GENOTOXIC AND CARCINOGENIC ACTIVITY OF OIL REFINERY EFFLUENTS

by

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CARCINOGENIC POTENTIAL OF REFINERY EFFLUENTS

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ABSTRACT

Concentrated extracts were prepared from particulate, dissolved, and volatile components of effluents from three low-temperature cracking refineries. Extracts were tested for genotoxic activity using the Ames bacterial mutagenicity assay, and an *in vitro* assay for sister chromatid exchange. The dissolved and volatile components of effluents showed little genotoxic activity, but particulate extracts from two of three refineries sampled were significantly mutagenic. One particulate sample gave a positive response in the sister chromatid exchange (SCE) assay. All samples required exogenous activation with rat-liver microsomes (S-9) for expression of genotoxic activity. Subfractionation of particulate extracts indicated that mutagenic activity was concentrated in neutral, polar fractions.

The mutagenic/carcinogenic hazard associated with the chlorination of water contaminated with oil refinery effluents was also investigated. Non-volatile agents with mutagenic (Ames test) and clastogenic (SCE) activity were formed by chlorination of dilute refinery effluents. These compounds were direct-acting, in that the addition of S-9 was not required for genotoxic activity. Contact time with chlorine, chlorine concentration, effluent concentration, and the

pH of the reaction mixture were found to vary the mutagenicity of the extracts.

The effluent extracts were tested for carcinogenicity using an *in vivo* embryo assay developed for these studies. Microlitre volumes of extracts were injected into eyed rainbow trout embryos, and the fish raised for 12 months before necropsy. Refinery extracts were not directly carcinogenic. Coinjection of aflatoxin B_1 with extracts from both the particulate and dissolved components of effluents significantly increased the incidence of hepatic carcinomas. This co-carcinogenic effect was most pronounced when the extracts and aflatoxin B_1 were preincubated with rat S-9 before embryo injection. Effluent extracts coinjected with a direct-acting carcinogen, N-methyl -N'-nitro-N-nitrosoguanidine (MNNG), did not increase the incidence of hepatic carcinomas.

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PART 1: GENERAL INTRODUCTION

I) Carcinogens in Drinking Water:

The aquatic environment is one of the ultimate recipients of man's chemical wastes and effluents. As a result, a major portion of the world's surface and groundwater resources contain organic contaminants (Zoeteman, 1977; Pye and Patrick, 1983). At present, some 1300 organic contaminants have been identified in drinking water (Garrison, 1976), and this has prompted concern over the possible health effects of long-term exposure to these compounds.

Epidemiological studies have indicated an association between cancers of the gastrointestinal tract and the drinking of contaminated water (Crump and Guess, 1980). Page et al (1976) found excess mortality rates for gastrointestinal cancers in U.S. counties using the Mississippi River for drinking water. Increased mortalities from stomach and bladder cancers were correlated with the drinking of Ohio River basin water (Kuzma et al, 1977). In a case-control study of cancer mortalities in Louisiana, Gottlieb and Carr (1982) reported an association between, mortality rates for rectal cancers and the use of Mississippi River water. The risk of rectal cancer increased with proximity to the river mouth. In a case-control study of populations in North Carolina, Struba (1979) found a positive association between

mortalities due to bladder, rectal, and colon cancers, and the use of surface water rather than well water for drinking. This association was significant only among rural populations.

Epidemiological studies have indicated an association between cancer mortalities and the chlorination of public drinking water. Water chlorination has been shown to produce high levels of halomethanes, such as chloroform (Bellar et al, 1974). The concentration of halomethanes in chlorinated water has been used as the independent variable in many of these epidemiological studies. Cantor (1982) correlated levels of halomethanes in the drinking water of urban U.S. counties with excess mortality rates for bladder cancer. Hogen et al (1979) found a positive association between colon and rectal cancers (combined) and the concentration of chloroform in the drinking water of several U.S. counties.

In a case-control study, Alavanja et al (1978) associated the chlorination of surface water in New York state with increased mortalities from gastrointestinal and urinary tract cancers. In a similar study of Illinois communities, Brenniman et al (1980) found a positive association between chlorination of ground water and mortalities due to cancers of the colon, rectum, and total gastrointestinal tract. Kanarek and Young (1980) extensively studied the relationship between drinking water chlorination and cancer in a case-control study based on mortality data for white Wisconsin females. The degree of exposure to chlorinated drinking water was

represented by the mean daily chlorine dose applied to each water source for a 20 year period prior to the study. Cancers of the colon were the only type for which there was a significant association with water chlorination. There was a strong interaction between the presence of rural runoff in the chlorinated water and increased risk of colon cancer. This is consistent with the theory that volatile halomethanes formed through the action of chlorine on humic substances (humic acids are leached from decaying organic material) are the causal factors in cancers related to drinking water (Morris and Johnson, 1976). When data were stratified for the presence or absence of pre-chlorination water purification systems, there was a strong association between the use of non-purified water and excess mortalities from colon cancer.

In a review of epidemiological data relating drinking water and cancer, Crump and Guess (1980) found a significant association between the incidence of cancers of the rectum and total gastrointestinal tract, and the quality of drinking water. It must be recognized that the odds ratios in these studies were small by traditional epidemiological standards (1.37-1.93). In contrast to rectal cancer data, results for cancers of the colon and bladder were not consistent in all studies.

In a national population-based (U.S.A.) case-control study designed to examine the putative association between bladder cancer and chlorination of surface water, Cantor (1984) found no overall elevation in bladder cancer risk among populations utilizing surface,

or chlorinated surface water. However, when the data were stratified into smokers and non-smokers, there was a slight elevation of bladder cancers (relative risk=1.4) among the non-smoking population drinking chlorinated surface water. This association may have been obscured among smokers and the general population by the greater effects of cigarette smoking.

The epidemiological data for carcinogenic risk associated with poor drinking water quality is supported by experimental evidence. Analysis of organic compounds in raw and treated drinking water have identified many volatile and non-volatile compounds which are known or suspected carcinogens (Garrison, 1970; Zoetemann et al, 1977; Loper, 1980; Coleman et al, 1980; Otson et al, 1982). Laboratory studies have demonstrated that halomethanes increase the incidence of cancers in animal models (Dowty et al, 1975; Page et al, 1976). Studies using laboratory animals show an increased risk of cancer associated with chlorination of drinking water (De Rouen and Diem, 1975; Hill et al, 1975).

In addition to animal assays for carcinogenicity, a number of recent studies have shown that concentrates of non-volatile organics in raw and treated drinking water are mutagenic in bacterial assays (Dutka and Switzer-House, 1978; Nestman et al, 1979; Cheh et al, 1980; Flanagan and Allen, 1981; Zoeteman et al, 1982; Kool et al, 1982). Loper et al (1978) found city-specific patterns of bacterial mutagenicity in drinking water concentrates which correlated with epidemiological patterns of cancer risk. One sample, prepared from

New Orleans drinking water, was shown to transform mouse fibroblasts. Gruner and Lockwood (1979) found no bacterial mutagenic activity for Miami drinking water concentrates, but these samples were mutagenic for mammalian cells and induced cellular transformation in human fibroblasts. Grabow et al (1981) found that the mutagenicity of concentrates following chlorination was dependent upon raw water quality; as measured by the total organic carbon content of the raw water sample.

Chlorination has been shown to dramatically increase the mutagenic activity of non-volatile extracts from drinking water (Chen et al, 1979; Fallon and Fliermans, 1980; Dolara et al, 1981; Zoeteman et al, 1982), but the mechanisms by which mutagen/carcinogens are formed are largely unknown. Volatile alkyl halides (including halomethanes) can be formed by chlorination of the phenolic compounds comprising humic, tannic, and fulvic acids (Youssefi et al, 1978; Rook, 1977; Dowty et al, 1976), as well as by chlorination of contaminants such as phenols and anilines (Hirose et al, 1982). Non-volatile compounds with genotoxic activity are also formed by chlorination of humic acids (Fallon and Fliermans, 1980; Watts et al, 1982), and by chlorination of polynuclear aromatic hydrocarbons (Oyler et al, 1982).

II) Oil Refineries- Contamination of Drinking Water:

An average of 3,000 litres of water is required to refine each 42 gal barrel of crude oil (Overgaard, 1960). Of this, approximately 200 litres of water is used in the "processing" of each barrel of oil, and actually comes into contact with the petroleum products. The balance is used as cooling water. Since Canada refines about 2 million barrels of oil per day (EPS, 1979), the volume of water used for crude oil processing amounts to 400 million litres per day . As a result of this large water requirement, the petroleum and petrochemical industry was ranked third in its worldwide potential for pollution of water resources (Gattelier, 1971). The worldwide discharge of oily material by refineries totals approximately 300,000 metric tons per year (Cote', 1976), and petroleum hydrocarbons have been identified at ppm concentrations in waters recieving refinery discharges (Keith, 1974).

There is little information on the carcinogenic risk among populations living near petroleum industries. Hoover and Fraumeni (1975) determined that cancer mortalities, particularily for cancers of the lung, bladder and liver, were elevated in U.S. counties where the density of petrochemical industries was high. Blot et al (1977) found elevated mortality rates for cancers of the stomach, rectum, lung, testes, skin, and nasal cavity/sinuses in counties where refineries were heavily concentrated. These associations were more pronounced in counties with high population densities. Many of these

mortalities may have been a result of occupational exposures, although there is no clear evidence of excess cancer risk in refinery workers (Blot et al, 1977; Rushton and Alderson, 1980). Excess lung cancers among the general population could be related to the release of airborne carcinogens by refineries. Carcinogenic polynuclear hydrocarbons (PAH's) have been found in soots and air surrounding refineries (Blot et al, 1977).

Although the results of these epidemiological studies are difficult to interpret, they do raise some intriguing questions concerning the modes by which petrochemical industries exert influence upon cancer rates in surrounding populations. Cancers of the urinary and gastrointestinal tracts (stomach, rectum, bladder) may be related to the effect of refineries on local drinking water quality. The effluents discharged by refineries have total organic carbon concentrations of 2 to 380 mg/l (PACE, 1981). Petroleum derived compounds have been identified in drinking water sources near refineries (Burnham et al, 1972). Hueper and Ruchhoft (1954) found that extracts from recieving waters contaminated with oil refinery effluents caused skin tumors when painted on mice. Flanagan and Allen (1981) reported high mutagenic activity in river water recieving refinery discharges.

Crude oil contains several PAH compounds, including benzo[a]pyrene, in concentrations of 450-1800 mg per ton (Blumer, 1972). However, the concentrations of PAH's in refinery effluents are in the low ppb range (Ershova, 1967; Andelmann and Suess, 1970; PACE,

1981). The concentration of benzo[a]pyrene in effluents increases with the cracking temperature of the refining process, and is not detected at all from operations under 500°C (Andelmann and Suess, 1970). Many of the PAH's identified in refinery effluent samples are methylated derivatives (Keith, 1974; PACE, 1981). Although little is known about the carcinogenicity of methyl-PAH's, several monomethyl benzopyrene derivatives are carcinogenic, and 11-methylbenzo[a]pyrene exceeds the parent compound in carcinogenic activity (Iyer, 1980). Diamond et al (1984) reported that the mutagenicity of dibenz[a,h]anthracene increased progressively with the substitution of a methyl group at one or both non-benzo bay-region sites.

There is little data on the concentrations of other potentially carcinogenic compounds in refinery effluents. Concentrations of benzene may be at ppm levels, and alkyl halides may exceed concentrations of 100 ppb (PACE, 1981). Various alkyl halides (e.g. chloroform) are carcinogenic in in vivo rodent bioassays (Page and Saffiotti, 1967), and benzene is a recognized leukemogen in humans (Laskin and Goldstein, 1977).

In vitro genotoxicity testing of effluent concentrates has been used to assess the carcinogenicity of effluents from a variety of refining facilities. Since the mutagenicity of fuels produced synthetically from coal and oil-shales is high (Pelroy et al, 1981), effluents from synfuel refineries have been tested for genotoxicity. Effluent concentrates from shale oil production facilities contain potent bacterial mutagens (Epler et al, 1979). These agents gave a

a positive response in the Ames bacterial mutagenesis assay with metabolic activation, and were concentrated in a basic fraction of the effluent extract. Shale oil process waters were also mutagenic in an in vitro mammalian cell mutagenesis assay, but only when extracts were photoactivated with near-UV light or natural sunlight (Strniste and Brake, 1980). Genotoxic, photoactive fractions of the shale-oil effluent were neutral fractions, and were found to include two genotoxic subfractions containing the more hydrophilic furans, furfurals, pyrazines, pyridines, quinolines, and ketones, and the more hydrophilic alkylated aromatic hydrocarbons, aromatic amines, and amides (Strniste et al, 1983). Even though synthetic fuels produced by coal liquifaction have high genotoxic activity (Pelroy et al, 1981), no reports have been published on the genotoxicity of the process effluents from these facilities. The effluents from bitumen upgrading processes in Canadian oil sands refineries (Hrudey et al, 1976) have potential for genotoxic activity, but there is no published information on this subject.

Commoner (1977) found that effluent concentrates from conventional ("low-cracking") refineries were mutagenic in the Salmonella assay, but activity was relatively low. In a study of 7 refinery effluents (PACE, unpublished), concentrates were not mutagenic in the Salmonella assay, did not cause chromosomal aberrations in mammalian cells, and did not elicit DNA repair synthesis in human fibroblasts. However, 4 of 7 samples did inhibit the repair of UV-induced DNA damage. Volatile organic components from

petroleum refineries were reported to give little activity in the Salmonella assay (OME, 1981), but no attempt was made in this study to modify the assay procedure to allow for the volatile nature of extracts.

The character of refinery effluents may vary considerably, depending upon the crude oil being refined, the refining process, and the degree of effluent treatment. A large amount of water is used for cooling of distillation processes, but this "cooling water" is only contaminated when there is an accidental leak in the heat exchanger. "Process water" is in contact with the crude oil and originates from crude desalting processes, as overhead condensate from crude distillation and cracking processes, as wash water from caustic soda treatment, storm water from processing areas, and flushings from lines and tanks (Cote', 1976). In addition to phenolics, sulfides, ammonia, cyanides, nitrogen compounds, and suspended solids, the process water contains high concentrations of oil and grease. Oil and grease concentrations are usually reduced to between 2 and 24 mg per litre in final effluents (PACE, 1981), for a total discharge in Canadian refineries of about 6,000 kg. per day (EPS, 1979). Depending upon the type of refinery, the oil and grease component may consist of gasolines, naphtha, kerosene, fuel oil, bunker C oil, diesel oil, mineral oils, waxes, lubricating oils, asphalt, coke, mercaptans, detergents, and sulfonic acids (Cote', 1976).

Process waters are treated by a variety of methods, but most treatment facilities include: 1) Primary treatment by gravity

separation of oil and water (API separator). 2)Intermediate treatment by air flotation and/or chemical coagulation. 3) Biological treatment in oxidation ponds, trickling filters, or activated sludge plants for removal of organic compounds.

There is potential for the formation of a wide range of harmful compounds when refinery effluents come into contact with chlorine. For instance, phenols, which occur in refinery effluents at ppm levels (PACE, 1981), have been shown to react with chlorine to produce halomethanes (Hirose et al, 1982). Effluent chlorination can occur when refineries do not treat their own effluents, but use municipal sewage treatment facilities for processing (Glaze and Henderson, 1975). The use of municipal water supplies for refining processes may also result in the formation of chlorine reaction products in final effluents (Cote', 1976). Finally, there is potential for chlorination of effluent residues when the discharge of large amounts of refinery effluents into freshwater resources results in significant quantities of effluent residues circulating into drinking water treatment facilities.

Studies which show that benzo[a]pyrene is present at only low concentrations in effluents from refineries operating at low cracking temperatures (Andelman and Suess, 1970) indicate that the refining process may effect effluent carcinogenicity. The majority of refineries are "low cracking" facilities using relatively low catalytic cracking temperatures (approximately 500^{o}C) to produce gasoline and other fuels. Facilities with increased potential for the

formation of carcinogenic pyrolysis products include "high cracking" refineries (producing fuels, ethylene, and organic feedstocks), petrochemical refineries, lube oil reprocessing refineries, upgrading operations for heavy oils, and facilities for the production of synthetic crudes.

III) Study Strategies:

In order to prevent cancers with an environmental etiology, the physical or chemical agents responsible for the disease must be detected and identified. Thus, human exposures to these agents can be limited and the incidence of associated cancers reduced.

There are two basic strategies available for identifying the carcinogenic agents within the complex mixture of compounds comprising environmental samples. In the first strategy, samples can be extracted and concentrated, and subjected to chemical analysis to determine the concentration of carcinogenic compounds. However, the shear number of compounds present in most complex mixtures confounds this strategy. For instance, the number of organic compounds identified in cigarette smoke condensate over many years of research totals greater than 2200 (Schmeltz and Hoffman, 1977). The literature that distinguishes between compounds that are carcinogenic and those

that are not is incomplete or ambiguous. In an attempt to reduce the amount of analytical work, certain groups of carcinogens are often chosen for analysis based upon *a priori* assumptions of relative carcinogenic importance. Groups of unexpected or unknown carcinogens may be ignored in this way. Furthermore, analytical data tells us little about the synergistic activity of compounds in mixtures. These types of chemical interactions have been shown to be important in the carcinogenicity of cigarette smoke (Wynder and Hoffman, 1968).

A second approach to assessing complex environmental mixtures is to test concentrates for carcinogenic and/or genotoxic activity by using *in vitro* or *in vivo* bioassays. These tests require no presumptive decisions on the classes of carcinogens present in the sample, and may give an indication of the synergistic activity of constituent compounds. By applying bioassays to fractionated samples (e.g. "mutagenicity-directed fractionation"), carcinogenic components may be isolated and identified by much less laborious chemical analysis.

Chemical fractionation and bioassay protocols were first used in experimental carcinogenesis studies with cigarette smoke condensate. A mouse skin-painting assay was used to isolate the majority of tumor-initiating activity in a neutral, non-polar fraction of the condensate (Wynder and Hoffman, 1968). Chemical analysis of this fraction indicated that PAH's, nitrogen heterocyclics, and DDT metabolites were the major constituents. Tumor-promoting activity was identified in acidic, phenolic and neutral fractions of the condensate

(Bock, 1972; Wynder and Hoffman, 1969).

The carcinogenic potential of extracts from diesel exhaust particulates have been extensively studied using in vitro assays for bacterial mutagenicity. Mutagenicity-directed fractionation indicated that, while some genotoxic activity was present in neutral, non-polar fractions, the majority of activity was found in more polar fractions (Huisingh et al, 1978; Hites et al, 1981). One polar, mutagenic fraction contained oxygenated compounds which included aromatic ketones and aldehydes (Hites et al, 1981). Mutagenicity-directed fractionation has been used to isolate genotoxic agents from organic concentrates of drinking water. Using column chromatography and preparative HPLC techniques, Loper and Tabor (1983) isolated a highly mutagenic constituent of drinking water which they tentatively identified by GC-MS as a polychlorinated, unsaturated aliphatic ether.

The carcinogenic potentials of several industrial effluents have been characterized by mutagen-directed fractionation. Douglas et al (1983) reported mutagenic activity in chlorination-stage effluents from a bleached kraft pulp mill. Mutagenic activity was confined to three silica-gel subfractions containing compounds of intermediate to high polarity. Medium-polarity compounds included various catechols, chloroacetones, and substituted fatty acids. Pelroy et al (1981) found that Ames-positive mutagens from shale-oil synfuels and effluents were concentrated in a polar fraction, which was shown by GC-MS to be nitrogen rich. Epler et al (1979) found that mutagens in

shale-oil effluents were largely confined to a basic, ether-soluble fraction. Neutral, non-polar mutagens identified in the shale oil itself were not present in plant effluents.

It is interesting to note that many of the genotoxic compounds isolated from complex environmental mixtures are relatively polar compounds. This characteristic does not correspond with the physicochemical properties of many of the "classic" environmental carcinogens, such as PAH compounds.

IV Bioassay Methods for Determining Carcinogenic Potential:

Over the past 10 years, several *in vitro* assays have been developed for determining the genotoxic activity of chemicals. These include tests for mutagenicity with prokaryotic and eukaryotic cells, and assays for clastogenic activity (e.g. chromosomal aberrations, sister chromatid exchanges, micronuclei) with mammalian cells *in vitro*. Because chemicals which are reactive with nuclear DNA are usually mutagenic, clastogenic, and carcinogenic (Ashby, 1982), the major impetus for the development of *in vitro* genotoxicity assays has been their usefulness as rapid screening sysytems for chemical carcinogens.

a) Ames Test:

The present interest in chemical carcinogens as mutagens was inspired by the demonstration by Ames et al (1973) and McCann et al (1975) that the majority of mammalian carcinogens are also mutagenic to Salmonella bacteria. Since then, the Salmonella /mammalian-microsome mediated mutagenesis assay (Ames test) has become the most widely used in vitro test for screening chemical carcinogens.

The test organisms in the Ames test are several mutant strains of Salmonella typhimurium selected for specificity to reversion by mutagens from a histidine requirement to prototrophy. The tests are conducted on plates with histidine deficient agar, and the number of revertant colonies on the plates following incubation are scored. Because many non-electrophilic chemicals require metabolic activation to DNA-reactive species, homogenates of rat-liver (S-9) are included in the test mixture to metabolically transform promutagens to ultimate mutagens.

The tester strains used in the assay were selected from populations with spontaneous or induced mutations in the histidine operon (Hartman et al, 1971). The most commonly used mutant strains are TA1535, TA100, TA1538, and TA98. All of these mutants have deletions in the uvr B region of the chromosome, which eliminates excision repair systems. Other mutations (rfa) eliminate polysaccharide side chains of the lipopolysaccharide bacterial coat,

rendering the bacteria more permeable to chemical agents, and also non-pathogenic.

The strains TA1535 and TA100 contain the histidine mutation his G46, which substitutes GGG (proline) for GAG (leucine) in the gene coding for phosphoribosyl-ATP synthetase (his G), the first histidine biosynthesis enzyme.. It can revert by a direct substitution of the base, or by a variety of suppression mutations which prevent the transcription of the missense codon (Benner and Ames, 1971). This mutation is reverted by alkylating agents. The strains TA1538 and TA98 contain the histidine frameshift mutation his D3052, which has been extensively studied by Iosono and Yourno (1974). The mutation is a deletion of a G.C base pair in the gene coding for histidinol dehydrogenase. Proper coding of the gene is restored most commonly by a -2 deletion of a C.G sequence proximal to the original mutation. These mutations are reverted by metabolites of various aromatic amines, PAH's, aflatoxins, and nitroso compounds, which form adducts with DNA.

The TA100 and TA98 strains contain an R factor (included in a plasmid carrying ampicillin resistance gene) which is thought to enhance the error-prone recombinational (SOS) repair of damaged DNA (McCann et al, 1975). This increases sensitivity to reversion by base-pair and frame-shift mutagens (McMahon, 1979), presumably as a result of base deletions or additions brought about by SOS repair.

The primary criticism of the Ames test has been its failure to detect some well-known mutagens and/or carcinogens (false-negatives).

Haroun and Ames (1980) and McCann and Ames (1978) discussed some of the problems which may lead to false-negative results. They believe that most false negatives are due to inadequacies of the *in vitro* metabolic system in activating promutagens. Safrole is a carcinogen which is not detected in the standard Ames test, while acetoxy safrole, the carcinogenic metabolite, is detected. This suggests that the *in vitro* activation system does not efficiently metabolize safrole. While dimethylnitrosamine gives little response in the standard assay with S-9 activation, preincubation of dimethylnitrosamine with the S-9 gives a positive response. Several mutagens which are negative when tested with rat-liver S-9 (phenacetin, *para*-rosaniline) give positive responses when tested with hamster liver preparations.

Some compounds, such as mitomycin C, give a positive response only with strains having an intact *uvr* B repair system (TA92 and TA94). This mechanism suggests that modifications of DNA by mitomycin C result in improper base-pairing following excision repair. Other compounds, such as actinomycin D, may be negative because they do not accumulate intracellularily in the bacteria (Benedict et al, 1977).

Some compounds are only mutagenic in the presence of cofactors. For instance, azo and diazo dyes are mutagenic when tested with riboflavin (Haroun and Ames, 1980), and aniline and o-toluidine are mutagenic only when tested in the presence of the β-carboline derivative, norharmane (Umezewa et al, 1978). Several chlorinated compounds (e.g. 2,3,7,8-tetrachlorodibenzodioxin, dieldrin) may be

toxic to Salmonella at mutagenic concentrations.

Rinkus and Legator (1979) discussed some of the factors which may lead to false positives in the Ames assay. The role of the R-factor, which leads to error-prone repair and mutagenesis in the Ames assay, has not been sufficiently elucidated. This mechanism of mutagenesis has not been confirmed in mammalian cells. In the case of the alkylating agent, diethyl sulfate, which has dramatically attenuated activity in R-factor strains, there may be little mutagenic activity in mammalian systems.

False-positives may also occur in the Ames test when bacteria metabolize a chemical to a mutagen, but mammals do not metabolize the compound. The nitroreductase of Salmonella that activates nitroaromatic compounds to mutagens is not found in mammalian cells. Nitroreductase-deficient derivatives of TA100 and TA98 have been isolated for studies on nitro-carcinogens (Rosenkranz and Speck, 1975).

One of the major criticisms of the Ames test has been the negative response of the standard test strains to oxidative mutagens, including ionizing radiation, bleomycin, psoralins and light, mitomycin C, peroxides, and aldehydes. To meet this criticism, Levin et al. (1982) developed strain TA102, which has an A.T base-pair at the critical site for reversion within the his G gene. The strain contains the "ochre" his G428 mutation, but there is no uvr B mutation because many oxidizing compounds require excision-repair for expression of mutations (e.g. mitomycin C). The sensitivity of the

gene to reversion was increased by increasing the number of copies of the mutation in the cell. This strain detects a variety of oxidative mutagens, many of which have been shown to act specifically upon thymine bases in DNA.

A positive result in the Ames assay is based upon an observed dose-response, with at least one dose showing double the spontaneous reversion frequency (de Serres and Shelby, 1979). For most mutagens tested in the Ames assay, there is a concentration range that produces a linear dose-response, above which the sample is toxic to the test organism (Maron and Ames, 1983). A number of statistical models have been described (Margolin et al, 1981; Bernstein et al, 1982) which characterize the non-normal distribution of revertants in replicate Ames plates. These complex analyses are useful statistical tools for checking control data vs. historical values, identifying aberrent test results, and defining the linear portion of dose-response curves. However, the criteria for a positive mutagenic response remains the simple observation of a mean response which is double the spontaneous frequency.

b) Sister-Chromatid Exchange Assay:

There is a correlation between the ability of a chemical to act as a mutagen and/or carcinogen, and its ability to induce sister-chromatid exchanges (SCE's) in mammalian cells (Perry and Evans, 1975; Latt et al, 1981; Natarajan and Obe, 1982). Because of this relationship, and because abnormalities in SCE formation appear

to be involved in some chromosome fragility diseases associated with high rates of skin cancer (Chaganti et al, 1974; Latt et al, 1975), the SCE assay has increasingly been used to screen chemicals for carcinogenic potential. However, direct demonstration of a cause and effect relationship between SCE formation and mutagenic processes has not been demonstrated.

SCE's represent the exchange of DNA between sister-chromatids at apparently homologous loci. These exchanges, which are detected in cytological preparations of chromosomes in metaphase, presumably involve DNA breakage and reunion, although the molecular basis of this process is not known. SCE induction does not occur unless there is DNA synthesis in response to DNA damage. Wolff et al (1974) demonstrated that UV-damaged cells needed to pass through S phase for SCE induction to be detected. It has been suggested that SCE formation in metaphase chromosomes is somehow analogous to error-prone repair in bacteria (Latt et al, 1974). SCE's and chromosomal aberrations probably arise by different mechanisms, since agents such as X-rays and bleomycin produce aberrations without inducing SCE's (Sudharsan and Heddle, 1980).

Detection of SCE's in metaphase chromosomes requires some means of differentially labelling sister chromatids. When the halogenated nucleoside 5-bromodeoxyuridine (BrdU or BrdUrd) is incorporated into DNA, it can quench the fluorescence of DNA binding dyes, such as Hoechst fluorochrome 33258. Cells which are allowed to incorporate BrdU for one replication cycle, followed by a cycle in the presence

or absence of this analogue (depending on whether in vitro or in vivo protocols are used), show differential fluorescence of sister chromatids after staining with Hoechst 33258. SCE's are visible as reciprocal alterations in fluorescence along the chromosome.

Fluorescence preparations are not permanent, so Geimsa staining methods utilizing Hoechst 33258 as a photosensitizing agent have become the common technique for routine SCE analysis. The Hoechst 33258 increases photodegradation of BrdU-substituted DNA at wavelengths between 350-400 nm. This procedure produces single strand breaks in the DNA, which after elution of single stranded fragments with buffer, decreases Geimsa staining of DNA.

Monolayer cultures are generally used for in vitro SCE assays. Chinese hamster ovary (CHO) cells are the most suitable cell line for these protocols (Latt ett al, 1981) because of the short generation time (12-14 hr), and low number of relatively large chromosomes (21-22). Furthermore, CHO cells are deficient in excision repair, and there is no need to trypsinize cultures to harvest mitotic cells, since they can be shaken from the vessel walls. Like most monolayer cultures, CHO cells are deficient in metabolic activation enzymes. Therefore, in vitro metabolic transformation of chemicals to electrophiles requires addition of rat-liver microsomes (S-9) to the culture.

Latt et al (1981) reviewed the correlation between SCE induction and the carcinogenicity of chemicals. Of 40 compounds for which there was combined data, 25 showed both positive SCE induction

and carcinogenicity, 4 showed carcinogenicity but no SCE induction, 3 showed only SCE induction, and for 8 compounds the SCE or carcinogencity data was ambiguous. BrdU itself is clastogenic, and may be responsible for most of the baseline SCE's observed in the assay. The assay is very sensitive to the clastogenic effects of mono- and bi-functional alkylating agents, such as nitrogen mustards, mitomycin C, and psoralins plus UV (Allen and Latt, 1976). The SCE assay is less sensitive to other classes of adduct-forming chemicals, such as PAH's and aromatic amines. However, in <u>in vitro</u> systems, this may often be a result of incomplete metabolic activation, since longer periods of incubation with S-9 often increases SCE induction (Takehisa and Wolff, 1978). Many carcinogenic compounds which are not mutagenic give a positive response in SCE assays. These compounds include diethylstilbesterol (Natarajan and Obe, 1981), and the tumor promotor, 12-O-tetradecanoylphorbol-13-acetate (Schwartz et al, 1982).

Latt et al (1981) used a two-tailed t-test to statistically analyse the response in SCE assays. The strength of the response was characterized by the confidence statistic associated with the t-test: $0.01<P1<0.05$; $0.001<P2<0.01$; $P3<0.001$. The agents most effective at inducing SCE's were those with a three-point dose-response curve, with at least one SCE value in the P3 category. In general, the SCE assay system is a rapid and sensitive method for screening chemicals for carcinogenic potential. Because it is a mammalian test system, it is often used in conjunction with bacterial mutagenicity assays as part of a multi-step carcinogen testing

program.

c) In Vivo Carcinogenicity Assays- Fish as Assay Organisms:

In vitro genotoxicity assays do not always give a reliable indication of the carcinogenic potential of a compound (Rinkus and Legator, 1979; Bartsch et al, 1982). For example, urethane, chloroform, sodium saccharin, and β-estradiol are carcinogenic in vivo , but give negative responses in in vitro genotoxicity assays. Moreover, the relative genotoxicity of a compound does not always correlate with its relative carcinogenic potentcy in vivo (Bartsch et al, 1982). Therefore, in vivo carcinogenesis bioassays must remain the definitive test of carcinogenicity.

Rodent bioassays are the standard method used in in vivo carcinogenesis testing. Unfortunately, rodent assays are expensive, labor intensive, and require large amounts of space. Moreover, the testing of compounds by these assays require that relatively large amounts of test material be available. For instance, hepatic tumors were only induced in mice fed DDT for 567 days at concentrations of 140 mg/kg (Innes et al, 1969). Similarily, in the mouse-skin carcinogenesis model, coke oven extract applied at a weekly dose of 0.5 mg per mouse for 52 weeks gave a cumulative skin tumor incidence of 33% (Nesnow et al, 1983). Negative results using one rodent species do not prove that a compound is non-carcinogenic. For instance, bromomethylbenzanthracene is non-carcinogenic in hooded rats, but produces lung and liver tumors in mice (Roe et al, 1972).

Clearly, *in vivo* bioassays are an important component of a comprehensive carcinogenesis study. However, methods are required which are inexpensive, and require small amounts of test material. As an approach to this problem, we developed a bioassay method using rainbow trout embryos which requires single exposures to nanogram quantities of test material.

The suitability of this assay depends upon the similarity in response of fish and mammals to carcinogen exposure. An epidemic of hepatocellular carcinomas in hatchery-reared trout led to the discovery of the potent carcinogen, aflatoxin B_1, in mouldy fish food (Sinnhuber et al, 1977). This mycotoxin was subsequently shown to be carcinogenic to rodents, and implicated in the high incidence of hepatomas among human populations in some developing countries (Shank et al, 1972). The epidemic of aflatoxin B_1-induced carcinomas in hatchery-raised trout initiated research into carcinogenesis in fish. Hart and Setlow (1975) used fish as a test organism to demonstrate that damage to cellular DNA is directly related to carcinogenesis. They treated isolated cells from a clonal molly species (*Poecilia formosa*) with carcinogenic agents (UV-light, 2-acetylaminofluorene), and measured the levels of damage to DNA. When treated cells were injected into isogenic recipients, the fish developed thyroid tumors within 6 to 9 months. A period of photoreactivation following UV treatment caused a decrease in DNA damage (thymidine dimers) and a decrease in tumor incidence. Subsequent studies have indicated that fish species are proficient at photoreactivation of UV-induced damage,

but they are deficient in the excision-repair pathways used to repair the DNA damage induced by most chemical carcinogens.

Stanton (1965) was the first to use small aquarium fish for carcinogenicity studies. He induced hepatomas and cholangiomas in zebra fish (Brachydanio rerio) by aqueous exposure to diethylnitrosamine. As summarized in Table 1, several investigators have subsequently induced tumors in aquarium fish by exposing them to a variety of chemical carcinogens, including 7,12-dimethylbenzanthracene, benzo[a]pyrene, 2-acetylaminofluorene, o-aminoazotoluene, ethylnitrosourea, dimethylnitrosamine, and methyl azoxymethanol acetate. In all cases, the exposure of fish to carcinogenic compounds in water or food resulted in the development of hepatic neoplasms (Table 1). An exception to the specificity of the liver to chemical carcinogenesis is the induction of chromatophoromas in the marine croaker (Nibea mitsukurii) after aqueous exposure to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) or 7,12-dimethylbenzanthracene (Kimura et al, 1981). Matsushima and Sugimura (1976) listed the advantages of aquarium fish as carcinogenesis assay organisms, including their resistance to the toxic effects of the carcinogen, the relative rapidity with which they develop tumors, and the ease with which large numbers of animals can be maintained in the laboratory. However, tumor induction in aquarium fish often requires prolonged exposure to high concentrations (ppm) of the carcinogen.

Fish species have also been used as assay organisms for skin-painting carcinogenesis models. Ermer et al (1970) induced

epithelioma in Gasterosteus aculeatus by repeatedly painting with benzo[a]pyrene or 3-methylcholanthrene. Black et al (1984) reported papillomas on the skin of brown bullheads (Ictalurus nebulosus) after repeated application of extracts from contaminated river sediments.

The outbreak of hepatocellular carcinomas among hatchery-raised trout also stimulated research on carcinogenesis in salmonid species. Early studies on the etiology of the trout liver cancer epizootic, and the use of trout as an animal model for chemical carcinogenesis were performed under the direction of Halver and Ashley (Ashley and Halver, 1968; Ashley, 1973). Further studies on the carcinogenicity of aflatoxin B_1 (AFB1) in rainbow trout, as well as the development of trout as a carcinogenesis assay organism, were conducted by Sinnhuber and co-workers (Sinnhuber et al, 1977; Greico et al, 1978; Wales et al, 1978; Hendricks et al, 1980a). One of the features of rainbow trout which enhances its value as a carcinogenesis research animal is the extreme rarity of spontaneous liver neoplasms among control fish. Any neoplasm observed in experimental animals can normally be attributed to a test chemical.

The rainbow trout is the most sensitive salmonid species to AFB1 carcinogenicity, and the Shasta strain of rainbow trout appears to be the most sensitive of the various genetic strains(Hendricks, 1982). Lee et al (1968) fed AFB1 at 0.4 ppb to rainbow trout for 60 weeks and produced a 14% incidence of liver carcinomas. In a similar experiment with rats, Wogan et al (1974) fed AFB1 to Fischer rats at

15 ppb for 96 weeks and produced liver carcinomas in 19% of the animals. Hepatic carcinomas have been induced in trout by dietary exposure to other aflatoxin analogues (Hendricks et al, 1980a), dimethylnitrosamine (Ashley and Halver, 1968; Greico et al, 1978), p,p'-DDT (Hendricks, 1982), urethane, carbon tetrachloride, carbazone, aminoazobenzene, thiourea, diethylstilbesterol, and tannic acid (Halver, 1967). However, the dietary method of exposure to carcinogens has several weaknesses; including the unequal ad libitum exposure of the animal to the chemical, and the considerable amounts of chemical required for extended feeding trials.

Sinnhuber and co-workers (Wales et al, 1978) recently developed a new technique for chemical carcinogenesis in rainbow trout, in which fertile rainbow trout embryos (egg stage) were exposed to aqueous solutions of a carcinogen for a single, short period. They found hepatic carcinomas in up to 60% of trout necropsied 12 months after exposure to 0.5 ppm of AFB1 for 1 hr. Using this assay, Hendricks et al (1980a) found that other aflatoxin metabolites and mycotoxins produced hepatic neoplasms. MNNG produced both hepatomas and nephroblastomas, and high concentrations of dimethylnitrosamine (500 ppm) produced hepatic carcinomas. However, several other potent carcinogens (ethylnitrosourea, diethylnitrosamine) did not give a positive response. The embryo may not have been adequately exposed to the carcinogen because of the impermeability of the embryo chorion to these compounds.

The tumor promoting effects of various compounds have been

studied using the trout embryo immersion protocol. Cyclopropenoid fatty acids fed to trout following embryo exposure to AFB1 produced a large increase in hepatic tumor incidence (Hendricks, 1981). In a similar experiment with Aroclor 1254 (a polychlorinated biphenyl mixture), there was no promotional effect upon AFB1 initiated tumorigenesis (Hendricks et al, 1980c). However, when gravid female rainbow trout were fed Aroclor 1254, there was enhancement of hepatocarcinogenesis among the offspring exposed to AFB1 by the embryo immersion protocol (Hendricks et al, 1981).

The liver is the predominant site of chemically-induced tumorigenesis in fish (Table 1), but tumors in rodents may be situated in a variety of other tissues, depending on the route of administration. While 7,12-dimethyl- benzanthracene (DMBA) induced liver tumors in fish (Schultz and Schultz, 1982), DMBA-induced tumors in rodents occur in the skin, intestine, kidney, or mammary gland, depending on the route of administration (Carrol and Khor, 1970; Toth, 1970). Obviously, tissue-specific homology cannot be expected in chemically-induced tumors in fish and rodent species. Factors which have been cited as explanations for intraspecific differences in mammalian carcinogenesis (Bartsch et al, 1982) would certainly apply to comparisons between fish and rodents. These include differences in binding reactions of carcinogens to macromolecules, DNA repair, cellular replication, and stimuli to tumor growth (e.g. hormones).

Since many of the compounds listed in Table 1 are not direct-acting carcinogens, it can be assumed that there are similar

metabolic activation processes for fish and rodents. Several studies have indicated that fish and rodent species metabolize xenobiotics by similar pathways (Chambers and Yarbrough, 1976; Sundstrom et al, 1976; Lech, 1981). However, fish and rodents may differ in the quantitative aspects of biotransformation which effect the concentration of an active carcinogen at the target site. For instance, the in vitro metabolism of benzo[a]pyrene to dihydrodiols and hydroxymetabolites by trout liver microsomes is actually 5 to 10 times higher than rates with rat liver microsomes (Ahokas et al, 1975).

Table 1: Experimental chemical carcinogenesis studies with aquarium fish species.

Compound	Exposure Route	Species	Target Organ	Reference
Aflatoxin B_1	Food	Guppy	Liver	1
	Food	Medaka	Liver	2
Aflatoxin G_1	Food	Medaka	Liver	2
Sterigmatocystin	Food	Guppy	Liver	3
	Food	Medaka	Liver	2
Cycasin	Food,Water	Zebrafish	Liver	4
Diethylnitrosamine	Water	Guppy	Liver	5
	Water	Guppy	Liver,Esophagus	6
	Water	Guppy	Liver,GI-tract	7
	Water	Medaka	Liver	8
	Water	Medaka	Liver	9
	Water	Medaka	Liver	10
	Water	Medaka	Liver	2
	Water	Poeciliopsis	Liver,Hematopoietic	11
	Water	Rivulus	Liver	12
	Water	Zebrafish	Liver,Esophagus	6
	Water	Zebrafish	Liver	13
Dimethylnitrosamine	Food	Guppy	Liver	1
	Water	Guppy	Liver	5
	Water	Guppy	Liver	6
	Water	Zebrafish	Liver,Esophagus	6
	Water	Zebrafish	Liver	13
Nitrosomorpholine	Water	Guppy	Liver,Esophagus	6
	Water	Zebrafish	Liver,Intestine	6
	Water	Zebrafish	Liver,Intestine	5
N-Dinitrosopiperazine	Water	Guppy	Liver,GI-tract	7
2-Acetylaminofluorene	Food	Guppy	Liver	1
	Food	Guppy	Liver	5
o-Aminoazotoluene	Food	Guppy	Liver	5
	Food	Medaka	Liver	2
4-Dimethylamino-azobenzene	Food	Guppy	Liver	5
7,12-Dimethylbenz-anthracene	Water	Poeciliopsis	Liver	14
Methylazoxymethanol acetate	Water	Medaka	Liver	15
	Water	Medaka	Liver	2

1) Sato et al, 1973; 2) Hatanaka et al, 1982; 3) Matsushima et al, 1975; 4) Stanton, 1966; 5) Pliss and Khudoley, 1975; 6) Khudoley, 1984; 7) Simon and Lapis, 1984; 8) Kyono, 1978; 9) Ishikawa and Takayama, 1979; 10) Klaunig et al, 1984; 11) Schultz and Schultz, 1984; 12) Koenig and Chasar, 1984; 13) Aydin and Bulay, 1983; 14) Schultz and Schultz, 1982; 15) Aoki and Matsudaira, 1977.

PART 2: GENOTOXIC ACTIVITY OF PETROLEUM REFINERY EFFLUENTS

PURPOSE

The purpose of this section of the study was to evaluate the levels of genotoxic agents in liquid effluents discharged by petroleum refineries. Concentrates of three effluent components (particulates, dissolved non-volatiles, and dissolved volatiles) were prepared and tested by *in vitro* assays (Ames test, SCE assay) to determine genotoxic activity. Mutagen-directed fractionation was used to isolate and partially characterize the genotoxic agents in effluents.

If significant quantities of organic contaminants from refinery effluents circulate into public drinking-water treatment facilities, chlorination of the water may produce hazardous compounds. Therefore, we also evaluated the effect of chlorination on the genotoxic activity of whole and dilute effluent samples. Comparisons were made of the relative contribution of refinery effluents and natural organic

compounds (e.g. humic acids) to genotoxicity following chlorination.

MATERIALS AND METHODS

I) Chemicals and Solvents

Distilled-in-glass or HPLC-grade solvents were purchased from Caledon Laboratories Ltd. (Georgetown, Ont). The n-alkanes, phenol, aniline, o-toluidine, and norharmane were purchased from Sigma Chemical Co. (St. Louis, Mo.). Mitomycin C, napthalene, 1-chloronapthalene, phenanthrene, benzo[a]pyrene, benzo[e]pyrene, 1-aminoanthracene, 2,6-dimethylphenol, hexachlorophenol, tetrachloro biphenyl, dimethylphthalate, diethylphthalate, dibromomethane, 1,2-dichloroethane, carbon tetrachloride, chloroform, and methylene chloride were purchased from RFR Corp. (Hope, R.I.). Epichlorhydrin and tannic acid were purchased from Fisher Scientific Co. (Fairlawn, N.J.). The 1-nitropyrene and 2-acetylaminofluorene were gifts from D. McCalla, McMaster University, and p-chlorophenol was a gift of M. Fox, Canada Dept. Environment. R. Gupta, McMaster University, supplied N-methyl-N'-nitro-N-nitrosoguanidine. Histidine, biotin, NADP, and glucose-6-phosphate were purchased from Sigma. Humic acid, Hoechst

fluorochrome 33258, and BrdU were purchased from Aldrich Chemical Co. (Milwaukee, WI). "Amberlite" XAD-2 resin was purchased from BDH Chemicals (Toronto), and Tenax-GC resin (30/60 mesh) was purhased from Applied Sciences Laboratories (State College, PA).

II) Sample Collection:

Effluents were collected from three refineries in Ontario, Canada. Refinery 1 was sampled several times during 1981 to 1983. Refinery 2 was sampled once during the winter of 1983, and Refinery 3 was sampled in February and June of 1983. "Grab" samples of 4 litres were collected in glass solvent bottles (non-volatiles), or in stoppered 500 ml glass bottles (volatiles). At all refineries, final effluent samples were taken at spillways just prior to discharge into adjacent bodies of water. Samples of intake water were also taken from sampling valves in the refinery cooling towers. At Refinery 1, in October, 1981, samples were taken at two additional sites within the effluent treatment facility (after oil separation, and after secondary clarification). All samples were stored at $4^{o}C$ under nitrogen, and processed within 96 hr of collection.

III) Water Quality Analysis:

Effluents were analyzed for ammonia-nitrogen, pH, total suspended matter, oil and grease, and phenol. Ammonia was determined colorimetrically by the method of Verdouw et al (1977), in which ammonia ion in a 38 ml effluent sample is reacted with 2 ml of sodium hypochlorite solution (2% chlorox bleach in 0.1 N NaOH), and 5 ml of sodium salicylate solution (40% in distilled water) to form blue-colored indophenol. Potassium ferrocyanide solution (2%) was used as a catalyst for the reaction, and sodium citrate solution (10%) was added to prevent precipitation of ammonium ions at the high pH of the reaction mixture. Absorbance was measured at 660 nm in 1 cm cuvettes after 1 hr, and compared to a calibration curve with ammonium sulfate in distilled water.

All other water quality parameters were determined by the methods outlined in "Standard Methods for the Examination of Water and Wastewater" (14th edition). Briefly, oil and grease was determined by extracting the material retained when a 1 litre water sample was passed through a 0.45 µm glass fibre filter. The filter was dried at 100°C for 30 min and extracted into petroleum ether (130-160°C) in a soxhlet apparatus for 4 hr. The petroleum ether was evaporated in a rotary evaporator, and the residue weighed. Total suspended matter was determined by passing 100 ml of sample through a 0.45 µm glass fibre filter, drying the filter at 103°C for 1 hr,

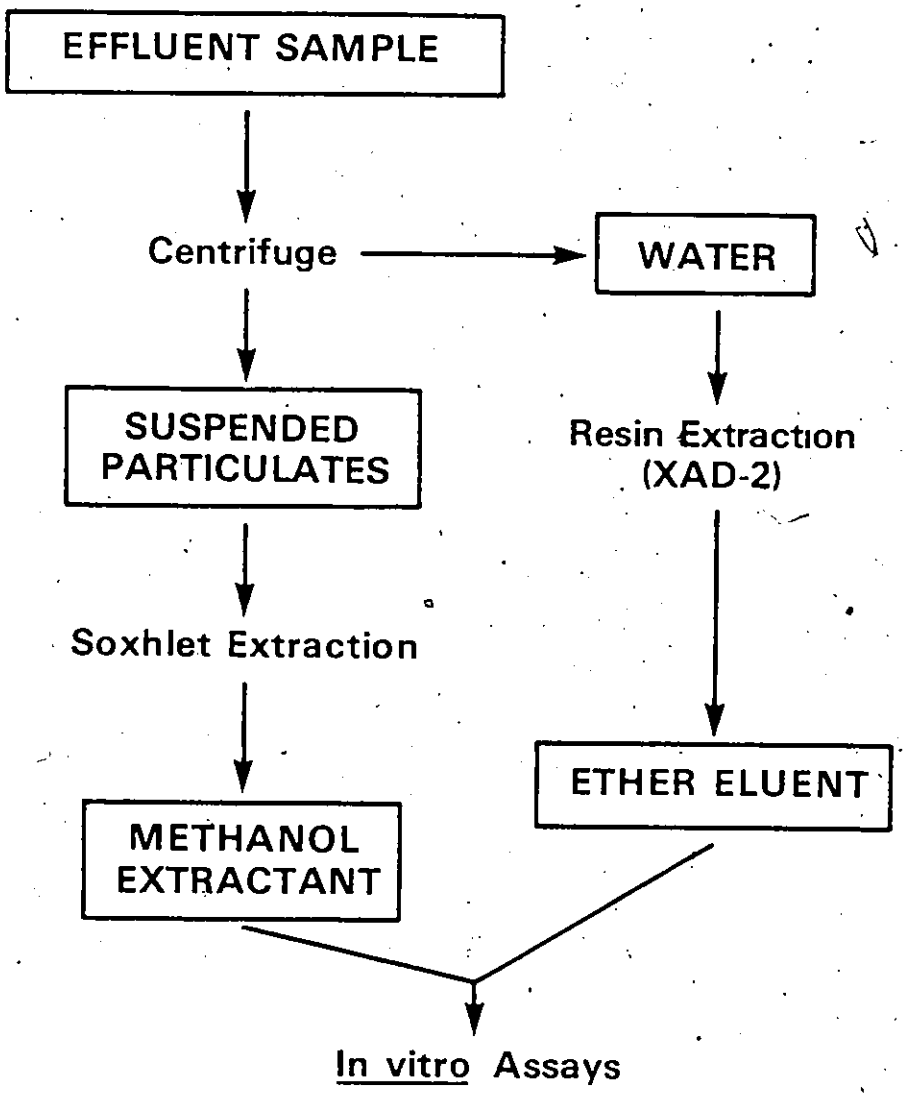
and weighing the residue on the filter. Phenol concentrations were determined after distillation of phenols from nonvolatile impurities in a 100 ml effluent sample. The distilled phenol was reacted with 2 ml of 4-aminoantipyrene solution (2%) after adjusting the pH of the distillate to 10 with ammonium hydroxide solution. The pink-colored reaction product was concentrated by extracting with 25 ml of chloroform. Chloroform extracts were dried by passing through sodium sulfate, and the absorbance read at 510 nm in a 1 cm cuvette. A Corning 610A pH metre and a Perkin-Elmer "Lambda 3" spectrophotometer were used for measurement of water quality parameters.

IV) Sample Extraction and Concentration:

The procedures for extraction of residues from effluent samples are summarized in Figure 1. Particulates were separated from effluent samples by centrifuging on a Cepa-Schnell continuous centrifuge (10,000 rpm) at a flow rate of 250 ml/min. Particulates were washed from the centrifuge rotor with 150 ml methanol, and the particulate/methanol slurry was filtered through a fritted glass extraction thimble (0.4 μm frit). The methanol passing through the thimble was saved. The particulates retained in the thimble were extracted by soxhlet (6 hr) into the methanol saved from the rotor wash. In order to test the efficacy of extraction using methanol,

Figure 1: Flow-diagram of procedures for the extraction and concentration of non-volatile components of oil refinery effluents.

Extraction . - Non-Volatiles



particulates from February, 1982 samples were extracted into methanol as described above, then further extracted for 6 hr into 150 ml. of methylene chloride.

The supernatant collected after continuous centrifugation was passed in volumes of 2 litres through columns (8 cm x 2.3 cm I.D.) of "Amberlite" XAD-2 resin (8 gm dry wt) at a flow rate of 25 ml/min. Organic compounds retained on the resin were eluted with 100 ml of diethyl ether (containing 1/4% ethanol as preservative). The ether was equilibrated in the column for 15 min prior to elution. In order to test the completeness of extraction using ether, samples collected in February, 1982 were extracted into ether as described above, and further eluted with 100 ml of methanol after equilibration of the solvent in the column for 1.5 hr.

All extracts were evaporated to approximately 15 ml on a rotary evaporator, and dried by passing through approximately 20 gm of sodium sulfate in a Buchner funnel. The sodium sulfate was then washed with 20 ml of the same solvent used in the extraction. The combined volume was again rotary-evaporated to approximately 2 ml, and this was evaporated just to dryness under a stream of nitrogen. After weighing the residue, the extract was made up to volume in acetone. All extracts were stored at $4^{\circ}C$ in amber glass vials, capped with air-tight septa. The sodium sulfate used to dry extracts had been previously washed with n-hexane (glass-distilled) to remove contaminants.

Volatile organic compounds were extracted from effluents by a

modification of the purge-and-trap technique of Pagel and Smillie (1980). An effluent sample (500 ml) was placed in a gas wash-bottle and heated to 80°C in a water bath. Volatiles were stripped from the sample by bubbling with nitrogen at a flow rate of 100 ml/min. The stripped volatiles were adsorbed by two glass traps in series, each containing 0.4 gm of 30/60 mesh Tenax GC packing. Traps were made from the bulb sections of 15 ml glass, volumetric pipettes, and Tenax resin was sealed into the traps with silanized glass wool. After 0.5 hr of extraction, the initial two traps were replaced and extraction continued for another hour. The four traps were each placed in a 15 ml glass, graduated centrifuge tube, and 0.5 ml of methanol was pipetted into the top of each trap. The tubes were centrifuged at 10,000 rpm for 5 min. on an IEC centrifuge at 4°C. A further 0.5 ml of methanol was added to each trap, and the tubes re-centrifuged for 5 min. The 0.75 ml volumes of methanol eluted from each trap into the centrifuge tubes were pooled, and the final volume (3 ml) stored in air-tight glass septum-vials at 4°C.

Non-volatile dissolved extracts and particulate extracts were subfractionated into acid, base, and neutral fractions by the method reported by Bryant and McCalla (1982), with the exception that diethyl ether was used instead of cyclohexane as the organic solvent. The effluent extract in 100 ml of diethyl ether was successively extracted into 100 ml of mild base(5% Na_2CO_3), and mild acid (5% acetic acid) solutions. The pH of the acid solution was then adjusted to pH 11 with 0.1 M NaOH, and the pH of the base solution adjusted to pH 1

with 0.1 M HCl. Base and acid fractions were then partitioned back from the aqueous phase into diethyl ether. The original organic (ether) phase retained neutral compounds.

Extracts were also subfractionated by silica gel column chromatography according to the method of Coleman et al (1980). Extract residue with a dry weight of between 20 and 25 mg was dissolved in 0.5 ml. of "hot" hexane, and applied to the top of a 14 cm x 5 mm I.D. micro-chromatographic column (disposable pasteur pipette) packed with 0.2 gm of 5% deactivated silica gel. Silica gel was activated before use for 2 hr at 130°C, cooled, and deactivated by adding distilled water (5% by weight). The silica gel was stored in a stoppered, glass Ehrlenmyer flask, and used within a week of activation. Organics were partitioned into 7 subfractions by eluting with the following solvents: 1) 0.5 ml hexane, 2) 1 ml hexane, 3) 4 ml hexane, 4) 4 ml hexane-benzene (1:1), 5) 4 ml benzene, 6) 4 ml methylene chloride 7) 4 ml methanol.

In order to determine the efficiencies of the extraction procedures, 2 litres of distilled water and 1 gm of activated alumina (representing particulates) were spiked with non-volatile compounds dissolved in 0.1 ml acetone (naphthalene, phenanthrene, benzo[a]pyrene, aniline, o-toluidine, 1-anthramine, dodecane, hexadecane, eicosane, phenol, dimethylphenol, p-chlorophenol), and extracted by the techniques previously described. These compounds were spiked at concentrations of 50 and 5 ug per litre for extraction by XAD-2 resin, and at 100 and 10 ug per gm for extraction by soxhlet

procedures. Volatile compounds (chloroform, benzene, 1,2-dichloroethane, dibromomethane, phenol) were spiked at concentrations of 2 and 0.2 mg per litre into 500 ml distilled water, and extracted by the purge-and-trap methods described previously.

We compared the system of XAD-2 resin extraction used in this study to another XAD-2 protocol used for concentrating drinking water samples (Nestmann et al, 1979). Using this method, we extracted mutagens from 200 litres of drinking water with an XAD-2 resin column described by LeBel et al (1979). Briefly, the XAD-2 column consisted of a copper tube (16 mm O.D.x 36 cm) filled with 15 gm of XAD-2 resin, which was connected by copper tubing to a drinking water tap. Water flow was controlled at a flow rate of 140 ml/min by a Nupro metering valve between the tap and cartridge. The XAD-2 resin was eluted with 300 ml of acetone-hexane (15:85) after 15 min. of solvent equilibration in the column. Extracts were dried and concentrated as described previously.

V) Chlorination of Refinery Effluents:

Undiluted effluents, and dilute effluent solutions (2.5%-20%) were chlorinated under various conditions of pH and chlorine concentration. The dissolved (volatile and non-volatile) organic components of the chlorinated solutions were then concentrated for in

vitro genotoxicity testing. Effluents used in chlorination tests were collected as described previously in June, 1982 and February, 1983 from the final holding-pond at Refinery 1.

Effluents were diluted with volumes of organic-free water, prepared by passing double-distilled water through 0.45 um membrane filters and "Norganic" resin cartridges (Millipore Ltd.). A stock chlorine solution was prepared by bubbling high-purity chlorine gas (Matheson Corp.) through organic-free water. The chlorine content of this solution was determined by the N,N-diethyl-p-phenylenediamine (DPD) colorimetric method described in "Standard Methods for the Examination of Water and Wastewater" (14th edition). DPD indicator solution (0.5 ml) and phosphate buffer solution (0.5 ml) were added to a 10 ml effluent sample (it was necessary to dilute chlorine stock solutions with distilled water), and free chlorine measured by absorbance at 515 nm in a 1 cm cuvette. Total chlorine was measured after addition of 0.2 ml of potassium iodide solution (0.1 gm/100 ml distilled water). Chlorine concentrations were calculated from a calibration curve of absorbance vs concentrations of potassium permanganate standards.

Effluent solutions were chlorinated by dispensing an appropriate volume of chlorine stock (2-8 ml) into 2 litres of effluent solution (non-volatile tests), or 500 ml of effluent solution (volatile tests) in stoppered amber-glass bottles. Solutions were incubated at 20°C, with gentle vortex mixing, at chlorine concentrations of 2, 4, 8, or 16 ppm for 1, 2, or 4 hr. The pH of the

solutions were periodically adjusted to 4, 6, or 8 with NaOH or HCl solutions (0.1M). Concentrations of total and free chlorine were determined by the DPD method at the beginning and end of the contact period.

In a separate experiment, tannic and humic acids, and refinery effluents in amounts equivalent to 10 mg of organic carbon were added to 2 litres of organic-free water (pH 6.0) and chlorinated at 8 ppm for 1 hr. The organic carbon content of acid residues and effluents was determined by the modified Walkley-Black dichromate oxidation method described by Bremner and Jenkinson (1960). In preparation for organic carbon determinations of refinery effluent, a 1 litre sample was lyophilized with a New Brunswick Scientific Model B64 freeze-dryer.

Volatile dissolved components of chlorinated samples were extracted by the purge-and -trap techniques previously described. The non-volatile, dissolved components of chlorinated samples were extracted by the XAD-2 methods previously described, without prior centrifugation of particulates. However, after elution of the column with 100 ml of diethyl ether, residual chlorine was removed from the eluent by passing it through a Buchner funnel containing approximately 15 gm of sodium sulfite. Non-volatile extracts were subfractionated into 7 fractions of increasing polarity by silica gel column chromatography, according to the method of Coleman et al (1980) outlined previously.

In a separate series of experiments, diluted effluent (2.5%)

made up in large volumes (20 litres) of surface water from the Hamilton Harbour area of Lake Ontario was chlorinated at 8 ppm for 1 hr (pH 6.0, 20°C), and extracted by an XAD-2 method, similar to the one described previously for 2 litre sample volumes. However, in this case the 20 litre sample was passed through the resin column at a faster flow rate of 120 ml/min. Sample elution, residual chlorine removal, sample drying, and evaporation of solvent were all as described above.

VI) Mutagenicity Assay:

The Salmonella /mammalian-microsome mutagenicity assay (Ames test) was conducted essentially as described by Ames et al (1975) for the plate-incorporation assay. Initially, samples were tested using Salmonella strains TA98, TA100, TA1535, and TA1538, but subsequent samples were tested using strains TA98 and TA100 only. All bacteria strains were tested by the methods outined by Ames et al (1975) for maintenance of histidine requirement, deep rough (rfa) character, ampicillin resistance (R factor), and UV sensitivity (uvr mutation). Stock culture was grown in Difco nutrient broth to a density of 1-2 x 10^9 cells per ml, and after addition of dimethylsulfoxide (3.5 ml per 40 ml of culture), the culture was frozen at -60°C in 1 ml Nunc provials.

Rat-liver microsomes (S-9) were prepared from Aroclor 1254-induced rats (Sprague-Dawley) according to the method outlined by Ames et al (1975). Male rats (200 gm) were i.p. injected with 0.5 ml of Aroclor 1254 in corn oil (200 mg/ml) and killed by decapitation after 5 days. Livers were placed in cold 0.15M KCl solution (1 ml/gm of liver) and homogenized using a Potter Elvehjem apparatus. The homogenate was centrifuged at 9000 g for 10 min on a Sorval RC2-B centrifuge (SS-34 head), and the supernatant decanted and centrifuged again. The second supernatant was frozen in 2 ml Nunc provials in liquid nitrogen.

Minimum agar plates were prepared a week prior to use, in order to dissipate condensation in the plate. Minimum agar was prepared by combining molten 3% Difco minimum bacto-agar (200 ml), 4% glucose solution (200 ml), and 10X Vogel-Bonner solution (45 ml). The agar was poured in 20 ml quantities into Fischer 8-757-12 (100 x 15 mm) plates, and the plates were stored upside down in a dust-free container. Vogel-Bonner solution (10X) was prepared from 2 gm $MgSO_4.7H_2O$, 35 gm $Na_2NH_4PO_4.4H_2O$, 100 gm KH_2PO_4 and 20 gm citric acid, made up to a volume of 1 litre with distilled water.

The S-9 mix (10 ml) was prepared by mixing 0.2 ml of $MgCl_2$ solution (0.4 M), 0.2 ml of KCl solution (1.65 M), 0.4 ml of glucose-6-phosphate solution (1.0 M), 0.5 ml of NADP solution (0.01 M), 5 ml of Na_2HPO_4 solution (0.2 M), and the balance of the volume as S-9 and sterile, distilled water (Gibco). Prior to use, S-9

mix was sterilized by filtration through a Millex-GS (0.22 μm) filter unit (Millipore Corp.). The optimum volume of S-9 required in the mix (5-20%) was determined for each batch of liver microsomes by optimizing the response of TA100 and TA98 to benzo[a]pyrene (BaP) and 2-acetylaminofluorene (2-AAF). In later batches of S-9, this volume was further confirmed by optimizing the response to effluent extracts.

To begin the assay, 0.1 ml of thawed, stock bacterial culture was placed in 10 ml of Difco nutrient broth in a culture tube with a loose-fitting cap. The culture was grown overnight (12 hr) at 37°C to a density of 1-2 x 10^9 cells per ml. The optical density of the overnight culture was determined by nephelometry at 600 nm wavelength, and compared to a calibration curve of O.D. vs bacterial density. Bacterial density was determined for the calibration curve by counting colonies in a dilution series (10^{-1} to 10^{-8}) grown up in nutrient agar.

A 0.1 ml volume of bacteria culture and 0.5 ml of S-9 mix were placed in an autoclaved glass culture tube (15 ml) with the test chemical or extract dissolved in 0.1 ml of solvent. Initially, test chemicals and extracts were dissolved in dimethylsulfoxide (DMSO), but because of the inadequate solubility of refinery extracts in this solvent, samples were later dissolved in acetone. Volatile extracts were dissolved in methanol. Both acetone and methanol are considered suitable solvents for use in the Ames assay (Mattern and Greim, 1978; Maron et al, 1981). The test mixture was added to 2 ml of moten top agar at 45°C, mixed thoroughly, and poured into minimal-agar

plates. The molten top-agar consisted of 0.6% Difco bacto-agar, and 0.5% NaCl in 100 ml of distilled water, to which was added 5 ml of sterile L-histidine.HCl solution (0.5 mM) and 5 ml of sterile biotin solution (0.5 mM). After cooling of the top agar, the plates were incubated upside-down for 3 days at 37°C.

Volatile extracts were tested for mutagenicity using a modification of the Ames protocol. In "aqueous phase" tests, extracts were combined with top agar and plated in plastic assay dishes. The dishes were sealed by covering with glass plates (10cm x 10cm x 0.6cm) rimmed with silicone stop-cock grease. The plates and pre-applied grease were previously autoclaved and dried at 90°C. Extracts tested in "gaseous phase" (extracts not mixed with top agar) were placed on the glass plate surface, and the plates were sealed onto a petri dish containing bacteria in the top agar. After 8 hr incubation at 37°C, the glass plates were removed and replaced by normal plastic dish covers for the remainder of the incubation period. Tests were conducted with S-9 activation in the aqueous phase protocol by pre-incubating the extract with bacteria and S-9 for 20 min at 37°C in air-tight septum vials, according to the method of Yohagi et al (1977). In the gaseous phase tests, S-9 was added to the bacteria in the top agar layer.

All samples were tested for a dose-response over a range of at least 4 doses. At each of these doses, mean revertants per plate were calculated from three replicate plates. In each test series, there were three replicate plates of sterility controls, spontaneous

revertants, and positive controls with BaP and 2-AAF. Plates were counted using a New Brunswick Scientific Model C111 automated colony counter.

In selected assays, estimates were made of the number of bacteria surviving the toxic effects of the test extract in the Ames plate. Dilution series (10^{-4} to 10^{-8}) were made in nutrient broth from an overnight culture of the test strain. These dilute bacterial cultures were plated with test compounds and S-9, as in experimental plates, with the exception that high concentrations of histidine (0.1M) were added to the top agar. The numbers of colonies present in the dilution series plates gave an estimate of the numbers of bacteria surviving the toxic effect of extracts.

VII) Comutagenicity Assays:

Particulate (soxhlet extract), and non-volatile dissolved (XAD-2 extract) components of effluent were tested with benzo[e]pyrene (BeP)and norharmane in the Ames assay to determine if these two known comutagens enhanced extract mutagenicity. Extracts were tested with BeP as described by Rao et al (1979). Preliminary tests showed that 1, 2.5, and 5 µg of BeP per plate produced a dose-dependent increase in the mutagenic response of TA98 and TA100 to BaP (Appendix 1). These same BeP concentrations and test conditions were used in

comutagenicity assays with XAD-2 and soxhlet extracts from Refinery 1 (June, 1982 sample).

The soxhlet and XAD-2 extracts were also tested with the comutagen norharmane, according to the method of Umezewa et al (1978); with the exception that test solutions were not pre-incubated for 30 min before plating. In our hands, the pre-incubation step was toxic to the test organism (Appendix 2). In preliminary trials, norharmane at a concentration of 0.45 µmoles per plate (in DMSO) was found to be most effective in enhancing the mutagenic response of TA98 to 2 µg of 2-AAF (Appendix 2). These same test conditions were used to determine the effect of norharmane on the mutagenicity of effluent extracts.

Effluent extracts (soxhlet and XAD-2) were also tested to determine if they enhanced the mutagenic activity of BaP (5 µg/plate) or 2-AAF (2 µg/plate). A range of extract concentrations were plated with the two known mutagens.

VIII) Sister Chromatid Exchange (SCE) Assay:

Chinese hamster ovary cells were cultured in αMEM medium with 10% fetal calf serum, 100 µg/ml streptomycin, and 100 U/ml penicillin. To start the assay, cells were inoculated into 25 ml of medium in 250 ml tissue culture flasks, and incubated for 12 hr.

When metabolic activation was required, fresh medium without

serum and 10% S-9/cofactor mix (prepared as in the Ames assay) were added after 12 hr incubation. Test extracts were added to culture flasks in 0.1 ml of acetone (non-volatiles), or in 0.1 ml of methanol (volatiles). After the flasks were incubated with extracts for 1 hr, or in some cases for 2.5 hr, the medium was changed and 10^{-5}M 5-bromodeoxyuridine (BrdU) was added. The flasks were wrapped in aluminum foil and incubated for 22 hr. After 22 hr, 0.25 ml of colchicine solution (200 µg/ml) was added to each flask, and 2 hr later cells were harvested by shake-off. The culture was approximately 50% confluent at this point.

The harvested cells were treated for 7 min with 10 ml of hypotonic solution at 37°C (0.075 M KCl), and hardened by the addition of 2 ml of 3:1 methanol-acetic acid fixative. After 15 min hardening, cells were centrifuged and fixed overnight at 4°C in 10 ml of fixative. Cells were centrifuged and an appropriate volume (0.25-0.5 ml) of fresh fixative added for spreading of metaphases.

Cells were spread on dry slides which had been cleaned overnight in 100 % ethanol at 4°C. Two drops of cell suspension were placed on the slide, and as the fixative evaporated, metaphases were spread by sharply blowing on the slides. Slides were dried for a week before staining.

Slides were stained in the dark for 12 min with Hoechst 33258 fluorochrome (5 mg per 100ml distilled water), rinsed with distilled water, and dried. Slides were mounted in Sorenson's buffer (pH 8.0), placed on a slide warmer at 50°C, and exposed at a distance of 5

cm to black light (Westinghouse F20 T12/BLB, 20W) for 2.5-3.0 min. Slides were rinsed in distilled water and stained in 4% Geimsa for 6 min; then dried and mounted with DPX mountant.

The mean number of SCE's per metaphase at each sample dose was calculated by scoring 25 metaphases. The significance of differences between the mean number of SCE's per metaphase in experimental and control tests were determined by Student's two-tailed t-tests. All samples were tested for a dose-response over a range of at least 3 concentrations.

IX) Analytical Instrumentation:

In order to determine the efficiencies of the extraction procedures (soxhlet, XAD-2, purge-and-trap), as well as the efficiency of acid/base/neutral fractionation, extracts from spiked samples were analyzed by gas chromatography using a Pye series 104 GLC with a flame ionization detector (FID). The 2.1 m x 2mm I.D. glass column was packed with 60/80 mesh Tenax GC for analysis of volatile extracts, and with 3% OV-101 on Chromosorb W-HP (80/100 mesh) for XAD-2 and soxhlet extracts. Chromatographs were run isothermally at temperatures ranging from 85° to 230°C, depending upon the compounds analyzed.

Analysis of phenols (phenol, dimethylphenol, p-chlorophenol)

required acetylation of samples prior to GLC. A 100 ml ether or methanol extract was evaporated to 10 ml, and 30 ml of 5% Na_2CO_3 were added. The aqueous layer was removed, and this procedure was repeated twice more. The organic layer was discarded, and 1 ml of acetic anhydride and 10 ml of hexane were added to the aqueous extract. The solution was incubated for 30 min at room temperature, and the hexane layer removed and evaporated to volume for analysis.

The efficiency of the silica-gel fractionation method, and characterization of effluent extracts were done by gas chromatography using a Varian 3700 GLC equipped with a cold, on-column injector and FID. A 30m x 0.32mm I.D., DB-5 (J&W Scientific, Inc.) fused silica capillary column was used. Analyses were performed with a temperature program of 60° to 300°C at 5°/min or 10°/min. High pressure liquid chromatography (HPLC) was performed on a Spectra-Physics 8000 liquid chromatograph equipped with a Beckman 153 UV detector (254 nm), and a Kratos FS950 fluorescence detector (excitation at 360 nm, fluorescence at > 440 nm). Samples were separated on a 25cm x 0.4cm I.D., 10um Vydac 201TP104 column using a gradient of 60:40 acetonitrile/water to 100% acetonitrile over 25 min. An Apple II Plus microcomputer equipped with a 12-bit analog-to-digital convertor was used for data acquisition, storage, and plotting.

GC-MS analysis was performed on a VG Micromass 7070F mass spectrometer equipped with a Varian 3700 gas chromatograph. Samples

were chromatographed on the fused silica capillary columns described above with the same temperature program. The column was connected directly to the mass spectrometer and inserted to within 2 cm. of the electron beam. Electron impact ionization with an electron energy of 70 eV was used. Data was acquired with a VG 2035 data system by scanning from 400 to 80 amu, at 1 sec/decade.

RESULTS

I) Sample Characterization:

The "process water" from refining processes and storm runoff was the effluent of interest in these studies, and effluent streams containing treated process water were sampled at all three refineries. Intake water, which was sampled before it entered the refinery cooling towers, was sampled as a control. The treatment of process water in Refinery 1 included an API separator for oil and sludge, flocculation

with aluminum sulfate, primary clarification, activated sludge treatment, secondary clarification, and discharge through a surge lagoon into Lake Ontario. Refinery 2 used API separators, sand filtration, and activated sludge treatment before discharging into Lake Ontario. In Refinery 3, process water was treated with a sour-water stripper, bio-oxidation unit, API separator, dual-media filtration unit, and activated sludge treatment. Treated process water was mixed with volumes of cooling water (1:3 ratio) before discharge into the St. Clair River. Final effluent samples taken at Refineries 1 and 2 consisted of treated process water, and samples from Refinery 3 consisted of mixed cooling and process water. In October, 1981 effluent samples were taken from Refinery 1 at three points within the effluent treatment process. Samples referred to as "process water", "secondary clarifier", and "surge lagoon" represent effluents after oil separation, after activated sludge treatment and clarification, and the final effluent, respectively. Also, a sample of intake water was taken as representative of uncontaminated Lake Ontario water.

Table 1 summarizes the catalytic-cracking temperatures, crude oil and effluent throughputs, and range of values for effluent quality parameters at the three refineries during sample collection. The oil refined in these facilities was primarily western Canadian light crudes, although tar sands synthetic crude was occasionally mixed into conventional crudes at Refinery 1. Water quality parameters for final effluents fell within federal effluent quality guidelines (EPS, 1979),

TABLE 1: Mean or range of values for parameters related to the operation and effluent discharge of the refineries sampled in this study. Data for intake water (Refinery 1) is presented for comparison.

PARAMETER	FINAL EFFLUENTS			INTAKE WATER
	Refinery 1	Refinery 2	Refinery 3	Refinery 1
Sample Dates	1981-83	1983	Feb, 1983	1981-83
Crude Thruput (Bbl/day)	60×10^3	45×10^3	75×10^3	-
Effluent Discharge (Litres/day)	4×10^6	12×10^6	24×10^6	-
Cracking Temperature (°C)	502-526	515	-	-
pH of effluent	7.4-7.7	7	7.0-7.7	7.6-8.1
NH_3-N (ppm)	3.4-10.8	1.7	0.6-8.0	< 0.1
Phenol (ppb)	32-42	17	28-34	< 1
Suspended Solids (mg/ℓ)	19-33	9	13-20	4-12
Oil and Grease (mg/ℓ)	1.9-8.7	1.4	7.2-8.0	0

and within the range of values reported for other Canadian refineries (PACE, 1981).

II) Method Verification:

(a) Sample Extraction:

The efficiency of recovery of spiked compounds by XAD-2 resin extraction was greater than 70% for all compounds, except aniline, dodecane, and three phenols (Appendix 3). Junk et al (1974) reported that the XAD-2 extraction procedure efficiently concentrates a wide variety of non-polar and medium-polarity organic compounds, but it was not suitable for extraction of phenols and acids. The recovery of compounds from alumina by soxhlet extraction into methanol was efficient for compounds with high boiling points (Appendix 3). The poor recovery of compounds with low boiling points may be due to volatilization during evaporation of the methanol carrier. Volatile compounds were efficiently recovered by the purge-and-trap technique (Appendix 3). Phenol, which is not considered a volatile compound (boiling point of 182^{o}C), was not extracted efficiently by purge-and-trap methods.

Figure 2, which summarizes the recovery data for extraction of dissolved compounds, and compounds associated with particulate material, indicates that a group of compounds with boiling points

between approximately 100° and 200°C would be lost from effluents during extraction. Non-acidic, dissolved compounds with boiling points greater than approximately 200°C would be extracted efficiently by XAD-2 resin, and compounds with boiling points greater than approximately 250°C would be efficiently removed from particulates by soxhlet extraction. The purge-and-trap procedure efficiently recovered compounds with boiling points < 100°C.

Because of the inefficient extraction of acidic compounds by XAD-2 resin, effluents passed through XAD-2 columns were subsequently passed through an Amberlite XAD-7 column. The recovery of spiked phenolic compounds by XAD-7 was found to be relatively efficient (Appendix 4). However, after several XAD-7 extracts failed to yield mutagenic activity, this step was eliminated.

To determine the compatability of extraction procedures with the Ames mutagenicity assay, extracts from samples spiked with 100 µg of BaP (XAD-2 and soxhlet extraction), or 6 mg dibromomethane (volatile extraction) were tested in the Ames assay. Recovery efficiencies calculated from assay results (Appendix 5) were 119% and 87% for BaP extracted by XAD-2 and soxhlet methods, respectively, and 75% for dibromomethane extracted by the purge-and-trap technique. There was no evidence of sample toxicity in the Ames plates.

Effluent samples consist of a much more complex mixture of organic compounds than the mixtures extracted in spiking studies. To ensure that samples were being completely extracted by soxhlet methods, February, 1982 effluent particulates (Refinery 1) were

"re-extracted" with methylene chloride for 6 hr. Similarily, elution of XAD-2 columns with ether was followed by a further wash with methanol. The concentrates from these "re-extraction" solvents were tested in the Ames assay for mutagenicity (Appendix 6). Although there were considerable amounts of material extracted by these solvents, none of the concentrates were appreciably mutagenic. However, there was often evidence of toxicity to the test strains.

(b) Fractionation:

The efficiency of fractionation of compounds into acid, base, and neutral components was tested by spiking 100 µg quantities of test compounds into 100 ml of diethyl ether, and fractionating the mixtures by the liquid-liquid partitioning methods described earlier. Phenolic compounds were detected in both the acidic and neutral fractions, and aromatic amines, excluding 1-anthramine, were found in the basic fraction (Table 2). Alkanes and PAH's remained in the neutral fraction of the sample. However, compounds with boiling points lower than approximately 200°C were inefficiently recovered, probably because of volatilization during sample concentration.

Mixtures of compounds spiked at concentrations of 100 ug into hexane were fractionated by silica-gel column chromatography. As summarized in Table 3, alkanes and PAH's were distributed between fractions 1,2, and 3; with the highest concentrations in fraction 2.

Figure 2: Relationship between recovery efficiency and boiling point for volatile and non-volatile compounds spiked into distilled water or onto alumina, and extracted by XAD-2 (○), soxhlet (●), and purge-and-trap techniques (▼).

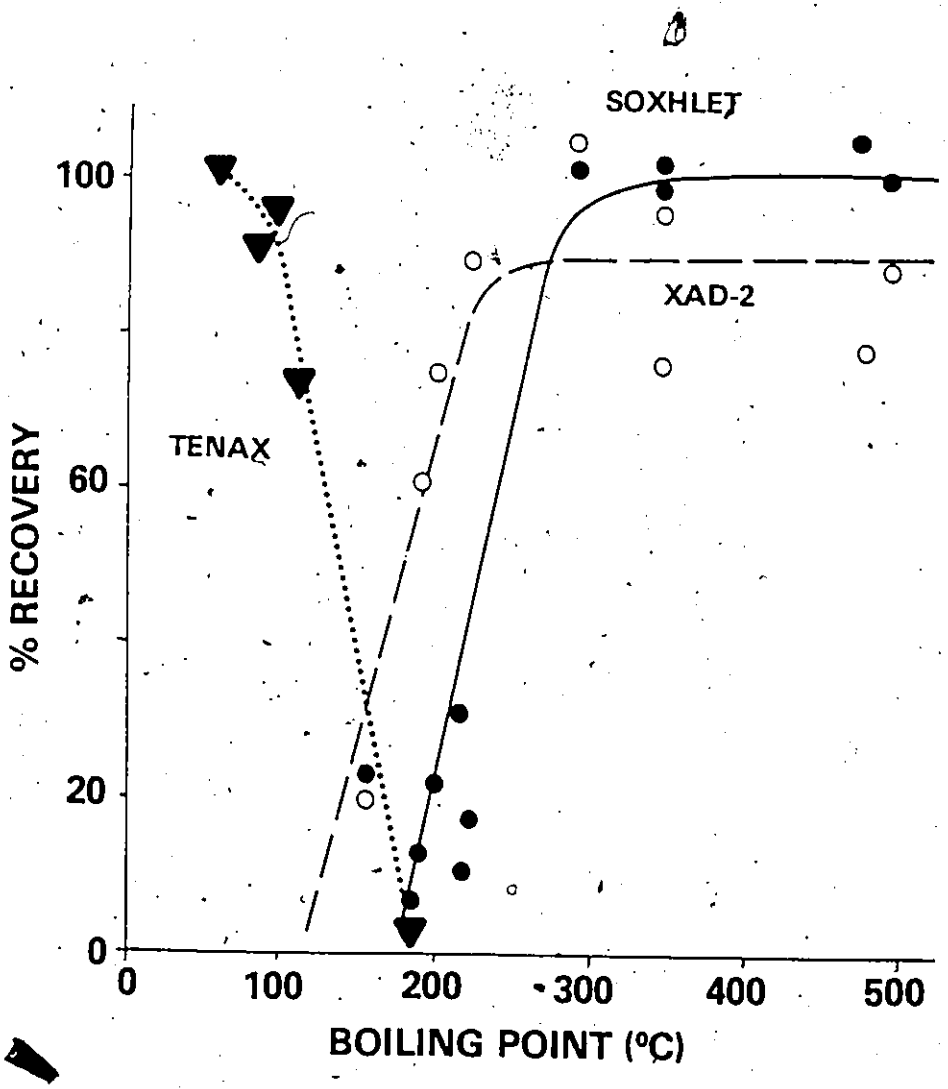

SOXHLET
XAD-2
TENAX
% RECOVERY
100
60
20
0
0
100
200
300
400
500
BOILING POINT (°C)

TABLE 2: Efficiency of recovery of spiked compounds in acid, base, and neutral subfractions. Percent recovery is calculated as the mean of two tests with ether (100 mℓ.) spiked at concentrations of 10 and 100 µg.

Compound	Fraction	% Recovery
1) PAH's:		
napthalene	Neutral	74
phenanthrene	Neutral	93
benzo'a'pyrene	Neutral	95
2) Aromatic amines:		
aniline	Basic	53
o - toluidine	Basic	70
1 - anthramine	Neutral	82
3) Alkanes:		
dodecane	Neutral	14
hexadecane	Neutral	103
eicosane	Neutral	95
4) Phenols:		
phenol	Acid & Neutral	38 & 52
dimethylphenol	Acid & Neutral	21 & 61
p-chlorophenol	Acid & Neutral	19 & 68

TABLE 3: Distribution of spiked compounds in 7 fractions separated by silica-gel column chromatography. Compounds were spiked at concentrations of 100 µg into hexane (0.5 mℓ.) and fractionated by the method of Coleman et al. (1980).

Compound	% Recovery in Silica-Gel Fractions						
	1	2	3	4	5	6	7
a) Alkanes:							
hexadecane	8	53	5				
eicosane	5	67	13				
tetracosane	5	69	17				
b) PAH's:							
napthalene	5	46	11				
phenanthrene	11	64	7				
benzo[a]pyrene	8	63	15				
c) Substituted PAH:							
2-acetylaminofluorene				87			
1-nitropyrene				79			
d) Phthalates:							
dimethylphthalate					71		
diethylphthalate					82		

Substituted PAH's were found in fraction 4, and phthalates in fraction 5. None of these relatively non-polar compounds were found in fractions 6 or 7. Coleman et al (1980) identified ethers, ketones, aldehydes, and alcohols in fractions 6 and 7 of a drinking water concentrate.

(c) Genotoxicity Assays:

Table 4 lists the mean spontaneous and positive-control activities for the Ames assays and SCE assays as conducted in this study. These data fall within the range of values reported in the literature. However, the spontaneous levels of SCE's in CHO metaphases are relatively low; possibly because of the low levels of 5-bromodeoxyuridine (BrdU) used in the test protocol.

Effluent extracts dissolved in DMSO showed less mutagenic activity than extracts in acetone (Appendix 7). This was presumeably a result of poor extract solubility in DMSO, since an insoluble precipitate was present in all DMSO solutions. Therefore, acetone was the solvent used in all Ames and SCE assays. However, it is important to note that the number of spontaneous revertants, and the number of revertants induced by BaP in the Ames assay were lower with acetone solvent than with DMSO (Appendix 7).

Non-volatile, dissolved components (XAD-2 extracts), and

TABLE 4: Mean values determined in this study for spontaneous and positive-control induced reversions in the Ames assay, and for spontaneous and positive-control induced exchanges in the sister-chromatid exchange assay (CHO).

a) Ames Assay:

Compound	Concentration	Assay System	Genotoxic Response
			Revertants/Plate
Control	-	TA98 without S-9	39
	-	TA98 with S-9	46
	-	TA100 without S-9	112
	-	TA100 with S-9	99
MNNG	0.5 µg/plate	TA100 without S-9	1253
		TA98 without S-9	897
Benzo[a]pyrene	5 µg/plate	TA100 with S-9	359
2-Acetylaminofluorene	5 µg/plate	TA98 with S-9	530

b) Sister Chromatid Exchange Assay:

			SCE's/Metaphase
Control	-	Without S-9	5.8
	-	With S-9 (1 h)	6.2
	-	With S-9 (2.5 h)	4.9
Mitomycin C	0.02 µg/ml	Without S-9	29.9
Benzo[a]pyrene	25 µg/ml	With S-9 (1 h)	16.3
		With S-9 (2.5 h)	17.3
2-Acetylaminofluorene	25 µg/ml	With S-9 (1 h)	5.6
		With S-9 (2.5 h)	15.2

particulate components (soxhlet extracts) were initially tested in the Ames assay with Salmonella strains TA98, TA100, TA1535, and TA1538. Because TA1538 and TA1535 were less sensitive to refinery extracts than the R-factor strains (Appendix 8), TA98 and TA100 were used exclusively throughout the study.

Ames mutagenicity assays with volatile compounds have previously been conducted in sealed containers (Simmon, 1981; Barber et al, 1981). Since these containers held several plates, and compounds were allowed to volatilize into the chamber, it was difficult to quantify the amount of compound to which each plate was exposed. The method devised for this study, in which individual plates were sealed, was an attempt to develop a simple and quantifiable technique for testing volatile extracts.

Initial trials using 6 volatile compounds indicate that the sealed-plate technique shows similar sensitivity to the sealed-container methods (Table 5). Dibromomethane (DBM), epichlorhydrin, and methylene chloride were confirmed as mutagens in this assay, while carbon tetrachloride and chloroform gave no response. Although 1,2 dichloroethane has been reported as a mutagen (Table 5), only a slight increase in revertant colonies (less than a doubling over spontaneous revertants) was noted in our tests.

Bridges (1978) reported that the distribution of a volatile mutagen between the gaseous and aqueous (agar) phases greatly affected the mutagenic activity. Some mutagens, like epichlorhydrin, were detected with equal facility in either phase, while dichlorvos was

TABLE 5: Comparison between Ames assay results for volatile compounds reported in the literature, and in this study using the sealed-plate protocol.

REFERENCE	COMPOUND	CONCENTRATION	EXPOSURE	REVERTANTS/PLATE (TA100 without S-9)
This Paper	Dibromomethane	1.2 mg/0.5µl per plate	Gaseous	132 (+)
			Aqueous	726 (+)
	Epichlorhydrin	1.6 mg/1.0µl per plate	Gaseous	1571 (+)
			Aqueous	1491 (+)
	Me Chloride[c]	5.5 mg/4µl per plate	Gaseous	783 (+)
	1,2 Dichloroethane	6.5 mg/4µl per plate	Gaseous	164 (±)
	Carbon Tet.[d]	3.7 mg/4µl per plate	Gaseous	104 (-)
	Chloroform	5.2 mg/6µl per plate	Gaseous	99 (-)
Simmon,[a] 1981	Dibromomethane	0.3 µl/dess	Gaseous	1245 (+)
	Epichlorhydrin	1 µl/dess	Gaseous	538 (+)
	Me Chloride[c]	0.6 µl/dess	Gaseous	426 (+)
	1,2 Dichloroethane	0.75 µl/dess	Gaseous	994 (+)
Barber,[b] et al, 1981	Dibromomethane	2.5 mg/plate	Gaseous	3380 (+)
	1,2 Dichloroethane	22.9 mg/plate	Gaseous	368 (+)
	Carbon Tet.[d]	1.6 mg/plate	Gaseous	100 (-)
	Chloroform	2.6 mg/plate	Gaseous	81 (-)

[a] Plates exposed to compound in sealed dessicator.

[b] Plates exposed to compounds in sealed container. Concentrations per plate determined by GC analysis of agar extracts.

[c] Methylene chloride

[d] Carbon tetrachloride

more readily detected in aqueous phase, and alkyl halides in the gaseous phase. In the sealed-plate assay, mutagens can be distributed in either phase. Glass petri dishes did not give an increase in sensitivity over polystyrene dishes, and DBM dissolved in methanol (0.1 ml) gave a slightly lower number of revertants than DBM without solvent (Appendix 9).

III) Genotoxicity of Effluents:

(a) Particulate Components:

Whole extracts:

Particulate extracts were initially tested over a wide range of concentrations to define the dose required to yield a maximum mutagenic response with minimum toxicity to the test organism. In this study, the extract dose in the Ames plate was defined by the volume of original effluent from which the extract was made. Thus, if particulates are centrifuged from a volume of 320 ml of effluent, soxhlet extracted, and concentrated to the plating volume of 0.1 ml, the dose applied to the Ames plate is 320 "ml equivalents". A range of 20 to 320 ml equivalents of particulate extract per plate gave a linear dose-response in the Salmonella assay (Fig. 3). The toxicity of sample concentrations above 320 ml equivalents was confirmed by

assays in which estimates were made of the numbers of surviving bacteria (Fig. 4). Of course, this toxicity assay does not give a true estimate of sample toxicity in the actual Ames plate, since the chemical dose per bacteria cell is higher in the dilute bacterial cultures of the toxicity assay. There are no simple techniques for accurately determining the numbers of revertants per surviving bacteria population in the actual Ames plate (Salmeen and Durisin, 1981; Green and Muriel, 1976). The problem of toxicity in a bacterial assay can only be circumvented by adjusting the mutagen dose to a range that produces a linear response below the toxic range (Maron and Ames, 1983).

A comparison of Ames tests of particulate extracts conducted with and without rat S-9 (Fig. 3) indicates that the numbers of revertants were often below spontaneous levels when no S-9 was added. This suggests that: (1) there were low levels of direct-acting mutagens in effluents, and (2) toxic activity was reduced by the addition of S-9; probably because of adsorption of toxicants to microsomes, or detoxification through metabolic pathways (Maron et al, 1981).

Strain TA100 was the most sensitive to reversion by particulate extracts from all three of the refineries. Figure 5 illustrates the relative mutagenic activity of refinery samples tested over the study period using strain TA100 with S-9 activation. Data presented are limited to the highest concentration within the linear portion of dose-response curves generated for each effluent sample

Figure 3: Mean mutagenic response of Ames test strains TA98 and TA100 (with and without S-9) to a particulate extract (soxhlet) of Refinery 1 effluent collected in October, 1981. The mean spontaneous revertants in the assay is represented by the line labelled "SR".

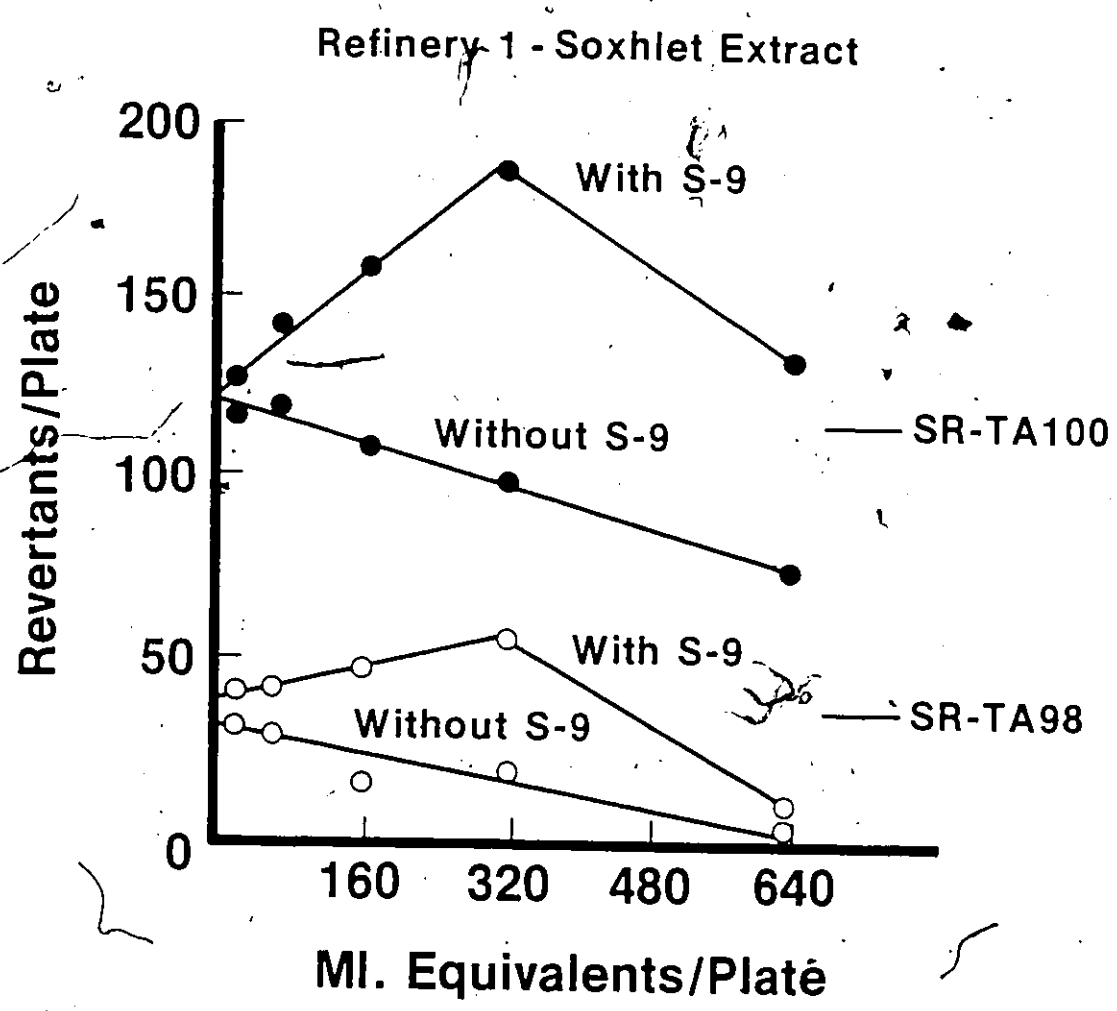
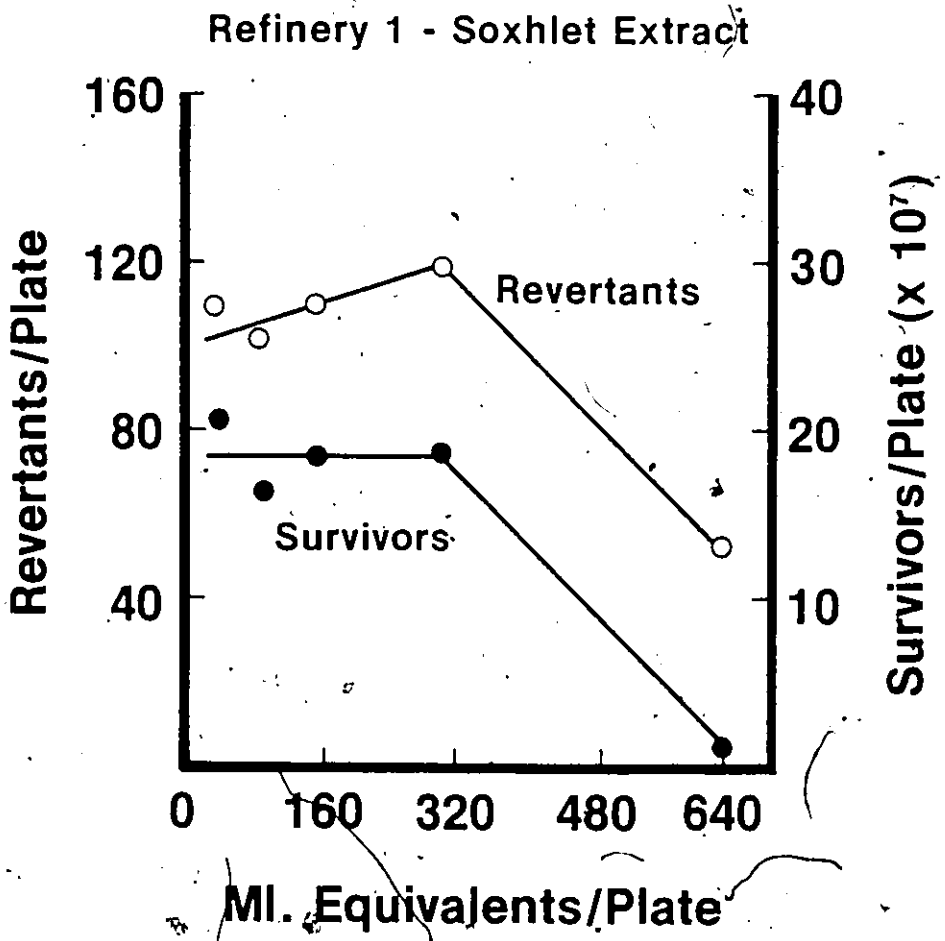

Refinery 1 - Soxhlet Extract
200
150
100
50
0
Revertants/Plate
With S-9
Without S-9
SR-TA100
With S-9
Without S-9
SR-TA98
160
320
480
640
Ml. Equivalents/Plate

Figure 4: Mutagenicity of a particulate extract (soxhlet) from Refinery 1 (February, 1983) using test strain TA100 with S-9, and estimates of the number of surviving bacteria in the test plate.


Refinery 1 - Soxhlet Extract
Revertants/Plate
160
120
80
40
Survivors/Plate (x 10⁷)
40
30
20
10
Revertants
Survivors
0
160
320
480
640
Ml. Equivalents/Plate

(320 ml equivalents).

The mutagenic activity of particulate extracts from Refinery 1 declined over the period between October, 1981 and February, 1983 (Fig. 5). This may reflect the improved effluent quality (e.g. oil and grease levels declined) at this refinery over the sampling period. Extracts from Refineries 2 and 3 were sufficiently mutagenic to yield a doubling over background revertants at the concentrations tested (Fig. 5). In Ames tests of pure compounds, a doubling of the numbers of revertants over spontaneous levels and a dose-response are considered evidence of significant mutagenicity (Johnson and Hopke, 1980). Extracts from intake water (controls) collected at all 3 refineries did not significantly elevate numbers of revertants above spontaneous levels at concentrations used to test effluent samples.

A mutagenic soxhlet extract from Refinery 3 (February, 1983 sample) significantly increased the numbers of SCE's in assays with S-9 activation (Table 6). Incubation of CHO cells with the extract and S-9 mix for 2.5 hr produced the greatest response. In contrast, intake water samples from Refinery 3 did not elevate SCE frequencies (Table 6). Other SCE assays of particulate samples (Refinery 1- June, 1982; Refineries 1,2, and 3- February, 1983) did not give a statistically-significant response in tests with or without S-9. Sample concentrations greater than 160 ml equivalents per flask retarded culture growth, which was an indicator of sample toxicity.

Figure 5: Mutagenicity of particulate extracts (soxhlet) from effluents sampled at three refineries (1981-1983), using strain TA100 with S-9 at a dose of 320 ml equivalents per plate. Error bars represent twice the standard deviation about the mean number of revertants per plate (n=3). The mean spontaneous revertants for all of the assays is represented by the line labelled "SR".

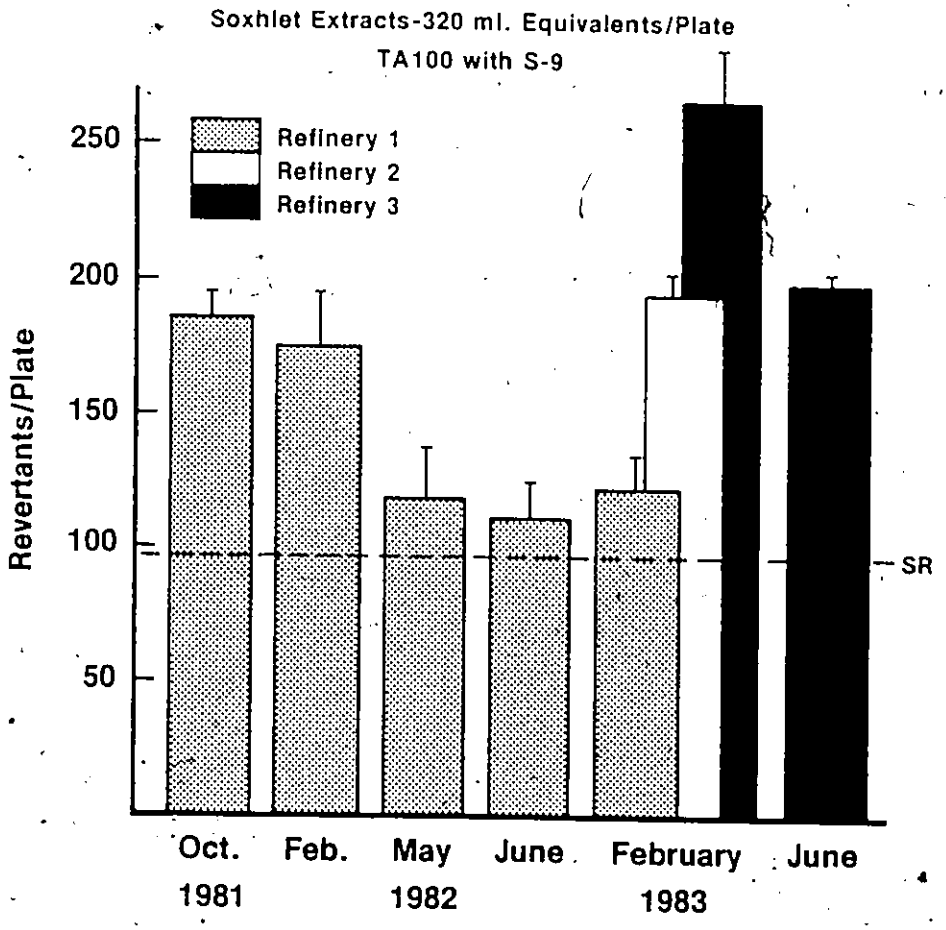

Soxhlet Extracts-320 ml. Equivalents/Plate
TA100 with S-9
Refinery 1
Refinery 2
Refinery 3
Revertants/Plate
250
200
150
100
50
SR
Oct. 1981
Feb.
May 1982
June
February 1983
June

TABLE 6: Results of a sister chromatid exchange assay with microsomal activation for a February, 1983 particulate extracts the final effluent and intake water of from Refinery 3. Extracts were incubated with S-9 in assay flasks with 25 ml of medium for 1 hr. or 2.5 hr. Standard deviations for mean SCE's (n = 25 metaphases) are presented in brackets.

SAMPLE	Mℓ. EQUIV/FLASK	MEAN AND S.D. OF SCE's PER METAPHASE	
		1h Incubate	2.5h Incubate
Solvent Control	-	4.3(2.2)	4.6(2.0)
Refinery 3 Effluent	160	6.4(1.8)*	10.7(2.0)**
	80	5.7(1.6)	9.1(2.9)*
	40	4.8(2.4)	8.8(1.8)*
Refinery 3 Intake	160	4.7(2.4)	4.8(1.8)
	80	4.2(1.9)	4.6(2.2)
	40	4.8(1.7)	4.6(2.3)

Mean SCE's significantly different from controls: * Confidence Interval <.05.
** Confidence Interval <.01

Mutagen-directed fractionation:

Mutagenic particulate extracts were subfractionated by acid/base/neutral fractionation, and silica-gel column chromatography. It was hoped that these procedures would separate toxic from mutagenic components, and allow partial characterization of the compounds giving a mutagenic response in the Ames assay.

The acid/base/neutral fractionation of a Refinery 3 sample indicated that mutagenicity was retained in the neutral fraction (Figure 6). However, a significant portion of the mutagenic activity, as well as 26% of the residue weight, was lost during the fractionation process.

Selected soxhlet extracts were subfractionated by column chromatography on silica gel into 7 subfractions of increasing polarity. In a particulate sample from Refinery 3 (February, 1983) activity was detected, in order of increasing mutagenicity, in fractions 1, 3, 5 and 7 (Fig. 7a). A sample from Refinery 2 (February, 1983) showed mutagenic activity in fractions 5 and 7, only (Fig. 7b). Dose-response data were generated for all fractions, but only mutagenic activity at 320 ml equivalents per plate is presented in these figures.

Table 7 indicates that those fractions with the highest mutagenic activity also contained the most residue material. Over 90% of the material applied to the top of the silica-gel column was recovered in the various fractions (Table 7). Since the most

Figure 6: Mutagenicity of whole particulate extract (soxhlet), and acid/base/neutral subfractions from a Refinery 3 sample (February, 1983) tested with TA100 and S-9 at a dose of 320 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assay is represented by the line labelled "SR".

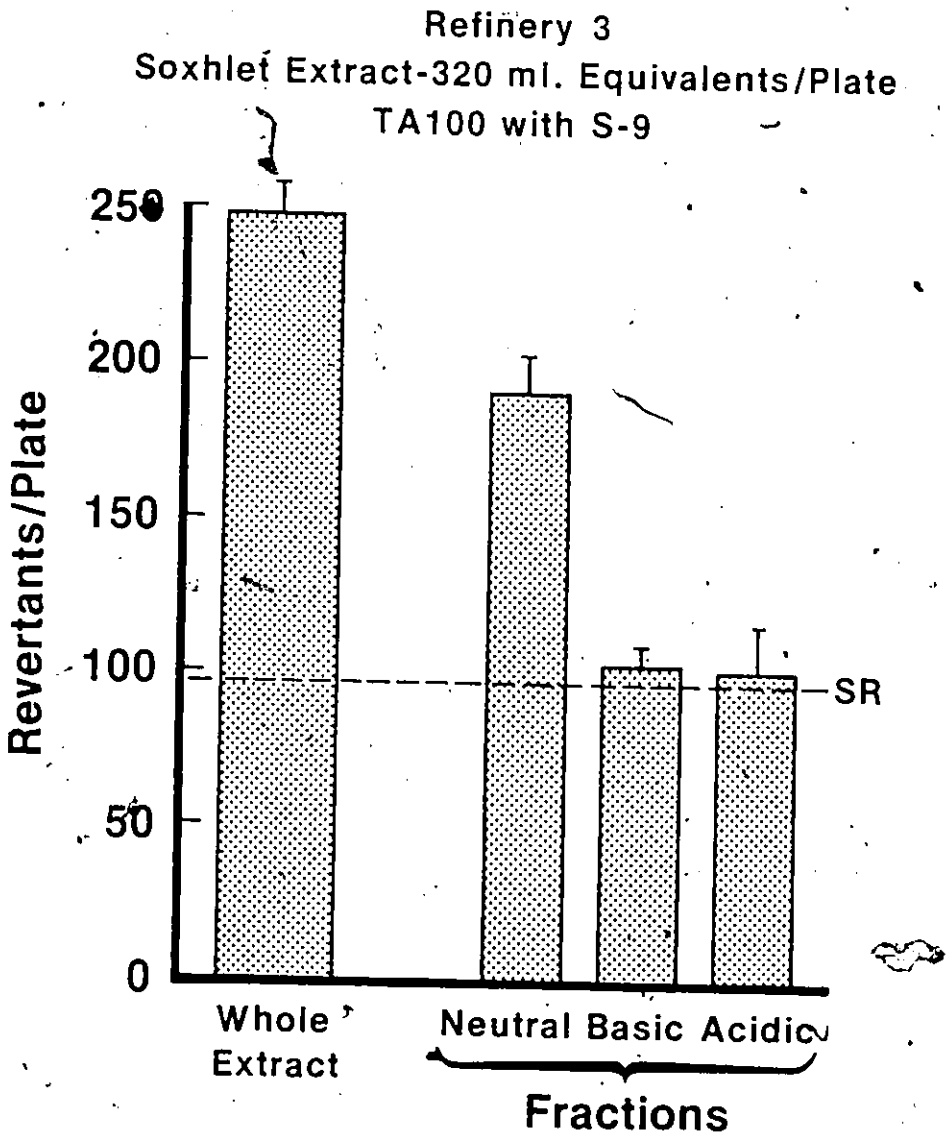
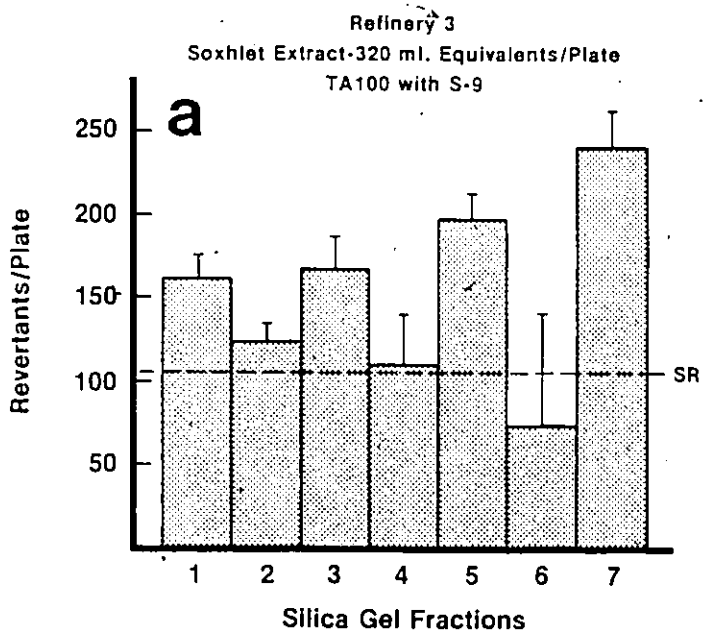

Refinery 3
Soxhlet Extract-320 ml. Equivalents/Plate
TA100 with S-9
Revertants/Plate
250
200
150
100
50
0
SR
Whole Extract
Neutral
Basic
Acidic
Fractions

Figure 7: Mutagenicity of silica-gel subfractions from particulate extracts (soxhlet) of effluents (February, 1983) from Refineries 3 (a) and 2 (b). Results are shown for test strain TA100 plated with S-9 at a dose of 320 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assays are represented by the lines labelled "SR".


Refinery 3
Soxhlet Extract-320 ml. Equivalents/Plate
TA100 with S-9
a
250
200
150
100
50
SR
Revertants/Plate
1
2
3
4
5
6
7
Silica Gel Fractions

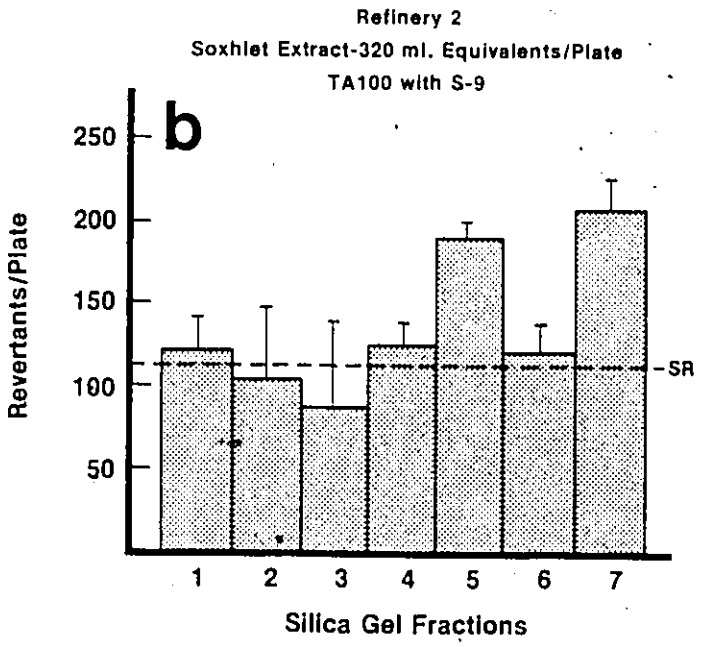

Refinery 2
Soxhlet Extract-320 ml. Equivalents/Plate
TA100 with S-9
b
250
200
150
100
50
SR
Revertants/Plate
1
2
3
4
5
6
7
Silica Gel Fractions

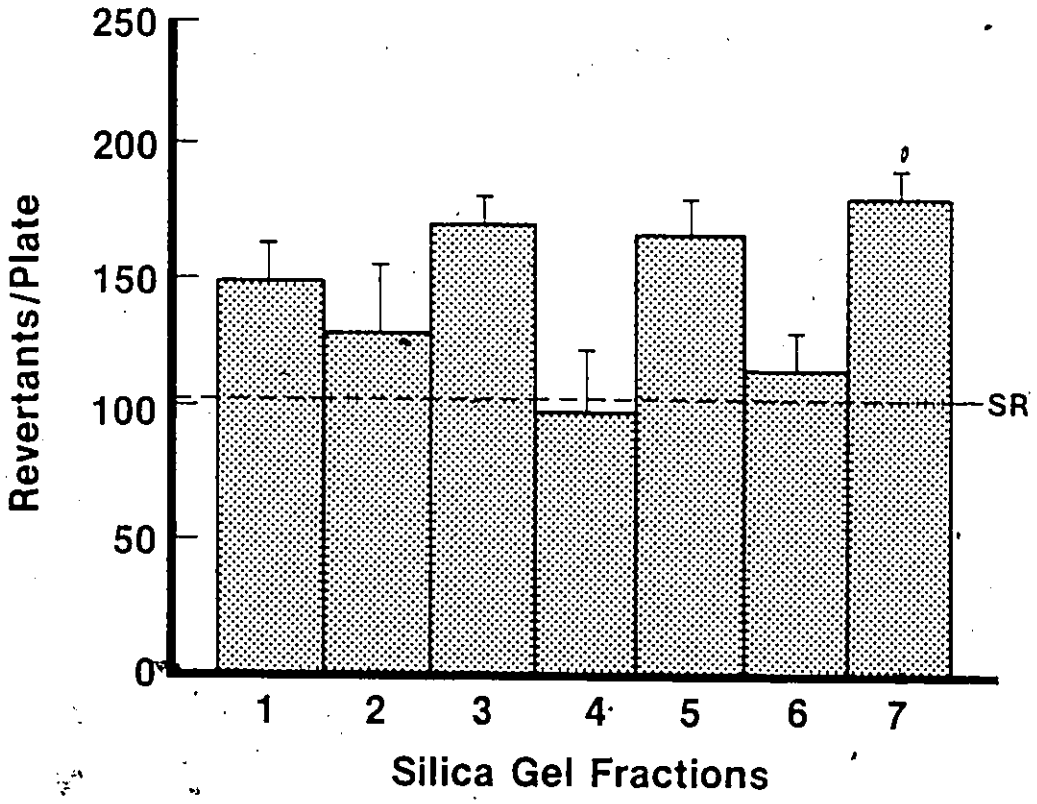
TABLE 7: Residue weights of silica gel fractions of effluent extracts from Refineries 1, 2 and 3. Residues from Refineries 1 and 2 were each separated on 2 columns, and fractions pooled. Residue from Refinery 3 was separated in 3 aliquots and pooled.

SAMPLE	FRACTION	RESIDUE WT.	
Soxhlet Extracts (Particulates):		Mg.	Percent
a) Refinery 2 - 2 litre Extract	1	1.1	3.0
	2	1.9	5.2
	3	2.7	7.5
	4	5.2	14.3
	5	7.4	20.4
	6	6.7	18.5
	7	11.2	30.9
	Total	36.2[a]	100.0
b) Refinery 3 - 2 litre Extract	1	1.4	1.9
	2	1.8	2.5
	3	12.2	16.9
	4	7.6	10.5
	5	14.9	20.7
	6	4.5	6.2
	7	29.7	41.3
XAD-2 Extracts (Dissolved):	Total	72.0[b]	100.0
a) Refinery 1 - 4 litre Extract	1	2.8	5.5
	2	1.1	2.1
	3	7.8	15.2
	4	9.0	17.5
	5	1.6	3.1
	6	19.4	37.8
	7	9.6	18.7
	Total	51.3[c]	100.0

a) 91% of amount applied to columns.
b) 96% of amount applied to columns.
c) 90% of amount applied to columns.

Figure 8: Mutagenicity of silica-gel subfractions from the neutral fraction of a particulate extract (soxhlet) prepared from effluent of Refinery 3 (February, 1983). Results are shown for test strain TA100 plated with S-9 at a dose of 320 ml equivalents. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assay is represented by the line labelled "SR".

Refinery 3
Soxhlet Extract, Neutral-320 ml. Equivalents/Plate
TA100 with S-9



mutagenic fractions were polar, it is possible that some of this material was lost during the liquid/liquid partitioning procedures used to generate acid/base/neutral subsamples. When a neutral subfraction of the February, 1983 sample from Refinery 3 was subfractionated by silica-gel chromatography, the distribution of mutagenic activity (Fig. 8) was similar to the whole extract (Fig. 7a), but there was reduced mutagenicity in polar fractions.

Analytical characterization:

Capillary column gas chromatography (GLC) with flame ionization detection was performed on neutral fractions of selected particulate extracts. As illustrated in Fig. 9, the samples were complex, with broad envelopes of unresolved components and only a few major peaks superimposed on the envelope. Differences were observed between particulate extracts from different refineries. For example, the neutral fraction of a Refinery 3 particulate extract, with relatively high mutagenic activity (Fig. 6), gave the GLC pattern illustrated in Fig. 9b. A neutral fraction from Refinery 1 with little mutagenic activity (February, 1983), contained less material, with a lower range of molecular weights (Fig. 9c).

With such complex mixtures, however, it is not possible to draw any correlations between GC patterns and genotoxic activity. Combined gas chromatography-mass spectrometry analyses have established some primary components of the Refinery 3 sample to be

Figure 9: a) Gas chromatogram of standards: n-alkanes (C8 to C40), naphthalene (1), phenanthrene (2), pyrene (3), chrysene (4), benzo[a]pyrene (5), and picene (6). b) Gas chromatogram of particulate extract (neutral fraction) from Refinery 3 effluent (February, 1983). Peaks marked "P" have been established by GC-MS to be di-alkyl phthalates. c) Gas chromatogram of particulate extract (neutral fraction) from Refinery 1 (February, 1983).

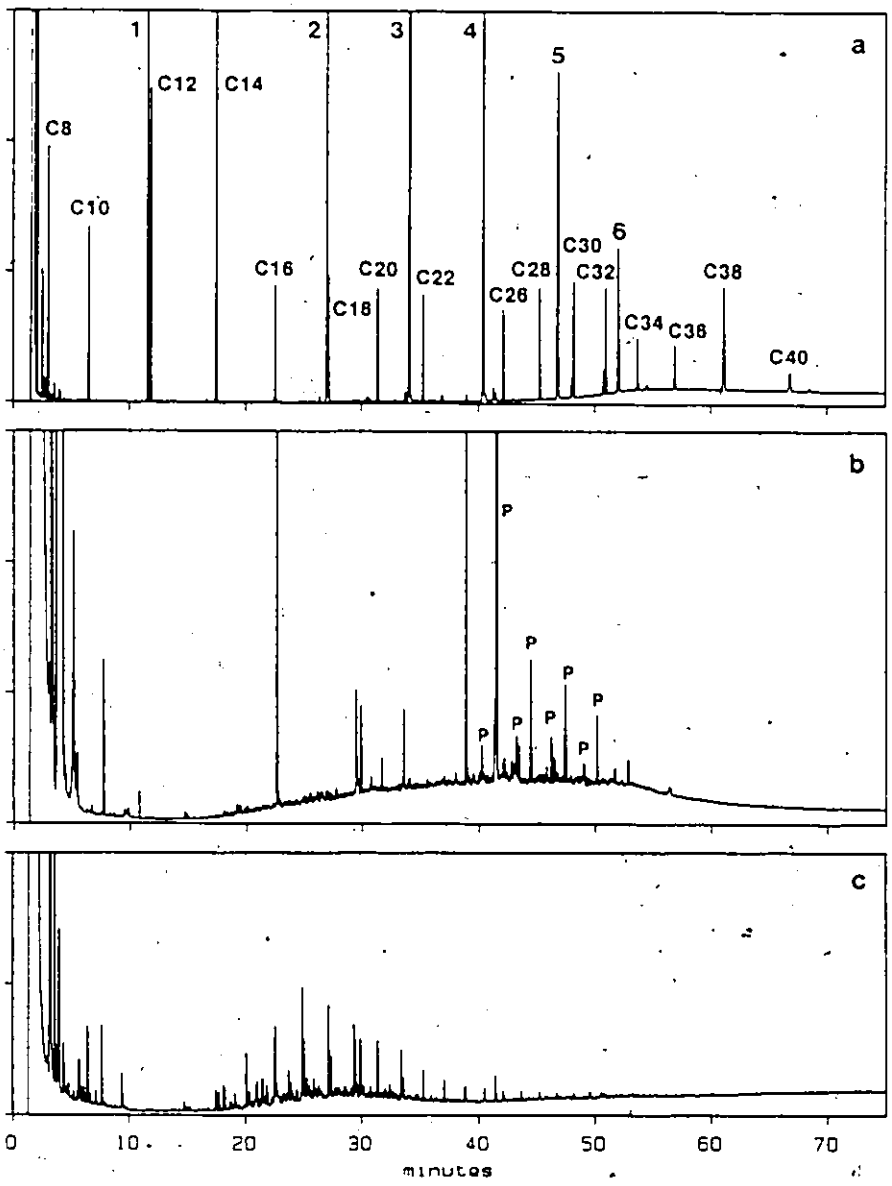
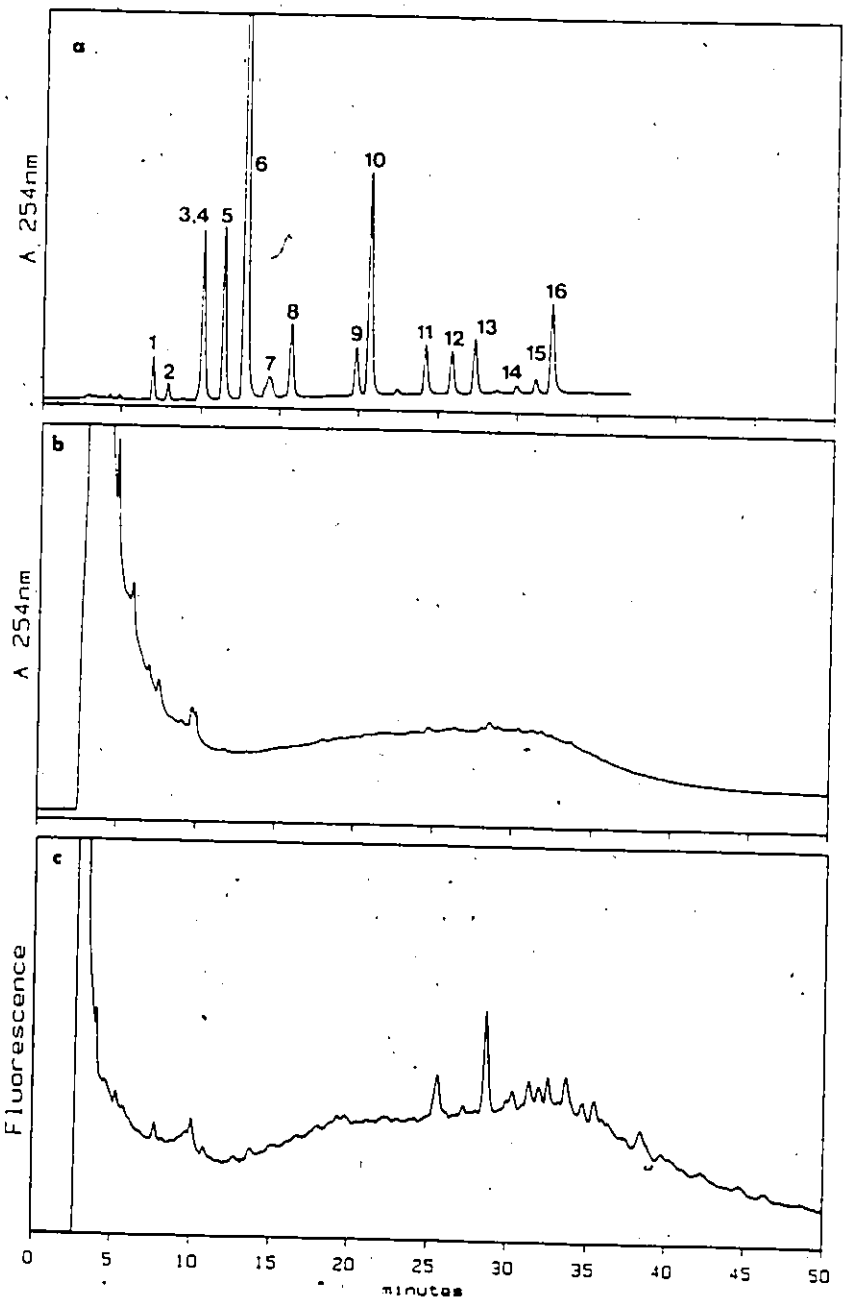

a
1
2
3
4
5
6
C8
C10
C12
C14
C16
C18
C20
C22
C26
C28
C30
C32
C34
C36
C38
C40
b
P
c
0
10
20
30
40
50
60
70
minutes

Figure 10: a) HPLC chromatogram (UV) for a mixture of PAH standards: naphthalene (1), acenaphthylene (2), acenaphthene (3), fluorene (4), phenanthrene (5), anthracene (6), fluoranthene (7), pyrene (8), benz[a]anthracene (9), chrysene (10), benzo[b]fluoranthene (11), benzo[k]fluoranthene (12), benzo[a]pyrene (13), dibenz[a,h]anthracene (14), benzo[g,h,i]perylene (15), and indeno[1,2,3]pyrene (16). b) and c) are HPLC chromatograms with UV absorption and fluorescence detection, respectively, for silica gel subfraction 7 of the particulate extract (soxhlet) of Refinery 3 effluent sampled in February, 1983.


a
A 254nm
1
2
3,4
5
6
7
8
9
10
11
12
13
14
15
16
b
A 254nm
c
Fluorescence
0
5
10
15
20
25
30
35
40
45
50
minutes

aliphatics, alkyl-substituted aromatics, and di-alkyl phthalates.

Gas chromatography proved unsuitable for the analysis of the most polar silica-gel fractions (Fraction 7) because of poor separation and elution of components from the GLC column. This fraction was of interest because it showed the most mutagenic activity in samples from Refineries 2 and 3 (Fig. 7). High performance liquid chromatography (HPLC) analyses show that this fraction generally contained a complex mixture of compounds, which may include aromatic hydrocarbons. For example, Fraction 7 from a Refinery 3 sample (February, 1983), gave chromatograms with largely unresolved envelopes with both ultraviolet (254 nm) absorption and fluorescence detectors (Fig.10).

(b) Dissolved, Non-Volatile Components:

Whole extracts:

The XAD-2 extracts of dissolved, non-volatile effluent components were acutely toxic at doses greater than 80 ml equivalents per plate (Fig. 11). A comparison of Ames tests of XAD-2 extracts with and without S-9 (Fig. 11) indicates that microsomal activation was required for expression of mutagenic activity. Figure 11 also indicates that strain TA98 was most sensitive to the mutagenic effects of XAD-2 extracts.

Figure 12 illustrates the relative mutagenicity of refinery samples tested over the study period using strain TA98 with microsomal activation. Mutagenicity is shown at the highest concentration within the linear portion of dose-response curves (80 ml equivalents per plate). The mutagenic activity of the February, 1982 extract from Refinery 1 approached a doubling over spontaneous levels, but activity of XAD-2 extracts was generally low for all refineries. Intake water extracts from all 3 refineries were not mutagenic at concentrations used to test effluents.

The XAD-2 extracts from all 3 refineries (Refinery 1- June, 1982; Refineries 1,2, and 3- February, 1983) gave negative responses in the SCE assay, both with and without S-9. Concentrations above 160 ml equivalents per culture flask were toxic.

Mutagen-directed fractionation:

Acid/base/neutral fractionation of a Refinery 1 sample indicated that the neutral fraction was mutagenic (Fig. 13). However, as in the fractionation of particulate samples, a significant proportion of the mutagenic acivity, as well as 37% of the residue weight, was lost during fractionation.

When an XAD-2 sample from Refinery 1 (June, 1982) was subfractionated by silica-gel column chromatography, mutagenic activity was confined to the relatively-polar Fraction 6 (Fig. 14).

Dose-response data was generated for all fractions, but only the mutagenicity at 160 ml equivalents per plate is presented in the figure. A large proportion of the toxic activity of the sample was confined to Fraction 1, but this fraction did not contain a large proportion of the residue weight applied to the column (Table 7). The bulk of the residue was present in fraction 6. The loss of mutagenic activity in the acid/base/neutral fractionation scheme may have been caused by loss of polar material during water/solvent partitioning.

Analytical characterization:

A neutral fraction from an XAD-2 extract of Refinery 1 effluent (February, 1983) was analyzed by gas chromatography. The GC profile was complex with retention times of the majority of hydrocarbons falling between C_{10} and C_{20} alkanes (Fig. 15). The presence of hydrocarbons with shorter retention times than those extracted by soxhlet methods (Fig. 9) may partially reflect a more efficient retention of low boiling-point compounds (200^{o}-250^{o}C) by XAD-2 extraction techniques.

Figure 11: Mean mutagenic response of Ames test strains TA98 and TA100 (with and without S-9) to an XAD-2 extract (dissolved, non-volatile compounds) of Refinery 1 effluent collected in October, 1981. The mean spontaneous revertants for the assay is represented by the line labelled "SR".

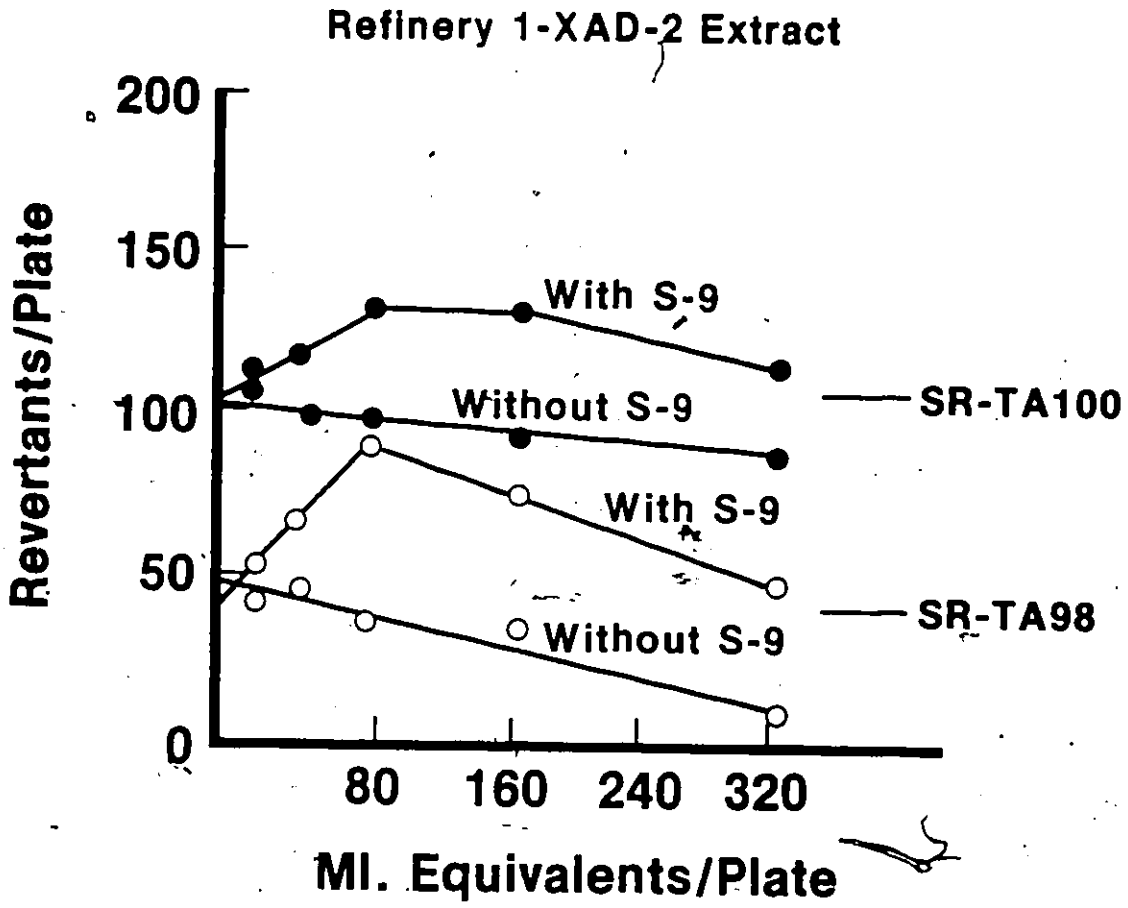

Refinery 1-XAD-2 Extract
200
150
100
50
0
Revertants/Plate
With S-9
Without S-9
SR-TA100
With S-9
Without S-9
SR-TA98
80
160
240
320
Ml. Equivalents/Plate

Figure 12: Mutagenicity of XAD-2 extracts from effluents sampled at three refineries (1981-1983), using strain TA98 with S-9 at a dose of 80 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for all of the assays is represented by the line labelled "SR".

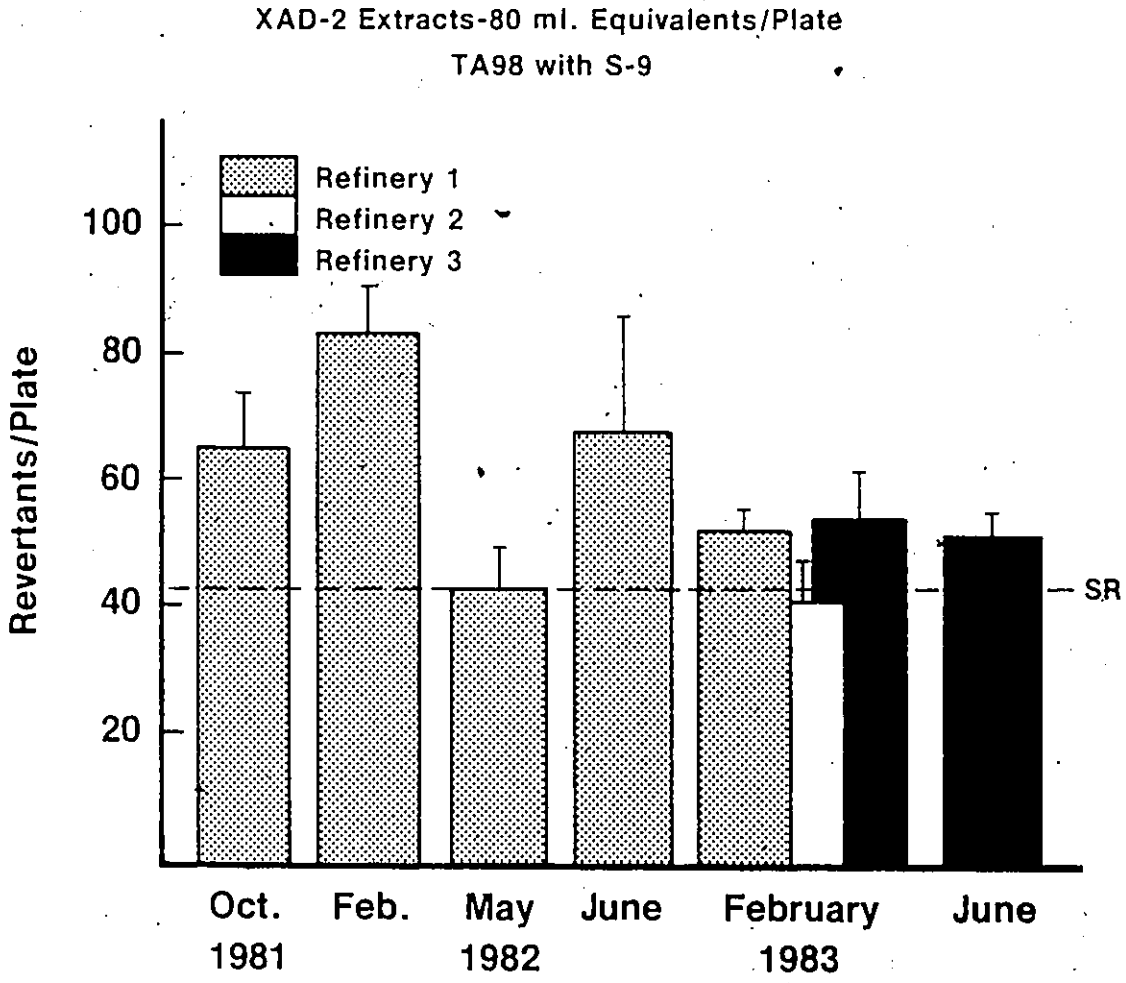

XAD-2 Extracts-80 ml. Equivalents/Plate
TA98 with S-9
Refinery 1
Refinery 2
Refinery 3
Revertants/Plate
100
80
60
40
20
SR
Oct. 1981
Feb.
May 1982
June
February 1983
June

Figure 13: Mutagenicity of whole XAD-2 extract, and acid/base/neutral subfractions from a Refinery 1 sample (February, 1983) tested with TA98 and S-9 at a dose of 80 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assay is represented by the line labelled "SR".

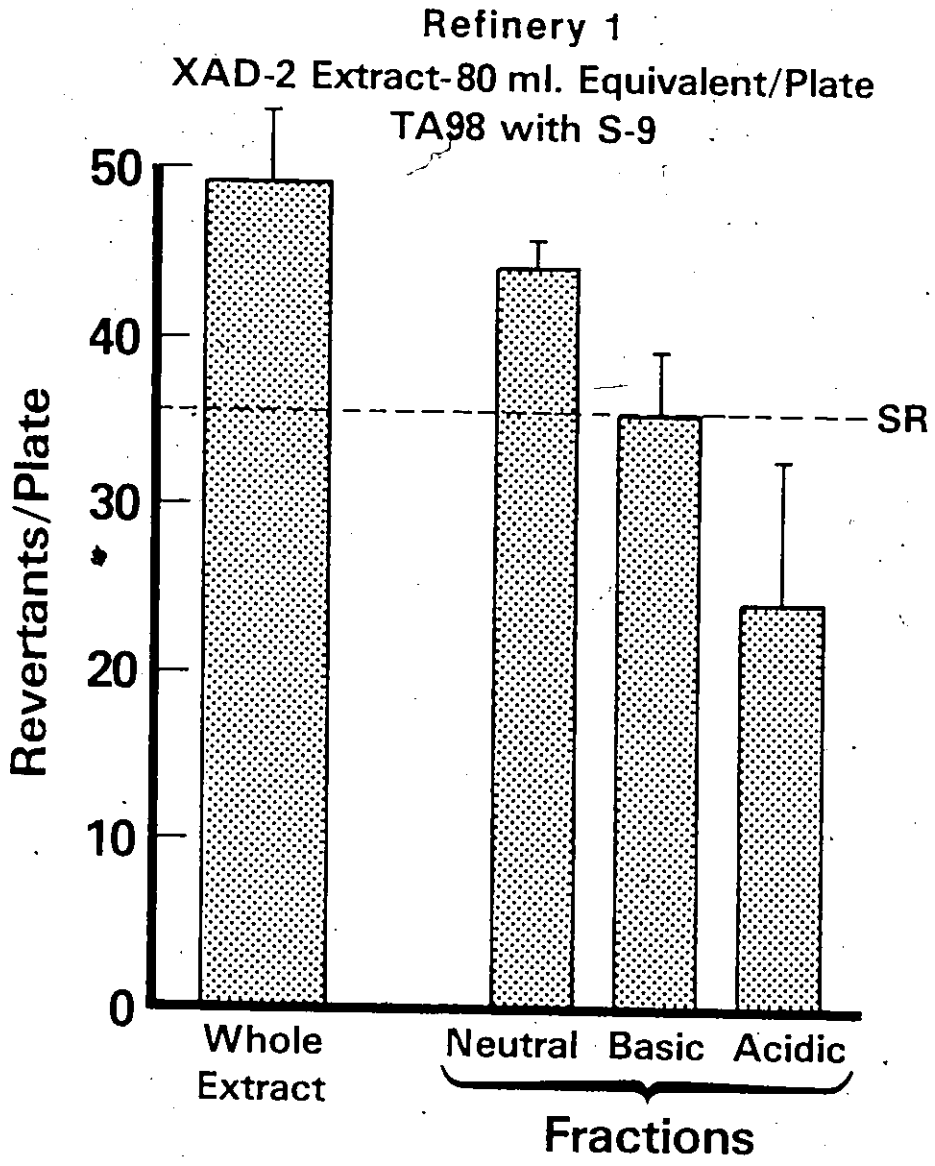

Refinery 1
XAD-2 Extract-80 ml. Equivalent/Plate
TA98 with S-9
Revertants/Plate
50
40
30
20
10
0
SR
Whole Extract
Neutral
Basic
Acidic
Fractions

Figure 14: Mutagenicity of silica-gel subfractions from an XAD-2 extract of effluent from Refinery 1 (February, 1983). Results are shown for the test strain TA98 plated with S-9 at a dose of 160 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assay is represented by the line labelled "SR".

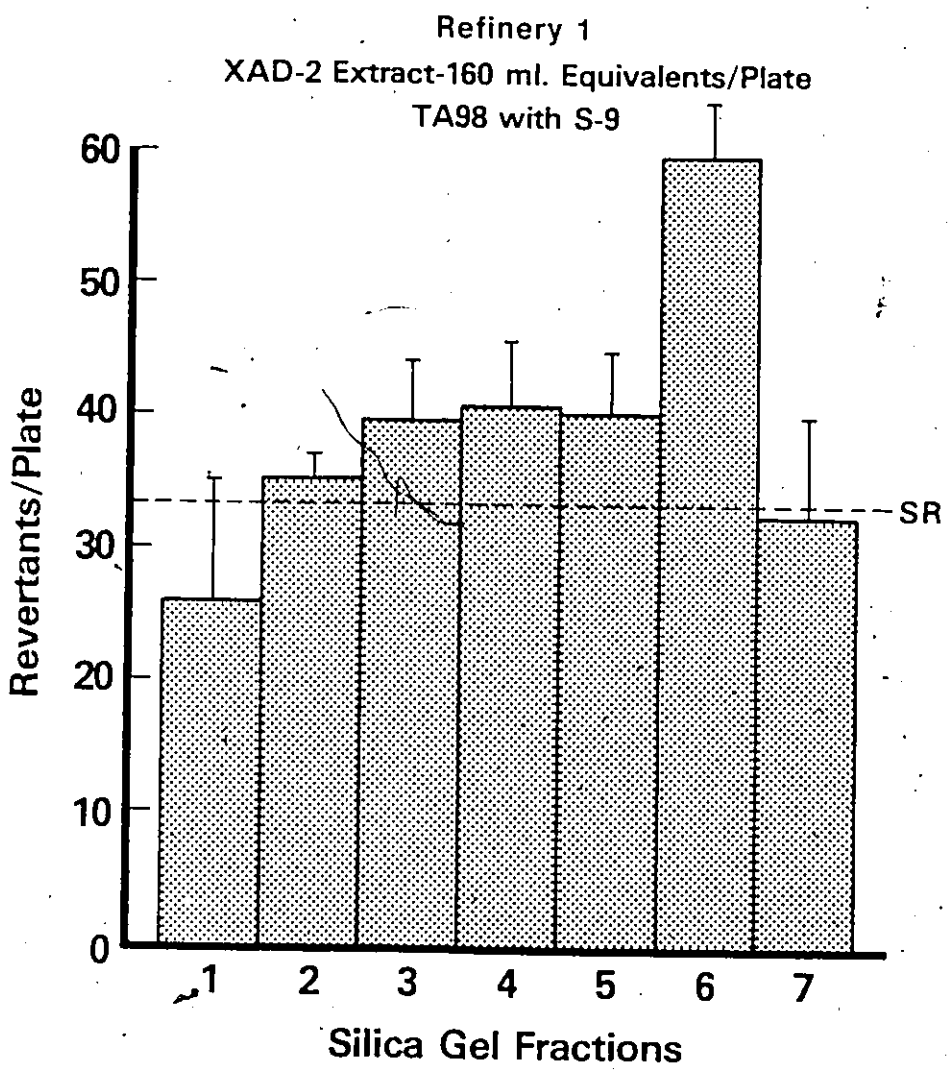
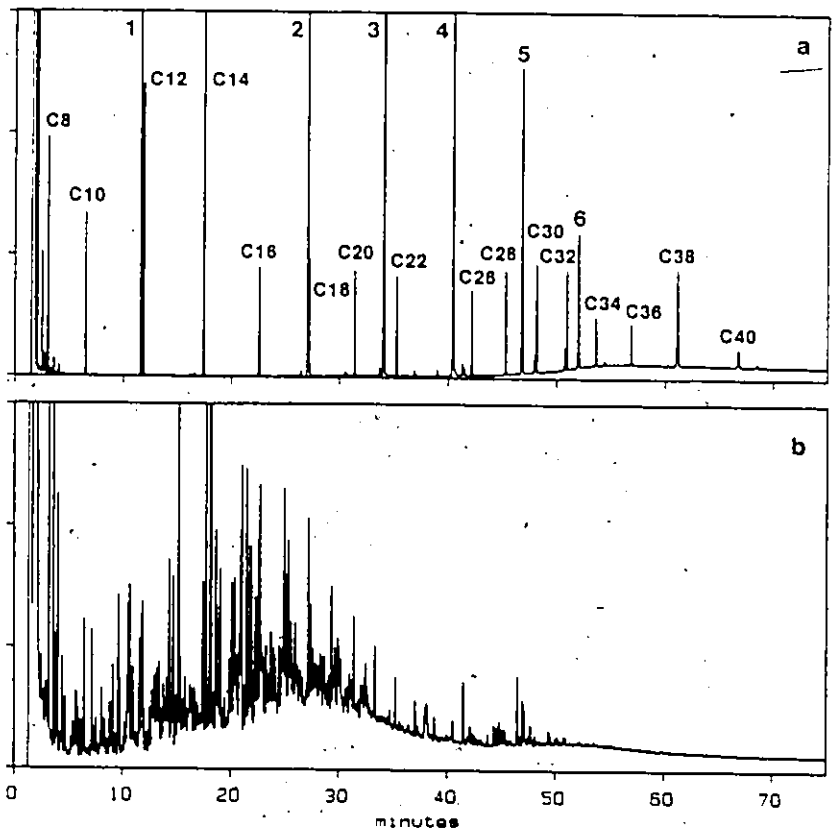

Refinery 1
XAD-2 Extract-160 ml. Equivalents/Plate
TA98 with S-9
Revertants/Plate
60
50
40
30
20
10
0
SR
1
2
3
4
5
6
7
Silica Gel Fractions

Figure 15: a) Gas chromatogram of standards: n-alkanes (C8 to C40), naphthalene (1), phenanthrene (2), pyrene (3), chrysene (4), benzo[a]pyrene (5), and picene (6). b) Gas chromatogram of XAD-2 extract (neutral fraction) from Refinery 1 (February, 1983).


1
2
3
4
5
6
a
b
C8
C10
C12
C14
C16
C18
C20
C22
C26
C28
C30
C32
C34
C36
C38
C40
0
10
20
30
40
50
60
70
minutes

(c) Dissolved, Volatile Components:

Although the mutagenic activity of volatile extracts was minimal, these samples did give a slight dose-response with TA100 in the modified (sealed plate) Ames protocol (Table 8). Activity was detected in aqueous phase assays only with S-9 pre-incubation (20 min). In gaseous phase tests, there was no mutagenic activity detected unless S-9 was added to bacteria in the top agar (Table 8).

There was a dose-response at sample concentrations between 4 and 17 ml equivalents per plate, while more concentrated samples were toxic. Volatile samples from all 3 refineries did not show significant mutagenic activity (greater than a doubling above spontaneous levels) at a dose of 17 ml equivalents per plate (Fig. 16). Volatile extracts from the three refineries (February, 1983) gave no response in the SCE assay , with or without S-9.

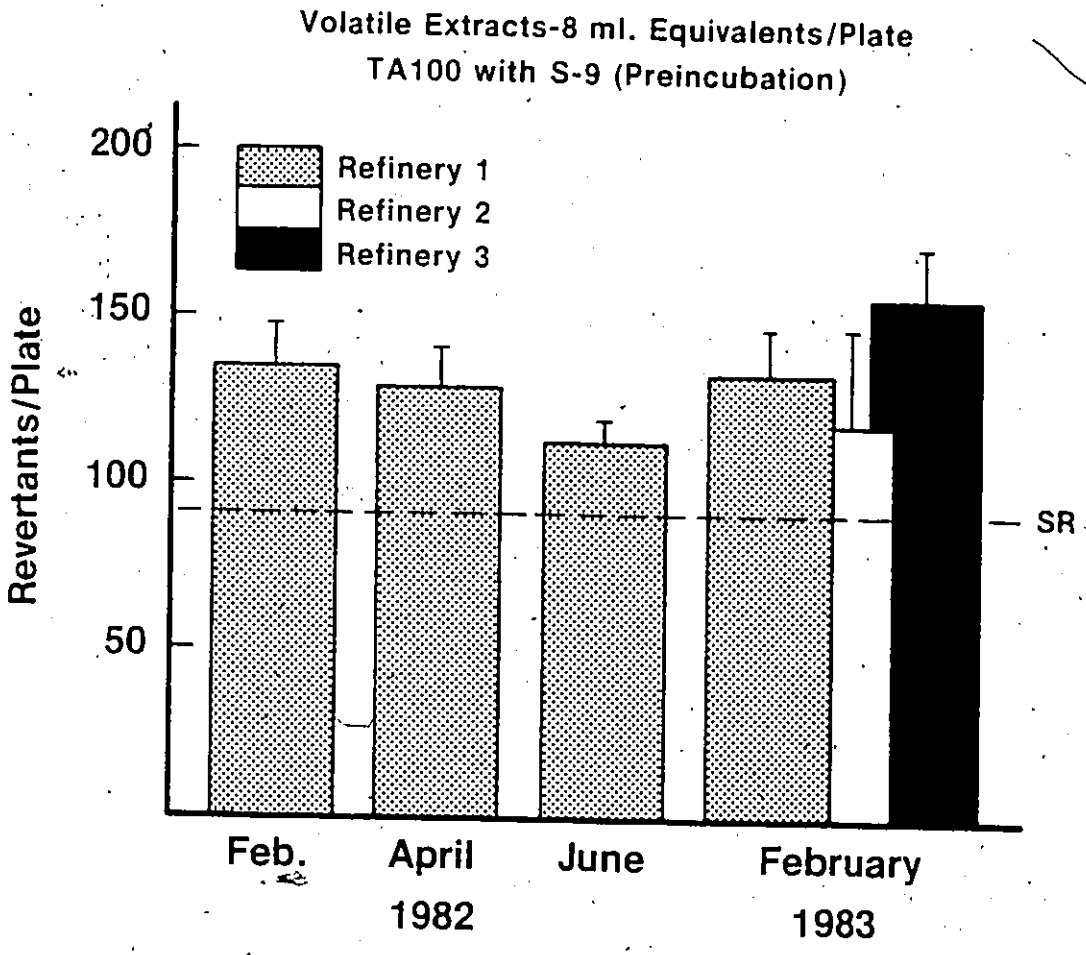
IV) Efficiency of Removal of Genotoxic Components by Effluent Treatment:

Ames assays of samples taken at several points in the effluent treatment process at Refinery 1 (October, 1981) indicate that

TABLE 8: Results of Ames assays of volatile samples (February, 1983) tested using strain TA100, and samples applied in aqueous and gaseous phases with and without S-9. SR refers to spontaneous revertants.

Sample	Mℓ. Equiv/Plate	Mean Rev./Plate			
		AQUEOUS		GASEOUS	
		With S-9	Without S-9	With S-9	Without S-9
Refinery 1	17	144	56	103	63
	8	143	83	133	89
	4	130	97	129	109
	2	113	90	114	101
	0(SR)	97	92	107	102
Refinery 2	17	140	73	134	77
	8	130	101	128	104
	4	127	98	116	99
	2	109	96	104	100
	0(SR)	97	92	107	102
Refinery 3	17	110	37	160	39
	8	161	82	156	82
	4	143	94	141	97
	2	123	92	132	105
	0(SR)	97	92	107	102

Figure 16: Mutagenicity of volatile extracts from effluents sampled at three refineries (1982-1983), using strain TA100 with S-9 at a dose of 8 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for all of the assays is represented by the line labelled "SR".


Volatile Extracts-8 ml. Equivalents/Plate
TA100 with S-9 (Preincubation)
Revertants/Plate
200
150
100
50
Refinery 1
Refinery 2
Refinery 3
SR
Feb.
April
1982
June
February
1983

effluents at all stages of treatment were more mutagenic than intake water (Fig. 17). The mutagenicity of 64 ml equivalent doses of particulate extracts (soxhlet) declined from 221 revertants per plate, for relatively untreated "process water", to 144 revertants per plate for final effluent in the "surge lagoon" (Fig. 17). The mutagenicity of XAD-2 extracts declined only marginally over the treatment process. Therefore, the effluent treatment process may be more efficient in removing genotoxic agents associated with suspended particulates.

V) Comutagenicity Assays:

In Ames tests in which non-volatile extracts were combined with norharmane, there was no enhancement of the mutagenicity of XAD-2 or soxhlet extracts (Table 9). Similarily, there was no enhancement of the mutagenicity of these extracts by the addition of BeP (Table 10). Neither XAD-2 or soxhlet extracts acted, themselves, as comutagenic agents, since the extracts decreased, rather than increased, the mutagenic activity of BaP and 2-AAF in the Ames assay (Table 11).

Figure 17: Mean mutagenicity of soxhlet and XAD-2 extracts of intake water, and of samples taken at three points within the effluent treatment system at Refinery 1 on October, 1981. Results are shown for test strain TA100 (particulates), and TA98 (XAD-2) plated with S-9 at a sample dose of 64 ml equivalents per plate. The mean spontaneous revertants in the assay is represented by the line labelled "SR".

Refinery 1 Effluents
Test Strain with S-9, 64 ml. Equivalents/Plate

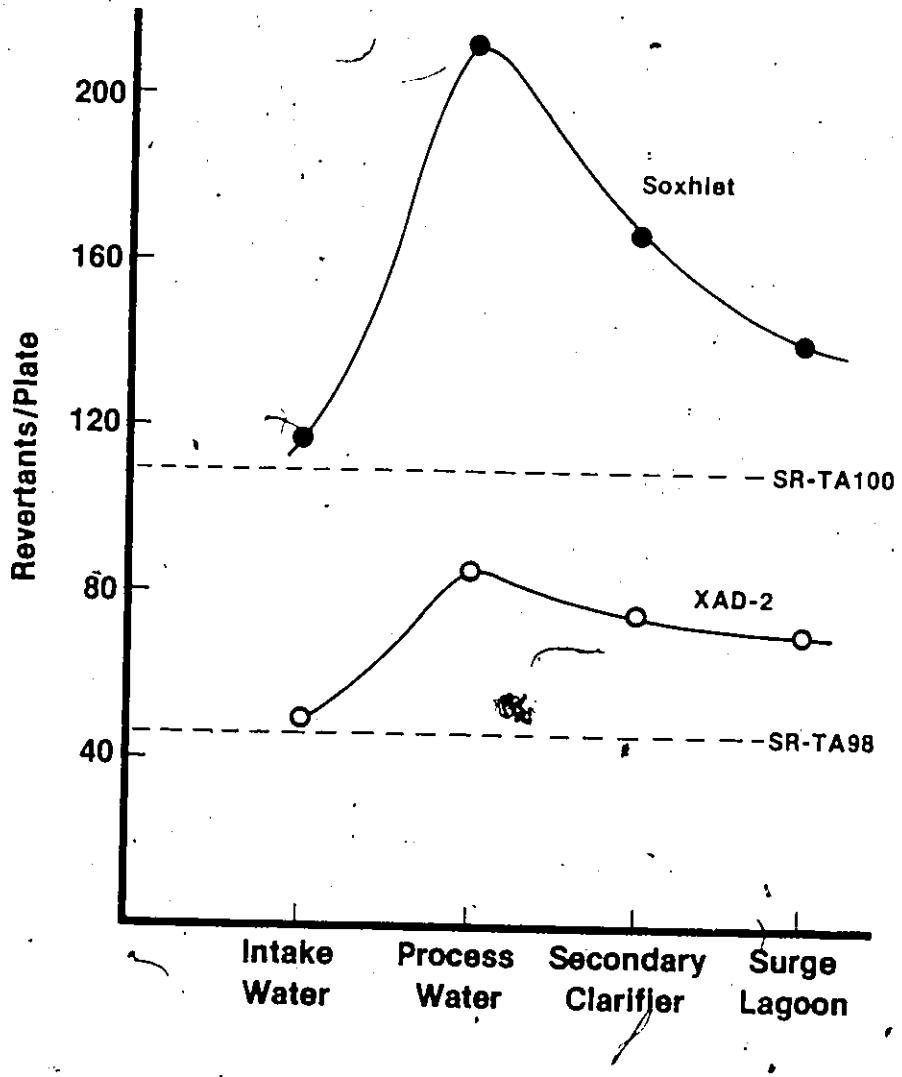


TABLE 9: Comutagenicity assays with norharmane, combined with either 2-acetylaminofluorene (2 µg), or soxhlet and XAD-2 extracts from Refinery 1 (June, 1982). Samples were tested in the Ames assay using TA98 with S-9, and 0.45 µmole of norharmane (no pre-incubation) in 0.02 mℓ. of DMSO.

Treatment	Mean Revertants/Plate
a) Control (DMSO):	
Spont. Revertants	39
2-AAF (2 µg)	266
Soxhlet - 320 mℓ. equiv.	91
160 mℓ. equiv.	40
80 mℓ. equiv.	42
XAD-2 - 80 mℓ. equiv.	73
40 mℓ. equiv.	66
20 mℓ. equiv.	57
b) Norharmane:	
Spont. Revertants	43
2-AAF (2 µg)	462
Soxhlet - 320 mℓ. equiv.	53
160 mℓ. equiv.	48
80 mℓ. equiv.	41
XAD-2 - 80 mℓ. equiv.	65
40 mℓ. equiv.	57
20 mℓ. equiv.	49

TABLE 10: Comutagenicity assays with benzo[e]pyrene (BeP) combined with either benzo[a]pyrene (BaP), or soxhlet and XAD-2 extracts from Refinery 1 (June, 1982). Samples were tested in the Ames assay using TA100 with S-9, and 0, 1, 2.5, or 5 µg of BeP. Mean numbers of spontaneous revertants were 107.

Sample	BeP Conc.	Mean Revertants/Plate
BaP (5 µg)	0 µg	306
	5 µg	421
Soxhlet (320 mℓ. equiv.)	0 µg	116
	1 µg	112
	2.5 µg	111
	5 µg	105
XAD-2 (80 mℓ. equiv.)	0 µg	119
	1 µg	108
	2.5 µg	113
	5 µg	115

TABLE 11: Results of Ames assays to test for comutagenic activity of effluent extracts from Refinery 1 (June, 1982) with benzo[a]-pyrene (5 µg/plate) and 2-acetylaminofluorene (2 µg/plate). All tests were done using strain TA100 with microsomal activation. The mean number of spontaneous revertants was 111.

Mutagen	Extract Dose (mℓ. Equiv./plate)	Revertants/Plate with XAD-2 Extract	Revertants/Plate with Soxhlet Extract
BaP (5 µg)	160	128	121
	80	143	156
	40	123	163
	20	163	177
	0	253	253
2-AAF (2µg)	160	86	73
	80	116	138
	40	193	132
	20	267	194
	0	362	362

VI) Chlorination of Effluent Residues:

Chlorination experiments with 2 litre volumes of organic-free water as diluent were conducted using a June, 1982 final effluent sample from Refinery 1. Tests with 20 litre volumes of surface water (Hamilton Harbour water) contaminated with effluents were conducted with an August, 1982 effluent sample from Refinery 1. Table 12 summarizes the water quality data for these effluent samples. The volatile and non-volatile components of the effluents showed little mutagenic activity before chlorination (Table 13).

Purge-and-trap techniques were used to extract volatile components from whole effluent samples chlorinated at 2, 8, and 16 ppm. In the modified Ames assay (strains TA98 and TA100) using gaseous and aqueous phase protocols, with and without S-9 activation, these volatile extracts failed to give a mutagenic response. Similarily, volatiles gave no response in SCE assays with and without microsomal activation.

When control solutions (organic-free water) were chlorinated and non-volatile extracts tested in the Ames assay, there was a slight increase above spontaneous levels of the mean number of revertants per plate (Table 14). The XAD-2 resin used for extraction contains naphthalenic preservatives (Junk et al, 1972) which may have reacted with residual chlorine in the water passing through the column. Chlorinated derivatives of these preservatives may have been

TABLE 12: Water quality parameters for effluent samples collected from Refinery 1 in June and August, 1982, and used in chlorination studies.

Parameter	Value	
	June	August
pH	8.0	7.5
NH_3-N (ppm)	17.4	3.9
phenol (ppb)	32.0	42.0
susp. solids (mg./l.)	34.0	29.0
oil and grease (mg./l.)	6.4	4.0

TABLE 13: Results of Ames Tests with and without microsomal activation (S-9) for extracts from Refinery 1 effluent samples collected in June and August, 1982, and used in chlorination experiments. Results are presented for the dose and the test strain which gave the maximum response to the extract. Spontaneous revertants are in brackets.

			Revertants/Plate			
			With S-9		Without S-9	
Extract	Strain	Mg. Equiv. per Plate	June	August	June	August
a) Non-Volatiles:						
Particulates	TA100	320	107(102)	122(100)	115(112)	102(115)
Dissolved	TA98	80	72(46)	50(42)	47(49)	50(46)
b) Volatiles:	TA100	17	110(99)	143(96)	98(102)	109(106)

TABLE 14: Revertants per plate for Ames tests (with TA100) of extracts from control solutions (organic-free water) chlorinated for 1 h. at 8 ppm chlorine (pH = 6).

Dose/Plate (ml. Equivalents)	Revertants/Plate	
	With S-9	Without S-9
160	138	56
80	118	91
40	108	112
20	120	119
10	106	106
Spontaneous Revertants	96	105

responsible for the slight mutagenic activity in the control samples. When control and experimental extracts were analyzed by gas chromatography, there were large peaks of unidentified material which interfered with GC analysis of effluent-derived compounds in the experimental (effluent) extracts.

In many water chlorination studies reported in the literature, sodium sulfite was added at the end of the contact period to terminate the chlorination process (Youssefi et al, 1978; Oyler et al, 1982). However, because Cheh et al (1979) reported that this procedure greatly reduced the mutagenic yield of chlorinated samples, this treatment was eliminated in our tests. However, in order to reduce toxicity in the <u>in vitro</u> assays, it was necessary to pass the ether eluent through a bed of sodium sulfite <u>after</u> elution from the XAD-2 column. This did not appear to reduce the mutagenicity of the samples. Moreover, when a 15 ml sample of ether containing 100 ug each of chloronaphthalene, hexachlorophenol, and tetrachlorobiphenyl was passed through sodium sulfite, gas chromatographic analysis revealed that the three compounds were recovered with efficiencies of 72%, 87%, and 89%, respectively.

A non-volatile extract prepared from a dilute effluent solution (10%) chlorinated at 8 ppm was mutagenic in Ames assays both with and without S-9 activation (Fig. 18). Test strain TA100 was more sensitive than TA98 to all of the chlorinated extracts tested. Extracts tested without S-9 were toxic at higher concentrations, but were mutagenic at very low doses (Fig. 18). The different

Figure 18: Mean mutagenicity of extracts from a chlorinated (8 ppm chlorine for 1 hr at pH 6.0) solution of 10% effluent tested using strain TA100 with and without S-9. The mean spontaneous revertants for the assay is represented by the line labelled "SR".

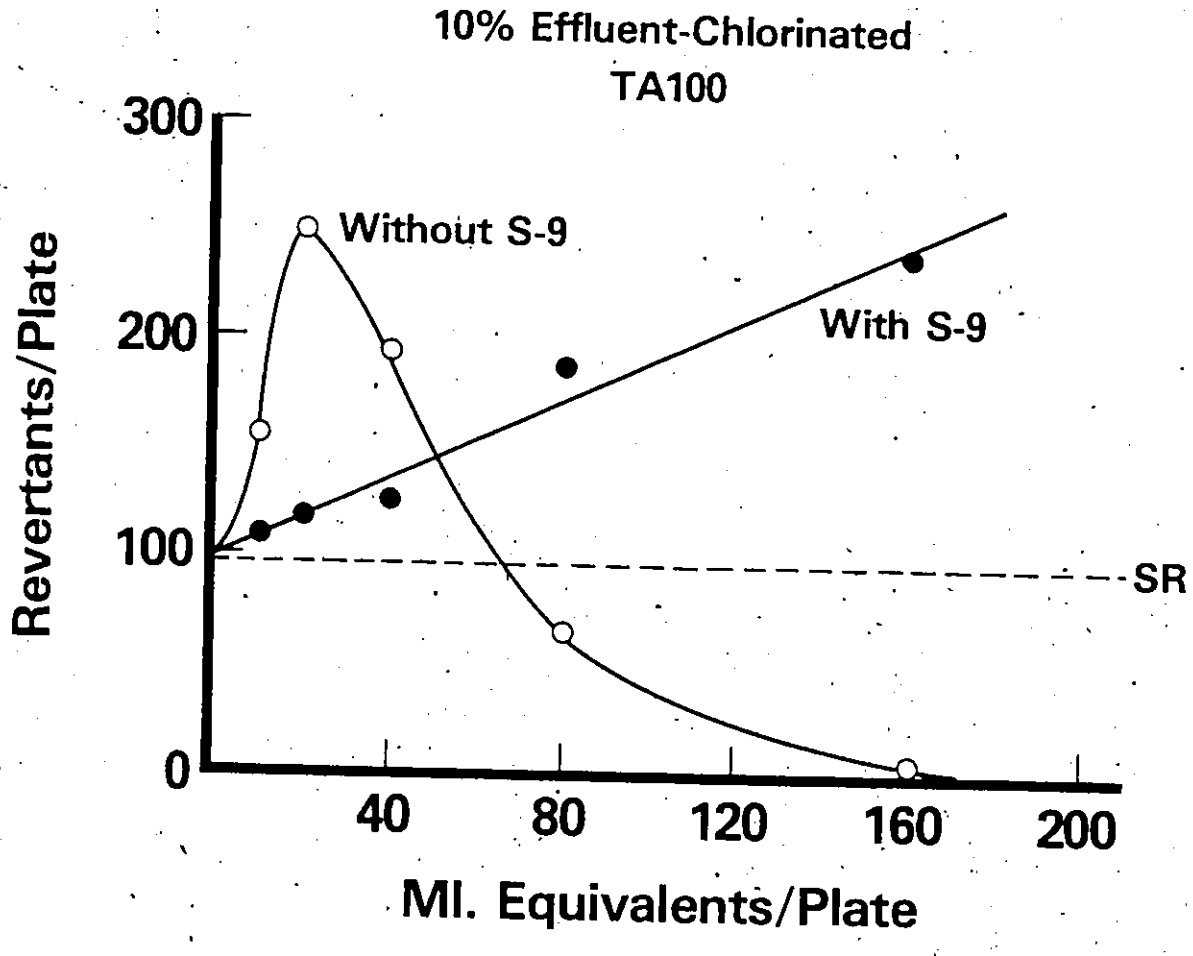

10% Effluent-Chlorinated
TA100
300
200
100
0
Revertants/Plate
Without S-9
With S-9
SR
40
80
120
160
200
Ml. Equivalents/Plate

TABLE 15: Mean SCE's induced by extracts from a 10% effluent solution (organic-free water as diluent) chlorinated at 8 ppm for 1 hr. (pH = 6). Assays were run with and without microsomal activation (S-9). Extracts were incubated with S-9 in assay flasks with 25 ml of medium for 1 hr. or 2.5 hr. Standard deviations for the mean SCE's (n = 25) metaphases) are presented in brackets.

Sample	Mℓ. Equiv. per Flask	Mean SCE's per Metaphase: Without S-9	With S-9 (1hr)	With S-9 (2.5hr)
Solvent Control	-	4.7 (2.0)	4.9 (1.8)	5.2 (2.0)
10% Effluent Sol'n	320	Toxic	5.7 (1.8)	Toxic
	160	11.6 (2.8)*	4.6 (2.0)	4.9 (3.1)
	80	8.3 (2.0)*	4.7 (2.3)	5.3 (2.2)
	40	6.9 (2.1)*	5.2 (1.6)	5.0 (1.7)
Control Sol'n	320	4.9 (2.2)	-	4.8 (1.7)
	160	4.6 (2.3)	-	4.7 (2.0)
	80	4.6 (1.9)	-	5.0 (2.3)
	40	4.8 (2.7)	-	4.8 (1.6)

*Mean SCE's significantly different from controls ($t_{.05}$).

dose-response curves for tests with and without S-9 may represent the mutagenic activity of two different agents; one direct-acting, and the other requiring metabolic activation. The non-volatile extracts also gave a positive response in the SCE assay, only without S-9 activation (Table 15).

When the chlorinated 10% effluent extract was subfractionated by silica-gel column chromatography, mutagenicity in the Ames assay without S-9 activation was confined to fractions 3 and 5 (Fig. 19a). In tests with S-9, activity was lower, but mutagenicity was detected in fractions 5 and 7 (Fig. 19b). The two patterns of mutagenic activity in tests with and without S-9 suggest that two different classes of genotoxic agents are present in the chlorinated sample.

The chlorinated solutions containing dilute effluents (2.5% to 20%), as well as undiluted effluent, were mutagenic without S-9 activation at doses as low as 20 ml equivalents per plate (Fig. 20). Linear dose-response curves were generated for all of these chlorination tests, but only the mutagenicity at the highest dose is shown. Since the amount of total and free chlorine present in the solutions after chlorination is inversely proportional to the amount of effluent (Table 16), it appears to be the reaction of chlorine with effluent which governs the mutagenic yield of the sample.

Tests were made to determine the effect of various chlorination conditions upon the mutagenic activity of non-volatile extracts. The mutagenicity of a 10% effluent solution increased with the chlorine concentration over a range of 2 to 8 ppm chlorine (Fig.

Figure 19: Mutagenicity (TA100) of silica-gel subfractions from extracts of chlorinated (8 ppm) 10% effluent. Figure (a) illustrates results of Ames tests without S-9 at a dose of 160 ml equivalents per plate, and figure (b) shows results with S-9 at a dose of 320 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assays is represented by the line labelled "SR".

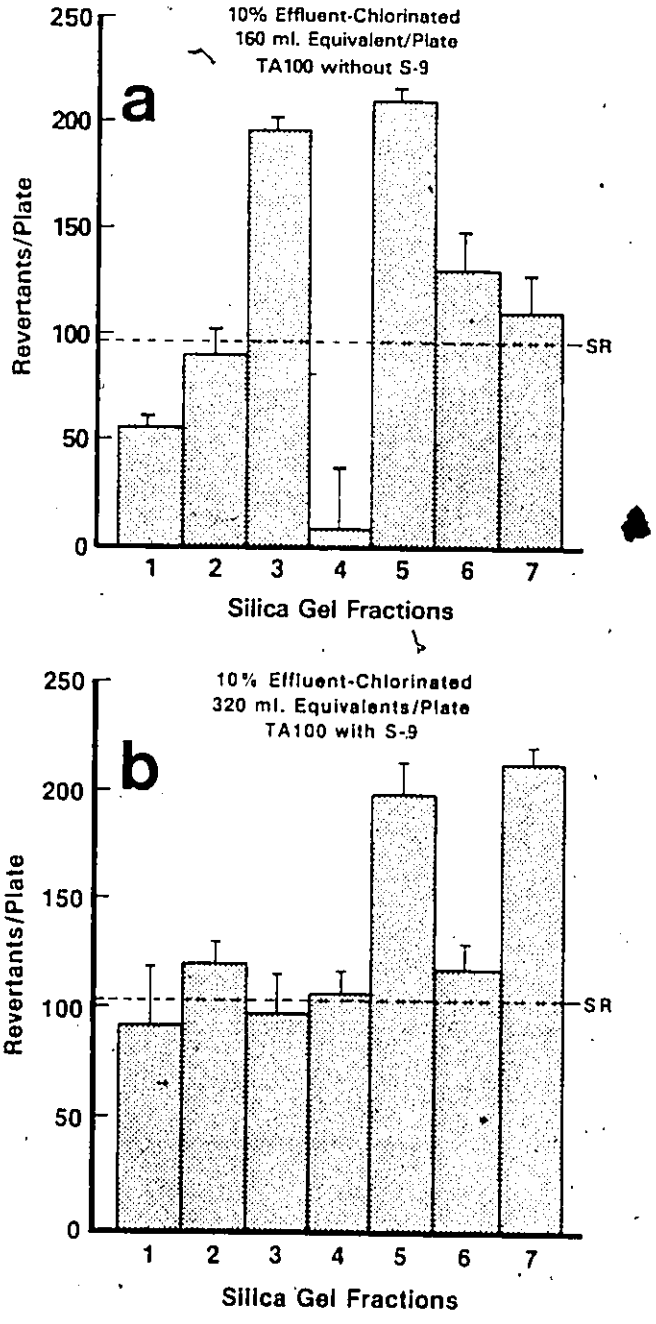

10% Effluent-Chlorinated
160 ml. Equivalent/Plate
TA100 without S-9
a
Revertants/Plate
250
200
150
100
50
0
SR
1
2
3
4
5
6
7
Silica Gel Fractions
10% Effluent-Chlorinated
320 ml. Equivalents/Plate
TA100 with S-9
b
Revertants/Plate
250
200
150
100
50
0
SR
1
2
3
4
5
6
7
Silica Gel Fractions

Figure 20: Mutagenicity (TA100 without S-9) of extracts from whole effluents, and diluted effluents (2.5 - 20%) chlorinated at 8 ppm, and plated at a dose of 20 ml equivalents per plate. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assay is represented by the line labelled "SR".

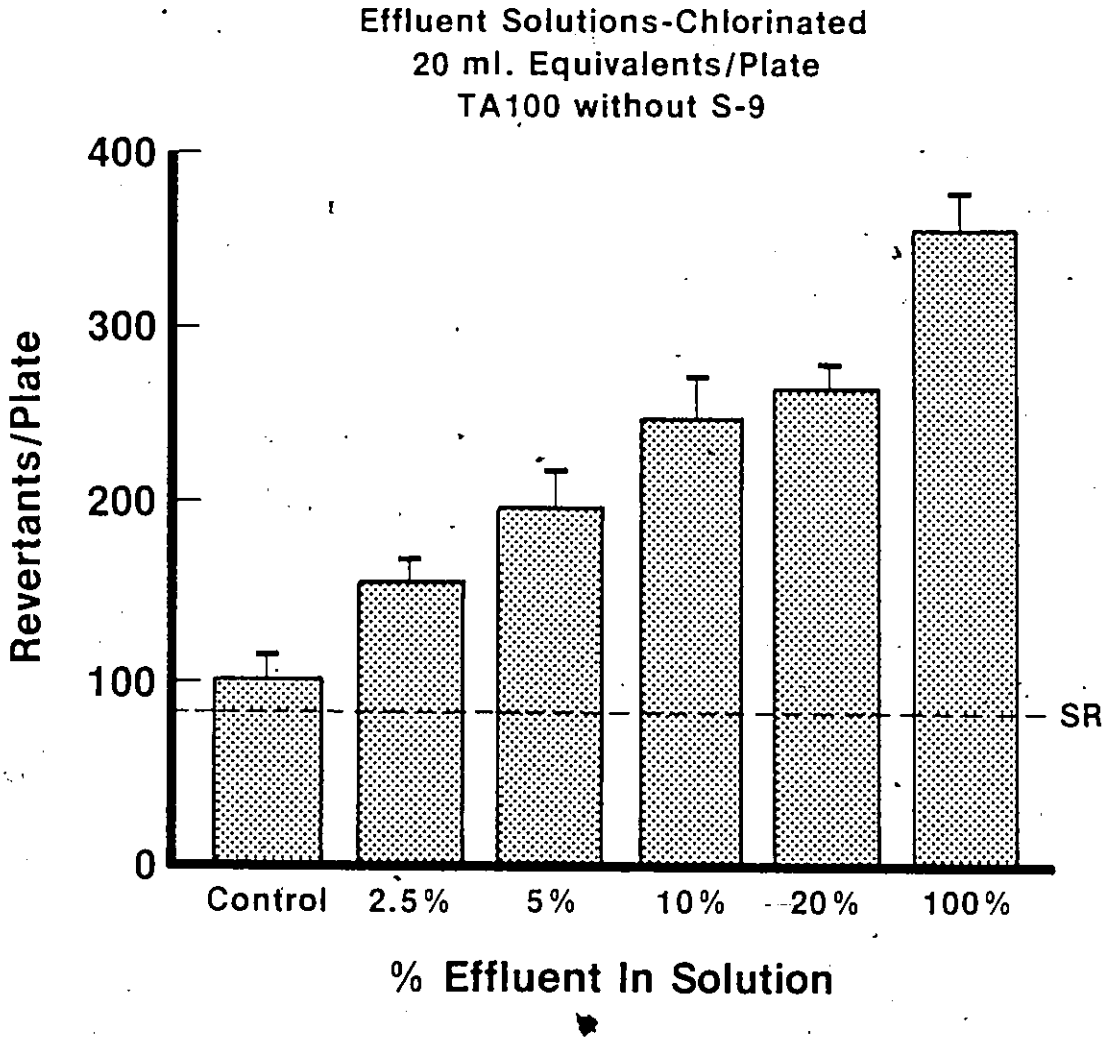

Effluent Solutions-Chlorinated
20 ml. Equivalents/Plate
TA100 without S-9
Revertants/Plate
400
300
200
100
0
SR
Control
2.5%
5%
10%
20%
100%
% Effluent In Solution

TABLE 16: Total and free chlorine concentrations after 1 hr. chlorination at 8ppm (pH = 6.0) of solutions containing 2.5% to 20% refinery effluent in organic-free water.

Treatment	Final Chlorine Conc. (ppm)	
	Total	Free
20% Effluent	1.9	0.4
10% Effluent	2.5	0.7
5% Effluent	2.8	0.9
2.5% Effluent	3.0	1.5
Control	5.8	3.2

21a). The mutagenicity of chlorinated effluent solutions also increased with contact time (Fig. 21b), with the greatest increment in mutagenic activity occurring in the first hour of contact with chlorine. There was little difference between the mutagenic activity of solutions chlorinated at pH 6 and pH 4, but mutagenicity was reduced in tests at pH 8 (Table 17).

While effluents diluted with organic-free water were mutagenic when chlorinated, it was not clear whether effluents diluted with surface water would give similar results. Results are summarized in Table 18 for a group of tests in which effluents were diluted with surface water (20 litres) taken from an embayment of Lake Ontario (Hamilton Harbour) which receives industrial and domestic wastewater discharges.

Following chlorination at 8 ppm, the mutagenicity of 2.5% effluent in surface water was greater than the mutagenicity of a sample containing surface water, alone (Table 18). Extract prepared from 2.5% effluent in 20 litres of organic-free water was slightly more mutagenic (Table 18); probably because of the absence of organic contaminants which competitively react with the chlorine (e.g. humic acids). The XAD-2 extraction protocol in which 2 litre volumes of sample were passed through the column recovered most compounds with > 70% efficiency (Fig.2), but the recovery efficiency using 20 litre samples was not determined. While the recovery of mutagens in tests using 20 litre solutions may not be quantitatively accurate, comparisons between treatments are valid.

Figure 21: Mutagenicity (TA100 without S-9) of extracts (20 ml equivalents per plate) from 10% effluent chlorinated at: a) 2,4, and 8 ppm chlorine concentrations; b) 8 ppm chlorine concentration for a 1, 2, or 4 hr contact time. Error bars represent twice the standard deviation about the mean (n=3). The mean spontaneous revertants for the assay is represented by the line labelled "SR".

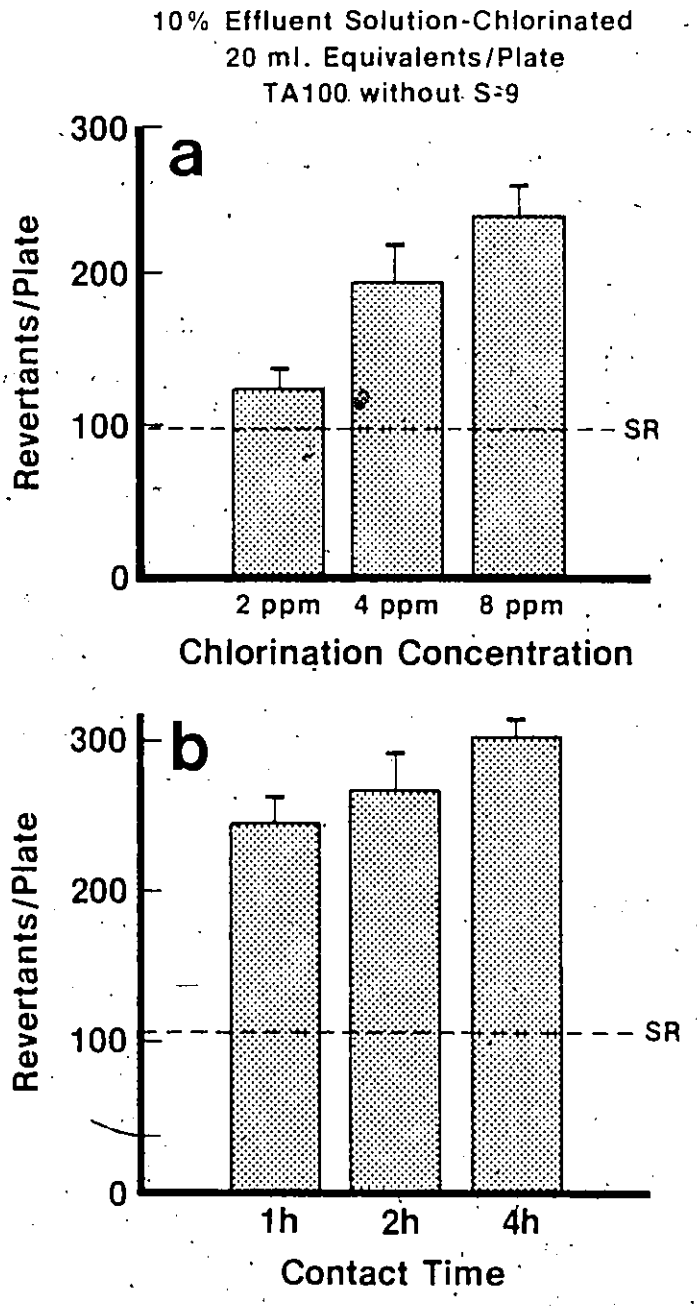

10% Effluent Solution-Chlorinated
20 ml. Equivalents/Plate
TA100 without S-9
a
Revertants/Plate
300
200
100
0
SR
2 ppm
4 ppm
8 ppm
Chlorination Concentration
b
Revertants/Plate
300
200
100
0
SR
1h
2h
4h
Contact Time

TABLE 17: Revertants per plate for Ames tests (TA100) without S-9, of extracts from a 10% effluent solution chlorinated for 1 h. at 8 ppm chlorine and at pH 4, 6 and 8. The mean number of spontaneous revertants was 102.

	Revertants per Plate			
	10 % Effluent			Control
Dose per Plate (Mℓ. Equivalents)	pH 4	pH 6	pH 8	pH 6
80	146	173	124	91
40	232	223	162	112
20	196	244	145	119
10	190	153	108	106
5	122	117	106	108

Subfractionation of the extracts from these surface water tests indicate that mutagenicity is confined to fractions 3 and 5 in tests without S-9 (Fig. 22). These are the same fractions which yielded activity in extracts from 2 litre chlorinated solutions (Fig. 19). Chlorinated surface water alone contained some mutagenic activity in Fraction 3, as well (Fig. 22).

Naturally-occurring organic compounds such as humic, tannic, and fulvic acids have been shown to contribute to the formation of non-volatile genotoxic compounds after chlorination (Fallon and Fliermans, 1980; Watts et al, 1982). The relative mutagenic potential of refinery effluents, tannic acids, and humic acids were determined by chlorinating samples of organic-free water spiked with these substances at concentrations of 4 mg/litre organic carbon. The extracts of chlorinated solutions were toxic in the Ames tests at doses greater than 40 ml equivalents per plate, but at a dose of 20 ml equivalents it is obvious that the relative mutagenicity of the chlorinated effluent solution was greater than solutions containing chlorinated humic and tannic acids (Table 19).

TABLE 18: Revertants per plate for Ames tests (TA100), without S-9, of extracts from a 2.5% effluent solutions in 20 l. of surface water or organic-free water, and chlorinated for 1 h. at 8 ppm (pH = 6.0). Sample dose per plate was 400 ml equivalents, and the mean number of spontaneous revertants was 96.

Treatment	Revertants per Plate
Organic-free water	82
Organic-free water plus effluent	227*
Surface water	97
Surface water plus effluent	191*

*Statistically significant differences ($t_{.05}$) between mean revertants per plate in tests with and without effluents (n = 3 per treatment).

Figure 22: Mean mutagenicity (TA100 without S-9) of silica-gel subfractions (20 ml equivalents per plate) from extracts of chlorinated (8 ppm) surface water (Hamilton Harbor water), and chlorinated surface water plus 2.5% effluent. The mean spontaneous revertants for the assay is represented by the line labelled "SR".

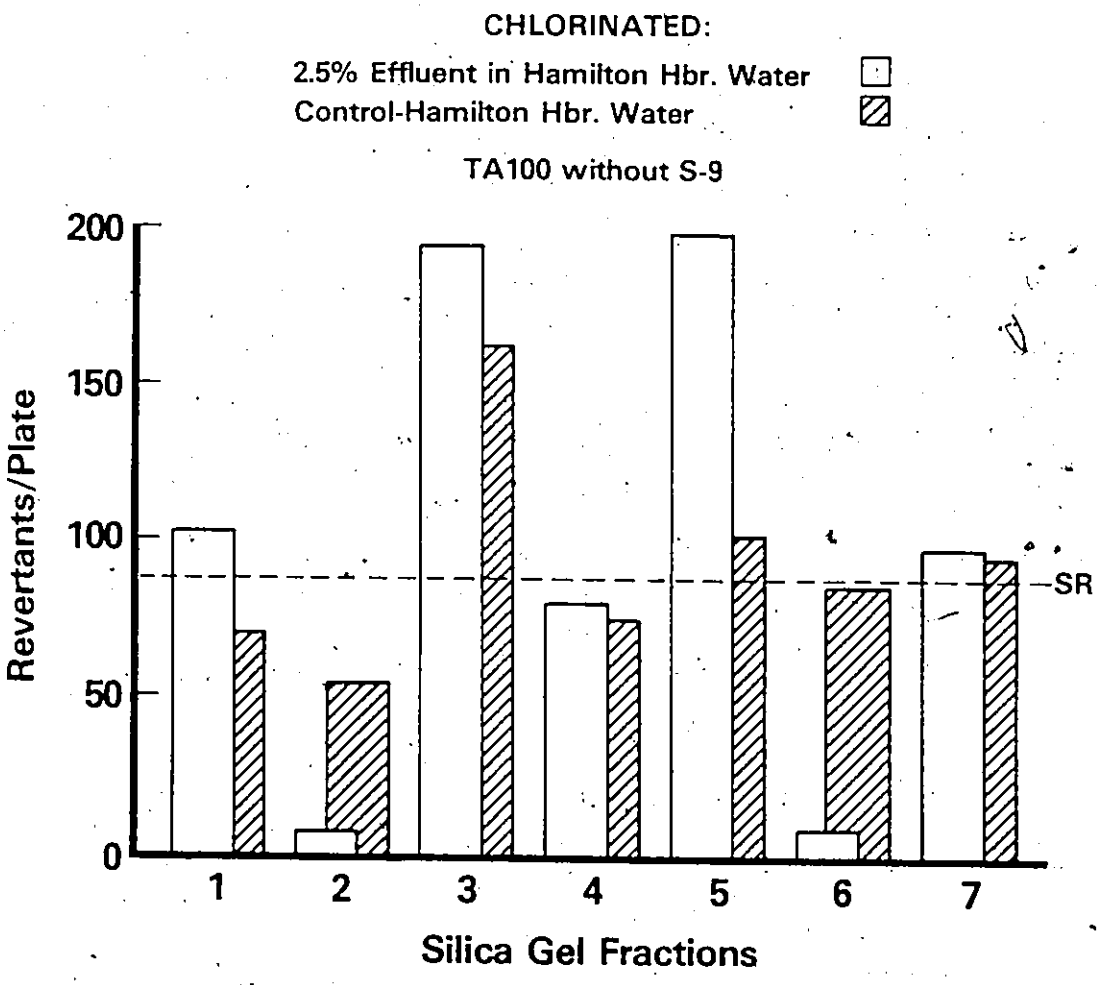

CHLORINATED:
2.5% Effluent in Hamilton Hbr. Water
Control-Hamilton Hbr. Water
TA100 without S-9
Revertants/Plate
200
150
100
50
0
SR
1
2
3
4
5
6
7
Silica Gel Fractions

TABLE 19: Mutagenicity of control solution (organic-free water), and diluted refinery effluents, tannic acid, and humic acid (4 mg./l. organic carbon) chlorinated at 8 ppm for 1 hr. (pH = 6.0). The mean number of spontaneous revertants for strain TA100 (without S-9) was 104.

	Revertants/Plate			
Dose per Plate (Ml. Equivalents	Effluent	Tannic Acid	Humic Acid	Control
160	Toxic	66	102	56
80	61	147	146	91
40	185	155	162	112
20	244	148	145	119
10	153	100	140	106
5	126	98	111	108

DISCUSSION

I) Effluent Genotoxicity:

(a)Particulate component:

Significant mutagenic activity was found in the particulate fraction of effluents from 2 of the 3 refineries tested. The mean mutagenic activity of extract residues, calculated on a revertants per mg basis (65-101 revertants per mg; Table 20), is much lower than the activities reported in the literature for several other complex mixtures. For example, neutral tar extracts from shale oil, and coal distillates induced 50-5000 revertants per mg, and 8500 revertants per mg, respectively, with TA98 (Pelroy et al, 1981). Kier et al (1974) found that cigarette smoke condensate induced 1240 revertants per mg with strain TA98. We extracted mutagens from two drinking water samples (200 litres) which gave a response with TA98 of 116 and 172 revertants per mg of residue (Table 21). Drinking water samples required no S-9 activation, as compared with refinery extracts. Mutagens present in most drinking water extracts are direct-acting (Nestmann et al, 1979; Zoeteman et al, 1982; Kool et al, 1982).

When a Refinery 3 particulate sample was tested in the SCE

assay with microsomal activation (Table 6), 3 mg of residue per ml of medium increased the mean SCE's per metaphase to 10.7 from a control value of 4.6. Raat (1979) reported that exposure of CHO cells to 0.3 mg/ml of cigarette smoke condensate (without microsomal activation) increased mean SCE's to 22 from a control value of 12. Latt et al (1981) suggested that compounds effective at inducing SCE's should produce a three-point dose-response curve, with t-test confidence intervals <0.001 for at least one dose. Since the induction of 10.7 SCE's per metaphase using 160 ml equivalents of Refinery 3 soxhlet extract was significant within confidence intervals of $0.001<P<0.01$ (Table 6), this sample borders on being an "effective" clastogenic agent.

At first glance, the low relative mutagenic activity of the extract residues from refinery effluent particulates would indicate that the discharge of mutagens in refinery effluents is minimal. However, the amount of mutagenic material released in particulates from a refinery may total tonnes per day (Table 20). To illustrate the cumulative hazard of these discharges, if BaP gives a response of 52 revertants/µg in the Ames test with TA100 and S-9 (Table 4), 1 mg of particulate extract from Refinery 3 contains the mutagenic equivalent of 1.9 µg of BaP. If the daily discharge of particulate extract residues was converted to BaP equivalents, it would amount to approximately 1.9 Kg of BaP released per day. It is possible that long-term accumulation of genotoxic compounds from refineries may represent a hazard to sources of drinking water. The magnitude of

TABLE 20: Mean and range of mutagenic activity (revertants per mg) of residues extracted from refinery effluents, and estimates of the rate of discharge of this material from the refineries. Mutagenicity is calculated after subtraction of spontaneous revertant values. Soxhlet residues were tested with TA100 (with S-9) at a dose per plate of 320 ml equivalents per plate, and XAD-2 residues were tested with TA98 (with S-9) at 80 mℓ. equivalents per plate.

Refinery	No. Samples	Residue Mutagenicity (Revert./mg)	Residue Per Litre (mg)	Residue Discharged (kg/day)
1) Soxhlet Extracts:				
1	5	64(52-73)	25(17-37)	100[a]
2	1	123	24	290[b]
3	2	101(97-105)	40(36-43)	960[c]
2) XAD-2 Extracts:				
1	5	47(4-72)	24(12-29)	95[a]
2	1	0	15	180[b]
3	2	36(34-38)	18(15-21)	430[c]

a) Effluent discharge from refinery 1 averages 4×10^6 ℓ/day

b) Effluent discharge from refinery 2 averages 12×10^6 ℓ/day

c) Effluent discharge from refinery 3 averages 24×10^6/day

TABLE 21: Mutagenicity of two drinking water samples extracted from 200 ℓ. of Hamilton, Ontario tap-water by the XAD-2 extraction method of Nestmann et al, (1979). SR refers to spontaneous revertants.

Dose/Plate (mg)	Mean Revertants/Plate			
	TA98		TA100	
	With S-9	Without S-9	With S-9	Without S-9
a) Sample I:				
1.0	26	154	127	213
0.5	50	85	110	178
0.25	47	51	115	143
0.12	40	48	102	127
0 (SR)	40	38	94	99
b) Sample II:				
1.0	56	215	113	197
0.5	67	173	102	176
0.25	40	52	105	138
0.12	43	55	99	126
0 (SR)	41	43	102	104

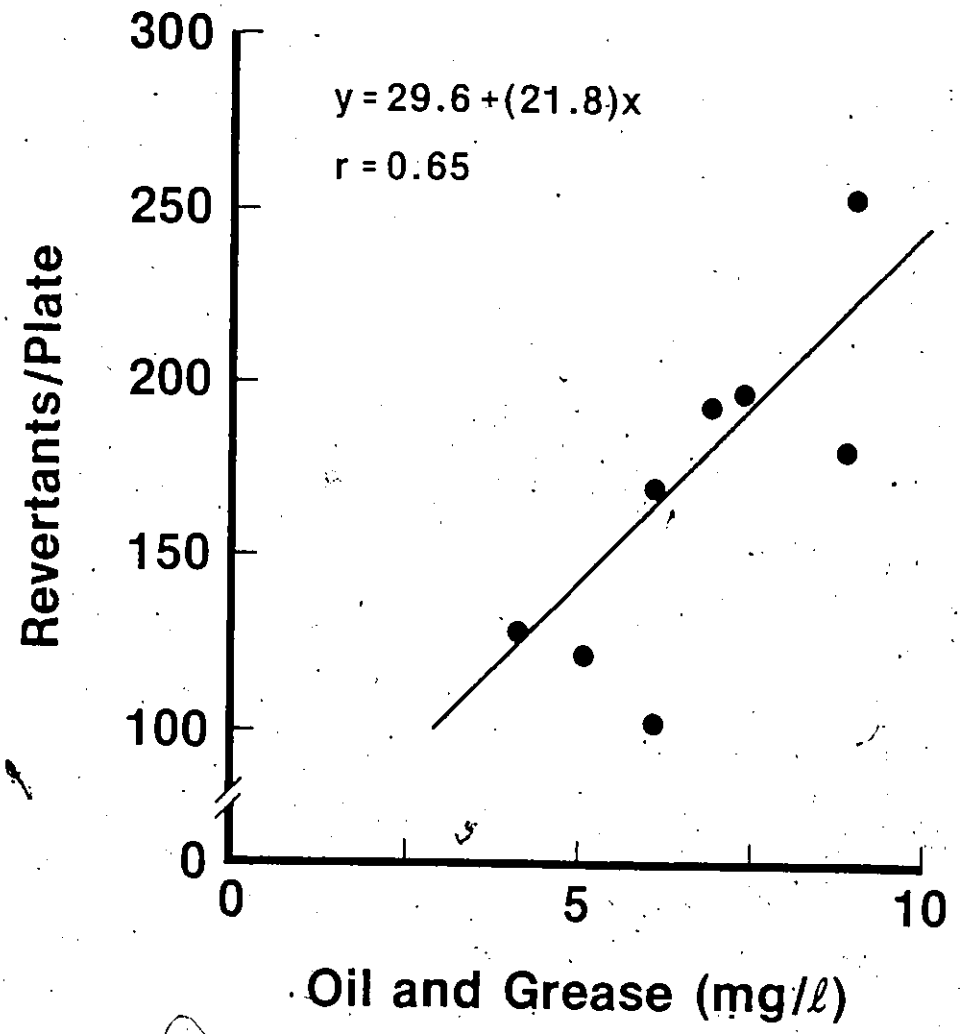
this hazard would depend upon parameters affecting the fate of particulates and associated genotoxic compounds. Rates of deposition of particulates, rates of desorption of organics from particulates, and rates of microbial and chemical degradation of genotoxic agents are all important factors.

When particulates were removed from effluent samples by centrifugation, over 95% of the total suspended solids, and oil and grease were removed from the effluent. Therefore, it is probable that the material classified as particulates in this study consists of immiscible oil and grease, and inorganic and organic suspended material. Figure 23 indicates that the mutagenic activity of extracts prepared from these particulate components is positively associated with the levels of oil and grease in the sample ($r=0.65$). No correlation was found between mutagenicity and other effluent quality parameters tested (ammonia, phenol, suspended solids).

Subfractionation facilitated the identification of some of the chemical characteristics of the genotoxic agents present in the particulate component of the effluents. These compounds were in the neutral, and polar fractions of the extracts. They were not direct-acting mutagens, and the sensitivity of TA100 to the extracts suggests that they act through base-pair substitution mechanisms of mutagenesis. An extract from Refinery 3 induced SCE's with S-9 activation.

Gas chromatographic analyses have shown that neutral fractions of effluent extracts contain complex mixtures of hydrocarbons,

Figure 23: Relationship between mutagenicity of particulate extracts (soxhlet) at a dose of 320 ml equivalents per plate (TA100 with S-9) and the concentration of oil and grease extracted from effluents of the three refineries (1981-1983).


300
250
200
150
100
0
0
5
10
y = 29.6 +(21.8)x
r = 0.65
Revertants/Plate
Oil and Grease (mg/ℓ)

including aliphatic and aromatic compounds. Di-alkyl phthalates are a major component of a mutagenic effluent sample from Refinery 3. These compounds are commonly found in ppb quantities in effluents from conventional refineries (PACE, 1981) and tar-sands facilities (Hrudey et al, 1976). Although differences in patterns were observed, it is not possible to correlate these with genotoxic activity. The majority of mutagenic activity in extracts from Refineries 2 and 3 was present in the most polar silica gel fraction (Fraction 7). HPLC analyses showed that these fractions contained complex mixtures of aromatic hydrocarbons. Characterization of components for eventual correlation with genotoxic activity would require further fractionation and genotoxicity testing.

(b)Dissolved Components:

The non-volatile components dissolved in refinery effluents (XAD-2 extracts) showed little genotoxic activity. Most extracts did elicit a dose-response in the Ames assay, but the number of revertants was never double the spontaneous levels. The mutagenicities of these XAD-2 extracts are similar to the values reported by Commoner (1977) for refinery, steel-mill, and industrial waste-water effluents discharging into the Houston ship channel (Table 22). The mutagenicity of the non-volatile, dissolved components is low in terms of revertants per mg of extract residue (Table 20). However, the large

TABLE 22: Comparison of the mutagenicity of effluents from industries discharging into the Houston ship channel (from Commoner, 1977) and the range of mutagenicities of XAD-2 extracts (non-volatile, dissolved components) from refinery effluents. Mean spontaneous revertants are presented in brackets.

SOURCE	Mℓ. Equiv./Plate	Revertants/Plate
HOUSTON:[a]		
Pulp Mill	1	36(31)
Steel Mill	62.5	64(42)
Oil Refinery	125	86(26)
Industrial Waste Plant-A	125	58(36)
-B	125	182(31)
ONTARIO:[b]		
Refinery 1	80	42-82(42)
Refinery 2	80	39(42)
Refinery 3	80	48-55(42)

a) Test strain TA1538, with S-9

b) Test strain TA98, with S-9.

amounts of this material discharged from each refinery may represent a cumulative environmental hazard. None of the XAD-2 extracts showed induction of SCE's in tests with or without S-9 activation.

Non-volatile, dissolved mutagens consisted of neutral, relatively polar fractions of the extract (Fraction 6). Test strain TA98 (sensitive to frame-shift mutagens) was most sensitive to these extracts, but S-9 activation was required for mutagenic activity.

Volatile components dissolved in refinery effluents showed a slight dose-response in the Ames tests, but activity was always less than a doubling over spontaneous revertants. The toxicity of the samples at low concentrations confounded genotoxicity testing of the volatile components. It is interesting that volatile extracts required S-9 activation for expression of the slight mutagenic activity in the modified Ames assay. Volatile alkyl halides give a positive response in the Ames assay without S-9 activation (Simmon, 1980). However, a volatile chloroallyl ether compound [3-(2-chloroethoxy)-1,2-dichloropropene] isolated from organic residue of drinking water requires S-9 activation for expression of mutagenic activity (Distlerath et al, 1984). Perhaps a similar class of compounds is present in low concentrations in volatile effluent samples.

(c) General discussion of effluent genotoxicity:

The genotoxic activity of dissolved components of refinery effluents are low, but some extracts from particulate components showed significant mutagenic (Ames test) and clastogenic (SCE) activity. The mutagenicity of particulates is not high when expressed on the basis of revertants per mg of extracted residue. However, the large amounts of this material released from refineries on a daily basis may represent a cumulative risk to the environment. The mutagenic activity of particulate extracts was greatly reduced by effluent treatment processes at Refinery 1, but dissolved components appeared to be less efficiently removed. The discharge of mutagenic particulate components in the other two refineries could probably be decreased by more efficient effluent treatment procedures.

Like the majority of North American refinery facilities, the refineries sampled in this study use "low-cracking" temperatures (500°C) to produce gasoline and other fuel products. "High-cracking", petrochemical, and lube-oil reprocessing refineries, as well as upgrading operations for heavy oils, and synthetic crudes are facilities with greater potential for the formation of carcinogenic pyrolysis products. Future studies of refinery effluents should include genotoxicity testing of samples from these types of facilities.

The polar nature of the mutagenic subfractions from soxhlet

and XAD-2 extracts indicates that previous studies of the genotoxic potential of refinery effluents (Andelman and Suess, 1970; Ershova, 1967) may have been too selective in limiting analysis to PAH compounds. Studies of the mutagenicity of various synthetic fuels have indicated that Ames-positive mutagens are also concentrated in polar fractions (Pelroy et al, 1981). Analytical characterization of compounds present in refinery effluents should concentrate on the more polar fractions in extracts.

II) Chlorination of Effluents:

Chlorination of water containing various concentrations of refinery effluent increased the mutagenic activity of the sample. The mutagenicity of chlorinated solutions increased with effluent concentration, contact time with chlorine, and chlorine concentration. Since most surface waters are disinfected with chlorine doses greater than 6 ppm (Cantor, 1982), the chlorine concentrations used in these tests (2-8 ppm) represent the lower range of concentrations used in drinking water treatment.

Chlorination at pH 4 and pH 6 produced high mutagenic activity, but mutagenicity was reduced in tests at pH 8. Substitution, oxidation, and amination reactions during aqueous

chlorination are highly pH dependent (Pierce, 1978). Oyler et al (1982) found that aqueous chlorination of PAH's at pH >6 resulted in the production of oxygenated compounds, whereas reactions at pH <6 yielded some oxygenated (quinones) and chlorinated compounds.

The differences in the dose-response curves for chlorinated extracts tested in the Ames assay with and without S-9 (Fig. 18) indicate that there may be two groups of mutagens (one direct-acting, and one requiring metabolic activation) in chlorinated samples. This is further supported by the the mutagenicity of subfractions 3 and 5 in Ames tests without S-9, and subfractions 5 and 7 in tests with S-9 (Fig. 19). SCE induction only occurred in assays without S-9 activation. The clastogenic activity of the direct-acting agent must have been destroyed by metabolic activity (De Flora, 1978), or by adsorption onto S-9 proteins.

Although chemical fractionation of chlorinated effluent extracts identified three fractions with genotoxic activity, the identities of the active compounds are unknown. Many non-volatile compounds identified in refinery effluents which are not considered genotoxic (eg. naphthalene, anthracene, phenol), may react with chlorine to form compounds with carcinogenic potential. It is known that aromatic hydrocarbons readily undergo substitution reactions during aqueous chlorination (Gaffney, 1977; Snider and Alley, 1979).

Volatile extracts from chlorinated effluent samples were not mutagenic in the modified Ames assay, and gave no response in the SCE

assay. However, even with the modified test protocols which take account of sample volatility, the Ames assay is insensitive to many of the carcinogenic alkyl halides produced by chlorination reactions (e.g. chloroform). In our modified Ames procedure, carbon tetrachloride, chloroform, and 1,2 dichloroethane were not mutagenic, but these compounds are all carcinogenic in rodent bioassays (Page and Saffiotti, 1976). To date, alkyl halides and many other classes of volatile compounds have not been tested by in vitro SCE assays (Latt et al, 1981). Therefore, negative results for volatile extracts may be due to the insensitivity of the assay systems to the classes of compounds produced by chlorination.

Without studies utilizing pilot-scale waste treatment systems (i.e. charcoal and sand filtration, coagulation, flocculation, etc.), it is difficult to assess the biological implications of the formation of genotoxic, non-volatile compounds during chlorination of refinery effluent residues. Contaminants originating from an oil refinery may be eliminated by water treatment systems before the application of chlorine. The contribution of naturally-occurring contaminants of drinking water (humic acids, tannic acids, fulvic acids) to the yield of genotoxic compounds following chlorination may exceed the contribution of effluents.

Refinery effluents may come into contact with chlorine by other mechanisms. Effluents at some refineries are not treated on-site, but are discharged into municipal sewage treatment facilities (Glaze and Henderson, 1975). Also, municipally-treated drinking water

supplies may be used as cooling and process water in some refineries (Cote', 1976). In both of these instances, there is potential for the formation of genotoxic compounds by the reaction of effluents with chlorine.

PART 3: DEVELOPMENT OF A TROUT EMBRYO INJECTION CARCINOGENESIS ASSAY AND CARCINOGENIC ACTIVITY OF PETROLEUM REFINERY EFFLUENTS

PURPOSE

The purpose of this section of the study was to evaluate the carcinogenic activity of petroleum refinery effluents using *in vivo* bioassays. Rodents were ruled out as test organisms because of the complexity and high cost of *in vivo* rodent assays. A carcinogenesis bioassay using fish as a test organism was developed in which ng quantities of compound or extract were microinjected into rainbow trout embryos. The utility of fish as an *in vivo* carcinogenesis test organism depends upon the similarities in response of fish and mammals to carcinogen exposure; in particular, the enzyme activation potential of fish vs. rodents. As an approach to this problem, we activated test compounds exogenously by pre-incubation with rat-liver microsomal preparations (S-9). The embryo microinjection assay was used to test the carcinogenic activity of

some of the refinery effluent extracts previously tested for genotoxicity in the *in vitro* section of this study (Part 2).

This portion of the study is divided into three general areas:

a) Development and evaluation of the trout embryo microinjection assay as an *in vivo* carcinogenesis test. The assay was evaluated by exposing embryos to three known carcinogens (aflatoxin B_1, 2-anthramine, 7,12-dimethylbenzanthracene). This section also includes a description of the criteria used to classify neoplasms induced by test carcinogens.

b) Statistical evaluation of the necropsy and histological survey methods used to determine the incidence of neoplasms in trout.

c) Evaluation of the carcinogenicity of petroleum refinery effluents in the embryo microinjection assay. This section also includes tests to determine whether effluent extracts act synergistically with aflatoxin B_1 and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG).

MATERIALS AND METHODS

I) Injection Procedures and Pathology:

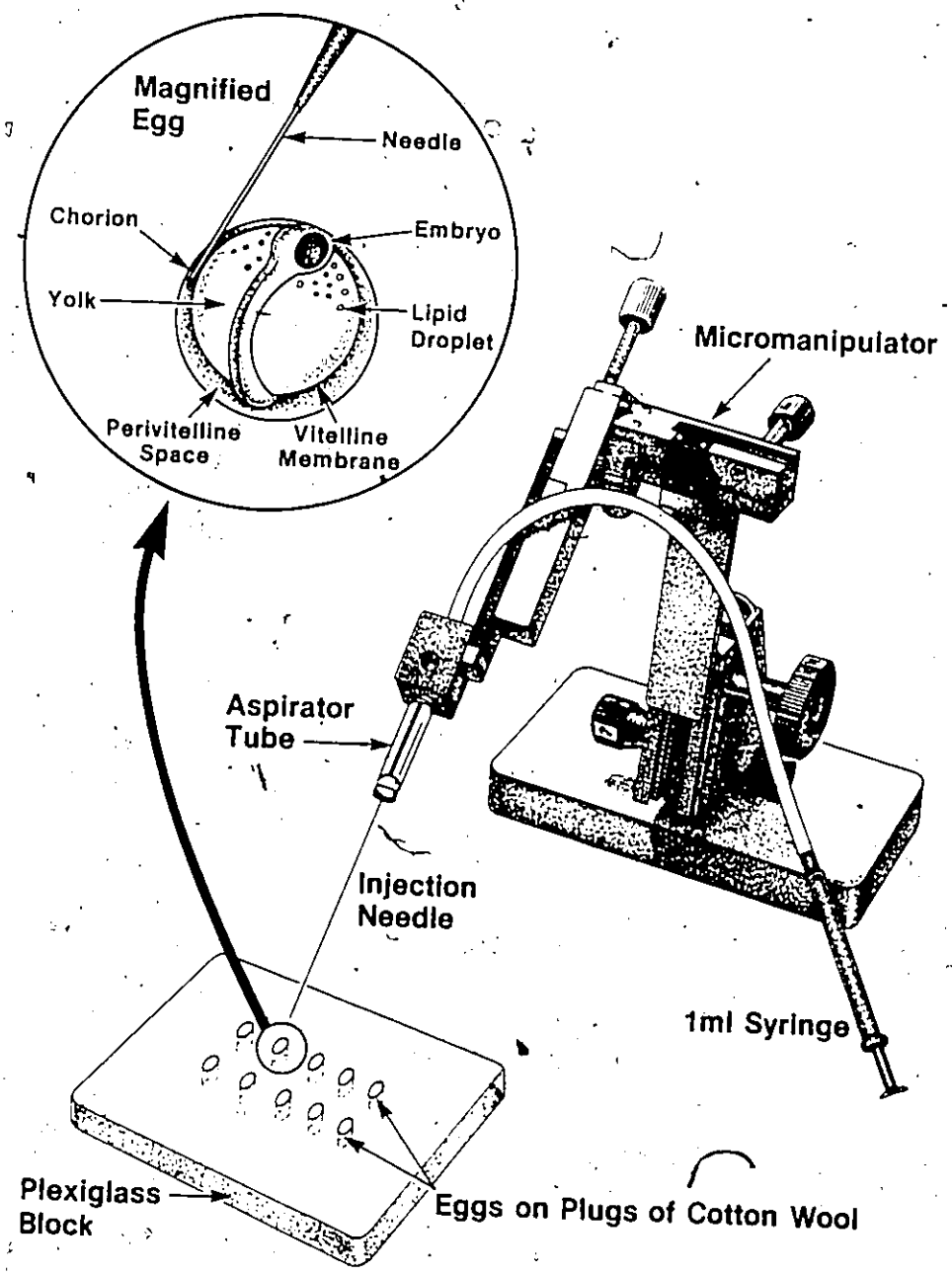
Rainbow trout (*Salmo gairdneri*) of the Kamloops strain were obtained from a commercial supplier at the "eyed" stage of development

(eye pigments visible). The embryos were incubated, without antibiotic treatment, for 20 days in 10°C well water before transfer to the laboratory. Embryos were then held in 6°C water to slow development over the week-long injection period.

Embryos were injected with solutions using graduated (5 x 1 ul) glass capillary micropipettes (Drummond Scientific Co.) with the tips drawn out over flame to a diameter of approximately 150 um. Needles were preloaded with injection solution by placing the needle in an aspirator tube (Drummond Scientific) attached to a 1 ml syringe. Solution was drawn up into the needle by suction produced by the syringe. For embryo injections, loaded needles were inserted into an aspirator tube fitted into a Pryor wormdrive micromanipulator (Fig. 1). Solutions were injected into the embryo by puncturing the chorion, and forcing a 0.5 ul aliquot into the perivitelline space of the embryo with pressure from a 1 ml syringe. A total of 10 embryos were treated with each loaded needle. Embryos were placed on dampened filter paper in a sealed container for 2 hr at 12°C to facilitate absorption of the test chemical prior to being returned to 12°C water. A total of 200 embryos were injected per treatment.

After injection, the embryos were raised in 12°C dechlorinated municipal water through hatching, "swim-up" (start of exogenous feeding after yolk absorption), and then raised for one or two years in 100 gal circular tanks at ambient water temperatures (8°-17°C). The trout were fed ad libitum commercial trout diets (Martin Feed Mills, Ontario).

Figure 1: Diagram of the apparatus used in embryo injection assays, and a magnified trout embryo showing the position of the needle at injection.


Magnified Egg
Needle
Chorion
Embryo
Yolk
Lipid Droplet
Perivitelline Space
Vitelline Membrane
Micromanipulator
Aspirator Tube
Injection Needle
1ml Syringe
Plexiglass Block
Eggs on Plugs of Cotton Wool

At the end of one year, trout were killed with an overdose of the anaesthetic, ethyl m-aminobenzoate (MS-222). The external surface of the fish, and the liver, spleen, kidney, and gastric caeca were examined for grossly visible neoplasms. In all treatments, the livers were dissected into 4 or 5 pieces, and fixed in Bouin's fixative and embedded in paraffin. The spleen, anterior, mid, and posterior kidney, and the gastric caeca from fish exposed to refinery effluent extracts were also prepared for histological examination.

In a histological survey of liver tissues, two sections (10 um each) were taken from the proximal face of each block and stained with haemotoxylin and eosin (H&E) for microscopic examination. The efficiency of the visual and histological survey methods for determining the frequency of hepatic neoplasms was evaluated by intensively examining liver tissues from one treatment. The liver tissues were sectioned at 250 um intervals ("step" sectioned) throughout the paraffin block (10-15 sections per block) and examined after H&E staining.

II) Statistical Analysis:

Comparisons of the frequency of hepatic lesions in trout treated with different injection solutions were analyzed as outlined by Armitage (1971), using Chi-square contingency analysis with a continuity correction. Comparisons were made between the incidence of neoplasms in fish injected with carcinogens and control solutions, between fish injected with carcinogens incubated with and without S-9,

and between fish exposed to aflatoxin with and without refinery effluent extracts.

III) Chemicals, Extracts, and Solutions:

Aflatoxin B_1, 2-anthramine (2-aminoanthracene), and 7,12-dimethyl- benzanthracene were purchased from Ultra Scientific Ltd. R. Gupta, McMaster University, supplied the MNNG. The anaesthetic, MS-222 was purchased from Sigma Chemical Co. (St. Louis, Mo.). Radiolabelled [^{3}H]-benzo[a]pyrene was purchased from New England Nuclear Ltd. Phosphate buffered saline (PBS) was prepared without calcium or magnesium (0.14 M NaCl, 8mM $Na_2HPO_4 .7H_2O$, 1.5 mM KH_2PO_4, 2.7 mM KCl). Rat-liver microsomes (S-9), and microsome-cofactor mix (S-9 mix) were prepared according to the methods reported by Ames et al (1975) and described in detail in Part 2 for the Salmonella -mammalian microsome mutagenicity assay.

Refinery extracts were prepared from two effluent samples collected at Refinery 1 in October, 1981, and June, 1982. These samples were tested for carcinogenicity in two separate in vivo assays carried out during 1981-82, and 1982-83, respectively. Effluents had passed through the full effluent treatment process, and were collected at the outlet to the refinery holding pond, just before the point of discharge into Lake Ontario. The particulate components of the extracts were extracted into methanol by the soxhlet extraction techniques described in Part 2. The non-volatile components dissolved

in the effluents were extracted by XAD-2 resin and diethyl ether elution according to the methods described in Part 2. All extracts were evaporated to dryness as outlined previously, dissolved in acetone, and stored in the dark in air tight septum vials at 4°C.

Injection solutions without S-9 consisted of the carcinogen or extract dissolved in 0.1 ml acetone, and 1.9 ml PBS. Solutions with S-9 consisted of the carcinogen dissolved in 0.1 ml acetone, and 0.5 ml of S-9 mix. This solution was incubated for 2 hr in the dark at 37°C, after which 1.4 ml of PBS were added. All injections were done under yellow light. Carcinogenic compounds injected into embryos included aflatoxin B_1, 2-anthramine, 7,12-dimethylbenzanthracene (DMBA), and MNNG. Soxhlet and XAD-2 extracts were injected into embryos, alone and in combination with aflatoxin B_1 or MNNG.

IV) Retention of Radiolabelled Benzo[a]pyrene in Embryos and Fry:

Embryos were injected as described previously with 0.5 ul aliquots of radiolabelled benzo[a]pyrene (BaP) solution. The [^{3}H]-BaP stock (5 mCi in 1 ml hexane) was cleaned-up by extracting three times with 0.25 M NaOH in 40% ethanol. The polar phase was discarded and the hexane phase evaporated to dryness. The residue was dissolved in 5 ml acetone to yield a stock solution of 1 mCi (150 ng) of [^{3}H]-BaP per ml of acetone. Injection solutions were prepared

by combining 1.0 mCi of radiolabelled BaP, and 5.0 mg of unlabelled BaP in 1.0 ml of acetone. This acetone solution (0.1 ml) was combined with 1.9 ml of PBS for injections without S-9, or incubated with 0.5 ml of S-9 mix for 2 hr and combined with 1.4 ml of PBS, as previously described for injections with S-9.

Embryos were also immersed in radiolabelled solutions by placing 200 embyos in 100 ml of water, to which had been added 0.1 mCi of [^{3}H]-BaP and 0.2 mg of unlabelled BaP dissolved in 0.1 ml acetone. After 2 hr, the embryos were removed from this solution and transferred to clean water.

Embyos were dissolved in 1 ml NCS tissue solubilizer (Amersham/Searle Co.), neutralized with 50 ul glacial acetic acid, and individually counted after addition of 10 ml of OCS liquid scintillation cocktail (Amersham/Searle Co.). The counting efficiency of embryos sham-injected with [^{3}H]-BaP and counted in the NCS/OCS matrix was 31%. Viable embryos (n=20) were sampled at 2, 24, 48, and 96 hr post-exposure, and one group of fry was sampled at 120 hr.

V) Ames Tests:

Effluent extracts were tested for mutagenicity by the Salmonella /mammalian microsome assay (Ames assay) according to the methods described in detail in Part 2 of this report. The effluent extracts were also tested for comutagenic activity with aflatoxin

B_1 and MNNG. The extract dose was calculated from the volume of original effluent which was extracted and concentrated to the 0.1 ml plating volume (ml equivalents per plate).

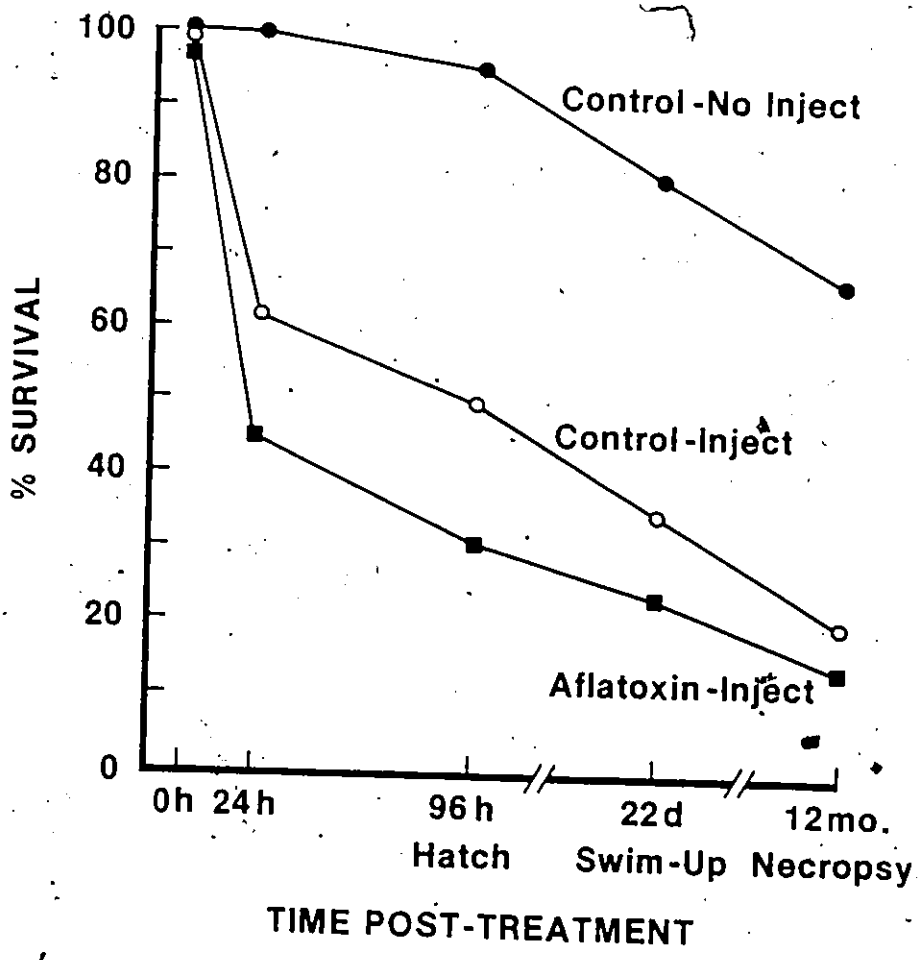
VI) Anaphase Aberrations:

As an in vivo assay for genotoxic activity, squash preparations of trout embryos were examined for anaphase aberrations. After injection with carcinogens and/or effluent extracts, embryos were fixed in 10% buffered formalin at 6, 15, 30, 48, and 96 hr post-injection. The chorion was removed, and the embryo dissected from the yolk. The embryo was placed in 70% acetic acid for 24 hr, and then transferred to 2% aceto-orcein stain with proprionic acid for 24 hr. Aceto-orcein stain was prepared by dissolving 2g of orcein in 45 ml glacial acetic acid, and diluting with 55 ml distilled water. The ratio of aceto-orcein to proprionic acid was 19:1.

The stained embryo was placed in acetic acid for 5 min to remove excess stain, and then teased into small pieces. The pieces of embryo were placed on a glass slide with a drop of acetic acid. The tissue was squashed by tapping the coverslip with the eraser-end of a pencil. The squashes were sealed with nail polish around the edges of the coverslip. Slides were scored for anaphase aberrations within 48 hr of preparation.

For each sample, 25 anaphases were scored from each of three

Figure 2: Survival to necropsy of rainbow trout injected with control solution and aflatoxin B_1 (13 ng; without S-9), in comparison to the survival of non-injected, control fish.


100
80
60
40
20
0
% SURVIVAL
Control-No Inject
Control-Inject
Aflatoxin-Inject
0h 24h
96h
Hatch
22d
Swim-Up
12mo.
Necropsy
TIME POST-TREATMENT

squash preparations (n=75). Anaphases were scored according to the criteria of Nichols et al. (1977) as normal, or containing acentric fragments, attached fragments, anaphase bridges, side-arm bridges, or multipolar bodies.

RESULTS

I) Embryo Injection and Fish Development:

The embryo injection procedure was sufficiently rapid that one person could inject 600 embryos per day. Because of the resilience of the chorion, very fine needles could not be used for injections. The solutions were injected into the perivitelline space because injections into the yolk quickly clogged the needle. The embryos appeared to imbibe water and took on a milky cast when placed in water after injection.

The majority of embryo mortalities occurred between injection and hatch; particularily within 24 hr post-injection (Fig. 2). Dead embryos turned a characteristic opaque, white colour, and there was

TABLE 1: Survival to hatch of rainbow trout embryos (n = 50) injected with 0.5 µl of test solutions.

Injection Solution	% Survival to Hatch
2.0 ml PBS	52
1.5 ml PBS, 0.5 ml S-9 mix	54
1.9 ml PBS, 0.1 ml acetone (5%)	52
1.4 ml PBS, 0.5 ml S-9 mix, 0.1 ml acetone (5%)	48
1.4 ml PBS, 0.5 ml S-9 mix, 0.1 ml DMSO (5%)	33
0.4 ml PBS, 0.5 ml S-9 mix, 0.1 ml acetone (10%)	37
Controls (uninjected)	94

often extrusion of yolk through the puncture wound in the chorion. Often this loss of yolk material seemed to be caused by violent movement of the embryo. Figure 2 indicates that a large percentage of the mortalities of both injected and uninjected embryos occurred at hatch and "swim-up". Many deaths at "swim-up" occur because fry do not begin proper exogenous feeding following yolk absorption. Mortalities between "swim-up" and necropsy (12 months) were low in all treatment groups, and were often a result of accidental death.

Acetone and dimethylsulfoxide (DMSO) were evaluated as solvents for injected compounds and extracts. A 5% solution of acetone in PBS was selected, since it induced the lowest mortalities between injection and hatch (Table 1).

In order to synchronize the developmental stage during carcinogen exposure, embryo development was retarded prior to injection by holding at 6°C. For example, eyed embryos held at 6°C hatched after 27 days, while embryos transferred to 12°C hatched within 8* days. No increase in mortalities or developmental anomalies were noted as a result of this treatment. Embryos received from the trout hatchery were at the "eyed" stage of development, when the embryo has pigmented eyes, a full complement of 60 somites, a beating heart, gill buds, and a liver bud (Knight, 1961). Development from this point is chiefly an increase in size.

The test concentrations of carcinogens were selected after preliminary lethality assays. Concentrations of 13 ng/egg aflatoxin B_1, 250 ng/embryo of DMBA, and 500 ng/embryo of 2-antnramine were

the calculated LC_{50} values over the period between injection and hatch (Appendix 10). The LC_{50}'s were calculated after subtracting control mortalities (50%) due to injection procedures. Table 2 lists the per cent survival of embryos to hatch after treatment of 200 embryos with carcinogens. Control mortalities approximated 50%, while 22 to 46% of embryos injected with carcinogens survived to hatch.

The uptake of [^{3}H]-BaP by embryos at 2 hr post-injection (Table 3) averaged 82% and 93% of the amount injected for solutions with and without S-9, respectively. In both cases the mean levels of BaP decreased slightly to 70 - 80% after hatching of the fry. The variability of counts among individual embryos was high, but appeared to decrease with time. Some of the variability was due to error in metering out the solutions, since standard deviations for sham-injections were 10% of the mean. Embryos immersed in BaP solutions retained relatively high levels of BaP, with low sample variability (Table 3). However, the large decrease in BaP concentrations after the immersed embryos hatched indicates that most of the compound was adsorbed to the chorion, and was not penetrating to the developing embryo. This is in marked contrast to the efficient retention of BaP by fry in the injection treatments.

Trout examined 12 months after injection were 20-25 cm long (fork length) and weighed between 100 and 200 gm. There was no clear indication of the effect of carcinogen exposure on fish growth, since growth was strongly affected by individual aggressiveness during feeding. Sex differences in response to carcinogens could not be

TABLE 2: Survival to hatch of rainbow trout embryos (n = 200) injected with carcinogens.

Injected Compound	Concentrations	% Survival to Hatch	
		Without S-9	With S-9
Aflatoxin B_1	25 ng/egg	22	28
	13 ng/egg	31	33
7,12-DMBA	250 ng/egg	23,28	32
2-anthramine	500 ng/egg	46,22	35
Control	-	56,48	54

TABLE 3: Retention of benzo[a]pyrene (BaP) by rainbow trout eggs and fry after exposure by injection and immersion procedures to solutions containing [^{3}H] benzo[a]pyrene. The mean and standard deviation of BaP concentrations are tabulated (n = 20).

a) Injection - Total BaP concentration = 125 ng/egg.

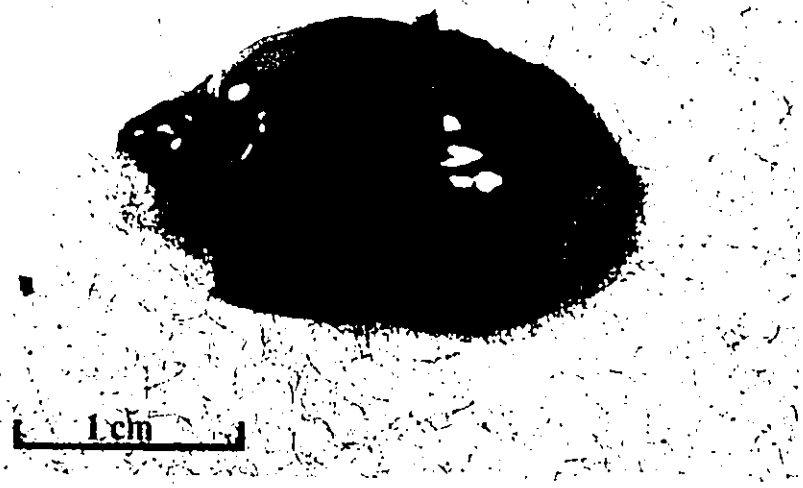
	Hr.	Concentration (ng/egg)			
		Without S-9		With S-9	
		Mean	S.D.	Mean	S.D.
Egg	2	103	± 33	116	± 26
	24	106	± 29	121	± 19
	48	81	± 27	109	± 23
	96	92	± 31	111	± 29
Fry	120	92	± 26	107	± 15

b) Immersion without S-9 - Total BaP concentration = 2 µg/ml.

	Hr.	Concentration (ng/egg)	
		Mean	S.D.
Egg	2	56	± 19
	24	49	± 5
	48	43	± 10
	96	48	± 5
Fry	120	9	± 3

Figure 3: A hepatic neoplasm grossly-visible on the liver (arrow) of a rainbow trout (x3 magnification) necropsied 12 mo after exposure by embryo injection to aflatoxin B_1 (13 ng with S-9).

Figure 4: A hepatic neoplasm grossly-visible on the liver (arrow) of a rainbow trout (x0.8 magnification) necropsied 24 mo after exposure by embryo injection to aflatoxin B_1 (13 ng) plus refinery extract (soxhlet).


1 cm

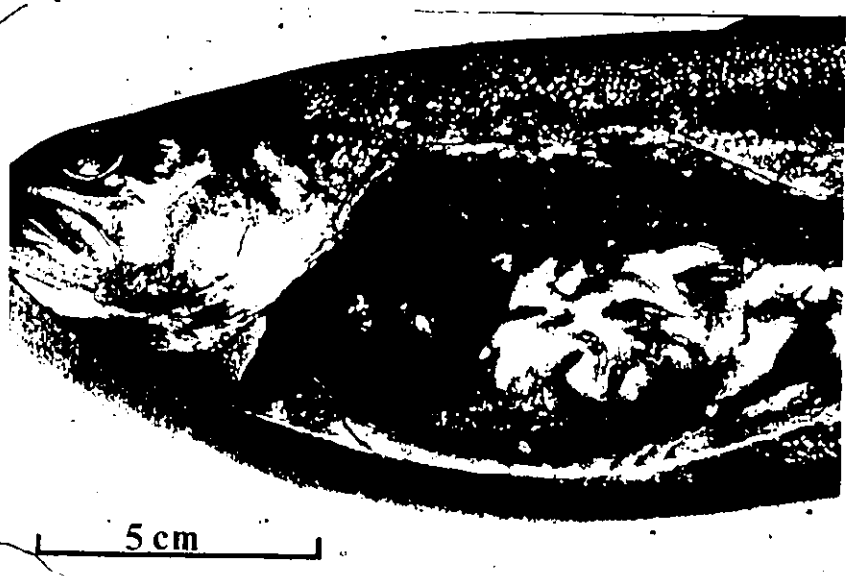
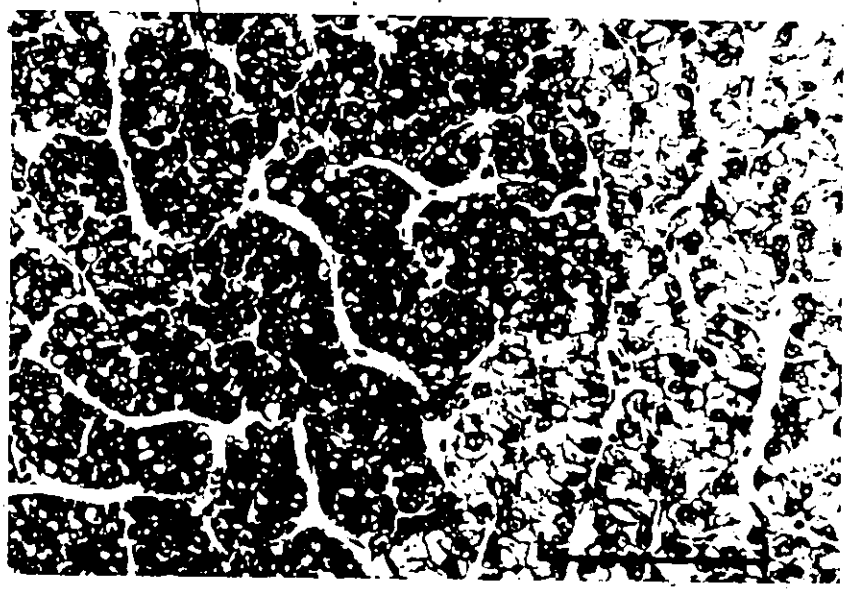

5 cm

Figure 5: High magnification photograph (x540) of a hepatocellular carcinoma in rainbow trout exposed to aflatoxin B_1 (25 ng, with S-9) by embryo injection. Normal liver parenchyma is situated on the right side of the figure, and the enlarged trabecular liver cords of the carcinoma are situated on the left (H&E staining).

Figure 6: Low magnification photograph (x62) of a hepatocellular carcinoma in a 1 yr-old rainbow trout exposed to aflatoxin B_1 (13 ng), showing the trabecular architecture of hepatic cords (H&E staining).



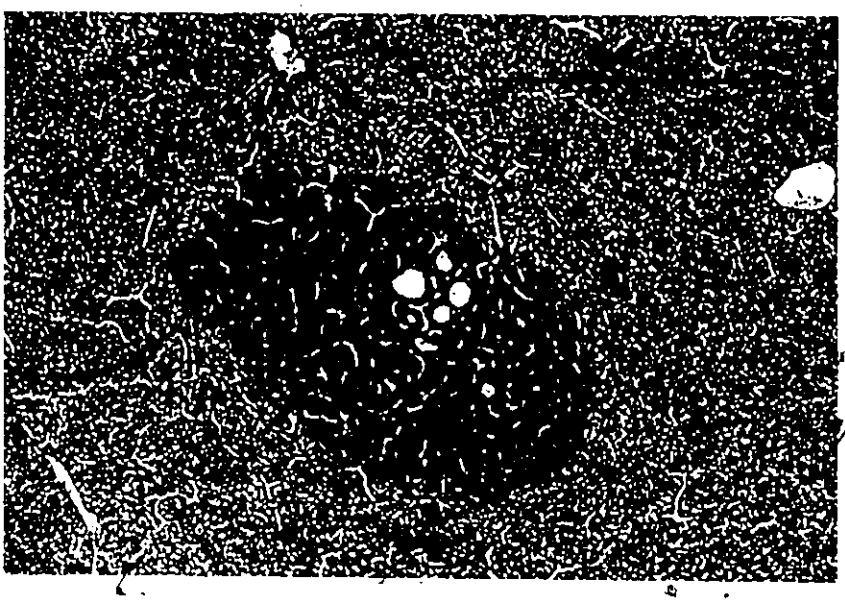
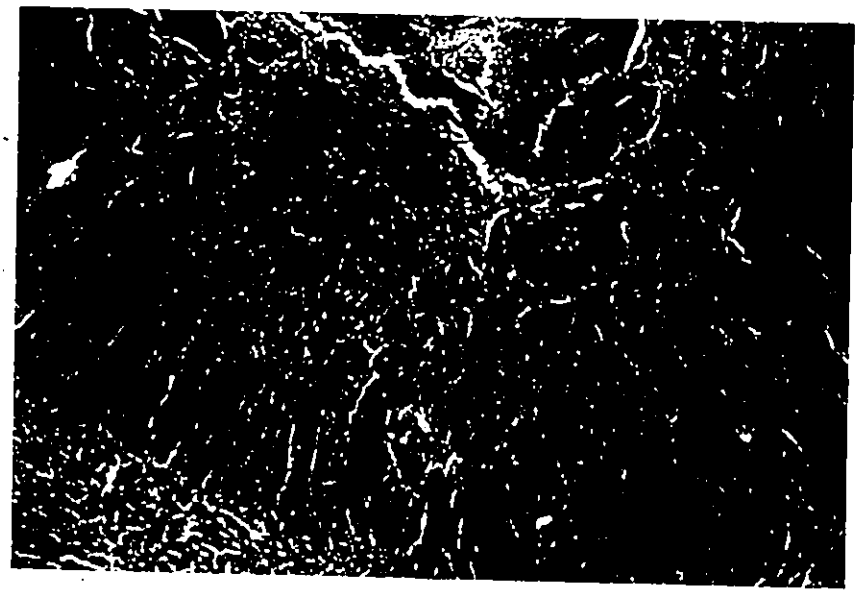


Figure 7: Low magnification photograph (x70) of a hepatocellular carcinoma in a 2 yr-old trout exposed to aflatoxin B_1 (13 ng) plus refinery extract (soxhlet), showing peripheral trabecular carcinoma tissue in the lower area, and a central fibrous stroma in the upper area of the photograph (H&E staining).

Figure 8: A low magnification photograph (x62) of a basophilic nodule in a rainbow trout necropsied 12 mo after exposure by embryo injection to 13 ng aflatoxin B_1 (H&E staining).



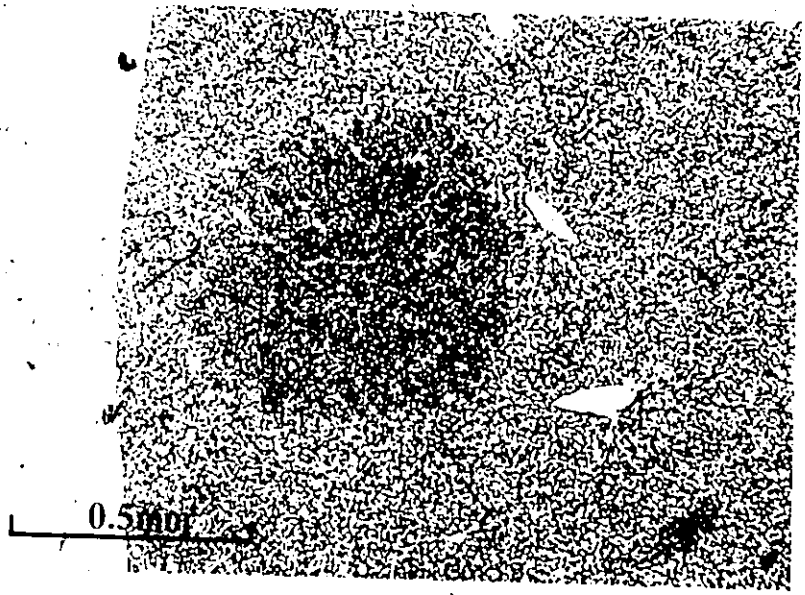
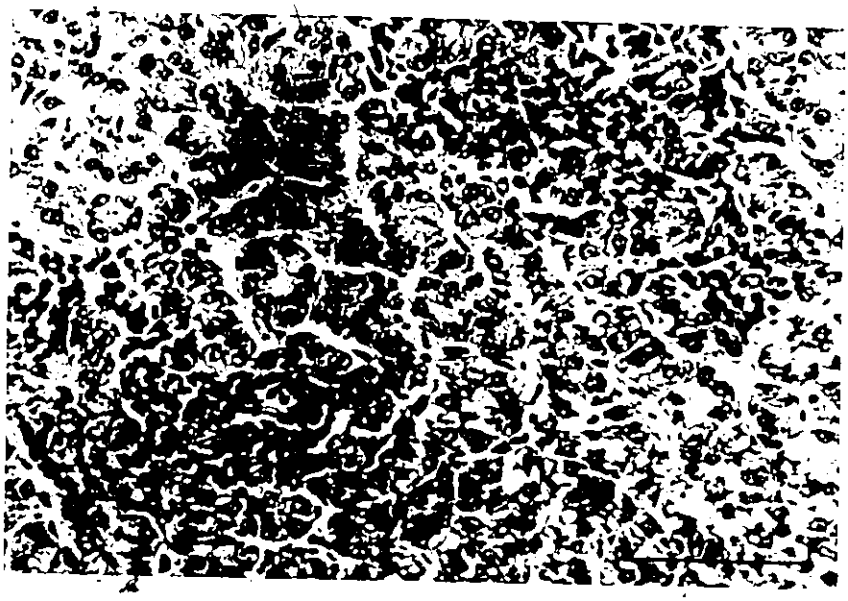

0.5mm

Figure 9: A high magnification photograph (x520) of an eosinophilic nodule in liver of rainbow trout necropsied 12 mo after exposure by embryo injection to DMBA (250 ng, with S-9). Enlarged eosinophilic cells are located on the upper left of the figure, and normal tissue on the lower right. Invading lymphocytes are present on the left and right portions of the nodule (H&E staining).



determined since only a small percentage (< 5%) of fish were sexually mature at necropsy. Trout examined 24 months after injection were 24-38 cm. long, and weighed over 300 gm.

II) Classification of Neoplasms:

The incidence of hepatic neoplasms was determined by gross examination at necropsy, and histologic examination of preserved tissues. No gross neoplasms were observed on the external surface, kidney, spleen, or gastric caecae of rainbow trout at necropsy. However, the round white lesions (1-6.5 mm diameter) observed on the liver surface of 1 yr-old fish (Fig. 3) closely resembled the small liver carcinomas reported by Sinnhuber et al (1977) for trout exposed to aflatoxin B_1. More than one gross lesion was rarely observed on a liver from fish necropsied 1 yr after embryo injection, but multiple surface lesions were common in fish examined after 2 yr. Surface neoplasms on 2 yr fish were often large (>1 cm), with a more vascularized appearance (Fig. 4).

The histological classification of hepatic neoplasms was based upon the criteria outlined by Sinnhuber et al(1977). Basophilic carcinomas were distinguished from preneoplastic basophilic nodules by increased hepatic cord width (>2 cells wide), compression of surrounding tissue, and increased mitoses (Fig. 5). Nuclear diameters

were not measured , since, in non-neoplastic trout, this parameter varies with sex and body weight in a manner independent of age (Simon et al, 1967).

Basophilic hepatocellular carcinomas (0.5-5.0 mm diameter) were observed histologically in liver sections prepared from fish held for 1 and 2 years. No cholangiomas were observed in these trout livers. The architecture of liver cords in carcinomas was usually distorted into a trabecular pattern (Figure 6), and in larger carcinomas (>3mm) there was often a sparse fibrous stroma near the center of the lesion (Fig. 7).

Basophilic nodules varied in size from islets of a few cells to nodules of up to 1.7 mm in diameter (Fig. 8). Several nodules were often present in one section, and nodules were occasionally found in the same section with basophilic carcinomas. Nodules were common in 1 yr trout, but only two were identified in fish necropsied after 2 yr.

Eosinophilic nodules (0.9-4.4 mm) were also present in liver sections. Within these nodules, cells were hypertrophic, although liver architecture was not altered markedly (Fig. 9). All eosinophilic nodules were characterized by the presence of lymphocytes. A variety of other pathological changes were observed in liver sections; including "fatty liver", foci of invading lymphocytes, and areas of cellular hypertrophy and necrosis. No hepatic carcinomas, basophilic nodules, or eosinophilic nodules were observed grossly or histologically in any of the control fish examined in these experiments (n=105).

III) Incidence of Hepatic Neoplasms:

a) Exposure to known carcinogens:

The incidence of liver neoplasms in fish exposed to aflatoxin B_1, 2-anthramine, and DMBA are presented in Table 4 under the headings of lesions observed in an initial visual survey, and those observed in a subsequent histological survey. Fish were visually surveyed at necropsy for gross liver neoplasms, and the fixed liver tissues were later examined histologically (2 sections per tissue block) for nodules and carcinomas. In some cases, the histological survey did not confirm the presence of grossly visible neoplasms (e.g. treatment with 2-anthramine without S-9), but subsequent serial sectioning of these blocks confirmed the presence of carcinomas in all of these cases. These lesions are tabulated as grossly visible neoplasms, but are not included as positives in the histological survey (Table 4).

Fish exposed to 25 and 13 ng per embryo of aflatoxin B_1, had a low incidence of grossly visible hepatic lesions, and histologically visible basophilic nodules and carcinomas (Table 4). In fish exposed to DMBA, hepatic carcinomas were observed grossly and

TABLE 4: Incidence of hepatic lesions in trout injected in the egg stage with carcinogens (with and without microsomal activation with S-9), as determined by a visual survey for gross lesions and a histological survey for nodules and carcinomas.

Treatment	Visual Survey	Histological Survey	
	Nos. Neoplasms	Nos. Nodules	Nos. Carcinomas
a) Injection:			
Aflatoxin - 25 ng:			
with S-9	5/20[a]	Baso. 4/20[b]	5/20[a]
without S-9	3/18[b]	Baso. 0/18	2/18[b]
Aflatoxin - 13 ng:			
with S-9	1/24	Baso. 3/24[b]	3/24[b]
without S-9	1/28	Baso. 3/28[b]	3/28[b]
DMBA - 500 ng:			
with S-9	2/32	Eosin. 2/32	1/32
without S-9	1/28	Eosin. 1/28	0/28
2-Anthramine - 250 ng:			
with S-9	0/21	0/21	0/21
without S-9	1/25	0/25	0/25
Control:			
with S-9	0/25	0/25	0/25
without S-9	0/25	0/25	0/25
b) Immersion:			
Aflatoxin-0.5 µg/ml	4/25[a]	Baso. 5/25[a]	2/25[b]

a) Frequency significantly different from controls at χ^2 (0.01)
b) Frequency significantly different from controls at χ^2 (0.10)

histologically , but liver nodules were all eosinophilic. These nodules contained foci of lymphocytes, and there were also numerous lymphocytic foci in the surrounding parenchyma. In the fish exposed to 2-anthramine, a single specimen exhibited a grossly visible lesion. The initial histological survey did not confirm the presence of this gross lesion, but subsequent serial sectioning of the block confirmed the presence of a small basophilic carcinoma (1.2 mm).

It should be emphasized that none of the control fish, injected as embryos with PBS solutions and solvent, contained grossly visible or histologically visible hepatic neoplasms (Table 4). Furthermore, livers sections from 25 of these control fish were later step sectioned, and no carcinomas or preneoplastic nodules were observed in these tissues.

At the high dose of aflatoxin B_1 (25 ng), Chi-square tests indicated that the incidence of histologically visible carcinomas in treatments with S-9 was statistically elevated ($X^2_{0.10}$), but at the lower dose (13 ng) there was no difference between treatments with and without S-9 (Table 4). In tests with DMBA, the incidence of carcinomas was slightly higher with S-9 treatment , but this was not statistically significant. Aflatoxin B_1, DMBA, and 2-anthramine were tested in the Ames mutagenesis assay using a modified protocol which mimicked the S-9 pre-incubation procedure used for embryo injections. The carcinogens were incubated with S-9 mix for 2 hr at 37°C. After addition of 0.1 ml of bacterial culture and 2 ml of top agar, the mixture was plated as

TABLE 5: Revertants per plate in Salmonella/mammalian microsome mutagenicity assay using normal test protocol, and a test protocol mimicking the egg injection pre-incubation step with rat S-9. Aflatoxin was tested with Salmonella strain TA100, and DMBA and 2-anthramine were tested with strain TA98.

Treatment	Mean Revertants per Plate	
	Normal Protocol	Pre-Incubation
Spontaneous Revertants:		
TA98	44	39
TA100	121	115
Aflatoxin (0.5 µg)	625	1017*
DMBA (5 µg)	405	92*
2-anthramine (10 µg)	346	238*

*Mean revertants per plate in pre-incubation tests significantly different from normal protocol tests ($t_{0.05}$).

described by Ames et al (1975). The numbers of revertants induced by the compounds after 2 hr pre-incubation with S-9 were compared to the revertants induced in the normal assay with S-9. The pre-incubation steps with S-9 increased the mutagenicity of aflatoxin B_1 (0.5 ug/plate), but decreased the mutagenicity of DMBA and 2-anthramine (Table 5).

(b) Evaluation of survey methods for neoplasm incidence:

In order to evaluate the carcinogenic activity of compounds or extracts in the embryo injection assay, the incidence of hepatic neoplasms must be determined accurately. Table 6 lists the incidence of: (1) nodules only; and (2) carcinomas (or carcinomas plus nodules) observed following histologic examination of step sections. These liver tissues were prepared from fish exposed by embryo injection to 13 ng aflatoxin B_1 plus soxhlet extract (with S-9), and necropsied at 1 yr (n=50), or 2 yr (n=16). If step section data represents the true incidence of hepatic neoplasms in this group of fish, then the numbers of nodules and carcinomas observed by the histological survey (2 sections per block) considerably underestimates the incidence of both types of lesions (Table 6). The visual survey detects a large percentage of hepatic carcinomas, but does not detect any of the preneoplastic nodules. A combination of visual and histologic surveys gives an accurate indication of the carcinoma incidence, but the

TABLE 6: Incidence of hepatic neoplasms in rainbow trout determined by histologic examination of "step" sections (at 250 µm intervals) of liver tissue, in comparison to the incidence of neoplasms determined in the same tissues by visual and histologic surveys. Fish had been exposed by embryo microinjection to aflatoxin B_1 (13 µg) plus refinery soxhlet extract (100 µl. equivalents) and held for 1 or 2 years before necropsy.

Survey Method	Neoplasm Incidence	
	Nodules Only	Carcinomas
1) 1 yr. Necropsy:		
Step Sectioning	26	15
Histological	11	6
Visual	-	11
Visual plus histological	11	14
2) 2 yr. Necropsy:		
Step Sectioning	1	14
Histological	2	7
Visual	-	11
Visual plus histological	1	13

incidence of nodules cannot be accurately determined. However, it is essential that the neoplasms observed visually are confirmed as carcinomas by subsequent histological examination.

The relative accuracy of the visual survey suggests that a large proportion of the hepatic carcinomas induced by this treatment are located near the liver surface. The histological survey is surprisingly poor at detecting hepatic neoplasms. Since many of the carcinomas are at the liver surface, the orientation of tissue in the block obviously affects the accuracy of the histological survey. Since the mean diameter of carcinomas, as determined by step sectioning, is 1.8 mm for 1 year fish, and 2.7 mm for 2 year fish, there is a relatively low probability of detecting a carcinoma in 2 slides from a block of liver tissue (approximately 0.3 cm thick). A combination of visual and histological observations is an accurate indication of the incidence of hepatic carcinomas.

Between the 1 and 2 yr necropsy , the incidence of carcinomas increased, the incidence of nodules declined, and the total incidence of hepatic lesions remained relatively constant (Table 6). This suggests that many of the preneoplastic nodules progressed to the carcinoma stage in the interval.

(c) Exposure to effluent extracts:

The carcinogenicity of effluents was evaluated by the embryo

TABLE 7: Incidence of grossly visible, and histologically visible hepatic lesions in rainbow trout injected at the egg state with aflatoxin B_1 (13 ng/egg), and aflatoxin (13 ng/egg) plus soxhlet and XAD-2 extracts (100 µl equivalents/egg) from an October, 1981 effluent sample (Refinery 1).

Treatment	Number Gross Neoplasms	Number Histologic Lesions		Total Carcinomas (Gross plus Histologic)	
		Nodules	Carcinomas	Number	%
a) With S-9 Preincubation:					
Aflatoxin	1/24	3/24	3/24	4/24	17
Aflatoxin + Soxhlet	7/25	9/25	3/25	9/25	36*
Aflatoxin + XAD-2	6/39	4/39	4/39	10/39	26*
b) Without S-9 Preincubation:					
Aflatoxin	1/28	3/28	3/28	4/28	14
Aflatoxin + Soxhlet	4/25	4/25	3/25	7/25	28*
Aflatoxin + XAD-2	4/27	2/27	1/27	5/27	19

*Frequency significantly different [$\chi^2_{(0.10)}$] from treatment with aflatoxin alone.

TABLE 8: Incidence of grossly visible, and histologically visible hepatic lesions in rainbow trout injected at the egg state with aflatoxin B_1 (13 ng, 25 ng), MNNG (250 ng), or these compounds plus soxhlet extract (100 µl equivalents) from a June, 1982 effluent sample (Refinery 1).

Treatment	Number Gross Neoplasms	Number Histologic Lesions		Total Carcinomas (Gross plus Histologic)	
		Nodules	Carcinomas	Number	%
a) With S-9 Preincubation:					
Aflatoxin (13 ng)	7/27	4/27	5/27	11/27	41
Aflatoxin + soxhlet	8/25	2/25	7/25	14/25	56*
Aflatoxin (25 ng)	10/20	1/20	4/20	14/20	70
Aflatoxin + soxhlet	10/18	3/18	5/18	14/18	78
b) Without S-9 Preincubation:					
Aflatoxin (13 ng)	5/20	2/20	2/20	7/20	35
Aflatoxin + soxhlet	6/21	1/21	3/21	9/21	43
MNNG	4/35	1/35	1/35	4/35	11
MNNG + soxhlet	2/34	3/34	2/34	2/34	6

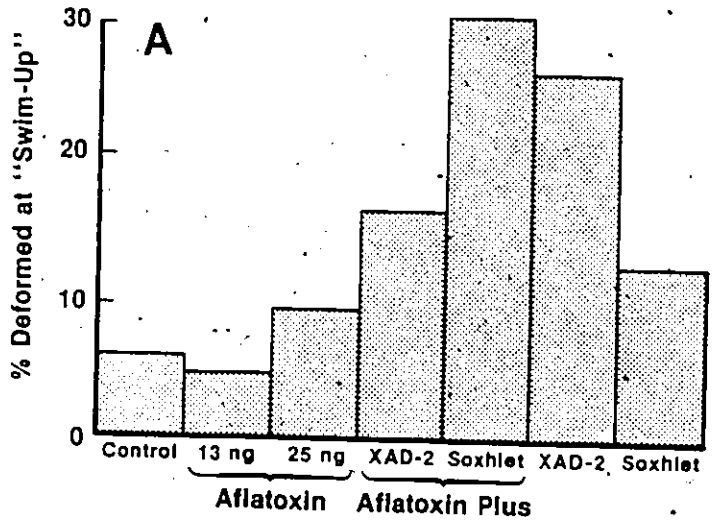
*Frequency significantly different [$\chi^2_{(0.10)}$] from treatment with aflatoxin alone.

injection assay in two separate trials using extracts prepared from samples taken in October, 1981, and June, 1982. No grossly or histologically visible hepatic neoplasms were observed in fish (n=30 per treatment) injected with dissolved (XAD-2 extract) or particulate (soxhlet extract) extracts prepared from an October, 1981 refinery effluent with and without S-9 pre-incubation. Kidney, spleen, and pyloric caeca tissues from these fish were also examined histologically (2 sections per block), and no neoplastic or preneoplastic lesions were observed. The injection concentration, which was equivalent to 200 ul of effluent concentrated to the injection volume of 0.5 ul, was selected in preliminary lethality tests as half of the 96 hr LC_{50} (Appendix 10). The 200 ul equivalents corresponded to a residue weight injected into each embryo of 6 ug of soxhlet extract and 4 ug of XAD-2 extract. In the second trial, no hepatic neoplasms were observed (n=25 per treatment) in fish exposed to 200 ul equivalents of particulate extracts from a June, 1982 effluent sample prepared with or without S-9 pre-incubation. There was less residue material in this soxhlet extract, so the amount injected into each embryo was 4.5 ug.

However, when trout were injected with 13 ng of aflatoxin B_1 plus 100 ul equivalents of refinery extracts (October, 1981) there appeared to be a higher incidences of hepatic neoplasms than in fish exposed to aflatoxin B_1 alone. This response was analyzed by comparing the incidence of hepatic carcinomas in the various treatments, as determined by visual plus histologic (2 sections per

Figure 10: Incidence among rainbow trout fry at "swim-up" of skeletal deformities following injection of: a) aflatoxin B_1; October, 1981 effluent extracts from Refinery 1 (soxhlet, XAD-2); or combined aflatoxin plus extract.

b) aflatoxin B_1; MNNG; June, 1982 effluent extract from Refinery 1 (soxhlet); or the carcinogens combined with extract.


A
% Deformed at "Swim-Up"
30
20
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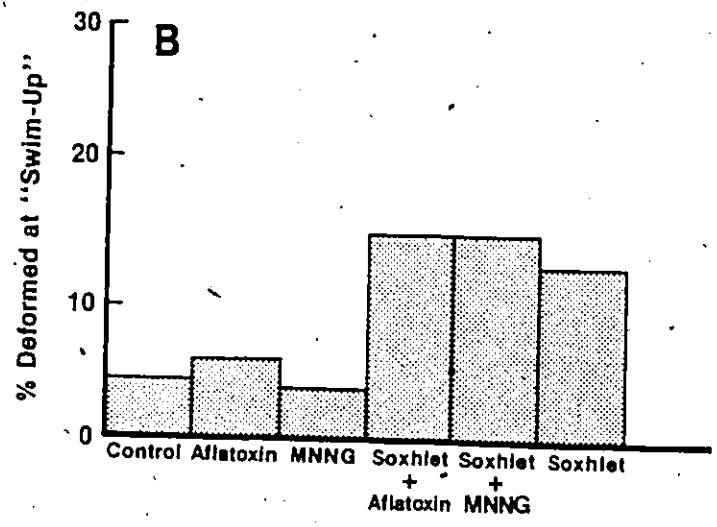
Control
13 ng
25 ng
XAD-2
Soxhlet
XAD-2
Soxhlet
Aflatoxin
Aflatoxin Plus


B
% Deformed at "Swim-Up"
30
20
10
0
Control
Aflatoxin
MNNG
Soxhlet + Aflatoxin
Soxhlet + MNNG
Soxhlet

block) surveys. All unconfirmed, grossly visible carcinomas were confirmed by subsequent step sectioning. There were statistically elevated numbers of hepatic carcinomas in fish exposed to aflatoxin B_1 plus refinery extracts (Table 7). This effect was most pronounced among fish injected with aflatoxin B^1 plus soxhlet extract. The incidence of carcinomas was higher among all treatments when injection solutions were pre-incubated with S-9.

In a series of experiments designed to re-test the observed synergistic effect of effluent extracts, fish exposed by embryo injection to 13 ng aflatoxin B_1 plus a June, 1982 soxhlet extract (100 ul equivalents) had a higher incidence of carcinomas than fish exposed to aflatoxin alone (Table 8). These differences were statistically significant only in treatments with S-9 pre-incubation. There was also a slight (non-significant) increase in carcinoma incidence among fish exposed to 25 ng aflatoxin plus soxhlet extract (with S-9 pre-incubation). Conversely, when soxhlet extracts were injected in combination with MNNG, the incidence of tumors in trout seemed to decrease, but this response was not statistically significant (Table 8).

In the carcinogenesis assays using refinery extracts, it was observed that there was an increase in the incidence of "deformed" fish. These deformities were various curvatures of the spine (scoliosis, lordosis, kyphosis) commonly found in low frequencies among hatchery-reared rainbow trout (Mauck et al, 1978). Elevations in the frequency of spinal curvatures were observed when embryos were

injected with XAD-2 or soxhlet effluent extracts; both alone, or in combination with carcinogens (Fig. 10). This occurred with injections of both October, 1981 effluent extracts (Fig. 10a), and with June, 1982 effluent extracts (Fig. 10b), but it is important to note that spinal deformities were only elevated in treatments without pre-incubation of extracts with S-9. When samples were incubated with S-9, the incidence of curvatures was the same as control treatments.

When the effluent extracts which were tested for carcinogenicity were tested in the Ames assay, none of the extracts were appreciably mutagenic (Table 9). Activation with S-9 was required for a mutagenic response, but the number of revertants per plate was not double the spontaneous levels at any of the extract doses. The October, 1981 soxhlet extract gave a slightly greater response than the June, 1982 soxhlet extract. When effluent samples were plated with aflatoxin B_1 (0.1 ug) or MNNG (0.5 ug), the extracts showed no comutagenic activity (Table 10). In fact, the effluent extracts decreased the mutagenicity of both compounds.

Table 11 summarizes the anaphase aberration data for embryos injected with aflatoxin B1, or aflatoxin B1 plus the June, 1982 soxhlet extract. Among embryos injected with mitomycin C (positive control), there was a large increase in aberrations at 15 h, followed by a toxic response, as indicated by the low mitotic index. In comparison to controls, embryos injected with aflatoxin B1, with or without soxhlet extract, had a slightly elevated incidence of anaphase aberrations at 30 to 48 hr post-injection. However, there

TABLE 9: Mutagenicity of extracts (soxhlet and XAD-2) from Refinery 1 effluents (October, 1981; June, 1982) used in tests for carcinogenicity by the embryo injection assay. Ames assays were run with TA98 (XAD-2 extracts) and TA100 (soxhlet extracts) with microsomal activation (S-9). SR refers to spontaneous revertants.

Sample	Ml Equiv./Plate	Mean Rev./Plate
a) October, 1981:		
Soxhlet -	320	184
	160	164
	80	141
	40	135
	0(SR)	110
XAD-2 -	80	76
	40	62
	20	65
	10	57
	0(SR)	40
b) June, 1982:		
Soxhlet -	320	144
	160	134
	80	132
	40	121
	0(SR)	105

TABLE 10: Results of tests for comutagenic activity of effluent extracts (soxhlet and XAD-2) in the Ames test with aflatoxin B_1 (0.1 µg/plate) or MNNG (0.5 µg/plate). Strain TA100 was used in the Ames test, with S-9 (aflatoxin B_1) and without S-9 (MNNG). Spontaneous revertants were 101 and 112 for tests with and without S-9, respectively.

Effluent Sample	Mutagen	Effluent Conc. (Mℓ. Equiv./Plate)	Mean Rev./Plate
a) October, 1981:			
Soxhlet	Aflatoxin B_1	320	412
		160	517
		80	704
		0	690
XAD-2	Aflatoxin B_1	80	503
		40	682
		20	713
		0	709
b) June, 1982:			
Soxhlet	Aflatoxin B_1	320	519
		160	753
		80	764
		0	811
	MNNG	320	912
		160	1072
		80	923
		0	1104

Table 11: Incidence of anaphase aberrations in trout embryos injected with mitomycin C, aflatoxin B_1, or aflatoxin B_1 plus June, 1982 soxhlet extract from Refinery 1 (100 ul equivalents per embryo).

Treatment	Number of Aberrations				
	6 hr	15 hr	30 hr	48 hr	96 hr
Mitomycin C (100 ng)	22/75	41/75	6/21	*	*
Aflatoxin B_1 (25 ng)					
-without S-9	4/75	2/75	12/75	5/75	7/75
Aflatoxin B_1 (13 ng)					
-without S-9	-	5/75	7/75	10/75	6/75
-with S-9	-	8/75	4/75	10/75	7/75
Aflatoxin (13 ng)/Soxhlet					
-without S-9	-	2/75	6/75	7/75	4/75
-with S-9	-	4/75	9/75	6/75	5/75
Control					
-without S-9	-	5/75	3/75	3/75	4/75
-with S-9	-	3/75	4/75	2/75	5/75

* Low mitotic index.

was no obvious difference between the aberration incidence for embryos injected with aflatoxin B_1 alone, and aflatoxin plus extract; either with or without S-9 preincubation. It appears that effluent extracts did not increase the genotoxic activity of aflatoxin B_1. However, the incidence of anaphase aberrations is too close to control levels to state this definately.

DISCUSSION

These studies demonstrate that a single microinjection of nanogram quantities of aflatoxin B_1, MNNG, DMBA, and 2-anthramine into rainbow trout embryos induced liver neoplasms after 1 yr. The liver histopathology of the aflatoxin B_1 injected embryos is similar to the liver neoplasms described for rainbow trout exposed to aflatoxin B_1 in diet (Sinnhuber et al, 1977) and by embryo immersion (Hendricks, 1981). It could be argued that the low incidence of hepatic carcinomas in fish exposed to 2-anthramine does not represent a positive response. However, because of the rarity of spontaneous hepatic neoplasms among rainbow trout (Hendricks, 1982), the one carcinoma observed was probably a consequence of exposure to

2-anthramine.

The Shasta strain of rainbow trout used in studies by Sinnhuber and co-workers (Wales et al, 1978; Hendricks et al, 1980a) has been shown to be very sensitive to aflatoxin B_1 induced carcinogenesis. When Shasta strain trout were immersed in aflatoxin B_1 (0.5 ug/ml) at several stages of embryo development, approximately 50% to 70% of fish examined after one year had grossly visible liver neoplasms. When we repeated the embryo immersion protocol with the Kamloops strain, the incidence of grossly visible neoplasms was 16% (Table 4). The optimal period for exposure of embryos to aflatoxin B_1 was 4 days prior to hatch (Hendricks et al, 1980a), whereas in this test, embryos were injected at eight days before hatch. The sensitivity of the embryo injection assay may be improved by using the Shasta strain of rainbow trout, and by injecting at a later stage of embryo development.

The microinjection bioassay offers several advantages for in vivo carcinogenesis testing. In comparison to the trout embryo immersion protocol, embryo injection facilitates the testing of compounds which may not penetrate the chorion, or are not water-soluble. In comparison to rodents, extremely small quantities of material can be used to perform a bioassay (ng per animal). These quantities compare favourably to the amounts of material used in in vitro genotoxicity assays (e.g. Ames test).

The suitability of embryo injection as a carcinogenesis bioassay depends upon the similarities in response of fish, laboratory

rodents, and man to carcinogen exposure. The relative carcinogenicity of aflatoxin B_1, DMBA, and 2-anthramine in the embryo injection assay decreased in that order. This agrees with the relative carcinogenicity of these compounds in skin-painting assays with rodent species (Lennox, 1955; Shubick et al, 1960; Dickens and Jones, 1965). MNNG is a potent rodent carcinogen, but produced only a low incidence (6-11%) of hepatic carcinomas in the embryo injection assay. Low mortalities in MNNG experiments (41% survival to hatch) indicate that the dose may have been too low.

The enzyme activation potential of fish may differ significantly from rodents, although similar metabolic activation processes have been observed for a wide range of compounds (Stott and Sinnhuber, 1978; Chambers and Yarbrough, 1976; Thornton et al, 1982; Ahokas et al, 1975). The practicality of using exogenous rat-liver microsomal activation in conjunction with the egg injection assay requires further testing, although there is evidence that aflatoxin B_1 carcinogenesis is enhanced by S-9 pre-incubation. There is potential for pre-incubating injection solutions with microsomal preparations from human livers.

Except for fish exposed to refinery effluent extracts, kidney, spleen, and gastric caeca tissues were not examined for histological evidence of neoplasia. Liver tissues were processed because hepatic lesions were grossly visible, and because studies indicate that the liver is the predominant target organ in rainbow trout exposed to a variety of chemical carcinogens (Hendricks, 1981; Kligerman, 1982). A

statistical analysis of the survey methods used to determine the incidence of neoplasms in trout liver indicates that, while histological observations of preneoplastic nodules are indicators of carcinogenic activity, combined tabulations of visually and histologically visible carcinomas are the most accurate data for quantitative comparisons. These data were used to assess the carcinogenic effects of refinery effluent extracts.

The refinery effluent extracts tested in the trout embryo model were not carcinogenic. These results are supported by in vitro Ames bacterial mutagenicity tests in which these same extracts gave only a weak response. In two experiments, the injection of effluent extracts in combination with aflatoxin B_1 increased the incidence of aflatoxin-initiated hepatic tumors (13 ng). This effect was most pronounced in treatments with S-9 pre-incubation. Although the tumor-enhancing effect of extracts was less pronounced in the second experiment, the higher overall incidence of hepatic neoplasms in this test (Table 8) may be due to the more advanced development of embryos at injection (4 days prior to hatch).

It is interesting to note that the effluent extracts were not comutagenic in the Ames test. The mutagenicity of both aflatoxin B_1 and MNNG was decreased in the Ames assay when tested with the effluent extract. It was previously shown in Part 2 of this report (Table 11) that the June, 1982 soxhlet and XAD-2 extracts from Refinery 1 were not comutagenic with BaP or 2-acetylaminofluorene; and, in fact, reduced the activity of these mutagens. Petrilli et al

(1980) attributed the reduced mutagenicity of BaP in combination with crude oil to mechanical trapping by the oil hydrocarbons. Haugen and Peake (1983) reported that aromatic hydrocarbons isolated from coal-derived oil reduced the activity of indirect-acting mutagens, but not of direct-acting mutagens. In this case, oil hydrocarbons were thought to bind to microsomal cytochrome P450 and inhibit metabolic activation processes.

The enhancing effect of extracts upon tumorigenesis in trout suggests that non-volatile effluent components have co-carcinogenic activity. Co-carcinogens are agents that are not themselves carcinogenic, but act to increase the overall carcinogenic process when administered at the same time as carcinogens (Weisburger and Williams, 1980). Criteria for accelerated carcinogenesis include increased tumor incidence or numbers of tumors per individual and accelerated appearance of tumors. Only increases in tumor incidence could be adequately evaluated in these studies since investigations of accelerated tumor appearance involve sampling of animals with time.

Co-carcinogenic agents in the mouse skin-painting assay include mineral oil, pyrene, fluoranthene, benzo[e]pyrene, resorcinol, n-dodecane, and phorbol myristate acetate (Van Duuren, 1976). The latter two compounds also have tumor promoting ability. The mechanisms of co-carcinogenic activity are unresolved, but they may include one or more of several possibilities (Weisburger and Williams, 1980):

1.) The co-carcinogenic agent alters metabolic processes to increase

the levels of the active ultimate carcinogenic metabolite, or to decrease the detoxification process.

2.) The co-carcinogens may increase non-specifically or specifically the growth of cells with an altered neoplastic genotype.

An interesting feature of the bioassays using refinery effluent extracts was an increase in the incidence of spinal curvatures among fish exposed to extracts from both the October, 1981 effluent and June, 1982 effluent. This occured after exposure to extracts alone, or in combination with aflatoxin or MNNG, but was noticeable only among treatments without pre-incubation with S-9. Sprague et al (1978) noted similar spinal curvatures among the offspring of flagfish held in diluted refinery effluents. Spinal deformities are seen in high frequencies among fish exposed in embryo and fry stages to cadmium (Bengtsson et al, 1975), organophosphate insecticides (Wildish et al, 1971), and organochlorines (Mauck et al, 1978). With the latter class of compounds, spinal deformities have been related to increased use of vitamin C for synthesis of hepatic detoxification enzymes, and a resultant deficiency of collagen synthesis in the backbone.

The increased incidence of spinal deformities suggests that there is induction of hepatic detoxification enzymes in the embryos and fry of trout exposed to refinery effluent extracts. Addition of rat S-9 to the extracts reduced the number of deformities to control levels; presumably as a result of enzymatic detoxification. It is possible that the mechanism for the co-carcinogenic activity of

effluent extracts is through alteration of the metabolism of aflatoxin B_1. Reports of the induction of hepatic metabolic activity (MFO) in fish exposed to oil refinery wastewaters *in situ* (Ridlington et al, 1982) further support this hypothesis.

The co-carcinogenic activity of the refinery effluents was not observed with a direct-acting carcinogen (MNNG), but was expressed with a compound requiring activation to a carcinogenic metabolite (aflatoxin B_1). This again indicates a metabolic mechanism for co-carcinogenesis, as opposed to an enhancement of the growth of tumor cells. Purely physical factors, such as effluent extracts acting as a surfactant for aflatoxin B_1 in the injection solution, may also explain these results. However, in the Ames assay, the effluent extracts reduced, rather than increased the mutagenic activity of aflatoxin B_1. Further studies are required to define the mechanism of co-carcinogenesis.

PART 4: CONCLUSIONS

It can be concluded from this study that organic contaminants released by petroleum refineries in liquid effluents are not carcinogenic in the trout embryo injection assay. Compounds associated with the particulate components of some refinery effluents show weak mutagenic (Ames test) and clastogenic (SCE) activity. The discharge of this material may constitute a public health hazard only if long-term accumulation of the active agent(s) in water resources led to the appearance of high concentrations of the material in drinking water. The release of mutagenic material from refineries may be reduced by improving effluent treatment procedures for the removal of particulates. Levels of oil and grease in effluents may be a useful water quality parameter for indicating potential genotoxicity associated with suspended particulates.

Chlorination of refinery effluent residues increases the genotoxic activity of this material. It is unknown whether the appearance of low concentrations of effluent residues in drinking water treatment facilities would constitute a health risk. However,

these results do indicate that refinery effluents should be regarded as potential reactants with chlorine for the production of genotoxic/carcinogenic compounds in drinking water. Pragmatically, the oil industry should be discouraged from using municipal sewage treatment facilities, or municipal water supplies for refining processes, since both practices may lead to the formation of carcinogenic reaction-products.

Refinery effluents were not directly carcinogenic, but the co-carcinogenic activity of effluent extracts in the trout embryo assay is of some concern. Refinery effluents contain high concentrations of phenolic and long-chain aliphatic hydrocarbons; many of which are known to have co-carcinogenic potential. Several of these compounds are thought to act as co-carcinogens and/or promotors through their activity as irritants. Petroleum products and effluents also contain high concentrations of aromatic hydrocarbons, which can act as co-carcinogens through alteration of metabolic enzyme levels. Subfractionation of effluent extracts (e.g. phenolics, aliphatics, and aromatics) and subsequent carcinogenesis testing may help define the mechanism involved in this process.

There is a possibility that compounds present in effluent extracts alter metabolic processes in fish. Co-carcinogenic compounds, which act through metabolic changes, may increase carcinogenesis in one species, and decrease it in another. For instance, in hamsters the administration of 3-methylcholanthrene increases the concentration of a carcinogenic metabolite of

2-acetylaminofluorene and thus increases carcinogenesis, while in rats this treatment has the opposite effect (Weisburger, 1978). Therefore, the relevance of results with the trout embryo assay should be assessed by testing with other animal models. It remains to be determined whether effluents show a co-carcinogenic effect with other indirect-acting carcinogens besides aflatoxin B_1. Another important factor to be determined with mammalian species is whether a co-carcinogenic response is detectable at environmentally-relevant concentrations of effluent in drinking water.

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APPENDIX I: Results of Amos tests (TA100 with S-9) to determine the optimal concentration of benzo[a]pyrene (BeP) to be used in comutagenicity tests. The mean number of spontaneous revertants was 102.

Mutagen	Comutagen (BeP) Conc.	Mean Rev./Plate
Benzo[a]pyrene (5 µg)	10 µg	312
	5 µg	404
	2.5 µg	382
	1 µg	343
	0 µg	350

APPENDIX II: Results of Ames tests (TA98 with S-9) to determine the optimal concentration of norharmane to be used in comutagenicity tests. Tests were conducted with and without pre-incubation of norharmane with test mixtures.

Treatment	Comutagen (norharmane) Conc. (µmole)	Mean Rev./Plate
a) Without pre-incubation:		
Spontaneous Revertants	2	42
	1	47
	0.45	43
	0.3	43
	0	48
2-acetylaminofluorene (2µg)	2	304
	1	339
	0.45	396
	0.3	307
	0	292
b) With pre-incubation (20 min.):		
Spontaneous Revertants	2	Toxic
	1	27
	0.45	31
	0.3	34
	0	41
2-acetylaminofluorene (2µg)	2	Toxic
	1	83
	0.45	89
	0.3	117
	0	309

APPENDIX III: Boiling points and mean recovery of spiked compounds by soxhlet, XAD-2, and purge-and-trap (P & T) extraction methods.

Compound	Boiling Point (°C)	% Recovery		
		Soxhlet	XAD-2	P & T
chloroform	61	-	-	103
benzene	80	-	-	91
1,2 - dichloroethane	84	-	-	97
dibromomethane	100	-	-	72
phenol	182	6	22	4
aniline	184	13	61	-
o - toluidine	200	22	75	-
p - chlorophenol	213	32	47	-
dimethylphenol	213	32	41	-
dodecane	216	23	23	-
napthalene	218	11	90	-
hexadecane	280	103	106	-
phenanthrene	340	98	76	-
eicosane	343	103	97	-
benzo(a)pyrene	>350	100	88	-
1 - anthramine	>350	106	77	-

APPENDIX IV: Mean recovery of phenols from distilled water by XAD-7 resin extraction.

Compound	Concentration	% Efficiency
phenol	100 µg/ℓ	95
dimethylphenol	100 µg/ℓ	81
p-chlorophenol	100 µg/ℓ	102

APPENDIX V: Recovery efficiency of known mutagens by XAD-2, soxhlet, and purge-and-trap methods, as determined by Ames assays of extracts: a) Extraction of 100 µg BaP in distilled water (1 litre) by XAD-2 resin, and 100 µg BaP in activated alumina (1 gm) by soxhlet. b) Extraction of 6 mg dibromomethane (DBM) from distilled water (300 mℓ.) by purge-and-trap methods. Mutagenicity was determined by Ames tests using strain TA100 with S-9 (a), or without S-9 (b). SR refers to spontaneous revertants.

Treatment		Mean Revertants/Plate
a) Non-volatile methods:		
Calibration - BaP:	15 µg	697
	10 µg	590
	5 µg	373
	1 µg	293
	0 µg(SR)	112
Extracts - XAD-2 (1 mℓ. final volume):		
	(I) Control Extract (0.1 mℓ./plate)	107
	(II) BaP Extract (0.1 mℓ./plate)	652 (119%)*
- Soxhlet (1 mℓ. final volume):		
	(I) Control Extract (0.1 mℓ./plate)	115
	(II) BaP Extract (0.1 mℓ./plate)	475 (87%)*
b) Volatile methods:		
Calibration - DBM:	0.4 mg	612
	0.2 mg	543
	0.1 mg	410
	0 mg(SR)	102
Extract - Purge-and-Trap (3 mℓ. final volume):		
	(I) Control Extract (0.1 mℓ./plate)	96
	(II) DBM Extract (0.1 mℓ./plate)	492 (75%)*

*Per cent recovery calculated from regression line for calibration results.

APPENDIX VI: Mutagenicity of a Refinery 1 (February, 1982) effluent sample prepared by re-extracting XAD-2 resin with methanol (MeOH), and re-extracting residue in a soxhlet thimble with methylene chloride ($MeCl_2$). The effluent sample had been previously extracted using solvents normally used in those protocols (ether and methanol). SR refers to spontaneous revertants.

Extract	Residue Wt.	Test Strain	Mℓ. Equiv./ Plate	Rev./Plate With S-9	Rev./Plate Without S-9
XAD-2 (MeOH)	8 mg/ℓ	TA100	320	Toxic	Toxic
			160	102	Toxic
			80	98	108
			40	99	113
			(SR)0	100	109
		TA98	320	52	34
			160	38	34
			80	40	49
			40	41	44
			(SR)0	42	40
Soxhlet ($MeCl_2$)	23 mg/ℓ	TA100	320	Toxic	Toxic
			160	Toxic	Toxic
			80	110	132
			40	102	112
			(SR)0	100	109
			320	51	Toxic
			160	47	Toxic
			80	54	Toxic
			40	45	38
			(SR)0	42	40

APPENDIX VII: Comparison between the mutagenicity of samples dissolved in DMSO and acetone. Samples tested in these solvents are controls, benzo[a]pyrene (BaP), and XAD-2 and soxhlet extracts from a refinery 1 effluent sample (October, 1981).

Sample	Test Strain (with S-9)*	Mean Rev./Plate
a) Control:		
DMSO	TA98	48
	TA100	113
Acetone	TA98	46
	TA100	99
b) BaP (5 µg):		
DMSO	TA100	442
Acetone	TA100	359
c) XAD-2 (80 mℓ. equiv.):		
DMSO	TA98	58
Acetone	TA98	76
d) Soxhlet (320 mℓ. equiv.):		
DMSO	TA100	119
Acetone	TA100	149

APPENDIX VIII: Comparison of the mutagenicity of refinery effluent extracts (Refinery 1, October, 1981) as determined in Ames assays (with microsomal activation) using four tester strains. Spontaneous revertants are tabulated in brackets.

Extract	Mg. Equiv./Plate	Strain	Rev./Plate
XAD-2	80	TA1538	54(38)
		TA98	71(43)
		TA1535	50(40)
		TA100	122(104)
Soxhlet	320	TA1538	41(38)
		TA98	47(43)
		TA1535	53(40)
		TA100	145(104)

APPENDIX IX: Mutagenicity of dibromomethane (DBM) in Ames tests with TA100, using various assay protocols. Spontaneous revertant values are presented in brackets.

Treatment	Test Conc.	Mean Rev./Plate
I) Without S-9		
a) Normal Ames protocol	2 mg	243(112)
b) Dessicator - Plastic plate	10 mg*	2835(107)
- Glass plate	10 mg*	2683(107)
c) Sealed plate: (i) Aqueous phase		
- Plastic plate	2 mg	1427(111)
- Glass plate	2 mg	1472(108)
- Plastic plate	2 mg (in MeOH)	1267(98)
(ii) Gaseous phase		
- Plastic plate	2 mg	1836(114)
- Glass plate	2 mg	1818(111)
- Plastic plate	2 mg (in MeOH)	1557(102)
II) With S-9		
a) Sealed plate: Aqueous phase		
- No preincubation	2 mg	613(105)
- Preincubation	2 mg	619(97)

* 10 mg. DBM refers to amount of compound placed in bottom of dessicator and allowed to volatilize.

APPENDIX X: Lethality data (96 hr.) and calculated LT_{50}'s for rainbow trout embryos (20 per treatment) injected with various concentrations of carcinogens and effluent extracts. All injection solutions were prepared without pre-incubation with S-9. LT_{50} values were calculated from experimental minus control mortality data, as the geometric mean of the times before and after which 50% of the fish died as a result of exposure to compounds or extracts.

Compound	Conc.	% Mortality						LT_{50}
		0h.	12h.	24h.	49h.	76h.	96h.	
a) Carcinogens (Conc. as ng/egg):								
Control	-	0	40	55	60	60	60	-
2-anthramine	1000	0	70	80	85	95	95	17hr.
	500	0	35	60	60	60	65	>96hr.
	250	0	35	55	55	55	55	>96hr.
DMBA	1000	0	95	100	100	100	100	3hr.
	500	0	75	75	80	80	85	34hr.
	250	0	30	30	60	65	65	>96hr.
aflatoxin B_1	50	0	100	100	100	100	100	3hr.
	25	0	65	65	70	70	85	85hr.
	13	0	35	40	55	70	70	>96hr.
MNNG	500	0	70	75	75	95	100	61hr.
	250	0	35	40	60	60	65	>96hr.
	100	0	40	40	55	55	55	>96hr.
b) Refinery Extracts; October, 1981 (Conc. as mℓ. equiv./egg):								
XAD-2	400	0	100	100	100	100	100	3hr.
	200	0	35	65	65	65	65	>96hr.
	100	0	30	40	45	60	60	>96hr.
Soxhlet	400	0	100	100	100	100	100	3hr.
	200	0	60	65	65	70	85	85hr.
	100	0	40	45	45	55	60	>96hr.