

The Chironomid Acute Toxicity Test: Development of a New Test System

Lennart Weltje,*† Hans Rufli,‡ Fred Heimbach,§ James Wheeler,|| Marco Vervliet-Scheebaum,# and Michael Hamer||

†BASF SE, Crop Protection—Ecotoxicology, Speyerer Strasse 2, D-67117 Limburgerhof, Germany

‡ecotoxsolutions, Schwarzwaldallee 215, Basel, Switzerland

§Bayer CropScience AG, Department of Ecotoxicology, Monheim, Germany

||Syngenta Environmental Safety, Jealott's Hill International Research Centre, Bracknell, Berkshire, United Kingdom

#University of Freiburg, Faculty of Biology, Plant Biotechnology, Schänzlestrasse 1, Freiburg, Germany

(Submitted 8 July 2009; Returned for Revision 22 September 2009; Accepted 18 October 2009)

ABSTRACT

This paper describes the basis for a water-only acute chironomid toxicity test guideline using first-instar larvae. The method is based on the OECD test guidelines for the acute *Daphnia* sp. immobilization test and the long-term tests with *Chironomus* sp., reflecting the common test procedures currently used by the European agrochemical industry. Development of this guideline proposal is important under the European Plant Protection Products Directive (91/414/EEC), under which an insect species may be required to be tested, particularly for certain insecticides, for which *Daphnia* sp. may not be representative of the sensitivity of nontarget aquatic invertebrates. *Chironomus* sp. is a freshwater insect currently used in different international test guidelines. Because their ready availability as a test organism, with culturing conditions and certain test methods already established, *Chironomus* sp. is regarded as a suitable additional freshwater invertebrate species for regulatory testing. Integr Environ Assess Manag 2010;6:301–307. © 2009 SETAC

Keywords: New guidelines Acute toxicity Aquatic Invertebrates *Chironomus*

INTRODUCTION

Acute chironomid immobilization tests have been performed by the agrochemical industry for a number of years. This has been driven by European testing requirements, under which a second test with an aquatic invertebrate species (i.e., an insect) may be required for insecticides with a specific mode of action (EC 2002). The Guidance Document on Aquatic Ecotoxicology (EC 2002) specifically mentions the neonicotinoid insecticides for which the acute toxicity for *Daphnia* may be up to 3 orders of magnitude lower than for aquatic insect larvae (Yokoyama et al. 2009). An insect species test is required when *Daphnia* sp. is not sensitive (defined by 48 h EC50 > 1 mg/L or 21 d NOEC > 0.1 mg/L) and may not be expected to represent the full range of sensitivities of nontarget aquatic invertebrate species.

Chironomus sp. is a widespread freshwater dipteran species, the sediment-dwelling larvae of which have commonly been used in long-term laboratory toxicity tests. Therefore, the *Chironomus* sp. acute immobilization test presents an ideal opportunity to enhance the currently available suite of test methods. In addition, chironomids are considered ecologically relevant, because of their widespread distribution, their numerical abundance, and their importance as prey for fish. Currently, there is no internationally validated test guideline (e.g., OECD) available to assess acute toxic effects of chemicals to chironomids. However, there is a Japanese chironomid acute toxicity guideline (JMAFF 2005) and a

generic test guideline for mosquito larvae (WHO 2005). This paper presents a proposal that could form the basis of a future OECD test guideline for the acute toxicity testing of chemicals to larvae of *Chironomus* sp. A water-only chironomid acute immobilization assay would complement the chronic chironomid OECD test guidelines 218 and 219 (OECD 2004a, 2004b) and the full life-cycle toxicity test method currently under validation in the Test Guidelines Programme of the OECD. Full life-cycle testing methods have been described for *C. riparius* (Taenzler et al. 2007; Tassou and Schulz 2009) and for *C. dilutus* (previously named *C. tentans*; Benoit et al. 1997; USEPA 2000).

Here, we present methodology based on the OECD test guideline 202, the 48-h acute *Daphnia* sp. test (OECD 2004c). Also, information from existing long-term sediment toxicity test protocols for *C. riparius* and *C. dilutus* developed in Europe and North America (Hill et al. 1993; Fleming et al. 1994; USEPA 1996a, 1996b, 2000; Environment Canada 1997) and included in previous ring-test experiments (Streloke and Köpp 1995; Milani et al. 1996; USEPA 2000) is taken into account. Other well-documented chironomid species such as *C. yoshimatsui* (Kawai 1986; Sugaya 1997) may also be used. This guideline proposal is supplemented with toxicity data for lindane and 3,5-dichlorophenol as reference substances.

Principles of the test

First-instar *Chironomus* sp. larvae are exposed to a range of concentrations of the test substance in water-only vessels for a period of 48 h. Immobilization is recorded at 24 and 48 h, and the EC50 is calculated at 24 and 48 h (if data allow).

* To whom correspondence may be addressed: lennart.weltje@basf.com

Published on the Web 10/30/2009.

DOI: 10.1897/IEAM.2009-069.1

Reference substances

A reference substance may be tested regularly as a means of ensuring that the test system and conditions are reliable. Toxicants that have been used in international ring-tests and validation studies are recommended for this purpose. Examples of reference toxicants used successfully with *Chironomus* sp. are lindane, pentachlorophenol, cadmium chloride, and potassium chloride (Fleming et al. 1994; Environment Canada 1995, 1997; Streloke and Köpp 1995; USEPA 2000), whereas potassium dichromate is typically used in the comparable acute test with *Daphnia* sp. (OECD 2004c). The laboratories involved in developing this guideline proposal have also used 3,5-dichlorophenol as reference substance. Tests with a reference substance should be conducted as required, for example, after introducing new stock organisms into the culture. However, at least 1 reference test per year should be conducted.

Validity of the test

For a test to be valid, the following performance criteria apply: 1) In the control, including the solvent control, if appropriate, not more than 20% of the larvae should show immobilization or other signs of disease or other stress (e.g., discoloration or unusual behavior, such as trapping at the water surface) at the end of the test. 2) The dissolved oxygen concentration at the end of the test should be ≥ 3 mg/L ($>60\%$ of the air saturation value) in control and test vessels.

MATERIALS AND METHODS

Apparatus

Test vessels and other apparatus that will come into contact with the test solutions should be made entirely of glass or other chemically inert material. Suitable test vessels are 50-mL Petri dishes (height 3 cm, inner diameter 4.7 cm) or 100-mL beakers (height 7 cm, inner diameter 4.5 cm) which should be cleaned before each use using standard laboratory procedures. Test vessels should be loosely covered to reduce the loss of water from evaporation and to avoid entry of dust into the solutions. Volatile and other test substances that might be difficult to test should be handled according to the recommendations described in the Guidance document on aquatic toxicity testing of difficult substances and mixtures (OECD 2000).

In addition, some or all of the following equipment will be used: oxygen meter (with microelectrode or other suitable equipment for measuring dissolved oxygen in low-volume samples), pH meter, equipment for the determination of hardness, adequate apparatus for temperature control, etc. If the dilution water is from a surface or groundwater source, equipment for the determination of the total organic carbon (TOC) concentration or equipment for the determination of the chemical oxygen demand (COD) will be necessary.

Test organisms

First-instar larvae are used in this test, because they represent the most sensitive larval stage (see *Discussion* section). Furthermore, larvae of this instar are free swimming and therefore are not stressed by the absence of sediment. The species is preferably *C. riparius*, but *C. dilutus* or *C. yoshimatsui* may also be used. Details on culturing

methods are available for *C. riparius* (OECD 2004b), *C. dilutus* (USEPA 2000), and *C. yoshimatsui* (Kawai 1986). Test organisms should come from a source (preferably in-house culture) where identity of the test species has been confirmed.

The larvae should be derived from a healthy stock (i.e., showing no signs of stress such as high mortality, discolored animals) with a known history (breeding method, culture conditions). All organisms used for an individual test should have originated from the same culture. The cultures should be maintained under conditions (light, temperature, and medium) similar to those to be used in the test. If the *Chironomus* sp. culture medium to be used in the test is different from that used for routine *Chironomus* sp. culture, it is good practice to include a pretest acclimation period by placing egg masses for hatching and maintaining the first-instar larvae in test dilution water at test temperature until the start of the exposure.

Test medium

Natural water (surface or groundwater), reconstituted water, and dechlorinated tap water are acceptable as holding and dilution water if *Chironomus* sp. will survive in it during culture, acclimation, and testing without showing signs of stress. Any water that conforms to the chemical characteristics of acceptable dilution water as listed in Table 1 is suitable as a test water. It should be of constant quality during the period of the test. Reconstituted water can be made up by adding specific amounts of reagents of recognized analytical grade to deionized or distilled water. Examples of reconstituted water are given in OECD (2004b, 2004c).

In the case of natural water, the water quality characteristics listed in Table 1 should be measured at least twice per year or when it is suspected that these characteristics might have changed significantly. Measurements of heavy metals (e.g., Cu, Pb, Zn, Hg, Cd, Ni) should also be made. If dechlorinated tap water is used, Cl analysis is desirable. If the dilution water is from a natural source, conductivity and TOC or COD should be measured.

Table 1. Some chemical characteristics of an acceptable dilution water

Substance	Concentration
Particulate matter	<20 mg/L
Total organic carbon	<2 mg/L
Unionised ammonia	<1 μ g/L
Hardness as CaCO ₃	<400 mg/L ^a
Residual chlorine	<10 μ g/L
Total organophosphorus pesticides	<50 ng/L
Total organochlorine pesticides plus polychlorinated biphenyls	<50 ng/L
Total organic chlorine	<25 ng/L

^aIt should be noted that, if an interaction is suspected between hardness ions and the test substance, water of lower hardness should be used (so Elendt Medium M4 must not be used in this situation).

To avoid the necessity of adaptation prior to the test, it is recommended that the water used in the test be the same as that used for culturing. The dilution water may be aerated prior to use for the test so that the dissolved oxygen concentration has reached saturation.

Preparation of test solutions

Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. Stock solutions should preferably be prepared by dissolving the test substance directly in the dilution water. As far as possible, the use of solvents or dispersants should be avoided; however, solvents may be required in some cases in order to produce a suitably concentrated and homogeneous stock solution. Guidance for suitable solvents as well as other aspects of handling difficult substances, such as biodegrading, complexing, ionizing, or multicomponent substances and preparations is available (OECD 2000). For active ingredients or other single chemicals (i.e., not preparations), the test substance concentration in the test solutions should not exceed the practical limit of solubility in the dilution water. If solubilizing agents are used, the concentration of solvent should be equal in all test concentrations and in the solvent control. In addition, the maximum allowed solvent concentration is 100 $\mu\text{L/L}$ or 100 mg/L, whichever is lowest (OECD 2000). The test should generally be conducted without adjustment of pH. If the pH does not remain in the range of 6 to 9, then a second test should be conducted, adjusting the pH of the stock solution to that of the dilution water before preparing the test solutions. The pH adjustment, preferably with 0.1 M HCl and 0.1 M NaOH, should be made in such a way that the stock solution concentration is not changed to any significant extent and that no chemical reaction, such as precipitation of the test substance, is caused. Adjustment within the pH range 6 to 9 may be appropriate for chemicals that show a difference in stability over this range, in order to maintain test concentrations for 48 h.

Test concentrations

A range-finding test may be conducted to determine the range of concentrations for the definitive test. For this purpose, the larvae are exposed to a series of widely spaced concentrations of the test substance. At least 10 larvae should be exposed divided over 2 replicates of each test concentration for 48 h.

In the definitive test, at least five test concentrations should be used, with a dilution water control and solvent control (if appropriate). The solvent control contains the solvent in a concentration identical to the level used in treatments. Concentrations should be arranged in a geometric series with a spacing factor preferably not exceeding 2.2. Justification should be provided if fewer than 5 concentrations or a larger spacing factor is used, e.g., a shallow slope of the concentration–response curve. The highest test concentration should preferably result in 100% immobilization, and the lowest test concentration should preferably give no observable effect. However, these effect levels are secondary to adequately defining the EC50. For each concentration, at least 20 larvae are used, divided into 4 groups of 5.

Alternatively, a limit test with a single concentration may be performed at 100 mg/L of test substance or up to the practical limit of solubility in the test medium (whichever is

lowest) in order to demonstrate that the EC50 is greater than this concentration. The limit test should be performed using 20 larvae (preferably divided into 4 groups of 5), with the same number in the controls. If the percentage of immobilization is $>20\%$ at the end of the test, a full dose–response study should be conducted. Any observed abnormal behavior should be recorded.

Incubation conditions

The water temperature should be within the range of 18 to 22 °C, and for each individual test it should be constant within ± 1 °C. A 16-h light and 8-h dark cycle is recommended, and the light intensity should be between 500 and 1,000 lux. Complete darkness is also acceptable, especially for test substances that are unstable in light.

Preparation of test organisms prior to exposure

Four or 5 d before adding first-instar larvae to the test vessels (test initiation), fresh egg masses (<24 h) should be taken from the culture and placed in small vessels in culture medium with a small amount of food. Aged medium from the stock culture or freshly prepared medium may be used. Normally, the larvae begin to hatch within a few days after the eggs are laid (2–3 days for *C. riparius* at 20 °C and 1–4 days for *C. dilutus* at 23 °C and for *C. yoshimatsui* at 25 °C). First-instar larvae should be used in the test. If necessary, the instar of midge larvae can be checked by measuring the head capsule width (USEPA 2000). Figures 1 and 2 show a freshly laid egg mass and an egg mass that has almost completed hatching, respectively.

Chironomids should not be fed during the test. However, feeding prior to exposure (i.e., directly after hatching) is important in order to ensure $\geq 80\%$ survival of the larvae in the controls at the end of the exposure period. A few droplets of filtrate from finely ground suspension of flaked fish food (e.g., Tetra-Min or Tetra-Phyll; see also OECD 2004b) in the amount of 0.05 to 0.5 mg per larva should be sufficient for young larvae. Green algae have also been suggested as a food source (OECD 2004a, 2004b), but care must be taken when



Figure 1. A freshly (<24 h) laid egg mass of *C. riparius*.



Figure 2. First-instar larvae from an almost completely hatched egg mass of *C. riparius*.

larvae are added to the test vessels not to “inoculate” the test medium with algae, which may influence test compound availability. As an alternative to flaked fish food, plant material may be used, for example, stinging nettle (*Urtica dioica*), mulberry (*Morus alba*), white clover (*Trifolium repens*), or spinach (*Spinacia oleracea*). Other plant material (cerophyl or alpha-cellulose) may also be used. However, as the amounts of food recommended in the literature vary considerably, it is recommended that individual laboratories determine the amount of food required prior to exposure to ensure adequate survival in the test. Note that fish food extract, which can be prepared by boiling the fish food suspension, followed by removal of the larger particles, e.g., by filtration or by discarding the settled fraction, would typically have about 3.7 g TOC/L, of which very small amounts would be carried over to the test vessels.

Loading of test system and exposure

Test vessels should be filled with appropriate volumes of test solutions. *Chironomus* sp. first-instar larvae should be randomly allocated to the test vessels using a blunt pipette. At least 20 animals, preferably divided into 4 groups of 5 animals each, should be used for each test concentration and controls. At least 2 mL of test solution should be provided for each animal (i.e., at least a volume of 10 mL for 5 larvae per test vessel; which should also be sufficient to allow for adequate samples to be taken for analytical determination of the test

concentrations). The exposure commences with the addition of larvae to the test solutions and lasts for 48 h.

Observations

Each test vessel should be checked for immobilized larvae at 24 and 48 h after the beginning of the exposure. Those animals that are not able to change their position within 15 s after mechanical stimulation (e.g., by subjecting the larvae to a gentle stream of water from a Pasteur pipette or agitation of the test vessel) are considered to be immobilized. Immobility is used as surrogate parameter for lethality, similar to the definition for neonate daphnids in the 48-h acute test guideline (OECD 2004c), because it is difficult to establish mortality in first-instar larvae. In addition to immobility, any abnormal behavior or unusual appearance should be reported. Immobilized larvae observed at 24 h are not removed from the test vessels so that their immobility may be confirmed at test end.

Physicochemical measurements

The dissolved oxygen and pH are measured, as a minimum, in the controls and in the highest test substance concentration at the beginning and end of the test. The dissolved oxygen concentration in the controls should be in compliance with the validity criterion. The oxygen level in control and test vessels should be reported as the air saturation value.

The pH should normally not vary by more than 1.5 units over the course of the test. Temperature is usually measured in control vessels, and it should be recorded, preferably continuously, throughout the test or, as a minimum, at the beginning and end of the test.

The concentration of the test substance should be measured, as a minimum, in the controls, at the highest and lowest test concentration, but preferably in all treatments, at the beginning and end of the test. It is recommended that results be based on measured concentrations. However, if evidence is available to demonstrate that the concentration of the test substance has been satisfactorily maintained within $\pm 20\%$ of the nominal or measured initial concentration throughout the test, then the results can be based on nominal or measured initial values.

Data and reporting

Data should be summarized in tabular form, showing for each replicate of each treatment the number of larvae exposed and immobilization at each observation. The percentages immobilized at 24 and 48 h are plotted against test concentrations. Data are analyzed by appropriate models (e.g., probit analysis) to determine the concentration–response curves, their slopes, and the EC50 values with 95% confidence limits (Stephan 1977; Finney 1978). The NOEC may be calculated by using Fisher’s exact test (Fisher 1922).

RESULTS

The laboratories involved in developing the present guideline proposal have used 3,5-dichlorophenol and lindane as reference substances. The data generated in 2 laboratories yielded 48-h EC50 values for 3,5-dichlorophenol in the range

Table 2. Acute toxicity data for 3,5-dichlorophenol for *C. riparius* first-instar larvae. Data were generated according to in-house protocols

48-h EC50 (mg/L)	Reference
0.55 0.68	BASF, Limburgerhof, Germany (L. Weltje, personal communication)
3.0 2.8	Bayer CropScience, Monheim, Germany (M. Dorgerloh and E. Bruns, personal communication)

of 0.55 to 3.0 mg/L ($n = 4$; see Table 2). For lindane, 48-h EC50 data from 2 laboratories and from the literature were in the range of 7 to 98 $\mu\text{g/L}$ ($n = 22$; see Table 3). However, these data also include tests performed with *C. plumosus* and *C. dilutus* and include tests with first- and second-instar larvae. Furthermore, it should be noted that the test results reported in Tables 2 and 3 were generated according to roughly the same protocol, but deviations with respect to water quality, age of the larvae, acclimation, temperature, test substance type (active ingredient or formulation), etc., are noted. Also, analytical confirmation of test concentrations was not performed. It is expected that the results will be in a narrower range when an identical protocol is followed and when corrections for analytically measured values are made. A detailed inspection of the data in Table 3 reveals that, with a few exceptions (i.e., the EC-formulation data from Strelake and Köpp [1995] and 2 values from the BASF laboratory), the EC50 values are actually quite similar. Therefore, for the pure active ingredient lindane the 48-h EC50 value for first-instar larvae of *C. riparius* obtained by using the present protocol is expected to be in the range of 20 to 55 $\mu\text{g/L}$.

DISCUSSION

The EC50 rather than the LC50 was chosen as the endpoint for this test method, analogously to the *Daphnia* sp. guideline (OECD 2004c), because immobility can be assessed with greater certainty than actual death in arthropods. Furthermore, for aquatic invertebrates, immobility can conservatively be considered indicative of death. Immobile

chironomids can be described as merely twitching and not actually performing deliberate swimming movements. In contrast, the Japanese chironomid acute toxicity guideline (JMAFF 2005) and the WHO mosquito acute test guideline (WHO 2005) use the LC50 as the endpoint. However, the WHO guideline distinguishes between dead and moribund larvae. An additional description of the typical swimming behavior of the larvae and their movements may further help to distinguish between mobile and immobile larvae. This may be important for certain toxicant modes of action with respect to reversibility of effect.

This guideline proposal recommends the use of first-instar larvae and an exposure period of 48 h, thus resembling the choices made for the acute daphnid test (OECD 2004c). The Japanese guideline (JMAFF 2005) uses the same exposure period, but with second- and third-instar larvae, whereas the WHO guideline (WHO 2005) advises exposure of third- and fourth-instar mosquito larvae for a period of 24 h (for slowly acting insecticides, 48 h or longer may be required). For the acute chironomid test, the use of first-instar larvae is easy, insofar as these larvae follow hatch immediately and are not or are only minimally stressed by the absence of sediment. This is in contrast to older larvae, which must be removed from their sediment tubes first, which undoubtedly poses great stress on the animals. More importantly, first-instar larvae are of higher sensitivity than older instars (see Gauss et al. 1985; Williams et al. 1986; Robinson and Scott 1995; Larrain et al. 1997), which is at least partially caused by their higher surface-to-volume ratio. In addition, when using early instars, there is no risk that larvae pupate and (consequently) a reduced risk for cannibalism during the 48-h exposure. This is in agreement with the USEPA (1975) recommendation of a test duration of 48 h in view of effects of starvation on larvae and their possible development into succeeding instars during the course of the experiment.

The validity criterion of maximally 10% control immobility in the *Daphnia* sp. acute test has been increased to 20% for chironomids, based on the experience of groups who have conducted these studies. The increase to 20% in the acute chironomid test is essentially to avoid an unnecessarily high test failure rate. For chronic OECD tests with chironomids and daphnids, there is also a 10% difference in the validity criteria, with at minimum 70% control emergence in the case of the chironomids against at least 80% control survival for

Table 3. Acute toxicity data for lindane and *Chironomus* sp. Data were generated according to various different protocols

Species	Instar	48-h EC50 ($\mu\text{g/L}$)	Reference
<i>C. riparius</i>	First	11; 32; 33 98;	BASF, Limburgerhof, Germany (L. Weltje, personal communication)
<i>C. riparius</i>	First	43	University of Landau, Germany (K. Tassou, personal communication)
<i>C. riparius</i>	First	16 (7–40) ^a	Strelake and Köpp (1995)
<i>C. riparius</i>	Second	44	Watts and Pascoe (2000)
<i>C. riparius</i>	Second	40	Hooftman et al. (1989)
<i>C. dilutus</i>	Second	40	Watts and Pascoe (2000)
<i>C. plumosus</i>	Second	32	Hooftman et al. (1989)

^aGeometric mean and range of 13 tests performed at five laboratories with a 200 g/L EC lindane formulation. Some studies were rejected as a result of control mortality. Original data made available by Peter Dohmen.

daphnids. The main reason for the higher background or control mortality of first-instar larvae is starvation (Bécharé et al. 2008), which typically is not an issue for the neonates in the acute *Daphnia* sp. test. Older larval stages of chironomids show lower control mortality rates and were therefore recommended for acute testing by Larrain et al. (1997), despite their lower sensitivity to toxicants (see above). Other guidelines, such as the WHO mosquito larvae guideline, require less than 20% control mortality, whereas the Japanese chironomid guideline allows a control mortality rate of at maximum 10% (but uses second and third instars). In addition, in the acute chironomid toxicity study of Jeyasingham and Ling (2000), a similar validity criterion of >80% control survival was suggested. Larrain et al. (1997) determined a median ($n=20$) control mortality of 19% for first-instar chironomid acute tests. Considering all of the above, there is a sound practical basis to use an acceptability criterion of maximum 20% immobility in the control. Nevertheless, a possible change in the validity criterion for control mortality, in consideration of the number of larvae per replicate and number of replicates, may be subject to study when the protocol presented here is internationally ring tested.

In the chronic chironomid OECD test guidelines 218 and 219 (OECD 2004a, 2004b), in which the test systems contain sediment and exposure is approximately 28 days, the amount of fish food fed is stated as 0.25 to 0.5 mg per larva per day for the first 10 days. For feeding of first instars, in the absence of sediment prior to acute testing, based on the experience of the authors, this amount is excessive. One of the laboratories successfully used a fish food suspension (50 g Tetra-Phyll/L deionized water), adding 0.01 mL to each test beaker containing 10 first-instar larvae in 25 mL test solution prior to exposure. This results in 0.02 mg food/mL and 0.05 mg food per larva. According to the Japanese guideline (JMAFF 2005), second- or third-instar larvae of *Chironomus* sp. are not fed during 48-h tests. The WHO protocol for mosquito larvae recommends no feeding in acute tests of 24-h duration, but food may be required if the exposure period is longer (WHO 2005). In those cases, finely ground yeast extract or ground mouse or rabbit pellets suspended in water are added to the water in test vessels at 10 mg/L. Inasmuch as 25 larvae are held in 100 to 200 mL, this corresponds to 0.04 to 0.08 mg food per larva. For long-term tests with juvenile hormone analogues or chitin synthesis inhibitors, this amount of food is added every 2 d until mosquitoes emerge (WHO 2005). In comparison, the detailed review paper on aquatic arthropods in life cycle and 2-generation toxicity tests (OECD 2006) recommends slurry prepared with commercial fish food flakes at a concentration of approximately 0.04 mg dry food/mL culture water. In 25 mL test solution with 10 chironomid larvae, this would correspond to 0.1 mg per larva. Furthermore, the form of the food is important. The OECD test guidelines 218 and 219 (OECD 2004a, 2004b) specify finely ground fish food suspended in water. However, this may still contain large particles that larvae can find difficult to eat. Large particles also aggravate the problem of dirtiness and reduction of oxygen content. Therefore, it is important to discard the large particles by filtration or by leaving solutions to settle and using only the supernatant.

With regard to the frequency of using reference substances, generally an event-driven use is preferred, e.g., after introduction of a new *Chironomus* sp. test culture in the laboratory, rather than a given frequency of testing. However,

at least 1 reference test per year is given as the minimum requirement.

Another issue is the acclimation phase for the adaptation of the larvae to the test medium. Because some larvae may moult into second instar during an extended length of acclimation, the acclimation phase can be disposed of by hatching egg ropes in the culture medium and maintaining the larvae in it for 24 h. By removing the egg rope 24 h after hatching of the first larvae, the age of larvae can be estimated very accurately.

The use of semistatic renewal and flow-through systems is not mentioned in this proposed guideline, and the recommended method is therefore a static exposure. Generally, toxicity tests can be performed in any of these systems, depending on the stability of the test substance and the duration of the test. Likewise, the *Daphnia* sp. guideline OECD 202 mentions semistatic renewal and flow-through systems explicitly, as does the Japanese guideline for chironomid acute tests (JMAFF 2005). Furthermore, the Guidance document on aquatic toxicity testing of difficult substances and mixtures (OECD 2000), which in principle applies to all OECD toxicity testing guidelines, gives detailed guidance on the use of the different test systems and the corresponding expression of the biological results, e.g., as geometric or arithmetic mean measured concentrations. However, for the short-term static test proposed here, the simplest technical approach is to use the geometric mean in case the test substance dissipates from the water rather than using a semistatic renewal or flow-through system.

Similarly to *Daphnia* sp., chironomids may get trapped at the water surface and might not be able to move back into the water column. This depends mostly on the physicochemical properties of the test substance and/or test medium but can also be related to the cleaning process of the glass vessels. Introduction of the larvae into the test system by releasing them below the water surface is one way to reduce trapping. Because this phenomenon may often result in unacceptable studies (i.e., high control mortality), some guidance on ways to reduce this is discussed here. A possible solution is the addition of a tiny amount of a dispersant, e.g. Tween 80 at 2 $\mu\text{L/L}$, to reduce the surface tension of the medium, although the use of dispersants is not generally advocated. Another way might be physically to keep the individuals from getting to the air–water interface, e.g., by using sealed vessels without air space or using a mesh to retain organisms below the surface.

The development of the present proposal to an OECD guideline would require proper validation and international ring testing, following the criteria set out in the guidance document on validation of new test methods (OECD 2005). This process could be accelerated by wider testing of the reference substances for which data are already available. Thereafter, a validated range for the 48-h EC50 of at least one reference compound should be determined.

CONCLUSIONS

The described guideline proposal for a chironomid acute toxicity test presents a methodology for an additional suitable freshwater invertebrate test species. This can be considered a superior option to testing a saltwater species (i.e., mysid shrimp), which are less relevant in terms of potential exposure to agrochemicals, are much harder to breed and test, and are taxonomically of lower importance, because

crustaceans are already present in the test suite (daphnids). *Chironomus* sp. is a freshwater insect already used in laboratory test methods. For these reasons, *Chironomus* sp. is regarded as a suitable second freshwater test species for insecticides, where *Daphnia* sp. may not be wholly representative of nontarget species sensitivity.

Acknowledgment—The authors are grateful to Ina Müller, Gabriele Janson, Stefanie Bergemann, and Daniela Belz for conducting acute tests at the BASF Ecotoxicology Laboratory; to Peter Dohmen (BASF) for helpful discussions; and to Eric Bruns and Michael Dorgerloh (Bayer CropScience) and Koffi Tassou (University of Landau) for providing data. The European Crop Protection Association (Brussels, Belgium) provided financial support for the preparation of this paper.

REFERENCES

- Bécharé KM, Gillis PL, Wood CM. 2008. Acute toxicity of waterborne Cd, Cu, Pb, Ni, and Zn to first-instar *Chironomus riparius* larvae. *Arch Environ Contam Toxicol* 54:454–459.
- Benoit DA, Sibley PK, Juenemann JL, Ankley GT. 1997. Chironomus tentans life-cycle test: Design and evaluation for use in assessing toxicity of contaminated sediments. *Environ Toxicol Chem* 16:1165–1176.
- [EC] European Commission. 2002. Guidance document on aquatic ecotoxicology in the context of the Directive 91/414/EEC. SANCO/3268/2001, rev. 4 (final), 17.10.2002, 62 p.
- Environment Canada. 1995. Guidance document on measurement of toxicity test precision using control sediments spiked with a reference toxicant. Report EPS 1/RM/30.
- Environment Canada. 1997. Test for growth and survival in sediment using larvae of freshwater midges (*Chironomus tentans* or *Chironomus riparius*). Biological Test Method. Report SPE 1/RM/32.
- Finney DJ. 1978. Statistical methods in biological assay 3rd ed. London (UK). Griffin, Weycombe.
- Fisher RA. 1922. On the interpretation of χ^2 from contingency tables, and the calculation of *P*. *J R Stat Soc* 85:87–94.
- Fleming R, Crane M, van de Guchte C, Grootelaar L, Smaal A, Ciarelli S, Karbe L, Borchert J, Westendorf J, Vahl H, Holwerda D, Looise B, Guerra M, Vale C, Castro O, Gaudencio MJ, van den Hurk P. 1994. Sediment toxicity tests for poorly water-soluble substances. Final Report to the European Commission. Report No. EC 3738. WRC (UK).
- Gauss JD, Woods PE, Winner RW, Skillings JH. 1985. Acute toxicity of copper to three life stages of *Chironomus tentans* as affected by water hardness–alkalinity. *Environ Pollut (series A)* 37:149–157.
- Hill IR, Matthiessen P, Heimbach F, editors. 1993. Guidance document on sediment toxicity tests and bioassays for freshwater and marine environments. Brussels (BE). Society of Environmental Toxicology and Chemistry–Europe.
- Hooftman RN, Adema DMM, Kauffman-van Bommel J. 1989. Developing a set of test methods for the toxicological analysis of the pollution degree of waterbottoms. Report No. 16105, Netherlands Organization for Applied Scientific Research, 68 p.
- Jeyasingham K, Ling N. 2000. Acute toxicity of arsenic to three species of New Zealand chironomids: *Chironomus zealandicus*, *Chironomus* sp. and *Polypedium pavidus* (Diptera: Chironomidae). *Bull Environ Contam Toxicol* 64:708–715.
- [JMAFF] Japanese Ministry of Agriculture, Farming and Fisheries. 2005. Chironomid acute toxicity studies (2-7-6), 3 p.
- Kawai K. 1986. Fundamental studies on chironomid allergy. I. Culture methods of some Japanese chironomids (Chironomidae, Diptera). *Jpn J Sanit Zool* 37:47–57.
- Larrain A, Riveros A, Bay-Schmidt E, Roa R. 1997. Evaluation of three larval instars of the midge *Chironomus petiolatus* as bioassay tools using a computationally intensive statistical algorithm. *Arch Environ Contam Toxicol* 33:407–414.
- Milani D, Day KE, McLeay DJ, Kirby RS. 1996. Recent intra- and inter-laboratory studies related to the development and standardisation of Environment Canada's biological test methods for measuring sediment toxicity using freshwater amphipods (*Hyalella azteca*) and midge larvae (*Chironomus riparius*). Technical Report. Burlington (ON): Environment Canada, National Water Research Institute.
- [OECD] Organization for Economic Cooperation and Development. 2000. Guidance document on aquatic toxicity testing of difficult substances and mixtures. Series on Testing and Assessment 23, Paris (FR).
- [OECD] Organization for Economic Cooperation and Development. 2004a. Guideline for the testing of chemicals 218: Sediment-water chironomid toxicity test using spiked sediment. Adopted 13 April 2004, Paris (FR).
- [OECD] Organization for Economic Cooperation and Development. 2004b. Guideline for the testing of chemicals 219: Sediment-water chironomid toxicity test using spiked water. Adopted 13 April 2004, Paris (FR).
- [OECD] Organization for Economic Cooperation and Development. 2004c. Guideline for the testing of chemicals 202: *Daphnia* sp., acute immobilisation test. Adopted 13 April 2004, Paris (FR).
- [OECD] Organization for Economic Cooperation and Development. 2005. Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment. Series on Testing and Assessment 34, Paris (FR).
- [OECD] Organization for Economic Cooperation and Development. 2006. Detailed review paper on aquatic arthropods in life cycle and two-generation toxicity tests with an emphasis on developmental, reproductive and endocrine disruptive effects. Series on Testing and Assessment 55, Paris (FR).
- Robinson PW, Scott RR. 1995. The toxicity of cyromazine to *Chironomus zealandicus* (Chironomidae) and *Deleatidium* sp. (Leptophlebiidae). *Pest Sci* 44:283–292.
- Stephan CE. 1977. Methods for calculating an LC50. In: Mayer FI, Hamelink JL, editors. Aquatic toxicology and hazard evaluation. Philadelphia (PA). American Society for Testing and Materials. STP 634, p 65–84.
- Strelöke M, Köpp H, editors. 1995. Long-term toxicity test with *Chironomus riparius*: Development and validation of a new test system Mitteilungen aus der Biologischen Bundesanstalt für Land- und Forstwirtschaft, No. 315, BBA, Berlin (DE).
- Sugaya Y. 1997. Intra-specific variations of the susceptibility of insecticides in *Chironomus yoshimatsui*. *Jpn J Sanit Zool* 48:345–350.
- Taenzler V, Bruns E, Dorgerloh M, Pfeifle V, Weltje L. 2007. Chironomids: suitable test organisms for risk assessment investigations on the potential endocrine disrupting properties of pesticides. *Ecotoxicology* 16:221–230.
- Tassou K, Schulz R. 2009. Effects of the insect growth regulator pyriproxyfen in a two-generation test with *Chironomus riparius*. *Ecotoxicol Environ Saf* 72:1058–1062.
- [USEPA] U.S. Environmental Protection Agency. 1975. Methods for acute toxicity tests with fish, macroinvertebrates and amphibians. Washington DC: EPA report 660/3-75-009.
- [USEPA] U.S. Environmental Protection Agency. 1996a. Whole sediment acute toxicity invertebrates. U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances. Washington DC: OPPTS 850.1735.
- [USEPA] U.S. Environmental Protection Agency. 1996b. Chironomid sediment toxicity test. U.S. Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances. Washington DC: OPPTS 850.1790.
- [USEPA] U.S. Environmental Protection Agency. 2000. Methods for measuring the toxicity and bioaccumulation of sediment-associated contaminants with freshwater invertebrates 2nd ed. EPA 600/R-99/064. Washington DC: USEPA.
- Watts MM, Pascoe D. 2000. A comparative study of *Chironomus riparius* Meigen and *Chironomus tentans* (Diptera: Chironomidae) in aquatic toxicity tests. *Arch Environ Contam Toxicol* 39:299–306.
- [WHO] World Health Organization. 2005. Guidelines for laboratory and field testing of mosquito larvicides. Geneva (CH) WHO/CDS/WHOPES/GCDPP/2005.13, 41 p.
- Williams KA, Green DWJ, Pascoe D, Gower DE. 1986. The acute toxicity of cadmium to different larval stages of *Chironomus riparius* (Diptera: Chironomidae) and its ecological significance for pollution regulation. *Oecologia* 70:362–366.
- Yokoyama A, Ohtsu K, Iwafune T, Nagai T, Ishihara S, Kobara Y, Horio T, Endo S. 2009. A useful new insecticide bioassay using first-instar larvae of a net-spinning caddisfly, *Cheumatopsyche brevilineata* (Trichoptera: Hydropsychidae). *J Pestic Sci* 34:13–20.