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ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · JUNE 1982

Impact Factor: 5.33 · DOI: 10.1021/es00100a009

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Occurrence of Brominated Alkylphenol Polyethoxy Carboxylates in Mutagenic Wastewater Concentrates

Martin Reinhard* and Naomi Goodman

Environmental Engineering and Science, Department of Civil Engineering, Stanford University, Stanford, California 94305

Kristien E. Mortelmans

Microbial Genetics Department, SRI International, Menlo Park, California 94025

Removal and formation of mutagenic activity during advanced wastewater treatment were investigated by using the *Salmonella typhimurium* strain TA100. The activity of direct acting mutagens appeared to increase at the recarbonation stage, where 6–10 mg/L of chlorine was added. Subsequent activated carbon treatment removed activity effectively but was apparently ineffective in removing precursors. Final chlorination typically with 25 mg/L of chlorine led to the reoccurrence of mutagenicity. Organic concentrates obtained by XAD-8 adsorption were separated by liquid–solid chromatography on silica gel. Mutagenicity was consistently found in the methanol fractions, which were further examined by GC/mass spectrometry. These fractions contained primarily carboxylic acids including brominated and nonbrominated alkylphenol polyethoxy carboxylic acids (BrAPECs and APECs). APECs were brominated during chlorination. Correlation between the occurrence of mutagenicity in the methanol fraction and the formation of BrAPECs suggested potential mutagenicity of these brominated compounds. However, preliminary experiments failed to confirm this hypothesis.

Introduction

An increasing number of communities in the arid Southwest and elsewhere are facing immediate or predicted water shortages. The pressing need for additional water has led to several large-scale research projects to evaluate ground-water recharge with treated wastewater as an alternative to augment ground-water supplies (*1*). In Orange County, Southern California, Water Factory 21 (WF 21), which is operated by the Orange County Water District, has been treating secondary municipal effluent for groundwater recharge since 1976 (*2*). The effluent of WF 21 is injected directly into a freshwater aquifer to prevent seawater intrusion. The potential of using highly treated wastewater for potable purposes led to a study to assess the reliability of the performance of WF 21 (*3–6*). Among the water-quality characteristics evaluated were those included in the primary drinking water standards, general organic and inorganic parameters, viruses, and trace organic contaminants. The removal of a broad spectrum of organic pollutants including chlorinated and nonchlorinated hydrocarbons, phenols, and carboxylic acids has been determined (*5, 6*). However, only a small fraction of the total organic content has been specifically characterized, and questions remain as to the composition and significance of the unknown fraction. In this report we evaluate organic concentrates of WF 21 effluents with respect to their mutagenic activity as determined by the Ames *Salmonella*/microsome test (*7*).

Many mutagens have been shown to be carcinogens, and their presence in water is therefore considered a potential public health hazard (*8*). The occurrence of mutagens has been documented in secondary and tertiary treated wastewater effluent (*9–12*), in drinking water (*13–15*), and in the effluent of paper pulp mills (*16–21*). The fact that mutagens may accumulate in fish (*21*) and in sediments (*16, 22*) indicates that the discharge of mutagens could be significant even at low levels. The origins of environmental mutagens are manifold; for example, byproducts of dye manufacturing (*22*), petroleum-derived hydrocarbons (*23*), diesel exhaust (*24*), certain pesticides (*25*), industrial solvents (*13*), and byproducts of water chlorination (*8, 13*) have been shown to be mutagenic. Mutagens belong to a broad variety of compound classes including nitroaromatics, polynuclear aromatics, nitrosamines (*26*), resin acids (*20*), volatile halogenated hydrocarbons (*13*), and chlorinated aldehydes (*27*).

The knowledge of the chemical properties of the mutagens present in advanced treated wastewater is limited at present, and no specific treatment technology is applied to remove them. However, it has been indicated that activated carbon (AC) adsorption (*9*), sulfite dechlorination (*15, 19*), biological treatment (*18–20*), and storage at high pH (*19*) reduce mutagenicity produced by chlorination. Some of the mutagenicity formed during chlorination appears to be associated with relatively nonvolatile compounds (*15, 20, 21, 28*), but none of the organics identified so far has been potent enough to account for the total activity detected in the samples. Apparently the residues causing the mutagenicity have not been detected with the analytical procedures applied.

Mutagenic concentrates of drinking water and of advanced wastewater treatment (AWT) plant effluent have been characterized in detail by combined gas chromatography/mass spectroscopy (GC/MS) (*9, 29*), but definitive conclusions as to the identity of the mutagens involved could not be drawn. In an effort to isolate specific mutagenic compounds, Tabor and Loper separated fractions by high-performance liquid chromatography (HPLC) to assay for mutagenic activity (*30*). Repeated separation and testing led to the isolation of a compound that was suggested to be a polychlorinated ether. Numerous compounds have been identified in mutagenic paper-pulp mill concentrates (*16, 17, 20, 21*), many of them chlorinated. The list of compounds identified includes aliphatic and aromatic hydrocarbons, phenols, aldehydes, quinones, and various classes of carboxylic acids. Polychlorinated acetone isomers identified in paper-pulp mill effluents were recently shown to be direct-acting, alkali-labile mutagens (*27*).

So that organic residues in biological assays could be tested, various enrichment procedures have been employed. Kopfler et al. used reverse osmosis (RO) to concentrate and isolate gram quantities from large samples up to 15 m^3 in size (*31*). Baird et al. developed a resin sampler for processing samples up to 200 L (*12*). In the studies on mutagenicity in drinking water reviewed by Loper (*14*), adsorption by Amberlite XAD resins (Rohm

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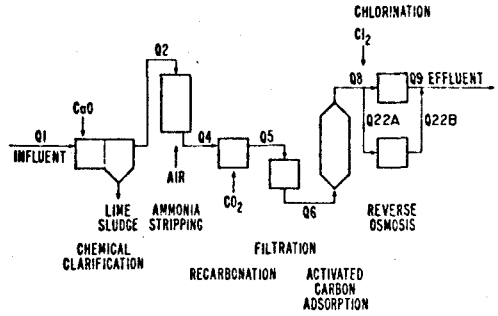

Figure 1. Schematic of WF 21 (this study period).

and Haas, Philadelphia) was the preferred concentration method, and 1–20-L samples were tested.

This study summarizes mutagenicity data obtained during a one and a half year study on WF 21 effluents. Organics were concentrated by adsorption on XAD-8, ion-exchange resins, ultrafiltration, and gaseous purging (*6*). Only in concentrates from XAD-8 were we able to detect mutagenicity; in all other concentrates, the response was negative. The samples tested were up to 1 gallon in size. In this report we emphasize the data obtained from XAD-8 concentrates and discuss the chemical characterization of the mutagenic extracts.

Experimental Section

Water Factory 21. WF 21 is an advanced wastewater treatment facility (*2*) which treats 0.66 m^3/s secondary effluent from an activated-sludge treatment plant. Figure 1 shows a schematic of the plant and indicates the sampling locations. The influent (Q1), was treated by high-lime treatment (Q2), ammonia stripping (Q4), recarbonation with the addition of 6–10 mg/L of chlorine for algae control and nitrogen removal (Q6), dual-media filtration, and granular AC adsorption (Q8). One-third of the AC effluent underwent reverse osmosis (RO) (Q22B); the rest was disinfected with chlorine, typically 25 mg/L (Q9). A detailed description of the plant and its performance has been given previously (*2–6*). Composite samples of 1 L and 1 gallon were taken by WF 21 personnel and shipped to the Stanford University Water Quality Research Laboratory by air freight, where they were received on the same day and refrigerated for up to 4 days until analyzed.

Extraction and Concentration. Methanol (MeOH), dimethyl sulfoxide (Me_2SO), acetonitrile, methylene dichloride ($MeCl_2$), and ethyl ether were "distilled in glass" quality and used as received (Burdick and Jackson, Muskegon, MI 49442). Clean water (MQ water) was produced by a MilliQ water-purification system (Millipore Corp., Bedford, MA 01730). A diagram of the concentration and fractionation procedure developed is depicted in Figure 2. Samples were acidified to pH 2 with concentrated hydrochloric acid. After pH adjustment, the chlorinated 1-L samples were purged with nitrogen for 2 h and the 1-gallon samples for 6 h to remove free chlorine (*18*). Amberlite XAD-8 was purified as described by Leenheer and Huffman (*32*). The XAD-8 columns were packed in acetone in preparative chromatography columns (9 × 250 mm) (Altex, Berkeley, Ca 94710) to 20 mm below the top. After packing, 1 L of water was passed through the columns, followed by two cycles of 100 mL of 0.1 N hydrochloric acid and 0.1 N sodium hydroxide. After a final rinse with 100 mL of water, the columns were ready for use.

The samples were forced through the columns from the

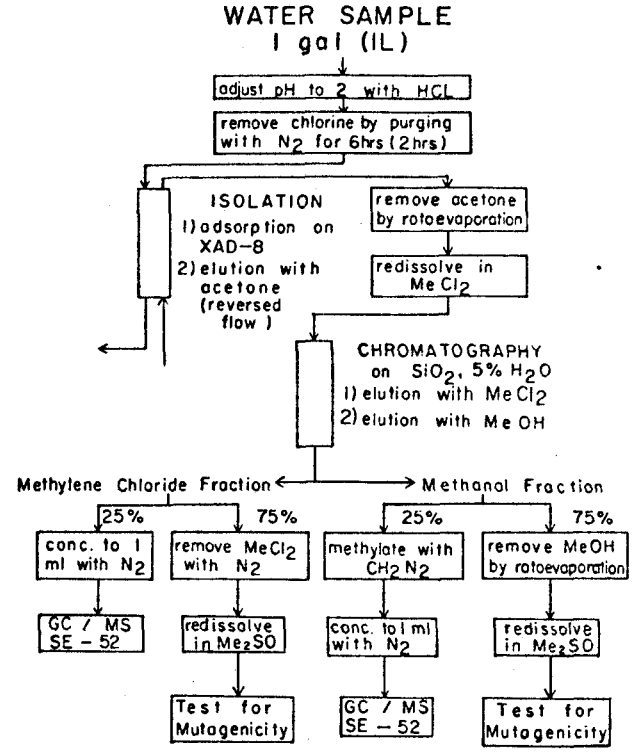

Figure 2. Concentration and fractionation scheme.

sample bottles by means of high-purity nitrogen and specially fabricated adapters (*6*). A nitrogen pressure of 10 psi resulted in a flow rate of approximately 8 mL/min. After the sample passed through the column, nitrogen was used to push the residual water out. Then an adjustable plunger was inserted into the column, and 50 mL of acetone was pumped through in reversed direction by means of a one-stroke metering pump (Model IVEK, ALLTECH, Deerfield, Il 60015) at the rate of 1–2 mL/min. Acetone and residual water were removed from the extract by rotoevaporation at 50 °C (thereby losing the volatiles). The concentrate was redissolved in 5 mL of acetone and divided into portions for mutagenic testing and chemical characterization.

Mutagenic Testing. The fractions intended for mutagenic testing were evaporated to dryness and redissolved in either 1 or 2 mL of Me_2SO. Extracts were tested for mutagenicity by SRI International, Menlo Park, with the procedure developed by Ames, Yamasaki, and McCann (*7*). Aliquots of 50, 100, 200, and 400 μL were applied per plate. Negative controls were run with 100 μL of Me_2SO. Positive controls without metabolic activation (MA) were run with sodium azide (NaN_3); positive controls with MA were run with 2-anthramine. Initial blanks were run with MQ water. Then procedural controls were obtained by extracting the precleaned XAD-8 columns with acetone. These extracts obtained from several columns were combined and tested along with the samples. An experiment was performed in which distilled water containing 5 mg/L of chlorine was passed through the columns without prior dechlorination in order to verify that residual chlorine did not produce mutagens during adsorption.

An extract was considered to be mutagenic (positive) when (i) a positive dose–response relationship was observed and (ii) when the number of revertant colonies at the highest dose exceeded the average background value by at least 100%. The net-revertant number (NR) was calculated by subtracting the average background value (determined with 2–4 doses of 100 μL of Me_2SO) from the number of revertant colonies observed. The NR concen-

tration (NR/L) was calculated from the positive slope of the NR–dose relationship by linear regression after considering appropriate concentration factors.

Silica Gel Chromatography. Fractions of the extract intended for chemical characterization were evaporated to dryness and redissolved in 200 μL of $MeCl_2$. The concentrate was then transferred to the top of a 0.5 × 10 cm silica gel column packed in $MeCl_2$. The silica (80-200 mesh, J. T. Baker, Phillipsburg, NJ 08865) was deactivated with 5% w/w water prior to use. A first fraction was eluted with 10 mL of $MeCl_2$. After passing 2 mL of 25% MeOH in $MeCl_2$ through the column, a second 10-mL fraction was eluted with MeOH. The combined MeOH eluates constituted the second fraction. Both fractions were concentrated to 1 mL under a stream of nitrogen purified with a Gas Purifier (Alltech Associates, Arlington Heights, IL 60004). Typically 25% of each concentrate was set aside for GC/MS analysis. The remainder was evaporated to dryness, redissolved in Me_2SO, and submitted for mutagenic testing.

Methylation. The MeOH fractions were derivatized with diazomethane prior to GC/MS analysis. Diazomethane was produced from Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide, Aldrich Chemical Co., Milwaukee, WI 53233) by using a Diazald kit. Approximately 2.15 g (10 mmol) of Diazald were transfered to a 250-mL round flask along with a magnetic stirring bar, followed by the addition of 30 mL of ether. As the round flask was cooled in an ice-water bath, the Diazald was stirred into solution, and 13.0 mL of a 0.81 N solution of potassium hydroxide in 95% ethanol was slowly added. Upon completion of the addition, the flask was allowed to warm to room temperature, and the etherial diazomethane solution was slowly distilled into the dry ice cooled receiving flask.

The fractions to be methylated were evaporated to dryness under a nitrogen stream and redissolved in 1 mL of a 1:1 (v/v) ether–methanol solution. Methylation was accomplished by adding an excess (~3 mL) of reagent solution. After mixing, the sample was allowed to sit overnight at room temperature. Prior to GC/MS analysis, the sample was reconcentrated to ~100/μL.

GC/MS Analysis. GC/MS analyses were done on a Finnigan 4000-INCOS system (Finnigan, Sunnyvale, CA 94086). The GC was equipped with a Grob-type injector (E. M. Becker Co., Bala Cynwyd, PA 19004) and a 50-m (0.3 mm i.d.) fused silica capillary column coated with SE-52 (J & W Scientific, Rancho Cordova, CA 95670), which was connected directly to the mass spectrometer. A 2-μL sample of extract was injected splitless for 36 s. The temperature program used was 50 °C isothermal for 3 min and then increased to 300 °C at a rate of 3 °C/min. The injector temperature was set to 260 °C; the interface oven and the transfer line were set to the maximum temperature of 300 °C. The mass spectra were acquired under the following conditions: ion source temperature, 220 °C; electron energy, 70 eV; electron current, 0.5 mA; preamplifier setting, 10^{-7} A/V. Mass spectra were acquired in the multiple-ion detection mode at a rate of one scan/2 s, mass range 35–235 in 0.666 s (3.3 ms/amu) and mass range 236–430 in 1.222 s (6.3 ms/amu).

GC/Triple Stage Quadrupole MS Analysis. A Finnigan triple-stage quadrupole (TSQ) mass spectrometer was used, coupled to a Finnigan 9610 Model GC. The GC was equipped with a 6-ft column packed with 3% OV-1 on 60/80 mesh Chromosorb W A/W. A daughter ion experiment was performed with the first mass spectra in the positive-electron-impact (EI+) mode (*33*). For collision-activated decomposition (CAD) the nitrogen pressure in the collision cell was adjusted to 2.35 mtorr; the collision energy was 12 V. A 2-μL extract was injected at 120 °C, and then the temperature was increased at a rate of 5 °C/min to 250 °C. Injector and interface were set at 250 °C; the flow rate was 16 mL/min.

GC/High-Resolution (HR) MS Analysis. A MAT 711 instrument was used, which was interfaced to a Hewlett-Packard 7610-A GC by means of a Watson–Biemann separator. The GC was equipped with a 6-ft column ($^1/_8$ in. i.d.) packed with 3% OV-17 on Chromosorb Q 80/100 mesh. The resolution was 5000. The mass spectra were acquired from 500 amu downward to 40 amu at a rate of 8 s/decade. The extract (3 μL) was injected at 100 °C, and then the temperature was increased at a rate of 4 °C/min to 300 °C.

Preparation of Brominated Alkylphenol Polyethoxy Carboxylate Test Mixture (BrAPECs). "*tert*-Octylphenol polyethoxylate" (3 mol of ethylene oxide units (ETO)/molecule) was purchased from Chem Service, West Chester, PA 19380. For bromination approximately 400 mg of the alkylphenol polyethoxylate (APE) was dissolved in 20 mL of glacial acetic acid. To this solution was added 160 mg of elemental bromine dissolved in 5 mL of glacial acetic acid (*34*). After the reaction mixture stood overnight, excess bromine was destroyed by adding 0.1 N sodium thiosulfate solution. Then 200 mL of water was added and the solution was extracted two times with 100 mL of ether. The combined extracts were dried over anhydrous sodium sulfate, filtered, and concentrated to about 10 mL. For oxidation, i.e., conversion of the alcohol into the corresponding carboxylic acid, 10 mL of Jones reagent (0.4 mol of potassium dichromate in 700 mL of 2 M sulfuric acid) was added to an APE and a BrAPE solution (*35*). The solution was warmed at 60 °C for 2 h, then diluted with 25 mL of water, and extracted two times with 50 mL of ether. The combined extracts were washed three times with 100 mL of water. After being washed, the ether extract was dried, filtered, and concentrated. Half of it was set aside for Ames testing, and the other was used for chemical characterization.

Results

Mutagenic Response of Concentrates. The net reversion rate/L (NR/L) was used to compare the mutagenic activity at different locations and in different fractions. So that an estimate for the significance of quantitative comparisons could be obtained, the data obtained from running negative and positive controls were statistically evaluated. The average of all background reversion rates observed with 100 μg/L of Me_2SO was 110 (n = 48); the coefficient of variation was 26%. Data from plates run simultaneously were less variable. Within a set, the coefficient of variation was only 12%. A similar observation was made for the positive controls obtained with 1 μg of NaN_3. When tested within a set, the coefficient of variation was 5%. However, the number of total inversions induced by NaN_3 fluctuated considerably over the study period. The mean value was 497, the coefficient of variation was 32%, and the observed values ranged from 227 to 706. Therefore, caution must be exercised when data obtained at different times are compared.

The initial experiments were designed to establish a procedure suitable for routine monitoring. XAD-8 resin was used as the adsorbent and both TA98 and TA100 were used as tester strains. The effect of metabolic activation was tested with concentrations of samples from the plant influent (Q1) and from the two plant effluents (Q9 and Q22B). These data are summarized in Table I.

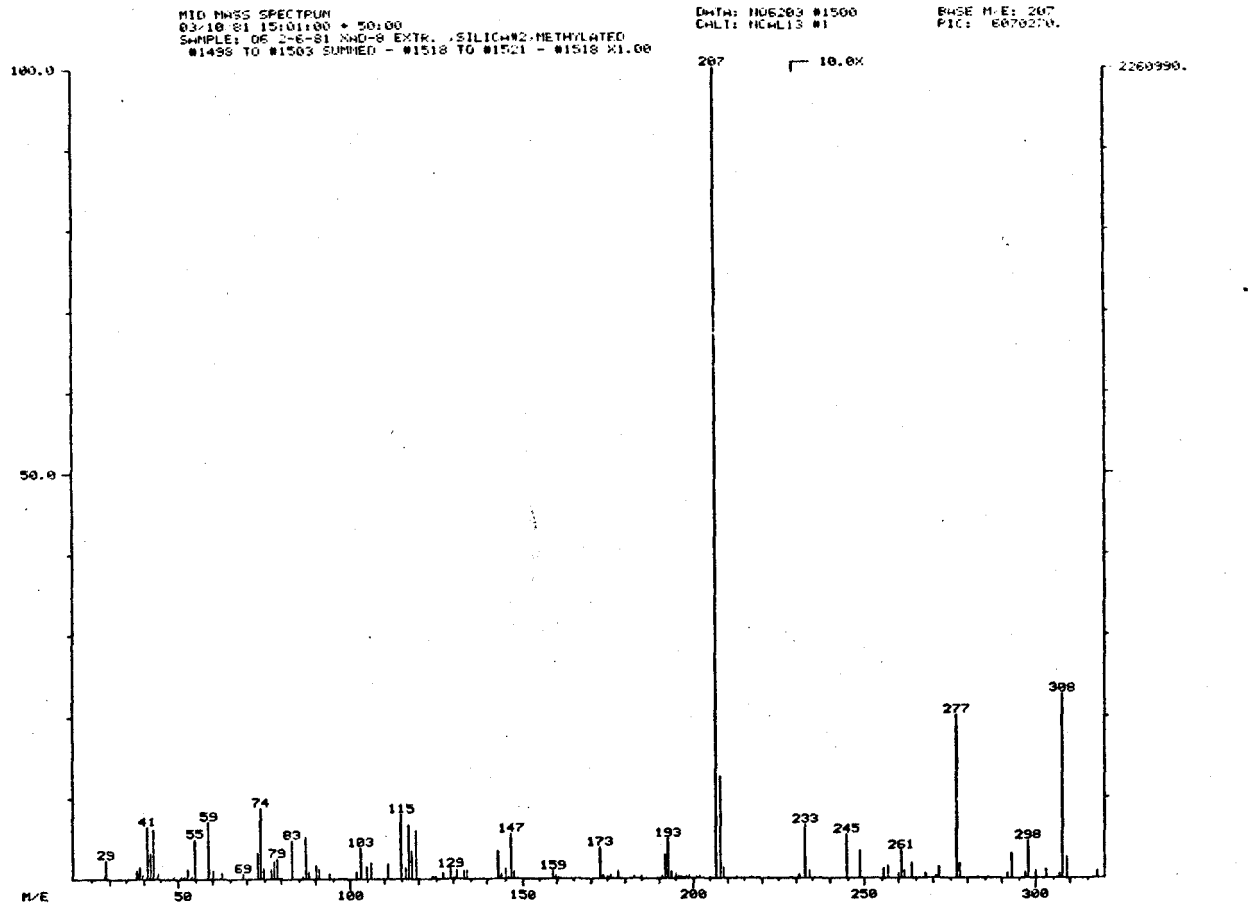

Figure 3. Mass spectrum of a representative APEC compound of the 207 group.

Table I. Mutagenic Activity at Q1, Q9, and Q22B against TA98 and TA100, without and with Metabolic Activation, in Net Revertants/L[a]

	sample	TA98		TA100	
influent	Q1	−MA	+MA	−MA	+MA
	12/25/79	280	0	1240	360
	12/31/79	290	0	670	320
Cl effluent	Q9				
	12/25/79	130	0	820	420
	12/31/79	650	0	2480	1110
	4/14/80	150		1190	
RO effluent	Q22B				
	12/25/79	0	0	0	0
	12/31/79	0	0	0	0
	1/25/80	0	0	0	0

[a] −MA, no metabolic activation; +MA, with metabolic activation; 0, no activity above detection limit.

The data suggest that the mutagens present in Q1 and in Q9 were detectable with both strains TA100 and TA98. In contrast, the organics present in Q22B did not induce activity in either strain. Several characteristics of the mutagenicity observed were similar to those reported in chlorination-stage effluents of pulp-mill effluents (*16–21*) and in chlorinated drinking water (*15*): the reversion rate was higher in TA100 than in TA98 and was reduced by MA. In TA100 the reduction was approximately 50%, and in TA98 it was 100%.

For evaluation of the impact of individual processes in more detail, concentrates from various stages of treatment were tested. MA was discontinued at this point because most mutagens present appeared to be direct acting. TA100 was selected for further investigations because of its high reversion rates.

Table II. Mutagenic Activity against TA100 at WF 21 Sampling Locations, Extracted with XAD-8 (Acetone Eluate)[a]

	location						
date	Q1	Q2	Q4	Q6	Q8	Q9	Q22B
4/25/80	970					500	
5/01/80	1080					830	
5/22/80	T[b]					540	0
5/28/80				2360 (T)	0	2400	0
6/17/80			0	570	0	1620	
6/21/80			0	2260	0	1840	
6/27/80	0	0		1080			
7/04/80	0	0	0			610	

[a] Net revertants/L. [b] T, toxicity.

The data indicating mutagenic activity at seven treatment stages of WF 21 are summarized in Table II. During the period April 25 to July 4, 1980, eight sets of samples were tested. Only two of the five influent samples tested were positive during this period, one showed signs of toxicity, and two were negative. At Q6 all samples tested were positive, with activities ranging from 570 to 2360 NR/L. No Q8 sample was found to be positive, but mutagenicity reappeared at Q9 where all samples tested were positive. In the chlorinated effluent, the activities ranged from 500 to 2400 NR/L.

In three instances direct quantitative comparison of Q6 and Q9 was possible. In two cases (May 28 and June 21, 1980) the activities at Q6 and Q9 were remarkably close, whereas in the sample set taken June 17, 1980, the activity appeared to be significantly higher in Q9 in spite of activated-carbon adsorption between Q6 and Q9. This suggested that precursors were not removed completely and that perhaps the chlorination conditions were gov-

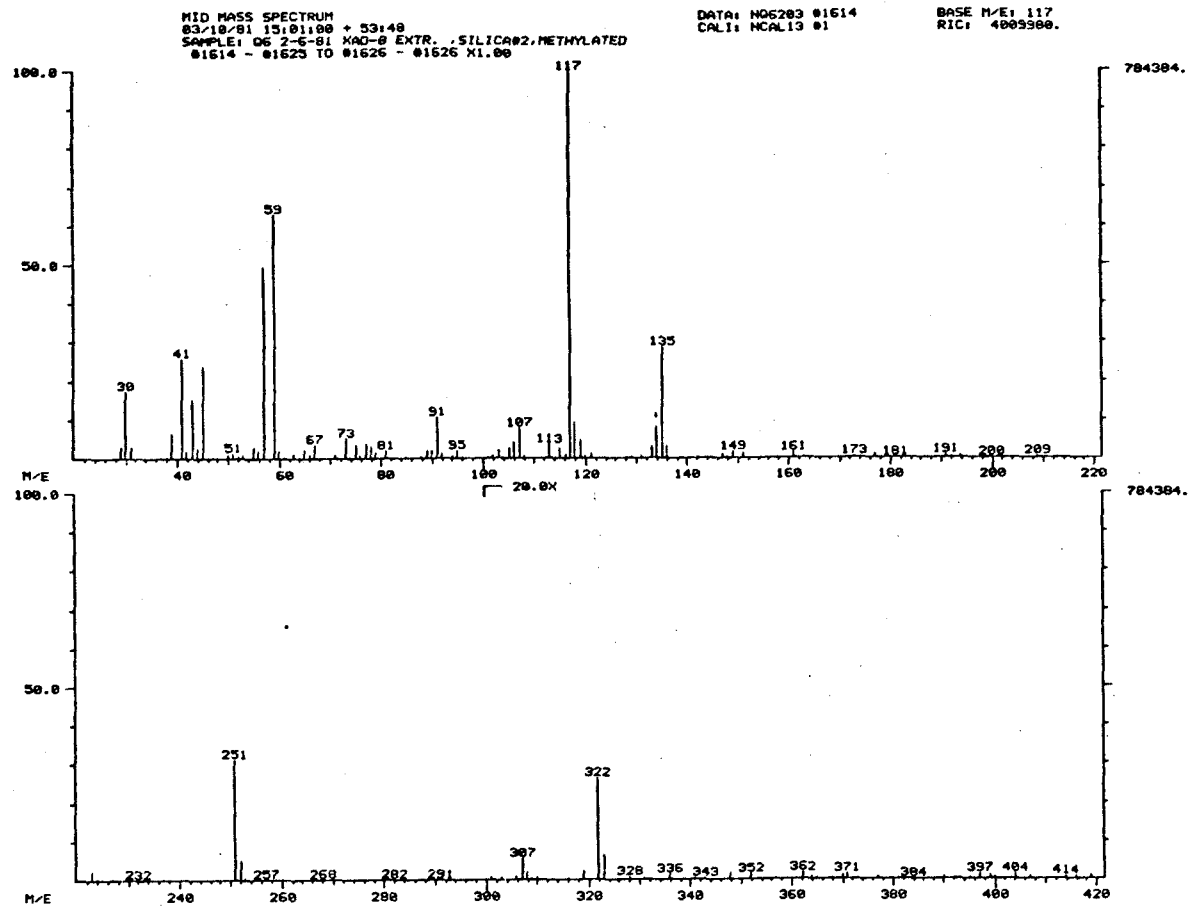

Figure 4. Mass spectrum of a representative APEC compound of the 117 group.

erning the amount of activity produced.

Mutagenic Response of Fractions. The XAD-8 concentrates were separated into nonpolar and polar fractions by silica gel chromatography. A nonpolar fraction was eluted with $MeCl_2$, and a more polar fraction was eluted with MeOH. An unfractionated aliquot of the XAD-8 concentrate was tested along with the corresponding $MeCl_2$ and MeOH subfractions. Samples were taken from Q6, Q8, and Q9, where the impact of chlorination was most easily observed. The results are summarized in Table III. In all cases the mutagenicity present in the unfractionated controls appeared in the MeOH fractions, whereas the fractions eluted with $MeCl_2$ were negative. In the set taken January 26, 1981, activity was not detected in the controls but was detected after fractionation at low levels. This suggested inhibition of activity by materials separated in the $MeCl_2$ fraction. The activity detected in the MeOH fraction of Q8 (January 26, 1981) was the only case where weak activity appeared to break through the activated carbon columns.

Chemical Characterization of Active Fractions. Methylated MeOH fractions were subjected to GC/MS analysis for chemical characterization. Numerous peaks were detected, most of which appeared to be carboxylic acids. Manual (*36*, *37*) and computer-assisted mass spectra comparisons suggested the presence of the methyl esters of the following acids: dehydroabietic acid and other unidentified compounds with similar spectra, fatty acids, toluenesulfonic acid, benzoic acid, and clofibric acid. Clofibric acid, which has previously been found in wastewater, is a metabolite of the drug clofibrate (*38*, *39*). However, the largest group of constituents belonged to a complex mixture of brominated and nonbrominated compounds. On the basis of their spectra, two main groups could be distinguished. The first group (the 207 group), which eluted between 45 and 60 min, had spectra with a base peak of m/z 207. The spectrum of the main peak of this group is depicted in Figure 3. The second group (the 117 group) eluted between 55 and 70 min and had spectra with a prominent fragment at m/z 117. The spectrum of the largest peak of this pattern is given in Figure 4.

A fraction of the 117 group appeared to contain bromine, as was suggested by the presence of characteristic isotope clusters. The degree of bromination appeared to vary widely from sample to sample but clearly increased at the chlorination stages. The spectra of these compounds appeared to belong to either of two subgroups, one characterized by a cluster at m/z 210–216 (the 213 group) and one by a cluster at m/z 200–206 (the 203 group). In Fig-

Table III. Mutagenic Activity (in NR/L) against TA100 in XAD-8-Acetone Concentrates and $MeCl_2$ and MeOH Subfractions

date	location	XAD-8 con[a]	$MeCl_2$	MeOH
8/12/80	Q6	3040	0	930
	Q9	2910	0	3100
10/10/80	Q9	970	0	550
11/25/80	Q8	0	0	0
	Q9	0	0	0
12/9/80	Q6	3710	0	T[b,c]
	Q8	0	0	0
	Q9	370	0	1590
1/26/81	Q6	0		420
	Q8	0		210
	Q9	0		550

[a] Unfractionated XAD-8 concentrate. [b] Questionable positive response. [c] T, toxicity.

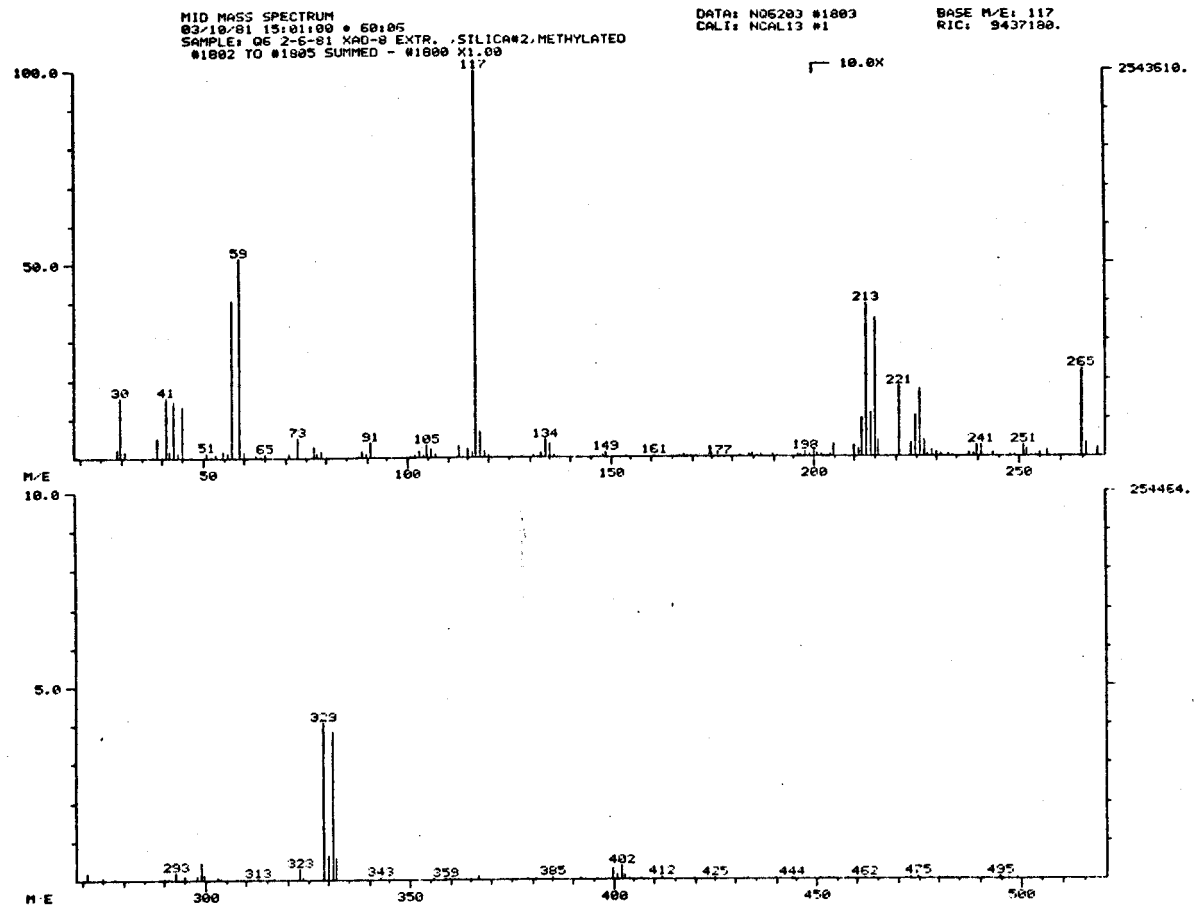

Figure 5. Mass spectrum of a representative BrAPEC compound of the 213 group.

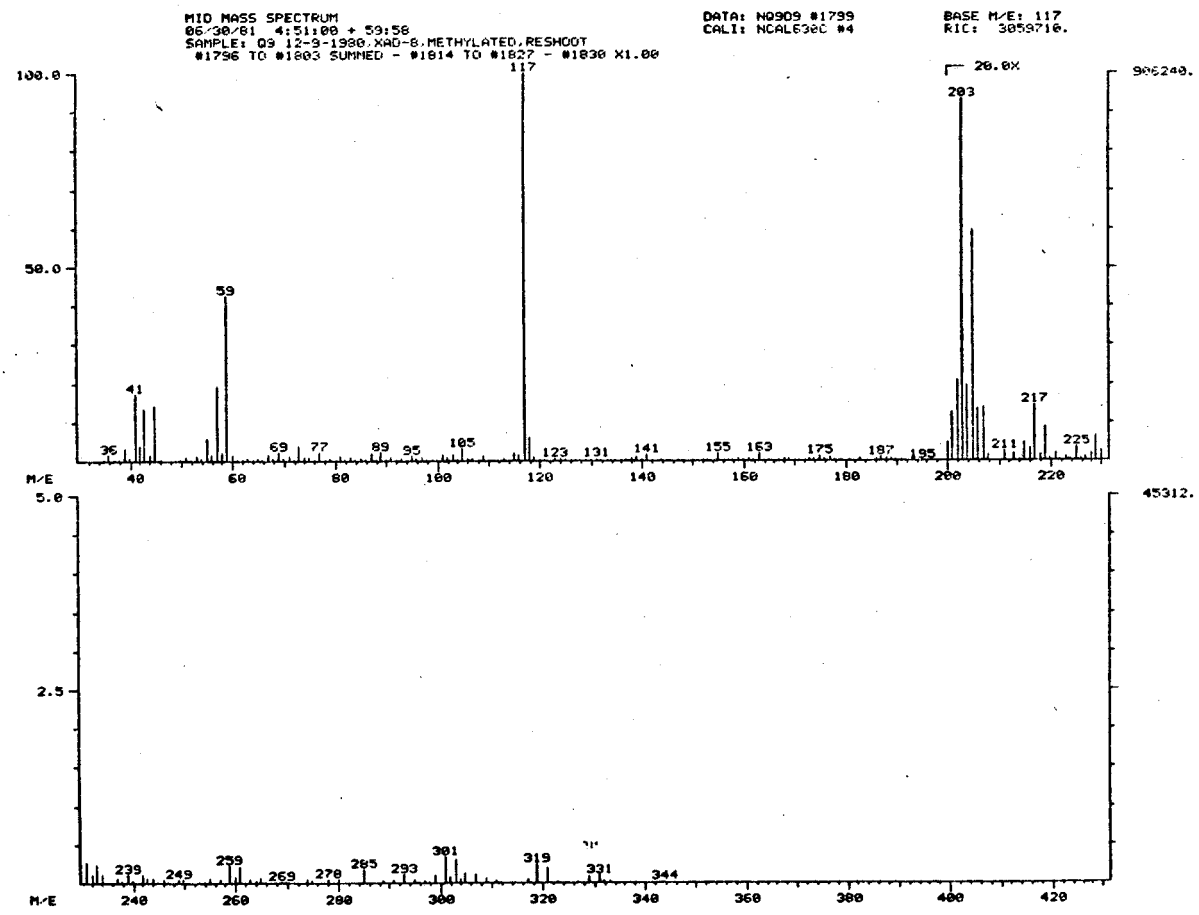

Figure 6. Mass spectrum of a representative BrAPEC compound of the 203 group (Br tentative).

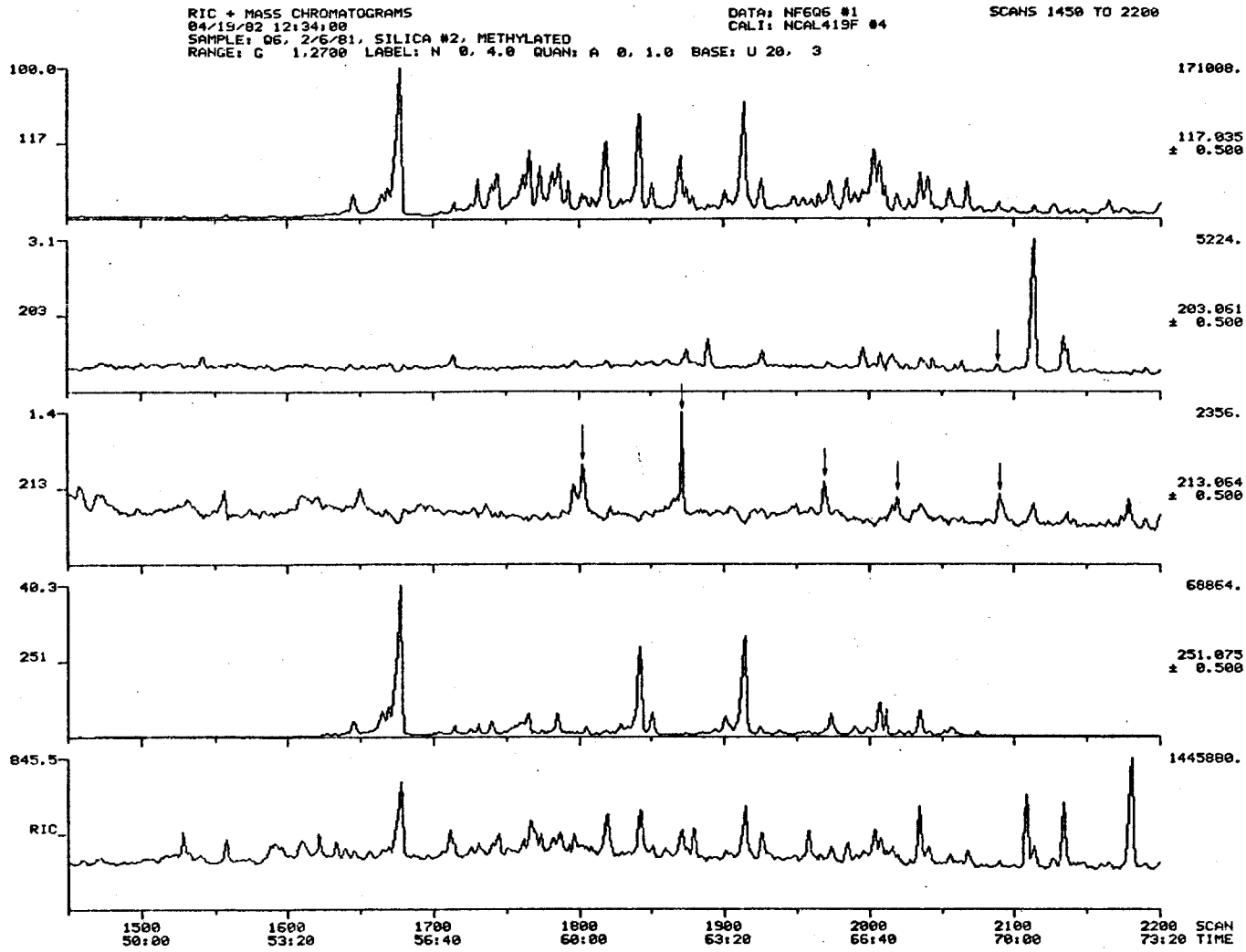

Figure 7. Mass chromatograms m/z 117, 203, 207, 213, 251, and reconstructed ion current (bottom) of methylated MeOH fraction; extract of Q6 February 6, 1981. Brominated peaks are marked.

ures 5 and 6 typical spectra of both groups are presented. For characterization of the unknown mixture, mass chromatograms m/z 117, 207, 203, 213, and 251 appeared to be suitable. Figure 7 shows these mass chromatograms along with a reconstructed ion current chromatogram of the methylated MeOH fraction of Q6 (February 6, 1981). In this sample the compounds are mostly unbrominated; trace amounts of brominated compounds occur between scans 1800 and 2100 (213 group) and at scan 2089 (203 group).

The spectra of these groups were absent in commercially available spectra compilations (*36–37*) but seemed to match very closely spectra of compounds detected previously in mutagenic concentrates of the Palo Alto Water Reclamation Facility (PAWRF) (*9*). This prompted us to undertake a new effort to elucidate their structure and to reexamine previously acquired high-resolution spectra. A 1-L sample of (PAWRF) chlorinated secondary effluent (sample C, February 27, 1979) had been extracted with $MeCl_2$ (3 × 20 mL) at pH 2. The methylated extract was then subjected to GC/MS and GC/HRMS. A scan was acquired in which some of the typical fragments were present. In Table IV the exact masses of these fragments are given along with matching elemental compositions and relative intensities.

The HRMS data suggested that the cluster at 400/402 consisted of $C_{19}H_{29}O_4Br$. The cluster at 329/331 ($C_{14}H_{18}O_4Br$) appeared to originate from a loss of a pentyl group. Other fragments detected were m/z 117 and the cluster m/z 134/135, which were matched to $C_5H_9O_3$ and $C_9H_{10}O$ + H, respectively. Fragment 59.047 was matched to C_3H_7O, fragment 43.056 to C_3H_7, and 41.038 to C_3H_5. However, the two clusters present in the low-resolution spectra suspected to contain bromine, m/z 200–210 and m/z 210–216, were missing in the HRMS scan. It was therefore suspected that the HR mass spectrum was incomplete and possibly consisted of overlapping spectra of two closely eluting GC peaks. The slow scan time (8 s) and the insufficient resolution of the packed column may have been inadequate for analysis of the complex mixture at hand.

The crux of spectrum identification was fragment m/z

Table IV. Exact Masses, Relative Abundance, MMU Error, and Suggested Elemental Composition of Major Fragments

mass	% area	MMU error	composition
41.0385	7.8	−0.652	C_3H_5
43.0561	5.2	1.349	C_3H_7
57.0340	1.8	−0.073	C_3H_5O
57.0705	17.1	0.087	C_3H_9
59.0471	11.8	−0.567	C_3H_7O
117.0563	100.0	1.175	$C_5H_9O_3$
134.0759	3.5	2.742	$C_9H_{10}O$
135.0802	2.9	1.034	$C_9H_{11}O$
329.0342	41.2	−8.176	$C_{14}H_{18}O_4{}^{79}Br$
331.0391	34.9	2.233	$C_{14}H_{18}O_4{}^{81}Br$
400.1243	5.8	−0.608	$C_{19}H_{29}O_4{}^{79}Br$
402.1187	6.4	−4.194	$C_{19}H_{29}O_4{}^{81}Br$

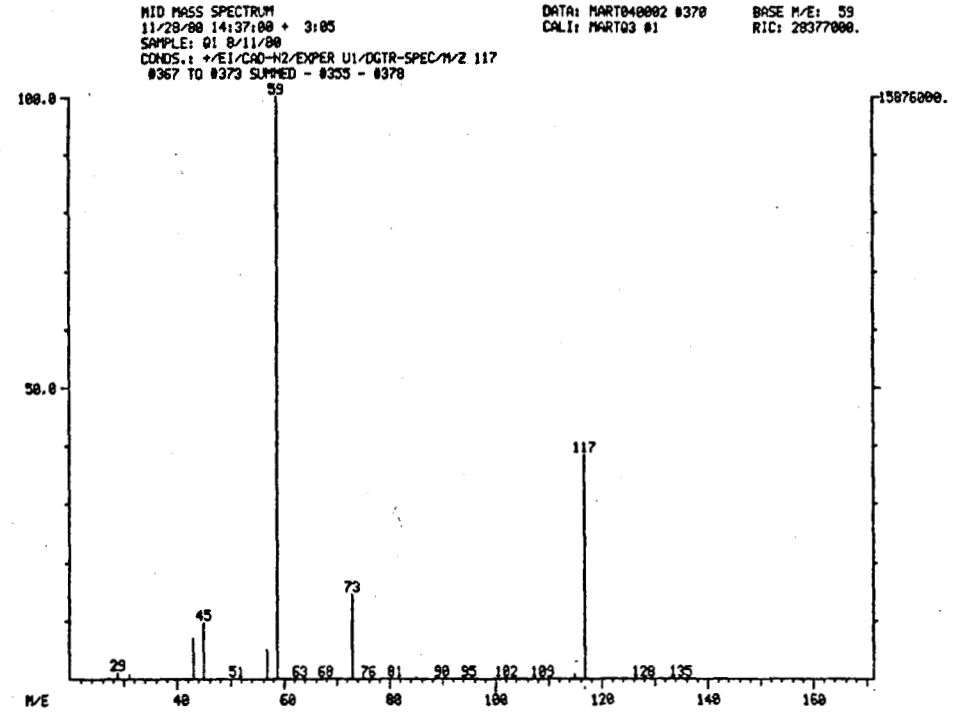

Figure 8. EI^+ daughter-ion spectrum of fragment ion m/z 117.

117. For aid in structural elucidation, an EI^+ daughter-ion experiment was conducted with GC/TSQ MS. Figure 8 depicts the daughter-ion spectrum of fragment m/z 117 produced by CAD with N_2. It indicates that this fragment loses neutral fragments of mass 44 and 58, resulting in the fragment ions m/z 73 and 59, respectively. These losses were tentatively assigned to C_2H_4O and $C_2H_4OCH_2$, which leaves $C_2H_3O_2$ for m/z 59. This is consistent with the elemental composition of a methylated carboxyl group. By assignment of the loss of 71 from the apparent molecular ions 322 and 400 to a pentyl group and by recognition of the aromatic and phenolic elements of the spectrum, structures I and II depicted in Figure 9 could be proposed. The relatively high intensity of m/z 135 was taken as an indication of the presence of two methyl groups (or an ethyl group) at the α-carbon in the aliphatic side chain. The fragmentation patterns proposed in Figure 9 may explain the presence of the major fragments. For the group with m/z 207 as a base peak no HRMS or TSQ MS data was available, and these spectra are consequently less understood. A structure of type III is suggested as a possibility. There appeared to be three reasons for the great complexity of the mixture. The presence of compounds differing in their apparent molecular weight by 14 amu indicated the presence of a series of homologues. The presence of compounds differing in their apparent molecular weight by 44 suggested the presence of compounds having side chains with different numbers of ETO units. The presence of compounds with the same apparent molecular weight but with slightly different GC retention times and with similar spectra suggested the occurrence of differently branched alkyl side chains.

So that spectra of compounds similar to those proposed in Figure 9 could be studied, commercially available alkylphenol polyethoxylates (APEs) were converted to the alkyphenol polyethoxy carboxylates (APECs) and brominated alkylphenol polyethoxy carboxylates (BrAPECs). The APE standard used was specified by the supplier as "polyethoxylated *tert*-octylphenol" with three ETO units per molecule. But GC/MS analysis indicated that a mixture of APEs were present, with the number of ETO

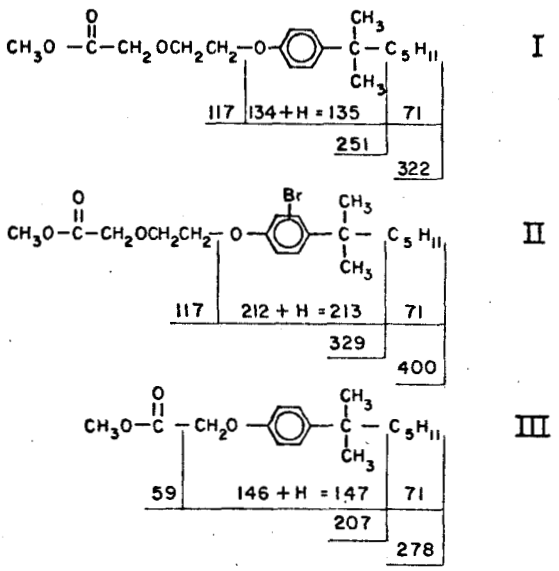

Figure 9. Proposed structures of APEC and BrAPEC compounds representative of 117 group (I), 213 group (II), and 207 group (III); fragmentation is tentative.

units/molecule ranging from one to greater than three. Spectra apparently belonging to the 207 group and the 117 group (Figures 10 and 11) were found in the APE mixture after oxidation. Visual comparison of the spectra suggested that the compounds produced chemically were of the same type as those present in the standard mixture. Bromination followed by oxidation of the APE mixture produced compounds with spectra similar to those found in the concentrates. Figure 12 depicts a spectrum matching closely the one depicted in Figure 5. Chemically produced methylated BrAPEC mixture was coinjected with an extract, and it was observed that BrAPECs with apparent MW of 400 coeluted. This seemed to confirm that the mixture produced chemically contained the same or closely related compounds as the extracts. Further characterization of the composition of the complex APEC and

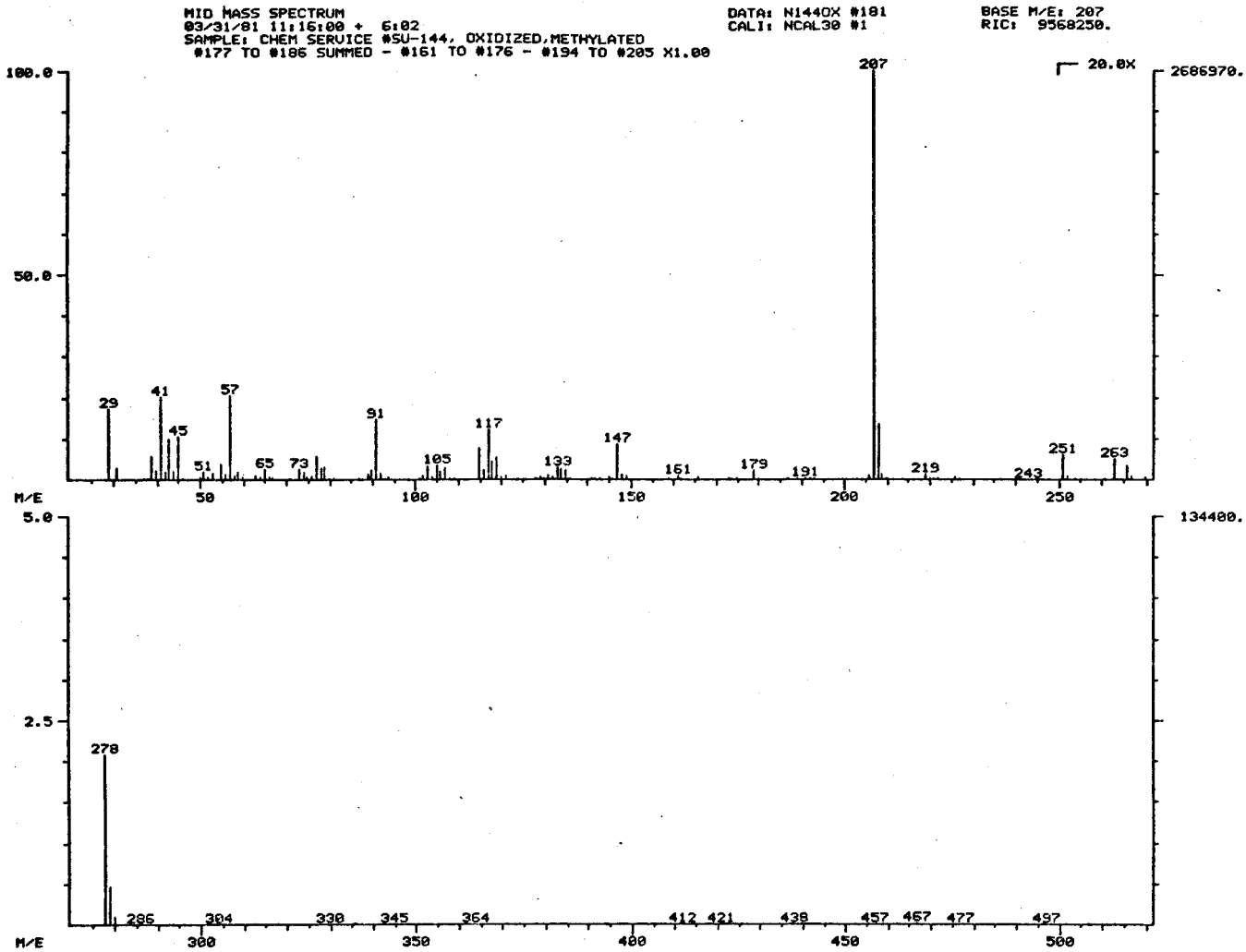

Figure 10. Spectrum of compound found in the chemically oxidized APE mixture apparently belonging to the 207 group.

BrAPEC mixture present in the water extracts was beyond the scope of this work.

Mutagenic Response of BrAPECs. Because of the prevalent occurrence of the APEC and BrAPEC compounds in the mutagenic fraction, an attempt was made to relate their presence to the mutagenicity data. BrAPECs were clearly products of chlorination, and it was suspected that they were the cause of the mutagenicity in the chlorinated samples. In Table V we present an attempt to correlate qualitatively the occurrence of mutagenicity with the occurrence of BrAPECs.

Nonbrominated isomers detected with MC 207 and 251 were present in both Q8 and Q6 samples tested. Their presence in Q8 indicated that they were breaking through the activated-carbon column. They were not found in either of the Q9 extracts, however. No BrAPECs were detected in the Q6 sample taken on December 9, 1980, where the mutagenic response was questionable (two values above twice background, but toxicity and no dose–response). However, BrAPECs of the 203 type were present in Q9, where a clear mutagenic response was observed. In the second set BrAPECs were present in Q6 (203 and 213 type) and Q9 (203 type), which were both positive, but were absent in Q8, which was negative. This indicated that bromination of APECs occurred during chlorination and suggested that BrAPECs produced the observed mutagenic response.

For further exploration of the BrAPECs as potential mutagens, the mixture prepared in the laboratory was subjected to Ames testing. It did not produce a mutagenic effect but was toxic at higher doses. In another experiment, the XAD-8/acetone extract of a 1-gallon Q6 sample (sampled May 20, 1981) was split into two halves, A and B. A was tested as previously; B was redissolved in 10 mL of 0.1 N NaOH. The next day, the organics were reextracted by using the XAD-8 adsorption method. The extract was split into two portions, one for Ames testing and one for chemical characterization. Comparison of the responses to A and B revealed that treatment of B with base apparently destroyed the mutagens that gave a positive response in A. However, comparison of the MC 213 profiles in A and B did not indicate an observable difference, and in both A and B the 203 profile was absent. These

Table V. Qualitative Correlation between Mutagenic Response and the Presence of APEC and BrAPEC Isomers

date	sample	mutagenicity	207[a]	251[a]	203[b,c]	213[b]
12/9/80	Q6	T[d]	+[e]	+	0[f]	0
	Q8	0	+	+	0	0
	Q9	+	0	0	+	0
2/6/81	Q6	+	+	+	+(w)[g]	+(w)
	Q8	0	+(w)	+(w)	0	0
	Q9	+	0	0	+(w)	0

[a] Indicative of APECs. [b] Indicative of BrAPECs. [c] Bromine substitution suggested. [d] Questionable positive response; T = toxicity. [e] + = characteristic indicated was present. [f] 0 = characteristic indicated was absent. [g] Weak.

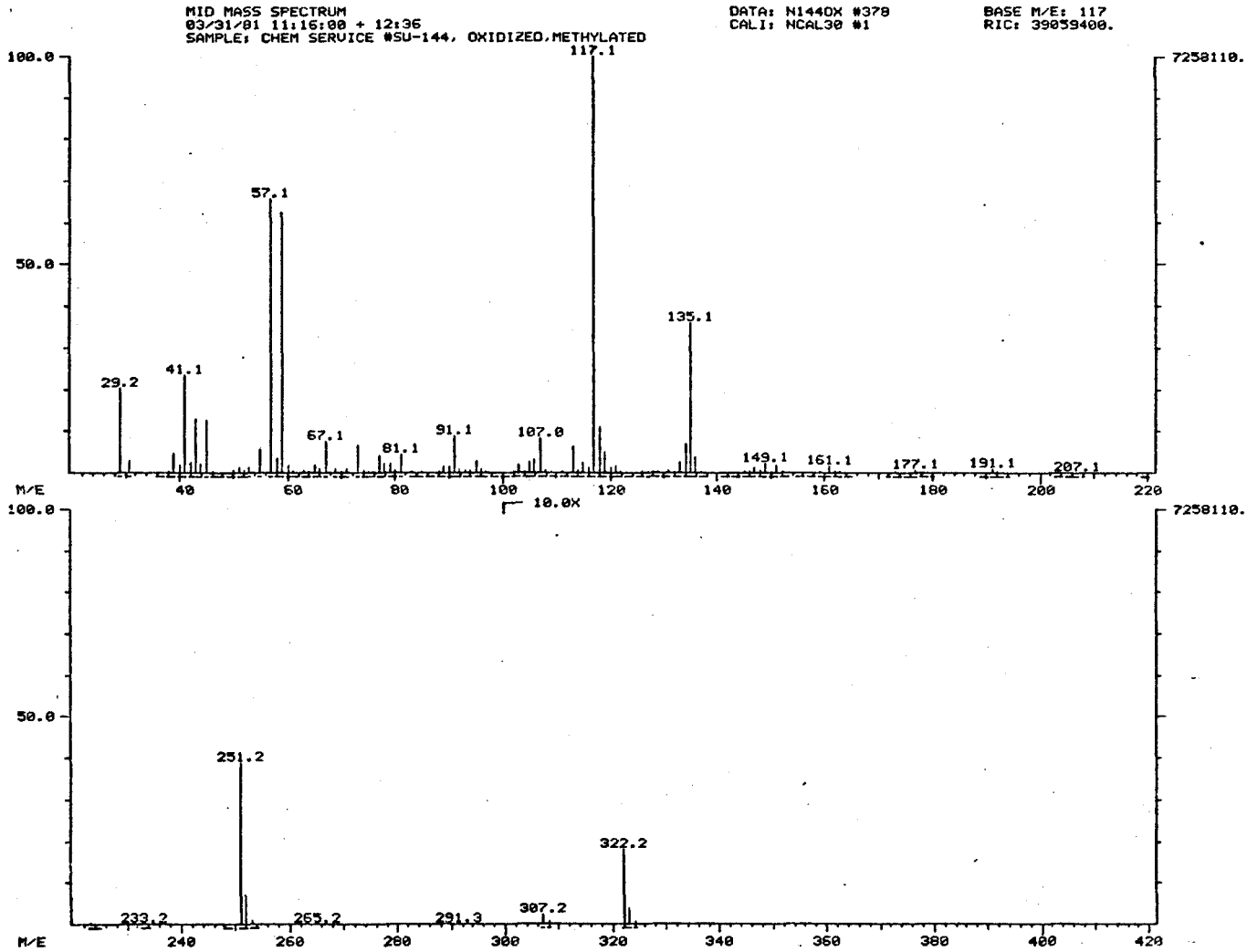

Figure 11. Spectrum of compound found in chemically oxidized APE mixture apparently belonging to the 117 group.

two experiments seemed to counter the hypothesis that the mutagenicity observed at Q6 and Q9 was due to BrAPEC compounds detected with MC 203 and 213.

Discussion and Conclusions

WF 21 received a secondary effluent in which direct-acting mutagens were detected intermittently. Our data suggest that the mutagens present in the influent were removed or destroyed during treatment, as indicated by the absence of mutagenicity in the activated carbon and reverse-osmosis effluents. WF 21 influent also appeared to contain precursors that were converted into direct-acting mutagens during chlorination. Increased activities were found at Q6, where chlorine was added for algae control and nitrogen removal. The mutagens formed there were removed during subsequent AC adsorption. In the AC effluent, only one marginal case of activity was detected. However, the precursors were not sufficiently removed by AC adsorption, as was indicated by the reoccurrence of mutagenicity during final chlorination for disinfection. By and large, activity appeared to be as strong or stronger in the final chlorinated effluent as in the chlorinated recarbonation basin effluent. None of the RO effluent samples tested exhibited a mutagenic response. Because the RO effluent was not chlorinated, it is not known whether the precursor materials were removed by RO.

The nonvolatile mutagens formed during chlorination at WF 21 appeared to be associated with rather polar or surface active materials. Analysis of the mutagenic fraction revealed the presence of a variety of organic acids, APECs and BrAPECs being the major constituents. In Q1, the concentrations of the major components were estimated to be in the 100 μg/L range. A significant fraction of each major component was removed during treatment, but significant quantities, probably in the μg/L range, remained even after AC adsorption.

The fact that BrAPECs were formed during chlorination suggested that these compounds were involved in producing a mutagenic response. But preliminary experiments with chemically produced BrAPECs did not support this hypothesis. Thus the coincidence of mutagenicity and BrAPECs might have been accidental. Various reasons could explain this apparent discrepancy: (i) the chemically produced mixture did not reflect the mixture found in the mutagenic fractions, (ii) the difference in halogenation conditions might have led to different products (for instance small amounts of side-chain brominated products might have been formed under plant chlorination conditions), or (iii) the active compounds were not detected with the analytical procedure used. Clearly, an evaluation of these questions is complex and requires much more study.

APEs, from which APECs and BrAPECs are suspected to derive, have been detected previously in wastewater effluents (*40*, *41*) but are not presently being monitored as such in the environment (*42*). Little is known about their health and environmental significance, and only limited data are available pertaining to their occurrence and fate in the environment. APEs are presently in use

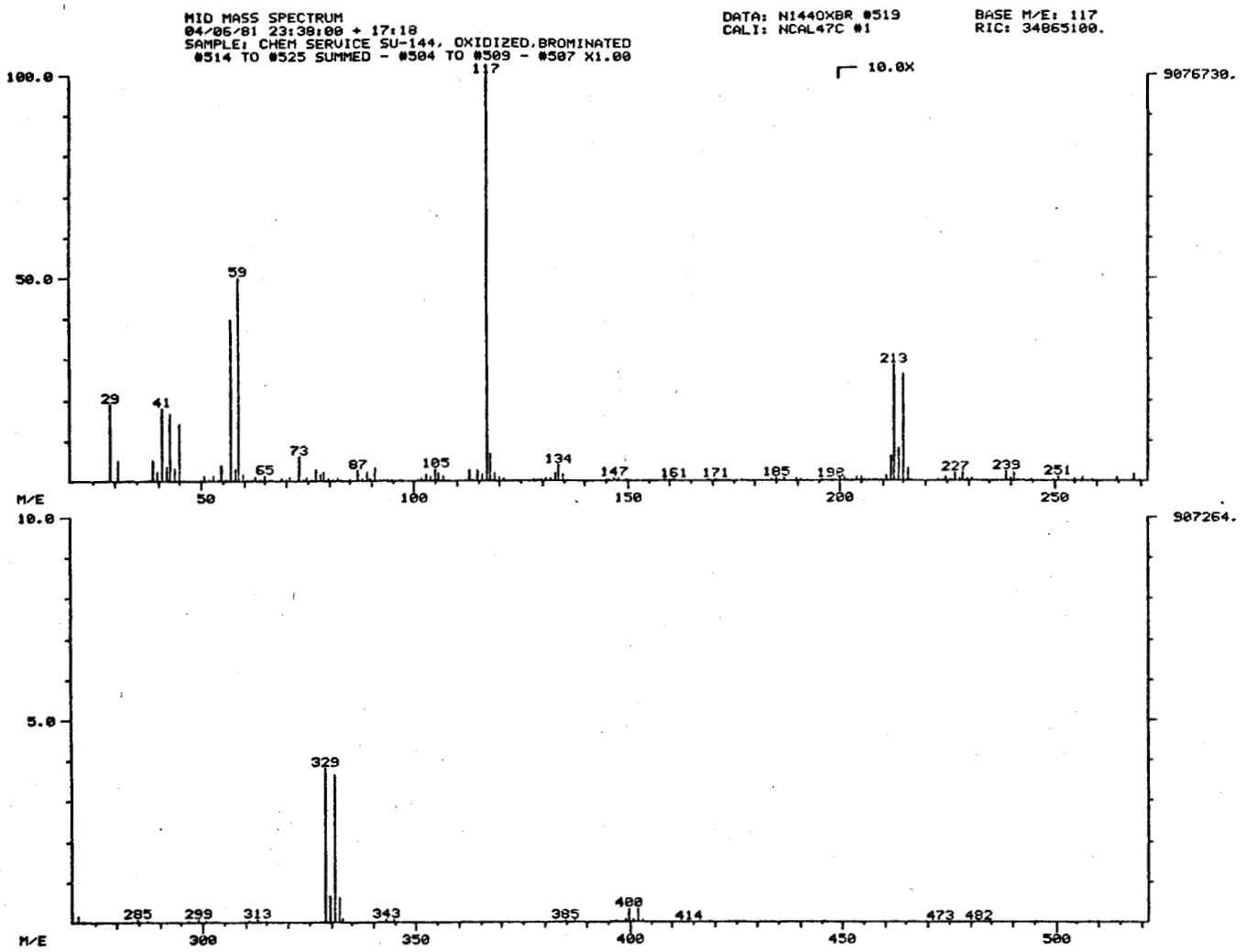

Figure 12. Spectrum of compound found in the chemically oxidized brominated APE mixture apparently belonging to the 213 group.

as industrial and domestic surfactants. The current amount of APEs produced is not known, but in 1962 about 50 000 tons were used in laundry-detergent formulations (*43*). APEs are reported to undergo primary biodegradation during activated sludge treatment and trickling filter sewage treatment (*42*), but it is known that APE residues are rather recalcitrant, particularly when the hydrophobic alkyl groups are highly branched (*43*). Long-chain APEs (10 ETO) were shown to undergo biological degradation to short-chain APEs (1–2 ETO) by adapted microorganisms in a laboratory investigation (*44*), but ultimate biodegradation is known to be very slow (*45*). It also seems likely that bromination (and chlorination if it should occur) will render these molecules even more refractory and more lipophilic. Both of those characteristics are likely to cause greater persistence in the environment and possibly accumulation in the food chain. It seems necessary to address these questions as well as the public health significance of these residues in more detailed studies.

The fact that brominated but not chlorinated species were detected is interesting. It suggests the presence of highly reactive brominating agents in the chlorinated water, possibly bromine (Br_2), bromine chloride (BrCl), or bromine hypochlorite (BrOCl). Most studies of chlorination effects in the past have been directed toward chlorinated byproducts (*8*). The facts that all brominated haloforms are mutagenic (*13*) and that aromatics appear to be preferentially brominated during wastewater chlorination suggests that bromine incorporation into organic materials should receive more attention.

Acknowledgments

Appreciation is extended to David Argo and his staff of the Orange County Water District for all assistance given, Ann Griffin for conducting the mutagenicity assays, Urs Steiner from Finnigan Corp. for performing TSQ MS analysis, and Annemarie Wegmann, Chemistry Department, Stanford University, for performing HRMS analysis. We also thank P. L. McCarty for helpful suggestions in preparing the manuscript.

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Received for review August 26, 1981. Accepted February 2, 1982. This study was supported by the Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, OH, through Grant EPA-S-803873, and the Orange County Water District.