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Sensitivity of microscale ecotoxicity tests and their suitability to measure toxicity of environmental samples

M.A. Vaal & A.J. Folkerts

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National Institute of Public Health and the Environment (RIVM),
P.O. Box 1, 3720 BA Bilthoven, the Netherlands
Telephone: +31 - 30 - 274 91 11, telefax: +31 - 30 - 274 29 71

MAILING LIST

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ABSTRACT

Aquatic microbiotests were selected and evaluated for their potential usefulness in measuring ecotoxicity in monitoring programs. Microbiotests are tests with aquatic invertebrates that demand a shorter exposure period and a smaller test volume than conventional aquatic ecotoxicity test methods. Microbiotests evaluated were the Thamnotox F test, the Rotox F test, the Algaltoxkit F test, the Microtox test and the Daphnia IQ test. They were tested with a reference toxicant (a metal salt), and, except for the Algaltoxkit F, with two defined mixtures of chemicals. One mixture contained toxicants with a non-specific mode of action and the other pesticides. The sensitivity and reproducibility of the microbiotests was compared with the conventional short-term tests on *Daphnia magna* and a fish species. Since the loss of even moderately volatile organic compounds from the original microbiotest containers appeared to be considerable, it could therefore result in a serious underestimation of toxicity when used in monitoring programmes. Modification of the test containers of the Thamnotox F and Daphnia IQ tests resulted in an improvement of the test performance. Along with the Microtox test, they form a good base for a test battery. The Rotox F test in its present form, appeared to be less useful because of its low sensitivity. At present, no suitable algal microbiotest is operational. Because of their ecological function as primary producers, algae need to be included in the test battery.

SUMMARY

In our laboratory research is being carried out to develop a method for measuring the toxicity of environmental samples. Briefly, the method consists of the extraction and concentration of toxicants from water samples to measure toxicity in the laboratory using different aquatic organisms. The purpose of the present study was to evaluate aquatic microbiotests for their reproducibility, sensitivity and in toxicity measurement.

Microbiotests are tests with aquatic invertebrates that demand a shorter exposure period and a smaller test volume than conventional aquatic ecotoxicity tests. The test organisms are kept in a dormant stage (cysts) and are hatched directly before the performance of the test. The microbiotests, Thamnotox F, Rotox F, Algaltoxkit F, Microtox and the Daphnia IQ test, were tested with a reference toxicant (potassium dichromate) and, except for the Algaltoxkit F, with two defined mixtures of chemicals, one containing organic compounds with a non-specific mode of action, the other pesticides. Their sensitivities to the mixtures were compared with the results of the conventional short-term tests with *Daphnia magna* and with the three-spined stickleback. Toxicity experiments with potassium dichromate were repeated 7 to 9 times to judge upon the reproducibility of microbiotest results.

The loss of even moderately volatile organic compounds from the original Thamnotox F and Rotox F test containers appeared to be considerable. Applying these tests in monitoring programmes may therefore result in a serious underestimation of the local toxicity. The Thamnotox F test can be improved by the use of glass vials with a snap cap and a larger test volume. This modification increases its sensitivity. The Rotox F test appeared very insensitive to the type of compounds used in both mixtures. No satisfying improvements could be found. More information on the sensitivity of this test method is needed to decide on its definite exclusion in the test battery. The sensitivity of the other microbiotests was found in the same range as the traditional tests. However, the *D. magna* acute toxicity test was 60 times more sensitive to the pesticide mixture than the most sensitive microbiotest, the Daphnia IQ test. The reproducibility of microbiotest results is comparable to traditional test methods.

The Microtox test and the modified Thamnotox F and Daphnia IQ tests are reproducible, feasible and sensitive. They form a good base for a test battery to measure environmental toxicity. At present, no suitable algal microbiotest is operational. Because of their ecological function as primary producers, algae need to be included in the test battery.

SAMENVATTING

In ons laboratorium ontwikkelen we een methode om de toxische druk op een lokatie te meten. Uit watermonsters worden de toxische stoffen gehaald waarna de toxiciteit in het laboratorium wordt gemeten met een set van verschillende soorten waterorganismen. Microbiotesten lijken geschikte testen voor toepassing in deze methode. Het zijn namelijk eenvoudig uit te voeren testen met ongewervelde waterorganismen en met een kortere blootstellingsperiode en een kleiner testvolume dan de traditionele testen met waterorganismen. Bovendien kunnen de testorganismen in rusttoestand worden opgeslagen tot de uitvoering van een test.

Een aantal microbiotesten is onderzocht op hun bruikbaarheid, reproduceerbaarheid en gevoeligheid voor toepassing in de methode. Microbiotesten met een kreeftachtige (de Thamnotox F test), een raderdiertje (de Rotox F test), een bacterie (de Microtoxtest), een alg (de Algentoxkit F) en een watervlo (de Daphnia IQ test) zijn getest met een metaalzout (kalium dichromaat) en (behalve de Algentoxkit F) met twee mengsels van chemicaliën. Het ene mengsel bestond uit organische stoffen met een niet-specifieke werking, het andere mengsel bestond uit bestrijdingsmiddelen. De gevoeligheid van deze testen werd vergeleken met die van de traditionele testen met de watervlo *Daphnia magna* en de stekelbaars. De reproduceerbaarheid van de resultaten van de microbiotesten werd gemeten door de testen met het metaalzout zeven tot negen maal te herhalen.

Het verlies van mengselstoffen uit de originele testvaten van de Thamnotox F test en de Rotox F test bleek behoorlijk groot te zijn. In monitoring programma's kan dat, bij toepassing van de originele methode, tot een ernstige onderschatting van de lokale toxiciteit leiden. De gevoeligheid van de Thamnotox F test kan worden vergroot door afsluitbare, glazen buisjes te gebruiken met een groter testvolume. Voor de Rotox F test is geen bevredigende aanpassing gevonden. Deze test is dus erg ongevoelig voor het type stoffen dat in onze mengsels zat. De gevoeligheid van de andere microbiotesten is vergelijkbaar met die van de traditionele testen. De traditionele watervlotest was echter 60 maal gevoeliger voor het bestrijdingsmiddelenmengsel dan de meest gevoelige microbiotest, de Daphnia IQ test. De reproduceerbaarheid van de microbiotestresultaten is vergelijkbaar met die van traditionele testen.

De Microtox test en de verbeterde Thamnotox F en Daphnia IQ testen zijn een goede basis voor een testbatterij voor het meten van de lokale toxische druk, door hun gevoeligheid,

reproduceerbaarheid en de relatief eenvoudige methode. Een kortdurende test met algen zou aan de batterij moeten worden toegevoegd, vanwege hun ecologische functie.

1. INTRODUCTION

1.1 Measuring environmental toxicity with the pT-method

In almost all environmental compartments monitoring systems have been implemented to assess the actual quality of the environment. These monitoring systems are usually directed at the evaluation of the concentration of a relatively limited number of chemicals. Such an approach does not allow for a realistic assessment of biological effects that might occur because of the wide variety of potentially occurring contaminants and the low availability of ecotoxicological data.

In addition to these chemical monitoring activities biological investigations are ongoing, on e.g. the abundance of species, to gain insight into the 'health' of some ecosystems. Due to the laborious character of this approach such investigations are limited to a small scale. Moreover, it is most often impossible to establish a causal relationship with, for example, toxicity.

In our laboratory research is carried out to develop a method for the measurement and risk evaluation of the local ecotoxicity. Environmental samples are taken into the laboratory to evaluate their toxicity with bioassays. The method is referred to as the pT-method (De Zwart et al., 1996).

The pT-method consists of three items:

- The environmental chemistry part

  Techniques are applied to extract and concentrate the fraction of organic toxicants from surface waters. Concentration of environmental samples is needed to facilitate measuring biological effects in short-term experiments and extractions is desirable to separate toxic stress factors from other stress factors. For practical reasons they may not have a too high volume.

- The ecotoxicological part

  The evaluation of toxicity is based upon a number of observed toxic effects on test organisms that are different with respect to their ecological function.

  The application of so-called microbiotest systems is required because of the small volume of the concentrated water sample that is available for testing. In order to develop a method that is suitable for monitoring purposes, toxicity tests with short exposure times are required and, if possible, sensitive sublethal criteria. This is demanded both for practical and financial reasons as for artefacts caused by unfavourable area to volume ratio due to the small scale of

the microbiotests.

- The risk assessment part

  Data of the aforementioned toxicity tests allow for calculations of the ecotoxicological risk, in a similar way as calculations for individual compounds are made. Such calculations result in HC_5 values, the hazardous concentration for 5% of the species (Aldenberg and Slob, 1993). In the Dutch method for risk evaluation, this value is in use in the regulation of individual substances. It is based on the policy allowing for a maximum permissible fraction of species in an ecosystem that may undergo an adverse effect due to the presence of that substance. Considering this, it is obvious to make use of a toxicity index in which results of toxicity tests are converted into a number that reflects the fraction of species that are not fully protected at the time and place where the sample was taken.

In recent years the pT-method has been further developed and refined. Modifications and improvements of the water concentration and extraction procedures are described in Collombon et al., 1997. The method used for the calculation of the unprotected fraction of species is presented in Chapter 5 of Roghair et al. (1997).
This report describes the applicability of previously selected microbiotests for the pT-method.

1.2 Development of a test battery of toxicity tests

To determine the toxicity of concentrates of water samples a test battery has to meet the following criteria:

- the tests have to be easy to perform, standardised and demand a short exposure period (hours rather than days) which makes them suitable for monitoring purposes;
- the battery should contain at least four tests with different species, varying in anatomy and ecological function;
- the tests have to be performed with a small volume of test medium ;
- toxicity parameters should be preferably sublethal, cover a broad range of toxicological mechanisms and be relevant to the population.

A literature study has been performed to select some potentially useful tests that can be performed with a very small test volume (Willemsen et al., 1995). Because of the undefined cocktail of toxicants in water samples the sensitivity of the selected test organisms should cover

a broad range of toxicological mechanisms. The tests that were proposed to form the backbone of the test battery were: the Microtox test, the *Selenastrum capricornutum* microplate test, the Thamnotox F, and the Mutatox test or SOS chromotest.

The Rotox F test was added to the first selection of tests for further study, because of earlier experimental experiences in our laboratory. Further, the Mutatox test and SOS chromotest were excluded because at present determining genotoxicity has no priority in the Dutch environmental policy.

The S*elenastrum* microplate test was excluded because of the high expenses. The ^{14}C incorporation photosynthesis test of Tubbing et al. (1993) that was used in an earlier stage demands too much specialised expertise to be practical. The results of a small scale study with a recently developed algal toxkit (Persoone, in press a) is included in this report, although the test duration is 72 hours which is rather long for application in the pT-method.

1.3 The aim and set up of this study

The purpose of this research was to evaluate a selection of microbiotests with respect to usefulness, reproducibility and sensitivity. If necessary, improvements or adaptations of test methods are defined. The aim is to develop an operational test battery suitable for the detection of toxic effects of mixtures of compounds present in small volumes of concentrated water samples.

The microbiotests that have been evaluated are: the Rotox F test, the Thamnotox F test, the Algaltoxkit F, the Daphnia IQ test and the Microtox test. They were tested with two defined mixtures of chemicals, one with a non-specific mode of action and one containing pesticides. These surrogate water samples were meant to simulate the complex nature of environmental samples. The sensitivity of the microbiotests is compared with the sensitivity of "classical" ecotoxicological tests like the Daphnia and fish acute toxicity tests. Besides the two mixtures, potassium dichromate was tested as a reference toxicant. From previous experiences it was known that the loss of toxicants from the microbiotest containers can be considerable. Therefore, special attention was paid to exposure concentrations during the test period.

In a later stage each test in the battery will be evaluated to which extend it is able to measure the toxicity of a concentrated water sample in comparison with the original, not concentrated water sample.

2. METHODS

2.1 Ecotoxicological tests

2.1.1 Description of test methods

The microbiotests that have been used are:

The Microtox test

Test organism is *Vibrio harveyi* (formerly known as *Photobacterium phosphoreum*) a marine species. Test parameter is the reduction of bioluminescence, a physiological parameter. The method is internationally accepted. After 5 and 15 minutes exposure the light output is measured in a photometer. The endpoint of the test is the concentration that decreases the light emission by 50% (the EC_{50}). The test requires 3 ml of water sample. Details on the experimental design are given in Table 1.

Table 1. Experimental design and observations in an acute toxicity experiment with *Vibrio harveyi* (Microtox).

Microtox test	
exposure regime	static
exposure time	5 and 15 minutes
age organisms	ca. 1.5 hours after reconstitution of the freeze-dried bacteria in 200g NaCl/l
individuals per group	10 µl of bacteria suspension
groups/concentration	2
test volume, dilution water	1 ml, diluent medium (20g NaCl/l)
test vessel	cuvette
food	none
aeration	none
temperature	15.0 ± 0.2 °C
lighting	none
oxygen level and measurement	none
pH level and measurement	dilution medium: 6-8, none
toxicological observations	inhibition of bioluminescence, measured with Microbics M500 toxicity analyser
toxicological parameters	EC_{50} after 5 or 15 minutes exposure
standard method	SOP ECO/064/01 (1996)

The Rotox F test

Test organism is the rotifer *Brachionus calycifloris*. The test parameter is lethality which is measured after 24 hours exposure. The test is commercially available. The rotifers are available as cysts. They hatch after incubation in hatching medium during a period of 16-18 hours under continuous light. They are exposed in a disposable plastic multiwell test plate. The total test sample volume needed is 6 ml. After 24 hours exposure in darkness the percentage mortality is determined by observing the animals with a binocular. The toxicity is expressed as the concentration that leads to 50% mortality (the LC_{50}) and is determined with the Spearmann-Kärber method (Hamilton et al., 1977). Details on the experimental design are given in Table 2.

Table 2. Experimental design and observations in an acute toxicity experiment with *Brachionus calyciflorus* (Rotox F).

Rotox F test	
exposure regime	static
exposure time	24 hours
age organisms	< 2 hours after hatching from cysts in EPA medium (see Annex 1)
individuals per group	5
groups/concentration	6
test volume, dilution water	300 µl, EPA medium
test vessel	Multiwell Test Plate (PVC) sealed with parafilm
food	none
aeration	none
temperature	25 ± 2 °C
lighting	darkness
oxygen level and measurement	>5.5 mg/l; t=0, highest test concentration
pH level and measurement	7.5-8.5; t=0, highest test concentration
toxicological observations	mortality after 24 hours exposure
toxicological parameter	24h - LC_{50}
standard method	SOP ECO/295/00

The Thamnotox F test

Test organism is the crustacean *Thamnocephalus platyurus*. The test parameter is lethality which is measured after 24 hours exposure. The test is commercially available. The crustaceans are available as cysts. They hatch after 20-24 hours incubation in a special hatching medium under continuous light. After hatching, the organisms have to acclimatise to the dilution medium for 4 hours. Hereafter the test can start. Instead of the original disposable multiwell test

plate, 5 ml glass vials with crimp caps are used to reduce loss of chemicals due to volatilisation. The total test sample volume needed is 30 ml. After 24 hours exposure in darkness the percentage mortality is determined by observing the animals with a binocular. The LC_{50} value is determined with the Spearmann-Kärber method (Hamilton et al., 1977). Details on the experimental design are given in Table 3.

Table 3. Experimental design and observations in an acute toxicity experiment with *Thamnocephalus platyurus* (Thamnotox F).

Thamnotox F test	
exposure regime	static
exposure time	24 hours
age organisms	second or third instar, hatched from cysts in EPA medium (see Annex 1) diluted 1:8 with demi water.
individuals per group	10
groups/concentration	3
test volume, dilution water	5 ml, EPA medium
test vessel	5 ml vials with crimp cap seals
food	none
aeration	none
temperature	25 ± 2 °C
lighting	darkness
oxygen level and measurement	> 5.5 mg/l; t=0, highest test concentration
pH level and measurement	7.5-8.5; t=0, highest test concentration
toxicological observations	mortality after 24 hours exposure
toxicological parameters	24 h - LC_{50}
standard method	SOP ECO/294/00

The Daphnia IQ test

The test organism is the waterflea *Daphnia magna*. In the Daphnia IQ test (Aqua Survey, Inc., 1993) hungry young daphnids are exposed for one hour to a range of test concentrations. Hereafter a biomarker, 4-methylumbelliferyl, β-D-galactoside is added as food to the test vessels. The biomarker consists of a fluorescent compound of which the light emitting properties are inhibited by binding to a saccharide. The compound is taken up by healthy daphnids that cleave the fluorescent tag from the substrate. As a result they start emitting visible light when exposed to UV radiation. Toxicant exposure causes inhibition of the enzymatic

reaction or ingestion rate. The test requires 30 ml test sample. The endpoint is the reduction in light emitting daphnids compared to the control and expressed as the EC_{50}, determined with the Spearmann-Kärber method (Hamilton et al., 1977).

Daphnids were obtained from the in house culture, where they are maintained on Dutch Standard Water medium (DSW, see Annex 1). They were acclimated to the dilution medium (EPA, see Annex 1) during the deprival period (about 24 hours). The test is preceded by a pre-test in which 15 out of 18 daphnids have to emit light. Details on the experimental design are given in Table 4.

Table 4. Experimental design and observations in an acute toxicity experiment with *Daphnia magna* (*Daphnia magna* IQ test).

Daphnia IQ test	
exposure regime	static
exposure time	1 hour and 15 minutes
age organisms	3 to 5 days old; daphnids born within 24 hours
individuals per group	6
groups/concentration	3
test volume, dilution water	5 ml, EPA medium (see Annex 1)
test vessel	5 ml glass vials with snap cap
food	none; daphnids deprived from food at 22-26 hours before testing
aeration	none
temperature	21 ± 2 °C
lighting	light
oxygen level and measurement	> 5.5 mg/l; t=0, highest test concentration
pH level and measurement	7.5-8.5; t=0, highest test concentration
toxicological observations	light emitting daphnids by visual observation after 1¼ hours exposure
toxicological parameters	EC_{50} (E = not emitting light)
standard method	SOP ECO/304/00

The Algal toxkit F

The test organism is *Selenastrum capricornutum*. The algae are immobilised in gel beads which are activated in 20-40 minutes with algal medium. The test is performed in disposable 10 cm synthetic long cells covered by an air exchanging lid. The total volume of the test sample needed is 150 ml. The optical density of the cell suspension is measured with a spectrophotometer (680 nm) at 24, 28 and 72 hours to calculate the growth rate and increase of

biomass. The endpoint of the test is the reduction in growth and biomass after 72 hours compared to the control and is expressed as the EC_{50} growth and EC_{50} biomass. Details of the test method are presented in Table 5.

The conventional ecotoxicity tests used were:

The *D. magna* acute toxicity test

The test are performed with juvenile daphnids (< 24 hours), obtained from the in house culture. Effects on behaviour (immobility) and appearance is measured after 4, 24 and 48 hours. The EC_{50} immobility after 48 hours exposure is determined with the Spearmann-Kärber method (Hamilton et al., 1977). Details of the test method are presented in
Table 6.

The fish acute toxicity test

The test organism used is the stickleback *Gasterosteus aculeatus*. Juvenile fish are obtained from the in house culture. Effects on behaviour, appearance and mortality is measured after 4, 24, 48, 72 and 96 hours. The LC_{50} value after 96 hours exposure is calculated with the Spearmann-Kärber method (Hamilton et al., 1977). Details of this test method are presented in Table 7.

Table 5. Experimental design and observations in an acute toxicity experiment with *Selenastrum capricornutum* (Algaltoxkit F).

Algaltoxkit F	
exposure regime	static
exposure time	72 hours
organism, age	*Selenastrum capricornutum* mobilised from algal beads
individuals per group	25 ml algae suspension of ± 1 * 10^6 cells/ml
groups/concentration	3
test volume, dilution water	25 ml, Algal toxkit culturing medium
test vessel	disposable 10 cm long-path cells with lid
temperature	23-25 °C
lighting	continuously illumination of 8000 lux
oxygen measurement	none
pH-measurement	none
toxicological observations	growth and biomass inhibition, daily, spectrophotometer (680 nm)
toxicological parameters	72h - EC_{50} growth and biomass
standard method	SOP for the Algaltoxkit F V110396 (1996)

Table 6. Experimental design and observations in an acute toxicity experiment with *Daphnia magna*.

Daphnia acute toxicity test	
exposure regime	static
exposure time	48 hours
organism, age	*Daphnia magna*, < 24 h
individuals per group	25
groups/concentration	2
test volume, dilution water	1 l Dutch Standard Water (see Annex 1)
test vessel	1,1 l glass flasks with lid
food	none
aeration	none
temperature	20 ± 2 °C
lighting	12h light/12h dark
oxygen level and measurement	> 5.5 mg/l; t=0 in dilution medium, at t=48 h in all concentrations
pH level and measurement	7.5-8.5; t=0 in dilution medium, at t=48 h in all concentrations
toxicological observations	immobility, appearance and behaviour at 4, 24 ,48 h
toxicological parameters	EC_{50} (E = immobility)
standard method	SOP ECO/071/01 (1994)

Table 7 Experimental design and observations in an acute toxicity experiment with *Gasterosteus aculeatus*.

Fish acute toxicity test	
exposure regime	semi-static (renewal at 48 hours)
exposure time	96 hours
organism, age	*Gasterosteus aculeatus*, 28 days
individuals per group	10
groups/concentration	2
test volume, dilution water	1 l Dutch Standard Water (see Annex 1)
test vessel	2 l glass beaker covered with parafilm
food	none
aeration	none
temperature	20 ± 1 °C
lighting	12h light/12h dark
oxygen level and measurement	> 5.5 mg/l; t=0 in dilution medium, at t=48 h in all concentrations
pH level and measurement	7.5-8.5; t=0, in dilution medium, at t=48 h in all concentrations
toxicological observations	mortality, appearance and behaviour at 2-4, 24, 48, 72, 96h
toxicological parameters	96h - LC_{50}
standard method	SOP ECO/073/01 (1994)

2.1.2 Set up of the experimental research

Two defined mixtures were tested. The first mixture consisted of 10 organic substances (chlorobenzenes, chloroanilines, nitrobenzenes and a chlorotoluene) with a non-specific mode of toxic action (the narcotic mixture). The second mixture consisted of six pesticides (the organophosphorous insecticides methyl-parathion, triazofos, azinfos-methyl and diazinon, and the phenyl urea herbicides diuron and linuron). The mixtures and compound characteristics are described in Table . Selection of chemicals in each mixture was based on environmental relevance, toxicity in relation to water solubility and availability of analytical methods.

Table 8. Physical-chemical characteristics of compounds in the narcotic and pesticide mixture.

Compounds	**Abbr.**	**Type[1]**	**Solubility in water (mg/l) (20-25°C)**	**Log K_{ow}[2]**	**Mol.w**	**H^3 (Pa m^3/mole) calculated**	**VP^5 (Pa) (20-25°C)**
Narcotic mixture							
1,4-dichlorobenzene	14DCB		83	3.5	147	160	90
1,2,3-trichlorobenzene	123TCB		17	3.8	181.4	72[4]	28
1,3,5-trichlorobenzene	135TCB		25	4	181.4	190[4]	28
1,2,3,4-tetrachlorobenzene	1234TCB		4.3	4.3	215.9	62[4]	0.7
pentachlorobenzene	PCB		0.6	4.8	249.4	59[4]	2.2
3-chloronitrobenzene	3CNB		501	2.6	157.6	0.09	0.3
1,2-dichloro-4-nitrobenzene	34DCNB		96.8	3.3	192	0.084	0
3,4-dichlorotoluene	34DCT		10.5	4.2	161	650	42
2,4 dichloroaniline	24DCA		213.8	2.8	162	0.67	0.9
2,4,6-trichloroaniline	246TCA		25.7	3.6	196.5	2.2	0.3
Pesticide mixture							
azinphos-methyl		op	25	2.75	317.1	$<1.3 \times 10^{-5}$	$< 1 \times 10^{-6}$
diazinon		op	45	3.81	304.3		9.7×10^{-5}
diuron		fu	38	2.68	233.1	5.6×10^{-5}	9.2×10^{-6}
linuron		fu	75	3.2	249.1	6.3×10^{-3}	1.9×10^{-3}
parathion-methyl		op	55	3.4	269.1	9.8×10^{-4}	0.017
triazofos		op	30				2×10^{-4}

[1] op: organophosphate insecticide, fu: fenyl urea herbicide

[2] Derived from either QSAR calculations (Aster, 1994) or from Slooff et al. (1991).

[3]: Henry coefficient

[4] : Measured values by Ten Hulscher et al. (1992).

[5]: vapor pressure

The concentration of each compound in the mixture was based on toxicity data. For the narcotic mixture each concentration was $2*1/n*EC_{50}$, where n is the number of chemicals in the mixture (10) and the EC_{50} is calculated from a Microtox QSAR (Hermens et al., 1985). However, due to water solubility problems these concentrations could not be realised and they had to be lowered, see Table. The mixture with these concentrations is referred to as the 100% concentration, the highest tested concentration.

Table 9. Concentrations and toxicological information of components in the narcotic mixture.

Compounds	**A 100% concentration (mg/l)**	**B EC_{50} (mg/l) from QSAR[1] Microtox**	**ratio A/B**
1,4-dichlorobenzene	0.98	3.14	0.31
1,2,3-trichlorobenzene	0.45	2.41	0.19
1,3,5-trichlorobenzene	0.20	1.93	0.11
1,2,3,4-tetrachlorobenzene	0.23	1.89	0.12
pentachlorobenzene	0.03	2.31	0.01
3-chloronitrobenzene	1.59	39.07	0.04
1,2-dichloro-4-nitrobenzene	1.50	6.06	0.25
3,4-dichlorotoluene	0.43	1.46	0.29
2,4 dichloroaniline	4.36	21.07	0.21
2,4,6-trichloroaniline	1.61	3.51	0.46

[1] QSAR is equation no.31 from Hermens et al., 1985.

Table 10. Concentrations and toxicological information of components in the pesticide mixture.

Compounds	**A 100% concentration (mg/l)**	**B *D. magna* EC_{50}[1] (mg/l)**	**ratio A/B**
azinphos-methyl	0.009	0.0011	8.18
diazinon	0.013	0.0015	8.67
diuron	11.65	1.4	8.32
linuron	6.25	0.75	8.33
parathion-methyl	0.04	0.0048	8.33
triazofos	0.025	0.003	8.33

[1] EC_{50} values from Van Rijn et al., 1995.

For the pesticide mixture it was expected that interspecies differences in sensitivity are larger than for the narcotic mixture. Therefore, the concentration of each component was chosen as $50*1/n*EC_{50}$, where n is the number of chemicals in the mixture (6) and EC_{50} is the sensitivity of *Daphnia magna* which was derived from literature, see Table . Thus, this 100% concentration allowed for measuring toxicity of less sensitive species.

Each experiment was performed with a dilution series starting at the 100% concentration. Range-finding experiments were performed for the pesticide mixture to decide upon the range of test concentrations in the definite test.

Some test designs (Rotox F, Thamnotox F and Daphnia IQ) are sensitive for the loss of organic chemicals. It is assumed that this is caused by a combination of a large area/volume-ratio, a relative long exposure time (Rotox F and Thamnotox F), high test temperatures and the plastic test plates that are the original test vessels. These factors make even moderate volatile organic contaminants to disappear from the test concentrations. For that reason we modified the design of the Thamnotox F and the Daphnia IQ test from their commercially available version. In the modified design, air-tight locked glass vials are used. For the Rotox F test such a modification was not applicable due to the small size of the test organisms which complicate the observation of effects in vials, whereas pouring of the vial content on a plate appeared to complicate the retrieval of all individuals and was too time-consuming. Therefore the Rotox test was carried out in its original multiwell plate, and loss of contaminants was accepted as part of the test method.

2.1.3 Reference toxicity experiments

Potassium dichromate ($K_2Cr_2O_7$) was used as reference toxicant. During the project a number of experiments with Rotox F, Thamnotox F and Daphnia IQ have been carried out, spread over a three year period, performed under identical conditions but with different batches of cysts or daphnids, and performed by different persons. The variation in the results is a measure of the reproducibility of each test. To allow for comparison of results among the test methods the coefficient of variation (CV) is calculated.

2.2 Preparation of test concentrations

Stock solutions of the mixtures were prepared in acetone as a solvent. A micropipet (Hamilton) was rinsed with acetone and used to prepare the 100% test concentration with the dilution medium. Heating and sonification were used to enhance the solubility process. In all tested concentrations the acetone concentration was 1 ml/l.

2.3 Chemical analyses

2.3.1 Narcotic mixture

Samples of the 50% test concentrations of the Microtox test were pooled for chemical analyses. For the other microbiotests some extra 100% test concentrations were prepared in order to obtain a volume sufficiently large for analyses. These test concentrations were prepared without test organisms. They were run parallel to the toxicity test and conditions were identical.

Samples were taken at the beginning and the end of the test or renewal period: at 0 and 24 hours (Rotox F, Thamnotox F), at 0 and 48 hours (Daphnia acute toxicity test and Fish acute toxicity test). Samples of the Daphnia IQ test and Microtox test were taken at the end of the experiment, after 1 hour.

The samples were analysed at the Laboratory for Ecotoxicology. Hexane (10 ml) with an internal standard was added to the sample (9 ml and 1 ml methanol). This mixture was shaken vigorously during three minutes. After separation, the upper layer (hexane) was analysed directly with a gas chromatograph with an electron capture detector. The detection limit ranged from 0.04 to 10.0 µg/l for respectively hexachlorethane and 2,4-dichloroaniline. The recovery was higher than 90% for all compounds. Analysed concentrations are not corrected for recovery of the analytical procedure.

2.3.2 Pesticide mixture

Samples were taken of the 100% test concentrations of the Rotox F test, the Thamnotox test and the Fish acute toxicity test. For the Daphnia IQ test, Microtox test and the Daphnia acute toxicity test the 50%, 50% and 25% test concentration were sampled, respectively. Test concentrations meant for chemical analyses of the Daphnia acute toxicity test and the Fish acute toxicity test were prepared without test organisms. Conditions were kept similar to the toxicity test.

Samples were taken at the beginning and the end of the test or renewal period: at 0 and 24 hours (Rotox F, Thamnotox F), at 0 and 48 hours (Daphnia acute toxicity test and Fish acute toxicity test), at 0 and 1.3 hours (Daphnia IQ test), and only once, at 0 hours, for the Microtox test. The samples were analysed at the Laboratory of Organic Chemistry.

Diuron and linuron were directly analysed by injecting an aliquot of the aqueous sample onto an HPLC-system. Separation was performed on a C18 column and detection was carried out with UV at 244 nm. Quantification was performed by external calibration with standard solutions.

The four organophosphorous insecticides were extracted with a micro-extraction procedure. 1 ml sample and 1 ml hexane was added to 0.5 g NaCl in a round bottom tube. The tube was closed and vigorously shaken by hand during 2 minutes. The hexane phase was dried over sodium sulphate and sampled in an autosampler vial. A volume of 60 µl was injected and analysed on a gas chromatograph with a large volume injection system and a flame photometric detector. Quantification was performed by comparing the signal obtained in the sample with that of a standard solution which followed the same sample pre-treatment procedure.

3. RESULTS AND DISCUSSION

3.1 Feasibility of the microbiotests

3.1.1 Influence of dilution medium on Thamnotox F response

Thamnotox F appeared to be extremely sensitive for the hatching and dilution medium used. For practical reasons we prefer an identical test medium for all microbiotests. However, in the preliminary potassium dichromate experiments it appeared that the Thamnotox F test cannot be performed in DSW (see Annex 1) which is our conventional culture and test medium. A high percentage of mortality (40-50%) was observed in the control, and this percentage was not increased by the toxicant in the test concentrations. Thus, no concentration-effect curve could be calculated. Simultaneously performed experiments with identical test conditions in EPA medium (see Annex 1) as hatching and dilution medium gave zero control mortality and a clear concentration-effect curve. This was observed in several repetitive experiments (see Annex 2).

A decrease of heavy metal toxicity in DSW compared to EPA can be explained by its hardness, since DSW contains more calcium and is harder than EPA medium. Competitive interactions with cations, particularly calcium, seem to be the cause of the decrease in toxicity. However, no appropriate explanation could be found for the high control mortality in DSW. Comparative experiments with 'softened' DSW (75% DSW and 25% demi water) did not improve the crustacean control survival. Because of these results EPA is used as the Thamnotox F dilution medium.

No such difference in medium sensitivity was observed in the Rotox F experiments. They were carried out in either DSW or EPA, as indicated.

3.1.2 Pre-testing and age of daphnids in the Daphnia IQ test

In the Daphnia IQ test method a pre-test is prescribed to check if sufficient daphnids (15 out of 18) feed on the substrate, if not the test should not be performed. However, results in this pre-test may vary considerably from results obtained with the control group during the test. Pre-tests repeated several times still gave various results after changing the prescribed pre-test conditions to conditions similar to the definite test (with respect to the number of animals, volume and substrate concentration). Results of pre-tests repeated within several hours varied from 11 to 15 daphnids out of 18 fluorescing brightly. Their performance was often better in the definite test. In most tests at least 15 out of 18 daphnids were fluorescing. Because of the varying results we

did not use the pre-tests to decide upon the validity of the definite test results. Pre-test results of experiments described below are given in the corresponding Annex.

Tests with 5 or 6 days old daphnids appeared to be easier to perform than with younger daphnids. Their larger size facilitates the scoring, probably because the consumed amount of substrate is larger. Still, the visual scoring of fluorescing daphnids is a subjective case. Improvement of the scoring will be gained by quantification of the fluorescence which can be achieved with the use of a fluorometer. We will search the possibility to apply this improvement in our test method.

3.2 Reference toxicity experiments

Results of the experiments with the reference toxicant $K_2Cr_2O_7$ are pictured in Figure 1. Results of individual tests are presented in Annex 2. Test conditions as pH, oxygen level and temperature are only reported when they deviate from test conditions mentioned in Tables 2-7.

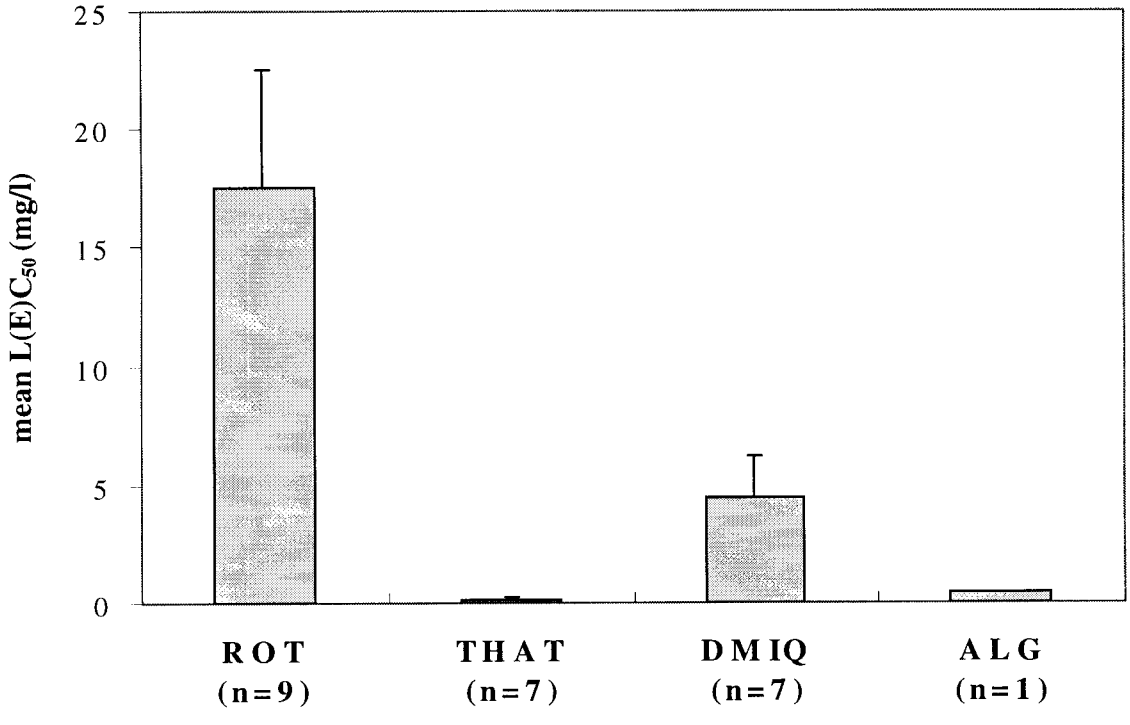

Figure 1 Toxicity (mean and standard deviation) of potassium dichromate in the Rotox F (ROT), Thamnotox F (THAT), Daphnia IQ (DMIQ) and Algaltoxkit F (ALG).

Algae were tested once and appeared to be very sensitive to potassium dichromate, similar to the Thamnotox F test. The Rotox F test was the least sensitive test. The mean LC_{50} of the

Thamnotox F, 0.14 ± 0.04 mg/l, was similar to the values reported by Centeno et al. (1995) which was 0.11 ± 0.006 mg/l, and to values of the two laboratories reported by Persoone et al. (1994): 0.14 mg/l (n = 10, CV = 14%) and 0.15 mg/l (n = 8, CV = 26%) . The CV's for Thamnotox F, Rotox F and Daphnia IQ in our studies were 26%, 29% and 38%, respectively. Considering the three years time span in which the experiments were carried out, these values indicate well reproducible test results (Grothe and Kimerle, 1985).

3.3 Loss of organic toxicants during the test

The toxkits showed a severe loss of compounds of the narcotic mixture whereas the loss in the conventional toxicity tests was limited (Table 11). In the Algal toxkit after 72 hours all compounds of the narcotic mixture, except 2,4-dichloroaniline, disappeared for more than 90% from the test concentrations. The water volume decreased with 6%. This evaporation is probably due to a combination of the following test conditions: the containers cannot be airtight locked because of the assimilation of the algae, the unfavourable area to volume ratio causes large evaporation and the high test temperature speeds this process. Loss of these organic compounds may also be caused by the plastic material of the test containers. In the development of this algal toxkit special emphasis has been paid to its comparability with the traditional flask algal assay. Comparisons of both assays were mainly based on their sensitivity for metal salts and were found to be well in concordance (Persoone, in press a). From our results it is suggested, however, that their comparability may be less evident when organic substances or environmental samples are tested, since loss of organic compounds in the standard flask algal assay will be considerably smaller because the area to volume ratio is not so unfavourable and the material is more inert.

The loss of narcotic compounds in the Rotox F test was considerable as well, ranging from 70% to about 100% of the original measured test concentration at the start of the test. In the Thamnotox F test the mean measured concentrations at the end of the test varied in two ranges: 6 to 11% for the polar narcotics and 44 to 99% for the non polar narcotics.

The measured concentrations in the Microtox test ranged from 54% to 99% of the nominal test concentrations at t =0. Considering the open test system this loss is rather limited. The combination of a short test duration and a low test temperature probably prevents larger losses.

This loss of the narcotic compounds from the containers can be related to their Henry coefficients (Table 8). In all microbiotests (except the Microtox) severe losses (40%-100%) were measured for the compounds with a Henry coefficient larger than 59 $Pa.m^3$/mole. For the

compounds with a Henry coefficient smaller than 2.2 Pa.m^3/mole the losses were less extreme. The difference between the Thamnotox F test and the Daphnia IQ test is remarkable, because both tests are performed in 5 ml glass vials with a snap cap. It may indicate the influence of handling: the period needed for preparation of the vials with dilution concentrations and test organisms is larger for the Thamnotox F than for the Daphnia IQ test. A repeated analysis experiment with 5 ml vials, carefully handled and as shortly opened as possible resulted after 24 hours in measured concentrations not lower than 70% of the measured concentration at t=0 hours (results not shown).

Table 11. Measured concentrations of 10 narcotic compounds at the end of the test, expressed as percentage of measured concentration at t=0. Details of test conditions in Tables 1- 7.

compound	nominal concentration (mg/l)	(mean) measured concentration at t (% of measured concentration at t=0)						
		ROT	THAT	DMIQ[1]	ALG	MICT[1]	DM48	FISH
14DCB	0.98	<1	11	54	<5	60	100	41
123TCB	0.45	0	7	62	<1	69	99	40
135TCB	0.20	<1	6	50	<3	54	99	31
1234TCB	0.23	<1	6	53	<1	60	123	35
PCB	0.03	<1	9	57	<5	70	93	32
3CNB	1.59	13	66	99	7	90	107	99
34DCNB	1.50	5	60	97	10	97	96	86
34DCT	0.43	<4	11	55	<20	63	106	38
24DCA	4.36	29	99	104	40	99	105	104
246TCA	1.61	2	44	88	3	84	103	85
t (hrs)		24	24	1	72	0.25	48	48
n		1	3	3	1	3	1	1

ROT: Rotox F; THAT: Thamnotox F; DMIQ: Daphnia IQ test; ALG: Algaltoxkit F; MICT: Microtox test; DM48: Daphnia acute toxicity test; FISH: Fish acute toxicity test

[1] : Expressed as percentage of nominal concentration at t=0.

- : Not measured

Analytical results of the Daphnia acute toxicity test demonstrate that under 'classical' test conditions (large volume, relative small surface) with, additionally, locked test containers no loss occurs of even moderate volatile test compounds. In the fish acute toxicity test the losses were more enhanced, probably because of the use of parafilm instead of a lid.

The loss of pesticides from the test containers was much smaller than the loss of the narcotic compounds (see Table 12). Evaporation and surface effects are low, due to the low Henry coefficients of the pesticides (Table 8). Analytical data on triazofos are not useful because decomposition appeared to occur during the analytical procedure, resulting in an unreliable calibration curve. The Microtox analytical data are not available.

Compared to the other tests, the Rotox F test demonstrated the largest losses. For diazinon and parathion-methyl 33% and 71%, respectively, was detected at the end of the test period.

Table 12 Measured concentrations of six pesticides at the end of the test, expressed as percentage of measured concentration at t=0. Details of test conditions in Tables 2-4, 6 and 7.

compound	**nominal concentration (mg/l)**	**(mean) measured concentration at t (%) (% of measured concentration at t=0)**				
		ROT	**THAT**	**DMIQ**	**DM48**	**FISH**
azinphos-methyl	0.009	70	71	127	100	86
diazinon	0.0125	33	89	100	83	95
diuron	11.7	103	100	99	104	102
linuron	6.25	101	100	98	103	101
parathion-methyl	0.04	71	115	104	71	88
triazofos[/]	0.025	-	-	-	-	-
t (hrs)		24	24	1.2	48	48
n		2	2	1	1	1

ROT: Rotox F; THAT: Thamnotox F; DMIQ: Daphnia IQ test; DM48: Dapnia acute toxicity test; FISH: Fish acute toxicity test.

[/]Data not available due to decomposition during analytical procedure.

3.4 Toxicity of the narcotic mixture

Results of the toxicity experiments with the narcotic mixture are presented in Figure 2. No effects were measured in the Rotox F test. This can be largely related to the loss of almost all compounds in the mixture (see Table 11). More than in other test methods, results of this test is dominated by the test conditions determining the (bio)availability of these test compounds rather than by the sensitivity of the rotifers.

Toxicity in the other microbiotests varied from an EC_{50} of 16 ± 5.7% in the Daphnia IQ test to

an LC_{50} of 75 ± 25% in the Thamnotox F test. The Thamnotox F test showed the largest standard deviation, in a third experiment no LC_{50} could be calculated because the highest measured effect was 21%. The toxicity measured in the Microtox test was similar to the toxicity calculated from the QSAR (the 100% concentration being 2 times the Microtox EC_{50}).

The sensitivity of the Daphnia and fish acute toxicity tests was similar to that of the microbiotests.

Detailed results of individual tests are given in Annex 3.

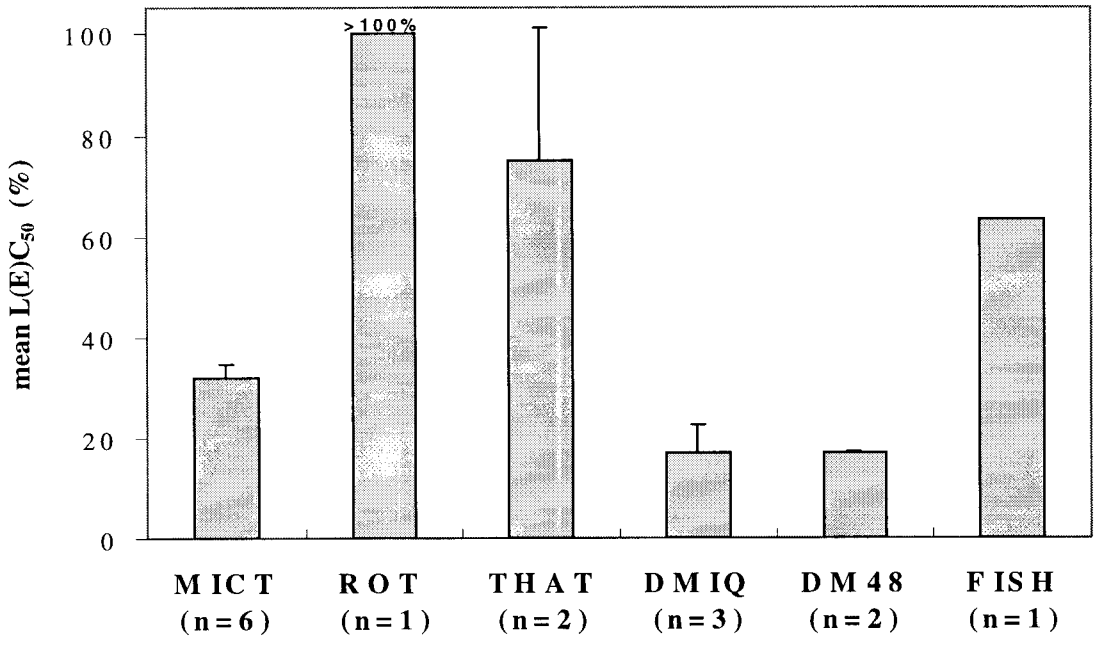

Figure 2 Toxicity (mean and standard deviation) of the narcotic mixture to Microtox (MICT, 15 min.), Rotox F (ROT), Thamnotox F (THAT), Daphnia IQ (DMIQ), Daphnia acute (DM48) and Fish acute toxicity test (FISH).

3.5 Toxicity of the pesticide mixture

As expected, differences in toxicity for the pesticide mixture were much larger than for the narcotic mixture (Figure 3). The loss of compounds in all tests was quite similar. Therefore differences are more representative for the differences in species sensitivity than in the experiments with the narcotic mixture.

The conventional test methods appeared to be the most sensitive. Daphnids in the acute toxicity test were 130 times more sensitive than the Microtox-bacteria. They were also 60 times more sensitive than the daphnids in the IQ test, the most sensitive microbiotest. Most likely, this

reflects the difference in sensitivity of the observed parameters in combination with the exposure period. The difference between the fish acute toxicity test and the Daphnia IQ test was smaller, a factor 3.

No LC_{50} could be calculated for the Rotox F test. The highest observed effect was 10% at the highest test concentration. Because the loss of compounds was not so extreme as for the narcotic mixture compounds, this absence of effects can be explained by a relatively low sensitivity of the rotifers. However, the studies of Calleja et al. (1994) and Toussaint et al. (1995) show in general no clear insensitivity of the Rotox F test for individual tested pesticides (malathion, paraquat, lindane, pentachlorophenol, 2,4-D) when compared to the Microtox test or the Streptoxkit F (which was later replaced by the Thamnotox F test, see Centeno et al., 1995). Compared to the *Daphnia magna* acute toxicity test the Rotox F test was much more insensitive for malathion and lindane but its sensitivity was in the same order of magnitude for the other pesticides.

Detailed results of the experiments are presented in Annex 4.

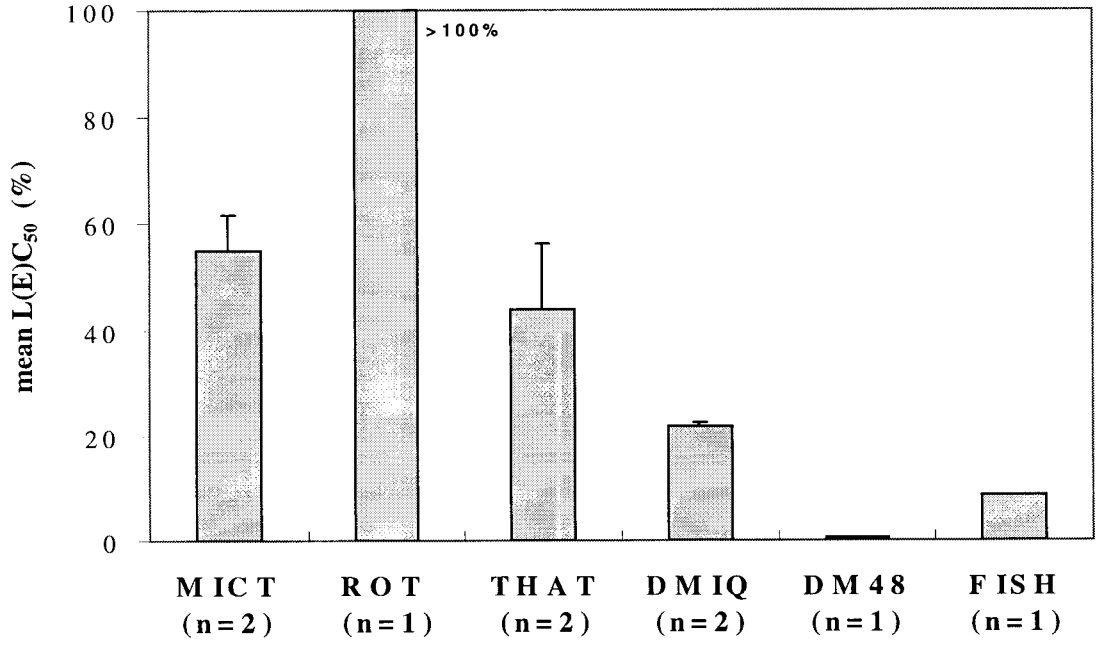

Figure 3 Toxicity (mean and standard deviation) of the pesticide mixture to Microtox (MICT, 15 min.), Rotox F (ROT), Thamnotox F (THAT), Daphnia IQ (DMIQ), Daphnia acute toxicity test (DM48) and Fish acute toxicity test (FISH).

3.6 Usefulness of microbiotests for the pT-method

The use of microbiotests is growing exponentially. In comparison to conventional tests their advantage is their short test period, low-cost, use of cysts and relative simplicity. Persoone (in press b) estimated that by 1995 nearly 14,000 toxkits assays would have been performed.

The results of our studies indicate some implications for the use of these small scale assays. The loss of even moderately volatile organic compounds can be considerable, which may result in a serious underestimation of the toxicity of these type of compounds. This loss is probably the result of the combination of a large area to volume ratio, plastic containers, a high test temperature and a long test duration. The Thamnotox F test and Daphnia IQ test can be modified by using glass vials with a snap cap. No satisfying modification for the Rotox F test was found, due to the small size of the organism which demands test containers that do not hinder the visual effect scoring. With this test no toxicity was measured with the narcotic mixture or with the pesticide mixture. This is probably due to a combination of the test method and a low sensitivity of the rotifers.

Although Calleja et al. (1993) already mentioned the use of larger volume glass vials with teflon screw-caps as an adaptation of the original toxkit method in order to test volatile compounds, the aspect of the loss of compounds from the microscale test containers has been largely underexposed. The use of multiwells may result in a serious underestimation of toxicity when organic compounds are tested. Especially in testing field samples of unknown constitution one should be aware of the limitations of these methods. However, in an evaluation of the toxicity of 350 sampling sites (including industrial effluents, waste dumps, rivers) Persoone (in press b) did not find the Rotox F test and Thamnotox F test to be extremely insensitive when compared to the Microtox test, the Daphnia microbiotest or the conventional Daphnia acute toxicity assay. This may be explained to the presence of contaminants like heavy metals or organic compounds with a low Henry coefficient that determined the toxicity.

Similarly, in a pT- monitoring program the Rotox F test appeared to be on average as sensitive as most of the other tests (Thamnotox F, Microtox, algae ^{14}C incorporation test, Daphnia IQ) (Roghair et al., 1997). The applied extraction and concentration procedure does not cover compounds that are more than moderately volatile (Collombon et al., 1997) nor heavy metals. It is remarkable that toxicity of field samples was measured with the Rotox F test whereas no toxic responses could be detected in our study with either the narcotic mixture or the pesticide mixture. Except for detergents, it is not very likely that the tested surface waters contained

pollutants very different from the ones in our mixtures, or certain (non-volatile organic) compounds in relatively high concentrations for which rotifers happens to be more sensitive. However, because also the blanks showed a comparable response, it is very likely that artefacts of the applied extraction and concentration procedures (e.g. concentrating humic acids) have caused mortality to the rotifers.

The findings indicate the importance of knowledge of the 'toxicological profile' of each test. In other words: how sensitive is the method for different compounds or groups of compounds? The sensitivity of a test contains three elements: the specific sensitivity of the tested organism; the behaviour of the substance in the test system determining the extent to which the organism is exposed; sensitivity of the method for other factors than toxicity. Such a sensitivity profile facilitates the interpretation of toxicity measurements of environmental samples and may link the observed effects to pollutants causing the toxicity.

More information is needed on the sensitivity of the Rotox F test for different groups of chemicals and on the influence of the extraction and concentration procedure on their sensitivity to decide whether this test should be definitively excluded from the pT test battery.

Persoone (in press b) detected toxicity in nearly all 350 environmental samples originating from industries, waste dumps, monitoring wells, purification plants and river stretches with either Daphnia microbiotests, the Rotox F test, Microtox or the conventional Daphnia acute toxicity test. The detection potential of individual tests revealed that the Microtox test detected toxicity in nearly 80% of the samples, whereas the Rotox F test detected toxicity in only 40% of the samples. However, when ranked by sensitivity per sample, each individual test was found to be 'the most sensitive' in less than half of the cases. This study supports the idea that a test battery is needed to detect the toxic impact of monitoring samples.

At present no useful algae test is available. Because of their ecological function it is felt that they should be included in the test battery. In the near future we will study the possibility to include a fluorescence algal assay with a test duration of several hours in the pT test battery.

4. CONCLUSIONS AND RECOMMENDATIONS

The loss of even moderate volatile organic compounds from the microbiotest containers is considerable. It may result in an serious underestimation of toxicity for these compounds when applied in monitoring programmes. The performance of the Thamnotox F test can be improved after modification of the test method. The use of glass vials with a snap cap with a larger test volume than the original test method increases its sensitivity. For the Rotox F test no satisfying improvement could be found.

In the Rotox F test no toxicity was measured for both the narcotic mixture and the pesticide mixture. Differences in sensitivity among the other microbiotests for the narcotic mixture were within a factor five and similar to the conventional Daphnia and fish acute toxicity test. Differences in sensitivity among these microbiotests for the pesticide mixture was within a factor two, whereas the conventional toxicity tests were more sensitive: a factor 3 to 60 compared to the Daphnia IQ test, the most sensitive microbiotest. The reproducibility of the microbiotests was comparable to traditional test methods.

Definite exclusion of the Rotox F test should be based on additional information for other groups of chemicals than those tested in this study (e.g. detergents) and on the influence of the extraction and concentration procedure on its sensitivity.

The modified Thamnotox F and Daphnia IQ tests, and the Microtox test form a good base for a test battery for the pT method due to their sensitivity, reproducibility and feasibility. In addition, an algal test need to be included because of their ecological function.

ACKNOWLEDGEMENTS

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ANNEX 1 COMPOSITION OF DSW AND EPA MEDIUM

Composition of Dutch Standard Water (DSW)

In demineralized water:

Ca^{2+}	54.5 mg/l	1.36 mmol/l
Mg^{2+}	17.8 mg/l	0.73 mmol/l
Na^{+}	27.4 mg/l	1.19 mmol/l
K^{+}	7.8 mg/l	0.20 mmol/l
Cl^{-}	96.3 mg/l	2.72 mmol/l
HCO_3^{-}	84.8 mg/l	1.39 mmol/l
SO_4^{2-}	70.2 mg/l	0.73 mmol/l

Hardness is 11.7 DH, 210 mg/l as $CaCO_3$

pH is 8.2 ± 0.2

Composition of EPA-medium

In demineralized water:

Ca^{2+}	14.0 mg/l	0.55 mmol/l
Mg^{2+}	12.0 mg/l	0.50 mmol/l
Na^{+}	26.2 mg/l	1.14 mmol/l
K^{+}	2.1 mg/l	0.05 mmol/l
Cl^{-}	1.9 mg/l	0.05 mmol/l
HCO_3^{-}	69.5 mg/l	1.14 mmol/l
SO_4^{2-}	81.5 mg/l	0.85 mmol/l

Hardness is ca. 6 DH, 84 mg/l as $CaCO_3$

pH is 7.6 ± 0.2

ANNEX 2 RESULTS OF TOXICITY TESTS WITH POTASSIUM DICHROMATE ($K_2CR_2O_7$)

Results of individual experiments. Further details of test methods are described in Tables 2-5.

test:	Thamnotox F		
concentrations:	0, 0.032-0.056-0.1-0.18-0.32 mg $K_2Cr_2O_7$ /l		
medium:	EPA		
container:	multiwell		
testcode	**LC_{50}**	**95% conf.lim.**	**remarks**
94/p021	-		75% DSW/ 25% demi, blanc 43% mortality, no concentration effect curve
94/P022	-		DSW, blanc 43% mortality, no concentration effect curve
94/P023	0.22	0.18-0.27	
95/156	0.14	0.12-0.17	
95/p134	0.12	0.11-0.14	
95/p132	0.14	0.12-0.17	temp: 23.7-24.8
95/p118	-		DSW, blanc 43% mortality, no concentration effect curve
95/p119	0.15	0.13-0.18	0,0.05,0.1,0.2,0.4,0.8
96/p021a	0.11	0.09-0.13	
96/p021b	0.12	0.10-0.14	temp: 21.4-27.8
mean	0.14		
std	0.04		
n	7		
CV (%)	25.8		

test:	Rotox F
concentrations:	0-3.2-5.6-10-18-32 mg $K_2Cr_2O_7$ /l
medium:	EPA
container:	multiwell

testcode	**LC_{50}**	**95% conf.lim.**	**remarks**
95/p167	16.9	15.0-19.1	
95/p138	23.7	16.7-33.7	
95/p127a	23.7	18.9-29.6	
95/p127	18.9	16.5-21.6	
95/p099	19.4	16.6-22.6	DSW
95/p073	14.0	12.3-15.9	DSW, temp.=25.6-26.6
96/p020	19.7	16.9-22.8	
	8.8	6.9-11.1	temp= 21.2-27.2
94/p067	12.6	10.7-15.0	DSW
mean	17.5		
std	5.0		
n	9		
CV (%)	28.5		

test:	Daphnia IQ
concentrations:	0,1.0-1.8-3.2-5.6-10 mg $K_2Cr_2O_7$ /l
medium:	DSW
container:	5 ml vials

Test code	**EC_{50}**	**95% conf.lim.**	**remarks**
94/p036	-		No prestest; blanc 28% mortality, no concentration effect curve
94/p037	2.1	1.6-2.9	0,0.32-0.56-1-1.8-3.2, DSW, age: 2 days; no prestest
94/p040	4.2	-	0,1.8-3.2-5.6-10-18, age: 5 days; No pretest
94/p041	3.6	2.9-4.4	0,1.8-3.2-5.6-10-18, age: 5 days; No pretest
95/p133	3.9	2.7-5.6	Prestest: ?
95/p131	>3.2		0, 0.32-0.56-1-1.8-3.2, age: 5 days. Prestest 18/18
96/p024a	5.6	4.9-6.4	
96/p024b	7.5	4.9-11.4	
96/p024c	4.7	3.4-6.5	
mean	4.5		
std	1.7		
n	7		
CV	37.6		

ANNEX 3 RESULTS OF TOXICITY TESTS WITH THE NARCOTIC MIXTURE

Results of individual experiments. Further details of test methods are described in Tables 1-4, 6 and 7.

test:	Thamnotox F		
concentrations:	0, 6.25, 12.5, 25, 50, 100% narcotic mixture		
medium:	EPA		
container:	5 ml vials with snap cap		
Testcode	**LC50**	**95% cl**	**remarks**
97/P016a-160497	56.7	40.5-79.6	
97/P016b-160497	93.9	72.1-122.3	
97/P016c-230497	>100		effect max. 21%, T 23.5-28.3
mean	75.3		
std	26.3		
n	2		
CV	34.9		

test:	Daphnia IQ		
concentrations:	0, 6.25, 12.5, 25, 50, 100% narcotic mixture		
medium:	DSW filtered over coal		
container:	5 ml vials with snap cap		
Testcode	**EC50**	**95% cl**	**remarks**
97/P015-200297	16.7	13.5-20.8	pretest: 15 out of 19; control: 5.6% inhibition
97/P015-270297	22.2	18.0-27.4	pretest: 12 out of 18; control: 11.1% inhibition
97/P015-060397	10.7	6.3-18.2	pretest: 14/18, 11/18, 14/18, 15/18; control: 5.5% inhibition
mean	16.5		
std	5.8		
n	3		
CV	35.2		

test:	Rotox F		
concentrations:	0, 6.25, 12.5, 25, 50, 100% narcotic mixture		
medium:	EPA		
container:	multiwell		
Testcode	LC50	95% cl	remarks
97/p014	>100%		effects <10%

test:	Microtox		
concentrations:	0, 6.25, 12.5, 25, 50% narcotic mixture		
medium:	diluent		
container:	3 ml cuvet		
Testcode	**EC50**	**95% cl**	**remarks**
97/p013a	36.1	29.6-44.1	t=5 min
97/p013a	41.4	32.0-53.6	t=15 min
97/p013a	28.0	26.0-30.2	t=5 min
97/p013a	30.9	28.9-33.1	t=15 min
97/p013b	30.8	29.9-31.8	t=5 min
97/p013b	33.0	31.2-34.9	t=15 min
97/p013b	31.7	28.5-35.2	t=5 min
97/p013b	33.4	29.6-37.6	t=15 min
97/p013c	30.9	22.6-42.3	t=5 min
97/p013c	32.9	25.8-41.9	t=15 min
97/p013c	33.8	24.2-47.2	t=5 min
97/p013c	37.9	21.9-65.6	t=15 min
mean (t=5 min)	31.9		
std	2.8		
n	6		
CV	8.8		

test:	D. magna 48hr		
concentrations:	0, 6.25, 12.5, 25, 50, 100% narcotic mixture		
medium:	DSW filtered over coal		
container:	1.1l glas beaker with lid		
Testcode	LC50	95% cl	remarks
97/P018	17.1	14.5-20.1	
95/P189	17.2	16.6-17.8	
mean	17.2		
std	0.2		
n	2		
CV	1.1		

test:	G. aculeatus 96 hr		
concentrations:	0, 6.25, 12.5, 25, 50, 100% narcotic mixture		
medium:	DSW		
container:	2l beaker with parafilm		
Testcode	**LC50**	**95% cl**	**remarks**
	63.7	57.1-72.2	Day 4: T 15.7-20.7

ANNEX 4 RESULTS OF TOXICITY TESTS WITH THE PESTICIDE MIXTURE

Results of individual experiments. Further details of test methods are described in Tables 1-4, 6 and 7.

test:	Thamnotox F
concentrations:	0, 6.25, 12.5, 25, 50, 100%
medium:	EPA
container:	5 ml vials with snap cap

Testcode	LC50	95% cl	remarks
TOX2-090997	35.7	30.2-42.4	
TOX2-160997	52.9	46.0-60.9	T: 25.0-27.1C
mean	44.3		
std	12.2		
n	2		
CV	27.6		

test:	Rotox F
concentrations:	0, 6.25, 12.5, 25, 50, 100%
medium:	EPA
container:	multiwell

Testcode	LC50	95% cl	remarks
TOX2-090997	>100%		effects < 10%
TOX2-160997	>100%		effects < 3%, T 25.0-27.1

test:	Daphnia IQ
concentrations:	0, 3.2, 6.25, 12.5, 25, 50%
medium:	EPA
container:	5 ml vials with snap cap

Testcode	**EC50**	**95% cl**	**remarks**
TOX2-100997	22.4	14.6-34.5	pretest: 14/18,15/18 control:16/18
TOX2-230997	23.2	15.9-33.9	pretest:17/18, T18.7-19.1
mean	22.8		
std	0.6		
n	2		
CV	2.5		

test:	Microtox
concentrations:	0, 6.25, 12.5, 25, 50%
medium:	diluent
container:	3 ml cuvet

Testcode	**EC50**	**95% cl**	**remarks**
TOX2-110997	60.4	50.0-73.0	t=5 min
TOX2-110997	50.7	4.2-64.0	t=15 min
TOX2-110997	71.6	45.4-113.2	t=5 min
TOX2-110997	60	45.1-79.9	t=15 min
mean (t=5 min)	55.3		
std	6.6		
n	2		
CV	11.9		

test:	D. magna 48hr		
concentrations:	0, 0.03, 0.06, 0.12, 0.25,0.5,1.0,2.0%		
medium:	DSW filtered over coal		
container:	1.1l glas beaker with lid		
Testcode	**LC50**	**95% cl**	**remarks**
TOX2-141097	< 1.6%		conc: 1.6,3.2,6.3,12.5,15%
TOX2-201097	0.4	0.4-0.5	

test:	G. aculeatus 96 hr		
concentrations:	0,6.25,12.5,25, 50, 100%		
medium:	DSW		
container:	2l beaker with parafilm		
Testcode	**LC50**	**95% cl**	**remarks**
TOX2-061097	8.6	7.6-9.8	