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Developing a chronic test for field-collected mayflies: The influence of laboratory conditions on development and survival of *Cloeon dipterum*

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Abstract

In recent years, the interest has increased for testing nonstandard species in the environmental risk assessment of pesticides. Especially mayflies seem to be of interest, because of their relevance in aquatic ecosystems, their potential sensitivity, and their vulnerability. So far there is a lack of standardized methodologies in acute and chronic testing of mayfly species. While acute testing of field-collected nonstandard species represents few challenges, especially chronic testing needs better and more detailed evaluation regarding the robustness and suitability of testing methods. Therefore, we conducted an inter-laboratory comparison of chronic experimental designs for the mayfly *Cloeon dipterum*. These methods were established in the two participating laboratories and were tested using a cross-design experiment. The test designs differed mainly in the feeding and light conditions. In addition, the influence of species origin was investigated and the Cloeon Development Index (CDI) was established to allow for a standardized evaluation of mayfly development. Our study showed that experimental design, especially the method of feeding, had a huge impact on larval development, while the origin of the organisms played a minor role. However, it was demonstrated that the mortality rate was higher for larvae that were transported between the participating laboratories than for larvae collected near each testing site. The presented work is not meant to be input for an Organisation for Economic Co-operation and Development test guideline but tries to provide necessary basic information for the performance of robust chronic studies with the mayfly *C. dipterum*. Based on these results, recommendations can be provided for an experimental design of a chronic test and on validity criteria regarding control survival and development rates for chronic testing of *C. dipterum*.

Keywords mayfly, chronic laboratory testing, periphyton, Cloeon Development Index

Introduction

The aquatic risk assessment for pesticides is based on a set of acute and chronic studies with defined test organisms. While the focus of acute tests is on mortality or immobility, chronic toxicity tests additionally aim at the quantification of sublethal endpoints related to, e.g., growth, development, and reproduction. The standard data requirements are laid out by the European Commission in regulation 283/2013 and regulation 284/2013 for active substances and product registrations, respectively (European Union [EU] 283, 2013; European Union [EU] 284, 2013). The mandatory set of tests cover primary producers, invertebrate species such as *Daphnia magna* and *Chironomus riparius*, and vertebrates (fish; European Food Safety Authority [EFSA], 2015). The selection of those invertebrate species for standardized water and sediment testing was originally based on practical reasoning as well as ecological relevance (Sibley et al., 2020).

In recent years, there is a renewed interest in testing nonstandard species in ecotoxicology and the risk assessment of pesticides. Species belonging to the orders of Ephemeroptera (mayflies) are generally considered sensitive toward aquatic contaminations by nutrients and organic chemicals (Mebane et al., 2020; Sibley et al., 2020). They have been of particular interest due to their high relevance for both aquatic and terrestrial ecosystems and their chronic sensitivity to certain pesticides, especially neonicotinoid insecticides (Merga & Van den Brink, 2021; Roessink et al., 2013; Sumon et al., 2018). There are, however,

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challenges in culturing the appropriate amount and quality of mayfly larvae and the testing of these larvae for longer periods in the laboratory. Physical and biological requirements such as, for instance, temperature and food preferences are often unknown or at least poorly understood, and life cycles cannot easily be completed in the laboratory or are too long for laboratory testing. Thus, regulatory test acceptability criteria cannot be met (Sibley et al., 2020). Good progress on the development of acute and chronic toxicity tests has been made in North America, based on two mayfly taxa, that is, *Neocloeon triangulifer* and *Hexagenia* spp. (Buchwalter et al., 2019; Raby et al., 2018; Soucek & Dickinson, 2015; Struewing et al., 2015; Sweeney et al., 2018; Weaver et al., 2015). The focus in Europe is, however, on *Cloeon dipterum* (e.g., Almudi et al., 2019; Beketov & Liess, 2005; Roessink et al., 2013; Van den Brink et al., 2016). Previously, Almudi and coworkers (2019) succeeded in reproducing *Cloeon* mayflies in a laboratory setting for embryogenetic and transcriptomic investigations for this species. Here the larvae are grown under standardized conditions until subimago and imago. As the swarming flight needed for mating could not take place in the laboratory, it was necessary to perform single forced copulas to close the life cycle and gain new eggs from which the new generation can hatch. Although successful, only a limited number of viable offspring could be generated, and this technique does not allow the rearing of enough larvae to populate an ecotoxicological test. In the present work, we focused on the comparison of two already existing and applied test protocols from the two participating laboratories. Therefore, larvae from semi-field facilities of the two participating laboratories were used as before and the establishment of an own breeding was discarded.

Cloeon dipterum is distributed throughout the Holarctic realm and is one of the most common mayflies in ponds in Europe. In temperate climates, the species usually exhibits two generations per year, referred to as bivoltinism (Cianciara, 1980; Van den Brink et al., 2016). *Cloeon dipterum* is also the only ovoviviparous mayfly species in Europe. The aquatic larvae of *C. dipterum* are well known grazers of (benthic) algae but also feed on fine (and to some extent also coarse) particulate organic matter (Banegas et al., 2020; Brown, 1960; Cianciara, 1980). However, some studies revealed that algae are a more beneficial food than detritus or leaves (Gupta et al., 1993; Sweeney & Vannote, 1984) and that mayflies develop well when reared with periphyton or diatoms (Sweeney et al., 2018; Webb & Merritt, 1987). The larval development times, first instar to subimago, may vary between 2 and >30 weeks depending on environmental conditions such as ambient temperature or food type (Cianciara, 1980; Šupina et al., 2016; Sweeney et al., 2018).

The aim of the present study was to consolidate test protocols from two different laboratories and to provide first recommendations for a future standardized chronic test design with *C. dipterum.* The two participating laboratories had experience in mayfly testing prior to the current study and had developed distinct test protocols for this species. Both methods were already applied successfully given the good control survival of >80%. However, larval growth and development rates differed considerably between the test setups. Here, we explored to which extent the two different protocols are suitable for investigating potential chronic effects on the mayfly species *C. dipterum*. The factors tested for the optimization of the test protocols were the ambient temperature, the origin of the mayfly larvae used for testing, and the food provided during the experiment.

Material and methods

Two established test designs were available. Laboratory 1 (Lab 1) in general uses laboratory-grown biofilm on unglazed tiles as a food source for the larvae, diffuse light ($<1\ \mu E\ m^{-2}\ s^{-1}$), and an external aeration. This biofilm on tiles was grown from an inoculum collected in field independently of the actual test to a dense, stable biofilm over 2–4 weeks, which was placed in the test vessels. The tiles thus served as a substrate for the food and also as a habitat structure. Details of gathering the field-collected inoculum for the biofilm are given in the section "Food." To ensure sufficient food, the tiles were renewed weekly. The setup of the second laboratory (Lab 2) comprises jars on whose glass surface a biofilm had developed over one week (Roessink et al., 2013). Here, the algae coating on a 5 cm branch of *Elodea nuttallii* served as an inoculum for the biofilm. Furthermore, a continuous regrowth of the biofilm and photosynthesis of the Elodea strand was facilitated during the test as stronger illumination (100 $\mu E\ m^{-2}\ s^{-1}$) was applied, thus making external aeration obsolete, as the Elodea continuously produced oxygen during daytime and morning measurements indicated that dissolved oxygen levels remained between 8.3 and 9.7 mg/L (see online supplementary material "Water Quality Parameters"). Furthermore, a steel mesh was added to each jar to provide habitat structures.

Comparison study—General experimental setup and test procedure

Eight different combinations of larvae-food-lab were tested in a matrix design (Table 1). Here, both laboratories used their own and each other's larvae as well as both test procedures. The two setups differ mainly in the food conditions and illumination (Table 2). In order to compare the test designs directly, both setups were always tested in parallel in both laboratories with larvae from the same batch.

For the study, larvae of *C. dipterum* were collected from uncontaminated artificial outdoor ponds and experimental ditches, respectively, near the two laboratories during May and June 2019. For the tests, larvae of the developmental stages L3/L4 according to Cianciara (Cianciara, 1979b) were used. In this work, the author determined 10 aquatic stages (L0 to N2) in the development of *C. dipterum*, based on well-differentiated morphological characters. For hemimetabolic species like mayflies that develop uniformly with numerous molts, the developmental stages define distinct biometrical stages during their larval development. To test the larvae of the respective other test site, they were transported to the other laboratory in cooled conditions immediately after collection. All larvae were acclimatized to the test conditions over at least 48 hr before inserting in the respective tests.

The experiments were performed in both laboratories at 18°C (±2°C) and a 16:8-hr light: dark regime over 28 days. The test temperature was determined based on previous studies and the measured temperature in the waters of origin at the time of test start. For all experiments, M4 medium according to

Table 1 Schematic setup of the tested matrix design at the two test locations.

Test location	Design	Number of replicates	Food	Origin larvae	Results
Lab 1	5 larvae	6	Biofilm on tiles	Lab 1	Figure 2C
	5 larvae	6	Biofilm on tiles	Lab 2	Figure 2A
	10 larvae	3	Biofilm + Elodea	Lab 1	Figure 2G
	10 larvae	3	Biofilm + Elodea	Lab 2	Figure 2E
Lab 2	5 larvae	6	Biofilm on tiles	Lab 1	Figure 2D
	5 larvae	6	Biofilm on tiles	Lab 2	Figure 2B
	10 larvae	3	Biofilm + Elodea	Lab 1	Figure 2H
	10 larvae	3	Biofilm + Elodea	Lab 2	Figure 2F

Table 2 Comparison of the general test designs in Lab 1 and Lab 2.

Experimental setup	Lab 1	Lab 2
Origin of the larvae	Outdoor artificial ponds located at the experimental facility	Outdoor artificial ponds located at the experimental facility
Temperature	18°C (+/-2°C)	18°C (+/-2°C)
Test medium	M4 medium	M4 medium
Test vessels	1 L beaker with 800 ml medium	1.5 L beaker with 1,000 ml medium
Number of larvae/beakers	5	10
Number of replicates	6	3
Oxygenation	External aeration	Not actively, by Elodea
Food	Tiles with laboratory grown biofilm (named: biofilm on tiles)	Preincubated jars with biofilm + Elodea (named: biofilm + Elodea)
Light-dark regime	<1 µE m-^{2}s-1. 16:8 hr	100 µE m-^{2}s-1. 16:8 hr
Additional structures	–	Stainless-steel mesh

Elendt and Bias (1990) was used. A number of 30 larvae were tested in each test design at both laboratories. Due to the already existing protocols, the number of replicates and individuals/replicate differed between the two general setups. The setup developed in Lab 1 comprised six replicates with five individuals in 800 ml medium. The protocol originating from Lab 2 refers to 10 individuals/replicate and 1,000 ml medium, resulting in a total of three replicates (Table 2). The light conditions were also different, with low illumination in the test protocol from Lab 1 and a higher illumination in the setup from Lab 2 to allow oxygen production by Elodea.

While medium and food were refreshed weekly, the instar development was assessed three times per week by visual inspection of each individual according to Cianciara (1979a) based on the ratio of the wing-pads to the abdominal segment. Therefore, first the tiles' steel mesh and Elodea shoot, respectively, were carefully removed out of the test vessel. Then, the larvae were carefully placed in a small drop of water in a Petri dish and examined using a binocular laboratory microscope. Afterwards, the tiles or steel mesh and Elodea sprout were returned to the vessels before adding the larvae again. Emergence was monitored daily.

Food

Food items comprised either pre-grown bioflim on tiles or pre-grown jars with biofilm on the glass walls of the beakers and a shoot of *Elodea nuttallii*.

The biofilm grown on tiles was actively seeded with field-collected biofilm and incubated for at least 14 days. The biofilm was collected in a section of a small nearby stream close to its source (Siebenquellen, Aachen). Stones covered with algae were brushed to obtain a highly concentrated algae suspension. This suspension was sieved through 55 µm mesh size before further use. For the cultivation, clean unglazed stoneware tiles (4.5 cm × 4.5 cm) were placed on the bottom of plastic containers (60 cm × 40 cm), filed with 5 L aerated M4 medium, and enriched with 10 ml of an additional nutrient solution ($NaNO_3$ 5.2502 g/L, KH_2PO_4 1.1470 g/L, Na_2SiO_3 2.9797 g/L). Approximately 200 ml of the algae suspension was distributed in each of the containers. The biofilm culture was cultivated at 23°C with an illumination of 25–40 µE $m^{-2}s^{-1}$ and a 16:8-hr light: dark regime. The medium enriched with the nutrient solution was exchanged once a week. Only tiles with uniform, well-developed biofilm without irregularities (e.g., chironomid larvae, fungal infestation, conspicuous smell) were used for the test. Food was provided ad libitum, usually four well-grown tiles per test vessel. All tiles that were used in the study were prepared in Lab 1 and delivered to Lab 2 weekly.

For the formation of the biofilm directly on the glass walls, 1.5 L glass beakers with 1 L M4 medium and a shoot of *Elodea nuttallii* were preincubated under the test conditions for up to 7 days. The jars used in the experiment were prepared in Lab 2 and delivered to Lab 1 weekly. Therefore, the incubation medium was discharged, and the jars were closed with parafilm to keep them moist. In Lab 1, aerated M4 medium was added carefully.

Data analysis and evaluation

Endpoints calculated from the comparison experiments were the development stage composition and the mortality over time. From these data mortality rates, development rates and the **C**loeon **D**evelopment **I**ndex (CDI) were calculated. To calculate the CDI, each developmental stage [L] is assigned an ascending value from 1 to 10, a score of 1 for L1 instar up to a score of 10 for the finally emerged subimago. This value is multiplied by the number of larvae of a stage for each day of examination:

$$CDI = \sum_{L=1}^{L=10} Days_L \times L \ \times \ s$$

In which CDI is the Cloeon Development Index, Days_L represents the number of days between larval examinations, and L is the development stage larvae; S = 1 for living larvae, and S = 0 for dead larvae.

The CDI result represents the development of the larvae over time. Higher CDI values indicate a faster development of the larvae.

Statistical evaluation

All statistical analyses were performed using R (version 4.4.2; R Core Team, 2025). To analyze the development (total number of nymphs over time) depending on the feeding, survival analysis was applied—function survfit from package survival (version 3.8-3; Therneau, 2024) and function ggsurvplot from package survminer (version 0.5.0; Kassambara et al., 2024)—with a logrank-test (function survdiff from package survival) to test for significant differences between feeding types (biofilm on tiles vs. biofilm + Elodea). The influence of transport on the survival of the individuals (number of individuals surviving over time) was also tested with survival analysis and subsequent logrank-test (no transport vs. transported). To test whether the CDI significantly changed over time between the different feeding types (biofilm on tiles vs. biofilm + Elodea), linear models were used (function lm from package stats [R Core Team, 2025]: CDI∼feeding) separately for both laboratories. Normal distribution of CDI values was tested using Shapiro-Wilk test (function shapiro.test from package stats). Slopes of regression models were tested against each other with a *t*-test (function t.test from package stats) for biofilm on tiles vs. biofilm + Elodea. The proportion of individuals emerged at the end of the test was modeled using a beta-distributed Generalized Linear Model (GLM; function betareg from package betareg [version 3.2-3; Zeileis 2025]) with feeding as independent variable (ratio∼feeding) and regression residuals assessed for systematic bias.

Temperature dependent development of *C. dipterum*

The temperature-dependent development of the larvae was investigated in a second study that was originally conducted to generate data for modeling purposes. The experiments were performed at Laboratory 1 starting with *C. dipterum* larvae of the development stage L1. The test organisms were collected in artificial outdoor ponds located near the experimental facility, acclimatized to the test conditions over 48 hr, and cultivated in the general experimental setup of Lab 1 (Table 2) until emergence at five temperatures (8°C, 13°C, 18°C, 22°C, 26°C). For every temperature, 20 larvae distributed in four replicates were examined. Their development during the test was monitored by the determination of the development stage according to Cianciara (1979a), and the larvae were fed with laboratory-grown biofilm on tiles as described before. Development stage determination in the higher-temperature experiments (18°C, 22°C, 26°C) was conducted three times a week. In the lower temperatures (8°C, 13°C), the development stages were monitored once a week. The monitoring of emergence was performed daily during periods when increased emergence was expected. To allow for sufficient food, the food tiles were exchanged at least once a week.

A standard mean development time (SMDT) from L1 to emergence was calculated for each temperature as time weighted average of the number of days until emergence of each surviving larva.

Results of this study were used to derive the standard test conditions in terms of temperature.

Results and discussion

Derivation of a test temperature for future standardized tests

The comparative tests were carried out at a constant temperature of 18°C as this temperature was already used in both existing test protocols and the test period was limited to 28 days. To determine an appropriate temperature for a future standardized chronic testing of *C. dipterum*, data from an additional experiment where the temperature-dependent development of *C. dipterum* was assessed were analyzed (Figure 1). On this basis, 18°C appears to be a suitable temperature even if, for full development to imago, more than the 28 days surveyed here are needed. When investigating the temperature-dependent development of *C. dipterum*, the SMDT from L1 to subimago was reduced from 37 days (27 to 47 days) at 18°C to 25 days (22 to 29 days) at 22°C. In addition, the variability also decreased (Figure 1). From the linear regression a SMDT of 29 days (L1 - subimago) at 20°C can be derived. In the experiments underlying this evaluation, the average development time from L1 to L2 was 6 days (2 to 10 days) and from L1 to L3 10 days (8 to 17 days). Thus, increasing the temperature to 20°C in combination with starting with L2 or L3 larvae would reduce the duration of the test until full emergence below 30 days. Sweeney and coworkers (Sweeney et al., 2018) report a mean development time of about 29 days until emergence at 20°C starting with larvae that were not older than 24 hr, corresponding with L0 larvae in the classification of Cianciara. This indicates that further optimization should be possible of the method used in our test setup. But this also indicates that a temperature of 20°C can be a suitable standard for future test protocols when including emergence as an endpoint. Furthermore, rearing and test temperatures of 20°C and 25°C are reported for the two North American mayfly species *Neocloeon triangulifer* and *Hexagenia* sp. (Buchwalter et al., 2019; Soucek & Dickinson, 2015; Weaver et al., 2015). In addition, testing *Cloeon* mayflies at 20°C facilitates the comparison of observed sensitivities with standard test organisms that are tested at the same temperature (e.g., *Daphnia* or *Chironomus riparius*).

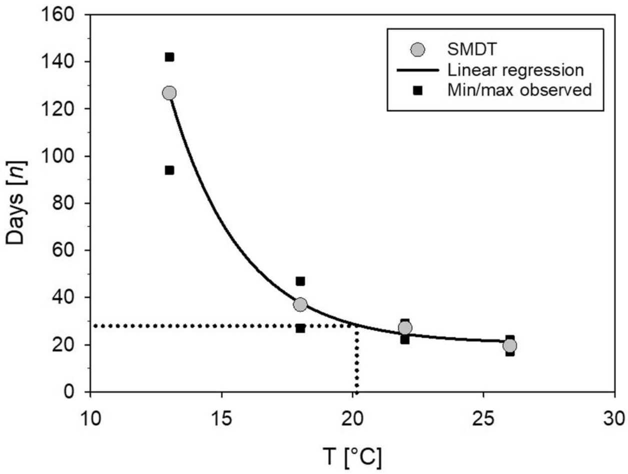

Figure 1 Temperature-dependent development of *Cloeon dipterum* derived from experiments at four different temperatures (13°C, 18°C, 22°C, 26°C). The gray dots show the standard mean development time (SMDT; as time weighted average of the number of days until emergence of each surviving larvae) for each respective temperature. The linear regression allows for the prediction of SMDT for additional temperatures. The dotted lines show the estimated SMDT at 20°C of approximately 29 days from L1 to emergence.

Comparison study—Influence of different test designs

In the present study, we used 18°C set in previous experiments to stay close to the two original test protocols. During the tests, the growth and survival of the larvae were recorded regularly over 28 days. Figure 2 illustrates the development stage composition for every day of examination in each of the eight tests A–H as well as the number of dead and emerged mayflies.

Interestingly, larval survival was significantly reduced when test organisms were transported between the participating laboratories (log rank test on the cumulative number of survivors: p = 0.0001; for more detailed information see online supplementary material “Statistics”), reaching a maximum of 48% mortality after 28 days (Figures 2A, D, E, H and 3 and see online supplementary material “Statistics”). Although performed with the utmost care, collection and subsequent immediate transport of approximately 2.5 hr to the other laboratory had apparently a negative impact on larval fitness. In contrast, the mortality of locally tested larvae comprised a maximum of only 20% (Figures 2B, C, F, G and 3 and see online supplementary material “Statistics”). This finding was independent of the test design used or the performing laboratories, indicating that the transport itself considerably influenced the results of the tests. This could not be compensated for by the fact that, as with the locally collected larvae, acclimatization over 48 hr followed transport. Thus, locally collected test organisms may be better suited for chronic tests. We assume that this also applies to larvae from any laboratory breeding and generally recommend transportation as short as possible.

Comparing the two test designs, it was observed that at both test locations the larvae develop faster when using the biofilm on tiles as a food source (Figure 2 and see online supplementary material “Statistics”). A log rank test on the cumulative number

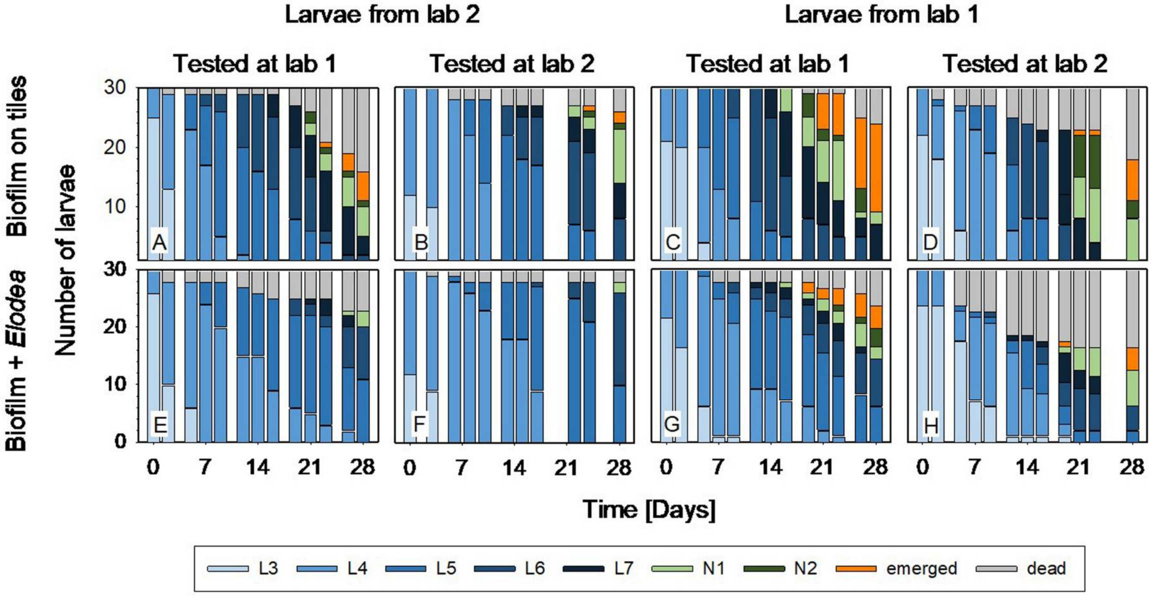

Figure 2 Development of *Cloeon dipterum* larvae in the different test setups. (A–D) Larvae provided with biofilm on tiles as a food source; (E–H) larvae raised on biofilm + elodea. For further details, see also Table 1. For each setup (panel), bars represent different census dates, and color codes refer to the different larval or survival, respectively.

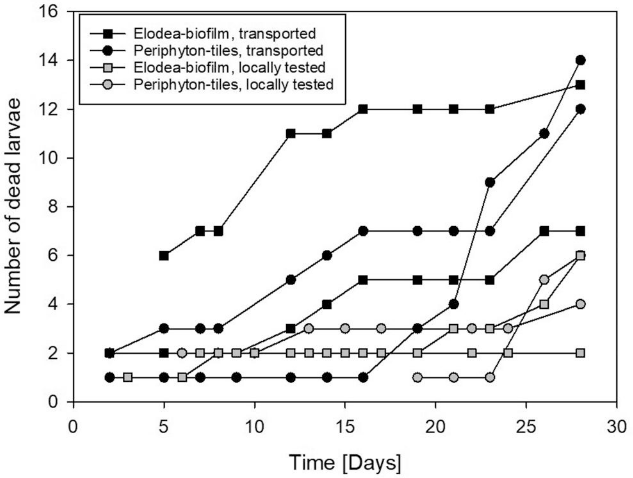

Figure 3 Total number of dead larvae in the eight different test setups over time. Black color: Larvae provided with biofilm on tiles as a food source. Gray color: Larvae raised on biofilm + Elodea. Squares: The larvae originated from the respective other laboratory and were delivered before the test began. Dots: locally samples larvae.

of nymphs and emerged mayflies revealed a significant difference between both food options ($p = 7\text{e}^{-09}$; see online supplementary material "Statistics"), and the GLM confirms a significant influence of the food on the percentage of emerged mayflies at the end of the test ($p = 0.0246$; see online supplementary material "Statistics"). No dependence on the source of the larvae was observed ($p = 0.0756$; see online supplementary material "Statistics"). Furthermore, a high control survival of 80% and 87% was observed, when using biofilm on tiles and locally captured larvae, in both laboratories, respectively (Figure 2B, C). This indicates that of the two feeding sources, the use of biofilm grown on tiles provides better results. We assume that although both offered food types originate from natural biofilm, the better performance on biofilm grown on tiles can be due to the overall amount and perhaps also the diversity of food available. However, neither the species composition, the microbiology, nor further parameters that potentially alter the nutritional quality of the food provided were investigated in this study. However, visual observations did show a distinct difference between the amount of biofilm grown on the tiles and the biofilm on the glass walls as on the tiles a denser layer of biofilm was visible, while the growth on the glass wall was less well developed. When investigating the development of *Cloeon* larvae at different temperatures, Sweeney and coworkers (Sweeney et al., 2018) observed a median development time of around 30 days and a survivorship of more than 85% when keeping the larvae at 20°C and feeding ad libitum with a 1 to 3 mm-thick coating of biofilm grown on acrylic plates. Here, the larvae development was somewhat faster than in our test design with biofilm on tiles. From the description, however, the total food amount seems to correspond well with that offered in the Lab 1 test design, indicating that in addition to the quantity also the quality of the food is critical for the development of the larvae. A relation between the quality and/or quantity of the feed and the larval development rate of mayflies was also observed in other studies (Cianciara, 1979a; Gupta et al., 1993; Sweeney & Vannote, 1984; Webb & Merritt, 1987). Different food sources as Detritus, Spirogyra (Cianciara, 1979a, 1979b), and not further specified algal filaments (Gupta et al., 1993) as well as diatom dominated periphyton (Sweeney & Vannote, 1984; Sweeney et al., 2018) were used in the studies mentioned. As in our case, this works fine within a specific research context. To further improve a future test protocol for *C. dipterum* and to ensure comparability of different studies, it will also be important to standardize and optimize the food. Weaver et al. (2015), for example, describe a standard food source consisting of three different diatoms for *N. triangulifer*. This might also work for *C. dipterum* but has not been investigated yet.

Regarding mortality, it was noted that when feeding with biofilm on tiles, mortality occurred predominantly in the late development stages and nymphs. In the tests with biofilm + Elodea, even younger larvae died. It appears that the larvae of a later stage are more likely to have a higher mortality. Figure 2 shows that mortality increases from the time when the first larvae develop to the nymphal stage. The reason for this, however, cannot be clarified here and needs to be looked at specifically in future studies.

CDI

The CDI summarizes the observed findings well. The values with biofilm on the glass surface, more intense light, and Elodea are consistently lower than those with biofilm grown on tiles, diffuse light, and aeration (Figure 4 and online supplementary material Figure S3.1), reflecting a generally slower development. The difference between both test protocols is particularly pronounced in the experiments performed at Lab 1, where the larvae generally showed faster growth and thus larval development was more advanced overall after 28 days. The linear models showed a highly significant dependency of the CDI on time for both laboratories (see online supplementary material "Statistics"), feeding types, and replicates (see online supplementary material Table S3.1). Regression coefficients are significantly higher when feeding biofilm grown on tiles instead of biofilm grown on the glass walls in both laboratories ($p = 0.0003065$ and $p = 0.0004033$; see online supplementary material Figure S3.2). Since one of the six replicates with biofilm on tiles in Lab 2 already started to show an uncommonly high mortality of the larvae at the end of the first week, this replication was excluded from the analysis. The proposed approach to condense the data over time into one Index of Development Instars allows an integrated concentration response relationship of all effects in an experiment. It should be evaluated with a compound if this calculated endpoint is only valid to determine control performance or would even be a possible endpoint to assess the effects of a treatment on *C. dipterum.*

Above all, adequate dietary conditions influence the quality and reproducibility of chronic tests with *Cloeon*. However, biofilm as used here is a variable food source that cannot be kept consistent if pulled from a natural stream ecosystem. In addition, the quantification and qualification of biofilm might be a challenge, which can result in differences between laboratories. Thus, there is a need to put further effort into the standardization and possibly simplification of food to further standardize this kind of assay. Besides food, it was important to offer the animals somewhere to hide during the test to minimize stress that can further increase sensitivity and control mortality. The

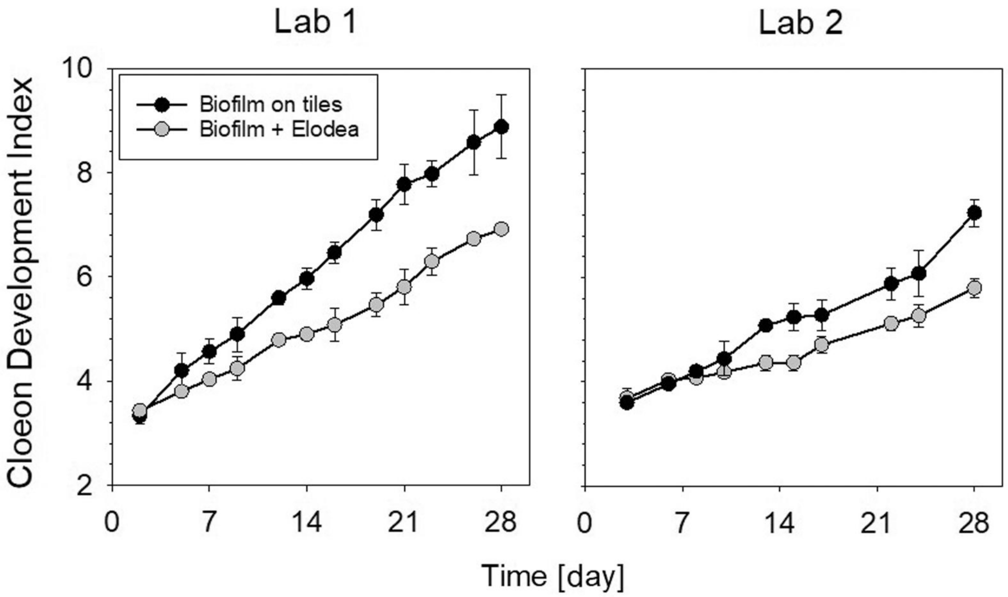

Figure 4 Cloeon Development Index, a measure of performance of the larvae in the test (experiments performed with locally captured larvae). Note that due to excess mortality in one biofilm + Elodea replicate, this was considered to be an outlier and consequently the biofilm + Elodea test in Lab 2 comprised five replicates for this analysis only.

experimentalists had the impression that the larvae move out of the hiding place more and show a more typical behavior at lower light conditions.

Looking at the tests with the same larvae and the same test design we found some variability between the laboratories, and it appears that the development in Lab 1 is always faster than in Lab 2 (Figure 2). To minimize this variability and thus increase the comparability of further tests, it must be assured that the combination of temperature and test duration is sufficient to allow emergence in the test.

As expected, the test conditions strongly influenced the development of *C. dipterum.* Based on the results shown here, recommendations for a future test protocol can be made, enabling further testing of an optimal experimental design. For this protocol, we suggest a temperature of 20°C ± 1°C, diffuse light (<1 µE m^{-2} s^{-1}), and a 16:8-hr light: dark regime. As the medium for the preparation of the test solutions, negative control, and the acclimatization of the larvae, artificial water type “Elendt M4” has proven to be successful. As long as no laboratory culture is available, larvae should be collected from a nearby (semi-)field source and adapted to the test conditions at least 48 hr.

Within the chironomid test (Organisation for Economic Co-operation and Development 218, 2004; Organisation for Economic Co-operation and Development 219, 2004), an emergence rate of 70% in the control is recommended for a valid test. For *Cloeon* we would propose separate validity criteria for control survival of larvae and emergence rates. Ongoing ring testing will provide further validation criteria for control survival and the use of the Instar Development Index. Ideally, a future test protocol should be tested using some reference toxicants in different laboratories to identify possible further endpoints as well as the variability and power of the method. The work presented here thus represents a first step toward a more consistent and standardized protocol to testing *C. dipterum* and highlights the differences between two already existing and applied methods.

Supplementary material

Supplementary material is available at *Environmental Toxicology and Chemistry* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contributions

Silke Classen (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing—original draft), Ivo Roessink (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Writing—original draft), Eric Bruns (Conceptualization, Writing—review & editing), Katrin Gergs (Conceptualization, Investigation, Writing—review & editing), Andre Gergs (Formal analysis, Writing—review & editing), Jutta Hager (Conceptualization, Writing—review & editing), Richard Ottermans (Formal analysis), and Thomas Günther Preuss (Conceptualization, Supervision, Writing—review & editing)

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Conflicts of interest

The authors declare that they have no known competing (financial) interests that could have appeared to influence the work reported in this paper. Bayer is a company that both produces and sells agrochemicals.

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