Development of a testing method simulating running waters for chronic testing of invertebrate species

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Background and Aim

Background

- Principal toxicity testing:
  - Model organisms which are easy to handle under laboratory conditions
  - Usually standard test organisms originate from standing water bodies (e.g. *Daphnia magna*)[1]
- Higher number of lotic species, especially potential sensitive species, which originate from running waters[2]

Aim

- Broaden the range of available test systems for chronic toxicity testing of lotic species which can be chosen for a specific issue
- Development of a test system for chronic testing of lotic invertebrate species under stream conditions

[1] Weaver et al., 2015; [2] Struewing et al., 2015

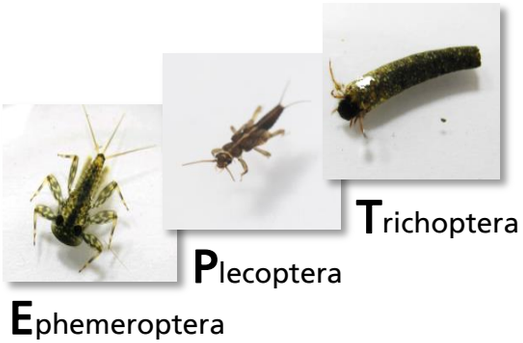
Challenge

- Design a test system which fulfils different criteria
- Easy to handle under laboratory conditions
- Mimics the natural habitat conditions of lotic organisms regarding
  - Flow: 0.1 – 0.3 m/s
    (typical flow velocity in a small streams[3]: 0.03 – 2.0 m/s)
  - Temperature: 10 – 12.5 °C
  - High oxygen level: 100%
  - Light regime: 16:8 h light / dark
  - Food supply: Algae, Detritus

[3] Laborbücher Biologie, Praktische Limnologie, Diesterweg-Salle-Sauerländer

Test organisms

- Largest group within invertebrate species are insects
- Three groups of sensitive lotic organisms: mayfly, stonefly and caddisfly (EPT-Taxa)[1]
- Field collected larvae, adapted to laboratory conditions
- Diversity and availability of e.g. mayfly and stonefly larvae during the course of the year



	Mayfly larvae				Stonefly larvae	
	Baetis sp.	*Epeorus sylvicola*	*Ecdyonurus venosus*	*Ephemerella ignita*	*Protonemura sp.*	*Isoperla sp.*
Spring	Y	Y	Y	XY	XY	X
Summer	XY	Y	Y	Y	Y	Y
Autumn	X	X	X	Y	XY	Y
Winter	XY	X	XY	X	X	n.a.

X = early stages Y = older stages;
n.a.: no information available

[1] Weaver et al., 2015;

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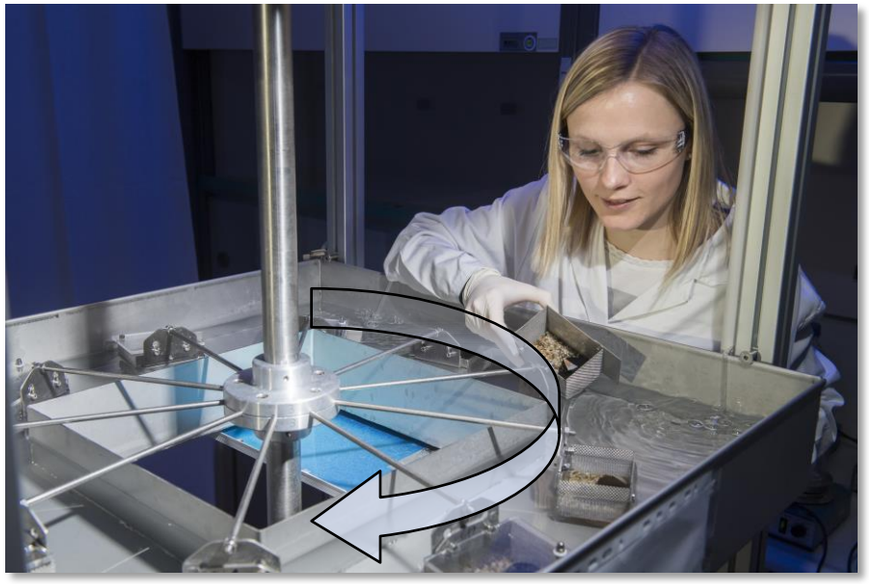
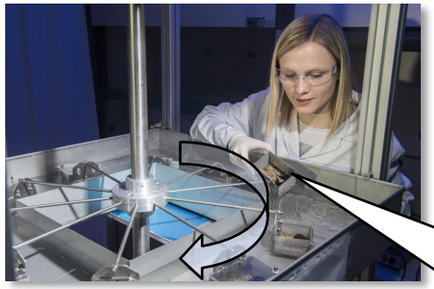
Test System



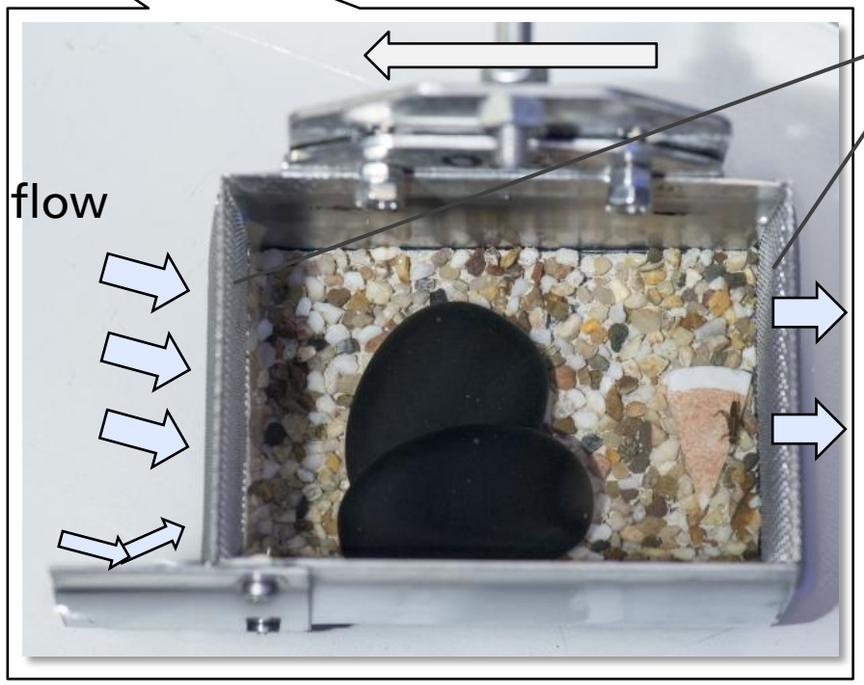
- Small cages circulate inside test containers to obtain the target flow: 0.1 m/s
- Aeration of test medium with air pumps provide high oxygen level: 100%
- Cooling system regulates temperature: 10.0 – 12.5 °C
- Light sources regulate the light regime: 16:8 h light/dark

Test System

- Test vessels:



- Small cages circulate inside test containers to obtain the target flow



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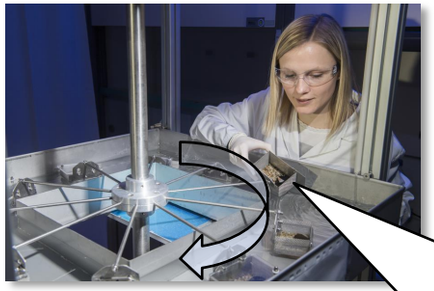
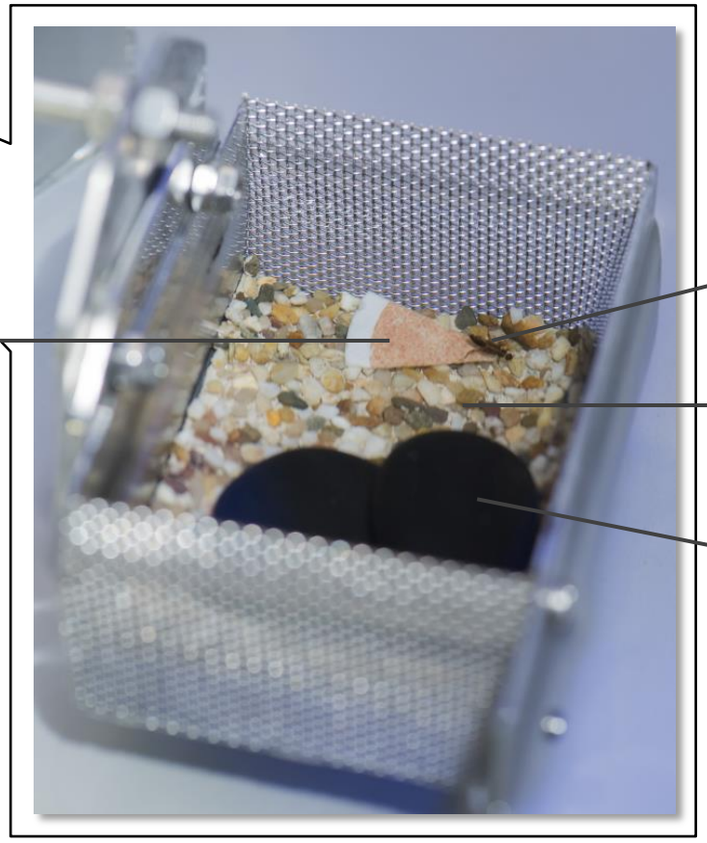
Test System

- Test vessels:



- Simulation of natural habitat of test organisms
- Individual testing of organisms (one organism per test vessel)



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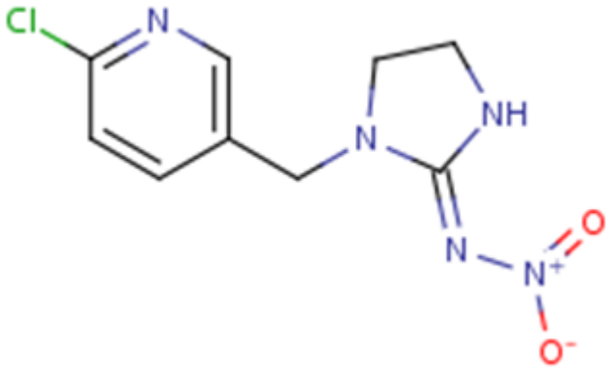
Test Design

- Treatments: Control + 5 Concentrations
- Replicates: 10 replicates per treatment,
  One individual per replicate
- Acclimation
  to test system: 48 hours
- Medium: Cu-free dilution water
- Medium renewal: Once per week (semi-static)
- Test duration: 21 days
- Endpoints: Growth (total length)
  Emergence
  Mortality
  → determined for each
  individual organism

Test item

- Chemical Identity
- Test item: Imidacloprid
- Class: Neonicotinoid
- Use: Insecticide



$C_9H_{10}ClN_5O_2$

- Mode of action[4]:
  - uptake by insects via contact and ingestion
  - binds to nicotinic acetylcholine receptor
  - Disrupt nerve impulses

- Exposure mainly via spray drift, leaching or runoff

[4] Roessnik et al., 2013

Pilot Studies

- **Test organisms:** **Stonefly (*Protonemura sp.*)**
- **Test substance:** **Imidacloprid**

- Study I:
  - Test concentrations: 1.0 3.0 9.0 27.0 and 81.0 µg/L
  - Test performance: November – December 2016
  - Endpoints: Growth (total length), Emergence, Mortality

- Study II:
  - Test concentrations: 3.0 9.0 27.0 81.0 and 243.0 µg/L
  - Test performance: February – March 2017
  - Endpoints: Growth (total length), Emergence, Mortality

Pilot Studies

- Results Study I (November/December 2016):
  - No control mortality (100% survival)
  - Statistically significant, dose-dependent effect on growth
  - No statistically significant effect on mortality
  - No evaluation of emergence, since larvae did not emerge until test end
  - Chemical analysis: test item remains stable over the whole test course: 93 - 111% of nominal after 7 days

Endpoint	Length	Mortality
EC_{10} / LC_{10} [µg/L]	11.2	23.1
EC_{20} / LC_{20} [µg/L]	19.5	61.1
EC_{50} / LC_{50} [µg/L]	56.4	n.d.
NOEC [µg/L]	9.0	n.d.

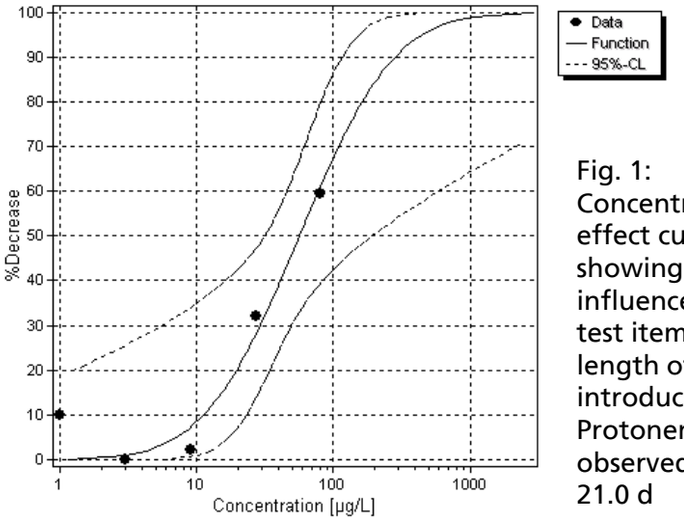


Fig. 1: Concentration-effect curve showing the influence of the test item on length of the introduced Protonemura as observed after 21.0 d

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Pilot Studies

- Results Study II (February/March 2017):
  - No control mortality (100% survival)
  - Statistically significant, dose-dependent effect on emergence and survival
  - No evaluation of growth due to 100% emergence in the control
  - Chemical analysis: test item remains stable over the whole test course: 93 – 99 % of nominal concentrations

Endpoint	Emergence	Mortality
EC_{10} / LC_{10} [µg/L]	6.0	6.1
EC_{20} / LC_{20} [µg/L]	11.2	12.2
EC_{50} / LC_{50} [µg/L]	37.1	47.5
NOEC	3.0	9.0

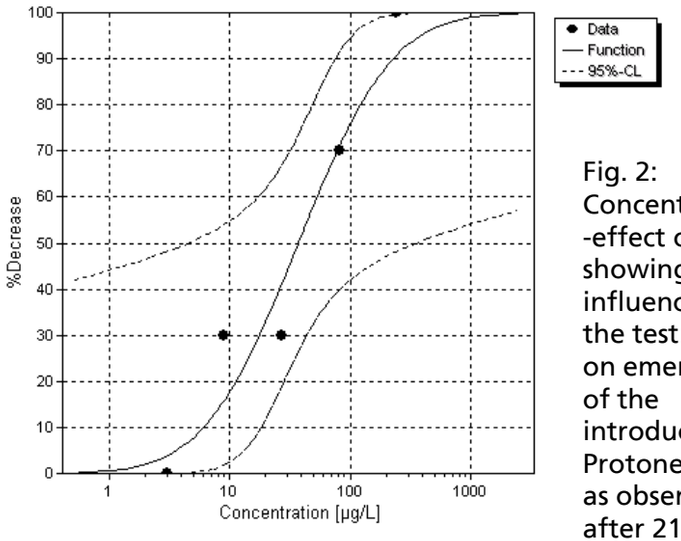

Fig. 2: Concentration -effect curve showing the influence of the test item on emergence of the introduced Protonemura as observed after 21.0 d

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Pilot Studies

- Comparison of Study I and Study II:

	Study I	Study II
Control Mortality	0%	0%
Endpoints	Growth, Mortality	Emergence, Mortality
EC_{10} [µg/L] (95%-CL)	11.2 (0.14 – 23.1)	6.0 (1.07 – 12.1)
EC_{50} [µg/L] (95%-CL)	56.4 (31.33 – 193.2)	37.1 (22.1 – 62.8)
NOEC [µg/L]	9.0	3.0

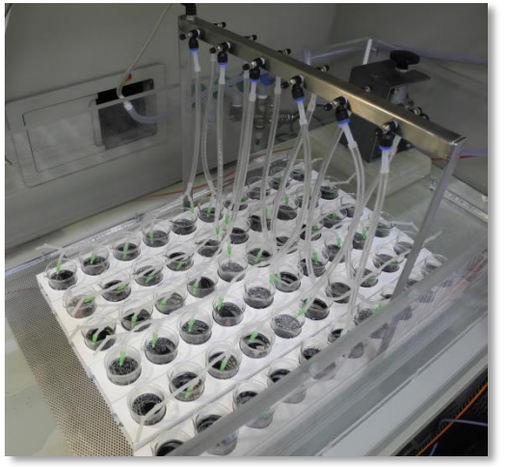
used for assessment of chronic toxicity

- Test system works well with both growth and emergence as endpoint
- Endpoints are season dependent (e.g. growth in winter; emergence in spring)
- Results of both studies are in the same order of magnitude regarding EC and NOEC independent of the observed endpoint

Comparative Study

- **Test system:** **50 mL aerated glass vessels**
- **Test organisms:** **Stonefly (*Protonemura sp.*)**
- **Test substance:** **Imidacloprid**

- Test concentrations: 3.0 9.0 27.0 81.0 and 243.0 µg/L
- Replicates: 10 replicates per treatment,
  One organism per replicate
- Test duration: 21 days
- Endpoints: Growth, Emergence, Mortality



- <u>Results:</u>
  - Since the larvae were more stressed, they were much more sensitive (higher mortality in controls and treatments)

Conclusion - Evaluation of the test system

- Test system works well for testing of stoneflies
- Test requirements (flow, oxygen level, temperature, light regime, food supply) are fulfilled
- Dependent on the season different endpoints have to be considered:
  - In autumn and winter we found organisms in early development stages
    → chosen endpoint growth
  - In spring we found organisms in a further development stage
    → chosen endpoint emergence

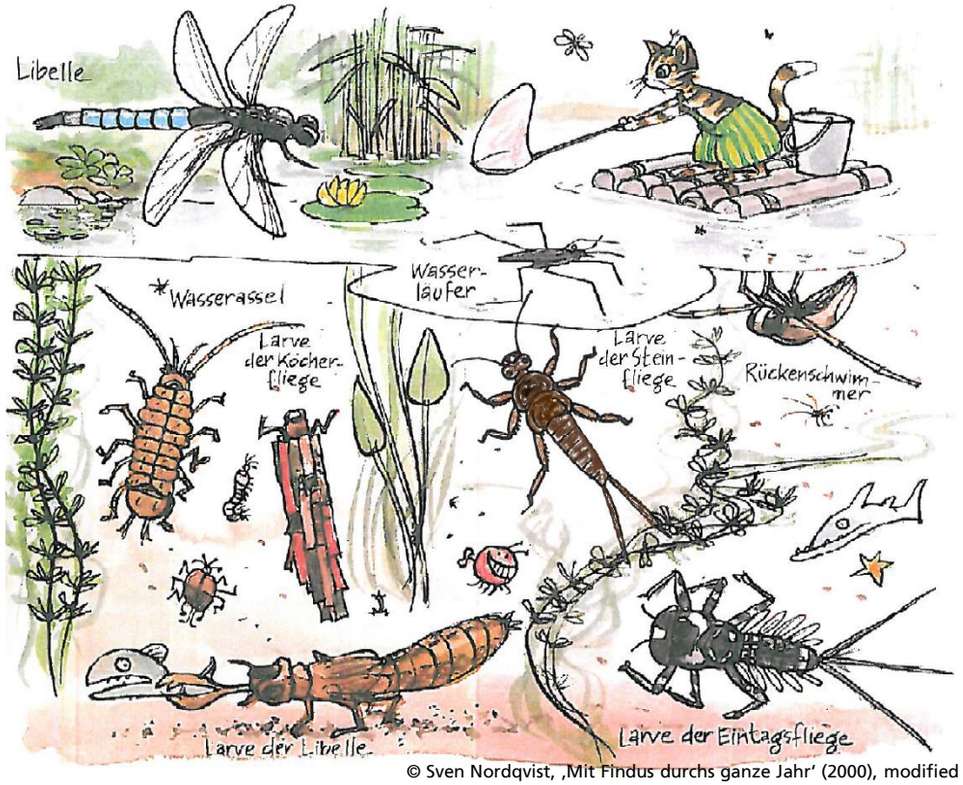
→ Both endpoints provide comparable results

Conclusion - Evaluation of the test system

- Advantages over usual stream systems
  - Individual testing of single organisms possible
  - One system can be used for different species (stonefly, mayfly, caddisfly -> EPT Taxa)
  - Flow, temperature and light can be easily regulated
  - Besides a semi-static exposure a test under flow-through exposure conditions or with peak exposure would also be possible
  - Low potential sorption of test item
    - → test system is built out off inert materials
    - → No water pumps are needed to reach the aimed flow

- New testing method can provide toxicity data of chronic testing with different aquatic insect larvae which can be used for a SSD (Species Sensitivity Distribution) approach

Thank you for your attention!



© Sven Nordqvist, ‚Mit Findus durchs ganze Jahr' (2000), modified

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Session 'Cost effective and ecological relevant testing using invertebrate species: new insights for environmental risk assessment'
Platform presentation
Meeting Studio 311 & 312, Thursday May 11, 2017, 9:35 a.m., ID: 468.

Development of a testing method simulating running waters for chronic testing of invertebrate species

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1. Introduction

Invertebrate species compass a various number of organisms which are already used in ecotoxicological tests including amphipods, choronomids, cladocerans or gastropods. However, the largest group within the invertebrate species are insects. Common laboratory toxicity testing is conducted with a relatively small number of model organisms which were often chosen because of their simplicity in culturing and testing. There are still several stream-dwelling invertebrate species including mayflies and stoneflies which can be more sensitive than standard test organisms. Because of their habitat adaptions testing of those organisms is more difficult than testing of organisms living in standing water bodies. Mayflies and stoneflies are hemimetabolic insects with a complex life cycle divided in a larval aquatic phase and a short adult phase as a flying insect. They occur in cold, fast moving streams which provide a high oxygen level.

The main goal was to develop a testing method for chronic testing of mayfly and stonefly larvae under stream conditions. The biggest challenge was to design a test system which is easy to handle under laboratory conditions but at the same time mimics the natural habitat conditions of the larvae regarding flow, temperature, oxygen level, light intensity and food supply. The developed test system provides a novel approach for the chronic testing of invertebrates particularly with regard to provide more organisms for a species sensitivity distribution.

2. Materials and methods

A pilot study with the mayfly *Ecdyonurus venosus* and the stonefly *Protonemura ssp.* exposed to the neonicotinoid imidacloprid for 28 days was performed in November 2016. The test system and the test animals are presented in Figure 1.

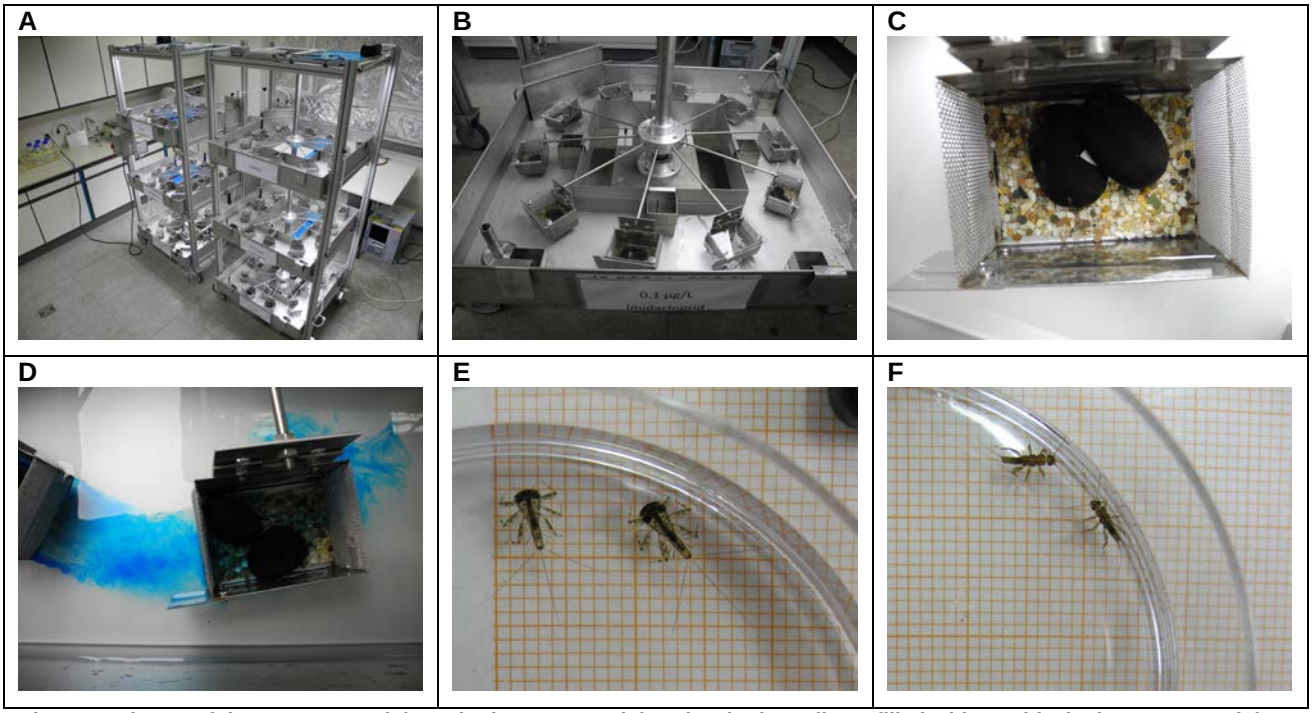

Figure 1: Photos of the test system (A), a single treatment (B) and a single replicate filled with two black glass nuggets (C), another photo of the test system showing the flow going through the test vessel visualized with blue colour in the water phase (D) and additionally photos of the test organisms used in the test, the mayflies Ecdyonurus venosus (E) and the stoneflies Protonemura ssp. (F).

Test design: For our study we used field collected larvae which were adapted to laboratory conditions before test start. In contrast to usual indoor stream systems not the water body itself but vessels insight the test containers were moved and circulated through each treatment to obtain the aimed flow. The test design consists of six treatments with ten replicates. As food source small filters were prepared with algae food which could be grazed by the larvae. Black glass nuggets were added to simulate the natural habitat. Cu-free dilution water was used as medium. Medium renewal was conducted once per week. Nitrate, nitrite and ammonium concentrations and the pH-value and oxygen level in the water phase were measured once per week. As endpoints total length, head width and length of wing pads were measured at test start and end. Mortality was recorded once per week.

Substance: Imidacloprid is a systemic insecticide which belongs to the class of chloronicotinoid insecticide and has been used for insect control since the early 1990s. Insects were affected via contact or ingestion. The substance effects the central nervous system of insects by binding to the nicotinic acetylcholine receptor (nAcChR), thus disrupting nerve impulses which are transmitted by acetylcholine [1].

The pilot study was conducted with five different concentrations of 0.03, 0.1, 0.3, 1.0 and 3.0 µg/L. A control with Cu-free dilution water only was run in parallel.

3. Results and discussion

3.1. Chemical analysis

During the study samples of fresh and aged medium were taken for chemical analysis and were analysed by LC/MS immediately afterwards. According to the results of the measurements test item concentrations remained stable until day 14. Results of the chemical analysis as available so far are shown in Table 1. Further results will be presented in the presentation.

Table 1: Chemical analysis of imidacloprid samples taken at day 0 (D0), day 7 (D7) and day 14 (D14) of fresh and aged test medium. Measured values are given in µg per litre and in % of nominal.

	Nominal Concentration [µg/L]									
	0.03		0.1		0.3		1.0		3.0	
	µg/L	%	µg/L	%	µg/L	%	µg/L	%	µg/L	%
Fresh samples D0	0.025	83.3	0.106	106	0.291	97.0	0.987	98.7	3.01	100.2
Aged samples D7	0.03	100	0.128	128	0.375	125	1.15	115	3.30	110
Fresh samples D7	0.033	110	0.105	105	0.308	103	0.947	94.7	2.89	96.4
Aged samples D14	0.032	107	0.106	106	0.302	101	0.964	96.4	2.96	98.7

3.2. Results of the chronic test

Over the period of 21 days, we found that for the stoneflies no control mortality was observed, indicating, that the test system works well. The different imidacloprid treatments indicate a slight dose-dependent effect on the mortality.

Mayflies had a control mortality of 50% after 21 days, suggesting that some modifications of the test system are necessary before performing the next test to improve the test system for that test species.

Since the test is still running until day 28, the results on the observed endpoints total length, head width and length of wing pads are not available yet.

4. Conclusions

To conclude on the novel test system and its applicability as well as the advantages and disadvantages is not completely possible based on the actual data. Mortality of the controls indicates that the novel test system works fine for stoneflies, but need to be adapted for mayflies. A full conclusion will be included in the presentation.

5. References

[1] Roessink, I., Merga, L. B., Zweers, H. J., Van den Brink, P. J., (2013). "The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs." Environ Toxicol Chem **32**(5): 1096-1100.