The Isolation and Identification of Mutagens in Drinking Water

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THE ISOLATION AND IDENTIFICATION OF MUTAGENS IN DRINKING WATER

RESEARCH PROJECT FOR THE
WATER RESEARCH COMMISSION

by

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EXECUTIVE SUMMARY

Exploratory research into the isolation and identification of mutagens in drinking water was conducted over a three-year period.

The research was aimed at firstly developing an extraction/separation scheme to isolate mutagens from drinking water in quantities and with a purity suitable for identification and, secondly, at developing and applying chemical and spectroscopic methods to identify the isolated mutagens.

A mutagen is a chemical or physical agent that can induce a permanent, transmissible change in the genetic material of a cell, usually in a single gene. Mutagens are therefore important compounds as they can initiate irreversible illnesses such as cancer; a cancer cell is a mutant. Not all carcinogens are mutagens but mutagenic carcinogens are more potent than non-mutagenic carcinogens and human carcinogens are mainly mutagenic in nature.

Throughout the world mutagenicity is mainly introduced into drinking water by chlorinating water that contains organic material which is mainly humic material. Establishing the identity of these mutagens is important for a number of reasons. Once identified, the pure compounds can be obtained, their toxicity determined and quantitative methods developed. These methods can then be used to determine the origin and formation of the mutagens, to determine their distribution in South Africa and to evaluate the efficacy of drinking water purification processes. In addition, these results can also be used to develop drinking-water quality standards.

The identification of the mutagens in water is, however, a complex problem. The mutagens are present in very low concentration, are probably bound to humic material and present together with a large variety of other low concentration organic compounds. The literature on the identification of mutagens in water is also very limited.

A new isolation strategy was investigated in this research project. This involved the isolation of mutagen precursors (compounds that, on chlorination, form mutagens) rather than the mutagens themselves. As an organic compound can form more than one compound on chlorination, chlorination of the mutagen precursors i.e., a fraction of the original sample, should result in a simpler mixture than chlorination of the whole original sample.

A number of mutagenicity extraction experiments, each based on the results of the previous one, were done on water from the Rietvlei Dam and on clarified water from the Rietvlei Dam Waterworks. The following observations were made:

1. A higher nitroarene precursor mutagenicity was detected in the clarified water from the water works than in water from the dam.

2. About 45% of the nitroarene mutagenicity precursors and 69% of the non-nitroarene precursors could be removed from the dam water by means of strong acid cation exchange while very little of the humic material was removed; i.e., an enrichment of precursors.

3. The percentage removal of nitroarene precursors from the clarified water by means of cation exchange was 60%.

4. About half of the non-nitroarene precursor mutagenicity could be removed either by non-ionic or by cation exchange resin while the other half could not be removed by either of these techniques; i.e., at least two types of non-nitroarene mutagenicity occurs.

5. No precursors could be recovered from the cation exchange column with hydrochloric acid. Relative to what was removed from water, a 166% recovery was obtained with a methanol/ 10% hydrochloric acid mixture. This suggests the formation of organic mutagenicity without chlorination.

6. The eluate from the cation exchange column was shown to contain both nitroarene and non-nitroarene mutagenicity without the need to chlorinate this eluate. This observation explains the 166% recovery obtained with chlorination.

7. A gas chromatographic analysis of the cation exchange column eluate indicated the presence of nitropyrene; the most potent carcinogen known.

The results of the experiments can be explained by assuming that a large part of the mutagenicity was already present in the water but undetectable due to chelation to humic material. Chlorination and/or cation exchange served to release the mutagens from the humic material. A study on the mechanisms of binding between mutagens and humic material did not progress beyond a literature survey.

A mathematical model was designed by which the role of chlorination and cation exchange could be calculated. One of the results obtained with this model was that the mutagenic potential of the waters studied was more than double that which could be achieved by chlorination and that the nitroarene mutagenicity can be destroyed by an excess of chlorine.

Further research should include the development of a chemical instrumental method to determine nitroarenes in waters. Negative-ion chemical ionization mass spectroscopy could be the right technique.

WIDER IMPLICATIONS OF THE RESULTS

1. The organic composition of waters

The humic material of water consists of fulvic and humic acids. This research has indicated the binding of the mutagens to the humic acids. Any study of the micro-organic pollution of a water should consequently be based on a knowledge of the humic acids present. For this purpose a convenient method has to be developed to determine the humic concentration of water.

2. The Ames test

This test is mainly used to obtain an indication of the quality of drinking water. The possibility that some of the mutagenicity cannot be detected with the Ames test - due to binding to humic acids - implies that the test in its present form underestimates the mutagenic potential of a sample of water.

3. Water purification

As the humic acids in water carry mutagens - as well as other harmful substances - the production of drinking water should optimize the removal of these acids. Chlorination - which forms as well as destroys mutagenicity - should also be optimized in order to achieve disinfection with a low amount of mutagenicity.

This research has indicated nitroarenes as the major contributor to the mutagenicity of Rietvlei Dam clarified water. A number of important observations were made of which the interpreted results have implications for our knowledge of the organic pollution of waters as well as for water purification.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

A mutagen is a chemical or physical agent that induces a permanent, transmissible change in the genetic material of a cell, usually in a single gene (Dorland's, 1981). Mutagens are important compounds as they can initiate irreversible illnesses such as cancer; a cancer cell is a mutant.

Not all mutagens are carcinogens and a distinction can be made between genotoxic and non-genotoxic carcinogens. The latter show no mutagenicity with the Ames test, do not induce tumours in reference tissues, have no DNA reactivity and are very tissue and species specific (Brown *et al.*, 1990). Trans-species carcinogens tend to be mutagens and these mutagenic carcinogens are more potent carcinogens than non-mutagenic carcinogens. Furthermore, human carcinogens are mainly mutagens (genotoxicants) and human non-carcinogens are generally non-genotoxic although some can be rodent carcinogens (Rozenkranz *et al.*, 1990).

Mutagens can enter the human body via tobacco smoke, food, medicine, cosmetics, air, drinking water, etc. Air and drinking water are essential to life but differ from the other vectors in that a person normally has no control over their quality and has no choice but to use the air and drinking water available. As large groups of people use the same supplies of drinking water, not only should compounds which are known to be a potential health hazard be monitored regularly, but additional research is needed into their largely unknown organic composition. The use of drinking water from a given source could mean an almost continuous exposure to a low concentration of one or more mutagens which would eventually have an adverse effect on the more susceptible part of a population.

Some epidemiological studies seem to indicate that the occurrence of illnesses in a certain population, such as cancer of the bladder, correlates with the type of the water consumed (Black *et al.*, 1980); Kuzma *et al.*, 1977; Cantor *et al.*, 1978; Alvanja *et al.*, 1978).

The method used in this project to determine the mutagenicity of a sample is the one developed by Bruce Ames of the University of California (Maron, 1983; Van der Gaag, 1985). This test uses a bacterial strain, *Salmonella typhimurium*, which requires histidine in order to grow and to multiply. In the presence of a mutagen some of the bacteria placed on an agar plate mutate in a way that enables them to produce their own histidine i.e. they start multiplying. After 2 days at 37°C small colonies of bacteria - each originating from a single mutant bacterium - become visible. As some bacteria mutate spontaneously, a blank plate i.e. an agar plate containing only bacteria and the solvent used to dissolve the mutagen, is used. Samples and blanks are tested at least in triplicate. A measure of the mutagenicity of a sample is the mutation ratio (MR) where

$$MR = \frac{\text{mean number of colonies on sample plate}}{\text{mean number of colonies on blank plate}}$$

At low MR values, the MR is linear with the concentration of the mutagen. An MR of two is sufficient for a meaningful detection of mutagenicity and $F \times [MR-1] + 1$ (at low MR-values) can be used to calculate the MR at different concentrations. F is either the concentration factor ($F>1$) or the dilution factor ($F<1$).

At high concentrations of mutagens, toxic effects take place i.e. the MR/concentration line curves downwards and the "background lawn" - formed by bacteria which did not mutate but multiplied for a short while only because of the lack of histidine - disappears. On dilution of the sample the measured MR will be higher than the calculated MR. Because of these complications, Ames tests were also done on 1:1 dilutions of extracts.

The specificity of the Ames test is increased by using different strains of *Salmonella typhimurium* which differ in their sensitivity for different mutagens. (Strains TA98, TA100, TA153, etc).

Rozenkranz (*et al.*, 1990) has developed strains of *Salmonella* deficient in the enzymes that reduce nitro aromatic compounds to the hydroxylamine compounds. As the hydroxylamines are the ultimate mutagens, these strains do not mutate with the nitroarenes. A higher MR with the normal strains (TA98, TA100) than with the enzyme deficient strains (TA98NR, TA100NR) indicates the presence of nitroarenes.

Some organic compounds (promutagens) can be metabolized by enzymes to mutagens and by incorporating rat liver enzymes (S9 mixture) in the Ames test, these promutagens can be distinguished from the direct acting mutagens.

Organic mutagens are - or can be enzymatically converted to - electrophilic reactants which can bind to the purines or pyrimidines of the nucleic acid. With the Ames test two types of mutations can be distinguished:

1. **Frame-shift mutation:** detectable with, for instance, TA98. This is a mutation which results from the deletion or addition of one or more bases from the DNA.

2. **Base pair substitution:** detectable with, for instance, TA100. With this mutation a base pair in the DNA is substituted by another pair.

Direct acting mutagens: include alkylating agents such as S-mustards, N-mustards, epoxides, aziridines, alkyl alkane sulphonates, strained ring lactones, N-nitrosamines and nitroarenes.

Promutagens: include polycyclic hydrocarbons, aromatic amines, N-nitrosamines, hydrazines and some hormones (McCann, 1975; Dean, 1985; Fishbein, 1980; Holzer, 1980; Reeves, 1981).

The concentrations of the mutagens in drinking water are usually too low to detect directly on a sample of drinking water, i.e. the mutagens have to be concentrated. It is possible that the concentration of the polar mutagens in drinking water is higher than the concentration of the non-polar mutagens but as non-polar, rather than polar organic substances tend to accumulate in the human body (Hutzinger *et al.*, 1978; MacKay, 1982), this investigation into the mutagenic composition of drinking water is limited to non-polar mutagens.

Because large volumes of water have to be extracted for isolation purposes, adsorption-extraction rather than liquid-liquid extraction is used to concentrate the mutagens from drinking water.

In practice a volume of water is passed through a column of a non-ionic adsorbent. Inorganic salts and highly polar organic compounds are not adsorbed. The non-polar and weakly polar adsorbed substances are eluted from the column with a suitable solvent. Until about 1975, activated carbon was used for the adsorption step. Activated carbon can, however, irreversibly adsorb certain organic compounds such as polynuclear aromatic hydrocarbons. The non-ionic XAD resins do not suffer from this disadvantage (Van Rossum, 1978) and, since they have become commercially available, they have replaced the activated carbon columns.

For an investigation into the non-volatile mutagens, a volatile solvent like acetone is used as eluent. After evaporation of the acetone on a rotary vacuum evaporator, a light yellow residue is left which can be used as the starting material for a separation scheme.

Using XAD adsorption/extraction - and to a limited extent reverse osmosis or freeze-drying - to concentrate the organic compounds from water, mutagenicity has been detected in the drinking waters of the following countries: England (Foster, 1983), Finland (Pahlman, 1983; Vartiainen, 1986), Israel (Neeman, 1980), Italy (Monarca, 1985), South Africa (Denkhaus *et al.*, 1980; Grabow *et al.*, 1980, 1981; van Rossum *et al.*, 1982), The Netherlands (Kool, 1981, 1983; van der Gaag, 1984) and the USA (Coleman, 1980; Glatz, 1978; Gruener, 1978; Loper, 1978; Schwartz, 1979).

Mutagenicity is mainly introduced into drinking waters world-wide by the chlorination processes at the water works (McCarty, 1981; Fallon, 1980; Kool, 1982; Wilcox, 1984). The humic substances present in the sources of drinking waters play a role in the formation of mutagenicity; waters with a low humic acids content show a low mutagenic activity.

Humic acids are polyelectrolytes. They consist of oxidized vegetative matter and are soluble in alkali. The humic material in drinking water sources consists mainly of fulvic acids. Fulvic acids constitute that fraction of humic material which - in contrast to humic acids - is soluble in water at pH 2. Fulvic acids are more acidic and less aromatic and have a lower molecular mass than humic acids (Christman, 1985). The yellow colour of some waters, as well as the main part of the ultraviolet (UV) light absorbence above 220 nm, are due to fulvic acids.

Fulvic acids are the main contributors to the total organic carbon concentration in drinking water. Ultrafiltration of Pretoria drinking water showed that the main portion of the UV light absorbing material has a molecular mass between 10 000 and 100 000 (NIWR, 1974).

The composition of humic material is dependent on the climate. At a mean annual temperature lower than 25°C, the rate of production of humic material is faster than the rate of its destruction by aerobic bacteria. Above 25°C the reaction rates reverse (Strahler, 1973). It is very likely that the composition and concentration of the related fulvic acids are also climate dependent. Apart from mean annual temperature, the seasonal variations influence the fulvic acids composition: in South Africa the UV light abs/dissolved organic carbon relation of some waters showed seasonal fluctuations (Van Steenderen, 1985). Mutagens originating from fulvic acids should consequently also show climatic and seasonal changes in concentration and/or composition and these variations in mutagenicity have indeed been detected (Grim-Kibalo, 1981). The isolation and identification attempts of mutagens originating from humic material in countries differing in climate i.e. Finland (cold climate, humic rich waters) and South Africa, could consequently lead to different results i.e. to the isolation of those members - of a family of compounds which are present world-wide - which have a high concentration locally.

The following three aspects of humic acids should be considered when studying their relation to mutagens:

1. Commercial humic acids as well as humic acids isolated from waters can form mutagens on chlorination.

2. Humic acids do interact with smaller molecules such as pesticides, dialkyl phthalates and polycyclic aromatic hydrocarbons and could, consequently, also interact with mutagens (McCarthy, 1985; Caron, 1985; Ogner, 1970; Wershaw, 1969).

3. Humic acids form molecular aggregates in solution. The degree of aggregation depends on the pH, ion strength and concentration of the acids. Disaggregation and reaggregation can occur when these parameters are changed (Wershaw, 1969). These interactions could influence the results of an attempt to isolate the mutagens from water.

The following characteristics have been observed of mutagens concentrated with XAD resins (Monarca, 1983; van der Gaag, 1985; Kool, 1984, 1985; Gruener, 1978; Wilcox, 1984; Backlund, 1985; Flanagan, 1981; Wigilius, 1985; Jenkins, 1983; Fawell, 1985; Loper, 1985):

1. Activity with TA100 and TA98. The activity with TA100 is often higher than with TA98.

2. Rat liver enzymes are often not needed. Sometimes the mutagenic activity is reduced, i.e. the detected mutagens were direct acting.

3. Low volatility.

4. Complete removal from water with virgin activated carbon.

5. Ozone treatment reduces the mutagenicity of some waters. With other waters ozone increases the mutagenicity.

6. Reduction of mutagenicity with dechlorinating agents such as sodium thiosulphate or sodium sulphite.

7. Higher yields of mutagenicity with XAD resins from acidified drinking water than from neutral drinking water.

8. Heating a mutagenic extract at 250°C destroys the mutagenicity. The mutagens are, however, resistant to boiling in water.

9. The mutagens are more readily eluted from XAD resin with acetone than with ether. These observations show that the mutagens in drinking water are weakly-polar compounds and that it is not unlikely that the same mutagens are present world-wide but in varying concentrations.

The literature on the isolation and identification of mutagens in drinking water is very limited; probably due to the complexity of the problem. The mutagens are not only present in drinking waters at very low concentrations but are also part of a very complex organic matrix. These two aspects are actually related; the number of organic compounds present at a concentration level increases with a decrease in concentration level.

It was soon realized that today's best separation/ identification technique eg. capillary gas chromatography/ mass spectroscopy, is unable to account for the major fraction of the mutagenicity of a water extract (Fawell, 1985). Furthermore, the resolution of today's high performance liquid chromatography (HPLC) is too low to achieve a satisfactory separation between mutagens and non-mutagenic material.

Tabor (1983) has identified 3-(2-chloroethoxy)-1,2-dichloropropene in an extract prepared in 1962 by means of activated carbon adsorption followed by desorption of the organic material with chloroform. This promutagen could be detected only with TA100 and TA1535 and probably originated from the herbicide diallate (2,3-dichloro alkyl NN-diisopropylthiolcarbamate).

Tabor (1985) has also isolated a fraction containing a TA98 direct-acting mutagen by applying C_{18}-reversed phase HPLC on an XAD-2 extract obtained from drinking water. Kool (1982) made the following fractions:

1. A concentrated water extract - obtained by adsorption on a mixed bed column (XAD-4 and XAD-8) followed by elution with acetone - was fractionated with thin layer chromatography. Mutagenicity (direct acting : TA98) was located in one band (Kool, 1982).

2. Fractionation with LH-20 produced a separation between the main portion of the organic material absorbing at 263 nm and TA98 mutagenic activity. A large part of the TA100 mutagenicity could also be separated from the organic material (Kool, 1984). Ames tests with TA98NR and TA100NR indicated the presence of nitro-compounds.

3. Fractionation of a drinking water concentrate with bondapak C_{18} and a water/acetonitrile gradient resulted in one broad peak of UV light (254 nm) absorbent material which consisted of numerous peaks and which spread over the whole length of the chromatogram. Mutagenicity was mainly concentrated in two neighbouring fractions out of the six that were collected.

A field of research which could give clues for the characteristics of the mutagens in drinking water, is the identification of the product formed by chlorinating paper-mill effluent.

Holmbom (1984) has tentatively identified the direct acting mutagen (TA100) 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone in a chlorinated Kraft effluent. This compound is stable at a pH lower than 6 but is completely destroyed above pH 8. McKague (1981) and Carlberg (1986) have identified a number of direct acting mutagens in chlorinated paper-mill effluents which produced responses in the *Salmonella* strains TA98 and TA100. These compounds included chloroacetones and tri- and tetrachloro-ethenes. Alkali destroyed the mutagenic carbonyl-containing compounds.

None of these identified mutagens can, however, explain the major part of the mutagenicity of drinking water (Fawell, 1985). Van Rossum (1986) using selected ion mass spectroscopy on isolated mutagenic fractions, indicated the presence of nitroarenes in Pretoria drinking water.

Nitro polynuclear aromatic hydrocarbons such as nitropyrene are probably the most potent mutagens - and probably also carcinogens - known. To obtain a ratio of two with the Ames test, only 0,5 nanogram of nitrofluoranthene is needed per petri dish.

As mentioned before, epidemiology has shown that it is likely that there is a correlation between the use of chlorinated water and the incidence of cancer of the bladder. From the dye industries it is known that these cancers can be caused by aromatic amines. In the human body, aromatic amines as well as aromatic nitro compounds are metabolized to the same ultimate mutagens i.e. the hydroxylamines. (Alvanja *et al.*, 1978; Daudel, *et al.*, 1966; Rosenkranz, *et al.*, 1985; Fishbein, 1984).

1.2 JUSTIFICATION OF THIS PROJECT

As mentioned before, drinking waters contain detectable amounts of mutagenicity which is mainly introduced by the chlorination process and these mutagens could initiate carcinogenesis. (For this reason, for example, Amsterdam (The Netherlands) has stopped the chlorination of drinking water and other cities are following).

Chlorination is still the best disinfection method and as there are no intentions to stop chlorinating drinking water in South Africa, the incidence and behaviour of the mutagens should be investigated.

Identification of these mutagens is, however, the first essential step. Once identified, the pure compounds can be obtained and used to establish their toxicity by means of bioassays and to develop quantitative methods. These methods can then determine the origin and formation of the mutagens to evaluate/improve drinking water purification plants and to determine the nation-wide distribution of these mutagens. The results of bioassays and surveys can be used to develop standards for waters.

1.3 AIMS OF THIS PROJECT

1. To develop an extraction/separation scheme to isolate mutagens from drinking water in quantities and with a purity suitable for identification.

2. To develop and apply chemical and spectroscopic methods to identify the isolated mutagens.

1.4 WORK PROGRAMME

Based on our experience in this field of research, the following tasks could be identified:

1. Determination of the contribution of the breakdown and/or chlorination products of known water pollutants such as endosulfan and steroids to the mutagenicity of drinking water.

2. Determination of the contribution of chlorinated, heterocyclic acids to the mutagenicity of drinking water.

3. Development of a quantitative method for nitroarenes.

4. Development of mathematical techniques to increase the information that can be obtained from the matrices of mass spectra of mutagenic isolates.

5. A systematic investigation into the large loss of mutagenicity which occurs at some of the separation steps in the isolation schemes we have developed.
6. The determination of the mutagenicity of the very polar organic material.

7. The identification of the mutagens formed from fractionated precursors.

8. Our investigation concerned Pretoria drinking water only. At least a small survey of the drinking water of other cities will have to be undertaken in order to determine the distribution of the types of mutagens i.e. to establish the relevance of the findings on Pretoria drinking water to the rest of the country.

9. Improvement of the pyrolysis gas chromatography/mass spectrometry techniques to obtain mass spectra from mutagen-humic material complexes.

Each of these tasks (except task 8) faces with the following problems:

1. The presense of a large variety of irrelevant organic compounds of about the same low concentration as the mutagens.

2. The presense of a - relative to the mutagens - large amount of humic material to which the mutagens are probably chelated.

The tasks were consequently redefined to the following two:

A. **A LITERATURE SURVEY ON HUMIC MATERIAL**

This survey can be found in Appendix A.

B. **THE ISOLATION OF MUTAGEN PRECURSORS** i.e. material which on chlorination form mutagens, rather than the isolation of the mutagens themselves. Experience has shown that present day isolation techniques are inadequate to identify the mutagens in waters by the extraction/purification/ identification sequence which is normally used in organic chemistry. On chlorination an organic compound usually forms more than one product. Therefore chlorination of the mutagens precursors - i.e. a fraction of the original sample - should result in a simpler mixture than the chlorination of the whole, original sample.

CHAPTER 2

THE ISOLATION OF MUTAGEN PRECURSORS

2.1 PART 1: FRACTIONATION OF THE PRECURSORS IN WATER ACCORDING TO POLARITY AND pKa-VALUE

2.1.1 Introduction

Due to presence of a large variety of low concentration organic compounds (micropollutants) in waters, the complexity of humic material and the uncertainty about the relevance of commercial humic acids as mutagen precursors, the isolation of mutagen precursors from waters seems an attractive alternative to identify mutagens.

Organic compounds can be extracted from sources of drinking water according to polarity and pKa.

2.1.2 Fractionation according to polarity

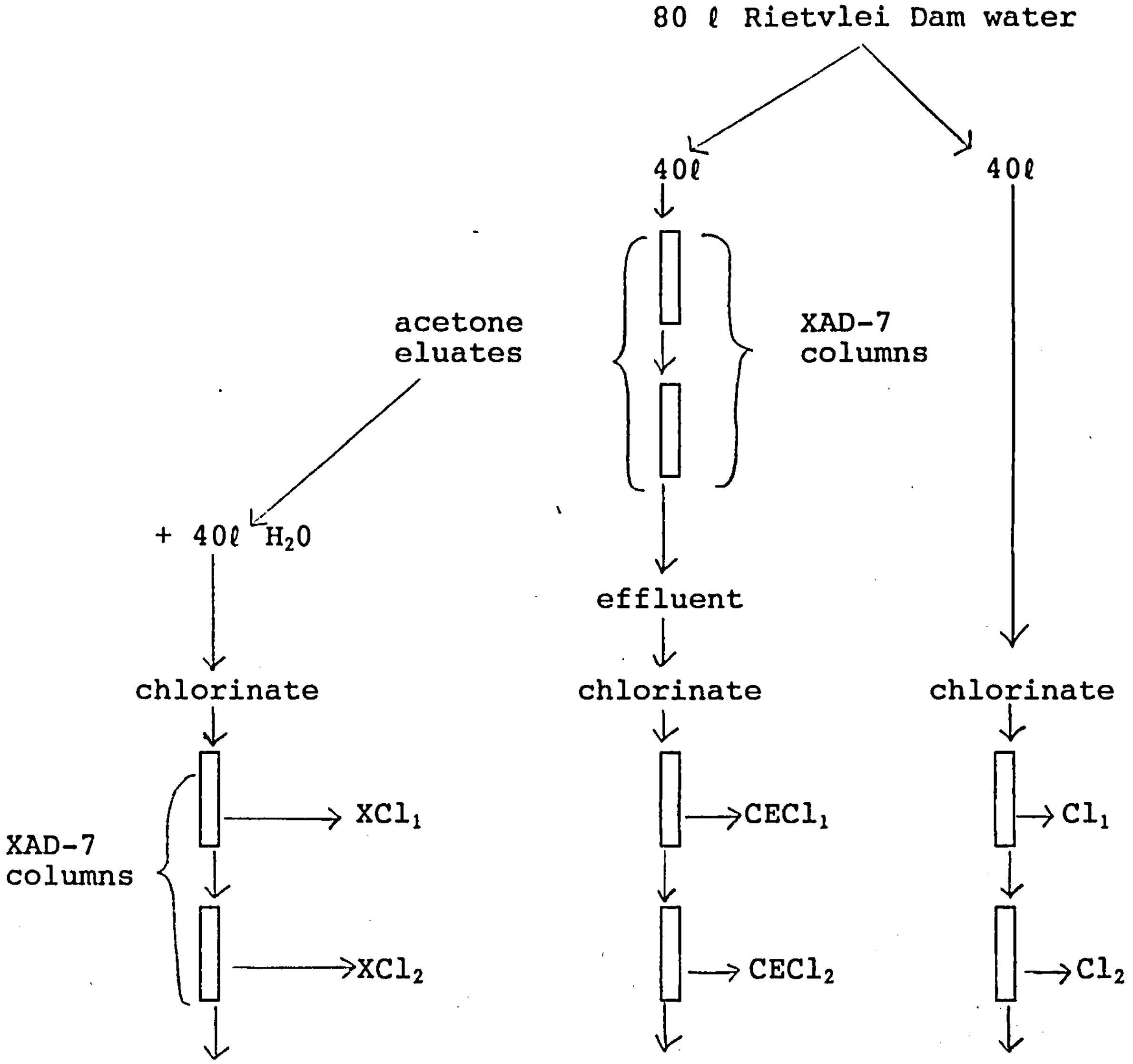
2.1.2.1 Experimental:

A sample of water (40 ℓ) from the Rietvlei dam was passed, under nitrogen pressure, at the rate of 60 mℓ/min, through a 5 µm Sartorius glass fibre filter and two glass columns (diameter 15 mm); each column was packed with 15 mℓ of XAD-7 resin. The resin had a particle diameter of between 500 and 710 µm and was purified before use by washing with distilled water (to remove inorganic salts and fine material) followed by soxhlet extraction with acetone. The column effluent (40 ℓ) was chlorinated with 2 mℓ of 6% sodium hypochlorite. The acetone eluate - 60 mℓ from each column - was evaporated to dryness in a rotary vacuum evaporator. The residue was dissolved in 5 mℓ of 95% EtOH and added to 40 ℓ of distilled water. This sample was also chlorinated with sodium hypochlorite. After 24 hours of reaction time, the formed mutagens were extracted by passing the water through two XAD-7 columns and eluting with acetone. (Same technique as used for the water sample).

The acetone eluates were dried with anhydrous Na_2SO_4 and concentrated to 2 mℓ in a rotary vacuum evaporator. Ames tests for mutagenicity were done on these acetone extracts (and 1:1 dilutions) with 0,1 mℓ per Petri dish.

Mutagen precursors were also determined by chlorinating Rietvlei Dam water without a fractionation step.

Experimental scheme:



2.1.2.2 Results

Table 1: Fractionation of mutagen precursors from Rietvlei Dam water according to polarity

Sample	XAD-7 column	Ames test for mutagenicity			
		Salm. t. strain TA98		TA98NR	
		MR*	1:1 dil.	MR*	1:1 dil.
Non-polar precursor (XC1)	1	2,5	2,8+	2,4	2,3
	2	1,6	1,3	1,3	1,3
Polar precursors (CEC1)	1	6,2**	3,6	4,2	2,2
	2	2,3	1,3	1,6	1,4
Total precursors (C1)	1	3,1**	4,1+	3,6	2,8
	2	2,8	2,0	2,3	1,3

* MR = Mutation ratio. Each MR is the mean of 3 Ames tests.

** The higher MR in the fractionated sample was possibly due to insufficient chlorination of the original sample.

\+ Increase of MR with dilution of the acetone concentrate indicates non-polar toxicity which inhibited the Ames test.

The polar organic compounds (CEC1) made a larger contribution to the formation of mutagens than the non-polar compounds (XC1). This means that for the preparation of mutagens precursors from the Rietvlei Dam, XAD-7 resin can be used to remove irrelevant organic material such as the organic micropollutants.

A difference between the MR of strain TA98 and TA98NR indicates the presence of nitroarenes in the sample. As this difference is larger in the polar material (CEC1) than in the non-polar material (XC1), polar organic material seems to make the main contribution to nitroarene precursors.

2.1.3 Fractionation according to pKa

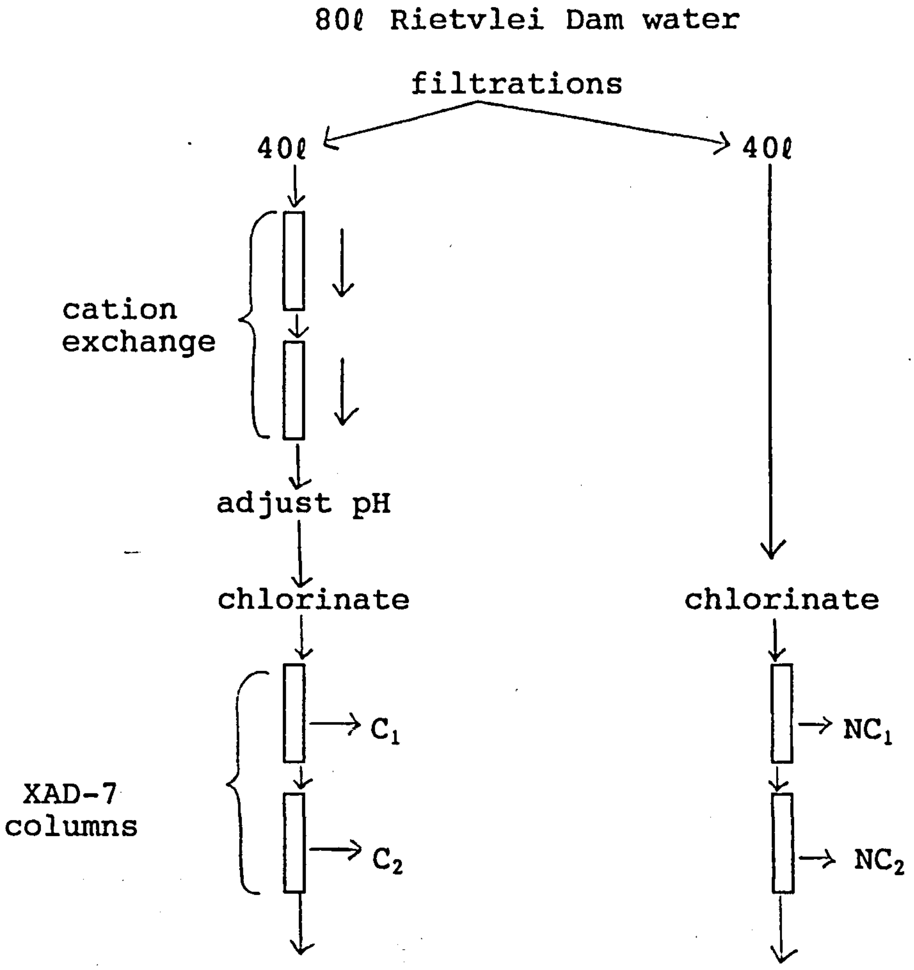
If XAD-7 is used to remove non-precursors, the soluble material in the column effluent consists mainly of inorganic salts and these salts can be removed by ion exchange. In order to determine the effect of ion exchange on mutagen precursors, the relative precursor concentrations of water from the Rietvlei Dam were determined before and after cation exchange.

2.1.3.1 Experimental

Particulate material was removed from 80 ℓ of Rietvlei Dam water by filtration through glass fibre filter, 0,45 µm and 0,22 µm membranes. Fourty litres of the filtrate were used to determine mutagen precursors in the normal way, i.e. chlorination followed by XAD-7 extraction of the mutagen and the Ames test. The cations were removed from the other 40 ℓ by means of a column (370 mm length, 35 mm i.d.) packed with Dowex 50W-X8 (100 - 200 mesh) in the H-form. (Atomic absorption showed that this column removed sodium from 40 ℓ of Rietvlei Dam water completely).

The pH of the column effluent was adjusted with NaOH to the original pH of the Rietvlei Dam water i.e. pH = 8,2. After chlorination, the mutagenicity was determined on the XAD-7 resin extracts.

Experimental scheme:



2.1.3.2 Results

Table 2: Fractionation of mutagen precursors from Rietvlei Dam water with cation exchange

Sample	XAD-column	Ames test for mutagenicity			
		Salm. t. strain TA98		TA98NR	
		MR	1:1 dil.	MR	1:1 dil.
Cation exchange pre-treatment (C)	1	3,6	2,5	2,1	1,4
	2	1,8	1,2	1,1	0,9
No cation exchange pre-treatment (NC)	1	7,8	4,3+	4,0	2,3
	2	2,1	1,5	1,2	1,0

+ The MR of the 1:1 dilution can be predicted: (7,8-1)/2+1=4,4. This shows that - in contrast to the previous experiment - the dam water did not contain toxicity to the Ames test.

The large loss of mutagen precursor (including nitroarene precursor) due to cation exchange treatment, was unexpected and this technique can, consequently not be used to remove cations in a mutagen precursor isolation scheme. In contrast to this large change in mutagen precursor, the cation exchange treatment had little effect on the UV spectrum of the water. This small effect was to be expected as the UV spectrum of water is mainly due to fulvic acids (Fig. 1).

If no mutagen precursor destruction had taken place during the cation exchange treatment, separation between them and the large bulk of humic material had taken place. The mechanism of precursor removal from water could have been through cation groups such as amine, pyrilium or metals (Fe, Cu, etc.) complexed to the precursors.

Attempts should be made to establish whether mutagen precursor destruction or separation had taken place.

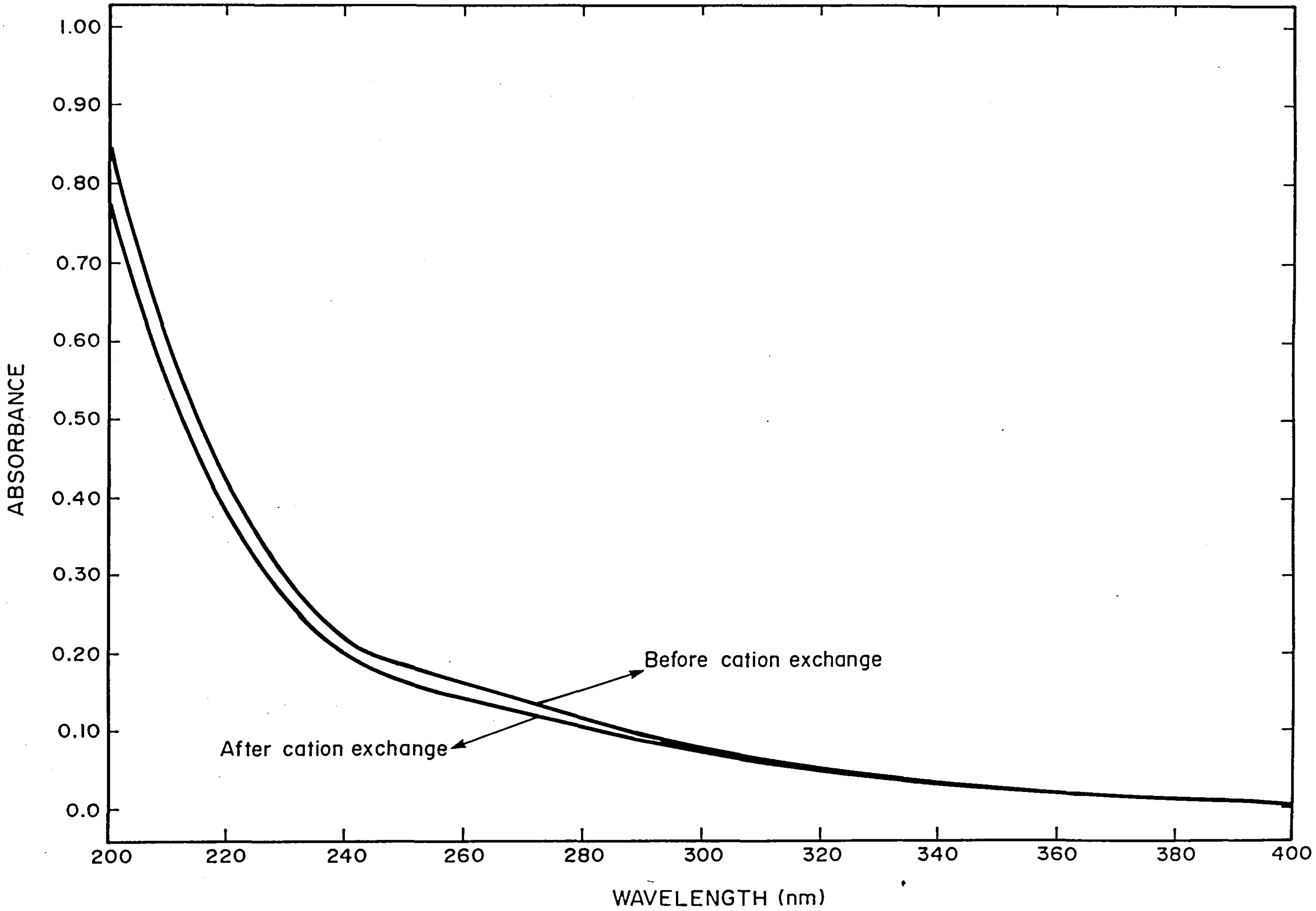

1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.0
ABSORBANCE
200
220
240
260
280
300
320
340
360
380
400
WAVELENGTH (nm)
Before cation exchange
After cation exchange

Fig. 1

2.2 PART 2: AN ESTIMATION OF THE DISTRIBUTION OF THE MUTAGENICITY AMONG MUTAGEN PRECURSORS FRACTIONATED ACCORDING TO POLARITY AND pKa-VALUES

2.2.1 Introduction

By using different combinations of the non-ionic XAD-7 resin and the Dowex 50W-X8 strong acid cation exchange resin mutagen precursors can be classified according to whether they can be removed from water by:

1. XAD-7 resin alone
2. strong cation exchange alone
3. XAD-7 and strong cation exchange
4. Neither XAD-7 nor strong cation exchange

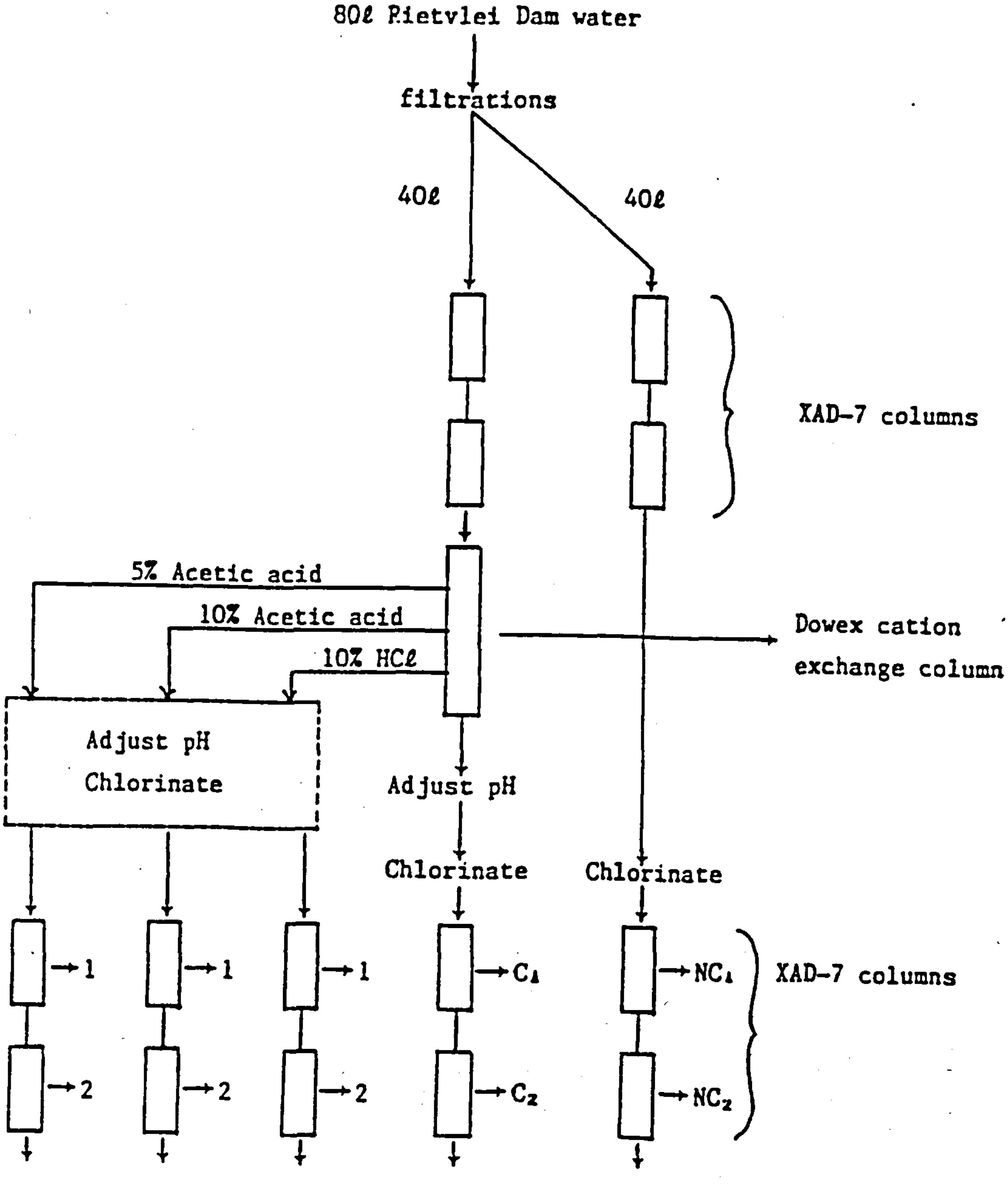
This information is needed to design a precursor isolation scheme and will also give some indication of the characteristics of these precursors.

2.2.2 Experimental

Due to alterations at the Rietvlei Dam water works, water samples could not be collected at the water works and were consequently collected from the Rietvlei Dam itself. Particulate matter was removed from a 80ℓ sample by means of filtration under nitrogen pressure through 5 µm glass fibre filter and 0,45 µm filters. Forty litres of the filtrate was passed at a flow rate of 60 mℓ min^{-1}(under N_2 pressure) through two glass columns (diameter 13 mm) in series; each column packed with 15 mℓ of cleaned XAD-7 resin (500-700 µm). The effluent (40ℓ) of these columns was chlorinated with 2 mℓ of 3,5% sodium hypochlorite. After 24 hours of reaction time the organic material was extracted by passing the water through two XAD-7 columns placed in series. After this adsorption step, the columns were separated, each washed with 60 mℓ of distilled water and the adsorbed material eluted with 60 mℓ of acetone. The acetone eluates were dried with anhydrous Na_2SO_4 and concentrated to 2 mℓ in a rotary vacuum evaporator. Ames tests were done using 0,1 mℓ of these acetone concentrates (and 1:1 dilutions) per petri dish.

The remaining 40ℓ of the 80ℓ of filtered Rietvlei Dam water was also passed through two XAD-7 columns placed in series. The effluent was passed through a strong acid cation exchange column (length: 370 mm; i.d.: 35 mm; Dowex 50W-X8; 100-200 mesh). The pH of the Dowex column effluent was adjusted to the pH of the original water sample (pH = 8,6). After chlorination, the organic material in this effluent was extracted as before i.e. adsorption onto two XAD-7 columns, desorption with acetone followed by concentration. The material collected by the cation exchange column was fractionated into three fractions by eluting with 50 mℓ each of 5% acetic acid, 10% acetic acid and 10% hydrochloric acid. The pH of each fraction was adjusted to the pH of the original water sample and after chlorination the organic material in each fraction was extracted with two XAD-7 columns as before.

Experimental scheme:



2.2.3 Results and calculations

Table 1: Fractionation of mutagen precursors with non-ionic resin (XAD-7) and cation exchange (Dowex 50W-X8).

		Mutation ratio (MR)			
		S. typhimurium strain			
		TA 98		TA 98NR	
Fraction	XAD-7 column		1:1 dil		1:1 dil
Polar precursors (XAD-7 pre-treatment)	1 (NC_1)	6,5+	5,2	5,0*	3,0
	2 (NC_2)	2,8*	1,9	2,4*	1,6
Polar and non-cationic precursors (XAD-7 & cation exchange pre-treatment)	1 (C_1)	5,8*	3,4	3,0+	2,6
	2 (C_2)	1,7	1,7	2,0+	2,0

\+ Contains toxicity (Difference between calculated and observed MR of 1:1 diluted extract)

* No indication of toxicity to the Ames test.

Table 2: Recovery of cationic mutagenicity precursors

		Mutation ratio (MR)			
		S. typhimurium strain			
		TA98		TA98NR	
Cation exchange column fraction	XAD-7 colum		1:1 dil		1:1 dil
5% Acetic acid	1	1,4	1,1	1,6	1,3
	2	1,4	1,2	1,2	1,3
10% Acetic acid	1	1,5	1,3	1,2	1,2
	2	1,3	1,1	1,3	1,0
10% HCl	1	1,9	1,3	2,3	1,3
	2	1,5	1,2	1,4	1,0

Estimation of the distribution of the mutagenicity among the fractions obtained by XAD-7 and cation exchange.

Table 3: Summary of experimental results used for the calculations (First XAD-7 column extract used for Ames test, extracts 1:1 diluted)

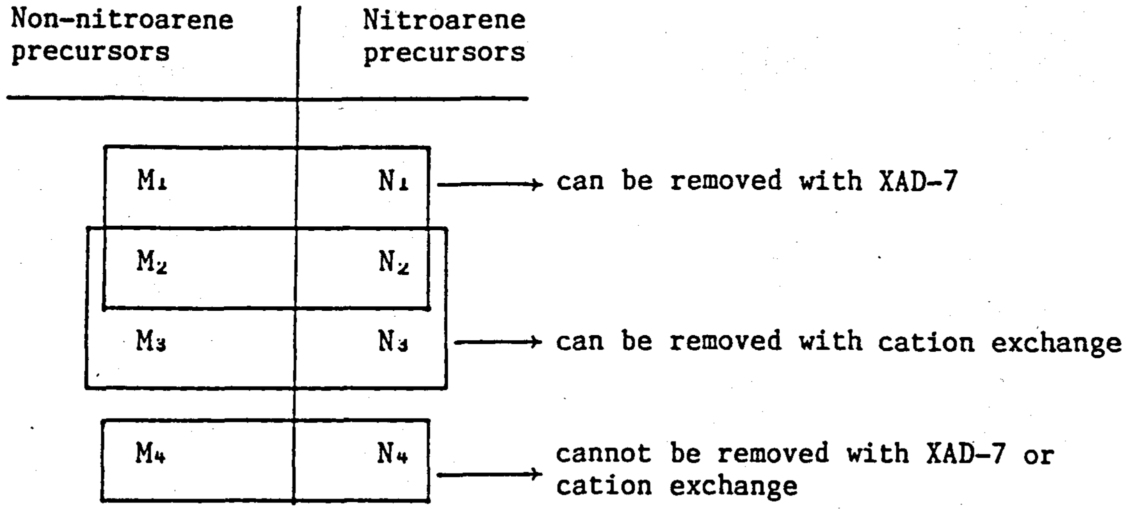
		Mutation Ratio (MR)	
		S. typhimurium strain	
Expt.	Fraction	TA98	TA98NR
1	Polar precursors Total precursors	3,6 4,1	2,2 2,8
2	Non-cationic precursors Total precursors	2,5 4,3	1,4 2,3
3	Polar non-cationic precursors Polar precursors	3,4 5,2	2,6 3,0

The following calculations are based on the assumption that the total mutagenicity of a sample can be resolved into the mutagenicities of the different mutagens in the sample i.e. that the MR-1 (at a low MR value) values of fractions can be linearly combined. Possible synergistic or antagonistic effects are thus ignored.

The opposite process i.e. calculating the total mutagenicity of a mixture of mutagens by means of linear combination could give the wrong answer; some mutagens could chemically react with each other.

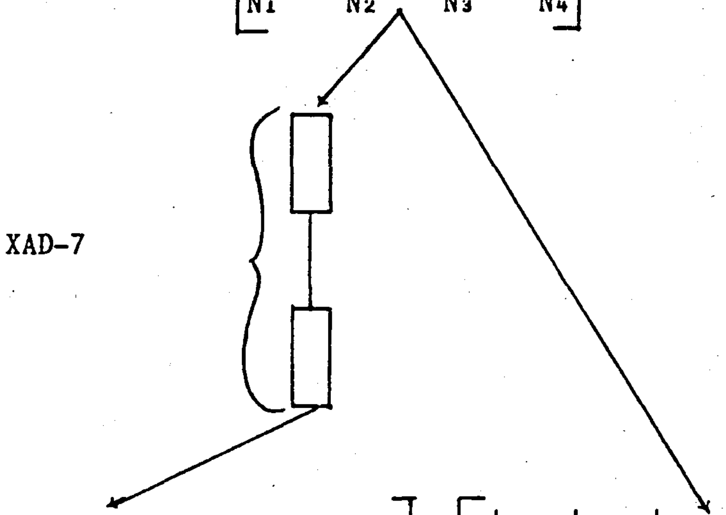
The difference between the MR obtained with TA98 and TA98NR is due to nitroarenes while the MR obtained with TA98NR is due to non-nitoarenes.

The mutagenicity (MR-1) of the following groups (M_1-M_4 and N_1-N_4) of mutagenicity precursors has to be determined.



Experiment 1

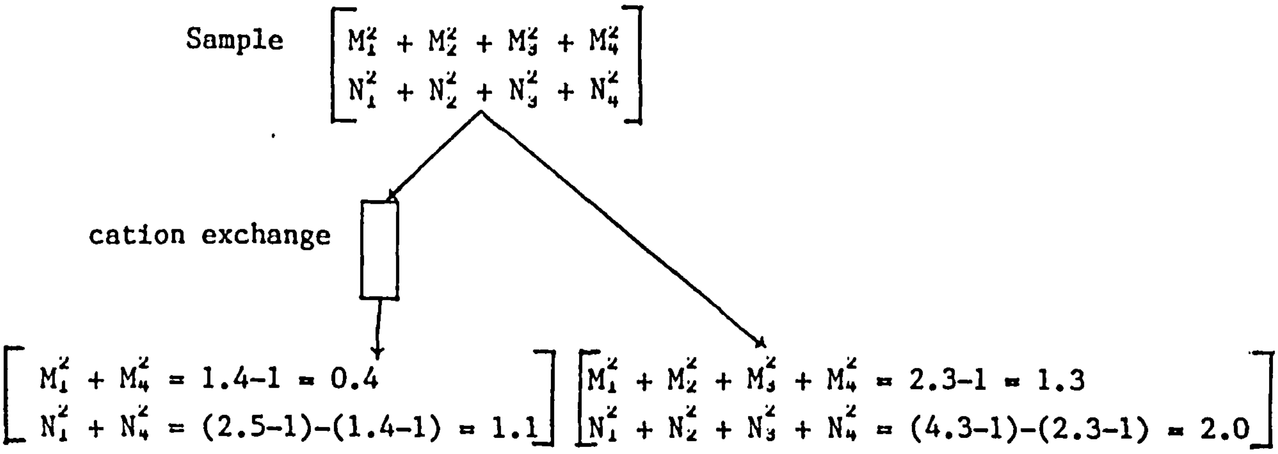
Sample: $\begin{bmatrix} M_1^1 & M_2^1 & M_3^1 & M_4^1 \\ N_1^1 & N_2^1 & N_3^1 & N_4^1 \end{bmatrix}$



$$\begin{bmatrix} M_3^1 + M_4^1 = 2.1-1 = 1.2 \\ N_3^1 + N_4^1 = (3.6-1)-(2.2-1) = 1.4 \end{bmatrix} \qquad \begin{bmatrix} M_1^1 + M_2^1 + M_3^1 + M_4^1 = 2.8-1 = 1.8 \\ N_1^1 + N_2^1 + N_3^1 + N_4^1 = (4.1-1)-(2.8-1) \\ = 1.3 \end{bmatrix}$$

$$\therefore \quad M_1^1 + M_2^1 = 1.8-1.2 = 0.6$$
$$N_2^1 + N_2^1 = 1.3-1.4 = -0.1$$

Experiment 2

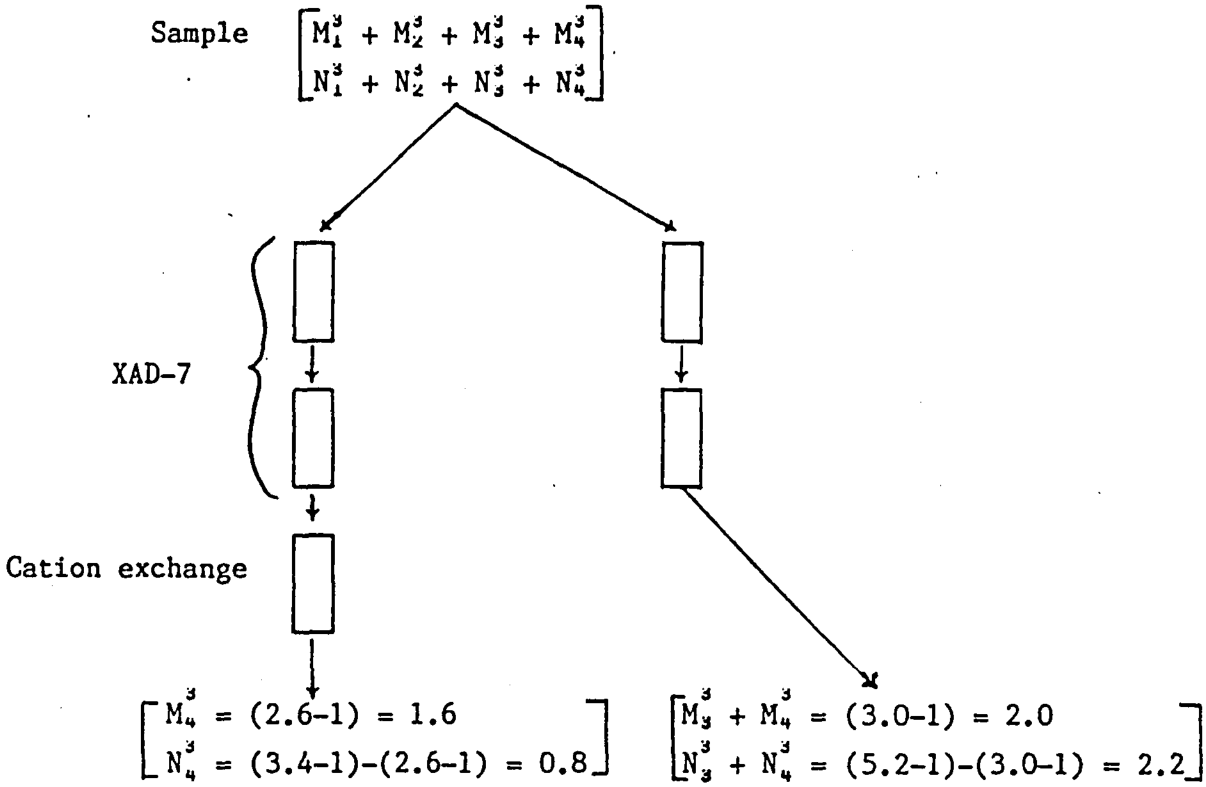


$$\text{Sample}\quad\begin{bmatrix} M_1^2 + M_2^2 + M_3^2 + M_4^2 \\ N_1^2 + N_2^2 + N_3^2 + N_4^2 \end{bmatrix}$$

$$\begin{bmatrix} M_1^2 + M_4^2 = 1.4-1 = 0.4 \\ N_1^2 + N_4^2 = (2.5-1)-(1.4-1) = 1.1 \end{bmatrix} \quad \begin{bmatrix} M_1^2 + M_2^2 + M_3^2 + M_4^2 = 2.3-1 = 1.3 \\ N_1^2 + N_2^2 + N_3^2 + N_4^2 = (4.3-1)-(2.3-1) = 2.0 \end{bmatrix}$$

$$\therefore \quad M_2^2 + M_3^2 = 1.3-0.4 = 0.9$$
$$N_2^2 + N_3^2 = 2.0-1.1 = 0.9$$

Experiment 3



$$\text{Sample}\quad\begin{bmatrix} M_1^3 + M_2^3 + M_3^3 + M_4^3 \\ N_1^3 + N_2^3 + N_3^3 + N_4^3 \end{bmatrix}$$

$$\begin{bmatrix} M_4^3 = (2.6-1) = 1.6 \\ N_4^3 = (3.4-1)-(2.6-1) = 0.8 \end{bmatrix} \quad \begin{bmatrix} M_3^3 + M_4^3 = (3.0-1) = 2.0 \\ N_3^3 + N_4^3 = (5.2-1)-(3.0-1) = 2.2 \end{bmatrix}$$

$$\therefore \quad M_3^3 = 2-1.6 = 0.4$$
$$N_3^3 = 2.2-0.8 = 1.4$$

Calculations for nitroarenes

MR-1

Expt 1

$$\left.\begin{array}{l}N_1^1\\ N_2^1\end{array}\right] - 0.1 \qquad \left.\begin{array}{l}N_3^1\\ N_4^1\end{array}\right] 1.4$$

$$\Sigma(MR_i^1-1) = 1.3$$

Expt 2

$$1.1\left\langle\begin{array}{l}N_1^2\\ \left.\begin{array}{l}N_2^2\\ N_3^2\end{array}\right\rangle 0.9\\ N_4^2\end{array}\right.$$

$$\Sigma(MR_i^2-1) = 2.0$$

Expt 3

N_1^3

N_2^3

$N_3^3 = 1.4$

$N_4^3 = 0.8$

$$\Sigma(MR_i^3-1) = 2.2 + N_1^3 + N_2^3$$

Normalize $\Sigma(MR_i-1)$ of experiment 1 to 2.0

$$\left.\begin{array}{l}N_1^1\\ N_2^1\end{array}\right] - 0.15$$

$$\left.\begin{array}{l}N_3^1\\ N_4^1\end{array}\right] 2.15$$

$$\Sigma(MR_i^1-1) = 2.0$$

$$\therefore \quad \begin{aligned} N_1 &= 0.32\\ N_2 &= -0.47\\ N_3 &= 1.37\\ N_4 &= 0.78\\ \Sigma N_i &= 2.0 \end{aligned}$$

$\therefore$ MR-1

Expt. 1

$N_1^1 = 0.208$

$N_2^1 = -0.306$

$N_3^1 = 0.890$

$N_4^1 = 0.507$

$\Sigma(MR_i-1) = 1.3$

Expt. 2

$N_1^2 = 0.32$

$N_2^2 = -0.47$

$N_3^2 = 1.37$

$N_4^2 = 0.78$

$\Sigma(MR_i-1) = 2.0$

Expt. 3

$N_1^3 = 0.33$

$N_2^3 = -0.48$

$N_3^3 = 1.4$

$N_4^3 = 0.8$

$\Sigma(MR_i-1) = 2.0$

Mutation ratio's of nitroarene precursors

	Expt. 1	Expt. 2	Expt. 3
	$N_1^1 = 1.2$	$N_1^2 = 1.3$	$N_1^3 = 1.3$
	$N_2^1 = 0.7$	$N_2^2 = 0.5$	$N_2^3 = 0.5$
Highest MR →	$N_3^1 = 1.9$	$N_3^2 = 2.4$	$N_3^3 = 2.4$
	$N_4^1 = 1.5$	$N_4^2 = 1.8$	$N_4^3 = 1.8$
	Total $MR^1 = 2.3$	Total $MR^2 = 3.0$	Total $MR^3 = 3.0$

Calculations for non-nitroarenes

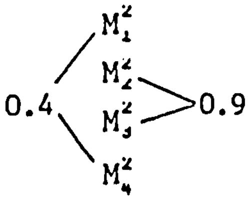
MR-1

Expt 1

$$\left.\begin{array}{l}M_1^1\\M_2^1\end{array}\right]0.6 \qquad \left.\begin{array}{l}M_3^1\\M_4^1\end{array}\right]1.2$$

$$\Sigma(MR_i^1-1) = 1.8$$

Expt 2



$$\Sigma(MR_i^2-1) = 1.3$$

Expt 3

M_1^3

M_2^3

$M_3^3 = 0.4$

$M_4^3 = 1.6$

$$\Sigma(MR_i^3-1) = 2 + M_1^3 + M_2^3$$

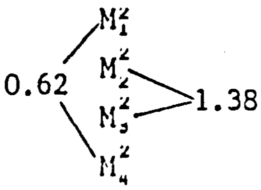
Normalize $\Sigma(MR_i-1)$ of experiments 1 and 2 to 2.0

Expt. 1

$$\left.\begin{array}{l}M_1^1\\M_2^1\end{array}\right]0.\bar{6}7 \qquad \left.\begin{array}{l}M_3^1\\M_4^1\end{array}\right]1.33$$

$$\Sigma(MR_i^1-1) = 2.0$$

Expt. 2



$$\Sigma(MR_i^2-1) = 2.0$$

$\therefore$ MR-1

$M_1 = -0.44$
$M_2 = 1.11$
$M_3 = 0.27$
$M_4 = 1.06$
$\Sigma(MR_i-1) = 2.0$

$\therefore$ MR-1

Expt. 1	Expt. 2	Expt. 3
$M_1^1 = -0.396$	$M_1^2 = -0.286$	$M_1^3 = -0.652$
$M_2^1 = 0.999$	$M_2^2 = 0.722$	$M_2^3 = 1.644$
$M_3^1 = 0.243$	$M_3^2 = 0.176$	$M_3^3 = 0.400$
$M_4^1 = 0.954$	$M_4^2 = 0.689$	$M_4^3 = 1.570$
$(MR_i^1-1) = 1.8$	$(MR_i^2-1) = 1.3$	$(MR_i^3-1) = 3.0$

Mutation ratio's of non-nitroarene precursors

	Expt. 1	Expt. 2	Expt. 3
	$N_1^1 = 0.6$	$N_1^2 = 0.7$	$N_1^3 = 0.3$
High MR ⟶	$N_2^1 = 2.0$	$N_2^2 = 1.7$	$N_2^3 = 2.6$
	$N_3^1 = 1.2$	$N_3^2 = 1.2$	$N_3^3 = 1.4$
High MR ⟶	$N_4^1 = 2.0$	$N_4^2 = 1.7$	$N_4^3 = 2.6$
	Total MR = 2.8	Total MR = 2.3	Total MR = 3.9

2.2.4 Conclusions

1. The nitroarene mutagenicity precursors were removed from the water samples mainly by cation exchange.

2. Two groups of non-nitroarene mutagenicity precursors which differed significantly in polarity could be detected. One group could be removed from water either by XAD-7 or by cation exchange. The other group could not be removed by either of these techniques.

3. MR<1 (N^2_2 of the nitroarene and N^2_1 of the non-nitroarene precursors) indicates toxicity to the Ames test. Removal of these toxic fractions from a sample of dam water will thus increase the MR of this water.

4. The recovery of mutagen precursors from the cation exchange column was unsuccessful. Although catalytic destruction of the precursor by the strong acid cation exchange cannot be excluded, extreme retention of organic material by ion exchange is a common problem. Elution should be improved by:

    (1) using a weak instead of a strong acid cation exchanger;

    (2) optimizing the pH of the eluent i.e. a pH 2 units lower than the pKa of the exchanger but 2 units higher than the pKa of the mutagen precursor. As the last mentioned pKa is unknown, the pKa of an aromatic amine can be used initially;

    (3) using a higher ionic strenth eluent; and

    (4) using a eluent containing a high selectivity cation such as Ba.

5. The catonic group present in the mutagen precursor could be an amine, a pyrillium compound or a metal complexed to the precursor. The possibility of an amine precursor which on oxidation with chlorine forms a nitro compound should also be explored.

2.3 PART 3: THE REMOVAL OF MUTAGEN PRECURSORS FROM CLARIFIED DAM WATER WITH CATION EXCHANGE AND THE EXTRACTION OF THESE PRECURSORS FROM THE CATION EXCHANGER

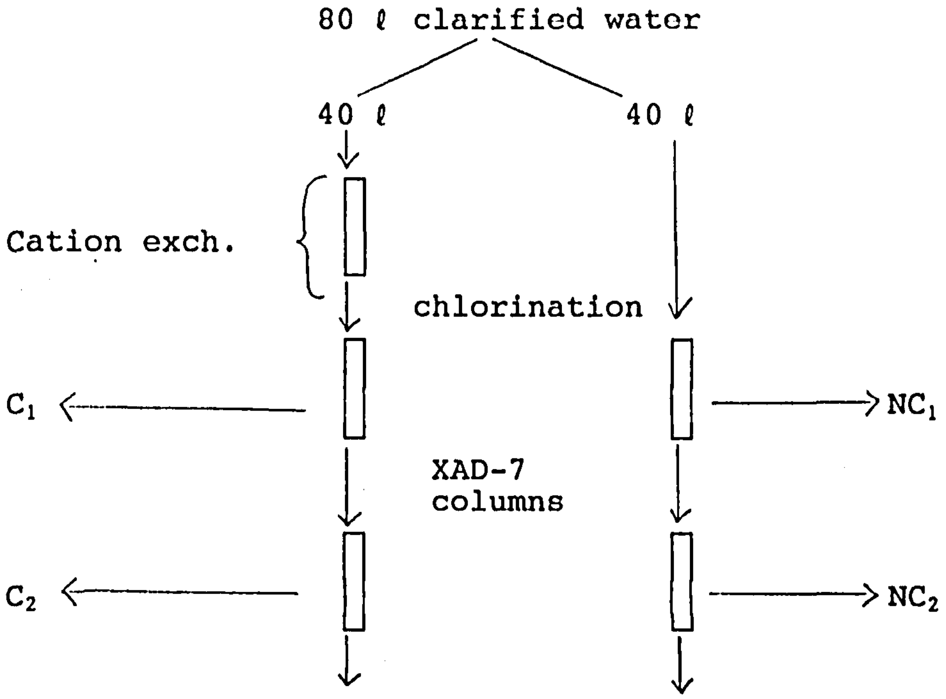
2.3.1 Introduction

The experiments described in parts 1 and 2 of this report were done on water from the Rietvlei Dam as no water could be obtained from the Rietvlei Dam Water Works due to construction activities. The results presented in this part were obtained with water sampled at the water works between the clarification and the chlorination steps. The composition of these samples is less complicated than those taken from the dam and the removal of mutagen precursors found with the dam water had to be established with the clarified water sampled at the water works.

2.3.2 Experimental

Eighty liters of water were sampled at the Rietvlei Dam Water Works between clarification and chlorination. Forty litres were passed through a strong cation exchanger (column length: 370 mm; i.d.: 35 mm; Dowex 50W-X8; 100-200 mesh). Before use the cation exchange column was cleaned with MeOH/10%HCl and distilled water. The pH of the column effluent was adjusted to the pH of the water sample and chlorinated for 24 hours with 2 mℓ of 3,5% sodium hypochlorite. The other 40 ℓ of water from the water works was chlorinated without a cation exchange pre-treatment.

The mutagens were extracted from these chlorinated samples by passing, under N_2 pressure and at a rate of about 60 mℓ/min, through two glass columns (i.d.: 13 mm) in series; each column containing 15 mℓ of cleaned XAD-7 resin (500-700 mesh). After this adsorption step the columns were separated, each washed with 60 mℓ of distilled water and the adsorbed organic material eluted with 60 mℓ of distilled acetone. The acetone eluates were dried with anhydrous Na_2SO_4 and concentrated to 2 mℓ on a rotary vacuum evaporator. Ames tests were done on 0,1 mℓ of these acetone concentrates (and 1:1 dilutions) per Petri plate in triplicate.



2.3.3 Results

TABLE 1 Removal of mutagen precursors with strong acid cation exchange (Dowex 50W-X8)*

		Mutation ratio			
		S. typhimurium strain			
		TA 98		TA 98NR	
Pre-Treatment	XAD-7 column		1:1 dil		1:1 dil
No cation exch.	NC_1	11,1	8,1	3,8	2,4
	NC_2	1,9	1,3	1,2	1,2
Cation exch.	C1	7,6	3,9	2,2	1,6
	C_2	1,8	1,2	1,5	1,0

* The final extract used for the Ames test was 2mℓ for column effluent experiments. For column eluents this volume was 1 mℓ.

(The difference between TA98 amd TA98NR for NC1 was 7,3. For C1 the difference was 5,4).

TABLE 2 Comparison of the mutagen precursor removal between Rietvlei Dam water and clarified water from the Rietvlei Dam Water Works.

		Mutation ratios of 1:1 dil. concentrates			
		Dam water+		Clar. water	
	XAD-7 col	TA98	TA98NR	TA98	TA98NR
No cation exch.	1 2	4,3 1,5	2,3 1,0	8,1 1,3	2,4 1,2
Cation exch.	1 2	2,5 1,2	1,4 0,9	3,9 1,2	1,6 1,0
% mut. precursor removal*		69		57	

+ Taken from Part 1: Table 2
* For non-nitroarenes: $((MR)_{nc}-(MR)_{c})/((MR)_{nc}-1x100$ for XAD-7 column fraction 1

Table 1 shows that the cation exchanger removed nitroarene as well as non-nitroarene mutagenicity precursors from clarified dam water.

Removal of nitroarene mutagenicity:

Dam water: $((4,3-2,3)-(2,5-1,4))/((4,3-2,3))x100=45\%$

Clarified water: $((8,1-2,4)-(3,9-1,6))/((8,1-2,4))x100=60\%$

The percentage removal of nitroarenes from the clarified water is high enough to be useful for isolation purposes.

If it is assumed that clarification removes from the dam water that fraction of the non-nitroarenes precursors which can be removed with XAD-7 but not with cation exchange then the calculated* and observed values correspond very well:

MR calculated for clarified water from dam water:
$(2,3-1)-(0,7-1)+1=2,6$

MR observed in clarified water: 2,4

Percentage removed by cation exchange from the clarified water as calculated* from dam water:
100 x ((1,2-1)+(1,7-1))/((2,3-1)-(0,7-1))=56%

Percentage removed by cation exchange from the clarified water as observed:
100 x (2,4-1)-(1,6-1)/(2,4-1) = 57%

* The values used for these calculations were obtained from the calculated fractions of Part 2 of this report (page 25).

Similar calculations cannot be made for the nitroarenes as the clarified water seems to contain more nitroarene mutagenicity than the dam water.

2.3.4 Isolation of mutagen precursors by means of cation exchange

2.3.4.1 Experimental

The Dowex cation exchanger was washed in a conical flask with MeOH/10%HCl and extracted with 400 mℓ batches of methanol. Each of these methanol extracts were evaporated to dryness under vacuum and the residues dissolved in 1 mℓ of acetone and tested for the absence of mutagenicity and toxicity with the Ames test. After these tests had shown that the resin was clean, it was repeatedly washed with water till no further swelling could be observed+ and packed into the glass column. Forty litres of clarified water from the water works were passed through the column. The resin was transferred to a conical flask, washed with 400 mℓ of distilled water and extracted twice with 400 mℓ of methanol and twice with 400 mℓ of MeOH/10%HCl. Each of these 4 fractions was added to 40 ℓ of distilled water (the waters containing the HCl were neutralized with KOH) and chlorinated with 2 mℓ of 3,5% of sodium hypochlorite for 48 hours. Each of these four samples was passed through a XAD-7 column. After elution with acetone and concentration to 1 mℓ, Ames tests were done.

2.3.4.2 Results

TABLE 3: Mutagenicity obtained from chlorinated cation exchange fractions from clarified dam water

		Mutation ratio		
		S.typhimurium strain		Difference (MR-1)
		TA98	TA98NR	
Cation exchange fraction				
MeOH	1	4,9	3,6	1,3
	2	5,7	4,0	1,7
MeOH/10%HCl	1	3,2	1,9	1,3
	2	4,9	2,9	2,0
Total (MR-1)			8,4	6,3

\+ Due to swelling, which at one stage broke the glass column, changes from methanol to H_2O was done outside the column.

The recovery of mutagenicity was higher than that shown in Table 2 of this report; indicating not only that the MeOH/10%HCl combination was a better eluant than 10% acetic acid or 10% HCl, but also that the mutagenicity was due to organic material.

The percentage recovery of the nitroarenes can be estimated as follows:

Nitroarenes removed by cation exchange (from Table 1):

2[+]x(((11,1-1)-(3,8-1))-((7,6-1)-(2,2-1)))=3,8

\+ This factor of 2 has to be included because the final extract was 2 mℓ.

Nitroarenes recovered from the cation exchanger:

((4,9-1)-(3,6-1))+((5,7-1)-(4,0-1))+((3,2-1)-(1,9-1))+((4,9-1)-(2,9-1))=6,3

(MR-1 is at low mutation ratios proportional to the amount of mutagens. The difference between

TA98 and TA98NR mutagenicity is proportional to the amount of nitroarenes).

The percentage recovery from the cation exchange column is 6,3/3,8x100=166%

As a methanol extract from the cation exchanger was tested by the Ames test before this experiment and found to contain no mutagens, ion exchange of material from the water followed by elution with methanol and MeOH/10%HCl seemed to have introduced additional mutagenicity or additional mutagenecity precursors.

In order to distinguish between these two possibilities cation exchange without chlorination was done next.

2.3.5 Isolation of mutagens by means of cation exchange without chlorination

2.3.5.1 Experimental

The cation exchange column was washed four times with 400 mℓ of MeOH/10%HCl. The last 400 mℓ was used as a control fraction to establish the absence of mutagenicity or toxicity. Forty litres of clarified water from the Rietvlei Dam Water Works was passed through the column. The resin was washed with 400 mℓ of distilled water and eluted with MeOH/10%HCl; three 400 mℓ fractions were collected. Each of these fractions and the control fraction were added to 40 ℓ of distilled water and the pH adjusted to about 8. Each was passed through a XAD-7 column, the adsorbed material eluted with acetone and the acetone extracts concentrated to 1 mℓ followed by the determination of mutagenicity with the Ames test.

2.3.5.2 Results

TABLE 4: Mutagenicity obtained from unchlorinated cation exchange fractions from clarified dam water

		Mutation ratio (MR) S.typhimurium strain			
	TA98		TA98NR		Diff.
		1:1 dil		1:1 dil	MR-1
Cation exch. col. fraction					
Control fraction	0,9		1,3		
Fraction 1	4,8	2,9	2,1	1,5	2,7
Fraction 2	7,0	3,8	3,5	2,3	3,5
Fraction 3	5,9	3,3	4,4	2,3	1,5
Fraction 4*	2,2		0		1,2
Total MR-1			7,0		8,9

* The MR of this fraction was estimated by extrapolation of the MR of the previous fraction by means of the Poisson distribution.

The presence of mutagenicity in unchlorinated cation exchange eluates explains the 166% mutagenicity precursor recovery of the previous experiment.

2.3.6 Analysis of material removed by cation exchange from clarified water

2.3.6.1 Experimental

The removal of organic material from clarified dam water with a combination of cation exchange and elution with MeOH/10%HCl was repeated but this time two cation exchange columns in series were used with a total amount of resin equal to the amount used in the previous experiments. After cation exchange the columns were separated, each column washed with 400 mℓ of distilled water and eluted with 400 mℓ of MeOH/10%HCl. Each extract was added to 40 l of distilled water, the pH adjusted to about 8 and passed through a XAD-7 column. The adsorbed material was eluted with acetone, the extracts concentrated to 2 mℓ and analyzed with electron capture gas chromatography.

2.3.6.2 Results

The chromatograms (Figs. 1 and 2) show only small differences. As the amount of material adsorbed from water should be higher in the first column, peaks present only in the eluate from the first column are candidates for mutagens. The extra peaks near the end of the first chromatogram coincide with some of the peaks of a synthetic nitropyrenes mixture (Fig. 3).

The concentrations are too low for further identifications with electron impact mass spectroscopy. Negative-ion chemical ionization mass spectroscopy, which is available at the Institute for Chromatography of the University of Pretoria, has about the same sensitivity as electron capture gas chromatography and is consequently a promising technique for further research in this field.

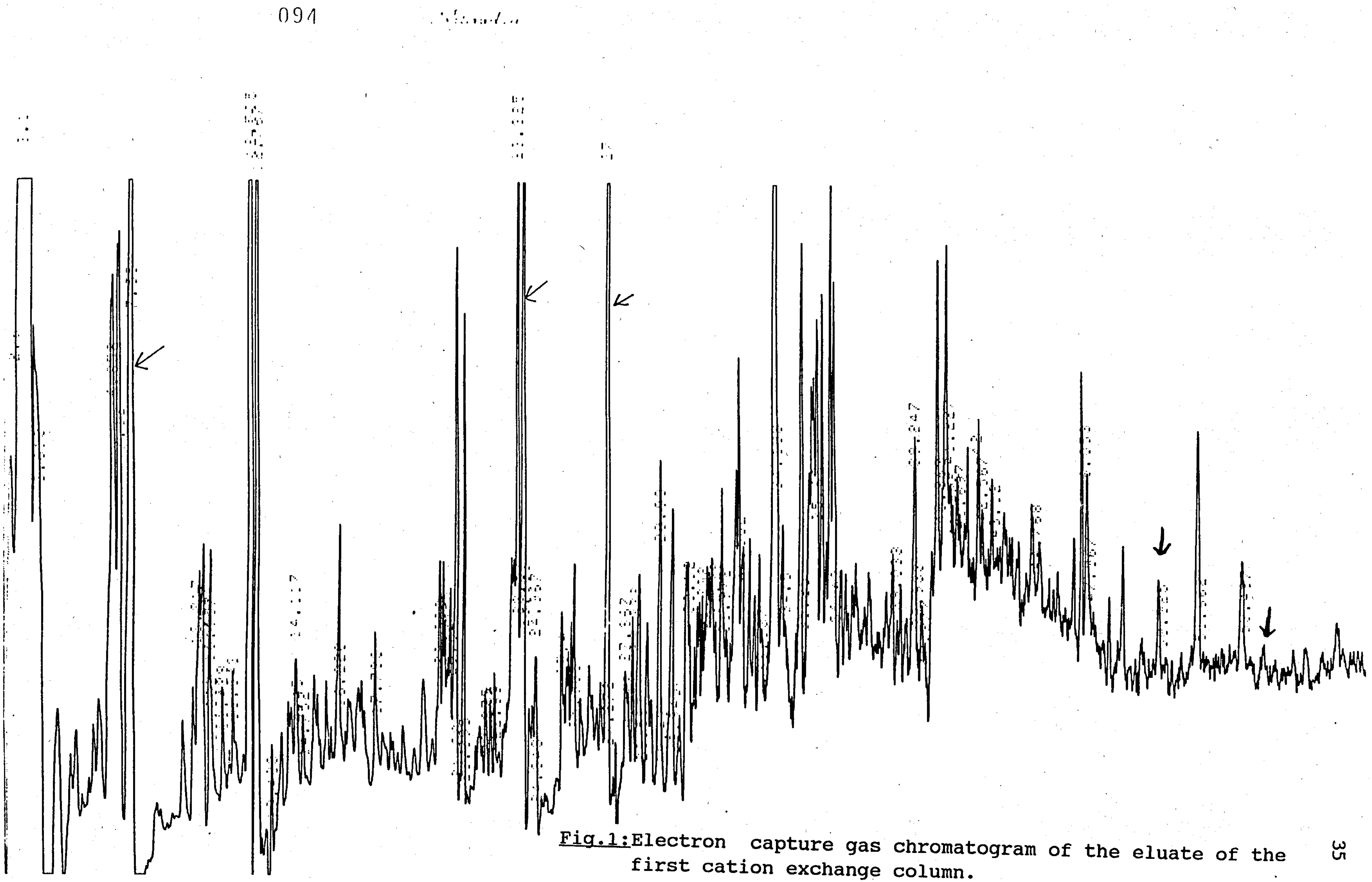


Fig.1: Electron capture gas chromatogram of the eluate of the first cation exchange column.

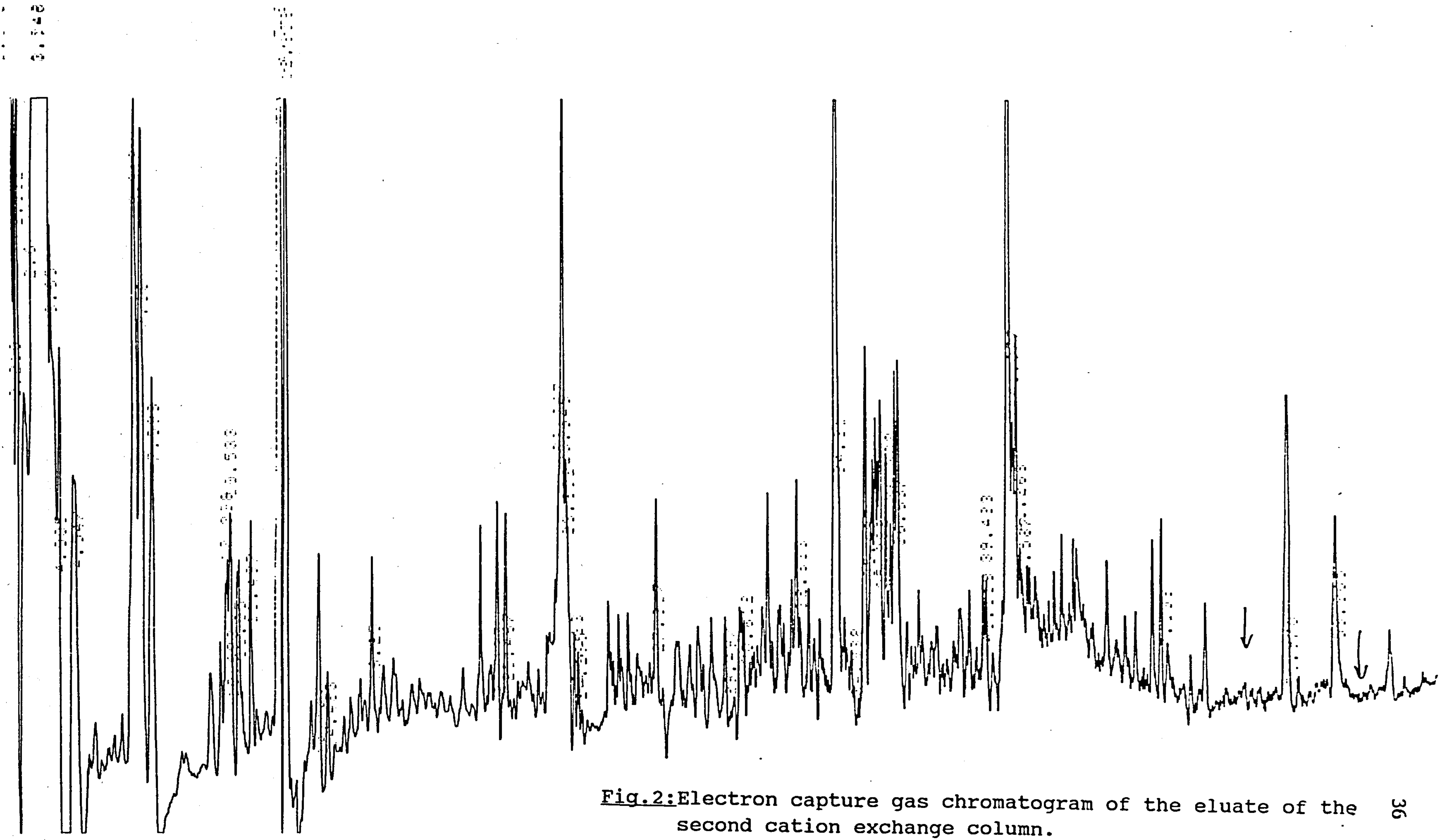


Fig.2: Electron capture gas chromatogram of the eluate of the second cation exchange column.

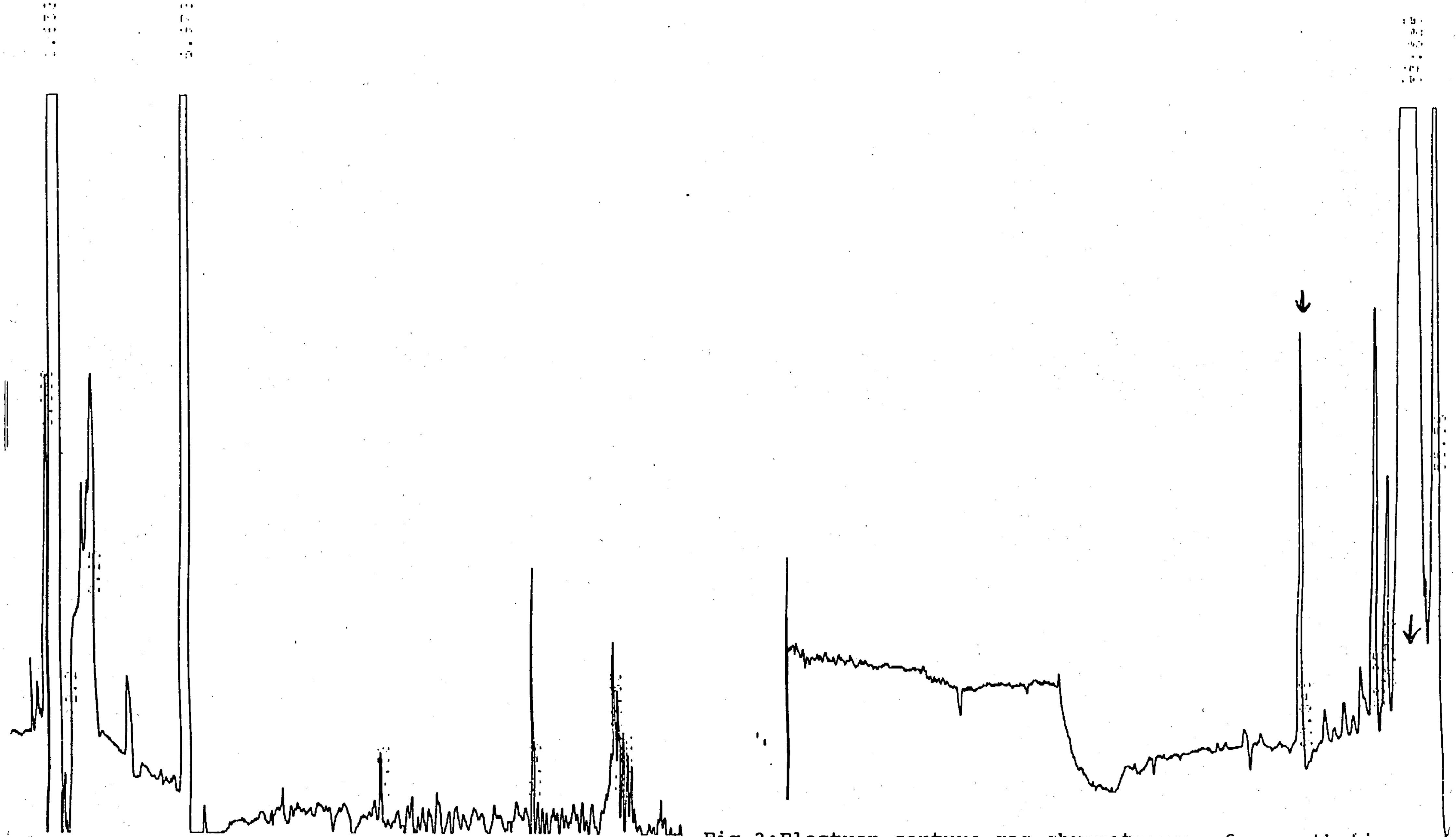


Fig.3:Electron capture gas chromatogram of a synthetic mixture of nitropyrenes.

CHAPTER 3

DISCUSSION OF RESULTS

According to UV-light absorption, the cation exchange column removed only a small amount of organic material (Fig. 1, Part 1 of this report) from water. Also, the pH of the column effluent was set to the pH of the original sample of water before this effluent was chlorinated. This means that the chlorination conditions of the water before and after cation exchange were about the same.

An important observation is that the total non-nitroarene mutagenicity in the chlorinated eluate (8,4) is higher than in the unchlorinated eluate (7,0) but that the total mutagenicity of the nitroarenes is lower in the chlorinated eluate (6,3) than in the unchlorinated eluate (8,9). This was not the case with the cation exchange effluents. The same concentration of chlorination agent was used but the total concentration of organic material was very low in the eluants; i.e., the ratio of chlorine to organic material was much higher in the cation exchanger eluates than in the effluents.

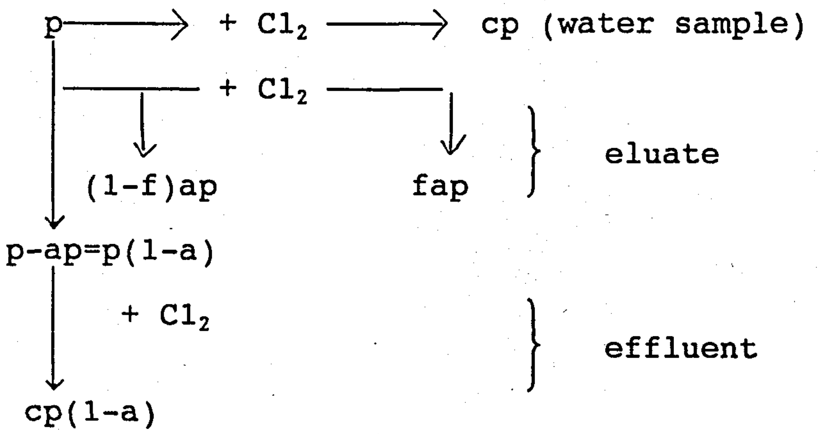
In the following models it is consequently assumed that before and after cation exchange the same numerical fraction (c) of precursors reacted with chlorine. MR-1 is used as a measure for mutagenicity.

3.1 NON-NITROARENES

p: total non-nitroarene mutagenicity potential in clarified water
c: fraction of p forming mutagenicity on chlorination
a: fraction of p collected through cation exchange
f: fraction of "adsorbed" material forming mutagenicity on chlorination

It is assumed that all the "adsorbed" mutagenicity potential is eluted. Each of p, c, a and f has a value between zero and one.

Mutagenicity balance:



Without cation exch	$cp = 5{,}6$	Table 1	[2(3,8-1)=5,6]
With cation exch.	$cp(1-a) = 2{,}4$	Table 1	[2(2,2-1)=2,4]
Elution with Cl_2	$ap = 8{,}4$	Table 3	
Elution without Cl_2	$(1-f)ap = 7$	Table 4	

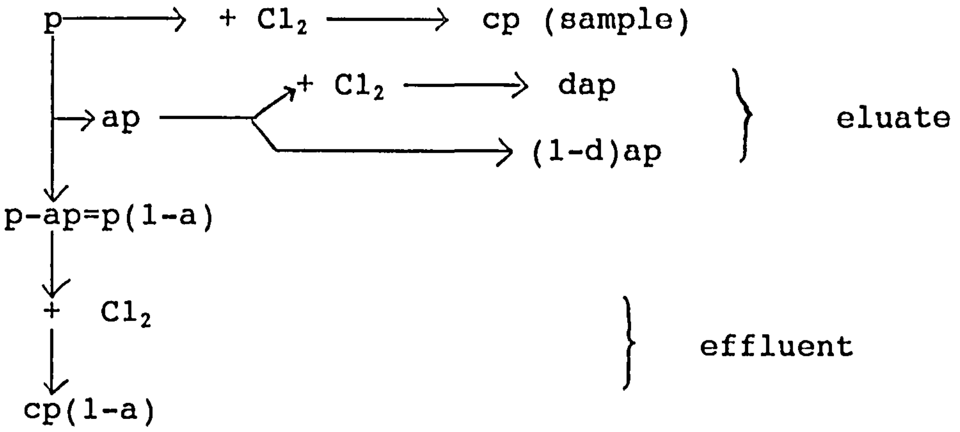
* A factor 2 has to be included as the final extract volume was 2 mℓ

Solving these equations: $a = 0{,}57$; $f = 0{,}17$; $p = 14{,}7$; $c = 0{,}38$

3.2 NITROARENES

p: total nitroarene mutagenicity potential in clarified water
c: fraction of p forming mutagenicity on chlorination
a: fraction of p which, through cation exchange, became mutagenic
d: fraction of mutagenicity formed through cation exchange destroyed by chlorination
ap: mutagenicity eluted from cation exchange column without chlorination

Mutagenicity balance:



Without cation exch	cp =14,6	Table 1	[2(11,1-3,8)=14,6]
With cation exch.	cp(1-a) =10,8	Table 1	[2(7,6-2,2)=10,8]
Elution with Cl_2	(1-d)ap = 6,3	Table 3	
Elution without Cl_2	ap = 8,9	Table 4	

* A factor 2 has to be included as the final extract volume was 2 mℓ

Solving these equations: a = 0,26; c = 0,43; p = 34,2; d = 0,29

Table 5: Mutagenicity (MR-1) distribution due to cation exchange and chlorination

Treatment	Non-nitroarenes	nitroarenes
Total potential	p = 14,7	p = 34,2
Formed by chlorination in water sample	cp = 5,6	cp = 14,6
Recovered after adsorption and elution without Cl_2	(1-f)ap = 7,0	ap = 8,9
Reacted with Cl_2 after ads. and elution	fap = 1,4	dap = 2,5
Formed in cation exch. effluent by chlorination	cp(1-a) = 2,4	cp(1-a) =10,8

1. According to these models, the mutagenicity potential (p) in the clarified water was much higher than could be generated by chlorination (cp).

2. Both nitroarene and non-nitroarene mutagenicity was formed due to the cation exchange/elution step without chlorination.

3. Some non-nitroarene mutagenicity was formed with chlorination of the cation exchange eluate while some nitroarene mutagenicity was destroyed. Concerning other observations nitro- and non-nitroarene mutagenicity behaved similarly.

3.3 A HYPOTHESIS FOR THE OBSERVATIONS AND CALCULATION RESULTS

A. Some of the nitroarene as well as non-nitroarene mutagens are already present in water but are bound to humic material and cannot be detected by the Ames test in this state.

B. Chlorination releases some of these mutagens which can, consequently, be detected by the Ames test.

C. The humic material in water consists mainly of fulvic acids and a small amount of humic acids. One of the differences between these acids is their solubilities at a low pH; fulvic acids are soluble at a pH of 2 while humic acids precipitate at this pH. If it is assumed that some of the mutagens are chelated to the humic acids, during strong acid cation exchange humic acids together with their mutagens are precipitated in the cation exchange column. During elution these mutagens are released and detected by the Ames test. For non-nitroarenes this mutagenicity released by cation exchange was higher than the mutagenicity formed by means of chlorination.

D. Some of the nitroarene mutagens in the cation exchange eluate were destroyed by the large excess of chlorine.

E. Some of the material in the cation exchange eluate consisted of non-nitroarene mutagen precursors which on chlorination formed mutagens; probably chlorinated mutagens.

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I. LITERATURE SURVEY ON HUMIC MATERIAL

Although the subject of the isolation and identification of the mutagens in drinking-water can be divided into a number of research tasks, the humic material normally found in waters will play a key role in each of these tasks.

Some of the important aspects of humic material are :

A. Humic material can bind with smaller organic molecules which could include mutagens. The concentration of mutagens in water could consequently be higher than the water solubility of the pure mutagens.

   Complexing of mutagens with humic material also prohibits the isolation of pure mutagens from waters. A successful isolation scheme must be able to dissociate organic complexes as well as prevent the formation of new complexes.

B. Humic material has a wide range of molecular masses and polarities i.e. separation of weakly or polar mutagens with liquid chromatography cannot be achieved in the presence of humic material.

C. Mutagenic isolates obtained so far consisted mainly of humic material which prevented the usual identification with spectrocopic techniques.

D. Humic material forms mutagenic, chlorinated heterocyclic compounds on chlorination. Mutagens can also be formed by chlorinating commercial humic acids. In this manner a clearer mutagenic sample is obtained than from a drinking-water. Elemental analysis and infrared spectroscopy show little difference between these commercial humic acids and humic material isolated from waters. Recently, new techniques such as cross-polarization and magic-angle spinning ^{13}C NMR show outstanding differences between commercial and other humic aicds i.e. the commercial humic acids are not true representatives of the humic material present in waters.

The literature survey is presented in a format suitable for a quick reference on the following items:

1. Purpose of research
2. Source of humic material
3. Interactions
4. Chemical reactions
5. Characterization
6. Fractionation
7. Articles

The format is : Author of article (abridged detail of item)

HA = humic acids
FA = fulvic acids
HS = humic substances

1. PURPOSE OF RESEARCH

Aiken (Comparison of XAD macroporous resins for the concentration of FA from aqueous solution)

Alberts (HA interaction Fe, Al, Mn studied to see effect metal ions on UV spectra. Assess consequences of metal organic interactions on quantification HA)

Alberts (Trace metal-natural occuring humic material interaction-models)

Alcaniz (Py-GC, Py-MS of soil used to discriminate between soil type)

Anderson (Ozone's reaction with isolated aquatic fulvic acid)

Arai (Combination effect of ionizing radiation and ozone to degrade HA in H_2O)

Barron (Humic soil and coal structure with magic-angle spinning ^{13}C CP-NMR)

Boening (Activated carbon versus resin adsorption of humic substances HA/FA)

Bollag (Cross-coupling of humus constituents and xenobiotic substances - model for probable binding mechanism of pollutants to humic matter)

Bracewell (A pyrolysis-gas chromatography method for discrimination of soil humus type)

Buffle (The use of ultrafiltration for the separation and fractionation of organic ligands in fresh water)

Bulman (Interactions of transuranics and cerium with humates)

Campbell (Inorganic and organic ligand binding of lead and cadmium)

Cammarata (Simple introduction to micellar phenomena)

Carlberg (Influence of aquatic humus with time on alkanes, PAH and chlorinated hydrocarbons. Feasibility of liquid/liquid extraction or resin (XAD-2) adsorption for analysing pollutants in humus water have also been studied)

Chiou (Water solubility enhancementof some pollutants, pesticides by dissolved humic/fulvic acids)

Choudhry (Humic substances, structural aspects, photophysical, photochemical and free radical characteristics)

Choudrhy (Environmental photoincorporations of polychlorobenzes into several humic model monomers using UV light

Christman (Priorities in humic research)

Christman (Increase understanding of chemical structure of aquatic humic material, and behaviour during chemical oxidation in particular chlorination)

Cleven (Heavy metal (Cd, Pb, Zn)/humic acid interactions studied by polarography, conductometry)

Collins (Characteristics of organic matter in water appear to affect removal of organic matter by employed water treatment processes)

Curtis (Humic acid fractionation using a nearly linear pH gradient)

De Haan (UV spectroscopy, gel filtration, Curie-point pyrolysis/MS of FA from lakes)

De Leer (Production of cyano compounds on chlorination of humic acid)

Dempsey (Proton and calcium intraction of four fulvic acid fractions)

De Nobili (Possibility of employing 2D electrophoresis to study humic substan - molecular weight determination)

Donard (Corrected fluorescent excitation spectra of FA-comparision with UV/visible absorption spectra)

Dzombak (Theory underlying discrete and continuous multiligand models for metal-humate binding critically examined)

Ertel (Difference spectroscopic properties, marine, terrestrial and model HA)

Evans (Method for carboxyl determination for submilligram quantities FA)

Evans (Removal of humic acids from river water with weak base resins)

Eward (Spectrofluorimetry of HS)

Farrah (Concentration of humic acid from tap water)

Fish (Models metal-humate interactions are compared/analysed using synthetic and experimetal data)

Francko (Release of phosphate from humic-iron complexes)

Frimmel (Influence of photochemical rxns on optical properties of HS)

Gabbita (DBCP residues detected in commercial Na humate significance discussed)

Gadel (Structural analysis of HS-deposits blue lake Greenland influence blue algae)

Gardner (Characterization of UV - absorbing dissolved humic material by liquid chromatography (HP SEC)

Gauthier (Fluorescence quenching method for determining equilibrium constants for PAH binding dissolved humic materials)

Gillam (Structual elucidation of dissolved marine HS by ^{13}C-NMR)

Gloor (Exclusion chromatograph with carbon detection used characterising DOC)

Goodman (Characterization of Fe - fulvic acid complexes Mosbauer and EPR spectroscopy)

Gregor (Effect of extraction procedures FA properties)

Hänninen (Synthesis and characterization of humic acid - like polymers)

Hänninen (Fulvic acids extracted peat studied ^{13}C - NMR - Cu oxdn)

Hatcher (Solid-state ^{13}C - NMR on sedimentary HS)

Heiden (HA prevents loss of trace Hg(11) from aqueous solns)

Herrmann (Photoreactivity of HA in natural waters)

Hirose (Separate fulvic acid by RP-HPLC)

Ho (Amino acid compositions and enantiomeric ratio of humic-like substances extracted fossils and seawater by SEP-PAK C_{18} and Amberlite XAD-2 resins)

Huck (Assessment of products of rxns of water disinfectants with HS and trace organic contaminants)

Hunter (Role of aqueous HA/FA as natural surface active organic compounds and their interaction with other surface-active substances-polarographic study)

Jensen-Korte (Photodegradation of pesticides in the presence of HS)

John (Influence of aquatic humus and pH on uptake of Cd by salmon)

Johnsen (Variation of physical, chemical properties of aquatic humic substance in one year)

Jouany (Wetting properties of Fe and Ca humates)

Kango (Investigate interaction properties of HA with certain heavy metals Ca_1, Zn, Fe, Mn)

Khan (Role of HS in predicting fate and transport of pollutants in the environment)

Kögel (Decomposition in forest humus layers studied by various techniques)

Kortelainen (Acidity and humic matter in lakes)

Kotzias (Factors affecting photoinduced formation of O_2 species, and their rxns with organic chemicals in aqueous solutions were examined)

Kulovaara (Complexation of chlorophenolics with aquatic humus)

Kunin (Removal of humic material from drinking water by anion exchange resins)

Lamy (Binding strenth evaluation of Cu(11) complexation with organic ligands)

Langford (Interaction of metal ions with well characterized FA)

Lawrence (Semi-quantitative determination of FA using UV)

Lee (Demonstrates potential of RPLC for separation of high molecular weight natural water organics)

Levitz (Flourescence decay study of the adsorption of non-ionic surfactants at the solid-liquid interface. Influence of polar chain length)

Lochmüller (Conformational changes in a soil fulvic acid measured by time-dependent fluorescence depolarization)

Love (Characteristics of surfactant aggregates advances of use in analytical schemes)

Mantoura (Optimal conditions evaluated for the effecient recovery of HA/FA with adsorbent columns XAD-2)

Martin (Composition/property changes in HS with diborane reduction)

Mc Knight (Changes in dissolved organic material in Spirit Lake result volcano)

Melcer (Characterization HA, comparison HS different sources, ^{13}C, ^{1}H NMR, IR, UV, GPC, ash content, TOC, COD)

Metelerkamp (Investigate influence of HA on three organo phosphorous pesticides)

Metelerkamp (2 Enzyme systems used to investigate HA-organophosphate and HA-metal interactions)

Mirave (Fractionation, radioiodination, mobility in soil plates of different molecular weight fractions of HA)

Müller-Wegener (Electron donor acceptor complexes between organic nitrogen heterocycles and humic acid)

Pempkowiak (Phenolic aldehydes as indicators of origin of HS in marine environments)

Pennanen (Use of dilution to overcome quenching effects when measuring fluorescence)

Perdue (Critical examination of metal-ligand complexation models)

Perdue (Association of organic pollutants with humic substances partitioning equilibria and hydrolysis kinetics)

Piccolo (Molecular weight distribution of peat HS extracted with different inorganic/organic solns)

Pilkington (GPC - for determining molecular weight distribution, + applications)

Plechanov (Investigations of HS in natural water)

Prakash (Environmental and human health significance of humic materials)

Randtke (Effect of salts on activated carbon adsorption of fulvic acid)

Reuter (Oxidative degradation studies on aquatic humic substances)

Saiz-Jimenez (Chemical structure of two acid hydrolysed soil HA investigated - plant components identified in soil HA)

Saleh (RP-HPLC provides more unformation on the reference Suwannee River FA)

Sato (Adsorption of mutagens by humic acid)

Schoor (THM yield as a function of precursor molecular weight)

Sedlacek (Isotope exchange between inorganic Fe and Fe naturally complexed by aquatic humus)

Sedlacek (Effect of aquatic humus on uptake and toxicity of cadmium to algae)

Senesi (Theoretical, experimental aspects - humic substances/herbicides bind by charge transfer mechanisms)

Senesi (Cu, Fe complexation by humic acid-like polymers)

Shinozuka (HA increase water solubility of hydrocarbons)

Shuman (Copper-organic complexes)

Skogerboe (Reduction of ionic species by fulvic acid, Hg(111) → Hg(0), Fe(111) → Fe(11), I_2, I_3 → I^-)

Sontheimer (Analysis for determining organic substances in water, particular emphasis UV extinction)

Speteller (Isolation and characterization of dissolved organic carbon from natural water by ultrafiltration)

Steelink (^{13}C NMR probes humic and fulvic acid structures soils)

Steelink (^{1}H-NMR, ^{13}C NMR, ESR spectroscopic studies of humates/fulvates)

Steelink (Structural features of liginins and HS - ^{13}C - NMR)

Summers (Molecular size distribution and spectroscopic characterization of humic substances)

Thorn (Structural characteristics of river HA, FA - ^{13}C NMR)

Thorn (^{13}C NMR characterization of humic materials - isolated by an MIBK partitioning procedure)

Thurman (Separation, purification HS by LC; degradation by oxidative procedures + methylation; identification of products GC/MS, ^{13}C NMR → structure)

Thurman (Preparative isolation of aquatic humic substances)

Tramonti (Interaction of lindane/FA: Effect of solution pH, ionic strength)

Tuschall (Complexation of heavy metals by aquatic humus - a comparitative study of five analytical methods)

Vartiainen (Use of TSK size exclusion columns - quality/quantity of humus in raw waters and drinking waters)

Visser (Effects on fluorescence spectra of molecular weight, functional group, pH, temperature, redox potential)

Visser (Crystal formation by low molecular weight fulvic/humic acids)

Weber (Potential mechanism for removal of HA from water by activated carbon)

Weber (Metal ion speciation studies in the presence of humic material)

Werner (Cation exchange proposed as a method for speciation of Cd^{2+}/Zn^{2+})

Wilson (Compositional, solid state ^{13}C NMR study of humic, fulvic acid)

Yonebayashi (Buffer solution as eluent for GPC - association of HA in buffer solution related to surface active properties of HA)

Zhang (Assessment of separation, analysis of HS by isoelectric focusing)

OVERVIEWS

1. Humic Substances by G G Choudhry. Structural, photophysical, photochemical, and free radial aspects and interactions with environmental chemicals.

2. Aquatic and Terrestrial Humic Materials by R F Christman, E T Gjessing Ch.1.

3. Dynamics, Exposure and Hazard Assessment of Toxic Chemicals Ch.16. Role of HS in predicting fate and transport of pollutants in the environment.

2. SOURCE OF HUMIC MATERIAL

2.1 Water

Aiken (Comparison of XAD macroporous resins for the concentration of FA from aqueous solution)

Anderson (FA - XAD - 8 extraction technique referenced)

Anderson (Trace organic removal by anion exchange resin)

Boening (Activated carbon versus resin adsorption for the removal of humic and fulvic acids from water)

Buffle (Separation and fractionation of organic ligands - in fresh water. Fractionation, centrifugation, ultracentrifugation, GPC, membrane filtration, ultrafiltration, separation, ultrafiltration)

Campbell (Dialysis membrane - separate large molecular weight organically bound metals, from free inorganically bound metals, pH very important)

Carlberg (Humic material. Solvent extraction, resin adsorption, determination concentration of pollutants)

Chiou (Concentrated by the XAD-8 resin technique, fractionated into HA/FA at pH=1. Reference Thurman, Malcom)

Christman (HA/FA isolated referenced. Precipitation of HA, concentration through settling and centrifugation . FA concentrated adsorption XAD-2 followed by base elution)

Chriswell (Comparison of macroreticular resin (XAD-2) and activated carbon as sorbents for isolating organic compounds from water. Recovery organic compounds from resins/carbon discussed)

Collins (Adsorption chromatography using XAD-8 resin was utilised to fractionate the dissolved organic matter into hydrophobic/hydrophilic fractions)

Dempsey (Concentration, cleaning, fractionation, storage of FA Acidified, filtered, XAD-7 resin, eluted HCℓ)

Evans (Removal of organic acids from water with weak base resins)

Farrah (Concentration of humic acid from tapwater, water acidified, passed through filters, HA adsorbed - removed NaOH, HA precipitated using HCℓ, collected centrifugation)

Fox (HA - filtered sample acidified, filtered HA residue extracted filters NaOH)

Gillam (Seawater HS. Filtered, acidified, XAD-2 column, rinsed cold water HS eluted mathanol/ammonium hydroxide, evaporated dryness)

Gloor (Organics concentrated - high capacity weak acid ion exchanger (Sephadex in the Na form), rotary evaporated, redissolved in water))

Herrmann (HS - acidic adsorption to a XAD-2 resin followed by alkaline desorption)

Hunter (Adsorption on XAD-2 of acidified water, adsorbed HA eluted NH_3, concentrated by rotary evaporation and dried in a laminar flow clean bench)

Johnsen (HS, membrane filtered, dialysed towards distilled water, rotary evaporation, freeze-dried, Soxhlet extrated methylene chloride, HPSEC)

Kunin (Removal of humic material from drinking water by anion exchange resins)

Lawrence (Ref. given for water extraction by XAD adsorption method)

Lee (Natural water sample components were separated by RPLC)

Mantoura (Optimal conditions evaluated for the efficient recovery of HA/FA with adsorbent columns, XAD-2 (pH, ionic strength, desorption))

Mc Knight (Aquatic fulvic acid isolated, XAD-8 ref. given)

Melcer (Ref. Thurman. XAD-8 resin preparation. Sample filtered (ultrafiltration-removal suspended particles). Sample acidified, passed through resin NaOH elute organic matter. Extract acidified, passed through ultrafiltration membrane (remove suspended solids), concentrated on membrane, freeze-dried)

Pempkowiak (Acidified water passed through XAD-2, resin washed water, eluted NH_4OH in a water/methanol mixture)

Plechanov (Water-soluable HS extracted using anion exchange and XAD-7. HA/FA isolated. Detailed concentration procedures given)

Reuter (HS adsorption XAD-7, elution triethylamine, extracted chloroform. Aqueous solution-cation, exchange resin (H^+) then freeze-dried)

Schoor (Organics concentrated - centrifuged, freeze-dried (in a lyophilizer). Dissolved in water. Fractionation of organic compounds according molecular weight-GPC)

Spiteller (Isolation of DOC from natural and lysimeter waters by ultrafiltration. Membrane better than XAD - less time consuming - no contact acid/base)

Thurman (Adsorption chromatography, size - exclusion chromatography to obtain HS. Fractionation HA/FA by acid precipitation)

Vartianen (Isolation humus for HPSEC, XAD-8 adsorption - elution ethyl acetate)

Visser (FA/HA separated aquatic origin - ref. given)

2.2 Soil and sediment

Alberts (Sediment, ref given to another paper for HA isolation)

Bracewell (NaOH, acidification referenced)

Bulman (Sediment and soils fractionated by 3 non-destructive procedures)

Chiou (NaOH extraction followed by established fractionation and purification procedures referenced)

De Nobli (NaOH extract, extracts purified with H^+ saturated ion exchanger. Neutralised before filtering through membrane)

Ertel ($NaOH/Na_4P_2O_7$, acidified to precipitation HA, centrifuged)

Gauthier (Dilute base, acidification, purified extraction HF/HCℓ)

Goodman (HCℓ extraction, acidifying solution HA/FA separated by centrifuging. FA isolated by adsorbung on polycar, desorbed NaOH, treated with resin to remove Na, filtered, freezedried)

Gregor (FA, HA - traditional NaOH method described, effects of extraction procedures on FA properties, alternate extraction solvent proposed)

Hänninen (FA - Alkali extraction, acidification, concentration and desalting by dialysing or by sorption on non-ionic and ionic resins)

Hatcher (Treated water, benzene/methanol, HCℓ. Extracted NaOH, centrifuged, ion-exchange separation to separate FA/HA cation exchange, elute acidified to precipitate HA)

Kango (Sediment, $NaOH/Na_2CO_3$, dialysis, HA precipitated HCℓ, basic solution upon adding NH_3)

Melcer (NaOH, centrifuged, acidified. Precipitated HA removed centrifugation FA in solution concentrated by ultrafiltration)

Metelerkamp (Daspoort soil HA, NaOH, HA precipitated upon acidification HA washed acid, HF/HCℓ, water Sac dialysed H^+ resin and freeze-dried)

Pempkowiak (Washed HCℓ, water. Extracted NaOH, centrifuged, acidified. HA precipitate, FA passed through XAD-2)

Piccolo (Molecular weight determination of peat humic substances extracted different inorganic/organic solutions)

Randtke (FA - extracted $K_4P_2O_7$. Acidified HCℓ, centrifuged to remove HA, passed through XAD-8, FA eluted resin with NaOH)

Skogerboe (Ref. given for isolation of FA from soil)

Thorn (Peat/stream sediment - MIBK partitioning procedure, FA/HA humic separated. Ref. given + diagram partition procedure in paper)

Tramonti (NaOH extract, FA containing extract on cation exchange resin, eluted, freeze-dried)

Wilson (NaOH extract, hydrogen - exchanged, freeze-dried, solid products extracted water to obtain water soluable samples (FA), water insoluable samples HA)

Yonebayashi (HA were extracted from these soils and purified according to the IHSS procedure)

2.3 Synthetic

Choudhry (Humic model monomers e.g. benzoic acid)

Cleven (Polyacrylic acid (PAA) - model HA)

Curtis (Hydroquinone - prepare synthetic humic acid)

Ertel (Melanoidin and phenolic humic acid synthesis)

Hanninen (Synthesis and characterization of humic acid - like polymers ^{13}C NMR)

Lamy (PCTG - synthetic humic like substance synthesis referenced)

Metelerkamp (Model humic substance - polymaleic acid (PMA))

Müller-Wegener (Synthetic HA-prepared alkaline oxidation of hydroquinone)

2.4 Commercial

Cleven (Fluka humic material, dialysed, treated ion exchanger)
Curtis (Humic acid - Aldrich)
Gabbita (Sodium humate - sodium salt humic acid - Aldrich)
Gauthier (Humic acid - Aldrich)
Jensen-Korte (HA potassium salt - Roth)
Steelink (Aldrich, Fluka HA - Tridom Chemical (Inc))
Summers (Aldrich humic acid)

3. INTERACTIONS

3.1 With organic compounds

Bollag (Probable binding mechanism of pollutants to humic material)
Buffle (Interaction HS with other dissolved molecules, make the fractionation by ultrafiltration more difficult)
Carlberg (Aquatic humus interactions with alkanes, PAH and chlorinated hydrocarbons investigated. Liquid/liquid extraction, and resin (XAD-2) adsorption method for analysing pollutants - also studied)
Chiou (Solubility enhancement of some organic pollutants, pesticides by dissolved humic/fulvic acid looked at appears related dissolved organic matter molecular size, polarity)
Chiou (Solubilization ability of HA with pesticides)
Choudhry (Sorptive interactions with environmental chemicals, other interactions solubilizing effects, catalytic effects on hydrolysis, effects on microbial processes, photosensitizing and quenching effects)
Choudhry (Investigations of the photochemical interactions of six PCBzs with several humic model monomers in aqueous acetonitrile)
Gabbita (DBCP in Na humate, GC, GC/MS, used to study)
Gauthier (Flourescence quenching used for equilibrium constants PAH and dissolved humic matter, HA/FA)
Herrmann (Reactive oxygen species formed by radiation, may react with organic pollutants initiating break down processes)

Hunter (Interaction between HA/other surface active organics studied by polarography)

Hunter (Polarographic studies study surface active properties as a result of interaction HA/other surface active organics)

Jensen-Korte (Photodegradation of pesticides in the presence of HS)

Johnsen (Ability of HS to complex/adsorp organic micropollutants as function contact time studied. PAH studied as well)

Khan (General discussion. Discusses humic substances - toxic chemical interactions, solubilizing, pesticides can form stable complexes humic materials)

Kotzias (Formation oxygen species and their reactions with organic chemicals in aqueous solution)

Kulovaara (Complexation chlorophenolics with aquatic humus)

Metelerkamp (HA/organophosphorous pesticides studied using enzyme acetylcholinesterase (ACHE))

Metelerkamp (HA/organophosphate using acetlycholinsterase (ACHE) and urease (toxicity looked at))

Müller-Wegener (Electron donor acceptor complexes between organic nitrogen heterocycles and humic acid, UV-VIS spectroscopy)

Perdue (Association of organic pollutants with HS partitioning equilibria and hydrolysis kinetics)

Sato (Adsorption of mutagens by humic acid)

Senesi (Humic substances to bind herbicides by charge transfer mechanisms)

Shinozuka (Humic acids increase water solubility hydrocarbons)

Tramonti (Solution phase intraction of lindane/FA studied GC. Effect solution pH, ionic strength. Ultrafiltration separates free lindane in solution from FA having bound lindane)

Tramonti (GC - used to destinguish between bound/free lindane))

3.2 With metals

Alberts (Effect of metal ions on UV spectra HA)

Alberts (Models, trace metal - naturally occurring humic material interaction)

Bulman (Interaction of transuranics, cerium with humates - Irving Williams series given: Cu(11) >Ni(11) >Pb(11) >Co(11) etc)

Campbell (Organic ligand binding of Pb, Cd - Dialysis membrane separate large molecular weight bound metals from free and inorganically bound metals)

Christman (Metal-ion interactions. Priorities in humic research)

Cleven (Heavy metal/HA - studied normal pulse polarography, conductometry)

Dempsey (Proton and calcium complexation of four FA fractions)

Dzombak (Models for metal - humate binding critically examined)

Fish (Models for metal-humate interactions compared using experimental and synthetic data)

Francko (Release of phosphate from humic-iron complexes)

Gardner (Effect Ni, Cu,Ca to UV - absorbing properties dissolved organic matter) (mechanism of metal-interactions))

Goodman (Iron fulvic acid complexes: dependence pH, iron-fulvic acid ratio) (Mossbauer, EPR spectroscopy))

Gregor (Metal-binding properties of FA compared as a result of two different extraction methods)

Heiden (HA prevents loss of trace Hg(11) from aqueous solutions)

John (Influence of aquatic humus and pH on the uptake of Cd by fish)

Jouany (Wetting properties of Fe and Ca humates, studied characterised DTA, IR)

Kango (Solubilization of Ca, Zn, Fe, Mn by humic acid)

Kango (Solubilization ability of HA with respect to Ca, Fe, Zn, Mn)

Khan (General discussion)

Lamy (Variation with pH of both proton and metal binding by a humic-like substance. pH, ion selective electrode titration data, binding strength evaluation)

Langford (Interactions metal ions with well characterized FA, aggregation induced by metal ions is explored)

Lee (In RPLC metal (Cu, Co) eluted with more polar fractions water)

Metelerkamp (HA/metal interacts lower toxic effect of metals)

Perdue (Critical examination of metal-ligand complexation models)

Randtke (Effect of salts on activated carbon adsorption of FA, Ca, Mg, Na)

Sedlacek (Isotope exchange between inorganic Fe and Fe complexed by aquatic humus)

Senesi (Cu, Fe complexation by HA-like polymers from soil fungi, amount metal adsorbed, type of complex formed, water, HCℓ stability, AAS, IR, ESR)

Shuman (Copper - organic complexes, stability constants dissociation rate constants)

Skogerboe (Reduction of ionic species, Hg, Fe, I by fulvic acid)

Tuschall (Complexation of heavy metals by aquatic humus, Zn, Cu, Cd)

Weber (Metal ion speciation studies in the presence of HS - model proposed)

Werner (Ca^{2+}, Zn^{2+} interactions, pH dependence)

3.3 With activated carbon

Randtke (Effect of salts on activated carbon adsorption of FA (influence inorganic anions, organic anions also discussed))

Weber (Adsorptive affinity of GAC for HA is dependent on the presence and concentration of water constituents including Ca^{2+}, Mg^{2+}. Proposed mechanism ion-humate carbon complexes)

3.4 Aggregation

Buffle (Aggregates affecting ultra filtration-difficult)

Cammarata (Micellar phenomena - introduction critical micelle concentration)

Hunter (Studied using polarography)

Langford (Aggregation induced by metal ions briefly explored FA)

Levitz (Fluorescence decay study of the adsorption of non-ionic surfactants at the solid-liquid interface. Influence of polar chain length adsorption above/below cmc discussed)

Lochmüller (FA disaggregation, molecular expansion favoured dilute solution at high pH. Contraction of molecular units, aggregation promoted higher concn, or when sample is protonated in a medium of high ionic strength)

Love (Charactersitics of surfactant aggregates, recent advances in analytical schemes)

Perdue (Micelles - first approximation to study humus pollutant interactions)

Yonebayashi (Large humic molecules associate with small ones to form micelles-like aggregates. Surface tension measured)

4. CHEMICAL REACTONS

4.1 Disinfection

Anderson (Extent of ozone's reaction with isolated aquatic fulvic acid)
Arai (Ionizing radiation/ozone degrade HA, then chlorinate)
Christman (Chlorination of aquatic humic substances)
Christman (Disinfection reactions-priorities in humic research)
De Leer (Production of cyano compounds on chlorination of HA)
Huck (Products of reactions drinking water disinfectants with HS and trace organic contaminants)
Pempkowiak (Nitrobenzene oxidation of HS)
Reuter (Oxidative degradation studies on aquatic HS)
Schnoor (Trihalomethane yield as function of molecular weight, chlorination)
Thurman (Chlorination)

4.2 Irradiation

Choudhry (Photochemical interactions of 6 PCBzs with several humic model monomers in aqueous acetonitrile)
Francko (Photodependent release of phosphate from humic-iron complexes)
Frimmel (Influence photochemical reactions on HS, UV-VIS, fluorescence, FT-IR laser flash)
Gloor (Reaction organic matter ozone. Effect on molecular weight)
Herrmann (Photoreactivity of humic acids in natural water)
Jensen-Korte (Photodegradation of pesticides in the presence of humic substances)
Kortzias (Humic materials react O_2 generation of reactive oxygen species upon illumination of HS, radicals, reactions with organic chemicals)
Mc Knight (Oxidative change in DOC as a result of exposure to sunlight and oxygenated conditions)

4.3 Other reactions

Choudhry (Free radical characteristics)
Martin (Compositional/property changes on diborane reduction HS)
Reuter (Methylation)
Thurman (Methylation)

5. CHARACTERISATION

Choudhry (Molecular weight, elemental, functional, NMR, structure, UV-VIS, fluorescent, origin)

5.1 Elemental composition, functional groups and properties

Anderson (Characterisation of aquatic organics, pH)
Anderson (Characterised FA, TOC, UV-VIS, XAD-8, ultrafiltration)
Choudhry (Typical elemental composition, functional groups)
Collins (Potentiometric titrations to determine carboxylic acidity + general characteristics)
Evans (Carboxyl group determination, new method discussed, old method ref)
Ewald (HS for estuary characterised by spectrofluorimetry)
Fox (Salinity, HA carbon, salt precipitated carbon, dissolved organic carbon, high molecular weight carbon, total amino acids, individual amino acids, elemental analyses etc.)
Gregor (Effect of extraction procedures on molecular weight distribution, equivalent weight (mass per mol COOH), acid dissociation constants (pKa) and metal bunding strength of FA)
Hänninen (Total carbohydrate content, CuO oxidation method referenced)
Kango (C,H,N - dry combustion method, potentiometric studies, pH)
Khan (Degradative/nondegradative characterisation - general discussion)
Kögel (CuO oxidation - sum phenolic compounds, acid/aldehyde ratio method ref.)
Kortelainen (Acidity, ion balances and humic matter in lakes)

Kunin (Typical, N etc)

Martin (Composition, property changes due to diborane reduction HS solubility, ionic mobility, elemental composition, spectral character)

Mc Knight (Elemental analysis, potentiometric titraction + acidic, ^{13}C-NMR functional group content and Cu-binding capacity FA, ref. given)

Melcer (Ash content, TOC, COD)

Plechanov (Potentiometric titrations, functional group, elemental analysis)

Saleh (RP-HPLC study on FA polarity components, solubility parameters, hydrocarbonaceous surface areas, % hydrophobic neutral constituents)

Sontheimer (Summary parameter for determining organic substance water)

Summers (Chemical composition, determined by IR, not differ between molecular weight-ultrafication)

Thurman (Characterisation methods - carbohydrate, phenolic, carboxylic acids)

Tramonti (Preparation and characterisation of FA referenced)

Vartiainen (Peaks areas separated HPSEC-related TOC, $KMnO_4$, colour values)

Visser (Crystal formation by low molecular weight fulvic/humic acid)

Wilson (Characterisation of sample by elemental/functional group - used method to place structural limits on composition + estimate probable distribution of aliphatic, aromatic, and "excess" C)

Zhang (IEF method for characterisation of HS rather than a separation technique)

5.2 Visible and ultraviolet light-absorbing characteristics

Alberts (Effect of metal ions on UV spectra)

Buffle (Concentration of organic matter)

De Haan (UV-VIS spectra of FA from lakes related humification)

Donard (Comparison corrected fluorescence spectra with UV-VIS spectra FA)

Ertel (Comparison marine, terrestrial, model HA)

Frimmel (Influence photochemical reactions on HS - UV-VIS spectra)

Gardner (UV adsorbing dissolved organic matter - fractionated by HPSEC. Characterisation of UV absorbing dissolved humic meterials from natural water)

Jensen-Korte (Photodegradation of pesticides)

Johnsen (Relation between DOC and UV-VIS for membrane filtered and dialysed humic water samples)

Kotzias (Influence of UV - irradiation of HA, production stable free radicals)

Lawrence (Semi-quantitative determination of FA using UV)

Love (Applications of micelles in analytical chemistry)

Muller-Wegener (Complexes between organic nitrogen heterocycles and humic acid studied)

Plechanov (Characteristics HS in natural water)

Saleh (Fractionate HA using RP-HPLC -UV-spectra fractions)

Sontheimer (UV extinction discussed with respect to characterisation of organic substance in water)

Summers (Differences in UV-VIS spectra (E_4/E_6 related molecular size UV absorbance good surrogate parameter)

Weber (UV adopted for routine quantitative analysis of HA concentration)

5.3 Fluorescence

Boening (Fluorescence used to quantitatively analyse humic materials)

Buffle (Concentrations of organic matter)

Donard (Corrected fluorescence extraction spectra FA comparison UV-visible spectra)

Ewald (Fluorescence from photic zone water - Atlantic ocean) compared fluorescing of estuary water)

Ertel (Comparison marine, terrestrial, model HA)

Frimmel (Influence photochemical reactions on fluorensce HS)

Gauthier (Fluorescence quenching method equilibrium constants between PAH and dissolved humic material)

Jensen-Korte (Photodegradation of pesticides)

Levitz (Fluorescence decay study of the adsorption of non-ionic surfactants at the solid-liquid interface 2. Influence of polar chain length)

Lochmüller (Time-dependent fluorescence depolarization spectroscopy used study conformational changes in soil FA)

Pennanen (Use of dilution to overcome quenching effects when measuring fluorescence of humic lakes)

Plechanov (Natural water)

Saleh (Fluorescence chromatogram of FA fractionated RP-HPLC)

Tuschall (Fluorescence quenching - study metal - humate interactions)

Visser (Fluorescence of humic material of aquatic origin - dependence on pH, molecular weight, functional groups, pH, temperature, redox potential FA/HA)

Weber (Fluorescence quenching - study metal - humate interactions)

5.4 Structure studies

Alcaniz (Py-GC, Py-MS of soil - discrimination between soil type)

Bracewell (Polycarboxylic acids as the origin of some pyrolysis products characteristic of soil organic matter)

Bracewell (Pyrolysis - gas chromatographs - mass spectra of soils)

Bracewell (Pyrolysis - mass spectrometry studies of humification in peat)

Barron (Humic soil and coal structure with magic angle spinning ^{13}C CP-NMR)

Choudhry (NMR spectrometry, products obtained degradation, physical separation. Working hypothesis on chemical structure)

Christman (Identification of degradation products of aquatic humus on chlorination)

De Haan (Pyrolysis MS studies of FA)

Ertel (Comparison of marine, terrestrial, model HA, IR, UV-VIS, fluorescence, ESR)

Gillam (^{13}C NMR - structure elucidation of dissolved marine HS)

Gadel (IR, NMR, Py-GC/MS of HS from deposits of blue lake Greenland - influence algae)

Hänninen (Synthesis and characterisation (^{13}C-NMR) of humic acid-like monomers)

Hänninen (^{13}C-NMR spectra, Cu oxidation - of FA from peat)

Hatcher (^{13}C-NMR of sedimentary HS)

Kögel (Decomposition forest humus layer studied by CPMAS, ^{13}C-NMR pyrolysis-field ionization-mass spectrometry and CuO oxidation)
Kunin (Chemical degradation products of aqueous humic substances)
Martin (Effect on IR spectra of HS due to diborane reduction)
Mc Knight (^{13}C-NMR spectra of aquatic FA)
Melcer (IR, ^{1}H NMR, ^{13}C NMR)
Plechanov (^{1}H NMR, IR, HS in natural water)
Reuter (Oxidative degradation studies of aquatic humus, GC/MS)
Saiz-Jimenez (Structure acid hydrolysed soil HA - pyrolysis plant components identified in HA)
Spiteller (Fractions isolated by ultrafiltration characterised by ^{13}C-NMR)
Steelink (Magnetic resonance studies of (^{1}H-NMR, ^{13}C NMR, ESR) of humates and fulvates)
Steelink (^{13}C NMR probes humic and fulvic acid structures soil)
Steelink (Structural features lignin, HS - ^{13}C-NMR)
Thorn (Structural characteristics of river FA/HA determined by ^{13}C-NMR)
Thorn (^{13}C NMR characterisation of humic material isolated by an MIBK partitioning procedure)
Thurman (^{13}C-NMR, GC/MS)
Wilson (Solid state ^{13}C-NMR study of HA/FA fractions of soil organic matter)

6. FRACTIONATION

6.1 Apparent molecular mass/size fractionation

6.1.1 Ultrafiltration

Collins (Ultrafiltration - stirred cell membranes)
Mirave (Fractionation of HA by ultrafiltrate membranes into high, medium molecular weight fractions)
Spiteller (Isolation of DOC obtained by membrane-ultrafiltration)

Summers (Ultrafiltration-membranes)

Visser (HA/FA separated, purified and fractionated into 8 molecular weight fractions by ultrafiltration)

Visser (Molecular weight separation by ultrafiltration Amicon Diaflo ultrafiltration membranes, fractionations by ultrafiltration were also done by Anderson, Pilkington, Plechanov and Randtke)

Fractionations by ultrafiltration were also done by Anderson, Pilkington, Plechanov and Randtke.

6.1.2 Gel permeation/Size exclusion chromatography (SEC)

Bulman (GPC - alternative to Sephadex for radionuclides)

De Haan (Sephadex gel filtration - most successfully applied fractionation technique of HS from fresh water - FA example)

Gardner (Water fractionation into chemical distinct groups by HPSEC-TSK 3000 sw size exclusion columns - UV detection)

Gloor (Sephadex, TSK SW improvement, elution dependent on ionic strength and pH eluent)

Johnsen (HP-SEC to determine molecular weight distribution,TSK-G 3000 SW)

Melcer (Soil/water samples characterised, Sephadex)

Piccolo (Sephadex-gel columns molecular weight distribution of peat HS extracted different inorganic/organic solvents)

Pilkington (Recent developments, improvement on sephadex, principle given - molecular weight distribution organic compounds in aqueous media, no sample preconcentration)

Plechanov (Gel filtration - Sephadex)

Schnoor (Sephadex)

Spiteller (Isolation of DOC obtained by membrane ultrafiltration characterised gel filtration - Sephadex)

Vartiainen (Use of TSK size exclusion columns - HPSEC - in determining quality and quantity of humus in raw water/drinking water GPC - Sephadex, HPSEC - macroporous silica-particle TSK column)

Yonebayashi (Neutral phosphate butter containing urea recommended eluent-Sephadex)

6.1.3 Other separation techniques based on molecular size/mass

Anderson (Molecular size separation of HS using XAD-8)

De Nobli (Possibility of employing 2D electrophoresis to study molecular weight distribution of HS)

Mantoura (HA/FA can be fractionated on a molecular weight basis using desorption from XAD-2, by serial elution at selected pH)

6.2 Other fractionation techniques

Anderson (XAD-8 exclusion chromatography - characterise changes in FA molecule during ozonation)

Curtis (HA fractionation using a nearly linear pH gradient to elute from XAD-8 method could be used finger printing technique for HA and as a means of fractionating these materials prior to further characterisation)

Collins (XAD-8 resin used to fractionate dissolved organic matter in water samples into hydrophobic/hydrophilic fractions)

Dempsey (FA was separated into 4 fractions based on desorption from a non-ionic resin (XAD-7) with increasing pH)

Gregor (XAD resins for desalting FA)

Hirose (Separate FA into freactions using RP-HPLC)

Saleh (Fractionate FA using RP-HPLC)

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