ASSOCIATION OF RHINE AND MEUSE WATER SUPPLY COMPANIES

RIWA

February 1997

The Toxicological and Ecological Study of the Rhine River in 1994

In relation to the preparation of drinking water

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By order of

Association of Rhine and Meuse Water Supply Companies RIWA

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List of Abbreviations

Several abbreviations are used regularly in this report. These abbreviations and their full meaning are given in alphabetic order below.

AWBR	=	Organization of Water Works Bodensee-Rhine
DSW	=	Dutch Standard Water
EINECS	=	European Inventory of Commercial Chemical Substances
EPA	=	Environmental Protection Agency (USA)
GC	=	Gas Chromatography
GWA	=	City Water Supply Amsterdam
HPLC	=	High Performance Liquid Chromatography
IAWR	=	International Organization of Water Works in the Rhine Basin
Kiwa	=	Kiwa Research and Consultancy Ltd.
MS	=	Mass Spectrometry
PWN	=	PWN Water Supply Company North Holland Ltd.
RIVM	=	(Dutch) National Institute for Public Health and Environmental Protection (Rijksinstituut voor Volksgezondheid en Milieuhygiëne)
RIWA	=	RIWA Cooperative Rhine and Meuse Water Supply Companies
RIZA	=	(Dutch) National Institute for Inland Water Management and Waste Water Treatment (Rijksinstituut voor Integraal Zoetwaterbeheer en Afvalwaterbehandeling)
TOR '94	=	Toxicological and Ecological Study of the Rhine River 1994
VCI	=	Consortium of German Chemical Industries
WBB	=	Water Storage Company Brabants Biesbosch Ltd.
WRK	=	Water Transport Company Rhine Kennemerland Ltd.
XAD	=	synthetic resins with a macroreticular structure composed of styrene and divinylbenzene

Preface

In 1994, the Toxicological and Ecological Study of the Rhine River 1994 (TOR '94) was organized by Kiwa as commissioned by RIWA. Water samples were collected from 10 locations along the Rhine River between Rheinfelden (about 17 km upstream from Basel) and Hagestein, as well as from the Bodensee and the IJsselmeer. For the toxicological study, the samples were concentrated using XAD isolation. Using the prepared concentrates, a genotoxicity test and various ecotoxicity tests were conducted. For the ecological study, exuviae (cast skins of insect pupae and nymphs) were collected and identified.

The toxicological and ecological study of the Rhine came about due to discussions of the 'Rhine campaign group' and the 'Biotesting group' of IAWR and representatives of the German chemical industry (VCI). Further preparation and overview of the study were conducted by the steering committee of TOR '94 consisting of:

- dr. J. van Genderen (Kiwa);
- Mrs. ir. A.J.M. van Grol (RIWA);
- drs. C. van de Guchte (RIZA);
- dr. W.F.B. Jülich (RIWA);
- dr. ir. J.A. Schellart (GWA)
- dr. P.G.M. Stoks (WRK);
- Mrs. dr. D.M.J. Tubbing (RIVM, currently at the Free University of Amsterdam);
- Mrs. ir. J. Willemsen-Zwaagstra (PWN, chairwoman);
- drs. D. de Zwart (RIVM).

Several additional people played an important role in this study. They are (in no particular order):

- John Pijl, captain of the motor ship 'Vios';
- Jan Wijkstra, former colleague of the Netherlands Energy Research Center, operator of the flow-through centrifuges;
- Astrid Spanjaardt and Judith van Hesselingen of PWN, who helped with sample collection for toxicological tests and field measurements;
- Lizette Kamp-Wagemaker and Astrid de Bouter of WRK, who helped with sample collection for toxicological tests and field measurements;
- Harry Polman, Evert-Jan van de Brandhof and Colinda Daane, formerly of RIVM, who conducted ecotoxicity tests;
- Mart de Groot of GWA, who conducted cholinesterase inhibition analyses;
- Jacqueline de Graaf-Ouwerkerk and Josje de Roos-Pouw of PWN who collected samples for the ecological study and identification of exuviae;
- Miranda Vink and Ad Kuijpers of WBB, who collected samples for the ecological study and identification of exuviae;

- Wolter Siegers of Kiwa, who helped with sample collection for the toxicological studies as well as with the related sampling logistics;
- Hans van Beveren and René van Doorn of Kiwa, who conducted XAD isolations;
- Ton Braat and Anke Brouwer-Hanzens of Kiwa, who conducted Ames-tests;
- Wim Hoogenboezem of PWN who coordinated the ecological study;
- Henk Ketelaars of WBB, who coordinated the ecological study.

Martin Meerkerk (Kiwa) was the project leader, especially concerning the sample collection for the toxicological study, and Theo Noij (also of Kiwa) had final responsibility for the study as a whole.

This report has been compiled from contributions by various authors, namely:
- John van Genderen (Kiwa) for the Ames-test;
- Dick de Zwart (RIVM) for the ecotoxicity testing;
- Jon Schellart (GWA) for cholinesterase inhibition test;
- Wim Hoogenboezem (PWN) and Henk Ketelaars (WBB) for the ecological study and the related sample collection.

Martin Meerkerk and Theo Noij wrote the remaining sections and coordinated the editing of the report as a whole.

Summary

For more than thirty years, RIWA has had its own water quality monitoring programme in the Rhine and Meuse Rivers. They regularly analyze river water samples to check the quality of the drinking water source for millions of people. To a large extent, the monitoring programme is focused on measuring the concentrations of several dozens of chemical compounds. This list of substances is growing, as new methods of analysis become available every year for relevant compounds.

In addition to chemical parameters, biological parameters are increasingly being measured due to awareness that chemical parameters alone do not give a complete indication of river water quality. One biological test which has been conducted for more than ten years in Rhine and Meuse waters is the Ames-test (reverse mutation assay in the bacterium *Salmonella typhimurium*). It was the results of this test that initially led to the plans for an extended Rhine campaign focusing on biologic testing.
Historical results of chemical analyses in the Rhine and Meuse Rivers clearly showed that major changes were occurring and were having positive results on the water quality. In the 1970's, sanitation measures were called for by the governments, environmental groups and cooperatives like RIWA and were set into place in the Rhine and Meuse River basins. Improved industrial production methods and more environmentally friendly agricultural practices were adopted, and complex treatment plants for municipal and industrial waste water were constructed. These combined actions led to a sharp decrease in concentrations of many relevant chemical compounds.

In contrast to these improving chemical trends, the results of the Ames-test in the Rhine River (at Lobith) and the Meuse River (at Eijsden and Keizersveer) remained relatively constant. Even more surprising, the differences between the two rivers remained the same: the mutagenicity (genotoxicity) of the Meuse River for bacteria remained very low, while for the Rhine River it was a factor of five to ten higher. In both cases, it is still not possible to identify the substances causing the mutagenic effects.

From the point of view of drinking water production, these biological test results were not very disturbing. During river water purification, the mutagenic substances which give a positive result in the Ames-tests are removed, as can be seen by testing drinking water. Also, the results of the Ames-tests cannot be directly extrapolated to humans. Positive results in this test and other additional genotoxicity tests suggest that genetic and related effects (possibly carcinogenic effects) may occur in mammals. However, the carcinogenicity of a compound still has to be determined in experimental animals.

Despite the limitations of the Ames-test, the water supply companies affiliated with RIWA felt it necessary to further investigate the causes of the mutagenic effects of river water on bacteria. The results of the Ames-tests in Rhine and Meuse river water were repeatedly discussed in great detail with representatives of government, industry, and research institutes. However, no

convincing explanation of the measured results could be found. It also remained unclear where in the river the mutagenicity was increasing and whether or not this was due to natural or anthropogenic sources.

RIWA decided to conduct a Rhine campaign, which would include not only the Ames-test, but also other biological tests, in order to obtain a broader overview of the biological and the toxicological conditions of the river water. To correlate results with chemical parameters, it was decided to collect the water samples at the established sampling locations between the Bodensee and the IJsselmeer where longterm water quality measurements are available. The study was conducted in order to answer the following questions:

1. Based on the results of the conducted tests, where does a change in water quality of the Rhine River occur?
2. What toxicological and ecological quality can be attributed to the Rhine River based on the test results?
3. Do the different parts of the study give a consistent picture of the changes in water quality?
4. Does this study confirm the previously identified differences between the rivers Rhine and Meuse?
5. Do the corresponding chemical analyses give an explanation for the changes in water quality found by the ecological and toxicological studies?

Thus, in 1994 the 'Toxicological and Ecological study of the Rhine 1994' was conducted. Water samples were collected at twelve different locations in the Rhine basin between the Bodensee and the IJsselmeer. These samples were then used for a genotoxicological study (Ames-test), an ecotoxicological study (5 different toxicity tests), an enzyme inhibition study (cholinesterase inhibition), and an ecological study (exuviae, cast coverings of insects). For the first three studies, samples were collected with a ship at three different times during the year. Samples for the ecological study were collected once in August 1994 from the river banks; this sampling trip was made by car.

For the short-term mutagenicity and ecotoxicity tests, the water samples were first concentrated in the laboratory using the XAD isolation technique, after which the toxicity tests were conducted. For the ecotoxicity tests, the 'toxic potential' parameter (pT-value), which gives an overall indication of the ecotoxicity, was determined. The samples for the exuviae study were preserved in the field and were later identified and counted in the laboratory.

For both the sample collection and the subsequent laboratory analyses, quality assurance practices were established and followed in order to have as accurate results as possible.

Of the biological parameters, the Ames-test with *Salmonella typhimurium* strain, TA98, both with and without metabolic activation, the pT-value (both measured in XAD concentrate at neutral pH) and the macro-invertebrate diversity were selected as the most representative of the water quality.

The results of the different studies give a surprisingly consistent picture concerning the water quality of the Rhine River from Switzerland to the Netherlands. Thus, it is possible to give some answers to the questions posed at the beginning of the study:

1. Based on the results of the conducted tests, where does a change in water quality of the Rhine River occur?
   The water of the Bodensee, collected from a depth of 60 meters, is chemically and toxicologically uncontaminated. Also, the Rhine River upstream of Basel has little or no measurable toxicity. Downstream of Basel, the level of pollution increases slowly up to the Ruhr region, where the highest toxicological and ecological effects are measured. Further downstream, a gradual improvement of water quality is seen, most likely due to processes such as dilution, adsorption to suspended matter and biological degradation. Based on results of the toxicity tests, the water in the IJsselmeer at Andijk can be considered unpolluted.

2. What toxicological and ecological quality can be attributed to the Rhine River based on the test results?
   On the basis of previous studies, it seems that the quality of the Rhine water has improved since the 1980's in terms of the cholinesterase inhibition and the ecological water quality. The water quality has also improved in terms of the ecotoxicity, though there are few historic measurements for the specific tests used in the present study. For the Ames-test, however, different conclusions must be drawn, namely: the measured values at Lobith and Hagestein are similar to those measured in previous RIWA studies. A summary of the water quality in the Rhine River is shown in figure 1 by means of a colour code for the three different parts of the study.

3. Do the different parts of the study give a consistent picture of the changes in water quality?
   It was expected that there would be a wide range of results from the different biological tests using a variety of organisms such as bacteria, algae, water fleas, crayfish and rotifers, as well as from the exuviae identification of macroinvertebrates. There are in fact distinct differences between the results of the different tests, depending on the individual sensitivity of the test organisms and their different reactions to the same substances. However, the toxic potential calculated from a combination

of different test results was in relatively good agreement with the results of the Ames-test and the exuviae study.

4. Does this study confirm the previously identified differences between the rivers Rhine and Meuse?
   While it was not specifically part of the study, a comparison of the Rhine and the Meuse Rivers with respect to ecological and ecotoxicological results is not consistent with the previously identified differences based on the genotoxicity (Ames-test) results. While the Meuse River had significantly lower scores in the Ames-test compared to the Rhine River (a factor five to ten lower), a comparison of ecological and ecotoxicological (cholinesterase inhibition) test results indicates that the Rhine River actually has a higher water quality.

5. Is there, on the basis of the corresponding chemical analyses, an explanation for the changes in water quality found by the ecological and toxicological studies?
   The samples for this study were collected at the established locations for chemical water quality monitoring in the Rhine River, thus extensive analytical measurements of water quality were available. However, the chemical parameters give no explanation for the measured toxicity of the river water. It is probable that not all the chemical substances responsible for the observed effects can be measured. Unknown compounds or synergistic effects between known substances may be the cause. Further chemical studies in combination with biological test methods may yield more information.

The results from the various tests used in this investigation indicate that large differences in water quality are measured in the Rhine River. Downstream of Basel there is a gradual deterioration of the water quality which seems to have a direct correlation with the degree of urbanization. The water quality only begins to improve again downstream of the Ruhr region.

It is obvious that unpolluted surface water is the preferred source for the production of drinking water. Therefore, further study as to the causes of the measured toxicological effects is necessary, and, from the point of view of drinking water supply, the water quality of the Rhine River must continue to receive significant attention.

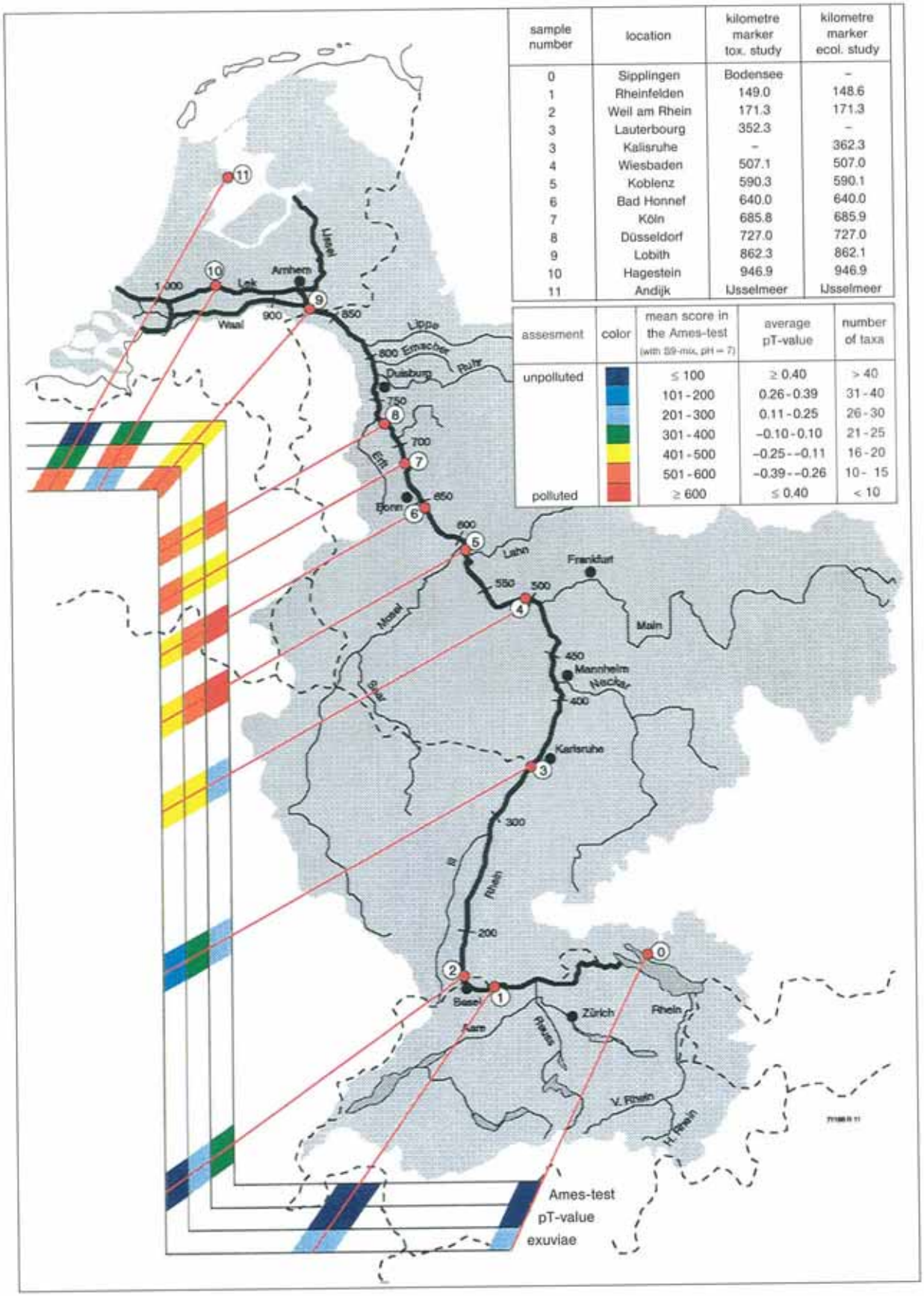


Figure 1 The water quality of the Rhine River in terms of the averaged results of the three separate studies of the TOR '94, given by a colour coding.

1. INTRODUCTION

Roughly two decades ago, the presence of organic micropollutants in drinking water and in the raw water used to prepare drinking water was realized and began to be of interest. Consequently, the toxicological importance of organic micropollutants became a subject of study. It was thus found that relatively high concentrations of toxicologically relevant trihalomethanes (chloroform, bromoform, bromodichloromethane and dibromochloromethane) were present in water that had been treated by chlorination [Rook, 1977]. In addition, it was shown that chlorination resulted in a higher score in the reverse mutation assay in *Salmonella typhimurium*, (called hereafter: Ames-test), especially for the sensitive test strain TA100 [Van der Gaag *et al.*, 1982].

Studies to identify the individual micropollutants responsible for the observed toxicological effects as measured in the Ames-test have rarely been successful. At the end of the eighties, the discovery in Scandinavia of the compound 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (also known as MX or Mutant X) was seen as a breakthrough in the identification of mutagenicity resulting from chlorination [Hemming *et al.*, 1986]. However, this compound is usually found only in waters affiliated with the bleaching of paper pulp [Wondergem and Backlund, 1989], therefore this explanation for toxicity remained limited to waste waters from the paper industry.

Currently, the focus of water quality studies is mainly on individual substances (the so-called substance specific approach). This approach offers many advantages, especially in terms of specifying water treatment measures. One great disadvantage with this approach, however, is that only a small number of substances potentially present in the surface water is measured. In the EINECS-list there are about one hundred thousand different substances, of which, at the moment, less than one percent are routinely monitored. Based only on the viewpoint of cost, it is not feasible to significantly increase the monitoring of other substances.

An important addition to the substance specific approach is the 'effects approach', in which indicator tests are used to measure biological effects such as mutagenicity, teratogenicity and toxicity of (multiple) chemical substances in water. The advantages of this approach are evident: the tests give an indication of the integrated water quality, in which both synergistic and antagonistic effects of the substances are incorporated. Although there has been interest in these water quality tests for some time, particularly as a they can identify the presence of known toxic substances, little is known about the spatial and temporal variation of such test results in river systems.

Currently there are several toxicity tests which have been developed and are available for water quality studies. Depending on the nature of the test, a

specific response of the test organism to the toxic substances present is measured. Due to the differences in the responses, the diversity of the test organisms and the large diversity of (types of) toxic compounds, there is still no clear correlation between scores of a toxicity test and the relevance for humans.

In the 'Toxicological and Ecological Study of the Rhine 1994', a combination of a genotoxicity test, several ecotoxicity tests and an applied ecological study were conducted for the first time, and the results were compared and correlated.

Concentrating organic micropollutants

Using the currently available toxicity tests, the concentrations of organic pollutants in surface water are generally too low to generate measurable effects in short exposure times. Environmental concentrations causing acute effects are only expected by point discharges (e.g. effluents or calamities). However, toxicity is caused by a combination of the chemical compound properties, concentration, and exposure time. Thus, from the point of view of cost and practicality of toxicity screening, it is possible and preferable to increase the sample concentration in order to minimize the necessary exposure time. By so doing, the actual ecotoxicological risks can be given as a relative toxicity value.

With the current isolation techniques, only a part of the organic substances can be isolated. As a result, the composition of the concentrate may deviate from that of the original sample. With the usual techniques such as adsorption to XAD resin, the non-polar and slightly polar compounds are isolated, while the most polar compounds remain in the water. By conducting XAD isolation under neutral or slightly acidic conditions, approximately 50% of the organic material can be isolated [Van Beveren and Noordsij, 1992]. In addition to having a relatively high specificity for biologically active compounds, XAD isolation is compatible with chemical analysis methods for organic substances (gas chromatography and liquid chromatography, potentially in combination with mass spectrometry), and is robust, efficient and reproducible.

Correlation between toxicity and individual substances

In order to protect the water sources for drinking water, it would be desirable to relate the scores of toxicity tests to individual organic micropollutants. Unfortunately, this correlation is difficult to establish. The concentrates produced by the isolated techniques consist mainly of naturally occurring compounds in drinking water, groundwater and surface water and only a very small fraction of these can be identified using a gas chromatograph coupled with a mass spectrometer (GC/MS) (an estimation is 1 - 10%) [Noordsij and Van Genderen, 1991].
The reasons for this are:

a. especially the more polar substances cannot be analyzed by gas chromatography;
b. most mass spectra are not specific enough to give a positive identification.

However, several compounds that were identified in drinking water and surface water concentrates using GC/MS are toxicologically relevant [Noij *et al.*, 1989]. Thus, Kiwa was commissioned by RIWA to evaluate approximately one thousand known organic micropollutants potentially found in drinking water and/or surface water [Van Genderen *et al.*, 1993]. From a literature review, 140 substances were shown to be toxicologically relevant. Of these, thirty were regularly occurring in both surface water and the produced drinking water, and twenty were found in surface water whereby drinking water was not investigated. However, it seemed that even here, no relation between the positive scores in the Ames-test and the toxicologically relevant compounds in the surface waters could be made [Puijker and Van Genderen, 1992; Janssen and Van Genderen, 1993].

The effects-approach, using different toxicological tests can supplement the substance specific approach for the assessment of (surface) water quality. For many years Kiwa, commissioned by RIWA, has been measuring Ames-test mutagenicity in concentrates of the Rhine and Meuse water prepared by XAD isolation [Puijker and Van Genderen, 1992; Janssen and Van Genderen, 1993]. The score for the test strain TA98 is systematically a factor two to ten higher in the Rhine River than in the Meuse. Since the score for the test *Salmonella typhimurium* strain TA98 is particularly well correlated with industrial pollution [Noij *et al.*, 1989], it initially seemed that the different natures of the two rivers could be the explanation. However, the improved quality of the Rhine and the worsening of the Meuse in the last years with respect to organic micropollutants has not affected the Ames-test scores for the two rivers. Also, the difference could not be explained from the results of chemical analyses such as GC/MS studies, analysis of pesticides, group parameters, etc. In addition, it was still unclear if the increased score in the Ames-test occurred gradually along the course of the Rhine, or if there were one or more locations where a sudden increase could be pinpointed, and if so, where. A study conducted by RIWA in 1989 near chemical industry discharges has provided a great deal of data in the form of Ames-test results and substance concentrations [Van Dorenmalen, 1990]. The results of this study have led to more biologically oriented research.

The use of microbiotest systems

When concentration techniques are used for toxicity tests, usually only a limited amount of concentrate is available, due to high costs and logistical problems associated with making large amounts of concentrate. In order to be

able to perform toxicity tests with limited amounts of concentrate, a number of so-called microbiotest systems is applied [Willemsen *et al.*, 1995]. A microbiotest is a complete toxicity test which uses very small organisms, thus allowing tests to be performed in a short time with a small amount of test medium. In most cases, the detection of an effect is not based on a gross response such as lethality, but rather on the measurement of a sub-lethal physiological response (for example enzyme inhibition or inhibition of photosynthesis).

Toxicity studies on surface waters aim to show findings about the water quality and to make correlations in terms of toxicity, without necessarily identifying which individual substances are responsible for the toxicity. Because all organisms and all biological processes have different sensitivity for various toxic compounds, and the types and concentrations of the toxic compounds in surface water can vary significantly, the preference in toxicity studies is to make use of various test systems, a so-called test battery. The effects that appear in the exposed community resulting from the presence of toxic compounds can only be approximated by using a series of toxicity measurements that reflect the species-specific variations in sensitivity. Because the application of microbiotest systems requires a minimum investment of time, material and space, the cost for conducting multiple tests remains relatively low.

Risk assessment

In the Netherlands, the ecotoxicological Maximum Tolerable Risk for ecosystems (MTR) for an individual compounds is defined as the concentration which can cause an adverse effect on a maximum of five percent of all species potentially present in an ecosystem during continuous exposure. The environmental condition whereby the MTR appears is also known as the HC_5 (5% Hazardous Concentration). If under the prevailing environmental conditions, a larger percentage of the species show negative effects, then this is considered unacceptable. For the risk assessment of individual compounds it is arbitrarily defined that a concentration corresponding to 1/100 of the MTR represents a negligible risk to the ecosystem.

Where multiple micropollutants exist, such as by the surface water discharges of toxic compounds, the ecotoxicological risk can be defined in a similar way. The MTR-level is the level which affects a maximum of five percent of the potentially present species. Based on the experience that the differences in the sensitivity of species are significantly reduced by exposure to complex mixtures [De Zwart and Slooff, 1983], a smaller safety margin (for example 1/10 instead of 1/100 of the MTR) is sufficient for a negligible environmental risk.

If a minimum of three acute toxicity observations is available, then the HC_5 value for acute effects can be estimated [Aldenberger and Slob, 1993]. Where the MTR concentration is defined as the limit value for the appearance of marginal effects in a maximum five percent of the potentially present species by chronic exposure, the calculated acute HC_5 value requires a safety margin or extrapolation factor. Based on the findings of the EPA in the United States [US-EPA, 1991], a factor of ten is used.

Toxic Potential

The toxicity of an environmental sample of unknown composition can be given as a 'pT-value' (toxic potential) which sets the actual toxicity of the sample on a scale showing the possibility of ecosystem damage. The pT-value is essentially set equal to: $^{10}\log HC_5$, where the chronic HC_5-value is given as the dilution or concentration factor with respect to the original conditions in the surface water. A pT-value equal to '0' corresponds to the MTR. With values larger than 1, the chance of environmental damage is considered to be negligible, while with negative values there is the possibility of acute effects. In table 1, the numerical pT-values are related to the above interpretations of the ecotoxicological environment quality.

Table 1 The pT-value with respect to the risk of ecosystem damage

pT-value	ecotoxicological environmental quality
>1	negligible risk for ecosystem damage
1	target value
0	Maximum Tolerable Risk (MTR) for the ecosystem by chronic exposure (limit value)
0 < pT-value < 1	chance of incurring acute effects in **less** than 5% of the potentially present species
< 0	chance of incurring acute effects in **more** than 5% of the potentially present species

Significance for the consumption of drinking water

It is not yet possible to relate the different toxicity test scores for untreated surface water to the possible health implications for drinking water consumers. However, the results of the different aspects of the study do give a general indication of the quality of the untreated surface water.
Due to the uncertainties concerning the eventual human health effects,

particularly in the longer term, it is preferable to use surface water with a low toxicity test score for the production of drinking water. It is therefore the desire of the drinking water companies to use surface water that is unpolluted in every respect.

Macro-invertebrates

In addition to the assessment of the water quality based on substance specific analyses or toxicological tests, the composition of the aquatic fauna can also indicate the quality and conditions of ecological communities and thus general water quality. The study of macro-invertebrates is considered to be the most informative for ecological studies.

Macro-invertebrates are often used for biomonitoring because they are ubiquitous, have varying reactions to disturbances in water quality and habitat, and have a basically sedentary nature. Also, due to their relatively long life cycles, they give an indication of the water quality integrated over a long period. The macro-invertebrates thus act as continuous monitors of their habitat [Rosenberg and Resh, 1993].

Since 1983, the composition of macro-invertebrate in large rivers has been determined by sampling artificial substrates and cast skins of insects (exuviae) [Frantzen, 1991; Ketelaars *et al.*, 1993; Ketelaars and Frantzen, 1995]. For determining the macro-invertebrate composition in the entire river-basin of the Rhine, the exuviae method was chosen out of practical considerations. The sample collection and determination of exuviae can be done relatively quickly and no special permission is required from the authorities for sample collection. With the artificial substrate method, baskets must be fastened to weirs, sluices or other structures, and permission is required. Another advantage of the exuviae method is that the macro-invertebrate fauna of all habitats are sampled. This in contrast to the artificial substrate method, which only samples the fauna of hard substrate.

The method also has disadvantages. In general, the collected exuviae originate from regions about 1-2 kilometres upstream of the sampling location [Wilson and Wilson, 1984]. With high river discharges, exuviae from even further upstream can be sampled [Klink and Moller Pillot, 1982]. Also, due to inflow from tributaries and streams upstream of the sampling location, unrepresentative samples of the studied river can be obtained. Another disadvantage is that weather conditions can influence the sample collection. If it has rained heavily in the period before the sample collection then no insects will have flown out and exuviae will possibly have sunk in a rain-shower. Also, the period in which exuviae can be found is short in comparison to the period in which larvae can be found. Therefore, a representative sample of the insect fauna present cannot always be gained. The final disadvantage is that only a part of the macro-invertebrates are sampled. On the other hand, all habitats are sampled and the exuviae can be identified to a lower taxonomic level.

Only a few studies are known in which the macro-invertebrates in the entire Rhine basin have been investigated. Lauterborn [1916; 1917; 1918] described the fauna of the Alpine Rhine up to the mouth in the Netherlands in what is now a valuable reference volume. In Knöpp [1957] a general overview of the water quality based on river fauna is given, without going into the species composition. Wilson and Wilson [1983; 1984] have given an accurate description of the chironomid fauna in the whole river-basin based on exuviae. Most other researchers have restricted themselves to only parts of the river basin (among others: Caspers [1980[a], 1980[b]; 1990], Klink and Moller Pillot [1982], Jatzek [1990], Titizer *et al.*, [1990], Schiller [1990], Van den Brink [1990], Van der Velde *et al.*, [1990], Van Urk and Bij de Vaate [1990], Frantzen [1991; 1993], Bij de Vaate and Greijdanus-Klaas [1993] and Ketelaars [1993]).

Goal of 'TOR' '94'

The 'Toxicological and Ecological Study of the Rhine 1994' which is described in this report, attempts to answer the questions that arose from previous (toxicological) water quality studies performed by RIWA. For this purpose, a series of (eco- and geno-) toxicity tests and ecological parameters have been used in order to gain as complete a picture as possible of the water quality related to these effect parameters. The specific research questions of TOR '94 are as follows:

1. Based on the results of the conducted tests, where does a change in water quality of the Rhine River occur?
2. What toxicological and ecological quality can be attributed to the Rhine River based on the test results?
3. Do the different parts of the study give a consistent picture of the changes in water quality?
4. Does this study confirm the previously identified differences between the rivers Rhine and Meuse?
5. Is there, on the basis of corresponding chemical analyses, an explanation for the changes in water quality found on the basis of the ecological and toxicological studies?

Set up of the study

The 'Toxicological and Ecological Study of the Rhine 1994' consisted of two main parts which were conducted more or less independently of each other:

- The toxicity study: Using a motor ship, three sampling series were conducted and the water samples were processed using XAD isolation. Various toxicity tests were then performed using the prepared concentrates.
- The ecological study: one sampling series was conducted to study the presence of exuviae. The collected samples were preserved on-site and were identified and counted later in the laboratory.

This report describes the set-up, organisation, execution, results and conclusions of the study. In addition, comparisons are made between the results of the different parts of the study. Finally, some recommendations for follow-up research are made.

Responsibility

The study was performed according to the proposal '936017/JvdB' dated September 21, 1993, and the contract dated October 20, 1993 with reference '3/2: 21748'. Where necessary, the actual research performed was adapted with respect to the proposed work, after consultation with the steering committee.

2. SET-UP

In order to meet the objectives of the study, the 'Toxicological and Ecological Study of the Rhine 1994' was designed as follows:

First, RIWA selected research methods that could be used to give a sufficiently clear picture of the toxicological and ecological status of the Rhine, and help answer the study questions. Furthermore, they selected twelve locations in the Swiss, French, German and Dutch regions of the Rhine River basin for sample collection.

For the toxicological study, samples were collected at three separate times during 1994, in order to gain an understanding of eventual seasonal influences and to see if the results over a year were consistent. The water samples for the toxicity tests were concentrated using XAD isolation techniques. Samples for the ecological study were collected once, during the period when the life cycle of the relevant macro-invertebrates would result in the most reliable research results. The ecological and the toxicological aspects of the study were performed separately; however, it was decided to combine the reporting and interpretation of these two studies.

2.1 Sample locations

The twelve sampling locations selected by RIWA are shown on the (fold-out) map of the Rhine River basin in appendix 11 of this report.
The Bodensee was sampled once during the toxicological study, as a 'negative control', that is to say that it was assumed beforehand that no toxicity would be present. The sampling location "Rheinfelden" was chosen because the bridge over the Rhine that connects the German and the Swiss parts of the city marks the most upstream point of the river which is navigable for inland ships. The IJsselmeer was included in the study because it is made up of sixty percent Rhine water, which makes it possible to determine the effect of dilution and biological degradation of toxicity. The (10) sample locations in between Rheinfelden and the IJsselmeer were specifically selected because of the availability of long-term chemical water quality monitoring results at these points.

The sample collection for the toxicological and ecological study was performed at essentially the same locations, as shown in appendix 11. For the ecological study, an extra sample was collected from the river Waal at Vuren, because this is a standard monitoring location.

2.2 Research Methods

The following research methods were selected by RIWA because they are practical to conduct and results can be readily interpreted:
- genotoxicity:
  - Ames-test;

- ecotoxicity:
  - *Daphnia*-IQ;
  - Rotoxkit-F;
  - Artoxkit-M;
  - algae-photosynthesis;
  - Microtox;

- enzyme inhibition:
  - cholinesterase inhibition;

- ecology:
  - exuviae.

These tests are described in more detail in Chapter 3.

The results of these tests, give a good indication of the toxicological and ecological quality of the Rhine water from the Bodensee to the IJsselmeer. The toxicological quality is expressed in a so-called toxicity parameter. In addition, the usefulness of the various performed toxicity tests is also specified.

2.3 Analytical Laboratories

The different water quality tests were conducted by various laboratories. The XAD isolation and the Ames-test were performed by the Laboratory for Organic Analysis and the Laboratory for Microbiology, Kiwa. The colour spectra were also measured here. The ecotoxicity tests were conducted by the Laboratory for Ecotoxicology and the Laboratory for Water and Drinking Water (Algae-photosynthesis), RIVM. The Laboratory of GWA determined the cholinesterase inhibition. The identification of exuviae was performed by PWN and WBB.

3. EXECUTION

In this chapter, a description of the research methods is given and the sample collection and the sample pre-treatment are discussed. After the description of the ecotoxicity tests, the method for calculating the pT-value, the toxicological potential, is presented. A more detailed description of the sample collection with respect to the toxicological study is given in appendix 1.

In order to guarantee as much as possible the quality of the study, the following were used:
- (inter)national directives for sample collection;
- (STERLAB) recognized analyses and tests;
- professional literature.

3.1 Genotoxicity

3.1.1 Ames-test

In the neutral and acid fractions that are obtained using the XAD isolation procedure, the presence of mutagenic or promutagenic substances is established by means of the Ames-test. The principle of the Ames-test is described in detail by Maron and Ames [1983] and with slight modifications is made suitable for the testing of water [Kool *et al.*, 1982; Van der Gaag, 1985; Van der Gaag and Oranje, 1984]. For testing the above mentioned fractions, only the TA98 strain of *Salmonella typhimurium* is used. The test is performed both with and without the addition of S-9 mix of homogenated rat liver [Ames *et al.*, 1975].
From each pH-fraction, a concentration series is set up that corresponds to the tests of 0.3, 0.5, 1.0, and 2.0 litre equivalents per plate. The tests are conducted in duplicate within four week after the sample collection. The presence of substances that could influence the growth of the bacteria is checked in routine controls [Kiwa, 1993]. For quality control in the Ames-test, the following aspects are checked:
- per batch, and additionally on a yearly basis, the strains are controlled for sensitivity;
- per test, the culture medium is tested for sterility and acidity;
- per test, positive controls are included: for the TA98 strain, picrolonic acid is used for the test without S9-mix, and benzo(a)pyrene for the test with S9-mix;
- per test, a negative control is included: a blank ethanol or an blank batch XAD concentrate.

A sample is considered mutagenic if the number of revertants counted per plate, after subtracting the number of spontaneous revertants, is at least twice the number of spontaneous revertants, and if there is a dose-effect relation in the tested series of concentrations. If the correlation coefficient is larger than 0.7, then a dose-effect relationship is considered to be present.

The following values are used for the scores with the strain TA98:

- < 100 revertants/l : slightly or not mutagenic
- 100 - 500 revertants/l : moderately mutagenic
- > 500 revertants/l : strongly mutagenic

3.2. Ecotoxicity

3.2.1 *Daphnia*-IQ

The *Daphnia*-IQ [ASTM, in press; Aqua Survey Inc., 1993[a], 1993[b]; Janssen *et al.*, 1993[a]] evaluates toxicity by measuring in-vivo the inhibition of an enzymatic reaction in the water flea *Daphnia magna*. In this toxicity test, young unfed individuals of the species *Daphnia magna* (age 24 - 48 hours) are exposed for one hour to a dilution series of an environmentally toxic concentrate prepared in Dutch Standard Water (DSW) (see table 2, section 3.6.2). After this exposure, a solution of the biomarker methylumbelliferyl-galactosidase (MUF) is added directly to the test container. This biomarker consists of a fluorescing compound whereby the fluorescence is inhibited by being bound to a sugar. The hungry water fleas quickly ingest this compound, after which the organisms with a functional enzyme system release the fluorescence by degradation of the sugars. The fluorescence can easily be seen in so-called black light.

The test determines the percentage of organisms that diminish in fluorescence within fifteen minutes after the adminstration of the biomarker, compared to the organisms in the control. The toxicity is expressed as the concentration whereby fifty percent of the animals show this effect (EC_{50}). This value should be interpolated within the series of test concentrations. The quality criterium for the validity of the test specifies that in the blank, a minimum of 5/6 of the number of exposed organisms should fluoresce.

3.2.2 Rotoxkit-F

The Rotoxkit-F is a bioassay developed for measuring acute toxicity in water samples. Newly hatched juvenile rotifers of the species *Brachionus calyciflorus* are used as test organisms [Janssen *et al.*, 1993[b]; Snell and Persoone, 1989]. As a measure of the toxicity, the percentage mortality is determined after 24 hours of exposure to a dilution series of a sample.

The hatching of *Brachionus calyciflorus* occurs after the cysts (eggs) are watered with DSW during fifteen minutes in the light (1000 - 4000 lux) and 16 - 18 hours in the dark. The standard fresh water medium as well as the cysts and the other requisites for the test can be found in a commercially available kit. Directly after hatching, the larvae are exposed to a dilution series of the environmental concentrate. After 24 hours, the percentage mortality for each concentration is determined.

The toxicity is expressed as the concentration whereby fifty percent mortality appears with regard to the control (LC_{50}). This value should be interpolated within the series of test concentrations.

3.2.3 Artoxkit-M

The Artoxkit-M is a bioassay for measuring the acute toxicity of chemicals or effluents in water samples [Van Steeregem and Persoone, 1993]. Newly hatched juvenile crustaceans of the species *Artemia Salina* are used as test organisms. The percentage mortality is determined as a measure of the toxicity of a sample.

The hatching of *Artemia Salina* occurs after the cysts (eggs) are watered with a synthetic seawater medium during one hour in the light (1000 - 4000 lux) and 16 - 18 hours in the dark. The standard medium and the necessary salts as well as the cysts and the other requisites for performing this test can be found in a commercially available kit. The hatched larvae are exposed for 24 hours to a dilution series of an environment concentrate. After 24 hours, the percentage mortality in each concentration is determined.

The toxicity is expressed as the concentration whereby fifty percent mortality appears with regard to the control (LC_{50}). This value should be interpolated within the series of test concentrations.

3.2.4 Algae-photosynthesis

The toxicity is determined by measuring the inhibition of the photosynthesis rate of populations of the algae *Selenastrum capricornutum* which are exposed under continuous light to a concentration series of a toxin at a temperature of 20°C [Tubbing *et al.*, 1993].

A set of algae cultures is exposed to a dilution series of an environmental concentrate for four hours. In addition, a known amount of radioactive bicarbonate is added to the algae test media as well as a known amount of dissolved normal bicarbonate. After a short incubation in light, the test media are filtered through membrane filters. After drying the filters, the radioactivity assimilated by the algae is measured by liquid scintillation counting. The total amount of carbon production is given by the product of the fraction of radioactive carbon production and the total amount of inorganic carbon present directly after the labelling. The photosynthesis rate during the incubation is equal to the amount of carbon production divided by the incubation time. By correlating the resulting photosynthesis rate with the exposure concentrations, a concentration-effect relation is determined. From this, via interpolation, the concentrations that cause photosynthesis inhibition of respectively ten and fifty percent (EC_{10} and EC_{50}) can be calculated.

3.2.5 Microtox

The Microtox is a standardised test system developed for measuring acute toxicity in water samples [Bulich, 1979; Bulich and Isenberg, 1980; Bulich *et al.*, 1981; Chang *et al.*, 1981; Levy *et al.*, 1989; Isenberg, 1993]. The bio-luminescent bacteria strain *Photobacterium phosphoreum* is used as a test organism. This marine bacteria produces the energy rich substance luciferin as a by-product of the citric acid cycle. Under influence of the enzyme luciferase this substance releases energy in the form of light (luminescence). If the bacteria come into contact with a toxic substance that interferes with the cell metabolism, the cell membranes are disturbed and the production of luciferine is inhibited, which can be seen as a decrease in luminescence. The level of reduction in bio-luminescence is proportional to the toxicity.
As a marine bacteria, *Photobacteria phosphoreum* is an obligatory halophile. Therefore, before the test, the environmental concentrate and the prepared dilution series are brought to the correct osmotic value. During the test the bacteria are exposed for fifteen minutes to various concentrations of the sample. After five and fifteen minutes, the bio-luminescence is compared to that of the control.
The concentration-effect relation is determined by statistical analysis. The toxicity of a sample is defined as the concentration whereby a twenty percent reduction in luminescence occurs compared to the blank ($EC_{20,t}$). This value should be interpolated within the series of test concentrations.

3.2.6 Calculation of the pT-value

For calculating the acute pT-value following the method of Aldenberg and Slob [1993] the computer programme ETX 1.3a [Aldenberg, 1993] is used. According to the log-log extrapolation model, the median value of the acute HC_5 is calculated from the available EC- and LC-values. The acute pT-value is then calculated using the formula:

$$pT\text{-}value_{acute} = {}^{10}log\ HC_5$$

An estimate of the chronic pT-value is calculated by:

$$pT\text{-}value_{chronic} = {}^{10}log(HC_5 / 10)$$

3.3 Enzyme inhibition

3.3.1 Cholinesterase inhibition

This analysis measures the inhibition of the enzyme cholinesterase caused by the sum of all inhibiting substances present in the sample. The contribution of each specific substance is a function of both the concentration and the inhibiting power of that substance.

In order to concentrate the samples, a three-step extraction with dichloromethane is first made, followed by an evaporation step. Not only the samples but also the standards are treated with bromide in order to convert the various substances into an 'active' form (ethylparathion is changed into paraoxon using bromide). The excess bromide is bound by albumen protein. In the auto-analyzer, the enzyme cholinesterase is mixed with the sample at 37°C, and depending on the sample, a part of the enzyme is inhibited. This mixture is then mixed with the substrate butyrylthiocholineiodide, again at 37°C.

The non-inhibited cholinesterase enzyme will convert the substrate into thiocholine. This substance is then dialysed with a membrane and reacted with chromogen (2,2-dinitro-5,5-dithio-dibenzoic acid) after which a yellow coloured compound is formed which can be measured at 420 nm. With more cholinesterase inhibiting substances in the sample, less thiocholine is formed, whereby a decrease in coloration takes place.

3.4 Ecology

3.4.1 Exuviae

Exuviae are the cast skins of animals having an exoskeleton (for example arthropods). In the current study, exuviae are defined as the cast skins of aquatic insects (nymphs or pupae). Exuviae are relatively easy to identify to genus or species level. After the insects have flown, the exuviae remain floating on the water surface for a certain period. By skimming the water surface, the exuviae can easily be collected. After identification and counting, some insight of the diversity of the insect fauna and the dominant taxa can be gained.

The composition of the river fauna present is determined by a number of factors. First of all 'natural' factors such as bottom composition, current speed, temperature, geographical orientation of the river, etc have an influence on the composition of the insects-fauna. The influence of human activity on the river ecosystem is actually the most important aim of this study. In the interpretation of the collected data it is assumed that in places with a large diversity and a large number of pollution sensitive species, relatively undisturbed conditions are found. In addition, based on the available information about the ecology of a collected species (or genus), which is unfortunately limited, it is checked if that species can tolerate chemical pollution. The dominant presence of species that can tolerate chemical pollution or heavy loads of organic substances indicates pollution in that part of the river.

The species diversity and whether or not dominant presence of a pollution insensitive taxa occur is evaluated for each sample location in the river. Comparing the results between the different locations gives a relative assessment of the ecological condition of the Rhine River. In this analysis, historical data of the Rhine fauna and data from other rivers are also taken into account.

Identification

In the laboratory, the collected sample was rinsed with distilled water in a fifteen litre bucket and was homogeneously mixed with a stirring spatula. From this mixture, two sub-samples consisting of about two hundred exuviae each were taken using a glass beaker. The remainder of the sample was put in a sample jar and preserved with 80% ethanol. The two sub-samples were put into separate white dishes and analyzed independently.

The white dish was placed on a light tray and using tweezers, all exuviae were put into 20 ml sample containers, filled with ethanol (80%) and given a label. In some cases, it turned out that less than four hundred exuviae were collected. In table B10 (appendix 9) the number of exuviae identified per location is given.

For exuviae identification, the sub-sample was flushed with ethanol (80%) into a glass petri dish. From here all exuviae were placed in a glycerine-filled petri dish using a pair of tweezers. Then, all exuviae were identified and counted. The main groups were identified using Wilson and McGill [1982]; Chironomidae were identified using Langton [1991; 1992] and Wiederholm [1986]; Trichoptera were identified using Ulmer [1909], and Lepneva [1964; 1966]; and Ephemeroptera using Schoenemund [1930] and Elliot *et al.*, [1988].

The main groups of exuviae were kept separately in 80% ethanol. For each new taxon at least one preparation was made. For the Chironomidae, the exuvium (on the ventral side) was placed on an glass slide in a drop of glycerine. Under the stereo microscope (magnification 6-200 times) the lower body (abdomen) was separated from the rest (head and thorax) and these were put side by side and covered with a cover slip. Exuviae of the other main groups were placed on the dorsal side in a drop of glycerine on a glass slide. These preparations were then sealed with clear nail polish and labelled.

A taxon was designated as dominant if the percentage composition of the taxon was $\geq$ 20% of the total. A taxon was designated as sub-dominant if the percentage composition of that taxon was $\geq$ 20% of the remaining sample.

Quality assurance

In order to assure the quality of the identifications, all species were controlled by both PWN and WBB. Problem cases were checked by an external expert

(Ir. A.G. Klink, 'Hydrobiologisch Adviesburo Klink', Wageningen, The Netherlands).

3.5 Sample Collection

3.5.1 Sample collection for the toxicological study

The three sampling series for the toxicological study were conducted by specially organised boat trips. In each sampling series, the samples were collected in a downstream progression (travelling from Switzerland to the Netherlands). The sample collection was performed in accordance with national and international directives [NPR 6600, 1993; ISO 5667-6, 1990].
A detailed description of the equipment and procedures used during the sample collection is given in appendix 1 of this report.

In order to immediately assess if there were any possible abnormal circumstances, field measurements were taken at each location. Based on these measurements, it was determined that there were no abnormal circumstances.

The three sampling series took place during the following periods:
- February 22 to March 1;
- June 7 to June 14;
- October 4 to October 11.

During each sampling trip, the largest part of the sampling route was generally covered in a period from Tuesday to Friday. The following Monday and Tuesday the last locations were sampled. No samples were collected at night or during the weekend. On the basis of actual data on current speed it was checked if there was question of a 'Fliessende Welle' (accidental spill) situation. From these data, which are further explained in appendix 4 of this report, it appears that in each sampling series there was a Fliessende Welle situation at two or three locations relevant to one of the more upstream sampling locations. Given the fact that there were only minor fluctuations in the water quality, the results of all of the sample locations may be interpreted as Fliessende Welle. Therefore, is it possible to assess the instantaneous quality changes.

During every sampling series the collected samples were kept refrigerated on the boat (see appendix 1). After the last samples were collected at 'Hagestein', the samples were taken for processing to the Kiwa laboratory. The sample collected at location 'IJsselmeer' was transported to the laboratory on the same day.

Because the samples were collected by boat, while the sample processing was performed in the laboratory, the time between collection and analysis was relatively long. Thus, the influence of a long holding time on the toxicity of the water samples was investigated. It was found that the measured toxicity in the samples held for fourteen days was essentially the same as that in samples held for only one day, and if anything it was slightly less. From this it can be concluded that there is no fear of having an overly negative image of the water quality in the Rhine.

During some periods of sample collection there was some question of unusual circumstances, such as described in the last section of appendix 1. However, with the exception of the high water discharge during the second sampling series there were no indications that these circumstances were of any influence on the study results. The high discharge of the river did have a dilution effect, but this was accounted for by the calculation of the load (see section 4.4).

3.5.2 Sample collection for the ecological study

For the ecological study on the presence of exuviae in the Rhine River, one sampling trip was conducted in the period between August 8-11, 1994. This was the most suitable time period for the determination of exuviae with respect to the life cycle of the water fauna. This sampling was also done in a progressively downstream direction, from Switzerland to the Netherlands. The sample locations were visited by car.

The different timing of the sampling periods was a reason to separate the sampling of the ecological and toxicological studies.
In addition, the sampling procedures also played an important role: sample collection for toxicological studies should preferably be performed in the middle of a river [NPR 6600, 1993], whereas samples for the exuviae study should be taken at river banks.

Exuviae were collected by skimming off the water surface with a 300 mm hand net. The sampling was conducted where possible in sheltered areas and between stones.
The samples thus collected were rinsed in a white dish (PVC) in order to assess whether or not sufficient specimens (about four hundred exuviae) were collected. In case insufficient whole specimens were collected, sampling was repeated. Any large material collected was carefully removed by flushing. The total sample was collected in the net, and was flushed into a sample jar using a funnel and siphon with 80% ethanol. The sample jar was then further filled with the ethanol and labelled (sample location, number and date).

At each location, the acidity (pH), oxygen concentration, water temperature and the flow velocity were also measured.

3.6 Sample processing

For the toxicological study, the organic material was first isolated under neutral and acid conditions using an XAD resin, and then concentrated. In this section, XAD isolation is described. For the ecotoxicological tests, the prepared concentrates were subjected to an additional process, which is described after the XAD isolation (section 3.6.2).

3.6.1 XAD isolation

The processing of the samples in the stainless steel containers took place using the XAD isolation and concentration technique developed by Kiwa [Noordsij *et al.*, 1983; Noordsij *et al.*, 1984]. For this purpose, one hundred twenty litres of centrifuged sample water from every location were used. This sample volume was divided into equal parts and put into two glass reservoirs of sixty litres each.

Via a siphon system the water from a reservoir was fed through a purified Amberlite XAD-4 type resin. The adsorbent was in a vertical column with a bed height of 20 cm, and a bed volume of 60 ml. The rate at which the actual isolation took place was 1 bed volume/min., so that the total isolation time of a sample of 60 litres took almost seventeen hours. After the water of neutral acidity (pH=7) passed from a reservoir through the first column, it was acidified in-line with hydrochloric acid to pH=2 and then fed through a second, identical XAD column. After the isolation, the XAD resin from the two neutral columns was collected into one larger column. The same was done with the two "acid columns".

Both the loaded 'larger' XAD columns were then eluted with ten bed volumes of ultra pure water. In the case of the acid column, the ultra pure water was acidified beforehand with a hydrochloric acid to pH=2. This 'clean-up' took about an hour.

After evaporating any remaining water in the resin columns with nitrogen, both columns were eluted with five bed volumes of pure ethanol and five bed volumes of an azeotropic mixture of 30% (v/v) pure ethanol in cyclohexane. During this step, the eluate was filtered through a PTFE filter of 0.45 mm, whereby any spores of bacteria were removed. The elution took 2-3 hours.

The eluates from both fractions were concentrated in two steps. First, distillation took place to a final volume of 250 ml. Then in another set-up, a

further distillation was performed to a final volume of 4-6 ml. In the second distillation, a small calibrated test tube was used. Finally the volume was brought to 4.8 ml by evaporating the ethanol with nitrogen or supplementing with ethanol. By concentrating 120 liters of water to an extract of 4.8 ml, the final concentration factor was 25,000.

The volume of 4.8 ml from each of the two fractions was divided into equal portions; one part was used for the genotoxicity test (Ames-test), the other part was used for the ecotoxicological tests, after following the procedure for further processing described below.

During the processing of the samples there was one noteworthy point in the sample 'Weil am Rhein' from the third sampling series. The first portion of the XAD column for the neutral fraction turned a pink colour during the isolation step. This will be discussed further with the results of the Ames-test (section 4.2).

3.6.2 Processing of the concentrates for the ecotoxicity tests

On account of the toxicity of the ethanol used, the concentrates prepared using XAD isolation could not be directly used in the ecotoxicological tests. In order to remove the solvent, 30 ml Dutch Standard Water (DSW [NPR 6503, 1981], see table 2) was added to the concentrate, after which nitrogen gas was bubbled through the mixture at room temperature for 6 hours (flow=1 l/min). The remnant was then made up to 120 ml with DSW. This result was a water concentrate with a concentration factor of five hundred relative to the original water sample. The water concentrates thus obtained were tested for toxicity according to procedures within 48 hours.

Due to the extra processing of the concentrates (adding of water and bubbling with nitrogen gas) the composition of the concentrates obtained in this way differs with respect to the concentrates that are tested for mutagenicity using the Ames-test.

The concentrate obtained from the neutral isolation was used as the highest concentration for each of the performed ecotoxicity tests. The Microtox was an exception to this. Due to the nature of the salinity adjustment and the test procedure, it was not possible with the Microtox to test a concentration greater than 45% of the water concentrate (the highest test concentrate was therefore 225 times concentrated). The concentrates obtained by means of acid isolation were exclusively tested for toxicity using Microtox.

Table 2 *The composition of Dutch Standard Water (DSW).*

ingredient	amount
distilled water	1000 ml
$NaHCO_3$	100 mg
$KHCO_3$	20 mg
$CaCl_2.2H_2O$	200 mg
$MgSO_4.7H_2O$	180 mg

The EC_{50}- and LC_{50}-values determined in all tests are calculated with respect to a method blank (2.4 ml ethanol added to 30 ml DSW). Just as with the samples, the blank is bubbled with nitrogen gas for six hours and then supplemented with DSW to 120 g.

4. RESULTS AND DISCUSSIONS

In this chapter the results of the various research methods performed are presented and discussed. Hereby, the meaning of the results for the quality of the Rhine water is also examined and in as far as possible comparisons are made with the results from previous RIWA studies. The following tests will be successively presented: Cholinesterase inhibition, Ames-test, ecotoxicological tests, and exuviae research. Finally, the consistency of the combined research results are examined.

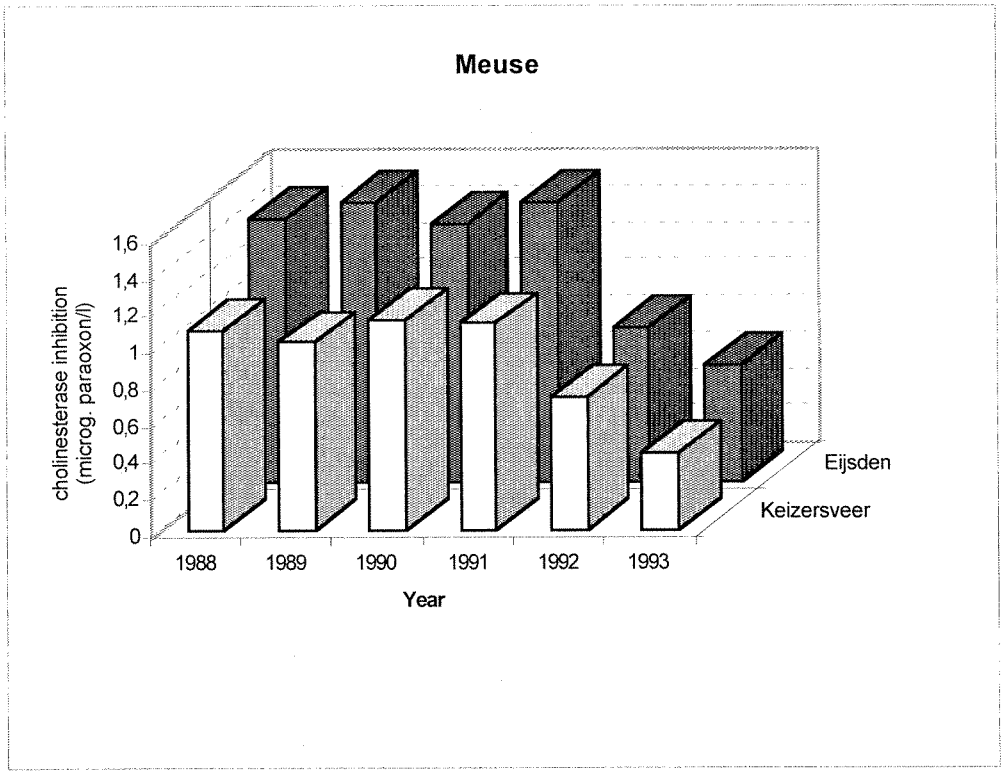
4.1 Cholinesterase inhibition

The results of the cholinesterase inhibition are shown in appendix 5 of this report. From these it appears that between 'Lauterbourg' and 'Hagestein', there is a question of cholinesterase inhibiting activity.

Upstream of 'Lauterbourg' and in the IJsselmeer at the location 'Andijk' there was no activity detectable (<0.10 mg/l = detection limit). Over the three sampling series, a decreasing activity was observed. The cause of this is not known, though it is likely that the difference in season/water temperature plays a role. Furthermore, it can be concluded from the results that cholinesterase inhibition activities do not occur much above the detection limit.

From a comparison with previous RIWA research it appears that for the locations 'Lobith', 'Hagestein' and 'Andijk' there has been a gradual decrease in the cholinesterase inhibition in the period 1988 to 1992 (see figure 2). The values found in the present study are in agreement with this trend. It can also be stated that the cholinesterase inhibition of Rhine water is significantly less than that of the Meuse ('Eijsden' and 'Keizersveer').

The cholinesterase inhibition activities in the period from 1988 for the Rhine and the Meuse were determined by two different laboratories. It is not expected that this is of any influence on the differences in the activities found in the two rivers.



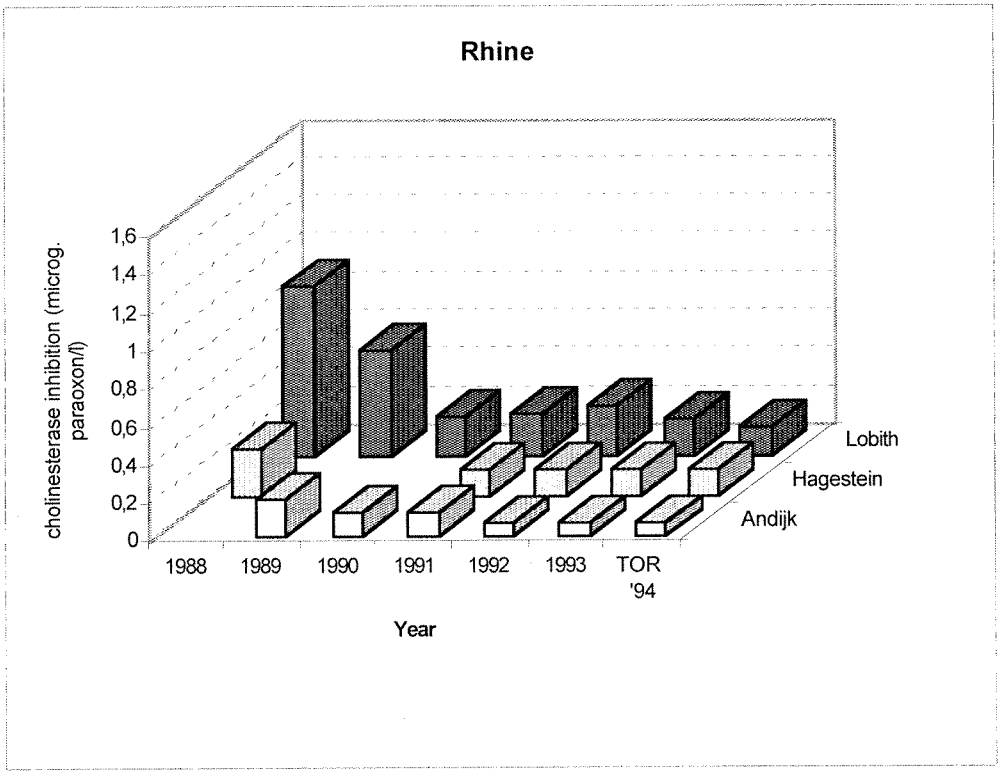


Figure 2 *Yearly average values for cholinesterase inhibition in the Meuse and Rhine Rivers from 1988-1993, including the average value of the 3 sampling events conducted for TOR '94.*

4.2 Ames-test

A complete summary of the Ames-test results for the three sampling series is given in appendix 6 of this report. These results are also shown graphically in figure 3; the results of the three successive sampling series are presented one below the other. The name of the sample locations shown by numbers can be found in the fold out page in appendix 11 of this report. For comparison of results, the same vertical scale is used for all three of the sampling series. The arrow (on the Y-axis) shows the direction of increasing toxicity.

As stated in the description of the Ames-test in section 3.1.1., a sample in the test is considered mutagenic if the number of induced revertants per plate (the total number of revertants minus the number of spontaneous revertants), is at least twice the number of spontaneous revertants, and if there is a dose-effect relation in the tested concentration series (correlation coefficient > 0.7).

If the above mentioned definition is adhered to then it can be stated that in most cases there was little or no mutagenic activity found with the strain TA98 in the Ames-test without S-9 mix. Only the samples from 'Weil am Rhein' from the first and third sampling series are an exception.

The mutagenic activity found in the samples from 'Weil am Rhein' with the Ames-test was the most pronounced in the sample from the third sampling series. During the processing, this sample caused a pink coloured band in the XAD column, which was eluted with ethanol. The eluate was further analyzed using GC/MS. The following substances (in order of decreasing concentrations) were found:

- C_2-benzene;
- possibly 2,5-dihydro-2,5-dimethoxyfuran;
- benzylbutylphthalate;
- dimethylnitrobenzene;
- a 'xanthine';
- isothiocyano-cyclohexane;
- ethylmethylbenzoate;
- possibly pyridylcarbinol-acetate;
- a urea compound;
- N-phenyl-1-napthaleneamine;
- tributylphosphate.

These compounds were found at a concentration of approximately 0.03 - 1.1 mg/l in the original water sample. By further fractioning of the eluate, it appeared that the pink colour did not occur in the fraction that could be analyzed via gas chromatography. The substance(s) that caused this colour appeared to be more polar in character. This sample was probably collected in the plume of a discharge.

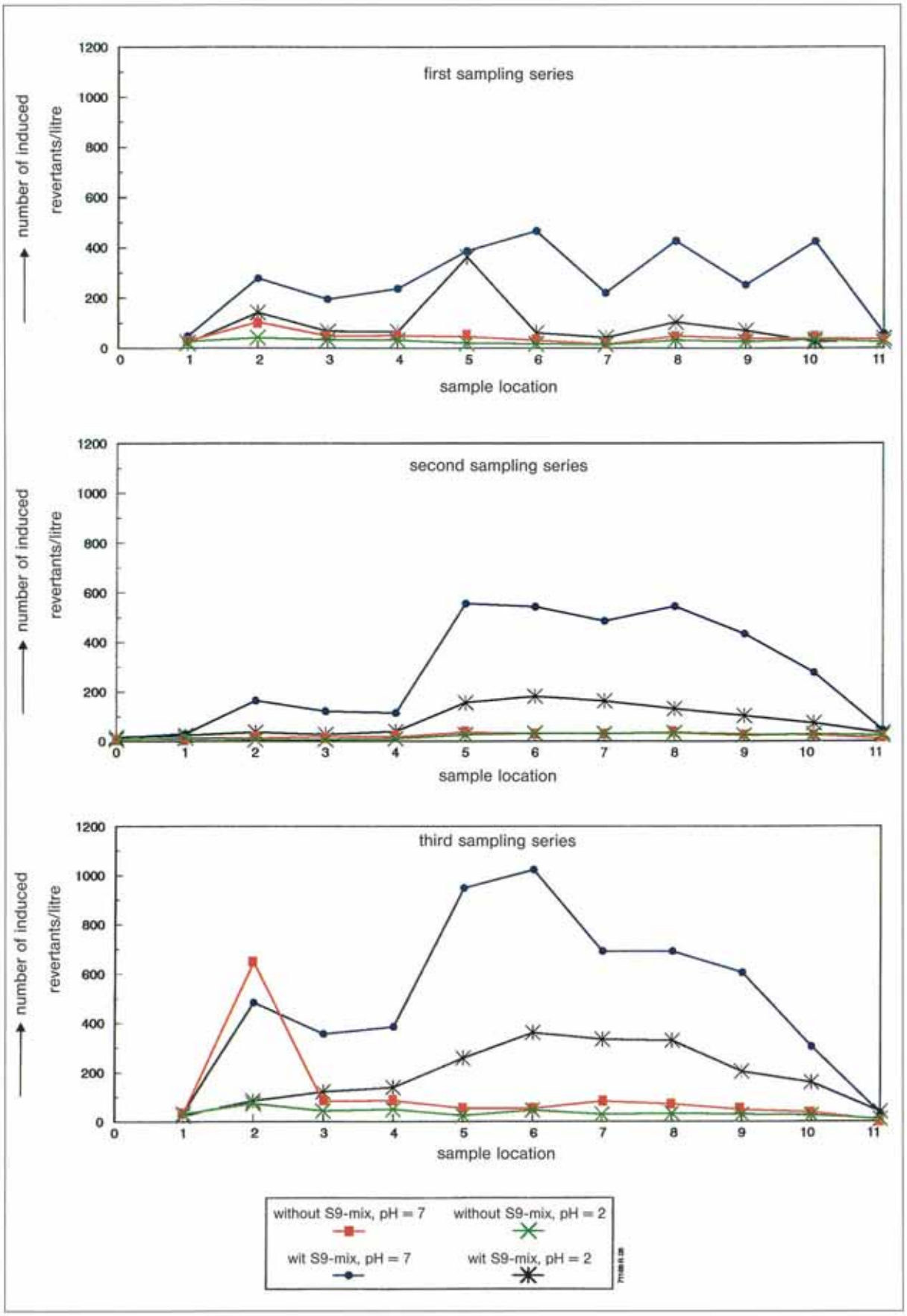


Figure 3: Graphical representation of the results of the Ames-test between the Bodensee and the IJsselmeer.

There was nothing in the results of the ecotoxicity tests indicative of the relatively high results of the Ames-test at the location "Weil am Rhein' from the third sampling series.
In comparison with the Ames-test without S9-mix, in the tests with S9-mix there was a clearly measurable mutagenic activity. This is particularly true in the neutral fraction of the samples from 'Weil am Rhein' to 'Hagestein'. This suggests the presence of so-called promutagenic substances in the Rhine water that can be converted in the body into mutagenic combinations. Considering the fact that no mutagenic activity was found in samples from the 'Bodensee' and 'Rheinfelden', the conclusion can be drawn that the mutagenic activity is mainly caused by human activity.

The absence of mutagenic activity in the IJsselmeer is probably due to a strong dilution of the concentrations present in the Rhine water and biological degradation. The absence of mutagenic activity in the Ames-test with S9-mix also applies for the acid fractions of the samples that were collected during the first sampling series at the locations 'Wiesbaden', 'Bad Honnef' and 'Köln'. The same applies for 'Weil am Rhein', 'Lauterbourg', 'Wiesbaden' and 'Hagestein' during the second sampling series and 'Weil am Rhein' from the third sampling series.

Per sampling series, a trend can be seen whereby the mutagenicity more or less slowly increases after 'Rheinfelden' until the Ruhr-area, and thereafter decreases. Also, it can be seen that the mutagenic activity in all samples from the third series was clearly higher than that in the samples from the first sampling series. This trend, as well as the level of mutagenic activity, agrees with the results of earlier studies performed on samples from the Rhine by Lobith [Puijker and Van Genderen, 1991; Puiker and Van Genderen, 1992]. It should be noted that the scores in the Ames-test for 'Lobith' showed a large spread depending on the time of sample collection. Thus, a slight to moderate increase or decrease of the score over a few years is difficult to perceive. A clear explanation for the increase of the mutagenic activity during the autumn and winter is still unknown. In relation to the Meuse, the TA98 strain in the Rhine had a score that was generally a factor five to ten higher. Finding an explanation for this is one of the research objectives of this project.

In general it can be stated that between 'Rheinfelden' and 'Andijk' a relatively moderate to high mutagenic activity was found. Further research should show what the meaning of this mutagenic activity is. In other words: it should be studied which individual compounds cause the mutagenic activity, whether these compounds can pass through water purification steps, and if so, what could be the consequences for the consumer. From previous research it has been shown that the purification techniques used in the Netherlands for the production of drinking water from surface water remove the mutagenicity as measured with the TA98 strain with S9-mix [Noij *et al.*, 1989]. At this moment, no conclusive answers can be given to the above mentioned questions. Thus, the need to press for a decrease of the discharge of mutagenic compounds remains undiminished.

4.3 Ecotoxicity tests

The results of ecotoxicity tests from the three sampling series are shown graphically in figure 4. The actual measurement results are given in appendix 7 of this report. The names of the (numbered) sample locations are given in appendix 11. The direction of the increasing toxicity is also shown (on the Y-axis) in this figure. For the sake of readability, the calculated EC- and LC-values are converted (inverted) to toxic units (Toxic Units, TU = 1/EC-value or 1/LC-value). In this manner, a higher value corresponds to a higher toxicity.

The reproducibility of the test results

For a correct interpretation of the toxicity data it is important to have some insight into the reproducibility of the applied tests. The literature reports that for repeated measurements of the *Daphnia*-IQ, the Rotoxkit-F and the Artoxkit-M on the same sample a variation of maximum fifty percent can occur [Persoone *et al.*, 1993; Hayes, 1994]. This considerable variability is in agreement with the standard *Daphnia* and fish tests and can be attributed to the use of just a small number of test organisms per test concentration. By the Microtox and the algae-photosynthesis, for which the response is integrated over a larger number of organisms, a variation of approximately twenty percent can in general be found. Therefore, it is possible to calculate a lower effect percentage with these two tests.

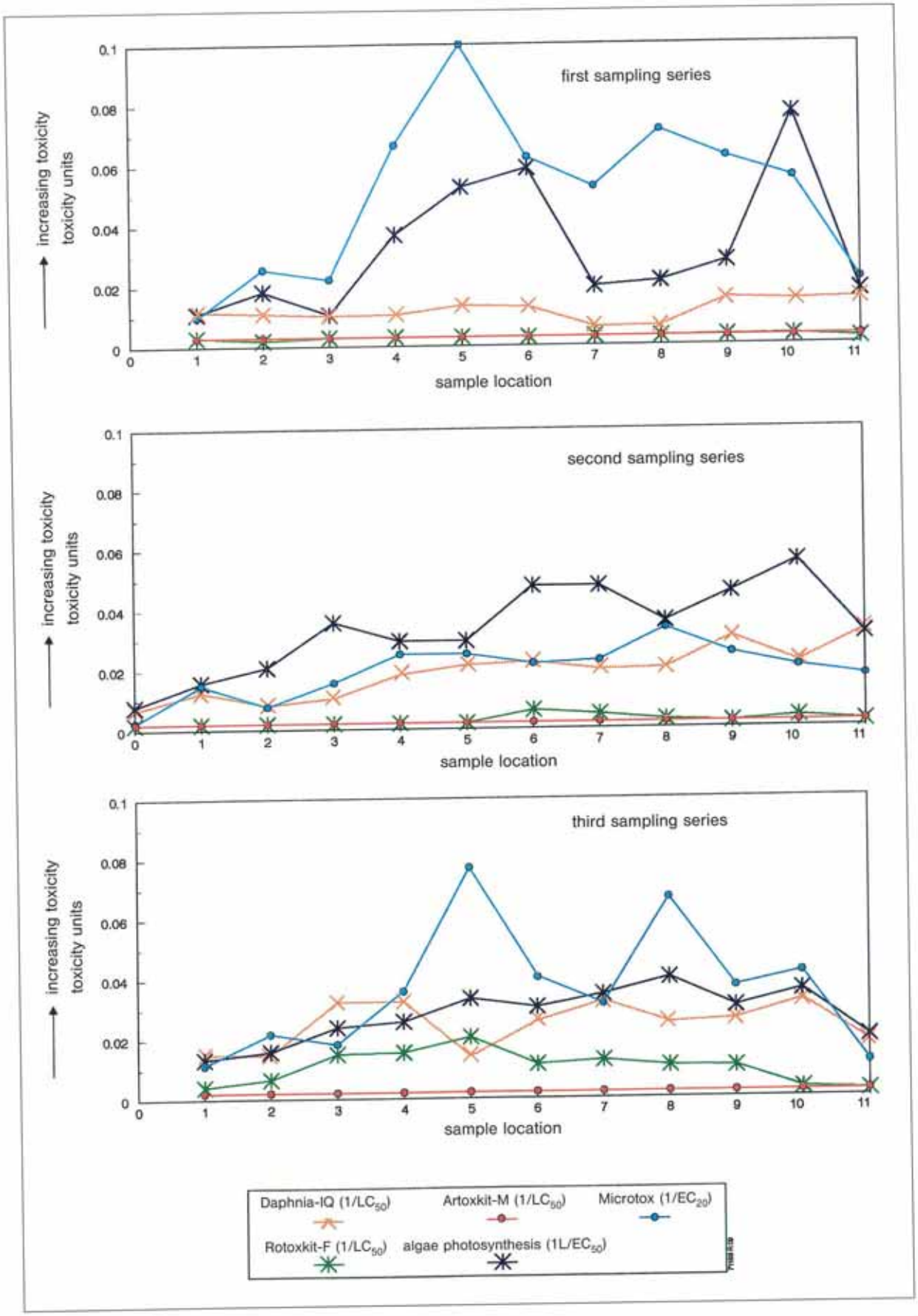


Figure 4 Graphical representation of the ecotoxicity test results between the Bodensee and the IJsselmeer.

Sensitivity and sensitivity spectra of the test systems
One of the first things noticeable when reviewing the test results is the low sensitivity of the Artoxkit-M. Not one of the five hundred times concentrated Rhine samples caused a single lethal effect on the *Artemia* larvae.

If all the toxicity results are converted to numerical values and statistically analyzed (see appendix 8), a ranking order of the test sensitivities can be drawn up. This is possible by comparing the median toxicity of all the measurements (hereby all the measurements for which median effect concentrations can be converted to numerical values (e.g. EC_{50}- or LC_{50}-values) are considered). This gives the following order of relative sensitivity:

1. Algae-photosynthesis;
2. *Daphnia*-IQ;
3. Microtox;
4. Rotoxkit-F;
5. Artoxkit-M.

The EC_{20}-values of the Microtox can be compared with the other EC_{50}- and LC_{50}-values. It appears that the Microtox in the first and third sampling series had a higher sensitivity than the algae-photosynthesis. In the second sampling series the algae-photosynthesis was only slightly more sensitive than the Microtox.

The low correlation coefficients in the correlation matrix of all converted numerical test results point to the fact that the sensitivity spectra of the four weighted test systems complement each other.

The ecotoxicological quality of the Rhine water
In all the measuring series, most of the ecotoxicological tests give, just as the Ames-test, more or less the same picture (figure 4). These show that the most upstream sampling locations have less toxicity than the other locations in the German and Dutch part of the Rhine. From 'Lauterbourg' to 'Düsseldorf' or 'Lobith' the toxicity is generally the highest. By the sample location 'IJsselmeer' a slight decrease of the toxicity was again measured. The river water in the first sampling series appears to have the highest toxicity with a clear increased toxicity by 'Koblenz' and 'Düsseldorf', as shown by the Microtox test. The third sampling series shows a somewhat lower toxicity, with similar peaks in levels of toxicity by 'Koblenz' and 'Düsseldorf'. The Rhine clearly has the lowest level of toxicity in the summer period.

In the beginning of the eighties, the untreated Rhine water in the Netherlands was acutely toxic for water organisms [De Zwart and Slooff, 1993]. Although slightly biased by the selectivity of the XAD isolation, the results from the current toxicity measurements show that the Rhine water must be concentrated a few dozen times before an acute toxic affect can be brought about.

In the tables below (tables 3-5), the results of the pT-value calculation are given for the three sampling series, together with the measurement results on which they are based.

If the measured toxicity from a specific toxicity test is given as '>500' or '<31' (see appendix 7), this indicates the estimated percentage effect is outside of the range of tested concentrations. For the purpose of calculating the pT-value, these results are replaced by 600 and 25 (number of times concentrated), which are equal to 120% of 500 and 80% of 31.

Table 3 Estimate of the median HC_5-values and the pT-values on the basis of the logarithmic extrapolation for the first sampling series

sample location (km marker)	*Daphnia*-IQ EC_{50} (*concen-trated)	Rotoxkit-F LC_{50} (*concen-trated)	Artoxkit-M LC_{50} (*concen-trated)	Algae photo-synthesis EC_{50} (*concen-trated)	Microtox EC_{20} 15 min (*concen-trated)	Acute HC_5-value (*concen-trated)	Chronic pT-value
Rheinfelden (149,0km)	86	316	600[1]	91	102	33.9	0.53
Weil am Rhein (171,3km)	91	600[1]	600[1]	55	39	13.0	0.11
Lauterbourg (352,3km)	99	338	600[1]	95	45	22.0	0.34
Wiesbaden (507,1km)	96	330	600[1]	27	15	5.1	-0.29
Koblenz (590,3km)	75	329	600[1]	19	10	3.0	-0.52
Bad Honnef (640,0km)	78	353	600[1]	17	16	3.8	-0.42
Köln (685,8km)	166	333	600[1]	51	19	9.5	-0.02
Düsseldorf (727,0km)	157	340	600[1]	47	14	7.0	-0.15
Lobith (862,3km)	65	319	600[1]	36	16	7.0	-0.1
Hagestein (946,9km)	68	316	600[1]	13	18	3.4	-0.47
Andijk (IJsselmeer)	66	600[1]	600[1]	56	46	13.0	0.11

[1]) result reported as '>500'

Table 4 Estimate of the median HC_5-values and the pT-values on the basis of the logarithmic extrapolation for the second sampling series

sample location (km marker)	*Daphnia*-IQ EC_{50} (*concen-trated)	Rotoxkit-F LC_{50} (*concen-trated)	Artoxkit-M LC_{50} (*concen-trated)	Algae photo-synthesis EC_{50} (*concen-trated)	Microtox EC_{20} 15 min (*concen-trated)	Acute HC_5-value (*concen-trated)	Chronic pT-value
Sipplingen (Bodensee)	149	600[1]	600[1]	125	403	75.3	0.88
Rheinfelden (149,0km)	80	600[1]	600[1]	63	67	18.5	0.27
Weil am Rhein (171,3km)	122	600[1]	600[1]	48	129	25.1	0.40
Lauterbourg (352,3km)	96	600[1]	600[1]	28	64	11.3	0.05
Wiesbaden (507,1km)	55	500	600[1]	34	40	8.5	-0.07
Koblenz (590,3km)	47	600[1]	600[1]	34	40	7.5	-0.12
Bad Honnef (640,0km)	45	163	600[1]	21	46	7.3	-0.14
Köln (685,8km)	51	211	600[1]	21	44	7.4	-0.13
Düsseldorf (727,0km)	50	359	600[1]	28	30	6.7	-0.17
Lobith (862,3km)	33	600[1]	600[1]	22	40	4.8	-0.32
Hagestein (946,9km)	47	277	600[1]	18	49	6.5	-0.19
Andijk (IJsselmeer)	25[2]	600[1]	600[1]	32	58	6.1	-0.21

[1]) result reported as '>500'
[2]) result reported as '<31'

Table 5 Estimate of the mean HC_5 values and the pT-values on the basis of logarithmic extrapolation from the third sampling series

sample location (km marker)	*Daphnia*-IQ EC_{50} (*concen-trated)	Rotoxkit-F LC_{50} (*concen-trated)	Artoxkit-M LC_{50} (*concen-trated)	Algae photo-synthesis EC_{50} (*concen-trated)	Microtox EC_{20} 15 min (*concen-trated)	Acute HC_5-value (*concen-trated)	Chronic pT-value
Rheinfelden (149,0km)	67	244	600[1]	75	88	25.3	0.40
Weil am Rhein (171,3km)	68	155	600[1]	63	46	16.6	0.22
Lauterbourg (352,3km)	25[2]	67	600[1]	42	55	7.8	-0.11
Wiesbaden (507,1km)	25[2]	65	600[1]	39	28	5.7	-0.24
Koblenz (590,3km)	71	49	600[1]	30	13	4.3	-0.37
Bad Honnef (640,0km)	39	88	600[1]	33	24[3]	6.3	-0.20
Köln (685,8km)	31	79	600[1]	29	32	6.2	-0.21
Düsseldorf (727,0km)	40	94	600[1]	25	15	4.2	-0.38
Lobith (862,3km)	38	97	600[1]	33	27	6.8	-0.17
Hagestein (946,9km)	25[2]	345	600[1]	28	24	4.3	-0.37
Andijk (IJsselmeer)	55	600[1]	600[1]	50	86	14.9	0.17

[1]) result reported as '>500'
[2]) result reported as '<31'
[3]) replaced by EC_{20}-value, 5 min

All measurement data of the ecotoxicity tests per sampling series and sample location have been converted to a chronic pT-value. This chronic pT-value is presented graphically in figure 5. From this it appears that only in the first sampling series and in the river segment between 'Bodensee'/'Rheinfelden' and 'Koblenz'/'Bad Honnef' can a gradual decline of the ecotoxicological water quality be seen. The river water downstream of 'Wiesbaden' has an ecotoxicological quality that lies slightly below the limit value (pT < 0). Therefore, the chance of some ecosystem damage cannot be ruled out. There is hardly any difference in the overall toxicity to be seen between the three sampling series.

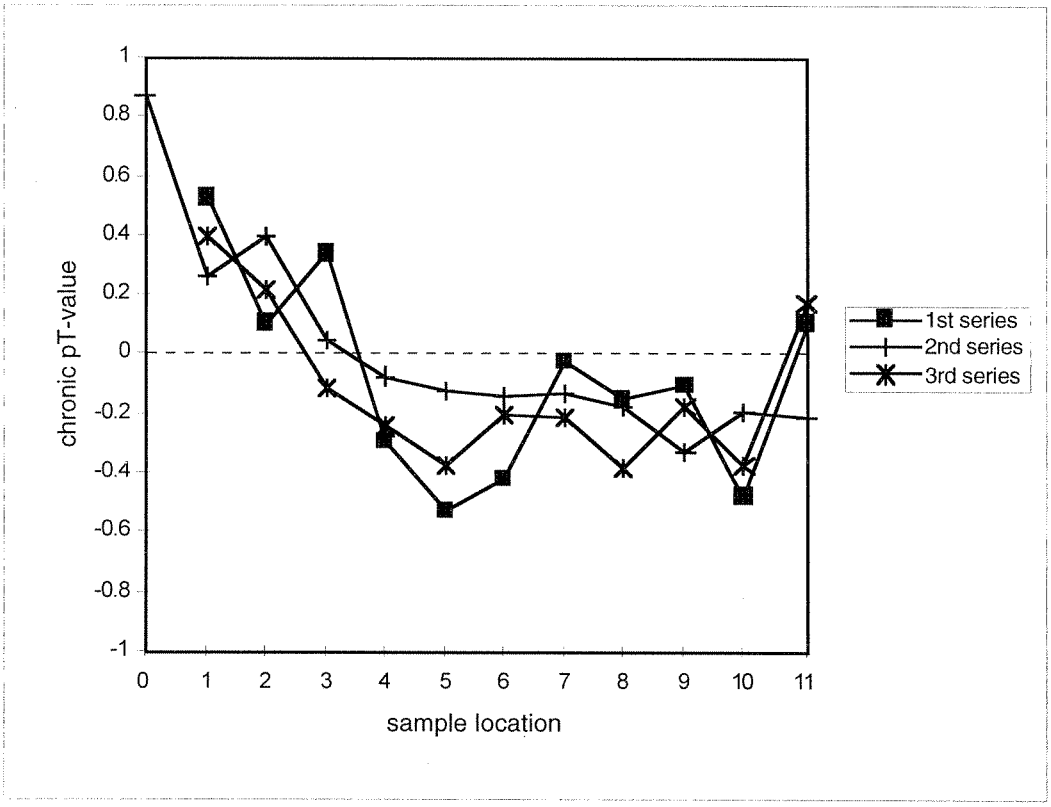


Figure 5 *Graphical presentation of the chronic pT-value calculated from the third sampling series.*

In comparison with the Meuse, the present study in the Rhine River has determined a pT-value that is slightly less negative than that for the catchment area of the Meuse in 1993. At the location Luik, a minimum pT-value of -1 was measured. For the sample locations between Tailfer and Keizersveer the pT-value was also negative [De Zwart and Polman, 1994]. It should be noted that the pT-value in this reference was calculated based exclusively on the Microtox test.

4.4 Calculating the load

In comparison with the measured scores based on toxicity tests, given as a concentration (number of induced revertants per litre for the Ames-test, or EC_{50} or LC_{50}-value for the ecotoxicity tests), the load gives a better indication of the amount of introduced toxicity or mutagenicity. For the Ames-test this is calculated

as a product of the concentration term and the Rhine discharge at the time and location of the sample collection; for the ecotoxicity tests it is the product of the 10th power of the -pT (10^{-pT}) and the discharge. Discharge data from the Bundesansalt für Gewässerkunde in Koblenz were available for the region in Germany downstream of Karlsruhe, and data from RIZA were available for locations in the Netherlands (see appendix 10). No discharge data were obtained for the stretch Basel - Karlsruhe. The results for the Ames-test (neutral pH with S9-mix) and the ecotoxicity tests are shown graphically in figures 6 and 7 espectively.

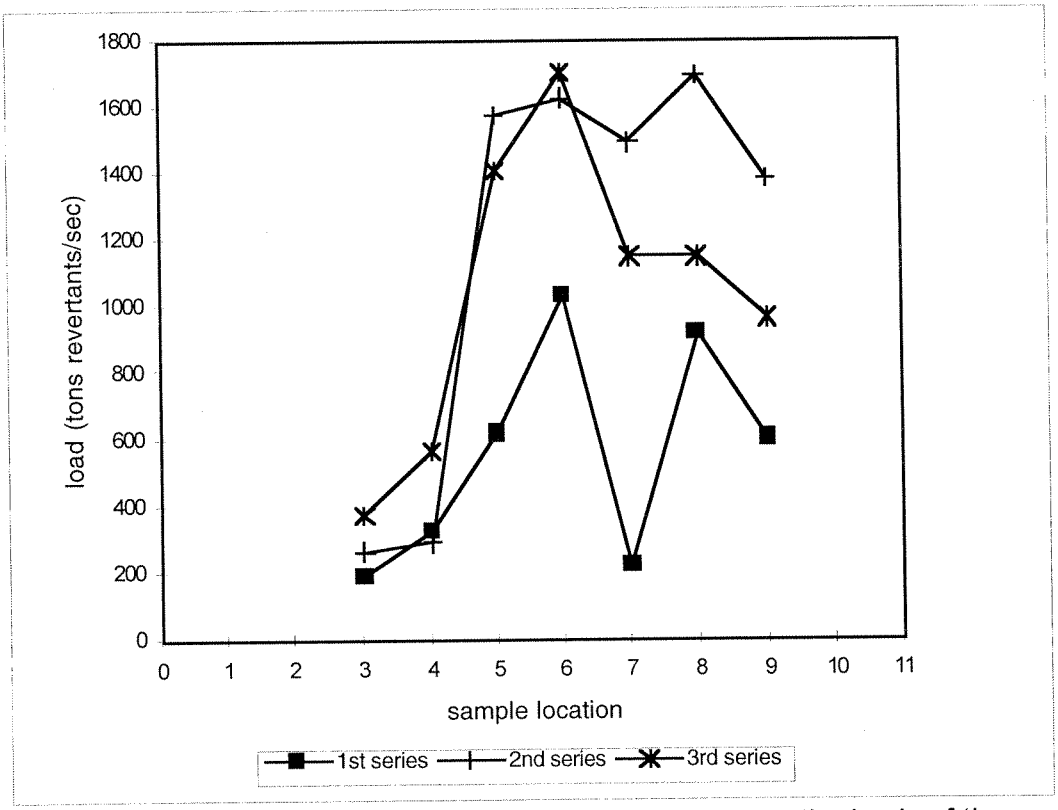


Figure 6 *The load of toxic substances in the Rhine on the basis of the Ames-test (neutral pH, with S9-mix) for the three sampling series along the trajectory 'Lauterbourg' - 'Lobith'.*

From figure 6 it can be seen that the introduction of mutagenic activity into the river occurs predominantly between 'Wiesbaden' and 'Bad Honnef'. This introduction was most prominent between 'Wiesbaden' and 'Koblenz' in the second and third sampling series. This result is striking because there is little industrialisation present in the section between 'Wiesbaden' and 'Koblenz'. It is unknown if the incomplete mixing of the water from the Rhine and the Main at 'Wiesbaden' plays a role, so that the influence of the Main is only measured at the location 'Koblenz' instead of at 'Wiesbaden'. This also applies to the mixing of the water from the Rhine and the Lahn at 'Koblenz'. Introduction of mutagenic activity between 'Weil am Rhein' and 'Lauterbourg' hardly occurs, because the increase of the discharge is in agreement with the slight decrease of the number of revertants/litre. In the Ruhr-area, there is hardly any further introduction of mutagenic activity.

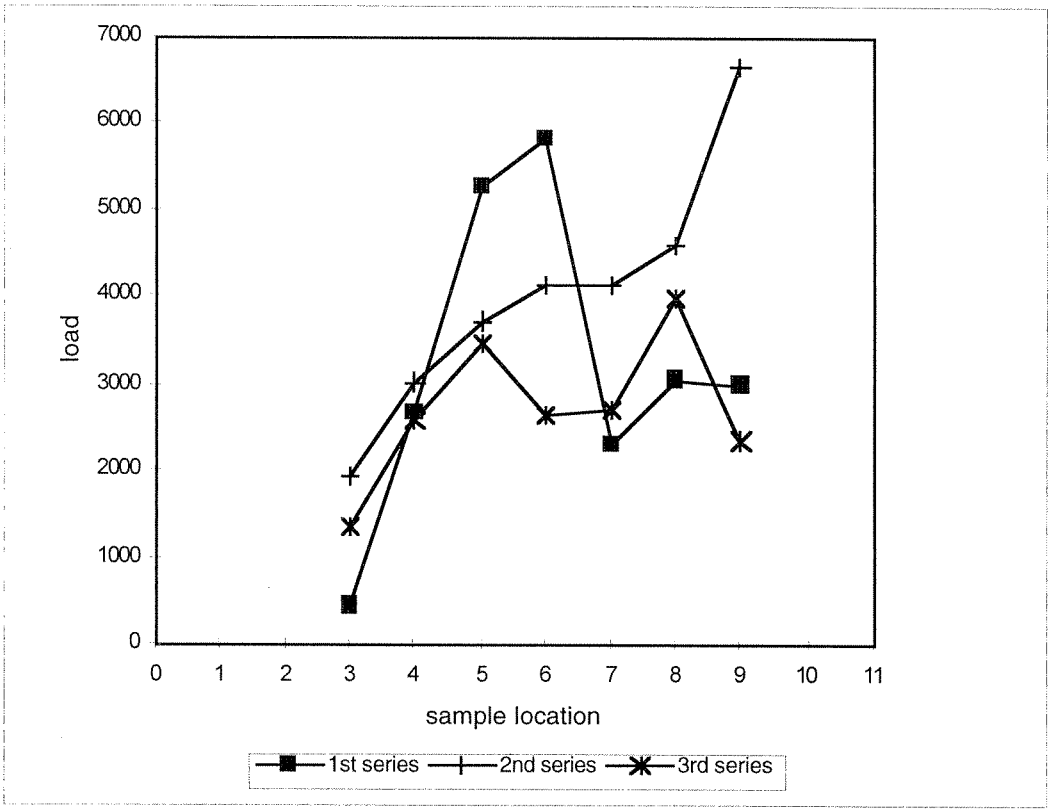


Figure 7 *The load of toxic substances in the Rhine on the basis of the pT-values for the three sampling series along the trajectory 'Lauterbourg' - 'Lobith'.*

From the ecotoxicological activity it is clear that introduction of toxicity does take place between 'Lauterbourg' and 'Wiesbaden'. This also applies for the stretch between 'Wiesbaden' and 'Koblenz' and to a lesser extent between 'Koblenz' and 'Bad Honnef' (figure 7). It is also true that there is hardly any introduction of ecotoxicity in the Ruhr-area.

In a general sense, the presentation of the results as loads gives some refinement of the water quality results as based on the Ames-test score and the toxic potential. The conclusions, however, remain unchanged.

4.5 Ecological study

The basic results are presented in appendix 9 of this report. A review of the results per location is given in section 4.5.1. In section 4.5.2 a more detailed interpretation of the results is given. In section 4.5.3 the results are compared with previous results for the Meuse River. In appendix 9 and in section 4.5.1 the extra location 'Vuren' is also discussed. Instead of a number, this sample location is indicated in appendix 9 as 'Vuren'.

4.5.1 Review of the results per location

Several different figures are used in presenting the results of the exuviae study. In figure 8, the total number of taxa divided over the groups 'rheophilons', 'indifferent' and 'stagnant' is shown for each location. In figure 9, the percentage distribution of the number of rheophilons taxa, with respect to the flow-indifferent taxa and stagnant water taxa is presented. In figure 10, the total number of taxa in each of the main taxonomic groups is shown. In figure 11, the percentage distribution of the main taxonomic groups is presented.
The total of the percentage distribution is not always 100% because not all taxa are sufficiently well known to be divided into one of the main groups. For this reason, the total number of taxa per sample location in figures 8 and 10 is different.

Rheinfelden (148.6 km)
At Rheinfelden, 25 taxa from five main taxonomic groups were found. This number of taxa is low in comparison with that found by Wilson and Wilson [1984] who, in 1981, sampled exuviae in the Rhine beginning at the source. At one sample location upstream of Basel, they found 37 taxa belonging to the Chironomidae. From the 25 taxa found in the current exuviae study, 21 belong to the Chironomidae. A further striking difference compared with the research of Wilson and Wilson is the absence of Chironomini. Chironomini are in general stagnant water inhabitants, detritus eaters [Fittkau and Reiss, 1978] and sediment dwellers [Wilson and Wilson, 1984]. The absence of Chironomini in 1994 could indicate an improved flow in the river bed and a decrease in the organic load [Wilson and Wilson, 1984].

The autecology of the dominant species also points in this direction. At Rheinfelden *Rheocricotopus chalybeatus* (42.6%) and *Tvetenia verralli* (synonymous with *Eukiefferiella verralli*, 26.8%) were dominant. These two species together formed approximately 70% of the total. Caspers [1980[a]] considers *Rheocricotopus chalybeatus* as a rheophilons species that is present mostly in large rivers. Klink [1989] characterises this species as living on stones (lithorheon). Moller Pillot and Buskens [1990] mention this species as being associated with large rivers and lowland streams with a stable oxygen concentration (> 50% saturation). According to Cranston *et al.*, [1983] *Rheocricotopus* is a rheophilons genus of which the different species are found on stones and plants in streams and rivers. Wilson and McGill [1982] consider this species to be relatively sensitive to organic pollution. Caspers [1990] mentions *Rheocricotopus chalybeatus* as a river species that has no strict demands on its environment and is found mostly in the lower course of rivers. *Tvetenia* was considered by Caspers [1980[a]] as a rheophilons genus and was classified by Klink [1989] as being a lithorheophilous genus. Moller Pillot and Buskens [1990] mention five species from the genus that have a preference for fast running waters. Wilson and McGill [1982] mention that *Tvetenia verralli* is slightly sensitive for organic pollution. In the study of Caspers [1980[b]] in 1978-

1979 and the study of Wilson and Wilson [1984] in 1981, no exuviae of *Tvetenia verralli* were found. In the Meuse River, *Tvetenia verralli* was only found in the relatively unpolluted sample location Hastière [Ketelaars and Frantzen, 1995].

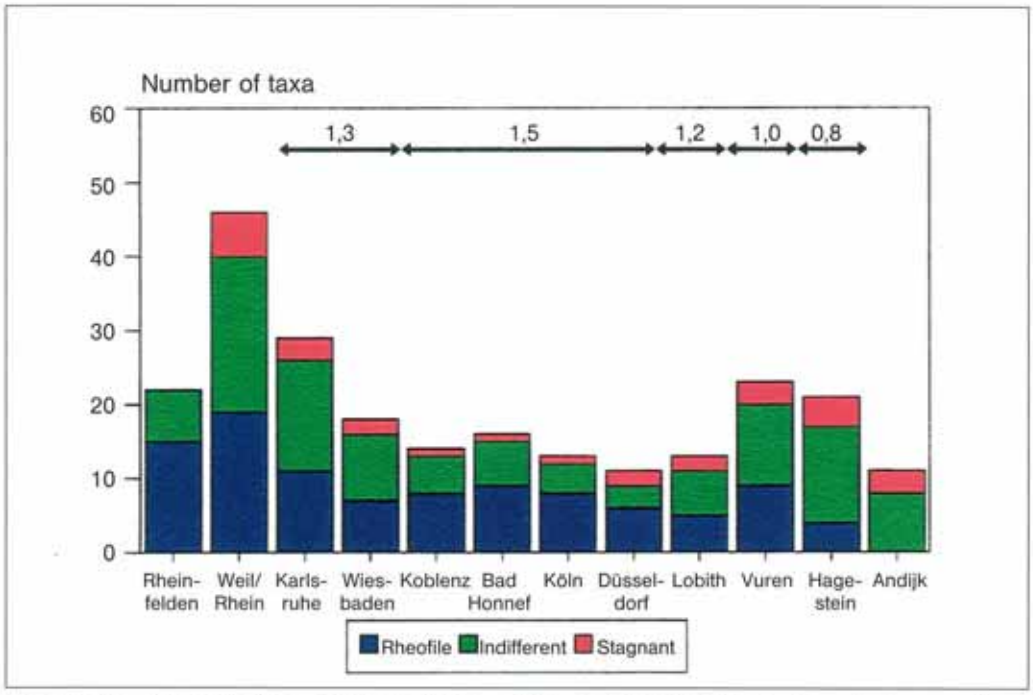


Figure 8 Cumulative number of rheophilons, flow-indifferent and stagnant water exuviae-taxa in the Rhine River in August 1994. (Flow velocities (m/s) are given at the top of the graph [after Streit, 1992]).

In contrast to Wilson and Wilson who found very few *Rheocricotopus chalybeatus*, Caspers [1980[b]] found a large number of this species. However, Caspers did not find any of this species in bottom samples.

At Rheinfelden some characteristic river taxa were found that were not present at any of the other sample locations. These concern the Trichoptera families Psychomyiidae and Rhyacophilidae and the chironomid *Eukiefferiella ilkleyensis*. Of the total number of exuviae, 89% can be characterised as flowing water taxa, while none of the found exuviae are exclusively stagnant water types (figure 9).

In conclusion, it can be stated that the fauna at 'Rheinfelden', despite consisting of a relatively low number of taxa, possess the characteristics of an essentially undisturbed river ecosystem. Knöpp [1957] also found in 1956 that the upper Rhine between the Bodensee and Basel was 'biologically healthy'.

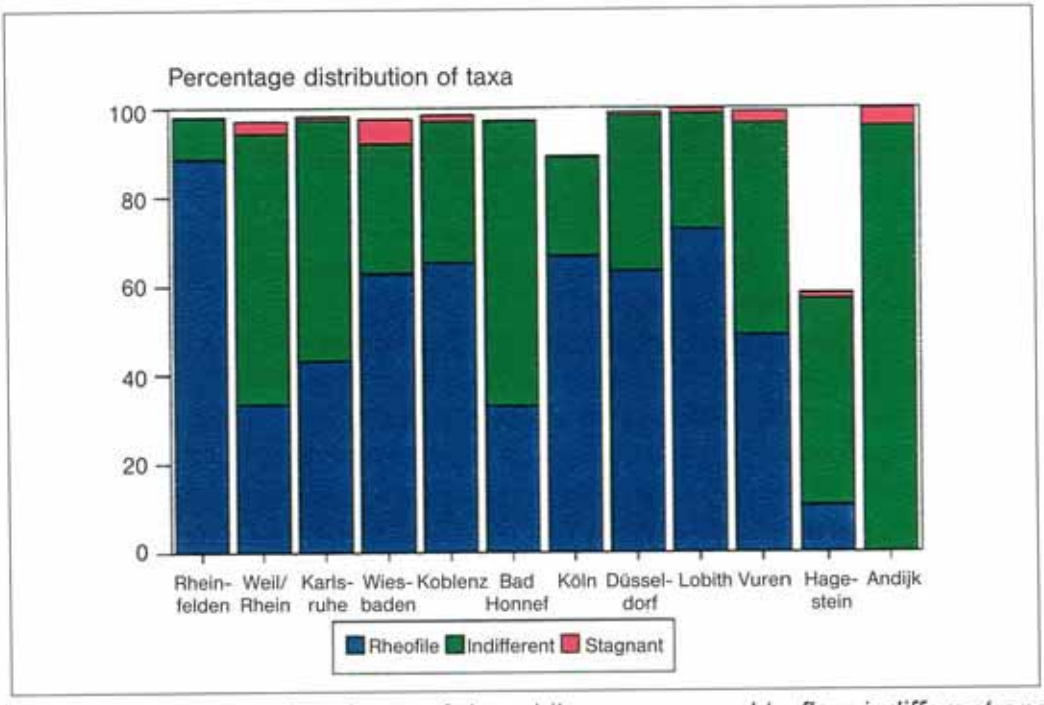


Figure 9 Relative abundance of rheophilons, compared to flow-indifferent and stagnant water exuviae-taxa in the Rhine River in August 1994.

Weil am Rhein (171.3 km)

The most diverse sample location was 'Weil am Rhein' (55 taxa, of which 52 were Chironomidae). This is more than twice as many as found at 'Rheinfelden' (upstream from Basel). The results from Wilson and Wilson [1984] who sampled the Rhine in 1981 are quite different. They found an equal number of taxa (30-35) both upstream and downstream of Basel. There are a number of possible explanations for this difference. Firstly, in relation to 1981, the water quality could have improved so much that there are now more taxa present. Also, Wilson and Wilson could have found less taxa because they determined only two hundred exuviae, while in the current study at 'Rheinfelden', 382 exuviae were determined. In a study on the number of exuviae that should be identified, Ouwerkerk [1992] states that a minimum of three hundred exuviae must be identified in order to get a representative picture of the diversity in a sample. This explanation is reinforced by the fact that for 25 of the taxa, only one specimen (0.3%) was found.

At 'Weil am Rhein', *Neozavrelia* pe 1 (23.0%) was dominant and *Synorthocladius semivirens* was sub-dominant (16.5%). *Neozavrelia* was found only at this sample location. Wilson and McGill [1982] found *Neozavrelia* pe 1 only in the upper Rhine and further upstream. The number found by them is considerably lower than the values found in this study (maximum 5%). Very little additional ecological information about this taxon is available.

Synorthocladius semivirens lives in slime cases, on stones or plants [Moller Pillot and Buskens, 1990] and is according to Klink and Moller Pillot [1982] not rare in the large rivers, even though it is not a typical river species [Fittkau and Reiss, 1978]. Wilson [1989] observed that the occurrence of this species in the Bristol river increased when organic pollution increased slightly.

The largest number of Tanytarsini of all the investigated locations was found at 'Weil am Rhein'. These were the already mentioned *Neozavrelia* and *Rheotanytarsus rhenanus* (8.9%). No Ephemeroptera were found at this sample location. Two species of Trichoptera were found in large quantities. The number of flow-indifferent taxa was considerably greater compared to 'Rheinfelden'. There were even six taxa that are usually only found in stagnant water; these actually make up only a small proportion of the total number of exuviae.

In comparison to 'Rheinfelden' there is in less evidence of a typical river fauna at 'Weil am Rhein'. No clear conclusions can be drawn regarding pollution.

Karlsruhe-Maxau (363.3 km)

At this sample location 32 taxa were found. From eleven taxa only one specimen was found. Compared to 'Weil am Rhein', the number of taxa has sharply decreased, while the number of Chironomini in the total is higher than at 'Rheinfelden'. The dominant species was *Rheocricotopus chalybeatus* (27.0%). Sub-dominant were *Nanocladius bicolor* (1%) and *Cricotopus triannulatus* (19.1%). *Rheocricotopus chalybeatus* is relatively sensitive to organic pollution and is found mostly in large rivers.

Cricotopus triannulatus is a species typical of eutrophic to hypertrophic large rivers having a hard substrate and a stable oxygen level (> 50% oxygen saturation) [Moller Pillot and Buskens, 1990]. According to LeSage and Harrison [1980] *Cricotopus triannulatus* can withstand heavy organic pollution and highly turbid water. Caspers [1980[a]] found large numbers in the Rhine by Bonn in the period between 1977 - 1979. Wilson and Wilson [1984] did not find this species anywhere in the Rhine River basin in August 1981. Strangely enough, this species is also found in brackish waters [Hirvenoja, 1973; Cranston, 1982]. Moller Pillot and Buskens [1990] report that *Nanocladius bicolor* occurs in rivers with organic enriched bottom sediments. According to them this species can endure relatively low oxygen levels (10- 50% oxygen saturation) and lives in eutrophic to hypertrophic waters.

The relative number of rheophilons and flow-indifferent taxa is comparable with 'Weil am Rhein'. The exuviae composition at Karlsruhe-Maxau indicates a larger number of tolerant taxa than in the upstream region of the river. The study of Mauch [1988] conducted shortly before and shortly after (respectively 19-10-1986 and 30-11-1986) the Sandoz accident at Schweizerhalle (2-11-1986) provided, according to the author, no indication of radical changes in the fauna. In this context, it is noteworthy that only *Rheotanytarsus* sp. was mentioned. This species was found in great density at Karlsruhe, both before and after the accident.

Furthermore, Mauch [1988] reports a relatively large density of Trichoptera (*Ceraclea dissimilis*, *Ceraclea nigronervosa* and *Hydropsyche contubernalis*). The sample taken from 'Karlsruhe-Maxau' consisted of only 5.0% *Rheotanytarsus* sp, whereas 5.5% belonged to the Trichoptera.

Wiesbaden (507.0 km)

At the sample location 'Wiesbaden' the number of taxa decreased sharply in relation to 'Karlsruhe-Maxau. From each of the main taxonomic groups one or more taxa were found. *Rheocricotopus chalybeatus* (29.2%) and *Kloosia pusilla* (20.4%) were the dominant taxa. *Nanocladius bicolor* (14.6%) was classified as sub-dominant.

The proportion of the Chironomini at this location increased sharply. This points to an increase of fine sediment and organic material in the bottom, possibly as a result of sedimentation. *Nanocladius bicolor* appears in organic rich sediment. The number of taxa that appear only in running water is rather high (63%). The large number (8.8%) of *Ephemeroptera* (cf. *Caenidae*) is striking; *Caenidae* live mainly in fine sediment (mud).

Koblenz (590.1 km)

At 'Koblenz', *Tvetenia verralli* (32.5%) and *Cricotopus triannulatus* (24.0%) were the two dominant species. *Rheocricotopus chalybeatus* (10.5%) and *Rheotanytarsus rhenanus* (12.5%) were classified as sub-dominant. *Rheocricotopus chalybeatus* and *Tvetenia verralli* are both rheophilons species and are sensitive for organic pollution. In the Meuse River, *Tvetenia verralli* was only found at the relatively unpolluted sample location Hastière. *Rheocricotopus chalybeatus* was also found in large numbers at this site, but downstream of Hastière it was found only sporadically [Frantzen, 1991]. This points to sensitivity of both species to both organic and chemical pollution, since there are discharges of both domestic and industrial waste water in the Meuse in the Liege region.

Cricotopus triannulatus is insensitive for organic pollution. The environmental requirements for *Rheotanytarsus rhenanus*, a species that is described comparatively recently [Klink, 1983], are not yet known. Caspers [1980[a]] considers *Rheotanytarsus photophilous* and *Rheotanytarsus musicicloa* as characteristic for beta-mesosaprobic water. Klink [1989] states that the species from this genus live among stones in rivers.

Compared to 'Wiesbaden', the number of taxa found at this location further decreased to sixteen (table B10, appendix 9). Six of the seven main taxonomic groups found during this study of the Rhine occur at this site (figure 10). The large majority of these species belong to the Orthocladiinae (figure 11). Only a very small percentage can be considered as characteristic for stagnant water (some specimens from *Polypedilum acifer* and *Stempellinella bausei*; table B10, appendix 9). All other species are considered as river dwellers or show no preference for running or stagnant water. The appearance of some specimens

of *Ephoron virgo*, a mayfly that appears in large rivers with a sandy bottom [Schoenemund, 1930] or coarser material [Bij de Vaate *et al.*, 1992] and that requires a good oxygen supply was noteworthy. *Ephoron virgo*, which has been absent in the Rhine for some time, was first found again in the Main in 1987 [Titizer *et al.*, 1987]. Thereafter, this mayfly was frequently found in the Lower Rhine [Titizer *et al.*, 1990; Bij de Vaate *et al.*, 1992]. In spite of the relatively small diversity, other species typical of oxygen rich water were also found.

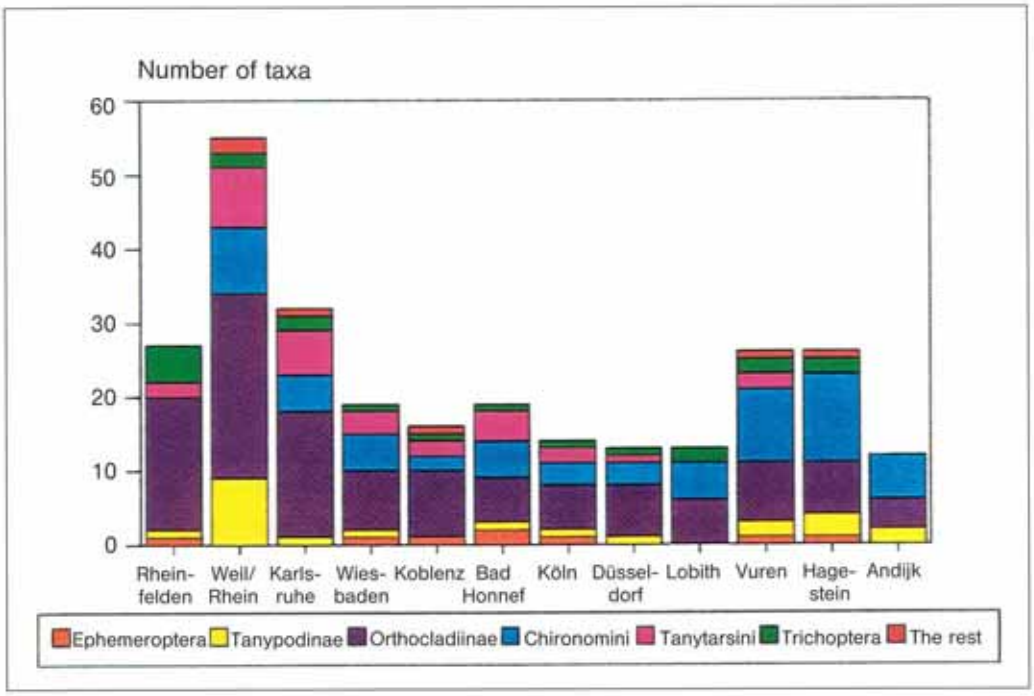


Figure 10 Cumulative number of taxa from the different taxonomic groups of exuviae in the Rhine River, August 1994.

Bad Honnef (640.0 km)

At this location in the river three dominant species were found: *Nanocladius bicolor* (25.8%), *Synorthocladius semivirens* (29.4%) and *Tvetenia verralli* (22.1%). *Cricotopus triannulatus* (7.8%) and *Rheotanytarsus rhenanus* (6.3%) were found to be sub-dominant. The (known) environmental requirements of all five species are described above. Nineteen taxa from six main taxonomic groups were found (table B10, appendix 9).

Just as at 'Koblenz', only a few specimens of stagnant water organisms (*Polypedilum acifer*) were found. The percentage of rheophilons taxa was considerably lower than at 'Koblenz' (figure 9). With increasing organic pollution, *Nanocladius bicolor* and *Synorthocladius semivirens* increase in numbers, though the latter tolerates only slight organic pollution [Moller Pillot and Buskens, 1990; Wilson, 1989]. *Nanocladius bicolor* was not found in the most polluted part of the Meuse (the region downstream of Luik) in the period 1983 - 1993 [Ketelaars and Frantzen, 1985]. Because *Nanocladius bicolor* is the least

pollution sensitive (sub)dominant taxon found at this location it can be concluded that 'Bad Honnef' is less polluted than the Meuse at Liege.

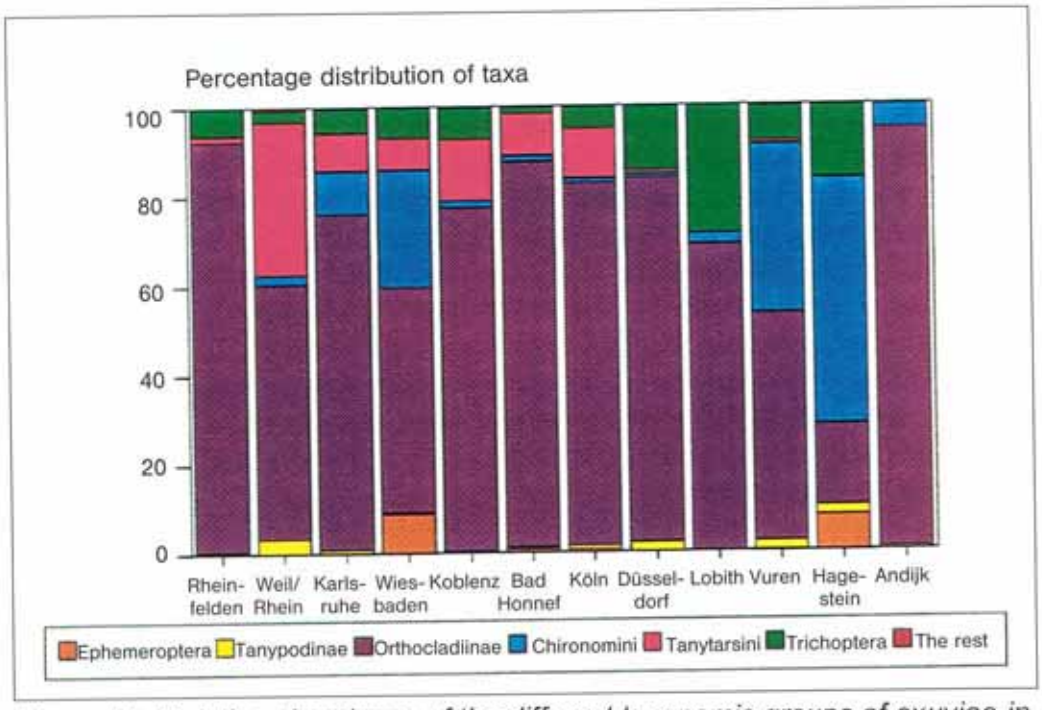


Figure 11 Relative abundance of the different taxonomic groups of exuviae in the Rhine River, August 1994.

Köln (685.9 km)

The species composition at this sample location strongly resembles that of 'Bad Honnef': twelve species appear at both locations (table B10, appendix 9). At Köln, *Tvetenia verralli*, a species that is relatively sensitive for organic pollution [Wilson and McGill, 1982] was dominant (54.7%). *Nanocladius bicolor* (10.3%) was found to be a sub-dominant species. This species is known from organically polluted water. The number of taxa found further decreased to only fourteen (figure 10). The proportion of Orthocladiinae was also high at this sample location. It is noteworthy that two specimens of *Ephoron virgo* were found.

Düsseldorf (727.0 km)

At Düsseldorf, *Tvetenia verralli* (40.7%) and *Cricotopus triannulatus* (32.3%) were dominant. *Hydropsyche* (14.6%) was sub-dominant. *Cricotopus triannulatus* is evidence of organically polluted water. In contrast, *Tvetenia verralli* is more sensitive to pollution. *Hydropsyche* pupae cannot be identified to the species level based on the existing literature. They were probably *Hydropsyche contubernalis*, because this is the most common species in the Middle and Lower Rhine [Caspers [1980[b]; Van Urk, 1981; Higler and Tolkamp, 1984; Van Urk and Bij de Vaate, 1990]. According to Caspers [1980[b]], *Hydropsyche contubernalis* is extremely tolerant for thermal and inorganic pollution. According to Caspers [1980[a]] this species was abundant in the polluted Middle Rhine in the period 1977 - 1979. Higler and Tolkamp [1984] on the other hand

report that *Hydropsyche contubernalis* has just returned in the Rhine after an improvement in the water quality (since 1976). In the Netherlands the first larvae have been found in the IJssel after a long absence [Van Urk, 1981]. *Hydropsyche contubernalis* is the most tolerant species of the genus *Hydropsyche* and endures slightly polluted water [Higler and Tolkamp, 1983], but disappears under conditions of high organic pollution [Meurisse-Genin *et al.*, 1987]. In the Meuse, *Hydropsyche contubernalis* was only found once in the period 1983 - 1993, at the polluted sample location 'Borgharen'. In the year concerned, the discharge was relatively high, which resulted in more dilution of the discharges and a higher oxygen level compared to the other years [Ketelaars and Frantzen, 1995].
The diversity is the same as found at the other locations (thirteen taxa). Representatives of five main taxonomic groups were found. Of the thirteen taxa, nine were also present at the location 'Köln'. Four taxa were represented by only a few specimens. The Ephemoptera were completely absent.

Based on the above information, the part of the Rhine in the Düsseldorf region can be classified as moderately polluted.

Lobith (862.1 km)
Location 'Lobith' was dominated by *Rheocricotopus chalybeatus* (32.9%) and *Hydropsyche* sp. (28.1%). *Tvetenia verralli* (11.1%) and *Cricotopus triannulatus* (18.7%) were found to be sub-dominant. Eight of the species identified were also found at 'Düsseldorf'; the total number of taxa (thirteen) was as low as at the preceding locations. The diversity at this location was very limited: Ephemeroptera, Tanypodinae and Tanytarsini were completely absent. All the species identified belong to only three groups (Orthocladiinae, Chironomini and Trichoptera).

About 69% of the specimens belonged to the Orthocladiinae. The quality of the Rhine water at the sample location corresponded with the quality of the two upstream locations. The dominant appearance of *Hydropsyche* indicates a slight improvement of the water quality.

Vuren (955.0 km)
The sample at Vuren was dominated by *Kloosia pusilla* (30.5%) and *Nanocladius bicolor* (20.4%). *Cricotopus bicinctus* (14.2%) was sub-dominant.

Kloosia pusilla is described as a species from large rivers having a mineral bottom and an oxygen saturation of more than 50%. For a relatively short period of time (24 hours) a lower oxygen saturation can be endured (10 - 50%) [Moller Pillot and Buskens, 1990]. Smit *et al.*, [1994] found that it was evident that *Kloosia pusilla* is characteristic for the littoral river sand (55% of the grains > 210 μm). In May 1993 larvae of this species were found in large numbers in the Lower Rhine at Opheusden for the first time in twenty years [Smit *et al.*, 1994]. Earlier *Kloosia pusilla* were also found by Van Urk and Bij de Vaate [1990] in

light traps in the IJssel (1985 - 1987). At Vuren, this species was observed once in August 1989 (unpublished data). No literature references were found regarding their behaviour with respect to pollution.

Nanocladius bicolor, as previously mentioned, is a species that is perfectly able to live in polluted waters.

Cricotopus bicinctus is a species from large rivers, however, it can also be found in stagnant water. *Cricotopus bicinctus* lives mostly on a hard substrate but can also be found between plants. It can also tolerate a reasonably high chloride level (1000 mg/l) [Moller Pillot and Buskens, 1990]. Caspers [1980[a]] reports a widespread appearance of this species in the Rhine. Strangely enough, this species was absent in the samples taken between Wiesbaden and Düsseldorf. According to Surber [1959] *Cricotopus bicinctus* is extremely tolerant of oxygen deficiency or high concentrations of chromium, copper and cyanide. The diversity at this sample location has increased with respect to the upstream locations (figure 10).

Hagestein (946.9 km)

At Hagestein *Chironomus* sp. was dominant (39.5%). *Ecnomus tenellus* was found as sub-dominant (15.5%). Most species of the genus *Chironomus* are known as an indictor for polysaprobic water [Caspers 1980[a]]. Moller Pillot and Buskens [1990] described this genus as being attached to degraded bottoms and having little sensitivity for oxygen deficiency. Most species avoid strong current.

Ecnomus tennellus is described by Klink [1986] to be a lithorheophilous species. Lepneva [1964] considers this caddis larva as a typical species of lowland rivers. According to Edington and Hildrew [1981] this taxon can also be found in stagnant water. This species is found in the Netherlands in amongst others the IJssel and the Rhine; the larvae are found particularly in the area of stones [Van Urk and Bij de Vaate, 1990]. Ketelaars and Frantzen [1995] found *Ecnomus tenellus* as the subdominant part of the macro-invertebrate fauna in the lowland region of the Meuse River (Keizersveer).

Dicrotendipus nervosus and *Lymnophyes* sp. were found in reasonable numbers (both 10.7%). Caspers [1980[a]] called *Dicrotendipus nervosus* a eurytherm and eurytope species. Caspers [1980[a]] found this species in moderate numbers in accretions on stones in the Rhine. This species decreases in numbers near organic discharges [Smit and Gardeniers, 1986]. By reasonable oxygen supply *Dicrotendipus nervosus* is one of the most numerous chironomids in the Netherlands [Moller Pillot and Buskens, 1990]. In the Meuse *Dicrotendipus nervosus* was often dominant at locations influenced by chemical and municipal waste water in the lowland part of the river [Ketelaars and Frantzen, 1995]. The number of rheophilons species at this sample location strongly decreased (figure 8). The proportion of Chironomini, in general sediment dwellers, was high, which can be expected in the lowland part of the river with a slow current velocity.

Andijk (IJsselmeer)

Because of its location in a large lake, the sample location at Andijk possesses no river characteristics. The results of the study are therefore not directly comparable with other locations. The exuviae sample from this location was dominated by *Cricotopus intersectus* (90.8%). These larvae are often numerous on stones and plants in lakes and rivers [Thiennemann, 1954; Reiss, 1968; Lehman, 1971; Caspers, 1980[a]]. In the Netherlands *Cricotopus intersectus* is very common in the large rivers and in not too small eutrophic waters [Moller Pillot, 1984[b]]. In Southern Sweden, this species is extremely characteristic of very eutrophic water [Brundin, 1949]. The dominance of *Cricotopus intersectus* shows the clearly eutrophic character of the water in the IJsselmeer. In view of the habitat of *Cricotopus intersectus*, the sample was probably taken in the region by the river bank.

Glyptotendipes pallens (3.4%) was sub-dominant. It is according to Reiss [1968] a species from lakes and ponds that lives on crusts of cyanobacteria. Some species of the genus *Glyptotendipes* live in Bryozoa colonies or in sponges [Pinder and Reiss, 1983]. *Glyptotendipes pallens* does appear in rivers but not in the main flow channel [Klink Moller Pillot, 1982], which points to a preference for the slowly flowing or stationary parts of the river. According to Moller Pillot and Buskens [1990] this species is not very sensitive for organic pollution and low oxygen levels. The diversity at the sample location Andijk is low (twelve taxa).

4.5.2 Further discussion of exuviae

The riverine species composition (diversity) and abundance is determined by a number of factors:

a. natural factors such as flow, current velocity, sedimentation, substrate, etc. In the whole river basin different zones exist which create a variety of habitats;
b. factors caused by human activity, such as chemical or organic pollution, which in turn can affect factors such as transparency, oxygen levels, temperature, amount of dissolved salts, etc.;
c. factors that cannot with certainty be classified into one of the two groups above.

The dominant appearance of a certain taxon can be seen as the result of a number of factors. It is, unfortunately, not always possible to make a distinction between natural factors (a) and anthropogenic factors (b). This complication is caused by the fact that ecological information given in the literature is very limited, and in addition is often only of a qualitative nature.

The first step required in the interpretation of the collected study data consists of differentiating between the influence of natural factors and those caused by human activities. In view of the fact that there is also a large group of factors (c) that are in part determined by causes mentioned in (a) and (b), it is clear that uncertainties appear already by the first step.

The dominant appearance of a certain species can be caused by a combination of influences. Dominance can also occur in biocoenoses not influenced by man, for example in a habitat with a uniform substrate. In such a case, the abiotic environment causes the occurrence of only a few taxa. One probable explanation for this is that the most important competitors are suppressed by the prevailing environmental circumstances. In addition, with the absence of competitor species there is in principle more food available.

Differentiating between dominance in an undisturbed environment and in a polluted environment cannot reliably be made without rather detailed knowledge of the environmental conditions required by the dominant species. Autecological knowledge is therefore necessary to determine the cause of the dominance. The autecological data of the other taxa present are also of importance for the interpretation.

The advantage of using autecological data for the assessment of water quality is the fact that water quality is integrated over a longer period. The fauna present has sufficient time to establish themselves in that part of the river, to develop and to complete their reproduction cycles.

The river geomorphology, abundance and diversity must be included as part of the data collected in this exuviae study. For the geomorphology aspects, the current velocity of the water is first considered (figure 8), as reported by Streit [1992]. The Rhine upstream from Basel has weirs at many locations, whereby large variations in current velocities occur. The changing character of the river offers a place to species that require varying environmental conditions. At 'Rheinfelden' no species that are characteristic of stagnant water were found (figure 8). The high diversity at 'Weil am Rhein' found in this study can possibly be explained by the fact that exuviae from both fast and slow flowing regions were collected, and there were also species found that are characteristic for stagnant waters (figure 8).

Presumably rheophilons species are more sensitive to pollution because dwellers of (fast) flowing water mostly live in clear oxygen rich water. These are exactly the properties that the water quickly loses due to organic pollution. Considering the above, the low number of rheophilons species at 'Bad Honnef' (a sample location where the current velocity is relatively high) can be attributed to increased pollution with respect to the upstream sample location.

In slower flowing regions of the river, organic sediment will accumulate on the bottom. In these areas sediment dwelling species will be numerous or even dominant. Sediment dwellers are more apparent in the group of the Chironomini. In figure 11 it can be seen that Chironomini are completely absent at Rheinfelden, and thereafter in the slower flowing region up to Wiesbaden, they increase in numbers. In the fast flowing section from Koblenz - Lobith their proportion is small, after which their numbers at 'Hagestein' increase considerably. At the sample location at Andijk, the number of Chironomini is unexpectedly low. This is explained by the assumption that this sample consisted of many stone dwelling species. The sample was taken in the direct surroundings of a stone sheetpiling.

4.5.3 Comparison with the Meuse River

A comparison of the (sub)dominant exuviae found in this study with the results of the research in the Meuse in the period 1984 - 1989 [Frantzen, 1991] shows that essentially the same taxa were found at the relatively less polluted location Hastière. The fauna at the more polluted sample location in the Meuse (Hermalle) were dominated by species that were not (sub)dominant in the Rhine. The proportion of rheophilons species in the Meuse study was considerably lower. This suggests that the Rhine fauna is more indicative of river elements and that the Rhine is probably less polluted than the Meuse. This applies principally for the Upper Rhine and the upper region of the Lower Rhine.

4.6 Combination of the study results

To use the results of the genotoxicity study, the ecotoxicity study and the ecological study for a more quantitative description of the water quality of the Rhine, the following parameters have been taken as the most relevant quality parameters:

- Ames-test at pH = 7 with S9-mix;
- pT-value;
- number of taxa.

In order allow a comparison of the results of the three parts of the study, it is important that the scale and the units of the three parameters are comparable. The Ames-test gives a score expressed as 'revertants/litre', and is equivalent to a concentration term (because there is a dose-effect-relationship in the Ames-test, the Ames-test score relates to concentrations of pollutants or mutagenic compounds). By the combined parameter for the ecotoxicity tests, the pT-value, there is a logarithm of a concentration factor at which a certain effect appears. In fact the 10th power of the negative pT-value (10^{-pT}) must be used in order to obtain a concentration term that linearly expresses the level of pollution. To correlate the results of the different tests this is not of real importance.

For the ecological study it is more difficult to define a representative concentration term that can express the level of pollution. In general, a good ecosystem quality results in a larger diversity of species than in a comparable biotope of poor quality. In this respect, (water) quality is not the only factor that determines the number of species. However, if species diversity is a measure of the water quality, then it can be supposed that the inverse of the number of species is a measure for the amount of pollution. In this case '1/number taxa' should be used in comparison with the Ames-test and the pT-value.

4.6.1 Correlation between the various study parts

Based on the above considerations then the following adapted results are numerically compared:

- score in the Ames-test with S9-mix (pH = 7);
- pT-value (pH = 7);
- 1/number of taxa.

For the Ames-test and the pT-value, the complete numerical results of all 34 observations from the three sampling series were compared with each other. From figure 12 it appears that there is, indeed, some correlation, but there is also a fairly large spread of results. If the three sampling series are assessed separately, such as was done for the first sampling series in figure 13, it appears that within a sampling series a clear correlation exists between the score in the Ames-test and the pT-value. Because the sensitivity of each of the tests probably varies between the three series, and other external factors also play a role (such as nature of the pollution), the combined results from the different sampling series provide a (visually) less consistent picture than within in each individual series. On this basis it can be concluded that a clear correlation exists between the results of the Ames-test and those of the ecotoxicity tests.

The exuviae study was conducted in August, which means the water quality in the months before was the most important for determining the species diversity. A comparison of the Ames-test results for the sampling series of June (second sampling series) with the results of the exuviae study in August, shows a large degree of agreement (figure 14). The same applies for the relation between the pT-value and 1/number of taxa (figure 15).

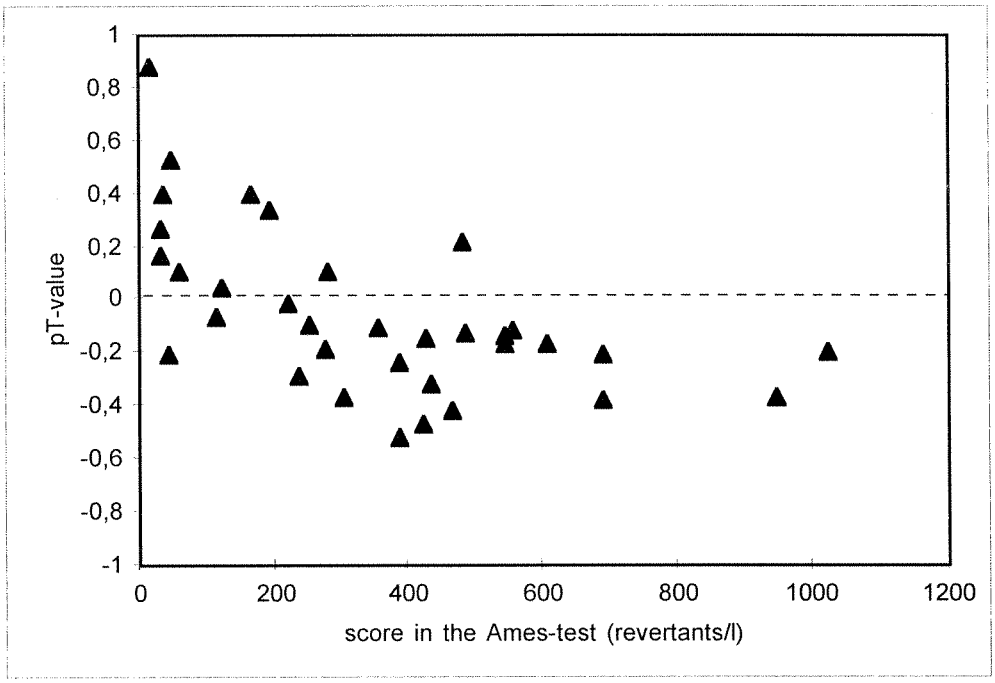


Figure 12 Correlation between the results of the Ames-test (TA98 with S9-mix at neutral pH) and the pT-values from the three sampling series

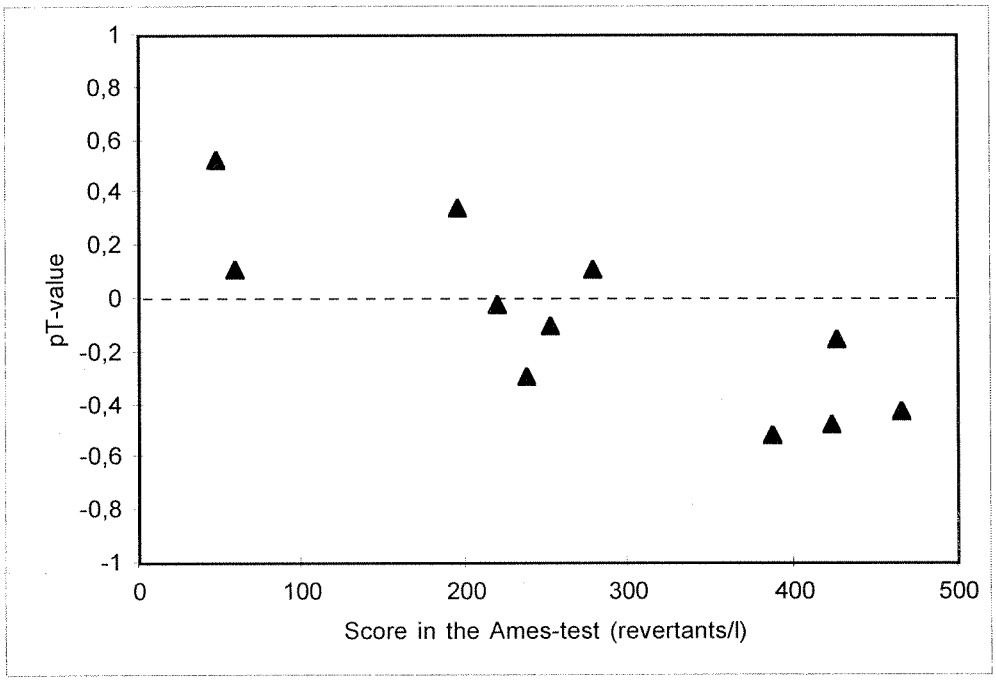


Figure 13 Correlation between the results of the Ames-test (TA98 with S9-mix at neutral pH) and the pT-values for the first sampling series.

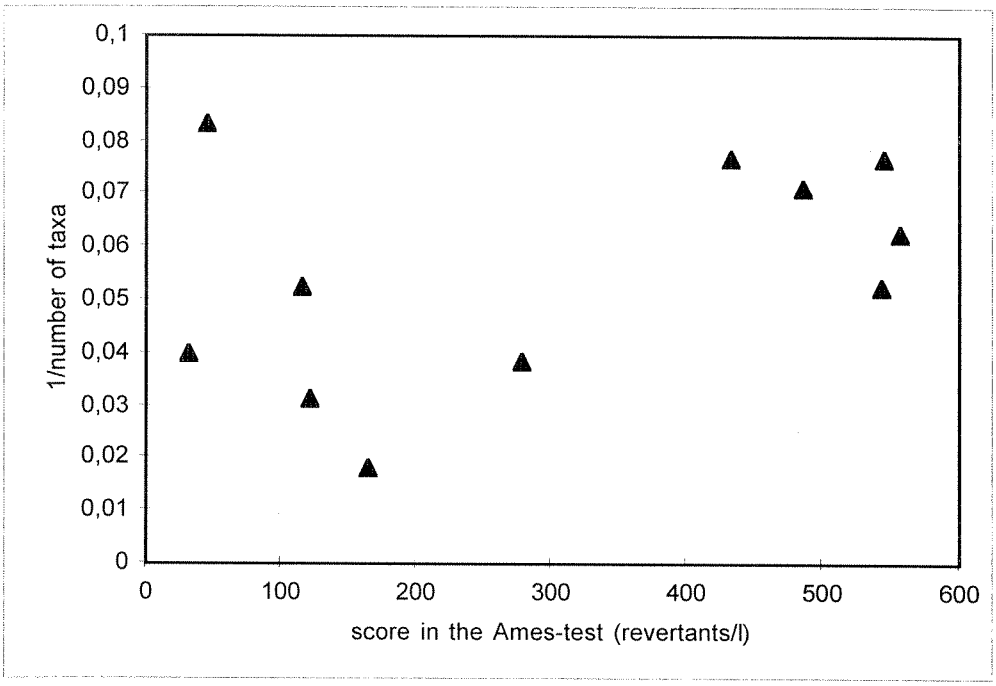


Figure 14 Correlation between the results of the Ames-test (TA98 with S9-mix at neutral pH) and the inverse of the number of taxa for the second sampling series.

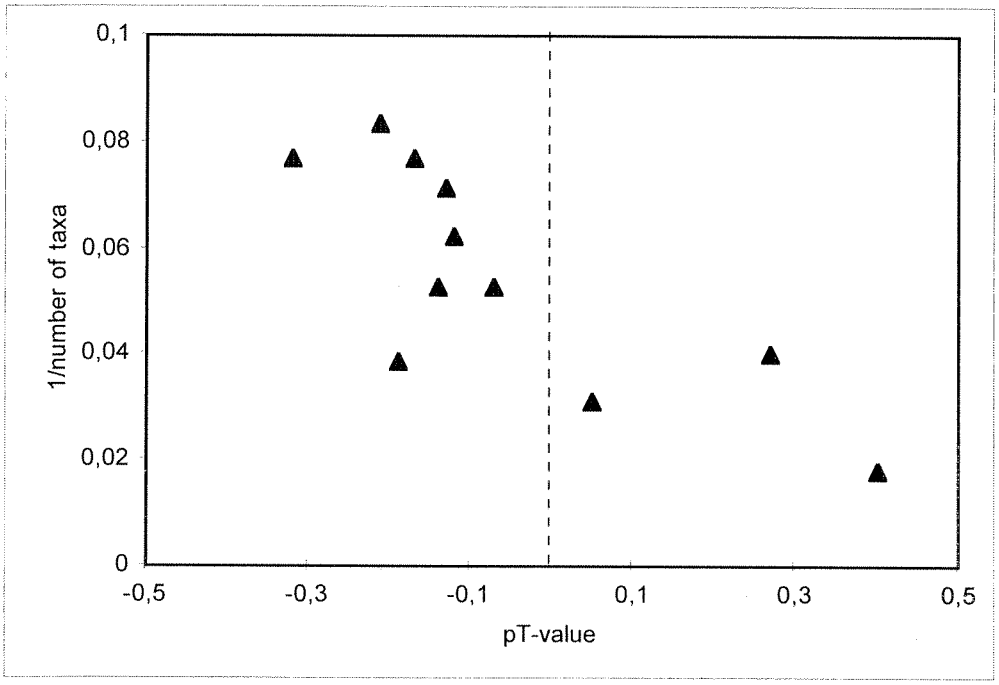


Figure 15 Correlation between the pT-values and the inverse of the number of taxa for the second sampling series.

5. CONCLUSIONS

Given that the mechanisms and pollutants that generate effects are very different for the various tests, it cannot be expected that the results of the Ames-test, the toxic potential (pT-value) and the number of exuviae taxa have a clear relationship with each other. This is in fact confirmed if all individual pairs of observations are considered. In spite of this, it can be still concluded that a fairly consistent picture of the water quality is obtained from the three aspects of the study.

Upstream from Basel the water quality of the Rhine is generally good. In the region around Basel, the pollutants affecting the genotoxicity (as measured by Ames-test), and the water quality (as measured by the ecotoxicity), are introduced. For the ecological water quality, however, no adverse effect is measured. It is noteworthy that in the second sampling series which should have a good correlation with the exuviae study, the ecotoxicity was not any worse downstream than upstream of Basel. Further downstream of Basel, the Ames-test indicated a slight improvement in the water quality, which then became gradually worse with increasing urbanisation.

The worst water quality was measured in the Ruhr area. This result was given by all three parts of the study, as can be seen in figures 3, 5, and 10, presented previously. Some of the individual test results sometimes showed a slight improvement or fluctuation. Downstream of the Ruhr area a gradual improvement is seen, and eventually at 'Andijk' both the Ames-test and the ecotoxicity tests indicate the water is essentially unpolluted. Due to the large differences in the nature of the biotope, the results of the ecological study at 'Andijk' are not comparable with those of the river itself.

Because the various parts of the study show a considerable amount of consistency, and all the performed tests and methods measure a different effect and are therefore related to different (types of) substances, it can be concluded that a wide range of substances is responsible for the measured changes in the water quality.

In the interpretation of the ecological data it should be realised that a certain fauna can only develop or maintain itself at a specific location if the water quality is relatively constant in time. Hereby a minimum time scale of months can be considered. In contrast, the toxicity tests give information about the water quality at a specific moment in time. Because there is a relatively good agreement between the different parts of the study, it can be concluded that the discharges to the river are reasonably constant in nature and probably do not fluctuate greatly.

For the separate parts of the study the following conclusions can be drawn:

Cholinesterase inhibition

- From 'Weil am Rhein' to 'Hagestein' it appears that there is some enzyme inhibition, although the measured effect is marginal (at most twice the detection level). The inhibition measured in the first sampling series is higher than that in the second and third series.
- In samples from 'Bodensee', 'Rheinfelden', 'Weil am Rhein' and 'Andijk' there was consistently no inhibition detected.
- In comparison with earlier RIWA studies over the period 1986 - 1992, a gradual decrease of the cholinesterase inhibition can be observed at the locations 'Lobith', 'Hagestein' and 'Andijk'. In comparison with the cholinesterase inhibition of the Meuse, it appears that the water quality of the Rhine for this parameter is significantly better.

Genotoxicty

The number of mutated bacteria (i.e. revertants) in the Ames-test is an indication of the rate of a mutagenic effect. The number of natural revertants should always be subtracted from the (total) number of revertants. A score is considered marginal if the number of induced revertants (after subtraction of the spontaneous revertants) is only a factor two or three greater than the spontaneous level.

- By the Ames-test with the TA98 strain without metabolic activation either no score or only a low score (approximately 20-50 revertants per litre) was measured at all locations, with the exception of 'Weil am Rhein' during the first and particularly the third sampling series (respectively, 104 and 647 revertants per litre). The mutagenicity found in the sample from 'Weil am Rhein' from the third sampling series could not be correlated with the results of the supplementary GC/MS-study on specific organic micropollutants. The mutagenicity seems to be related to an intense pink colouring of the XAD column which occurred during the isolation.
- By the Ames-test with the TA98 strain with metabolic activation, a medium to high score was measured in samples from 'Weil am Rhein' to 'Hagestein'. The score increased from 'Weil am Rhein' up to and including the Ruhr area (approximately 200 - 1000 revertants per litre by pH = 7), and thereafter decreased gradually. For locations 'Bodensee', 'Rheinfelden' and 'Andijk' no significant score was found (33-58 revertants per litre at pH = 7).
- It is clear that between Basel and the Ruhr area an increase in the mutagenic activity of the Rhine water takes place. Based on discharge data it can be concluded that the introduction of mutagenic activity takes place primarily between 'Wiesbaden' and 'Bad Honnef'.
- Promutagenic substances are present in the Rhine water. After metabolic activation in the liver these substances can generate mutagenic effects. This explains the high scores in the Ames-test with S9-mix.

- A similar picture of the mutagenicity along the Rhine River is obtained from the Ames-test results of all three sampling series. There are some fluctuations in the results from the first sampling series.
- The results for the location 'Lobith' are in agreement with measurements from the previous ten years. This includes not only the actual scores in the Ames-test but also the seasonal pattern of higher scores in winter and lower scores in summer.
- Because the mutagenicity as measured with the Ames-test is approximately a factor five to ten higher in the Rhine than in the Meuse (at Eijsden and at Keizersveer), it can be concluded that the pollution in the Rhine from organic and inorganic substances is of a different nature than that in the Meuse. The DOC concentrations in the Rhine and the Meuse are similar: Rhine at Lobith 2.9 - 4.0 - 5.5 mg/l (in 1993), Meuse at Eijsden 2.2 - 3.4 - 5.0 mg/l (in 1993). These values for the Rhine and Meuse are respectively the minimum, the average, and the maximum value.
- Whether or not a connection can be made between the increased mutagenicity of the Rhine water and the amount of industrialisation and urbanisation of the area between Basel and Lobith remains to be investigated. Possible natural causes in the extensive river basin of the Rhine cannot be ruled out. It should also be investigated which factors are responsible for the decrease in the Ames-test score between Lobith and the IJsselmeer (at Andijk).

Ecotoxicity/toxic potential

- The individual ecotoxicity tests do not among themselves show a consistent correlation. This points to the fact that different substances and conditions influence the water quality as measured with these tests.
- With the pT-value, in which the responses of the various ecotoxicity tests are integrated, a more consistent picture is obtained. The results based on the pT-value are in agreement with the conclusions of the other two parts of the study.
- A decrease in the sensitivity of the tests used can be seen in the following order: algae photosynthesis, *Daphnia*-IQ, Microtox, Rotoxkit-F, and Artoxkit-M. This last test is too insensitive for measuring any effects in the Rhine water. The first three or four named tests give sufficient results on which to base the pT-value.
- The results of this study indicate that the pT-value based on several ecotoxicological tests clearly has an additional value for the monitoring of large water systems above measuring the response of only a single test organism. For a good interpretation, sufficient measurements are necessary, and a certain degree of expertise is required.

- In a general sense, the pT-value shows that there is an increase of toxicity from 'Rheinfelden' to 'Köln' after which there is a gradual decrease.
- Based on the pT-value it can be concluded that there is an increased risk for damage to the ecosystem between 'Wiesbaden' and 'Hagestein'.
- With regard to the discharge data, it can be concluded that the introduction of ecotoxicity occurs primarily between 'Lauterbourg' and 'Koblenz'.
- In the beginning of the eighties, the untreated water of the Rhine was acutely toxic for certain water organisms. Based on the fact that the Rhine water now only generates an acute toxic effect after concentration, it can be concluded that the ecotoxicological water quality has improved in the last decade.

Exuviae

- The species diversity of macro-invertebrates can be used as an indicator for the water quality. In addition, the sensitivity of different species for organic pollution also gives information over the water quality.
- Based on the above it can be concluded that the upstream section of the Rhine (location 'Rheinfelden') was the least influenced by organic pollution; the section by Karlsruhe and Bad Honnef was the most influenced.
- With respect to studies performed in the beginning of this century, the current diversity of species is small. However, compared to the situation in the beginning of the eighties, an increase of (organic) pollution sensitive species can be seen. It can thus be concluded that the ecological water quality of the Rhine has improved in the last decade.
- In comparison to the Meuse in the period 1984 - 1989, the Rhine exhibits, on the basis of this study, more river-specific species; also the species found in the Rhine are consistent with species found in the less polluted section of the Meuse.

Conclusions in relation to the research questions

1. Based on the results of the conducted tests, where does a change in water quality of the Rhine River occur?

   A change in the water quality along the length of the Rhine River can be seen as shown by the results of the biological tests conducted. This change is most prominent near 'Weil am Rhein' and in the stretch from 'Lauterbourg' to 'Bad Honnef' (worsening), and by 'Lobith' and 'Andijk' (improving).

2. What toxicological and ecological quality can be attributed to the Rhine River based on the test results?

   On the basis of ecotoxicity tests it can be concluded that there is an increased risk of ecosystem damage between 'Wiesbaden' and 'Hagestein'. With the Ames-test, a slight to high mutagenicity was also measured in this region. Also, from the ecological point of view, the samples along this course were the most effected.

3. Do the different parts of the study give a consistent picture of the changes in water quality?
   Despite some differences between the results of the individual tests, there is a good agreement in the assessment of the water quality based on the genotoxicity (Ames) test, the ecotoxicity tests (toxic potential) and the ecological study.

4. Does this study confirm the previously identified differences between the rivers Rhine and Meuse?
   A detailed comparison with the Meuse is considered beyond the scope of the study. However, the results of earlier studies in the Meuse have been compared with the results of the present Rhine study. The earlier results from the RIWA-research in which the Meuse shows less mutagenicity (lower Ames-test score) than the Rhine have been confirmed. However, the results of the ecological and ecotoxicological (cholinesterase inhibition) studies indicate that the quality of the Rhine River is better than that of the Meuse River.

5. Is there, on the basis of the corresponding chemical analyses, an explanation for the changes in water quality found on the basis of the ecological and toxicological studies?
   A supplementary chemical analysis was made for only one sample collected in the study; a sample of XAD concentrate was further investigated with GC/MS. The results showed a number of characteristic organic micropollutants, but provided no further explanation for the observed effect in the Ames-test for that sample. Further comparisons of all the biological test results with the chemical results available from the Rhine River monitoring network gave no clear explanation for the measured toxicity of the river water.

In Summary

The low mutagenic activity found in the river water with the Ames-test is eliminated during the water purification process. In spite of this, the drinking water companies would prefer to use water that is unpolluted in all respects. Thus, the surface water used for drinking water production should have a low score in the toxicity tests and should have a good ecological quality.

It is important to apply a wide range of (biological, chemical and toxicological) analytical methods when monitoring water quality. In this way, the best possible indication of the presence of a large number of different substances and their significance for (drinking) water quality can be obtained.

The measured mutagenic effects, although slight, should be further investigated. The origin and removability of the responsible substances must be determined by co-operation with research institutes and industries.

6. RECOMMENDATIONS

The 'Toxicological and Ecological Study of the Rhine 1994' has supplied a large amount of data and has contributed to answering the questions that formed the basis of the study. Nevertheless, some questions could not be sufficiently answered, and other questions that were inherent in the design of the study remain; also, the techniques and methods are not yet sufficiently developed to give a complete toxicological and ecological description of the quality of the Rhine River. In addition, some study results raise new questions. Thus, a number of recommendations can be made for a follow-up study. Based on these recommendations, the RIWA-project-group 'Evaluation on behalf of a follow-up of the Rhine campaign 1994' under chairmanship of Mrs. Ir. J. Willemsen-Zwaagstra (PWN), will formulate supplementary research goals and develop research plans.

Policy

- The results and conclusions of this study as well as from the eventual follow-up research should be made known to all relevant parties. It must be promoted that the responsible parties should take measures for a longterm improvement of the water quality of the Rhine River, with a view to restoring the ecological value and safeguarding the Rhine as a source for drinking water supply.

Toxicological and ecological studies

- The cause for the higher scores in the Ames-test between Basel and Lobith must be further investigated. If possible, sanitation measures leading to a decrease in the mutagenic effects in the Rhine water should be taken.
- Due to the applied XAD isolation technique, only a fraction of all the organic compounds in the original water sample is included in the toxicity tests. Specifically, the polar compounds remain out of consideration. In the framework of, amongst others,the VEWIN-research programme conducted by Kiwa, isolation techniques that aim to isolate the more polar compounds and make them available for chemical and toxicological research are being developed. If from this research it appears that the polar compounds are relevant from a toxicological viewpoint, then a study similar to TOR '94, in which these new techniques are applied, should be conducted.
- Given the results of this above recommended study and the possibilities they offer regarding an emission-based approach for assessing surface water pollution, it could be desirable to regularly perform similar studies in the whole Rhine River basin using both the 'old' and 'new' isolation techniques.
- In addition to the regular RIWA-research into the quality of the Rhine and Meuse Rivers using the Ames-test, it is recommended to expand the periodic analyses

with a few ecotoxicity tests, specifically algae photosynthesis, *Daphia*-IQ, and Microtox, which are the three most sensitive tests.

Implications for drinking water

- The degree to which water purification techniques, including bottom infiltration, are capable of removing toxicity must be further studied. The existing simple purification procedures as described in the Rhine and Meuse Memorandum should form the starting point for this evaluation.
- In the event that compounds can pass through the purification process, there must be a better understanding of implications of positive toxicity scores on public health. It is especially important to establish the relation between the level of the score and the significance for humans as consumers.

Chemical-analytical study

- From the study results, it seems that toxicologically relevant compounds are introduced in the Rhine River downstream of Basel. There is a number of sample locations where the increase in toxicity is especially prominent: introduction takes place upstream of 'Weil am Rhein', 'Wiesbaden' and 'Koblenz', and to a lesser extent 'Bad Honnef' and 'Düsseldorf'. It is recommended to use GC/MS to investigate the remaining extracts for the presence of toxicologically relevant compounds. This could be done either with or without chemical derivatization of the concentrate in order to enlarge the spectrum of detectable compounds. In addition, it is important to monitor the concentrations of these substances by sampling further downstream, and to check if they are present by the intakes of drinking water companies. Studies should be conducted to monitor the fate of these substances in the water purification process and to check if they are still present in the drinking water.
- The degradation products of toxicologically relevant substances should be assessed on the basis of toxicological criteria, irrespective of whether these degradation products can be isolated or analyzed with the existing techniques.
- Quantitative and reliable analysis methods must become available for the above mentioned compounds if they do not already exist.
- The scores in toxicity tests are in general caused by compounds that are not detectable with GC but rather with HPLC. Thus, the above mentioned research should preferably be performed with HPLC/MS. At the moment, however, this technique is not well enough developed to allow the identification of unknown compounds. It is important that in the HPLC/MS research being conducted by WRK and Kiwa, amongst others, methods for the identification of unknown substances are developed in the future. If suitable, these methods must be used in further water quality research.

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Appendix 1

Sample collection for the toxicological study, including the equipment used

1.1 Equipment used

The various pieces of equipment used to collect samples for the toxicological study are described in the sections below. The field conditions during the sample collection, including any unusual circumstances, were noted in the so-called sample report, an example of which is given in appendix 2.

In selecting the sampling equipment, a great deal of attention was given to the equipment material, due to concerns about sample contamination from desorption and/or loss of organic substances due to absorbtion and/or adsorption. For these reasons, equipment made of the inert materials PTFE (PolyTetraFluoroEthene or 'Teflon'), glass and stainless steel was used as much as possible.

To store the samples for extended time periods under controlled conditions, the suspended sediments were removed, and samples were stored and transported in refrogerated, dark, and airtight containers.

1.1.1 The ship

The ship used for collecting the samples was set up especially for this type of work. It had a positioning pole at the bow which was used to keep the ship at a fixed location. Also, the ship was fitted with a car crane for collecting the water samples. The captain was experienced with shipping in the Rhine River. A picture of the ship is given in figure B1.

Figure B1 The motor ship 'Vios' with the cooling wagon in the back

1.1.2 The pump

In the literature [NPR6600, 1993; ISO 5667-6, 1990], it is advised to collect surface water samples from a depth of 30-50 cm. Samples can be collected from a specific depth using special equipment, but the use of a pump is recommended. With a pump, it is relatively easy to collect a sample from a specific depth without disturbing the water. Another advantage is that samples can be collected at different velocities if required. On the basis of these recommendations, it was decided to use a pump for sample collection. In addition, the amount of time needed to collect the required amount of water (approximately 200 l) from each location influenced this decision.

The capacity and the material characteristics led to the selection of a submersible pump. Finally, a choice was made for a specialized submersible pump designed for sample collection, a so-called monitor pump (type MP1 made by Grundfos). With this pump, the sample water comes into contact only with stainless steel and Teflon. The capacity of the pump is continuously variable to a maximum of 2 m^3/hr.

Before the first sampling trip, the new sampling system consisting of the pump and hose was thoroughly flushed with cold drinking water. Before the second and third sampling trips, the hose was flushed with first hot (approximately 80 °C) and then cold drinking water.

1.1.3 The sampler

During the first sampling trip, the pump was hung directly from the car crane using a stainless steel cable supplied with the pump. At several sample locations air was sucked in due to the combined effect of the flow velocity and relatively high waves caused by the shipping traffic.

In order to prevent this problem in the following trips, the pump was built into a special sampler (see figure B2) before the second boat trip. This sampler consisted of a large steel middle plate with removable plastic covers on the upper and lower sides. A horizontal pipe with a tail fin was attached to the back end of the plate. The sampler had a total weight of approximately 150 kg.

Inside the plastic cover, on the underside of the steel middle plate was a clamp to hold the pump. By attaching the pump here, it was held in place at the front end of the sampler, right next to an opening. This opening in the lower half of the cover allowed the water to be drawn up into the pump.

This set-up worked very well in the second and third sampling series: no air was sucked into the pump during the sampling.

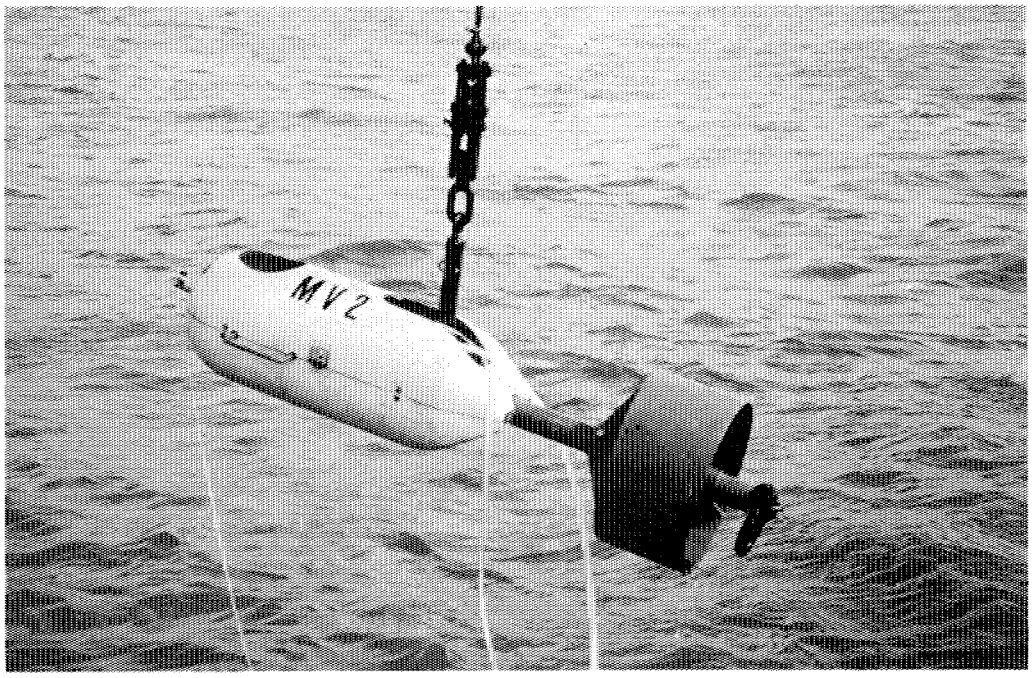

Figure B2 The sampler

1.1.4 The sample collecting vessel

In order to collect the amount of water necessary for centrifuging at each sampling station, a stainless steel sample collecting vessel with a volume of two hundred

litres was used. This vessel had holes in the bottom so that it could be easily emptied. The bottom of the vessel also had stainless steel pipes with two or three faucets and flexible hoses. The vessel was kept outdoors and was sealed with a cover so that the sample could not be contaminated from e.g. bird droppings or rain.

Before every boat trip the sample collecting vessel was cleaned with an alcohol base solution (900 ml ethanol and 100 ml 40% m/m sodium hydroxide solution) and then rinsed with hot drinking water (± 80 °C). Between samples and during the boat trip, the vessel was not cleaned. However, at each sampling station the vessel was thoroughly flushed with water from that location.

1.1.5 The centrifuges

From the sample collecting vessel, the water was centrifuged with two so-called flow through centrifuges, with the goal of removing the suspended sediment from the sample. Sample preservation was the foremost reason for doing this, because biological activity is usually affiliated with the sediment. The second reason for centrifuging the water was that the study was focused on the toxic compounds in the water (i.e. exclusive of sediment).

The capacity of the two centrifuges was approximately 0.7 l/min with roughly 10,000 r.p.m. Centrifuging 150 litres using one centrifuge required approximately 4 hours. According to personal communication with the owner (Netherlands Energy Research Centre) studies have shown that particles larger than 0.45 mm are removed in this process.

The centrifuges were fixed to the sample collecting vessel using stainless steel hoses and a Teflon hose. During the first trip, use was made of connectors, probably made of hard PVC. For the second and third trip, these were replaced with stainless steel parts. For practical reasons, PVC hose was used to connect the Teflon hoses to the centrifuges during all three trips. In so doing, both the centrifuged and non-centrifuged water came into contact with a few centimetres of this artificial material.

1.1.6 The sample containers and bottles

The necessary litre bottles from GWA for the cholinesterase samples were prepared by the water supply company laboratory. For determining the absorption spectra, the centrifuged water samples were collected in polyethylene bottles.

The centrifuged water was collected in specially made sample containers with a volume of 25 litres. A schematic drawing of a sample container is given in figure B3.

The containers were made of stainless steel with the DIN classification '1.4404'. The percentages of the different elements in this material and thus the resistance to corrosion are similar to the stainless steel with the better known (American) classification '316'. The containers could be sealed (from air and light) with a cover of the same material. The inside of the cover and the containers were 'brushed'. Brushing is a metal removing technique that gives a surface roughness of approximately 0.3 mm. With this relatively small roughness, the loss of substances due to adsorption is minimized.

As can be seen in figure B3, the sample container contains no seams. The contact between the bottom and the side was rounded. Thus, there could be no accumulation of substances at this point.

The containers were sealed airtight with a combination of an O-ring and a clamp. The O-ring was made of the Teflon-like material Viton. The surface area of contact between the water in the container and the O-ring was relatively small.

The new stainless steel containers were thoroughly washed with an alcohol base solution (900 ml ethanol and 100 ml 40% m/m sodium hydroxide) and rinsed clean with hot drinking water (± 80 °C) before the first trip. Finally, the containers were dried with tissue paper and the surface of the containers and the covers were checked with water to see if there was any attraction to grease. If the water on certain parts of the cover did not form a uniform film, then the cleaning procedure with alcohol base solution was repeated until there was a uniform film. The containers were closed after being dried with tissue paper and cooled. While they were being wiped dry, attention was paid to the giving off of 'black material' from (some of) the surfaces.

After being emptied for XAD isolation, the containers were immediately cleaned again. The containers were then sufficiently rinsed with drinking water (approximately 80 °C) and dried with tissue paper.

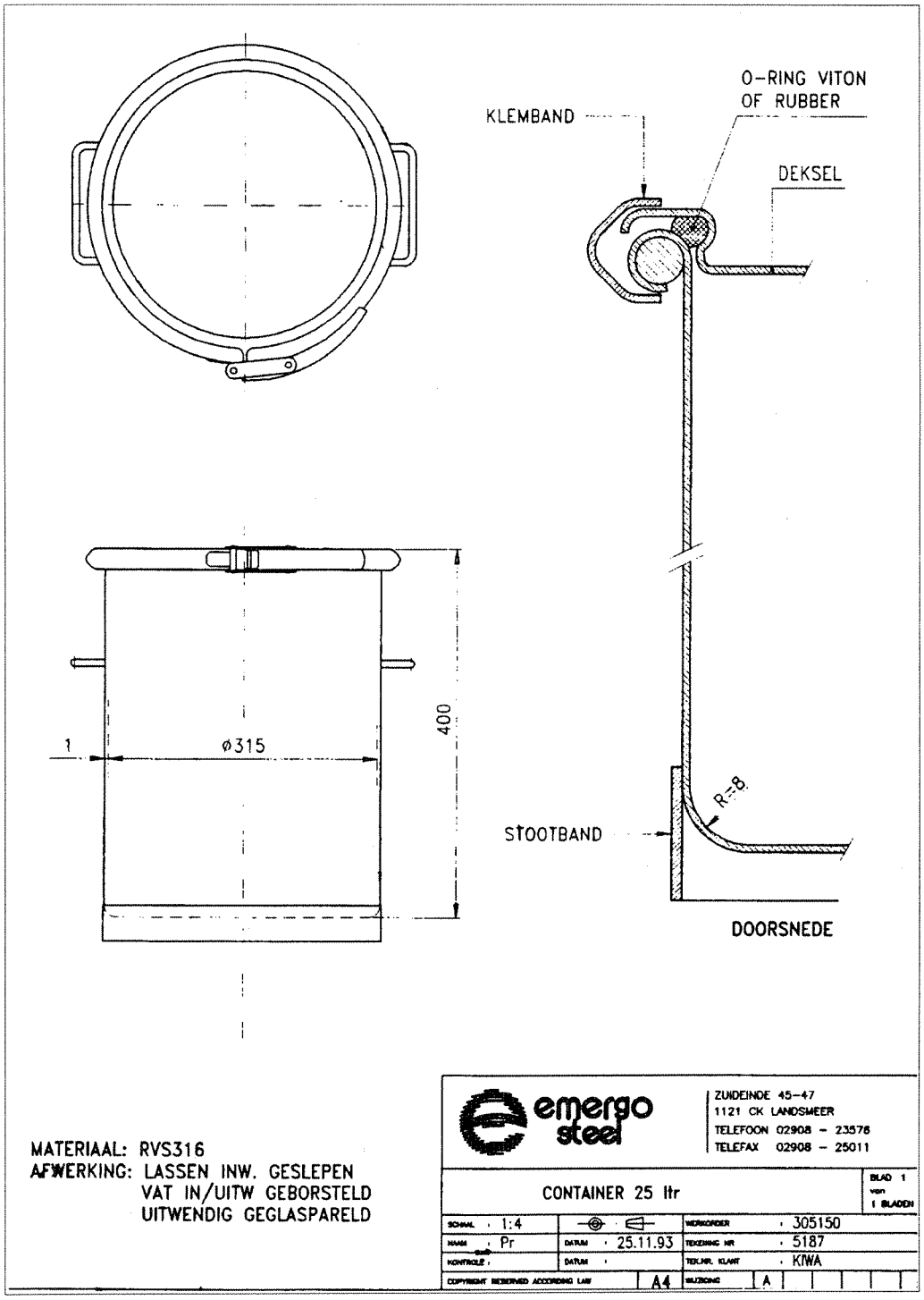

Figure B3 Schematization of a sample container

1.1.7 The cooling wagon

During the boat trip the sample containers and bottles were put in the cooling wagon directly after sampling or directly after centrifuging. The cooling wagon was on the

deck of the ship and the temperature within was maintained at 4-5 °C. During the boat trip, this temperature was checked several times a day with a thermometer in the cooling wagon.

1.1.8 The field measuring equipment

In the sampling report (appendix 2 of this report) it is stated that certain unusual conditions during the sampling can be of importance at a later time for the interpretation of results. Unusual conditions can often be visually observed. Another possibility is to conduct field measurements. For the toxicological study of the Rhine, the following field measurements were made at each location:

- water temperature;
- acidity;
- electric conductivity;
- oxygen concentration and percent saturation;
- turbidity.

In addition, the barometric air pressure and the local air temperature were recorded as part of the weather observations.

All the field measuring equipment was checked thoroughly as part of the quality control procedure before being brought on board in the Netherlands. The thermometers used were hand-calibrated with a certified thermometer. The buffer solutions for the pH-meter were controlled. For the turbidity meter, the values of the secondary standard were fixed from freshly prepared primary standards. The barometer was checked before the boat trip. The air pressure measured with this instrument was used for the calibration and adjustments of the oxygen meter.

Also during the boat trip as much as possible was done for the quality control of field measurements. The pH-meter was calibrated (and adjusted if necessary) at each sample location before being used. The same is true for the oxygen and the turbidity meters.

1.2 Sample collection

The actual sample collection for the toxicological study is described in more detail in this section. The description of the sample collection for the toxicological study is divided into several different parts. The positioning of the ship is described first. Then, the sample collection and the field measurements are described. The various activities related to processing the samples are described next. Finally, all the unusual conditions encountered during the sampling trips are discussed. Specifically, these are the conditions and circumstances that could influence the quality of the samples.

1.2.1 Positioning the ship

At a sample location, the bow of the ship was set facing upstream. In this way, the sampling crane, which was in the bow of the ship, was pointed upstream in order to avoid any sample contamination from the ship itself. During the sample collection, everything possible was done to prevent contamination from the ship. At each location, the ship was fixed in place using the positioning pole.

In the literature [NPR 6600, 1993] it is advised that sampling surface water should be done from the middle of the river. With all the shipping traffic in the Rhine, this is virtually impossible to do, especially since the shipping channel is narrow in most locations. The only exception to this was the sample location 'Rheinfelden', the most upstream navigable point of the Rhine for inland ships. At all other sampling locations, it was attempted to collect the samples from as close to the middle of the river as possible.

1.2.2 Sample collection

At each sampling location, during the three sampling series, three types of samples were collected:

- 150 litres of centrifuged water for the XAD-isolation, for conducting the Ames-test and the ecotoxicity tests;
- 1 litre of water for determining the cholinesterase inhibition;
- approximately 0.25 litres of centrifuged water for measuring an absorption spectrum from 400-800 nm.

In order to make visual observations, all samples were collected during daylight hours. Collecting the necessary samples required approximately twenty minutes. The dates and times of the sample collection are given in appendix 3 of this report.
It must be assumed that in the twenty minutes necessary to collect all samples, there was always some shipping traffic. The only exceptions to this were at the locations 'Rheinfelden' and 'Andijk'. However, there are no indications that this had a negative influence on the quality of the samples.

The sampling pump was attached to the sampler as described in section 1.1.3 of this appendix. The sampler was suspended in the water using the crane, and the pump intake was then at a depth of approximately 40 cm. This depth is in accordance to the recommended 30-50 cm sampling depth [NPR 6600, 1993; ISO 5667-6, 1990].
The samples were collected two to three meters beyond the bow of the ship. At this distance, in combination with the strong current at the sampling locations, the potential influence of the ship itself on the quality of a sample is expected to be minimal.

The pump and the Teflon hose on the outlet side of the pump were flushed (by pumping water through) for a few minutes before collecting a sample. During the

flushing in some cases the flow was measured with an available flow meter and if necessary set to the desired value of 960 l/hr. This flow corresponds with a velocity of 2 m/s in the hose at the outlet side of the pump. This sampling speed was chosen before-hand by the review committee and is based on a recommendation from the literature [ISO 5667-6] for maintaining a velocity of 0.5-3 m/s at the pump inlet.

During the flushing, the oxygen concentration and the temperature necessary for calculating the oxygen saturation were measured. These measurements were made in flowing water. During the first boat trip, this was done in an overflowing plastic bucket. In the last two trips, a stainless steel sampling can of ten litres was used. The water flowed very slowly into the bottom side of the bucket or can, and the oxygen measurements were made in the upper portion of the sampling bucket or can.

The sample for cholinesterase inhibition during the first and third sampling series was collected after the measurement for oxygen. The sample bottle was filled from the bottom directly from the pump, and thus was filled without any air bubbles. During the second sampling series, the sample for cholinesterase inhibition analysis was collected from the first filled sample container. Immediately after collection, the samples were placed in the cooling wagon.

After sufficient flushing of the pump and the hose, the sample collecting vessel was rinsed. After emptying the vessel and setting the correct flow, the vessel was completely filled. The water flowed in from above and along the side of the vessel. During the filling, the vessel was kept covered as much as possible in order to prevent any contamination. After finishing the sample collection, the colour was determined visually, and the sample collecting vessel was then closed with the cover.

During the filling of the vessel, the conditions at the sample location were noted (first page of the sample report). The water surface was constantly checked. During the second and third boat trip, the river banks were observed with binoculars for any unusual conditions. In addition, unusual conditions such as oil, foam, or other irregularities on the water surface were also noted. During the second boat trip, slide photos were taken of the sampling locations. In addition, the weather conditions during the sampling trip were noted (second page of the sampling report) while the sample collecting vessel was being filled.

1.2.3 Field measurements

The field measurements were made in the remaining water in a bucket or the sampling can in which oxygen was measured. The results of the field measurements were immediately written in the sampling report.

1.2.4 Further processing

Immediately after finishing with sample collection, the boat trip was continued, and the centrifuging of the water in the sample collecting vessel was started. As described above, the centrifuged water was put into sample containers. Per sampling location, 6 sample containers of approximately 25 litres each were filled with centrifuged water. The filled sample containers were placed in the cooling wagon together with the sample bottles.

All the sample containers were given double sample labels in case one label was lost or damaged. One label was set on the side and one on the cover. The labels on the cover were usually safe from damage, because the sample containers could not be stacked on top of each other.

The sample bottles were given either one or two sample labels. The sample labels only needed to filled out with the date of sample collection. A pen with waterproof ink was used in order to prevent the ink from running due to water or condensation, and in order to avoid any uncertainty over the collected samples.

1.2.5 Unusual conditions during sample collecting

During the first boat trip, the positioning pole was only used at the sample location 'Rheinfelden'. Due to the rocky bottom, the pole was bent and rendered useless. Later, this was repaired, so that by stations 'Lobith' and 'Hagestein' it could be used again. At the sampling stations in between, there was thus no possibility of a 'fixed' sample location. At these locations, the captain held the ship in place as best as possible using the motor, with the bow pointing upstream .

In the description of the sampler (section 1.1.3 in this appendix) reference is made to the surfacing of the sampling pump during the first boat trip. In order allow continued sampling with the pump at the remaining locations, this problem was solved by hanging the pump from the crane in such a way that it was horizontal with the inlet facing directly upstream. As a result of sampling in this way, the water was not collected from the desired depth of 30-50 cm. Despite the adapted procedure, air would still occasionally enter the pump/hose system, so that the filling of the vat proceeded in steps. The samples collected in this manner were from a depth of 0-60 cm.

Only during the first trip was there an unusual condition: at the sample location 'Bad Honnef' there was oil on the water surface.

The field measurements made during the sampling do not indicate any unusual conditions.

The second boat trip took place in a period in which there was a lot of fresh water in the upper layer as a result of high rainfall in the previous weeks. Because there was a possibility that the stretch Rheinfelden-Basel would be closed to shipping traffic when the ship left Rheinfelden, the second sampling trip started at the second sample location ('Weil am Rhein'). In order to still be able to collect samples at 'Rheinfelden', this location was visited by car and sampled from the river bank. The sample was collected from a platform that hung over the water, so that the water sample was collected several meters from the river bank. A sample container on a rope was used to collect the samples. Due to the high turbulence of the water at the location, there was no dependence of the water quality on the sample depth.

The one sample from the Bodensee (see section 2.1) was collected during the second sampling trip. The sample was collected on June 6 by a staff member of the 'Zweckverband Bodensee-Wasserversorgung' from a depth of sixty meters. The six sample vats were brought on board on the same day. This very clear, colourless water was not centrifuged. This seemed pointless based on the visual observation of the water.

During the first two days of the second sampling trip, gasoline vapour was noted in the vicinity of the centrifuge. After two days, the cause of this was discovered and the problem was fixed. In the period of the two days, two samples were centrifuged: 'Rheinfelden' and 'Weil am Rhein'. The possible influence of the vapour on the quality of the samples was minimized as much as possible by ventilating the location for the whole day.

During the second trip, the cooling wagon was not functioning for a few hours, due to an electric malfunction. However, the temperature never rose above 10 °C.

The IJsselmeer was always sampled by the inlet of the reservoir of the water supply company PWN at Andijk. The first time, use was made of a stainless steel faucet in the inlet pipe between the IJsselmeer and the reservoir. The second and third time, water was pumped directly from the IJsselmeer.

Appendix 2
The Sample Report

In relevant standards and directives for sampling of surface water [NPR 6600, 1993; ISO 5667-6, 1990] it is stated that several details concerning the location and conditions at the time of sampling should be determined and officially recorded. The reason is that certain events or unusual conditions can be of importance for the interpretation of sampling results. Some of the conditions at the time and location of sampling can be observed visually. Others can be determined with field measurements. The local weather conditions can also influence the quality of a sample, and should be recorded as well.

For the purpose of the toxicological study, a so-called sampling report for recording all the relevant information was developed prior to the first sampling trip. An example is given below. The sample report consists of four pages for recording the following information:

- general information about the time of sampling, the sample collector and relevant visual observations;
- the local weather conditions, including air pressure;
- field measurements, including a visual estimate of the colour of the water;
- a page of 'Observations', for recording any other important conditions.

On the last page of this appendix the table which is used to estimate the wind speed at the sample location is given.

Sample collection TOR '94

Conditions during sampling

sample location	....
sample collector	Martin Meerkerk (Kiwa N.V. Onderzoek en Advies)
date	day : ..... month : ..... year : 1994
day of the week[1]	0 Monday 0 Tuesday 0 Wednesday 0 Thursday 0 Friday 0 Saturday
time of sample collection - begin time - end time	 ....
sample depth	30 - 50 cm
smell	unusual smell: yes / no[2]
foam	foam on the water and/or at the water edge: yes / no[2]
oil	oil on the water and/or at the water edge: yes / no[2]
surface	frozen: yes / no[2] snow layer[3]: yes / no[2]
visual observations	unusual conditions: yes / no[2]

1) check appropriate day
2) cross out what is not relevant
3) if 'yes', see 'comments'

Local weather conditions:

ambient temperature[1]	..... °C
air pressure	..... mbar
wind speed[2]	..... Beaufort
wind direction[3]	0 north 0 northeast 0 east 0 southeast 0 south 0 southwest 0 west 0 northwest 0 variable
cloud cover	..... %
precipitation	yes / no[4]
type of precipitation[3]	0 not applicable 0 mist 0 drizzle 0 rain 0 snow 0 hail

1) in the shade
2) estimated using the table
3) check appropriate option
4) cross out what is not relevant

Surface water field measurements

temperature	..... °C
acidity	..... pH-units
conductivity	..... mS/cm/mS/cm[1]
redox potential	..... mV
oxygen saturation	..... %
oxygen concentration	..... mg/l
turbidity	..... NTU
color	0 clear 0 clear light yellow 0 clear yellow 0 clear yellow/brown 0 light yellow/brown 0 yellow/brown 0 brown 0 brown/grey 0 grey 0 clear yellow/green 0 light yellow/green 0 yellow/green 0 green 0 other color: .....

1) cross out what is not relevant
2) check appropriate color

Comment(s):

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Estimate of wind force

wind force (Beaufort)	average wind speed at 10m above sea level (km/hr)	description
0	<1	calm: smoke rises (essentially) straight up; no movement of leaves
1	1-5	light air: smoke plume indicates wind direction
2	6-11	light breeze: leaves and windvanes move
3	12-19	gentle breeze: leaves and twigs move continuously
4	20-28	moderate breeze: small branches begin to move; dust and paper swirl upwards
5	29-38	fresh breeze: leafed branches move; white caps on water surfaces
6	39-49	strong breeze: large branches move; wind whistles and buzzes
7	50-61	moderate (near) gale: trees move; wind is a hinderance
8	62-74	gale: twigs break off; forward movement is difficult
9	75-88	strong gale: light damage; roof tiles are blown off
10	89-102	storm: trees are uprooted; visible damage
11	103-117	violent storm: widespread damage
12	>117	hurricane: serious destruction

Appendix 3
Dates and Times of Sample Collection

The dates and times of sample collection are recorded in tables B1 and B2. These concern the data of the one sampling event for the ecological study and the three sampling events for the toxicological study respectively.

In table B2, the 'date' and 'begin time' and 'end time' are given for each sample location for each sampling event. The two recorded times give the time interval in which the sample for XAD-isolation is collected. The sample for cholinesterase inhibition was collected either immediately before or immediately afterwards.

Table B1 Dates and times of sample collection for the ecological study

sample location (kilometre marker)	date	time
Rheinfelden (148,6km)	08-08-'94	08:30
Weil am Rhein (171,3km)	08-08-'94	11:10
Karlsruhe (362,3km)	08-08-'94	16:15
Wiesbaden (507,0km)	09-08-'94	11:30
Koblenz (590,1km)	09-08-'94	16:45
Bad Honnef (640,0km)	10-08-'94	08:10
Köln (685,9km)	10-08-'94	10:20
Düsseldorf (727,0km)	10-08-'94	12:45
Lobith (862,1km)	10-08-'94	16:40
Vuren	11-08-'94	09:20
Hagestein (946,9km)	11-08-'94	11:35
Andijk (IJsselmeer)	11-08-'94	14:30

Table B2 Dates and times of sample collection for the three sampling series of the toxicological study.

sample location (kilometre marker)	first sampling series			second sampling series			third sampling series		
	date	start time	end time	date	start time	end time	date	start time	end time
Rheinfelden (149,0km)	22-02-'94	08:54	9:07	07-06-'94	07:32	07:47	04-10-'94	08:26	08:39
Weil am Rhein (171,3km)	22-02-'94	14:59	15:12	07-06-'94	10:26	10:39	04-10-'94	11:44	11:56
Lauterborg (352,3km)	23-02-'94	15:47	16:00	08-06-'94	13:00	13:13	05-10-'94	16:26	16:38
Wiesbaden (507,1km)	24-02-'94	11:17	11:33	09-06-'94	09:48	10:02	06-10-'94	13:35	13:47
Koblenz (590,3km)	24-02-'94	16:05	16:26	09-06-'94	14:19	14:31	06-10-'94	18:01	18:12
Bad Honnef (640,0km)	25-02-'94	07:40	07:52	09-06-'94	17:04	17:17	07-10-'94	06:41	06:54
Köln (685,8km)	25-02-'94	10:32	10:44	09-06-'94	19:38	19:51	07-10-'94	09:17	09:30
Düsseldorf (727,0km)	25-02-'94	13:02	13:14	10-06-'94	09:22	09:35	07-10-'94	11:42	11:54
Lobith (862,3km)	28-02-'94	11:31	11:43	10-06-'94	16:26	16:39	10-10-'94	09:40	09:51
Hagestein (946,9km)	28-02-'94	18:25	18:50	13-06-'94	11:50	12:07	10-10-'94	15:38	15:50
IJsselmeer (Andijk)	01-03-'94	14:10	14:28	14-06-'94	13:00	13:13	11-10-'94	13:54	14:07

Appendix 4

Comparison between the sample collection times and the actual water level and flow conditions in the Rhine River

In order to determine whether there was any possibility of a so-called 'Fliessende Welle' during any of the three sampling series for the toxicological study, the time periods of sample collection were further calculated with the 'Rhine Alarm Model'. The calculation gave an overview at the moment where the Rhine water was after sample collection at the location 'Rheinfelden'. These moments are shown graphically in figures B4 through B6.

Because sampling was not conducted at night or during the weekends, there can be a 'Fliessende Welle' between the different points. All sample locations in the figures are therefore compared with each other. On the basis of the comparison, it can be concluded that there was a possibility of a Fliessende Welle between the locations:

- for the first sampling series:
  - 'Rheinfelden' and 'Weil am Rhein';
  - 'Lauterbourg' and 'Lobith'
  - 'Wiesbaden' and 'Hagestein';

- for the second sampling series:
  - 'Weil am Rhein' and 'Hagestein';
  - 'Köln and 'Düsseldorf';

- for the third sampling series:
  - 'Wiesbaden' and 'Lobith';
  - 'Koblenz' and 'Bad Honnef'.

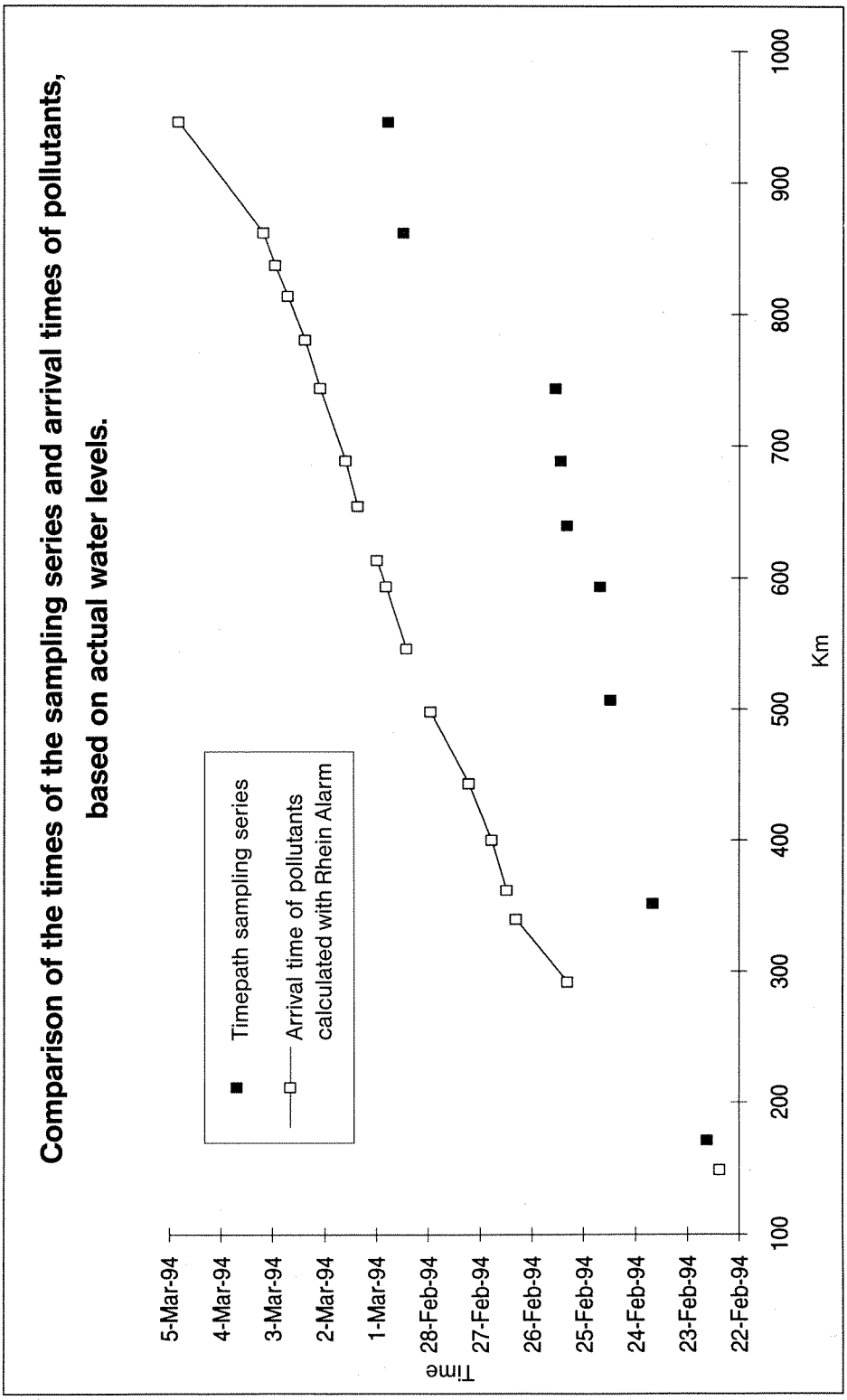


Figure B4 *Comparison of the time periods of the first sampling series and the arrival times of pollutants, based on actual water levels.*

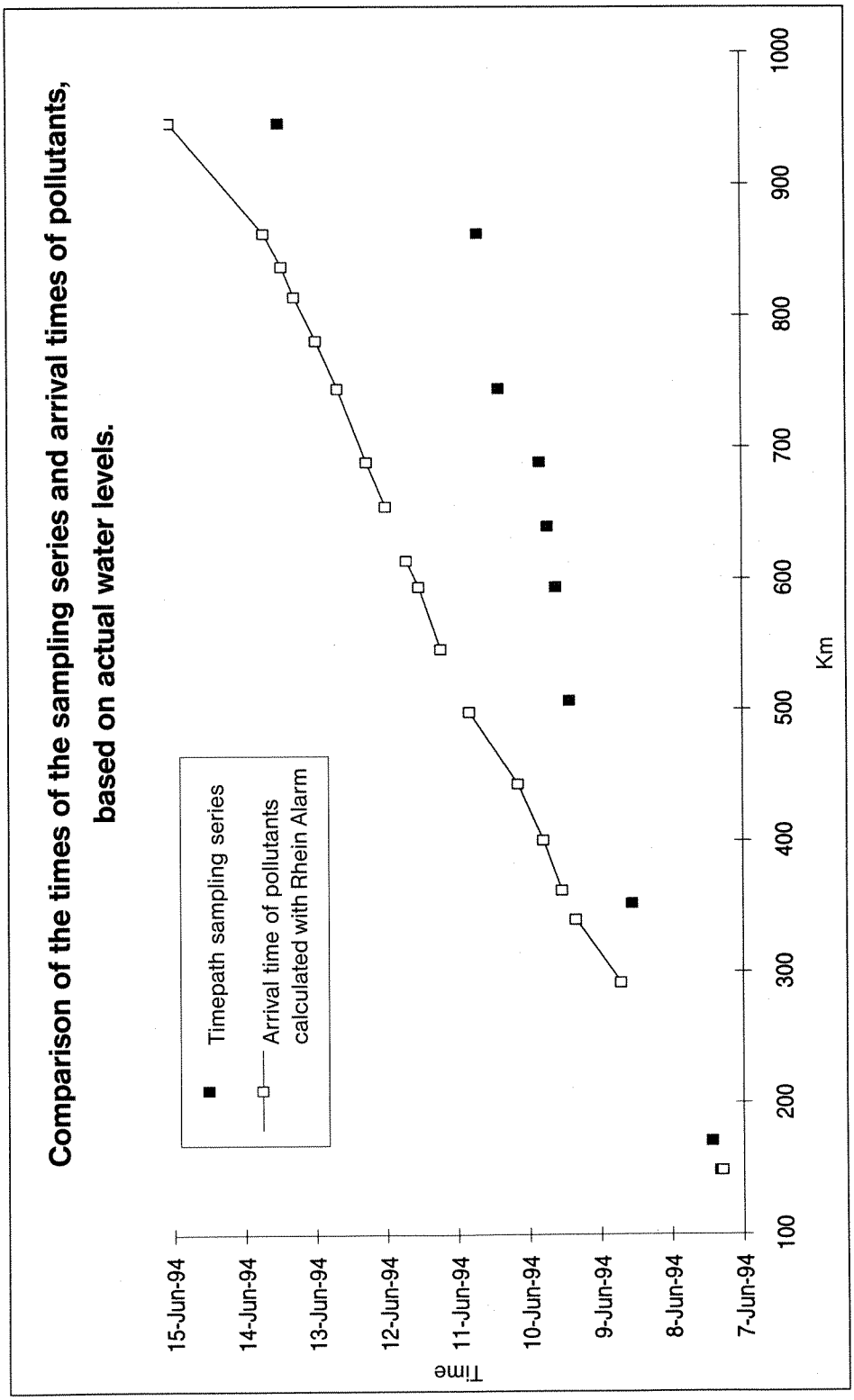


Figure B5 *Comparison of the time periods of the second sampling series and the arrival times of pollutants, based on actual water levels.*

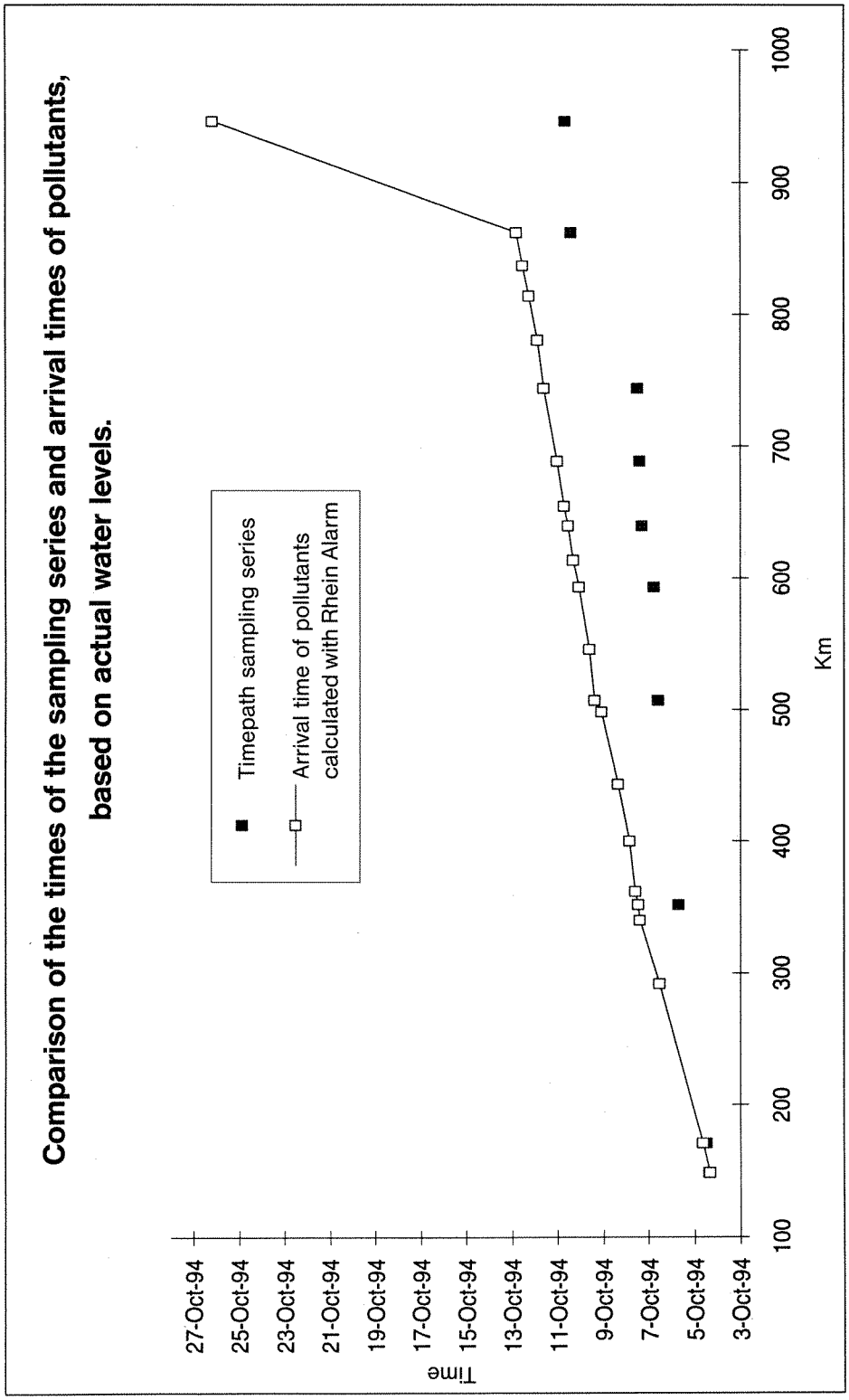


Figure B6 *Comparison of the time periods of the third sampling series and the arrival times of pollutants, based on actual water levels.*

Appendix 5

Results of the cholinesterase inhibition

The results of the cholinesterase inhibition from the three sampling series are given in Table B3.

Table B3 *Results of the cholinesterase inhibition*

sample location (kilometre marker)	concentration of cholinesterase inhibiting compounds (µg paraoxon/l)		
	1st sampling series	2nd sampling series	3rd sampling series
Sipplingen (Bodensee)	-	< 0,10	-
Rheinfelden (149,0 km)	0,11	< 0,10	< 0,10
Weil am Rhein (171,3 km)	0,11	< 0,10	< 0,10
Lauterborg (352,3 km)	0,.13	0,12	0,10
Wiesbaden (507,1 km)	0,15	0,14	0,12
Koblenz (590,3 km)	0,17	0,12	0,12
Bad Honnef (640,0 km)	0,14	0,11	< 0,10
Köln (685,8 km)	0,19	0,12	0,11
Düsseldorf (727,0 km)	0,17	0,14	0,13
Lobith (862,3 km)	0,21	0,14	0,12
Hagestein (946,9 km)	0,18	0,14	0,12
Andijk (IJsselmeer)	< 0,10	< 0,10	< 0,10

-) not sampled

Appendix 6
Results of the Ames-test

The results of the Ames-test for the first, second, and third sampling series are given in tables B4, B5 and B6, respectively. In each table, the results of the Ames-test both with and without S9-mix are given next to the sampling location. The results of both these tests are split into a neutral ('pH=7') and an acidic ('pH=2') fraction. The recorded number of 'introduced revertants' is the number of revertants after subtracting the number of spontaneous revertants from the total number of revertants:

'Induced revertants' = 'Total number of revertants' - 'Spontaneous revertants'

In order to show if there was a dose-effect relationship, a 'correlation coefficient' is given next to every test.

Table B4: Results of the Ames-test from the first sampling series.

sample location (kilometre marker)	TA98 without S9-mix				TA98 with S9-mix			
	pH = 7		pH = 2		pH = 7		pH = 2	
	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})
Rheinfelden (149,0 km)[1]	0.95	29	0.96	27	0.92	48	0.79	21
Weil am Rhein (171,3 km)[1]	0.98	104	0.94	43	0.99	279	1.00	142
Lauterborg (352,3 km)[1]	0.98	49	0.94	35	0.99	195	0.99	67
Wiesbaden (507,1 km)[2]	1.00	50	0.92	33	0.97	237	0.95	66
Koblenz (590,3 km)[2]	0.93	46	0.88	21	0.85	387	0.85	363
Bad Honnef (640,0 km)[2]	0.97	40	0.99	38	0.77	41	0.85	61
Bad Honnef[a] (640,0 km)[4]	0.98	32	0.89	19	0.99	466	1.00	93
Köln (685,8 km)[2]	0.89	16	0.72	16	0.90	220	0.79	43
Düsseldorf (727,0 km)[3]	0.99	49	0.99	34	0.98	427	0.92	104
Lobith (862,3 km)[3]	1.00	39	0.98	27	0.95	252	0.99	70
Hagestein (946,9 km)[3]	0.99	46	0.98	29	0.98	420	0.98	25
Hagestein[a] (946,9 km)[4]	0.99	39	0.99	35	0.99	424	0.98	115
Andijk (IJsselmeer)[3]	0.99	38	0.97	25	0.99	58	0.99	38
blanco XAD[3]	0.81	5	0.44	3	0.41	2	0.79	5

[a]) Ames-test repeated
[1]) 16 spontaneous revertants without S9-mix, 34 spontaneous revertants with S9-mix
[2]) 17 spontaneous revertants without S9-mix, 77 spontaneous revertants with S9-mix
[3]) 23 spontaneous revertants without S9-mix, 38 spontaneous revertants with S9-mix
[4]) 26 spontaneous revertants without S9-mix, 41 spontaneous revertants with S9-mix

Table B5: Results of the Ames-test from the second sampling series.

sample location (kilometre marker)	TA98 without S9-mix				TA98 with S9-mix			
	pH = 7		pH = 2		pH = 7		pH = 2	
	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})
Sipplingen (Bodensee)[1]	0.92	10	0.91	8	0.94	15	0.98	17
Rheinfelden (149,0 km)[1]	0.84	12	0.96	15	0.91	31	0.92	25
Weil am Rhein (171,3 km)[2]	0.93	13	0.77	7	0.99	165	0.97	38
Lauterborg (352,3 km)[2]	0.93	20	0.78	8	0.99	122	0.93	28
Wiesbaden (507,1 km)[2]	0.96	18	0.93	11	1.00	115	0.98	40
Koblenz (590,3 km)[3]	0.95	37	0.93	27	0.98	556	0.97	156
Bad Honnef (640,0 km)[3]	0.96	32	0.99	33	0.98	543	0.97	182
Köln (685,8 km)[3]	0.99	32	0.98	32	0.98	486	0.97	163
Düsseldorf (727,0 km)[4]	0.99	37	0.98	35	0.98	545	0.97	132
Lobith (862,3 km)[4]	0.98	30	0.91	25	0.98	434	0.98	104
Hagestein (946,9 km)[1]	0.94	32	0.98	32	0.99	362	0.99	125
Hagestein[a] (946,9 km)[4]	0.98	27	0.97	30	0.99	278	0.99	74
Andijk (IJsselmeer)[4]	0.90	12	0.99	24	0.99	45	0.94	33
blanco XAD[2]	0.25	1	-0.10	-1	0.37	2	-0.20	-1

[a]) sample processed within 24 hours
[1]) 31 spontaneous revertants without S9-mix, 38 spontaneous revertants with S9-mix
[2]) 27 spontaneous revertants without S9-mix, 30 spontaneous revertants with S9-mix
[3]) 26 spontaneous revertants without S9-mix, 53 spontaneous revertants with S9-mix
[4]) 22 spontaneous revertants without S9-mix, 35 spontaneous revertants with S9-mix

Table B6: Results of the Ames-test from the third sampling series.

sample location (kilometre marker)	TA98 without S9-mix				TA98 with S9-mix			
	pH = 7		pH = 2		pH = 7		pH = 2	
	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})	correlation coefficient	induced revertants (l^{-1})
Rheinfelden (149,0 km)[1]	0.96	31	0.95	32	0.99	36	0.91	25
Weil am Rhein (171,3 km)[1]	0.99	647[a]	0.97	74	0.99	483	0.99	85
Lauterborg (352,3 km)[1]	0.99	83	0.97	44	0.98	357	0.99	121
Wiesbaden (507,1 km)[1]	0.93	87	0.98	51	0.99	385	0.98	139
Koblenz (590,3 km)[2]	0.99	55	0.98	25	0.98	949	0.98	258
Bad Honnef (640,0 km)[2]	0.99	55	0.95	47	0.98	1023[4]	0.97	362
Köln (685,8 km)[2]	0.99	83	0.83	30	0.99	692	0.95	335
Düsseldorf (727,0 km)[3]	0.99	72	0.96	33	0.99	692	0.98	329
Lobith (862,3 km)[3]	0.98	50	0.99	30	0.98	606	0.98	204
Hagestein (946,9 km)[3]	0.98	40	0.95	27	0.98	305	0.99	159
Andijk (IJsselmeer)[3]	0.78	6	0.84	10	0.99	33	0.92	40
blanco XAD[2]	0.76	5	0.98	22	0.65	3	0.81	7

[a]) fraction analyzed with GC/MS
[1]) 38 spontaneous revertants without S9-mix, 44 spontaneous revertants with S9-mix
[2]) 25 spontaneous revertants without S9-mix, 51 spontaneous revertants with S9-mix
[3]) 31 spontaneous revertants without S9-mix, 40 spontaneous revertants with S9-mix
[4]) the Ames-test was repeated: 40 spontaneous revertants

Appendix 7

Results of the ecotoxicity tests

A summary of the results from the ecotoxicity tests from the first, second and third sampling series is given in tables B7, B8, and B9 respectively. In these tables, the results are presented for the *Daphnia*-IQ, Rotoxkit-F, Artoxkit-M, algae photosynthesis and Microtox tests for each sample location.

For all the tests the results are in the form of an Effect or Lethal Concentration (EC- or LC-value) given as a concentration factor, that is to say the number of times that the original water sample had to be diluted (1/*) or could be concentrated (*) in order to cause the given percentage effect or percentage mortality. These results are corrected for the method blank, which is the analytical measurement of 2.4 ml pure ethanol treated with the same procedures as the prepared surface water concentrate.

Table B7: Results of the ecotoxicity tests for the first sampling series.

sample location (kilometre marker)	*Daphnia*-IQ	Rotoxkit-F	Artoxkit-M	algae-photosynthesis		Microtox					
						pH = 7				pH = 2	
						EC_{50}		EC_{20}		EC_{20}	
	LC_{50} (*concen-trated)	LC_{50} (*concen-trated)	LC_{50} (*concen-trated)	EC_{50} (*concen-trated)	EC_{10} (*concen-trated)	5 min (*concen-trated)	15 min (*concen-trated)	5 min (*concen-trated)	15 min (*concen-trated)	5 min (*concen-trated)	15 min (*concen-trated)
Rheinfelden (149,0 km)	86	316	>500	91	8	350[1]	127	127	102	37	30
Weil am Rhein (171,3 km)	91	> 500	>500	55	5	167	48	48	39	19	15
Lauterborg (352,3 km)	99	338	>500	95	32	137	48	48	45	32	27
Wiesbaden (507,1 km)	96[2]	330	>500	27	8	71	18	18	15	23	21
Koblenz (590,3 km)	75[2]	329	>500	19	5	57	15	15	10	24	22
Bad Honnef (640,0 km)	78	353[2]	>500	17	3	66	18	18	16	39	32
Köln (685,8 km)	166[2]	333[2]	>500	51[3]	17[3]	71	19	19	19	42[1]	37[1]
Düsseldorf (727,0 km)	157	340[2]	>500	47[3]	8[3]	53	15	15	14	23	21
Lobith (862,3 km)	65	319[2]	>500	36	13	84	21	21	16	22	21
Hagestein (946,9 km)	68	316	>500	13	2	74	22	22	18	20	19
Andijk (IJsselmeer)	66	>500	>500	56	11	185	48	48	46	28	29
blanco XAD	> 500	>500	>500	>500	124	>225	>225	>225	>225	258[1]	87

[1]) extrapolated value
[2]) the effects did not increase uniformly; corrections were conducted for Spearman-Karber-estimate
[3]) longer holding time (one week)

Table B8: Results of the ecotoxicity tests for the second sampling series.

sample location (kilometre marker)	*Daphnia*-IQ	Rotoxkit-F	Artoxkit-M	algae-photosynthesis		Microtox					
						pH = 7				pH = 2	
						EC_{50}		EC_{20}		EC_{20}	
	LC_{50} (*concen-trated)	LC_{50} (*concen-trated)	LC_{50} (*concen-trated)	EC_{50} (*concen-trated)	EC_{10} (*concen-trated)	5 min (*concen-trated)	15 min (*concent rated)	5 min (*concen-trated)	15 min (*concen-trated)	5 min (*concen-trated)	15 min (*concen-trated)
Sipplingen (Bodensee)	149[2]	>500	>500	125	13	>225	>225	>225	403	71	48
Rheinfelden (149,0 km)	80[2]	>500	>500	63	11	321[1]	277[1]	78	67	71	65
Weil am Rhein (171,3 km)	122[2]	>500	>500	48	7	465[1]	349[1]	166	129	93	82
Lauterborg (352,3 km)	96[2]	>500	>500	28	7	446[1]	312[1]	83	64	88	79
Wiesbaden (507,1 km)	55	>500	>500	34	10	224	184	43	40	57	46
Koblenz (590,3 km)	47[2]	>500	>500	34	11	236[1]	185	47	40	60	54
Bad Honnef (640,0 km)	45[2]	163	>500	21	6	225	172	58	46	29	24
Köln (685,8 km)	51[2]	211	>500	21	6	245[1]	198	62	44	22	20
Düsseldorf (727,0 km)	50[2]	359	>500	28	8	138	136	31	30	27	25
Lobith (862,3 km)	33	>500	>500	22	7	155	138	40	40	40	35
Hagestein[a] (946,9 km)	68	301	>500	15	4	182	130	31	23	15	12
Hagestein (946,9 km)	47	277[2]	>500	18	5	165	137	48	49	45	44
Andijk (IJsselmeer)	<31	>500	>500	32	9	315[1]	259[1]	65	58	36	25
blanco XAD	>500	>500	>500	>500	90	>225	>225	>225	>225	>225	237[1]

[a]) sample analyzed within 24 hours
[1]) extrapolated value
[2]) the effects did not increase uniformly; corrections were conducted for Spearman-Karber-estimate

Table B9: Results of the ecotoxicity tests for the third sampling series.

sample location (kilometre marker)	*Daphnia*-IQ	Rotoxkit-F	Artoxkit-M	algae-photosynthesis		Microtox					
						pH = 7				pH = 2	
						EC_{50}		EC_{20}		EC_{20}	
	LC_{50} (*concen-trated)	LC_{50} (*concen-trated)	LC_{50} (concen-trated)	EC_{50} (*concen-trated)	EC_{10} (*concen-trated)	5 min (*concen-trated)	15 min (*concen-trated)	5 min (*concen-trated)	15 min (*concen-trated)	5 min (*concen-trated)	15 min (*concen-trated)
Rheinfelden (149,0 km)	67	244	>500	75	13	374[1]	319	101	88	55	45
Weil am Rhein (171,3 km)	68	155	>500	63	15	200	181	50	46	46	44
Lauterborg (352,3 km)	<31	67	>500	42	10	239[1]	181	58	55	46	37
Wiesbaden (507,1 km)	<31	65	>500	39	14	143	110	36	28	50	38
Koblenz (590,3 km)	71[2]	49	>500	30	9	93	72	15	13	70	67
Bad Honnef (640,0 km)	39[2]	88	>500	33	8	107	107	24	-[3]	37	25
Köln (685,8 km)	31[2]	79	>500	29	7	128	106	26	32	46	36
Düsseldorf (727,0 km)	40[2]	94	>500	25	6	98	75	17	15	36	25
Lobith (862,3 km)	38[2]	97	>500	33	11	142	106	31	27	40	30
Hagestein (946,9 km)	<31	345	>500	28	7	122	89	30	24	20	17
Andijk (IJsselmeer)	55[2,4]	>500	>500	50	11	332[1]	265	113	86	46	39[1]
blanco XAD	> 500[4]	69	>500	97	8	>225	>225	344[1]	>225	107	59

[1]) extrapolated value
[2]) the effects did not increase uniformly; corrections were conducted for Spearman-Karber-estimate
[3]) problem in measurement
[4]) background effect > 16.6%; test does not conform with validity criteria

Appendix 8

Statistical analysis of measurements

Statistically analyzed measurements:

TRIP	= sampling series number	FS	= algae photosynthesis
STN	= sample location	MTX	= Microtox (EC_{50}, neutral)
DA_IQ	= *Daphnia*-IQ (EC_{50})	R_TOX	= Rotoxkit-F (LC_{50})

TRIP	STN	DA_IQ	FS	MTX	R_TOX
1	1	86.000	91.000	288.00	316.00
1	2	91.000	55.000	141.00	M
1	3	99.000	95.000	111.00	338.00
1	4	96.000	27.000	58.000	330.00
1	5	75.000	19.000	43.000	329.00
1	6	78.000	17.000	53.000	353.00
1	7	166.00	51.000	68.000	333.00
1	8	157.00	47.000	46.000	340.00
1	9	65.000	36.000	65.000	319.00
1	10	58.000	13.000	62.000	316.00
1	11	66.000	56.000	167.00	M
2	0	149.00	125.00	M	M
2	1	80.000	63.000	277.00	M
2	2	122.00	48.000	349.00	M
2	3	96.000	28.000	312.00	M
2	4	55.000	34.000	184.00	500.00
2	5	47.000	34.000	185.00	M
2	6	45.000	21.000	172.00	163.00
2	7	51.000	21.000	198.00	211.00
2	8	50.000	28.000	136.00	359.00
2	9	33.000	22.000	138.00	M
2	10a	68.000	15.000	130.00	301.00
2	10b	47.000	18.000	137.00	277.00
2	11	M	32.000	259.00	M
3	1	67.000	75.000	319.00	244.00
3	2	68.000	63.000	181.00	155.00
3	3	M	42.000	181.00	67.000
3	4	M	39.000	110.00	65.000
3	5	71.000	30.000	72.000	49.000
3	6	39.000	33.000	107.00	88.000
3	7	31.000	29.000	106.00	79.000
3	8	40.000	25.000	75.000	94.000
3	9	38.000	33.000	106.00	97.000
3	10	M	28.000	89.000	345.00
3	11	55.000	50.000	265.00	M

Summary statistics:

	DA_IQ	FS	MTX	R_TOX
CASES	31	35	34	25
MEAN	73.84	41.23	152.6	242.7
S.D.	35.23	24.88	86.85	124.9
S.E. (MEAN)	6.327	4.206	14.89	24.98
LOWER 95.0% C.I.	60.92	32.68	122.3	191.2
UPPER 95.0% C.I.	86.76	49.78	183.0	294.3
C.V.	47.71	60.35	56.90	51.46
MEDIAN	67.00	33.00	136.5	301.0
MINIMUM	31.00	13.00	43.00	49.00
MAXIMUM	166.0	125.0	349.0	500.0

Correlations:

	DA_IQ	FS	MTX	R_TOX
DA_IQ	1.0000			
FS	0.3867	1.0000		
MTX	-0.2387	0.5108	1.0000	
R_TOX	0.4488	0.1115	0.0048	1.0000

CASES INCLUDED: 22 MISSING CASES: 13

Appendix 9

The identified exuviae

The percentage distributions for the taxa found per sample location are given in table B10. In addition, information is given per taxon as to whether the taxon is representative of flowing water, stagnant water, or if it occurs in both water types. The sample locations given here with a number can be found in appendix 11.

In table B11 an overview of the dominant and sub-dominant taxa per sample location is given. The sample locations are also given here with a number.

Table B10 Percentage distribution of exuviae in the Rhine River in 1994.

sample location	1	2	3	4	5	6	7	8	9	Vuren	10	11	habitat[1]	reference[2]
kilometre marker	148.6	171.3	362.3	507.0	590.1	640.0	685.9	727.0	862.1	955.0	946.9	IJssel meer		
	High Rhine		Upper Rhine		Middle Rhine		Lower Rhine							
Ephemeroptera														
Ephemeroptera	0.2					0.2								
cf. Caenidae				8.8									I	1
Ephoron virgo					0.3	0.5	0.7			0.2	7.9		R	1
Diptera														
Diptera			0.3							0.2				
Ceratopogonidae														
Ceratopogonidae					0.3									
Chaoboridae														
Chaoborus sp.		0.4											S	2
Chironomidae														
Chironomidae											0.2			
Chironomidae, Tanypodinae														
Conchapelopia sp.		0.2											I	3
Conchapelopia pe 1		0.2											I	3
Procladius sp.		0.7									0.6	0.2		
Procladius choreus		0.2												
Procladius pe 4		0.9									0.5		S	3
Procladius sagittalis pe 3[3]	0.2	0.2										0.2	I	3
Rheopelopia ornata		0.2	0.8	0.3		0.5	0.7	2.1		1.7	1.1		P	3
Tanypus punctipennis pe 1[3]		0.4											S	3
Telopelopia fascigera										0.2			P	3
Thienemannimyia fusciceps		0.2											S	3
Chironomidae, Buchonomyiinae														
Buchonomyia thienemanni		0.2											RP	3

sample location	1	2	3	4	5	6	7	8	9	Vuren	10	11	habitat[1]	reference
kilometre marker	148.6	171.3	362.3	507.0	590.1	640.0	685.9	727.0	862.1	955.0	946.9	IJssel meer		
	High Rhine		Upper Rhine		Middle Rhine		Lower Rhine							
Chironomidae, Orthocladiinae														
Orthocladiinae		0.2												
Cardiocladius fuscus	4.5		1.3		1.5		1.1	0.6	0.2				P	3
Corynoneura coranata			0.3										S	3
Cricotopus annulator		0.4		1.2									I	3
Cricotopus bicinctus	0.2	1.1	0.5						2.7	14.2		2.9	I	3
Cricotopus curtus		0.2	1.0		1.0	0.2							RP	3
cricotopus intersectus		0.9	0.5	0.6						0.2	1.1	90.8	I	3
Cricotopus pe 1	0.6		0.3											
Cricotopus sylvestris		0.4	1.0							6.7	0.6	0.2	I	3
Cricotopus triannulatus	0.9	6.1	19.1		24.0	7.8	4.0	32.3	18.7	0.2	0.8		I	3
Cricotopus trifascia	4.3	0.2	0.3										I	3
Cricotopus cf. *tristis*		0.4											R	3
Cricotopus vierriensis					0.5								I	3
Eukiefferiella clypeata		2.0											R	3
Eukiefferiella coerulescens	0.6	0.4											I	3
Eukiefferiella cf. *fuldensis*	0.4												R	3
Eukiefferiella fuldensis	0.2	0.7											R	3
Eukiefferiela ilkleyensis	3.0												R	3
Limnophyes sp.											10.7	0.2	I	3
Nanoclaudius bicolor	1.1	7.4	14.6	16.1	6.0	25.8	10.3	1.4	3.3	20.4			I	5
Nanocladius distinctus/rectinervis	2.2	0.98	0.5	0.6				0.2		4.9			R	5
Orthocladius cf. *oblidens*		0.2											I	3
Orthocladius rubicundus	0.2	0.2	0.5										R	3
Paracricotopus niger pe 2	1.5	2.2											R	3
Parametriocnemus stylatus		5.9											R	3
Paratrichocladius rufiventris		2.0	2.5	0.9	1.0						0.5		I	3
Pseudosmittia recta pe 1[3]											3.2		I	4
Rheocricotopus chalybeatus	42.6	6.7	27.0	29.2	10.5	1.0	4.0	5.1	32.9	3.2			RP	3
Synorthocladius semivirens	1.9	16.5	4.3	0.3	0.3	29.4	7.2	1.2					I	3
Thalassosmittia thalassophila											1.3		B	3
Thienemanniella majuscula		0.2	0.3										R	3
Thienemanniella pe 2b	0.2	0.2											R	4
Tvetenia calvescens	0.4	0.4											R	3
Tvetenia verralli[5]	26.8	1.3	1.5	1.5	32.5	22.1	54.7	40.7	11.1	1.3			R	3

sample location	1	2	3	4	5	6	7	8	9	Vuren	10	11	habitat[1]	reference[2]
kilometre marker	148.6	171.3	362.3	507.0	590.1	640.0	685.9	727.0	862.1	955.0	946.9	IJssel meer		
	High Rhine		Upper Rhine		Middle Rhine		Lower Rhine							
Chironomidae,Chironomini														
Chironomini											0.2			
Chironomini genus D[4]	0.2													
Chironomus sp.						0.2		0.2		0.2	39.5			
Cryptochironomus albofasciatus	0.2												I	3
Cryptochironomus obreptans											0.2	0.2	S	3
Cryptochironomus rostratus				0.3						0.2	2.1		I	3
Cryptotendipes usmaensis											0.3		S	3
Demicryptochironomus pe 1		0.2											R	4
Dicryptotendipes nervosus		0.2	3.8			0.2			0.2	1.5	10.7		I	3
Einfeldia cf. *pagana*												0.2	I	3
Endochironomus albipennis												0.9	I	3
Fleuria lacustris												0.2	S	3
Glyptotendipe pallens								0.2	0.5	0.2	0.2	3.4	S	3
Glyptotendipes paripes											0.2	0.4	I	3
Harnischia curtilamellata		0.4	4.8	0.3						1.7	0.8		I	3
Kloosia pusilla				20.4	0.3		0.2		0.5	30.5	0.8		P	5
Microchironomus tener										0.6			S	5
Parachironomus frequens (longiforceps)[3]						0.2			0.7	0.2			I	3
Polypedilum acifer				4.9	1.3	0.2	0.2	0.4	0.7	1.5			S	3
Polypedilum convictum		0.2											I	3
Polypedilum pe 10		0.2												
Polypedilum pullum/scalaenum			0.3	0.6						1.1	0.2		I	3
Polypedilum sordens			0.3										S	3
Stenochironomus sp.[4]		0.2											S*	5
Xenochironomus xenolabis		0.2	0.8			0.5	0.7				0.3		I	3

sample location	1	2	3	4	5	6	7	8	9	Vuren	10	11	habitat[1]	reference
kilometre marker	148.6	171.3	362.3	507.0	590.1	640.0	685.9	727.0	862.1	955.0	946.9	IJssel meer		
	High Rhine		Upper Rhine		Middle Rhine		Lower Rhine							
Chironomidae, Tanytarsini														
Cladotanytarsus cf. pe 6	0.2	0.7	1.3	2.4	1.3	2.2	10.8	0.8		0.4				
Neozavrelia pe 1		23.0											I	6
Rheotanytarsus musicola/photophilus						0.6							R	4
Rheotanytarsus pentapoda		0.4				0.5				0.2			R	4
Rheotanytarsus rhenanus	1.1	8.9	5.0	4.0	12.5	6.3	0.2						R	4
Stempellina almi pe 1[3]			0.3										S	3
Stempellina bausei				0.6									S	3
Stempellina minor			0.3										I	3
Tanytarsus eminulus		0.4	1.3										I	4
Tanytarsus heusdensis		0.2	0.3										R	4
Tanytarsus cf. pe 23		0.2												
Virgatanytarsus pe		0.2												
Trichoptera														
Trichoptera	0.6													
Cyrnus flavidus		0.4											S	7
Ecnomus tenellus			0.3						0.5	1.3	15.5		I	7
Hydroptilidae	0.2													
Hydropsyche sp	1.9	2.2	5.3	7.0	7.0	1.7	5.2	14.6	28.1	6.7	0.8		R	7
Psychomyiidae	0.6												R	7
Rhyacophila sp	2.8												R	8
Total number determined	462	460	397	329	400	411	446	486	584	465	633	445		

*) probably 'S': according to literature study no preference can be made with certainty

1) R = Rheophilons
I = Indifferent to flow conditions
S = Stagnant water
P = Potamon (river)

2) 1 = Puthz [1978]
2 = Hynes [1970]
3 = Fittkau and Reiss [1978]
4 = Langton [1991]
5 = Moller Pillot and Buskens [1990]
6 = Wilson and McGill [1982]
7 = Edington and Hildrew [1981]
8 = Botosăneanu and Malicky [1978]

3) old name according to Langton [1984]

4) name according to Pinder and Reiss [1984]

5) at 'Rheinfelden' and 'Weil am Rhein' 57 and 1 specimen(s) respectively were found with slightly unusual characteristics; these were sent to Langton for further study.

Table B11 *Dominant (D) and sub-dominant (S) taxa in the exuviae samples of the Rhine in August 1994*

taxon	sample location											
	1	2	3	4	5	6	7	8	9	Vuren	10	11
Cricotopus bicinctus										S		
Cricotopus intersectus												D
Cricotopus triannulatus			S		D	S		D	S			
Nanocladius bicolor			S	S		D	S			D		
Rheocricotopus chalybeatus	D		D	D	S				D			
Synorthocladius semivirens		S				D						
Tvetenia verralli	D				D	D	D	D	S			
Chironomus sp.											D	
Glyptotentipes pallens												S
Kloosia pusilla				D						D		
Cladotanytarsus cf. pe 6							S					
Neozavrelia		D										
Rheotanytarsus rhenanus					S	S						
Ecnomus tellenus											S	
Hydropsyche sp.								S	D			

Appendix 10
Determining the actual river discharge

Measurements of the flow in the Rhine were collected for the periods in which the three sampling series for the toxicological study were conducted. Measurements for the locations Maxau (362.327 km), Kaub (546.230 km), Andernach (613.780 km), Köln (688.000 km), and Rees (837.380 km) were furnished by the Bundesanstalt für Gewässerkunde in Koblenz. The flow measurements for locations Lobith and Hagestein were obtained from RIZA.

The flow measurements from these stations for the three sampling periods are given in tables B12, B13, and B14. These are daily averaged flow values. These measurements are used to calculate the flow in the Rhine at each sampling location for the day of the sample collection. The procedure for each specific location is described below. The measurement values which were used for this are given in bold face in the tables.

The flow measurements from sampling locations between 'Lauterbourg' and 'Lobith' are calculated on the basis of the provided measurements. For 'Lauterbourg', the measurements from the location Maxau were used directly, since the difference in the locations is only 10 km. 'Wiesbaden' was calculated by interpolating the flow measurements from Maxau and Kaub. The sample collection point 'Koblenz' is upstream of the Mosel. Because the measurement point Andernach also includes water flowing into the Rhine from the Mosel, the daily average flow at this sample location (Koblenz) was calculated by interpolation from the measuring locations at Maxau and Kaub, and not from interpolation from the locations Kaub - Andernach.

The daily average flow for 'Bad Honnef' was calculated from interpolation in the reach Andernach - Köln . The daily average flow for 'Köln' was taken directly from the flow measuring station, due to the small distance (2 km) between the two locations for measuring flow and collecting water quality samples. For calculating the flow at location 'Düsseldorf', the flow measurements at Köln and Rees were interpolated. At 'Lobith', the data from measurement point Lobith were used directly.

The measurements from the location 'Hagestein' are given in the following tables, but were actually not used. The sample location is in the Rhine Delta where only part of the river flows, and therefore cannot be used for calculating a load.

In the period of the third sampling series, no flow measurements for the locations Maxau, Köln, or Rees were obtained. Due to the small difference in flow at the measuring stations during this sampling period, the daily average flow from Kaub was used for locations 'Wiesbaden' and 'Koblenz'. For 'Bad Honnef', 'Köln', and 'Düsseldorf', the measurements from Köln were used directly. For 'Lauterbourg', an estimate was made.

For the stretch of the Rhine upstream of Maxau, there were no flow measurements available. In order to get some idea of the flow in the river for the stretch Basel-Karlsruhe, the yearbooks of the AWBR for 1992 and 1993 [AWBR, 1993; AWBR, 1994] were examined. The average flow by Birsfelden (163.9 km) in 1992 was about 85% of the flow by Karlsruhe (359.3 km). In 1993, it was approximately 89%.

The flows determined for the sampling locations from 'Lauterbourg' to 'Lobith' are given in table B15.

Measurement location (kilometre marker)	daily average flow (m^3/s)							
	22-02-'94	23-02-'94	24-02-'94	25-02-'94	26-02-'94	27-02-'94	28-02-'94	01-03-'94
Maxau (326.327km)	976	**1000**	**1010**	1120	1180	1160	1140	1180
Kaub (546.230 km)	1360	1400	**1480**	1550	1630	1760	1770	1750
Andernach (613.780 km)	1730	1790	1970	**2210**	2320	2450	2400	2320
Köln (688.000 km)	1840	1820	1940	**2200**	2350	2470	2500	2420
Rees (837.380 km)	2050	2000	1990	**2070**	2240	2390	2520	2580
Lobith	2027	1966	1923	1961	2119	2268	**2382**	2497
Hagestein	302	329	327	329	337	446	-	-

N.B. The values in **bold type** were used to calculate the daily average flow in the Rhine at a sample location.

Table B12: Flow measurements in the Rhine River during the first sampling series.

Measurement location (kilometre marker)	daily average flow (m^3/s)							
	07-06-'94	08-06-'94	09-06-'94	10-06-'94	11-06-'94	12-06-'94	13-06-'94	14-06-'94
Maxau (326.327km)	2270	**2170**	**2140**	2380	2430	2370	2320	2160
Kaub (546.230 km)	2760	2750	**2700**	2660	2690	2800	2780	2720
Andernach (613.780 km)	3070	3010	**2950**	2980	2980	2980	2980	2920
Köln (688.000 km)	3160	3140	**3070**	**3060**	3090	3070	3060	3020
Rees (837.380 km)	3170	3200	3200	**3200**	3220	3200	3150	3130
Lobith	3110	3180	3180	**3188**	3188	3211	-	-
Hagestein	518	516	529	540	567	544	-	-

N.B. The values in **bold type** were used to calculate the daily average flow in the Rhine at a sample location.

Table B13: Flow measurements in the Rhine River during the second sampling series.

Measurement location (kilometre marker)	daily average flow (m^3/s)							
	04-10-'94	05-10-'94	06-10-'94	07-10-'94	08-10-'94	09-10-'94	10-10-'94	11-10-'94
Maxau (326.327km)	-	-	-	-	-	-	-	-
Kaub (546.230 km)	1370	1410	**1480**	1490	1410	1330	1280	1220
Andernach (613.780 km)	1510	1560	1650	**1660**	1610	1510	1450	1400
Köln (688.000 km)	-	-	-	-	-	-	-	-
Rees (837.380 km)	-	-	-	-	-	-	-	-
Lobith	1604	1634	1576	1571	1617	1621	**1590**	1480
Hagestein	150	200	167	143	153	158	174	133

N.B. The values in **bold** type were used to calculate the daily average flow in the Rhine at a sample location.

Table B14: Flow measurements in the Rhine River during the third sampling series.

Table B15: Calculated flows during the three sampling series for sample locations 'Lauterbourg' to 'Lobith'

Sample location	daily average flow (m^3/s)		
	1st series	2nd series	3rd series
Lauterborg	1000	2170	1050[1]
Wiesbaden	1370	2581	1480
Koblenz	1593	2834	1480
Bad Honnef	2205	2992	1660
Köln	2200	3070	1660
Düsseldorf	2166	3096	1660
Lobith	2382	3188	1590

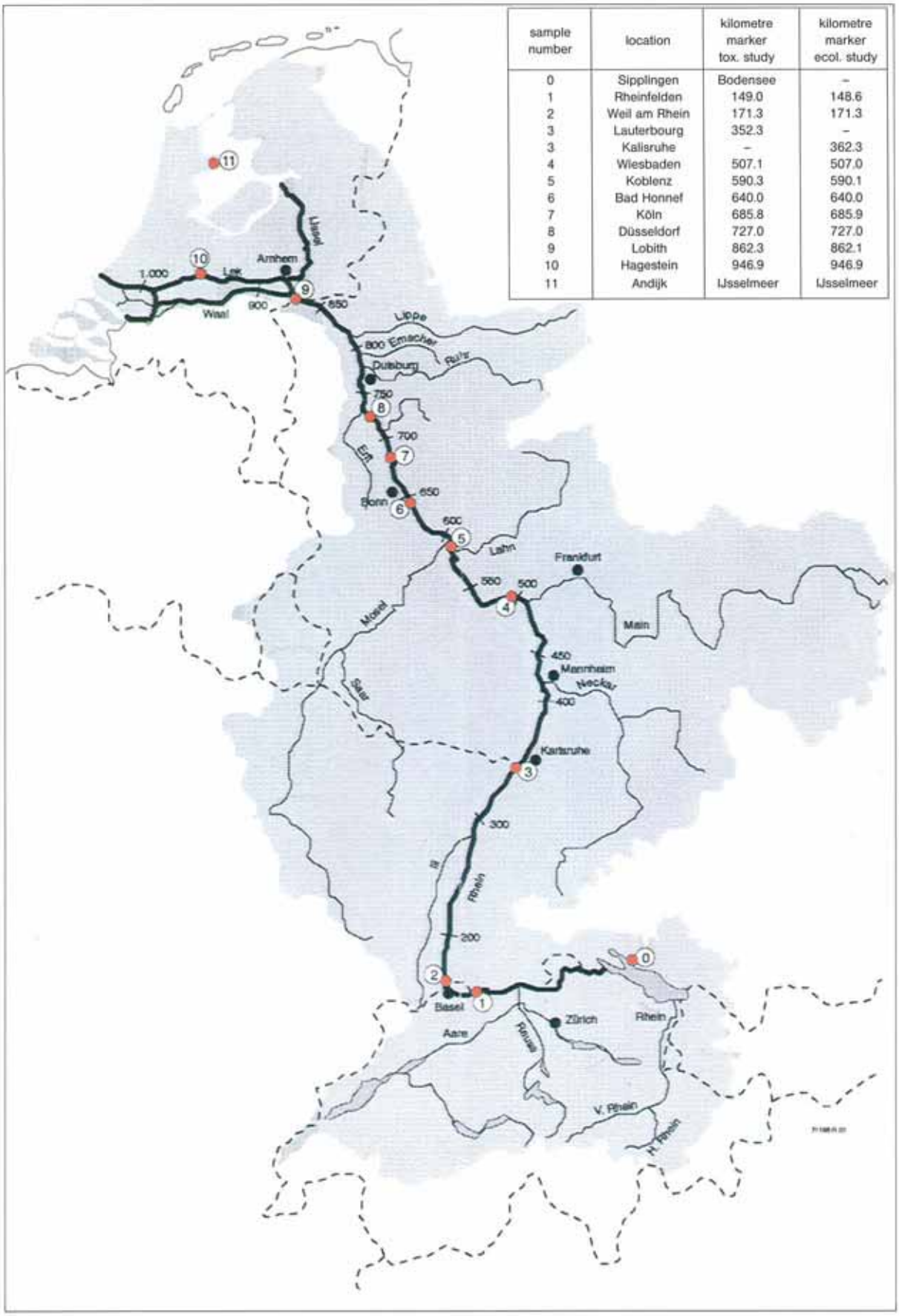
[1]) estimated value

Appendix 11

Overview of the Rhine River basin showing the sampling locations

The overview of the Rhine River basin is shown here on the fold-out page (figure B7). The table in the upper right-hand corner gives the numbers and location names of the sampling points. The sample locations are denoted by means of the 'kilometre marker'. The kilometre marker refers to the number of flow kilometres, calculated from the Rhine bridge at Konstanz. The kilometre marker is given for both the toxicological and the ecological study, because in some cases there was a (small) difference in the sampling location. The difference for sample location '3' was relatively large; this location is therefore given twice.

The geographic position of the sample locations is shown on the map by the circled numbers along the river.



sample number	location	kilometre marker tox. study	kilometre marker ecol. study
0	Sipplingen	Bodensee	–
1	Rheinfelden	149.0	148.6
2	Weil am Rhein	171.3	171.3
3	Lauterbourg	352.3	–
3	Kalisruhe	–	362.3
4	Wiesbaden	507.1	507.0
5	Koblenz	590.3	590.1
6	Bad Honnef	640.0	640.0
7	Köln	685.8	685.9
8	Düsseldorf	727.0	727.0
9	Lobith	862.3	862.1
10	Hagestein	946.9	946.9
11	Andijk	IJsselmeer	IJsselmeer

Figure B7 Sampling locations in the Rhine River basin