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Toxic Effects of Pollutants on the Mineralization of Acetate in Methanogenic River Sediment

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The highly industrialized drainage basin of the river Rhine has a total area of 160,000 km^2 before it enters the Netherlands (Van der Weijden and Middelburg 1989). About 58% of the river's load of suspended matter is deposited in sedimentation areas in the Netherlands (Admiraal and Van Zanten 1988) where the river eventually reaches the North Sea. These sediments are heavily polluted with persistent organic compounds and heavy metals and form a major environmental problem in the Netherlands. The Dutch research program on the 'Ecological Rehabilitation of the River Rhine' included research on the effects of toxic compounds on the biodegradation in river sediments. The input of organic matter into the sediment is estimated to be 10^8 kg C yr^{-1} (Admiraal and Van Zanten 1988). High numbers of bacteria are present (Van Beelen and Fleuren-Kemilä 1989) and oxygen supplied by the overlying water will be rapidly consumed in the top layer of the sediment. The underlying part of the sediment is anoxic and there biodegradation of natural as well as anthropogenic organic substrates is dependent on anaerobic processes. Anaerobic mineralization of organic matter by micro-organisms is an essential component of the carbon cycle. Acetate is a key intermediate in anaerobic degradation of organic matter and a major component of anaerobic waste water. Organic polymers like proteins, carbohydrates, and lipids are hydrolysed to amino acids, sugars and fatty acids. These are converted by acidogenic bacteria which produce hydrogen, acetate, propionate, butyrate, lactate, and alcohols (Gorris 1987). Acetogenic bacteria convert these products to acetate. Hydrogen can be oxidized by hydrogenotrophic acetogens with the use of carbon dioxide to form acetate. Hydrogen and carbon dioxide are also used directly to form methane but about 70% of the methane formation in anaerobic freshwater sediments is derived from acetate (Lovley and Klug 1986). The following reaction is performed:

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad \Delta G^{\circ\prime} = -32.5 \text{ kJ/mol}$$

The energy liberated by this reaction is only 4% of the energy which is liberated upon the aerobic oxidation of acetate (Thauer *et al.* 1977). Hence the fraction of the acetate which is converted to biomass is small. During the aerobic mineralization of [^{14}C] acetate about 50% of the label is converted to biomass while during the anaerobic mineralization 50% of the label is converted to methane.

The mineralization of acetate under methanogenic conditions is performed by a few specialized methanogenic bacteria like

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Methanosarcina sp. since most methanogens are not able to split acetate into methane and carbon dioxide (Van der Meijden 1984). This is in contrast with the aerobic mineralization of acetate which can be performed by a multitude of species.

This paper reports the effect of 6 toxicants on the anaerobic mineralization of acetate in methanogenic sediment microcosms. The toxicants used in this study (benzene, chloroform, 1,2-dichloroethane, pentachlorophenol, mercury and zinc) are priority pollutants in the Rhine Action Program and are produced in high quantities and are relatively toxic and persistent.

MATERIALS AND METHODS

Sediment samples were taken with a core sampler at a depth of 0.5 to 1 m below the sediment-water interface at the location Gorichem, the Netherlands (in a harbor in the estuary of the river Rhine: E.L. 4°57′50", N.L. 51°49′46"). The sampling device and techniques have been described before (Van Beelen and Van Keulen 1990). The sediment cores were primarily composed of methanogenic mud; their properties are presented in Table 1. The cores were kept under nitrogen during transport and then brought into a glove box (N_2 atmosphere) with less than 1 ppm O_2 (v/v). Incubation bottles (60 mL) were filled with 1:1 (w/w) wet sediment/water suspension (20 mL) and toxicants were added: two bottles per concentration. The bottles were stoppered, crimp cap sealed and preincubated for 2 hr at 20°C.

Table 1. Properties of the Gorinchem sediment samples.

Code	G-1		G-2	
date (mmddyy)	11-13-89		03-14-90	
log AODC/g dry sedim.[a]	10.0	(0.03)[b]	10.1	(0.04)
wet density (kg/L)	1.45		1.32	
% dry weight	44.2		45.8	
M-50 (µm)[c]	63		110	
%clay	23		25.8	
pH H_2O	7.9		7.8	
pH KCl	7.4		7.6	
% org.C	4.6		4.6	
% $CaCO_3$	9.3		11.3	
total N (mg/kg)	3620		2970	
mineral NO_3^--N (mg/kg)	<0.1		0.3	

a) log AODC is the number of bacteria per gram dry sediment, determined by fluorescence microscopy with the Acridine Orange method (Ghiorse and Balkwill 1983). b) s.d. is standard deviation. c) M-50 is the median of the particle size distribution.

The following toxicants and solvents were used. Benzene (Merck, Germany, purity ≥ 99.7%) was added pure. Chloroform (Merck, Germany, purity ≥99.0%) was dissolved in methanol. 1,2-Dichloroethane (DCE, Instra Analyzed, J.T. Baker Chemical Co., the Netherlands) was added pure at higher concentrations or solved in methanol. Pentachlorophenol (PCP, Fluka, Switzerland, purity ≥ 99%), zinc ($ZnCl_2$, Merck, Germany, purity ≥ 98%) and mercury ($HgCl_2$, Merck, Germany, purity ≥99.5%) were added as portions of a 100 g/L or a 1 g/L stock solution. PCP (100 g/L) was dissolved in 0.5 N NaOH, zinc chloride (100 g/L) was dissolved in 0.1 N HCl and mercury chloride was dissolved in 1 N HCl (100 g/L). Acetate was [U-^{14}C]-acetate (Amersham, England), radiochemical purity 99%. The final concentration was 0.7 µg/L (180 Bq per bottle).

After preincubation a half-life determination and toxicity experiments were started simultaneously by injecting radiolabelled acetate (0.5 mL from a sterile aqueous stock solution) through the stoppers. Bottles that were provided for a half-life determination of acetate contained 20 mL suspension only. Two bottles with addition of the highest concentration of solvent were incorporated in toxicity experiments as controls. Incubation was performed at 20°C in a rotary shaker at 225 rpm. Bottles used for the half-life determination were measured in duplicate for $[^{14}C]$-CO_2 production and $[^{14}C]$-acetate disappearance at different times. The toxicity experiments were terminated by freezing the bottles upside down (to prevent leakage of radioactive gas) at -80°C, after a specific incubation time when a measurable amount of CO_2 was produced in the half-life determination experiment.

To quantify $[^{14}C]$-CO_2, the bottles were thawed, heated to 80°C, acidified, and flushed with nitrogen gas and shaken (300 rpm) for 8 min. The gas was led trough an ethanolamine based absorbent (Carbosorb, Packard) to trap $[^{14}C]$-CO_2. Radioactivity was counted with a Tricarb liquid scintillation counter (Packard, type 1500 or 1550). The extraction efficiency of carbon dioxide from the sediment slurry was 90%, which was measured by extraction of $[^{14}C]$-$NaHCO_3$ (Van Beelen and Van Keulen 1990). The sediment was settled for 1 day and 1 mL of waterphase was pipetted into 10 mL of Instagel (Packard, scintillation fluid) in order to count the water-soluble radioactivity. The total recovery of radioactivity was 90%. For calculations an efficiency of 90% for $[^{14}C]$-CO_2 and of 85%-95% for $[^{14}C]$-acetate (aqueous phase radioactivity) was used.

For the half-life determination both the carbon dioxide and the acetate curve were fitted separately with a first order kinetic model using non-linear regression (Van Beelen *et al.* 1991). The parameters of the calculated first order curves are given in Table 2. The effect-concentrations (EC10 and EC50) of the toxicity experiments were estimated by fitting a logistic dose-response curve (Haanstra *et al.* 1985) using nonlinear regression (Van Beelen *et al.* 1991). The effect-concentration (EC10 or EC50), is dependent on the incubation time. At toxicant concentrations where partial inhibition of the mineralization occurs, the not-inhibited part of the microflora will continue to mineralize the substrate. This will lead to an underestimation of the inhibiting effect at extended incubations (and low substrate half-life). When the EC10, the EC50, the incubation time of the toxicity experiment and the half-life of the substrate mineralization are known, a time-independent inhibiting concentration (IC10 or IC50) can be calculated. The IC50 value is defined as that toxicant concentration which decreases the uninhibited acetate mineralization rate by 50%. The mathematical derivation is described in detail by Van Beelen *et al.* (1991). Since duplicate measurements were performed in all experiments, it is possible to consider one complete data set as consisting of two separate experiments and calculate the standard deviation of the IC10 and IC50.

RESULTS AND DISCUSSION

Figure 1 shows that the anaerobic mineralization of acetate in sediment is rapid and follows first order kinetics. Only 40% of the acetate is converted to carbon dioxide; the remainder is methane and biomass (addition of 10 µmol of acetate to 20 mL sediment slurry in a separate experiment yielded 12 µmol of methane). Figure 1 shows that at t=zero, a small amount of $[^{14}C]$-CO_2 was already formed due to rapid utilization of the substrate and the imperfect stopping procedure (thawing of the frozen bottles). This accounts for the small negative lag time mentioned in Table 2. The determination of carbon dioxide is more reliable than the quantification of acetate which is measured as soluble radioactivity. Therefore the half-life of the carbon dioxide production curve was used for further

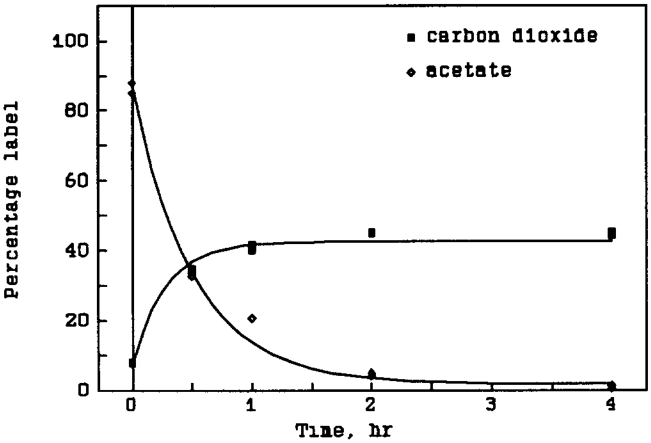

Figure 1. The mineralization of 0.7 µg/l acetate in sediment G-2. Halflife of the carbon dioxide production is 0.2 hr. Two bottles per time were used.

calculations.

Table 2. First order mineralization kinetics: parameters for sediment samples.

Sample	carbon dioxide				acetate			
	$t_{1/2}$[a]	s.d.[b]	%max[c]	tlag[d]	$t_{1/2}$	s.d.	%base[c]	tlag
G-1	0.5	0.02	49.3	-0.1	0.5	0.01	0.1	0
G-2	0.2	0.01	42.5	-0.1	0.4	0.05	1.6	-0.1

a) Half-life values are given in hours. b) s.d. = standard deviation. c) '%max' and '%base' are the amount of CO_2 formed and the amount of substrate left, at the end of the incubation. They are expressed as percentages of the total amount of labelled substrate added. d) tlag is lag time. G-1 and G-2 are codes of the sediment samples used, and refer to Table 1.

Because of the short half-life of the acetate mineralization, the incubation time of the toxicity experiments was also kept short (Table 3). Therefore substantial degradation of the organic toxicants by the sediment microflora is not expected. Radioactivity measured in CO_2 traps after extraction of autoclaved samples that were incubated for 48 hr, was 0.5% of the total amount of label added.

Figure 2 shows the effect of DCE on the mineralization of acetate, measured at t=4 hr (Table 3). The mineralization of acetate in uninhibited samples is shown in Figure 1. DCE can be reductively dehalogenated under methanogenic conditions (Egli *et al.* 1987) but its transformation rate is reported to be slower than that of chloroform (Bouwer and McCarty 1983). Note that the CO_2 and acetate curve could not be fitted simultaneously. DCE can act as an alternative electron acceptor instead of CO_2 so that less methane is formed. Therefore more CO_2 can be formed from the same amount of acetate. This effect was not present in the other dose effect curves.

The IC10 of 0.7 mg DCE/kg sediment determines an equilibrium water

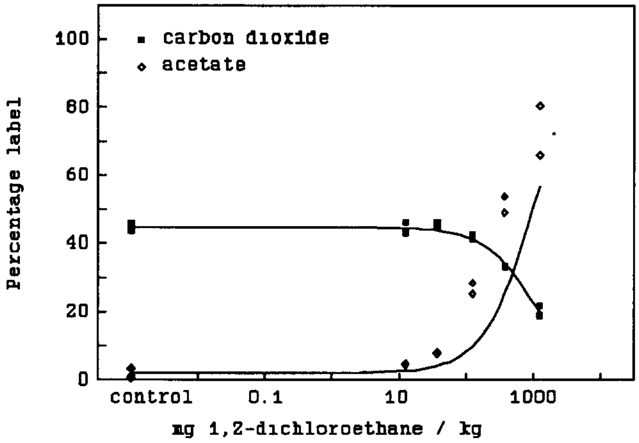

Figure 2. The effect of 1,2-dichloroethane on the mineralization of acetate in sediment G-2. EC10 = 158 mg/kg, EC50 = 1037 mg/kg. Two bottles per concentration were used.

concentration. Above this concentration toxic effects on the anaerobic acetate mineralization in sediments can occur. For 1,2-dichloroethane this water concentration was calculated from the IC10 using the Karickhoff equation (Karickhoff 1981):

$$Cw = 100 * Cs / (\%C \times Kow \times 0.411) \qquad \{1\}$$

Where Cs = sediment IC10 concentration in µg/kg dry weight, Cw = corresponding water concentration in µg/L, %C = 4.6% organic carbon (Table 1). Using a Kow of 1.45 a water concentration of 26 mg/L can be calculated. This is much higher than 0.1 µg DCE/L Rhine water (RIWA 1992).

The effect of PCP on the acetate mineralization (figure 3) shows that both the CO_2 and acetate curve follow the data points more closely than in Figure 2. This was also true for all the other dose effect curves summarized in Table 3. The concentration of pentachlorophenol in sediment of the river Rhine was maximally 34 µg/kg (Wegman and Van den Broek 1983). Effect of PCP on acetate mineralization was found at much higher concentrations (Table 3).

Table 3. The effect of toxicants on the mineralization of acetate.

Toxicant	EC50	EC10	t_i[a]	$t_{1/2}$[b]	IC50	s.d.[c]	IC10	s.d.	sample
benzene	3500	1200	2	0.5	1700	500	480	250	G-1
chloroform	0.5	0.2	3	0.5	0.16	0.05	0.04	0.01	G-1
DCE	1000	160	4	0.2	15	4	0.7	0.3	G-2
PCP	130	46	2	0.5	64	24	19	15	G-1
mercury	>1500	>1500	2	0.5	--	--	--	--	G-1
zinc	>3500	>3500	4	0.2	--	--	--	--	G-2

a) t_i is the incubation time of the toxicity experiment (hours). b) $t_{1/2}$ is the half-life of the carbon dioxide production without toxicant (hours). c) s.d. = standard deviation, calculated from four possible IC-values using the unbiased (n-1) method. All EC and IC values and standard deviations are given in mg toxicant per kg dry sediment.

Benzene is the least toxic of the organic compounds for the acetate

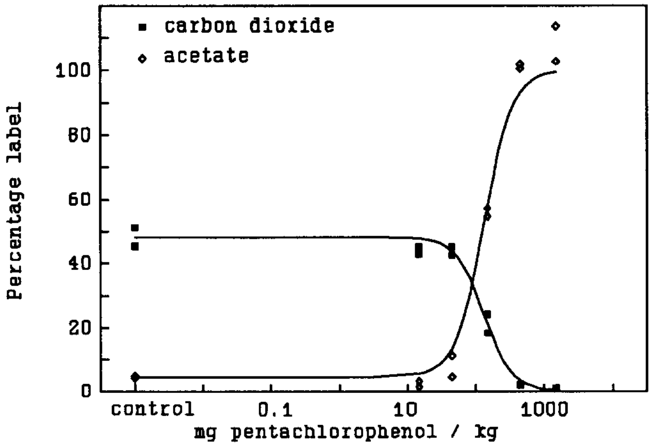

Figure 3. The effect of pentachlorophenol on the mineralization of acetate in sediment G-1. EC10 = 46 mg/kg, EC50 = 132 mg/kg. Two bottles per concentration were used.

mineralization (Table 3). Sierra-Alvarez and Lettinga (1991) found an IC50 for acetoclastic methanogenesis at 1500 mg/L benzene in a granular sludge study. Such high IC values suggest that benzene exhibits minimum toxicity and thus is a narcotic toxicant.

The equilibrium water concentration can be calculated from the Kow of 2.13 and the IC10 shown in Table 3 using Equation 1. The calculated equilibrium water concentration is 12 g/L which is much higher than the actual concentration of 0.1 µg/L measured in Rhine water (RIWA 1992).

At the highest additions of mercury or zinc no effects on the anaerobic acetate mineralization were observed. The background concentrations in this sediment were 1 mg/kg mercury and 800 mg/kg zinc, the effect-concentrations for both mercury and zinc exceed these levels. Added mercury or zinc might precipitate as HgS or ZnS respectively when sulfide is present, since their solubility products are lower than that of FeS (Di Toro *et al.* 1990).

Of the six compounds tested, chloroform is most toxic to the acetate mineralization under methanogenic conditions. Although the Kow of benzene and chloroform are comparable, benzene exhibits minimum toxicity and chloroform is four orders of magnitude more toxic (Table 3). A low redox potential seems to enhance the toxicity of chloroform. The formation of reactive intermediates (radicals) during reductive dechlorination (Klecka and Gonsior 1984; Krone *et al.* 1989) may explain the enhanced toxicity of chloroform in a reducing environment. For comparison, van der Heijden *et al.* (1986) quote a value for initial reduction of cell multiplication of a *Pseudomonas putida* strain under aerobic conditions at 125 mg/L chloroform. In this sediment, [^{14}C]-labelled chloroform was shown to be mineralized to [^{14}C]-CO_2 at low concentrations (3 µg/L) without a lag phase (Van Beelen and Van Vlaardingen 1993).

The equilibrium water concentration can be calculated from the Kow of 1.97 and the IC10 shown in Table 3 using Equation 1. The calculated equilibrium water concentration of 1 mg/L is much higher than the actual concentration of 0.5 µg/L, measured in Rhine water (RIWA 1992). Hence chloroform will not inhibit the anaerobic acetate mineralization at the present field concentrations.

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REFERENCES

Admiraal W, Van Zanten B (1988) Impact of biological activity on detritus transported in the lower river Rhine: an exercise in ecosystem analysis. Freshw. Biol. 20:215-225

Bouwer EJ, McCarty PL (1983) Transformations of 1- and 2-carbon halogenated aliphatic organic compounds under methanogenic conditions. Appl Environ Microbiol 45:1286-1294

Di Toro DM, Mahony JD, Hansen DJ, Scott KJ, Hicks MB, Mayr SM Redmond MS (1990) Toxicity of cadmium in sediments: the role of acid volatile sulfide. Environ Toxicol Chem 9:1487-1502

Egli C, Scholtz R, Cook AM, Leisinger T (1987) Anaerobic dechlorination of tetrachloromethane and 1,2-dichloroethane to degradable products by pure cultures of *Desulfobacterium* sp. and *Methanobacterium* sp. FEMS Microbiol Lett 43:257-261

Gorris L (1987) Analysis of methanogenic populations in anaerobic digesters. PhD thesis Catholic University Nijmegen, The Netherlands

Haanstra L, Doelman P, Oude Voshaar JH (1985) The use of sigmoidal dose response curves in soil ecotoxicological research. Plant Soil 84:293-297

Karickhoff SW (1981) Semi-empirical estimation of sorption of hydrophobic Pollutants on natural sediments and soils. Chemosphere 10:833-846

Klecka GM, Gonsior SJ (1984) Reductive dechlorination of chlorinated methanes and ethanes by reduced iron (II) porphyrins. Chemosphere 13:391-402

Krone UE, Thauer RK, Hogenkamp HPC (1989) Reductive dehalogenation of chlorinated C1-hydrocarbons mediated by corrinoids. Biochemistry 28:4908-4914

Lovley DR, Klug MJ (1986) Model for the distribution of sulfate reduction and methanogenesis in freshwater sediments. Geochim Cosmochim Acta 50:11-18

RIWA (1992) The composition of the Rhine water in 1988 and 1989. Co-operating Rhine and Meuse Waterworks Companies, Amsterdam, the Netherlands

Sierra-Alvarez R, Lettinga G (1991) The effect of aromatic structure on the inhibition of acetoclastic methanogenesis in granular sludge. Appl Microbiol Biotechnol 34:544-550

Thauer RK, Jungermann K, Decker K (1977) Energy conservation in chemotropic anaerobic bacteria. Bacteriol Rev 41:100-180

Van Beelen P, Fleuren-Kemilä AK (1989) Enumeration of anaerobic and oligotrophic bacteria in subsoils and sediments. J Contam Hydrol 4: 275-284

Van Beelen P, Van Keulen F (1990) The kinetics of the degradation of chloroform and benzene in anaerobic sediment from the river Rhine. Hydrobiol Bull 24:13-21

Van Beelen P, Fleuren-Kemilä AK, Huys MPA, Van Montfort ACP, Van Vlaardingen PLA (1991) The toxic effects of pollutants on the mineralization of acetate in subsoil microcosms. Environ Toxicol Chem 10:775-789

Van Beelen P, Van Vlaardingen PLA (1993) The mineralization of chloroform in river sediments. Neth J Aquat Ecol, in press

Van der Heijden CA (ed) (1986) Criteria document Chloroform. National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, rep. no. 738513004

Van der Meijden P (1984) Methanol conversion by methanogenic and acetogenic bacteria. PhD thesis Catholic University Nijmegen, The Netherlands

Van der Weijden CH, Middelburg JJ (1989) Hydrogeochemistry of the river Rhine: long term and seasonal variability, elemental budgets, base levels and pollution. Wat Res 23:1247-1266

Van Vlaardingen PLA, Van Beelen P (1992) Toxic effects of pollutants on methane production in sediments of the river Rhine. Bull Environ Contam Toxicol 49:780-786

Wegman RCC, Van den Broek HH (1983) Chlorophenols in river sediments in the Netherlands. Wat Res 17:227-230

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