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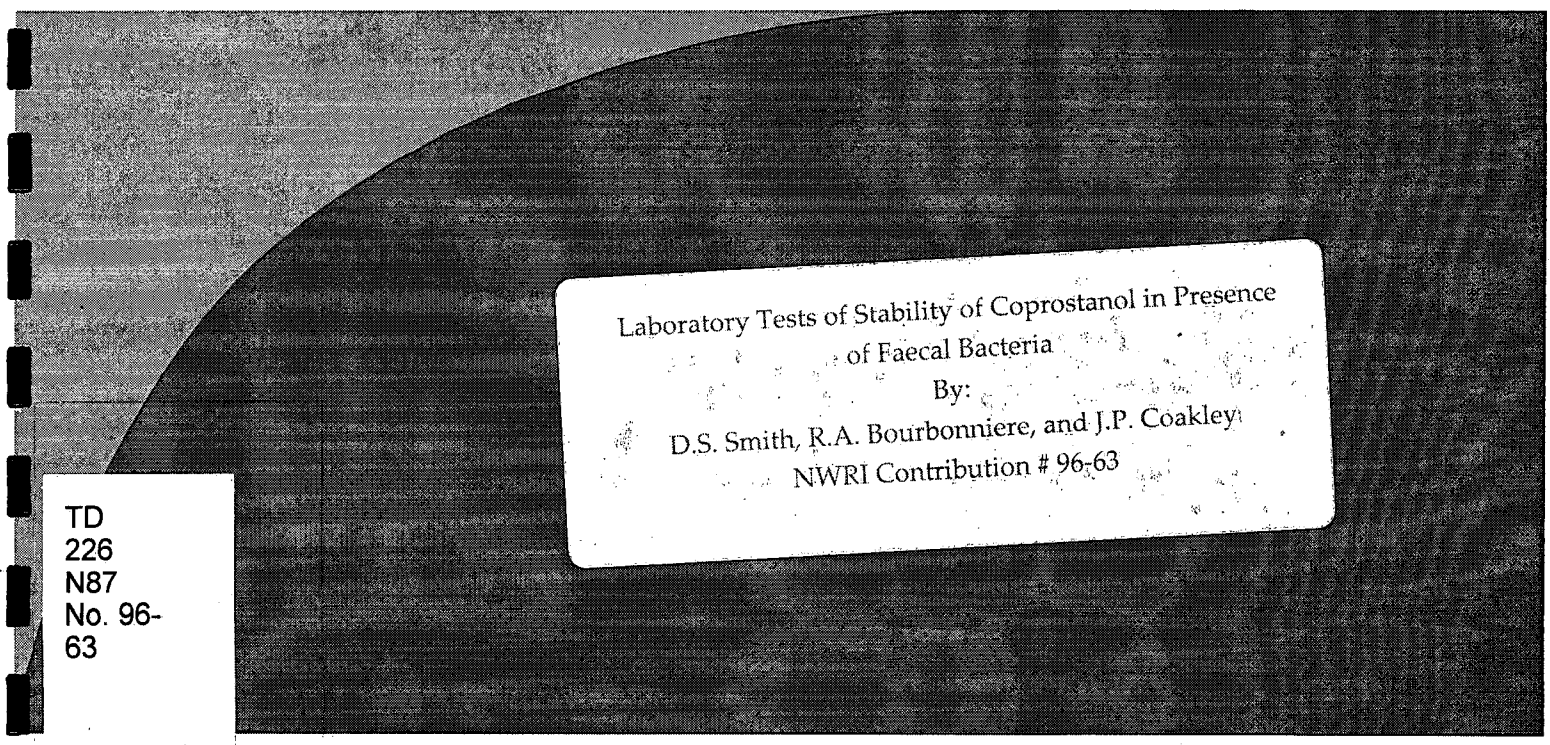

Laboratory Tests of Stability of Coprostanol in Presence of Faecal Bacteria

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# Laboratory tests of stability of coprostanol in presence of faecal bacteria.

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## ABSTRACT

This paper examines the stability of sediment-bound coprostanol in laboratory bench-top tests over 50 days of exposure to a vigorous bacterial flora. The experiments were carried out in triplicate at room-temperature and under aerobic conditions. For comparative purposes, the microbial degradation of other organic additives was monitored. They were: n-C20 alkene, n-C21 alkane, Benzo (a) anthracene, erucic acid, tricosanoic acid, n-C26 alcohol, and another steroid stigmasterol. Extraction solvent was methanol / dichloromethane and analysis was by GC-MS with FID detector. The results show that coprostanol was resistant to degradation over the 50-day period.

## MANAGEMENT PERSPECTIVE

Title: Laboratory tests of stability of coprostanol in presence of faecal bacteria.

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Issue: Remedial Action Plans for various degraded ecosystems in the Great lakes require that sources and transport properties of priority contaminants be identified as well as possible. The use of specific organic compounds as tracers of hydrophobic contaminants has been used for this purpose in Hamilton Harbour, an Area of Concern. A requirement of the technique is that the compound used as a tracer be stable over extended periods in the sediment medium.

Current status: This phase of the study is a collaboration between NWRI and McMaster and was initiated in 1993. Comparable investigations of coprostanol stability over longer periods (decades) are in progress using core data.

Next steps: When the stability question is resolved, the technique will be applied to other similar sites in the Great Lakes.

## INTRODUCTION

Coprostanol is used extensively as a spacial tracer for sewage in sediment. This faecal steroid (5β-cholestan-3β-ol) was studied in marine environments, Antarctica (Green et al. 1992, and Venkatesan et al. 1992), New York Bight (Hatcher et al. 1979) and Venice Italy (Sherwin et al. 1993). Coprostanol has also been applied as a sewage indicator in fresh water environments, ie, Lake Constance in central Europe (Müller et al. 1979). An intensive investigation on the extraction and use of coprostanol was done by Kirchmer (1971).

The basis for the use of coprostanol as a sewage indicator is that its only known source is intestinal microbial reduction of stenols in mammals, including humans (Midtvedt et al. 1993). The assumption in the use of coprostanol as a temporal as well as spacial tracer of sewage is that this compound is resistant to biodegradation. Measurements taken down core in Lake Constance were used to investigate the history (1800 to 1977) of sewage input (Müller et al. 1979); coprostanol stability being assumed because sterols are insoluble in water and compared to other lipids are very resistant to microbial degradation. Coprostanol degradation was studied experimentally by Bartlett (1987), Kirchmer (1971) and Nishimura (1982).

The stability of coprostanol and seven other compounds with respect to a mixed culture of four randomly selected bacteria under aerobic conditions is investigated here. The other compounds used included a normal alkene and alkane (C20 and C21 respectively), a polycyclic aromatic hydrocarbon (benzo(a)anthracene), an unsaturated fatty acid (C22 erucic acid), and saturated fatty acid (C23 tricosanoic acid), an saturated alcohol (C26), and stigmasterol. The experiment involved loading clean sediment in water with known amounts of these organic compounds, including coprostanol, and measuring the compound concentrations with time in the presence of bacteria.

## EXPERIMENTAL METHODS

The experiment was performed in loosely stoppered 1000 mL Erlenmeyer flasks, kept at room temperature throughout. All glassware was rinsed with methanol followed by dichloromethane and oven dried prior to use.

### Sediment media

The sediment used in these experiments was very low in organic compounds

initially, especially those of high molecular weight. The sediment was a very fine-grained glacial clay (median diameter 1 micrometer). A known mass (10 g) of sediment was placed in three different Erlenmyer flasks and 500 mL of MilliQ water was added to create a slurry. The slurry was then placed on a shaking table and one day allowed for equilibration before anything was added.

## Organic Spike

A solution was prepared to spike the clean sediment with. The solution was in methanol/toluene (1:1). The compounds selected and their concentrations in the stock solution are presented below in Table 1.

**Table 1:**

| Compound | Concentration (µg/mL) |
|---|---|
| n-C20-alkene | 222 |
| n-C21-alkane | 200 |
| Benzo(a)anthracene | 201 |
| Erucic Acid | 243 |
| Tricosanoic Acid | 203 |
| n-C26-alcohol | 200 |
| Coprostanol | 1000 |
| Stigmasterol | 180 |

This organic solution was injected (1.00 mL) onto the sediment slurry and allowed to equilibrate 24 hours before the bacteria were added. The resultant original dry sediment concentration was about 20 µg/g for all compounds except coprostanol which was 100 µg/g.

## Bacteria used

A sample of Burlington Sewage Treatment Plant effluent before treatment was

collected in a glass jar. Agar plates were used to grow the bacteria present in this sample. There were at least 30 dominant different species. Four of these species were chosen for the experiment. The chosen species were cultivated in four separate nutrient broths. Prior to inoculation of the sediment sample the four separate broths were combined so that a mixed culture of bacteria would be injected. The bacteria concentration in the Erlenmyers was originally $10^7$ organisms/mL. The bacteria were quantified after 1081 hours by using serial dilutions and agar plates.

## Sampling

Sampling was performed first after the sediment and water had mixed for 24 hours; this was the blank. The organic spike was then added and the slurry equilibrated for 24 hours before the bacteria were added. A day after the bacteria were added the second sample was obtained. Subsequent samples were taken at increasing intervals of time. The values for time zero were obtained in a separate experiment using the same methods but smaller amounts, and no bacteria.

The sampling procedure involved pipetting out 25 mL of the slurry while it was being homogenized using a magnetic stir bar.

## Extraction

The extraction was performed using methanol/dichloromethane (1:1) as the solvent. Initially the sample was centrifuged and the supernatant water decanted off into another centrifuge tube. This water was then acidified (pH < 2) using conc. HCl to flocculate the very fine materials. The water was then centrifuged and the sediment plug transferred to the original centrifuge tube and the water discarded (testing subsequent to extraction showed only 0.4% of the total coprostanol retained in the aqueous fraction).

15 mL of the solvent was added to the sediment plug and this was stirred for two minutes. The sample was centrifuged and the solvent decanted and saved. This process was repeated two more times, combining the subsequent extractions with the first. Anhydrous granular sodium sulphate was added to the solvent for an hour to remove the water. The extract was then filtered.

The extract was evaporated to dryness and then silylated with 100 µL of N-methyl-N-(trimethylsilyl)-trifluoroacetamid (MSTFA) for 20 minutes at 130°C. This was allowed to cool and 1 mL of heptane was added and the sample transferred to a vial for injection into the gas chromatograph.

## Chromatography

The compound concentrations were determined using an HP autoinjector model (HP 5890A) gas chromatogram with an FID detector and on column injection to a DB5 fused silica column (0.32 mm X 35 m). The initial temperature was 94° C and it was increased at a rate of 10° C per minute up to 238°, thereafter by 5° C per minute up to 300°, where it was held for 5 minutes. Quantization was performed using n-alkane C23 as an injection standard; a known amount of C23 was added to the GC vial after silylation.

# RESULTS

## Bacteria

Initially four different bacteria were added, $10^7$ organisms/mL in the slurry. After 1081 hours only two species had survived; one was still $10^7$ organisms/mL and the other was $10^5$ organisms/mL. In addition to these species 8 other species $<10^5$ organisms/mL were found in all three slurries.

## Sampling

Figure 1 shows the sample mass for all the samples taken. Three points lie outside two standard deviations about the mean; the results from these points were not included in the final analysis because the sampling was obviously inconsistent. A preliminary experiment in which the sample was not homogenized with a magnetic stir bar during sampling showed that coprostanol had a strong preference for the smaller particle sizes.

## Compounds

The results for the 8 different compound concentrations with time are summarized in Figures 2 and 3. The concentrations are plotted as the determined concentration divided by the original load versus time. The concentration ratios are consistently less than one because of the injection standard method will tend to underestimate the concentrations and the quick extraction technique is not 100% effective for all compounds. The standard deviation is approximately 5-10% for compound concentration in µg per gram of sediment.

The only compounds that have definitely decreased are the alkane and the alkene, possibly benz(a)anthracene has decreased. The values for coprostanol do not

decrease; in fact, almost without exception they oscillate between the mean and the standard deviation lines in Figure 2 and show no increasing or decreasing trend. The same evidence of stability can be seen for the fatty acids, C26 alcohol and stigmasterol.

## DISCUSSION

Almost all the previous experiments on coprostanol stability took place in environments where anaerobic conditions were the rule (Bartlett, 1987; Hatcher and McGillivary, 1979). Largely because of these experiments, it is now accepted that coprostanol is very resistant in anaerobic conditions typical of burial in marine sediments. Experiments in aerobic conditions, show that degradation to some extent may occur.

In this experiment the conditions were essentially aerobic; air was available during sampling times and the stoppers were not air tight, yet coprostanol did not decrease. The conditions were meant to model stirred up sediments, hence the constant shaking. The bacteria chosen would be bacteria available to sewage contaminated sediment. In other experiments coprostanol has been found to degrade under aerobic conditions, but not under anaerobic or reducing conditions.

Kirchmer (1971) shows that coprostanol decreased more rapidly in non-chlorinated versus chlorinated sewage effluent. An explanation for this could be that the bacteria causing degradation are killed in the chlorinated effluent. These experiments were performed in 20 L tanks open to the atmosphere and stirred; more aerobic than the conditions in this experiment.

Bartlett (1987) also showed that coprostanol is unstable under aerobic conditions. Raw primary sewage sludge in an aerated 50 L tank showed a rapid decrease in sludge coprostanol concentration in the first 29 days followed by a slower decrease until 54 days. Artificial sediment (4:1 clean sand to sewage sludge) in a similar tank in which the aeration did not extend to the sediment bed, showed little or no decay over 54 days. The conclusions from this were that coprostanol is stable under anaerobic conditions but unstable under aerobic conditions.

Finally, an experiment by Nishimura (1982) shows that coprostanol is stable under reducing conditions. In this experiment microbiologically active surface sediment from Lake Suwa (Japan) was incubated with coprostanol under intensely reducing conditions and coprostanol was 90% recovered after 45 days and 89% recovered after 450 days.

Although for logistical reasons, neither redox potentials nor dissolved oxygen

were measured, conditions in this experiment were essentially aerobic, as air was available during sampling times and otherwise, the stoppers on the flasks were not air tight. The bacteria chosen would be bacteria available to sewage-contaminated sediment. The experiment was intended to duplicate the dynamic conditions of sediment-bound coprostanol in freshwater conditions, either on the sediment surface or resuspended in the bottom part of the water column, hence the constant shaking. We believe that the varying oxygen levels in the flask (ranging from saturation during sampling to low levels otherwise) are representative of conditions on or close to the bottom in shallow Great Lakes waters (Carlton et al., 1989; M.N. Charlton, National Water Research Institute, personal communication, 1995).

## CONCLUSIONS

Coprostanol is very resistant to biodegradation even under mildly aerobic conditions. This implies that coprostanol can be used as a temporal as well as a spatial sewage tracer.

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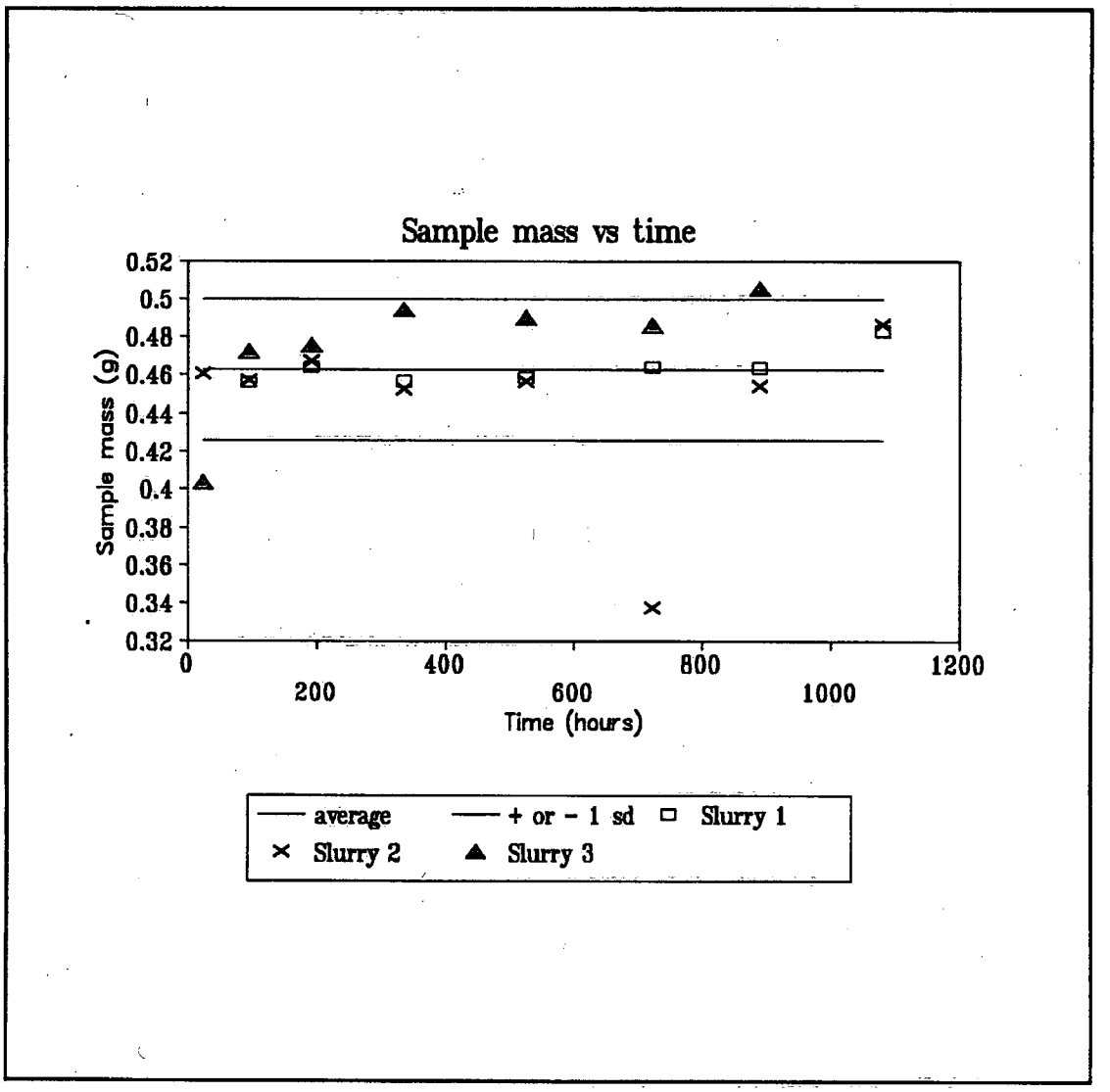
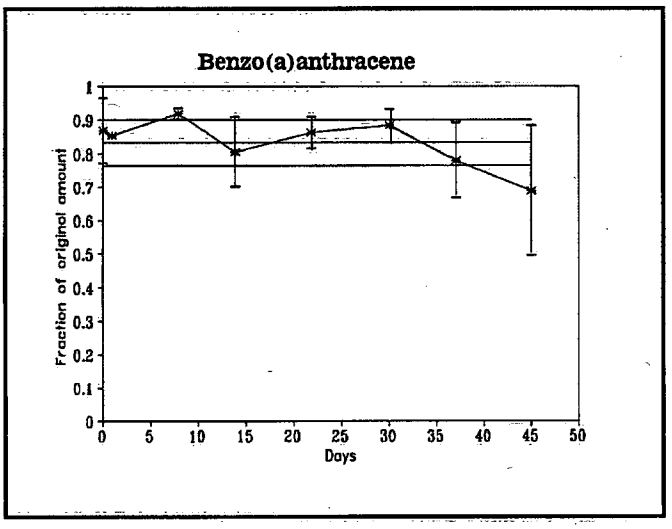
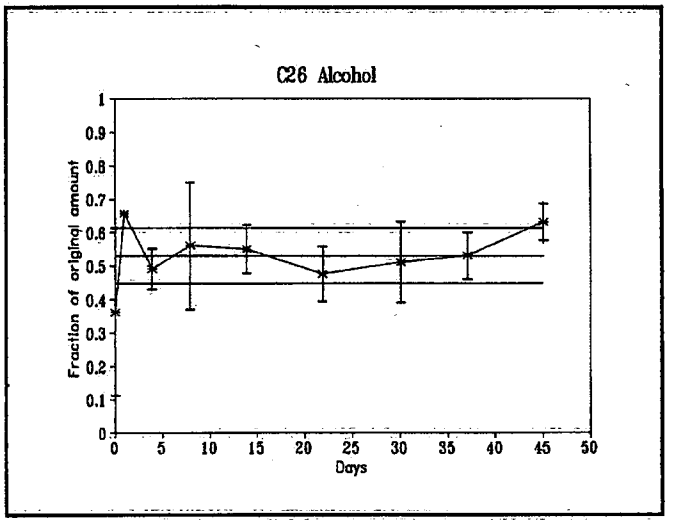
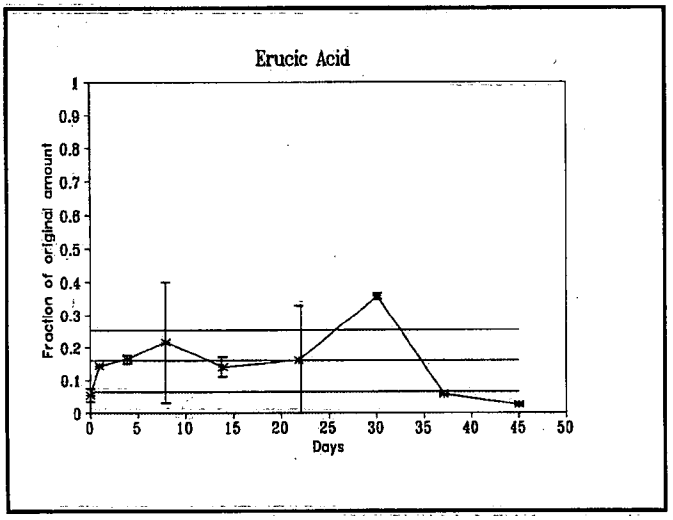



**Figure 1:** **Sediment mass for every sample with time. The horizontal lines indicate the mean plus and minus one standard deviation.**



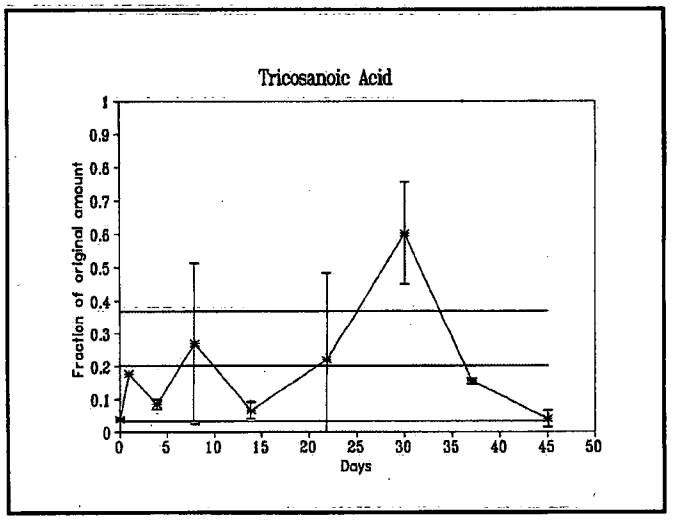
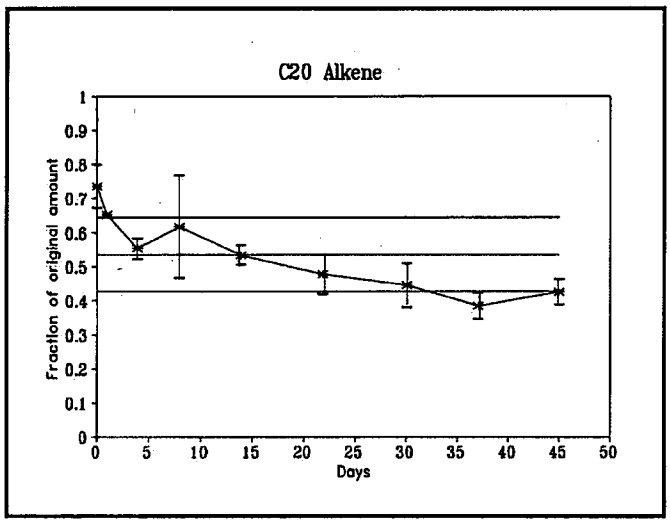
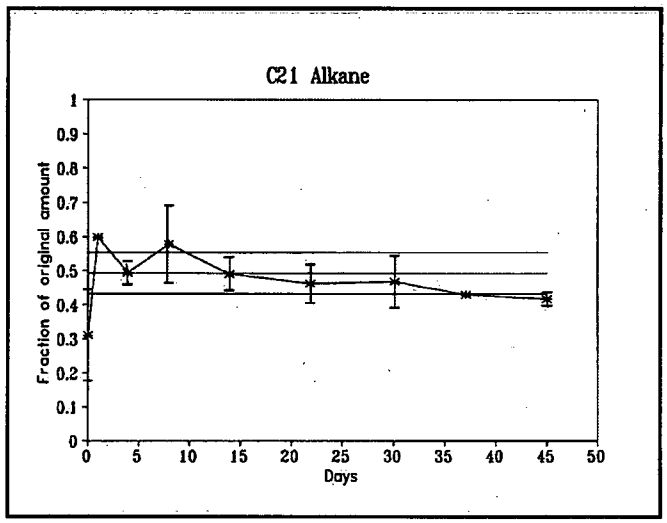
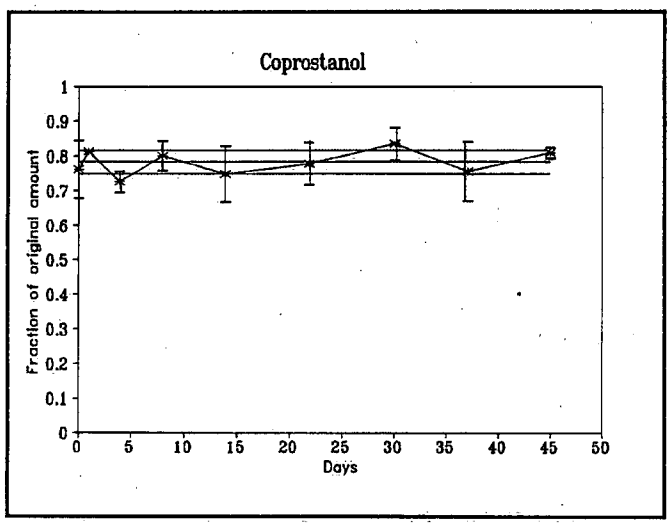




Fig. 3 Plot of changes vs.time in concentration of various spiked compounds in test sediment.



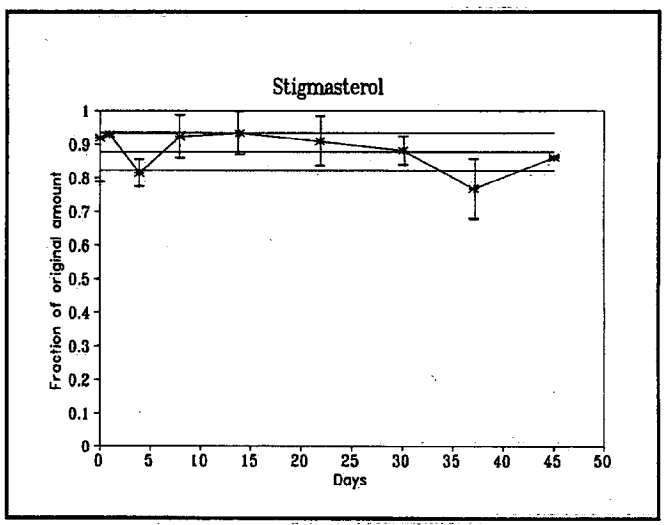




Fig. 2 Plot of changes vs.time in concentration of spiked compounds in test sediment.

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