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Original Article

# Affordable chironomid housing: proposed modifications of standard OECD substrate for testing of chemicals on aquatic midges

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*Abstract:*

This study was conducted in order to formulate a modification of standard substrate for laboratory bioassays (OECD protocol number 218: "Sediment water chironomid toxicity test using spiked sediment"), with as few constituents as possible, yet enabling the highest larval survival of model organism: *Chironomus tentans* (Chironomidae, Diptera). Laboratory experiment consisted of two bioassays: first with substrate mixture of two and more ingredients tested: standard OECD substrate, standard substrate with medical clay, peat + clay, peat + sand, clay + sand; second with every ingredient individually tested: coarse sand, fine sand, peat, clay and no substrate. Highest larval survival was observed in sand + peat (≈85%) and coarse sand substrate (≈82%), whilst clay + peat and peat substrate caused the highest larval mortality (≈65% and 46% respectively). No larvae survived in treatment without any substrata, indicating the absolute necessity and importance of substrate presence for larval survival.

*Key words:*
*Chironomus tentans,* bioassay, life traits, OECD guidelines, sediments, substrate, toxicity tests

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*Apstract:*

**Pristupačno stanovanje za hironomide: predlog izmene standardne podloge OECD-a za ispitivanje hemijskih agenasa na vodenim komarcima**

Ovo istraživanje sprovedeno je radi formulisanja modifikovane standardne podloge namenjene laboratorijskim biološkim testovima (protokol OECD-a broj 218: "Test toksičnosti na hironomidama u sistemu sediment-voda, sa supstancom u sedimentu"), sa što manje sastojaka koja omogućava visoku stopu preživljavanja larvi model organizama: *Chironomus tentans* (Chironomidae, Diptera). Laboratorijski eksperiment sastojao se od dva biološka testa: u prvom je testirana mešavina dva ili više sastojaka: standardna OECD podloga, standardna podloga sa medicinskom glinom, treset + glina, treset + pesak i glina + pesak; u drugom je testiran svaki sastojak podloge individualno: krupan pesak, sitan pesak, treset, glina i tretman bez podloge. Najveće preživljavanje uočeno je u podlozi sa sastavom pesak + treset (≈85%) i podlozi sa krupnim peskom (≈82%), dok su podloge sastavljene iz mešavine gline i treseta ili samo treseta izazvale najveći mortalitet larvi (≈65% i 46% respektivno). Ni jedna larva nije preživela u tretmanu bez ikakve podloge, ukazujući na potpunu neophodnost i značaj prisustva podloge za preživljavanje larvi.

*Ključne reči:*
*Chironomus tentans, biološki test, životni parametri, OECD smernice, sedimenti, podloga, testovi toksičnosti*

## Introduction

In aquatic ecosystems, sediments represent a vital and specific habitat for many organisms providing feeding, spawning, hiding, and rearing areas. On the other hand, sediments in aquatic habitats can accumulate anthropogenic chemicals and waste materials, which can be directly toxic to sediment-dwelling organisms or bioaccumulate in the food chain contributing to a variety of environmental problems (den Besten and Munawar, 2016; Savić-Zdravković et al. 2020). Considering this enormous environmental problem, ecotoxicological testing of water and sediment based on information from chemical analyses, but also on effect measurements, i.e. bioassays or toxicity tests, has been developed over the years (den Besten and Munawar, 2016). These tests have been standardized and numerous regulations and guidelines have been developed, providing direct, quantifiable evidence of biological consequences of sediment contamination (Taylor and Scroggins, 2013). Great efforts has been invested to harmonize testing and to facilitate the mutual recognition of data by American Public Health Association (APHA), American Society for Testing and Materials International (ASTM), Environment Canada (EC), European Committee for Standardization or Comite´ Europe´en de Normalisation (CEN), International Organization for Standardization (ISO), Organisation for Economic Co-operation and Development (OECD) and United States Environmental Protection Agency (US EPA) (Taylor and Scroggins, 2013). In Europe, toxicity tests are conducted by the guidelines developed by OECD (OECD, 2020). The results from OECD test guidelines are intended to be accepted by all EU member states by the MAD principle (OECD agreement on Mutual Acceptance of Data in the Assessment of Chemicals, http://www.oecd.org/env/ehs/mutualacceptanceofdatamad.htm). A wide range of toxicity tests and test organisms exist for both freshwater and marine organisms, but there is a need for adjusting the existing protocols in a sense of new chemical substances and new possible tested endpoints (Hund-Rinke et al. 2016) and there is allowance for case-specific final modifications of the testing methodology.

Very important part of the macrozoobenthos in the freshwater ecosystems is the group of chironomids, known as non-biting midges (Dickman and Rygiel, 1996). Chironomidae are a family of the order Diptera, representing world-wide distributed midge flies with exceptional species richness and abundance (Ferrington, 2008). The family is divided into 11 subfamilies and 22 nominal tribes (Ferrington, 2008). Chironomids could be found in all sorts of habitats, from aquatic to terrestrial ones, but a total of 339 genera and 4,147 species are strictly aquatic in the larval stage (Ferrington, 2008). Occupying almost every type and conditions of aquatic habitats and having extraordinary ecological range and sensitivity makes them very good environmental indicators (Dickman and Rygiel, 1996). Chironomids usually exhibit specific phenotypes as a response to the presence of different stressors (Wiederholm, 1984). In polluted areas midges with deformed phenotypes were found, which lead to conclusion that aquatic pollutants cause deformities in chironomids, thus making them widely used as indicators of environmental stress in studies assessing the ecological impact of human activities (Lenat, 1993). Hamilton and Saether (1971) were the first to propose the usage of chironomid deformities as biological indicators of freshwater quality, and a lot of research has been done ever since. In fact recent meta-analysis found 41 studies on chemical effects on chironomid deformities in laboratory experiments, especially larval mouthpart deformities (Gagliardi et al. 2016). This study, by Gagliardi et al. 2016, also emphasizes the inconsistency in the published data due to several factors, amongst which are mortality effects and background stressors (referring to all stressors apart from the one that is tested in the particular study, including inbreeding, parental effects, inadequate substrates and unknown stressors in substrates and food), that influence the "reliability" of conducted studies.

The design of the appropriate bioassay depends on the intended application of the test. When examining chemicals' effects on structures in chironomid larvae, especially mouthparts, it is of great importance to have minimal wear of the structures and minimize additional deformities caused by sediment they are reared in. In few papers, substrate consisting only on quartz sand has been proposed as suitable for this kind of studies (Bird, 1997; Langer-Jaesrich et al. 2010), but no detailed studies have been conducted.

In this study, modifications of the standard OECD protocol number 218, entitled "Sediment water chironomid toxicity test using spiked sediment", designed to assess the effects of prolonged exposure of chemicals to the sediment-dwelling larvae, have been made. This was done in order to formulate a substrate with as few constituents as possible for bioassays where morphological changes on the larvae are examined as an endpoint. The aim of this study was to compare the effect of different standard substrate modifications on the survival on *C. tentans* larvae and to propose a simplified substrate formulation (lowering the possibility of damage or deformation of the larval mouth apparatus) that will enable favorable larval survival. The proposed modifications of sediment standards are based upon the laboratory experiments on *Chironomus tentans* species.

## Material and methods

### *Experimental organisms*

Specimens of *Chironomus tentans*, Fabricius, 1805, (Diptera, Chironomidae), used in the experiments were from the stock cultures reared in the laboratory of the Faculty of Science and Mathematics, University of Niš (Niš, Serbia). Stock cultures were reared under the OECD guidelines (OECD; 2004). Specimens were reared in glass tanks with water temperature 21.0±0.5 °C, a light-dark cycle of 16:8h artificial daylight and under constant aeration. The artificial sediment used for rearing the laboratory cultures was shredded paper sediment. Larvae were fed with TetraMin® fish food flake mixture. About a week before the start of the experiment, fresh egg cases have been isolated from the stock aquaria into petri dishes filled with water from the stock culture aquaria for hatching.

### *Experimental design*

Sediment bioassays were performed following OECD guidelines for the Testing of Chemicals, Section 2, specifically the test number 218: Sediment-Water Chironomid Toxicity Using Spiked Sediment (OECD, 2004), further referred to as OECD Test 218. Larvae were exposed to sediment in the test vessels (glass 600 ml beakers measuring 8 cm in diameter) which were composed of 2 cm sediment layer surrounded by a mixture of deionized and tap water (pH 6.5±0.4; hardness 7.3 °dH; electrical conductivity 325 µS/cm3). Twenty freshly hatched 1st instar larvae were placed in each test vessel (larvae were 2 days old), at 21 ± 1 °C, with gentle aeration and 16:8 light-dark cycle. Three replicas were made for each treatment, i.e. tested substrate type. On termination of the experiment (each bioassay was terminated after 12 days when larvae reached 4th stadium), the number of larvae recovered was recorded from each substrate. Survival (S) was calculated by the formula: S=ne/na, where ne is the sum of midge larvae survived at the end of the experiment per vessel and na is number of larvae introduced per vessel. In order for test to be valid, the average survival of *C. tentans* must be greater than or equal to 70% in the control at the end of the test, according to the protocol.

### *Modification of the formulated substrate*

The following formulated substrate, based on the artificial soil used in OECD Test 218 (OECD, 2004), is recommended:

1. 4-5% (dry weight) peat: as close to pH 5.5 to 6.0 as possible; it is important to use peat in powder form, finely ground (particle size less than 1 mm) and only air dried.
2. 20% (dry weight) kaolin clay (kaolinite content preferably above 30%).
3. 75-76% (dry weight) quartz sand (fine sand should predominate with more than 50 percent of the particles between 50 and 200 µm).

Deionised water is added to obtain a moisture content of the final mixture in a range of 30 to 50 % and calcium carbonate of chemically pure quality ($CaCO_3$) is added to adjust the pH of the final mixture of the sediment to 7.0 ± 0.5. Organic carbon content of the final mixture should be 2% (±0.5%) and is to be adjusted by the use of appropriate amounts of peat and sand, according to the proposed proportions.

The experiment consisted of the two identically structured bioassays with different modifications of thhe recommended standard OECD substrate. The modifications consisted of simplification, i.e. excluding one or more of the requested constituents. In the first bioassay we tested the mixture of two and more ingredients, whilst in the second we tested every ingredient individually (for sterilization each ingredient was thoroughly rinsed with water, than incubated 3 h at 500 °C, except peat, which was air dried as suggested by the protocol). Each tested substrate (treatment) had three replicas and in each replica the ratio of the depth of the substrate layer to the depth of the overlying water was 1:4 (overlaying water consisted of mixture of dechlorinated tap and deionized water in 1:1 ratio).

First bioassay was conducted with following five treatments, i.e. substrate mixtures:

1. Standard OECD substrate (sand + peat + clay) (further referred to as STANDARD)
2. Standard substrate with medicinal clay (sand + peat + medicinal clay) (further referred to as S+P+MC)
3. Standard substrate without sand (peat + clay) (further referred to as P+C)
4. Standard substrate without clay (peat + sand) (further referred to as P+S)
5. Standard substrate without peat (clay + sand) (further referred to as C+S)

Second bioassay was conducted with following five treatments, i.e. individual ingredient substrates:

1. Coarse quartz sand (canary grain sand which is available from pet stores with >50% of the particles were in the range of 400–1000 µm) (further referred to as CS)
2. Fine sand (commercially available sandbox sand with >50% of the particles were in the range of 50–200 µm) (further referred to as FS)
3. Peat (further referred to as P)
4. Clay (further referred to as C)
5. Without substrate (vessels with only water) (further referred to as 0)

### *Data analysis*

The datasets from both bioassays were of normal distribution (Kolmogorov-Smirnov with Lilliefors Significance Correction and Shapiro-Wilk test of normality, significance <0.05), therefore the data was analyzed with parametric tests (One way ANOVA). In order to test the significant differences in mortality rates between all tested combinations of substrates, the datasets from both experiments were analyzed separately, than were pooled together for the analysis. For *post hoc* comparison of larval mortality between groups, i.e. tested substrates, Tukey's honestly significant difference (Tukey HSD) test was used, with significance set at $p<0.05$. All statistical analyses were conducted in IBM SPSS Statistics software version 19 © SPSS Inc. 1989, 2010.

**Table 1.** Survival mean values ± SD in % of *C. tentans* larvae in different ingredient mixture substrate types. Calculated as the mean values of three replicas for each tested substrate.

| Code | Substrate type | Mean Survival ± SD% |
|---|---|---|
| STANDARD | Standard OECD substrate (sand + peat + clay) | 76.7 ± 7.6 |
| S+P+MC | Standard substrate with medicinal clay (sand + peat + medicinal clay) | 61.7 ± 15.3 |
| P+C | Standard substrate without sand (peat + clay) | 53.3 ± 12.6 [b] |
| P+S | Standard substrate without clay (peat + sand) | 85 ± 15. [a] |
| C+S | Standard substrate without peat (clay + sand) | 63.3 ± 2.9 |

Treatment a is statistically significantly different from treatment b (Tukey HSD $p<0.05$)

## Results

### *Bioassay with ingredient mixture substrate types*

The 1st bioassay was terminated after 12 days. According to protocol propositions, the average survival of *C. tentans* larve must be greater than or equal to 70% in the control at the end of the test, which was the case for only two tested substrates: STANDARD and P+S substrate.

One way ANOVA showed statistically significant differences in mean survival values among the groups, i.e. tested substrates ($p=0.049$, $F=3.518$). Post hoc test showed that survival of chironomid larvae in substrate P+C was the lowest and significantly different ($p<0.05$, mean difference = – 0.316) than in the substrate P+S, where the highest survival was recorded (**Tab. 1**).

### *Bioassay with individual ingredient substrate types*

The 2nd bioassay was terminated after 12 days. According to protocol propositions, the average survival of *C. tentans* larve must be greater than or equal to 70% in the control at the end of the test, which was the case for only two tested substrates: CS substrate and FS substrate.

One way ANOVA showed statistically significant differences among the groups, i.e. tested substrates ($p=0.001$, $F=11.019$). Post hoc test showed statistically significant differences between the following groups (Tukey HSD $p<0.05$): none of the larvae survived in the substrate 0, which was significantly lower survival than in all other substrates, accept in substrate P. Survival of chironomid larvae in substrate P was the second lowest and significantly different than in substrate CS, where the highest survival was recorded (**Tab. 2**).

### *All substrate types*

When combining the results from both bioassays (**Fig. 1**), and excluding the treatment 0 (in which no larvae survived), one way ANOVA showed statistically significant differences among the groups, i.e. tested substrates ($p=0.025$, $F=2.2998$). Larval survival in the substrate CS and P+S was the highest among all substrates and significantly higher only than survival in substrate P, which was the lowest (Tukey HSD $p<0.05$).

## Discussion

When conducting eco-toxicological tests on chironomids it is essential to propose a simple and effective bioassay design that will enable detecting the influence of examined stressor with desired specific endpoints. If we consider sublethal effects of the tested stressor as specific endpoints we desire to investigate (i.e. larval deformities, stress enzyme activity, DNA damage etc.), the bioassay that ensures survival of at least 70% of the population in the control, with proper substrate formulation that reduces the morphological alteration, variability of important population parameters and other background stressors, is essential. To propose optimal simplified substrate formulation for laboratory bioassays designed to assess the effects of prolonged exposure of chemicals to the sediment-dwelling freshwater chironomid

**Table 2.** Survival and mortality mean values ± SD in % of *C. tentans* larvae in different individual ingredient substrate types. Calculated as the mean values of three replicas for each tested substrate.

| Code | Substrate type | Mean Survival±SD % |
|---|---|---|
| CS | Coarse sand | 81.67 ± 17.55 [a] |
| FS | Fine sand | 73.3 ± 12.5 |
| P | Peat | 35 ± 31.2 [b] |
| C | Clay | 61.6 ± 7.6 |
| 0 | Without substrate | 0.0 ± 0.0 [c] |

The treatment a is statistically significantly different from treatments b and c, whilst treatment c is statistically significantly different from all other treatments accept b (Tukey HSD $p<0.05$)

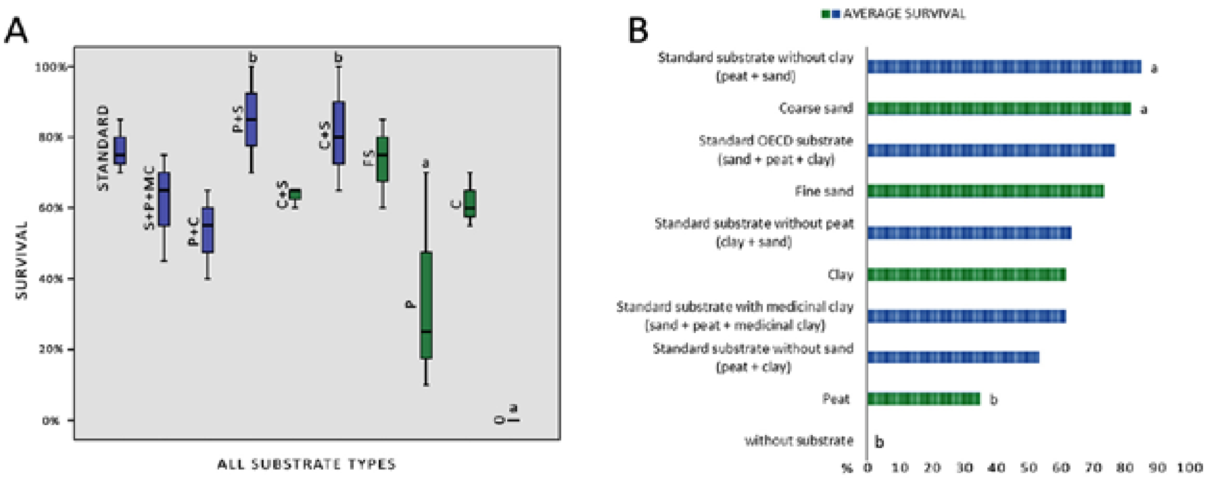


**Fig. 1.** Larval survival in all tested combinations of substrate types, i.e. treatments, presented together. Treatments from the 1st bioassay are colored blue and treatments form the 2nd bioassay are colored green. A – boxplots of survival rates with SD values; B – average survival rates sorted from highest to lowest. Treatments a are statistically significantly different from treatments b (Tukey HSD p<0.05).

larvae, we compared the effect of different standard OECD substrate modifications on the survival on *C. tentans* larvae. A total of 10 substrate types, i.e. treatments, were tested: 9 combinations of substrate constituents and one treatment with no substrate at all. Acceptable survival (70% and above) was recorded in only following four substrates (listed by average survival in descending order): P+S > CS > STANDARD > FS. In all other substrates the recorded survival was lower than 70%, whilst the survival in treatment with no substrate was 0%.

Most of the existing internationally harmonized ecotoxicological bioassays were developed through research and development, validation and peer review before they were officially published as documents with standard conditions, procedures, and guidance (Davis, 1977). Typically, several years of development precedes establishment of bioassay protocols and new additions to existing rules and regulations are regularly made in line with contemporary trends and market demand. Therefore, these protocols can be modified with respect to their function depending on the specific requirements of the tested species and the tested hypothesis (ISO, 2003). Sediment dwelling larvae of chironomids represent ideal test organisms for sediment ecotoxicity tests, especially since they usually exhibit specific phenotypes as a response to the presence of different stressors (Wiederholm, 1984) and are most diverse and abundant group out of all macroinvertebrates (Milošević, 2013). The standard OECD Test 218, recommends using formulated substrate because of several advantages: reduced experimental variability, reduced costs in comparison to using field sediments as substrate, replicability and comparison of results between studies (Davis, 1977; OECD, 2004). However, modifications of the recommended substrate are approved, as long as clean and uncontaminated substrate which provides satisfactory environment for larval development and 70% or higher survival (OECD, 2004). Providing more economical and simpler design which leads to same results in a more cost-effective way is always encouraged. This is why in this study we investigated the possibility of creating a simplified substrate that provides sufficient survival rate, with as few ingredients as possible to reduce possible influence on larval mouth apparatus morphology, reduce contamination, cost and time of substrate preparation and therefore increase the efficiency of laboratory bioassays.

To simplify the existing OECD formulated substrate, we firstly excluded only one of the standard three recommended substrate constituents, formulating the substrate out of two mixed ingredients. Thereunto we tested the standard OECD substrate and standard substrate with medicinal clay (commercially available and less expensive clay than proposed kaolin clay). We recorded the highest survival in the S + P substrate (≈85%) and in the STANDARD (≈77%), whilst the lowest survival was in C + P sediment (≈53%) (**Tab. 1**).

We then excluded two out of three OECD substrate constituents, formulating the substrate out of only one ingredient. Thereunto we tested the larval survival in vessels containing only water and no substrate at all. We recorded the highest survival in CS substrate (≈82%), following by the high survival in the FS substrate (≈73%). No larvae survived the end of the experiment in treatment 0, the jars without any substrata (**Tab. 2**), indicating the absolute necessity of substrate presence for larval survival.

Just few previous studies conducted the usage of different substrate formulations in the experiments with chironomids as model organisms: the study of Bird, (1997), where *C. tentans* larvae were cultured on four different substrates (shredded paper, coarse sand, fine sand and silty clay), showed the highest survival in paper sediment. This study of Bird suggests that substrates with sand are less suitable for chironomids because some of the food is lost between the sand grains and is not accessible to the larvae. However, this study encourages the use of coarse sand if the bioassay is designed to examine larval mentum deformities, since larvae reared in coarse sand had less worn ($p$<0.05) teeth than those reared in the other substrates. *Chironomus tentans* larvae belong to gatherers/collectors Functional Feeding Group (FFG), meaning that they collect small food particles (<1mm) that get lodged between rocks or in deep pools (Brabec et al., 2017). Given the size of the particles they can eat, it is possible for the larvae to ingest the substrate particles and mistake them for food, if they are less than 1 mm in size, therefore lowering the amount of food eaten and increasing mortality rates. The substrate composed of coarse sand (with particles around 1 mm) is too large to be ingested by the larvae and therefore does not cause higher mortality, deformation and damage of the oral apparatus. In our study, the survival recorded on both coarse and fine sand substrate was within the allowed survival rate, and in the coarse sand it was even greater than in a STANDARD substrate.

When combining the results from both bioassays and comparing all possible substrate combinations (**Fig. 1**), the descending order of suitable substrates (from highest to the lowest larval survival recorded) is the following: C+S > STANDARD > F+S > C+S > C > S+P+MC > P+C > P > 0. Significant variation in larval survival on different substrates was observed, but the only significant difference was between P substrate (where the lowest survival of ≈35% is recorded) and CS and P+S substrate (where the highest survival is recorded: ≈82% and ≈85% respectively). The average larval survival in the STANDARD substrate was among the highest of all treatments (≈77%), but when excluding clay form the standard mixture or excluding clay and peat (leaving only coarse sand sediment) the larval survival increased, justifying our aim to simplify the substrate formulation.

Considering that acceptable survival of above 70% was recorded in only four substrates: C+S, CS, STANDARD and FS, only these combinations of ingredients could be recommended for further usage as substrata in further laboratory bioassays. Those four tested substrates were not mutually statistically significantly different in terms of larval survival in the present experiment. Using the substrate composed of peat and sand sediment has shown to be the most convenient in the terms of survival of the larvae, but sediments that consist of only peat, or peat mixed with other ingredients, such as clay, have shown to cause higher mortality. High survival is evident in the sediments containing sand, which is in accordance with research of Langer-Jaesrich et al. (2010) which suggested the use of quartz sand substrate in bioassays (particle size 0.1–0.3 mm) as free of possible organic contaminations, and proposed by several other authors (Bird, 1997, Meregalli and Ollevier, 2001; Meregalli et al., 2001).

There has never been a systematic proposal for modification of OECD Test 218 (OECD, 2004; OECD, 2005). This could be due to the fact that research and development of a test method is typically the most time-consuming aspect of the standardization process, as it requires one to become familiar with all aspects of the test organism and experimental design. The importance of uniformity and consistency in ecotoxicological studies is indisputable, but in order to have solid comparative framework there are still a lot of problems that need to be overcome in the future. On the basis of this study, conducted under controlled laboratory conditions, we could propose further usage of substrate consisting only of quartz sand (especially coarse canary grain sand which is available from pet stores with >50% of the particles in the range of 400–1000 μm) as substrate in laboratory bioassays on *C. tentans* species. The substrate consisting only of one ingredient could be lowering possible negative effects and "background noise" in bioassays and increasing the reproducibility and ease of performing experiments: sand could be obtained by cheaper prices in the pet stores, could be easily sterilized (thoroughly rinsed with water, than burned 3 h at 500 °C) and chironomid larvae could be better visible and more easily separated from the sediment at the end of the experiment. Further study on the influence of substrate to more specific, sublethal endpoints, such as mentum and mandible ware, deformity rates, body weight, larval shape and size has to be conducted on all four proposed suitable substrates in order to confirm the preposition of their usage in bioassays where sublethal effects on the larvae are examined.

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