ASSOCIATION OF RIVER WATERWORKS

RIWA

March 2003

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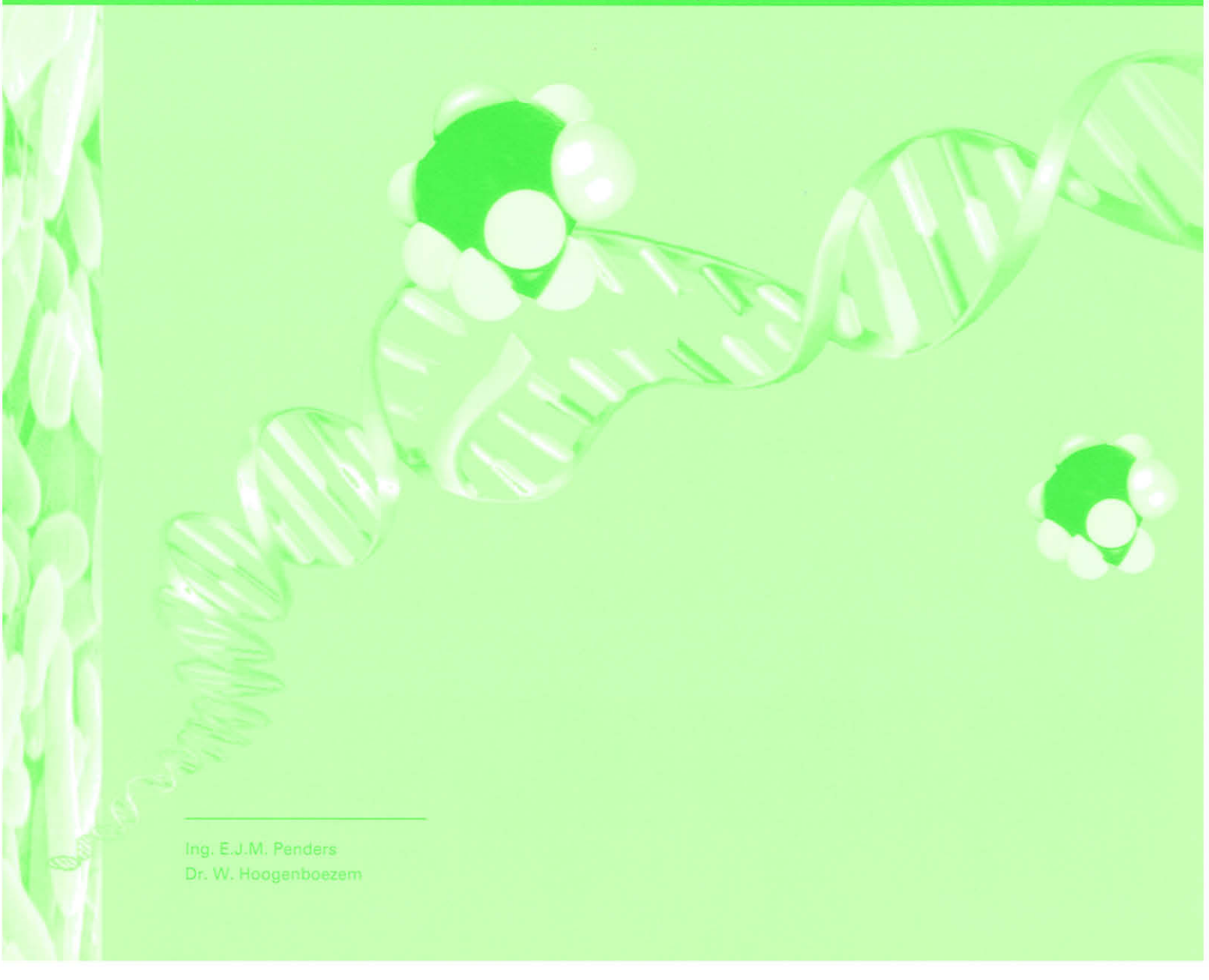

Ing. E.J.M. Penders
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Abbreviations

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

PCA	=	Principal Component Analysis
PWN	=	N.V. PWN Waterleidingbedrijf Noord-Holland (PWN Water Supply Company North Holland)
RIWA	=	Vereniging van Rivierwaterbedrijven (Association of River Waterworks)
VITO	=	Vlaamse Instelling voor Technologisch Onderzoek (Flemish Institute for Technological Research)
WRK	=	N.V. Watertransportmaatschappij Rijn-Kennemerland (Water Transport Company Rhine-Kennemerland Ltd.)
XAD	=	Synthetic Resin with a macroreticulate structure based on a styrene and divinyl benzene

Summary

Several studies have been conducted to determine the genotoxicity of surface water used for drinking water production. In a previous study (Penders & Hoogenboezem, 2001), performed by the Association of River Waterworks (RIWA), a wide range of bioassays including several genotoxicity tests were carried out in riverwater samples. The results of this study did not allow to present some conclusions in respect to the suitability of genotoxicity tests. Additional measurements using three genotoxiticity tests were therefore carried out via a new study and are presented here.

Aim

The aim of this RIWA study was:

- to find the most suitable genotoxicity test for detecting mutagenity in the Dutch rivers;
- to compare water quality data of the Ames TA98 with the UMU and Comet assay applied to the same sample;
- to define the restrictions of the genotoxicity tests studied here;
- to suggest what kind of approvements can be made in the procedure of the current tests;
- to present the status of quality in both rivers and what can be expected in the near future.

Study setting

Samples were taken from the River Meuse at Eijsden and from the River Rhine at Lobith and Nieuwegein from March to November 2000.

Methods

Large volumes of surface water samples (100 litre) adjusted to pH 7, were concentrated using XAD-4 in a column as adsorbant and a gradient of ethanol in cyclohexane as eluent. After distillation of the eluent and reducing the volume of ethanol by evaporating with nitrogen, an ethanol extract of the sample with a concentration factor of 25000x was obtained for further analysis with the genotoxicity tests Ames TA98, UMU and Comet.

Results

Only the evaluation of results from the battery of genotoxicity test compared to the Ames TA98 test are presented in this summary. For more detailed information about the different methods and their results, the reader is referred to sections 2.2 and 3 in this report.

By combining all the results from the different genotoxicity tests, in all samples at location Lobith (Rhine), genotoxic compounds were present. At the location Nieuwegein (also Rhine), all samples were positive when S9 mix was used in the test and in 4 out of 5 samples genotoxic compounds were present when S9 mix was not applied. At the location Eijsden (Meuse), 4 out of 5 samples were positive without the use of S9 and 3 out of 5 samples were positive with the use of S9 mix. When the measurement of genotoxicity was estimated on the use of the Ames TA98 test only (as has been done in the past), the degree of genotoxicity varied considerable. With the use of S9 mix, the number of genotoxic samples slightly decreases from 13 samples to 12 samples (table 2). Without the use of the S9 mix, the differences are clearly visible. No samples were considered genotoxic when information was used

from the Ames TA98 test only. In contrast, 13 samples were considered genotoxic using the UMU and the Comet assay.

The Ames TA98 assay displays a significant difference between Rhine and Meuse, when S9-mix was introduced into the samples. Only at sample location Lobith, there was a significant difference when only the Ames TA98 test was used with or without the S9-mix. Further, no significant differences were found between the measurements of location Lobith and Nieuwegein from the same river when tested with the Mann-Whitney-U test. Via the Principal Component Analysis, using the three genotoxicity test, little redundant information was obtained.

Overall conclusions

- It is not possible to select a single genotoxicity test for monitoring purposes in Dutch rivers.
- The best possible set of genotoxicity assay are the combination of Ames TA98 or UMU assay and the Comet Assay, due to their different points of impact for genotoxic compounds. Via this study, for the time being, the Ames TA98 is preferred above the UMU assay due to the higher number of positive extracts of surface water samples when the S9 mix was applied.
- Concentration of surface water samples is still required when genotoxicity tests are used. Due to the use of the XAD concentration technique, the measured genotoxicity can only be related to the non-polar compounds present in surface water. No techniques are available to concentrate all components (also metals and polar organics) from the water sample into the desired level in which the genotoxicity tests will give a response.
- Additional studies may elucidate why different tests perform so unexpectedly different. The Ames TA98 test seems to be the most sensitive test, but on theoretical grounds UMU-test is expected to be much more sensitive. It is reasoned that the sensitivity of the detection method of the response products in the UMU-assay is far too low. Reifferscheid & Zipperle (2000) showed a more or less ten times lower detection limit in UMU-assay using luminometric and fluorometric techniques, demonstrating that improvements are possible indeed.
- Distinct genotoxic differences between Rivers Rhine and Meuse have been demonstrated based on the results from the Ames TA98 test and a significant decrease of toxicity has been observed in both rivers in the period 1994 – 2000. However, the level of genotoxicity can be perhaps as low as 15 revertants per litre which has been measured in 1994 at the location of Sipplingen (Bodensee), by preventing input of chemicals into the rivers.

Recommendations

- Removal capacity of the present sewage treatment plants is not known. Since a distinctly higher toxicity was still measured in the River Rhine, it is recommended to investigate in a limited pilot study whether the compounds in the raw sewage are removed during the treatment process or not.
- Using a small series of samples testing River water and raw sewage water, the Ames TA98 test, with the use of S9 at pH = 7, is probably the best assay for this evaluation.
- Additional bio-assays (e.g. Sister Chromatid Exchange test) detecting chromosome damage in vertebrates (fish) exposed to unconcentrated river water for an intermediate period of time, reveals perhaps better knowledge on the genotoxicity of river water.
- Due to significant differences in genotoxicity of both rivers, it is still recommended to monitor genotoxicity profoundly.

1. Introduction

By using genotoxicity tests since 1986, RIWA acquired new information that could not be obtained from standard chemical or biological tests . The mutagenity of the River Rhine in comparison with the River Meuse was very different and high when measurements started, and could not be explained by the obtained chemical results (e.g. Veenendaal & Van Genderen, 1997). However when RIWA included genotoxicity in their monitoring program for rivers and did publish the results, it did give a possible approvement of water quality of the Rhine and the Meuse in the last decade.

To measure the mutagenity of the Rhine and the Meuse, the Ames test with mutant TA98 was being used since the early 80-ies. In 1998, RIWA conducted a new investigation to measure the water quality of both rivers by using bioassays, in which a number of genotoxicitytests was included (Penders & Hoogenboezem, 2001). Apart from the Ames TA98 genotoxicity test, other tests (like the UMU and Comet assay) were also conducted to get additional information.
Meanwhile, the procedures of the UMU and the Comet assays were less laborious compared to the procedure of the Ames TA98 assay, so both assays could be conducted in a laboratory of waterworks in the near future. However, during this investigation it became clear that the Ames TA98 test had a distinct detection limit compared to other genotoxicity tests. An explanation for the differences in results at that moment, were the limited and ambiguous procedures of the genotoxicity tests for extract of surface waters. Often, the results obtained by other genotoxicity tests were just on the detection limit and interference or toxicity problems were observed by using the required extraction solutions. As a recommendation in that report, further research to the use of the UMU and Comet assay was suggested to verify the water quality of the rivers by using the Ames TA98 test.

In this report, results from a new RIWA investigation are presented, in which the Ames TA98, UMU and Comet assays were used on samples from the rivers Rhine and Meuse during the year 2000. One extra sample point on the River Rhine (Nieuwegein) was included in the investigation and only extracts from surface water at pH=7 on the XAD column were studied here, since earlier experiments showed hardly positive testresults at pH=2 and pH=11 (Puijker *et al.* 1988-1992, Veenendaal *et al.*, 1995-1999, Penders & Hoogenboezem, 2001).
The aim of this study was to obtain answers to the following questions:

- What is the most suitable genotoxicity test to detect mutagenity in the Dutch rivers?
- Are Ames TA98 results fully comparable to the results of UMU or Comet assay applied to the same samples?
- What are the restrictions when genotoxicity tests are being used?
- What kind of approvements can be made in the procedure of the present tests?
- What is the status of quality in both rivers and what can be expected in the near future?

2. Description of the methods used and the logistics

2.1. Logistics of sampling and sample processing

For this project, the locations Eijsden (Meuse), Lobith (Rhine) at the border of the Netherlands and Nieuwegein (Rhine) were chosen as sampling locations (figure 1). For each sample, a volume of 100 litres of surface water was transported to KIWA in Nieuwegein. After concentration of the sample, the extracts in ethanol were sent to the laboratories.

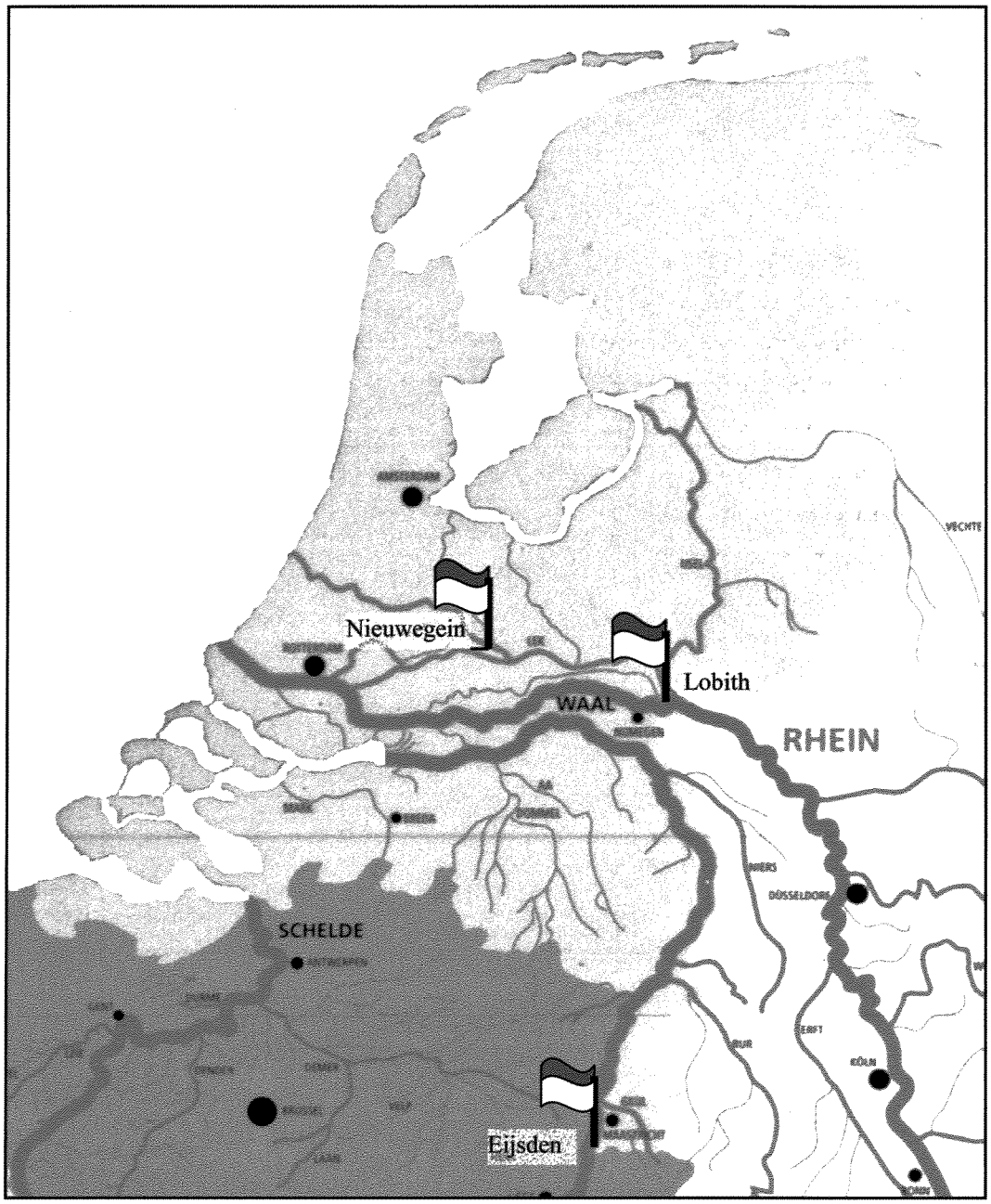


Figure 1: Sampling locations genotoxicity tests.

Table 1: Sampling date's of the genotoxtests programme for 2000 with their sample code.

River	Location	Date	Sample code
Meuse	Eijsden	March 21st, 2000	E2103
		May 16th, 2000	E1605
		July 11th, 2000	E1107
		September 5th, 2000	E0509
		October 31st, 2000	E3110
Rhine	Lobith	March 22sc, 2000	L2203
		May 17th, 2000	L1705
		July 12th, 2000	L1207
		September 6th, 2000	L0609
		November 1st, 2000	L0111
	Nieuwegein	April 19th, 2000	N1904
		June 14th, 2000	N1406
		August 9th, 2000	N0908
		October 5th, 2000	N0510
		November 29th, 2000	N2911

The samples with a pH set to 7, were concentrated according to a procedure developed by Kiwa (Noordsij et al., 1983 and 1984), using XAD-4 (Amberlite) in a column as absorbant and a gradient of ethanol in cyclohexane as eluent. After distillation of the eluent and reducing the volume of ethanol by evaporating with nitrogen, an ethanol extract of the sample with a concentration factor of 25000x was obtained for further analysis with the genotoxicity tests.

2.2. Description of the genotoxicity tests used

Ames TA98 test

The Ames TA98 genotoxicity test is carried out with a mutated strain of *Salmonella typhimurium* bacteria. These mutants do not grow on a histidine-free medium. Individuals whose histidine gene has been recovered by means of reverted mutations due to, for instance the presence of a mutagenic substance, are able to form colonies on this medium (revertants). After a 3-day incubation period, these revertants can be counted as colonies (see figure 2a). A sample is considered to be mutagenic when the number of revertants counted per plate is at least twice the number of spontaneous revertants (obtained from control plates without samples), and when there is a dose-effect relationship. Different strains of mutated *Salmonella typhimurium* can be used (e.g. TA98, TA100, TA1535 or TA1538). Some will detect base pair (smallest element of DNA) substitutes, while others will detect the removal or addition of a base pair. In this project the TA98 strain was used, by means of which frameshift mutations can be registered. This test was performed at Kiwa (Veenendaal, 2001).

UMU test

In the UMU genotoxicity test (Oda *et al.*, 1985, Reifferscheid *et al.*, 1991; Reifferscheid & Heil, 1996) a modified strain of *Salmonella typhimurium* TA1535/pSK1002 bacteria is used, whereby an enzyme gene (ß-galactosidase) is linked to the SOS-DNA recovery system. In the case of DNA damage, the SOS-DNA system is induced, whereby production of the enzyme also takes place. The more DNA damage occurs, the more ß-galactosidase is produced. After an incubation period of 1½ hours, the amount of enzyme produced is determined by means of the application of the 0-nitrophenol galactopyranoside substrate (ß-galactosidase produces a yellow colour, which can be quantified spectrophotometrically, see figure 2b). To correct for the spontaneous mutations, the extinction measured is corrected on the basis of the measurements of the blanks. The amount of enzyme product measured is a measure of the sample's mutagenicity, while taking into account the test strain's growth speed. If this is too low, the sample is toxic rather than mutagenic. A sample is considered positive if the induction rate is higher than 1,5. The test is controled using 4-nitroquinoline-N-oxide (4NQO), when no S9-mix was used, and 2-aminoanthracene (2-AA) with the use of S9. On both controls, the induction rate must be 2 or higher. Negative controls are the use of no bacterial suspension and the use of ethanol and DMSO. This test was performed at Aquasense (Aquasense, 2000).

Comet test

The Comet test (Tice, 1995), a very recent technique, measures a very different genetic endpoint. The alkaline comet test used in this study detects both single and double strand breaks and alkali labile sites. Alkali labile sites in DNA are 'vulnerable' parts of DNA, which lead to breaks in the DNA under alkaline circumstances. They are not present as such, and will probably result in an abnormality.
In the Comet test, lymphocytes from human blood after 2 hours of exposure to the sample are lysed in gel on a microscope slide, in order to release and denature DNA, and to subject this to a gel-electrophoresis. Under the influence of the electrical field created, the DNA will undergo a certain migration, whereby

small DNA fragments migrate further than larger fragments or intact DNA. A 'comet'-shaped pattern is formed (figure 2c), whereby the length and content of the comet tail provides a measure for the DNA damage. The 'comets' can be analysed after coloration with a fluorochrome (e.g. ethidium bromide) by means of a fluorescence microscope. In this study, a sample is considered genotoxic when in the tail of the comet more than 10 % DNA is present. Benzo(α)pyrene and cyclophosphamide are used as positive control solutions when S9 mix test are performed. Potassiumdichromate (10^{-4} M) is used as a positive control solution when no S9 mix is introduced into the samples. Two negative controls are used; a not treated blood sample and a in ethanol diluted sample (1/32). The test was performed at VITO (Verschaeve & Van Gorp, 2000).

The used genotoxicity tests are expected to provide different information depending on the mode of action of the genotoxic substance (alteration of a base-pair, inducing DNA damage repair system, or fragmentation of chromosomes).

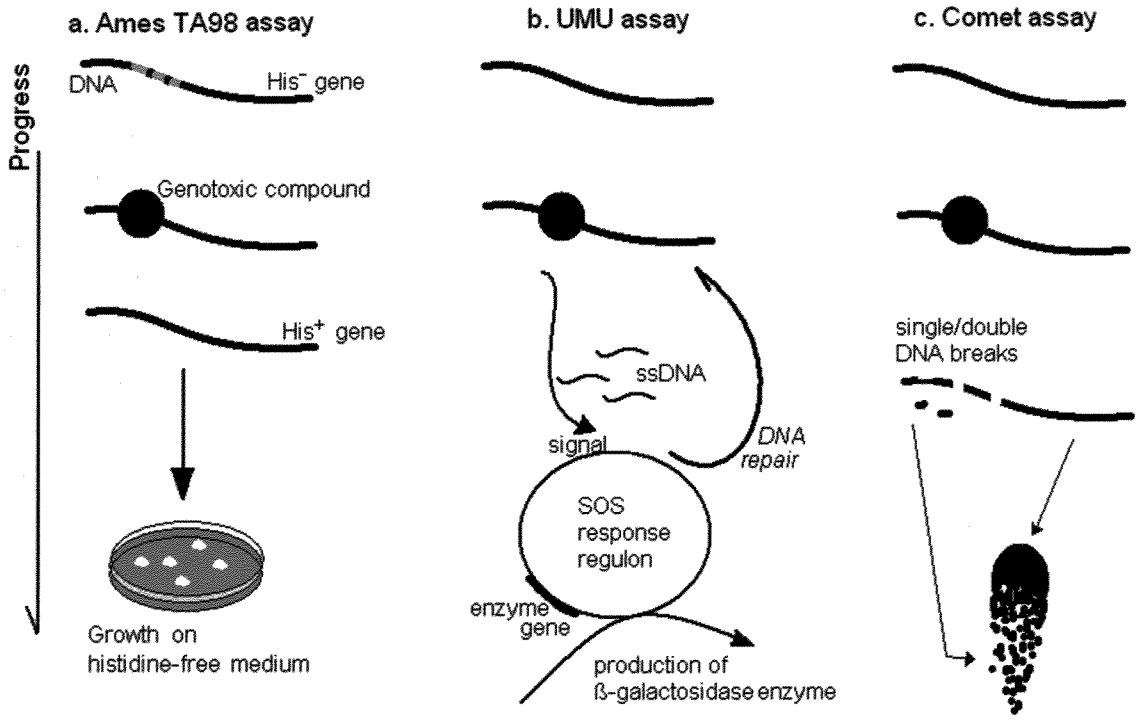


Figure 2: Different setup of the used genotoxicity tests

Different genotoxic compounds may be capable to induce DNA damage on different sites on the DNA. The UMU is assumed to detect any DNA damage that occurs, by induction of the SOS repair mechanism. The Ames TA98 test gives a response only when one single specific gene has changed. In an optimal situation, it is therefore expected, that the UMU assay is more sensitive compared to the Ames TA98 test.
In the Comet assay, chromosome fractures are detected, the induction of many particles in the comet tail require a higher number of genotoxic components intruding each cell. It is therefore assumed that the comet assay will be less sensitive compared to the Ames TA98 test and the UMU-test.

3. Results and evaluation

In the present study three locations were studied: Eijsden (Meuse) Lobith and Nieuwegein (Rhine). All genotoxicity tests were carried out in test series with and without addition of S9, an extract from the liver of rats (*Rattus norvegicus*). Enzymes in this extract are supposed to simulate human physiology and may show increase or decrease of toxicity due to digestive activity.
Table 2 presents the combined results from the Ames TA98, UMU- and Comet assay, in which the sample is considered positive when one or more genotoxicity test indicate the presence of genotoxic compounds. In all samples of the River Rhine genotoxic compounds were present when the S9 mix was applied.

Table 2: Combined results from the used genotoxicity tests (Ames TA98, UMU and Comet) when applied to concentrated surface water. Positive AmesTA98 results are displayed in brackets.

Sample location	Number of samples tested	Number of samples positive without S9	Number of samples positive with S9
Lobith (Rhine)	5	5 (0)	5 (5)
Nieuwegein (Rhine)	5	4 (0)	5 (5)
Eijsden (Meuse)	5	4 (0)	3 (2)
Total	15	13 (0)	13 (12)

From the River Meuse, 3 out of 5 samples are considered to be genotoxic. Without the use of the S9 mix, at the location Lobith (Rhine), genotoxic compounds were present in all samples, but at the location Nieuwegein (Rhine), 4 out of 5 samples have genotoxic compounds. At Eijsden (Meuse), 4 out of 5 samples are positive for the presence of genotoxic compounds.
When the measurement of genotoxicity was based on the use of the Ames TA98 test only (as has been done in the past), the number of genotoxic positive samples is different. With the use of the S9 mix, the number of genotoxic samples decreases from 13 samples as stated in table 2 to 12 samples when information was obtained from the Ames TA98 test only (see annex 1). Without the use of the S9 mix, the differences are clearly visible. No samples were considered genotoxic when information was used from the Ames TA98 test only, in contrast using the UMU and the Comet assay, 13 samples were considered genotoxic.
The Ames TA98 test results applying S9 showed higher toxicity. Both Rhine locations showed higher level of toxicity than those from the River Meuse and exceeded the toxicity level set by a double number of revertants compared to the control test (figure 3).

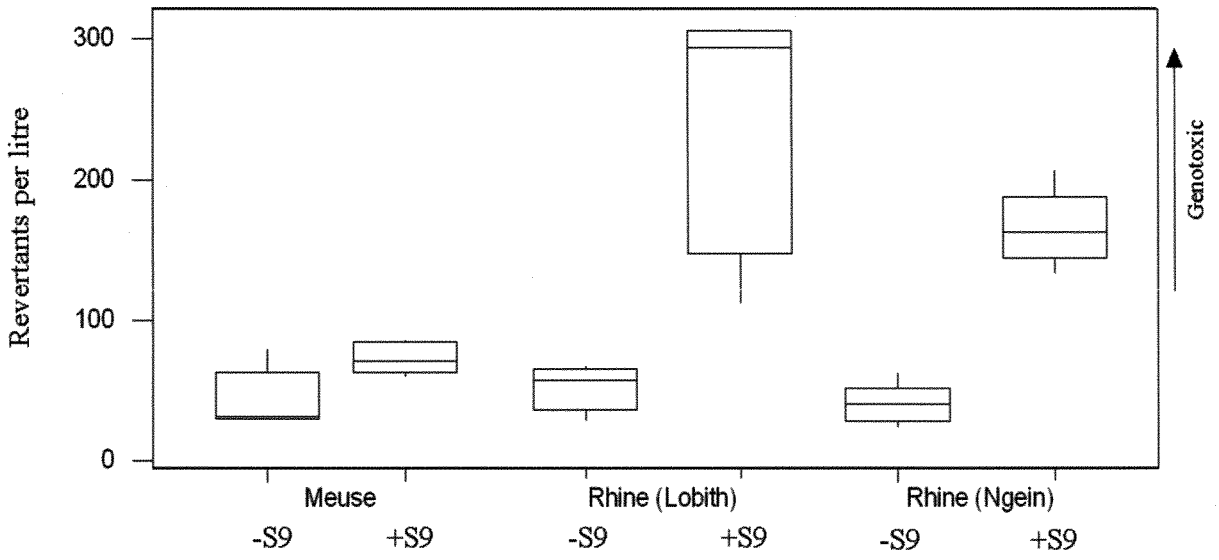


Figure 3: Boxplot of the results Ames TA98 test (n=5) on different sample locations.

The UMU test showed more or less opposite results; here the runs without S9 are usually higher than those with S9 (figure 4). The number of samples considered to be genotoxic is higher in this series of tests compared to the Ames test. At the Ames TA98 test results of only two locations and only with S9 were above the minimum toxicity level, while at the UMU test series all types of tests and at all locations were occasionally above the minimum toxicity level. In general, the genotoxicity detection level and the kind of response measured via the UMU assay were different compared to the results obtained with the Ames TA98 test.

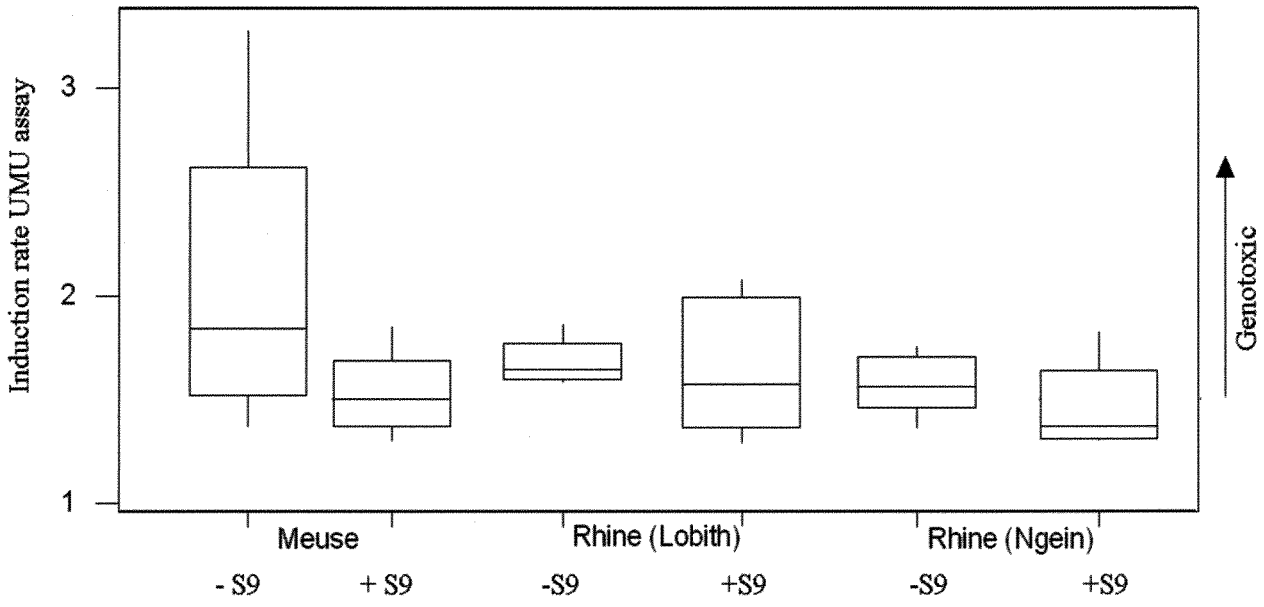


Figure 4: Boxplot of the results from the UMU assay (n=5) on different sample locations

The analysis of the Comet assays (figure 5) conducted in the River Meuse is showing a relatively low level of genotoxicity compared to those studied in the Rhine where much higher values were observed in both runs with and without addition of S9. Using this test location Nieuwegein seems to be the most genotoxic river area. After a short evaluation of the three assays applied to the same sample concentrates, each test appears to point to another river area having the worst genotoxicity quality: Ames TA98 to Lobith, UMU in the Meuse samples while the Comet assay indicated Nieuwegein as most genotoxic locality.

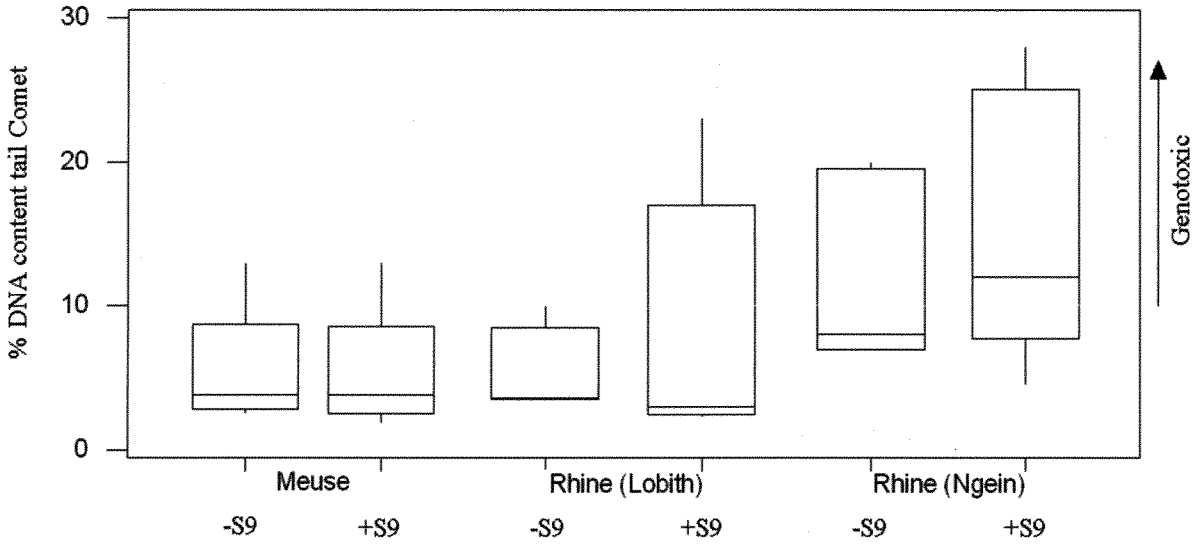


Figure 5: Boxplot of the results from the Comet assay on different sample locations (n=5)

In order to compare the results of various tests applied to the samples, the number of positive samples is listed for each test and location in tables 3 and 4. When applying the S9-mix (table 3), it appeared that the Ames TA98 test considered 12 samples out of 15 (80%) samples to be genotoxic. It is therefore more sensitive than the UMU test with only 7 samples positive (47 %) and the least sensitive one, the Comet assay, with only 5 samples (33 %) considered as being genotoxic. The combination Ames TA98 and UMU assay did provide both for 53 % the same result. The Comet assay with one of the other tests was for 47 % of the tested samples similar.

Table 3: Number of samples per genotoxicity test (and combinations of them) out of 15 samples found to be positive, with the use of a S9 mix.

+S9	Ames TA98	UMU	Comet	AU@	AC@	UC@	AUC@
Lobith	5	3	2	3 (3)	2 (2)	1 (2)	1 (1)
Nieuwegein	5	1	3	1 (1)	3 (3)	1 (3)	1 (1)
Eijsden	2	3	0	2 (4)	0 (2)	0 (2)	0 (2)
Total	12	7	5	6 (8)	5 (7)	2 (7)	2 (4)
Total (%)	80	47	33	40 (53)	33 (47)	13 (47)	13 (27)

@ = The number given in brackets is the number of combinations with the same test result;
AU = Combination Ames TA98 and UMU; AC = Combination Ames TA98 and Comet;
UC = Combination UMU and Comet; AUC = Combination Ames TA98, UMU and Comet

When the S9-mix was not applied (table 4), 86 % of all the sample were considered genotoxic according to the UMU test. This result could not be verified with other genotoxicity tests (see also figure 6). It was remarkable, that the Ames TA98 assay showed no genotoxicity at all. Due to the big differences in the results, it is not surprising to find that almost no similarity can be found between the UMU assay and the Ames TA98 or Comet assay. However, both the Ames TA98 assay and the Comet assay provided for 86 % the same information.

Table 4: Number of ethanol extracts per genotoxicity test (and combinations of them) out of 15 samples found to be positive, without the use of a S9 mix. (abbreviations, see table 2)

-S9	Ames TA98	UMU	Comet	AU@	AC@	UC@	AUC@
Lobith	0	5	1	0 (0)	0 (4)	1 (0)	0 (0)
Nieuwegein	0	4	1	0 (1)	0 (4)	1 (2)	0 (1)
Eijsden	0	4	0	0 (1)	0 (5)	0 (1)	0 (1)
Total	0	13	2	0 (2)	0 (13)	2 (3)	0 (2)
Total (%)	0	86	13	0 (13)	0 (86)	13 (20)	0 (13)

If S9 mix was used (figure 6), 80% of the samples tested with the Ames TA98 test became genotoxic. Via the Comet assay, 20 % of the samples tested became genotoxic and via the UMU assay, only 7 % of the samples were genotoxic when at first no genotoxicity was measured. Only the UMU assay displayed reduction in genotoxicity when the S9 mix was used. 46% of samples that were at first genotoxic became not genotoxic.

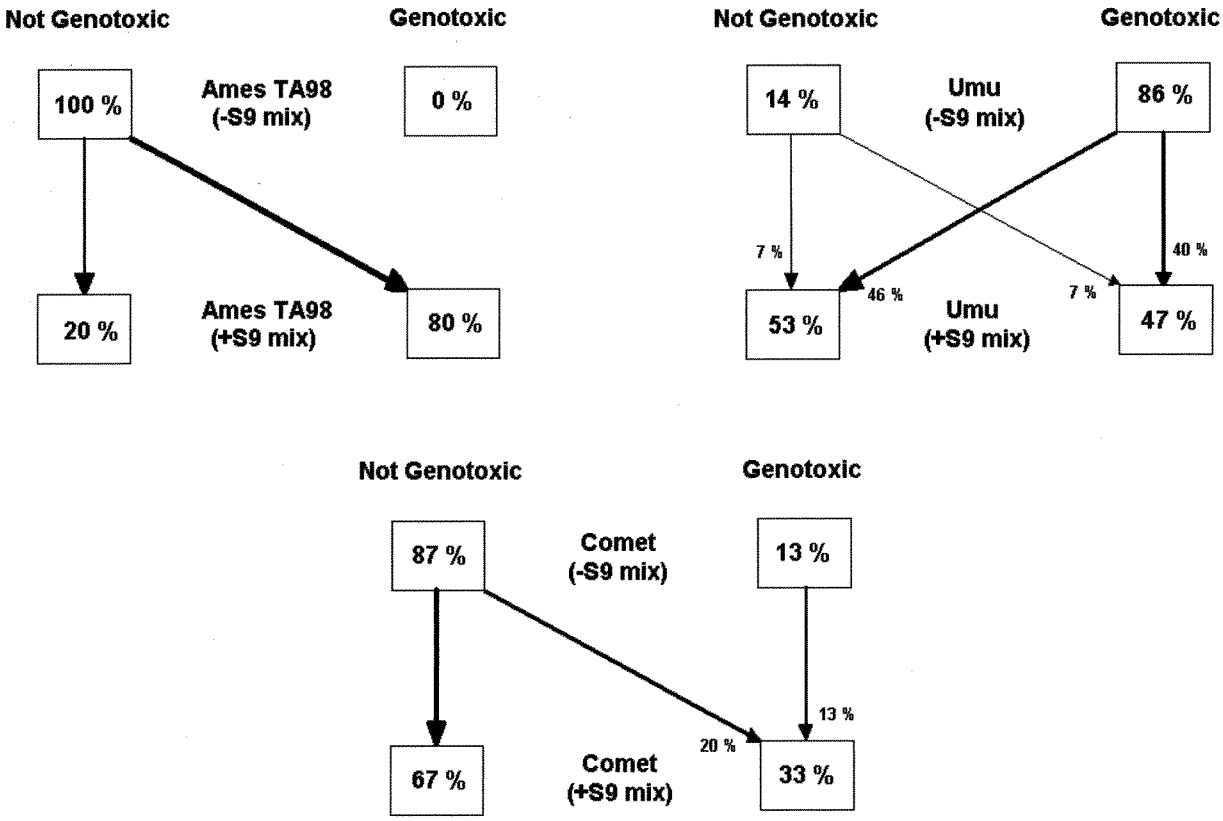


Figure 6: Diagrams of the genotoxicity tests used. Upper box were responses of the test without S9 mix; lower box with the application of S9. Arrows indicate the change in genotoxicity when S9 mix was applied.

Differences in response between the sample taken from the Rhine and the Meuse are stated in table 5. These observed differences were tested using the Mann-Whitney-U test.

Table 5: Signicifant Mann-Whitney-U test differences in response in the genotoxicity tests related to river and application of the S9 mix. The significance level is shown in brackets.

Genotoxicity test	Rhine vs. Meuse +S9 mix	Rhine vs. Meuse –S9 mix	+S9 vs. –S9 Rhine	+S9 vs. –S9 Meuse
Ames TA98 assay	Rhine > Meuse ($p<0.0061$)	-	+S9 > -S9 ($p<0.0061$)	-
UMU assay	-	-	-	-
Comet assay	-	-	-	-

By comparing the tests, only Lobith samples were used as a representative of the River Rhine. Only the Ames TA98 assay displays a significant difference between Rhine and Meuse, when S9-mix was introduced into the samples. Only at sample location Lobith, there was a significant difference when only the Ames TA98 test was used with or without the S9-mix. Further, no significant differences were found between the measurements of location Lobith and Nieuwegein from the same river when tested with the Mann-Whitney-U test (table 5). In order to determine the degree of overlap between different assays applied to the same samples, a Principal Component Analysis (figure 7) has been carried out. The calculation was based on the maximal values related toxicity data from table B1 in annex 1 for each test. Also the tests were correlated for samples with or without the use of the S9 mix.

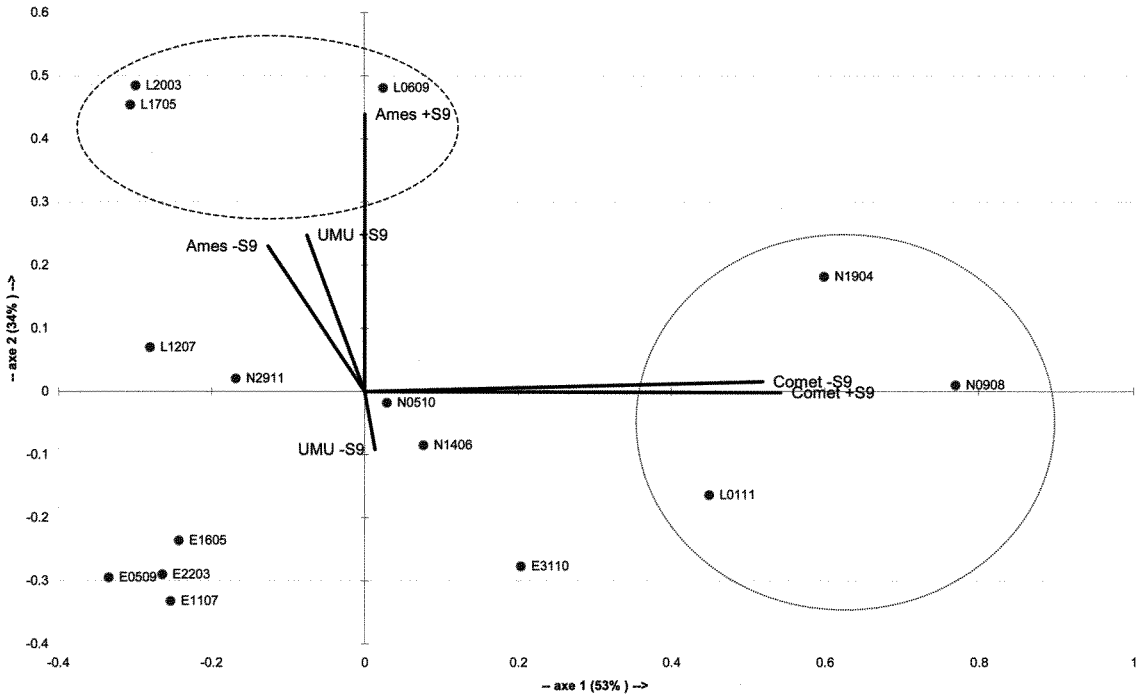
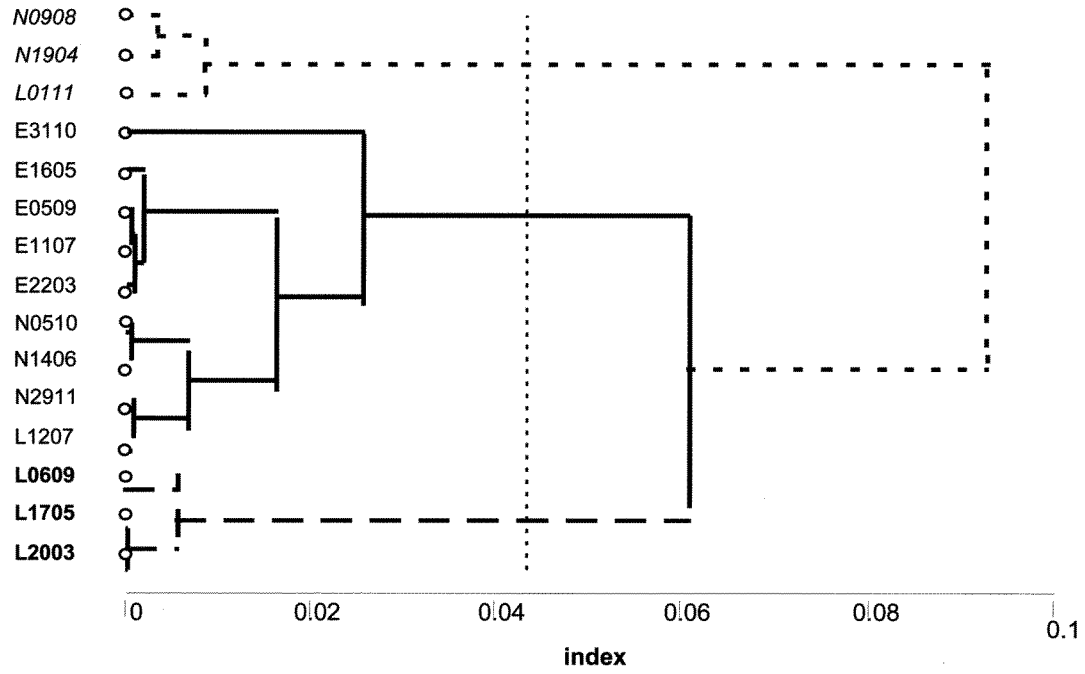


Figure 7: Principal Component Analysis of the genotoxicity tests used (covariant analysis), explanation see text.

The PCA explains 87 % of the covariance in the measurements obtained. When the lines in a PCA plot point in different directions, this indicates that there is little redundant information. Lines in opposite directions show a negative correlation as regards to the obtained values of genotoxicity of the tests used. The test with the most positively correlated tests are the Comet with or without the S9 mix.
Lines pointing towards the sample code indicate a relatively high genotoxicity for the relevant test, e.g. sample L0609 with regard to the Ames TA98 +S9 test.
Via ascendant hierarchical cluster analysis using Ward's method (see the dendrogram, figure 8), clusters of samples (site and date) are identified. Three clusters of samples are obtained, from which two are also displayed in figure 7 (dotted circles in figure).

One set of samples (N1904, N0908 and L0111) are clustered around the Comet assay and one cluster is found around the Ames TA98 test (samples L2003, L1705 and L0609). The samples E1605, E0509, E2203 and E1107 appears to have no relation to any genotoxicity tests and are therefore slightly genotoxic.

Figure 8: Clusteranalysis of all the obtained data from the genotoxicity tests.



4. Discussion

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

One of the goals of this study was to select an appropriate test procedure to monitor genotoxicity in river water samples. The present results however, do not permit the selection of a single test procedure since the tests revealed quite different results when applied to the same sample concentrate. A difficult matter is the fact that the Ames TA98 test and the Comet assay usually give most responses when S9 is applied in the test. The S9 is a mixture of proteins and enzymes of a mammal liver simulating what might happen when toxic compounds are ingested. On the other hand UMU assay results showed that more response is to be expected when no S9 is applied. The actual significance of the present results with and without S9 cannot be deducted here. Moreover, the quite different results obtained with these three tests indicate that no single special test can be selected for overall gene toxicity monitoring purpose. The fact that different tests applied to the same concentrate may indicate that different genotoxic substances occur in the concentrated samples inducing different test effects, implying that various tests may detect different genotoxic substances present in the concentrate and not necessarily all compounds are detected in a single assay.

Since it is not clear what significance the various results have for possible human mutagenity, for the time being a small test battery may be the best option.

It is remarkable that many tests with S9 give most positive responses. This may indicate that certain processes in the mammal (human) liver alter relatively harmless compounds into mutagenic substances. Whereas the Comet assay results may be interpreted that S9 does not influence genotoxicity considerable.

4.2. Water quality of the Rhine and the Meuse

The Ames TA98 and Comet values of the River Rhine are distinctly higher compared to those in the Meuse. With regard to the UMU assay, the Meuse values appeared to be higher. Sample points where high values were detected showed a distinctly larger variation, thus also relatively low values occur at these sites. At none of these sites a constant higher concentration was observed. This points to a more or less constant low pollution level with occasional peaks of toxic substances.

How did the pollution vary over time? RIWA conducted regularly Ames TA98 tests in the period 1986-2000 as part of the water quality monitoring program (Puijker, L.M. *et al.,* 1988, 1989, 1991 & 1992; Janssen, H.M.J. *et al.*, 1993; Veenendaal, H.R. *et al.*, 1995, 1997, 1999 & 2001). These results were reanalysed here to evaluate possible trends (figure 9). The River Meuse data generally have a lower value compared to those measured at Lobith, whereas the genotoxicity at the Rhine is distinctly higher especially in the 80-ies and early 90-ies. The genotoxicity of the River Rhine is still higher compared to the River Meuse; an observation that can be confirmed by the Comet assay. Based on existing chemical analysis, no explanations for this observation are available. It is therefore necessary to keep a very close look to the genotoxicity in the rivers.

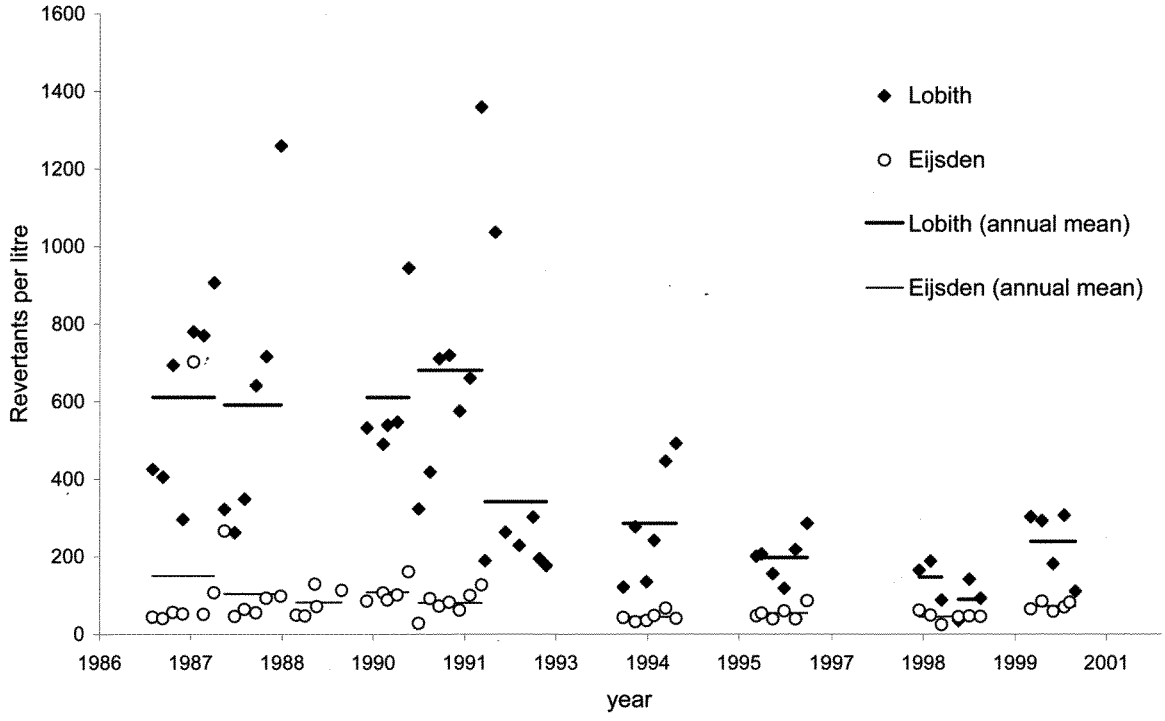


Figure 9: Results from the AmesTA98 assay (only at pH=7 and +S9 mix) of the River Rhine and Meuse.

Close observations of the individual measurements reveal a rather strong variation in water quality. This may be due to irregular industrial discharges or spills or extreme water levels. A similar pattern is discernable in the present data, although the variation is much smaller in recent years. Locations meeting constantly high toxicity values were not observed.
In figure 10 , the measurements of the Ames TA98 (1987-2000) are compared to the discharge of the rivers, in which a direct relationship is not visible.

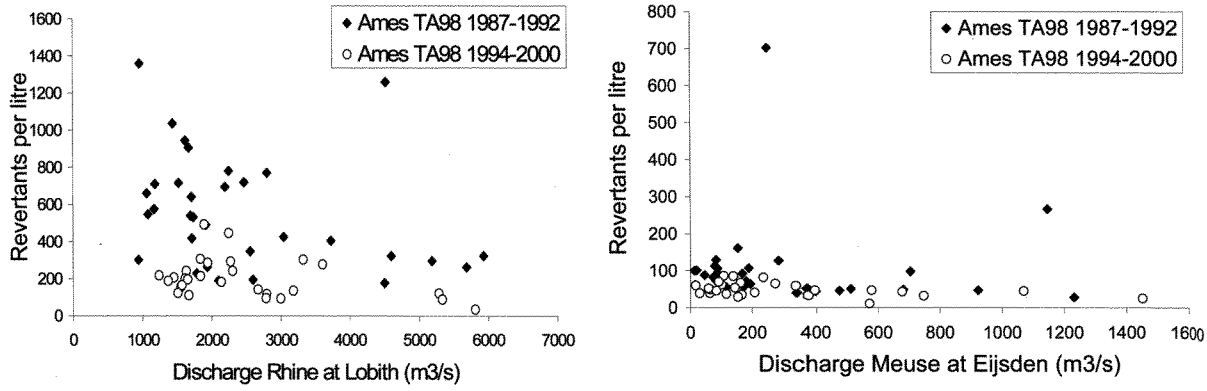


Figure 10: Ames TA98 genotoxicity (revertants per litre at pH7 with use of S9 mix) versus the discharge of the river Rhine (left) and Meuse (right). Plotted for two periods (1987-1992 and 1994-2000)

The River Rhine results show a different pattern in both study periods. In the first period relative high values are seen at low discharges, while in more recent years (1994-2000) no distinct differences are discernible. Although no statistical confirmation of this pattern has been calculated, it is reasoned that at low discharge volumes toxic compounds may be concentrated resulting in higher test results.
In the River Meuse, no discharge volume related pattern can be seen in the plotted data.

When the annual means are considered the number of revertants per litre decreases distinctly in the River Rhine (figure 9), the mean values before and after 1994 decreased in both Rhine (from 563 to 209 revertants/l) and Meuse (from 106 to 53 revertants/l). In both rivers genotoxicity was reduced roughly to half its original value. These differences were tested statistically, using Kolomogorov Smirnov two sample test, a non parametric test not requiring any special distribution of the data to be tested. The distribution of data measured in both periods (figure 11) differ significantly for both rivers (Rhine: $N_1 = 32$; $N_2 = 30$; $D_{max} = 0.556 > D_{0.05} = 0.345$; Meuse: $N_1 = 30$; $N_2 = 23$; $D_{max} = 0.402 > D_{0.05} = 0.38$).

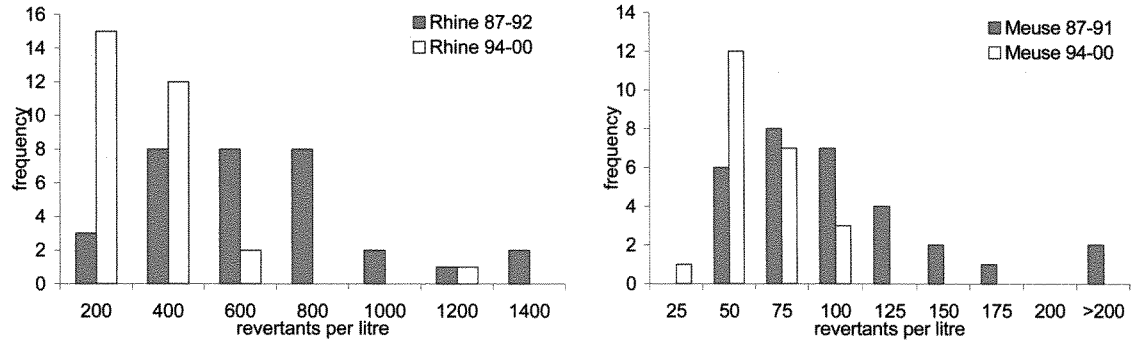


Figure 11: Distribution of genotoxicity measured with Ames TA98 over the periods until 94 and after 94 in the River Rhine (left) and Meuse (right).

To confirm the decrease of genotoxic compounds in the River Rhine, measured via the Ames TA98 test, another genotoxicity test with some historic data is available.
Alink et al. 1980. studied chromosome aberrations in fish (sister chromatid exchanges) exposed to river Rhine water and they observed distinct reactions compared to fishes exposed to drinking water prepared from natural ground water. These measurements were conducted in a period a few years before Riwa started Ames TA98 tests presented in figure 9. Although not actually measured, relatively high Ames test responses were likely in 1997 as well. It is recommended to conduct a similar fish test like sister chromatid investigation in order to demonstrate also a significant decrease in genotoxicity on vertebrate DNA. When these investigations show a significant genotoxicity decrease the Ames TA98 test may be regarded as a reliable indicator for genotoxicity in surface water. However, when these studies show a constant level or even an increase of genotoxicity the reliability of Ames TA98 test assays for monitoring mutagenic effects on vertebrates or mammals becomes at least doubtful.
An important difference between the fish test and the tests carried out in our program is that the fish were exposed to Rhine water, thus without any concentration step, while all tests in the present study were conducted in highly concentrated samples. An other advantage of the Sister Chromatid Exchange (SCE) test is that fishes are exposed for about ten days to unconcentrated river water, making this test a more or less intermediate between an acute (short term exposure) or chronic test in which much longer exposure periodes are applied. A positive respons of this test is still likely, because the Comet assay detected chemicals that interact with vertebrate DNA.

4.3. Restrictions and modifications related to the use of genotoxicity tests

Previous results were obtained from samples which had to be concentrated 25000x. Measurements on native water will mostly provide a negative results for all the used tests. The high concentration factor was required to minimize toxicity due to the use of organic extraction solutions. For instance, the UMU test can only be used when less than 3 % of the testvolume is the organic fraction. This means that the highest possible concentraction factor used in the test was 750x. Due to the concentration procedure with XAD, the

genotoxicity can only be related to the non-polar organic compounds present in water. At this moment, no techniques are available to concentrate all components (also metals, and polar organics) from the water sample into the desired level in which the tests will give a response. Genotoxicity tests that can give a response on native water are, at the moment, not available (Grummt, 2000). Therefore, to get an idea if the genotoxicity in the rivers will increase or decrease during the years, the use of a concentration procedure with all its restrictions is still required. There is no introduction of foreign chemicals from the used resins and extraction liquids. This was checked by using bottled spring water as a water sample, performing the same concentration and extraction procedure and testing it with the Ames TA98 test. The level of revertants per litre was very low, like the spontaneous revertants per litre. Figure 9 displays a trend that the genotoxicity of non-polar organics in surface water decreased over recent years; a trend that could not be presented if the tests would have been performed on native waters.

Several modifications are availabe to increase the detection level for some genotoxicity tests or to reduce the concentration factor. The detection limit of the UMU test can be improved by decreasing the amount of bacteria introduced to the sample (Reifferscheid and Zipperle, 2000). However, photometric detection of the product from the ß-Galaktosidase reaction is not possible. Only when luminometric or fluorometric substrates were used, measurement of genotoxic stress was possible. In table 6, the detection limits for standard genotoxins measured via the UMU test according to different procedures are displayed.
The amount of bacteria introduced into the sample should not be too low. An optimal ratio between the produced signal and the number of bacteria introduced into the sample is yet not determined.

Table 6: Detectionlimits for standard genotoxins with different variants of the UMU test (Reifferscheid and Zipperle, 2000)

Compound	Detection limit (µg/l) UMU DIN 28415-3	Detection limit (µg/l) Fluorometric UMU	Detection limit (µg/l) Luminimetric UMU
4-Nitro-chinolinN-oxid	10	0,8	1
Nitrofurantoin	14-20	3,5	7
Benzo(a)pyrene	200-235	95	250
N,N-dimethyl-nitrosamin	$6\text{-}14x10^6$	-	$4x10^6$
2-Amino-anthracen	18-45	30	-

The Ames TA98 test can optimized in a way that a lower concentration factor is required, although the same level of response is to be expected. At the moment, a sample was concentrated 25000x and a sample volume of 40 µl was used, so the amount of revertants measured was related to 1 litre of unconcentrated water sample. By increasing the volume from 40 µl to 4 ml, using a 9 cm petridish and modified agar (Erdinger *et al.*, 2000), the concentration factor can be reduced to 250x.
Apart from the modification of the test procedures itself, it is also possible to decrease the detection limit for all tests by using other evaluation limits when a sample is considered genotoxic. The standard limits in which a sample is considered genotoxic by, for instance, the Ames TA98 are, when a dose-effect

relationship is measured and when the amount of revertants is increased 2 times with respect to the obtained spontaneous revertants. The requested factor 2 is, however, not explained by science (Grummt 2000) and for tests like the UMU assay another factor is used (1,5). By using a more statistic approach in which the test results are compared to the obtained results from blanks, the use of fixed factors can be neglected. A statistical tool could be the modified T-test or the Mann Whittney-U test (see appendix B2). As an example for the use of statistics, the number of genotoxic samples (with and without the use of S9-mix) is 7 when for the Comet assay the criterium "more than 10 % DNA damage" is used. Using the Mann Whittney-U test, 25 samples are considered to be genotoxic. However, for the evaluation of samples via the Mann Whittney-U test, results from samples and blanks with its confidence levels are required and thus per assay more applications on one sample must be performed.

5. Conclusions

- By combining results from the Ames TA98, UMU- and Comet assay, genotoxic activity was shown to be present in all samples of the River Rhine when the S9 mix was applied. From the River Meuse, 3 out of 5 samples were considered to be genotoxic.

- Without the use of the S9 mix, at the location Lobith (Rhine), genotoxicity was present in all samples, but at the location Nieuwegein (Rhine), 4 out of 5 samples had genotoxic activity. At the location Eijsden (Meuse), 4 out of 5 samples were positive for the presence of genotoxic compounds.

- With the use of the S9 mix, the number of genotoxic samples decreased from 13 samples with information from all genotoxicity tests used to 12 samples when information was obtained from the Ames TA98 test only.

- Without the use of the S9 mix, the differences were clearly visible. No samples were considered genotoxic when information was used from the Ames TA98 test only, while using the UMU and the Comet assay, 13 samples were considered genotoxic. Thus the Ames TA98 test did provide insufficient information with respect to the use of the S9 mix.

- It is not possible to select a single genotoxicity test for monitoring purposes in Dutch rivers.

- The best possible set of genotoxicity assays are the combination of Ames TA98 or UMU assay and the Comet Assay, due to their different points of impact for genotoxic compounds. Based on this study, the Ames TA98 is preferred fot the time being above the UMU assay due to the higher number of positive extracts of surface water samples when the S9 mix was used.

- Concentration of surface water samples is still required when genotoxicity tests are used. Due to the use of the XAD concentration technique, the measured genotoxicity can only be related to the non-polar compounds present in surface water. No techniques are available to concentrate all components (also metals and polar organics) from the water sample into the desired level in which the genotoxicity tests will give a response.

- Distinct genotoxic differences between Rivers Rhine and Meuse have been demonstrated based on the results from the Ames TA98 test and a significant decrease of toxicity has been observed in both rivers in the period 1994 – 2000. However, the level of genotoxicity can perhaps still be reduced to around 15 revertants per litre as has been measured in 1994 at the location of Sipplingen (Bodensee) (Noij & Meerkerk, 1997), by preventing input of chemicals into the rivers.

- Over the period 1986 – 2000 the toxicity level of the River Rhine water was distinctly higher than that of the River Meuse.

- Genotoxicity occurs in variable concentrations, which may indicate industrial spills or discharges.

6. Recommendations

- Additional studies may elucidate why different tests perform so unexpectedly different. The Ames TA98 test seems to be a sensitive test. On theoretical grounds UMU-test is expected to be much more sensitive. It is reasoned that the detection of response products in the UMU-assay is much too low. Reifferscheid & Zipperle (2000) showed a more or less ten times lower detection limit in the UMU-assay using luminometric and fluorometric techniques, demonstrating that improvements are possible indeed. Thus modifications on different genotoxicity tests to obtain the optimal detection of genotoxic compounds in surface water is recommended.

- Removal capacity of the present sewage treatment plants is not known. A distinctly higher toxicity was measured in the River Rhine than in the Meuse. Therefore, it is recommended to investigate in a limited pilot study whether the compounds in raw sewage are removed during the treatment process or not.

- Using a small series of samples testing River water and raw sewage water, the Ames TA98 test, with the use of S9 at pH=7, is probably the best assay for this evaluation.

- Additional bio-assays (e.g. Sister Chromatid Exchange test) detecting chromosome damage in vertebrates (fish) exposed to unconcentrated river water for an intermediate period of time, reveals perhaps the same knowledge on the genotoxicicity of river water, as already obtained using the Ames TA98 test. If there is no difference between these tests, the Ames TA98 test is therefore suitable to detect genotoxic compounds in surface water. A positive respons of this test is still likely, because the Comet assay detected chemicals that interact with vertebrate DNA.

- Due to significant differences in genotoxicity of both rivers, it is still recommended to monitor genotoxicity profoundly.

Bibliography

Alink, G.M., Frederix-Wolters, E.M.H., Van der Gaag, M.A., Van de Kerkhoff, J.F.J. & Poels, C.L.M. (1980). Induction of sister-chromatid exchanges in fish exposed to Rhine water. Mutation Res., 78: 369-374.

Aquasense (2000). Genotoxiciteit van micro-verontreinigingen Resultaten UMU-C testen - In opdracht van: RIWA projectgroep Biotests. Report number: 2000.1602

Erdinger, L. Dürr, M. and Grummt T. (2000). Use of the Ames test for the examination of surface water. Article in Grummt, T (2000).

Grummt, T (2000). Forschungsverbundvorhaben "Erprobung, Vergleich, Weiterentwicklung und Beurteilung van Gentoxitätstests für Oberflächenwasser 1995-1999", Forschungszentrum Karlsruhe GmbH, Dresden.

Janssen, H.M.J. & J.van Genderen (1993). Organische microverontreinigingen in de Rijn en de Maas in 1992: bestrijdingsmiddelen en mutageniteit. Kiwa report SWO 92.283. pp 1-20.

Oda, Y., Nakamura, S., Oki, I., Kato, T. & Shinagawa H. (1985). Evaluation of a new system (*umu*-test) for the detection of environmental mutagens and carcinogens. Mutation Res., 147:219-229.

Noij, Th.H.M. & Meerkerk, M.A. (1997). The toxicological and ecological study of the Rhine river in 1994. A RIWA report.

Penders, E. & Hoogenboezem, W. (2001). Biological tests, a suitable instrument for the quality control of surface water?. A RIWA report.

Puijker, L.M., H.M.J. Jansen & J.van Genderen (1988). Organische microverontreinigingen in de Rijn (Lobith) en Maas (Keizersveer) in 1987: bestrijdingsmiddelen, adsorbeerbare organische zwavelverbindingen en mutageniteit. Kiwa report SWO 92.283. pp 1-29.

Puijker, L.M. & J.van Genderen (1989). Organische microverontreinigingen in de Rijn en Maas in 1988: bestrijdingsmiddelen en mutageniteit. Kiwa report SWO 89.245. pp 1-43.

Puijker, L.M. & J.van Genderen (1991). Organische microverontreinigingen in de Rijn en de Maas in 1990: bestrijdingsmiddelen en mutageniteit. Kiwa report SWO 91.285. pp 1-23.

Puijker, L.M. & J.van Genderen (1992). Organische microverontreinigingen in de Rijn en de Maas in 1991: bestrijdingsmiddelen en mutageniteit. Kiwa report SWO 92.283. pp 1-13.

Reifferscheid, G., Heil, J. & Zahn, R.K. (1991). Die Erfassung von Gentoxinen in Wasserproben mit dem *Umu*-Mikrotest. Vom Wasser, 76: 153-166.

Reifferscheid, G. & Heil J. (1996). Validation of the SOS/umu test using test results of 486 chemicals and comparison with the Ames test and carcinogenicity data. Mutation Res., 369: 129-145.

Reifferscheid, G. & Zipperle, J. (2000). Development and application of luminometric and fluorometric umu-test systems for the detection of genotoxic compounds in surface water. Article in Grummt, T (2000).

Tice. T.R. (1995). The single cell gel/Comet assay: a microgel electrophoretic technique for the detection of DNA damage and repair in individual cells. Environmental Mutagenesis, Phillips and Venitt (eds.), BIOS Scientific Publishers: 315-339.

Veenendaal, H.R. & L.M. Puijker (1995). Mutageniteit in de Rijn en Maas in 1994 Kiwa report SWO 95.095. pp 1-9.

Veenendaal, H. & Van Genderen, J. (1997). Mutageniteit in Rijn en Meuse in 1996. RIWA report.

Veenendaal, H.R. & J. van Genderen (1999). Mutageniteit in Rijn en Maas in 1998. RIWA report: pp 1-20.

Veenendaal, H.R. (2001). Mutageniteit in Rijn, Maas en Lekkanaal in 2000. Kiwa report: KOA 01.018, pp. 1-14.

Verschaeve, L. & Van Gorp, U. (2000). De komeettest voor studie van genotoxiciteit van waterstalen uit Eijsden, Lobith en Nieuwegein. VITO report.

Annex 1: Summary results from Aquasense, Kiwa and VITO

Table B1: Results of genotoxicity tests.

Sample	Ames TA98 +S9#	Ames TA98 -S9#	UMU +S9@	UMU -S9@	Comet +S9*	Comet –S9*
L2003	303	62	2.08	1.87	2.6	3.5
L1705	293	68	1.89	1.61	2.3	3.6
L1207	182	45	1.44	1.68	3.0	3.5
L0609	307	57	1.57	1.65	11.0	7.0
L0111	111	28	1.28	1.58	23.0	10.0
N1904	206	63	1.83	1.65	22.0	20.0
N1406	132	40	1.30	1.36	12.0	8.0
N0908	162	34	1.32	1.56	28.0	19.0
N0510	155	23	1.44	1.56	11.0	7.0
N2911	168	40	1.37	1.76	4.5	7.0
E2203	65	45	1.52	1.36	3.8	3.2
E1605	85	80	1.44	1.96	4.2	3.8
E1107	59	31	1.30	1.68	3.2	4.5
E0509	70	29	1.51	1.85	1.8	2.5
E3110	82	31	1.85	3.28	13.0	13.0

= data as revertants per litre
@ = data as obtained induction rate measured at the highest concentration factor (750 *)
* = data as the mean value of content DNA in the tail of the comet as percentage measured at the highest concentration factor (781 *)

Table B2: General overview of positive or negative respons of the genotoxicity tests

Sample	Ames TA98 +S9	Ames TA98 -S9	UMU +S9	UMU -S9	Comet sign. + S9	Comet sign. -S9	Comet DNA + S9	Comet DNA -S9
L2003	+	-	+	+	-	+	-	-
L1705	+	-	+	+	+	+	-	-
L1207	+	-	-	+	+	-	-	-
L0609	+	-	+	+	+	+	+	-
L0111	+	-	-	+	+	+	+	+
N1904	+	-	+	+	+	+	+	+
N1406	+	-	-	-	+	+	+	-
N0908	+	-	-	+	+	+	+	-
N0510	+	-	-	+	+	+	-	-
N2911	+	-	-	+	+	+	-	-
E2203	-	-	+	-	+	+	-	-
E1605	-	-	-	+	+	-	-	-
E1107	-	-	-	+	+	+	-	-
E0509	+	-	+	+	-	-	-	-
E3110	+	-	+	+	+	+	-	-

Colophon

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