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Long-term toxicity test with *Chironomus riparius*: Development and validation of a new test system

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Preface and Acknowledgements

Plant protection products can reach surface waters via several exposure routes but usually via the water surface. It is known from fate studies in water/sediment systems that several active ingredients partition into sediments. As the exposure of sediment organisms differs from that of inhabitants of the water column, the risk assessment for the relevant substances is incomplete. This gap should be closed by the method described herein, thus allowing our ecologists to perform a refined risk assessment for all important parts of surface waters.

This booklet is an excellent example how the "players" in the registration procedure of plant protection products can act together successfully. As with the German spray drift project which was presented in a previous publication of this series, I believe that the knowledge of industry, contract laboratories and authorities should be combined over the whole process of developing a new guideline. Because regulators of the BBA (Biologische Bundesanstalt für Land- und Forstwirtschaft, Federal Biological Research Centre for Agriculture and Forestry) were also involved in the practical scientific work, the implementation of the outcome of this project into the registration procedure is simplified.

The BBA is the registration authority for plant protection products in Germany. With respect to environmental issues we have to identify possible risks of the use of plant protection products, and the sediment organism test serves as a tool to get a more precise picture of the toxicity profile of a substance. This enables us to do a much better job on our most important task that is to generate appropriate risk management measures that can be observed under practical farming routine. To meet this objective, the staff of the BBA comprises scientists familiar with the practical problems of plant protection measures and those who are experienced in ecotoxicology. These scientists are organisationally and locally closely connected. Additionally, we have strong links with the extension services which are distributed all over Germany with permanent contacts to the farmers.

I appreciate very much that scientists from other European countries collaborated in this project and contributed to this booklet. Today, the development of test guidelines in the field of the registration procedure of plant protection products clearly is an international business. Much of this work is done through OECD, with strong support from the European Commission and many national governments and experts. The draft guideline presented here will be submitted to OECD in the hope that it will contribute to the international sharing of burden.

Hence, I wish to express my particular thanks to all colleagues who contributed scientific knowledge, time and laboratory resources to this joint effort to develop an idea into a working test method. The following individuals served as members of the working group and/or as authors of this booklet:
Katie Barrett, Liesel Buhr, Peter Dohmen, Annette Fliedner, Michael Hamer, Wolfgang Heger, Fred Heimbach, Rainer Heusel, Ian Hill, Herbert Köpp, Axel Mueller, Hans Toni Ratte and Martin Streloke. I am also indebted to all participants of the ring tests which are listed in the article of Fred Heimbach. The test substances were generously supplied by DowElanco and Rhône-Poulenc.

Braunschweig, November 1995

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History and Background of the Sediment Toxicity Test

Abstract

Standard testing on aquatic toxicity employs pelagic organisms in a water-phase exposure test design. However, substances which are readily absorbed by particulate matter and sediment particles and thus partition into the sediment may be of more concern to sediment organisms which can be exposed to such substances via several different routes (e.g. by contact to or ingestion of loaden particles or through the pore water). The increasing importance of such compounds in plant protection and the lack of methods to accurately predict exposure of benthic organisms triggered the development of an appropriate test design by a task force of Germany's regulatory agencies and agrochemical industry.

Zusammenfassung

Die Standardtests zur aquatischen Ökotoxikologie arbeiten mit pelagischen Organismen, die der gelösten Testsubstanz ausgesetzt werden. Wirkstoffe, die stark an Schwebstoffe und Sedimentpartikel adsorbieren und daher vorwiegend in das Sediment verlagert werden, stelle jedoch möglicherweise ein höheres Risiko für benthische Organismen dar, da diese auch über andere Wege (z.B. Kontakt mit oder orale Aufnahme von Partikeln mit adsorbierten Wirkstoffen oder durch das Interstitialwasser) exponiert sein können. Wegen der zunehmenden Bedeutung solcher Wirkstoffe im Pflanzenschutz sowie fehlender Methoden zur Vorhersage der Exposition im Sediment hat eine gemeinsame Arbeitsgruppe von Behörden (Biologische Bundesanstalt für Land- und Forstwirtschaft [BBA]; Umweltbundesamt [UBA]) und Pflanzenschutzmittelherstellern seit 1992 ein geeignetes Testsystem entwickelt.

Introduction

Germany's current Plant Protection Act entered into force in 1986, replacing the one from 1968. Among other changes, the new act put more emphasis on the environmental aspects of the use of plant protection products, thus asking the regulatory authorities for a more

sophisticated environmental hazard assessment. The implementation of the new act into regulatory practice lead to more data requirements, mainly with regard to the environmental fate and ecotoxicological effects of plant protection products. In the area of aquatic ecotoxicology, the standard data set, which until then consisted of the acute Daphnia and fish toxicity test, was extended to include also the sub-chronic fish toxicity test, the Daphnia reproduction study and the algal test (BBA 1990 a). All these test organisms live in the water column, thus being exposed mainly via their body surfaces to substances in solution.

Since 1986, the authorized registration for many pesticidal compounds has expired (at maximum, the authorization can be granted for 10 years) and had to be applied for again under the new requirements. Thus, many new data concerning environmental fate and ecotoxicology of plant protection products became available in the late 1980's, rapidly increasing the knowledge on these issues. One of the consequences was the sometimes rather vigorous discussion on the leaching of certain compounds.

At the same time, and possibly encouraged by the leaching issue, new active substances were being developed and submitted for authorization, many of them belonging to the chemical group of triazole fungicides. Their common properties which they shared (more or less) with the sulfonylurea herbicides, the pyrethroid insecticides and some other active substances or their metabolites included:

* rather long half-lives in soil and
* a high tendency to adsorption, thus showing only little if any mobility in soil.

Both triazol fungicides and pyrethroids and also other stable coumpounds were further intended for several applications per season. The increasing number of compounds with such characteristics pointed at some gaps in the evaluation process which are briefly described below and which needed to be addressed.

Fate and distribution of chemicals in water bodies

Once a chemical enters a surface water, for example by spray drift or surface runoff after agricultural use, it is subjected to several processes which strongly influence the exposure of organisms (Fig. 1; BURTON 1991). These processes can be divided into **transport** and **transformation mechanisms.** The latter can be divided further into chemical (e.g. hydrolysis), photochemical and biological transformation. Transport processes include volatilization, advection, diffusion, adsorption to surfaces and particulate matter and subsequently sedimentation to the bottom of the water body. Furthermore, compounds which strongly

adsorb to particulate matter are effectively protected against degradation and persist much longer than in overlying water (COTHAM & BIDLEMAN 1989).

Aquatic exposure scenarios

The water-column exposure scenario which was used at that time for the aquatic hazard assessment had to be adapted to deal adequately with these substances and to take account of the increased knowledge.

Water-column exposure scenario: In the late 1980's, exposure for water organisms was calculated only for the water column. This, together with the design of the standard aquatic toxicity tests, implied that only uptake from solution through body surfaces was addressed. A PEC (Predicted Environmental Concentration) was calculated using a given input from a crude overspray and spray drift model and assuming homogenous distribution within a water column with a depth of 30 cm. Degradation was considered if this PEC exceeded the NOEC/LOEC. Adsorption to particulate matter and sediment was only taken into account for some pyrethroids where toxicity tests with fish and daphnids with spiked sediment (to simulate both adsorption and runoff) were submitted by industry. However, such tests were designed to show that under real-world conditions toxicity to water-column organisms would be less than expected from the standard laboratory test (in clean water) (HILL 1985).

Exposure for sediment organisms: Substances with a strong tendency to adsorb to particles are removed from solution by adsorption (ideally until the stage of equilibrium partitioning is reached), thus reducing the exposure for water column organisms. The laden particulate matter is subjected to sedimentation and can potentially result in exposure of sediment-dwelling organisms. When bound to the sediment, such substances can further be protected against degradation and may be present in the sediment for long periods which easily exceed the life span of certain benthic organisms. Frequent application of many of these compounds can not only further prolongue their presence in the sediment but may also lead to a build-up of the concentration in the sediment over time during the spraying season, depending on the half-life of the chemical and the application frequency. The sediment serves as a sink for these substances, but also as a reservoir from which chemicals can be remobilised by resuspension of the sediment and by desorption in order to reach equilibrium again with the concentration in the water phase (CLARK et al 1989).

For sediment organisms, there are more possible routes of uptake (Fig. 2; POWER & CHAPMAN 1992) than for water column organisms. They can be exposed via their body surfaces to substances in solution in the overlying water and in the pore water, to bound

substance by direct contact or by ingestion of laden particles. Whichever of these routes is the most important is strongly influenced by compound properties, sediment properties (i.e. particle-size distribution, organic carbon content) and by species-specific feeding mechanisms and mode of existence (e.g. habitat preference and burrowing habits) in or on the sediment (ADAMS 1984). Instead of one predicted environmental concentration (the substance in solution for the water column organisms), the overall exposure of sediment dwellers may in principle be composed of up to four different PEC's. For certain stable substances, the exposure for sediment organisms is likely to be long-term rather than short-term. However, it must be noted that high peaks of short-term exposure can also occur, i.e. to the overlying water during the initial phase of distribution and adsorption.

For the time being, an accepted model for the calculation of these different PEC's and their change in time is still not available. The equilibrium partitioning approach alone is only considered adequate as a trigger for further testing, as was stated by the participants of the SETAC Workshop on Sediment Toxicity Assessment (HILL et al 1994).

Due to these rather complicated exposure, it is not appropriate to address sediment toxicity with water column organisms or with elutriate or pore water tests with benthic species (ANKLEY et al 1991). Thus, considering the ecological importance of benthic organisms for aquatic ecosystems they had to be included into the hazard assessment on their own right.

Regulatory implications

All these issues were not adequately dealt with in an aquatic exposure scenario and testing requirements that were designed for the water phase only (both environmental fate and ecotoxicology). Thus, the BBA took two steps to address these aspects:

Firstly, starting in 1989 a guideline for testing the distribution and degradation of active compounds in a water/sediment system (BBA 1990 b) was developed in co-operation with the UBA (Federal Environmental Agency) and the Technical University of Aachen. This test became obligatory in 1990 (BBA 1990 a) for every active substance which was not readily biodegradable. It was designed not only to evaluate a chemical endpoint like a DT90 but also specifically to provide important data for the aquatic hazard assessment by measuring how fast the substances adsorb to the sediment and disappear from the water column. These data had been identified as crucial for the determination of PEC's.

Secondly, in 1991, BBA required sediment toxicity tests for some very stable compounds. However, the tests methods which were available at that time had been developed in the context of sediment quality assessment, typically using field samples of contaminated sediment and testing either the sediment as a whole or elutriate water (BURTON 1992). They were designed to be used in a **retrospective** evaluation of chemicals already present in the sediment, not in a **predictive** hazard assessment for plant protection products before their authorization and use. For our purpose, it was important to design the test as close to reality as possible to avoid problems in calculating PEC's for the different routes of exposure. Therefore, the test was required to include a spray drift scenario by applying the test substance to the water column. Thus, the initial phase of distribution of the substance in the system could be included in the overall exposure of the test organisms. Further, it should be designed for long-term exposure.

In response to these requirements, a task force of agrochemical industry, BBA (Biologischen Bundesanstalt für Land- und Forstwirtschaft; Federal Biological Research Centre for Agriculture and Forestry) and UBA (Umweltbundesamt; Federal Environmental Agency) started working on the development of a suitable test protocol in January 1992. The results of this joint activity are presented in the following chapters of this booklet.

In the meantime, the necessity of sediment toxicity tests for predictive hazard assessment for plant protection products has achieved widespread acceptance, as can be concluded from the growing number of publications on this issue (for excellent review, see BURTON 1991). Further, a sediment toxicity test has been included as a standard data requirement in Annex II of the EU directive 91/414/EEC which harmonizes the authorization of plant protection products in the EU. The same regulation adopted the requirement concerning the fate study in water/sediment systems as obligatory for compounds which are intended for outdoor use.

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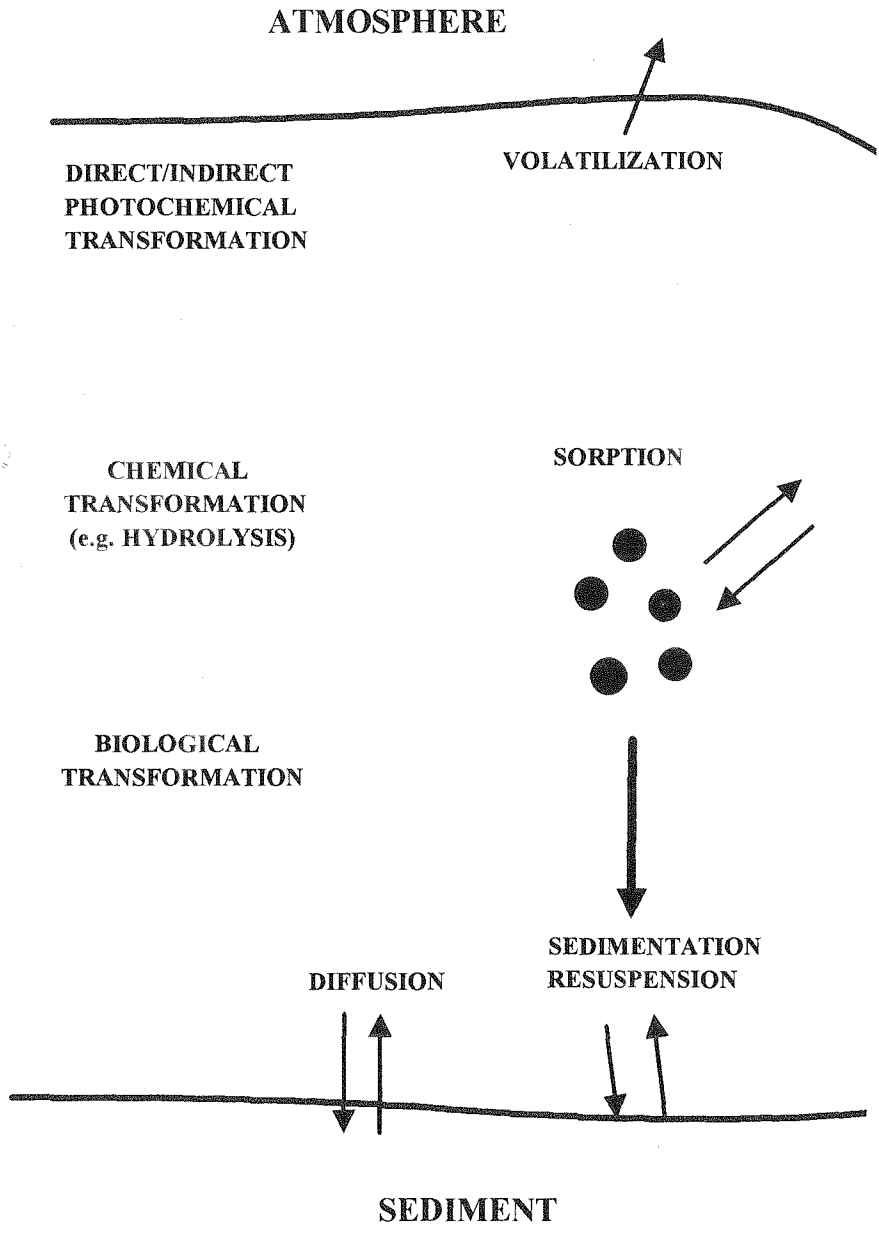
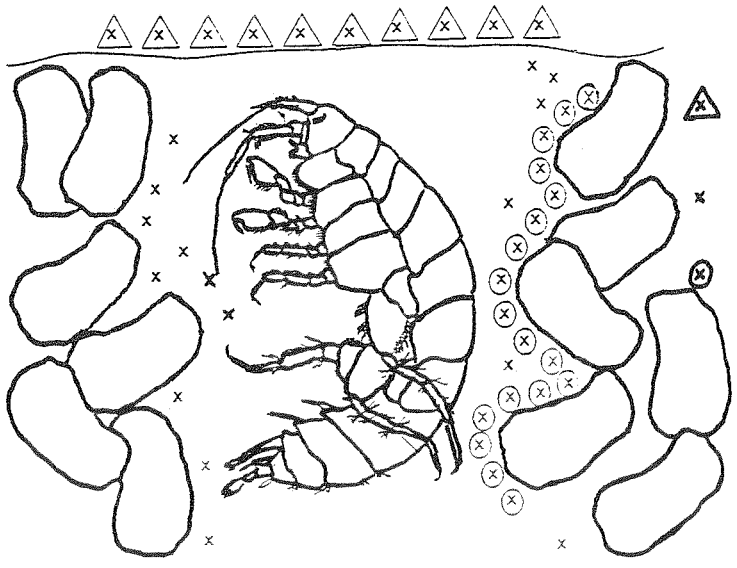
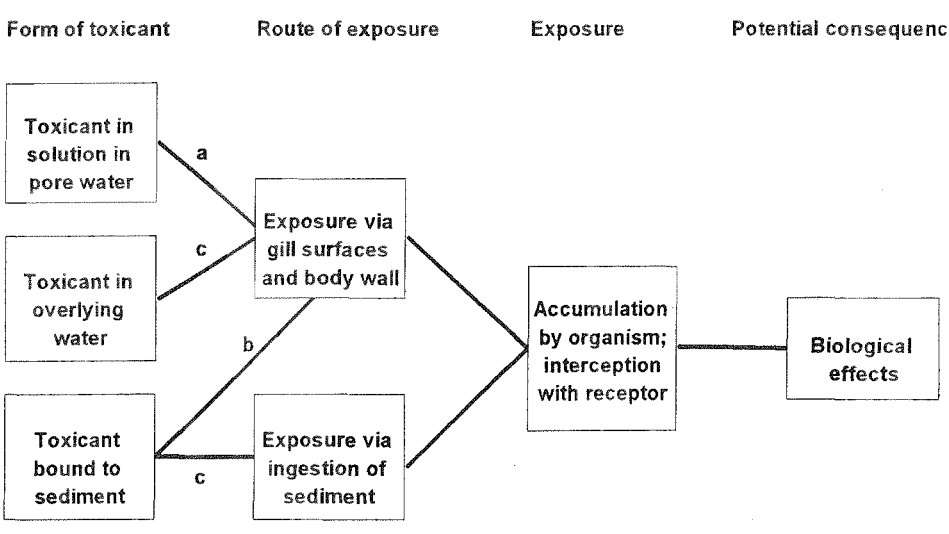


Figure 1: Fate and distribution processes in water bodies





a **Expected major route of uptake**

b **Not a major route of direct uptake; often estimated by normalization of bulk sediment contaminant data (e.g., total organic carbon [TOC] and acid volatile sulphides [AVS]**

c **Route of exposure; relative importance subject to debate and/or case-specific**

Figure 2: *Upper*: Schematic representation of benthic organism in sediment and routes of exposure; *Lower*: Interactions of contaminated toxic sediments with benthic organism. [Adapted from POWER & CHAPMAN (1992)]

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International Toxicity Ring-Test on Sediment Dwelling *Chironomus riparius* - Preliminary Small-Scale Test -

Summary

After the development of a sediment toxicity test had been initiated in 1992, a small-scale ring test was performed in 1993 by five German and UK laboratories. Emergence studies were conducted, exposing *Chironomus riparius* larvae to formulated products of both lindane and trifluralin in sediment-water systems using both natural and articial sediments. In addition, acute water-only tests were carried out. Results showed consistency among laboratories and similarity for the different sediments. Therefore both test design and the culturing method seemed robust enough to initiate a wider-scale international ring test.

Zusammenfassung

Im Rahmen der 1992 begonnenen Entwicklung einer Testmethode mit benthische Chironomidenlarven führten 1993 einige deutsche und britische Labors einen ersten Ringtest durch. Die Testsysteme wurden mit zwei handelsfertigen Pflanzenschutzmitteln mit den Wirkstoffen Lindan bzw. Trifluralin behandelt, wobei sowohl natürliches als auch künstliches Sediment eingesetzt wurde. Akute Toxizität wurde auch ohne Sediment bestimmt.
Die Ergebnisse der beteiligten Labors sowie für die verschiedenen Sedimente waren gut vergleichbar, sodaß die Testmethodik geeignet erschien, um in einem größeren internationalen Ringversuch weiterentwickelt zu werden.

Introduction

In 1992, in response to concerns from the Federal Biological Research Centre for Agriculture and Forestry [Biologische Bundesanstalt für Land- und Forstwirtschaft (BBA)] in Germany regarding the toxicity of some pesticides to sediment-dwelling organisms, representatives from German federal research and regulatory authorities together with the pesticide industry formed a working group to develop a suitable test method (BARRETT & DOHMEN 1992). Larvae of the midge *Chironomus riparius* were chosen as the test species, with application of the

chemical as the formulated product to the water phase of a sediment-water system. During 1993, a small-scale ring-test was undertaken by laboratories in Germany and the UK to evaluate the method as a preliminary to a wider-scale international ring-test. The method and results of the small-scale ring-test are discussed here.

Test Method

The test methods were described in two test protocols, one per test chemical. The methods were essentially the same as those of the international ring-test (HEIMBACH 1995) and used the same test chemicals, EC formulations of lindane and trifluralin. Six laboratories (Bayer, BASF, AgrEvo and BBA in Germany and AgrEvo and Zeneca in the UK) took part in this small-scale test. For both chemicals, each laboratory was asked to first conduct an acute toxicity test using first instar *Chironomus riparius* larvae in water alone, in order to aid selection of concentrations for the sediment-water toxicity test. The sediment-water toxicity test was conducted using both an artificial and natural sediments. Details of the preparation of artificial sediment are given in the protocol for the international ring-test (HEIMBACH 1995).

The concentrations applied to the water in the sediment-water tests were:

Lindane: 1600, 400, 100, 25 and 6.3 µg formulation/l
(although some tests were done using 25, 6.3 and 1.6 µg/l)

Trifluralin: 64, 16, 4, 1, 0.25 mg formulation/l

Analysis of Results

EC50 values were calculated from the acute test in water-alone. For the sediment tests, no observed effect concentrations (NOECs; p=0.05) were generated (in comparison to control organisms) for the total number of emerged midges (using a χ^2-test) and the mean emergence time (using the U-test, one-sided) in comparison to control organisms. In addition, an EC50 for total emergence was estimated by graphical interpolation.

Results

(a) Water only 48hr LC50 Tests

Five laboratories conducted a total of 13 tests with lindane, producing LC50s in the range 35-202 µg/l. Two of the tests were rejected due to control mortalities of 30 and 40%. In the remaining tests control mortality was in the range 0-15%.

Five labs conducted a total of 9 tests with trifluralin, 5 tests gave LC50s in the range 1.7-4.9 mg/l. Two tests gave values of 0.19 and 0.22 mg/l and another 66 mg/l, but these were considered outliers when evaluating the data for the determination of exposure levels in the sediment/water systems. One test was rejected due to control mortality of 47%, whilst in the remaining tests control mortality was in the range 0-16%.

(b) Sediment-water Toxicity Tests

The results received from the participating laboratories are summarised in Tables 1 and 2. One test failed to fulfil the control emergence criteria (70%) and was omitted from the results. Graphical representations of the results are shown in Figures 1 and 2.

Generally, the results from the participating laboratories were in good agreement as is apparent from Figures 1 and 2. The mean emergence time in the controls varied between 14 and 19 days. With both chemicals there was a tendency for total emergence to decrease and the mean emergence time to increase with increasing concentration.

Lindane gave NOECs ranging from <1.6 - 25 µg/l, although the biological response recorded by each laboratory was essentially the same, with only one outlying result (see Figure 1). The EC50s based on total emergence also reflected the similarity of the data. Ten out of the eleven valid results gave EC50s in the range 30-60 µg/l (including one laboratory, which only tested up to 25 µg/l giving an EC50 >25 µg/l), with the other giving an EC50 of 15 µg/l.

The trifluralin results showed more variability in the pattern of emergence, see Figure 2. NOECs ranged from 1-16 mg/l and EC50s from 9 - >64 mg/l. This greater variability is possibly associated with the fact that effect concentrations were greater than the water solubility of the active ingredient. However the results may not be as variable as they seem on first inspection. Six out of the ten EC50s fell in the range 15-38 mg/l. The 3 results where the concentration response was markedly different (giving EC50s >64 mg/l) were all from the same laboratory.

Table 1 : Summary of Lindane Results

Lab	Sediment Type	NOEC total emergence (µg/l) *	NOEC emergence time (µg/l) *	EC50 total emergence (µg/l) *
1	natural	25	25	55
	artificial	25	6.3	60
2	artificial	25	6.3	60
3	natural	25	6.3	40
	"	6.3	25	55
	"	6.3	25	30
4	natural	6.3	6.3	35
	"	6.3	<1.6	60
	artificial	6.3	6.3	45
	"	>25	<1.6	>25
5	natural	6.3	6.3	15

* concentrations given as µg formulation/l

Table 2 : Summary of Trifluralin Results

Lab	Sediment Type	NOEC total emergence (mg/l) *	NOEC emergence time (mg/l) *	EC50 emergence (mg/l) *
1	natural	16	4	38
2	artificial	16	4	38
3	natural	16	16	>64
	"	?[a]	?[a]	>64
	"	16	16	>64
4	natural	4	1	35
	"	4	1	9
	artificial	4	1	38
6	natural	1	1	25
	artificial	4	1	15

[a] inconsistent results, total emergence significantly lower at 0.25, 1 and 64 mg/l, but not at 4 and 16 mg/l

* concentrations given as mg formulation/l

Comparison of Toxicity Results in Natural and Artificial Sediments

One of the purposes of the small-scale ring-test was to evaluate the artificial sediment by comparison of the results with those of natural sediments. The data shown in Tables 1 and 2 clearly indicate that whilst there is some variation in results, there is no distinction between results obtained using natural or artificial sediments. All laboratories prepared the artificial sediment to the same recipe (HEIMBACH 1995), however no specifications were given for the natural sediments and although they were all obtained from freshwater environments, those sediments for which data are available showed a wide range in particle size distributions and organic matter contents (see Table 3). The similarity in the toxicity results obtained is reflected in the similar results obtained in studies comparing the distribution of ^{14}C-labelled pesticides between the sediment and water phases using natural and artificial sediments (BARRETT 1995).

Table 3 : Particle Size Distribution and % Organic Matter of Natural Sediments

Lab	Particle Size Distribution			% Organic Matter
	sand	silt	clay	
1	20	39	41	13.1
3	n/a[a]	n/a	n/a	n/a
4	39	55	6	3.6
5	99.9	0.04	0.01	0.2
6	86	0	14	0.6

[a] not available

Conclusions

The results of the preliminary small-scale ring-test showed consistency between laboratories and indicated that the test was likely to be robust enough to be used as a standard method for assessing the toxicity of pesticides to sediment dwelling organisms. This led to the proposal from the working group that an organising committee be set up to organise a wider international ring-test. Similar test results were obtained from a variety of natural sediments with a range of particle sizes and organic matters and also an artificial sediment. In the interests of standardisation, it was therefore proposed that the subsequent international ring-test should be conducted using artificial sediment only.

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A Comparison of the Partitioning of Pesticides in Natural and Artificial Sediment Water systems.

Summary

During the development of a test method to assess the impact on sediment-dwelling organisms of pesticides entering the water column (BARRETT & DOHMEN 1992), the possibility of using an artificial sediment and the need for analytical support of such a study were considered.

As part of the method development, a study was conducted using five different radiolabelled pesticides. The pesticides were applied to the water column of both natural and artificial sediment water systems, at a rate equivalent to that applied in previous environmental fate studies.

At timed intervals after treatment units were taken for analysis to determine the distribution of radioactivity.

The results showed the behaviour of the products tested to be sufficiently similar for the intended purpose of this method, in both the artificial and natural sediments, and similar to that observed in independent environmental fate studies.

Zusammenfassung

Bei der Entwicklung einer Testmethode zum Einfluß von Pflanzenschutzmitteln auf benthische Organismen (Chironomidenlarven) wurde auch untersucht, ob künstliches Sediment für diese

Versuche eingesetzt werden kann und welche Analysen dann erforderlich sind. Dazu wurden fünf radioaktiv markierte Wirkstoffe jeweils auf die überstehende Wassersäule von Testsystemen mit natürlichem und mit künstlichem Sediment appliziert. Die Dosierung entsprach dabei derjenigen in vorhandenen Studien zu Verteilung und Verbleib der Wirkstoffe in Wasser/Sediment-Systemen (ohne Makroorganismen). Zu festgelegten Zeitpunkten erfolgten Probenahmen zur analytischen Kontrolle der Radioaktivitätsverteilung.

Die Ergebnisse zeigten ein ähnliches Verhalten der untersuchten Wirkstoffe in allen Testsystemen (sowohl mit Chironomiden in natürlichem bzw. künstlichem Sediment als auch in den davon unabhängigen Verbleibstudien ohne Makroorganismen).

Introduction

Pesticides may enter surface waters by for example, spray drift. Products which are not readily degraded and which may also be adsorbed to sediments are of particular concern. There is a need for a reliable, reproducible test system for evaluating the effects of such products on sediment dwelling organisms. During the development of a protocol for a suitable test method, the option of using an 'artificial' sediment was considered. This would have several advantages over natural sediments:

- it would form a reproducible "standardised matrix", which would hopefully reduce experimental variability
- it would remove the need to find 'uncontaminated clean' sediment sources, for such studies
- with a standardised sediment being used in all studies this would allow some comparison and ranking of products, useful for regulators reviewing data
- studies could be initiated at any time without encountering seasonal variability in the test sediment
- there would be no need to pre-treat the sediment to remove indigenous fauna.

However it is important to know if products would demonstrate the same partitioning behaviour in the presence of the artificial sediment as demonstrated with natural sediments under the conditions of the proposed test. This was also an important factor when considering the need for analytical support on such a study. Since if the partitioning pattern was similar to that observed in natural water systems it was proposed that the results of natural sediment water fate studies may be extrapolated to predict the relative distribution of a product.

Methods

Sediment-water systems were prepared using natural sediment, 2 mm sieved and frozen at -18°C for a minimum of 48 hours, and artificial sediment prepared in accordance with OECD guideline 207 (OECD 1984). The freezing of natural sediments has proved to be an efficient method of removing indigenous fauna prior to use in toxicity tests (THIRKETTLE & BARRETT 1994).

Details of the physical and chemical properties of the sediments used are included in Table 1.

Table 1. Sediment Physical Chemical Properties

TEST SUBSTANCE	SEDIMENT CHARACTERISTICS						
	pH	CEC	% Org. C	Sand %	Silt %	Clay %	BBA Class
Diuron	6.40	13.31	2.10	38.7	55.50	5.80	Sandy silt
Epoxyconazole	7.90	0.80	0.30	90.0	0	10.0	Clayey sand
Isoproturon	7.40	0.92	0.09	99.9	0.02	0.01	Sand
Lindane	7.50	30.50	8.41	19.0	44.00	37.00	Sandy clay loam
Prochloraz 1.	8.20	3.00	0.17	97.0	1.00	2.00	Sand
2.	7.90	37.00	8.24	15.0	51.00	34.00	Silty clay loam
All (Artificial Sediment)*	7.50	11.70	4.23	70.0	1.43	28.57	Sandy clay

* Figures from one representative sample analysed as natural sediments

Each system comprised ca. 2.0 cm sediment with 20 cm of overlaying reconstituted water (ELENDT & BIAS 1990) in a glass beaker of ca. 13.0 cm diameter. Gentle aeration of the units was provided 2.5 cm above the sediment layer. These were allowed to settle and acclimate for 7-14 days, before addition of 25 first instar larvae (1-3 days after hatching), of the midge *Chironomus riparius*. These were fed daily on a diet of tetramin 1 mg/larva fed as a fine suspension in water.

Twenty-four hours after addition of the midge larvae the systems were treated with one of the test chemicals as detailed in Table 2. The test chemicals used in this study all had medium to high log K_{ow} values, between 2.0 and 4.5 and are relatively stable in aqueous environments. They were selected as it was considered they represented the class of compounds which may warrant this type of investigation.

Table 2. Test products

	CHEMICAL	CLASS	RELATIVE K_{ow}	APPLICATION RATE* MG/L
1	Diuron	Herbicide	medium	0.10
2	Epoxyconazole	Triazole fungicide	high	0.063
3	Isoproturon	Herbicide	medium	14.5
4	Lindane	Insecticide	high	0.0063
5	Prochloraz	Triazole fungicide	high	0.195

* Initial concentration in overlaying water.

For the purpose of this study ^{14}C-radiolabelled technical test material was used. It was applied to the water columns in a small volume of a carrier solvent.

The units were incubated at 20 ± 2°C with a 16:8 light:dark photoperiod.

At time intervals after treatment, 6 hours, 2, 7 and 14 days and at the end of the study (21-38 days), units were taken to determine the distribution of radioactivity in the overlaying water (OLW), interstitial water (ISW) and sediment (SED). The interstitial water fraction was extracted from the sediment by filtration under vacuum. The total recovery from the units (TOT) was also determined, from combining the radioactivity in the individual fractions.

Radioactivity in the overlaying and interstitial water was determined by liquid scintillation counting. The residue remaining in the sediment was determined by combustion, and liquid scintillation counting of evolved carbon dioxide.

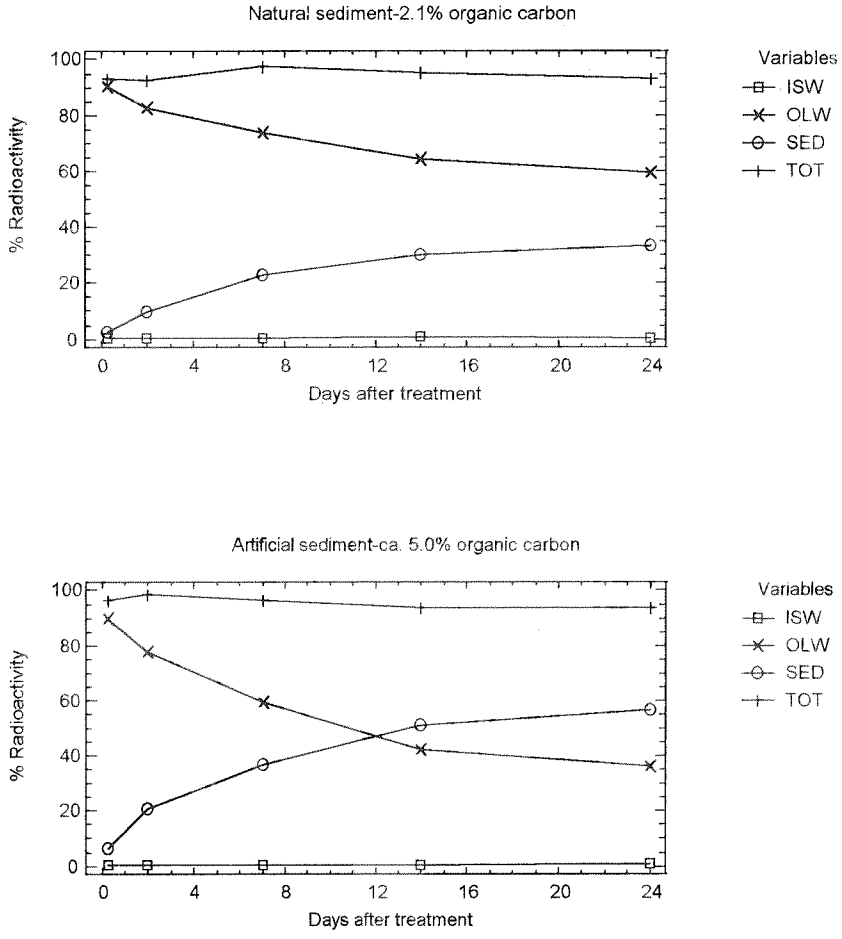
Results

The distribution of radioactivity in overlaying and interstitial water and sediment are presented graphically for each compound in artificial and natural sediment. The graphs show the pattern of distribution for a given compound to be similar, with greater adsorption to the higher organic matter sediments.

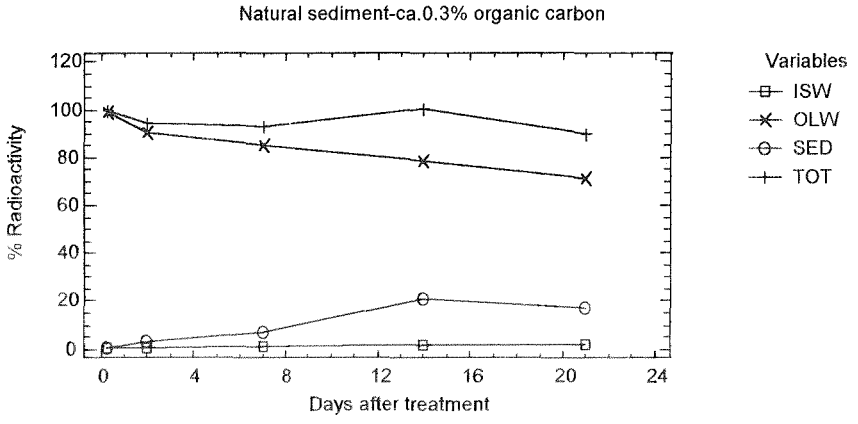
Recovery of radioactivity for four of the compounds used was good remaining close to 100% on all sampling occasions. Radioactivity recovery from the ^{14}C-Lindane treated units declined over time, this was considered to be due to degradation and mineralisation of the compound to CO_2.

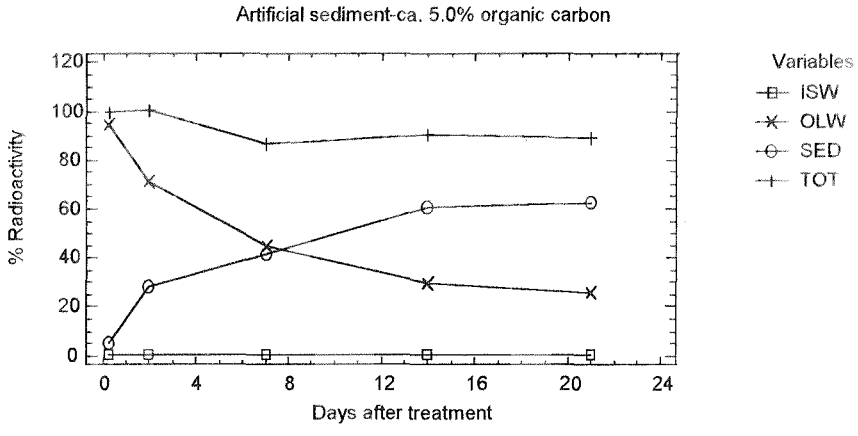
Fig. 1: Distribution of radioactivity in sediment water systems

a) Diuron

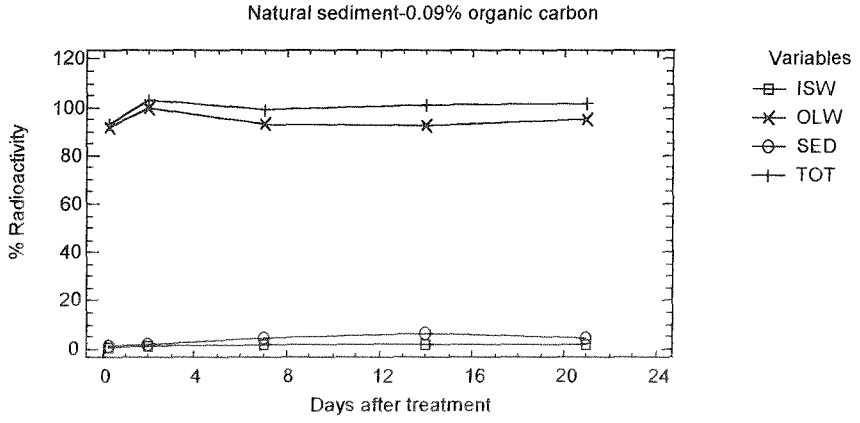


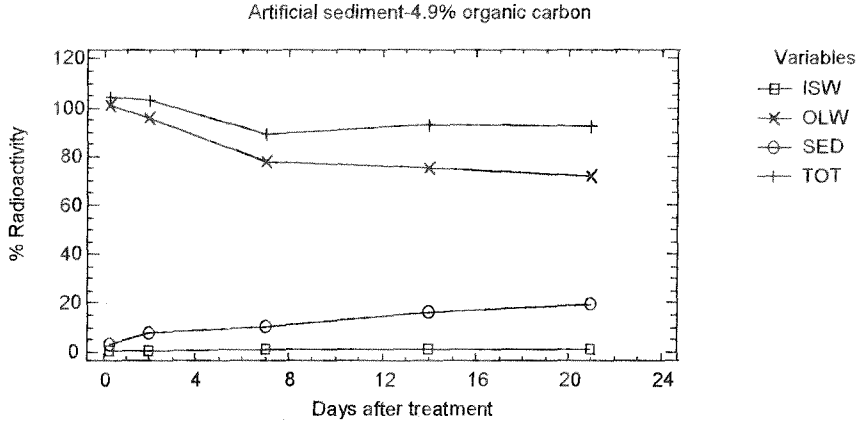
b) Epoxyconazole



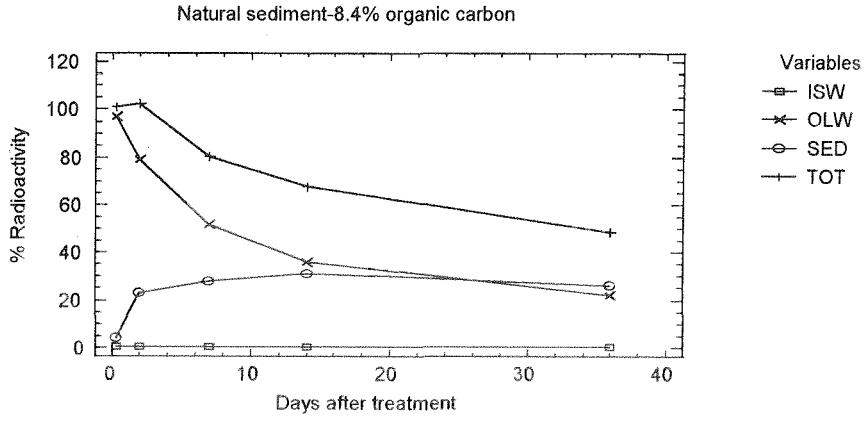


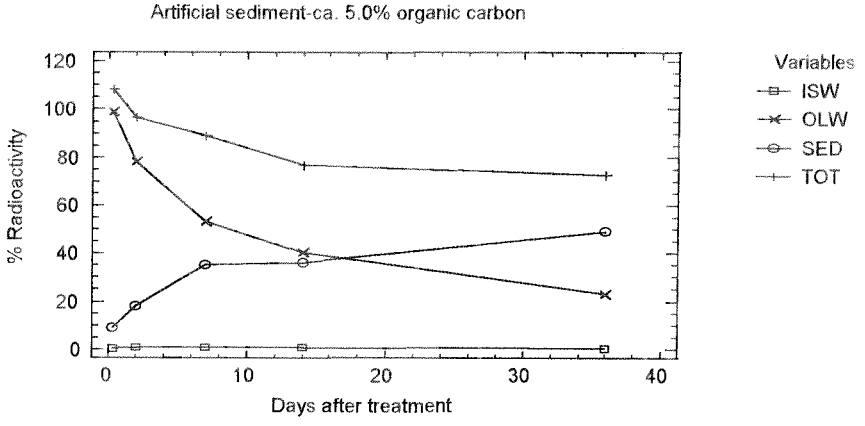
c) Isoproturon



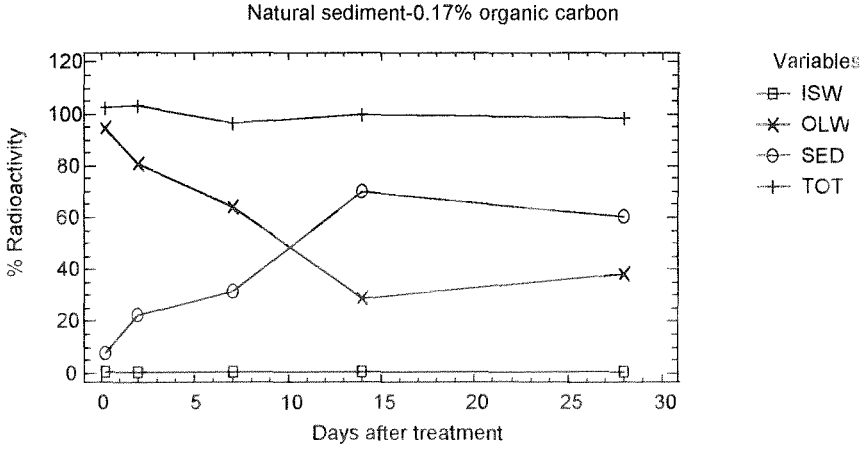


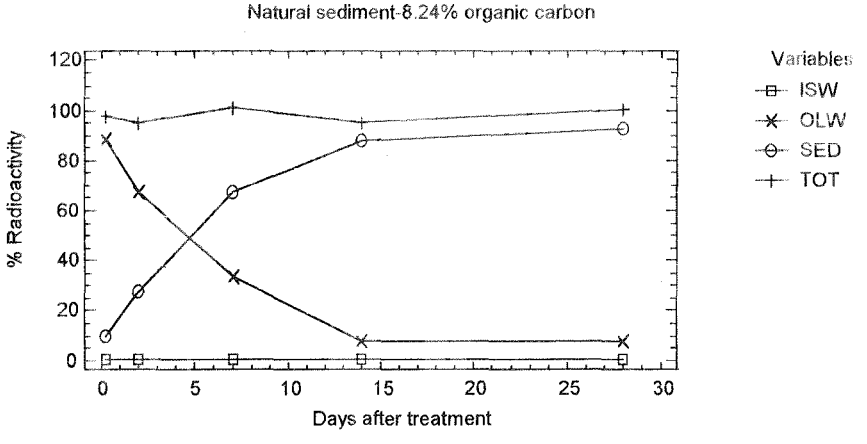
d) Lindane

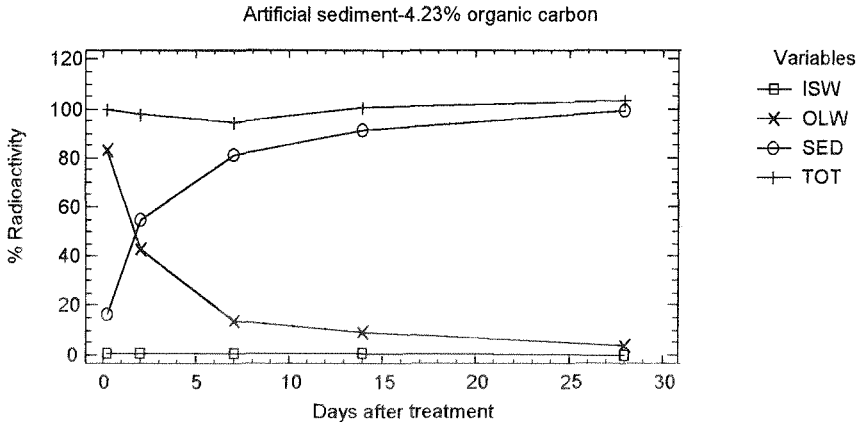

Natural sediment-8.4% organic carbon
% Radioactivity
120
100
80
60
40
20
0
0
10
20
30
40
Days after treatment
Variables
ISW
OLW
SED
TOT


Artificial sediment-ca. 5.0% organic carbon
% Radioactivity
120
100
80
60
40
20
0
0
10
20
30
40
Days after treatment
Variables
ISW
OLW
SED
TOT

e) Prochloraz







Conclusions and Discussion

The results of these studies indicate the behaviour of the compounds in the artificial and natural sediment water systems was sufficiently similar for the intended application of this method. The natural sediments used came from a variety of sources and varied in organic matter content. As expected the partitioning observed in the artificial sediment-water systems (organic carbon content ca. 5%) mimics that observed in the higher organic matter natural sediment water systems. It was also similar to the distribution observed in smaller scale sediment water fate studies carried out previously with some of the compounds. As expected the compounds with the higher K_{ow} values demonstrated the greatest affinity for the sediment.

The concentrations of the substances in interstitial water of the sediments with a low organic carbon content were higher than in the interstitial water of sediments with higher organic carbon content, however, in all systems it was significantly lower than the overlaying water concentration.

In conclusion, the artificial sediment appears to offer an acceptable alternative to natural sediment for toxicity evaluations to sediment dwelling organisms. With a comparable pattern of distribution within water/sediment systems with sediments of a similar organic carbon content. This may also be compared with data generated in small scale fate studies, hence reducing the need for additional analytical support.

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Mr Mick Hamer, Zeneca
Dr Rainer Heusel, AgrEvo GmbH

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Organisation, Methodology and Participation of the International Toxicity Ring-Test on Sediment-Dwelling *Chironomus riparius*

Summary

In 1994, an international ring-test was performed on sediment-dwelling larvae of the midge *Chironomus riparius*. In this test, the test substance was spiked into the overlying water of a water-sediment system to simulate the common routes of exposure of sediment-dwelling organisms to pesticides. For the test, a 2 cm layer of an artificial sediment was overlaid by 15 - 20 cm synthetic or natural water. One day before application, a defined number of first instar larvae were added to each test beaker. The test substance was spiked beneath the water surface of the overlying water and organisms were fed throughout the study. The numbers of emerged male and female midges were recorded daily for about 4 weeks after application. For the ring-test, two formulated pesticides with different chemical characteristics were selected: an insecticide containing 20 % lindane as active ingredient (test concentrations based on the overlying water: 1.6, 6.3, 25 and 100 µg formulation/l) and an herbicide containing 48 % trifluralin as active ingredient (0.25, 1, 4 and 16 mg formulation/l). Overall, thirty-eight laboratories registered for the ring-test, of which twenty-five sent results to the Organising Committee. Not all of the submitted studies fulfilled the validity criteria of at least 70 % emergence in the control and a mean control development time of organisms of less than 20 days. Finally, 18 studies on lindane and 12 studies on trifluralin were used for the evaluation of the ring-test.

Zusammenfassung

1994 wurde ein internationaler Ringversuch mit Sediment-bewohnenden Larven der Zuckmücke *Chironomus riparius* durchgeführt. Um die Expositionsbedingungen von Sediment-bewohnenden Organismen für Pflanzenschutzmittel zu simulieren, wurde die Testsubstanz in diesem Versuch in das Wasser eines Testsystems appliziert, das aus einer etwa 2 cm dicken Schicht eines künstlichen Sediments bestand, welches mit 15 - 20 cm synthetischem oder natürlichen Wasser überschichtet war. Eine festgelegte Anzahl Larven des ersten Larvenstadiums wurde einen Tag vor der Applikation in jedes Testgefäß gegeben. Die Applikation der Testsubstanz erfolgte unter die Wasseroberfläche des überstehenden Wassers. Die Tiere wurden während des Versuches gefüttert und die Anzahl der geschlüpften männlichen und weiblichen Mücken nach der Applikation täglich während der etwa 4 wöchigen Versuchszeit ausgezählt. Für den Ringversuch wurden 2 Pflanzenschutzmittel mit

unterschiedlichen chemischen Eigenschaften ausgewählt: ein Insektizid mit 20 % Lindan als 1.6, 6.3, 25, 100 ìg Formulierung/l) und ein Herbizid mit 48 % Trifluralin als Wirkstoff (0.25, 1, 4, 16 mg/l). Insgesamt meldeten sich 38 Laboratorien zur Teilnahme an diesem Ringversuch an, von denen 25 Laboratorien dem Organisationskomitee Ergebnisse zusandten. Nicht alle der eingereichten Untersuchungen erfüllten die Qualitätskriterien von mindestens 70 % Schlupferfolg in der Kontrolle und einer durchschnittlichen Entwicklungszeit der Kontrolltiere von weniger als 20 Tagen, so daß schließlich 18 Untersuchungen mit Lindan und 12 mit Trifluralin für die Auswertung des Ringversuches zur Verfügung standen.

Organisation of the Ring-Test

Some years ago, German authorities started to request sediment toxicity studies on sediment-dwelling organisms for the registration of certain pesticides (KÖPP 1995). In January 1992, the first meeting of a working group of scientists from academia, government authorities and industry was held to develop a suitable test method (BARRETT & DOHMEN 1992, DOHMEN & BARRETT 1994). In this method, the test substance is spiked into the overlying water of a water-sediment system to simulate the common routes of exposure of sediment-dwelling organisms to pesticides under worst case conditions. First instar larvae of *Chironomus riparius* are added shortly before the application of the test substance. The endpoint of the study is emergence of the test organisms. A small-scale ring-test was performed within the working group using lindane and trifluralin as test substances, the outcome of which seemed to be satisfactory (HAMER 1995, HAMER & HEIMBACH, in press). Thus, an Organising Committee (OC) was formed to organise a wider, international ring-test. Members of the OC were:

Peter Dohmen	BASF AG	Germany
Wolfgang Heger	Environmental Agency (UBA)	Germany
Fred Heimbach	Bayer AG	Germany
Ian R. Hill	Zeneca Agrochemicals	UK
Martin Streloke (Chairman)	Federal Biological Research Centre (BBA)	Germany

The OC met for the first time in September 1993. Participants in the International Ring-Test were contacted in October to December 1993. Protocols were sent to participants in February 1994, and test substances in March / April 1994. The results from participants were collected in September, before a final workshop on the outcome of the ring-test, which took place in Braunschweig (Germany) some weeks later in November 1994.

Methodology

Recommendations for culturing of *Chironomus riparius* and the test protocol, which were sent to ring-test participants, are appended (Appendices I and II). Participants were asked to use their own strain of test organisms, but OC-members provided egg masses of a common strain on request. Only minor deviations from the study protocol occurred, and these were not considered to have influenced the outcome of this ring-test.

The main characteristics of the test method were:

- A laboratory test in a water-sediment test system consisting of a 2 cm layer of an artificial sediment, overlaid by 15 - 20 cm synthetic or natural water.
- 1 day before application, a defined number of first instar larvae (L_1) of laboratory reared *Chironomus riparius* were added to each test beaker.
- The test substance (lindane or trifluralin) was spiked beneath the water surface of the overlying water. (The test concentrations were specified in the test protocol.)
- Organisms were fed throughout the study and numbers of emerged male and female midges were recorded daily for about 25 to 30 days after application.

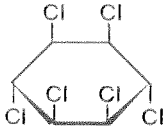
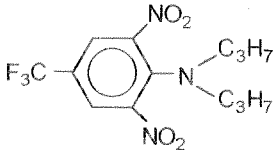
Test Substances

For the ring-test, two pesticides (with different chemical characteristics) were selected. The first test substance was

- the insecticide lindane, formulated as an EC, containing 20 % (w/v) active ingredient (AI).
- the herbicide trifluralin was also formulated as an EC and contained 48 % AI (w/v).

Lindane is moderately soluble and relatively stable in water, and its adsorptivity is high (Table 1). Trifluralin AI is also moderately soluble in water, however the test concentrations were greater than the water solubility. The adsorptivity of this substance was much higher than the adsorptivity of lindane. The vapour pressure was in the same range for both compounds, although trifluralin showed approximately 2-times higher volatility than lindane.

Table 1: Main physico-chemical characteristics of the test substances

Test Substance	lindane	trifluralin
Use:	insecticide	herbicide
Formulation:	EC 200	EC 480
Active ingredient (AI):	lindane	trifluralin
Chemical name of the AI:	1,2,3,4,5,6-hexachloro-cyclohexane	α,α,α-Trifluoro-2,6-dinitro-N,N-dipropyl-p-toluidine
Structural formula of the AI:		
Active ingredient content:	200 g/l	482 g/l
Density:	0.98 g/ml	1.04 g/ml
Solubility of the AI in water:	7.3 mg/l [1]	0.2 mg/l [1]
Hydrolysis stability of the AI in water ($t_{1/2}$) at pH 7:	191 d [1]	"stable" [1]
Vapour pressure:	5.6 mPa [1]	9.5 mPa [1]
Adsorption constant (K_{OC}):	1100 [2]	8000 [2]

[1] The Pesticide Manual (1994) [2] Di GUARDO et al. (1994)

Participation

Overall, thirty-eight laboratories registered for the ring-test, of which twenty-five sent results to the OC (= 66 % of registrants). The laboratories were from six countries: one from Canada, two from France, fourteen from Germany, one from Netherlands, five from UK and two from USA. The names and institutions of these participants are listed in Table 2.

Twenty-four of the twenty-five participants sent results of one study on lindane to the OC with one laboratory performing two studies on this pesticide (i.e. in total twenty-six studies on lindane are available). The number of studies on trifluralin, for which participants had been asked to perform studies at a second choice, was lower: thirteen laboratories performed one study each, 2 laboratories two studies each (i.e. in total seventeen studies available).

Table 2: Names of participants and their institutions of the international ring-test

Name	Institution	Country
Admiraal / Buckert-de Jong	University of Amsterdam	Netherlands
Barrett / Barber	AgrEvo Ltd.	UK
Day	Environment Canada	Canada
Dohmen	BASF AG	Germany
Fliedner	Fraunhofer Institut	Germany
Hamer	Zeneca Agrochemicals	UK
Handley	Safepharm Laboratories Ltd.	UK
Hansen / Warnecke	Technical University Berlin	Germany
Heimann-Detlefsen	Dr. Noack Laboratories	Germany
Heimbach	Bayer AG	Germany
Heusel	AgrEvo GmbH	Germany
Lewecke	Bundesanstalt für Gewässerkunde	Germany
Memmert	RCC GmbH & Co. KG	Germany
Müller	BBA (Kleinmachnow)	Germany
Naylor	University of Sheffield	Germany
Nusch	Ruhrverband	Germany
Pascoe	University of Cardiff	UK
Ratte / Hubbertz	Technical University Aachen	Germany
Sheahan	MAFF	UK
Streloke / Klose	BBA (Braunschweig)	Germany
Surprenant / Putt	Springborn Laboratories, Inc.	USA
Suteau	Rhone-Poulenc, Secteur Agro	France
Swigert / Roberts	Wildlife International Ltd.	USA
Tornier	GAB Biotechnologie GmbH	Germany
Vogel / Cerbelaud	Rhone-Poulenc Industrialisation	France

Not all of the submitted studies fulfilled the validity criterion of at least 70 % emergence in control chambers, which was specified for this ring-test. In addition, some of the studies had not been used for the final evaluation, as the mean development time of control organisms was too long (i.e. more than 20 days) or no dose-response was recognisable. The number of studies available for the final evaluation of the ring-test are given in Table 3. All further statistical evaluations were performed by RATTE (1995).

Participants were asked to analyse test concentrations in the water phase of the test systems on a voluntary basis only. The analysis required expertise and considerable resource. Thus, only a very few data sets on analysis of test concentrations were submitted and consequently will not be discussed here.

Table 3: Number of submitted and invalid studies

test substance	No. of studies submitted	with less than 70 % control emergence	more than 20 days development time in control	without dose-response	used for final evaluation of the ring-test
lindane	26	5	2	1	18
trifluralin	17	1	2	2	12

Acknowledgements

The Organising Committee is grateful to DowElanco and Rhône-Poulenc for providing the test substances to the participating laboratories.

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Appendices

Three protocols of February 11, 1994 which had been send to ring-test participants as instructions for the performance of the studies:

- Recommendations for Culture of *Chironomus riparius*
- Protocol for the toxicity ring-test of two pesticides to the sediment-dwelling larvae of *Chironomus riparius*
- Test solutions "M4" and "M7" for Sediment Toxicity Tests on Chironomids

International Toxicity Ring-Test on Sediment-Dwelling *Chironomus riparius*

Recommendations for Culture of *Chironomus riparius*

February 11, 1994

1. Introduction

Chironomus riparius is a non-biting midge, whose larvae are common to most aquatic environments. The larvae pupate and emerge from the water as adults. Adults usually breed within 24 hours post-emergence. Females extrude gelatinous strands of eggs into the water. Larvae hatch after 2 - 4 days. They go through four instar stages, pupate and emerge as adults in 13 - 25 days at 20 °C.

2. Culture Conditions

Chironomus larvae may be reared in crystallising dishes or larger containers. Fine quartz sand is spread in a thin layer of about 5 to 10 mm deep over the bottom of the container. Kieselgur (e.g. Merck, Art 8117) has also been shown to be a suitable substrate (a thinner layer of up to a very few mm is sufficient). A suitable water is then added to a depth of several cm. Water levels should be topped up as necessary to replace evaporative loss, and prevent desiccation. Water can be replaced if necessary. Gentle aeration should be provided. The larval rearing vessels should be held in a suitable cage which will prevent escape of the emerging adults. The cage should be sufficiently large to allow swarming of emerged adults, otherwise copulation may not occur (minimum is ca. 30 x 30 x 30 cm).

Cages should be held in a constant environment room at 20 ± 2 °C with a photo period of 16 hour light (intensity ca. 1000 lux), 8 hours dark. It has been reported that air humidity of less than 60 % RH can impede reproduction.

3. Dilution Water

Any suitable natural or synthetic water may be used. Well water, dechlorinated tap water and artificial media (e.g. Elendt "M4" or "M7" medium, see Protocol "Test Solutions "M4" and "M7" for Sediment Toxicity Tests on *Chironomus riparius*") are commonly used.

The water has to be aerated before use. If necessary, the culture water may be renewed by pouring or siphoning the used water from culture vessels carefully without destroying the tubes of larvae.

4. Feeding

4.1. Larvae

Chironomus larvae should be fed with a fish flake food (Tetra-Min® or other similar brand of proprietary fish food), at approximately 250 mg per vessel per day. This can be given as a dry ground powder or as a suspension in water: 1.0 g of flake food is added to 20 ml of dilution water and blended to give a homogenous mix. This preparation may be fed at a rate of about 5 ml per vessel per day. (Shake before use.) Older larvae may receive more.

Feeding is adjusted according to the water quality. If it becomes cloudy, feeding should be reduced. Food additions must be carefully monitored. Too little will cause emigration of the larvae into the water column, and too much will cause increased microbial activity and reduced oxygen concentrations. Both conditions can result in reduced growth rates.

Some green algae (e.g. *Scenedesmus subspicatus*, *Chlorella vulgaris*) cells may also be added when new culture vessels are set up.

4.2. Emerged adults

Some workers have suggested that a cotton wool pad soaked in a saturated sucrose solution may serve as a food for emerged adults.

5. Emergence

At 20 ± 2 °C adults will begin to emerge from the larval rearing vessels after approximately 13 - 15 days. Males are easily distinguished by having plumose antennae.

6. Egg Masses

Once adults are present within the breeding cage, all larval rearing vessels should be checked 3 times weekly for deposition of the gelatinous egg masses.

If present, the egg masses should be carefully removed. They should be transferred to a small dish containing a sample of the breeding water. Egg masses are used to start a new culture vessel (e.g. 2 - 4 egg masses / vessel) or are used for toxicity tests.

First instar larvae should hatch after 2 - 3 days.

7. Set-up of New Culture Vessels

Once cultures are established it should be possible to set up a fresh larval culture vessel weekly or less frequently depending on testing requirements, removing the older vessels after adult midges have emerged. Using this system a regular supply of adults will be produced with a minimum of management.

International Toxicity Ring-Test on Sediment-Dwelling *Chironomus riparius*

Protocol for the toxicity ring-test of two pesticides to the sediment-dwelling larvae of *Chironomus riparius*

February 11, 1994

1. INTRODUCTION

The methods described here assess the potential impact of pesticides on the sediment dwelling life stage of the organism *Chironomus riparius*, a common "nonbiting" midge. The ring test with this protocol is designed to evaluate the technique for reproducibility and ease of use.

2. STUDY OUTLINE

The study will be conducted using a range of concentrations of the test material applied to the water column of a sediment-water system containing first instar larvae, for a period sufficient to assess the impact on full maturation of the larvae to adult midge.

3. MATERIALS

3.1. Test Species

The tests will be initiated using first instar larvae obtained from in-house cultures of *Chironomus riparius* (syn. *Chironomus thummi*). Details of culture methods are given in a separate paper. The egg masses used to start the culture may be from in-house or from members of the Organising Committee. Identification of in-house cultures must be confirmed before testing. Details of culture and source of organisms must be provided on the attached forms.

3.2. Sediment

For the test an artificial sediment will be used. The artificial sediment (according to OECD test guideline No. 207) should be prepared as follows (on the basis of dry weights):

- 10 % sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, air dried and finely ground)
- 20 % kaolin clay (kaolinite content preferably above 30 %)

- 70 % industrial sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 microns)
- pH of the final mixture of the sediment is adjusted to 6.0 + 0.5 by addition of calcium carbonate (chemically pure quality)

The dry constituents are blended in the correct proportions and mixed thoroughly, either in a large-scale laboratory mixer or small electric cement mixer. Some deionised water is added to moisten the artificial sediment before it is used for the study.

3.3. Test Water

Any suitable natural or synthetic water is accepted, but the use of the reconstituted water "M4" or "M 7" according to Elendt is recommended (details provided).

3.4. Test Vessels

The study will be conducted in glass 2 to 3 l-beakers measuring 10 or 13 cm in diameter. Any other vessels are also suitable, but they should guarantee a suitable height of the overlying water (see 4.1.).

4. TEST METHODS

4.1. Preparation of Test Vessels

The amount of wet artificial sediment which is needed for each test container is filled into a beaker. To avoid a separation of the ingredients of the sediment and high turbidity of the test water, the sediment surface is covered by a plastic "plate", which floats as the water is poured onto it. The water is poured into the beaker very slowly, taking care not to disturb the sediment.

The vessels should be prepared at least 1 week before the study starts and be acclimatised under the test conditions. The vessels should contain 2.0 cm of sediment and a water column 15 to 20 cm deep. The exact volume of water added should be recorded, and the level marked outside on the test vessel.

Gentle aeration will be provided through a glass Pasteur pipette situated ca. 2.5 cm above the sediment layer (i.e. ca. 1 bubble / sec).

Beakers should be covered (e.g. by glass plates). If necessary, the water levels should be topped up during the study to the original starting volume to prevent concentration of the test material. Water levels should not change more than 10 %.

4.2. Incubation

All test should be conducted in a constant temperature room at 20 ± 2 °C with a photo period of 16 hours light (intensity about 1000 lux), 8 hours dark.

4.3. Inserting of Test Organisms

About four to five days before application (application = day 0 of the study) some egg-masses are taken from the cultures and are deposited into small vessels in culture medium. You may use aged medium from the stock culture or freshly prepared medium. If the later is used, add a small amount of food e.g. green algae or a finely ground fish flake suspension. Fresh egg-masses should be chosen only.

Normally the larvae begin to hatch after 2 to 3 days. Twenty-five larvae of the first larval stage (1 - 3 days old, i.e. 1 - 3 days after hatching) are allocated randomly to each test vessel with a blunt pipette (e.g. 5 collectives of 5 larvae each, per vessel). When adding the larvae and also for the following 24 hours, the aeration of the water must be stopped.

4.4. Application of the Test Substance

One day after adding the larvae, the slight aeration (ca. 1 bubble / sec) should be started again and the test material will be applied to the water column on the same day, in a small volume of water (about 10 ml per vessel) just below the water surface by using a pipette, and gently mixing to ensure homogeneous distribution, without disturbing the sediment.

Triplicate vessels will be treated with each rate.

The test concentrations (calculated as concentrations of the pesticide formulation in the overlying water) for this study are the following:

- lindane: control - 0.0016 - 0.0063 - 0.025 - 0.10 mg formulation / l
- trifluralin: control - 0.25 - 1.0 - 4.0 - 16.0 mg formulation / l

Some analytical data is desirable but is not an absolute requirement. As a minimum, we would prefer that samples of the test solutions (overlying water) are analysed 1 hour after application. An analytical method for water will be provided.

If only one test is done at a time, lindane should be carried out first. Also if only one test can be completed this should be one with lindane.

4.5. Food

It is necessary to feed the larvae at least 3 times per week. 1 mg fish-food (a suspension in water or finely ground food, e.g. Tetra-Min®, see information's given in culture instructions) per day and per larvae seems to be adequate for young larvae (i.e. 175 mg per vessels per week). Slightly more food may be necessary for older ones.

5. ASSESSMENT

5.1. Emergence of Midges

The endpoints of the study are the day of first emergence, the time distribution (peak) of emergence of male and female midges, and the total number of fully emerged male and female midges.

The test vessels should be observed at least three times per week to make a visual assessment of any behavioural differences compared with the control. During the period of expected emergence (normally starting at day 13 to 16 and lasting until day 25) a daily check of emerged midges is necessary. The sex and number of adults emerging should be recorded daily. After identification the midges are removed from the vessels. Any egg masses deposited prior to the termination of the test should be recorded and removed to prevent re-introduction of larvae into the sediment. Only the number of fully emerged male and female midges will be counted. The number of visible pupae that have failed to emerge will be counted separately.

Ten days after emergence of the last adult or after 90 % or more have emerged in the control vessels, the study is finished. The study should not continue longer than 30 days. The results will be used for statistical procedures by the ring-test organisers.

As a validity criteria, control emergence should be equal to or greater than 70 %. If you do not achieve this, please provide us with your final data set.

5.2. Physico-chemical Parameters

In all tests the oxygen concentration and pH should be recorded in all test vessels at the start and end of the study. These parameters (and water temperature) should also be recorded at least once weekly for the duration of the test.

6. REPORTING

The test results are to be reported on the enclosed data sheets separately for each study. The results will be used for statistical performances using the same procedures for all ring-test participants.

International Toxicity Ring-Test
on Sediment-Dwelling *Chironomus riparius*

Test solutions "M4" and "M7" for Sediment Toxicity Tests on Chironomids

February 11, 1994

General Remarks

Elendt (1990) has described the medium known as "M4". The medium "M7" is prepared as the "M4"-medium with the exception of the substances which are marked in Table 1, which are by a factor of 4 lower in "M7" than in "M4". A publication of the "M7"-medium is in preparation (Elendt, pers. communication). Do not prepare the solution as described in Elendt & Bias (1990), as in this paper the concentrations of sodium silicate ($NaSiO_3 \cdot 5\ H_2O$), sodium nitrate ($NaNO_3$), potassium dihydrogen phosphate (KH_2PO_4) and di-potassium hydrogen phosphate (K_2HPO_4) for the preparation of the stock solutions are wrongly given.

Table 1: Stock solutions of trace elements for medium M4 and M7

stock solutions (I)		amount (mg) made up to 1 litre deionised water	to prepare the combined stock solution II mix the following amounts (ml) of stock solutions (I) and make up to 1 l of deionised water		final concentrations in test solution (mg/l)	
			M4	M7	M4	M7
H_3BO_3	*)	57190	1.0	0.25	2.86	0.715
$MnCl_2 \cdot 4\ H_2O$	*)	7210	1.0	0.25	0.361	0.090
LiCl	*)	6120	1.0	0.25	0.306	0.077
RbCl	*)	1420	1.0	0.25	0.071	0.018
$SrCl_2 \cdot 6\ H_2O$	*)	3040	1.0	0.25	0.152	0.038
NaBr	*)	320	1.0	0.25	0.016	0.004
$Na_2MoO_4 \cdot 2\ H_2O$	*)	1260	1.0	0.25	0.063	0.016
$CuCl_2 \cdot 2\ H_2O$	*)	335	1.0	0.25	0.017	0.004
$ZnCl_2$		260	1.0	1.0	0.013	0.013
$CoCl_2 \cdot 6\ H_2O$		200	1.0	1.0	0.010	0.010
KJ		65	1.0	1.0	0.0033	0.0033
Na_2SeO_3		43.8	1.0	1.0	0.0022	0.0022
NH_4VO_3		11.5	1.0	1.0	0.00058	0.00058
$Na_2EDTA \cdot 2\ H_2O$	*) **)	5000	20.0	5.0	2.5	0.625
$FeSO_4 \cdot 7\ H_2O$	*) **)	1991			1.0	0.249

*) These substances differ in M4 and M7, as indicated in "General Remarks".
**) These solutions are prepared individually, then poured together and autoclaved immediately.

Preparation of the "M7"-medium

Each solution I is prepared individually according to Table 1 and from these solutions a combined stock solution II is prepared by the amounts indicated in Table 1. Fifty ml from the com-

bined stock solution II and the amounts of each macro nutrient stock solution which are given in Table 2 are made up to 1 l deionised water to prepare the "M7"-medium. A vitamin stock solution is prepared by adding 3 vitamins to deionised water as indicated in Table 3 and 0.1 ml of the combined vitamin stock solution are added to the final "M7"-medium shortly before use. (The vitamin stock solution is stored frozen in small aliquots.) The medium is aerated and stabilized.

Table 2: Macro nutrient stock solutions for medium M4 and M7

	amount (mg) made up to 1 litre deionised water	amount of macro nutrient stock solutions added to prepare medium M4 and M7 (ml/l)	final concentrations in test solution M4 and M7 (mg/l)
$CaCl_2 \cdot 2\ H_2O$	293800	1.0	293.8
$MgSO_4 \cdot 7\ H_2O$	246600	0.5	123.3
KCl	58000	0.1	5.8
$NaHCO_3$	64800	1.0	64.8
$NaSiO_3 \cdot 9\ H_2O$	50000	0.2	10.0
$NaNO_3$	2740	0.1	0.274
KH_2PO_4	1430	0.1	0.143
K_2HPO_4	1840	0.1	0.184

Table 3: Vitamin stock solution for medium M4 and M7

All three vitamin solutions are combined to make a single vitamin stock solution.

	amount made up to 1 l of deionised water (mg)	amount of vitamin stock solution added to prepare medium M4 and M7 (ml/l)	final concentrations in test solution M4 and M7 (mg/l)
Thiamine hydrochloride	750	0.1	0.075
Cyanocobalamin (B12)	10	0.1	0.0010
Biotine	7.5	0.1	0.00075

References

ELENDT, B.P. (1990): Selenium deficiency in Crustacea. Protoplasma 154, 25-33

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Statistical Analysis of the Results from the International Toxicity Ring-Test on Sediment-Dwelling *Chironomus riparius*

Summary

Prior to the determination of NOECs/LOECs statistical characteristics of the biological variable were investigated. Emergence rate (as measure of mortality) and development rate (1/development time) proved to be appropriate end-points. The final evaluation was conducted using 18 of 26 lindane data sets (reasons given in Tables 2, 3) and using 12 of 17 trifluralin data sets. The NOECs of lindane were found in the range of 1.6 to 25 µg/l (median 6.3 µg/l, Figures 3, 4). Here the emergence rate appeared more sensitive than development rate. In contrast, with trifluralin the development rate responded more sensitively. Here NOECs were found at any test concentrations (median 4 mg/l, Figures 8,9). The higher variability was probably due to problems concerning the correct adjustment and maintenance of test concentrations because of the physico-chemical properties of trifluralin.

Zusammenfassung

Bei der Auswertung der Ringtestergebnisse wurden zunächst die statistischen Eigenschaften der biologischen Meßvariablen untersucht. Die Emergenzrate (als Maß für die Mortalität) und die Entwicklungsrate (1/Entwicklungszeit) erwiesen sich als geeignete Testendpunkte. In die Schlußauswertung gelangten 18 von 26 Lindan-Tests und 12 von 17 Trifluralin-Tests (Kriterien siehe Abbildungen 2, 3). Bei Lindan lagen die NOECs beider Endpunkte im Bereich zwischen 1,6 und 25 µg/l (Median 6,3 µg/l, Abbildungen 3,4), wobei die Emergenzrate empfindlicher war. Dagegen reagierte in den Tests mit Trifluralin die Entwicklungsrate empfindlicher. Hier erstreckte sich die Verteilung der NOECs über den gesamten Konzentrationsbereich (Median 4 mg/l, Abbildungen 8,9). Die höhere Variabilität der Trifluralin-Tests wird in Schwierigkeiten bei der korrekten Einstellung und Einhaltung der Konzentrationen gesehen, die auf den physikalisch-chemischen Eigenschaften dieser Substanz beruht.

Introduction

The Organising Committee proposed a test design for two substances, lindane and trifluralin, in order to determine NOECs/LOECs by a statistical test procedure. Hence, the preliminary phase of the statistical evaluation of the ring-test results intended to find appropriate measures of the two end-points "emergence" and "development". These measures should exhibit low variability as well as a high degree of normal distribution and homogeneity of variance, in order to apply powerful parametric statistical test procedures allowing the detection of NOECs/LOECs as safely as possible. In the final evaluation, the results of the ring-test are presented by means of the most appropriate measures, emergence rate and development rate.

Preliminary Evaluation

As statistical measures the emergence rate and several measures for the development time were statistically investigated (Table 1).

Table 1: Statistical characteristics of the emergence rate and some measures of the development time, each calculated per vessel. The values were obtained from the data of all participants. L: lindane; T: trifluralin; %ND: percentage of data sets showing normal distribution; %VH: percentage of data sets showing variance homogeneity; CV%: coefficient of variation

Biological Measure		% ND	% VH	CV%
Emergence Rate	L	50.7	94.1	20.0
	T	69.0	100.0	23.9
Development Time Mean	L	90.6	88.2	6.3
	T	94.9	84.6	5.1
Development Time Median	L	85.5	88.2	6.2
	T	91.4	92.3	5.3
Development Time Mode	L	24.0	100.0	11.1
	T	29.4	100.0	7.6
Development Time of First	L	14.3	100.0	10.0
(Day of First Emergence)	T	23.5	88.9	5.4
Development Time	L	44.8	41.2	12.3
(Single Midge as Replicate)	T	60.0	23.1	11.3
Development Rate Mean	L	93.8	100.0	5.8
	T	98.3	100.0	4.5
Development Rate First	L	18.8	84.6	7.2
	T	25.6	60.0	3.9
Development Rate	L	43.3	41.2	11.6
(Single Midge as Replicate)	T	58.3	23.1	10.6

Except of single midges as replicates, the variables of Table 1 had been computed first on a per-vessel basis with 3 replicate values per treatment each. For presentation of the results

(Tables 2 through 5 and Figures 1, 2 , 6 and 7) and statistical inference, per-treatment arithmetic means and standard deviations were calculated. To increase variance homogeneity and normal distribution in the emergence rate, arcsin-transformed values were used. Two multiple t-tests (Williams, Dunnett) and a multiple sequential U-test (Bonferroni-Holm) were performed with all biological measures of Table 1. This preliminary study revealed that it is reasonable to pool sexes, use the emergence and the development rate as end-points and the Williams' test procedure to determine LOECs/NOECs[1] The development rate is a well accepted concept in insect biology and is computed as the reciprocal of the development time. The unit thus is 1/day. This rate showed a lower and more homogeneous variance as well as was more often normally distributed than the development time.

Final Evaluation

Table 2 through 5 show the per-treatment results obtained by all participants regardless of any validity criteria. The final evaluation, however, was conducted using only data sets which (1) fulfilled the validity criterion (emergence rate of control at least 70%) and which (2) did not show marked deviations from the development or emergence rates normally observed (mean development time less than 20 days). This was done in order to prevent a bias in the ring-test summary statistics due to experimental errors which might possibly have occurred with laboratories inexperienced in *Chironomus* testing. In so doing, with lindane 6 of 26 data sets were excluded according to the test validity criterion, and two additional sets were excluded because of the reasons given in Tables 2 and 3. With trifluralin 16 of 17 were valid in terms of the test criterion "emergence rate" and additional 4 ones which could not be evaluated for the ring-test (Tables 4 and 5, bottom part), so that 12 data sets have been taken for evaluation.

Dose-response curves for the remaining valid tests are shown in Figures 1, 2, 6 and 7, to which the ring-test mean curve was added together with the 95%-confidence interval, indicating that the true mean response curve is assumed in the given range with a probability of 95%. In contrast, a second range in the graphs represents the 95%-prediction range for individual laboratories, indicating that area in which 95% of the per-treatment results of the individual participants were found and, most important, are to be expected in future testing. The NOECs obtained can be immediately derived from the Tables 2 through 5. Their statistical distributions are illustrated by Figures 3, 4, 8 and 9. Additionally, Figures 5 and 10 show which of the end-points contributed to the lowest NOEC in the respective test.

[1]It seems also reasonable to the author to pool all containers of a treatment and use the single midges as replicates. The application of a multiple U-test procedure for development time and rate revealed the lowest NOECs/LOECs in some data sets. But, because this approach is not unequivocal among statisticians, it should not be recommended to date. Please follow the further debate on this topic in literature.

Results

The lowest NOEC with lindane was mainly determined by the emergence rate, demonstrating that emergence inhibition or mortality was the most sensitive end-point. Nevertheless, the development rate proved to be significantly inhibited mainly at 6.3 µg/l and higher concentrations. For both end-points, all NOECs were found in the range between 1.6 to 25 µg/l (Figure 5). The distribution of NOECs with trifluralin was less pronounced than with lindane and the NOEC/LOEC computation was impossible in several cases. From distribution of the lowest NOECs (Figure 10) one can conclude that the development rate was slightly more sensitive than the emergence rate. The varying results are probably due to the physico-chemical characteristics of trifluralin, which impeded an exact and equal adjustment of test concentrations.

From a statistical point of view, the guidance document for this ring-test did not consider the control of the type-II-error (false acceptance the null-hypothesis). Due to the limited number of replicates per treatment, the type-II error was probably relatively high which means that some effective concentrations have probably not been detected by the statistical test procedures. This aspect should be considered for the draft guideline of this test.

Table 2: Emergence rate from all reported studies as affected by lindane. Lab. Code: Code of laboratory. The shaded cells indicate treatments significantly different from control (Williams-test, α=0.05, one-sided). The treatment left hand of the shaded area marks the NOEC, the first shaded treatment the NOEC; n.d.: a NOEC/LOEC could not be determined by the statistical test.

Lab. Code	Control	1.6	6.3	25	100	µg/l
5	86.7	97.3	4.0	0.0	0.0	
7	76.0	76.0	48.0	12.0	0.0	
9	82.7	82.7	64.0	68.0	0.0	
13	78.7	80.0	72.0	56.0	2.7	
19	100.0	92.0	88.0	81.3	0.0	
4	84.0	85.3	85.3	34.7	0.0	
11	100.0	98.7	100.0	48.0	0.0	
14	92.0	97.3	76.0	26.7	0.0	
17	90.7	90.7	96.0	29.3	0.0	
21	74.7	61.3	80.0	44.0	0.0	
25	96.0	98.7	92.0	64.0	0.0	
10	78.7	74.0	93.3	46.7	0.0	
6	92.0	77.3	92.0	80.0	0.0	
13	96.0	92.0	86.7	93.3	1.3	
15	74.7	88.0	81.3	97.3	0.0	
16	93.3	72.0	92.0	85.3	6.7	
20	82.7	81.3	84.0	88.0	8.0	
23	96.0	93.3	89.3	96.0	0.0	
8*)	74.7	50.7	1.3	0.0	0.0	
1**)	53.3	49.3	58.7	42.7	0.0	n.d.
3**)	65.3	57.3	28.0	1.3	0.0	
2**)	57.3	46.7	65.3	36.0	0.0	
12**)	62.7	84.0	2.7	0.0	0.0	
18**)	37.3	32.0	25.3	33.3	6.7	
22**)	56.0	72.0	46.7	30.7	0.0	
24***)	96.0	78.7	53.3	37.3	21.3	

*) mean control development time > 20 days

**) control emergence rate < 70%

***) The dose-response curve of this study is different to all other studies and cannot be used for the evaluation of the ring-test without further findings as e.g. analysis of the test concentrations.

Table 3: Development rate (%/day) from all reported studies as affected by lindane; n.d.: a NOEC/LOEC could not be determined due to 100% mortality at the highest rate. Further explanations see Table 2.

Lab. Code	Control	1.6	6.3	25	100	µg/L
5	9.2	9.2	5.8			
25	7.6	7.5	7.1	6.8		
4	7.6	7.7	7.5	6.5		
9	7.5	7.4	7.3	6.9		
11	5.8	5.6	5.8	5.1		
14	7.5	7.4	6.9	5.4		
17	7.4	7.1	7.1	5.9		
19	6.6	6.6	6.6	6.3		
23	7.6	7.6	7.5	7.2		
13	5.7	5.9	6.1	5.6	4.3	
13	6.3	6.5	6.2	6.0	4.3	
16	6.3	6.5	6.6	6.1	4.9	
20	6.5	5.9	6.3	6.6	4.4	
6	6.0	6.1	6.2	5.8		n.d.
7	5.5	5.2	5.6	5.1		n.d.
10	5.4	6.2	5.3	5.0		n.d.
15	8.1	7.4	7.5	7.4		n.d.
21	7.7	8.2	8.4	7.1		n.d.
8*)	4.7	4.4	3.4			
3*)	4.6	4.4	4.3	3.6		n.d.
1**)	6.6	6.8	6.6	6.1		
2**)	6.9	7.1	6.8	6.5		n.d.
12**)	6.9	6.7	5.3			
18**)	8.1	8.2	7.9	7.5	6.3	
22**)	4.9	4.9	4.3	4.2		
24***)	5.8	5.6	5.7	5.5	5.7	

*), **), ***) see Table 2.

Table 4: Emergence rate from all valid studies as affected by trifluralin. Explanations see Table 2.

Lab. Code	Control	0.25	1	4	16	mg/l
24	96.0	60.0	42.7	14.7	2.7	
7	88.0	81.3	32.0	30.7	0.0	
17	88.0	92.0	90.7	68.0	0.0	
4	84.0	92.0	85.3	72.0	0.0	
5	90.7	81.3	89.3	81.3	28.0	
6	85.3	86.7	90.7	85.3	21.3	
10	70.7	66.7	88.0	65.3	0.0	
13	92.0	80.0	84.0	82.7	73.3	
15	84.0	72.0	80.0	76.0	54.7	
5	90.7	85.3	92.0	89.3	82.7	
13	98.7	96.0	97.3	96.0	96.0	
16	92.0	97.3	89.3	90.7	96.0	
8*)	74.7	73.3	60.0	6.7	0.0	
20*)	78.7	81.3	60.0	76.0	56.0	
2**)	57.3	46.7	65.3	36.0	0.0	
1***)	85.3	100.0	50.7	72.0	48.0	
18***)	93.3	37.3	54.7	28.0	37.3	

*) mean control development time > 20 days
**) control emergence rate < 70%
***) The dose-response curve of this study does not show a dose-response relationship which allows a statement on toxic threshold concentrations.

Table 5: Development rate (%/day) from all valid studies as affected by trifluralin. Explanations see Table 2

Lab. Code	Control	0.25	1	4	16	mg/l
17	7.6	7.1	6.9	5.5		
7	5.3	5.2	4.9	4.9		
10	5.5	5.6	5.3	4.5		
4	7.6	7.7	7.7	6.9		
6	6.6	7.1	6.8	6.0	5.2	
5	9.1	9.2	8.9	8.7	8.0	
5	9.6	9.8	9.8	10.1	8.5	
13	7.1	7.2	7.1	6.9	6.4	
13	7.2	7.3	7.3	7.1	6.3	
15	7.8	7.9	7.7	8.1	5.7	
16	6.1	6.4	6.3	5.6	5.2	
24	5.8	5.7	6.2	5.9	5.6	
8*)	4.7	4.3	4.3	4.6		n.d.
20*)	4.2	4.9	4.0	4.3	4.1	
2**)	6.9	7.1	6.8	6.5		n.d.
1***)	5.7	5.5	5.8	6.0	4.8	
18***)	5.7	5.1	5.0	4.6	5.2	

*), **), ***) see Table 4.

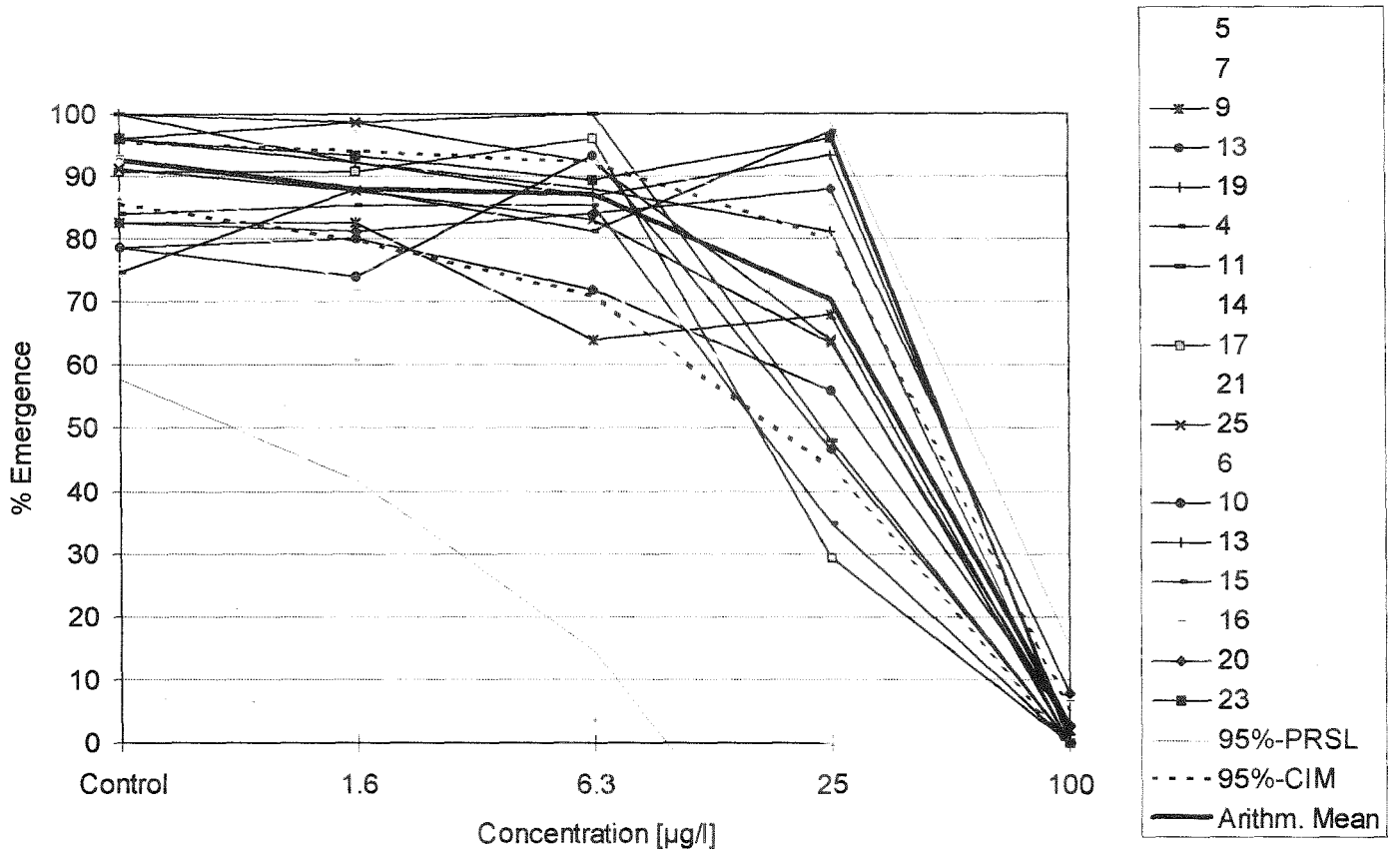

Figure 1: Emergence rate from all valid studies as affected by lindane (numbers on the right refer to the laboratory codes in Tables 2 and 3), 95% PRSL: 95%-Prediction range for results of individual laboratories; 95%-CIM: 95%- confidence interval for the mean curve.

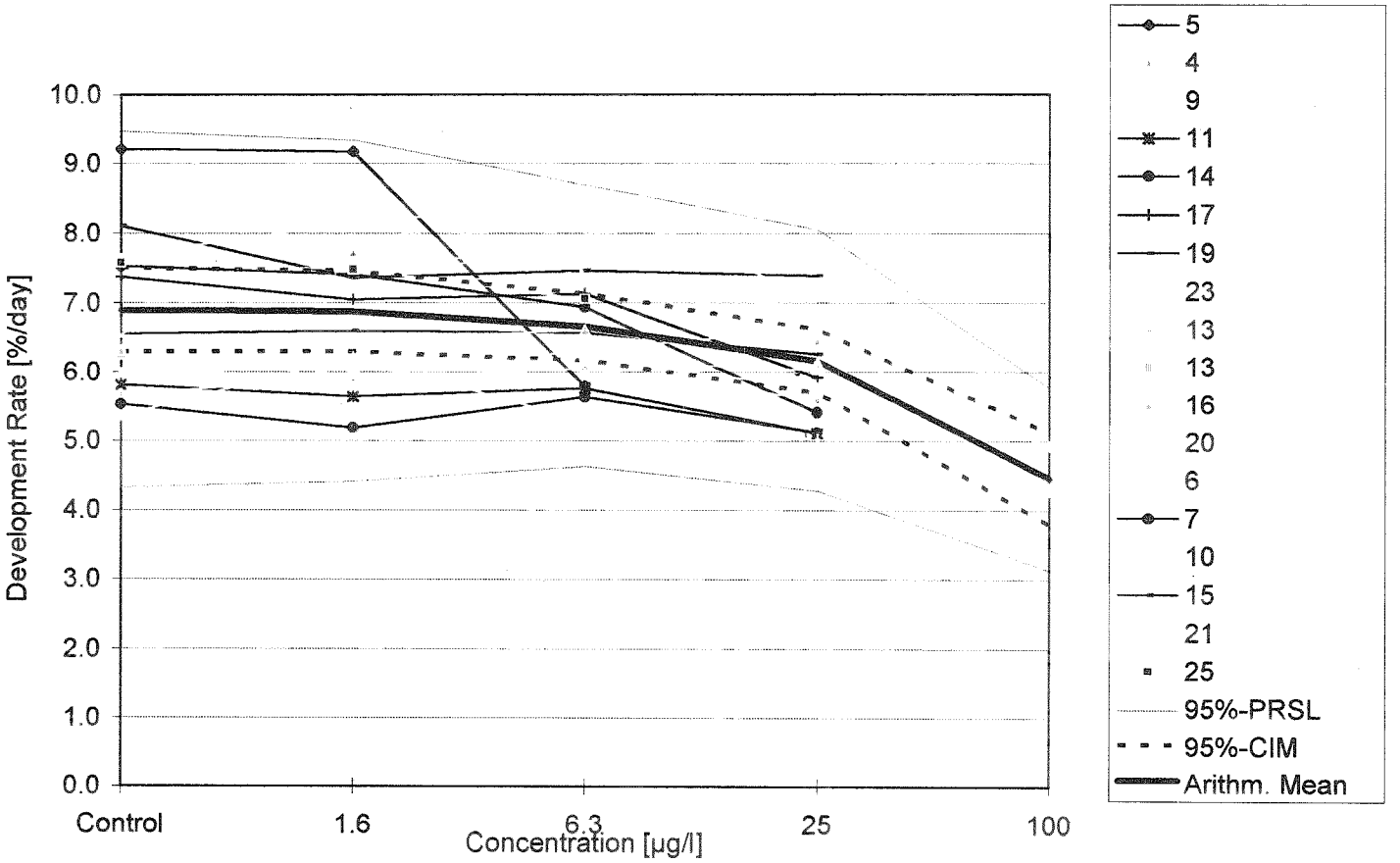

Figure 2: Development rate from all valid studies as affected by lindane. Further explanations see Figure 1.

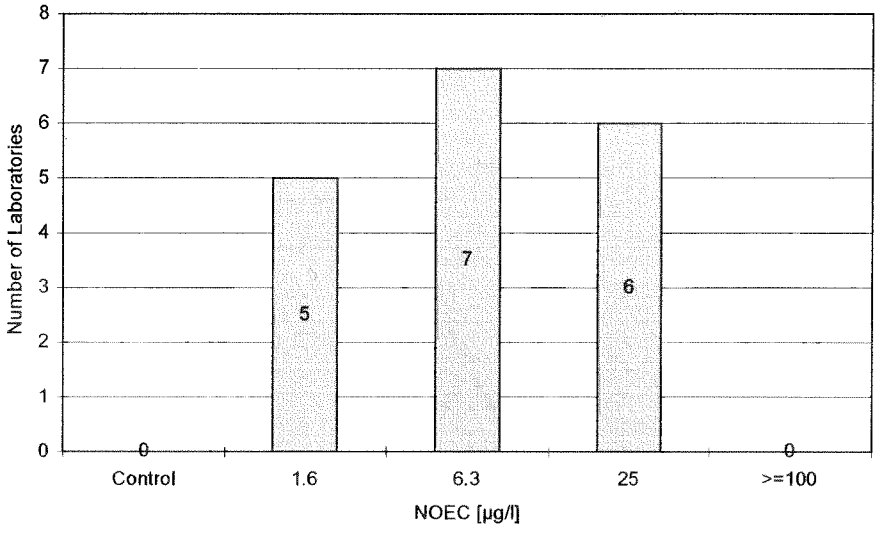

Figure 3: Distribution of NOECs of the emergence rate for all valid studies on lindane. The NOECs were obtained via the Williams-test.

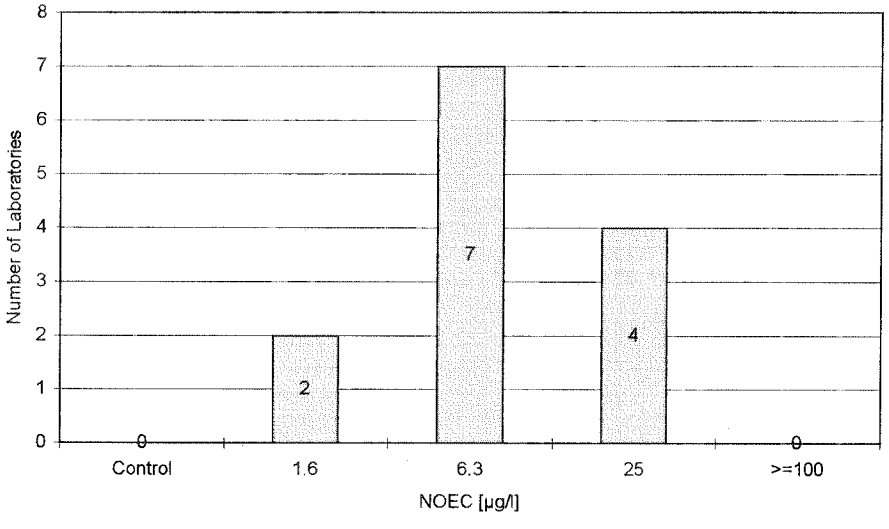

Figure 4: Distribution of NOECs of the development rate for all valid studies on Lindane. The NOECs were obtained via the Williams-test.

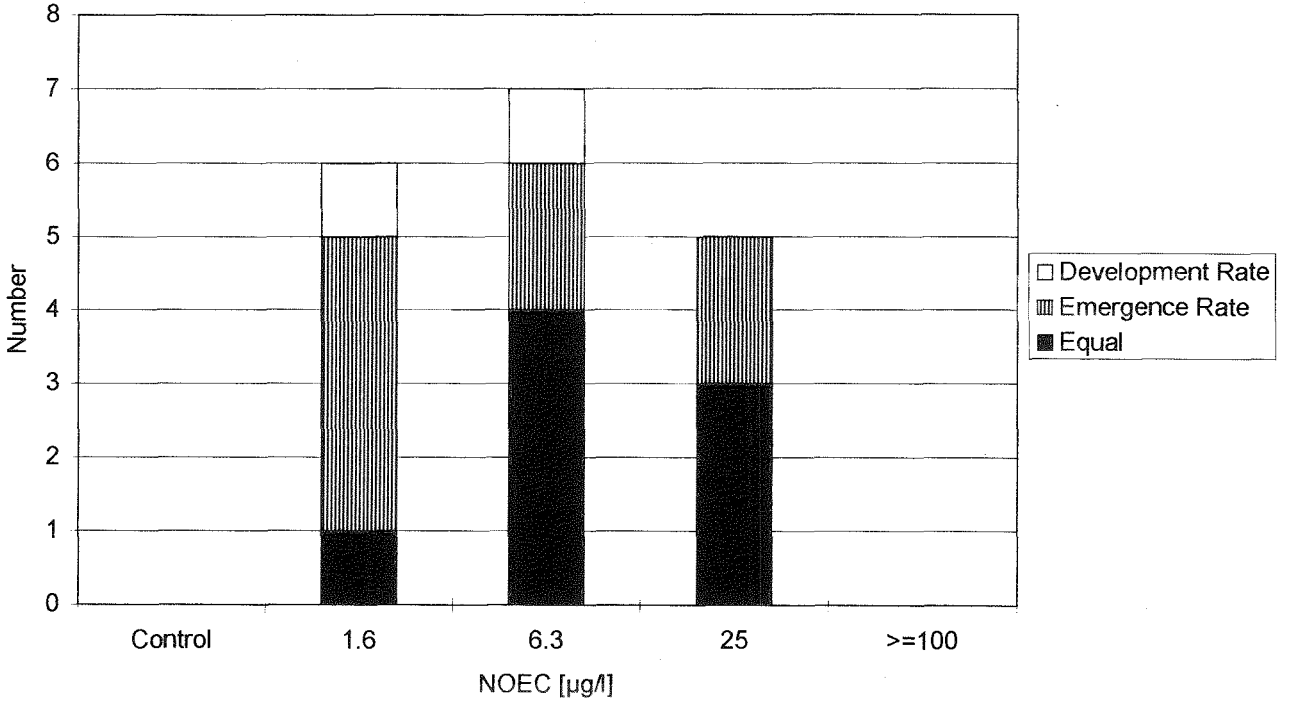

Figure 5: Distribution of the lowest NOECs of the development rate and the emergence rate for lindane. Black parts of the bars represent studies in which the NOEC was equal in both end-points, grey parts indicate those studies in which the emergence rate showed the lowest NOEC, and light parts stand for the lowest NOEC in the development rate.

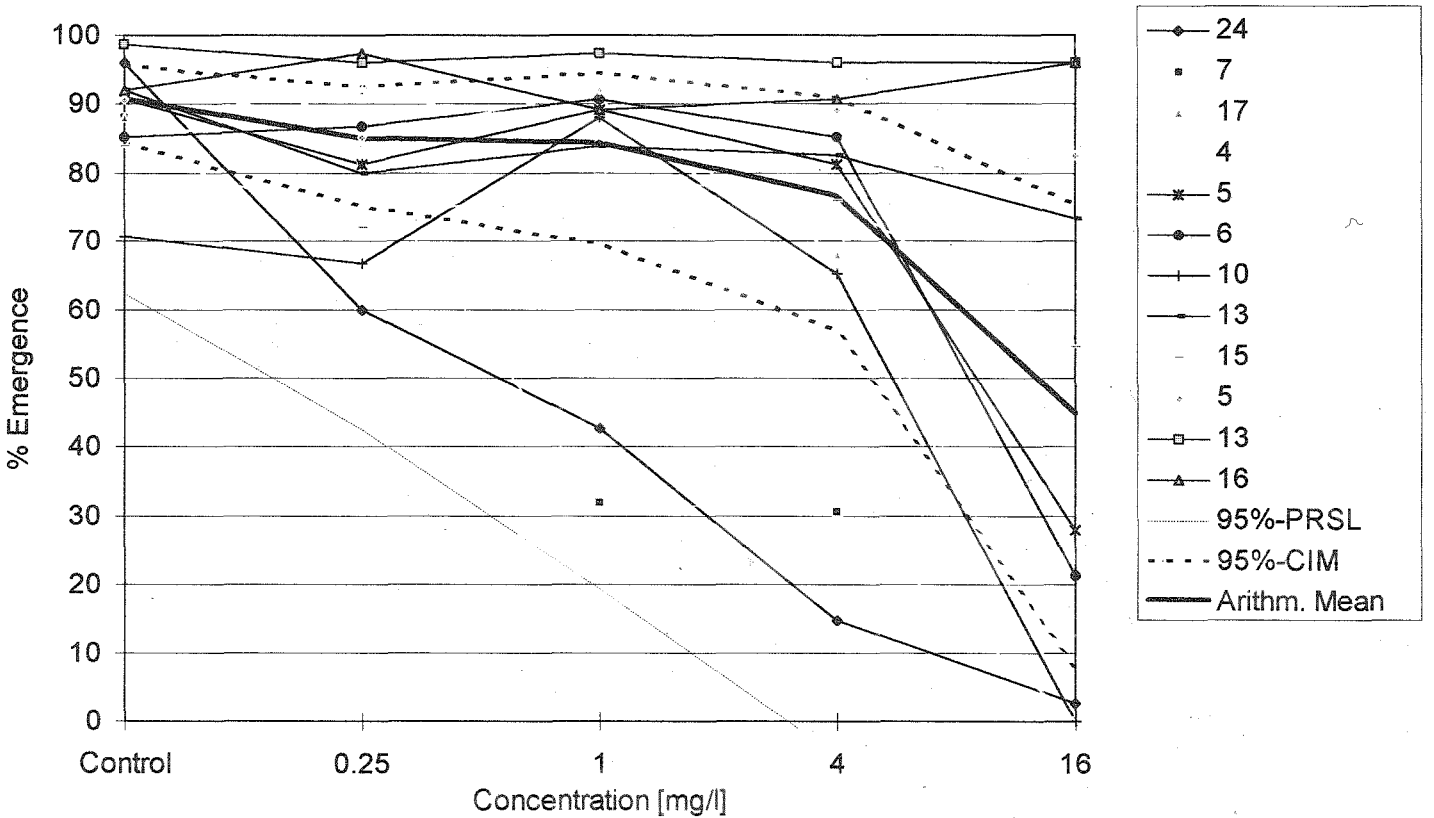

Figure 6: Emergence rate of all valid studies as affected by trifluralin (numbers on the right refer to laboratory codes in Table 4 and 5)

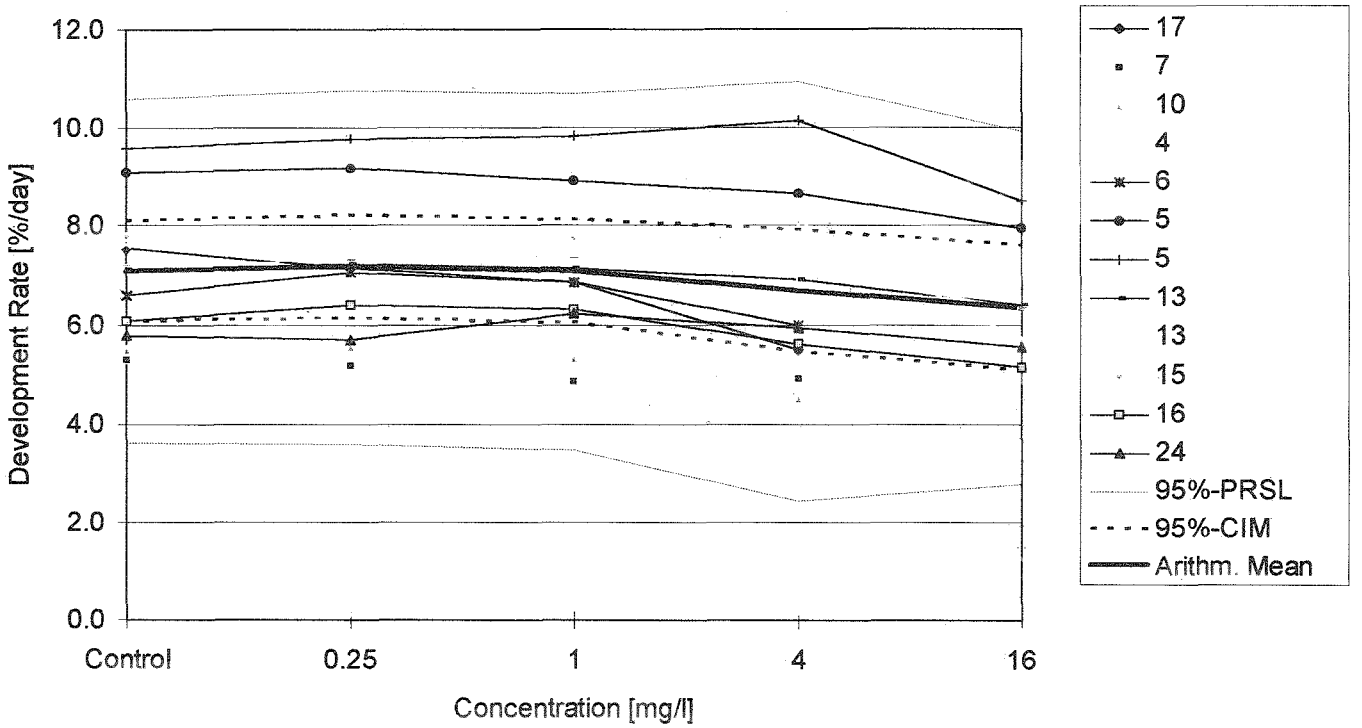

Figure 7: Development rate of all valid studies as affected by trifluralin. Explanations see Figure 6.

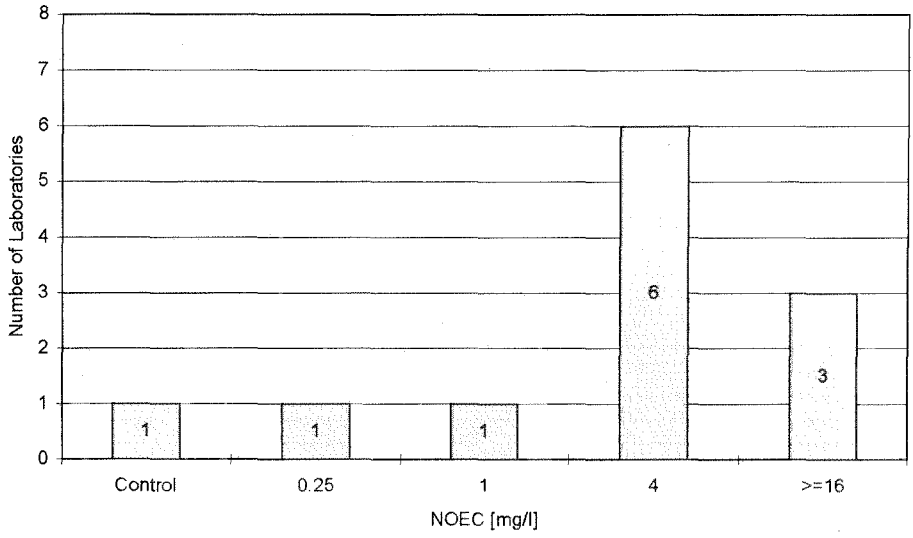

Figure 8: Distribution of NOECs of the emergence rate for all valid studies on trifluralin. The NOECs were obtained via the Williams-test.

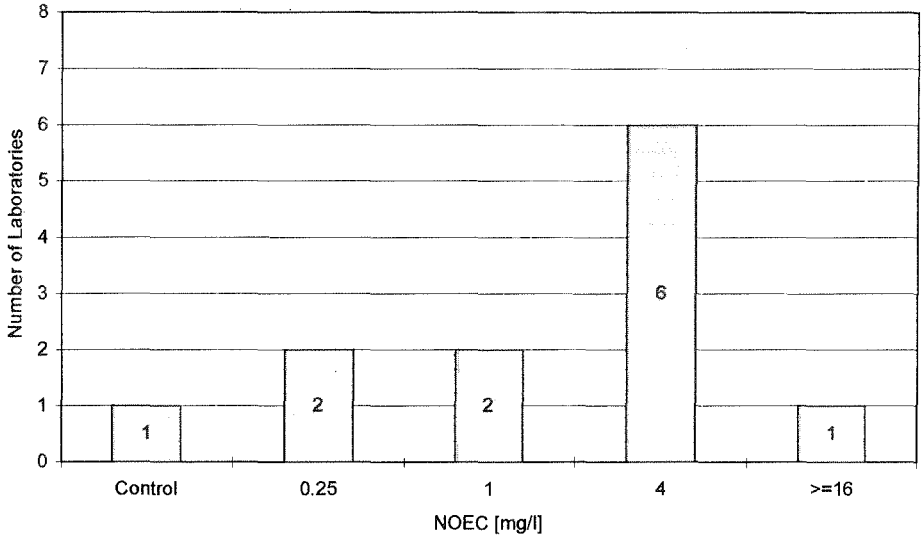

Figure 9: Distribution of NOECs of the development rate for all valid studies on trifluralin. The NOECs were obtained via the Williams-test.

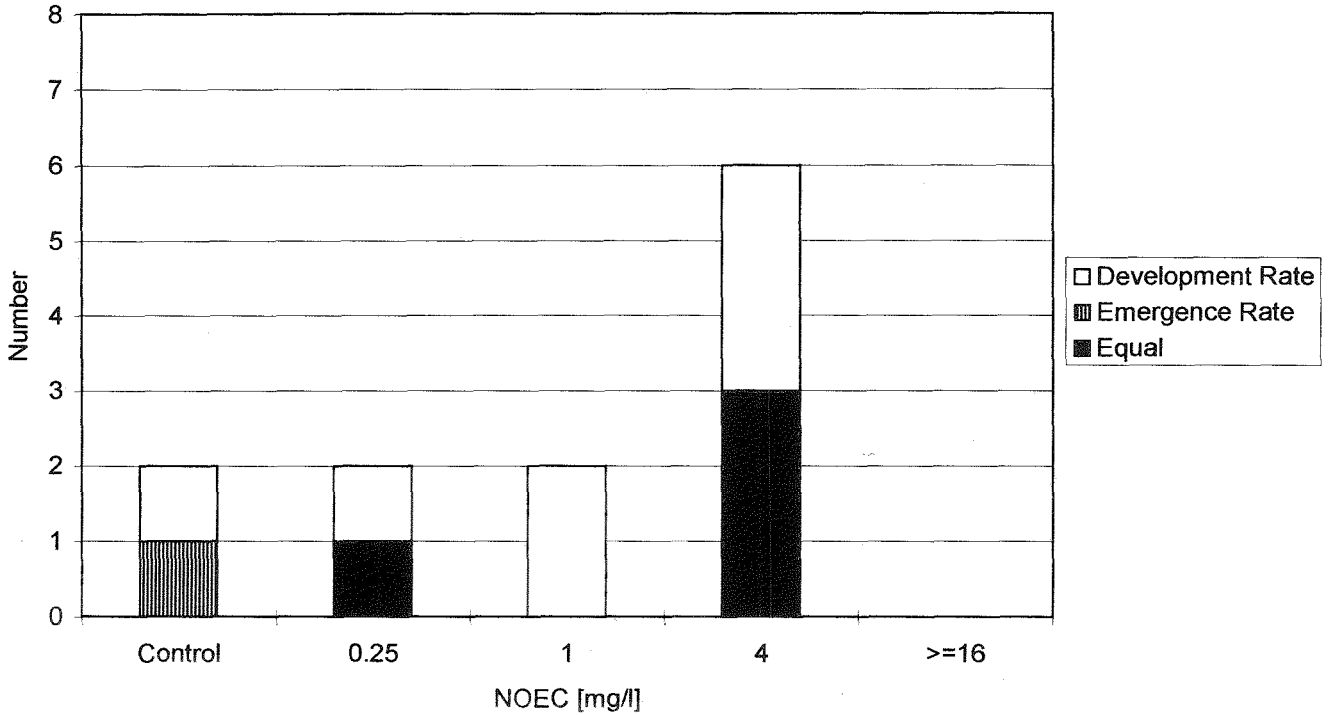

Figure 10: Distribution of the lowest NOECs of the development rate and the emergence rate for trifluralin. Black parts of the bars represent studies in which the NOEC was equal in both end-points, grey parts indicate those studies in which the emergence rate showed the lowest NOEC, and light parts stand for the lowest NOEC in the development rate.

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Test Method Development
- the Rationale behind it, Discussions and Conclusions -

Summary

This paper elucidates the development of the sediment toxicity test as described in this booklet. It explains the decisions made during this process based on intensive discussions and gives the rationale behind it.

Zusammenfassung

Dieser Beitrag erläutert die Entwicklung des in diesem Heft beschriebenen Sediment-Toxizitätstest. Die Entscheidungen, die nach intensiven Beratungen gefällt wurden, werden dargestellt sowie die wissenschaftlichen Grundlagen, auf denen diese Entscheidungen beruhen.

Introduction

The method for the benthic species *Chironomus riparius* described in this booklet has been developed to investigate the potential effects of these pesticides with rather long half lives and which have a high tendency to adsorption (KÖPP 1995). The method should also reflect the typical way of pesticide contaminations with input via the water phase, and which are rather sporadic events (in contrast to effluent inputs). Therefore the principal approach was to simulate a natural scenario as close to reality as possible including a sediment layer, an overlaying water column and spiking the water with the maximum predicted environmental concentration. Because of the longevity of the foreseen test substances the test should consider a chronic exposure of the organisms.

The final design of the test method is based on many discussions and experiences made during its development. The following section reflects these discussions and the conclusions, including to a large extend those made on the occasion of the meeting of participants of the international ring test. It is arranged according to the main discussion topics from the meeting.

Results of the ring tests

The variability in the results was lower in the small-scale ring-test as compared to the international one. This seems to be mainly due to the greater experience of the participants of the first test. This is supported by the fact that the acceptability of the second study within the international ring test was higher than the one from the first study.

The between laboratory variability of the study with trifluralin was larger than the one in the lindane test, partly because of the selected concentration range, and possibly due to the physical-chemical parameters of trifluralin which make this substance difficult to handle (e.g. the test concentrations were far above the water solubility of the substance, which in addition has a high volatility and high adsorptivity). In general, the outcome of the ring test has been quite satisfactory. In addition the variability of the results will likely be further reduced with increasing experience of the test laboratories. It is therefore recommended that laboratories demonstrate their ability to conduct such tests by presenting a minimum of three studies with known (reference) substances before they conduct tests for registration purposes.

Test species

It was generally accepted to have *Chironomus riparius* as the standard test species. Chironomids are an important group of sediment dwelling organisms. They are found worldwide in many ecosystems. The relative sensitivity of young larvae of *C. riparius* is high, and this species can easily be maintained in laboratory cultures. No need was recognized to use only one single laboratory strain of this species.

It has been reported that the handling of very young L1 larvae is difficult and can lead to unwanted damages and thus decrease emergence rate or enhance variability. It was therefore suggested to use L2 larvae for this test. This was opposed by others as L1 larvae seem to be more sensitive than L2 and the outcome of the ring tests indicated a sufficient rate of emergence. Finally it was recommended to test L1 larvae, but to use only 2-3 day (after hatching) old individuals.

Test method

It was found that a period for acclimatization of the test system of about one week is suitable.

To reduce some variability it has been suggested to increase the standardization of some of the test parameters and to give more detailed information. Acceptable levels should be defined for

the following parameters:

Oxygen (> 2.5 mg/l)
pH (for sediment: (6 ± 1) and water (6 - 9))
conductivity (150-1100 µS/cm)
total hardness (≤ 4 mmol/l)

The test method should contain detailed information on the set up of the artificial sediment (e.g. peat quality, peat grinding, mixing of constituents).

Aeration of the test vessels is regarded to be necessary. This should be done by placing a pasteur pipette approximately 2.5 cm above the sediment and providing gentle aeration (approx. 1 bubble/second) without disturbing the sediment surface. Coverage of the test vessels is required, to avoid evaporation of the water, to help to fix the pasteur pipette and to prevent emerging midges from escaping.

Feeding strongly influences larval development. Insufficient food induces the larvae to leave their tubes, to search for food and thus larval growth is reduced (accordingly the development time is increased). Too much food leads to a deterioration of water quality and can increase larval mortality and also impede development. Therefore more detailed guidance should be given to the appropriate feeding rates during the test.

Test vessels

The principal approach to this type of study was to assess the effect of a realistic worst case exposure of a pesticide to sediment dwelling organisms. It was assumed that a certain amount of spray drift (or overspray) hits a shallow water (basic scenarios often use a water depth of 30 cm, the nominal concentrations are based on the respective water volume). To simulate this type of contamination and to carry out the study as close to reality as possible, large 2-3 litre glass beakers, tall forms, are used. It was recommended that the sediment layer should be 2 cm and the water column 15-20 cm. The test substance is added to the water. It will then - according to its physico-chemical parameters - partition between the compartments water and sediment.

If the same test concentrations are used, smaller water columns can have reduced effects as preliminary results showed. Therefore these large vessels should be used when a realistic worst case scenario is simulated using maximum expected concentrations. On the other hand much smaller test units have also been shown to support *Chironomus riparius*. Therefore this type of test should be recommended, if different approaches are followed (e.g. testing several concentrations regardless of the PEC and calculating an EC50).

Test performance

Many of the expected test substances will have low water solubility. The need for solvents/ emulsifiers was discussed. It is recommended to use no such agents if the test substance is applied at concentrations below their water solubility. Otherwise these agents should be applied according to the recommendations given in the revised OECD guideline 305 E.

It is recommended to make daily observations of the test systems as soon as the midges start emerging (this is of particular importance during the peak emergence time).

Assessment of males/females

During the test development no differential effects on the two sexes could be detected. If the results are nevertheless evaluated separately for males and females, this will decrease the statistical power considerably, particularly since one cannot influence the sex ratio in the test vessels when adding the young larvae. On the other hand it cannot be excluded that certain test substances may have a differential impact on males and females (the sexes are easy to distinguish). Therefore it is recommended that the number of emerging males and females should be recorded separately. However generally for the evaluation, females and males will be pooled together. Only, if there is an indication for a differential effect, the evaluations should also be made separately.

Particular, unusual observations should be documented.

The test duration is 28 days. It can be finished at the earliest when more than 90% of the midges in the control have emerged and/or five days after the last midge emerged in the controls.

Quality criteria

Poor emergence rates in the control and unusually long or short development times are an indication of deviating experimental conditions (e.g. feeding rates, temperature or the initial age of introduced animals were incorrect). To reduce variability and increase reliability of the results the following quality criteria for the acceptability of the tests are set:

- a minimum emergence rate of 70% should be achieved as average of the controls,
- the mean development time in the controls should not be longer than "20" days (after test substance application, according to a development rate of 0.05) and not shorter than 10 days (i.e. a rate of 0.1)..

End points

The main endpoint for the assessment of the data is the emergence rate as an indicator of mortality in the test system. Additionally, as a sublethal parameter, the mean development time should be given, respectively the mean development rate (as this allows more confident statistical analysis, see RATTE 1995). The day of first emergence is not recommended as a suitable endpoint, both because of the poor statistical data underlying this factor and also because of the low ecological relevance of this singular event (in contrast to the mean development time).

Chemical analysis

Analytical measurements in the overlaying water should be conducted to assess the correct application of the test substance and to compare its fate in this test system with the water/sediment metabolism study. For this reason analytical samples should be taken at days 0 (shortly after application), 7 and 21 from two treated replicates. A more thorough estimation of the fate of the test substance in such a system can be derived from the water/sediment metabolism study. A preliminary series of tests have shown that this study also gives an indication for the distribution behaviour of the test substance in the chironomid study (BARRET et al. 1995). In addition, the results of the small-scale ring-test have shown that even large differences in the type of sediment used - which also means differences in the distribution behaviour of the test substances - had no measurable impact on effect of test substances on the chironomids (HAMER 1995).

Conceptual and statistical considerations

Some discussion centred around the problem of an "EC_X" versus "NOEC/LOEC" approach. The following recommendations were finely agreed upon:

It is advisable to perform a limit test first. This should be conducted using 4 replicates in the control and four replicates for the limit concentrations (this is based on an "overspray" scenario and a water depth of 30 cm to create a worst case situation). Only if significant effects are observed in this test, further tests with additional concentrations become necessary:

- To define a NOEC/LOEC with sufficient confidence, a test has to be conducted using four replicates per concentration; the spacing between the concentrations should not exceed a factor of two.

- As the second tier alternatively to the NOEC/LOEC approach an "EC_X" type study is recommended. This should be conducted with a minimum of six concentrations and 1 replicate in each. The control should be carried out with a minimum of three replicates. At least three of the concentrations should be in the range of $\geq 5\%$ $\leq 95\%$ effect. (This is not necessary, if 0 and 100% effect are within a concentration range of a factor of two.)

References:

BARRET, K.L.; STRELOKE, M.; HEIMBACH, F. (1995): A comparison of the partioning of pesticides in natural and artificial sediment water systems. In: Long-term toxicity-test with *Chironomus riparius*. Development and validation of a new test system, edited by M. Streloke and H. Köpp. Mitt. Biol. Bundesanst. Land- u. Forstwirtschaft Berlin-Dahlem **315**: 22-32.

HAMER, M.J. (1995): International toxicity ring-test on sediment-dwelling *Chironomus riparius* - preliminary test. In: Long-term toxicity-test with *Chironomus riparius*. Development and validation of a new test system, edited by M. Streloke and H. Köpp. Mitt. Biol. Bundesanst. Land- u. Forstwirtschaft Berlin-Dahlem **315**: 16-21.

KÖPP, H (1995): History and background of the sediment toxicity test. In: Long-term toxicity test with Chironomus riparius: Development and validation of a new test system, edited by M. Streloke and H. Köpp. Mitt. Biol. Bundesanst. Land-Forstwirtschaft Berlin-Dahlem **315**: 7-15.

RATTE, H.-T. (1995): Statistical analysis of the results from the international toxicity ring-test on sediment-dwelling *Chironomus riparius*. In: Long-term toxicity test with Chironomus riparius: Development and validation of a new test system, edited by M. Streloke and H. Köpp. Mitt. Biol. Bundesanst. Land-Forstwirtschaft Berlin-Dahlem **315**: 49-63.

Proposal for a BBA-Guideline:

Effects of plant protection products on the development of sediment-dwelling larvae of *Chironomus riparius* in a water-sediment system

1. INTRODUCTION

The methods described here assess the potential impact of pesticides on the sediment dwelling life stage of the organism *Chironomus riparius*, a common "nonbiting" midge. The study will be conducted using one concentration or a range of concentrations of the test material applied to the water column of a sediment-water system containing first instar larvae, for a period sufficient to assess the impact on full maturation of the larvae to adult midge.

2. MATERIALS

2.1. Test Species

The test should be initiated using first instar larvae obtained from in-house cultures of *Chironomus riparius* (syn. *Chironomus thummi*). Details of culture methods are given in Annex I. Identification of cultures must be confirmed before testing. Details of culture and source of organisms must be provided in the test report.

2.2. Sediment

For the test an artificial sediment will be used. The artificial sediment (according to OECD test guideline No. 207 (OECD 1984)) should be prepared as follows (on the basis of dry weights):

- 10 % sphagnum peat (as close to pH 5.5 to 6.0 as possible, no visible plant remains, air dried and finely ground)
- 20 % kaolin clay (kaolinite content preferably above 30 %)
- 70 % industrial sand (fine sand should predominate with more than 50 per cent of the particles between 50 and 200 microns)
- pH of the final mixture of the sediment is adjusted to 6.0 $\pm$ 0.5 by addition of calcium carbonate (chemically pure quality)

It is important to use only air dried and finely ground peat, which appears to be powdered.

The dry constituents are blended in the correct proportions and mixed thoroughly, e.g. in a large-scale laboratory mixer or small electric cement mixer. Some test water is added to moisten the artificial sediment before it is used for the study.

2.3. Test Water

Any suitable natural or synthetic water is accepted, but the use of the reconstituted water "M4" or "M 7" according to Elendt is recommended (Annex II). As quality criteria, the pH of the water should be between 6 and 9 at the start of the test, the conductivity between 150 and 1100 µS/cm and the total hardness not higher than 4 mmol/l.

2.4. Test Vessels

The study will be conducted in glass 2 to 3 l-beakers measuring 10 to 13 cm in diameter. Any other vessels are also suitable, but they should guarantee a suitable height of the overlying water (15 to 20 cm, see 3.1.).

3. TEST METHODS

3.1. Preparation of Test Vessels

The amount of wet artificial sediment which is needed for each test container is filled into a vessel at a layer of 2.0 cm deep. To avoid a separation of the ingredients of the sediment and high turbidity of the test water, the sediment surface is covered by a plastic "plate", which floats as the water is poured onto it. Other devices may be also appropriate. The water is poured into the beaker very slowly, taking care not to disturb the sediment. It is important not to disturb the sediment as the ingredients of the sediment might get separated into different sediment layers, which may influence the results of the test.

The vessels should be prepared one week before the study starts and be acclimatised under the test conditions. The vessels should contain 2.0 cm of sediment and a water column 15 to 20 cm deep. The exact volume of water added should be recorded, and the level marked outside on the test vessel.

Gentle aeration will be provided through a glass Pasteur pipette fixed 2 to 3 cm above the sediment layer (i.e. ca. 1 bubble / sec).

Beakers should be covered (e.g. by glass plates). If necessary, the water levels should be topped up during the study to the original starting volume to prevent concentration of the test material. Water levels should not change more than 10 %.

3.2. Incubation

The tests should be conducted in a constant temperature room at 20 ± 2 °C with a photo period of 16 hours light (intensity about 1000 lux), 8 hours dark.

3.3. Inserting of Test Organisms

About four to five days before application (application = day 0 of the study) some egg masses are taken from the cultures and are deposited into small vessels in culture medium. Aged medium from the stock culture or freshly prepared medium may be used. If the latter is used, a small amount of food e.g. green algae, a finely ground fish flake suspension or the filtrate of a finely ground fish flake suspension has to be added. Fresh egg masses should be chosen only.

Normally the larvae begin to hatch after 2 to 3 days. Twenty-five larvae of the first larval stage (2 - 3 days old, i.e. 2 - 3 days after hatching) are allocated randomly to each test vessel with a blunt pipette (e.g. five collectives of five larvae each, per vessel). When adding the larvae and also for the following 24 hours, the aeration of the water must be stopped.

3.4. Application of the Test Substance

One day after adding the larvae, the slight aeration (ca. 1 bubble / sec) should be started again and the test material will be applied to the water column on the same day, in a small volume of water (about 10 ml per vessel) just below the water surface by using a pipette, and gently mixing to ensure homogeneous distribution, without disturbing the sediment.

3.5. Test Concentrations

The test concentrations are calculated as concentrations of the test substance in the overlying water. If test concentrations are higher than the solubility of the test substance in water, solvents or emulsifiers have to be used in accordance with the OECD-Guideline 305E (draft) (OECD 1994).

If the study is performed on one test concentration and the control, the statistical power must be sufficient to detect differences from the control of 20 % as significant ($p = 0.05$), (i.e. usually at least 6 replicates are necessary). If the study is performed to yield the NOEC / LOEC, as a minimum four replicates have to be tested for each concentration and the control. The factor between concentrations should not be greater than 2.0.

If the study is performed to yield an EC_X, at least six concentrations have to be tested. At least three of these concentrations should have emergence between 10 and 90 % for the determination of the EC50 or between 5 and 30 % for an EC15, but the factor between concentrations should not be greater or lower than 2.0. For these studies, the minimum number of replicates is one for treatments and three for the control. Increasing this number lower the confidence intervals of the ECx which lead to a higher statistical power of the test.

3.6. Food

It is necessary to feed the larvae at least three times per week. 1 mg fish-food (a suspension in water or finely ground food, e.g. Tetra Min® or Tetra Phyll®, see information's given in Annex I) per day and per larvae seems to be adequate for young larvae (i.e. 175 mg per vessel per week). Slightly more food may be necessary for older ones.

4. EVALUATION

4.1. Emergence of Midges

The endpoints of the study are the development time and the total number of fully emerged male and female midges. Males are easily distinguished by having plumose antennae.

The test vessels should be observed at least three times per week to make a visual assessment of any behavioural differences compared with the control. During the period of expected emergence (normally starting at day 13 to 16 and lasting until day 25) a daily check of emerged midges is

necessary. The sex and number of adults emerging have to be recorded daily. After identification the midges are removed from the vessels. Any egg masses deposited prior to the termination of the test should be recorded and removed to prevent re-introduction of larvae into the sediment. Only the number of fully emerged male and female midges will be counted. The number of visible pupae that have failed to emerge will be counted separately.

4.2. Test Duration

The test duration is 28 days (after application). If midges emerge earlier, the study may be finished for a minimum of five days after emergence of the last adult in control.

4.3. Physico-chemical Parameters

In all tests the oxygen concentration and pH should be recorded in all test vessels at the start and end of the study. These parameters should also be recorded at least once weekly for the duration of the test in all test vessels. Water temperature should be recorded at least in one or two test vessels at the same times.

4.4. Analysis of Test Concentrations

As a minimum, samples of the overlying water, the pore water and the sediment must be analysed 1 hour, 7 days and 28 days after application in the highest test concentration and a lower one. The analytical measurements of the concentrations in sediment are not necessary if the partitioning of the test substance between water and sediment has been determined in a separate similar study. If analysis needs large samples which cannot be taken from test vessels without influences on the test system, analysis should be performed during the study (i.e. at least 1 hour and 7 days after application) on samples from additional parallel test vessels which are handled and exposed in the same way but which are not used for biological data evaluation. In some cases it might not be possible to analyse concentrations in pore water as the sample size is too small.

5. STATISTICAL ANALYSIS

If there are no indications on different sensitivities of sexes, male and female results are pooled for statistical analyses. The raw data are statistically evaluated in three steps:

1. The calculation of per-vessel statistics,
2. the calculations of per-treatment statistics,
3. the determination of effective concentrations such as the ECx or statistically significant threshold concentrations such as the NOEC/LOEC.

5.1. Per-Vessel Statistics

End-points are the emergence rate, which is related to mortality of the test cohort, and the development rate of the midges. Individual vessels are considered as replicates (independent observations).

5.1.1. Emergence Rate

Per vessel statistics for emergence rate are only necessary if statistical testing is intended to determine the NOEC/LOEC using ANOVA methods (e.g. t-test, Dunnett-test, Williams-test; for computation of the ECx see 5.2). The sum of midges emerged per vessel, n_e, is calculated and divided by the number of larvae introduced, n_i:

$$ER = \frac{n_e}{n_i} \qquad (1)$$

If $n_e > n_i$ then n_i is replaced by n_e. k values of ER (k = number of replicates) are calculated, the square root of which should be transformed by the arcsin-function to obtain an approximate normal distribution and to equalise variances. The arcsin-transformed values, ERarc, may be obtained from tables (e.g. Geigy Scientific Tables) or from the following function:

$$\text{ERarc} = \arcsin(\sqrt{\text{ER}}) \qquad (2)$$

or equivalently:

$$\text{ERarc} = \text{ATN}\left\{\frac{\sqrt{\text{ER}}}{\sqrt{1-\text{ER}}}\right\}$$

In contrast to arcsin, the ATN function (arc tangent) is usually available on desk calculators or PCs.

5.1.2. Development Rate

The mean development time represents the mean time span between the application of the test substance (day 0) and the emergence of the experimental cohort of midges. (For the calculation of the true development time the age of larvae at the time of application should be considered.) The development rate is the reciprocal of the development time (unit: 1/day) and represents that portion

of larval development which takes place per day. The development rate is preferred for the evaluation of these sediment toxicity studies as its variance is lower, more homogeneous and it is closer to normal distribution as compared to development time. Hence, powerful parametric test procedures may be used with development rate rather than with development time.

For the following statistical performances the number of midges observed on inspection day x are assumed to be emerged at the mean of the time interval between day x and day x-l (l = length of the inspection interval, usually 1 day). The mean of the development rate per vessel is calculated according to:

$$\bar{x} = \sum_{i=0}^{m} \frac{f_i \bullet x_i}{n_e} \qquad (3)$$

where i index of inspection interval

m maximum number of inspection intervals

f_i number of midges emerged in the inspection interval i

n_e total number of midges emerged until the end of experiment ($= \sum f_i$)

x_i development rate of the midges emerged in interval i

$$x_i = 1 \Big/ \left(day_i - \frac{l_i}{2} \right)$$

where day_i inspection day (days since application)

l_i length of inspection interval i (days, usually 1 day)

5.2. Per-Treatment Statistics

The arithmetic mean of both endpoints and the variance per treatment or control are calculated according to:

$$\bar{X} = \frac{\sum_{i=1}^{k} x_i}{k} \qquad (4)$$

$$s^2 = \frac{1}{k-1} \left(\sum_{i=1}^{k} x_i^2 - \frac{\left(\sum_{i=1}^{k} x_i \right)^2}{k} \right) \qquad (5)$$

where k number of replicates (vessels) per treatment or control

x_i emergence rate ERarc (Eq. 2) or development rate $\bar{x}$ (Eq. 3) per vessel

$\bar{X}$ per treatment mean

s^2 per treatment variance

$\overline{X}$ and s^2 are used for ANOVA procedures such as STUDENT t-test, Dunnett-test, or Williams-test as well as for the computation of 95%-confidence intervals according to:

$$\overline{X} \pm t_{0.05;df} \frac{s}{\sqrt{k}} \qquad (6)$$

where $\overline{X}$ per treatment mean (Eq. 4)

s per treatment standard deviation (Square root of Eq. 5)

$t_{0.05;df}$ tabulated t value for =0.05 (two-sided) and df = k-1

If an analysis of variance has been performed, s and df should be replaced by the pooled variance estimate obtained from ANOVA and by its degrees of freedom, respectively. Eq. 4 to 6 are usually calculated by commercial statistical software using the per-vessel results.

To compute the EC50 or any other ECx , the per-treatment statsitics should be used as data in regression analysis. Therefore, the original (not the transformed) numbers of emerged midges per treatment are summarised to calculate the number of midges per treatment which did not emerge This "mortality" should be corrected by the control "mortality" (according to Abbott's formulae, see Finney, D. Y. 1978. Statistical Method in Biological Assay. - Charles Griffin & Company Ltd, London) before it is used for probit analysis or similar statistics.

5.3. Statistical Testing and Inference

1. For a limit test (comparison of control and one treatment only) the STUDENT t-test is recommended.
2. For the calculation of NOECs/LOECs multiple t-tests such as Dunnett or Williams test (= 0.05, one-sided) should be performed.
3. In the emergence rate, 95%-confidence limits are calculated by probit analysis (or logit, weibit, etc.), or in case of failure, by non-parametric methods such as a moving averages.
   In the development rate, Eq. 6 should be applied.
4. In the emergence rate, ECx-values are calculated using probit analysis (or logit, weibit, etc.), or in case of failure, non-parametric methods such as a moving averages or simple interpolation.
   In the development rate, the per-treatment means, $\overline{X}$, or %inhibitions relative to the control are used for regression analysis. An ECx is obtained by inserting a value corresponding to x% of the control mean into the equation found by regression analysis. Confidence limits are calculated after Fieller (see Finney, D. Y. 1978. Statistical Method in Biological Assay. - Charles Griffin & Company Ltd, London).

6. CRITERIA OF VALIDITY

For the untreated control the following criteria have to be fulfilled:

- emergence should be equal to or greater than 70 %,
- the mean development time for larvae should not be more than 20 days after application (i.e. a development rate of 0.05) and not shorter than 10 days (i.e. a development rate of 0.1).

The oxygen concentrations should always be > 2.5 mg/l and the pH between 6 and 9 in all test vessels.

7. REPORTING

The test report should at least provide the following information:

- test substance (name, common name, chemical name, Batch-No., purity etc.)
- test animals used (species, source of organisms, breeding conditions)
- handling of egg masses and larvae
- age of test animals when inserted into test vessels

- ingredients and preparation of the artificial sediment
- pH and water holding capacity of the artificial sediment
- preparation of the test water
- oxygen concentration, pH, conductivity, hardness of the test water at the start of the test
- depth of sediment and overlying water
- volume of overlying and pore water; weight of wet sediment with and without pore water
- test vessels (material and size)

- test concentrations and number of replicates
- description of the preparation of stock and test concentrations
- method of application

- exposure conditions (temperature, light cycle and intensity)
- method of aeration and intensity
- food (kind of food, preparation, amount of food, feeding regime)

- number of emerged male and female midges per vessel per day
- number of pupae which failed to emerge (per vessel and day)

- percent emergence per replicate and treatment rate (male and female midges pooled)
- mean development rate of fully emerged midges per replicate and treatment rate (male and female midges pooled)

- kind and results of statistical evaluations
- results of statistical comparisons of treatments and control

- pH, oxygen concentration and temperature in test vessels during the study
- replacement of evaporated test water (if appropriate)
- results of analyses of test concentrations

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Annex I: Recommendations for culture of *Chironomus riparius*

Chironomus riparius is a non-biting midge, whose larvae are common to most aquatic environments. The larvae pupate and emerge from the water as adults. Adults usually breed within 24 hours post-emergence. Females extrude gelatinous strands of eggs into the water. Larvae hatch after 2 - 4 days. They go through four instar stages, pupate and emerge as adults in 13 - 25 days at 20 °C.

Culture Conditions

Chironomus larvae may be reared in crystallising dishes or larger containers. Fine quartz sand is spread in a thin layer of about 5 to 10 mm deep over the bottom of the container. Kieselgur (e.g. Merck, Art 8117) has also been shown to be a suitable substrate (a thinner layer of up to a very few mm is sufficient). A suitable water is then added to a depth of several cm. Water levels should be topped up as necessary to replace evaporative loss, and prevent desiccation. Water can be replaced if necessary. Gentle aeration should be provided. The larval rearing vessels should be held in a suitable cage which will prevent escape of the emerging adults. The cage should be sufficiently large to allow swarming of emerged adults, otherwise copulation may not occur (minimum is ca. 30 x 30 x 30 cm).

Cages should be held at room temperature or in a constant environment room at 20 ± 2 °C with a photo period of 16 hour light (intensity ca. 1000 lux), 8 hours dark. It has been reported that air humidity of less than 60 % RH can impede reproduction.

Dilution Water

Any suitable natural or synthetic water may be used. Well water, dechlorinated tap water and artificial media (e.g. Elendt "M4" or "M7" medium, see Annex II) are commonly used. The water has to be aerated before use. If necessary, the culture water may be renewed by pouring or siphoning the used water from culture vessels carefully without destroying the tubes of larvae.

Feeding Larvae

Chironomus larvae should be fed with a fish flake food (Tetra Min®, Tetra Phyll® or other similar brand of proprietary fish food), at approximately 250 mg per vessel per day. This can be given as a dry ground powder or as a suspension in water: 1.0 g of flake food is added to 20 ml of dilution water and blended to give a homogenous mix. This preparation may be fed at a rate of about 5 ml per vessel per day. (Shake before use.) Older larvae may receive more.

Feeding is adjusted according to the water quality. If it becomes cloudy, feeding should be reduced. Food additions must be carefully monitored. Too little will cause emigration of the larvae into the water column, and too much will cause increased microbial activity and reduced oxygen concentrations. Both conditions can result in reduced growth rates.

Some green algae (e.g. *Scenedesmus subspicatus*, *Chlorella vulgaris*) cells may also be added when new culture vessels are set up.

Feeding Emerged Adults

Some workers have suggested that a cotton wool pad soaked in a saturated sucrose solution may serve as a food for emerged adults.

Emergence

At 20 ± 2 °C adults will begin to emerge from the larval rearing vessels after approximately 13 - 15 days. Males are easily distinguished by having plumose antennae.

Egg Masses

Once adults are present within the breeding cage, all larval rearing vessels should be checked three times weekly for deposition of the gelatinous egg masses. If present, the egg masses should be carefully removed. They should be transferred to a small dish containing a sample of the breeding water. Egg masses are used to start a new culture vessel (e.g. 2 - 4 egg masses / vessel) or are used for toxicity tests.

First instar larvae should hatch after 2 - 3 days.

Set-up of New Culture Vessels

Once cultures are established it should be possible to set up a fresh larval culture vessel weekly or less frequently depending on testing requirements, removing the older vessels after adult midges have emerged. Using this system a regular supply of adults will be produced with a minimum of management.

Annex II: Preparation of test solutions "M4" and "M7"

Elendt (1990) has described the medium known as "M4". The medium "M7" is prepared as the "M4"-medium with the exception of the substances which are marked in Table 1, which are by a factor of 4 lower in "M7" than in "M4". A publication of the "M7"-medium is in preparation (Elendt, pers. communication). The solution should not be prepared as described in Elendt & Bias (1990), as in this paper the concentrations of $NaSiO_3 \cdot 5\ H_2O$, $NaNO_3$, KH_2PO_4 and K_2HPO_4 for the preparation of the stock solutions are not correctly given.

Preparation of the "M7"-medium

Each solution I is prepared individually according to Table 1 and from these solutions a combined stock solution II is prepared by the amounts indicated in Table 1. Fifty ml from the combined stock

Table 1: Stock solutions of trace elements for medium M4 and M7

stock solutions (I)		amount (mg) made up to 1 litre deionised water	to prepare the combined stock solution II mix the following amounts (mg) of stock solutions (I) and make up to 1 l of deionised water		final concentrations in test solution (mg/l)	
			M4	M7	M4	M7
H_3BO_3	*)	57190	1.0	0.25	2.86	0.715
$MnCl_2 \cdot 4\ H_2O$	*)	7210	1.0	0.25	0.361	0.090
LiCl	*)	6120	1.0	0.25	0.306	0.077
RbCl	*)	1420	1.0	0.25	0.071	0.018
$SrCl_2 \cdot 6\ H_2O$	*)	3040	1.0	0.25	0.152	0.038
NaBr	*)	320	1.0	0.25	0.016	0.004
$Na_2MoO_4 \cdot 2\ H_2O$	*)	1260	1.0	0.25	0.063	0.016
$CuCl_2 \cdot 2\ H_2O$	*)	335	1.0	0.25	0.017	0.004
$ZnCl_2$		260	1.0	1.0	0.013	0.013
$CoCl_2 \cdot 6\ H_2O$		200	1.0	1.0	0.010	0.010
KJ		65	1.0	1.0	0.0033	0.0033
Na_2SeO_3		43.8	1.0	1.0	0.0022	0.0022
NH_4VO_3		11.5	1.0	1.0	0.00058	0.00058
$Na_2EDTA \cdot 2\ H_2O$	*) **)	5000	20.0	5.0	2.5	0.625
$FeSO_4 \cdot 7\ H_2O$	*) **)	1991			1.0	0.249

*) These substances differ in M4 and M7, as indicated above

**) These solutions are prepared individually, then poured together and autoclaved immediately.

solution II and the amounts of each macro nutrient stock solution which are given in Table 2 are made up to 1 l deionised water to prepare the "M7"-medium. A vitamin stock solution is prepared by adding 3 vitamins to deionised water as indicated in Table 3 and 0.1 ml of the combined vitamin stock solution are added to the final "M7"-medium shortly before use. (The vitamin stock solution is stored frozen in small aliquots.) The medium is aerated and stabilised.

Table 2: Macro nutrient stock solutions for medium M4 and M7

	amount (mg) made up to 1 litre deionised water	amount of macro nutrient stock solutions added to prepare medium M4 and M7 (ml/l)	final concentrations in test solution M4 and M7 (mg/l)
$CaCl_2 \cdot 2\ H_2O$	293800	1.0	293.8
$MgSO_4 \cdot 7\ H_2O$	246600	0.5	123.3
KCl	58000	0.1	5.8
$NaHCO_3$	64800	1.0	64.8
$NaSiO_3 \cdot 9\ H_2O$	50000	0.2	10.0
$NaNO_3$	2740	0.1	0.274
KH_2PO_4	1430	0.1	0.143
K_2HPO_4	1840	0.1	0.184

Table 3: Vitamin stock solution for medium M4 and M7

All three vitamin solutions are combined to make a single vitamin stock solution.

	amount made up to 1 l of deionised water (mg)	amount of vitamin stock solution added to prepare medium M4 and M7 (ml/l)	final concentrations in test solution M4 and M7 (mg/l)
Thiamine hydrochloride	750	0.1	0.075
Cyanocobalamin (B12)	10		0.0010
Biotine	7.5		0.00075

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The Establishment of a Long-Term Toxicity Test on Sediment-Dwelling Organisms in the Registration Procedure of Plant Protection Products

Summary

This paper deals with general regulatory issues of toxicity-tests on sediment-dwelling organisms and with the particular test method presented in this booklet. There is a regulatory requirement for data from this type of study. The results of the international ring-test are discussed in terms of the authorization of plant protection products. Important details of the method are raised and explained. Finally a description is given of how the data will be included into the whole risk assessment for aquatic organisms.

Zusammenfassung

In diesem Beitrag wird die Bedeutung von Sedimentorganismen-Tests im allgemeinen für Zulassungsverfahren und speziell für die in diesem Buch vorgestellte Methode diskutiert. Die Notwendigkeit der Vorlage von Daten aus diesen Test wird begründet. Die Ergebnisse des internationalen Ringversuches werden bezüglich der Eignung der Testmethode für das Zulassungsverfahren eingeschätzt. Wichtige Details werden dargestellt und erläutert. Abschließend wird die Einbindung der Testergebnisse in das gesamte Verfahren der Bewertung der Auswirkungen auf Gewässerorganismen beschrieben.

Need for sediment toxicity tests

Risk assessments of plant protection products concerning aquatic organisms are mainly based on data from laboratory tests with algae, daphnia and fish. This approach is reasonable for most of the substances and there will be no significant changes in the near future. However, data for substances with strong adsorption to sediments can be difficult to interpret because the actual concentrations in the test vessels may differ strongly from nominal ones. To get comparable results

for different substances, flow-through test systems have to be used with those substances. But this exposure system is less realistic than a semi-static one with respect to the evaluation of monitoring data and for the setting of buffer zones or other risk managemet measures. In the latter case nominal concentrations are more appropriate whereas the measured ones are the most suitable to assess monitoring data.

In the field adsorptive substances partition into sediment and adsorb to other particulate matter in the waterbody. Thus, it can be difficult to predict their concentrations in the different compartments of the aquatic ecosystem. For species inhabiting the water column, it is reasonable to assess the exposure on the basis of the initial concentrations of these adsorptive substances in the water column, whereas the situation for sediment-dwelling organisms is more complicated. At the moment it cannot be decided whether the concentration in the sediment or in the porewater is more important for the toxicity to sediment-dwelling organisms. For substances with a moderate logPow-value it is assumed that the concentration in the porewater is the most important one (HEBERT&HAFFNER 1991). Substances with a high logPow may enter by other routes into the sediment organisms than in those living in the water column. Species dwelling in the sediment have direct contact of their integument to this matrix (KÖPP 1995). Furthermore they may ingest quantities of this material (many tend to selectively eat organic particles rather than whole sediment) leading to direct contact between contaminant and lipophilic membranes of the intestine (BOESE et al. 1990). During a considerable period after the contamination the concentration in the water column differs from that in the porewater even in the uppermost sediment layer (BARRET et al. 1995). Some partitioning models describe the concentration of the substance in different parts of the system after the equilibrium has been reached. However this equilibrium will frequently not be reached in natural systems as these systems frequently undergo natural fluctuations.

Sediments may act as sinks for those adsorptive compounds which are persistent (HAMER et al. 1992). The exposure of benthic organisms to these substances is different from pelagic ones living in the water column. Nevertheless for some compounds the remobilisation of substances from sediments (reestablishment of an equilibrium by desorption of adsorbed material, and as a consequence the exposure of pelagic organisms) has been demonstrated (HERBERT&HAFFNER 1991). The quantification of this type of exposure is extremely difficult (VAL KLUMP et al. 1991).

Considering all these aspects toxicity data on sediment organisms are necessary for a reasonable risk assessment for aquatic systems. It is important to use a standardized test method in order to

get comparable data and to rank substances. A long-term test may be more appropriate because relevant substances persist in sediments and subchronic effects have to be expected. Data available from sediment toxicity tests show that these endpoints are more sensitive than acute mortality (CHANDLER 1990).

Suitability of the proposed test method

The NOEC-values of the ring-test showed some variability (RATTE 1995). The likely major reasons were:

- the complexicity of the test system, e.g. sediment quality, handling of test organisms, application of the test substance.
- many participating laboratories were not familiar with the proposed test method and some were not familar with sediment toxicity testing at all.

The outcome of the first small-scale ring test of the members of the BBA/IVA working group showed that reproducible results can be generated after a short training period. Hence, the introduction of the proposed test method into the registration procedure of plant protection products seems to be justified.

Trigger values for toxicity tests on sediment-dwelling organisms in the registration procedure of plant protection products

Data from the metabolism study in water/sediment systems (BBA 1990) will be generally used as the basis for decisions on the need for a sediment toxicity test. The study should be required if the active ingredients, toxic metabolites or bound residues are found in the sediment in an amount higher than 10% of the applied test substance after day 14. Furthermore, the NOEC from the chronic toxicity test with Daphnia should be lower than 0.1 mg/l or the BCF in fish higher than 100. With respect to the latter cases it is important that the sediment-dwelling organism test is an additional test and data on the effects of the substance on aquatic invertebrates are available. Furthermore considering the exposure routes spray-drift and runoff and usual field conditions it is unlikely that higher concentrations will be reached. At the moment the calculation of predicted environmental concentrations is burdened with a lot of shortcomings. Therefore decisions upon the need of a study for a single compound should not be based on a special exposure assessment for the relevant plant protection product.

Test substance

Annex II of the directive 91/414/EEC which will be the basis of the registration of plant protection products in the EU requires a sediment toxicity test with the active ingredient in certain cases. The risk assessment procedure of this guideline is based mainly on tests with the active ingredient. Furthermore, the metabolism study in water/sediment systems is conducted using the active ingredient. The partioning of the radiolabelled test substance between sediment and water column is investigated in this study very thoroughly and the test system is nearly similar as that presented in this booklet. Therefore data from this study are going to be used as a surrogate for the analytical measurement of the concentration of the test substance in the sediment of the toxicity test with *Chironomus riparius* if the same test material is used.

Replicates and dosing

For regulatory purposes, a test design should be recommended which can be performed routinely by different laboratories to generate valid data. For animal welfare concerns the number of animals used in the test should be as low as possible. Taking these arguments into account and from a statistical point of view the concept of the "ECx-estimation" and the calculation of the slope of the concentration-effect curve seems to be the most effective proposal. Since the determination of the concentration-effect curve gives far more information on the toxicity of the test substance, regulatory authorities clearly favour a test design to determine an ECx and the slope. Future experience will show whether study directors will be able to choose the test concentrations routinely with the necessary precision. Otherwise even a test concentration where no effect was observed cannot be used for the risk assessment because the amount of replicates is insufficient.

As the test species is considered as representative of other benthic organisms, the test results must be sufficiently reliable in order to protect even more sensitive species. Considering these aspects the determination of an EC15 instead of the NOEC seems to give more information with higher precision than the NOEC/LOEC approach. Points lower than 15 should be used with care because the confidence intervals become too large. The data of the ring-test clearly showed the disadvantages of the NOEC/LOEC approach:

- high variability in the experiment corresponds to a low sensitivity of the test results (NOEC/LOEC).

- three replicates for each treatment are insufficient. The NOEC approach needs at least 6 replicates per concentration.

Nevertheless, the inclusion of the latter approach is reasonable because it is the common one at the moment. In order to get robust NOEC/LOEC-values to detect 20% difference from control with the Dunnett-test at least 6 replicates are needed, assuming a coefficient of variation of 10%. For regulatory purposes it is always helpful to get a test concentration where no effect was observed because this value is considered to be safe. However a NOEC should only be used in the risk assessment if it has sufficient statistical power.

In accordance with the introduction to the Annex II of the directive 91/414/EEC the objective of the test should be the generation of a concentration/response curve. Especially for substances with a shallow slope effects are to be expected at concentrations clearly below the EC15 whereas the range of effects for other substances may be easier to be determined. Considering the low water solubility of many of the relevant substances and their expected environmental concentrations, it is advisable to set an upper limit for the test concentrations above which testing will not be required. Otherwise the regulator has to discuss the test concentrations for each substance on the basis of the intended application rate and the fate data with the applicant. Changes in the use of the plant protection product may then require a new test. Considering the possible exposure scenario via spray drift in low and high growing cultures (30 cm deep water body, overspray application as a worst case assumption) a limit of 0.1 mg/l nominal concentration in the water column seems reasonable. This concentration is also suitable regarding data from field monitoring studies. Additionally, the highest test concentration is limited by the need of solubilising agents which have to be used in accordance with the draft OECD-guideline 305 E (paragraph 18 and 37). The acceptable concentrations of these agents in the test solutions limit the highest possible test concentrations. In any case, precipitation of the test substance or droplets on the water surface should be avoided.

Sometimes semi-static and flow-through test systems are recommended instead of static studies. Flow-through systems are appropriate if a continuous entry of a substance into water bodies can be anticipated. Many plant protection products are applied only once per season. Those which are applied several times and persist in aquatic systems between two applications have to be applied to the test system several times. This system offers also the flexibility which will be needed considering expected future developments in the field of exposure assessment.

The application of the test substance via the water surface is suitable to determine lethal and sublethal effects to benthic organisms caused by the entry of contaminants into water bodies. It is a different approach from those toxicity tests where the substance is spiked into the sediment. The method presented in this booklet covers mainly the exposure of sediment organisms via spray and

drift of plant protection products from adjacent fields. The entry of these substances via surface runoff and drainage systems is also covered if the contaminants are translocated in the water phase. The bioavailability and therefore the toxicity of compounds adsorbed to soil particles is lower (HAMER et al. 1992). It is expected that the intake of adsorbed substances leads to lower effects and the application of the test substance onto the water surface represents the worst case scenario. As compounds adsorbed to soil particles settle on the surface of the sediment even a test system with a spiked sediment would not cover the intake of adsorbed substances into waterbodies.

Test Organisms

Chironomids are insects and represent a taxonomic group which is not currently tested routinely in aquatic toxicology. The situation has changed recently as Annex II of the directive 91/414/EEC includes the requirement for a chronic test with insects. Studies generated in accordance with the test method described in this booklet generally fullfil this requirement. The data from this test enhance the risk assessment with respect to safety factors and statistical approaches of hazard assessment. Chironomids are part of the infauna of sediments, of the diet of fish and waterfowl and are therefore ecologically important (OLAFSSON 1992; TOWNSEND et al. 1981). They are widely distributed, have a short life-cycle and are easy to culture in the laboratory. Available data show that the first instar of *C. riparius* is the most sensitive developmental stage (WILLIAMS 1986).

Type of sediment

The advantage of an artificial sediment is the absence of possible contaminants or other constituents which may influence the test results (SUEDEL&RODGERS 1994). As a consequence the toxicity data are more comparable because the variability of natural sediments between laboratories is excluded. The studies on lindane and trifluralin demonstrated that in this tests the type of the sediment had no effect on the toxicity of the substances. Metabolism tests with five active ingredients showed that the partitioning in test systems using natural and artificial sediments is comparable if the organic carbon content is similar (BARRET et al. 1995). Although this database is still small, the use of artificial sediment is recommended.

Analysis of test concentrations

In aquatic testing test concentration are usually verified by analysis. This is also required for the proposed test method, at least in the water column. The Canadian EPA recommends analysis of sediment and porewater in all sediment toxicity tests, but the test substance is spiked into the sediment in these studies (draft guidelines). In order to provide comparable toxicity data for different substances, the concentration of the test substance in the pore water should be determined if a suitable method is available. In these cases the concentration of the test substance reaching the part of the test system where the chironomids dwell is measured.

Members of the BBA/IVA-working group performed studies with radiolabelled substances in toxicity tests on *C. riparius* using a natural and an artificial water/sediment system (BARRET 1995). The comparison of data from these studies and those from the metabolism study in a water/sediment system with the same substance shows similar partitioning between sediment and water column (Figure 1). The latter type of study is usually available for active ingredients of plant protection products and has to be conducted with radiolabelled material using two different natural sediments generally over a period of more than 100 days. As the partitioning between water and sediment for the relevant substances is therefore usually known, the concentration of the test substance in the sediment can be estimated.

Endpoints

Several endpoints (emergence, first day of emergence etc.) were examined in the course of the international ring-test for reproducibility and sensitivity. Emergence of the chironomids was the most important one. Other derived parameters like the development rate are sometimes even more sensitive (RATTE 1995) and should also be used for the risk assessment. Like data from other aquatic toxicity tests the NOEC/LOEC or EC15 from the sediment organism test are compared with the predicted environmental concentrations (PECs). As the test substance is applied to the water surface PECs calculated on the basis of the German **P**redicted **E**nvironmental **DRI**ft **VAL**ue (PEDRIVAL) (GANZELMEIER et al. 1995) can be used directly in order to assess the risk and to set restrictions of use like buffer zones. Furthermore in connection with the data from the metabolism study in water/sediment systems those concentrations in a sediment can be roughly calculated above which sediment organisms are potentially at risk.

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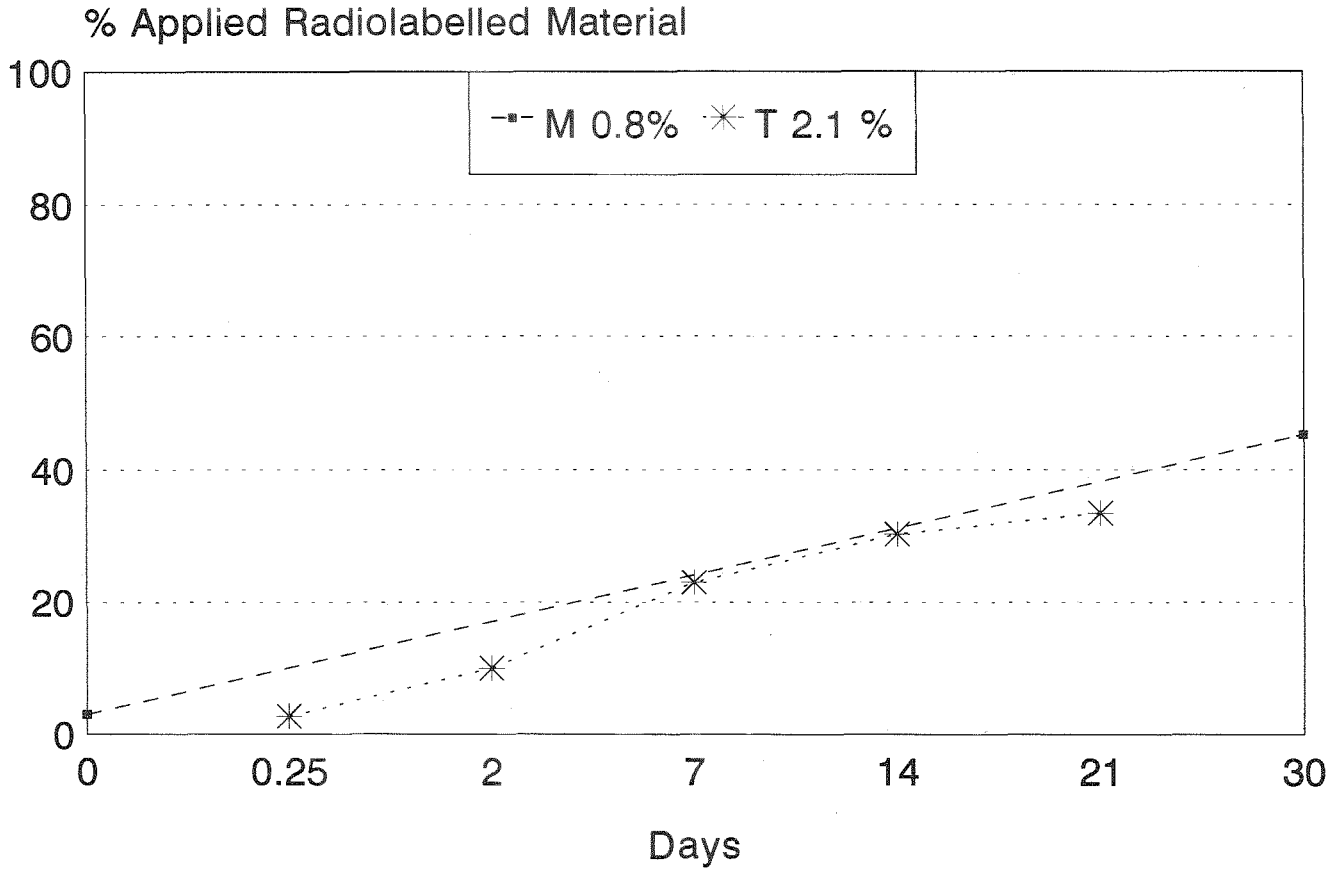

Figure 1: Amount of the same substance in the sediment of a metabolism-study (M) and a toxicity-test (T). Organic carbon content in percent.

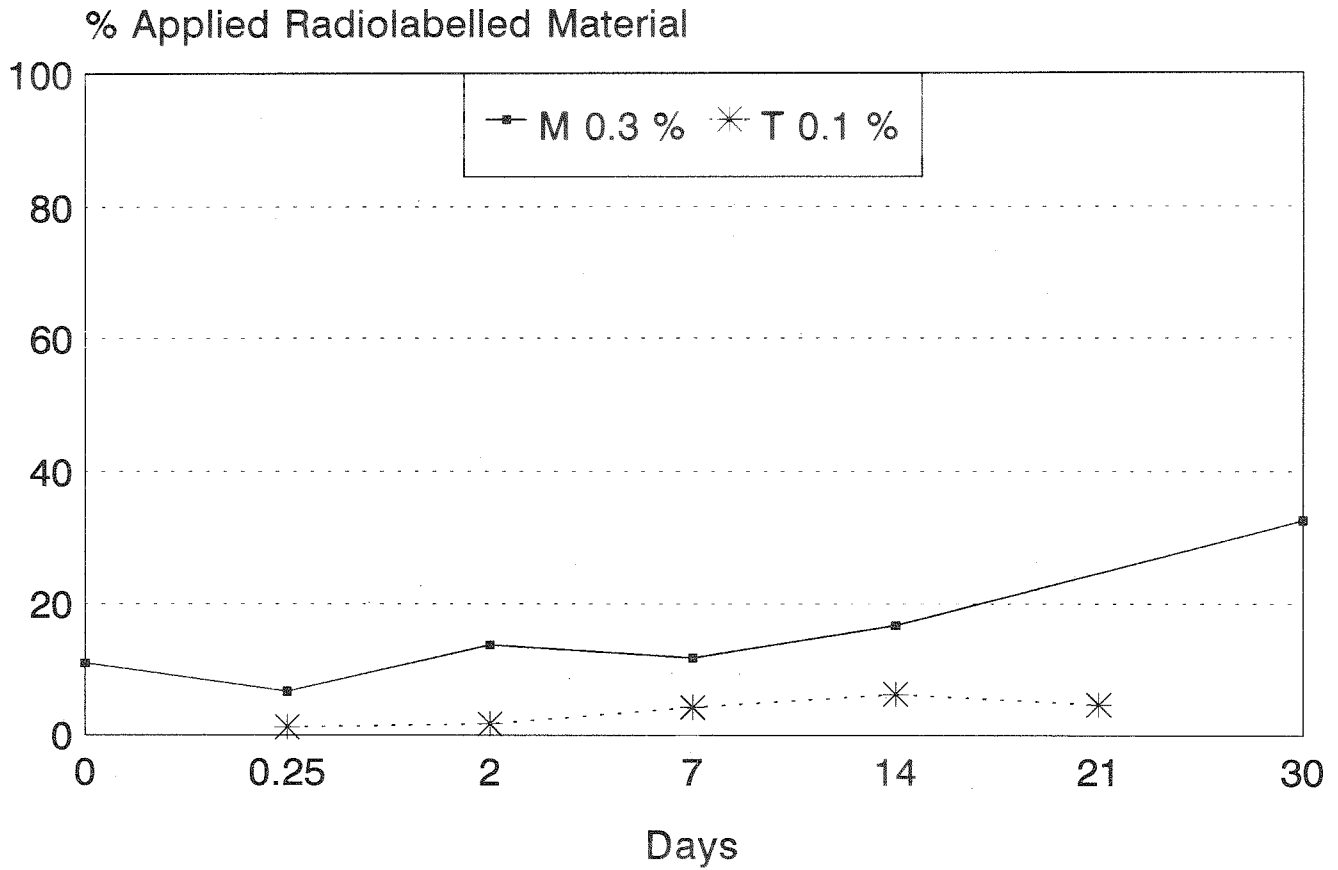

Figure 2: Amount of the same substance in the sediment of a metabolism-study (M) and a toxicity-test (T). Organic carbon content in percent.

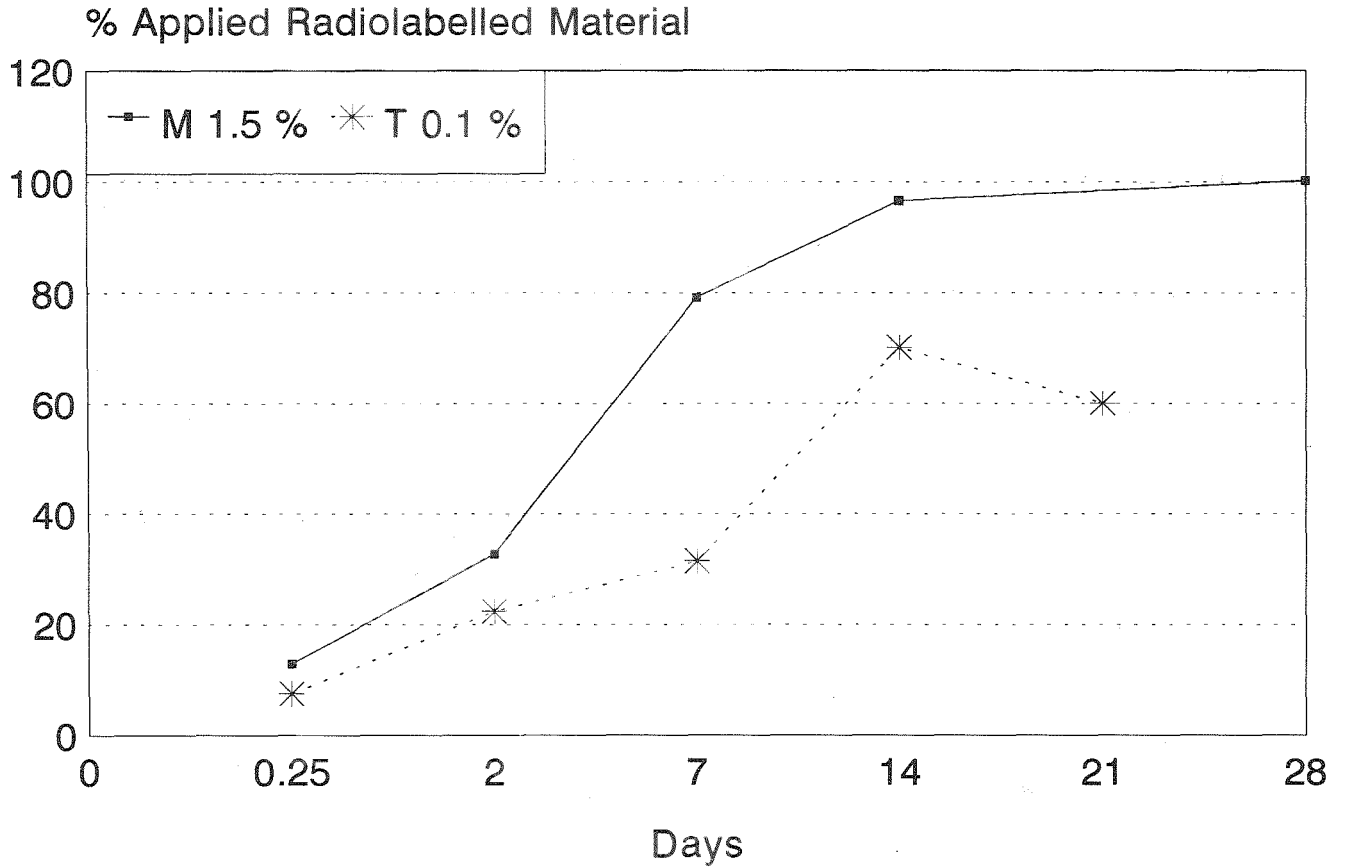

Figure 3: Amount of the same substance in the sediment of a metabolism-study (M) and a toxicity-test (T). Organic carbon content in percent.