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FORMATION OF MUTAGENIC ORGANIC BY-PRODUCTS AND AOX BY CHLORINATION OF FRACTIONS OF HUMIC WATER

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Abstract—Dissolved organic matter from a lake was isolated and fractionated by sorbtion on an XAD-8 column and then separated into humic and fulvic acids. After chlorine treatment of the fractions 2-chloro-3-dichloromethyl-4-oxo-butenoic acid, MX, and three related compounds were determined by means of GC/MS in the selected ion monitoring mode. Mutagenicity was determined in the Ames test and adsorbable organic halogen (AOX) was determined by adsorption to activated carbon followed by neutron activation analysis. AOX formation per unit organic carbon was similar in the hydrophilic and hydrophobic fractions, but the hydrophobic fraction produced more mutagenic activity and MX than the hydrophilic one. The humic acid fraction produced slightly more mutagenicity and MX than the fulvic acid fraction per unit organic carbon. However, there are more fulvic acids than humic acids in natural waters and therefore fulvic acids contribute more to the mutagenicity and MX formation in chlorinated natural waters.

Key words—humic water, fractions of, chlorine-treatment, mutagenic by-products

INTRODUCTION

Chlorine treatment of natural waters gives rise to mutagenicity and the potent mutagen 2-chloro-3-dichloromethyl-4-oxo-butenoic acid (MX) (Holmbom *et al.*, 1981; Loper, 1988; Meier, 1988). The mutagenicity is formed from reactions between chlorine and dissolved organic material in the water (Holmbom *et al.*, 1981; Meier *et al.*, 1983; Hemming *et al.*, 1986). Humic and fulvic acids constitute the major part of the organic material in natural waters. The structure of humic and fulvic acids has been studied by modern NMR-techniques (Norwood *et al.*, 1987; Reckhow *et al.*, 1990; Pihlaja *et al.*, 1991). These studies have focused especially on the content of aromatic and phenolic units, since it has been suggested that the formation of many halogenated by-products in the chlorination of natural waters is proceeding via chlorine attack on the aromatic nucleus with subsequent fragmentation (Norwood *et al.*, 1987; Reckhow *et al.*, 1990). However, few attempts have been made to correlate the chemical structure of this natural material to the formation of mutagenicity after chlorine treatment (Horth, 1989). Some studies with model compounds of humus (Horth, 1989; Norwood *et al.*, 1980; Peters, 1991; Långvik *et al.*, 1991a) and lignin (Rapson *et al.*, 1980) show that mutagenicity and/or MX as well as compounds chemically related to MX are formed by aqueous chlorination of mainly phenolic compounds. MX is the strongest mutagen identified in chlorine treated waters, thus far. It is a matter of discussion, though, if and to what extent these precursor structures are present in natural humus and/or lignin.

We fractionated dissolved organic matter in humic water from a lake (lake Savojärvi), free of industrial pollution, by sorbtion on XAD-8 according to a method developed by Thurman and Malcolm (1981). The formation of four organic, chlorinated by-products (Fig. 1), mutagenicity and adsorbable organic halogen (AOX) of the fractions and of unfractionated humic water were determined after aqueous chlorination. The construction of lake Savojärvi humic and fulvic acids has been studied by ^{13}C-NMR techniques (Pihlaja *et al.*, 1991) and the parameters studied were correlated to the aromaticity of the fractions. Chlorine doses were supplied in relation to the TOC (= total organic carbon) content of the waters. Milled wood lignin (MWL) was chlorinated as a reference. Lignin is a well-characterized material known to produce mutagenicity and MX during chlorination under acid conditions (Holmbom *et al.*, 1984; Holmbom, 1990). Lignin is assumed to be the main precursor of humic material (Sjöström, 1981).

EXPERIMENTAL

Isolation of humic material and TOC determinations

Natural humic water (138 l.) was collected from lake Savojärvi, situated in a marsh region in the southwest of Finland. The water was prefiltered through a Millipore Polycard 0.5 μm CRA 5 (71026) and a 0.45 μm Millicard

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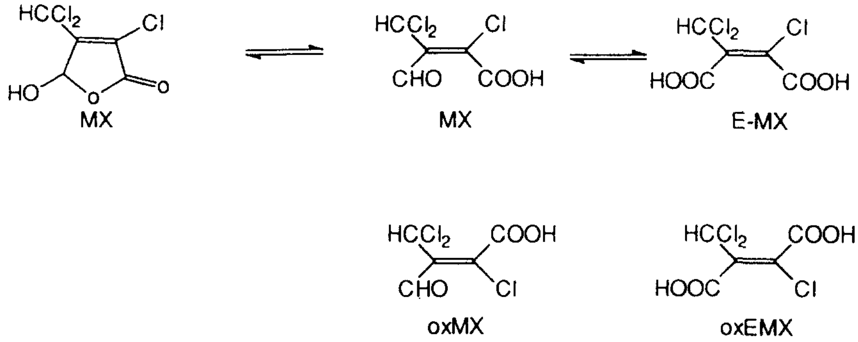

Fig. 1. Structures of the studied organic by-products formed by chlorination of humic waters.

membrane filter CWSC (71 TP3). Dissolved organic material in the water was adsorbed to an XAD-8 column (30 x 5.4 cm) in several runs (approx. 0.4 l/min.). The eluting water is called the hydrophilic fraction and the adsorbed material, eluted with 0.1 N NaOH, is called the hydrophobic fraction in accordance with generally used terminology. The hydrophobic fraction was further separated into humic (HA) and fulvic (FA) acids by the method of Thurman and Malcolm (1981). The spruce MWL studied was obtained from the Institute of Wood Chemistry, Riga Latvia.

The TOC content of the waters were analyzed with a Shimadzu carbon analyzer (model TOC 5050).

Chlorination and treatment of samples

The waters and the water solutions of humic and lignin material were chlorinated at ambient temperature in the dark with freshly prepared hypochlorous acid/sodium hypochlorite solution at pH 7.0 ± 0.2 until the residual chlorine was <0.15 mg/l, about 12–24 h depending on the chlorine dose. The chlorine doses used were 0.5:1, 1:1 and 2:1 (chlorine:TOC). HA, FA and MWL were dissolved in distilled deionized water at about 20 mg TOC/l at pH 10, whereafter pH was adjusted to 7.0. Further treatment of the chlorinated water samples were as described before, i.e. diethyl ether extraction after acidification to pH 2, evaporation to dryness and dissolution of the extract in ethyl acetate (Långvik *et al.*, 1991a).

AOX analysis

The adsorbable organic halogen analyses were performed at the Technical Research Centre of Finland (Espoo, Finland) with activation carbon concentration and instrumental neutron activation analysis (Manninen, 1990).

Mutagenicity

Mutagenicity of the extracts was determined with the Ames test, using tester strain TA 100 (*Salmonella typhimurium*) without metabolic activation (Ames *et al.*, 1975). Just before testing, the solvent was changed to dimethyl sulfoxide, which is relatively non-toxic to the bacteria (Tikkanen and Kronberg, 1991). The tests were performed in duplicates at three dose levels. The tests included positive (0.5 μg Na-azide per plate) and negative controls. Samples which doubled the spontaneous reversion rate and caused a dose-dependent increase in the revertant number (linear regression; $r \geqslant 0.97$) were considered to be mutagenic. The calculation of MX contribution to the total mutagenicity was based on a value of 6200 net revertants per nmol of MX (Tikkanen and Kronberg, 1990).

Analysis of MX, E-MX, oxMX and oxEMX

Two methylation procedures were employed. For the analysis of MX and *E*-MX the methylation was performed with acidic methanol at 70°C for 1 h (Hemming *et al.*, 1986). When the oxidized forms of MX and *E*-MX were analyzed the methylation was performed with 10% BF_3 in methanol for 17 h at 100°C. The reaction mixture was neutralized with 4% $NaHCO_3$ and extracted twice with 0.4 ml *n*-hexane. The combined hexane extracts were concentrated under nitrogen gas. Quantitative determinations were carried out relative to the standard mucobromic acid added to the extracts immediately before methylation. The GC/MS analysis was performed in the selected ion monitoring mode and the column used with a 60 m DB-1 (J & W Scientific, Palo Alto, Calif.). Identification of the compounds was based on positive matching of retention times as well as relative peak area ratios (Hemming *et al.*, 1986; Kronberg *et al.*, 1991).

RESULTS AND DISCUSSION

Isolation of humic material and TOC-values

After filtration the TOC value of the lake water was 21 mg/l. Only 6 mg/l was recovered in the hydrophilic fraction and about half of the original TOC was recovered in the hydrophobic fraction. Thus about 20% of the dissolved organic material was not recovered. The hydrophobic material was further fractionated into about 20% humic acids and 80% fulvic acids. Our TOC results are in accordance with those of a similar fractionation of lake Savojärvi water (Pihlaja *et al.*, 1991).

AOX analysis

AOX values generally increased with chlorine dose at the chlorine doses studied (Fig. 2). The hydrophilic fraction was approximately as potent in forming AOX (μg/mg TOC) as the raw water whereas the fulvic acid fraction, the humic acid fraction and MWL produced lower levels. As the TOC value of the hydrophobic fraction was higher than that of the hydrophilic fraction it naturally produced more AOX totally although the potentials for AOX formation were equal in the two fractions. It is somewhat surprising that the hydrophilic fraction had as high a potential to form AOX as the hydrophobic fraction since it has been suggested that most of the halogenated organics would be formed from the reactions between chlorine and aromatic structures (Norwood *et al.*, 1987).

Our results indicate that the more hydrophilic structures (like sugars and amino acids) present in the hydrophilic fraction react as well with chlorine as the

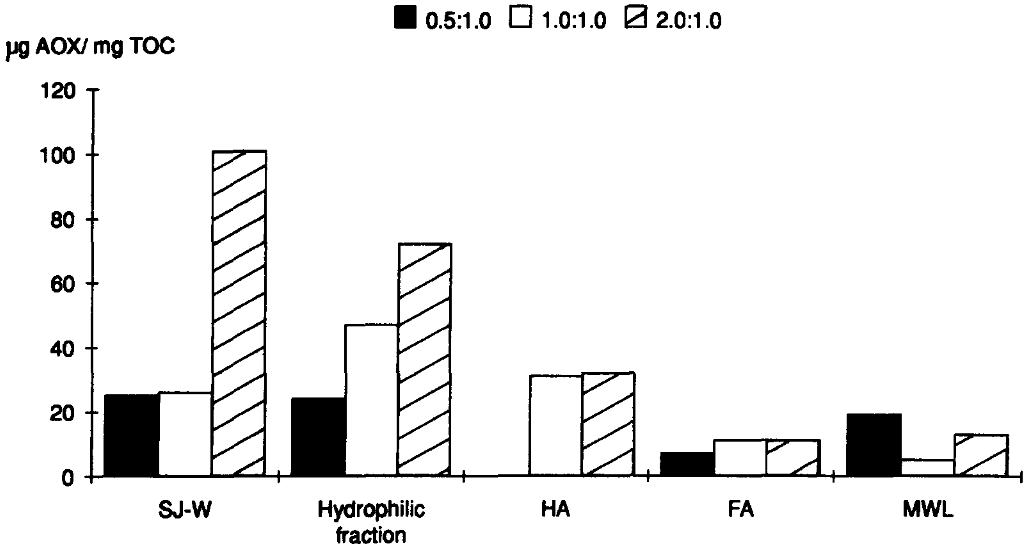

Fig. 2. AOX in chlorinated fractions of natural humic-rich water. SJ-W: Savojärvi water; HA: humic acids; FA: fulvic acids; MWL: milled wood lignin. The legend at the top indicates the chlorine doses (mg Cl_2/mg TOC) used.

humic acid fraction and fulvic acid fraction. The humic acid fraction produced more AOX per unit TOC than the fulvic acid fraction did. This was also found by Reckhow and co-workers (1990).

Mutagenicity

The raw water was mutagenic after chlorination at all chlorine dose levels used (Fig. 3). The response increased with chlorine dose up to equivalent chlorine to TOC doses (1:1; mg per mg). At higher doses (2:1) the mutagenic response decreased, which is in agreement with earlier findings (Kronberg *et al.*, 1985).

The hydrophilic fraction of the water had the lowest mutagenic activity of all fractions expressed per unit TOC as well as totally. The lowest chlorine dose was not mutagenic at all. Thus in the lake water studied this fraction contributes least to the mutagenicity. This is interesting in view of the fact that the AOX forming potential was similar in the hydrophilic and hydrophobic fractions. The hydrophobic fraction of the water showed a little stronger mutagenic potential than the raw water on a TOC basis. Thus it seems that the fractionation method concentrates the structures that produce most mutagenicity upon chlorination. The drop in activity at the highest chlorine dose, observed for the raw water was not seen for the hydrophobic fraction. After separation of the hydrophobic material into humic and fulvic acid

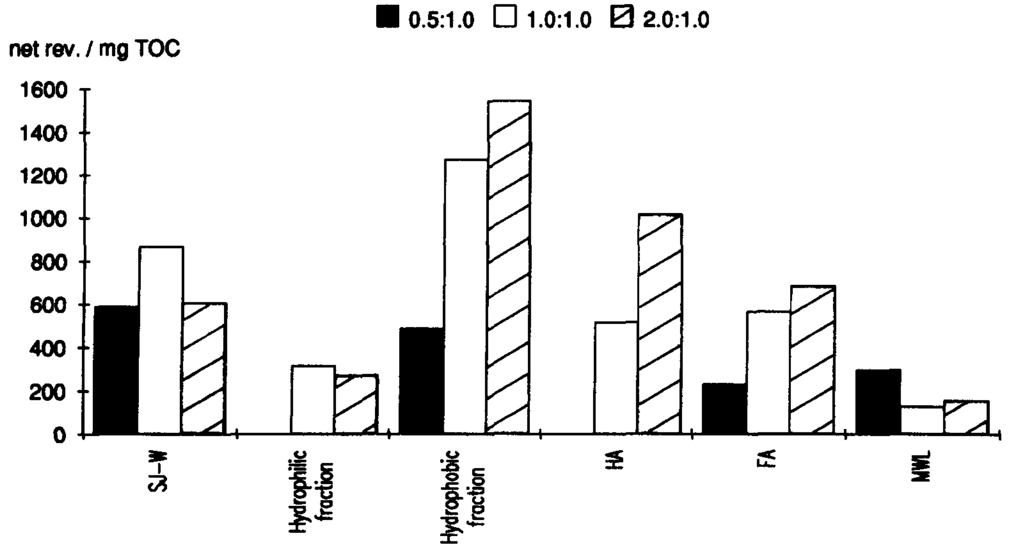

Fig. 3. Mutagenicity in chlorinated fractions of natural humic-rich water. For abbreviations see Fig. 2.

Table 1. Formation of MX and related by-products by aqueous chlorination of natural waters and the calculated mutagenicity contribution from MX

Sample	Cl-dose*	MX†	*E*-MX†	oxMX†	oxEMX†	Mutagenicity‡	MX contribution (%)§
SJ water	0.5:1	7	9	—	7	589	34
	1:1	36	171	4	159	866	115
	2:1	21	222	12	760	605	99
Hydrophilic fraction	0.5:1	—	65	—	78	nm	
	1:1	—	82	9	160	316	
	2:1	—	30	4	165	270	
Hydrophobic fraction	0.5:1	14	23	—	43	486	82
	1:1	20	106	6	586	1270	45
	2:1	29	142	13	792	1545	54
Humic acids	0.5:1	—	21	—	43	nd	
	1:1	10	409	3	223	514	56
	2:1	31	627	13	442	1016	87
Fulvic acids	0.5:1	—	44	4	232	232	—
	1:1	14	199	4	311	565	71
	2:1	12	209	14	593	686	50
Milled wood lignin	0.5:1	—	3	1	85	297	—
	1:1	1	5	3	71	129¶	22
	2:1	1	3	3	127	159¶	18

*Chlorine to TOC (mg/mg).
†ng/mg TOC; –, <1 mg/l.
‡Net revertants per mg TOC.
§Estimated from MX TA 100-S9 mutagenicity (6200 net rev. per nanomole). nm, Non-mutagenic; nd, not determined.
¶$r \leqslant 0.97$.

fractions and subsequent chlorination the humic acid fraction showed a slightly stronger mutagenic activity than the fulvic acid fraction (Fig. 3). Horth has reported equal levels of mutagenicity for chlorinated humic and fulvic acids (Horth 1989) on a TOC basis. There are much more fulvic acids than humic acids in water, which indicates that fulvic acids contribute more to the total mutagenic activity of chlorine treated natural waters (Pihlaja *et al.*, 1991).

Lignin is thought to be an important precursor to humic substances. It is known to produce mutagens, including MX, when bleaching pulp with chlorine (Holmbom *et al.*, 1984). MWL was only slightly mutagenic when chlorinated at neutral pH. This may be due to the chlorination pH. The lignin that is chlorinated in pulp bleaching is different from MWL because lignin is extensively modified in the pulping process (Sjöström, 1981). It may be that the conditions used in this study are too mild to fragmentate the lignin structure.

Formation of MX and related compounds

MX. MX was detected in all waters after chlorination, except for in the hydrophilic fraction (Table 1). The observed mutagenic activities correlated well with the concentrations of the very potent mutagen MX. The calculated contribution of MX to the total mutagenicity was between 34 and 115%. The decrease in the MX amounts at the highest chlorine dose for the raw water correlates well with the decrease in mutagenicity. The MX decrease can be explained by the observed further reaction of MX with chlorine at neutral pH (Schenck *et al.*, 1990; Långvik *et al.*, 1991b). This decrease was not seen for humic and fulvic acids, which might be because they have a higher chlorine consumption and thus the chlorine would not be accessable to the reaction with MX at the doses used.

The fractionation of natural humic material showed that most of the MX was formed from structures recovered in the hydrophobic fraction of the water. Humic acids formed slightly more MX on a TOC-basis than the fulvic acids. Fulvic acids are more abundant than humic acids in natural waters and therefore they most probably contribute more to the formation of MX.

The structure of Savojärvi humic and fulvic acids has been studied by ^{13}C-NMR (Pihlaja *et al.*, 1991). There is a difference in the aromaticity of humic and fulvic acid fractions (28.7 and 26.4%, resp.) and in the amount of aromatic hydroxy-groups (1.44 and 1.36 mequiv/l, resp.), with humic acids on a slightly higher level. It has been suggested that MX and mutagenicity may be formed by chlorination of phenolic structures in the humic and lignin material (Horth, 1989; Norwood *et al.*, 1980; Peters, 1991; Långvik *et al.*, 1991a; Rapson *et al.*, 1980). Norwood and coworkers (1980) showed that the aromatic peaks in ^{13}C-NMR of lake fulvic acids decreased after chlorination. To our knowledge there is no report on the formation of MX from non-aromatic compounds by aqueous chlorination. Therefore, it may be that the observed difference in the aromatic character of humic and fulvic acids is reflected in the small difference in MX and mutagenicity formation potential found in this study.

E-MX. The formation of *E*-MX increased with chlorine dose. Increased MX concentrations were generally accompanied by increases in *E*-MX concentrations (Table 1). The concentrations formed were much higher (about 10-fold) than the corresponding MX concentrations. Humic acids formed more *E*-MX than the other fractions. The mutagenicity of *E*-MX is not convincingly proven, but in acid conditions it isomerizes to MX (Holmbom *et al.*, 1989). However, it seems unlikely that *E*-MX will contribute much to the total mutagenicity of chlorine treated natural waters. MWL formed only a little MX and *E*-MX.

oxMX and oxEMX. Very low amounts of oxMX were formed (approx. 1/50 of the oxEMX) in all waters (Table 1). oxEMX amounts were much higher. oxMX and oxEMX are not mutagenic (Tikkanen and Kronberg, 1990). The hydrophobic fraction and fulvic and humic acids formed the highest amounts of oxEMX. In fact, the hydrophobic fraction formed more of all studied compounds and mutagenicity when compared to the hydrophilic fraction. The formation of oxMX and oxEMX were similar in the humic and fulvic acid fractions on a TOC basis. Again, as the amount of fulvic acids is greater than the amount of humic acids in natural waters (Pihlaja *et al.*, 1991), the fulvic acids probably contribute more to the formation of oxMX and oxEMX too.

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