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Project Summary

Fractionation of Mutagens from Municipal Sludge and Wastewater

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There are potential environmental concerns from the disposal of municipal wastewater effluents and sewage treatment plant sludges. This report summarizes the microbial mutagenic evaluation and chemical analysis of 13 sewage sludge samples from various sewage treatment plants located in Texas and Washington state. The sewage sludge samples were air-dried followed by sequential Soxhlet extraction with three organic solvents of increasing polarity, i.e., pentane, methylene chloride, and methanol. The organic extracts from three of the samples were further fractionated by normal phase high-pressure liquid chromatography (HPLC). The obtained extracts and fractions were bioassayed for microbial mutagenic response using the standard histidine reversion assay with *Salmonella typhimurium* strains TA98 and TA100, both with and without S9 metabolic activation. Extracts and fractions were chemically analyzed by high resolution gas chromatography (GC) using a variety of element-specific detectors, gas chromatography/mass spectrometry (GC/MS), and (to a lesser extent) liquid chromatography/mass spectrometry (LC/MS).

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Potential genotoxicity of sewage sludges may be of particular concern relative to agricultural uses and to land disposal; likewise, the disposal of treated wastewater is of considerable environmental concern because of the possible introduction of mutagens into the receiving water. The spectrum of mutagens (and organic compounds in general) found in the sludges is likely to be different than that found in treated wastewater. This is related to the fact that many of the incoming organic compounds become associated with the sludge, and the biological treatment and disinfection processes remove some compounds and synthesize many more in the treated wastewater. The focus of this project was on the separation and identification of mutagenic constituents in sewage treatment plant sludges. At the beginning of the project, ten different sewage sludges were examined. Six were from various locations in Texas, and four were from various locations in Washington state. The organic constituents of these sludges were isolated by organic extraction and were screened for microbial mutagenicity. All of the extracts were examined qualitatively by high resolution gas chromatography (GC) using a variety of detectors to evaluate similarities in gross chemical composition. In addition, selected samples were analyzed by gas chromatography/mass

spectrometry (GC/MS). These analyses were performed in an attempt to identify the principal components found in each of the extracts.

After the initial preliminary chemical characterization of the original ten sewage sludge extracts, it was decided that further fractionation would be necessary to separate and identify any mutagenic components of sewage sludge. Three new sewage sludge samples from Texas were provided from the same location, one was an original sludge, while the two others had been stored in a Lysimeter for varying amounts of time. These sludges were extracted in a similar manner as the previous ten sludges. The extracts were then subjected to further fractionation based on polarity using normal phase, high-pressure liquid chromatography (HPLC). The HPLC fractions were screened for microbial mutagenic activity as were the above extracts. Selected fractions were analyzed by GC/MS and liquid chromatography/MS (LC/MS).

Procedure

Samples of all 13 of the sewage sludges as received were air-dried in a laminar flow hood at room temperature until they achieved a constant weight; percent water compositions were determined. The total carbon contents of the air-dried samples were determined by complete combustion. The organic constituents of the dried sludges were isolated by sequential Soxhlet extraction using pentane, methylene chloride, and methanol solvents. Total amounts extracted by each solvent were calculated after solvent removal.

The pentane, methylene chloride, and methanol Soxhlet extracts from three sewage sludge samples were further fractionated by normal phase HPLC using a ternary mobile phase gradient of hexane, methylene chloride, and methanol. Three fractions were collected from the pentane extracts, four fractions were collected from the methylene chloride extracts, and five fractions were collected from the methanol extracts. Total amounts in each fraction were calculated after solvent removal.

Solutions of the extracts and HPLC fractions from the sewage sludge samples were tested for mutagenicity in the *Salmonella* histidine reversion assay after evaluations of solvents for maximized dissolution. *Salmonella typhimurium* tester strains TA98, which screens for frame shift mutagens, and TA100, which screens for point mutagens, were selected. Each of the extracts and HPLC fraction was tested with the standard plate incorporation method at five or greater concentration levels of test extract with Aroclor-induced rat liver homogenate (S9) metabolic activation. Two to five concentration levels of each extract were tested without S9 metabolic activation. The levels of test extracts and HPLC fractions ranged from 2 to 1250 µg/plate. The concentration of sludge extracts and HPLC fractions were chosen based on preliminary toxicity testing which indicated cytotoxicity concentrations at the level of 2000 µg/plate. Also, the higher concentrations of extracts (i.e., 1000 µg/plate or greater) consistently exhibited undissolved sludge particulates when plated with agar.

Revertant colonies per petri plate were counted electronically (or by hand if precipitates were present). An extract or HPLC fraction was considered to give positive microbial mutagenic response if it showed a two-fold increased number or revertants form background or if there was a positive linear dose response with a correlation coefficient of 0.75 or greater and an intercept on the ordinate axis within 20% of the negative control for the day as determined by linear regression analysis.

Selected sludge sample extracts were analyzed by GC using a HP 5880A gas chromatograph. The extracts were analyzed using multiple detectors including a flame ionization detector (FID), a nitrogen-phosphorus detector (NPD), an electron capture detector (ECD), and a sulfur-specific flame photometric detector (FPD). The percent of each extract that was chromatographable was estimated. Elemental sulfur was quantified in some of the sludge extracts by calibration of the FPD with an elemental sulfur standard. Some unknown nitrogen-containing components detected in the extracts were quantified based on the NPD response of an internal standard, benzo[b]carbazole.

Selected extracts and HPLC fractions were analyzed by GC/MS using an HP 5982 or 5987 quadrupole mass spectrometer interfaced to an HP 5710 or 5840 gas chromatograph. The mass spectrometers was operated in the electron impact mode at 70 e V and were scanned from 50 to 500 atomic mass units (amu). Some of the extracts and fractions were derivatized with diazomethane prior to analysis.

Two HPLC fractions were analyzed by LC/MS using an HP 5988A thermospray system. A binary mobile phase of 95:5 (v:v) 0.1 M ammonium acetate:acetonitrile and acetonitrile was used. The mass spectrometer was scanned from 140 to 600 amu. Prior to the analyses by LC/MS many of the extracts and fractions were analyzed by HPLC using a photodiode array UV detector. UV spectra were plotted from responses at wavelengths of 254 nm and 340 nm. Samples were selected for LC/MS if it was felt there was adequate response on the UV detector to be detected by the mass spectrometer.

Results

The water content of the sludges was highly variable, ranging from less than 1% water to nearly 85% water. The total carbon contents of the dried sludges were in the 22-31% range. The percent solvent extractable ranged from about 2% to 15%. There was a direct linear correlation between the carbon contents of the sludges and their percent extractable. The HPLC chromatograms showed there were components of increasing polarity in the extracts from solvents of increasing polarity.

The sludge extracts and HPLC fractions were difficult to bioassay mainly due to the presence of undissolved sludge particulates when the samples were plated with agar. Data resulting from assays in which the formation of precipitates occurs cannot be meaningfully expressed, and they cannot be useful for comparing the activities of different materials. The microbial mutagenic responses of all the sludge extracts and fractions was low, regardless of tester strain or metabolic activation. All extracts expressed less than 0.5 revertants per mg and all fractions expressed less than 1.2 revertants per mg microbial mutagenic activity. Fewer extracts exhibited microbial mutagenic activity when tested with strain TA100 compared to strain TA98. Some of the mutagenic responses were direct-acting. The microbial mutagenic activity of the extracts and fractions were not clearly separated into any one extract or fraction.

While there was considerable variability for the percent chromatographable amongst the extracts from the sludge samples, approximately two-thirds of the pentane-extractable components, one-third of the methylene chloride-extractable components, and 10% of the methanol-extractable components were chromatographable under the conditions employed. Three major components electron-capturing components were detected in all the extracts, regardless of origin. Elemental sulfur as well as three other sulfur-containing components were de-

tected in the low parts per thousand (mg/g) region in some of the sludge extracts when analyzed by FPD.In addition, some high molecular weight, nitrogen-containing components were detected in the parts per thousand region (mainly in the methanol extracts) in some of the sludge extracts when analyzed by NPD.

Generally, the same components were found in all the different sludge extracts, regardless of origin. The components did tend to be extracted preferentially into different solvents. The major components of all the sewage sludge extracts and fractions as identified by GC/MS were fatty acids having 16 or 18 carbon molecules. Overlap of these components between the extracts and in adjacent fractions was observed. There was also evidence of the presence of benzenedicarboxylic acids in some of the HPLC fractions. No compounds were identified by LC/MS due to lack of sensitivity.

Conclusions and Recommendations

Accurate assessments of mutagenic activities were difficult due to the formation of precipitates with the agar using the standard microbial mutagenicity assay. Extreme care must be taken in the evaluation of sewage sludge samples for mutagenic activity to prevent inaccurate reporting. Using the methods employed, the microbial mutagenicity, and hence, the mutagens of the sewage sludge samples were not clearly separated. The overall levels of mutagenicity in all extracts and fractions were low (less than 1.2 revertants per mg), and no specific mutagens were identified in any of the extracts of fractions using GC and GC/MS techniques.

Due to the extremely low levels of mutagenicity expressed by the extracts and fractions of the sewage sludge samples studied, further studies should concentrate on samples that exhibit higher levels of biological activity as determined by a screening method. Further characterization would only be done as mutagenicity dictated. This may increase the likelihood of identifying some mutagens present in sewage sludge samples. The estimated low amounts of the organic extracts and fractions that were amenable to analysis by GC and GC/MS indicates the need for other sensitive chemical analyses for high molecular weight biological and/or highly polar components to better characterize the sewage sludge.

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The complete report, entitled "Fractionation of Mutagens from Municipal Sludge and Wastewater," (Order No. PB 89-161 491/AS; Cost: $15.95, subject to change) will be available only from:
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