ASSOCIATION OF RIVER WATERWORKS

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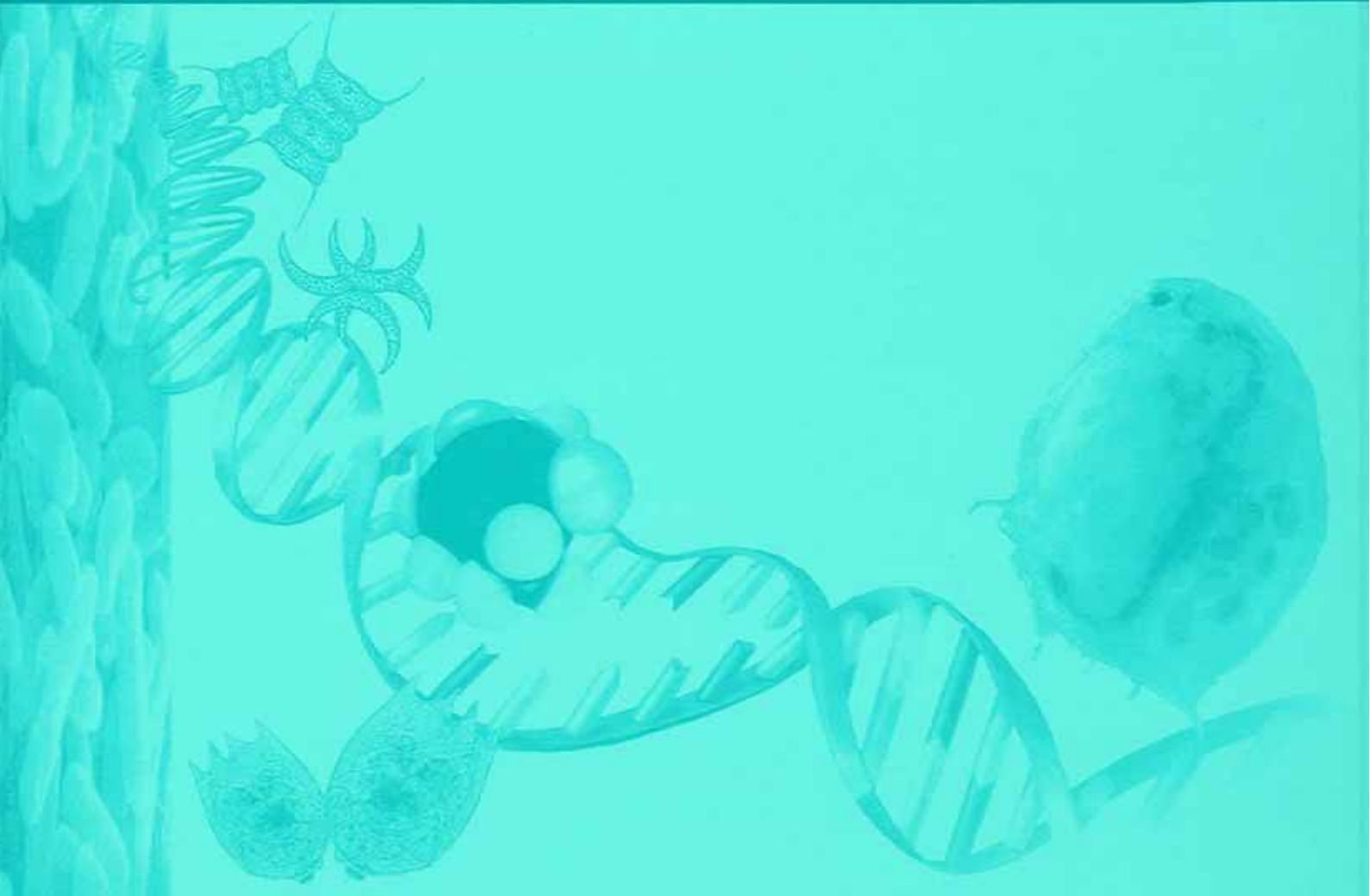
Biological tests, a suitable instrument for the quality control of surface water?



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Abbreviations

The list below gives the abbreviations used in alphabetical order, followed by their meaning.

AchE	=	Acetylcholine esterase
CLN	=	Confidence Limit
DMSO	=	Dimethyl sulphoxide
DZH	=	Duinwaterbedrijf Zuid-Holland (Dune Water Supply South Holland)
ECf_{50}	=	Effect Concentration factor in 50% of the organisms used
ERE	=	Estrogen Responsive Element
ER	=	Estrogen Receptor
EEQ	=	Estradiol Equivalents
GW	=	Gemeentewaterleidingen Amsterdam (Amsterdam Water Supply)
HPLC	=	High Pressure Liquid Chromatography
IVM	=	Instituut voor Milieuvraagstukken (Institute for Environmental Studies)
LCf_{50}	=	Lethal Concentration factor in 50% of the organisms used
LOECf	=	Lowest Observed Effect Concentration factor
NOEC	=	No Observed Effect Concentration
NECf	=	No Effect Concentration factor
pT	=	Toxic potency
PAF	=	Potentially Affected Fraction
PAM	=	Pulse-Amplitude-Modulation
PCA	=	Principal Component Analysis
PTFE	=	Polytetrafluoroethylene
PWN	=	N.V. PWN Waterleidingbedrijf Noord-Holland (PWN Water Supply Company North Holland)
RID	=	Rijksinstituut Drinkwatervoorziening
RIVM	=	Rijksinstituut voor Volksgezondheid en Milieuhygiëne (National institute of public health and the environment)
RIWA	=	Vereniging van Rivierwaterbedrijven (Association of River Waterworks)
RIZA	=	Rijksinstituut voor Integraal Zoetwaterbeheer en Afvalwaterbehandeling (Institute for Inland Water Management and Waste WaterTreatment)
TMoA	=	Toxic Mode of Action
VITO	=	Vlaamse Instelling voor Technologisch Onderzoek (Flemish Institute for Technological Research)
WBB	=	N.V. Waterwinningbedrijf Brabantse Biesbosch (Water Storage Company Brabantse Biesbosch Ltd.)
WRK	=	N.V. Watertransportmaatschappij Rijn-Kennemerland (Water Transport Company Rhine-Kennemerland Ltd.)
XAD	=	Synthetic Resin with a macroreticulate structure based on a styrene and divinyl benzene

Preface

Human society produces thousands of substances for a wide variety of purposes. In the sixties it became clear that too many of these compounds ended up in surface water, with serious consequences for the plants and animals living in the water. The consequence was a serious disruption of the aquatic environment; in fact, the situation was so bad that water fleas that were brought into contact with undiluted river water died within minutes! Since the seventies, the water quality of the Rhine and, to a lesser extent, the Meuse has improved gradually. Partly thanks to regulations and consultation with industries, the levels of compounds that can be measured with chemical techniques have been reduced considerably. However, there are all kinds of compounds that cannot be identified with the current measuring equipment. Moreover, it is difficult to interpret the significance of the presence of all kinds of measurable substances to organisms in the water, and it is even more difficult to identify their possible consequences for the consumer of drinking-water produced from surface water.

The significance of a number of individual compounds is known, but the concentrations currently measured in surface water are low, and often it is not clear whether such low concentrations still pose a threat. It is also often unclear whether simultaneous burdening with different substances causes a greater risk than the individual compounds separately, a phenomenon called synergy. As the significance of known and unknown contamination should ultimately be interpreted in terms of their effects on organisms, it is better to use a so-called effect-based measurement. Biological tests are used to measure directly whether there are any effects on organisms. All compounds present can be included in such tests, even those that cannot be identified with current chemical-analytical techniques.
Different compounds may have different effects on organisms. Some substances will damage an organism's genetic material, while others disrupt enzymatic processes in the cell. For this reason it is necessary to use a battery of biological tests for the assessment of the water to be investigated. In comparison with chemical analyses this approach is more direct, although it can still not resolve all questions. An important question, for instance, is how an identified effect on a certain type of bacteria or on water fleas should be interpreted in terms of risks to humans.

As appears from the above, effect-based measurement is a complicated issue which requires a broad approach. The RIWA Project Group on Biological tests may count itself lucky because of the partnership formed for this project. Thanks to the contribution and cooperation of institutions such as RIVM, RIZA, AquaSense, VITO, KIWA and Koeman en Bijkerk and a number of water companies (GW, WRK, PWN, WBB and DZH), it has been possible to undertake this study, interpret the data acquired and produce the report. By carefully coordinating the measuring programmes of the different participants, it has been possible to include a number of additional biological tests in the programme. Thus valuable data could be collected which the individual institutions would not have been able to obtain.
It was decided to ask two authors to write the final version of the report, but this was done with data, interpretations and the assistance of all the working group's members.

Wim Hoogenboezem (Project leader of the RIWA Working Group on Biological tests)

Summary

Introduction

In addition to the regular RIWA measuring network, a more effect-oriented approach to measuring water quality is needed. To test the suitability of biological tests, a battery of tests was examined at two sampling points at the Meuse (Eijsden) and the Rhine (Lobith). This investigation (1998-1999) was undertaken as a joint project of RIWA and RIVM, RIZA, KIWA, Vito and several RIWA associates. Once every two months a sample was taken from each river, from which organic pollutants were extracted using XAD resins (figures 1, 2 and 3, pages 20-22). These extracts were subjected to the following battery of tests (table 2, page 15):

- Bioassays (concentration factor: 1000*): Microtox®, the Microtitre plate test with two species of algae, the PAM algae test, the Rotox kit, the Thamnotox kit and the Daphnia IQ test.
- Genotoxicity tests (concentration factor: 25,000*): Ames test, UMU test, VITOTOX® and two comet tests.
- Effect-specific test: Acetylcholine esterase (concentration factor: 1000*) and the ER-Calux assay (concentration factor: 10,000-100,000*)

Bioassays

The results of this investigation show that in almost all cases only the more concentrated samples yielded a positive result (table 6, page 37). The test with the highest response rate differed considerably from sample to sample. Evaluation of all the measurements reveals that the different tests yielded little overlapping information (figure 14, page 42), thus clearly demonstrating the need to use a test battery. The bioassays most suitable for research in Dutch rivers appear to be: Microtox®, the Microtitre plate test with only one species of algae, the PAM algae test, the Thamnotox kit and the Daphnia IQ test. The Rotox kit only led to a response in some samples with a high concentration factor; this test does not appear to be very suitable for measuring the level of contaminants present in the rivers Rhine and Meuse. According to these tests, the general toxicity of the Rhine is lower than that of the Meuse (figures 11, 12 and 13, pages 40-41). There was more variation in the range of concentration factors in which positive samples were obtained in the Meuse, which implies a less constant water quality. Based on the ecotoxicologic risk/toxic pressure model of RIVM, two samples from the Meuse (September 1998 and June 1999) may be considered toxic (table 9, page 43). Using chemical measurements and the 'Toxic Units' calculated, six relevant organic compounds were found in the Meuse. Although the average concentration of these compounds was below 0.1 µg/l, they contribute significantly to the explained toxicity of Meuse water (table 12, page 46). The kind of effect these compounds can have on organisms mainly involves photosynthesis and a possible neurotoxic effect. Establishment of the Combi-pT (table 10, page 44), which, through the results from bioassays, represents the ecotoxicologic quality of the rivers for all organisms, reveals that the Meuse is approximately 7.5 times more toxic than the Rhine. No relationship is shown between the pT values (toxic pressure on the basis of measurements from bioassays) and the PAF values (toxic pressure via chemical analysis; figure 15, page 44). This appears mainly from the sharply divergent point where a high PAF value does not correspond to the relevant pT value, which emphasises the added value of using biological tests. Modification of the model used for the calculation of toxic pressure/ecotoxicologic risk is therefore recommended. To supplement this pT-model with data from genotoxicity tests, research is desirable in order to obtain a combination + end parameter for bioassays and genotoxicity tests. At

the moment it is not possible to predict toxic pressure from chemical measurements on the basis of models, as the biological tests show the effects that are the consequence of often very complex interactions between chemicals and organisms. Hence the added value of using biological tests (at least 1 test for each trophic level) for establishing the quality of surface water.

Genotoxicity tests

With respect to the genotoxicity tests, the Ames test most frequently demonstrated mutagenic activity (tables 13 and 14, page 47). This test also produced the best dose-response curves with the clearest results, and is therefore still preferable to the other genotoxicity tests. The VITOTOX® and UMU tests yielded less positive results. The values at which a response was obtained in these two tests were often close to detection level. Moreover, the level of extraction solution in the test medium had to be minimised in these tests because of interfering toxicity problems in the test organisms. Useful results were obtained with two additional tests: the SOS Chromotest and the Mutatox test. The Mutatox test could generate a genetic response at a relatively low concentration factor. As regards the use of the comet test, no unambiguous criteria have as yet been laid down to facilitate a proper evaluation. As the measurements of a number of genotoxicity tests are not very reliable, the quality picture produced by the Ames test is still to be verified in a follow-up study. Future genotoxicity research should also aim to establish any possible links between the toxicants detected and possible risks to humans.

When considering point mutations (e.g. the Ames test) and activation of the SOS–DNA repair system (e.g. VITOTOX®), the Rhine shows a significantly higher level of genotoxicity than the Meuse (table 15, page 48). However, when considering chromosomal damage (the comet test), the Meuse scores higher than the Rhine. There is (as yet) no explanation for this remarkable difference.

Effect-specific tests

The effect-specific tests showed that the Meuse has twice as much choline esterase inhibition as the Rhine (figure 18, page 51). Moreover, in the Meuse this parameter appears to fluctuate much more.

According to effect measurements by means of the ER-Calux test, related to the occurrence of substances with estrogenic activity (figure 19, page 52), only one sample from the Meuse clearly showed increased activity.

1. Introduction

RIWA considers it necessary to include biological tests in its measuring programme to supplement the chemical, physical and biological parameters. In 1994 RIWA undertook an extensive study into the toxicologic state of the Rhine (De Noij & Meerkerk, 1995). For the purpose of that study, samples taken from a large section of the Rhine basin weresubjected to a number of different analyses, including biological. In addition, a study was carried out into the composition of the insect fauna at various locations. RIWA has also been doing research into the extent of mutagenicity in Rhine and Meuse water by means of the Ames test. In spite of the fact that the measurable contamination burden in the Meuse is often higher than that in the Rhine, this test has demonstrated a higher mutagenicity level in Rhine water (e.g. Veenendaal & Van Genderen, 1997). Furthermore, a number of studies were undertaken in the two large river basins in order to characterize the substances that require further research (Van Genderen & Noordsij, 1998), and a number of inventory studies were undertaken on substances such as biocides (Groshart & Balk, 1998) and xenoestrogens (Dennemanet *al.*, 1998).

Biological tests are experiments that can make the effects of contamination on organisms visible. In biological tests, organisms or parts of organisms (e.g. bacteria, algae, animals, cell cultures or tissues) are exposed to the water to be investigated or a concentrate of this, after which reactions by test organisms (e.g. reduction in activity, reproduction or growth, or even death) are recorded, which are then compared with any reactions identified in control tests. The use of biological tests is more valuable in comparison with the usual chemical tests, as it directly shows any effects on organisms or parts of organisms. In the same way, the effects can be measured of compounds or mixtures containing compounds that cannot be analyzed or identified.

If, at a certain location, important effects are measured repeatedly with one or more biological tests, it may be important to try to identify the cause since, only when it is known which compound(s) caused the effect, will it be possible to try to trace its source. Only then will it be possible to remove the cause and so improve the quality of the water.

So far, experiences with biological tests have shown that it is necessary to use different biological tests in parallel for water samples. Such batteries are necessary because different substances may have different effect mechanisms. There are compounds which, for instance, influence enzymatic reactions, while others lead to damage to the genetic material. Such differences make it necessary to use a test battery. It is therefore recommended that a battery should becomposed which consists of tests that use organisms from different main classes and functions in the ecosystem (trophic levels). Such a battery will consist of tests for bacteria, animal and plant organisms and, preferably, also for vertebrates or their cells or tissues.

In 1997 the working group started on an inventory of possible suitable tests. The following preconditions were applied to the choice of the tests to be used:

1. The tests should be (broad range) tests with which good results have already been achieved, i.e. no tests that are still under development or that are still at an experimental stage should be used.
2. Preference should be given to tests that do not require preconcentration of water samples for the investigation of Dutch rivers.
3. The tests should be fairly straightforward laboratory tests, making it easy and relatively cheap to use them.

As regards the use of concentration techniques, it soon appeared that it is not to be expected that tests will be available that can be used without concentration. The second precondition was therefore abandoned. The concentration techniques available have been evaluated for the purpose of this project, briefly stating the advantages and disadvantages of each technique (see table 3).

In the second part of this project, the tests referred to were tried at sample points in both the Meuse and the Rhine. The results of this investigation are discussed and evaluated in this report, while giving an answer to the following questions.

1. Which biological tests are most suitable for research in Dutch rivers?
2. Which test battery is most suitable for obtaining measurements?
3. What is the best location for taking measurements, and with what frequency should measurements be taken?
4. Can any statement be made on the water quality of the Rhine and the Meuse on the basis of the data collected so far?
5. Is there a measurable difference between the two rivers?
6. Does the toxicity level vary during the season?

The results were evaluated simultaneously by means of assessment tools such as the principal component analysis (PCA), which was used to check the extent to which the different tests detect the same or different components. In addition, all results were subjected to an analysis of the ecotoxicologic risks. With this analysis, all individual tests can be compared objectively, and a conclusion can be drawn regarding the toxicologic state of the two rivers.
Within the framework of a joint research program of the Dutch water companies, similar investigation was started at KIWA in 1998, in which a number of genotoxicity tests were compared. Because of a partial overlap between this investigation and the study of this RIWA project group, KIWA decided that its study should be incorporated in the RIWA project.

2. Composition of test battery for biological tests with concentration techniques

2.1. Which biological tests are available?

The toxicity of a water sample can be determined by using biological tests focusing on the general effects on cells or organisms (bioassays), focusing specifically on changes in the DNA (genotoxicity tests) and/or focusing specifically on a certain effect (effect-specific tests). The sections below describe the biological tests available for research on surface water.

2.1.1. Bioassays

In current practice, a fairly large number of bioassays are used for the investigation of surface water and industrial effluents, and for assessing the toxicity of individual compounds. Usually such investigation involves three groups of test organisms (bacteria as the decomposers, algae as the primary producers and water fleas or other invertebrates as the consumers), in order to obtain a broad picture of the sample's toxicity. A number of these bioassays have now been standardised. The tests are adjusted regularly (by using other test organisms), and new developments (improvements in their practicability) occur frequently. In a test battery, bioassays are chosen from the three trophic levels mentioned. As there are quite a number of different tests and the sensitivity of these tests is usually insufficiently described, it does not appear to be easy to put together a reliable, sensitive combination.

2.1.2. Genotoxicity tests

One of the first genotoxicity tests available was the well-known Ames test (Maron & Ames, 1983). This test is used worldwide as a standard test (OECD471) for establishing the mutagenicity of samples from different matrices. However, the test is laborious and time-consuming, as a result of which universities/institutions quickly started looking for a suitable alternative to this test. Faster genotoxicity tests are now available, which can also provide more information about the nature of the abnormalities in the gene/chromosome. A selection is given in table 1. Many of the newly developed tests use the same mechanism, which means that the variety of tests available is smaller than it might seem. Although different genotoxicity tests have now been developed, only a few tests are on the market, which are available commercially or in a standardised form.

Table 1: Selection of the genotoxicity tests available.

Genotoxicity test	Response time	Comment
Ames	3 days	**
Mutachromo Plate test	5 days	**
Ames fluctuation test	2 days	**
Mutatox test	1 day	
Microsome rec-assay	1 day	
UMU-test	4 hours	*
VITOTOX®	4 hours	*
SOS Chromotest	1 day	*
SCE test	3 days	
Comet test	2 hours	Can be used on other cell materials

* = SOS response after DNA damage (see page 14 for explanation)

** = Same type of bacteria

2.1.3. *Effect-specific biological tests*

The effect on the cells of animals, humans and plants of a number of substances of non-natural origin has been established (Berndt, 1995). By measuring the inhibition of enzymes or the activation/blockage of receptors in terms of certain reactions in the metabolism, the level of those substances (usually as the number of equivalents of a known inhibitor/activator) in the sample can be established. Effect-specific tests aimed at establishing the quality of surface water were rarely undertaken in the past.

2.2. Selection criteria for the composition of a test battery

The following selection criteria were applied:

- It had to be easy to apply the test in a regular monitoring network.
  Following some training, it had to be easy for a lab technician to carry out the biological test at a 'water' laboratory. The results from the biological test had to be unambiguous.
- Brevity.
  In order to minimise changes in the testing medium during the exposure time as far as possible, preference was to be given to a short test.
- Small volume of testing medium.
  As the toxicity was measured in concentrated samples, with a very small extract volume, it had to be possible to carry out the tests with a very small volume (several millilitres) of testing medium.
- Low costs.
  As measurements were to be taken with a battery of tests at several locations, preference was to be given to cheap tests with comparable results.
- Standardisation.
  The tests were to be carried out according to (inter)national standardised instructions or according to clear instructions from the supplier.
- Sensitivity.
  The most sensitive tests among the biological tests available were chosen. In addition, various detection mechanisms and trophic levels were to be considered in the selection.

2.3. Composition of the test battery

2.3.1. Bioassays

A substance's capacity to cause damage or toxicity depends on the nature of the substance, the dose, the duration of exposure and the route along which the substance is absorbed by the body. By means of bioassays, it is possible to establish whether the surface water contains compounds or combinations of different compounds that have a toxic effect on organisms in surface water. It is possible in this respect that different compounds have a negative effect on different cell elements (enzymes, receptors, DNA), which together may lead to the organism's death. When compiling bioassays with organisms from different trophic levels, it is possible to establish whether there are compounds in the sample that have an effect on a specific group of targets (e.g. the enzymes involved in the photosynthesis when algae are used as test organisms). For this project it was decided to use three bioassays with algae as test organisms (producers), three bioassays with invertebrate test organisms (consumers) and one bioassay with a bacterium as a test organism (destructor) (table 2, page 18). As concentrates with small volumes were used, microtitre plates were used for a number of tests (for 2 tests with algae and invertebrates). As it is only possible to observe lethal effects or growth inhibition in microtitre plate tests, which requires a relatively long incubation period, two tests (the PAM and the Daphnia IQ) that are able to show a sublethal effect on the basis of a substantially shorter incubation period were also included. By using three tests on algae and invertebrates, it is possible to establish which tests are most sensitive. As the only destructor available was Microtox®, no comparison of the most sensitive test could be done for this group of organisms.

All bioassays chosen are acute toxicity tests, which show the harmful effect of compounds within a short period of time (within 72 hours). Chronic tests, by means of which long-term effects can be established, were not included in this project, as these require a complex procedure and involve high costs. Chronic effects are often the consequence of the presence of compounds in very low concentrations, whose negative effect can be proven only after a considerable period of time. By means of concentration techniques, it is possible to establish with acute bioassays whether many of these compounds are present in the water to be researched.

2.3.2. *Genotoxicity tests*

Genotoxicity tests demonstrate the presence of compounds that affect DNA (see table 2). The presence of certain compounds in the cell may lead to a change in the DNA (mutation) which is irreversible and may be expressed in the organism exposed, or may only appear in future generations. There are different types of mutation:

- Point mutation: A change in the single base pair in the DNA. Point mutations may be the consequence of errors during the replication of the DNA, recombination or repair. In the case of point mutations, a different amino acid is built into the protein chain, as a result of which the enzyme or protein required may no longer function properly.
- Frameshift mutation: This type of mutation takes place when a base pair or a number of base pairs are deleted from or added to the DNA. As the code for an amino acid consists of a sequence of 3 base pairs (triplets), the deletion or addition of a base pair will lead to the formation of an entirely different protein, which will usually no longer be functional to the cell.
- Deletion mutations: This involves the removal of thousands of base pairs and possibly of many genes from the DNA.
- Inversion mutations: Sometimes the inversion of a DNA sequence takes place, as a result of which all genes in the relevant region become oriented towards the opposite direction.
- Duplication mutations: In a duplication mutation, a sequence is copied from one DNA region to another region.
- Insertion mutations: Insertion mutations are caused by the addition of a large DNA part to another gene region.

Frameshift mutations account for a large percentage of all spontaneous mutations. The use of an Ames test with a TA98 strain shows the presence of substances that cause those frameshift mutations. As Dutch surface water has been monitored by means of the Ames test for some years, this test was included in the test battery as a reference.

The cell has a large number of mechanisms available to repair damage to its DNA as a consequence of the presence of mutagenic compounds. The VITOTOX® test and the UMU ® test, which specifically detect the induction of the SOS repair mechanism (as a consequence of serious DNA damage by mutagenic substances), were included in the test battery. The Comet test was used for the detection of breaks in single and double DNA strands and any alkali labile sites in the DNA. Alkali labile sites in the DNA are 'vulnerable' parts of the DNA that lead to breaks in the DNA under alkaline circumstances, which were not present as such before and which will probably lead to abnormalities. Two different tests were used in this context: a test whereby human lymphocytes were exposed to the extract, and a test whereby *Daphnia* cells were exposed to the extract. The above genotoxicity tests were also done on a sample to which an amount of liver extract (S9 preparation) was added. The S9 preparation contains a number of liver enzymes which simulate (part of) a mammal's metabolism. This preparation is able to show that certain mutagenic

compounds have been inactivated, or that certain changes in the liver may lead to the formation of genotoxic compounds.

2.3.3. *Effect-specific tests*

The effect-specific acetylcholine esterase test demonstrates the presence of organophosphates (including thiophosphates) and carbamate pesticides in extracts from surface water. The ER-Calux is used to detect specific substances that may disrupt the hormone system in animals and humans.

Table 2: The biological tests chosen for the measuring programme.

Biological test	**Instructions**	**Duration of exposure**	**Volume@ (ml) required**	**Costs per analysis# (Euro)**
Bioassays				
Microplate algae test (Raphidocelis sp./Scenedesmus sp.)	DIN 38412	72 hours	5	180
PAM algae test (Selenastrum sp.)	RIVM house-method	4.5 hours	3	250
Microtox®	NVN 6516	30 minutes	3	250
Rotox kit F	Supplier's instructions	24 hour	2.5	200
Thamnotox kit F	Supplier's instructions	24 hours	4	200
Daphnia IQ test	Supplier's instructions	1.25 hours	10	250
Genotoxicity tests				
Ames test	KIWA house method	72 hours	2	355
UMU test	DIN 38415	2.5 hours	3	155
VITOTOX®	VITO house method	4 hours	3	140
Comet test	VITO house method	2 hours	3	250
Effect-specific biological tests				
AchE inhibition	GWA house method	15-20 minutes	5-10	115
ER-Calux	LU Wageningen house method	24 hours	1 litre of surface water	340

Legend: # = estimated costs per analysis, excluding the concentration of surface water
estimated costs for concentrating samples for bioassays/effect-specific tests: Euro 4300 per sample
estimated costs for concentrating samples for genotoxicity tests: Euro 2700 per sample
@= volume based on 1000x concentrated samples

See section 3.1 for a more detailed description of the tests.

2.4. Concentrating the samples

In order to establish a toxicity trend and obtain an adequately comparable effect parameter (L(E)Cf50 value by means of a dilution procedure), the samples for the bioassays must be concentrated to a factor of 1000. For the Ames test, the water sample must be concentrated 25,000 times. To be able to compare the results of the other genotoxicity tests (UMU test, Comet test, VITOTOX®) with the Ames test to some extent, all genotoxicity tests are done on the same concentrate. Due to the low concentration of estrogenic substances in surface water (pg/l), it proved necessary to concentrate the samples for the ER-Calux test as well.

Concentration techniques have been used in chemical analyses in order to detect compounds for some time. Concentration techniques for biological tests must meet certain conditions, as they are used in tests with living (parts of) organisms. Table 3 gives an overview of the available concentration/isolation techniques and the type of substances concentrated/isolated by means of each technique, as well as the advantages and disadvantages of using the technology for biological tests.

Table 3: Concentration techniques available for biological tests.
(D. de Zwart, personal communication)

	Auxiliary substances	Types of substance	Advantages	Disadvantages
Isolation of contaminants by means of adsorption/elution: Solid phase extraction followed by elution by means of a solvent				
XAD 2/4/8#	Eluents: acetone, DMSO or ethanol/methanol	Organic compounds with varying polarity and hydrophobicity	Broad range, including: 'modern toxicants'; good 'model' for biologically active substances; possibility of exhaustive extraction; compound to large degree reversible; humous compounds are concentrated only to a very small extent (they are too polar)	Losses when toxic eluent is removed/replaced by water; costs of cleaning XAD
Polyurethane foam	Eluents: e.g. hexane	Hydrophobic organic compounds with low polarity		Batch differences in material; sometimes contamination in material, see XAD
Silicon dioxide (SiO_2)#	Eluents: acetone, ethanol/methanol	Hydrophilic organic compounds with high polarity	Quick	Unknown affinity with low loading degree; no exhaustive extraction possible, see XAD
Activated carbon#	Eluents: organic solvents	Unknown range of organic compounds	Forms of organic lipoid microcontamination are compounded very well	Recovery low through partially irreversible compound , see XAD; often many impurities are present
Chelex	Eluents: acids/salts	Metal ions	Selective for heavy metals	Salt burden in toxicity test
	Auxiliary substances	Types of substance	Advantages	Disadvantages
Isolation of contaminants by means of partition: Fixed phase extraction followed by elution by means of a solvent				
C18#	Eluents:	Hydrophobic organic	Reversible exhaustive	Losses when toxic eluent is

	hexane, cyclohexane	compounds	extraction possible	removed/replaced by water. Humous acids released with extraction
Isolation of contaminants by means of partition: Liquid/liquid extraction				
Hexane/water#	Solvent applied	Hydrophobic organic compounds	Exhaustive extraction possible	Losses when toxic eluent is removed/replaced by water.
Cyclohexane/water#	Solvent applied	Hydrophobic organic compounds	Exhaustive extraction possible	Losses when toxic eluent is removed/replaced by water.
MeCl/water#	Solvent applied	Fewer hydrophobic organic compounds	Exhaustive extraction possible	Losses when toxic eluent is removed/replaced by water.
Etc. , e.g. octanol#	Solvent applied	In general highly hydrophobic organic compounds	Exhaustive extraction possible	Losses when toxic eluent is removed/replaced by water.
Removal of water				
Freeze-drying#	None	All non-volatile compounds	Not very selective	
Freezing out	None	All compounds	Not very selective	
Reverse osmosis/hyperfiltration #	None	All compounds of minimum molecule size	Not very selective	
Dialysis: Concentration of substances by means of dialysis				
Dialysis membrane	Dialysing agents: water, hexane, dichloromethane, etc.	All compounds of minimum molecule size	Reversible exhaustive reaction possible	It takes very long for the balance to be restored. Losses when toxic eluent is removed/replaced by water.

= In the past, these techniques were tried out at the former RID; it turned out that the XAD technique was least effective in terms of DOC, but was most effective in terms of toxicity.

For the following reasons, the XAD 4/8 concentration technique was chosen for the genotoxicity tests, the bioassays and the AchE test:

- Currently the only technique by means of which a broad range of organic substances can be concentrated.
- A good 'model' for biologically active substances.
- RIVM and KIWA have years of experience of and data on this technique.
- The levels and presence of organic substances in surface water are very variable, as opposed to those of heavy metals and other salts.
- Organic substances may be the cause of the changed (geno)toxicity in water.

Due to the use of this concentration technique, it is only possible to make a statement on the toxicity of the **organic** compounds in surface water. No statement can be made as to the overall toxicity of the water sample (combination of organic compounds and metals).

For the ER-Calux, a separate, specific concentration technique was used (see § 3.2.2) in order to prevent contamination (xeno)estrogen properties, caused by compounds from plastics.

Table 4: The concentration factors used in the different types of biological tests.

Type of biological test	Concentration factor used	Acidity when reprocessed (pH)
Bioassays and AchE	1000	7
Genotoxicity tests	25,000	2 and 7
ER-Calux	10,000-100,000	7

3. Description of the methods used and the logistics

3.1. Logistics of sampling and sample processing

For the following reasons, Eijsden (Meuse) and Lobith (Rhine) were chosen as sampling locations:

- The availability of an extensive package of chemical-analytical data.
- The toxicity tests of RIVM/RIZA for 1998/1999 had already been planned for both locations.
- Further extension of the number of sampling points would be too expensive (the preparation of samples as extract accounts for most of the costs).
- For both locations, historic data on previous (geno)toxicity measurements is available.

For each sample, a volume of 100 litres of surface water was transported to the RIVM in Bilthoven, where concentration of the sample took place within 48 hours. Twenty-four hours before the extracts were sent to the laboratories carrying out the research, they were brought to the correct volume by means of the dilution medium required.
For the genotoxicity tests, 300 l surface water was concentrated on location or after transport to KIWA Nieuwegein, after which extracts in ethanol were sent to the laboratories.
The sample (1 litre) for the ER-Calux was transported to IVM for concentration on the sampling date. The extract was examined by the Agricultural University of Wageningen (LU Wageningen). A separate sampling procedure was used for this test, in order to prevent contamination with (xeno)estrogen compounds from the equipment.

Table 5: Sampling data of the biological testing programme for 1998-1999 with sample codes.

Date	Meuse sample code	Rhine sample code
July 1998	Eijs9804	Lob9804
September 1998	Eijs9805	Lob9805
November 1998	Eijs9806	Lob9806
February 1999	Eijs9901	Lob9901
April 1999	Eijs9902	Lob9902
June 1999	Eijs9903	Lob9903

3.2. Description of the concentration methods used

3.2.1. Concentration procedure for the bioassays

The XAD-4 (Rohm & Haas, Antwerp) and XAD-8 (DAX-8, Supleco) resins used were purified thoroughly before they were used for concentrating the samples (RIVM, 1998). By means of these resins, apolar or mildly polar components were isolated. Within 48 hours of sampling, the non-filtered sample was transferred to 10 l borosilicate bottles, to which a resin mixture (with an XAD4/XAD8 ration of 1:1) was added with a concentration of 2 ml mixture per litre water (see 1, figure 1). Mixing took place by rolling the bottles at 20 °C in the dark for 24 hours (2), after which the resin granules were sieved from the bottles (3). The granules were dried using an air current until the granules' weight had stabilised at < 6 grams per 20 ml (4). After drying, the different XAD batches of 1 sample were mixed and introduced into an elution column (5), after which elution took place on a bed of volume acetone (6). The 500* of concentrated concentrate was distributed among 20 ml vials, sealed off with crimp cramps and stored at –20 °C until further processing (7).

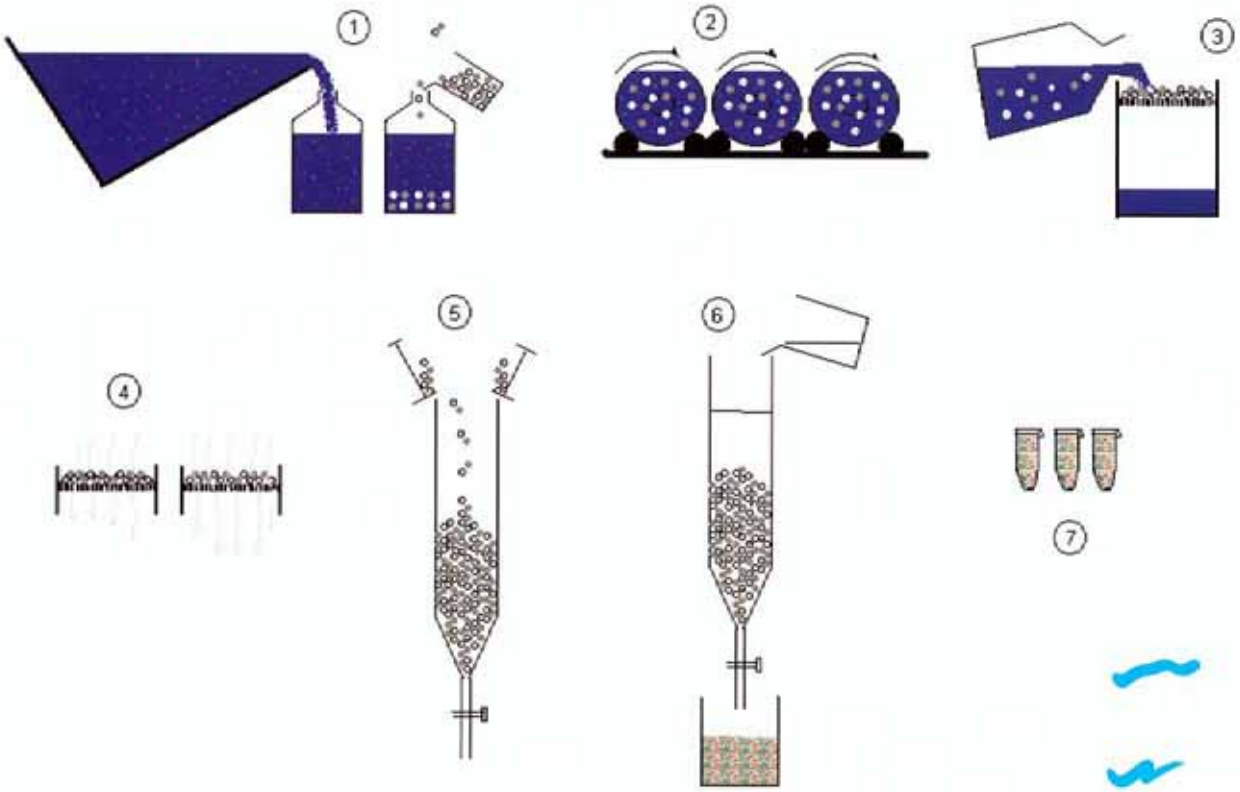

Figure 1: Sample preparation.

Before this concentrate was used for the test, the extract's acetone level was reduced to < 0.1 % (v/v) (see figure 2). For this purpose, the acetone concentrate from the 20 ml vial was transferred to a conic tube of a Kuderna-Dänish distillation device, to which 2 ml of mineral water (Spa Monopole BV, Spa, Belgium, 'Spa Reine') was added (8). The acetone was evaporated at 65 °C (9) until no further volume reduction took place and no boiling symptoms occurred. The residue was aerated with a nitrogen current, which was set exactly at the acetone level required of < 0.1 % (v/v) (10). Following this nitrogen aeration a dilution medium (the type of which depended on the test to be used) was added, until a total volume of 10 ml was reached. The water concentrate then had a concentration factor of 1000x (11). The water concentrate was stored at 5 °C until the test was carried out (within 24 hours) (12).

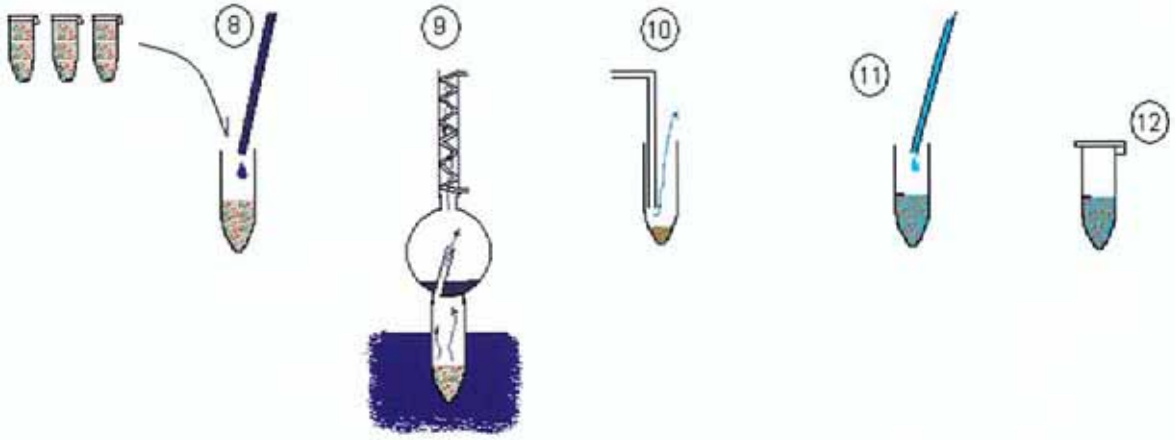

Figure 2: Preparation of the concentrate as a test solution.

Commercially available mineral water (Spa) was used as the procedure blank. The results of the toxicity tests on these procedure blanks are stated in the annexes (table B2).

3.2.2. *Concentration procedure for the genotoxicity tests*

In this procedure, developed by KIWA (Noordsij *et al.*, 1983, 1984), XAD-4 (Amberlite) was used in a column with a bed height of 20 cm and a bed volume 300 ml. Via a siphon system, the sample (300 litre) was conducted from a reservoir to the vertical resin column at a speed of 1 bed volume per minute (see 1, figure 3). After a neutral sample (pH=7) was obtained from the first column, this was brought in-line to pH=2 by means of hydrochloric acid, and conducted to another XAD column. Both loaded XAD columns were rinsed with 10 bed volumes of ultra pure water (in the acid column, the water was first brought to pH=2) (2). After drying the columns with nitrogen (3), elution took place with 5 bed volumes of pure ethanol and five bed volumes of azeotropic mixture with 30% pure ethanol in cyclohexane (4). In this elution, the eluate was filtered in a 0.45 µm PTFE filter, by means of which traces of bacteria were removed (5). The eluate of the two fractions was concentrated to 250 ml in the first phase (6), and then to 15-20 ml in the second distillation phase (7). In the second distillation phase a calibrated tube was used. Lastly, the extract volume was reduced to 12 ml by further evaporating the ethanol with nitrogen, leading to the ultimate concentration factor of 25,000x (8). The quantities of extract needed for the different genotoxicity tests were taken from this fraction (9).

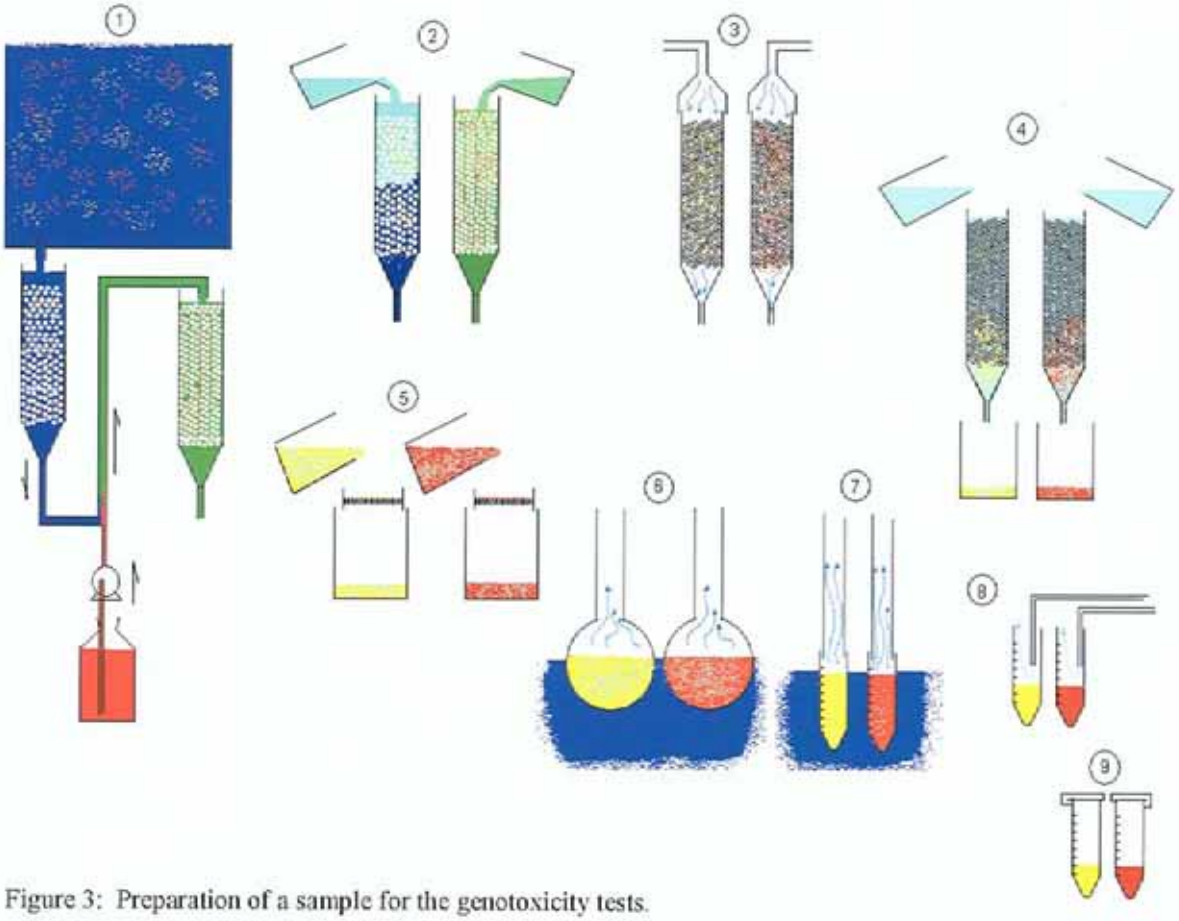

Figure 3: Preparation of a sample for the genotoxicity tests.

3.2.3. *Concentration procedure for the ER-Calux*

On arrival at the laboratory, the water was filtered through 1.2 µm and 0.45 µm glass filters (Whatman, GF/C filters), and extracted with an SDB-XC disc (a polystyrene benzene empore disc) (Murk *et al.*, 2000). The substances on the disc were eluted with 3 x 5ml methanol, after which the extract was condensed and absorbed in DMSO. For each (maximum amount of) 1.5 litre, 1 disc was used. All glass was rinsed with ethanol of HPLC quality beforehand to prevent background contamination.

3.3. Description of the bioassays used

Micro plate algae test

In this test, algae of the species *Raphidocelis subcapitata* and *Scenedesmus subspicatus* are added to a series of diluted extracts from the sample. During the 72 hours of incubation, the population growth is measured by determining the number of cells by means of the cytofluorescent meter. On the basis of the numbers obtained the ECf_{50} value was determined (see 3.6.1), whereby 50% growth inhibition occurred in comparison with the inspection (yield). A number of years ago, it was only possible to carry out this test with Erlenmeyers or other glass, which meant that a large amount of sample was required. The application of microtitre plates has made it possible to use small quantities of sample as well. However, it should be borne in mind that organic pollutants from the sample may be adsorbed onto the wall of the microtitre plates, causing erroneous results.

PAM algae test

Algae of the *Raphidocelis subcapitata* species are added to a series of diluted extracts, followed by an incubation period of 4.5 hours under continuous lighting at 20 °C. After this incubation period, the photochemical efficiency, or the photon yield, is determined with the Pulse-Amplitude-Modulation fluorescent meter (Genty, 1998; Hofstraat, 1994). The photochemical efficiency is expressed as a percentage of the inspection values. The ECf_{50} value is determined as the concentration factor, whereby 50% reduction of the photochemical efficiency occurs.

Microtox®

In this test, freeze-dried bacteria of the *Vibrio fisheri* strain are used, and applied to a series of diluted extracts after 1.5 hours of reconstitution (i.e. making viable again after freeze-drying). This strain of bacteria emits light when its metabolism is normal. After 5 to 15 minutes of incubation at 15 °C, the luminescence level is determined with a luminosity meter (Bulich, 1979; Bulich & Isenberg, 1981). When the bacteria are in a toxicologic stress situation, a reduction in light emission is established. For the ECf_{50} value, the lowest concentration factor is taken, whereby light emission has been reduced by 50% in comparison with the blank. The ECf_{50} values and confidence limits were obtained via a dose effect curve from a logistics response model (Haanstra *et al.*, 1985).

Rotox kit F

Rotifers of the *Brachionus calyciflorus* species are used as test organisms. These are obtained when the cysts from this kit (Rotoxkit F, Janssen *et al.*, 1993; Snell *et al.*, 1989, 1991) are placed in EEP medium with lighting for 16 to 18 hours. Within 2 hours, the rotifers are added to a dilution series of the extract in polystyrene multititre plates. After a 24-hour incubation period in the dark, the survivors are counted by means of a microscope.

The LCf_{50} value is determined by means of the Spearmann-Kärber method (Hamilton *et al.*, 1977). Again, it should be borne in mind that organic pollutants may be adsorbed on the wall of these microtitre plates.

Thamnotox kit F

Crustaceans of the *Thamnocephalus platyurus* species are used as test organisms. These are obtained when the cysts from this kit (Thamnotox kit F, Centeno *et al.*, 1993) are placed in a medium with lighting for 24 hours. These organisms are acclimatised to the dilution medium for 4 hours, after which the extract is added according to a specific dilution plan. The tests are carried out in glass vials that can be sealed off. After 24 hours of incubation in the dark, the number of living organisms is counted. Again the LCf_{50} value is determined.

Daphnia IQ test

Non-fed young *Daphnia magna's* (of less than 24 hours) are exposed to a dilution series from the water extract. After 1 hour of incubation a tracer compound is applied (4-methyl- umbelliferyl-ß-D-galactose) (Daphnia IQ, Aqua Survey Inc., 1993). After 15 minutes of incubation, the fluorescence of each *Daphnia* is measured by means of UV light. The toxicity is determined on the basis of the inhibition of the enzymatic division of the galactose from the tracer. The less light is measured, the more toxic the sample will be. The ECf_{50} value is determined on the basis of the data obtained.

3.4. Description of the genotoxicity tests used

Ames test

The Ames genotoxicity test is carried out with a mutated strain of *Salmonella typhimurium* bacteria. These mutants do not grow on a histidine-free medium. Only individuals whose histidine gene has been recovered by means of reverted mutations due to the presence of amutagenic substance are able to form colonies on this medium (revertants). After a 3-day incubation period, these revertants can be counted as colonies. A sample is considered to be mutagenic when the number of revertants counted per plate is at least twice the number of spontaneous revertants, and when there is a dose-effect relationship. Different strains of mutated *Salmonella typhimurium* can be used (e.g. TA98, TA100, TA1535 or TA1538). Some will detect base pair (smallest element of DNA) substitutes, while others will detect the removal or addition of a base pair. In this project the TA98 strain was used, by means of which frameshift mutations can be registered.

UMU test

In the UMU genotoxicity test (Oda *et al.*, 1985, Reifferscheid *et al.*, 1991; Reifferscheid & Heil, 1996) a modified strain of *Salmonella typhimurium* TA1535/pSK1002 bacteria is used, whereby an enzyme gene (ß-galactosidase) is linked to the SOS-DNA recovery system. In the case of DNA damage, the SOS-DNA system is induced, whereby production of the enzyme also takes place. The more DNA damage occurs, the more ß-galactosidase is produced. After an incubation period of 1 ½ hours, the amount of enzyme produced is determined by means of the application of the 0-nitrophenol galactopyranoside substrate (ß-galactosidase produces a yellow colour, which can be quantified spectrophotometrically). In order to establish the spontaneous mutations, the extinction measured is corrected on the basis of the measurements of the blank. The amount of enzyme product measured is a measure of the sample's mutagenicity, while taking into account the test strain's growth speed. If this is too low, the sample is toxic rather than mutagenic.

VITOTOX®

In the VITOTOX® genotoxicity tests (Van derLelie *et al.*, 1997) a modified *Salmonella typhimurium* bacterium is used, which has linked a lux gene to the SOS-DNA recovery system. Again, the SOS-DNA recovery system is induced when DNA damage occurs; in this case, however, light is produced. This can be measured directly and continuously, whereby the amount of light produced is a measure of the sample's mutagenicity. By using another test strain, it is checked whether the sample is toxic rather than mutagenic. This strain produces light under normal conditions. When this strain produces no light or little light when a certain dilution of the sample is used, and the VITOTOX® still has not produced any light, the sample is toxic rather than genotoxic.

Comet test

The comet test (Tice, 1995), a very recent technique, measures very different genetic ends. The alkaline comet test used in this study detects both single and double strand breaks and alkali labile sites. Alkali labile sites in DNA are 'vulnerable' parts of DNA, which lead to breaks in the DNA under alkaline circumstances. They are not present as such, and will probably result in an abnormality.
In the comet test, cells are lysed in gel on a microscope slide (VITO uses lymphocytes from human blood after 2 hours of exposure to the sample; RIZA uses living *Daphniae* after 48 hours of exposure), in order to release and denature DNA, and to subject this to a gel-electrophoresis. Under the influence of the electrical field created, the DNA will undergo a certain migration, whereby small DNA fragments migrate further than larger fragments or intact DNA. A 'comet'-shaped figure will occur, whereby the length and content of the comet tail provide a measure for the DNA damage. The 'comets' can be analysed after coloration with a fluorochrome (e.g. ethidium bromide) by means of a fluorescence microscope.

3.4.1. Description of the genotoxicity tests used in the KIWA study

In the context of the joint study by the water companies, an investigation was started at KIWA in 1998, which involved the comparison of a number of biological tests, including a number of genotoxicity tests. As this study partly overlapped with the RIWA study, it was decided to join forces; KIWA joined the research programme of the RIWA Working Group on Biological Tests.

The KIWA study had two objectives:

- to investigate which tests were to be carried out in addition to the Ames test in order to verify the results of the Ames test (additional genotoxicity tests);
- to investigate which newly developed tests might be able to replace the Ames test (alternative genotoxicity tests).

Additional genotoxicity tests

For the detection of genotoxic compounds in (drinking-)water, the Ames test with *Salmonella typhimurium* is usually carried out. However, the Health Council (1995) states that no genotoxocity test may be considered reliable when used on its own. As the Ames test produces a percentage of false negative and false positive results in the prediction of carcinogenic effects that cannot be neglected, the Health Council states that, in addition to Ames tests, tests with eukaryotic cells must be carried out for the detection of gene mutations and chromosome abnormalities.

On the basis of the advice of the Health Council, it was proposed to carry out an *in vitro* gene mutation test or an *in vitro* cytogenetic test in addition to the Ames test, depending on the result of the Ames test.

1. If the results of the Ames test are positive, they should be confirmed in a second *in vitro* gene mutation test. In this project, the TK test was done with L5178Y mouse lymphoma cells.
2. If the results of the Ames test are negative, an *in vitro* chromosome aberration test should be carried out to check whether the substance causes chromosome aberration. In this case, the chromosome aberration test was carried out with human lymphocytes.

Alternative genotoxicity tests

The execution of the Ames test involves the concentration of water samples. This may lead to the introduction of artefacts, and it cannot be ruled out entirely that relevant compounds may be lost in the process. Preference should therefore be given to a genotoxicity test which does not involve concentration. Over the past few years, several tests have been developed which claim that the water used does not need to be concentrated and/or that they provide more information, or that they are more sensitive, faster or simpler than the Ames test. These are the UMU test, the VITOTOX® test, the SOS Chromotest, the Mutatox test and the MutaChromoPlate test. It is not known with regard to all these tests what exactly their advantages are in comparison with the Ames test; it was therefore recommended that these tests should be further investigated.

The VITOTOX® test and the UMU test were used in the research programme of the Project Group on Biological Tests. In addition to this, KIWA carried out an *in vitro* gene mutation test (TK test), an *in vitro* chromosome aberration test, the SOS Chromotest, the Mutatox test and the MutaChromoPlate test.

Four samples from the Rhine were investigated (July 1998, September 1998, November 1998 and March 1999). In each case, this was the pH 7 fraction of the samples.
The additional genotoxicity tests and the SOS Chromotest are an exception to this. The additional genotoxicity tests were carried out as a pilot experiment, and were therefore done on two samples. In the SOS Chromotest, both the pH 2 and the pH 7 fractions of samples from the Rhine and the Meuse were tested (July 1998, September 1998, November 1998 and March 1999).

Gene mutation test with lymphoma cells from mice (OECD guideline 476)

Cells deficient in thymidine kinase (TK) are resistant to the cytotoxic effects of trifluorothymidine (TFT). TFT inhibits the cell metabolism and inhibits further cell division. In this test, a known number of cells are exposed to the sample to be investigated, both with and without metabolic activation (S9 mix). They are then transferred to a medium with TFT. Mutated cells will be able to grow in the presence of TFT, while normal, non-mutated cells (which contain thymidine kinase) are unable to do so. Cytotoxicity is established by establishing the survival of cultures after the treatment.

Chromosome aberration with human lymphocytes (OECD guideline 473)

By means of the *in vitro* chromosome aberration test, substances are identified that cause structural chromosome damage in cultivated mammal cells. Cell cultures are exposed to the substance with and without metabolic activation (S9 mix).

At fixed intervals after exposure to the test substance, the cell cultures are treated with a substance that inhibits metaphase. Following this, the cells are gathered, coloured and analysed for the presence of chromosome aberrations.

SOS Chromotest

The SOS Chromotest was developed by Quillardet *et al.* (1982) as an alternative to the Ames test. The SOS Chromotest measures the expression of certain genes that are induced by genotoxic substances. Bacteria such as *Escherichia coli* have a very sensitive enzyme system for the detection of DNA damage, the SOS system. In the SOS Chromotest, the*E. coli* strain has been modified; instead of the SOS genes, the gene for the ß-galactosidasis has been added behind the SOS promoter. In this way, activation of the SOS repair system by genotoxic substances can be measured by means of a photometric identification of the product formed from starch.

MutaChromoPlate test

The MutaChromoPlate test is an adjusted version of the Ames test, which is used for the evaluation of mutagenicity and the mutagenic potency of samples from the environment and chemicals. The test makes use of one (or more) mutated strain(s) of *Salmonella typhimurium* that contain(s) mutations in the operon which encodes for the biosynthesis of histidine. The Ames test has always been carried out on agar plates. An alternative test, which is carried out entirely in liquid medium, is the 'Ames fluctuation test' (Le Curieux *et al.*, 1996), which is based on several yes/no-coloured ends. This test principle is also applied in the MutaChromoPlate test; again a colour reaction is the end, with – in principle – a non-mutagenic sample colouring purple and a mutagenic sample colouring yellow.

The advantage of the MutaChromoPlate kit, as stated by the distributor, is that it is more sensitive to mutagenic compounds. This is based mainly on the fact that a relatively large volume of water can be tested in each well.

The MutaChromoPlate test was carried out according to the procedure as shown by the producer. *Salmonella typhimurium* of a TA98 strain was used instead of the TA100 strain supplied to make it easier to compare the test with the Ames test.

Mutatox

The Mutatox™ test is a relatively new test (Microbics Corporation, USA). This test makes use of a special dark variant of the luminescent bacteria *Vibrio fischeri* (M169). Like the SOS Chromotest, this test detects DNA damage.

The bacterium shows an increased luminescence level when it grows in the presence of sublethal concentrations of mutagenic compounds. The luminescence level shows the sample's relative genotoxicity. The test is carried out both in the absence and in the presence of S9 mix.

3.5. Description of the effect-specific tests used

AchE inhibition

This test measures the inhibition of the activity of the enzyme cholinesterase. The contribution of each inhibiting compound in the sample to the total inhibition depends on both the concentration and the inhibiting capacity of that substance. The extracts and different concentrations of ethyl parathion (standards) are treated with bromide in order to obtain the 'active' form of various substances (bromide converts ethyl parathion into paraoxon). The excess of bromide is then compounded by the protein albumin. The enzyme acetylcholine esterase is added at 37 °C to the extracts and standards treated, and after a specific period of time the substrate butyryl thiocholine iodide is added. The non-inhibited enzyme converts this substrate into thiocholine. This substance is dialysed, after which a yellow compound is formed with the chromogen (2.2-dinitro-5.5-dithio-dibenzoyl acid), which can be measured spectrophotometrically at 420 nm. The quantity of inhibiting substances in the extract is expressed as the quantity of paraoxon equivalents (µg/l).

ER-Calux

The ER-Calux assay is based on the effect-mechanism of estrogenic substances (see figure 4).

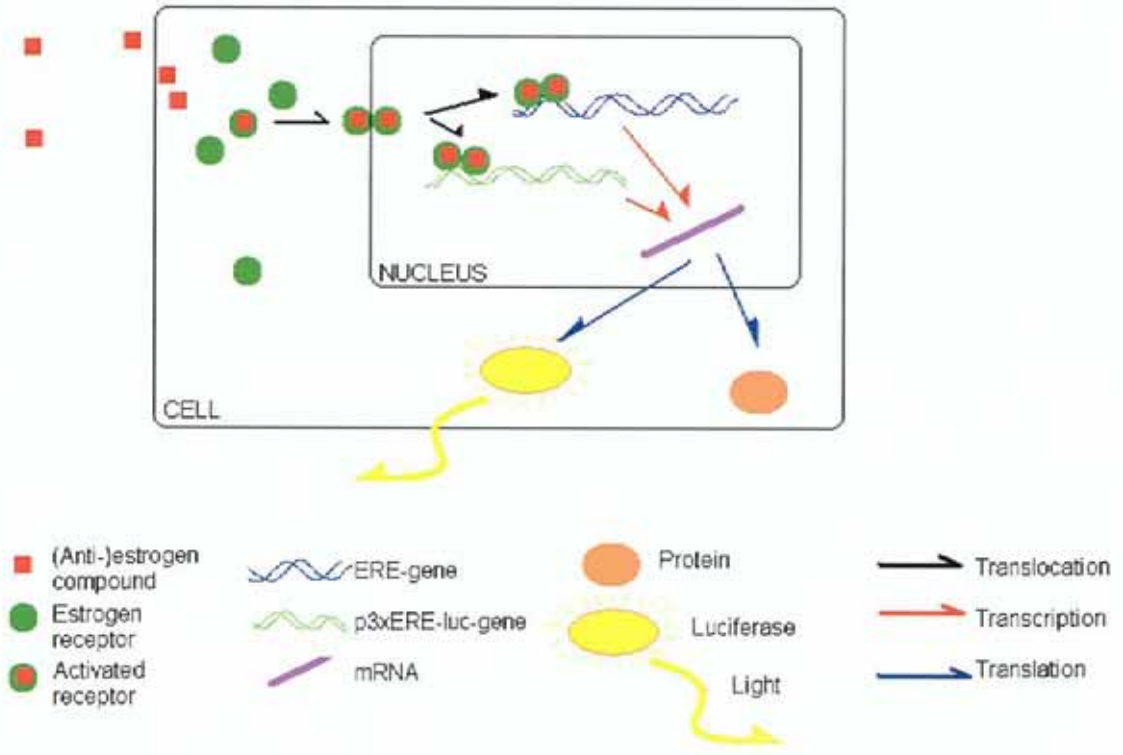

Figure 4. Diagram of ER-receptor related effects and luciferase expression.

When (pseudo)estrogen substances from a concentrate of the surface water sample enter a cell, these can bind to the estrogen receptor (ER) in the cell's protoplasm. The receptor is then activated, leading to a

complex of 2 joined loaded receptors. This complex migrates to the nucleus, where it binds to a specific DNA part, the ERE ('estrogen responsive element') gene. After this the reading of genes is stimulated, as a result of which ultimately the entire reaction of an organism on (pseudo)estrogen compounds from the concentrate is directed. In the ER-Calux assay, the entire process from binding to the receptor to activation of the genes is measured in a human breast cancer cell line. Binding and activation of the ER linked to a Lux gene results in the dose-related formation of luciferase. This luciferase can be measured easily with a luminosity meter.

The response is a resultant of both estrogenic and anti-estrogenic activity, i.e. the concentrate may contain estrogenic as well as anti-estrogenic substances, which will suppress the response. The ultimate response of the ER-Calux assay is expressed in estradiol equivalents (EEQ) in µg/l. Background information on the ER-Calux assay and its properties in comparison with other assays for estrogenicity can be found in Legler *et al.* (1999).

3.6. Calculation methods used/result-processing

3.6.1. Evaluation of bioassays

In bioassays, data is collected in the form of the number of surviving or dead organisms in each exposure concentration (Rand, 1995). This data yields a characteristic, S-shaped (sigmoid) curve (figure 5A). Each point in the curve indicates the average cumulative response for a specific concentration, whereby each average has an associated variation as a result of the different reactions of individual organisms. The least variability in the curve (figure 5B) is at the 50% level of the response. The concentration, whereby 50% of the organisms respond after a specifically defined exposure time (e.g. 24 or 48 hours), is therefore used as a measure of a sample's activity or toxicity.

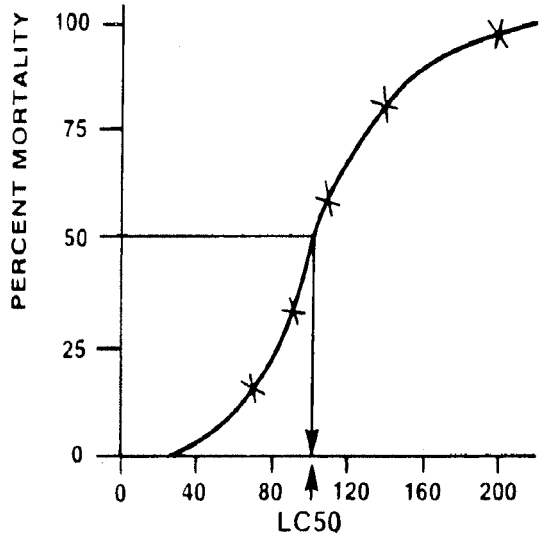

Figure 5A: Dose-response curve
(From: Rand, 1995)

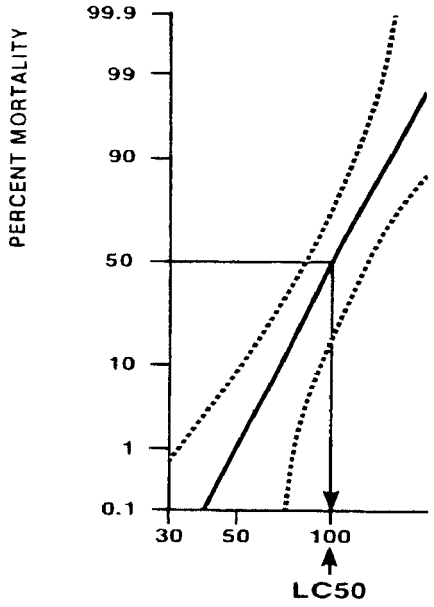

Figure 5B: Same data as 5A
The dotted line indicates the 95% confidence limits.
(From: Rand, 1995).

The LCf_{50} value is the concentration factor, whereby 50% of the test population is no longer alive after a specific period of time. The ECf_{50} value is the concentration factor, whereby 50% of the test population shows a specific effect after a specific period (immobility, abnormal development of organisms or abnormal behaviour. The lower the ECf_{50} or LCf_{50} values are, the more toxic the sample will be. In this project, the ECf_{50} or LCf_{50} values were usually calculated by means of the Spearmann-Kärber method (Hamilton *et al.*, 1977) or the GraphPad package.

3.6.2. *Evaluation of genotixicity tests*

In tests where the desired end parameter (effect) to be measured is influenced by another, undesired effect, it is often not possible to accurately calculate an ECf_{50} value. In the UMU test, for instance, an additional toxic effect will be measured if the concentration factor becomes too high. In order to nevertheless make a judgement with regard to the concentration factor at which, for instance, a genotoxic effect could only just be identified, the LOECf (Lowest Observed Effect Concentration factor) is used (see figure 6 as an example).

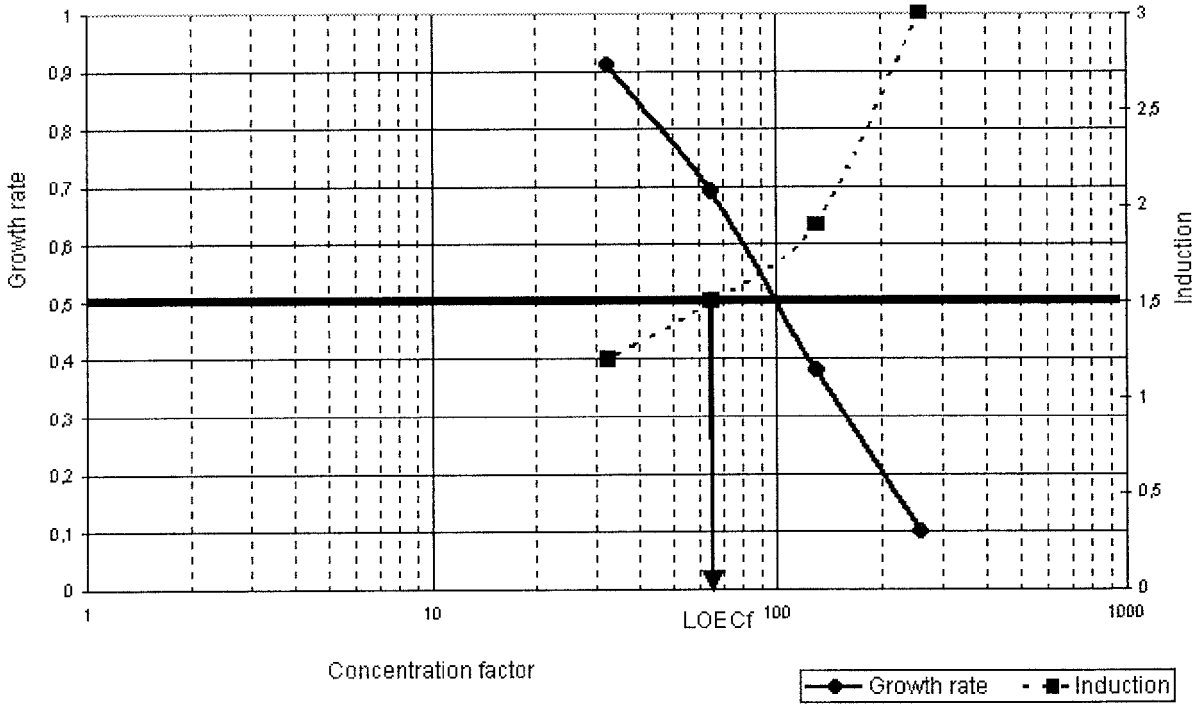

Figure 6: In the UMU test, the LOECf is determined when the induction requirement imposed is higher than or equals the factor 1.5, and the growth speed is higher than or equals 0.5.

3.6.3. *Establishing the ecotoxicologic risk*

It is not directly possible to estimate the effects of chronic exposure on the basis of the bioassays used, which only measure acute effects. By comparing the measuring results of acute and chronic toxicity tests carried out on the same complex effluents (US-EPA, 1991), it has nevertheless been demonstrated that the difference in sensitivity is considerably less than a factor of 10.

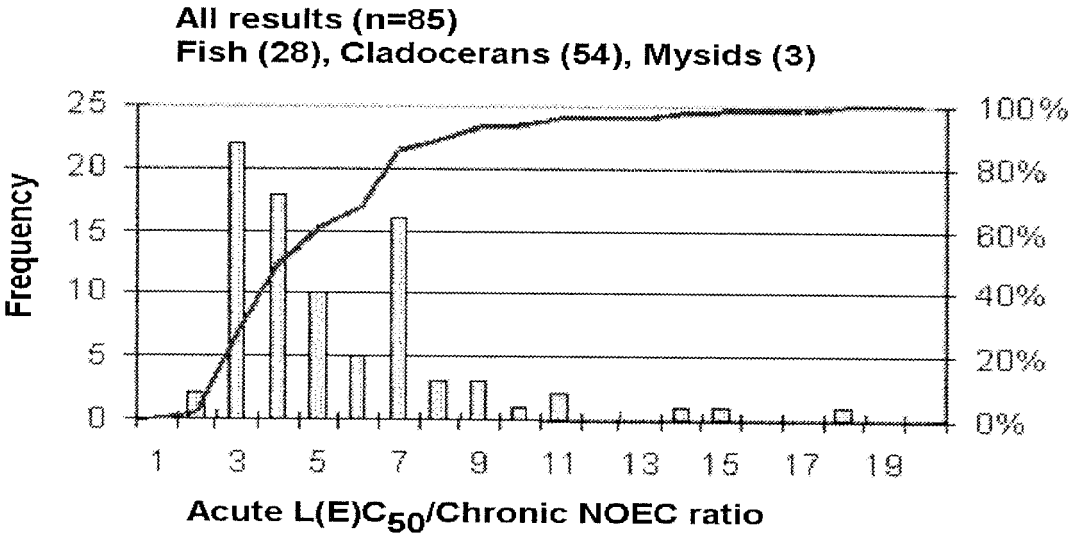

Figure 7: Acute / chronic toxicity ratio of complex mixtures of chemicals in industrial and domestic wastewater (US-EPA, 1991).

By means of an extensive analysis of internationally available toxicity data it has been established that individual contaminants of the average acute $L(E)C_{50}$ (median lethal or effect concentration) are approximately a factor of 10 higher than the average chronic NOEC (no observed effect concentration) (De Zwart, In press).

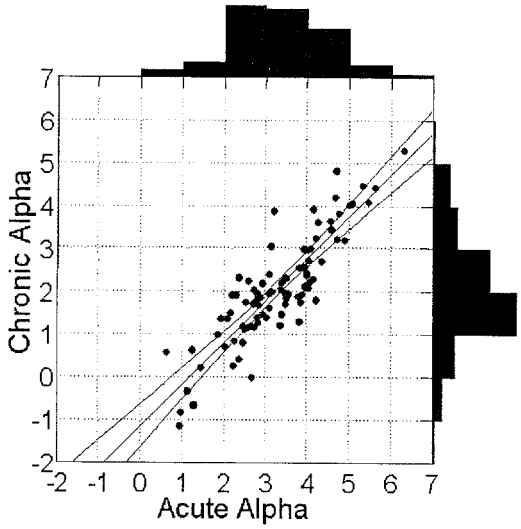

Figure 8: Regression of chronic and acute alpha values of individual contaminants (De Zwart, in press). Alpha is defined as the average ^{10}log transformed toxicity value of all types.

On the basis of the above findings, it was assumed on arbitrary grounds that a chronic NECf value (no effect concentration factor) can be estimated by dividing the ECf_{50} or LCf_{50} values measured by a factor of 10. According to De Zwart and Sterkenburg (in press), theecotoxicologic risk can be estimated by fitting a generic species collection (SSD: Species Sensitivity Distribution) log-logistically to the NECf values of the species measured. The log-logistic sensitivity distribution is characterised by only two values:

- The α (alpha), i.e. the average of the ^{10}log-transformed NECf values
- The ß (beta), i.e. the angle of inclination of the curve fitted. Beta is proportional to the standard deviation of the ^{10}log-transformed NECf values (ß $=\sqrt{3}/\pi$ * standard deviation)

The SSD fitted as shown in figure 9 can be calculated with the following formula:

$$F_x = \frac{1}{1 - e^{-\frac{^{10}\log C_f - \alpha}{\beta}}}$$

in which C_f stands for the concentration factor of the original surface water sample.
The ecotoxicologic of the organic contaminants present in the original surface water sample is estimated by filling in 1 for the C_f factor. The ecotoxicologic risk calculated (pT= Toxic potency expressed in PAF units) stands for the fraction of the aquatic species that is potentially exposed above the NEC of the cocktail of organic contaminants present (PAF=Potentially Affected Fraction).

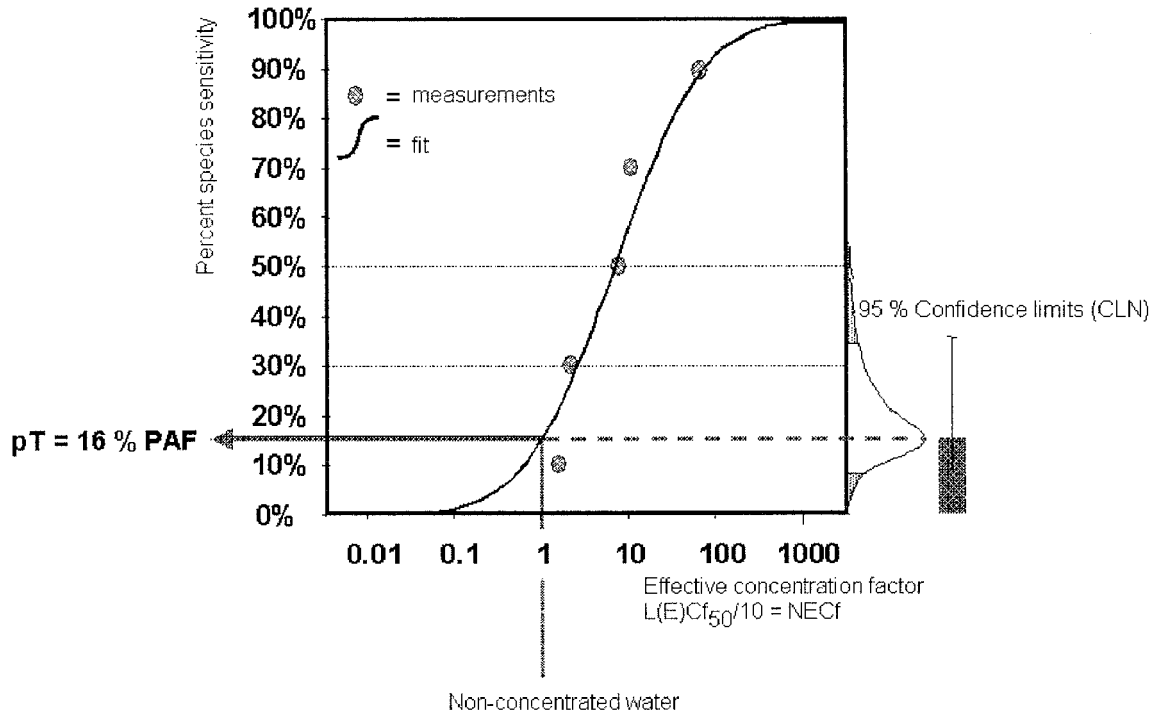

Figure 9: Determination of the pT value on the basis of data from the bioassays (From: De Zwart & Sterkenburg, in press).

The confidence limits for the pT values are obtained by means of a complicated set of statistical formulae based on the 'non-central t distribution' (De Zwart & Sterkenburg, in press). For the sake of readability, this is not shown here.

pT values of different sample points and/or different measuring periods can be aggregated to a combined pT value by calculating the geometric means of the individual pT values.

In addition to the ecotoxicologic risk derived by means of bioassays, the ecotoxicologic risk is calculated on the basis of the concentrations of organic contaminants measured. Calculation of the ecotoxicologic risk on the basis of measured concentrations is based on the same principles as the above SSD evaluation, on the understanding that the alpha and beta values are obtained from laboratory toxicity tests carried out with pure substances. Calculation of the ecotoxicologic risk is limited to substances for which one or more measurements were above the detection limit. Within this group of substances, concentrations below the detection limit were replaced by a quarter of the detection limit. By means of a pivot table in EXCEL, the ^{10}log-transformed concentrations were averaged on the basis of sample location and organic substance. For each component, the average (acute alpha) of the log-transformed $L(E)C_{50}$ values of a multitude of different test organisms were looked up in a table (De Zwart, in press). The relevant beta values, which indicate the distribution of sensitivity among species, were also looked up in this table. The table was prepared on the basis of a large number of internationally available toxicity data. On the basis of the acute alpha the chronic alpha was calculated (=acute alpha-1).
By means of the chronic alpha the concentration values measured could be converted into Toxic Units (TU):

$$TU_{\text{substance x}} = \frac{\text{Concentration}_{\text{substance x}}}{10^{\alpha\ \text{substance x}}}$$

The Toxic Units of different substances with the same effect mechanism (TMoA=Toxic Mode of Action) were added up (ΣTU_{TMoA}). Substances with a similar TMoA turned out to have a more or less equal beta value. With regard to each type of effect, 1-PAF was determined by:

$$1\text{-}PAF_{TMoA} = 1 - \frac{1}{1 + e^{-\frac{^{10}\log \Sigma TU_{TMoA}}{\text{av. } \beta}}}$$

The 1-Combi-PAF was calculated by multiplying the 1-PAF values for different effect mechanisms. On the basis of this, the Combi-PAF was calculated for each sample location.

4. Results and analyses

A detailed overview of all results obtained with genotoxicity tests, bioassays and effect-specific tests is given in the annex (tables B1-B3). In this chapter the test results are compared with each other.

4.1. Bioassays

The results of the bioassays were divided into three concentration factor areas: $1<=ECf_{50}<10$; $10<=ECf_{50}<100$ and $100<=ECf_{50}<1000$ (table 6). The number of positive responses per test were determined for each concentration factor area, on the basis of which it can be demonstrated which tests are most sensitive.

Table 6: Results of the toxicity tests for each concentration factor area

Toxicity test	Abbreviation used	Number of results $1<=ECf_{50}<10$	Number of results $10<=ECf_{50}<100$	Number of results $100<=ECf_{50}<1000$
Daphnia IQ	DIQ	0	7	5
Thamnotox kit	ThTox	0	8	4
Rotox kit	RoTox	0	0	12
Scenedesmus sp. MTP	ScenALG	0	11	1
Raphidocelis sp. MTP	RaphALG	0	12	0
PAM algae test	PAMALG	1	9	2
Microtox®	MTX	1	7	4

Only the PAM algae test and the Microtox® test appear to have responded in the least concentrated sample. Most of the tests gave positive reactions in the $10<=ECf_{50}<100$ concentration factor area. The Rotox kit was the only test which responded only to samples concentrated by more than a factor 100, and was therefore probably the least sensitive test of the battery.

The relative sensitivity of the bioassays was determined by converting the 12 measurements per test logarithmically, and by determining the median of this (table 7). In addition, the mean variation coefficient of the bioassays was determined for each measurement by means of the 95% confidence limits. The figures given between brackets indicate the number of measurements on the basis of which the mean variation coefficient was calculated. Only $L(E)Cf_{50}$ measurements, which yielded a confidence limit and whose variation coefficient in the measurement was less than 50%, were used.

Table 7: Determination of the relative sensitivity of the bioassays.

	Consumers			Producers			Decomposers
	DIQ	ThTOX	RoTOX	RaphALG	ScenALG	PAMALG	MTX
Eijs9804	1.260	1.958	2.419	1.719	1.754	1.326	1.776
Eijs9805	1.747	1.986	2.040	1.225	1.241	1.167	1.799
Eijs9806	1.934	1.954	2.332	1.711	1.928	1.913	0.907
Eijs9901	2.259	2.034	2.195	1.981	1.981	2.339	1.638
Eijs9902	1.685	1.939	2.406	1.815	1.929	1.737	2.180
Eijs9903	1.653	1.977	2.235	1.225	1.223	0.994	1.805
Lob9804	2.326	2.252	2.670	1.759	1.832	1.696	2.015
Lob9805	1.934	1.863	2.107	1.517	1.753	1.542	1.673
Lob9806	2.143	1.898	2.334	1.783	1.782	1.714	1.678
Lob9901	1.940	1.992	2.460	1.989	2.085	2.352	1.776
Lob9902	2.061	2.033	2.414	1.876	1.927	1.843	2.648
Lob9903	2.478	2.319	2.779	1.732	1.940	1.875	2.165
Average	1.952	2.017	2.366	1.694	1.781	1.708	1.838
Median	1.937	1.982	2.370	1.746	1.880	1.725	1.787
Variation coefficient	14% (10)	8.6% (12)	6.1% (11)	24% (9)	22% (6)	4.3% (12)	11% (11)
Order of sensitivity	1	2	3	2	3	1	1
Meuse median	1.716	1.968	2.284	1.715	1.841	1.531	1.787
Order of sensitivity	1	2	3	2	3	1	1
Rhine median	2.102	2.013	2.437	1.771	1.880	1.778	1.896
Order of sensitivity	2	1	3	1	3	2	1

At trophic consumer level it appeared, on the basis of the median of all measurements (Meuse and Rhine), that *Daphnia* IQ responded most sensitive. When all Rhine measurements were used to determine the relative sensitivity, the Thamnotox was more sensitive than the *Daphnia* IQ. However, there was only a very minor difference between the two tests. Among the producers, the PAM algae test appeared to be the most sensitive test.

For each trophic level, the measurements and the medians were printed in box whisker plots (figures 11, 12 and 13). On the basis of those plots, the concentration range in which the different bioassays demonstrate positive reactions at the different sample points could be established. It could also be established whether measurements were obtained that show a clear difference. These were marked with a star or a circle (for an explanation of the box plot, see figure 10). A low log LCf_{50} or ECf_{50} value indicates that the sample was toxic. The sample required less diluting in order to observe a toxic effect.

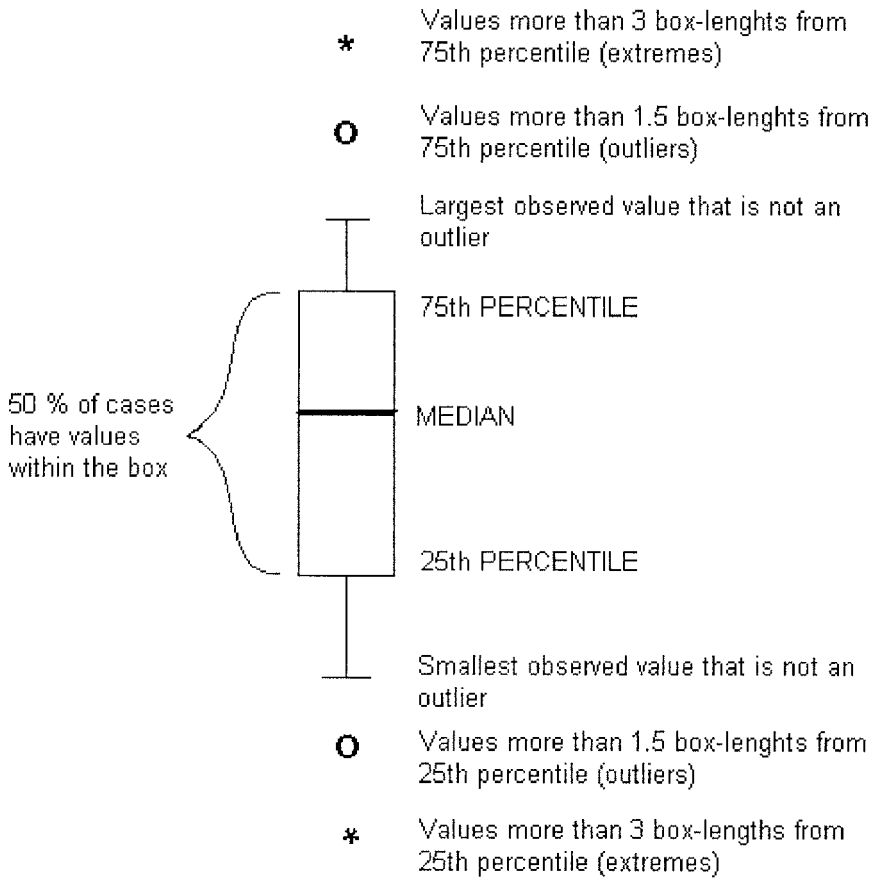

Figure 10: Explanation of the features of box plots.

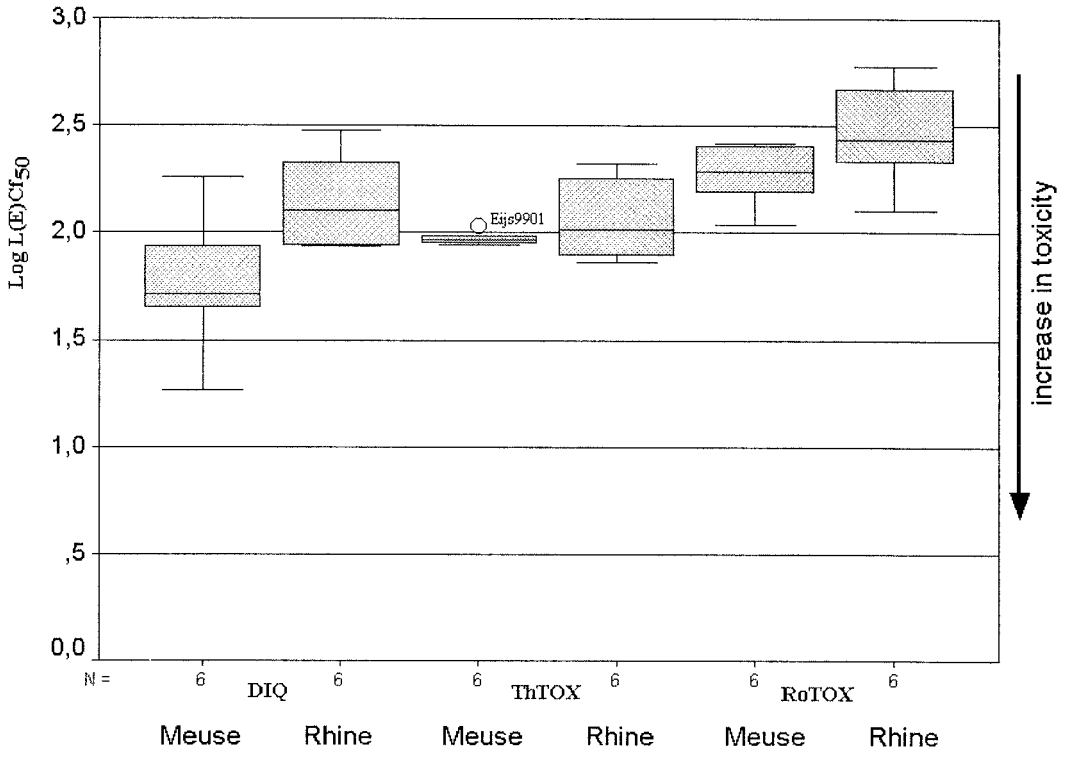

Figure 11: Box plot of the data provided by the bioassays with invertebrate organisms for the two rivers separately .

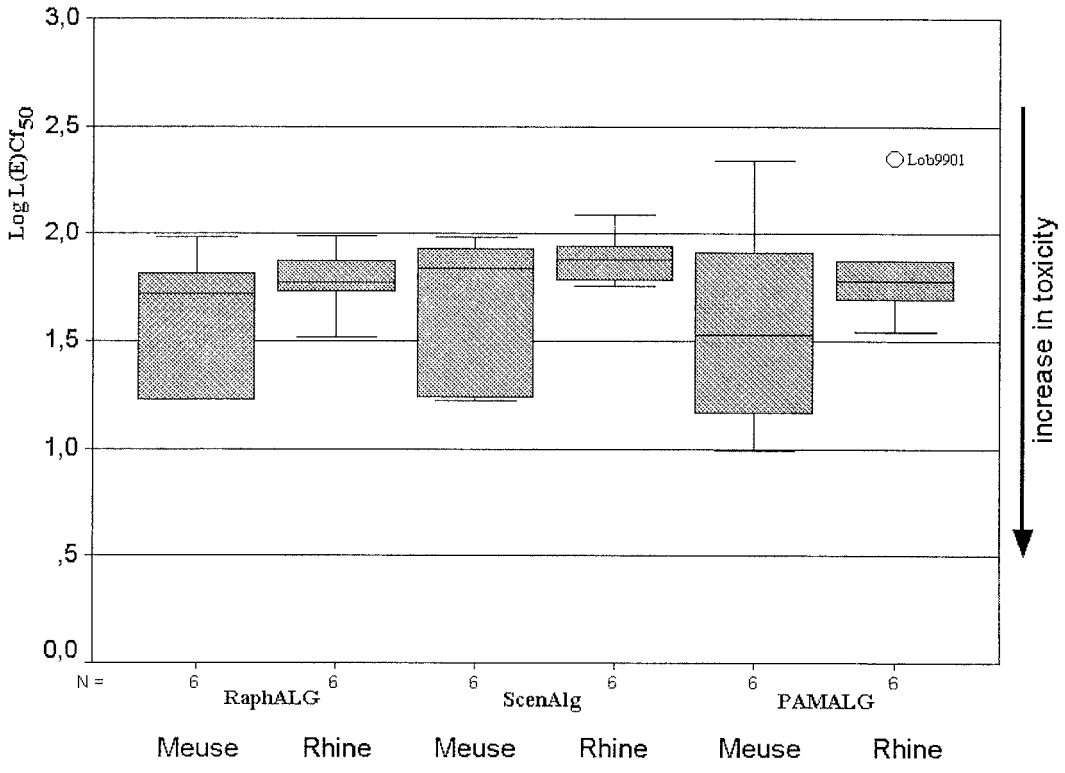

Figure 12: Box plot of the data provided by the bioassays with algae for the Meuse and the Rhine separately.

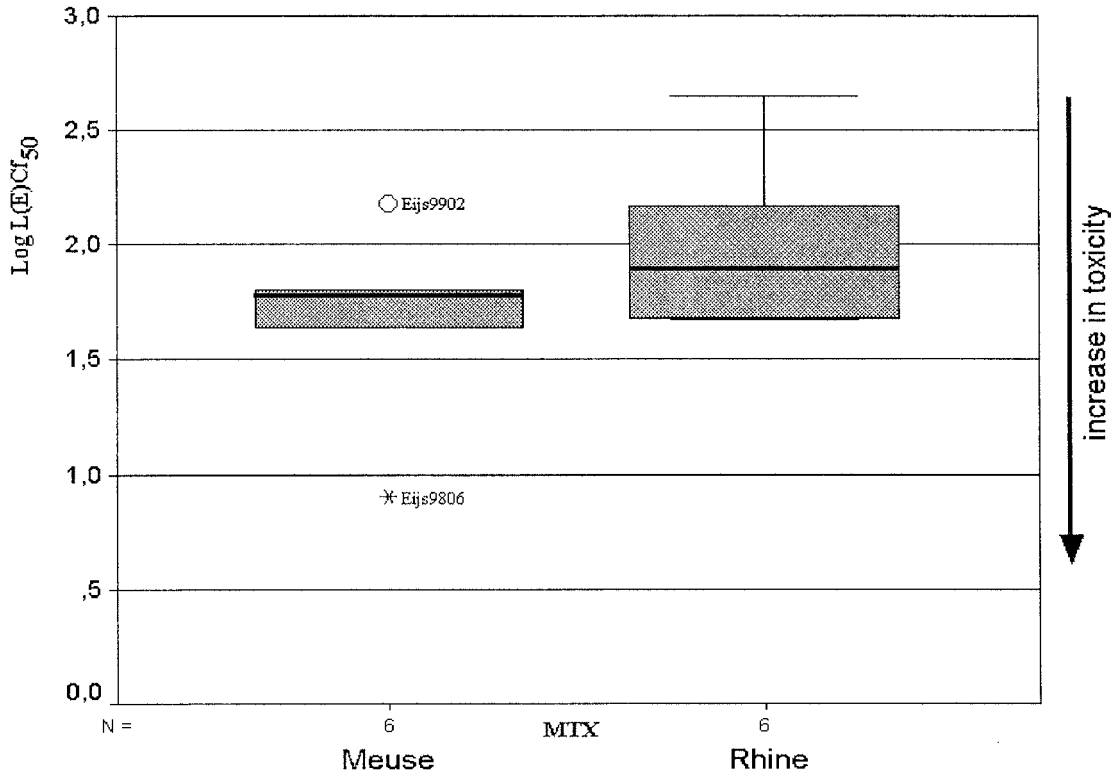

Figure 13: Box plot of the data provided by the Microtox®

As also appears from table 6, most of the results are in the area of concentration factor $10<L(E)Cf_{50}<100$ (log $L(E)Cf_{50}$=1-2). In one measurement, Eijs9806, the Microtox® is clearly below the area of the other observations. This shows that a significant change in the water quality took place that day (increased toxicity of the sample!). Moreover, the whisker plots show that some tests are in a fairly narrow concentration factor band, while others show more variation. The algae measurements of the Meuse in particular clearly show more variation than those of the Rhine.

In order to determine the extent to which tests are used that give equivalent or possibly overlapping information, a correlation analysis between the different biological tests was carried out (table 8), using log-transformed and centred toxicity data from table B2. The bold figures show the combination of tests that clearly have a positive correlation, such as, for instance, the Rotox kit and the Thamnotox kit. The figures in italics show a significant negative correlation between the bioassays, such as, for instance, the PAM algae test against the Thamnotox kit and the Rotox kit.

Table 8. Correlation between the different bioassays

	DIQ	ThTOX	RoTOX	RaphALG	ScenALG	PAMALG	MTX
DIQ	1.00						
ThTOX	0.16	1.00					
RoTOX	-0.18	**0.64**	1.00				
RaphALG	*-0.46*	*-0.72*	-0.21	1.00			
ScenALG	*-0.35*	*-0.76*	*-0.40*	**0.54**	1.00		
PAMALG	-0.05	*-0.44*	*-0.74*	0.29	**0.41**	1.00	
MTX	-0.22	0.10	-0.05	-0.30	*-0.36*	*-0.37*	1.00

The Principal Component Analysis (PCA) provides a more detailed picture (figure 14).

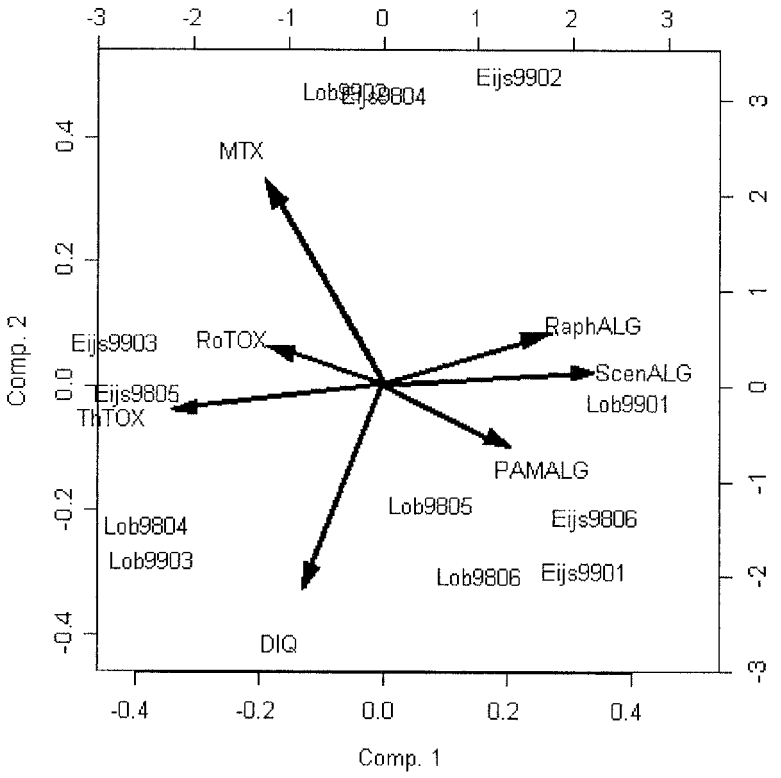

Figure 14: PCA of the bioassays used (covariant analysis).

The figure accounts for 67.3% of the covariance in the measurements obtained. The arrows in the PCA all point in a different direction, which indicates that there is little superfluous information. Arrows in opposite directions show a negative correlation as regards the sensitivity of those tests. The tests with the most positively correlated tests are the *Raphidocelis* and *Scenedesmus* microtitre plate tests, and to a lesser extent the PAM algae test. The invertebrates tests Rotox kit and Thamnotox kit point in the opposite direction.

Arrows pointing towards the sample code indicate a relatively low toxicity for the relevant test, e.g. Eijs9806 in the PAM algae test. Arrows in the opposite direction to the sample code indicate a relatively high toxicity for the relevant test, e.g. LOB9901 in the Thamnotox kit test.

To compare the general toxicity of the Meuse and the Rhine, the measuring values of the bioassays (Table B2) were compared by means of the Mann-Whitney-U-test. It was established, with a 95% level of certainty, that the water quality of the Meuse is substantially worse than that of the Rhine.

Table 9 describes the toxic pressure/ecotoxicologic risk on the basis of the pT values calculated (see 3.6.3) and the relevant confidence limits. While sampling for the biological tests, chemical analyses were also carried out extensively. The organic parameters were screened for their usability (see 3.6.3), on the basis of which for each sample the fraction of the population was determined, which was influenced by the quality of the water (PAF, toxic pressure).

Table 9: Toxic pressure/ecotoxicologic risk for each sample point/date.

Sample code	pT value (%) (logistic model)	5% CLN	95% CLN	PAF (%)
Eijs9804	3.0	0.2	21.7	4.71
Eijs9805	5.3	0.6	28.0	4.97
Eijs9806	3.4	0.2	22.8	2.59
Eijs9901	0.0	0.0	1.1	0.2
Eijs9902	0.1	0.0	3.4	1.79
Eijs9903	8.7	1.7	34.7	0.49
Lob9804	0.4	0.0	7.4	1.55
Lob9805	0.1	0.0	4.0	1.05
Lob9806	0.1	0.0	3.5	1.25
Lob9901	0.0	0.0	1.2	0.35
Lob9902	0.1	0.0	3.6	1.2
Lob9903	0.3	0.0	6.2	NB

NB=not determined

According to De Zwart (personal communication) a pT value of 5% is still acceptable. Two samples with a high pT value were found in the Meuse. The pT values of the Rhine are substantially smaller than the pT values of the Meuse

The relationship between the PAF values calculated by means of chemical measurements and the biological pT value is shown in figure 15. For a number of measurements no clear relationship between the two values can be defined.

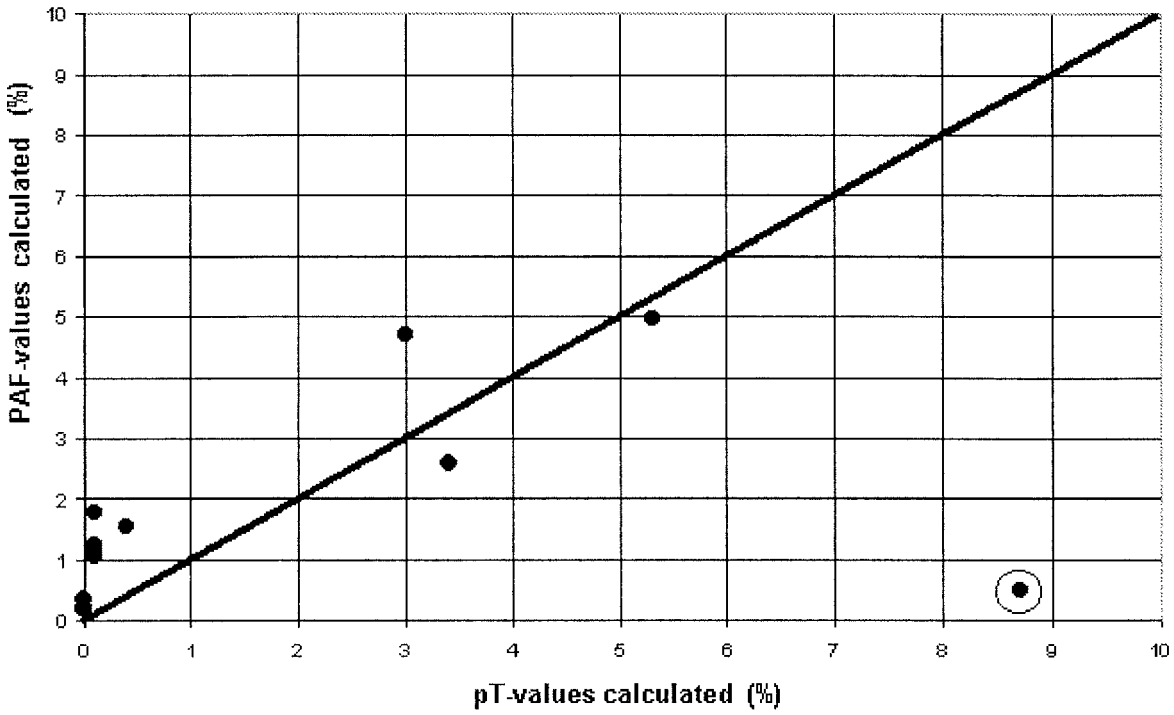

Figure 15: Relationship between the pT values and the PAF values.

On the basis of the pT values de Combi-pT value for each location as well as the Combi-PAF for chemical measurements (see table 10) were calculated.

Table 10: Determination of the general water quality of the Meuse and the Rhine via the Combi-PAF.

Location	Combi-pT (LOGISTIC Bioassays)	Combi-PAF (chemical measurements)
Meuse	1.07 %	3.0 %
Rhine	0.14 %	1.1 %

Comparison of the Combi-pT values of the bioassays used shows that the ecotoxicologic water quality of the Meuse i substantially worse than that of the Rhine. This fact can also be established via the Combi-PAF of chemical measurements.

On the basis of the chemical measurements, carried out by RIZA on samples taken at the same time as the samples for this study, it could be established which organic compounds have a substantial effect on the flora and fauna (Combi-PAF). It is likely that other substances, which also had an important effect on the tests, were also present in the water but were not measured chemically. Table 11 shows the organic compounds in bold, which account for a major share in the toxic end effect of the Rhine. In table 12 the organic compounds are listed that were measured in the Meuse.

Table 11: Poisonous organic compounds present in the Rhine.

Organic compound	CAS number	Effect mechanism	Average concentration (µg/l)	Relative share in the toxic end effect (%)
1,2-Dichloroethane	107062	Apolar narcosis	0.049	0.3
2-(2,4-Dichlorophenoxy) propionic acid, *2,4-DP*	120365	Inhibits plant growth	0.028	3.2
6,7,8,9,10,10 –Hexachloro--1,5,5a,6,9,9a-hexahydro-6,9-methanol-2,4,3-benzodioxathiepin, 3-Oxide, *Endosulfan*	**115297**	**Neurotoxicant: cyclodiene type**	**0.0004**	**27.0**
6-Chloro-N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine, *Atrazin*	**1912249**	**Inhibits photosynthesis**	**0.016**	**9.8**
Benzene	71432	Apolar narcosis	0.020	0.2
3-(1-Methylethyl)-1H,2,1,3-benzothiadiazin-4(3H)-1, 2,2-Dioxide, *Bantazon*	25057890		0.023	0.1
(1a,2a,3b,4a,5a,6b)-1,2,3,4,5,6-Hexachlorocyclohexane, *Lindane*	**58899**	**Neurotoxicant: cyclodiene type**	**0.002**	**13.4**
2-[[4-Chloro-6-(ethylamino)-S-triazin-2-yl)amino]-2-methylpropionitril, *Cyanizin*	21725462	Inhibits photosynthesis	0.016	1.7
Chlorotoluron	15545489	Inhibits photosynthesis	0.016	1.0
Fosforothioic acid, O,O-Diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) ester, *Diazinon*	**333415**	**Acetylcholine esterase inhibition: organophosphates**	**0.003**	**15.3**
N,N-Dimethyl-N'-[4-(1-methylethyl)phenyl]urea, *Isoproturon*	34123596	Inhibits photosynthesis	0.029	2.1
Chloro acetic acid	**79118**	**Alkylation or arylation reaction**	**0.026**	**16.0**
2-methyl-4-chlorophenoxy propionic acid, *MCPP*	7085190	Inhibits plant growth	0.023	0.2
Pentachlorophenol	**87865**	**Uncouples oxidative phosphorylation**	**0.004**	**6.5**
Xylene	1330207	Apolar narcosis	0.011	0.2
6-Chloro-N,N'-diethyl-1,3,5-triazine-2,4-diamine, *Simazin*	122349	Inhibits photosynthesis	0.006	0.4
Tetrachloroethene	127184	Apolar narcosis	0.027	1.7
Trichloroethene	79016	Apolar narcosis	0.017	0.3
Trichloromethane	67663	Apolar narcosis	0.026	0.5

Table 12: Poisonous organic compounds present in the Meuse.

Organic compound	CAS number	Effect mechanism	Average oncentration (µg/l)	Relative share in the toxic end effect (%)
1,1,1-Trichloroethene	71556	Apolar narcosis	0.023	0.0
1,2-Dichloroethane	107062	Apolar narcosis	0.210	0.3
Xylene	1330207	Apolar narcosis	0.012	0.1
2,4,5-Trichlorophenol	**95954**	**Polar narcosis**	**0.030**	**7.7**
(2,4-Dichlorophenoxy) acetic acid	94757	Inhibits plant growth	0.044	0.2
2-(2,4-Dichlorophenoxy) propionic acid, *2,4-DP*	120365	Inhibits plant growth	0.028	0.9
6-Chloro -N-ethyl-N'-(1-methylethyl)-1,3,5-triazine-2,4-diamine, *Atrazin*	**1912249**	**Inhibits photosynthesis**	**0.061**	**10.3**
Benzene	71432	Apolar narcosis	0.020	0.1
3-(1-Methylethyl)-1H,2,1,3-benzothiadiazin-4(3H)-one, 2,2-Dioxide, *Bantazon*	25057890	Inhibits photosynthesis	0.015	0.0
(1a,2a,3b,4a,5a,6b)-1,2,3,4,5,6-Hexachlorocyclohexane, *Lindane*	**58899**	**Neurotoxicant: cyclodiene type**	**0.003**	**6.8**
2-[[4-Chloro -6-(ethylamino)-S-triazin-2-yl)amino]-2-methylpropionitril, *Cyanazin*	21725462	Inhibits photosynthesis	0.020	0.6
Coumaphos	**56724**	**Acetylcholine esterase inhibition: organophosphates**	**0.025**	**34.5**
N'-(3,4-Dichlorophenyl)-N,N-dimethylurea	**330541**	**Inhibits photosynthesis**	**0.060**	**27.2**
N,N-Dimethyl-N'-[4-(1-methylethyl)phenyl]urea, *Isoproturon*	34123596	Inhibits photosynthesis	0.024	0.5
Chloro acetic acid	**79118**	**Alkylation or arylation reaction**	**0.034**	**5.5**
2-methyl-4-chlorophenoxy propionic acid , *MCPP*	7085190	Inhibits plant growth	0.030	0.1
Chloromethane	74873	Apolar narcosis	0.005	0.0
Pentachlorophenol	87865	Uncouples oxidative phosphorylation	0.003	1.7
Xylene	1330207	Apolar narcosis	0.013	0.1
6-Chloro -N,N'-diethyl-1,3,5-triazine-2,4-diamine, *Simazin*	122349	Inhibits photosynthesis	0.014	0.2
Tetrachloroethene	127184	Apolar narcosis	0.132	2.1
Tetrachloromethane	56235	Apolar narcosis	0.015	0.1
Trichloroethene	79016	Apolar narcosis	0.152	0.7
Trichloromethane	67663	Apolar narcosis	0.046	0.2
Methyl benzene	108883	Apolar narcosis	0.017	0.1

In the Rhine the presence of 10 organic compounds that may affect the flora and fauna was demonstrated. In the Meuse the presence of 26 compounds was demonstrated. In the Rhine mainly organic compounds were found, which may have disadvantageous effects on the nerve system. 56% of the total effect was nerve related. In the Meuse, 37.5% of the total effect was related to photosynthesis and 41.5% to the nerves.

4.2. Genotoxicity tests

Tables 13 and 14 show the number of ethanol extracts that were found to be positive for LOECf values (on the basis of tables B1A and B1B), isolated at different degrees of acidity.

Table 13: Number of ethanol extracts (pH=7) per genotoxicity test that were found to be positive.

Genotoxicity	Number found to be positive
Ames-test TA98[@]	12 (9)
UMU	8
VITOTOX®	9
Comet lymphocytes[1]	3
Comet Daphnia	6

Table 14: Number of ethanol extracts (pH=2) per genotoxicity test that were found to be positive.

Genotoxicity test	Number found to be positive
Ames-test TA98[@]	12 (3)
UMU	8
VITOTOX®	9
Comet lymphotcytes[1]	8
Comet Daphnia	6

1 = Instead of 12 extracts, only 11 extracts were examined for this test.

@ = The number given between brackets is based on exceeding the number of revertants per litre of sample.

On the basis of the LOECf values, all samples (pH 7 fraction and pH 2 fraction) were found to be positive in the Ame test. However, when the number of revertants per litre rather than the LOECf values were used as a criterion, the number of positive ethanol extracts in the Ames test of the 12 extracts tested was 9 and 3 (pH 7 fraction and pH 2 fraction respectively). The UMU test and the VITOTOX® yielded a positive signal in approximately 70% of the samples. In the UMU test, it was often impossible to distinguish from general toxic effects, as the growth speed of the bacteria culture was too slow. In the Comet *Daphnia* test, 50% of the measurements scored a positive reaction. The Comet test with lymphocytes was the only test that showed a distinction between the extracts. 70% of the extracts the pH 7 fraction were found to be positive in this test, while only 25% was found to be positive in extracts of the pH 2 fraction.

Table 15 shows the differences in response between the Rhine and the Meuse, between pH2 and pH7, and between tests for the presence or absence of a metabolising S9 fraction. This was done by means of the Mann-Whitney-U test

Table 15: Significant differences in response in the genotoxicity tests related to river, acidity and the use of the S9 fraction. The significance level is shown between brackets.

Genotoxicity test	Rhine vs. Meuse	+S9 vs. -S9	pH7 vs. pH2
Ames-test (TA98)	Rhine > Meuse (0.0067)	+S9 > -S9 (0.001)	pH7 > pH2 (0.005)
VITOTOX®	-	-S9 > +S9 (0.004)	-
UMU-test	-	-	-
Comet lymphocyte test	Meuse > Rhine (0.036)	-	pH2 > pH7 (0.031)
Comet Daphnia test	-	-	-

In the Ames test and the Comet test with lymphocytes, a difference between the Meuse and the Rhine was found. However, the Ames test gave a lower response for the Rhine than for the Meuse, while the Comet (lymphocyte) test gave the opposite result. With regard to the use of the S9 liver extract, the Ames test turned out to yield a higher response when the S9 mix was added to the extract. The VITOTOX®, on the other hand, gave more positive results in the extract without S9. The Comet test with lymphocytes responded mainly to hydrophilic compounds (acidity=2), while the Ames test detected more mutagenicity on hydrophobic compounds.

In terms of mutagenicity in the course of time, only the Ames test gave enough positive results to gain a picture of the development in the Meuse and the Rhine (see figures 16 and 17). Because of the higher mutagenicity level of Rhine water in comparison with Meuse water, this development is also most visible in the Rhine samples. In the Rhine, the lowest mutagenicity level was found in March. In the Meuse the differences were less evident; it appears that in the Meuse the lowest values occurred in November.

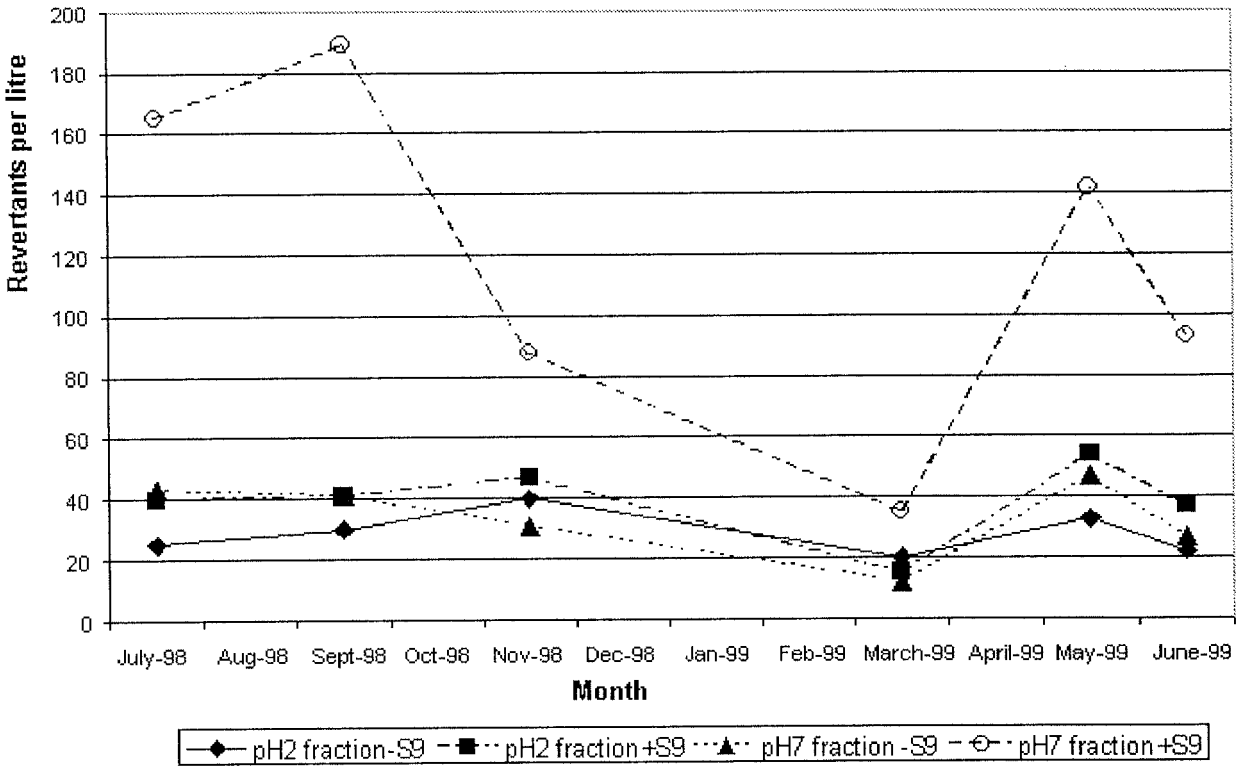

Figure 16: Trend in the mutagenicity of the Rhine over time (Ames test).

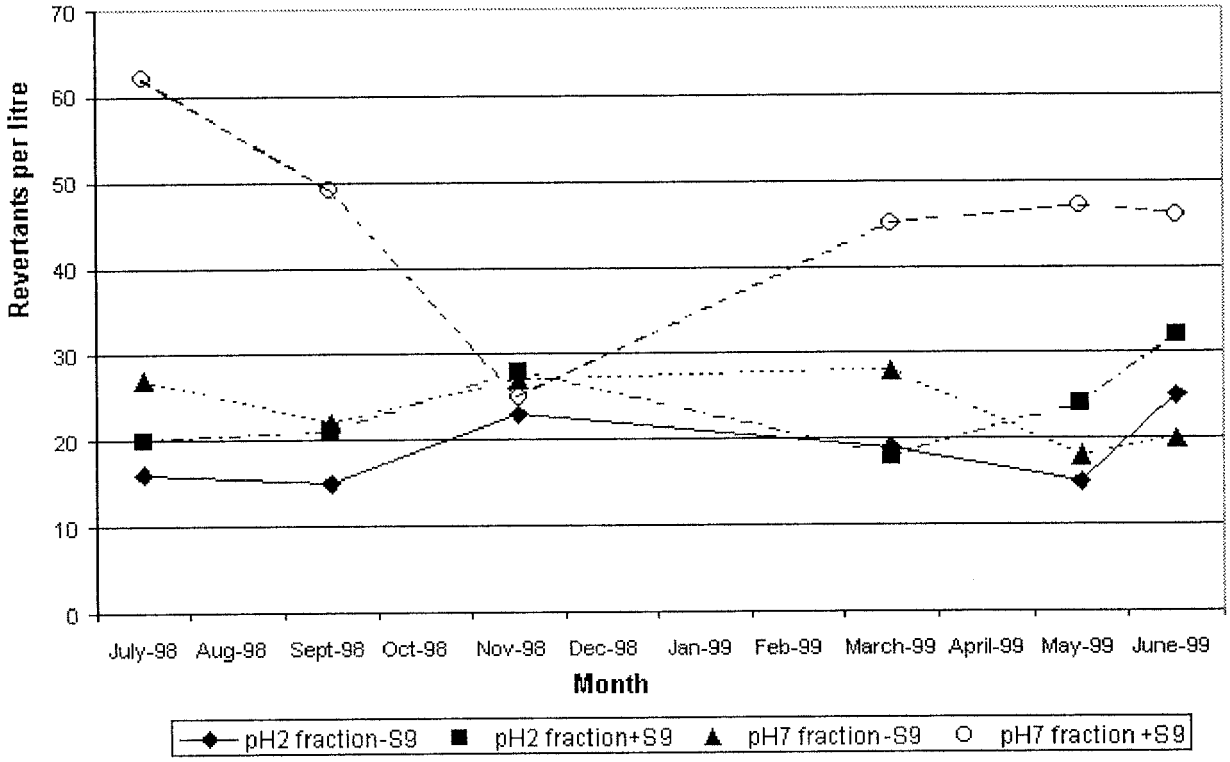

Figure 17: Trend in the mutagenicity of the Rhine over time (Ames test).

4.2.1. Results of the genotoxicity tests used in the KIWA study

Table 16 shows the concentration factors for which a genotoxic response was found. The results of the MutaChromoPlate test have not been considered, as they could not be used.

Table 16: Concentration factors for which a genotoxic response was found in the different tests (Rhine, pH7 fraction).

Rhine pH 7	**Ames**		**Gene mutation test (TK-test)**		**Chromosome aberration test**		**SOS Chromotest**		**Mutatox test**	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Lob9804	42	21	-	-	ND	ND	2500	500	4	31
Lob9805	42	21	ND	ND	-	-	2500	500	1	16
Lob9806	63	21	ND	ND	-	-	-	100	1	-
Lob9901	-	63	-	-	ND	ND	2500	100	1	1

Legend: ND = not determined

\- = no genotoxic response observed

It is impossible to pass an overall judgement as to which test is best on the basis of this, as the different tests test for different end results, and one test may therefore be more sensitive to a specific substance group than another.

4.3. Effect-specific tests

The results of the effect-specific tests (theacetylcholine esterase test and the Calux-ER-test) are shown in figures 18 and 19; for more detailed information see annex B3.

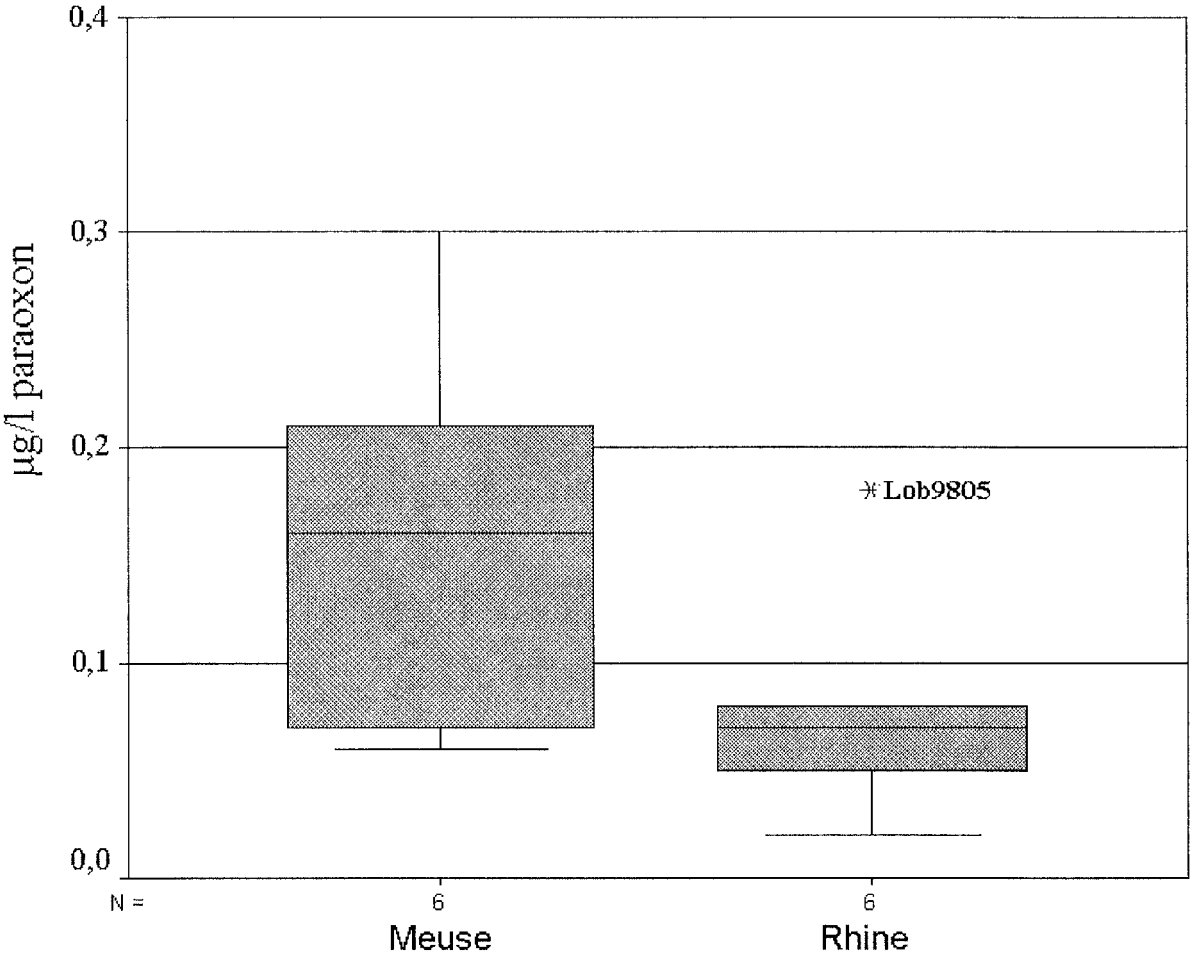

Figure 18: Box plot of results from the choline esterase inhibition test

The Meuse has substantially more pollutants that inhibit the choline esterase enzyme in water than the Rhine. However, one measurement from the Rhine (September 1998) contained a clearly increased number of choline esterase inhibiting compounds (0.18 µg/l) in comparison with the median (0.07 µg/l).

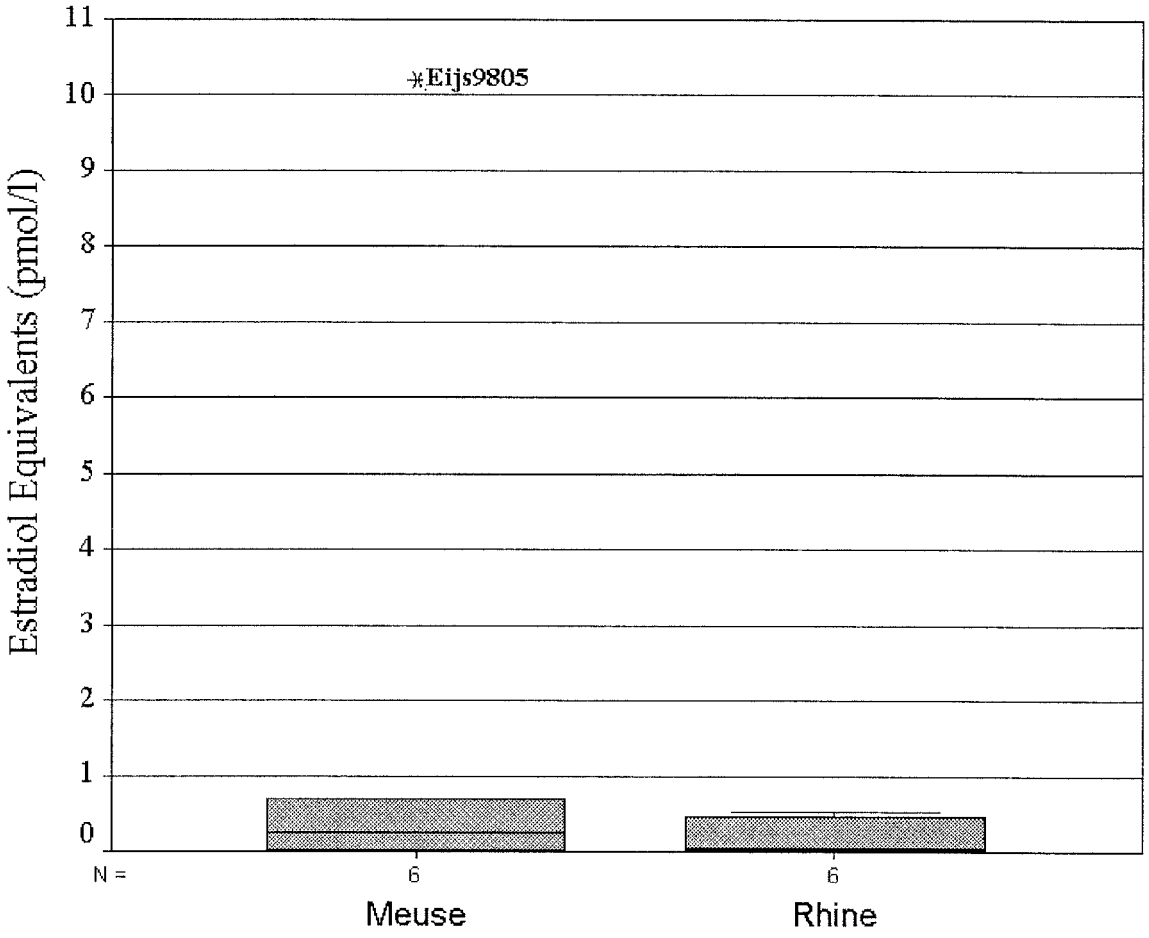

Figure 19: Box plot of results from the ER-Calux assay.

The Meuse has a higher number of hormone disrupters than the Rhine. In the Meuse, a relatively high concentration of hormone disrupters (10.2 pmol/l estradiol equivalents) were found in September 1998. However, the margin appears to be comparable in the rivers. The high value in the Meuse shows that relatively large fluctuations may occur in the levels from the Meuse. As only a few samples were examined, it cannot be ruled out that the same might apply to the Rhine.

5. Discussion and conclusions

5.1. Bioassays

5.1.1. The most suitable bioassays for research in Dutch rivers

The results of this study show that the algae as primary producers are the most sensitive organisms in effect measurements of ethanol concentrates of surface water. This is followed by the decomposers (bacteria) and the consumers (invertebrates).
In terms of the producers, the PAM algae test appears to be the most sensitive test, which is probably the consequence of an effect measurement at sublethal level. As far as the consumers are concerned, the Daphnia IQ is the most sensitive test; again, this may be due to the fact that this test measures a sublethal effect.

The Principle Component Analysis (figure 14) indicates that the arrows shown all point in a different direction. From this it may be concluded that little overlapping information is obtained with this test battery. The correlation analysis (table 8) shows that there is a clear correlation between the *Raphidocelis* sp. MTP/*Scenedesmus* sp. MTP and theThamnotox kit/Rotox kit. On the basis of the Principle Component Analysis and the sensitivity, it may be concluded that one of the two algae tests and the Thamnotox kit will suffice in future test batteries.
The use of the battery of biological tests chosen costs approximately Euro 1300 per sample (excluding sample preparation). When the Microtox®, the PAM, one of the two MTP algae tests, the Thamnotox kit and the Daphnia IQ are used, the costs per sample will be approximately Euro 900.

5.1.2. Water quality of the Rhine and the Meuse

When 7 bioassays are used, it can be established with a certainty of 95%, that the Meuse is significantly more toxic than the Rhine. According to the Combi-pT, with which the general quality of the rivers is shown in terms of all types of organisms by means of results from bioassays, the Meuse is approximately 7.5x more toxic than the Rhine. There is no relationship between the pT values (toxic pressure on the basis of measurements from bioassays) and the PAF values (toxic pressure on the basis of chemical measurements) (figure 15). This mainly appears from the highly deviating point where a high PAF value does not correspond to the relevant pT value. Adjustment of the model used for the calculation of toxic pressure/ecotoxicologic risk is therefore recommended. Predicting the toxic pressure from chemical measurements on the basis of models is not possible at the moment, as the biological tests show the effects as a consequence of very complex interactions between chemical substances and organisms. Hence the added value of using the biological tests for establishing the quality of surface water.

The chemical measurements (table 12) show that there are six organic compounds in the Meuse, which make a substantial contribution (92%) in terms of toxicity to the general water quality. On average, the levels of these substances are less than 0.1 µg/l (i.e. the limit defined for organic components in the

infiltration decree). The concentration of some compounds was more than 0.1 µg/l. The kind of effect that these six compounds may have on organisms is related mainly to photosynthesis, and they may have a neurotoxic effect. In the Rhine there are six organic compounds that could account for 88% of the toxic effect. The level of these compounds was also below 0.1 µg/l. These compounds mainly affect the nervous system.

Two samples from the Meuse (Eijs9805 and Eijs 9903) may be called toxic because of their high pT value (>5%). When, in the interpretation, the highest confidence limits calculated are also considered, two other measurements in the Meuse may also be regarded as suspected of being toxic (Eijs9804 and Eijs9806). In the measuring series of the Rhine no samples with an excessively high pT value (>5%) were found, although two samples suspected of being toxic were found (Lob9804 and Lob9903).

5.2. Genotoxicity tests

5.2.1. The most suitable genotoxicity tests for research in Dutch rivers

Tables 13 and 14 show that the Ames test demonstrates mutagenic activity most frequently. Given the fact that this test also yielded the best dose response curves and the clearest results, it seems clear to us that this test should still be preferred to the other genotoxicity tests. The VITOTOX® and the UMU test generated less positive results. The quantity of extraction medium in the test medium had to be kept low in the latter two tests due to toxicity problems. The results were often at the limit of the detection level.
It is possible that there were fewer compounds in the extracts that evoked an SOS response in the bacteria than those that generated a frameshift mutation. The use of surface water samples of a stronger concentration (of more than 25,000 times), with which the amount of extraction medium in the volume to be tested can be minimised, will be necessary in order to make a definitive statement in this respect. The number of positive samples in the two comet tests was lower than those in the other genotoxicity tests. This may be related to the fact that a comet test only demonstrates damage to DNA which is much more serious than that shown by the other tests, which are able to demonstrate minor damage. The test with lymphocytes has already been carried out many times, and has proved its usefulness in many different applications (Tice, 1995). However, no uniform criteria have as yet been laid down in order to arrive at a good evaluation. In concrete terms, this means that parameters such as average values, median values and distributions should be further investigated in order to consider the most relevant parameter with the most relevant static approach. The comet test with lymphocytes was based on average DNA tail contents. When, however, the distribution of the DNA contents was considered, more samples appeared to have mutagenic properties.
The comet test in *Daphnia* is fairly new and should certainly be validated further. In the procedure used, a pool of cells is studied, some of which may have a higher or lower sensitivity than others. In addition, the cell population largely consists of a quickly proliferating population, which may cause problems. Whether or not this is the case should be further investigated.

The advantage of the use of two comet tests is that the lymphocytes have a clear link to humans, while the *Daphnia* test is more directly related to animals living in the water. The latter test therefore gives more information about the ecological effects.

In the KIWA study, the SOS Chromotest and the Mutatox test in the Rhine yielded useful pH 7 fraction results (table 16). The MutaChromoPlate test did not yield any useful results. Although the SOS Chromotest investigates a different type of genotoxic response than the Ames test, the concentration factor at which a response was measured was considerably higher than in the Ames test. Another aspect of the SOS Chromotest is that it consists of a test kit which is supplied ready for use by the producer, which makes it difficult to optimally modify the test. This is, however, possible with regard to the UMU test and the VITOTOX®, but in these tests too a genotoxic result was generally observed only at a higher concentration factor than in the Ames test.

It is interesting to note, however, that the Mutatox test in the KIWA study was able to detect a genotoxic response at relatively low concentration factors. These concentration factors were also lower than in the Ames test. Neither the *in vitro* gene mutation test nor the *in vitro* chromosome aberration test detected a genotoxic response. According to the Health Council's advice, the relevant samples therefore did not contain any compounds that are genotoxic to humans (assuming that these tests are also suitable for testing water samples). To obtain more certainty in this respect, these tests should be carried out on a larger number of samples. On this occasion, each test was only done twice.

5.2.2. *Water quality of the Rhine and the Meuse*

As genotoxic measurements had been carried out on Rhine and Meuse water with the Ames test for a number of years (from 1981), this test was included as a reference in the test battery. The concentration factor of 25,000 times, normally used for the Ames test, was therefore also adopted; this concentrate was also used for the other genotoxicity tests. Due to the fact that the other tests did not yield a response for all samples, the course of genetoxicity in the Meuse and the Rhine can be followed only by means of the Ames test (figures 16 and 17). As in previous years (Veenendaal, 1999) the genotoxicity of the Rhine was considerably higher than that of the Meuse. The highest genotoxicity level was measured in the pH 7 fraction of the extracts, to which the S9 mix had been added. The Meuse and the Rhine, then, contain fairly apolar organic compounds, which are not genotoxic at first, and only become genotoxic after conversion by this liver extract (S9).

The genotoxic activity has gradually reduced in both rivers since 1981 (see figure 20).

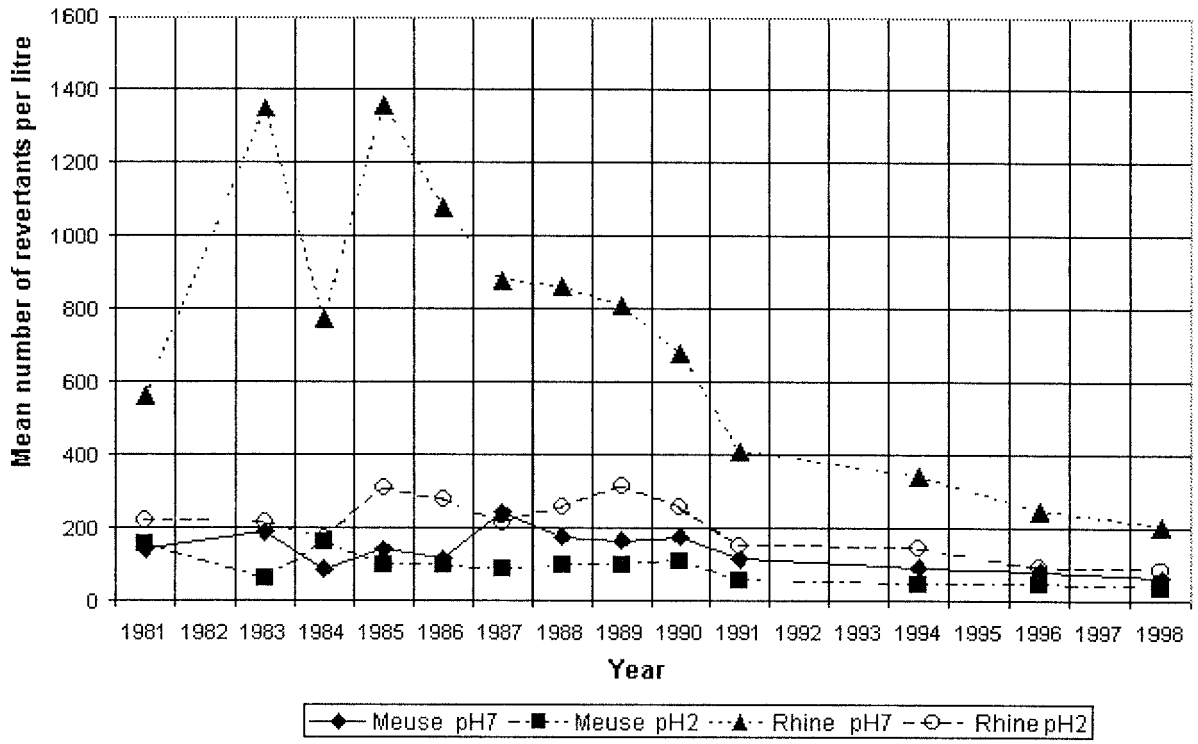

Figure 20: Trend in genotoxicity of the Meuse and the Rhine from 1981, measured with the Ames test. (Data from the report by Veenendaal & Van Genderen, 1998)

Figure 20 is based on the annual average levels of 6 samples per year, whereby the results of the extracts without S9 and those with S9 were aggregated.

The comet test, in which lymphocytes were used as a cell line, showed that the Meuse is more genotoxic than the Rhine, and that the more hydrophilic compounds are now more genotoxic than the more hydrophobic compounds. Furthermore, the VITOTOX®shows that the extracts without S9 are more genotoxic than the extracts with S9. However, due to the limited number of positive measurements, more research is required in order to verify the picture generated by the Ames test.

5.3. Effect-specific biological tests

5.3.1 Water quality of the Rhine and the Meuse

In the Rhine, a clearly increased paraoxon equivalent level was found once (figure 18). The concentration of choline esterase inhibitors has clearly declined in both the Rhine and the Meuse since 1988 (figure 21). On average, the Rhine contains 2x less choline esterase inhibitors than the Meuse.

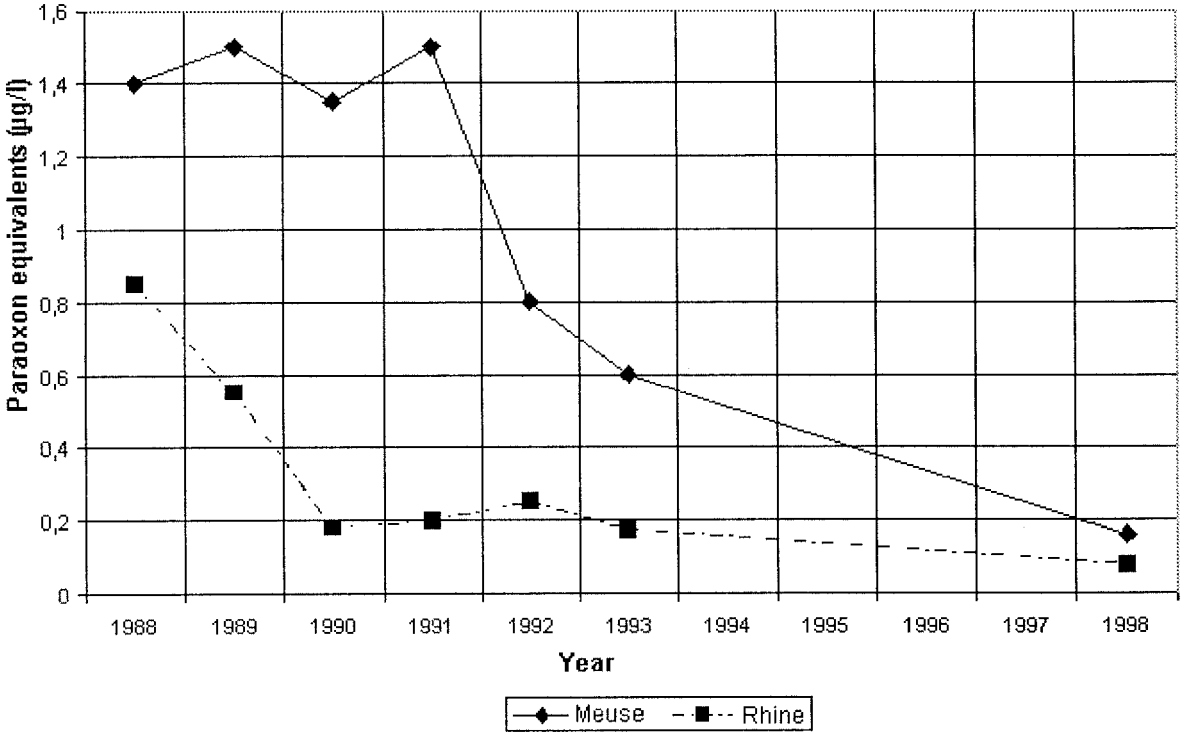

Figure 21: Trend in the number of compounds that inhibit the acetylcholine esterase enzyme, expressed in paraoxon equivalents in µg/l (mean values) from 1988-1998/1999 (data from 1988-1993 from Noij and Meerkerk, 1995).

On the basis of the ER-Calux test, a clear increase in the level of estrogen substances (10.2 pmol/l EEQ) was measured in only one sample from the Meuse (Eijs9805). This sample must certainly have had an effect on the fish population. The value at which an effect on fish is expected (LOEL of about 1 pmol/l EEQ) had clearly been exceeded in this sample (Murk, personal communication). It is not clear, however, how long such a burden must be present in the environment in order to have an impact. This sample was an exception (figure 19). It is also noticeable that the levels for 1999 (Annex, Table B3) were lower than those for 1998. As regards the execution of the measurement, the cells complied with the normal quality standards, and the calibration points showed normal responses.

6. Recommendations

The following recommendations have been formulated on the basis of the results of this study:

- The use of at least one bioassay for each trophic level is necessary in order to establish the ecotoxicologic effects of a large variety of organic pollutants. The battery should therefore at least consist of the PAM algae test, the Daphnia IQ test and the Microtox®.
- More? Several measurements should be carried out to obtain a uniform quality parameter for the further adjustment of the model used for the calculation of the toxic burden/ecotoxicologic risk.
- The use of genotoxicity tests that have a different approach to mutagenic substances is desirable in order to verify the quality picture generated by the Ames test. Preference should be given to the use of the UMU test and the Comet test. Moreover, it is recommended that the Mutatox test should be further investigated. A follow-up project for researching surface water with the Ames test, the UMU test and the Comet test was started in 2000.
- Research into the options for increasing the concentration factor is necessary in order to acquire a reliable, comparable LOECf value. It is important in this respect to investigate the levels of extraction medium that may be used, without measuring any toxicity in the UMU test and the Comet test.
- As regards the genotoxicity tests, the use of a uniform quality standard is necessary. This makes it easier to compare the findings of the different tests. LOECf values, for instance, could be used as a quality standard, in which case a confidence limit may also be determined. As regards determining the LOECf value in the comet test, the end parameter of the genotoxic effect should be established first.
- Integration of data from the genotoxicity tests into data from the bioassays is not yet possible due to the inadequate data available and/or due to the use of the current +/- assessment. Research into obtaining a combination + end parameter is desirable if, in addition to the bioassays, genotoxicity tests are to be carried out in the future.
- The KIWA study has resulted in the recommendation that additional research should be done on a larger set of samples with the *in vitro* gene mutation test and the *in vitro* chromosome aberration test. If such a study were to be carried out, this could give insight into the similarities of and differences between all the genotoxicity tests and the significance for humans.
- As there are still substances in the Rhine and the Meuse which generate inhibition of the acetylcholine esterase enzyme, the use of this test continues to be necessary.

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Annexes

Table B1A: Results of the genotoxicity tests (ethanol extract pH = 7)

Sample code[1]	Conc. setup	Conc. factor	Ames test				UMU		VITOTOX®		Comet test with lymphocytes		Comet test with Daphnia	
			-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
			Revertants/l	Revertants/l	LOECf[2]	LOECf[2]	LOECf[3]	LOECf[3]	LOECf[4]	LOECf[4]	LOECf[5]	LOECf[5]	LOECf[6]	LOECf[6]
Eijs9804	K#	25000	27	62	84	21	65	-	250	-	390	ND	31.2	ND
Eijs9805	L@	21259	22	49	66	33	520	-	250	-	390	ND	26.5	ND
Eijs9806	K	25000	27	25	84	84	260	-	-	-	-	ND	31.2	ND
Eijs9901	L	14000	28	45	70	47	-	-	-	-	-	-	8.7	ND
Eijs9902	L	25000	18	47	84	42	-	520	-	250	ND	ND	-	ND
Eijs9903	L	25000	20	46	84	42	-	-	125	250	-	-	-	ND
Lob9804	K	25000	43	165	42	21	-	-	250	-	-	ND	7.8	ND
Lob9805	K	25000	41	189	42	21	-	-	250	-	-	ND	31.2	ND
Lob9806	K	25000	31	88	63	21	260	260	-	-	-	ND	-	ND
Lob9901	K	25000	12	35	-	63	-	260	250	250	-	-	-	ND
Lob9902	K	25000	47	142	42	21	33	260	250	125	-	390	-	ND
Lob9903	K	25000	27	93	42	42	-	260	250	250	-	-	-	ND

1 = See table 5 for explanation of sample code
2 = Condition required for determining the LOECf: the sample has 2x the number of spontaneous revertants per litre
3 = Condition required for determining the LOECf: growth speed of more than 0.5 and induction factor of more than or equalling 1.5
4 = Condition required for determining the LOECf: factor signal from the test strain is 1.5x higher than the primary strain
5 = Condition required for determining the LOECf: lowest concentration factor with a static difference between control and treated cells on the basis of the Mann-Whitney U test ($p<0.05$)
6 = Condition required for determining the LOECf: lowest concentration factor with a static difference between control and treated cells on the basis of the Kruskal-Wallis test ($p=0,05$)
# = Surface water transported to KIWA for further processing
@= Surface water inserted locally in the XAD column, after which the column was transported to KIWA for further processing
ND = not determined

Table B1B: Results of genotoxicity tests (ethanol extract pH=2)

Sample Code	Conc. Setup	Conc. factor	Ames test				UMU		VITOTOX®		Comet test with lymphocytes		Comet test with Daphnia	
			-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
			Revertants/l	Revertants/l	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf
Eijs9804	K	25000	16	20	125	42	65	-	250	-	390	ND	7.8	ND
Eijs9805	L	21259	15	21	100	66	65	-	250	-	390	ND	13.3	ND
Eijs9806	K	25000	23	28	42	84	260	260	63	-	390	ND	15.6	ND
Eijs9901	L	14000	19	18	70	-	-	-	17	17	-	390	-	ND
Eijs9902	L	25000	15	24	84	84	-	-	-	-	ND	ND	-	ND
Eijs9903	L	25000	25	32	42	84	-	-	125	-	98	195	-	ND
Lob9804	K	25000	25	40	63	63	65	-	-	-	-	ND	31.2	ND
Lob9805	K	25000	30	41	63	42	55	-	250	-	-	ND	62.5	ND
Lob9806	K	25000	40	47	42	21	-	520	125	-	390	ND	-	ND
Lob9901	K	25000	20	15	84	-	130	260	125	-	-	-	-	ND
Lob9902	K	25000	33	54	63	42	260	260	-	-	-	195	31.2	ND
Lob9903	K	25000	22	37	63	42	-	-	250	-	195	390	-	ND

Table B1C: Results from the KIWA study: genotoxicity tests, the Rhine.

Sample code	Mouse lymphoma		Chromosome aberration test		Mutatox		SOS Chromotest		SOS Chromotest	
	pH 7 fraction		pH 7 fraction		pH 7 fraction		pH 7 fraction		pH 2 fraction	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf	LOECf
Lob9804	-	-	ND	ND	4	31	2500	500	2500	x
Lob9805	ND	ND	-	-	1	16	2500	500	2500	500
Lob9806	ND	ND	-	-	1	-	-	100	x	100
Lob9901	-	-	ND	ND	1	1	2500	100	x	x

Table B1D: Results from the KIWA study: genotoxicity tests, the Meuse.

Sample code	SOS Chromotest		SOS Chromotest	
	pH 7 fraction		pH 2 fraction	
	-S9	+S9	-S9	+S9
	LOECf	LOECf	LOECf	LOECf
Eijs9804	x	2500	2500	2500
Eijs9805	-	125	2000	2000
Eijs9806	-	100	-	x
Eijs9901	-	x	-	x

Table B1E: Results from the RIWA genotoxicity tests, general overview (+S9 and –S9)

Sample code	Ames test		UMU		VITOTOX®		Comet test with Lymphocytes		Comet test with Daphnia	
	pH2	pH7	PH2	pH7	PH2	pH7	pH2	pH7	pH2	pH7
Eijs9804	+	+	+	+	+	+	+	+	+	+
Eijs9805	+	+	+	+	+	+	+	+	+	+
Eijs9806	+	+	+	+	+	-	+	-	+	+
Eijs9901	+	+	-	-	+	-	+	-	-	+
Eijs9902	+	+	-	+	-	+	ND	ND	-	-
Eijs9903	+	+	-	-	+	+	+	-	-	-
Lob9804	+	+	+	-	-	+	-	-	+	+
Lob9805	+	+	+	-	+	+	-	-	+	+
Lob9806	+	+	+	+	+	-	+	-	-	-
Lob9901	+	+	+	+	+	+	-	-	-	-
Lob9902	+	+	+	+	-	+	+	+	+	-
Lob9903	+	+	-	+	+	+	+	-	-	-

Table B2: Results from acute bioassays.

Sample code	D. magna IQ		Thamnotox		Rotox kit		Raphidocelis sp.		Scenedesmus sp.		Microtox®		PAM	
	ECf_{50}	95%	LCf_{50}	95%	LCf_{50}	95%	ECf_{50}	95%	ECf_{50}	95%	ECf_{50}	95%	ECf_{50}	95%
Eijs9804	18.2	12-28	90.8	79-105	262.3	229-302	52.3	28.3-95.0	56.7	36.4-88.0	59.6	52-68	21.2	20-22
Eijs9805	55.8	43-74	96.9	90-105	109.6	100-121	16.8	12.2-23.4	17.4	12.2-25.4	62.9	56-72	14.7	13-16
Eijs9806	86.0	69-107	90.0	78-104	215.0	193-239	51.4	32.4-80.0	84.7	∞	8.1	3-22	81.8	74-92
Eijs9901	181.6	109-306	108.2	93-126	156.7	141-175	95.7	∞	95.8	∞	43.4	28-69	218.2	205-232
Eijs9902	48.4	40-59	86.9	72-104	254.8	229-284	65.3	35.4-120	85	∞	151.5	136-169	54.5	49-61
Eijs9903	45.0	37-55	94.9	82-111	171.8	151-197	16.8	13.0-21.9	16.7	13.0-21.9	63.8	54-75	9.9	8.6-11.3
Lob9804	211.9	176-254	178.6	152-209	467.9	416-526	57.4	39.6-82.2	67.9	52.8-88.3	103.5	94-113	49.7	47-53
Lob9805	86.0	72-104	73.0	64-83	128.0	114-145	32.9	22.0-48.0	56.6	16.0-194	47.0	41-54	34.8	31-39
Lob9806	139.0	105-184	79.0	66-93	216.0	192-244	60.7	43.6-83.2	60.6	40.5-90.3	47.7	40-58	51.7	48-55
Lob9901	87.0	69-109	98.2	79-124	288.4	261-318	97.4	∞	121.5	78.9-186	59.7	51-71	224.7	205-246
Lob9902	115.0	94-142	108.0	92-130	259.3	233-289	75.1	∞	84.6	∞	445.0	317-625	69.6	65-76
Lob9903	300.3	-	208.4	169-256	601.2	-	54	45.1-63.1	87.1	∞	146.1	122-175	75.1	69-81
Con9804@	>250	-	>500	-	>500	-	661	327-1339	>900	∞	100	80-124	2368	17-?
Con9805	>250	-	444	397-497	267	240-297	278	178-432	659	246-1760	327	235-456	251	226-279
Con9806	>250	-	>500	-	247	219-280	464	257-838	>900	∞	98	86-116	131	1-16000
Con9901	210	135-326	>500	-	>500	-	653	458-932	>900	∞	61	41-90	395	380-411
Con9902	>250	-	>500	-	>500	-	317	∞	811	719-914	118	104-135	463	0-8.6E17
Con9903	>250	-	>500	-	>500	-	315	∞	482	369-631	159	134-190	509	0.2-1.67E6

@ = Inspection measurements (blanks)
- = It was impossible to proceed with the calculation of confidence limits
∞ = Confidence limit from 0 to indefinite

Table B3: Results of effect-specific tests.

Sample code	Choline esterase inhibition			ER-Calux
	Cf	Extract	Surface water	Surface water
		(µg/l paraoxon)	(µg/l paraoxon)	EEQ pmol/l
Eijs9804	1011	302	0.30	0.43
Eijs9805	1015	210	0.21	2.5-10.2
Eijs9806	1009	201.9	0.20	0.39-0.69
Eijs9901	1003	56	0.06	0.08
Eijs9902	1010	68.3	0.07	0.027
Eijs9903	998	120.6	0.12	0.015
Lob9804	1015	86	0.08	0.02
Lob9805	1001	180	0.18	0.36-0.46
Lob9806	2023	37.6	0.02	0.32-0.53
Lob9901	1012	48.1	0.05	0.057
Lob9902	1009	67.4	0.07	0.025
Lob9903	1002	67.6	0.07	0.027
Con9804	1011	<0.1	0.00	0.01
Con9805	1013	<0.1	0.00	0.072-0.095
Con9806	1009	157.4	0.16	0.03
Con9901	1009	41.5	0.04	0.008
Con9902	1006	14.1	0.01	0.011
Con9903	995	1.1	0.00	0.008

The ER-Calux sometimes gives values as a range. The maximum values have been used in the results.

Colophon

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