.308)	0.038
Larval development after 10 days	0.030 (0.002 – 0.071)	1.174 (0.566 – 12.708)	0.075
Emergence after 38 days	n. d.	0.160 (0.067 – 0.426)	0.038
Mortality after 38 days	n. d.	0.185 (0.085 – 0.571)	0.038

n. d. = not determined

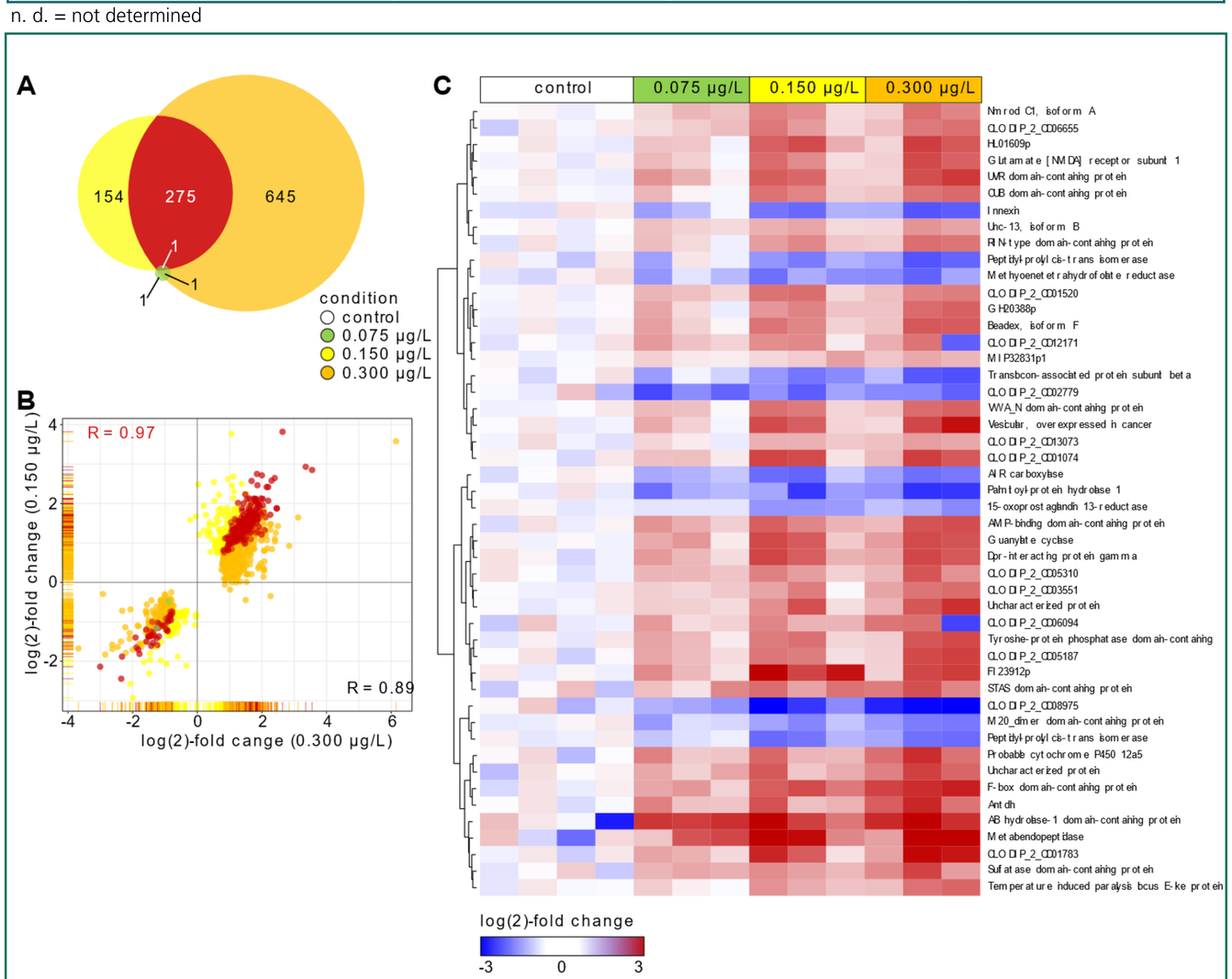

Figure 3: A: Venn diagram depicting the number of differentially regulated genes (DEGs) in *C. dipterum* larvae exposed to low (0.075 µg/L), mid (0.150 µg/L), and high (0.300 µg/L) concentrations of Fipronil. **B:** Scatter plots comparing DEGs log 2-fold change (lfc) values after mid and high exposure conditions. The common subset of both exposures is highlighted in red. **C:** Heatmap showing relative gene expression patterns of *C. dipterum* larvae post exposure to sublethal concentrations of Fipronil. Presented here is the top 48 core DEGs (rows). Relative expression signal for each gene is the variance-transformed normalized counts, centered around the control group's mean and scaled by the global standard deviation. The color red indicates an enhanced expression and the color blue denotes a suppressed expression relative to the global control's mean expression of a gene. The color above each column indicates the corresponding exposure condition.

Conclusion

- A test system for chronic toxicity testing with *C. dipterum* covering the life span from young larvae until emergence was successfully established and **larval development** was pointed out as appropriate sensitive endpoint.
- **High control emergence of 85 %** at test end and a reproducible effect of Fipronil on larval development of *C. dipterum* prove the functionality and suitability of the test system.
- Transcriptomics were successfully applied to field-collected *C. dipterum* larvae, thereby observing **concentration-dependent gene expression changes**.

➢ Concluding, we were able to show sensitive concentration-related effects in the sublethal endpoints larval development and emergence in a chronic toxicity test until emergence. Additionally, **comparable sensitive concentration-related effects** were observed on a molecular level already after a short test duration. Together with the full chronic toxicity test, this shortened assay allows **early identification of toxicity** of chemicals in *C. dipterum*, thereby providing a possibility for assessing chronic toxicity in this challenging non-standard organism as **representative for EPT-taxa**.

1 Gaiac – Research institute for Ecosystem Analysis and Assessment (05.05.2021). Chronic toxicity test with *Cloeon dipterum* – test protocol for the ringtest.
2 Cianciara, S. (1976). Some study on the biology and bioenergetics of *Cloeon dipterum* (L.), Ephemeroptera (pp. 175–195). Institute of Ecology PAS.
3 OECD. (2004). Test guideline No. 202: *Daphnia* sp., Acute Immobilisation Test. *OECD Guidelines for the Testing of Chemicals.*

Publication in preparation:
Germing, K., Ayobahan, S. U., Kosak, L. A., Vogt, M., Ringbeck, B., Eilebrecht, E., Eilebrecht, S., Schäfers, C. (2023) Establishment of Chronic Toxicity Testing with *Cloeon dipterum* including Transcriptomics-based Molecular Profiling.