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  19. Supported in part by NSF grants DEB-8001803 (T.N.T.) and DEB-7908607 (T.N.T. and E.L.S.), a grant-in-aid of research from Sigma Xi, an Ohio State University graduate school alumni research award, and a fellowship from the American Association of University Women Educational Foundation. We thank P. Van Faassen and G. L. Floyd for helpful comments.

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## 4-Nonylphenol in Sewage Sludge: Accumulation of Toxic Metabolites from Nonionic Surfactants

**Abstract.** *Anaerobically treated sewage sludge was found to contain extraordinarily high concentrations of 4-nonylphenol, a metabolite derived from nonionic surfactants of the nonylphenol polyethoxylate type. Concentrations in activated sewage sludge, in mixed primary and secondary sludge, and in aerobically stabilized sludge were substantially lower, suggesting that the formation of 4-nonylphenol is favored under mesophilic anaerobic conditions. Because 4-nonylphenol may be highly toxic to aquatic life, further research is needed on the fate of 4-nonylphenol after sludge is disposed of in the environment.*

4-Alkylphenol polyethoxylates (structure A in Fig. 1) are widely used nonionic surfactants. In 1982 their end-use market in the United States reached approximately 140,000 metric tons (1). Their degradation during aerobic treatment of wastewater by activated sludge leads to the formation of 4-alkylphenol mono- and diethoxylates (structure B in Fig. 1), which have been found as major refractory constituents of treated wastewater effluents (2) and river water (3). In addition, alkylphenol polyethoxy carboxylic acids have been detected in biologically treated domestic wastewater (4). This report concerns the degradation of the 4-alkylphenol ethoxylates during sludge treatment and, in particular, the high concentrations of the toxic compound 4-nonylphenol (structure C1 in Fig. 1) in anaerobically stabilized sewage sludge.

Sludge samples were collected from pipelines of mixed digesters during sludge transfer and extracted in a closed-loop steam distillation-extraction apparatus (5). Cyclohexane extracts were subjected to column chromatography on deactivated silica. The methylene chloride-eluted fractions were then analyzed by high-resolution capillary gas chromatography (Fig. 2A). Compounds were

identified on the basis of comparisons with a commercial, technical grade 4-nonylphenol. Since 4-nonylphenol is manufactured from tripropylene and phenol (6), it contains many isomers with variously branched structures of the nonyl substituents. The different alkyl branching gives rise to several incompletely resolved peaks in the capillary gas chromatogram of commercial 4-nonylphenol (2, 5). Major peaks in the gas

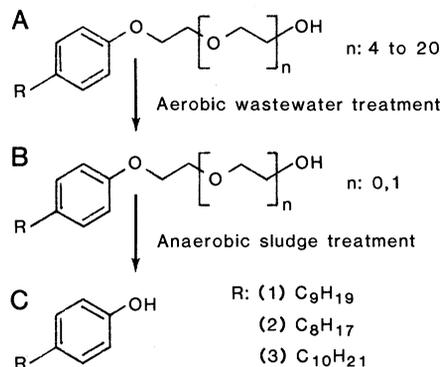


Fig. 1. Biological transformation of alkylphenol polyethoxylates (A) during wastewater and sludge treatment. Refractory metabolites are alkylphenol mono- and diethoxylates (B) and alkylphenol (C). Nonyl (1), octyl (2), and decyl (3) form the alkyl substituents (R).

chromatogram of the sludge extract (Fig. 2A) were assigned to isomeric 4-nonylphenols. In addition, 4-octylphenol and 4-decylphenols were detected.

Directly coupled gas chromatography-mass spectrometry provided the basis for identifying the alkylphenols. Molecular ions (mass/charge ratios, 206, 220, and 234) and base peaks (mostly 135, but also 107, 121, 149, and 163) were identical for corresponding peaks in the sludge sample and in the reference material. The mass spectra of 4-nonylphenols have been published elsewhere (5).

We also used high-performance liquid chromatography (HPLC) for further confirmation. Normal-phase HPLC [Lichrosorb-NH<sub>2</sub> column, hexane-isopropanol (9:1) eluent, 278-nm detection wavelength] allowed for a semipreparative separation of 2- and 4-nonylphenols from the standard nonylphenol. The major compounds in the sludge extracts coeluted with the 4-nonylphenols. Reversed-phase HPLC [Lichrosorb RP-8, methanol-water (3:1) eluent] provided a means for separating the alkyl homologues (structures C1, C2, and C3 in Fig. 1). Ultraviolet and infrared spectra of the sewage sludge extracts were identical to the corresponding spectra of the isolated 4-nonylphenols. Proton and carbon-13 nuclear magnetic resonance (NMR) spectra supported the identification of 4-nonylphenol: the aromatic proton signals (Fig. 2B) indicated 4-substitution of the nonylphenol.

For quantitative determinations, an internal standard (*n*-nonylbenzene) was added to the sludge extracts and concentrations were calculated from the sum of the electronically integrated areas of the 4-nonylphenol peaks in the chromatogram. Response factors were determined with technical grade 4-nonylphenol. Reproducibility was  $\pm 4$  percent (four determinations) at 1.18 g per kilogram of dry matter. Analyses of samples spiked with 0.4, 0.9, and 1.3 g of 4-nonylphenol per kilogram of dry matter revealed recoveries of 105, 104, and 93 percent, respectively.

The concentration of 4-nonylphenol in 30 anaerobically stabilized sludge samples (Fig. 3A) ranged from 0.45 to 2.53 g/kg (mean,  $1.01 \pm 0.52$  g/kg, or 4.7 mmol/kg). Eight samples taken from the same digester over 10 months (September 1982 to June 1983) contained 0.81 to 1.49 g/kg (mean,  $1.18 \pm 0.23$  g/kg). 4-Nonylphenol concentrations in eight aerobically stabilized sludge samples were significantly lower [Fig. 3B; range, 0.08 to 0.5 g/kg; mean,  $0.28 \pm 0.15$  g/kg;  $t(36) = 3.91$ ,  $P < 0.0005$ ]. Activated sludge and

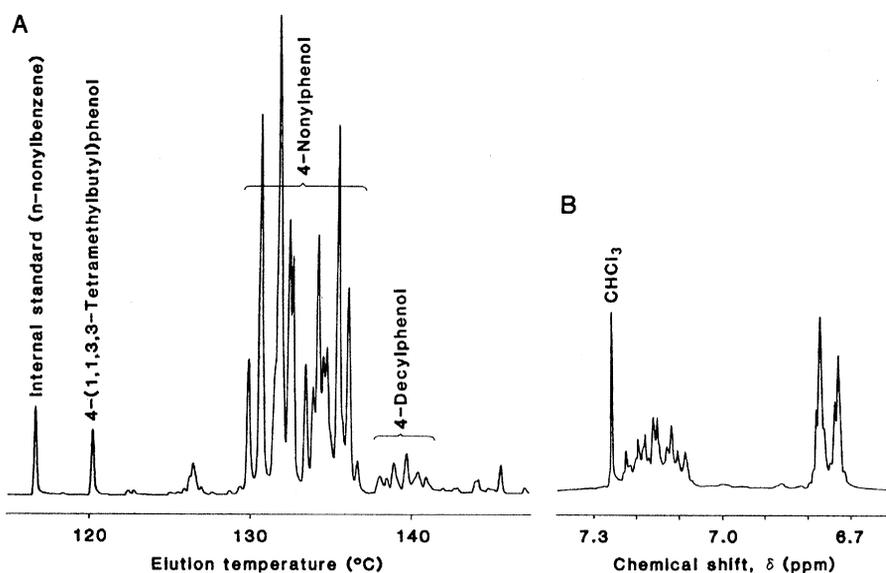


Fig. 2. Partial gas chromatogram (A) and proton NMR spectrum (B) of an extract of anaerobically treated sewage sludge. Gas chromatography was carried out with a 20 m by 0.31 mm (inner diameter) glass capillary, OV-73 stationary phase, hydrogen carrier gas at 0.4 atmosphere, splitless injection at ambient temperature, temperature program after elution of the solvent (methylene chloride) at 3° per minute from 80° to 270°C, and flame ionization detection. Nuclear magnetic resonance (Fourier-transformed proton resonance at 200.13 MHz) was performed with deuteriochloroform as the solvent and with trimethylsilane as the internal standard. The complex signal between 7.08 and 7.23 ppm is assigned to the 3- and 5-protons of the aromatic ring, while the signal between 6.71 and 6.80 ppm is attributed to the 2- and 6-protons. The chemical shift of the former is more strongly influenced by the various isomeric structures of the nonyl substituent.

mixed primary and secondary sludge also showed low concentrations of 4-nonylphenol (0.09 to 0.15 and 0.04 to 0.14 g/kg, respectively). These samples also contained nonylphenol mono- and diethoxylates (structure B1 in Fig. 1) at concentrations similar to their 4-nonylphenol content.

Our survey indicates that the large amount of 4-nonylphenol in stabilized sewage sludge originates from alkylphenol polyethoxylates (structure A in Fig. 1). We propose the following pathway for the formation of 4-nonylphenols. During aerobic treatment of wastewater, the polyethoxylate chains of the alkylphenol polyethoxylates are shortened by microbial transformations (7). The resulting alkylphenol mono- and diethoxylates (structure B in Fig. 1) appear to be less biodegradable. Because these metabolites have lost their hydrophilic moieties, they are less water-soluble and are partially removed from the wastewater stream by sorption to lipophilic flocs of sludge. Such nonbiological mechanisms of elimination in biological treat-

ment plants have been reported for other organic pollutants (8). When the sludge is stabilized, the alkylphenol mono- and diethoxylates are further degraded to alkylphenols (structure C in Fig. 1), which accumulate in the digested sludge. Our observation that significantly higher levels of 4-nonylphenol are consistently

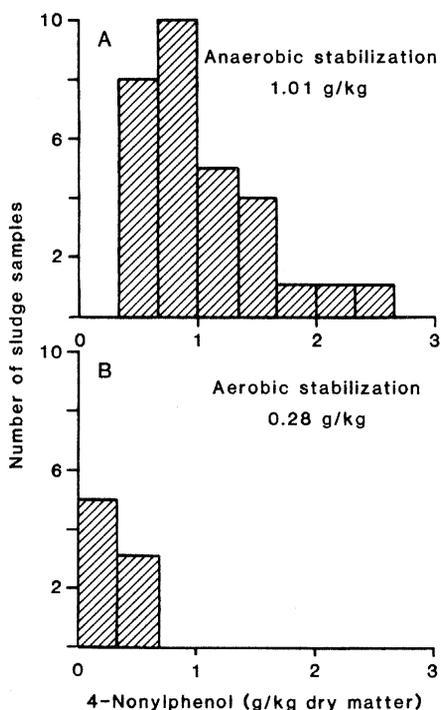


Fig. 3. Frequency distributions and arithmetic means of 4-nonylphenol concentrations in sewage sludge after anaerobic (A) and aerobic (B) stabilization. Samples are from Switzerland (33 treatment plants), Germany (three plants), and Finland (two plants).

found in anaerobically stabilized sludge indicates that anaerobic environments favor the accumulation of 4-nonylphenol. This suggestion is supported by several observations:

1) Garrison and Hill (9) reported increased concentrations of nonylphenol in industrial wastewater after anaerobic treatment.

2) In a treatment plant we found that samples of anaerobically treated sludge contained 0.89 g of 4-nonylphenol per kilogram of dry matter and aerobically stabilized samples 0.20 g/kg.

3) Raw and anaerobically stabilized sludge were mixed in equal parts and anaerobically digested for up to 40 days. In these controlled batch experiments in the laboratory we observed a four- to eightfold increase in the concentration of 4-nonylphenol related to the content already present in the raw sludge. In batch experiments involving aerobic incubation, 4-nonylphenol concentrations increased by a factor of only 2. 4-Nonylphenol concentrations did not increase in sterile controls.

Concentrations of 4-nonylphenol in digested sewage sludge are extraordinarily high compared to those of other contaminants. Heavy metals of similar toxicity are usually found at concentrations two orders of magnitude less. In sewage sludge from nonindustrial sources, polychlorinated biphenyls were found at concentrations of 0.35 to 23 mg per kilogram of dry matter (10) and polycyclic aromatic hydrocarbons at 20 to 30 mg/kg (11).

The toxicity of nonylphenol in aquatic ecosystems has been studied thoroughly (12, 13). Median lethal doses for fish and shrimp range from 0.13 to 0.3 mg/liter. Toxicity tests with *Daphnia magna* show a median effective concentration (EC<sub>50</sub>) for nonylphenol of 0.18 mg/liter. The maximum permissible concentration in sludge for substances of similar toxicity, such as cadmium (EC<sub>50</sub>, 0.35 mg/liter), is usually set at about 30 mg/kg.

Further research is needed to investigate the possible degradation of 4-nonylphenol when sewage sludge is applied to land and to study the effects on soil organisms and plants. The environmental risks of surface runoff and disposal of sludge at sea must also be assessed.

Past research has been devoted to measuring the concentrations of inorganic contaminants in sewage sludge and their impact on the environment. However, a recent report on mutagenic properties of sewage sludge (14) indicates that organic contaminants pose as many problems as do inorganic substances. It is therefore necessary to screen sewage

sludges for elevated concentrations of toxic organic chemicals and to study their formation and degradation during wastewater treatment, sludge treatment, and sludge disposal.

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## Mung Bean Nuclease Cleaves *Plasmodium* Genomic DNA at Sites Before and After Genes

**Abstract.** *Mung bean nuclease was found to cut the genomic DNA of the malaria parasite Plasmodium at positions before and after genes but not within gene-coding regions. This cleavage, which had nearly the preciseness of a restriction nuclease, required controlled conditions in the presence of formamide. Southern blot analysis showed that the coding areas for Plasmodium actin, circumsporozoite protein, histidine-rich protein, ribosomal RNA's, and tubulin are each cleaved from genomic DNA to yield a single major band on an agarose gel. DNA sequence data on several clones of mung bean nuclease cleavage products containing the gene for the circumsporozoite protein of Plasmodium falciparum confirmed that cleavage sites are before and after genes. Recognition and cleavage of DNA did not seem to be related to any primary sequence but may be related to structural features of the DNA duplex that demarcate genes. Mung bean nuclease-cleaved DNA could be inserted directly into a  $\lambda$  expression vector, yielding a representative but small gene bank of intact gene fragments.*

The conformation of double-stranded DNA has been shown to be variable. In addition to the familiar  $\beta$ -form helix, DNA can contain bends and kinks as well as have a Z-DNA configuration. Distinguishing these features along the DNA molecule undoubtedly plays a large part in the regulation of gene expression. Using species of the malaria parasites, *Plasmodium*, we show here that mung bean nuclease recognizes and cleaves the genomic DNA at specific locations before and after genes. The cleavage is directed by the structure of naked DNA since the nuclease cuts both cloned and genomic DNA to yield the same products. Because the cleavage points are related to the beginning and ending of genes and not to a primary sequence, we propose that the nuclease is recognizing defined conformational

structures that demarcate genes in the DNA of *Plasmodium* and of other organisms.

We cleaved genomic DNA with mung bean nuclease under a series of conditions and analyzed the products by the Southern blot techniques. Cloned genes isolated from *Plasmodium* and other organisms, or synthetic oligonucleotides, were used as probes. In each case, single major bands of *Plasmodium* DNA hybridized to each probe. Further, the bands were approximately the size needed to encode the corresponding gene. A number of genes were investigated in the same manner to see if the phenomenon was a general one or was specific to a single gene.

Four different DNA's were used as radioactively labeled probes for Southern blot analysis to investigate the re-

sults of mung bean cleavage of genomic DNA. Complementary DNA's (cDNA's) for chicken  $\beta$ -actin (1) and *Chlamydomonas* tubulin (2) were selected because they have been used to find corresponding genes in a broad range of organisms including parasitic protozoa (3). A series of different cleavage conditions was tried (4). Figure 1 shows an analysis of the reactions in 40 and 45 percent formamide. Southern blot analysis of *Plasmodium knowlesi* DNA after cleavage in 45 percent formamide shows a single predominant fragment at 1.6 kb hybridizing to the actin probe (Fig. 1A) and one at 2.6 kb hybridizing to the tubulin probe (Fig. 1B). These fragments are of adequate size to encode the corresponding genes. The 40 percent formamide reactions contain larger fragments that hybridize to the probes.

The area surrounding the histidine-rich protein (HRP) of *P. lophurae* (5) was investigated with a radioactively labeled oligonucleotide consisting of a sequence of five tandem histidine codon triplets as described (6). DNA from *P. lophurae* was made greater than 95 percent pure by passing it through a Hoechst dye CsCl gradient (7), and was then digested with mung bean nuclease in 30, 35, and 40 percent formamide. Analysis of the 30 and 35 percent reactions showed a band at 2.2 kb (Fig. 1C). This is the same size as the messenger RNA (mRNA) described (6) which codes for the HRP and hybridizes to the oligonucleotide probe. Again the mung bean nuclease seemed to excise a gene-sized fragment.

The gene for the circumsporozoite (CS) protein of *P. falciparum* was investigated by using as a probe the clone pMP5, which contains the complete coding area of the gene (8). In Southern blots only one band at 2.3 kb was seen in the 35 percent reaction. One major band of 1.3 kb and one minor species of slightly larger size were seen in the 40 percent reaction.

In general, the number of bands hybridizing to a probe did not increase as the formamide in the reaction mix was increased. The results indicated that the region of identity between each probe and the *Plasmodium* DNA was not cleaved under any of a series of conditions that reduce the size of the gene-containing fragments from large to "gene sized." We assume that this means that the coding regions are not cleaved even though, in the case of probes from other systems, sequence identity may not extend over an entire *Plasmodium* gene.

To determine the site of mung bean nuclease cleavage we cloned and se-