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NATIONAL INSTITUTE OF PUBLIC HEALTH AND THE ENVIRONMENT

RIVM report 607200 002

Toxicological validation of a procedure for extracting organic micropollutants from water samples

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July 1999

This investigation has been performed by order and for the account of the Directorate-General of the RIVM, within the framework of project 607200, pT methods.

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Abstract

A procedure was validated to extract the unknown cocktail of toxicants from water samples in order to measure its toxicity using different aquatic organisms. The purpose was to validate the extraction and concentration procedure toxicologically by determining how much toxicity is recovered after treatment of the water samples. It was found that the procedure was prone to the introduction of undesired toxicity. After a technical improvement of the procedure, the measured toxicity in concentrated water samples generally appeared consistent.

Acknowledgements

We would like to thank Carlo Strien who prepared the water concentrates. Dick Gielen is thanked for supplying the daphnids.

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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. In deze studie is de methode voor het extraheren en concentreren van de toxische stoffen toxicologisch gevalideerd. Dat wil zeggen: we hebben bepaald hoeveel toxiciteit kan worden terug gevonden in een geconcentreerd watermonster. Uit voorgaand onderzoek bleek dat de behandeling van het water mogelijk toxiciteit introduceert. Deze ongewenste toxiciteit werd ook onderzocht.

De extractie en concentreringsprocedure is ontwikkeld met het doel om microverontreinigingen uit water monsters in een concentraat te brengen dat geschikt is voor het uitvoeren van aquatische toxiciteitstesten. Dit betekent dat de toxische stoffen geconcentreerd worden tot een niveau waarop in kortdurende testen een toxisch effect gemeten kan worden. De toxiciteit wordt gemeten met microbiotesten met een kreeftachtige (de Thamnotox F test), een raderdiertje (de Rotox F test), een bacterie (de Microtoxtest) en een watervlo (de Daphnia IQ test). Deze kleinschalige aquatische toxiciteitstesten werden eerder geselecteerd vanwege hun korte blootstellingsduur en kleine testvolume. Ze zijn gevoelig en kunnen reproduceerbaar worden getest.

Het toxicologisch validatie onderzoek is uitgevoerd met een synthetisch watermengsel waaraan een mengsel van organische chemicaliën met een niet-specifieke werking is toegevoegd. De chemicaliën werden met behulp van XAD harsen uit het water geextraheerd. Door elutie werden de chemicaliën van de harsen in aceton geconcentreerd en bewaard. Direct voor de uitvoering van een toxiciteitsexperiment werden ze door middel van een Kuderna Danish destillatie in water gebracht.

De toxiciteit van het watermonster werd gemeten met de vier microbiotesten voor en na behandeling om het verlies aan toxiciteit te bepalen. Daarnaast werd een watermonster zonder toegevoegde chemicaliën op identieke wijze behandeld en getest om te bepalen of de behandeling ongewenste toxiciteit veroorzaakte.

Uit de resultaten bleek dat de behandelingsmethode een aanzienlijke hoeveelheid toxiciteit veroorzaakte. De toxiciteit van de blanco water monsters was zelfs net zo hoog als de toxiciteit van de watermonsters waaraan chemicaliën waren toegevoegd. Vanwege deze bevindingen is de opwerkingsmethode zo verbeterd dat het waterconcentraat minder aceton bevat dan in de voorgaande methode.

De toxiciteit van de monsters die met de verbeterde procedure werden opgewerkt, blijkt dan ook grotendeels verklaard te kunnen worden uit het gedoseerde testmengsel.

Uit bovenstaande resultaten blijkt de waarde van de toxicologische validatie: met enkel chemische analyses zou de ongewenste toxiciteit niet zijn aangetoond.

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 validate the extraction and concentration procedure toxicologically. In other words, we determined how much of the toxicity is recovered after treatment of the water samples. Further, it was measured if undesired toxicity were introduced by the procedure because earlier research demonstrated inexplicable toxicity after treatment of water samples.
The extraction and concentration procedure has been developed with the purpose to extract micropollutants from water samples into a concentrate that is suitable for the performance of aquatic toxicity tests. This means that the toxicants are concentrated to a level that toxicity can be measured in short term toxicity tests. Toxicity is measured with so called microbiotests. These aquatic toxicity tests were selected previously for there short test duration and small test volume. They were found to be reproducible and sensitive. The microbiotests used are the Thamnotox F test, the Rotox F test, the Daphnia IQ test and the Microtox test.
The toxicological validation was performed with a synthetic water sample prepared from a mixture of organic chemicals with a non-specific mode of action. The chemicals were extracted from the water sample with the aid of XAD resins. They were eluted from the resins and stored as acetone concentrates. Just before the performance of a toxicity test the concentrates were brought into a water fraction by means of a Kuderna Danish destillation. The toxicity of the mixture before and after the treatment was measured with the four toxicity tests to determine the loss of toxicity. A water sample without added toxicants was treated similarly to determine the toxicity possibly introduced by the treatment.
The results of the study showed that a considerable amount of toxicity is introduced by the treatment procedure. It was so high that toxicity of the blank water sample was as high as the toxicity of the water sample containing the mixture of toxicants. Because of these results the procedure was further improved so that the final concentrate used for the toxicity tests contains less acetone than in the former procedure. It appeared that the toxicity in the concentrated water samples, prepared according to the improved procedure, is almost entirely attributed to the applied toxic test mixture.
The value of the toxicological validation research is demonstrated by the results of this study. Chemical analysis alone would have failed to show the unexpected, artificial toxicity introduced during certain steps of the procedure

1. Introduction

1.1 Measuring environmental toxicity with the pT-method

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. Extraction is desirable to separate toxic stress factors from other stress factors. For practical reasons the environmental water samples 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 only a small volume of the concentrated water sample 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. Short exposure times are demanded both for practical and financial reasons. Moreover, the unfavourable area to volume ratio caused by the small scale of the microbiotests may lead to artefacts.

- 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. A toxicity index is calculated in which the results of toxicity tests in relation to the sample concentration factor are converted into a number. This index 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. Several modifications 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).

The ecotoxicological part is directed at the toxicological validation of the extraction and concentration procedure.

The purpose is to:

- determine the specific sensitivity of microbiotests for environmentally relevant micropollutants

- determine the loss of toxicity caused by the extraction and concentration procedure
- determine the reproducibility of the toxicological effects taking into account the whole procedure of water treatment preceding the toxicity experiment
- determine the sensitivity of the microbiotests for concentrated water samples, compared to the sensitivity of international acknowledged test methods with the waterflea Daphnia magna and fish for non treated water samples.

Earlier performed research described the selection of microbiotests (Willemsen et al., 1995) and the specific sensitivity of these microbiotests for two mixtures of toxicants in comparison with the sensitivity of the waterflea Daphnia magna and the stickleback Gasterosteus aculeatus (Vaal and Folkerts, 1998). The Microtox test and the modified Thamnotox F and Daphnia IQ tests appeared to be a good base for a test battery. The Rotox F test was very insensitive for the compounds in the mixtures compared to the other test methods. It was felt that algae as test organism were lacking from the battery. At present a rapid algae test (the PAM test) is being implemented.

This report describes the toxicological validation of the extraction and concentration procedure. The validation is used to determine the sensitivity of the selected biological test methods to measure toxicity in concentrated water samples in comparison to non treated water samples. Artificial water samples are prepared containing micropollutants of the narcotic type (including polar narcotics), covering a wide range of physico-chemical characteristics to simulate complex environmental water samples. The loss of toxicity due to the concentration and extraction procedure is measured with the microbiotests mentioned above. The toxicological recovery measurement also includes the possible, undesirable addition of toxicity that might be introduced by artefacts of the extraction and concentration procedure (Vaal and Folkerts, 1998). The set up of the study is described in 1.2.

1.2 The aim and set up of this study

Figure 1 shows the outline of the research. C stands for a surface water sample with respect to the concentration of the toxicants. It is prepared by dilution of a 1000 times more concentrated sample (1000C). This 1000C sample (Stock A) is used to perform toxicity tests because at lower concentrations no effects would be observed in the short duration of the tests.
After the extraction and concentration procedure (see 1.3) Stock B is obtained. It is hypothesized that when no toxicity is lost during the extraction and concentration treatment Stock A and B give the same level of toxicity.
Experiments carried out earlier (Vaal and Folkerts, 1998) indicated a possible increase of toxicity due to some artefacts in the extraction and concentration procedure. To search that possibility two control stock solutions were prepared. Stock C is prepared by adding the mixture of toxicants after the extraction and concentration procedure, Stock D is the blanc control of the extraction and concentration procedure. The tested hypothesis is that Stock A

and Stock C give the same toxicity levels and no toxicity is measured with Stock D. This will occur when zero toxicity is introduced by the procedure.
The characteristics of each stock are summarised as follows:
Stock A: non-treated water sample with known amount of toxicants added
Stock B: treated water sample with unknown amount of toxicants due to the concentration procedure
Stock C: treated water sample with known amount of toxicants added
Stock D: treated water sample without toxicants added

The Daphnia IQ test, the Microtox test and the Thamnotox F test were used because they were found to be sensitive and reproducible (Vaal and Folkerts, 1998). The Rotox F test is also included unless it appeared to be a relatively insensitive test method. However, because this test method was not so insensitive in a monitoring program (Roghair et al., 1997) it gave rise to the idea that the concentration procedure might need further refinement because of the introduction of toxic artefacts in the procedure.

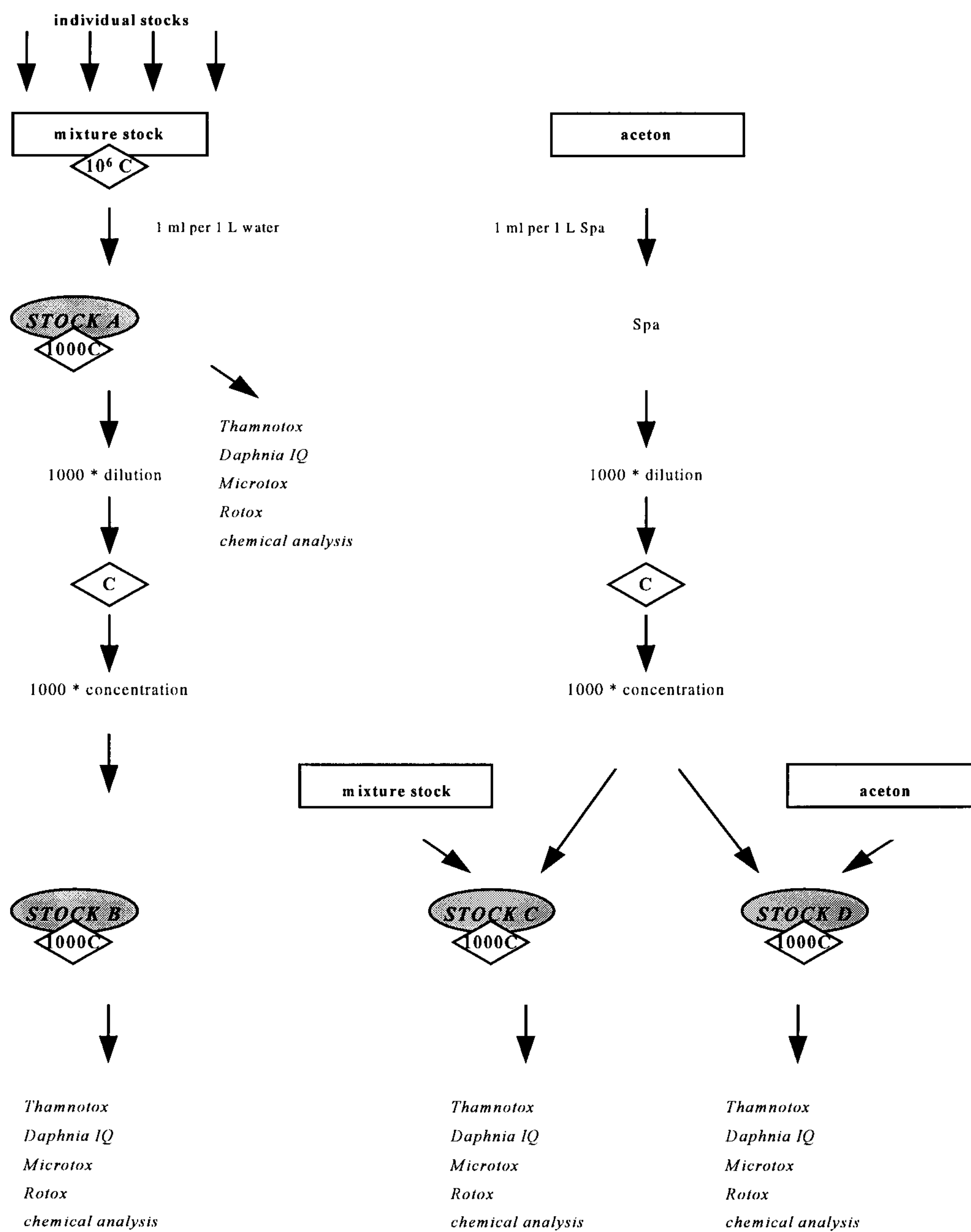

Figure 1. Outline of the research. Stocks A, Stock B, Stock C and Stock D represent differently prepared test concentrations that are used in the toxicity tests mentioned. C stands for a surface water sample with respect to the concentration of the toxicants. 1000C and 10^6 C indicated higher concentrations of toxicants, to allow for toxicity testing.

2. Methods

2.1 Ecotoxicological tests

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. 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 EC50). 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	EC50 after 5 or 15 minutes exposure
standard method	SOP ECO/064/01 (1996)

The Rotox F test

Test organism is the rotifer Brachionus calyciflorис. 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 a hatching medium during a period of 16-18 hours under continuous light. They are exposed in a disposable plastic multiwell test plate. 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 LC50) and is determined with the Spearmann-Kärber method (Hamilton et al., 1977). Details on the experimental design are given 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 - LC50
standard method	SOP ECO/295/00 (1998)

The Thamnotox F test

Test organism is the crustacean *Thamnocephalus platyurus*. The test parameter is lethality 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 LC50 value is determined with the Spearmann-Kärber method (Hamilton et al., 1977). Details on the experimental design are given in Table 3.

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 with light emitting properties, which 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 EC50, determined with the Spearmann-Kärber method (Hamilton et al., 1977).

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 - LC50
standard method	SOP ECO/294/00 (1998)

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	EC50 (E = not emitting light)
standard method	SOP ECO/304/00 (1998)

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.
Reference toxicity experiments with Rotox F, Thamnotox F and Daphnia IQ have been carried out using potassium dichromate ($K_2Cr_2O_7$) as a reference toxicant. The results were compared with toxicity criteria supplied with the test kit and to results obtained earlier with this toxicant.

2.2 Preparation of Stock A-D

A mixture was tested consisting of nine organic substances with a narcotic mode of action (chlorobenzenes, chloroanilines, nitrobenzenes and a chlorotoluene, see Table 5). It is based on the mixture used in Vaal and Folkerts (1998) but has been changed a little. Pentachlorobenzene was excluded because of its low water solubility and its limited contribution to the overall toxicity of the mixture. Concentrations of 1,3,5-trichlorobenzene, 2,4,6-trichloroaniline and 3-chlorobenzene were adjusted in favor of a more balanced mixture. The overall toxicity of the mixture was calculated assuming equitoxicity of all components. In Table 5 their contribution is expressed relative to the Microtox EC_{50} that was calculated using the QSAR presented by Hermens et al. (1985). In the present mixture all components contribute between 6% and 15% to the overall mixture toxicity.

For each compound in the mixture a stock solution was prepared in acetone (pa). They were stored in 20 ml vials with a crimp cap at -20°C in the dark for no longer than three months. The individual stock solutions were used to prepare a mixture stock solution in acetone with the aid of a microsyringe. This mixture stock solution was stored similar to the individual stock solutions.
Stock A-D were prepared according to the scheme presented in Figure 1. The mixture stock solution was diluted 1000 times using the proper test medium (see Table 1-Table 4) to prepare the so-called 100% concentration of Stock A. This 100% concentration was freshly prepared before the start of an experiment. The acetone concentration of each test concentration was adjusted to the level of the 100% concentration: 1 ml/l test medium in the Rotox F, Thamnotox and Daphnia IQ experiments and 0.5 ml/l test medium in the Microtox test.
To obtain Stock B, a sample of Stock A was prepared using commercially available mineral water (Spa blue). It was further diluted 1000 times by addition of a 10 ml sample of Stock A to 10 l mineral water. It was performed in ten-fold. Each 10 l sample was shaken during 24 hours with 20 ml of the resin mixture XAD 4/8 (prepared according to SOP/ECO/303/*00, 1996*). The XAD batches of each 10 l sample are sieved, collected and dried overnight in an open petri dish. The XAD is eluted with 600 ml acetone which is three times the acetone volume as prescribed by SOP/ECO/303/00. The acetone concentrate is stored in portions of

1996). The XAD batches of each 10 l sample are sieved, collected and dried overnight in an open petri dish. The XAD is eluted with 600 ml acetone which is three times the acetone volume as prescribed by SOP/ECO/303/00. The acetone concentrate is stored in portions of (20 ml) in the freezer until toxicity experiments were to be performed. An acetone eluate sample is distilled according to the Kuderna Danish technique to remove the bulk amount of acetone. After addition of an appropriate volume of water, the residual acetone is largely eliminated during 20 minutes of purging with nitrogen (SOP ECO/310/00, 1997). Finally, after adjusting the volume to 10 ml with mineral water, the chemicals in the original 10 l mineral water sample are concentrated 1000 times if 100 % recovery is assumed.
Stock C and D were prepared similarly but without the addition of Stock A to the 10 l mineral water samples. Instead, an identical amount of acetone was added. For Stock C 10 μl of the mixture stock was added to 10 ml water concentrate. Stock D was prepared by the addition of 10 μl acetone to 10 ml water concentrate.

Table 5 Characteristics of the chemicals in the mixture. The 100% concentration is the highest test concentration used in the toxicity experiments. To express the contribution of each component to the overall toxicity of the mixture, its nominal concentration is compared to the Microtox EC_{50}. The 100% concentration is calculated to be 2.5 times as toxic as the Microtox (MICT) EC_{50}.

	Solub. in water water (mg/L) 20-25°C	Log Kow	Mol w	H (Pa m^3/mol) calculated	VP (Pa) (20-25°C)	Stock A 100% conc. (mg/l)	ratio 100%/ MICT EC_{50}
1,4-dichlorobenzene	83.0	3.5	147.0	$1.60x10^{+2}$	90	1.224	0.39
1,2,3-trichlorobenzene	17.0	3.8	181.4	$3.00x10^{+2}$	28	0.563	0.24
1,3,5-trichlorobenzene	25.0	4.0	181.4	$2.00x10^{+2}$	28	0.499	0.26
1,2,3,4-tetrachlorobenzene	4.3	4.3	215.9	$2.50x10^{+2}$	0.7	0.288	0.15
3-chloronitrobenzene	501.0	2.6	157.6	$9.00x10^{-2}$	0.3	9.382	0.24
1,2-dichloro-4-nitrobenzene	96.8	3.3	192.0	$8.4 x10^{-2}$	0	1.863	0.30
3,4-dichlorotoluene	10.5	4.2	161.0	$6.50x10^{+2}$	42	0.538	0.36
2,4 dichlooraniline	213.8	2.8	162.0	$6.70x10^{-1}$	0.9	5.452	0.26
2,4,6-trichloroaniline	25.7	3.6	196.5	2.20	0.3	1.001	0.29
						total =	2.5 x EC_{50}

2.3 Chemical analyses

Samples were taken of the mixture stock solution and of the 100% concentration of Stock A of each test at the beginning of the experiment at t=0. The samples were analyzed at the Laboratory for Ecotoxicology according to De Groot et al. (1996). Hexane (5 ml) with an internal standard was added to 5 ml of the sample in a 20 ml vial and sealed with a crimp cap. Liquid-liquid extraction was performed through shaking this mixture during three minutes. The hexane samples were stored at 4°C until analyses. The sample was analyzed with a gaschromatograph with an electron capture detector. The recovery was more than 90% for all compounds.

3. Results and discussion

3.1 Toxicity experiments with Stock A- Stock D

The results of the toxicity experiments are presented in Figure 2, more details are presented in Appendix 3. The L(E)C_{50}'s of Stock A were higher than the L(E)C_{50}'s measured for Stock B-D in the Microtox test, the Rotox F test and the Thamnotox F test. This was not observed in the Daphnia IQ test were Stock B was less toxic than Stock A. Stock C was only slightly more toxic than Stock D. These findings indicate that a considerable amount of toxicity is introduced by the extraction and concentration procedure. It cannot be distinguished from the toxicity added to the samples.
The findings for the Rotox F test confirm the assumption made in Vaal and Folkerts (1998). In the experiments described here once more no toxic effects were detected when the untreated toxic mixture was tested (Stock A). However, treatment of the water sample caused toxicity even detectable by the Rotox F test. Results of the Rotox F test obtained with field samples in previous monitoring studies should be observed with this knowledge in mind.
The Thamnotox F test appeared to be the most sensitive test for the artificially introduced toxicity.

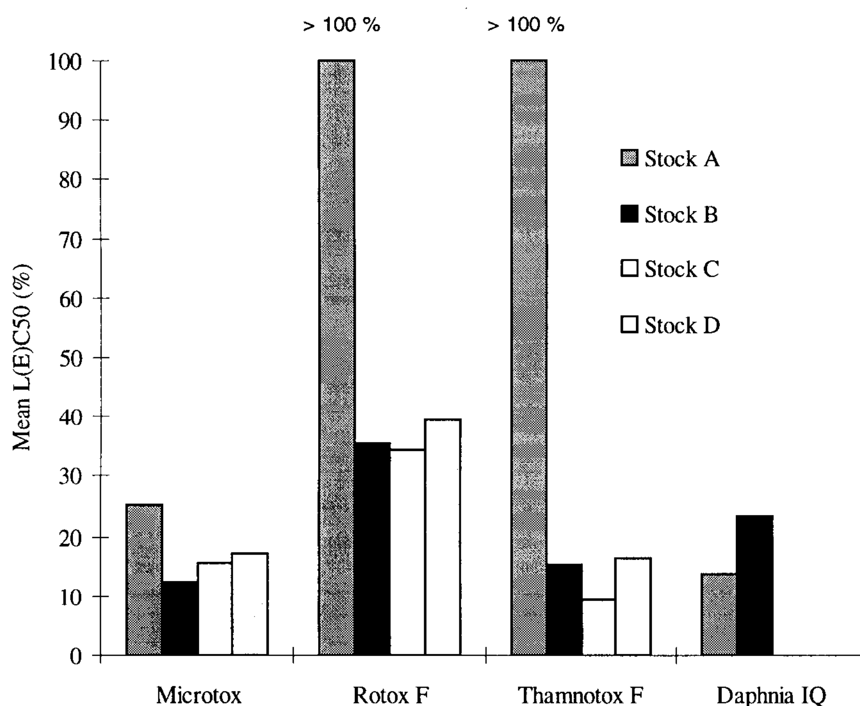

Figure 2 Toxicity of Stock A-D expressed as percentage of the highest tested mixture concentration, the 100% concentration, for four test methods.

For the Daphnia IQ test no data on Stock C and D could be obtained because of temporary problems in the culture of daphnids. These tests were cancelled until an improved extraction and concentration procedure was developed.

Table 6 shows the nominal and actual concentrations of the narcotic chemicals in the mixture stock solution in acetone and prepared as Stock A in the test medium at the start of the experiment. The loss of toxicants during the test has been reported in Vaal and Folkerts (1998). Table 7 shows how much of each narcotic chemical is recovered after the extraction and concentration procedure. These measurements were obtained from experiments not reported here, but the Stocks were prepared similar to the description in Chapter 2. 3-Chlorobenzene, 1,2-dichloro-4-nitrobenzene, 2,4-dichloroaniline and 2,4,6-trichloroaniline were detected in percentages between 29% and 63%. The other components could not be detected or were present in very low concentrations compared to the original concentrations in Stock A. Those findings are in accordance with frequently repeated measurements reported by Collombon et al. (1997).
These analytical results indicate that toxicity of Stock B is mainly determined by these four components. Based on these data it is expected that the toxicity of Stock B is lower than the toxicity of Stock A. Since our results show, however a lower L(E)50 (meaning a higher toxicity) of Stock B compared to Stock A, except for the Daphnia IQ test, it indicates that an unacceptable amount of toxicity is introduced during the water treatment procedure. Apparently, either the sensitivity for narcotics, or low sensitivity for introduced toxicity, may have caused the results as to Daphnia IQ.
It is assumed that this unintentionally added toxicity is caused by far too high concentrations of acetone. The solid phase extraction according to SOP/ECO/303/00, 1996) includes treatment of the collected XAD and subsequent elution with acetone. Unfortunately, SOP/ECO/303/00 was lacking a simple control measurement, which appeared of vital importance in the preparation of suitable acetone concentrates. If evaporation of water from the XAD during overnight drying in an open petri dish is insufficient, it can not be avoided that the acetone eluate contains a too high water concentration. In turn, this hinders the procedure to remove the bulk amount of acetone by means of the Kuderna Danish distillation according to SOP/ECO/310/00 (1997). As a result, the residue of the distillation has a relatively high volume, mainly consisting of water. However, it is still a water-acetone mixture and 20 minutes of purging with nitrogen appeared not long enough to remove the residual acetone to meet the requirement that the final product of the overall procedure contains acetone in a concentration not exceeding 0.2 % (v/v). Thus insufficient separation of water from the XAD will result in an eluate with a too high water/acetone ratio and as a consequence, after distillation and purging, the concentrated water samples may contain acetone concentrations too toxic to be appropriate for microbiotests.
Analysis of acetone concentrations showed that levels could be as high as 5 % v/v or 39 g/L. A Microtox experiment with acetone resulted in an EC_{50} of 2.1% v/v acetone in EPA medium (see Appendix 3). It explains the toxicity measured in the microbiotest with Stock B-D.

Because of these findings the water treatment procedure has been improved, by introducing two control measurements to prevent too high acetone concentrations. These control measurements on both cause and effect may eliminate a weakness in the procedure. First, the water content is bound to an upper limit, which is controlled through simply weighting the XAD after one night drying in a petri dish (or longer if necessary). This control prevents the cause of a flaw. Second, the concentrated water sample is GC-analyzed on the acetone concentration. This is the final check on the suitability of the sample for toxicity testing.

Table 6 Nominal and actual concentrations of the mixture stock (in acetone) and Stock A (in test medium).

	MIXTURE STOCK		STOCK A (mg/l)			
	nominal (mg/ml)	measured (mg/ml)	nominal (mg/l)	mean measured (mg/l) (n=4, t=0)	std.	% of nominal
1,4-dichlorobenzene	1.22	1.10	1.10	1.08	0.02	98
1,2,3-trichlorobenzene	0.56	0.56	0.56	0.54	0.02	96
1,3,5-trichlorobenzene	0.50	0.49	0.49	0.45	0.01	92
1,2,3,4-tetrachlorobenzene	0.29	0.29	0.29	0.28	0.01	97
3-chlorobenzene	9.38	9.77	9.77	9.82	0.57	101
1,2-dichloro-4nitrobenzene	1.86	1.99	1.99	1.86	0.05	93
3,4-dichlorotoluene	0.54	0.51	0.51	0.47	0.01	92
2,4-dichloroaniline	5.45	5.49	5.49	4.98	0.21	91
2,4,6-trichloroaniline	1.00	0.96	0.96	0.96	0.02	100

Table 7 Measured concentrations of compounds in the narcotic mixture before (Stock A) and after the extraction and concentration procedure (Stock B).

	Stock A (mg/l)	Stock B (mg/l)	% of Stock A
1,4-dichlorobenzene	0.84	<0.006	< 0.7
1,2,3-trichlorobenzene	0.33	<0.0006	< 0.2
1,3,5-trichlorobenzene	0.37	<0.014	< 3.8
1,2,3,4-tetrachlorobenzene	0.42	0.001	0.2
3-chlorobenzene	7.27	2.07	29
1,2-dichloro-4nitrobenzene	4.26	2.7	63
3,4-dichlorotoluene	0.22	0.001	0.5
2,4-dichloroaniline	1.53	0.65	43
2,4,6-trichloroaniline	0.75	0.28	37

3.2 Toxicological validation of the improved procedure

To prove that the concentration and extraction procedure was truly improved, some additional validation experiments were carried out. For this purpose Stock B, C and D were prepared once again but now according to the improved procedure. Toxicity experiments with the Microtox, Thamnotox F test, Rotox F test and the Daphnia IQ test were performed according to the methods described in Chapter 2. In Fig 3, results of the water treatment independent Stock A of the first experiment are combined with Stock B, C and D obtained with the improved procedure. The Rotox F series demonstrate the improvement of the water treatment procedure. Rotox F indeed was sensitive to the toxicity that was introduced during the former procedure (Fig 2). In Fig 3, Stock B, C and D display also a low toxicity. This means that, for Rotox F, the procedure did not ad significant toxicity compared to the untreated Stock A. Thamnotox F is not sensitive for introduced toxicity (Stock D). Results with Stock C are probably a combination of 1) the effect of high concentrations of the test cocktail, without loss due to the water treatment procedure (test cocktail added after the concentration procedure) and 2) the toxicity due to procedure. Instead of less toxic, stock B was more toxic than C; this result could not be interpreted. Probably artefacts during the Thamnotox F test played a role as the blank in this test was unsatisfactory. The Microtox and the Daphnia IQ series are rather consistent in showing nearly equal toxicity of Stock A and C, indicating that the procedure did not add a significant toxic effect to the test mixture. Lower toxicity for Stock B can be attributed to the loss of toxic test chemicals during the water treatment procedure. Both microbiotests are somewhat sensitive to a 1000-fold water concentrate without any test chemicals (Stock D), apparently due to toxicity introduced by the concentration procedure, even in the improved method.
The concentration of acetone did not exceed 0.15 % v/v with an average of 0.11 % v/v.
The EC50 value for Microtox was measured twice for acetone: 2.0 and 2.2 % v/v. Details are given in Appendix 3, where also EC50 values of K2Cr2O7 are given for Thamnotox F, Rotox F and Daphnia IQ. The results with this reference compound indicate that the performance of these three microbiotests was satisfactory. The values for K2Cr2O7 are within the range specified for the delivered batch of test species and did not significantly deviate from the sensitivity for similar species measured in other batches.

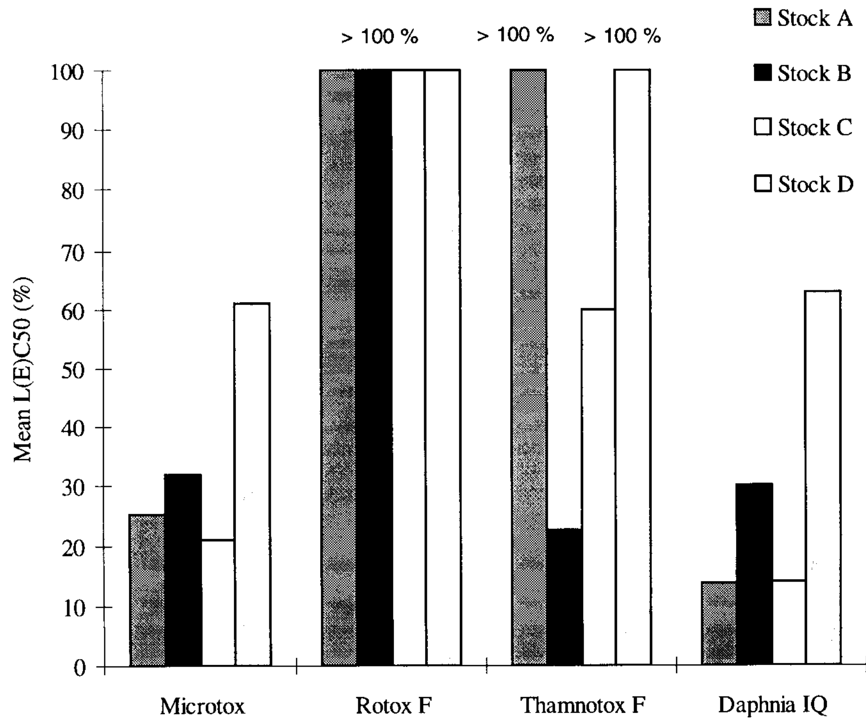

Figure 3 Toxicity of Stock A-D expressed as percentage of the highest tested mixture concentration, the 100% concentration, for four test methods.

4. Conclusions and recommendations

The procedure used for extraction and concentration of micropollutants from water samples, as described by SOP ECO/303/00 (1996) and SOP ECO/310/00 (1997), introduces toxicity to the water concentrates. This undesired, additional toxicity, could not be distinguished from and was even higher than the toxicity added as a toxic mixture to artificially prepared water samples. The Thamnotox F test was most sensitive for the toxicity introduced by the water treatment procedure. Even the relatively insensitive Rotox F test could detect toxicity in treated water samples. This means that results from previous field monitoring studies should be used with great caution, bearing this knowledge in mind.
Because of these findings the water concentration and extraction procedure has been refined to control for the high and variable levels of acetone in the water concentrates. Toxicological validation of this improved procedure showed that measured toxicity in several defined test mixtures gave more confidence that the toxicity introduced by the procedure can be constrained within acceptable limits. Now the Rotox F test appeared insensitive to all samples, which is consistent with the results of toxicity experiments previously conducted (Vaal & Folkerts, 1998). The results obtained with the Microtox test and the Daphnia IQ generally appeared consistent. However, two out of four samples tested with the Thamnotox F test showed results that could not be interpreted.

We recommend the following:

- to add an algae microtest to the set tox kits applied in this study. The recently developed PAM test (pulse amplitude modulation) seems a cost-effective candidate (Bas v. Beusekom, 1999). In the PAM test, the efficiency of the photosynthesis is determined fluorometrically and no use has to be made of ^{14}C-labeled substrates.
- to repeat this toxicological validation with a new extraction/concentration procedure. What has been denoted in this report as the "improved procedure" has recently been further optimized (Peijnenburg and Struijs, 1998). Firstly, the chemical recovery has been increased as 7 out of 11 compounds of the narcotic test mixture are found in the aqueous concentrate at levels of 60 % or higher (mostly between 70 and 85 %) and yet the four most volatile chemicals at levels between 20 and 50 %. Secondly, the new procedure is far less laborious and more suitable to treat field samples of high volumes. In a toxicological validation exercise also high volumes are required to obtain sufficient water extract to perform the microbiotests. Within the framework of this project this method ("the optimized procedure") will tentatively have the status "finalized".
- to include other test cocktails in a following toxicological validation. Two pesticide cocktails have recently been investigated in the optimized procedure (Peijnenburg and Struijs, 1998). Nine out of eleven showed recoveries 70 % or higher, one 50% and one 24%. In 1999 two detergents (LAS and alkylethoxylate) and two organotin compounds will be investigated. After evaluating the toxicological characteristics, an appropriate test cocktail could be designed for the next toxicological validation study.

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Appendix 2 Composition of test media

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

Appendix 3 Results of toxicity experiments

test:	Daphnia IQ		
concentrations:	0, 6.25, 12.5, 25, 50, 100% Stock		
medium:	EPA		
container:	5 ml vials with snap cap		
Testcode	**EC50**	**95% cl**	**remarks**
150798 Stock A	13.6	9.4-19.9	pretest: 16 out of 18
150798 Stock B	23.5	18.8-29.5	pretest: 16 out of 18
160299 Stock B	28.8	13.9- 69.7	pretest: 15 out of 18; improved procedure
081298 Stock C	14.0	10.1- 19.3	pretest: 16 out of 18; improved procedure
081298 Stock D	63.3	44.1 - 90.8	pretest: 16 out of 18; improved procedure

test:	Rotox F		
concentrations:	0, 6.25, 12.5, 25, 50, 100% Stock		
medium:	EPA		
container:	multiwell		
Testcode	**LC50**	**95% cl**	**remarks**
120598 Stock A	>100%		
190598 Stock B	35.4	-	no conf. limits because 0-100% effect in 25-50% Stock solution
240698 Stock C	34.4	32.6-36.3	
170698 Stock D	39.5	35.7-43.6	
170299 Stock B	> 100		improved procedure
091298 Stock C	> 100		improved procedure
091298 Stock D	> 100		improved procedure

test:	Microtox (t = 5 min)		
concentrations:	0, 6.25, 12.5, 25, 50% Stock		
medium:	diluent		
container:	3 ml cuvet		
Testcode	**EC50**	**95% cl**	**remarks**
130598 Stock A	25.2	21.5-29.5	
130598 Stock B	12.5	11.6-13.4	
150698 Stock C	15.7	13.2-18.7	
150698 Stock D	17.2	15.7-18.8	
160299 Stock B	32.2	26.4 - 39.3	improved procedure
091298 Stock C	20.6	19.1 - 22.3	improved procedure
091298 Stock D	60.6	28.6 - 128.4	improved procedure

test:	Thamnotox F		
concentrations:	0, 6.25, 12.5, 25, 50, 100% Stock		
medium:	EPA		
container:	5 ml vials with snap cap		
Testcode	**LC50**	**95% cl**	**remarks**
120598 Stock A	est. 100		15 of 31 died in 100% concentration
190598 Stock B	15.2	13.7-17.0	
240698 Stock C	9.5	8.6-10.4	
170698 Stock D	16.5	15.3-17.8	
170299 Stock B	22.6	11.1- 45.9	improved procedure, pore blank
101298 Stock C	60.4	52.7- 69.1	improved procedure
101298 Stock D	> 100		improved procedure

test:	Microtox (t = 5 min)		
concentrations:	0, 6.25, 12.5, 25, 50% of 2 % or 20% v/v acetone in EPA		
medium:	diluent		
container:	3 ml cuvet		
Testcode	**EC50**	**95% cl**	**remarks**
250698-aceton 2%	2.0	0.5-8.8	extrapolated EC_{50}
250698-aceton 20%	2.2	1.9-2.5	

test:	Thamnotox F		
concentrations:	0, 0.032-0.063-0.125-0.25-0.50 mg $K_2Cr_2O_7$ /l		
medium:	EPA		
container:	multiwell		
testcode	**LC_{50}**	**95% conf.lim.**	**remarks**
300698	0.08	0.07-0.09	

test:	Rotox F		
concentrations:	0-3.2-6.3-12.5-25-50 mg $K_2Cr_2O_7$ /l		
medium:	EPA		
container:	multiwell		
testcode	**LC_{50}**	**95% conf.lim.**	**remarks**
300698	9.2	7.3-11.4	

test:	Daphnia IQ		
concentrations:	0,1.6-3.2-6.3-12.5- 25 mg $K_2Cr_2O_7$ /l		
medium:	EPA		
container:	5 ml vials		
Test code	**EC_{50}**	**95% conf.lim.**	**remarks**
170798	3.3	2.0-5.4	