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The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge

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Summary. Benzene derivatives are important constituents of certain effluents discharged by pulp and paper, petrochemical and chemical industries. The anaerobic treatment of these waste-waters can be limited due to methanogenic inhibition exerted by aromatic compounds. The objective of this study was to evaluate the effect of aromatic structure on acetoclastic methanogenic inhibition. The toxicity to acetoclastic methanogens was assayed in serum flasks utilizing granular sludge as inoculum. Among the monosubstituted benzenes, chlorobenzene, methoxybenzene and benzaldehyde were the most toxic with 50% inhibition occurring at concentrations of 3.4, 4.2 and 5.2 mM, respectively. In contrast, benzoate was the least inhibitory: concentrations up to 57.3 mM were non-toxic. In general, the toxicity of aromatic compounds increased with increasing length of aliphatic side-chains, increasing the number of alkyl or chlorine groups. The logarithm of the partition coefficient octanol/water (log *P*), an indicator of hydrophobicity, was observed to be positively correlated with the methanogenic inhibition. The results indicate that hydrophobicity is an important factor contributing to the high toxicity of the most inhibitory aromatic compounds.

Introduction

Aromatic compounds are present in natural environments as derivatives of lignin, tannins, phenolic amino acids, and other aromatic plant components. Human activities also contribute to the presence of aromatics in the environment. Waste incineration, mining and the discharge of waste-water streams generated by petrochemical factories and paper manufacturing and chemical industries, among others, are important sources of aromatic pollution.

The presence of aromatic xenobiotics in the environment may create serious public health and environmental problems. Some aromatics are mutagenic or carcinogenic and some may bioaccumulate. Additionally, man-made aromatics are often resistent to biodegradation and toxic to microorganisms.

Traditionally, biological treatment of aromatic containing waste-waters was based on aerobic processes. Recently, anaerobic processes have been used more often for the treatment of these complex waste-waters (Salkinoja-Salonen et al. 1983; Suidan et al. 1988; Kim et al. 1986; Borghans and van Driel 1988). However, their application is restricted by the limited data available on the behaviour of aromatic compounds in anaerobic treatment systems.

Although the anaerobic biodegradation of aromatic compounds has been extensively investigated, as reviewed by Young (1984), Colberg (1988), Hollinger et al. (1988) and Schink (1988), little attention has been given to the toxic effects of these compounds on anaerobic microbial communities. The purpose of this study was to evaluate the toxicity of several aromatic compounds to acetoclastic methanogens in unacclimated methanogenic granular sludge. The inhibitory effects of homologous series of aromatic compounds were assessed to study the relationships between chemical structure and methanogenic toxicity.

Materials and methods

Biomass. Elutriated methanogenic granular sludge from a full scale upward-flow anaerobic sludge blanket reactor treating distillery waste-water was used as inoculum. The sludge was stored at 4° C, and reactivated by incubation at 30° C in the presence of volatile fatty acids. The sludge used was not acclimated to aromatic compounds prior to the experiments.

Basal medium. The basal medium used in the methanogenic toxicity assay contained (mg/l): $NaHCO_3$ (400), 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),

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$MnCl_2 \cdot 4H_2O$ (0.05), $CuCl_2 \cdot 2H_2O$ (0.03), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.05), $AlCl_3 \cdot 6H_2O$ (0.09), $CoCl_2 \cdot 6H_2O$ (2), $NiCl_2 \cdot 6H_2O$ (0.05), $Na_2SeO_3 \cdot 5H_2O$ (0.1), EDTA (1), resazurin (0.2) and 36% HCl (0.001 ml/l). All chemicals were of analytical grade (Merck, Darmstadt, FRG). The yeast extract was supplied by Gist-Brocades (Delft, The Netherlands).

Analyses. Acetic acid was determined with a HP5890A gas chromatograph (Hewlett-Packard, Avondale, Pa, USA) equipped with a 2 m × 4 mm glass column packed with Supelcoport (100–120 mesh), coated with 10% Fluorad FC 431 (Supelco, Bellefonte, Pa, USA). The temperature of the column, the injection port and the flame ionization detector were 130, 220 and 240° C, respectively. Nitrogen saturated with formic acid was used as carrier gas at a flow rate of 50 ml/min.

The methane content in the gas samples was determined by gas chromatography. The column was packed with a molecular sieve 5A (mesh 60–80). The temperature of the column, the injection port and the flame ionization detector were 60, 200, 220° C, respectively. The carrier gas was nitrogen at a flow rate of 14.3 ml/min.

All the other analytical determinations were performed according to standard methods (APHA, 1985).

Anaerobic toxicity assay. Specific acetoclastic methanogenic activity measurements were performed in 315-ml glass serum flasks. Methanogenic granular sludge (1 g volatile suspended solids/l) was transferred to the serum vials containing 100 ml basal medium and acetate was added from a neutralized stock solution to obtain a final concentration of 30 mM. Subsequently, the liquid and head space were flushed with nitrogen gas. The flasks were sealed with a rubber septum and a screw cap and placed in a reciprocating shaker at 30 ± 2° C.

After 1 day of incubation the acetate concentration in the assay media was measured and replenished to obtain a concentration of 30 mM. The required amount of inhibitory compound was added to each flask to provide the toxic concentration to be investigated. Acidic test compounds were neutralized prior to their addition to the assay medium. Substrate controls were based on assays where no toxicant was added. Finally, after flushing the head space with nitrogen gas, the flasks were incubated again.

After 2 days of exposure to the toxicants, the acetate concentration was replenished to 30 mM in order to assay the specific methanogenic activity. The head space was flushed with nitrogen gas and the bottles were reincubated for 1 h prior to determination of the methane production rate. The methane composition in the head space content of each serum flask was determined periodically during the 4–5 h period that followed. The maximum specific acetoclastic methanogenic activity was calculated from the slope of the methane production versus time curve. The inhibited activity was expressed as a percentage of the control activity (% ACT). The percentage inhibition (% I) was defined as: % I = 100 − % ACT. The compound concentrations that caused 50% and 80% inhibition of the methanogenic activity are referred to as 50% IC and 80% IC, respectively.

Chemicals. All chemicals were purchased from Janssen Chimica (Tilburg, The Netherlands) and Merck (Darmstadt, West Germany).

Results and discussion

The inhibitory effects of 34 aromatic compounds on the activity of acetoclastic methanogenic bacteria were evaluated in this study. The compound concentrations resulting in 50% and 80% IC of the acetoclastic activity are summarized in Table 1. Those compounds that were not inhibitory at a concentration lower than 40 mmol/l were considered to be non-toxic as shown in the table.

The various aromatic compounds caused different levels of inhibition as indicated by the wide range of the 50% IC values observed (Table 1). Pentachlorophenol was the most toxic compound studied, causing a 50% inhibition at 0.03 mM, whereas compounds such as benzoate were non-toxic at concentrations as high as 57.3 mM.

The experimental results suggest that some general relationships between the molecular structure of aromatic compounds and their inhibitory effects on acetoclastic methanogens can be established. Bioassay results indicated that ring substitution is an important factor determining the inhibition caused by aromatic compounds. The toxicity of the monosubstituted benzenes (Fig. 1) was found to increase in the following substituent order:

$$COOH < SO_3H < H < OH < CH_3 < CHO < OCH_3 < Cl$$

Structure-toxicity relationships were also evident for aromatic compounds with more complex substitution patterns. Structural features associated with increasing inhibition included increasing the number and the length of alkyl substitutions, as well as an increasing number of chlorine atoms on the aromatic compound. Figure 2 illustrates the higher toxicity of the methyl-substituted aromatics as compared to their homologous counterparts with less or no methyl groups. The inhibitory effect of alkylbenzenes with increasing carbon chain length is compared in Fig. 3. Benzene caused the least inhibition, whereas propylbenzene, with the longest alkyl side chain, was the most inhibitory of this homologous series. Figure 4 illustrates the distinct effect of chlorine atoms on increasing the toxicity of aromatic compounds. These structure-toxicity relationships are not unique to acetoclastic methanogens as similar results are evident in literature reports concerning the toxicity of aromatic compounds towards a wide range of organisms (Bringmann and Kuhn 1980; Chou et al. 1978; Jurd and Manners 1980; Ruckdeschel et al. 1987).

Our toxicity results show that the addition of a functional group containing an oxygen or sulphur hetero-atom, such as carboxylic and sulphonic substitutions, often decreased the toxicity of aromatics. However, the aldehyde group was an exception since it caused increased inhibition. The effect of the hydroxyl group on the inhibition exerted by the aromatic compounds tested in our study was diverse. Addition of the first hydroxyl group to the aromatic ring increased somewhat the toxicity of the aromatic compound. However, a dihydroxy-benzene (catechol) was less inhibitory than phenol, suggesting that further introduction of hydroxyl groups may lead to less inhibitory compounds. Decreasing methanogenic inhibition has been found to correspond with increasing hydroxyl substitutions in studies with phenolic monomers including phenol, catechol, resorcinol, pyrogallol and phloroglucinol (Chou et al. 1978; Field et al. 1987; Field and Lettinga 1989). Furthermore, we observed that adding a polar func-

Table 1. The 50% and 80% inhibitory concentrations (IC) observed in this study for various aromatic compounds

Compounds	Molecular weight	50% IC	80% IC	Log P^a
		← (mmol/l) →		
Miscellaneous				
(1[b]) Benzene	78.1	18.91	23.04	1.95
(2) 4-Methylbenzaldehyde	120.1	4.25	6.18	1.98
(3) Benzaldehyde	106.1	5.03	7.92	1.48
(4) Methoxybenzene	108.1	4.61	7.40	2.11
(5) 2-Methylanisole	122.2	2.74	3.69	2.61
(6) 1,3,5-Trimethoxybenzene	168.2	1.58	2.08	NA
(7) 4-Hydroxystilbene	196.3	0.33	0.43	4.31
Alkyl-benzenes				
(8) Methylbenzene	92.1	6.76	8.14	2.69
(9) Ethylbenzene	106.2	4.95	5.65	3.15
(10) 1,2-Dimethylbenzene	106.2	4.23	5.03	3.15
(11) 1,3,5-Trimethylbenzene	120.2	2.59	3.34	3.42
(12) Allylbenzene	118.2	2.13	3.40	3.23
(13) *n*-Propylbenzene	120.2	1.66	3.54	3.68
(14) Styrene	105.2	0.09	0.38	3.00
Chloro-benzenes				
(15) Chlorobenzene	112.6	3.38	4.18	2.84
(16) 1,2-Dichlorobenzene	147.0	1.22	1.77	3.53
(17) 1,2,4-Trichlorobenzene	181.5	0.52	0.66	4.26
Chloro-phenols				
(18) 2-Chlorophenol	128.6	3.19	4.01	2.17
(19) 2,4-Dichlorophenol	163.0	0.49	0.64	3.15
(20) 2,4,6-Trichlorophenol	197.6	0.59	0.91	3.38
(21) 3-Chloro-5-methoxyphenol	158.6	0.41	0.79	NA
(22) Pentachlorophenol	266.3	0.03	0.05	5.01
Apolar phenols				
(23) 1,2-Dihydroxybenzene	110.1	16.47	24.66	1.01
(24) 2-Methoxyphenol	124.1	9.39	14.95	1.48
(25) Phenol	94.1	11.69	18.38	1.46
(26) 4-Methylphenol	108.1	5.26	7.23	1.94
(27) 4-Ethylphenol	122.2	2.13	4.09	2.66
Alcohols				
(28) 2-Phenylethanol	122.2	46.53	66.46	NA
(29) Phenylmethanol	108.1	31.74	42.17	1.10
Carboxylic and sulphonic acids				
(30) Benzoic acid	122.1	NT	NT	1.87
(31) 3-Phenylpropionic acid	150.2	NT	NT	NA
(32) Phenylacetic acid	136.2	5.27	9.18	1.41
(33) 4-Phenolsulphonic acid	174.2	NT	NT	NA
(34) Sulphonic acid	158.2	36.86	NT	−2.25

[a] The logarithm of the partition coefficient *n*-octanol/water (log *P*) values were obtained from the literature (Keuning and Janssen 1987; Laane et al. 1986; Leo et al. 1971; Verscheuren 1983; Vighi and Calamari 1987)

[b] Compound identification number

NA, non-available (i.e. the log *P* value was not available); NT, non-toxic (compounds were considered to be non-toxic if the 50% IC was higher than 40 mM)

tional group to an alkylic side chain generally contributed to a significant decrease in the aromatic toxicity (Fig. 5). The latter observation suggests that the alkyl side-chain structure plays an important role in the antimicrobial activity of aromatic compounds. Similarly, literature reports show that the presence of polar func-

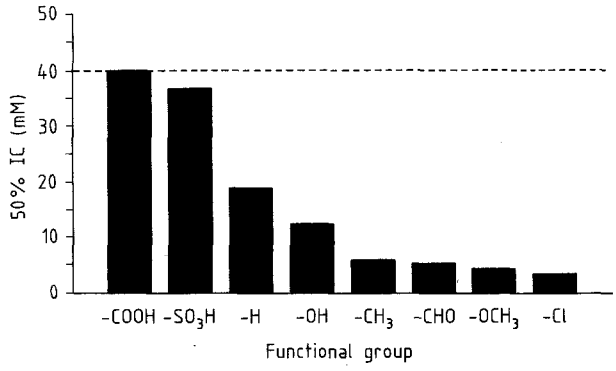

Fig. 1. Effect of the functional group on the toxicity exhibited by mono-substituted benzenes to acetoclastic methanogens in granular sludge; 50% IC, compound concentrations causing 50% inhibition of methanogenic activity

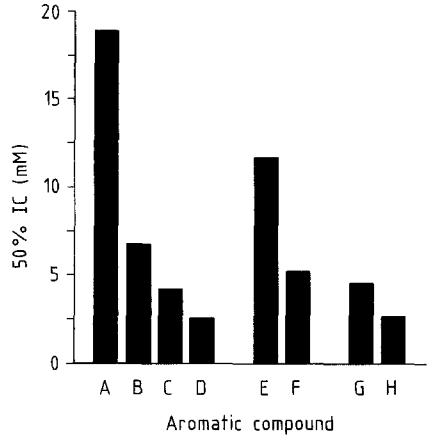

Fig. 2. Effect of methyl groups on the toxicity of aromatic compounds to acetoclastic methanogens in granular sludge: *A*, benzene; *B*, methylbenzene; *C*, 1,2-dimethylbenzene; *D*, 1,3,5-trimethylbenzene; *E*, phenol; *F*, 4-methylphenol; *G*, methoxybenzene; *H*, 2-methylanisole

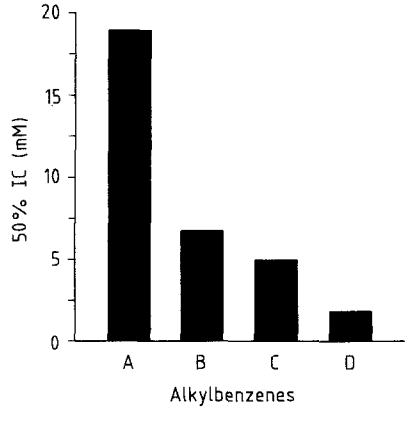

Fig. 3. The acetoclastic toxicity of several alkylbenzenes with increasing carbon chain length: *A*, benzene; *B*, methylbenzene; *C*, ethylbenzene; *D*, *n*-propylbenzene

tional groups on aliphatic side chain often results in a decrease in the microbial toxicity of aromatics (Zemek et al. 1979).

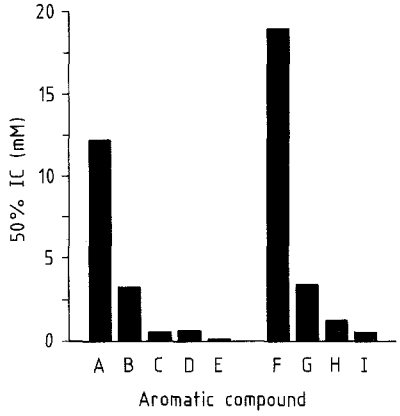

Fig. 4. The acetoclastic toxicity of several chlorinated aromatics with increasing chlorine atom number: *A*, phenol; *B*, 2-chlorophenol; *C*, 2,4-dichlorophenol; *D*, 2,4,6-trichlorophenol; *E*, pentachlorophenol; *F*, benzene; *G*, chlorobenzene; *H*, 1,2-dichlorobenzene; *I*, 1,2,4-trichlorobenzene

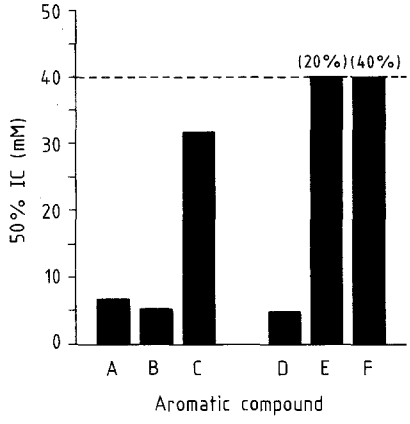

Fig. 5. Effect of adding a hydroxyl or carboxylic group to the alkyl side chain on the acetoclastic toxicity of aromatics: *A*, methylbenzene; *B*, phenylacetic acid; *C*, phenylmethanol; *D*, ethylbenzene; *E*, 3-phenylpropionic acid; *F*, 2-phenylethanol. Numbers in parenthesis indicate the inhibition (%) at the highest concentration tested

Correlations of toxicity with octanol/water partition coefficient

The logarithm of the *n*-octanol/water partition coefficient (log *P*) is widely used as an indicator of the hydrophobic character of organic compounds (Leo et al. 1971; Verscheuren 1983). Increasing hydrophobicity leads to easier passage of the organic compound through membranes and greater distribution to lipophilic areas of the organism (Florence et al. 1984; Galbraith and Miller 1973; Parsons and Opperhuizen 1987). Consequently, log *P* has been successfully used to predict partitioning and bioconcentration phenomena of hydrophobic organic pollutants in aqueous systems (Neely et al. 1974; Chiou et al. 1977; McKay 1984). It is also interesting to note that in numerous Quantitative Structure-Activity Relationship (QSAR)

studies (Hansch and Dunn 1972; Wayne Schultz et al. 1978; Kamlet et al. 1986) the toxicological properties of a wide array of organic chemicals correspond inversely with water solubility and directly with log *P*. The toxic effects observed in these studies were attributed to adsorption or bioaccumulation of the aromatic compounds on the organisms tested.

The results of this study indicated that hydrophobicity is also an important factor contributing to the toxicity of aromatic compounds to acetoclastic methanogens. As shown in Fig. 6, a moderate correlation was found between toxicity and hydrophobicity when log (50% IC) was plotted against log *P* for all the data points of this study. In contrast, very good correlations between toxicity and hydrophobicity were observed within series of homologous compounds such as alkylbenzenes, alkylphenols, chlorobenzenes and chlorophenols, respectively, as shown in Fig. 7. However, the high correlation disappeared when the compounds from each of these series were combined (Table 2). These results imply that log *P* is only strongly related to toxicity within homologous series of toxicants that have a similar mode of inhibitory action. Strong positive correlations between toxicity and partition coefficient within series of structurally related organic contaminants have also been reported earlier by several research groups using aerobic bacteria (Liu et al. 1982), yeast (Kwasniewska and Kaiser 1983), ciliates (Wayne Schultz et al. 1978), crustacea (Kopperman et al. 1974) and fish (Kaiser et al. 1984) as test organisms.

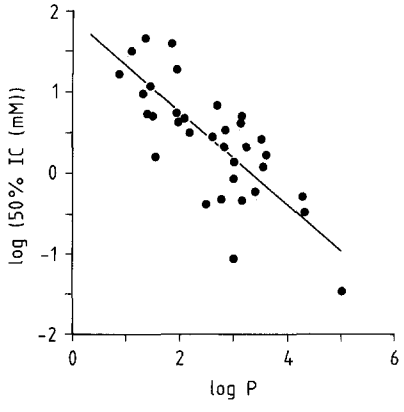

Fig. 6. The effect of hydrophobicity (log *P*) on acetoclastic toxicity [log (50% IC)] exerted by the aromatic compounds evaluated in this study

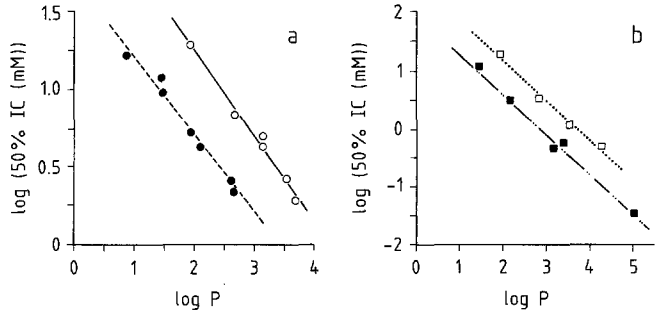

Fig. 7 A–B. The effect of hydrophobicity on the acetoclastic toxicity of homologous series of aromatic compounds: **A** Alkylbenzenes (—○—); Alkylphenols (- -●- -). **B** Chlorobenzenes (··· □ ···); Chlorophenols (— ·· ■ ·· —). Compounds included in the series were (1, 8–11, 13), (4, 5, 23–27), (1, 15–17) and (18–20, 22, 25), respectively (see Table 1)

Table 2. Linear relationships of hydrophobicity-methanogenic toxicity [log (50% IC) = *b* log *P* + *a*]

Homologous series	*b*	*a*	*n*	*r*
(A) Alkylbenzenes	−0.549	2.350	6	0.983
(B) Alkylphenols	−0.544	1.792	7	0.989
(C) Chlorobenzenes	−0.684	2.548	4	0.988
(D) Chlorophenols	−0.712	2.076	5	0.990
A, B, C and D combined	−0.551	1.951	20	0.735

b, regression coefficient; *a*, intercept; *n*, number of data points evaluated; *r*, linear correlation coefficient

The slope and the intercept values of the linear regression between log (50% IC) and log *P* provide numerical indexes for comparing structure-toxicity relationships. The slope is a measure of the response of the biological system to increasing hydrophobicity within a series of homologous toxicant compounds, whereas the intercept indicates the intrinsic toxicity of the series. The intercept is also related to the sensitivity of the test organism or biochemical system to the toxicants (Hansch and Dunn 1972).

In our study, the chloro-aromatics have similar slopes, regardless of whether they are derivatives of benzene or phenol. In a similar fashion the alkylbenzenes and alkylphenols had nearly equal slopes. However these were distinctly lower than those observed for chloro-aromatics. This results demonstrate that the increment of toxic activity resulting from increasing hydrophobicity clearly depends on the type of functional group added (i.e. chlorine or methyl). Therefore, log *P* is not the sole factor determining toxicity but rather it is a parameter indicating the response of bacteria to the hydrophobic effects of a determined toxicant series.

Furthermore, when considering the intercept values obtained, the toxicity of alkyl- or chlorophenols at a given level of hydrophobicity was superior to that of the corresponding benzene derivatives. This indicates that phenol derivatives have an additional mode of action beyond that which would be predicted from the hydrophobicity of the benzene derivatives. This additional toxicity mechanism is perhaps related to the hydrogen bonding phenomena of the phenol group (Kamlet et al. 1986).

It should be noted that the toxicity of some compounds differed distinctly from that expected on the basis of their hydrophobicity, suggesting that their toxic activity is not controlled by partitioning and that it may involve specific mechanisms. In this respect, carboxylic and sulphonic acids, with very low toxicities and intermediate log *P* values, deviate significantly from the cor-

relation. Log P may not be an appropriate hydrophobicity indicator for organic acids and bases since their aqueous solubility may vary drastically with changes in pH. Furthermore, compounds bearing aldehyde groups or containing side chains with double bonds caused significantly higher acetoclastic inhibition than anticipated when only considering their hydrophobic character. Likewise, greater toxicities to various organisms than predicted by QSAR have been reported for various aldehydes (Kamlet et al. 1986) and allylic organics (Hansch and Dunn 1972). Certain polar phenols also form an important exception to the trend relating increasing hydrophobicity with toxicity. Tannins, which are highly hydrophilic phenols, are highly toxic to methanogenic bacteria (Field and Lettinga 1987; Field et al. 1988). The inhibition results from strong hydrogen bonding interactions between bacterial proteins and tannins (Field et al. 1989).

In conclusion, strong hydrophobicity-toxicity correlations should only be expected for organic toxicants wherein factors such as partitioning and transport are controlling their toxic effects and within homologous series of compounds having a similar mode of action. Deviations from this trend should be anticipated for toxicants with biological effects that are mainly determined by specific toxicity mechanisms.

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