organic mutagens and drinking water in The Netherlands

A study on mutagenicity of organic constituents in drinking water in The Netherlands and their possible carcinogenic effects

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H.J. Kool

Organic mutagens and drinking water in The Netherlands

A study on mutagenicity of organic constituents in drinking water in The Netherlands and their possible carcinogenic effects

Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
hoogleraar in de veeteeltwetenschap,
in het openbaar te verdedigen
op vrijdag 8 april 1983
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen.

ISN : 183638

aan mijn ouders en oma Iet

NN08201,934

stellingen

1. Duininfiltratie en filtratie m.b.v. aktief koolfilters zijn zuiveringsstappen, die de mutagene aktiviteit (Ames test), veroorzaakt door organische stoffen drastisch kunnen reduceren.

dit proefschrift

2. Een behandeling van het water met chloor tijdens de bereiding van drinkwater uit oppervlaktewater resulteert meestal in een verhoging van de mutagene aktiviteit (Ames test) in het water.

dit proefschrift

3. De toegepaste zuivering in drinkwaterbedrijven, die oeverfiltratie van Rijnwater toepassen, is inefficiënt t.a.v. het verwijderen van organische verontreinigingen.

dit proefschrift

4. De bewering, dat een hoog koloniegetal van 37°C duidt op een faecale verontreiniging in drinkwater, is niet gefundeerd.

R.K. Dart and R.J. Stretton
Microbial aspects of pollution control
75-117 (1980).

5. De veronderstelling van Maruoka et al. dat het consumeren van drinkwater van de stad Kyoto geen nadelige effecten voor de gezondheid van de consument oplevert kan niet worden bewezen op grond van het uitgevoerde toxiciteitsonderzoek.

S. Maruoka, T. Nishio and S. Kawai
Bull. Environm. Contamin. Toxicol.
545-550 (1974).

6. De door Zoeteman et al. voorgestelde definitie van persistentie (persistence of an aquatic pollutant), kan tot een onderschatting van de eventuele risico's van de stof leiden.

B.C.J. Zoeteman, K. Harmsen, J.B.H.J. Linders
C.F.H. Morra and W. Slooff
Chemosphere, 9, 231-249 (1980).

7. Het bepalen van de biologische aktiviteit in humusrijke grond d.m.v. ATP, leidt met de huidige extractiemethoden tot misleidende resultaten.

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8. De conclusie van Schwartz et al. dat mutagene aktiviteit is aangetoond in drinkwater, dat zonder concentreren in de Ames assay werd getest, berust op een onjuiste interpretatie van de verkregen resultaten.

D.J. Schwarz, J. Saxena and F. Kopfler
Environm. Sci. Techmol., 13, 1138 (1979).

9. Op grond van de toxicologische risico's van cadmium, dient de industriële productie en toepassing van deze stof geheel te worden gestopt, teneinde de belasting van het milieu zoveel mogelijk te beperken.

10. Bij reorganisatie van de rijksoverheid fungeert Het Georganiseerd Overleg nog steeds als bezemwagen.

11. Het verlengen van een honkbalwedstrijd bij een gelijkspel na 9 innings tot eventueel 12 innings, om dan alsnog een gelijkspel toe te staan is, volstrekt onlogisch.

12. Het verrichten van wetenschappelijk onderzoek bij overheidsinstituten vereist naast ontstellende hoeveelheden papier een zeer positieve 'grond-houding'.

H.J. Kool
Organic mutagens and drinking water
Wageningen, 8 april 1983.

woord vooraf

Wetenschappelijk onderzoek is veelal samenwerken met anderen. Dit onderzoek vormt hierop geen uitzondering. In eerste instantie was het niet de bedoeling dat dit onderzoek tot een proefschrift zou leiden. Nu het ervan gekomen is, wil ik graag diegenen bedanken die dit mede mogelijk hebben gemaakt.

- In de eerste plaats wil ik prof. dr. J.H. Koeman bedanken voor zijn ondersteuning en begeleiding van het onderzoek alsmede prof. dr. H. van Genderen, dr. ir. B.C.J. Zoeteman en dr. H. A.M. de Kruijf voor het ondersteunen van het initiatief tot het promotie-onderzoek. De directies van het Rijksinstituut voor Drinkwatervoorziening en de Sector Drink- en Industriewatervoorziening bedank ik voor het geven van de gelegenheid tot dit onderzoek.

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Het onderzoek werd verricht op het Rijksinstituut voor voorziening (RID) en het Centrum Kleine Proefdieren (CKP) van de L.H. Wageningen in samenwerking met de Vakgroep Toxicologie van de L.H. Wageningen.
Het histopathologisch onderzoek werd uitgevoerd door het Centraal Instituut Voedings Onderzoek (CIVO-TNO) te Zeist en de Vakgroep Toxicologie van de Landhogeschool Wageningen.
Het onderzoek werd mede gefinancierd door het Koningin Wilhelmina Fonds, project RID 80-1.

curriculum vitae

Hendrik, Jacob Kool werd geboren op 30 juli 1942 in Amsterdam en behaalde het HBS-B diploma (Staatsexamen) in 1963 via avondstudie. Van augustus 1963 tot mei 1965 werd de militaire dienstplicht vervuld. In 1965 werd met een studie scheikunde aan de Gemeentelijke Universiteit van Amsterdam aangevangen. In juli 1971 werd het doctoraal examen scheikunde afgelegd met als hoofdvak biochemie en als bijvakken plantenfysiologie en microbiologie. In datzelfde jaar trad hij in dienst bij het Rijksinstituut voor Drinkwatervoorziening.

De laatste jaren houdt hij zich bezig met gedrag en effectenstudies van organische stoffen in water en bodem en met onderzoek aan drinkwater in welk kader het onderhavige onderzoek in 1979 werd aangevangen.

contents:

contents:

Parts of this study are published in detail in the following papers:

I. C.F. van Kreijl, H.J. Kool, M. de Vries, H.J. van Kranen and E. de Greef (1980)
Mutagenic activity in the river Rhine and Meuse in The Netherlands.
Sci. Total Environm., 15, 137-147.

II. H.J. Kool, C.F. van Kreijl and E. de Greef (1981)
The use of XAD-resins for the detection of mutagenic activity in water.
I. Studies with surface water.
Chemosphere, 10, 85-98.

III. H.J. Kool, C.F. van Kreijl and H.J. van Kranen (1981)
The use of XAD-resins for the detection of mutagenic activity in water.
II. Studies with drinking water.
Chemosphere, 10, 99-108.

IV. H.J. Kool, C.F. van Kreijl, H.J. van Kranen and E. de Greef (1981)
Toxicity assessment of organic compounds in drinking water in The Netherlands.
Sci. Total Environm., 18, 135-153.

V. H.J. Kool, C.F. van Kreijl, E. de Greef and H.J. van Kranen (1982)
Presence, introduction and removal of mutagenic activity during the preparation of drinking water in The Netherlands.
Environmental Health Perspective, 46, 207-214.

1.
general introduction

1.1. Mutagenicity and suspect carcinogenicity of drinking water

Trace amounts of several mutagenic and (suspect) carcinogic organic compounds have been identified in drinking water of several cities in The Netherlands, which prepare their drinking water mainly from surface water (Zoeteman, 1978). Similar findings have also been reported in the USA (NAS, 1977). Some data of these compounds identified in drinking water in The Netherlands are given in table 1.

Besides the finding of these kind of organic compounds in drinking water, several other factors viz. the use of heavily polluted river Rhine and Meuse water (Meijers, 1970; Van de Leer and Van der Meent, 1976; Morra et al., 1979) as water source for drinking water, the finding of cytogenetic changes in fish exposed to water from a specific Rhine (Lek) location in connection with mutagenic activity (Ames test) in organic extracts of the same water (Prein et al., 1978), the results of a study in which mutagenic activity (Ames test) and celtransformation (Balb/3T3) was produced by organic drinking water concentrates (Loper et al., 1978), results from several epidemiological studies suggesting a possible association between cancer mortality rates and the use of contaminated drinking water (NAS, 1979), the finding that several organic concentrates prepared from drinking water in the USA and France were able to produce carcinogenic effects in mice and rats (Hueper and Payne, 1963; Hémon et al., 1978; Truhaut et al., 1979), and finally some preliminary results of organic drinking water concentrates in The Netherlands showing mutagenic activity in the Ames test, which farly exceeded mutagenic activity based on the concentration of the identified organic mutagens in drinking water (Kool, unpublished data), have led to a decision to start an investigation for the presence of organic mutagens and/or carcinogens in drinking water in The Netherlands.

Table 1. A review of some (suspect) carcinogenic and mutagenic compounds detected in the rivers Rhine and Meuse and in drinking water prepared from these rivers in 1973-1977 (Kool et al., 1979)

Compound	maximum concentration (ug/l)		carcinogenicity in :		mutagenic[e] in :		
	surface water	drinking water	humans	animals	in vitro prokyaryotic	in vitro eukaryotic	in vivo eukaryotic
Benzene	0.03	0.005	o[a]			o	o
3,4 Benzene(a)pyrene	1	0.005		o[a]	o	o	o
Benzo(b)fluoranthene	0.21	0.045		o[a]			
Indeno(1,2,3 c-d)pyrene	1.4	0.0075		o[a]	o		
Bis(2chloroethyl)ether [d]	0.01	0.03		o[c]			
Bis(2chloroisopropyl)ether	25	3			o		
Bromodichloromethane [d]	2	55			o		
Chloroform	30	100		o[a]			
Tetrachloromethane	4.2	0.7		o[a]			
1,2 Dichloroethane	20	0.06		o[a]	o		o
Heptachlor	0.04	0.01		o[b]		o	
Lindane	0.03	0.01		o[b]		o	
DDE	0.2	0.2		o[c]		o	

a : sufficient evidence for carcinogenicity, see reference IARC, 1979
b : limited evidence for carcinogenicity, see reference IARC, 1980
c : suspect carcinogenic compound which do not fulfil requirements a and b
d : compounds currently tested at the National Cancer Institute, Bethesda, Maryland, USA
e : mutagenicity data obtained from NIH-EPA, CIS, NIOSH, RTECS and from reference Simmon et al., 1977.

1.2. Organic constituents in drinking water

The presence of organic materials in drinking water has been known for many years since these substances may influence the taste, colour and odour of the drinking water (Meijers, 1970; Drost and Zoeteman, 1976; Zoeteman, 1978).

The organics consist of compounds from both natural and industrial origin. The natural ones comprises by far the major portion and include mainly undefined fulvic and humic acid (Meijers, 1970).

Up till 1950, the organic substances in drinking water were characterized by $KMnO_4$ consumption since it was not possible to identify specific organic compounds in water at a microgram per litre level. Later on these organic substances were characterized by the total organic carbon content (TOC) and dissolved organic carbon content (DOC). In the fifties much progress was obtained in the separation of organics by gaschromatographic techniques (GC). The increased possibilities to identify many organic constituents in drinking water due to the development of capillary columns and the combination of this GC technique with massspectrometry (MS) analysis, draw the attention of toxicologists and the question raised whether these organic compounds found in drinking water pose a health risk.

To date hundreds of organic constituents have been detected in drinking water in many countries in the world, but these organics usually are present below the microgram per litre level (Dowly et al., 1975; Zoeteman, 1978; Aichele et al., 1979; Packham et al., 1981).

Limitations of time, manpower and scientific information have not permitted an indepth evaluation of most of the compounds recently found in drinking water and therefore relatively little is known about their toxic effects including their carcinogenic potential. In addition to this it is recognized that in fact the non purgeable fraction which comprises 90-95% of the total organics in the water have not been identified (NAS 1977, 1980, 1981), since this fraction cannot be readily volatilized for their subsequent separation and identification by GC/MS analysis.

The organics present in drinking water may be divided into 4 classes (see table 2).

Table 2. Organic composition of drinking water

Group	Property	TOC (Estimated % of total)	Compounds identified
I	Volatile/non-polar compounds	10	many (80%) THM, ethers etc.
II	Volatile/polar compounds	10	very few, trichloro-ethanol, formaldehyde etc.
III	Non volatile/non-polar compounds	80-90	few PAH, PCB, DDT, etc.
IV	Non volatile/polar compounds		very few, humic acid, dichlorophenols etc.

Most attention has been paid so far to the volatile/non-polar compounds. In part this was due to analytical (technical) restrictions and in part to the growing awareness since the publications of Rook and Bellar in 1974 (Rook, 1974; Bellar et al., 1974) showing that halogenated hydrocarbons are introduced as a result of a chlorine treatment. Later on a survey of the EPA in 1975 showed that approximately 50% of the volatile non-polar compounds in drinking water are halogenated (EPA, 1975). In particular one group among these, the trihalomethanes (THM) receive major attention by toxicologists since these group frequently is detected in relatively high concentrations (>100 µg/l) and one of these compounds viz. chloroform have to be considered as an animal carcinogen (IARC, 1979b). As already stated before, the majority of the identified organics are present in relative low levels and therefore it is very unlikely, that unless there are extremely toxic among them, these low levels of organics will induce acute toxic effects. There is however a possibility that certain organics may cause "long term effects" since drinking water is universally consumed at all ages and all

stages of health throughout the entire life span. In this respect special attention is nowadays paid to those compounds which have mutagenic and/or carcinogenic properties. The majority, however of the organics in drinking water has not been identified including the individual organic mutagens present in organic drinking water concentrates (Loper, 1980; Kool et al., 1982a) and therefore the possible carcinogenic potential of these mutagenic fractions can only be investigated with the fractions themselves. With this in mind, toxicological studies with complex (mutagenic) organic concentrates prepared from drinking water are needed to find out whether there exists a toxicity problem with respect to drinking water containing organic (micro)pollutants.

1.3. Objective of the present study

The present study has been carried out:

- to study in more detail the mutagenicity in water and in particular in drinking water including the influence of raw water purification treatment
- to obtain further information about the identity of potential mutagenic and/or carcinogenic compounds present in drinking water
- to assess carcinogenic properties of mutagenic drinking water concentrates by carrying out a carcinogenicity study with rats.

2.
materials and methods

2.1. Procedures for concentrating organic mutagens from water

Organic mutagens were concentrated using an adsorption/elution technique. Previous results have shown (Yamasaki and Ames, 1977) that the non-polar resin XAD-2 was able to concentrate mutagens from human urine and therefore it was suggested that this method might be applicable for concentration of organic mutagens from water. The choice for using a combination of XAD-4 and XAD-8 (XAD-4/8) instead of XAD-2 was based on results from Webb (1975) and Van Rossum and Webb (1978) which showed that this combination was most effective in concentrating a broad range of organic compounds in large volumes of water in a few days. Besides the XAD procedure, a freeze dry procedure was applied to find out whether the XAD procedure is a selective concentration procedure for organic mutagens.

2.1.1. XAD resins

Amberlite XAD-4 and 8 were obtained from Serva GmbH, Heidelberg F.R., Germany. The resins were purified by repeated Soxhlet extraction for 16 h in (consecutively) methanol, diethylether, acetonitril and again methanol. A subsample (column packed) of the resin was then eluted with diethylether and the eluate checked for purity by means of GC-analysis (no detectable impurities). The resins were stored in methanol at room temperature.

2.1.2. XAD concentration procedure

Surface water

For the concentration of organic constituents, depending on the concentration factor required, a volume of 5 to 40 litres of surface water was collected per sample. The samples were filtered (under nitrogen pressure) over a prefilter and a membrane filter with pore sizes of resp. 8 and 0.45 µm (Sartorius).

Concentration of the organic constituents via adsorption on XAD-resins was, except for the dimethylsulfoxide and acetone elution step, already described previously (Junk et al., 1974, 1976). A mixture (1:1) of XAD-4 and XAD-8 was used. Columns (25 x 1.5 cm) were packed with about 10 cm^3 of the XAD-mixture and washed subsequently with methanol, acetone, methanol and dimethylsulfoxide (Merck, für Spektroskopie). For a 200-fold concentration 5 litres of filtered water was passed through the washed column (upside down, under nitrogen pressure) with a flow rate of maximal 40 ml/min (4 bedvolumes/minute) and at a constant temperature of 15°C. The adsorbed organic material was then eluted with dimethylsulfoxide (DMSO). After discarding the first few ml which still contained water, about 25 ml of DMSO-eluate was collected. This was sterilized by filtration over 0.2 µm teflon filters (Millipore) and stored at -20°C.
For higher concentration factors, corresponding larger volumes of water were passed through the column until the desired water/eluate ratio (v/v) was obtained.

Drinking water

Drinking water samples were taken from the tap and in the 18 city survey just before the water left the treatment plant and filtered (under nitrogen pressure) through a 0.45 µm membrane filter before concentration. For about a 3000- to 7000-fold concentration 60-160 litres of the filtered water were passed over columns containing 20 cm^3 XAD at a flow rate of maximal 4 bed volumes/min. and at a constant temperature of 15°C. Elution of the adsorbed organic constituents has been carried out with the appropriate volume of either DMSO or acetone (≥1 bed volume), if indicated. After passing the XAD-column the XAD filtrate was collected if indicated, adjusted to pH=2 with HCl and readsorbed on XAD-4/8. Subsequent elution was carried out with DMSO or acetone (acid fraction). For lower or higher concentration factors, corresponding smaller or larger volumes of water were passed through the XAD column until the desired water/eluate ratio (v/v) was obtained.

2.1.3. Freeze drying procedure

Surface water

For a 300-fold concentration about 5 litres of (filtered) surface water were lyophilized in a Virtus Unitrap II. The dried residue was then packed in a small column (25 x 1.5 cm) with a sintered glass filter and the organic material was eluted with DMSO. About 16 ml of the DMSO eluate was collected, and sterilized by filtration over a 0.2 m teflon filter (Millipore).

Drinking water

For a 7000 fold concentration 70 litres of drinking water were lyophilized in a Virtus Unitrap II. The dried residue was then divided in equal weights and packed in 2 columns (25 x 1.5 cm) with a sintered glass filter and the organic material was eluted with DMSO. The DMSO concentrates were sterilized by filtration over a 0.2 m teflon filter (Millipore).

2.2. Mutagenicity testing

All organic concentrates prepared from water were tested in the Ames Salmonella/microsome assay (Ames et al., 1973, 1975) because this assay had proven to be successful in testing organic concentrates of surface and drinking water (Prein et al., 1978, Loper et al., 1978). The tester strains which have been used in this study were TA 98 and TA 100 (McCann et al., 1975a) because these strains were more sensitive to organic surface. and drinking water concentrates than strains TA 1535 and TA 1538 (Van Kreijl et al., 1980; Kool, unpublished data).

Bacterial strains

Salmonella typhimurium strains TA 98 and TA 100 (Ames et al., 1975), were obtained from Dr. I.E. Mattern, MBL-TNO, Rijswijk, The Netherlands. They were stored frozen at -80°C in nutrient broth containing 10% DMSO.

Ames Salmonella/microsome assay

The methods of bacterial culture, the verification of genetic markers and the plate incorporation assay were essentially as described previously (Ames et al., 1975). Petri dishes (Ø 90mm) containing about 20 ml of 1.2% Noble agar in minimal Vogel Bonner Medium E supplied with excess biotine and 2% bactodextrose (Difco) were used. They were seeded with 3 ml of molten top agar (45°C) to which the following was added consecutively: 0.1 ml of nutrient broth culture of the bacterial tester strain (containing about 5 x 10^8 bacteria/ml), up to 0.5 ml of DMSO concentrate or up to 0.25 ml of acetone concentrate, and 0.50 ml of S-9 mix (if indicated). The induction of microsomal enzymes and the preparation of the rat liver homogenates (S-9) has also been described previously (Ames et al., 1973, 1975). For surface water samples male Spraque-Dawley rats, obtained from the Biotechnical Department of MBL-TNO at Rijswijk, were intraperitoneally injected once with Aroclor 1254 five days before sacrifice.

For drinking water samples rat liver S-9 induced by Aroclor 1254 was obtained from Litton Bionetics. In the S-9 mix 0.075 ml of liver homogenate was added per ml of mix since this was found to give optimal results. The S-9 mix was sterilized before use by filtration over a 0.22 µm membrane filter (Millipore). All water concentrates were tested in 3-5 fold and the deviation of the mean in the figures was usually below 20%. The results were considered as significant when a 2-fold increase above the background and a dose-response effect were observed.

Routine controls were included to check for the presence of histidine and other possible toxic or (growth) stimulating effects in the sample. First, 0.5 ml of each DMSO concentrate or 0.25 ml of each acetone concentrate (max. volume tested) was plated out in the absence of histidine in the top agar and then compared with the normal spontaneous background level. Second, as a internal control, a fixed amount of test mutagen (Ethidium bromide or Nitrofurazon) was dissolved in 0.50 ml resp. 0.25 ml of each concentrate and tested for possible differences in the mutagenic response.

Finally, as a control for the concentration procedure, similar concentrates of tapwater (The Hague) were assayed for mutagenic activity; this control was àlways found negative.

2.3. Chemical analysis

Analysis of water quality parameters in the unconcentrated water samples were carried out to correlate these parameters with mutagenic activity in drinking water concentrates and were done according to routine procedures as previously described.
The selection of these parameters was mainly based on the presence of (chlorinated) hydrocarbons, because studies have shown an increase of chlorinated hydrocarbons and mutagenic activity after chlorination (Rook, 1974; Cheh et al., 1980; De Greef et al., 1980), which treatment frequently is applied in the preparation of drinking water.
Extractable organic halogens (EOCl) were analysed by microcoulometry (Wegman and Greve, 1977). Total nitrogen (Tot.N) according to the method by Koroleff (Koroleff, 1970) with some modifications (D'Elia et al., 1977). Adsorbable organic halogens (AOCl) were determined with the aid of active carbon (Sander, 1980). Total organic carbon (TOC) was analysed with a Beckman TOC analyser (Tocomaster model 915 B). Volatile organic halogens (VOCl) were determined by microcoulometry (Wegman and Hofstee, 1979) with a Texmar liquid sample concentrator. Trihalomethanes (THM) were analysed with a Carlo Erba 2900 analyzer containing a capillary column G.C. OV/225 diameter 0.5 mm, length 50 m and equipped with an automatic headspace sampler model 250.
Polynuclear aromatic hydrocarbons (PAH) were determined by thinlayer chromatography (Borneff and Kunte, 1969).

2.4. Fractionation techniques

To see whether organic mutagens responsable for the mutagenic activity in the drinking water concentrates could be separated from the rest of the organics and to obtain more information about the molecular weight of these organic mutagens, several fractionation techniques were carried out.

Thin layer chromatography (TLC)

For the TLC fractionation of acetone concentrates of drinking water preparative plates were used, precoated with 2.0 mm of Silicagel-G (PSG Merck, Fertigplatten). A volume of 2.0 ml acetone concentrate was applied on the plate with an automatic spraying device in the form of a small band (4.0 x 0.3 cm). Two solvent systems ethylacetate: iso-octane (1:1) and benzene: methanol (4:1) proved to yield the best separation. All solvents were of spectroscopic quality and the plates were air-dried between developments prior to further investigation. The developed plates were examined under UV light (366 nm) in order to mark the separate bands. The marked fractions were collected by scraping off and collecting the adsorbens with a Pasteur pipette connected to a vacuum-pump. The outlet of the pipette was fitted with a plug of glass-wool. The organic material was recovered by eluting the pipette with 5 ml DMSO. The eluates were stored at -20°C prior to mutagenicity testing.

Gelfiltration - Sephadex LH20

The glass column (height 40 cm, Ø 1 cm) was packed with Sephadex LH20 in dioxane-water (7:3) as described previously (Concin et al., 1980). About 1.0 ml of a DMSO/XAD concentrate of drinking water was layered on the column and subsequent gelfiltration was performed with dioxane-water (7:3) as solvent. Fractions of 1 ml were collected with an automatic fraction collector. After measuring the adsorbance at 263 nm the fractions were pooled, 5 fold diluted in water, reconcentrated on XAD-4/8 (bed volume 4 ml) and eluted with 5 ml DMSO. The concentrate was stored at -20°C prior to mutagenicity testing. Calibration of the column was performed using two coloured markers, viz. vitamin B12 (mol. weight 1355) and nitrofurazon (mol. weight 198).

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) separations of organic concentrates were performed as described previously (Wilson Tabor and Loper, 1980). Separations were carried out on a Hewlet Packard Model 10084 B equipped with an automatic sampling device, a solvent programmer, a variable absorbance detector and an automatically steered fraction collector. The instrument was fitted with a 3.9 mm by 30 cm prepacked analytical column of 10 µm silica particles bonded with octadecylisane (µ Bondapack -C18) for analytical scale. For semi preparative scale separations, the HPLC was fitted with a 7.8 mm x 30 cm prepacked column packed with 10 µm silica particles bonded with octadecylisane.

2.5. Preparation of organic drinking water concentrates for coupled bioassay/analytical fractionation

Organic drinking water concentrates (acetone) obtained from 2000-4000 litres of drinking water prepared with the XAD concentration procedure were used. The acetone in the drinking water concentrates was removed by rotary evaporation under reduced pressure at 30°C. The remaining aqueous sample was mixed with about 150 ml of non mutagenic water and subsequently extracted 3 times with 150 ml of diethylether. The ether extracts were pooled and the ether was removed by rotary evaporation under reduced pressure 30°C and the dry residue was dissolved in a few ml DMSO. The DMSO extract was used for HPLC fractionation and was also tested for mutagenicity in the Ames test.

HPLC and isolation of mutagenic fractions

Analytical and semipreparative reverse phase HPLC elution were performed using a water to acetonitrile linear gradient (Wilson Tabor and Loper, 1980). Samples for HPLC were injected as 20 ul (flowrate 1 ml/min) and as 80 ml (flowrate 4 ml/min) and the adsorption was measured at 254 nm. Fractions or subfractions were pooled as indicated and after reconcentration with ether assayed for mutagenicity in the Ames test.

2.6. Description of materials and methods used in the carcinogenicity study

Test substance

Weekly, organic concentrates (DMSO) were prepared from drinking water of city 18 using the XAD-4/8 concentration procedure and tested in the Ames assay. 1 Litre of drinking water corresponded to 0.11 ml of DMSO concentrate and contained on the average 115 µg organic material (OM). This values was obtained by weighting the freeze dried DMSO concentrate.

Animals and Treatment

Two hundred male and two hundred female SPF-reared albino rats (Wistar SSP TOX) were obtained from the National Institute of Public Health, Bilthoven, The Netherlands. The selection of this strain was based on the well documented tumour incidence of this strain (Kroes et al., 1976) and the use of this strain in a similar carcinogenicity study with chlorinated hydrocarbons (Van der Heijden and Van Esch, 1980).
The study was carried out at the CKP of the Agricultural University of Wageningen in collaboration with the Department of Toxicology.
At the beginning of the study the mean body weights were 165 g and 130 g for males and females respectively. They were housed five at a cage under conventional conditions at 22°C ± 1. Food and water was available ad libitium. Food was a semisynthetic diet SSP Tox. (Fa.Trouw and Co, Putten, The Netherlands). The composition of the diet is described in Appendix 1.
Organic drinking water concentrates of DMSO were mixed with non-mutagenic water. This was used as drinking water for the animals. The non-mutagenic water was refreshed every week with freshly prepared organic drinking water concentrates. The control group received non-mutagenic water with DMSO alone (0.24 % v/v), whereby DMSO was eluted with freshly prepared XAD-4/8.

Since the units for concentrating organic mutagens from drinking water had limited capacity as well as the fact that the organic composition of the water may vary considerably in time, no estimation of the maximum tolerated dose could be made.

Dose levels

Dose levels in this study were based on multiples of expected human exposure levels. It was assumed in the calculation that the average human weighed 70 kg and consumed 2 litres of water per day. Thus human exposure to the organic material (OM) in drinking water would be given approximately by 29 ml per kg body weight per day (3.3 µg OM/kg bw). The rats were divided in four groups (50 males and 50 females per group): a control group (group 1) and groups which received respectively 10x (group 2), 30x (group 3) and 90x (group 4) the human exposure level in their drinking water.

Experimental design and conduct

The animals were acclimatized for 4 weeks and treated during 106 weeks with organic drinking water concentrates in their drinking water at concentration of 0 (group 1), 0.27 (group 2), 0.81 (group 3) and 2.43 ml (group 4) per litre non mutagenic water. Water consumption was measured weekly. The general condition and behaviour of the rats were checked daily and body weights were recorded weekly in the first two months of the experiment and once a month thereafter. Animals in poor health or moribund were killed. At the end of the experiment all survivors were killed by exsanguination under anaesthesia, autopsied and examined for gross pathological changes.
The pathology was carried out at CIVO-Institutes TNO at Zeist and the Department of Toxicology of the Agricultural University of Wageningen. Samples of the following organs were preserved in a 4 % neutral, aqueous phosphate-buffered formaldehyde solution: adrenals, brain, gastrointestinal tract, heart, liver, lungs,

adrenals, brain, gastrointestinal tract, heart, liver, lungs, kidneys, lymph nodes, mammmary glands, pancreas, prostate, pituitary, ovaries, spleen, thymus, thyroid, testes, uterus, submaxillary salivary glands, sublingual salivary glands, exorbital lachrymal glands, urinary bladder, spinal cord, skeletal muscle, skin, eyes, nervus ischiadicus and all gross lesions. The organs and tissues to be examined histologically were embedded in paraplast, sectioned at 5 µm and stained with haematoxylin and eosin. Microscopic examination was performed on all organs of fifteen male survivors and fifteen female survivors of the control group and group 4 and on liver, spleen, adrenals, pituitary, thyroid and all lesions suspected of tumour of the other animals in control and group 4 and of all animals of the intermediate groups.

2.7. Statistical procedures

Computer assisted statistical analysis was carried out to determine possible relations between the mutagenic response of drinking water concentrates and the occurrence of some organic parameters in chlorinated and unchlorinated drinking water. An association between chlorinated drinking water and mutagenic activity in drinking water concentrates was described by calculating contingency coefficients (Siegel, 1956).
The Mann-Whitney test, a non parametric analogue of the t-test was performed (Daniel, 1978) to test whether the concentration of chemical parameters in mutagenic and non mutagenic samples are different or not.
To determine an association between the organic parameters and the mutagenic response in drinking water concentrates, Kendall's non-parametric correlation coefficients, which make no assumption about the distribution of values, were obtained as described previously (Daniel, 1978).
A straight line was fitted to the data points using mutagenic response (number of revertants at 0.5 ml concentrate divided by the spontaneous revertants) as a function of the concentration of

the chemical parameters (Tukey, 1977). For calculating least squares correlation coefficients between the considered chemical parameters (Green and Margerison, 1977), a square root transformation of these data was performed to obtain an approximately normal distribution (Mosteller and Tukey, 1977). Analysis of tumour incidence was carried out using Fisher's exact test and Chi-square analysis as described previously (Siegel, 1956) and according to the method of Peto (Peto et al., 1980).

3.

assessment of organic mutagens in water

3.1. The need for concentration

The investigation of the possible toxicity of drinking water due to the presence of organic mutagens and/or carcinogens may proceed along different lines : testing of the water as such, testing of organic concentrates and testing of individual compounds identified in drinking water. In principle, the possible toxicity should be studied in the water as such, since only then all the known and unknown constituents are present and all the possible cumulative, antagonistic or synergistic effects are accounted for. In this respect many epidemiological studies relating drinking water contaminants to health in man have been carried out the last decade. However, the results obtained in these studies do not allow a firm conclusion (NAS, 1979; Williamson, 1981) and therefore additional toxicity studies are needed.

Several facts render it unlikely that also toxicity studies with drinking water as such will lead to meaningful results. First, the total organic carbon content (TOC) of drinking water which meet drinking water standards generally is in the order of 1-5 mg C/ litre or less.

This means that all the known and unknown organic compounds add up to about 2-10 mg of organic matter per litre. Such a concentration of organics in general will be to low to exert effects. Only when the organic matter in drinking water consists of one or a few highly toxic compounds, which is very unlikely to happen, than an effect may be observed.

Second, a dose-response relationship seems very difficult to establish, because this relationship only can be obtained by diluting drinking water.

Third, a selection of a suitable control seems almost impossible.

Considering these factors it is not likely that direct testing of drinking water as such in chronic tests will provide suitable answers.

Moreover, the few animal studies that have been carried out with waters as such however with regard to reproductive toxicity in-mice, all with the exception of one, which result is questionable, showed no effects (McKinney et al., 1976; Chernoff et al., 1979; Stapler et al., 1979; Kool et al., 1982a). Loper (1980) also recently pointed out that most of the known organic mutagens and mutagens with carcinogenic properties would escape detection if drinking water containing trace amounts of these compounds (<1 µg/l) is tested directly in a sensitive in vitro system like the Ames Salmonella/microsome assay. Although some authors reported mutagenic activity of unconcentrated water samples (Pelon et al., 1977; Dutka and Switzer-House, 1978; Schwartz et al., 1979; Moore et al., 1980) these results are all marginal and probably subjected to intercurrent variables affecting bacterial growth such as toxic contaminants, histidine etc. With this in mind, concentration of organic constituents from the water are needed and this procedure offers the advantage of increased sensitivity upon subsequent biological testing. Another approach is to test the individual compounds known to occur in the water. With respect to the latter, it is recognized that many of the organic compounds present in drinking water cannot be readily volatilized for their subsequent separation and identification by GC/MS analysis. In fact it has been estimated as already stated that the non-purgeable fraction comprises in the order of 90% of the total organics in the water (NAS, 1977) and for this reason too, concentration of the organics from drinking water are needed.

3.2. Concentration of organic mutagens from water

3.2.1. Surface water

In The Netherlands the rivers Rhine and Meuse serve as important water sources for the drinking water supply and they provide either directly or indirectly the drinking water for about 5 million people in The Netherlands.

Since most organic pollutants including the genotoxic substances were shown to be present in concentrations at the microgram per litre level or less, it was clear (see part 3.1.) that direct testing of surface water samples up to 2 ml will yield negative results in the Ames test.
For this reason concentration of organics in surface water samples was carried out with a XAD resin procedure as firstly was suggested by Yamasaki and Ames (1977).

Results and Discussion

Using the XAD-4/8 concentration technique in combination with the Ames test, mutagenic activity could be demonstrated in different surface waters in The Netherlands (figure 1).

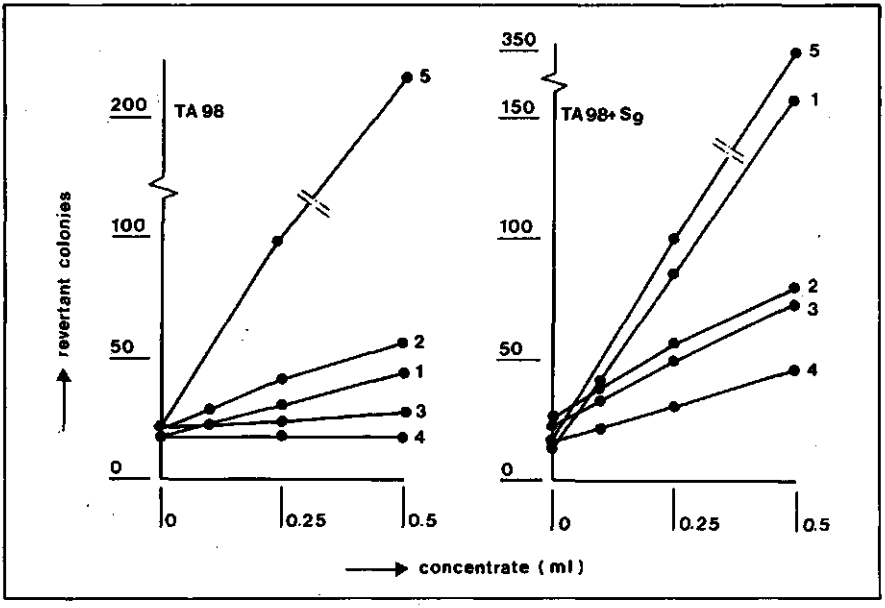

Figure 1. Mutagenic activity in XAD-concentrates of different types of surface water

The sampling, concentration with XAD-4/8, DMSO-elution and subsequent mutagenicity testing were as described in Methods. The types of surface water used and the corresponding concentration factors are: 1 Meuse water (4000); 2 Biesbosch water (4000); 3 biological effluent from a waste water plant (1000); 4 North Sea water, close to the Nieuwe Waterweg (1000); 5 Rhine water (1000). Each point represents the average of 3 plates.

It was shown that significant mutagenic activity could be demonstrated in XAD-concentrates of Rhine, Meuse and Biesbosch water, biological effluents from a waste water plant and even sea water. In all cases the activity was observed primarily with strain TA 98 and showed linear dose-response curves.
The highest mutagenic activity in the XAD procedure was obtained at a flowrate of maximal 4 times XAD bedvolume/minute and when an elution volume >1 XAD bedvolume was used (Kool et al., 1981a).
When samples of unknown organic composition like XAD concentrates of water are tested, alternative explanations like the presence of histidine, the generation of mutagenic activity during the concentration procedure and toxic effects have to be excluded. Besides the standard controls to check for factors affecting bacterial growth, XAD derived mutagenic activity was not likely by the negative results with control concentrates of tapwater or other surface waters (Van Kreijl et al., 1980; Kool et al., 1981a) and by the fact that comparable mutagenic activity was observed in a corresponding lyophilized Rhine water concentrate (figure 2).

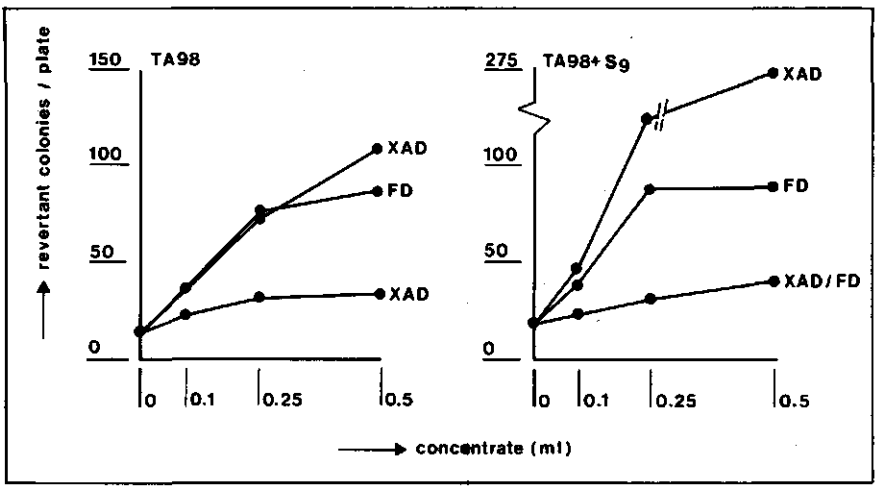

Figure 2. Comparison of mutagenic activity in lyophilized and XAD-concentrated Rhine water.
Sampling, 300-fold concentration using either XAD-4/8 (XAD) or freeze drying (FD), subsequent elution with DMSO (15 ml) and mutagenicity testing with strain TA 98 were as described in Methods. XAD/FD represents the lyophilized concentrate of the water collected after passage over the XAD-4/8 column. Each point represents the average of 3 plates.

This last experiment demonstrated the selective nature of the XAD method for organic mutagens. An other advantage of this concentration procedure is, that in contrast with other methods in this field, in which evaporation (Dutka and Switzer-House, 1978), solvent extraction (Prein et al., 1978; Kurelec et al., 1979; Grabow et al., 1980), reverse osmosis (Loper et al., 1978), adsorption on carbon or foams (Glatz et al., 1978; Schwartz et al., 1979) and bioaccumulation in mussels (Parry et al., 1976) this XAD procedure is not elaborating and time consuming because a high flowrate can be combined by direct testing the DMSO or acetone concentrate in the Ames test.

3.2.2. Drinking water

6 City survey.

Since in two important surface water sources for the drinking water supply in The Netherlands viz. the rivers Rhine and Meuse, mutagenic activity could be detected, a small scale study on the presence of mutagenic activity in drinking water in 6 cities, preparing their drinking water from these rivers, was carried out. In this study organic mutagens were concentrated with the XAD procedure which was succesfully applied for surface waters.

Results and Discussion

The results in the study showed (figure 3) that the XAD-4/8 concentration method in combination with the Ames test appeared to be a appropriate method to detect organic mutagens in drinking water. The optimal conditions for the adsorption of organic mutagens in drinking water were more or less similar to those observed in the surface water study and up to 200 litre of drinking water could be concentrated on a single XAD-4/8 column without a considerable run through of mutagenic activity (Kool et al., 1981c).

The limited survey of the drinking water of 6 cities showed that after 6000 fold concentration significant and dose-related mutagenic activity could be demonstrated in 4 out of 6 cities (figure 3).

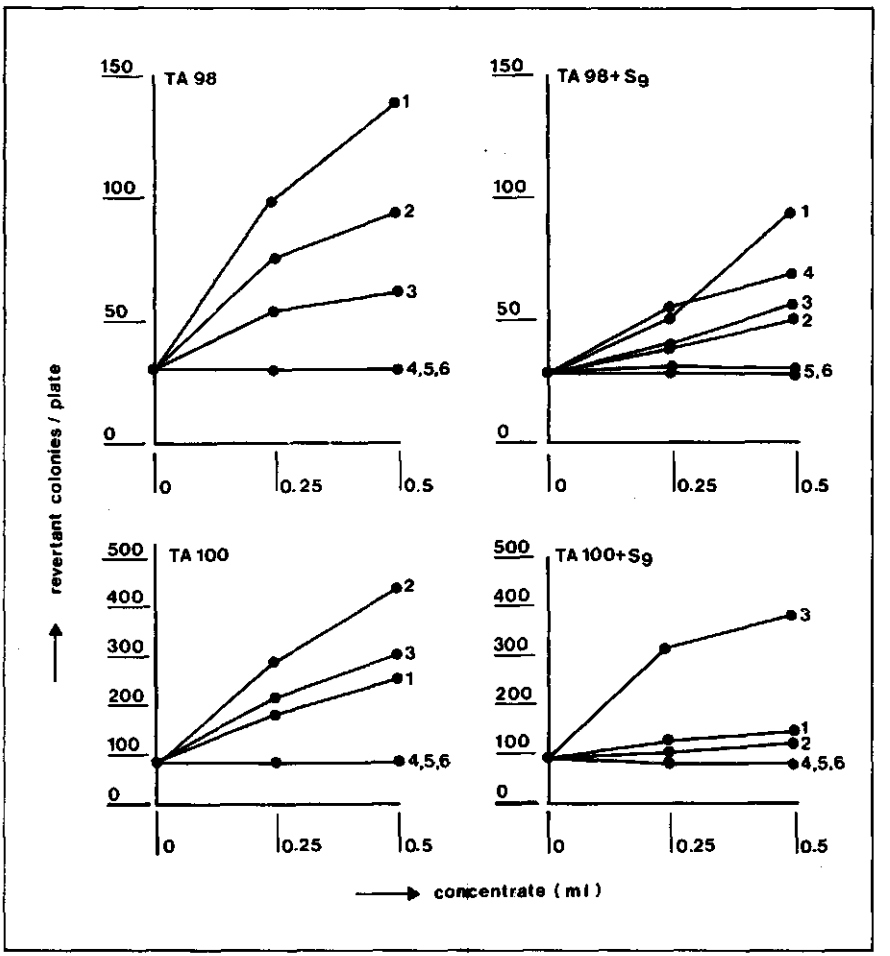

Figure 3. Mutagenic activity in different types of drinking water.

The sampling, 6000-fold concentration with XAD-4/8, elution with DMSO and subsequent mutagenicity testing with strain TA 98 and TA 100 were as described in Methods. Each point represents the average of 5 plates.

The mutagenic properties were not incidental ones since reproducible results were obtained in time. Possible artefacts could be excluded due to the standard controls (Kool et al., 1981a), the fact that dose related increases of up to 5 times the spontaneous level were observed, the negative results found in 2 out of 6 the cities and finally the mutagenic activity of a lyophilized and a XAD drinking water concentrate from city 1 which gave comparable result with strain TA 98+S-9 (figure 4).

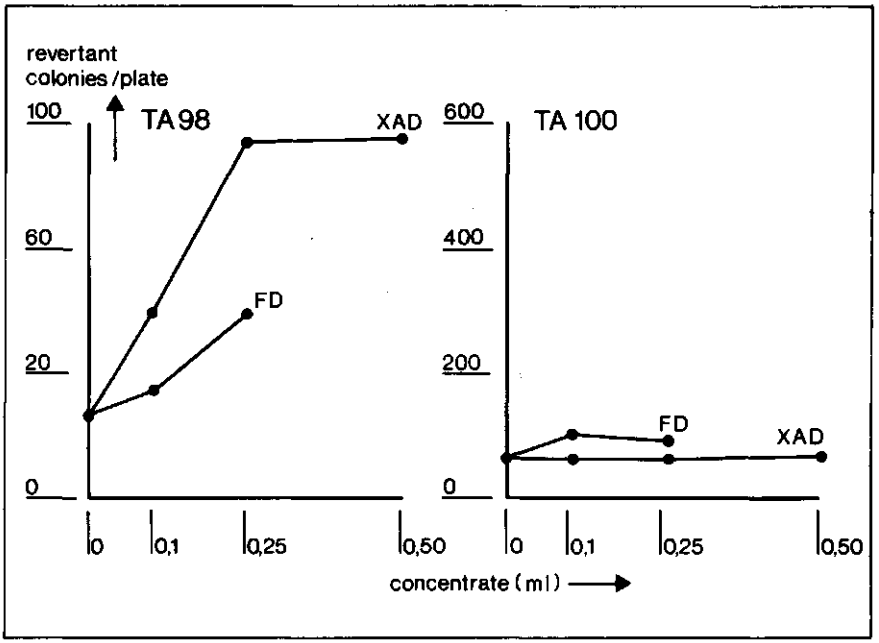

Figure 4. Comparison of mutagenic activity in lyophilized and XAD concentrated drinking water

Sampling, 3000 fold concentration on XAD-4/8 (XAD) and 4000 fold concentration by freeze drying (F.D.), subsequent elution with DMS and mutagenicity testing with strain TA 98 and TA 100 without S-9. Each point represents the average of 3 plates.

18 City survey

In the six city survey as described in the previous part, the presence of mutagenic activity in 0.5 to 3 litres of drinking water could be demonstrated in 4 cities out of 6. All six cities however, prepare their drinking water from surface water. To investigate whether the results are representative in The Netherlands, an extended survey in 18 cities (20 types of drinking water) was carried out three times, over a period of about two years.

As shown in table 3, these cities prepare their drinking water (chlorinated or unchlorinated) either from groundwater, surface water, or a mixture of both. In city 8 and city 16, drinking water after two different treatments were investigated and therefore they are indicated as city 8a. respectively city 16a.

Table 3. Types of drinking water involved in the 18 city survey

City	Treatment chlorine [a]		Raw water source
1.	-[b]	}	
2.	-	}	
3.	-	}	
4.	-	}	Groundwater
5.	-	}	
6.	+	}	
7.	+	}	
8.	+	} Dune filtration	Groundwater/
9.	+		Surface water
10.	+	}	
11.	-	}	
12.	-	} Bank filtration	Groundwater/
13.	-	}	Surface water
14.	+	}	
15.	-		
8a	+	}	
16.	+	} Storage reservoir	Surface water
16a	+	}	
17.	+	}	
18.	+	}	

a) +: chlorine treatment; -: no chlorine treatment
b) City 1 received chlorinated drinking water from city 16 during the third sampling period.

Results and discussion

The results of the three surveys are presented in figures 5-8.

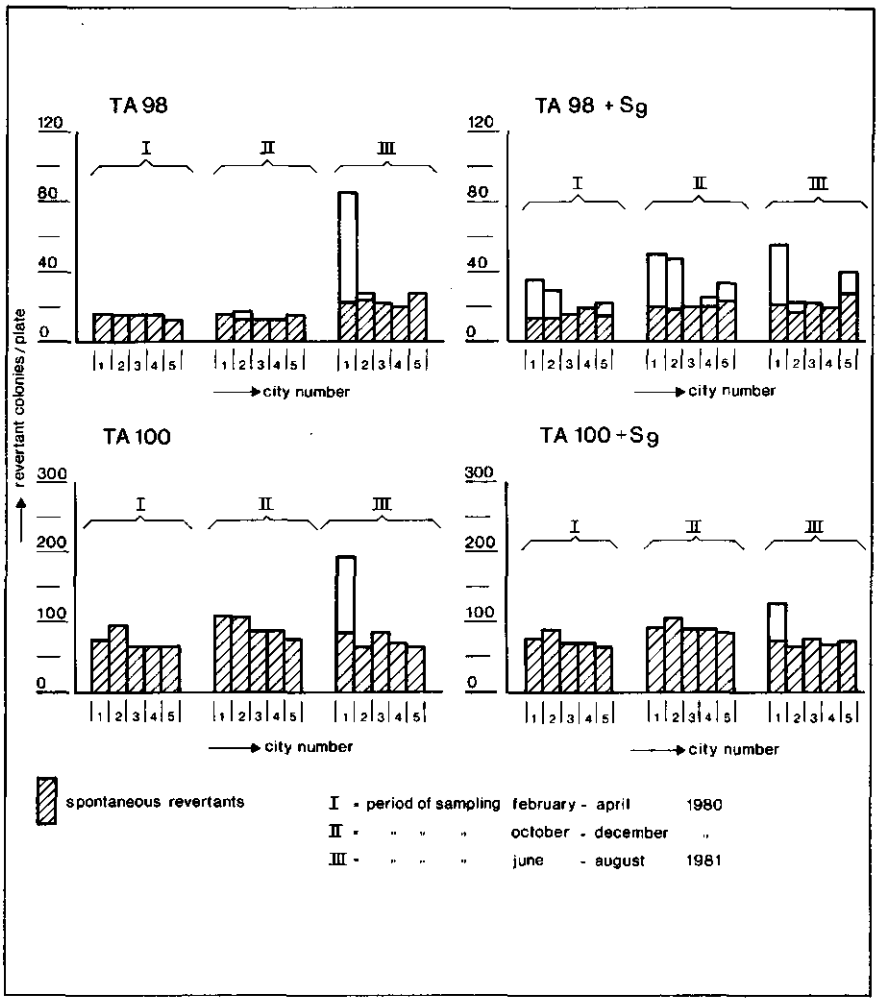

Figure 5. Mutagenic activity in drinking water prepared from ground water

The sampling, 7000 fold concentration with XAD-4/8, elution with DMSO and subsequent mutagenicity testing with strain TA 98 and TA 100 as described in Methods. The city numbers refer to the 18 cities depicted in table 3 and the results correspond to 3.5 litre of water per plate

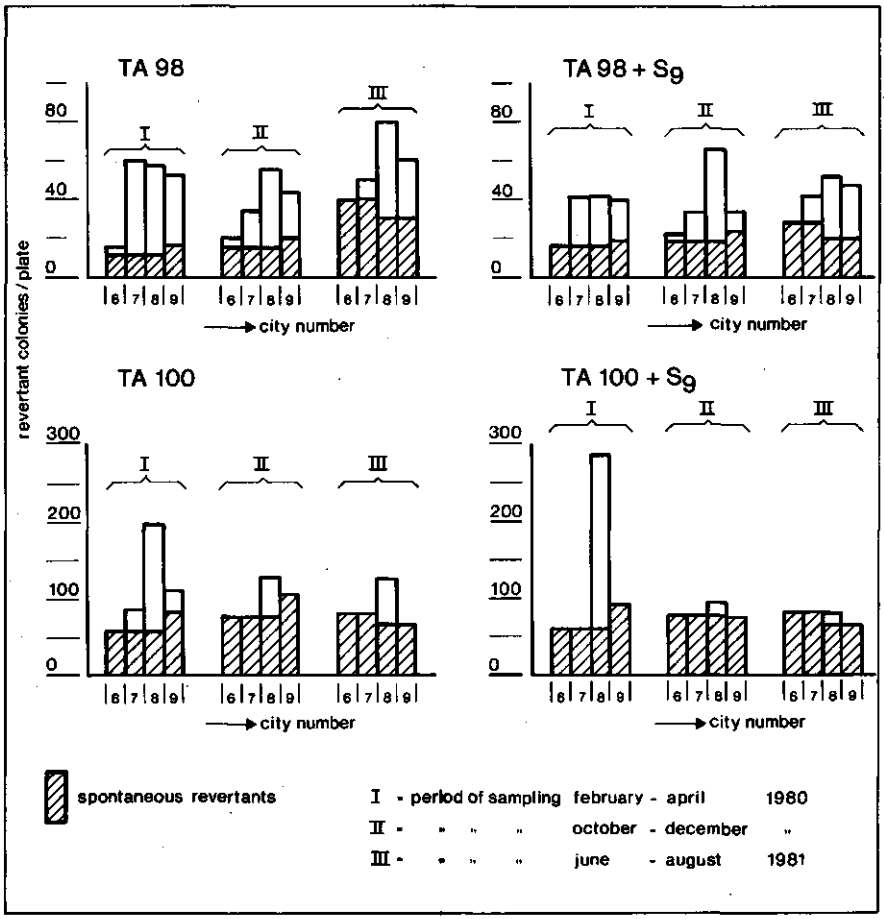

Figure 6. Mutagenic activity in drinking water prepared from dune filtrated surface water

The sampling, 7000 fold concentration with XAD-4/8, elution with DMSO and subsequent mutagenicity testing with strain TA 98 and TA 100 as described in Methods. The city numbers refer to the 18 cities depicted in table 3 and the results correspond to 3.5 litre of water per plate

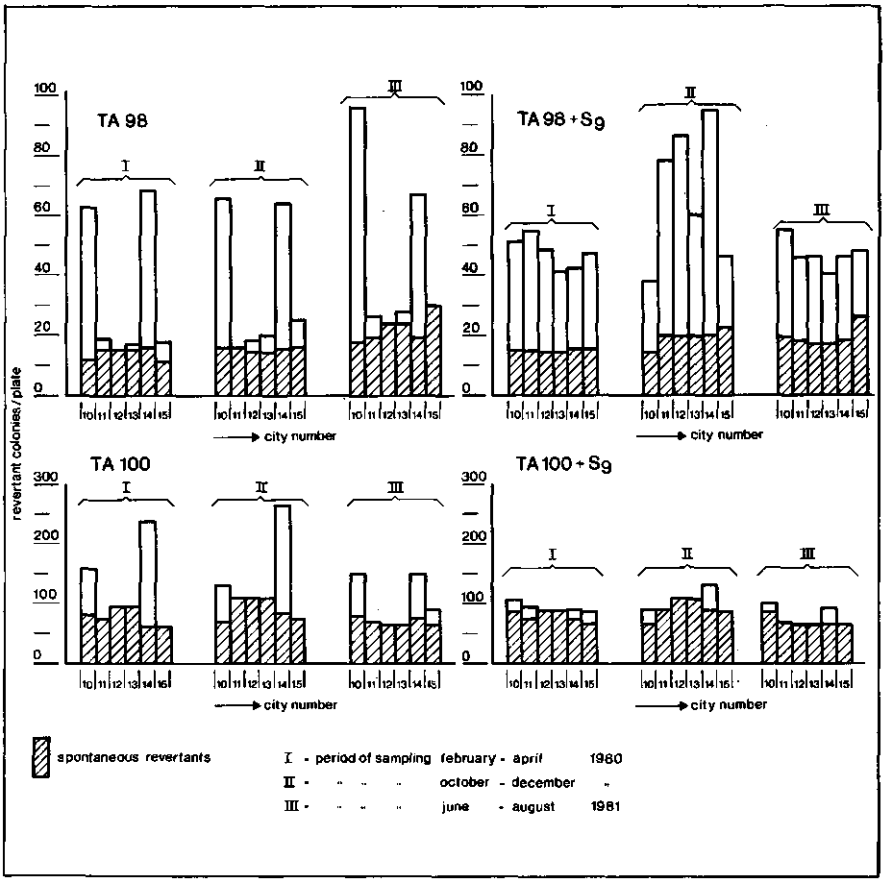

Figure 7. Mutagenic activity in drinking water prepared from bankfiltrated surface water.

The sampling, 7000 fold concentration with XAD-4/8, elution with DMSO and subsequent mutagenicity testing with strain TA 98 and TA 100 as described in Methods. The city numbers refer to the 18 cities depicted in table 3 and the results correspond to 3.5 litre of water per plate.

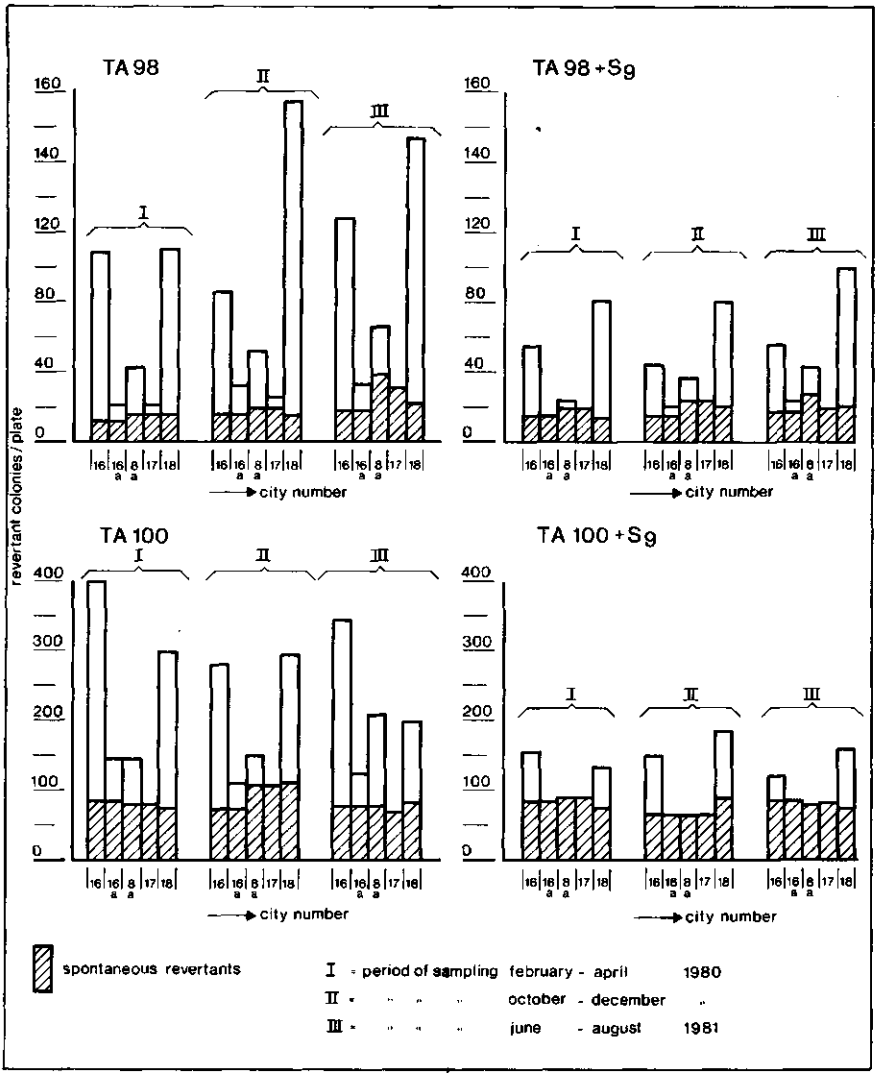

Figure 8. Mutagenic activity in drinking water prepared from stored surface water.

The sampling, 7000 fold concentration with XAD-4/8, elution with DMSO and subsequent mutagenicity testing with strain TA 98 and TA 100 as described in Methods. The city numbers refer to the 18 cities depicted in table 3 and the results correspond to 3.5 litre of water per plate

These results showed that in 14 drinking waters out of 20, mutagenic activity could be detected. When the cities are classified in four groups with regard to the raw water source and type of treatment, several features were observed.
First, the mutagenic activity observed in the 18 city survey obtained in three sampling periods showed to be a reproducable phenomenon with only two exceptions. The relatively high promutagenic activity with strain TA 100 of city 8 (figure 6) could only be detected in the first sampling period and the direct mutagenic activity with strain TA 98 and TA 100 of city 1 (figure 5) increased to a great extent in the third survey. The latter however, is explained by the fact that just before the third sampling time, the drinking water of city 1 was no longer prepared from groundwater. This city received in that period drinking water from city 16 which uses stored river water as water source.
Second, only two cities (city 1 and city 2) out of five which prepare their drinking water from groundwater showed significant promutagenic activity with strain TA 98. Third, only one city (city 9) out of four which prepare their drinking water with dune filtrated surface water showed no mutagenic activity. All six cities which use bankinfiltrated surface water showed promutagenic activity with strain TA 98 and two of them (city 10 and city 14) showed direct mutagenic activity with strain TA 98 and TA 100. From the category stored river water only city 16a and city 17 did not show mutagenic activity. Since previous investigations had revealed that another class of organic mutagens which only adsorb at pH=2-3 to the XAD-4/8, the so called acid fraction (Kool et al., 1981b; figure 18), the non mutagenic drinking waters were also screened for the presence of this type of mutagenic activity in the third survey. In none of these drinking waters this type of activity could be detected in the Ames microsome assay (not shown). Positive results with different organic extracts of drinking water have been observed in the USA, Canada and South Africa (Loper et al., 1978; Glatz et al., 1978; Schwartz et al., 1979; Nestmann et al., 1979; Grabow et al., 1980; Nestmann et al., 1982a; Williams et al., 1982). In most cases the mutagenic

activity was observed in drinking water prepared from chlorinated surface water (Nestmann, 1982b). In this study however, mutagenic activity could also be demonstrated in drinking water prepared from unchlorinated groundwater. Another remarkable result was that chlorinated drinking water of city 17 prepared from mutagenic river Meuse water did not show mutagenic activity. The latter result indicated that a specific combination of drinking water treatment processes is able to remove the organic mutagens detectable in the Ames test to a great extent.

3.3. Correlation of mutagenic activity in drinking water concentrates with water quality parameters and chlorine treatment

3.3.1. Introduction

From the previous part 3.2. it appeared that mutagenic activity in organic extracts of drinking water had been present in many drinking waters in The Netherlands. However, concentrating the organic mutagens from water and testing the concentrates in the Ames/microsome assay will take at least 3 days before the results will become available, which is a relatively long period with regard to water quality control. To overcome this time problem a search for chemical parameters which can be associated or related to mutagenic activity and which can be quickly and easily measured has to be made. To see whether such water quality parameter(s) could be found, several organic parameters were measured in drinking water samples and related to mutagenic activity in drinking water concentrates of the 18 cities. The choice of the water quality parameters viz. AOCl, EOCl, THM, VOCl, TOC, PAH and Tot.N had mainly been based on the presence of chlorinated hydrocarbon, since several studies have shown (Cheh et al., 1980, De Greef et al., 1980; Dolara et al., 1981; Kool et al., 1981b), that a chlorine treatment is able to introduce and increase the mutagenic activity. With respect to the latter a chlorine treatment versus no chlorine treatment therefore was also related with mutagenic activity in drinking water of the 18 cities.

3.3.2. Results and discussion

To find out whether chemical parameters present in drinking water and a chlorine treatment during the preparation of drinking water could be related with mutagenic activity in drinking water, several correlation studies were carried out.

In the correlation studies, the number of revertants (revertants of 0.5 ml concentrate minus spontaneous revertants) obtained from drinking water concentrates of 18 cities during the three sampling periods were used as estimates of mutagenic potency of each drinking water concentrate for each strain. The sampled population however, failed to meet the assumption of a normal or approximately normal distribution and equal variance. Therefore the classical parametric statistical tests could not be used. A number of non-parametric statistical procedures was used instead (Siegel, 1956; Daniel, 1978). The first hypothesis which had been tested was, whether a chlorine treatment and mutagenic response are positively correlated.

Mutagenic and non mutagenic samples, according to the criteria described in methods were compared with the presence or absence of a chlorine treatment. Based on these data the respective contingency coefficients (Siegel, 1956) were calculated (table 4).

Table 4. Relationship between chlorine treatment and mutagenic response in TA 98 and TA 100

Type of activity	Mutagenic effect a)	Number of samples chlorine treatment			Contingency coefficient
		Not applied	Applied	Total	
TA 98	-	26	11	37	
	+	0	23	23	0.57[b]
TA 98 + S-9	-	11	14	25	
	+	15	20	35	0.01
TA 100	-	26	22	48	
	+	0	12	12	0.40[b]
TA 100 + S-9	-	26	30	56	
	+	0	4	4	0.23

a) -: no mutagenic effect; +: mutagenic effect
b) significant at $p < 0.01$

Table 4 shows that only a significant positive correlation ($p < 0.01$) was observed between a chlorine treatment and direct mutagenic activity with strains TA 98 and TA 100. The highest correlation was observed with strain TA 98 ($r = 0.57$).

For testing the hypothesis whether the concentration of chemical parameters in mutagenic and non mutagenic samples were significant different or not, the Mann-Whitney test, a non-parametric analogue of the t-test was performed (not shown) (Daniel, 1978).

Significant higher concentrations of EOCl, AOCl and Tot.N ($p < 0.01$) were found in drinking waters which showed direct and promutagenic activity with strain TA 98 and direct activity with strain TA 100. VOCl and THM concentrations were significantly higher in these drinking waters which showed direct mutagenic activity with strain TA 98 and TA 100.

Considering the TOC and the PAH levels, it was shown that the direct and promutagenic activity in both strains did not show a relation with the TOC concentration, while the PAH concentration was below detection level (< 50 ng/l, not shown) in all samples so that these parameter was not considered for further analysis.

In addition to the Mann-Whitney test, Kendall's tau correlation coefficients were calculated (Daniel, 1978) in order to measure the extent of correlation between the chemical parameters and the mutagenic response (not shown).

These results demonstrated that the level of AOCl, EOCl, VOCl and Tot.N in drinking water showed a significant correlation ($p < 0.01$) with direct mutagenic activity with TA 98 and TA 100, in which AOCl showed the highest correlation $r=0.57$ respectively $r=0.49$. These parameters with the exception of AOCl (TA 100 + S-9) and VOCl (TA 98 + S-9) also showed a significant correlation with promutagenic activity with both strains whereby AOCl and Tot.N showed the highest correlation viz. $r=0.42$ respectively $r=0.34$. THM ($r=0.56$) and TOC only showed a significant correlation with the direct mutagenic activity with strain TA 98 respectively TA 100 but the correlation for the TOC was marginal ($r=0.26$).

In addition to the correlation studies, chemical parameters which showed a significant difference in concentration between mutagenic and non mutagenic samples (Mann-Whitney) and a significant

correlation (Kendall correlation coefficient), were used for straightline curve fitting and the intercepts and slope of the fitted regression lines for AOCl, the parameter with the highest correlation, are shown in figure 9.

From these fitted regression lines, the concentration levels were calculated at which a doubling of revertants may be expected (table 5).

Table 5. Concentration of chemical parameters in drinking water at which mutagenic activity in drinking water may be extected

Chemical parameter		Mutagenic activity in strain:			
		TA 98	TA 98+S-9	TA 100	TA 100+S-9
EOCl	(nmol hal/l)	25	26	59	-
AOCl	(µmol hal/l)	0.8	0.7	1.4	-
Tot.N	(mg N/l)	1.5	1.6	2.9	-
VOCl	(nmol hal/l)	142	-	222	-
THM	(µg/l)	21	-	-	-

- data not valid for calculating concentration levels

Table 5 shows that for EOCl, AOCl and Tot.N., an almost similar concentration in drinking water have to be present at which direct and promutagenic activity may be found with strain TA 98 and approximately twice this level for direct mutgenic activity with strain TA 100. This feature counts too for VOCl although for this parameter no concentration could by calculated at which a promutagenic response with strain TA 98 may occur. In none of the considered chemical parameters a concentration could be calculated at which promutagenic activity may be found with strain TA 100.

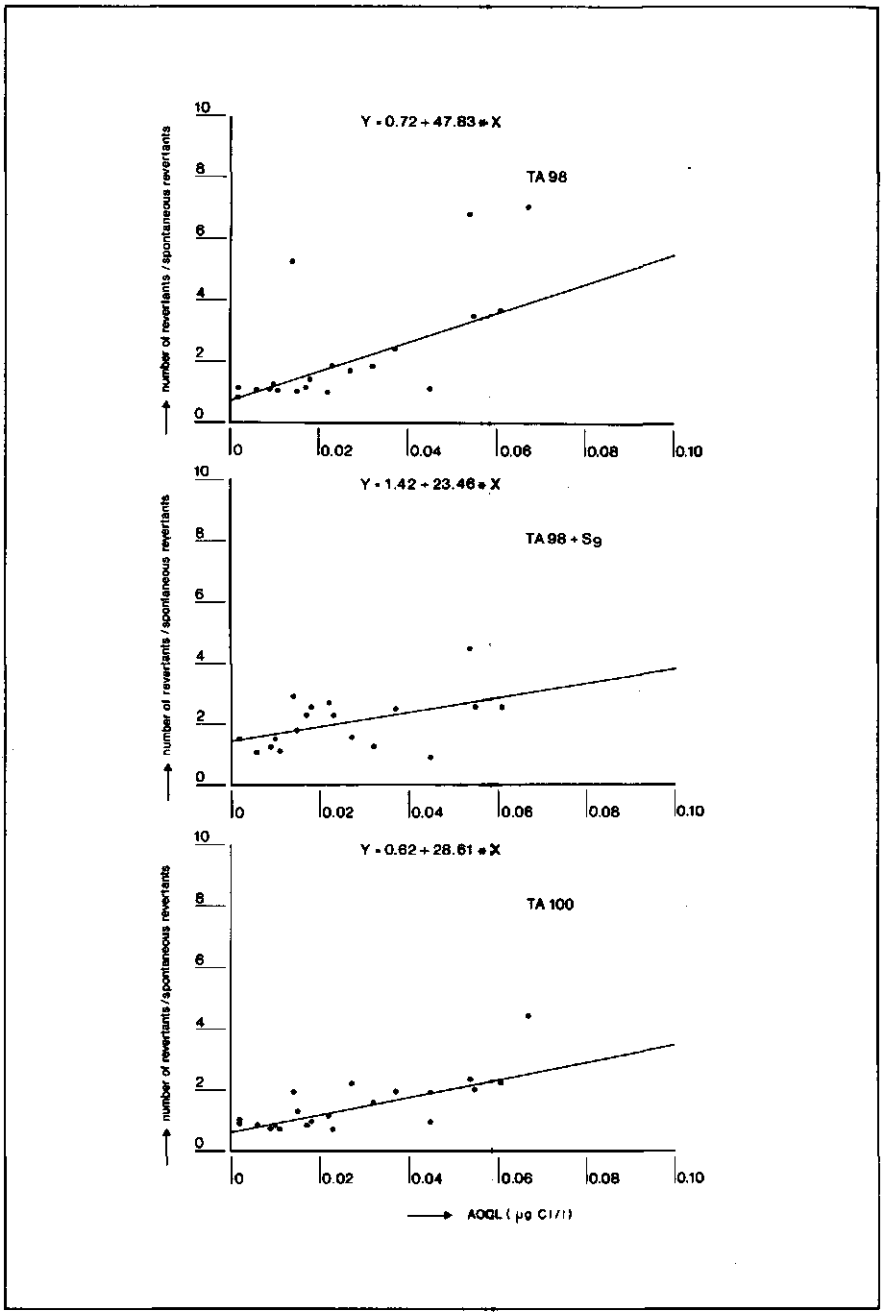

Figure 9. Relation between the level of AOCl and mutagenic response with strain TA 98 and TA 100.

Since EOCl, AOCl and Tot.N., behaved very similar with regard to the mutagenic respons in both strains, a correlation matrix was calculated (Green et al., 1977) for the considered chemical parameters after square root transformation (not shown).
As expected, a significant correlation ($p < 0.01$) existed between the chlorinated hydrocarbon parameters (EOCl, AOCl, VOCl, THM) from which the EOCl - AOCl showed the highest correlation ($r=0.76$). It was however, unexpected and no clear explanation is available that Tot.N. also showed a significant correlation with these chlorinated-hydrocarbon parameters whereby the correlation with AOCl appeared to be the highest ($r=0.59$). When TOC was considered, it appeared that no significant correlation was found between this parameter and the others.
When the results obtained in the correlation studies are evaluated it appeared that a significant positive correlation was observed between a chlorine treatment and the direct mutagenic activity with strain TA 98 and TA 100. When the levels of chemical parameters in drinking water were correlated with the mutagenic response it became clear that AOCl showed the highest correlation with the mutagenic response in both strains.
In a similar study, recently carried out in Canadian drinking waters (Nestmann et al., 1982a), it was shown that the highest correlation was obtained between levels of non adsorbed organics in the XAD filtrates and mutagenicity in concentrates. A confirmation of these results was however not possible since no chemical measurements were carried out in the filtrates in this study. A comparison of AOCl results also was not possible, since in the Canadian study this parameter was not measured.
The TOC and THM results in both studies however, showed a similar pattern. No correlation was observed between mutagenicity activity and TOC levels in drinking water while about the same level of correlation was observed with activity with strain TA 100 and THM.

From the results in this study it is clear, that AOCl in the first place should be considered as indicatpr for mutagenicity in drinking water, although further research with regard to this subject is needed.

In addition, the minimal concentration of AOCl and of the other relevant parameters had been calculated above which a mutagenic response may occur, when this level is found in drinking water. This calculation was based on straight line curve fitting. Whether this approach is valid or not is still in discussion, since quantitative comparison of the mutagenic results obtained in different samples in time is subjected to critizism because of the variation of spontaneous revertants in different controls. However, when in drinking water these parameters are found in concentrations above the calculated levels, the chance to find mutagenic activity in drinking water has been strongly increased.

3.4. Formation and removal of organic mutagens in drinking water supply

3.4.1. Introduction

Mutagenic activity of organic concentrates prepared from the rivers Rhine and Meuse was most pronounced in the presence of rat liver homogenate (S-9 mix) (figure 1). Results with concentrates of drinking water prepared from these rivers showed, however, that the mutagenic activity in the Ames test was most pronounced without metabolic activation in many of the investigated cities in The Netherlands (part 3.2.). These results indicate that during drinking water treatment formation and/or removal of mutagenicity has occurred.

A possible formation of mutagenic activity during drinking water treatment is supported by results from several pilot plant studies which have shown an increase activity in the Ames test after water chlorination (Cheh et al., 1980; Zoeteman et al., 1982).

These results were supported furthermore by some preliminary experiments in drinking water plants as reported previously (Kool et al., 1981b) and from results in another study (Dolara et al., 1981). In the present part the effects of different oxidation and filtration processes on the course of the Ames mutagenicity in several waterworks have been investigated in more detail. This included chlorination, ozonation, filtration through sand- and carbon-filters, and filtration in natural dunes, which are all processes employed on a routine basis in Dutch Waterworks.

In addition some chemical parameters AOCl, EOCl, THM and TOC were also analysed to see how these correlate with the observed mutagenic activity in the water during treatment.

3.4.2. Results and discussion

Effect of oxidation processes

In The Netherlands most drinking water treatment plants which use surface water for the preparation of drinking water, apply a chlorine treatment in the form of a transport, breakpoint or post-chlorination step. As stated in part 3.4.1., chlorine treatment clearly is suspect with regard to its possible formation of mutagenic activity during drinking water preparation. Therefore, effects of a breakpoint and postchlorination step have been studied more thoroughly in a few water supplies.
In figure 10 the results of two chlorination treatments are shown.

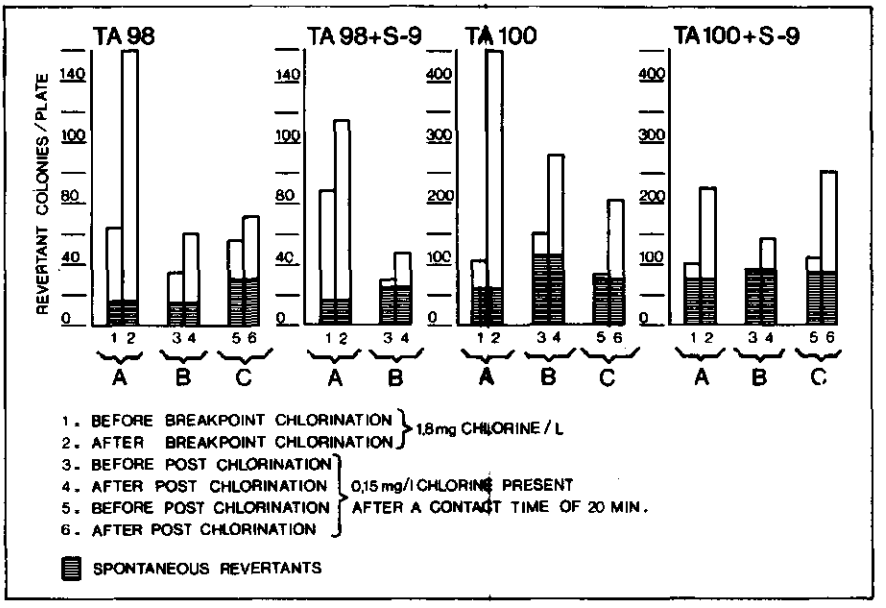

Figure 10. Effect of chlorination on mutagenic activity

The sampling, 7000 fold concentration of treated water in a water supply before and after a chlorine treatment (A and B Raw water source the river Meuse, C raw water source the river Rhine) on XAD-4/8, elution with DMSO and subsequent testing the DMSO concentrate in the Ames assay as described in Methods. Each value represents the average of 3 plates. The results correspond to 3.5 litre water per plate.

In water supply A a strong increase of direct and promutagenic activity in the neutral fraction and acid fraction (not shown) was observed in both strains after a breakpoint chlorination. Also the levels of the chemical parameters but one (TOC) showed a drastic increase after this chlorination step (table 6). Application of a postchlorination also appeared to be able to increase the mutagenic activity of the water and was even able to convert drinking water without detectable mutagenic activity into mutagenic drinking water as was demonstrated in water supply B and C (figure 10).

Table 6. Effect of a breakpoint chlorination on chemical parameters

Sampling site in Water Supply A	Level of organic parameters			
	TOC mg/l	THM µg/l	EOCl nmol hal/l	AOCl µmol hal/l
Before chlorination	3.5	0.1	30	0.4
After chlorination	2.6	30.7	80	3.5

Ozone, which is a powerful disinfectant is used increasingly in the preparation of drinking water.
Conflicting results regarding both the reduction and enhancement of mutagenic activity have recently been reported by several investigators (Gruener, 1978; Dolara et al., 1981; Zoeteman et al., 1982; Kool et al., 1982b; Van Hoof, 1982; Van der Gaag et al., 1982).
Differences relate either to the watertype studied or the concentration procedure employed (acid XAD eluates). In figure 11 the influence of an ozone treatment on the mutagenic activity in two water supplies is shown.

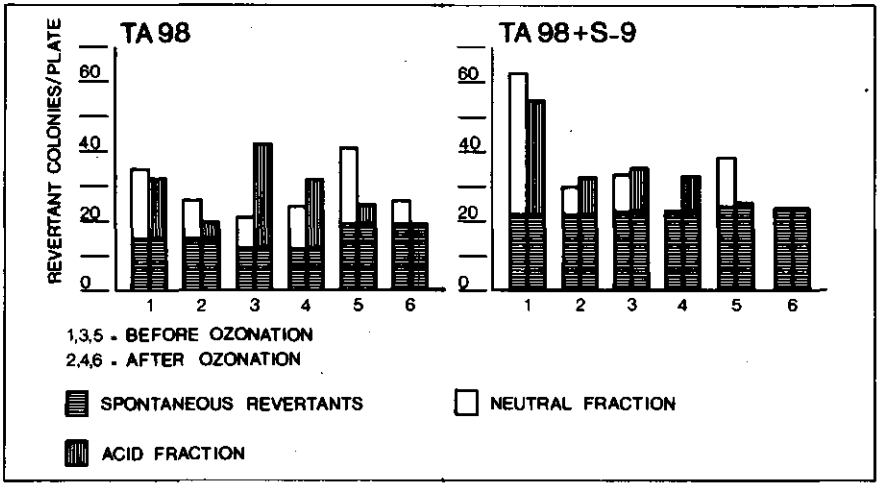

Figure 11. Effect of an ozone treatment on mutagenic activity

The sampling, 7500 fold concentration of (treated) water in a water supply and after ozonation (2 mg ozone/l, contacttime 10 minutes, A and B raw water source the river Meuse, C raw water source the river Rhine) on XAD-4/8, elution with DMSO and subsequent testing the DMSO concentrate in the Ames assay as described in Methods. Each value represents the average of 3 plates. The results correspond to 1.5 litre of water per plate.

The results showed that the direct and promutagenic activity with strain TA 98 in the normal XAD concentrate (neutral fraction) was reduced after an ozone treatment, with the exception of the direct activity in water supply B in which the number of revertants slightly seemed to increase. The reduction of the direct and promutagenic activity in the acid fraction was also demonstrable in the three water supplies, although the reduction of the promutagenic activity in water supply B and C was marginal. In the investigated waters no mutagenic activity could be demonstrated with strain TA 100 before and after ozonation. The chemical data showed (table 7) that the level of all parameters but one (THM) decreased after an ozone treatment. In contrast to the chlorinated waters however, these results were not consistent since the levels of TOC, EOCl and AOCl did not always decrease after an ozone treatment (not shown). In some case the level of all organic parameters remained unchanged while the mutagenic activity decreased slightly.

Table 7. Effect of an ozone treatment on chemical parameters

Sampling site in Water Supply C	Level of organic parameters			
	TOC mg/l	THM µg/l	EOCl nmol hal/l	AOCl µmol hal/l
Before ozonation	2.1	2.2	50	0.8
After ozonation	0.5	2.3	2.5	0.3

Effects of filtration processes

Filtration processes like dune filtration (artificial recharge), slow sand filtration and activated carbon filtration are used in The Netherlands in drinking water supply. Therefore these processes were also evaluated for their removal capacity towards organic mutagens. In figure 12 the results of dune filtration are shown.

The direct and promutagenic activity with strain TA 98 in the neutral and acid fractions were reduced to a great extent after dune filtration. A reduction in number of revertants was also observed in both fraction with strain TA 100 while no promutagenic activity was detectable with this strain. A similar result (not shown) was observed after dune filtration in another water supply, in which the water source was the river Meuse. The chemical data showed (table 8) that the levels of AOCl and THM decreased significantly while the TOC value increased and the EOCl level remained unchanged. Only the AOCl and EOCl parameters behaved consistently with mutagenic activity in several samples at different sampling times.

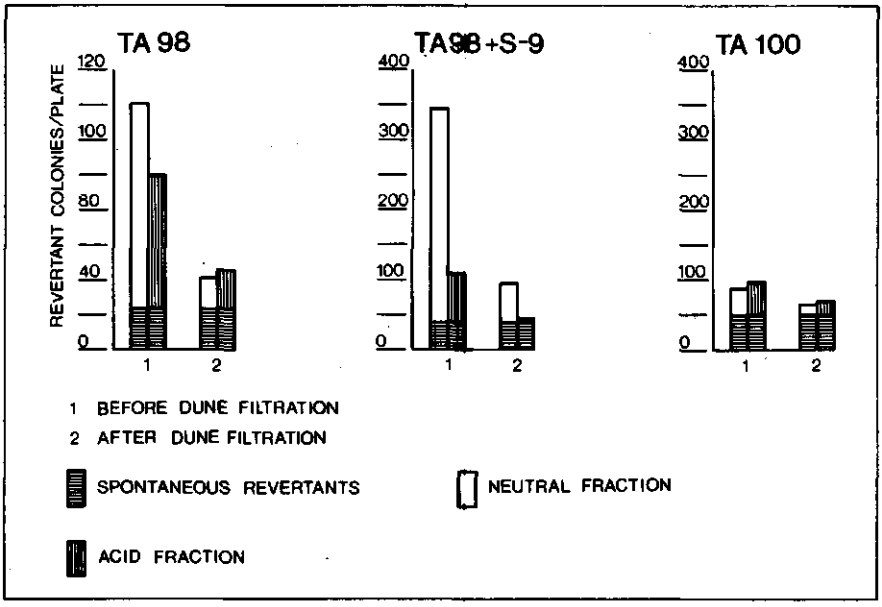

Figure 12. Effect of dune filtration on mutagenic activity

The sampling, 7500 fold concentration of water samples before and after dune filtration (detention time 3 weeks) on XAD-4/8, elution with DMSO and subsequent testing the DMSO concentrate in the Ames assay as described in Methods. Each value represents the average of 3 plates. The results correspond to 1.5 litre of water per plate.

Table 8. Effect of a dune filtration on chemical parameters

Sampling site in Water Supply A	Level of organic parameters			
	TOC mg/l	THM µg/l	EOCl nmol hal/l	AOCl µmol hal/l
Before dune filtration	1.3	1.6	30	2.2
After dune filtration	2.4	0.2	30	0.6

The results obtained with a slow sand filtration could not be evaluated, since the XAD-concentrates (neutral and acid fraction) after this treatment were toxic for both Ames tester strains (not shown).

The results of carbon filtration are shown in figure 13.

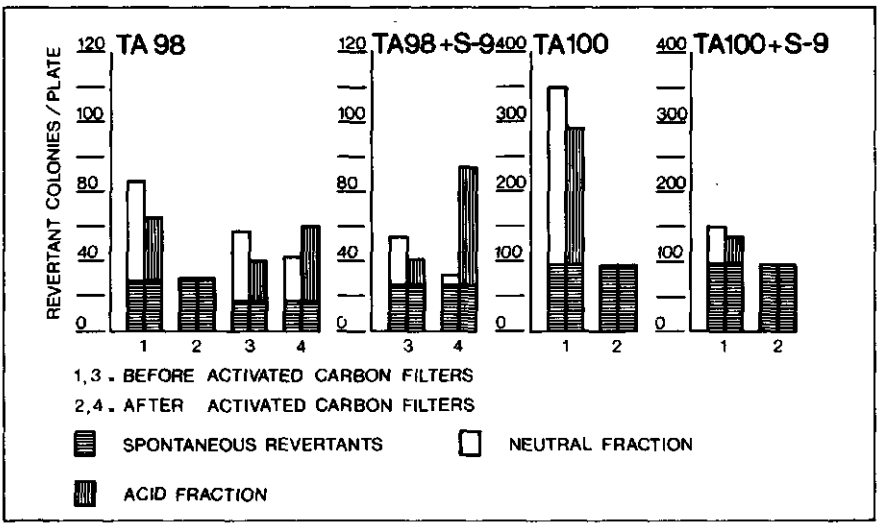

Figure 13. Effect of carbon filtration on mutagenic activity

The sampling, 7000 fold concentration of treated water in a water supply before and after activated carbon filtration (A, contacttime 10 minutes, filter used less than 1 year; raw water source the river Meuse; B, contacttime 15 minutes, filter used more than 1.5 year, raw water source the river Rhine) on XAD-4/8, elution with DMSO and subsequent testing the DMSO concentrate in the Ames assay as described in Methods. Each value represents the average of 3 plates. The results correspond to 3.5 litre of water per plate.

In water supply A the direct activity with strain TA 98 and the direct and promutagenic activity with strain TA 100 in the neutral and acid fraction was no longer detectable after carbon filtration (contacttime 10 minutes and the carbon filter is less than 1 year in operation in the water work). In water supply B however, in which the carbon filter had been operated for more than 1.5 year (contacttime 15 min.) in a pilot plant study, the direct and promutagenic activity with strain TA 98 still was reduced, but the direct and promutagenic activity of the acid fraction seemed even to

increase after this treatment. No direct and promutagenic activity was observed with strain TA 100 for supply B. The chemical data showed (table 9) that the level of all organic parameters with the exception of THM in water supply A decreased after carbon filtration.

The decrease of most of the organic parameters in water supply B however, contrasted the increase of the mutagenic activity in the acid fraction after carbon treatment.

Table 9. Effect of a carbon filtration on chemical parameters

Sampling site	Level of organic parameters			
	TOC mg/l	THM µg/l	EOCl nmol hal/l	AOCl µmol hal/l
Water Supply A:				
Before carbon filtration	1.9	0.10	5	0.3
After carbon filtration	0.6	0.18	0.3	0.06
Water Supply B:				
Before carbon filtration	2	2.2	50	0.8
After carbon filtration	1	2.0	30	0.4

In the present study it was shown, that different treatment processes which at present are applied in the preparation of drinking water in The Netherlands, were able either to remove or increase mutagenic activity in the water.

The observed increase of mutagenic activity by chlorine treatment confirms other investigations (Cheh et al., 1980; De Greef et al., 1980; Dolara et al., 1981; Kool et al., 1981b; Van der Gaag et al. 1982; Maruoka and Yamanaka, 1980; Zoeteman et al., 1982). Chlorination however, does not necessarily have to increase the mutagenic activity of drinking water prepared from surface water.

This could be demonstrated with drinking water of city 6. Mutagenic activity which was present in the river Meuse, was complete removed during treatment (storage reservoir, transportchlorination, dune filtration, activated carbon (powder), rapid and slow sand filtration (Kool et al., 1981b) and drinking water from this water supply receiving a chlorine dose up to a few milligrams per litre did not introduce mutagenic activity in the neutral and acid fraction (Kool, unpublished data). Ozonation of the water decreased or had little influence on the direct and promutagenic activity in the acid and neutral fraction. The latter results confirms previous results (Zoeteman et al., 1982; Kool et al., 1982b; Van Hoof, 1982, Van der Gaag et al., 1982). The effects of ozonation on the mutagenicity, does not seem consistent, since one drinking water study (Dolara et al., 1981) showed an increase of mutagenic activity with strain TA 100 in the neutral fraction while two other studies, revealed an increase in the acid fraction (Van Hoof, 1982; Van der Gaag et al., 1982). This may indicate that depending on the type of water, ozone in some cases is able to increase or generate mutagenic activity with strain TA 100. The results with dune filtration (artificial dune recharge), showed that with a detention time of about 3 weeks, a large reduction of the direct and promutagenic activity in the neutral and acid fraction occurred. This result confirms our earlier results (Kool et al., 1982b) and the results obtained in a recent Dutch study (Van der Gaag et al., 1982). Whether this reduction is due to (micro)biological activity or physical/chemical processes or a combination of both remains to be solved. From the mutagenicity results obtained from slow sand filtration it is clear that the evaluation of this filtration step is not yet possible because of the observed toxicity of the waterconcentrates for bacterial strains TA 98 and TA 100. Therefore further investigation viz. separation of the toxic part from the mutagenic fraction should be carried out.

Results with carbon filtration in drinking water treatment showed that mutagenic activity in water supply A no longer was detectable in the Ames test after this treatment. This confirms earlier results (Kool et al., 1982b) and results obtained in a recent Belgian study (Van Hoof, 1982).

In water supply B however, this treatment, although on a pilot plant scale showed quite different results. In the neutral fraction only a slight increase in activity was observed while in the acid fraction even an increase in activity was shown. Although this phenomenon should be investigated more thoroughly, this result indicates that a break through of organic mutagens may occur when the filter is to long in operation.

When the chemical data of the organic parameters are evaluated with respect to the behaviour of the organic mutagens in oxidation and filtration processes, the results showed that during a chlorine treatment the levels of AOCl, EOCl and THM as expected behaved similar as the mutagenic activity.

During ozonation EOCl, AOCl and TOC followed in general the pattern of the mutagenicity. In dune filtration processes only AOCl and THM behaved similar with the mutagenic activity and during activated carbon filtration the levels of AOCl, EOCl and TOC were in consistency with the mutagenic pattern in the neutral fraction but not in the acid fraction. The association between the organic parameters and the mutagenicity seems therefore only consistent for AOCl for the neutral fraction in the considered treatments.

The inconsistency of AOCl with the activity in the acid fraction seems not of great significance, since up till now organic drinking water concentrates showing mutagenic activity in the neutral fraction did not always show activity in the acid fraction. With this result and previous results (part 3.3.), AOCl seems from the considered group parameters the best indicator for describing mutagenic activity in drinking water although more investigation should be carried out in this respect, in particular with non chlorinated drinking waters.

Finally, the question has been raised whether treatment processes which are able to increase and reduce mutagenic activity should be stopped respectively introduced in drinking water preparation. Since however, an evaluation of bacterial mutagenicity (Ames test) due to drinking water concentrates at this moment is not possible, no conclusion may be drawn in this respect. Therefore additional research, like identification of the organic mutagens and toxicity studies have to be carried out to obtain more detailed information about the significance of the organic mutagens present in drinking water.

4.

characterization of the mutagenic fractions in drinking water concentrates and some physical-chemical properties of the responsable organic mutagens

4.1. Introduction

The majority of the organics present in drinking water has not been identified yet, and it is apparent that there is an advance to combine analytical procedures and biological testing in order to separate the biological active fraction. It will permit the isolation of bioactive subfractions which hopefully may lead to identification of the bioactive compounds. Coleman et al.(1980) investigated what kind of organic compounds could be identified in a mutagenic concentrate of Cincinnati tapwater. More than 700 organic compounds could be detected in an Ames test positive concentrate from which 460 could be identified. This result clearly shows that a more sophisticated coupled bioassay/chemical fractionation procedure in a sense that the major part of the organics which is not responsable for mutagenic activity will be separated from the organic mutagens, is necessary to identify the biological active organics in a complex mixture. Recently Wilson Tabor and Loper (1980) carried out initial partitioning by liquid/liquid extractions was followed by repeated high performance liquid chromatography (HPLC) for separation into smaller subfractions. Active subfractions (positive in the Ames test) were analysed by GC/MS and consequently for peak identification. Preliminary results obtained with a chloroform extract from a Cincinnati tapwater of 1962 showed that a polychlorinated aliphatic ether was responsible for the mutagenic activity. More recently the structure of this mutagenic compound has been identified and this compound 3-(2-chloroethoxy)-1,2-dichloropropene is probably derived from a herbicide diallate widely used in that period (Wilson Tabor, 1982).

The investigation of the organic mutagens in drinking water in The Netherlands is now moving along similar lines.

4.2. Characterization and some physical-chemical properties of organic mutagens

4.2.1. Adsorption/elution

An investigation of some positive drinking waters from the eighteen city survey (part 3.2.2) revealed that readsorption of the XAD filtrate on a second and third column at pH=2-3 respectively pH=10, revealed the presence of another class of organic mutagens at pH=2-3 the so called acid fraction. A representative example is shown in figure 14.

Further it was investigated whether an organic solvent like diethylether, which is widely used for analytical purposes for instance gaschromatography coupled with massspectrometry (GC/MS), can also eluate the organic mutagens from the XAD column. The results in figure 15 show that diethylether only elutes a minor part of the mutagenic activity from XAD-4/8. Subsequent elution with acetone however, eluted the major part of the activity.

This result indicated that the major part of the organic mutagens is present in the slightly polar fraction and therefore it is likely that these organic mutagens are not identical with the gaschromatographable organics already identified in this type of drinking water. This follows from the fact that diethylether was used as elution solvent for XAD-4/8 in chemical analysis of this drinking water described previously (Zoeteman, 1978).
The organic mutagens are not only less volatile but also resistent to boiling (figure 16) which almost completely eliminates the volatile organics like the THM's from the water (table 10), and therefore the mutagens obviously belong to group III and IV as presented in table 2.

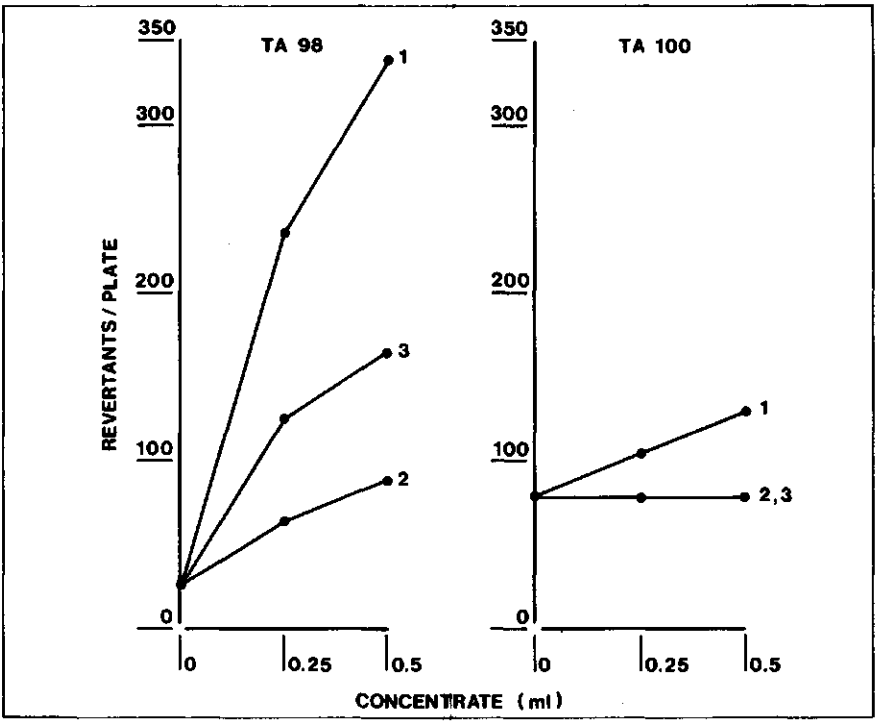

Figure 14. Effect of pH on the adsorption of organic mutagen in drinking water to XAD-4/8.

The sampling, 7000-fold concentration on XAD-4/8, elution with DMSO (20 ml) and subsequent Ames testing of drinking water was as described in Methods. After passing the XAD-column the XAD filtrate was collected and readsorbed on XAD-4/8 at either neutral pH (nr. 2) and pH=2-3 (nr. 3).
Subsequent elution was again with 20 ml DMSO and testing for mutagenic activity in the Ames was as described in Methods. Each point represents the average of 3 plates.

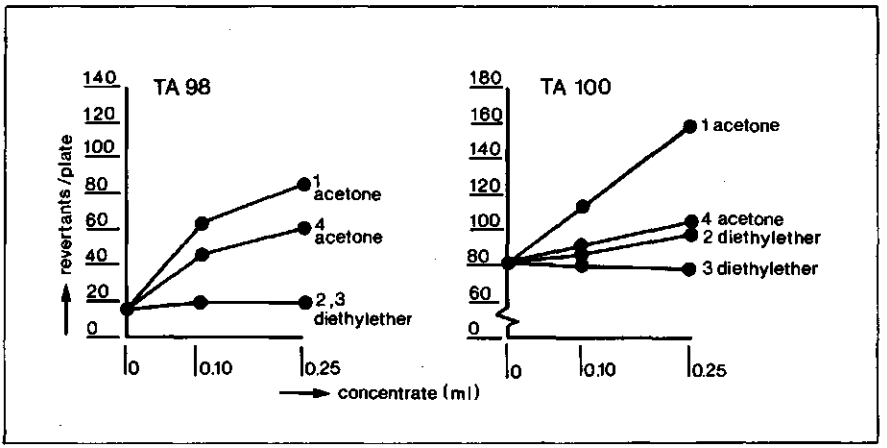

Figure 15. Behaviour of concentrated organic mutagens on XAD-4/8 by elution with diethylether.

The sampling, 8000-fold concentration of drinking water on XAD-4/8 (nr. 1). The elution was carried out as follows: XAD 4/8 was eluted with acetone (10 ml) (nr.1) followed by an elution with diethylether (10 ml) (nr.2). XAD-4/8 was eluted with diethylether (10 ml) (nr.3) followed by an elution with acetone (10 ml) (nr.4). The diethylether concentrates were mixed with equal volumes of H_2O and evaporated up to 10 ml with N_2. The concentrates were tested in the Ames test as described in Methods

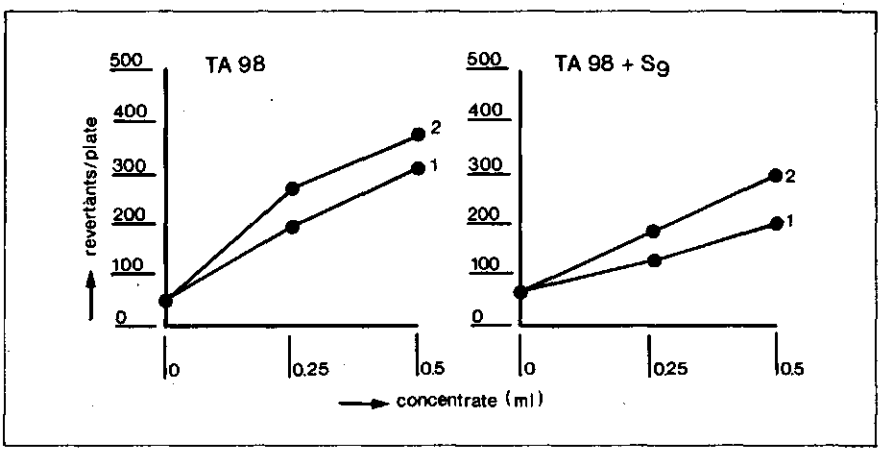

Figure 16. Effect of boiling on mutagenic activity in drinking water.

The sample, 50 litre drinking water (nr.1) and 50 litres boiled (60 seconds) drinking water (nr.2) were 7000-fold concentrated on XAD-4/8, elution with DMSO and assayed in the Ames test as described in Methods.

Table 10. The presence of haloforms in boiled drinking water.

Compound	Drinking water µg/l	Drinking water (Boiled) µg/l
Bromodichloromethane	20.8	0.01
Dibromochloromethane	18.6	nd
Dichloromethane	2.8	nd
Chloroform	12.6	0.3
Bromoform	3.4	nd

nd = not detectable

4.2.2. Fractionation

Another approach to obtain more information on the nature of the responsable mutagens is to apply thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) on concentrates of drinking water and look for possible fractionation of the activity. Figure 17 shows TLC results whereby six zones have been retested in the Ames test. The mutagenic activity is found predominantly in zone 2.

Experiments using gelfiltration on Sephadex LH20 with a DMSO concentrate of this drinking water indicate that these organic mutagens probably have a molecular weight in the order of 200 (figure 18).

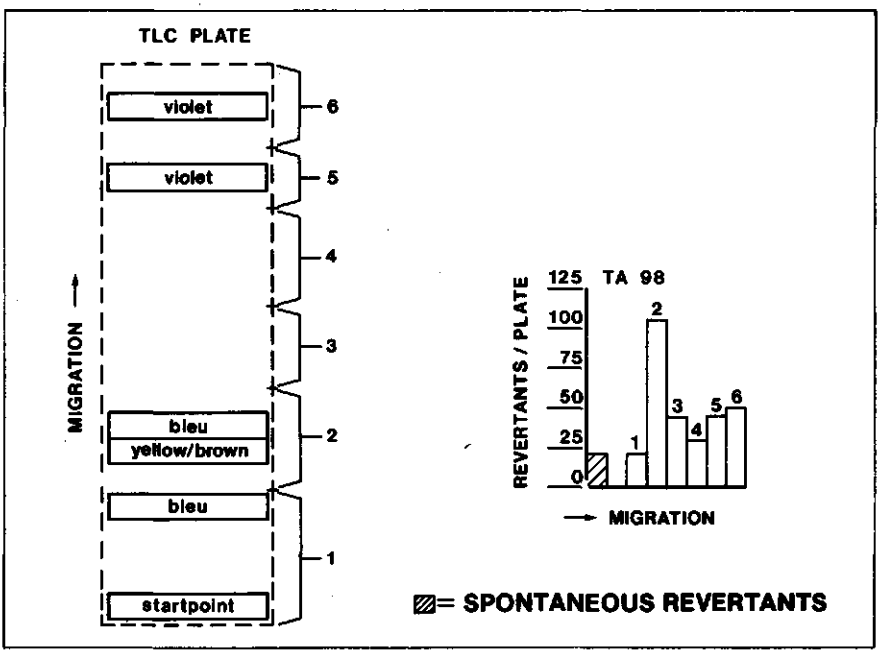

Figure 17. Fractionation of a mutagenic drinking water concentrate by thin layer chromatography.

The sampling, 90.000-fold concentration of drinking water on XAD-4/8, elution with acetone and subsequent TLC testing as described in Methods. After incubation the TLC plate was divided into six blocks and these blocks were scrapped off, eluted with 5 ml DMSO and assayed in the Ames test as described in Methods.

Additional fractionation and subfractionation is at present performed by means of repeated linear gradient HPLC-analysis (analytical and semipreparative) in combination with the Salmonella/microsome assay.

Results of this investigation (figure 19) namely showed that the mutagenic activity is found predominantly in two fractions. The results however, also showed that no good separation of the organic mutagens has occurred since the background due to the presence of many organics is much to high and consequently more fractionations steps have to be carried out. This is now under investigation. When this goal is reached, final identification of the organic mutagens present in these fractions is to be expected by means of mass-spectrometry.

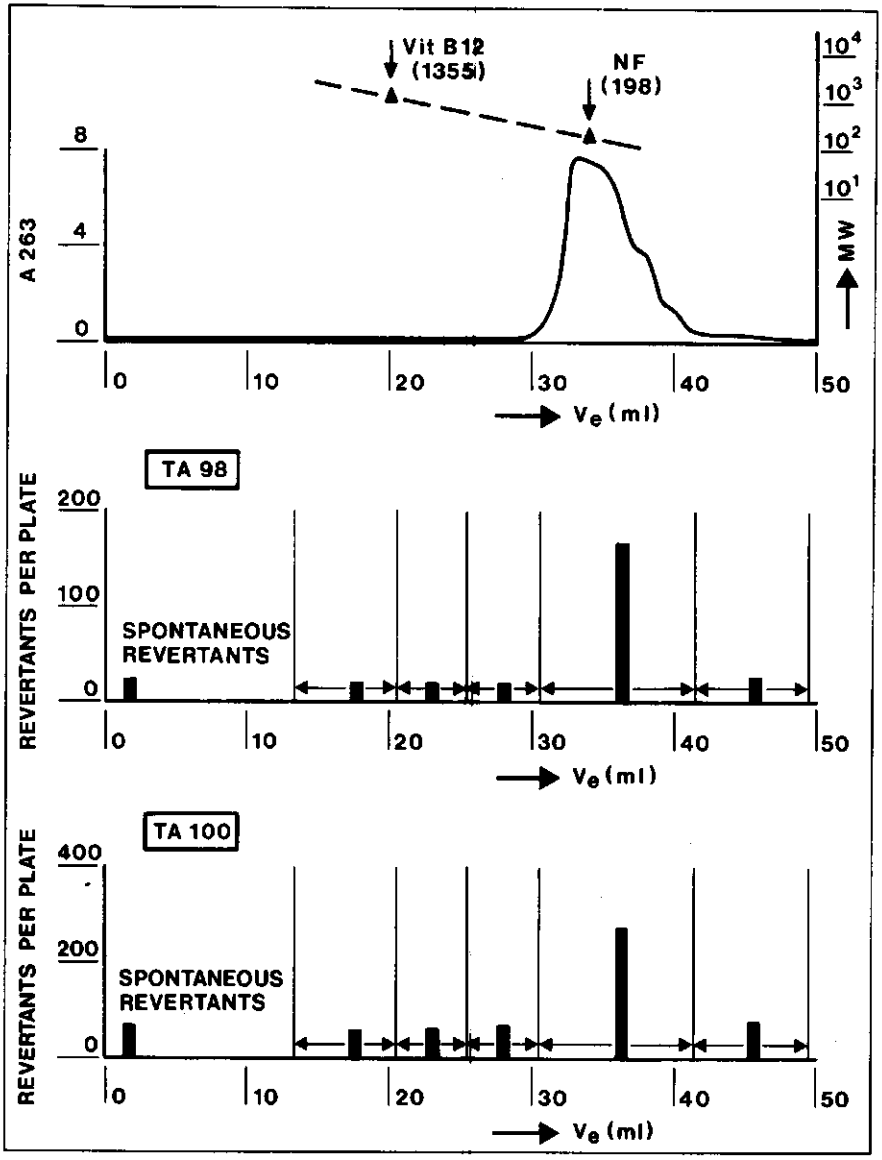

Figure 18. Gelfiltration of a drinking water concentrate with Sephadex LH20.

The sampling, 250.000 fold concentration of drinking water on XAD-4/8, elution with DMSO and subsequent gelfiltration was as described in Methods. After measuring the absorbance at 263 nm, the fractions were pooled and, after dilution in water, reconcentrated on XAD-4/8, eluted with 5 ml DMSO and assayed for mutagenic activity with TA 98 and TA 100.

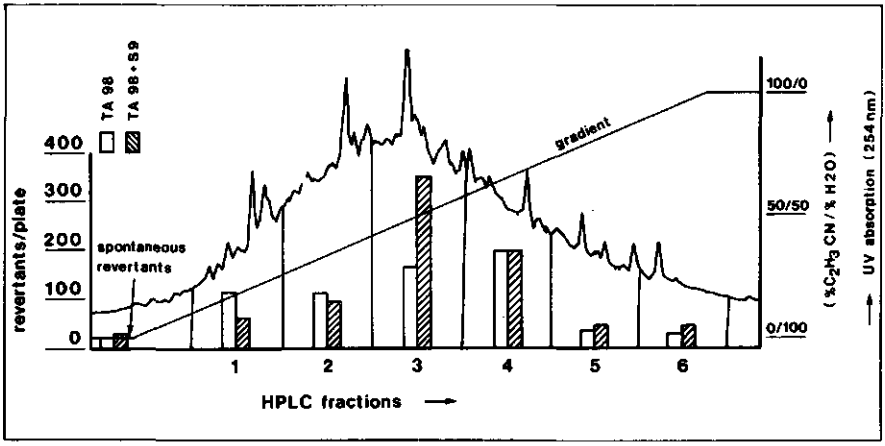

Figure 19. HPLC analysis of a mutagenic drinking water concentrate

On a bondapak C18 column 15 x 20 ul of a drinking water concentrate (12.5×10^5 fold concentrated) was separated using a H_2O-C_2H_3N gradient. Fractions were pooled as indicated and after reconcentration with ether extraction; assayed for mutagenic activity in the Ames test. Column: 30 cm, flow: 1 ml/min. (total: $\pm$ 1 h), detection: absorption at 254 nm.

5.

a carcinogenicity study with mutagenic drinking water concentrates

5.1. Introduction

The major part of drinking waters investigated for mutagenic activity (part. 3.2.2.) showed mutagenic activity in the Ames test. Organic drinking water concentrates of city 16 and city 18 which showed the highest number of revertants in the Ames test were also investigated in mammalian test systems for chromosome abberation, sister chromatide exchange and point mutation, but failed to give appropriate results because of the toxic properties of these concentrates towards these celsystems. Since the Ames test is considered as an useful screening test for predicting possible carcinogenicity of organic chemicals (McCann et al., 1975b, Purchase, 1982), only a long term study with mammals is accepted as a reliable testsystem for obtaining information about carcinogenic properties of compounds (Gezondheidsraad, 1978). Therefore this kind of study was carried out with mutagenic drinking water concentrates of city 18. This city was selected for its relatively high mutagenic response and its consistent mutagenic pattern obtained in the inventory study (figure 8). In this chapter results of this carcinogenicity study will be presented and discussed.

5.2. Results and discussion

Weights and mortality

In this study oral exposure to drinking water concentrates had no effect on the body weight of male and female Wistar SSP TOX rats (table 11).

Table 11. Average body weight of Wistar SSP TOX rats during exposure of drinking water concentrates

Treatment/ Exposure time (weeks)	Average weight ± standard deviation *						
	0	4	8	12	24	52	106
Females :							
group 1	131±15	179±19	207±20	221±23	239±24	268±31	277±35
group 2	133±14	180±13	210±16	227±18	247±20	282±28	277±43
group 3	127±11	185±16	213±20	231±21	251±24	282±34	297±40
group 4	121± 9	182±15	211±18	226±20	243±19	272±24	278±28
Males :							
group 1	163±27	245±27	310±28	335±29	368±29	404±33	373±35
group 2	167±29	246±29	312±33	340±33	374±34	416±39	383±57
group 3	160±25	256±33	321±33	349±34	384±36	420±45	376±49
group 4	163±23	252±29	318±27	345±30	378±28	411±33	388±46

* results expressed in grams

When the course of the mortality of the rats during the experiment is considered, it appeared that from week 29 till week 44, nine rats (3 males and 6 females) died. All animals showed trichobezoar in their stomach and blood in their feces. Their death was due to the high level of raw cellulose material (15%) because trichobezoar of this material was found in the stomach. Lowering the percentage of the cellulose material to 8.8 % stopped this cause of death, because rats dying after week 44, did not show these characteristic features. At the end of the experiment the mortality increased considerably in particular the females of group 4 (figure 20). All the exposed groups showed a somewhat higher mortality than the control, however no dose-effect relationship was observed. This effect is therefore, unlikely to be related to the levels of exposure.

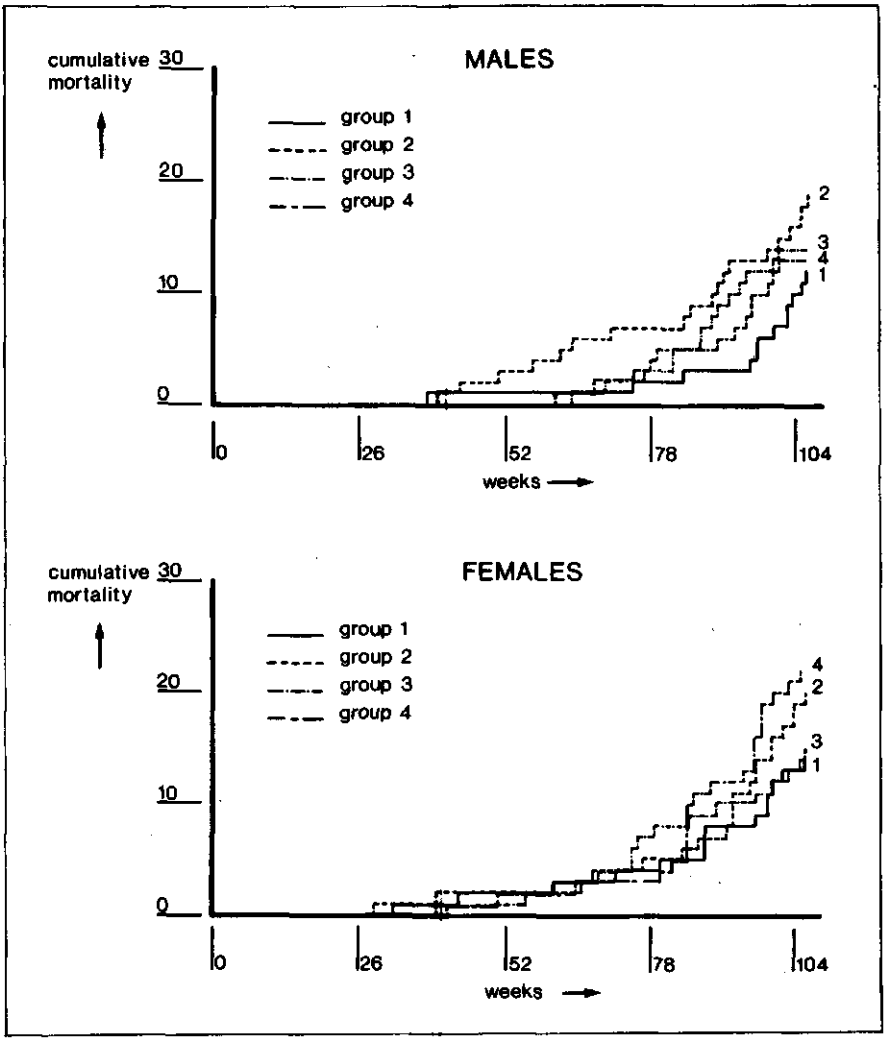

Figure 20. Cumulative number of dead or killed Wistar SSP TOX rats during exposure of drinking water concentrates.

Mutagenic activity and recovery of mutagenic activity from drinking water

During the experiment the mutagenic activity of concentrates prepared from drinking water of city 18 showed a similar pattern of activity as shown in figure 8, throughout the 2 years study. The mutagenic response (maximal number of revertants of a 0.5 ml

DMSO concentrate/spontaneous revertants) showed in general no much variation over the two years period with some exceptions of the direct mutagenic activity with strain TA 98. This value for TA 98 in general varied between 7-10 and only in a few cases a value of 4 and 14 were observed. Values for TA 98+S-9, TA 100 and TA 100+S-9 were respectively 4, 2-3 and 2.

In the study organic concentrates were prepared weekly and mixed with the drinking water of the rats.

Since the drinking water of the rats was refreshed every week, the mutagenic activity in the drinking water was regularly investigated to find out what amount of activity was still present after a week (figure 21).

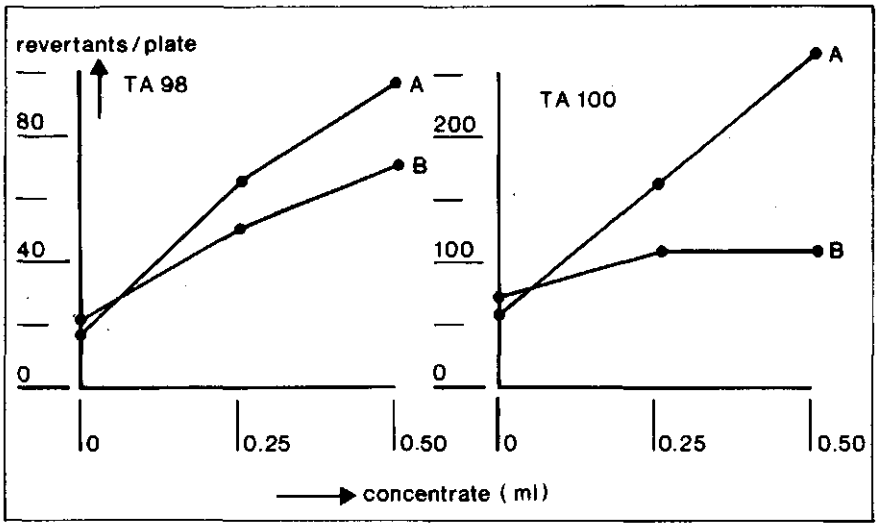

Figure 21. Recovery of mutagenic activity mixed with non mutagenic drinking water.

A sample of 50 ml DMSO drinking water concentrate (A) of city 18 was mixed with about 21 litres of drinking water of group 4. After a week the mutagenic activity in the drinking water left (5 litre) was concentrated on XAD-4/8, elution was carried out with DMSO (B) and both concentrates were tested in the Ames assay as described in Methods.

The results show (figure 21) that the direct mutagenic activity on strain TA 98 could be recovered for about 70 %, while hardly any direct activity with strain TA 100 could be recovered.

Many experiments showed similar results viz. 60%-90% recovery of the direct mutagenic activity with strain TA 98 and hardly any recovery of the direct activity with strain TA 100.
To find out whether the organic mutagens active on strain TA 100 will loose their activity after a week or cannot be reconcentrated on XAD-4/8, 50 ml of a DMSO drinking water concentrate was mixed with 5 litres of drinking water.
After a week the mutagenic activity was reconcentrated on XAD-4/8 and the XAD filtrate was freeze dried according to similar procedures as described in Materials and Methods. Both concentrates were assayed in the Ames test. No activity could be observed in the freeze dried concentrate. The recovery of the direct and promutagenic activity with strain TA 98 and TA 100 were 60 %, 60 % respectively 30 % and 60 % (not shown).
This result suggests that the organic mutagens active with strain TA 100 indeed loose some of their activity when they are mixed with this type of non mutagenic drinking water.

Actual dose levels

As described in Materials and Methods, the Wistar SSP TOX rats should be exposed to 10, 30 and 90 times the expected human exposure level. To obtain those levels, it was assumed and found in a preliminary experiment, that a rat of 250 grams consumed about 30 ml water per day. With this premiss, a fixed amount of DMSO drinking water concentrate per group was added to the drining water of the rats. However in the experiment it appeared that the animals became much heavier as expected and described previously (Kroes et al., 1976). The occurrence of the heavy weight may be due to the DMSO exposure of the rats in their drinking water, since in a preliminary experiment preceeding the carcinogenicity study this phenomenon also was observed, however only at a much high exposure level viz. 1% v/v (Kool unpublished data). The underlying mechanism however is unknown. In addition the water consumption of the rats was not 210 ml/week as expected but was on the average 160 ml/week.

Therefore the actual dose levels of the three treated groups were different from the expected ones and had to be recalculated (table 13).

Table 13. Actual dose levels of Wistar SSP TOX rats

Treatment	Actual dose level *			
	male		female	
group 1	0	(0)	0	(0)
group 2	4.5	(10)	7	(10)
group 3	14	(30)	22	(30)
group 4	40	(90)	68	(90)

* actual dose level based on measured average waterconsumption per rat per group per day and the average weight (kg) per rat per group

( ) dose level based on 120 ml water consumption per kg rat per day

The results in table 13, show that the actual dose levels of the females was somewhat lower than the expected levels, but the actual dose levels for the males were much lower approximately a factor 2 than the dose levels based on the assumed 120 ml water consumption per kg rat per day.

Effect of drinking water concentrates on the tumour incidence in Wistar SSP TOX rats

Table 14 shows the different tumour percentages which are obtained when Wistar SSP TOX rats are exposed to mutagenic drinking water concentrates through their drinking water.

Exposing Wistar SSP TOX male and female rats to mutagenic drinking water concentrates for 106 weeks, did not result in a significant increase ($p < 0.05$) in tumour induction.

From all tumour percentages shown in table 14, only the percentage of the malignant tumours in females showed a somewhat consistent pattern, because all treated groups show a higher tumour percentage than the control, although this increase in none of the treated groups was significant different ($p < 0.05$) from the control (group 1). The development and types of the individual tumours observed in the organs, in general were similar, although differences in the number of tumours among the groups did not show any relationship with the dose levels (see Appendix 2).
The relatively high percentage of tumours in the mamma and the pituitary (up to 50 %) was remarkable (see Appendix 2). These relatively high percentage of tumours in these organs is however characteristic for this Wistar strain (Kroes et al., 1976).
The tumour incidencies were analysed further according to the method of Peto (Peto et al., 1980). This type of statistical testing enables adjustment for differences in mortality. The analysis revealed that the differences between treated groups and controls were not significant ($p < 0.05$).
When the results of the carcinogenicity study are evaluated, it is clear that in this study mutagenic drinking water concentrates administered to Wistar SSP TOX male and female rats at a highest dose of 40 respectively 68 times the expected human exposure level, did not result in an elevated tumour induction. This result contrasts results obtained in a similar chronic test in which organic concentrates (chloroform extracts) prepared from drinking water of Paris were administered though their diet to 50 Spraque Dawley rats and CFLP Han mice (Truhaut et al., 1979). In that study indeed, an increase in tumour induction was observed in rats and mice exposed to 100 and 200 times the human dose expressed in mg/kg bw., whereby only in the rats a dose-response effect was seen. A specified list of tumours found in this experiment, however was not presented in the study.

Table 14. Tumour percentage in male and female Wistar SSP TOX rats treated with mutagenic drinking water concentrates

Treatment	Total Dose a)	% of animals with :					
	mg CM/kg bw.	tumours b)	single tumours b)	multiple tumours b)	benign tumours b)	malignant tumours b)	Number of animals
Males :							
Group 1	0	58	42	16	52	10	50
Group 2	11	48	40	8	40	10	47
Group 3	34	54	40	14	50	8	50
Group 4	97	68	58	10	58	12	50
Females :							
Group 1	0	74	50	24	72	10	49
Group 2	17	64	44	20	54	14	47
Group 3	53	70	48	22	64	16	47
Group 4	165	70	52	18	60	16	50

a) length of treatment 106 weeks

b) all percentages of the treated groups are not significant different from the control (group 1) ($p < 0.05$, Fisher's extact test, Siegel, 1956)

For the females the tumour incidence was much more pronounced than for males in particular in mice. In this French study however, the used human dose was relatively high (3 l waterconsumption/day at a human body weight of 60 kg). Also the concentration of the organic material (OM) in the chloroform extracts was on the average approximately two times higher per litre water compared to the present study. Consequently, the exposure of the animals to the organic material on a base of µg OM/kg bw/day in the present study was therefore 0.29 times the dose in the French study.
On the other hand one should bear in mind, first, that this study was set up to find out whether mutagenic drinking water concentrates are able to produce carcinogenic effects in rats and not just any given organic drinking water concentrate and second, that a comparison of the results with respect to a carcinogenic response in both studies on a base of µg OM/kg bw. may not be permitted, because of the different concentration procedures used which may result in a quite different organic composition of the organic concentrate.
Several other studies regarding, the carcinogenicity of drinking water organics have been carried out in which the mouse (skin) was exposed to drinking water concentrates. In somewhat older studies in the USA, chloroform-activated carbon extracts showed negative (Hueper and Ruchhoft, 1954) and positive results (Hueper and Payne, 1963). In the experiment with a positive result a total dose of 56 mg OM was subcutaneous (s.c.) tested. Using the same procedure, drinking water concentrates from two areas with respectively a high and low incidence of bladder cancer, did not induce more carcinogenic effect after s.c. injection in mice, than the control (Dunham et al., 1967). This negative result may however be due to the relatively low dose to which mice were exposed (total dose 5 mg OM). Applying another chloroform extraction procedure (Cabridenc and Sdika, 1975) organics in French drinking water indeed showed cancer (promoting) activity in a mouse skin test (Hémon et al., 1978). The positive results in three studies (Hueper and Payne, 1963; Hémon et al., 1978; Truhaut et al., 1979) out of five suggest that a chloroform extraction

procedures seems effective to concentrate those organic which may give a carcinogenic response, although in two studies (Hueper and Payne, 1963; Truhaut et al., 1979) still a matter of concern is the treatment of the control group because the control group in both studies did not receive a 'control' chloroform extract during the study (Kool et al., 1982a). Additional information whether these drinking water concentrates show mutagenic activity are unfortunately not available. That besides chloroform extracts, also mutagenic drinking water concentrates (Loper et al., 1979) obtained by reverse osmosis (RO) in combination with XAD (Kopfler et al., 1977) may give a positive result in a mouse skin test, in this case a mouse skin initiation/promotion test, was demonstrated in a recent study in the USA (Robinson et al., 1980). Drinking water concentrates of two cities out of five (one RO- and one XAD-concentrate) increased the number of pappilomas in the presence of the promotor phorbol myristate acetate proving iniating properties.
Tumour promoting potential of all ten samples (5 XAD and 5 RO-concentrates) was also tested and found to be negative. All samples failed to be complete carcinogens at a total dose up to 30 mg OM/mouse. This result however is not valid since the duration of the experiment viz. 38 weeks is certainly not long enough to test for complete carcinogenicity.

Hence, the results in this carcinogenicity study show that mutagenic drinking water concentrates to which rats were exposed did not induce carcinogenic effects at the doses used. This result indicated that these mutagenic drinking water concentrates did not contain very potent carcinogens in effective concentrations for instance compounds like N-Nitrosodiethyl-or dimethylamine, because when this kind of compounds should be present, the carcinogenicity study should be positive at the dose levels used (IARC, 1978). On the other hand weak carcinogens cannot be traced at the doses tested, thus the presence of this kind of carcinogens still cannot be excluded.

What significance has this negative carcinogenicity study for this type of drinking water. One of the problems in this respect is the fact that the organic mutagens concentrated only represents a small part, in the order of 1 %, of the total organic material present in drinking water, and therefore it cannot be excluded that the organic constituents left in drinking water may influence the results obtained in this present study.

However it is not unrealistic to assume that the mutagenic drinking water concentrates (XAD concentrates) contain most organic mutagens/carcinogens, since most organic mutagens and carcinogens tend to be non-polar (Yamasaki and Ames, 1977) and the XAD procedure is well known to concentrate these less polar compounds (Webb, 1975). If further is assumed that only the organics present in the mutagenic drinking water concentrate will contribute in carcinogenicity and an extrapolation of data from animals to man is allowed, than the negative results in this carcinogenicity study in which the highest group received 68 times the expected human exposure level, may be used to estimate a risk for people who consume this drinking water. Statistically, the absence of cancer in a group of 100 test animals means only that at a 99 % confidence level the true incidence is less than 5 % (Hoel et al., 1975). At 99 % confidence level the true incidence in this experiment with a 'natural' incidence of malignant tumours of 10% is less than 22×10^{-2} for females (Hoel et al., 1975).

In this study no malignant tumours were induced at a dose of 68 times the expected human exposure level. By linear extrapolation (Weinhouse, 1977) one can estimate that the risk of cancer at the expected human exposure level is less than $1/68 \times 0.22$, thus for the population of this city (1.1×10^5) this would imply $1/68 \times 0.22 \times 1.1 \times 10^5 = 356$, so the negative results in this experiment tells us, that less than 356 people might be at risk. On this basis the contribution of drinking water, if there is a contribution at all, should be relatively small viz. less than 1.1 % in comparison to the expected tumour incidence of 33.000. The latter value is based on an average tumour incidence of 30 per 100, due to background processes (Gezondheidsraad, 1978).

To improve however, the reliability of a risk estimation for people who consume polluted drinking water, organic concentrates of drinking water which have not been included in the present carcinogenicity study, should in combination with higher dose of concentrates of the organic mutagens as used in this study be examined for complete carcinogenicity.
Besides the examination for complete carcinogenicity, these organic concentrates should be tested for promotion and/or initiation activity and identification of the organics responsable for these effects should also be carried out.

6.
conclusions

- During an inventory study in which drinking water of 18 cities in The Netherlands was investigated for mutagenic activity, it appeared that in drinking water prepared from surface water, groundwater and a mixture of both, mutagenic activity (Ames test) was detectable in 0.5 to 3 litres of drinking water.
- The use of groundwater as a source of drinking water has to be preferred over other water sources, because from this study it appeared that only one city out of five which prepare their drinking water from this source showed significant mutagenic activity (Ames test). Six cities using bankfiltrated river water and four out of six cities using surface water as a drinking water source showed significant mutagenic activity.
- Application of a chlorine treatment will in general increase the mutagenic activity (Ames test) significantly.
- The use of an ozone treatment in this study showed a reduction of mutagenic activity with Salmonella strain TA 98. In a few other drinking water studies however, a slight increase in activity with Salmonella strain TA 100 after this treatment was observed. These results indicate that ozone is able to reduce and increase mutagenic activity.
- Dune filtration and carbon filtration (GAC) are able to reduce the mutagenic activity to a great extent.
- The results in this study show that a combination of different treatment steps remove the organic mutagens originating from the raw water source and oxidation processes during drinking water preparation.

- From the chemical parameters measured in drinking water the level of AOCl showed the best correlation with mutagenic activity in drinking water concentrates. Therefore this parameter should be considered for the time being as an indicator for mutagenic activity in drinking water.
- The organic mutagens in drinking water responsable for mutagenic activity in the Ames assay, are mainly observed in the slightly polar non volatile fraction. It is very unlikely that these compounds are the same organic compounds already identified in this drinking water with GC/MS. The fraction active with strain TA 98 has a molecular weight in the order of 200.
- The carcinogenicity study in which male and female Wistar SSP TOX rats were exposed to mutagenic drinking water concentrates with a maximum dose level of 68 times the expected human exposure level showed that the number of tumours in the exposed groups did not significantly exceed ($p < 0.05$) the number in the control groups.
  It appeared that the number of animals with tumours and the animals which died of tumours in the exposed groups, were not significantly higher ($p < 0.05$) than the number in the control groups.
- The negative results in the carcinogenicity study suggests that mutagenic drinking water concentrates do not contain effective concentrations of very potent carcinogens. The presence of weak carcinogens however, cannot be excluded.

summary

Several mutagenic and carcinogenic organic compounds have been detected in Dutch surface waters and in drinking water prepared from these surface waters. Although the levels of these compounds in drinking- and surface water are relatively low, in general below µg per litre, it appeared that organic concentrates tested in the Ames/microsome assay, showed mutagenic activity in 50 ml surface- and 500 ml drinking water.

Such a result however was not expected based on the concentration of organic mutagens identified in these waters. Therefore the conclusion had been drawn that a number of unknown organic mutagens in combination with the identified mutagens were responsable for the level of mutagenic activity. With this in mind, an extensive investigation was carried out in an attempt to answer the following questions :

- do drinking waters prepared from surface water, groundwater or a mixture of both show mutagenic activity;
- do different water treatment processes during the preparation of drinking water influence the mutagenic activity;
- what kind of physical-chemical properties have the organic compounds in drinking water concentrates showing mutagenic activity in the Ames test;
- do mutagenic organic concentrates prepared from drinking water show carcinogenic properties ?

Against this background an inventory study was made on the presence of mutagenic activity in drinking water of 18 cities in The Netherlands.

Besides this study, the influence of different water treatment processes on the mutagenic activity was examined in a number of water works.

Furthermore, an attempt was made to characterize the organic compounds which are responsable for the mutagenic activity. Finally, a carcinogenicity study was carried out to see whether mutagenic drinking water concentrates induce carcinogenic effects in rats.

Chapter 1, describes the aim of this investigation and the factors which have led to the present concern regarding the possible toxic effects of organic (micro)pollutants in drinking water in The Netherlands.
Furthermore a summary is given of the literature on the identification of individual compounds and some data are given of the mutagenic and (suspect) carcinogenic organic compounds which are detected in surface- and drinking water in The Netherlands.

In Chapter 2, materials and methods are described which were used for the inventory study on the presence of mutagenic activity in drinking water of 18 cities (Chapter 3), the characterization of organic mutagens (Chapter 4) and for a carcinogenicity study with mutagenic drinking water concentrates (Chapter 5).
This chapter describes the XAD resins which have been used for concentrating organic mutagens from water, the concentration procedures with the aid of XAD resins and freeze drying, a mutagenicity assay viz. the Ames Salmonella/microsome assay (Ames test). The methods for chemical analysis which are used in the 18 city survey, fractionating techniques like thinlayer chromatography (TLC), high performance liquid chromatography (HPLC) and gelfiltration are described. The treatment, dose levels and experimental design and conduct which are used in a carcinogenicity study are explained. Finally, statistical procedures which have been used for analyzing tumour incidence in the carcinogenicity experiment and relating chemical parameters in drinking water to mutagenic activity in organic concentrates prepared from these drinking waters are described.

In Chapter 3, the need for concentrating organic mutagens is explained. Mutagenicity results (Ames test) of five surface waters in The Netherlands are presented, in which the organic mutagens are concentrated with the XAD procedures. Depending on the concentration factor applied (10^3-4.10^3) it appeared that in all five surface waters mutagenic activity could be detected. The river Rhine showed the highest mutagenic activity viz. a doubling of revertants in 50 ml water.

To be certain that the XAD procedure is a selective concentration method for organic mutagens, this procedure was compared with a freeze drying technique. The results of this comparative investigation showed that the XAD procedure is a reliable method for concentrating organic mutagens from surface water. On the basis of the surface water results, the XAD procedure was also applied for concentrating organic mutagens from drinking water. It was found that drinking water of four out of six cities showed mutagenic activity in volumes varying from 0.5 to 3 litre.
To see whether the mutagenic results obtained in the six cities are representive for drinking water in The Netherlands, an extensive inventory study on the presence of mutagenic activity in several types of drinking water was carried out. In this study eighteen cities (twenty drinking waters) were investigated three times for the presence of mutagenic activity and the following chemical parameters : AOCl, EOCl, THM, VOCl, TOC, Tot. N, over a period of 2 years. The chemical parameters were measured to see whether a relationship could be found between one or more of these parameters and mutagenic activity.
The selection of the drinking water was based on the water source (ground water, surface water) the storage facility (dune filtration, bankfiltration, storage reservoir) and the application of a chlorine treatment during the preparation of drinking water. The results of this study showed that in fourteen of the twenty drinking waters, mutagenic activity could be detected in volumes varying from 0.5 to 3 litre. When the cities were classified according to their water source, storage facility and type of treatment, it appeared that only three of the fifteen cities

which prepare their drinking water from surface water or a mixture of surface- and groundwater, did not show mutagenic activity. Two of the five cities which use groundwater as a drinking water source showed mutagenic activity although in one city the activity was marginal.

Correlating the chemical parameters with the mutagenic activity, it was demonstrated that AOCl showed the highest correlation with the direct mutagenic activity in strain TA 98 and TA 100. It was also shown that a chlorine treatment applied during drinking water preparation correlated well with the direct mutagenic activity in strain TA 98 and TA 100. In addition, chemical parameters which showed a significant difference ($p < 0.01$) in concentration between mutagenic and non mutagenic samples (Mann-Whitney) and a significant correlation ($p < 0.01$, Kendall tau) with mutagenic activity were used for straight curve fitting. From these fitted regressions, concentration levels were calculated above which a doubling of revertants may be expected.

In the second part of Chapter 3 (3.4) the influence of different treatment processes on the mutagenic activity and some chemical parameters were investigated in three waterworks. Application of a chlorine treatment, generally increased the direct and promutagenic activity, but the amount of increase proved to be dependent on the type of water chlorinated. The use of ozone in the preparation of drinking water decreased the mutagenic activity in the water. The amount of reduction was dependent on the type of water ozonated. Dune filtration greatly reduced the mutagenic activity.

Slow sand filtration could not be evaluated, because of the toxicity of the organic concentrates for the bacterial strains. Filtration over active carbon filters which operated for about 1 year, reduced the mutagenic activity below the detection level. Carbon filters which operated more than 1.5 years in a pilot plant, showed a break-through of mutagenic activity. This result suggests that carbon filters are able to remove organic mutagens only for a short time.

From the results of the chemical parameters before and after the different treatment processes it appeared that the level of AOCl behaved very similar with the mutagenic activity in the neutral fraction. These results confirm those obtained in the eighteen city survey and support the idea that AOCl might be a useful indicator for mutagenic activity in drinking water.

The physical-chemical characterization of organic mutagens in drinking water concentrates is described in Chapter 4.
The first approach was to examine the influence of the pH on the adsorption behaviour of organic mutagens on the XAD resins. Lowering the original pH of drinking water with HCl to pH = 2-3, revealed the presence of another class of organic mutagens the so-called acid fraction which hardly adsorb on the XAD resins at pH = 7.5.
Further it was investigated whether different organic solvents are able to eluate the organic mutagens from the XAD column.
It was found that diethylether only eluted a minor part of the mutagenic activity from the XAD resins and subsequent elution with acetone eluted the major part of the activity.
This result showed that it is not likely that the organic mutagens in the acetone fraction are identical with the already identified organics in the ether fraction in this drinking water.
Furthermore it appeared that the organic mutagens are not only less volatile but also resistent to boiling. Another approach to characterize the organic mutagens was to apply fractionation techniques. Using TLC and HPLC, it was found with HPLC analysis that the mutagenic activity was present predominantly in two fractions. Finally, gelfiltration on Sephadex LH20, showed that organic mutagens which demonstrated mutagenic activity with strain TA 98, had a molecular weight in the order of 200.

Chapter 5, presents the results of a carcinogenicity study, in which Wistar SSP TOX rats were exposed to mutagenic drinking water concentrates of one city in The Netherlands. Drinking water concentrates were prepared every week using the XAD concentration

procedure and the organic concentrates (DMSO) were mixed with the non mutagenic drinking water of the Wistar SSP TOX rats.
Dosage levels were based on multiples of expected human exposure levels. In the calculation the average human exposure was assumed to be approximately 29 ml/kg bw./day. Furthermore it was assumed that a rat of 250 grams would consume about 30 ml water per day. In the experiment rats were divided into four groups (50 males and 50 females per group), a control group and groups which received respectively 10, 30 and 90 times the human exposure level in their drinking water.
During the experiment (106 weeks) the water consumption of the rats was measured weekly. Body weights were recorded weekly in the first two months of the experiment and once a month thereafter.
The mutagenic activity of concentrates which were mixed with non mutagenic drinking water were measured after a week. It was found that the mutagenic activity with strain TA 98 could be recovered for 60-90%, while the TA 100 activity hardly could be recovered. The latter was explained by the observed partial decrease of TA 100 activity after mixing the mutagenic drinking water concentrate with drinking water.
During the experiment it appeared that the assumed water consumption of 30 ml per day per rat, on which the dose level was based, not was reached.
Moreover, it appeared that Wistar SSP TOX rats became much heavier than was expected and therefore the actual dose levels in ml/kg bw. of the three exposed groups were not 10, 30 and 90 times the expected human exposure levels, but 4.5, 14 and 40 for male rats respectively and 7, 22 and 68 for female rats. Exposing Wistar SSP TOX rats to these dose levels for 106 weeks, did not result in a significant increase ($p < 0.05$) in tumour induction.
Furthermore it was shown, that the development and types of tumours were similar in the treated and control groups. The number of animals with tumours and the animals which died of tumours in the exposed groups was not significantly different ($p < 0.05$) from the control group. The negative results in this carcinogenicity study indicate that mutagenic drinking water concentrates did

not contain very potent carcinogens in effective concentrations like for instance N-Nitrosodiethylamine because when these kind of compounds are present, the carcinogenicity should be positive at the dose levels tested.

On the other hand one cannot exclude the presence of weak carcinogens in the mutagenic concentrates, because one cannot detect a carcinogenic effect at the dosis tested.

Based on the carcinogenicity results, an estimation of a risk factor was made for the people who consumed this mutagenic drinking water.

For the estimation the following three assumptions had been made:

- when organic carcinogens had been present in drinking water, they were present solely in the mutagenic drinking water concentrates
- results obtained in the present carcinogenicity study with rats may be extrapolated to man
- linear extrapolation of high dosis to low dosis give correct results.

When all these assumption are correct it appeared after statistical analysis that less than 356 of 110.000 people might be at risk. On this basis the contribution of drinking water, if there is a contribution at all, seems relatively small viz. less than 1.1 % in comparison to the expected tumour incidence of 33.000. The latter value is based on an average tumour incidence of 30 per 100, due to background processes.

To improve, however the reliability of an estimation of the risk factor for people who consume polluted drinking water, organic concentratres of drinking water which have not been included in the present carcinogenicity study should be examined for complete carcinogenicity in combination with higher doses of concentrates of organic mutagens as used in this study.

Besides this investigation it is recommended that both organic concentrates will be tested for tumour promotion and initiation activity as well as identification of the organic responsable for these effects should receive priority.

Finally Chapter 6 describes a number of conclusions emerging from this investigation. The data presented show that mutagenic activity was detectable in drinking water of many cities in The Netherlands.

The number of cities which showed mutagenic activity in their drinking water was the least where groundwater was used as a drinking water source. Therefore it is recommended that groundwater is preferable over other sources of drinking water and the quality of the groundwater should be protected very carefully so that oxidation and disinfection with a chlorine treatment will not be necessary. This recommendation is not only based on the eighteen city survey but also from data obtained in waterworks which prepare their drinking water mainly from surface water, because mutagenic activity is significantly increased by a chlorine treatment which may often result in mutagenic activity in the end product. It is recommended further that the organic mutagens should be identified in order to evalute them with respect to their possible toxic properties. Also higher dosis of mutagenic drinking water concentrates in combination with concentrates prepared from the rest of the organics in drinking water, should be tested for carcinogenicity and chronic toxicity for a more reliable risk estimation. Besides, this investigation it is recommended that both organic concentrates should be investigated for tumour promotion and initiation activity.

samenvatting

Diverse mutagene en carcinogene organische verbindingen zijn in het Nederlandse oppervlaktewater aangetoond evenals in drinkwater bereid uit dit oppervlaktewater. Hoewel de niveaus van deze type stoffen in drink- en oppervlaktewater relatief laag zijn, in het algemeen beneden de µg per liter, bleek, dat indien organische concentraten bereid uit drink- en oppervlaktewater werden getest in de Ames assay, mutagene aktiviteit kon worden aangetoond in 50 ml oppervlaktewater en 500 ml drinkwater.

Een dergelijke bevinding is echter op grond van de aangetoonde concentraties bekende mutagene componenten niet te verwachten. De conclusie is dan ook getrokken dat tal van nog onbekende organische componenten tesamen met de reeds geidentificeerde stoffen dit effect veroorzaken. Op grond van bovenstaande conclusie is dan ook een uitgebreid onderzoek verricht waarbij het onderzoek zo mogelijk een antwoord moest geven op de volgende vragen:

- is in de diversen typen Nederlands drinkwater mutageniteit aantoonbaar;
- wat is de invloed van diverse zuiveringsprocessen op de mutagene aktiviteit;
- wat voor fysisch-chemische eigenschappen hebben de organische stoffen in drinkwaterconcentraten die een mutagene response in de Ames test geven;
- vertonen mutagene drinkwater concentraten carcinogene eigenschappen?

Tegen deze achtergrond werd een inventarisatie onderzoek uitgevoerd naar de aanwezigheid van mutagene aktiviteit in diverse typen drinkwater. Hierbij werd het drinkwater van 18 Nederlandse steden onderzocht. Bovendien werd in een aantal waterleidingbedrijven het verloop van de mutagene aktiviteit gedurende diverse waterbehandelingsprocessen bekeken. Daarnaast werd getracht m.b.v. adsorptie/elutie en diverse scheidingstechnieken, de organische stoffen verantwoordelijk voor de mutagene aktiviteit nader te karakteriseren. Tenslotte werd m.b.v. een dierproef nagegaan in hoeverre mutagene drinkwaterconcentraten een verhoogde tumorinductie geven bij ratten.

Hoofdstuk 1 geeft een beschrijving van het doel van het onderzoek en de belangrijkste factoren die tot het onderzoek aanleiding hebben gegeven alsmede een overzicht wat voor soort organische stoffen in het water worden aangetroffen en welke groepen van stoffen het meest bestudeerd zijn. Tevens wordt een korte samenvatting gegeven over de identificatie van individuele componenten welke met de moderne analyse-apparatuur zijn aangetoond. Daarnaast wordt er een beknopt overzicht gegeven van mutagene en carcinogene organische verbindingen die zowel in Nederlands oppervlakte- als in drinkwater in de jaren zeventig zijn aangetoond.

In hoofdstuk 2 worden de materialen en methoden beschreven die voor het inventarisatie onderzoek van de 18 steden (Hfdst. 3), het karakteriseren van organische mutagene stoffen (Hfdst. 4), en een carcinogeniteitsstudie met mutagene organische drinkwater concentraten (Hfdst.5) zijn gebruikt.
In dit hoofdstuk zijn de XAD-harsen beschreven die nodig zijn voor het concentreren van organische stoffen uit water, de concentratieprocedure m.b.v. XAD-harsen en vriesdrogen, één mutageniteitstest nl. de Ames Salmonella/microsomale test (Ames test), methoden van chemische analyses die o.a. in het 18 steden onderzoek zijn uitgevoerd, fractioneringstechnieken zoals dunnelaagchromatografie, vloeistofchromatografie, gelfiltratie, de wijze waarop een carcinogeniteitsexperiment met drinkwaterconcentraten is uitgevoerd en tenslotte de statistische procedures welke zijn gebruikt voor het relateren van chemische parameters in drinkwater aan mutagene aktiviteit in drinkwaterconcentraten en het bepalen van de tumorincidenties bij de ratten in het carcinogeniteitsexperiment.

In hoofdstuk 3 wordt in de eerste plaats ingegaan op de noodzaak een concentratietechniek toe te passen om organische mutagenen in de Ames test te kunnen detecteren. Vervolgens worden de mutageniteits (Ames test) resultaten gepresenteerd van een vijftal oppervlaktewateren in Nederland waarin de organische stoffen m.b.v. een XAD procedure uit het water zijn geconcentreerd.

Afhankelijk van de gebruikte concentratiefactor (10^3-4.10^3) bleek dat alle onderzochte wateren mutagene aktiviteit vertoonden, waarbij de rivier de Rijn de hoogste aktiviteit bezat, nl. een verdubbeling van het aantal revertanten in ca. 50 ml water.
Om er zeker van te zijn dat de XAD-procedure voldoende selectief is in het concentreren van organische mutagenen uit water, zijn experimenten uitgevoerd waarbij deze procedure is vergeleken met een vriesdroog procedure. Uit dit onderzoek bleek de XAD-procedure een betrouwbare methode te zijn voor het concentreren van organische mutagenen.
Op grond van deze resultaten is deze procedure eveneens uitgeprobeerd op drinkwater van een zestal steden, die hun drinkwater bereiden uit oppervlaktewater. Uit dit onderzoek bleek, dat in 4 van de 6 steden mutagene aktiviteit in volumina van 0,5 liter tot 3 liter kon worden aangetoond. Om de selectiviteit van de XAD-procedure voor het drinkwater na te gaan, werd deze procedure eveneens vergeleken met de reeds eerder toegepaste vriesdroogmethode. Ook hier bleek dat beide concentratiemethoden globaal eenzelfde resultaat vertoonden.
Teneinde na te gaan of het eerdere verkregen beeld m.b.t. mutageniteit in drinkwater juist was, werd een uitgebreid inventarisatie onderzoek naar de aanwezigheid van mutageniteit in diverse typen drinkwater opgezet. Bij dit onderzoek waren 18 steden (20 drinkwatersoorten) betrokken en deze werden in een periode van ca 2 jaar driemaal onderzocht op mutagene aktiviteit en enkele chemische groepsparameters. De laatsten werden gemeten om na te gaan of één van deze parameters een duidelijke relatie vertoonde met de mutagene aktiviteit. Indien dit het geval was zou deze parameter als indicator van mutagene aktiviteit dienst kunnen doen.
De selectie van de verschillende soorten drinkwater was o.a. gebaseerd op de ruwwaterbronnen nl. grond- en oppervlaktewater, de zuiveringsprocessen, nl. duinfiltratie, oeverinfiltratie en het al of niet toepassen van een chloorbehandeling bij de bereiding van drinkwater.
Van de 20 onderzochte soorten drinkwater bleek na concentratie in 14 watertypen mutagene aktiviteit aantoonbaar in volumina van 0,5

tot 3 liter. Verder kwam uit het onderzoek naar voren dat slechts in 3 van de 15 onderzochte typen drinkwateren die bereid water uit oppervlakte- of een mengsel van grond- en oppervlaktewater, geen mutagene aktiviteit aantoonbaar was. Van 5 steden die hun drinkwater bereiden uit grondwater bleek dat in 3 steden geen aktiviteit kon worden aangetoond. Van de 2 positieve steden was er bij één stad sprake van marginale aktiviteit. Bij het correleren van de chemische groepsparameters met de mutagene aktiviteit in drinkwaterconcentraten werd duidelijk dat AOCl de hoogste correlatie gaf met de direct mutagene aktiviteit in TA 98 en TA 100 na toetsing volgens Mann-Whitney en Kendall tau.
Op grond van deze gegevens werd een lineaire regressie uitgevoerd, Uit deze regressies werden concentraties berekend van de relevante groepsparameters welke aangeven dat de kans aanmerkelijk groter wordt om mutagene aktiviteit in drinkwater aan te treffen, indien concentraties van de betrokken parameters boven een bepaald niveau in het drinkwater aanwezig zijn. Tenslotte werd nog aangetoond, dat een chloorbehandeling toegepast bij de bereiding van het drinkwater significant positief correleerde ($p< 0.01$) met de directe mutagene aktiviteit in stam TA 98 en stam TA 100.
In het tweede gedeelte van hoofdstuk 3 (3.4) werd de invloed van verschillende waterbehandelingsprocessen op zowel de mutagene aktiviteit als chemische parameters in een drietal waterleidingbedrijven nagegaan. Toepassing van een chloorbehandeling gaf in het algemeen zowel een verhoging van de directe als promutagene aktiviteit te zien, de toename bleek echter afhankelijk van het type water. Het gebruik van ozon tijdens de bereiding van drinkwater, verlaagde in het algemeen de mutagene aktiviteit in het water maar ook hier bleek de mate van reductie afhankelijk van het type water. Bij het beoordelen van diverse filtratieprocessen m.b.t. de mutageniteit, bleek dat een duinfiltratie de aktiviteit aanmerkelijk reduceerde. De filtratie d.m.v. langzame zandfiltratie kon helaas niet worden geëvalueerd vanwege de toxiciteit van het concentraat voor de test stammen. Filtratie m.b.v. aktief koolfilters die ca. 1 jaar in bedrijf waren, gaf aan dat de mutagene aktiviteit in het water na deze stap niet meer aantoonbaar was.

Koolfilters die daarentegen in een 'pilot-plant' studie langer dan 1,5 jaar in bedrijf waren vertoonden doorslag van mutagene aktiviteit in de "zure" fractie en geen volledige verwijdering in de neutrale fractie. Dit suggereert dat koolfilters slechts voor een beperkte duur de mutagene aktiviteit kunnen verwijderen.
Uit de analyse van de chemische parameters bleek dat evenals in het 18 steden onderzoek, AOCl zich geheel in overeenstemming gedroeg met de mutagene aktiviteit in de neutrale fractie, zodat de eerdere aanwijzing, dat AOCl als indicator voor mutagene aktiviteit dienst kan doen werd versterkt.

De karakterisering van de organische mutagenen in drinkwaterconcentraten is in hoofdstuk 4 beschouwd.
In de eerste plaats werd nagegaan in hoeverre de adsorptie van organische mutagenen wordt beinvloed door de pH. Door de natuurlijke pH van het drinkwater te verlagen met HCl tot pH=2-3, werd een andere klasse van organische mutagenen aangetoond, welke bij pH=7,5 niet of nauwelijks adsorberen aan de XAD harsen. Bovendien werd de op de XAD geadsorbeerde organische stof met verschillende organische oplosmiddelen geëlueerd. Hierbij kwam vast te staan, dat ether slechts in beperkte mate in staat was de mutagene aktiviteit te elueren. Op grond van deze resultaten is dan ook nauwelijks aannemelijk, dat de organische verbindingen met mutagene eigenschappen, dezelfde verbindingen zijn die reeds eerder m.b.v. GC/MS geidentificeerd zijn in een dergelijke ether fractie. De organische mutagenen bleken nogal persistent te zijn en niet erg vluchtig, daar de aktiviteit na het koken van water niet afnam doch eerder leek toe te nemen. Naast de beschreven experimenten is getracht de organische mutagenen nader te karakteriseren d.m.v. fractioneringstechnieken. Door het toepassen van dunnelaagchromatografie en vloeistofchromatografie is een eerste stap gezet om de fraktie met mutagene aktiviteit te scheiden van de rest van de organische stoffen.

Daarbij bleek dat de mutagene aktiviteit na HPLC fractionering voornamelijk in twee blokken kon worden aangetroffen. Verdere scheiding en zuivering van de mutagene componenten is noodzakelijk om uiteindelijk de componenten m.b.v. massaspectrometrie te identificeren. Tenslotte werd door middel van gelfiltratie inzicht verkregen wat betreft de grootte van het molecuulgewicht van een fractie die actief is in stam TA 98. Door het toepassen van een tweetal markers werd na gelfiltratie van mutagene drinkwaterconcentraten de aktiviteit in stam TA 98 aangetoond in een fractie met een molecuulgewicht in de orde van grootte van 200.

In hoofdstuk 5 wordt verslag gedaan van een carcinogeniteitsexperiment met mutagene organische concentraten afkomstig van één type drinkwater. De concentraten werden wekelijks aangemaakt m.b.v. de XAD-procedure en via het drinkwater aan Wistar SSP TOX ratten aangeboden. De dosis op ml/kg basis die de ratten werden toegediend waren 0, 10, 30 en 90 keer de hoeveelheid waaraan een 'standaard' mens wordt blootgesteld. Voor de berekening van de dosering werd voor de 'standaard' mens van een gemiddeld gewicht van 70 kg en 2 liter drinkwaterconsumptie per dag uitgegaan. Bovendien werd aangenomen dat een rat van ca 250 gram ca 30 ml drinkwater per dag consumeert.
De opzet van de proef was zodanig, dat 400 ratten (200 vrouwelijke en 200 mannelijke ratten) werden verdeeld in 4 groepen van 100 (50 vrouwtjes, 50 mannetjes) waarbij per kooi 5 ratten van gelijke sexe werden gehuisvest. Groep 1 was de controlegroep, groep 2 ontving 10 x; groep 3, 30 x en groep 4, 90 x de berekende hoeveelheid in hun drinkwater.
Gedurende het experiment (106 weken) werd wekelijks de waterconsumptie gemeten alsmede de mutagene aktiviteit van het drinkwaterconcentraat.
Maandelijks werden de gewichten van de dieren bepaald m.u.v. de eerste twee maanden waarin wekelijks werd gewogen. Zeer regelmatig werd de mutagene aktiviteit die met het drinkwater van de ratten werd gemengd na een week getest. Hierbij bleek, dat de aktiviteit met de stam TA 98 voor 60-90% kon worden teruggevonden terwijl de

aktiviteit met TA 100 nauwelijks werd teruggevonden. Het laatste werd verklaard door de gevonden gedeeltelijke afname aan TA 100 aktiviteit die na menging met het drinkwater optrad.
Tijdens het experiment bleek, dat de waterconsumptie van ca 30 ml per dag per rat, waarop de berekening van de dosering was gebaseerd niet werd gehaald. Bovendien bleek dat de ratten aanzienlijk zwaarder werden dan was voorzien. Dit had tot gevolg dat de expositie in ml/kg slechts in de eerste 5 maanden bij de vrouwelijke ratten overeenkwam met de berekende dosering, maar in de resterende periode lager uitkwam. Het laatste gold evenzeer voor de mannelijke ratten echter met dien verstande dat de werkelijke expositie voor de gehele periode ca een factor 2 lager was dan de berekende.
De expositie van de ratten was dan ook niet 10 x,30 x en 90 x de menselijke expositie, maar 4 1/2, 14 en 40 voor de mannelijke ratten respectievelijk 7, 22 en 68 voor de vrouwelijke ratten.
Bij het toepassen van deze doseringen in het carcinogeniteits-experiment bleek dat geen verhoogde tumorinductie werd waargenomen bij de groepen ratten die 106 weken werden geëxposeerd aan mutagene drinkwater concentraten.
Bovendien bleek, dat het aantal dieren met tumoren en dieren die aan tumoren overleden in de geëxposeerde groepen niet hoger te zijn dan in de controlegroep.
Het afwezig zijn van een verhoogde tumorinductie in deze carcinogeniteitsproef duidt erop, dat het mutagene drinkwaterconcentraat niet voldoende hoge concentraties aan zeer potente carcinogenen zoals b.v. N-Nitrosodiethylamine bevatten omdat in dat geval de carcinogeniteitsstudie waarschijnlijk positief was uitgevallen bij het testen van de toegepaste dosering. Anderzijds kan de samenstelling van het mutagene concentraat uit minder potente carcinogenen niet worden uitgesloten, omdat bij de geteste dosering geen verhoogde tumorinductie verwacht mag worden van dit soort carcinogenen.
Op grond van de verkregen resultaten in dit carcinogentiteitsexperiment: met name geen verhoogde tumorinductie, is een risicoschatting uitgevoerd m.b.t. de populatie die dit mutagene drinkwater consumeert.

Bij deze schatting is van de volgende aannames uitgegaan:

- indien organische stoffen met carcinogene eigenschappen in drinkwater aanwezig zijn, dan bevinden deze zich uitsluitend in het mutagene drinkwater concentraat
- resultaten verkregen in experimenten met ratten mogen geëxtrapoleerd worden naar de mens
- lineaire extrapolatie van hoge dosis naar lagere dosis is toegestaan.

Indien al deze aannames correct zijn dan blijkt op grond van een statistische berekening slechts de volgende uitspraak mogelijk, namelijk dat in ieder geval minder dan 356 mensen van de 110.000 consumenten een risico aan kanker lopen. Op basis hiervan lijkt de bijdrage van drinkwater, als er al sprake is van een bijdrage, relatief gering namelijk < 1,1 % in vergelijk tot de verwachte tumorincidentie van 33.000, welke waarde gebaseerd is op een gemiddelde tumorincidentie van 30 per 100 veroorzaakt door bijdragen van toegediende en achtergrond carcinogenen (Gezondheidsraad, 1979). Om de betrouwbaarheid van dit soort uitspraken echter te verhogen, zullen naast hogere doses van concentraten van mutagene stoffen als gebruikt in deze studie ook concentraten van de rest van de organische stoffen die in drinkwater aanwezig zijn op carcinogene activiteit onderzocht moeten worden.
Tevens is het zinvol om na te gaan, in hoeverre deze beide organische concentraten tumorpromotor en/of initiator aktiviteiten bezitten alsmede het identificeren van die stoffen die verantwoordelijk zijn voor dit soort effecten.

In hoofdstuk 6 worden een aantal conclusies gegeven, die uit deze studie naar voren komen.
De conclusies luiden :

- In een inventarisatie-onderzoek waarbij het drinkwater van 18 steden werd onderzocht op mutagene aktiviteit, bleek dat in drinkwater bereid uit oppervlaktewater, grondwater alsmede een mengsel van beide, mutagene aktiviteit werd aangetoond in volumina variërend van 0,5 tot 3 liter drinkwater.

- Op grond van deze studie blijkt, dat grondwater als bron voor drinkwater de voorkeur verdient boven de andere ruwwaterbronnen omdat slechts 1 van de 5 steden die zijn drinkwater bereidt uit deze bron een duidelijke mutagene aktiviteit vertoonde. Dit resultaat is aanmerkelijk beter dan de resultaten verkregen met de steden die hun drinkwater bereiden uit oevergeinfiltreerd oppervlaktewater en uitsluitend oppervlaktewater. Bij het gebruik van oeverinfiltraat als drinkwaterbron werd in alle 6 de onderzochte steden mutageniteit in het drinkwater geconstateerd terwijl bij het gebruik van oppervlaktewater als bron in 4 van de 6 steden mutagene aktiviteit in het drinkwater werd aangetoond.
- Toepassing van een chloorbehandeling bij de bereiding van drinkwater zal in het algemeen de mutagene aktiviteit in het water doen toenemen.
- Het gebruik van een ozonbehandeling gaf tijdens het bereiden van drinkwater een duidelijke reductie in mutagene aktiviteit te zien met stam TA 98. Op grond van andere drinkwaterstudies lijkt het er echter op dat ozon een geringe toename in aktiviteit kan veroorzaken met stam TA 100.
- Duinfiltratie en filtratie m.b.v. aktief koolfilters zijn zuiveringsstappen die de mutagene aktiviteit drastisch kunnen reduceren.
- Bij het toepassen van verschillende zuiveringsstappen in de juiste volgorde blijkt, dat ondanks het gebruik van een mutagene waterbron en het introduceren van mutagene aktiviteit tijdens de zuivering, de mutageniteit in het water zodanig gereduceerd kan worden, dat deze niet meer aantoonbaar is in 3,5 l in drinkwater.
- Van de onderzochte chemische parameters die in drinkwater werden gemeten, vertoonde het AOCl gehalte de beste relatie met de mutagene aktiviteit in drinkwaterconcentraten. Deze parameter komt dan ook in aanmerking als zg. indicator voor mutagene aktiviteit in drinkwater.

- De organische stoffen die verantwoordelijk zijn voor de mutagene aktiviteit in het drinkwater, worden voornamelijk in de licht polaire, niet vluchtige fraktie aangetroffen. Het is zeer waarschijnlijk dat het om andere organische stoffen gaat dan welke reeds in dit drinkwater m.b.v. GC/MS zijn geidentificeerd. Een belangrijke fractie die aktief is met stam TA 98 heeft een molecuul gewicht in de orde van grootte van 200.
- In een carcinogeniteitsstudie waarin Wistar SSP TOX ratten aan mutagene drinkwaterconcentraten werden blootgesteld en waarbij de maximale dosering die getest werd 68 x de hoeveelheid is waaraan een 'standaard' mens wordt blootgesteld, werd na statistische analyse geen significant ($p < 0.05$) hogere aantallen tumoren in de geëxposeerde groepen gevonden in vergelijk met de niet geëxposeerde groepen. Bovendien bleek, dat het aantal dieren met tumoren en dieren die aan tumoren overleden in de geëxposeerde groepen niet hoger te zijn dan in de controle groepen.
- Het negatieve resultaat met de mutagene drinkwaterconcentraten in deze carcinogentiteitsstudie bij alle geteste dosis duidt erop dat deze concentraten niet voldoende hoge concentraties aan zeer potente carcinogenen zoals b.v. N-Nitrosodiethylamine bevatten. Anderzijds kan de samenstelling van het mutagene concentraat uit voornamelijk minder potente carcinogenen niet worden uitgesloten omdat geen verhoogde tumorinductie verwacht mag worden van dit type carcinogenen bij de geteste dosis.

list of abbreviations

Ames test	Ames Salmonella/microsome assay
AOCl	Adsorbable organic halogens
bw	Body weight
C	Carbon
DMSO	Dimethylsulfoxide
EOCl	Extractable organic halogens
GC	Gas chromatograph
hal	Halogens
HPLC	High performance liquid chromatography
min	Minute
MS	Mass spectrometer
OM	Organic material
PAH	Polynuclear aromatic hydrocarbons
PCB	Polychlorinated biphenyls
r	Coefficient of correlation
RO	Reverse osmosis
S-9	Liver homogenate (9000 g supernatant)
s.c.	Subcutaneous
THM	Trihalomethanes
TLC	Thin layer chromatography
TOC	Total organic carbon
Tot.N	Total nitrogen
VOCl	Volatile organic halogens

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Appendix 1. Composition of semi-synthetic diet SSP-TOX (per kg), based on the information supplied by the manufacturer

Composition					
moisture	54	g	crude protein	217	g
raw fibres	88	g	vitamins, minerals		
carbohydrates	504	g	and spore elements	58	g

Vitamins, minerals and spore elements					
vitamin A	9500	i.u.	phosphor	6.7	g
vitamin D3	1500	i.u.	magnesium	0.7	g
vitamin E	25	mg	sodium	4.2	g
thiamine (B1)	7	mg	potassium	5.2	g
riboflavin (B2)	9.5	mg	chloride	7.9	g
pantothenic acid	13	mg	sulphate	0.15	g
nicotinic acid	49	mg	iron	51	mg
choline	1430	mg	copper	5.2	mg
meso-inositol	150	mg	zinc	57.9	mg
folium acid	3	mg	manganese	20	mg
biotin	0.3	mg	cobalt	0.8	mg
cyanocobalamine	0.02	mg	iodine	0.5	mg
(B12)			selenium	0.1	mg
calcium	7.7	g	molybdene	0.04	mg

Amino acids					
isoleucine	11.1	g	threonine	9.3	g
leucine	19.9	g	tryptophan	3.4	g
lysine	17.2	g	valine	14.1	g
methionine+cysteine	8.7	g	arginine	10	g
phenylalanine +			histidine	6.5	g
tyrosine	22.2	g			

Appendix 2. Chronic carcinogenicity study with drinking water in rats
Summary of tumours

Tumours	Incidence of tumours (numeric)							
	MALES				FEMALES			
Treatment	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Axillary lymph nodes :	(15)			(13)	(15)			(15)
Preputial/clitoral glands:	(15)			(15)	(11)	(1)		(12)
Squamous-cell carcinoma				2				
Skeletal muscle :	(14)			(15)	(15)			(14)
Mammary glands :	(12)			(10)	(20)	(8)	(16)	(20)
Fibroadenoma					11	5	12	9
Adenocarcinoma					2	1	1	2
Adenoma					1	1		1
Multiple (2) fibroadenoma							2	1
Fibroma						1		
Spleen :	(50)	(47)	(49)	(50)	(49)	(44)	(44)	(50)
Pancreas :	(15)	(1)		(15)	(16)			(15)
Islet-cell adenoma	1				1			
Islet-cell carcinoma		1						
Stomach :	(16)	(2)	(2)	(14)	(17)	(3)	(5)	(14)
Caecum :	(15)			(15)	(15)			(15)
Colon :	(15)	(5)	(4)	(20)	(18)	(4)	(6)	(17)

Appendix 2. Chronic carcinogenicity study with drinking water in rats
Summary of tumours

Tumours	Incidence of tumours (numeric)							
	MALES				FEMALES			
Treatment	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Small intestines :	(15)	(2)		(15)	(15)			(16)
Rectum :	(15)	(1)	(2)	(15)	(15)		(1)	(15)
Mesenteric lymph nodes :	(15)			(14)	(14)			(15)
Testes :	(15)	(1)	(1)	(15)				
Epididymides :	(15)		(1)	(14)				
Prostate :	(13)	(1)		(15)				
Seminal vesicles :	(14)	(1)	(1)	(15)				
Coagulating glands :	(15)			(15)				
Urinary bladder :	(15)	(2)	(2)	(14)	(15)			(15)
Uterus :					(19)	(4)	(3)	(17)
Fibromatous polyp					3	2		3
Endometrial stromal sarcoma								1
Adenocarcinoma					1			
Leiomyosarcoma							1	
Squamous-cell carcinoma							1	

Appendix 2. Chronic carcinogenicity study with drinking water in rats
Summary of tumours

Tumours	Incidence of tumours (numeric)							
	MALES				FEMALES			
Treatment	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Ovaries :					(30)	(17)	(13)	(22)
Malignant granulosa-theca cell tumour						1	1	
Granulosa-theca cell tumour						1		
Adrenals :	(49)	(45)	(47)	(49)	(46)	(42)	(42)	(49)
Small phaeochromocytoma	8	2	5	5	2		2	1
Malignant phaeochromocytoma	3	2		1		1	1	1
Medium size phaeochormocytoma	1	1	1	1		2		1
Large phaeochromocytoma		1	2	2		1		
Mixed phaeochromocytoma and ganglioneuroma	1	1						
Medium size cortical adenoma				1				
Ganglioneuroma	1	1	1	1				
Small bi-lateral phaeochromocytoma	1							
Small cortical adenoma					1			
Multiple (2) cortical adenoma						1		
Kidneys :	(15)	(1)	(2)	(17)	(15)			(15)
Tumours only recorded			2	2				

Appendix 2. Chronic carcinogenicity study with drinking water in rats
Summary of tumours

Tumours	Incidence of tumours (numeric)							
	MALES				FEMALES			
Treatment	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Liver :	(49)	(47)	(50)	(50)	(49)	(42)	(44)	(50)
Neoplastic nodule	1		1					1
Cholangioma (Cystic)								2
Hepatocellular carcinoma			1					
Sternum with bone marrow :	(15)			(14)	(15)			(15)
Heart :	(15)			(15)	(15)			(15)
Thymus :	(9)			(7)	(12)			(12)
Lungs :	(16)			(15)	(14)			(15)
Trachea :	(13)			(14)	(14)			(14)
Oesophagus :	(15)	(1)		(15)	(15)			(14)
Aorta :	(14)			(15)	(15)			(14)
Submaxillary salivary glands :	(16)			(15)	(14)			(15)
Sublingual salivary glands :	(12)			(12)	(13)			(12)
Parotid salivary glands :	(14)			(14)	(14)			(15)

Appendix 2. Chronic carcinogenicity study with drinking water in rats
Summary of tumours

Tumours	Incidence of tumours (numeric)							
	MALES				FEMALES			
Treatment	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Exorbital lachrymal glands :	(14)			(15)	(14)			(15)
Thyroid :	(47)	(40)	(44)	(45)	(45)	(39)	(43)	(43)
Medium size light-cell solid adenoma	1			1	2	2	1	
Small light-cell solid adenoma	1		1		1		1	
Light-cell solid carcinoma	1					1		
Eyes :	(15)			(15)	(14)			(15)
Brain :	(14)			(17)	(15)			(15)
Granular-cell tumour				1	1			
Sarcoma				1				
Pituitary :	(44)	(36)	(46)	(43)	(43)	(43)	(42)	(43)
Large haemorrhagic tumour	3	3	7	1	11	6	9	4
Medium size haemorrhagic tumour	2	5	2	4	4	7	3	5
Small solid tumour	7	3	6	5		1	5	5
Small haemorrhagic tumour	1	2	1	4	4	2	1	1
Medium size solid tumour	2	1		4	1	1	1	
Large solid tumour				1		1	1	
Small pars intermedia tumour						1		

Appendix 2. Chronic carcinogenicity study with drinking water in rats
Summary of tumours

Tumours	Incidence of tumours (numeric)							
	MALES				FEMALES			
Treatment	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Pituitary :	(44)	(36)	(46)	(43)	(43)	(43)	(42)	(43)
Medium spongiocytic tumour				1				
Small spongiocytic tumour			1					
Locally invasive haemorrhagic tumour							1	1
Small multiple (2) haemorrhagic tumour							1	
Skin/subcutis :	(8)	(5)	(7)	(7)		(2)	(5)	(4)
Fibroma	1	2	2	4			1	2
Fibrosarcoma		1	1	1		1	2	1
Lipoma				2			1	
Squamous-cell carcinoma	1					1	1	
Mesenchymal tumour		1						
Lymphoma (histiocytic)			1					
Trichoepithelioma	1							
Mediastinal lymph node :	(1)							
Mesentery :	(1)							
Abdominal cavity :		(1)	(2)	(2)	(1)	(1)		
Mesenchymal tumour			1	1	1	1		
Lipoma			1	1				

Appendix 2. Chronic carcinogenicity study with drinking water in rats
Summary of tumours

Tumours	Incidence of tumours (numeric)							
	MALES				FEMALES			
Treatment	group 1	group 2	group 3	group 4	group 1	group 2	group 3	group 4
Parathyroids :				(1)				
Cervical lymph nodes :								(1)
Thoracic cavity :								(1)
Carcinoma								1
Haematopoietic system :		(1)			(1)		(1)	(3)
Lymphoma					1			2
Generalised lymphoma		1						
Leukaemia							1	
Diaphragm :		(1)						

Figures in brackets represent the number of animals from which this tissue was examined microcospically.
In this table, a benign tumour is ignored if a malignant tumour of the same histogenetic origing is also present in the same tissue.
The absence of a numeral indicated that the lesion specified was not identified.