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Toxicity of N-Substituted Aromatics to Acetoclastic Methanogenic Activity in Granular Sludge

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N-substituted aromatics are important priority pollutants entering the environment primarily through anthropogenic activities associated with the industrial production of dyes, explosives, pesticides, and pharmaceuticals. Anaerobic treatment of wastewaters discharged by these industries could potentially be problematical as a result of the high toxicity of N-substituted aromatics. The objective of this study was to examine the structure-toxicity relationships of N-substituted aromatic compounds to acetoclastic methanogenic bacteria. The toxicity was assayed in serum flasks by measuring methane production in granular sludge. Unacclimated cultures were used to minimize the biotransformation of the toxic organic chemicals during the test. The nature and the degree of the aromatic substitution were observed to have a profound effect on the toxicity of the test compound. Nitroaromatic compounds were, on the average, over 500-fold more toxic than their corresponding aromatic amines. Considering the facile reduction of nitro groups by anaerobic microorganisms, a dramatic detoxification of nitroaromatics towards methanogens can be expected to occur during anaerobic wastewater treatment. While the toxicity exerted by the N-substituted aromatic compounds was closely correlated with compound apolarity (log P), it was observed that at any given log P, N-substituted phenols had a toxicity that was 2 orders of magnitude higher than that of chlorophenols and alkylphenols. This indicates that toxicity due to the chemical reactivity of nitroaromatics is much more important than partitioning effects in bacterial membranes.

N-substituted aromatic compounds, such as nitrobenzenes, nitrophenols, aminophenols, and aromatic amines, are widely used in the manufacturing of azo dyes, explosives, pharmaceuticals, and pesticides (8, 15). Nitrobenzene is produced annually in the order of 225,000 metric tons and it has been estimated that as much as 9,000 metric tons of nitrobenzene is discharged annually into natural waters (35). The presence of these aromatic xenobiotics in the environment may create serious public health and environmental problems. Some of these compounds have mutagenic or carcinogenic activity and may bioaccumulate in the food chain (8, 20). Many nitroaromatics have also been shown to be toxic or mutagenic to microorganisms (32, 39). The toxicity has been attributed to the fact that nitrophenols act as uncoupling agents in oxidative phosphorylation. Cell metabolism is affected at concentrations lower than 50 μM (32). Aerobic biodegradation of a variety of N-substituted aromatics has been well documented (13, 23, 31), whereas the anaerobic biodegradation and toxicity of these compounds have only recently been addressed (12, 26, 34). Some authors have reported on their toxicity as part of an overall study surveying the effects of xenobiotic compounds on anaerobic sludge (3, 10). However, the protocols employed in previous experiments were not fully adequate for N-substituted aromatics (3, 10, 19, 25, 34). In many of the assay procedures, the nitroaromatic test compounds were highly modified by reduction due to inappropriate selection of assay substrates (19, 25). Most authors used anaerobic media containing chemical reducing agents (sulfides) which have been shown to transform several nitroaromatic compounds (11); consequently, the bacteria were exposed only momentarily to the toxic compounds. In some of the previous protocols (25, 26, 34), toxicity was based not on methanogenic production rate but rather on comparison of the methane production of compound-amended cultures with that of controls within a given time period. Such comparisons may underestimate the true toxicity if incubation continues after exhaustion of the assay substrate in the control cultures.

The rationale behind the toxicity assay employed in this study was to minimize test compound biotransformation and to compare the rate of methane production in highly active methanogenic granular sludge. Acetate was used as the assay substrate since it is known to be a poor electron donor (9) and, thus, would result in minimal nitroaromatic modification. This is the first comprehensive study evaluating a wide range of N-substituted aromatics in a standardized batch toxicity assay. Such knowledge is essential in predicting the impact of these xenobiotics on anaerobic wastewater treatment, thereby preventing potentially costly upsets of treatment plant operations. A better understanding of the toxicity has made feasible the application of anaerobic treatment technologies to wastewaters containing other aromatic compounds (7, 12, 24, 40).

MATERIALS AND METHODS

Biomass. Methanogenic granular sludge from a full-scale upward-flow anaerobic sludge blanket reactor treating chemical industry wastewater of Shell Nederland Chemie at Moerdijk, The Netherlands, was used as the inoculum. The sludge was elutriated to remove fines and stored at 4°C before use. The sludge had not been previously acclimated to any of the N-substituted aromatics.

Basal medium. The basal medium used in the anaerobic toxicity assay contained the following (in milligrams per liter): $NaHCO_3$, 5,000; NH_4Cl, 280; $CaCl_2 \cdot 2H_2O$, 10; K_2HPO_4, 250; $MgSO_4 \cdot 7H_2O$, 100; yeast extract, 100; H_3BO_3, 0.05; $FeCl_2 \cdot 4H_2O$, 2; $ZnCl_2$, 0.05; $MnCl_2 \cdot 4H_2O$, 0.05; $CuCl_2 \cdot 2H_2O$, 0.03; $(NH_4)SeO_3 \cdot 5H_2O$, 0.05; $AlCl_3 \cdot 6H_2O$, 2; $NiCl_2 \cdot 6H_2O$, 0.05; $Na_2SeO_3 \cdot 5H_2O$, 0.1; EDTA, 1; resazurin, 0.2; as well as 36% HCl at 0.001 ml/liter.

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Analyses. The methane content in the gas samples was determined by gas chromatography (Packard-Becker, Delft, The Netherlands). The gas chromatograph was equipped with a steel column (2 m by 2 mm) packed with Poropak Q (80/100 mesh; Millipore Corp., Bedford, Mass.). The temperatures of the column, the injector port, and the flame ionization detector were 60, 200, and 220°C, respectively. The carrier gas was nitrogen at a flow rate of 20 ml/min.

Samples for measuring methane content (100 μl) in the headspace were determined with a pressure-lock gas syringe (Pressure-Lok series A-2), which was purchased from Dynatech Precision Sampling Corp., Baton Rouge, La. An isobaric precise proportion of the known headspace volume could be analyzed.

The pH was determined immediately after sampling with a model 511 pH-meter (Knick, Berlin, Germany) and a model N61 double electrode (Scot Gerade, Hofheim, Germany). The UV absorbance was measured with a Spectronic 60 spectrophotometer (Milton Roy/Analytical Products Division, Ostend, Belgium) and a model 100-QS (Hellma Benelux, The Hague, The Netherlands) 1-cm quartz cuvette. Absorption is reported as the absorption of the medium containing aromatic compounds minus the absorption of the control medium (which contained no test compounds). All samples were diluted to less than 0.8 absorbance unit in 0.2 M phosphate buffer (pH 7.0). Nitro group reduction of 2-nitrophenol and 4-nitrophenol was monitored at 370 and 400 nm, respectively. Aromatic ring absorption of 2-nitrophenol–2-aminophenol and 4-nitrophenol–4-aminophenol was monitored at 209 and 225 nm, respectively. All the other analytical determinations were performed as described in *Standard Methods for Examination of Water and Wastewater* (1).

Anaerobic toxicity assay. Specific acetoclastic methanogenic activity measurements were performed with 120-ml glass assay bottles sealed with 12-mm-thick butyl rubber septa (Rubber B.V., Hilversum, The Netherlands). Granular sludge (2 g of volatile suspended solids per liter) was transferred to vials containing 25 ml of the basal medium and acetate from a neutralized stock solution to yield a final concentration of 39.3 mM (2.5 g of chemical oxygen demand per liter). The maximum specific acetoclastic methanogenic activity of the control sludge was 890 mg of methane expressed as chemical oxygen demand per gram of volatile suspended solids per day. Assay bottles were then flushed with 70% N_2–30% CO_2 gas for 5 min and incubated overnight at 30°C. On the following day, vials which were still pink because of the lack of reduction of the redox indicator dye resazurin were discarded. The desired amount of the toxicant was added to duplicate vials from concentrated stock solutions. However, in the case of poorly soluble compounds, the compound was weighed out and introduced into the vials in solid form. Acidic test compounds were neutralized prior to their addition to the assay medium. Some aromatic amines (e.g., aminophenols), which are prone to autoxidation (7), were prepared fresh with 250 mg of ascorbic acid per liter to prevent oxidative coupling. Triplicate substrate controls were based on assays where no toxicant was added. Incubations were done in a temperature-controlled room at 30°C ± 2°C in an orbital-motion shaker (Gerhardt, Bonn, Germany) at 70 strokes · min^{-1}. After 3 days of exposure to the toxicant, the acetate concentration was replenished to 15.72 mM (1 g of chemical oxygen demand per liter) to assess the specific methanogenic activity. The headspace was reflushed with 70% N_2–30% CO_2 gas, and the assay bottles were reincubated for 1 h, prior to the determination of the methane production rate. The methane content in the headspace of each assay bottle was determined hourly during the subsequent 6- to 8-h incubation period.

The maximum specific methanogenic activity was calculated from the slope of the methane production versus time curve. To determine the degree of inhibition, the methanogenic activities of the control and samples containing inhibitory compounds were determined.

Chemicals. Chemicals were purchased from either Jannsen Chimica (Tilburg, The Netherlands), Merck (Darmstadt, Germany), or Sigma (Bornem, Belgium). All chemicals were of the highest purity available and were not purified further.

RESULTS

Biotransformation of test compounds during protocol. The nitro group absorbance maxima of nitrophenols were monitored during the 3-day exposure period of these test compounds with the anaerobic sludge. A small level of nitro group reduction did occur during the exposure period, accounting for 16 and 22% losses of the nitro group absorbance maxima of 2-nitrophenol and 4-nitrophenol, respectively. The use of ascorbic acid and the preincubation for the biological removal of dissolved oxygen were found to be sufficient measures in preventing the oxidative coupling of aromatic amines. No formation of visible light absorbance could be detected.

Effect of aromatic structure on methanogenic inhibition. The inhibitory effects of 25 aromatic compounds on the activity of acetoclastic methanogenic bacteria were evaluated in this study. The inhibition caused by each compound was tested at various levels, from concentrations that were nontoxic to those that were completely inhibitory concentration to acetoclastic methanogenic activity, as seen in a typical experiment with 3-nitrophenol in Fig. 1. Table 1 summarizes the 20, 50, and 80% inhibiting concentrations (ICs) of the aromatic compounds evaluated in this study. Those compounds which were not inhibitory at concentrations of 70 mM or less were considered to be nontoxic.

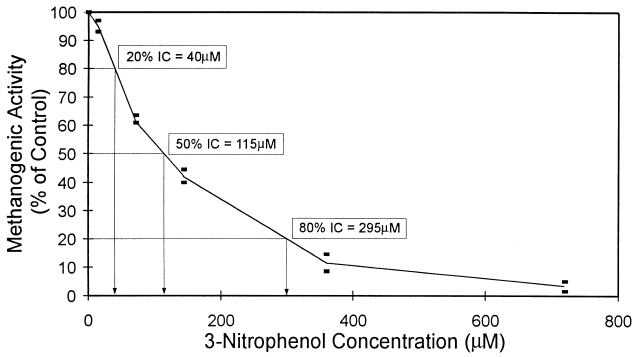

FIG. 1. Estimation of 20, 50, and 80% IC values of 3-nitrophenol to acetoclastic methanogens.

The least toxic compounds were benzene, benzoate, phenol, and the aromatic amines. Nitrobenzenes, nitrophenols, and nitroanilines were among the most toxic compounds. The most toxic compound tested was 2-nitroaniline, having a 50% IC of 14 μM.

TABLE 1. The 20, 50, and 80% IC values observed in this study for various aromatics

Compound no. and name	Mol wt	Log P[a]	IC (μM)		
			20%	50%	80%
1. Benzene	78	1.95	10,580	20,500	ND[b]
2. Aniline	93	0.9	5,000	9,670	14,100
3. Nitrobenzene	123	1.85	41	81	210
4. 2-Nitroaniline	138	1.83	7	14	70
5. 3-Nitroaniline	138	1.37	7	30	212
6. 2-Phenylenediamine	108	0.15	9,760	18,920	27,500
7. 3-Phenylenediamine	108	0.03	29,500	65,700	NT[c]
8. Phenol	94	1.46	7,140	13,830	20,510
9. 2-Aminophenol	109	0.57	1,650	3,210	4,920
10. 3-Aminophenol	109	0.16	9,700	18,810	27,100
11. 4-Aminophenol	109	0.104	7,330	14,220	21,100
12. 2,4-Diaminophenol	124	NA[d]	146	283	510
13. 2-Nitrophenol	139	1.79	46	89	200
14. 3-Nitrophenol	139	2.0	40	115	295
15. 4-Nitrophenol	139	1.91	31	61	180
16. 2,4-Dinitrophenol	184	1.67	22	43	130
17. 2,5-Dinitrophenol	184	1.80	6	114	270
18. Benzoic acid	122	1.87	34,900	NT[c]	NT[c]
19. 2-Aminobenzoic acid	137	1.21[e]	31,100	67,000	NT[c]
20. 3-Aminobenzoic acid	137	0.20[e]	NT[c]	NT[c]	NT[c]
21. 4-Aminobenzoic acid	137	0.68[e]	NT[c]	NT[c]	NT[c]
22. 2-Nitrobenzoic acid	167	1.28[e]	277	538	980
23. 3-Nitrobenzoic acid	167	1.83[e]	50	96	140
24. 4-Nitrobenzoic acid	167	1.89[e]	30	120	250
25. 2,4-Dinitrotoluene	182	2.0	5	27	59

[a] Log P values were obtained from the literature (21, 28, 36).
[b] ND, not determined; compound not soluble above 24 mM (30°C).
[c] NT, not toxic; compounds were considered to be nontoxic if this value was greater than 70 mM.
[d] NA, not available; log P value was not available.
[e] Not included in log P correlations because the carboxyl group dissociates at the assay pH of 7.

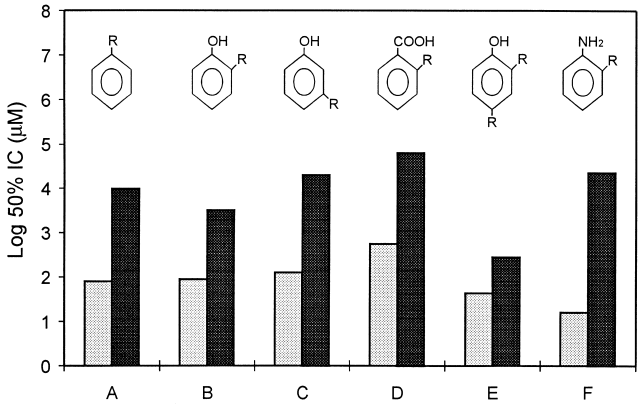

FIG. 2. Comparison of the toxicity of selected nitroaromatic compounds and their aromatic amine counterparts towards acetoclastic methanogens. Bars: A, nitrobenzene and aniline; B, 2-nitrophenol and 2-aminophenol; C, 3-nitrophenol and 3-aminophenol; D, 2-nitrobenzoic acid and 2-aminobenzoic acid; E, 2,4-dinitrophenol and 2,4-diaminophenol; F, 2-nitroaniline and 2-phenylenediamine. Symbols: ▨, R = NO_2 group; ■, R = NH_2 group.

The results obtained indicate that some general relationships exist between the aromatic structures and their inhibitory effects on methanogenic bacteria. N-substitutions were more toxic than other ring substituents or benzene itself. The toxicity of the monosubstituted benzenes was observed to increase in the following order: COOH < H < OH < NH_2 < NO_2.

The type of N-substitution had a profound effect on its toxicity. Figure 2 clearly demonstrates that aromatic amines were much less inhibitory than their corresponding nitroaromatic analogs. The nitroaromatics were from 6- to 1,350-fold more toxic than their amino-substituted counterparts.

Structure-toxicity relationships were also evident for aromatic compounds with more complex substitution patterns. The addition of an ortho carboxyl group to nitrobenzene greatly decreased the toxicity. In strong contrast, the addition of an amino group (i.e., 2-nitroaniline) increased the toxicity towards methanogens.

Correlation of toxicity with compound hydrophobicity. To determine if the lipophilic character of the aromatics tested could be correlated with their methanogenic toxicity, the logarithm of the 50% IC values of seventeen N-substituted aromatics were plotted against the logarithm of the octanol-water partition coefficient (log P) of the compounds. A significant correlation was obtained ($R^2 = 0.91$, $P < 0.001$), indicating that the partitioning of apolar N-substituted aromatics into lipophilic membranes in bacteria may have a role in the toxicity. However, certain functional groups might be expected to undergo chemical interactions with proteins, and consequently, enzymes could become inhibited as well. Therefore, when comparing compounds that possess different types of substitutions, a perfect correlation with the log P of the compound cannot be expected. A higher correlation could potentially be obtained by comparing compounds in a homologous series. Figure 3 illustrates the correlations determined with the toxicity data of the N-substituted phenols and benzenes, respectively. The methanogenic toxicity of the N-substituted phenols (aminophenols and nitrophenols) were even more highly correlated to the log P data ($R^2 = 0.95$, $P < 0.001$). However, the correlation with N-substituted benzenes (anilines, nitroanilines, nitrobenzene, and dinitrotoluene) was not as high ($R^2 = 0.876$, $P < 0.01$) because the nitroanilines exerted a higher toxicity than nitrobenzene did with a comparable log P value. In Fig. 3, regression lines are also plotted from the acetoclastic

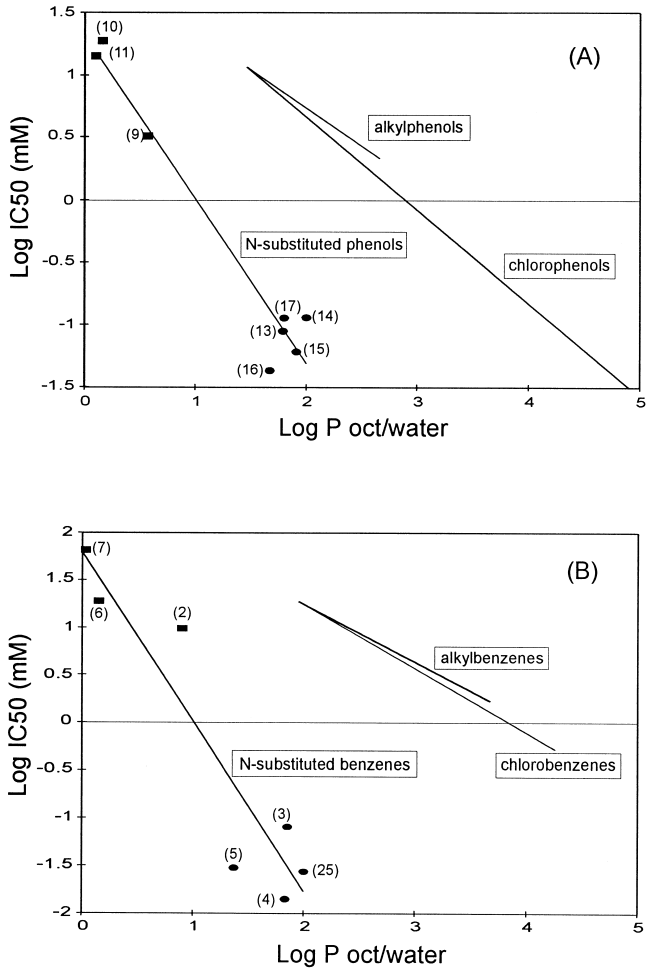

FIG. 3. (A) Effect of hydrophobicity on the methanogenic toxicity of homologous series of phenols. (B) Effect of hydrophobicity on the methanogenic toxicity of homologous series benzenes. Compounds are referred to by their compound numbers as reported in Table 1. Alkylphenols ($R^2 = 0.989$), chlorophenols ($R^2 = 0.990$), alkylbenzenes ($R^2 = 0.983$), and chlorobenzenes ($R^2 = 0.988$) are adapted from a previous study (29).

methanogenic toxicity data of alkyl- and chloro-substituted phenols and benzenes reported by Sierra and Lettinga (29). The measured 50% IC values of phenol and benzene from our study are plotted in the graphs and coincide with their data, indicating that the toxicity results from the two studies are compatible. At any given log P, the alkyl and chloro substituents of phenols and benzenes were approximately 2 orders of magnitude less toxic than the N-substituted analogs. This observation clearly indicates a higher chemical reactivity of aromatic nitro and amino groups compared with that of alkyl and chloro groups.

DISCUSSION

Preventing test-compound modification. Nitroaromatic compounds are easily reduced by microorganisms (6, 11, 14) and abiotic reducing agents (11, 16, 33). Even lysed cells of methanogenic bacteria reduce nitroaromatics (11). Consequently, several precautions were taken to minimize nitro group reduction during the toxicity assay. The practice of adding reducing agents (e.g., sulfide) to chemically remove dis-

solved oxygen was replaced with a medium preincubation step to biologically remove dissolved oxygen.

Anaerobic media were prepared with acetate as the substrate. Previous studies concerned with the reductive dehalogenation of chlorinated hydrocarbons indicate that acetate is a poor electron donor (9) compared with other substrates commonly used for methanogenic activity assays. In this study, we were also able to demonstrate that it was not very effective in reducing nitroaromatics. The reductive biotransformation of nitroaromatics was limited to 25% during the 3-day test-chemical exposure period. This can be viewed as a major improvement over assay substrates that provide interspecies electrons (e.g., H_2), such as ethanol, which was shown to completely convert 500 mg of nitrobenzene per liter into aniline in less than 1 day (19, 41). In experiments where ethanol or other good electron donors (e.g., propionate) have been used as an assay substrate, the 50% IC values of nitroaromatics were underestimated by approximately a factor of 10 (25, 41) because of the elimination of nitroaromatics occurring during the assay. Our nitroaromatic toxicity results are only in agreement with literature data in which acetate was used as the sole substrate (3, 5, 38).

Aromatic amines are more persistent to biotransformations in anaerobic environments (7). No losses in aromatic amines could be detected during the 3-day test-compound exposure period. Nonetheless, after acclimatization of sludge for longer time periods, anaerobic mineralization of 2- and 4-aminophenols occurs (25, 26). In one study (3), aminophenols were 60-fold more toxic than indicated by our data. A possible explanation for this deviance is the ease by which aminophenols could become partially auto-oxidized during the preparation of the experiment, leading to the formation of more toxic oligomers (7). The auto-oxidation reactions were prevented in our study by preparing stock solutions together with ascorbic acid and adding the stock solution to the culture only after all dissolved oxygen was removed.

Toxicity of N-substituted aromatics. Nitroaromatics were clearly very toxic compounds to methanogens, with 50% IC values generally ranging from 0.014 to 0.12 mM. Aromatic amines, in contrast, were less inhibitory; the 50% IC values were for the most part between 3.2 and 67 mM. Nitroaromatic compounds were, on the average, 500-fold more toxic than their corresponding aromatic amine analogs, indicating that the facile reduction of nitroaromatics known to occur in anaerobic environments (11, 26, 27, 41) would be responsible for a dramatic detoxification of nitroaromatics towards methanogens.

Increasing the number of nitro groups beyond one had little effect in altering the toxicity of nitrobenzenes. The addition of an extra amino group to aminophenol resulted in a more toxic compound, while the addition of an amino group to aniline resulted in less toxic phenylenediamines. However, the combination of nitro and amino groups, e.g., nitroanilines, was found to be the most toxic substituent pattern, with 50% IC values ranging from 0.014 to 0.030 mM.

Log P-toxicity correlations. The hydrophobicity of a compound as indicated by log P is directly related to the partitioning of the compound into bacterial membranes (17, 30). Compounds of greater hydrophobicity are expected to accumulate more efficiently in membranes, causing a greater disturbance to the membrane structure, and consequently, they are responsible for a higher toxicity. The accumulation of apolar pollutants in bacterial membranes causes the membrane to swell and leak, disrupting ion gradients and eventually causing cell lysis (17, 30). Methanogens rely almost entirely on membrane potential (H^+ and Na^+ gradients) to obtain energy during their metabolism (37). When the methanogenic toxicity data of N-aromatics were plotted as a function of the log P, a strong linear fit was obtained, indicating that partitioning into membranes was an important factor contributing to the toxicity of the most toxic N-substituted aromatics. In this study, the compounds ranged from highly polar aromatic amines of low toxicity to apolar nitroaromatics of high toxicity. The decrease in compound toxicity due to the presence of a carboxy group can also be rationalized in terms of compound polarity, since this group (pK_a = 2.16 to 6.94) would be highly dissociated at the assay pH of 7. Consequently, in accordance with a previous report (29), these compounds were not included in the overall correlation.

Many other types of aromatic and phenolic compounds have been reported to be inhibitory to methanogenic bacteria (3, 10, 29). High linear correlations of their methanogenic toxicity to the log P of the compound has also been observed in the case of alkyl- and chloro-substituted benzenes and phenols (29). However, these relatively nonreactive compounds are approximately 100-fold less toxic than N-substituted aromatics with the same log P values. Thus, when present at similar concentrations in bacterial membranes, the N-substituted aromatics exert a much higher toxic effect than that which can be accounted for by membrane toxicity alone.

Chemical reactivity of N-substituents. Nitroaromatics have been reported to be reactive toxicants (3, 18, 22). Nonaromatic nitrogen oxides were reported to inhibit the activity of some component in the methanogenic enzyme complex itself (2). The reactivity of the N-substituents could enable N-substituted aromatics to undergo sorptive and chemical interactions with proteins, thereby inactivating vital enzymes (4, 18). The toxicity of these compounds to methanogens has been suggested to involve interactions between nitroaromatics or intermediates of the reduction process (nitrosoamines or hydroxylamines) and the unique cell membrane of the methanogens (11). N-substituted aromatics may also interfere with the outcome of a biochemical conversion, such as the uncoupling of phosphorylation reactions (32) or interfering with physiological redox couples. The methanogenic toxicity of N-substituted aromatics was found to be the most pronounced for nitroanilines. The nitroanilines have the highest dipole moment of the compounds tested, making them the most chemically reactive compounds.

Conclusions. In this study, the aminoaromatics were determined to be considerably less toxic than the parent nitroaromatic compound. The ability of anaerobic consortia to remove and detoxify the nitro group in nitroaromatics would make anaerobic processes a useful treatment adjunct and/or alternative to conventional aerobic systems. In particular, anaerobic nitro group reduction may be an important initial step, which when followed by aerobic posttreatment, could result in complete mineralization of such highly nitrated compounds as trinitrotoluene and picric acid, which are highly resistant to aerobic degradation (7).

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