Bull. Environ. Contam. Toxicol. (1988) 41:936-942

Bulletin of Environmental Contamination and Toxicology

Effect of Hexachlorobenzene on Growth and Survival of Various Microorganisms

M. K. Hamdy

Food Science & Technology Department, University of Georgia, Athens, Georgia 30602

Hexachlorobenzene (HCB) has been used as a fungicide for control of smut infestation in barley, oat, and wheat seed (Bechard 1981). However, only ~6800 kg HCB were utilized for this purpose in 1971 (Isensee et al. 1976). Most HCB was found as a by-product in the waste from manufacture of vinyl chloride, chlorine, and chlorinated solvents (Niimi and Cho 1980). It has been estimated that 1.1 to 2.2 million kg of HCB were produced from these industrial processes (Mumma and Lawless 1975). The presence of HCB was reported in tissues of freshwater and marine fish (Johnson et al. 1974; Ofstad et al. 1978; Niimi 1979), birds and eggs (Gilberton and Reynolds 1972; Bechard 1981), and adipose tissues (Mes et al. 1982). These findings suggest that HCB has entered and contaminated our environment and thus demonstrates potential for accumulation in biological systems. As an environmental contaminant, HCB has been reported as resistant to both photodecomposition (Plimmer and Klingebiel 1976) and biodegradation by soil microbes (Isensee et al. 1976). Therefore, this investigation was conducted to examine the effect of HCB on growth, survival, and uptake, by selected microorganisms, and its use as sole source of carbon and energy by these bacteria. Efforts were also made to remove HCB from waste water by chemosorption on charcoal.

MATERIALS AND METHODS

Two Gram negative short rods isolated from a contaminated industrial lake in Pennsylvania (identified as mutants of Serratia liquefaciens and Pseudomonas aeruginosa); eight mutant Bacillus spp. previously isolated by Nunn (1979) from sediment samples in a sewer system in Rome, Georgia. (these cultures are resistant to high levels of Aroclor 1254); and nine Aspergillus spp. isolated from a contaminated flask containing basal salt broth (BSB) during a time-course uptake experiment. The cultures were stored at -85°C in glycerol and glucose basal salt broth (GBSB) containing 100 ug HCB/ mL broth and activated by repeat inoculation and incubation for 24 h at 37°C prior to use.

Three types media (all containing 0.1% Tween 80 to emulsify the HCB) were used: GBSB containing 1% glucose (HAMDY and NOYES 1975), BSB, and Difco tryptic soy broth (TSB). Glucose basal salt agar (GBSA) and tryptic soy agar (TSA) were prepared by mixing 1.5% agar with GBSB and TSB, respectively. All media were sterilized at 121.5°C for 15 min before use.

HCB (97% pure) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and 2 stock solutions made; the first had 1 g HCB/100 mL sterilize benzene-ethanol (1:1 v/v) and the second had 1 g HCB/100 mL sterile peanut oil. This HCB-oil preparation effectively kept the HCB in solution if heated to 100°C and then kept at 37°C.

S. liquefaciens and/or P. aeruginosa were allowed to grow in GBSB (24 h, 37°C), cells were harvested, washed with sterile saline, and suspended in sterile sodium phosphate buffer (SPB; 0.1 M, pH 7.0). For survival studies, tubes were prepared to contain desired levels of HCB (0 to 800 ug HCB/mL) in 7-mL cell suspension, varying amounts of sterile SPB, and sterile 0.1% Tween 80. One mL of the mixture was taken at intervals and viable cells scored by plating dilutions on GBSA. Colonies were counted after 24-48 h incubation at 37°C and survival fractions (N/N_o) determined where N_o is number of cells/mL initially, and N is number of cells surviving HCB exposure at specified time.

GBSB and TSB media containing desired levels of HCB were each inoculated with 10% of one of the test organisms, incubated 24 h at 37°C in shaker water bath, and cell counts (CFU/mL) determined at intervals by plating on GBSB and TSB as necessary. Cell counts were determined at specific time intervals to provide data for calculation of generation times.

GBSB flasks containing 300 ug HCB/mL were each inoculated with 10% of a test organism (eight Bacillus spp., nine Aspergillus spp.) and shaker-incubated (37°C) for desired times. The Bacillus spp. were each inoculated into flasks of BSB containing 1200 ug HCB/mL and incubated for six mon. The HCB level in each flask was analysed and compared to controls (HCB in BSB only) to estimate percentage HCB uptake and use as carbon source.

HCB was recovered from media by three consecutive extractions using equal volumes of benzene. Excess water was removed by adsorption using anhydrous sodium sulfate

and assayed (after proper dilution) for HCB level using a Tracor 560 gas liquid chromatograph (GLC) equipped with a Ni-63 electron-capture detector and pyrex column (0.4 x 180 cm) containing 5% OV-101 Chromosorb WHp (80-100) mesh. Operating conditions of GLC: carrier (5% methane in argon) with flow rate of 60 cm^3/min and column, detector, and injection port temperatures at 200, 350, and 225°C, respectively. An HCB standard curve was obtained by plotting peak area vs. HCB concentrations in benzene or in hexane. Efficiency of HCB recovery by benzene or hexane extraction was also made in GBSB spiked with 0.01, 0.1, and 1 mg HCB/mL.

Activated charcoal was used as an adsorbant to remove HCB from industrial aqueous waste containing (per mL): 1.5 ng hexachloroethane, 190 ng pentachlorobutadiene, 1600 ng HCB, 170 ng pentachlorobenzonitrile, 110 ng octachloro-styrene, and 460 ng decachlorobiphenyl. The waste (250 mL) was filtered through 10 g activated charcoal and gas chromatographic analyses conducted before and after treatment were compared.

RESULTS AND DISCUSSION

A linear relationship was noted between peak areas and HCB levels ranging from 0.05 to 1.0 ng with 70 ±1 sec retention time. When different levels of HCB were spiked in GBSB, recovery ranged from 94 to 102% indicating that benzene extraction and GLC gave excellent HCB recovery.

S. liquefaciens cells exposed up to 60 min to 200 ug HCB/mL showed little death (Fig. 1A), but when the level was 300 ug HCB/mL or more, survival fractions decreased drastically. *P. aeruginosa* was able to survive exposures up to 500 ug HCB/mL (Fig.1B), whereas 800 ug/mL progressively inactivated the cells. The use of HCB in either benzene-ethanol or in peanut form had no effect on cell survival. The increase in cell death rate at higher HCB levels can be attributed to its toxic effect and/or the benzene. Again, no effect was noted on cell survival due to the presence of solvent alone (benzene-ethanol mixture) or peanut oil at levels used in any experiments.

P. aeruginosa growth in absence or presence of up to 600 ug HCB/mL GBSB (Fig. 1C) showed some inhibition during 8 h but not after 24 h and generation time was 82 min compared to 66 min for control. The presence of 800 ug HCB/mL progressively delayed growth as compared to

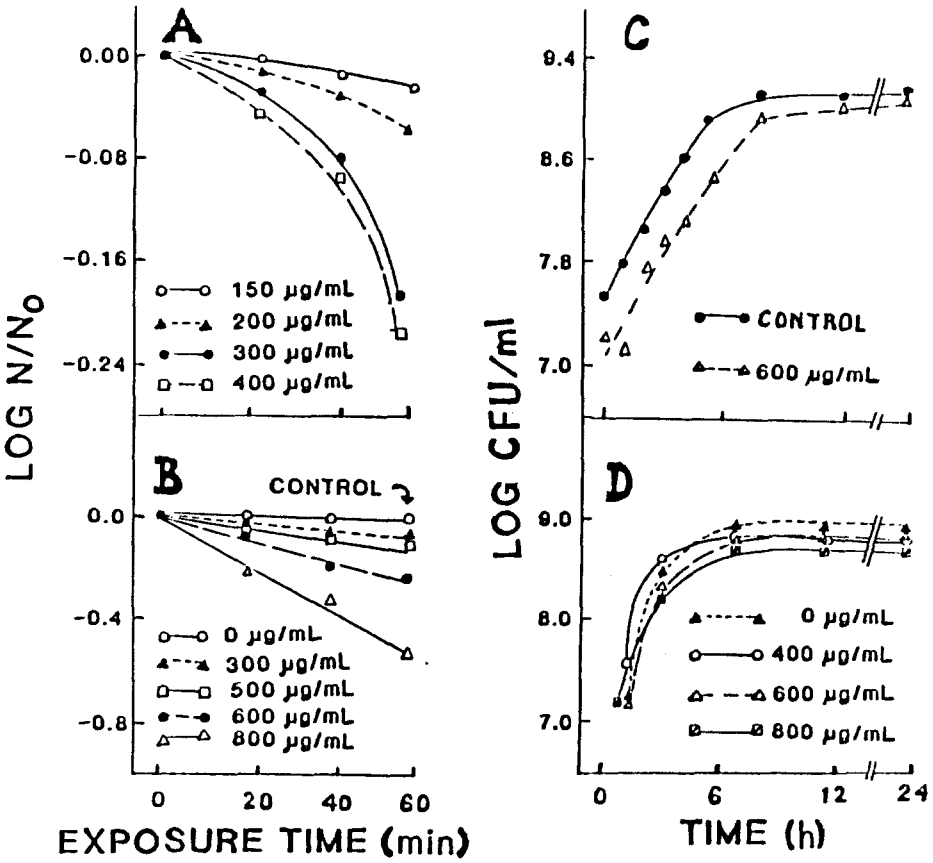

Figure 1. Effect of various levels of HCB on the survival of (**A**) Serratia liquefaciens and of (**B**) Pseudomonas aeruginosa in phosphate buffer. Growth of Pseudomonas aeruginosa in absence and presence of HCB in (**C**) **GBSB** and in (**D**) **TSB** at 37°C.

control (data not shown), but generation time was 328 min. In TSB media HCB showed only slight effect (Fig 1D) and generation time was the same (28 min) for 0, 200, and 400 ug HCB/mL but 34 and 46 min for 600 and 800 ug HCB/mL, respectively. Obviously, HCB affected growth of this culture and was dependent both on concentration of HCB and type of media used. However, it should be pointed out that the presence of HCB in media did not effect growth until the level reached 600 ug HCB/mL. The organic compounds in TSB protected growing cells against the toxicity of HCB more than GBSB, which had a generation 2.4 times longer than in the TSB.

The recovery of HCB from sterile controls ranged from 96 to 100% after 7 days incubation, and from 85 to 99% in media inoculated with any Aspergillus or Bacillus spp.

Table 1. HCB recovery from GBSB inoculated with Aspergillus spp. for 7 d and Bacillus spp. for 25 and 180 d.

Culture Number	% HCB recovery after incubation		
	Aspergillus	Bacillus	
	7 d	25 d	180 d
1	95	86	37
2	99	90	45
3	99	94	37
4	98	89	39
5	98	96	44
6	98	99	45
7	91	88	43
8	94	85	42
9	97	-	-

These data confirmed other investigators (Isensee et al. 1976; Tabak et al. 1981) in that HCB was not significantly degraded by test organisms in this experiment. The major problem was precipitation of HCB from medium after evaporation of the carrier-solvent during incubation. Crystalization of HCB may influence efficiency of uptake and biodegradation. However, a reduction in HCB recovery was noticed when HCB in peanut oil was used as the sole carbon source for the Bacillus spp. incubated in this emulsion for six months. HCB recoveries ranged from 37 to 45% (Table 1) indicating more than 55% of the HCB was used by the Bacillus cells. Further study is needed to prove whether this reduction of HCB recovery is due to binding with Bacillus cells or biodegradation.

HCB was extracted and assayed by GLC; recovery from control was 96 to 100%. GLC patterns of compounds in the aqueous waste before and after AC treatment showed removal of five of the six peaks (Fig 2) and reduction of the HCB peak area to >99%. However, hexachloroethane was not completely adsorbed by the AC. Repeated hexane extraction of the AC revealed that all compounds present

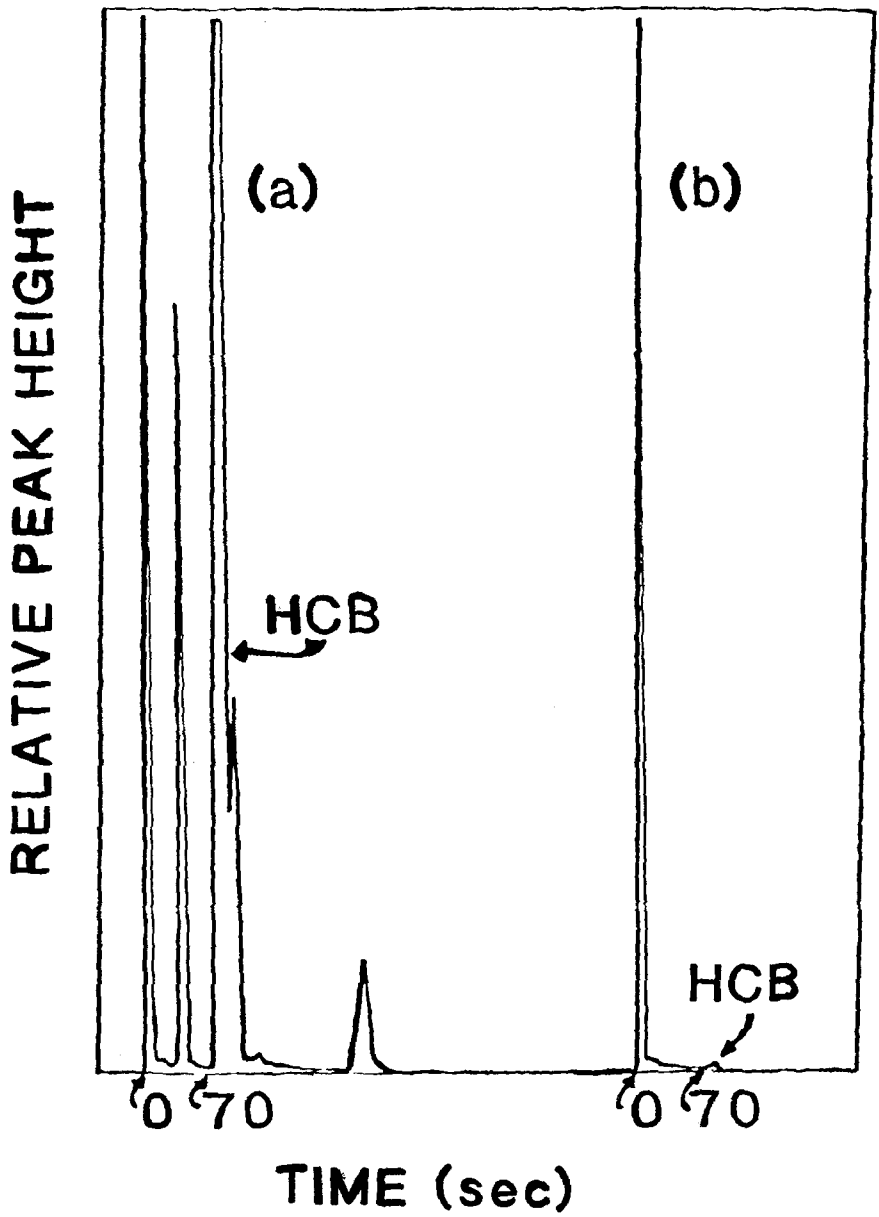

Fig.2. Gas chromatographic pattern for hexane extracts of industrial waste water before (a) and after (b) treatment with activated charcoal. Hexachloroethane (HCE) was not completely adsorbed by the charcoal.

in the waste were recovered from the charcoal. This suggests that adsorption on AC (due to low solubility and hydrophobic nature of HCB) is a possible approach to remove low levels of HCB from industrial waste water.

REFERENCES

Bechard, M (1981) DDT and hexachlorobenzene residues in Southeastern Washington Swainson's hawks (Buteo swainsoni). Bull Environ Contam Toxicol 26:248-253

Gilbertson M, Reynolds LM (1972) Hexachlorobenzene (HCB) in the eggs of common terms in Hamilton Harbor, Ontario. Bull Environ Contam Toxicol 7:371-373

Hamdy MK, Noyes OR (1975) Formation of methyl mercury by bacteria. Appl Microbiol 30:424-432

Isensee AR, Holden ER, Woolson EA, Jones GE (1976) Soil persistence and aquatic bioaccumulation potential of hexachlorobenzene (HCB). J Agric Food Chem 24:1210-1214

Johnson JL, Stalling DL, Hogan JW (1974) Hexachlorobenzene (HCB) residues in fish. Bull Environ Contam Toxicol 11:393-398

Mes J, Davis DJ, Turton D. (1982) Polychlorinated biphenyls and other chlorinated hydrocarbon residues in adipose tissue. Canadian Bull Environ Contam Toxicol 28:97-104

Mumma CE, Lawless EW (1975) Task I - Hexachlorobenzene and hexachlorobutadien pollution from chlorocarbon processes. EPA Report No. 530-3-75-003. EPA, Washington, DC

Niimi AJ (1979) Hexachlorobenzene levels in Lake Ontario salmonids. Bull Environ Contam Toxicol 23:20-24

Niimi AJ, Cho CY (1980) Uptake of hexachlorobenzene (HCB) from feed by rainbow trout (Salmo gairdneri). Bull Environ Contam Toxicol 24:834-839

Nunn TM (1979) Biodegradation of PCB and other chlorinated hydrocarbons by bacteria. M.S. thesis, University of Georgia

Ofstad EB, Lunde G, Martinsen K (1978) Chlorinated aromatic hydrocarbons in fish from an area polluted by industrial effluents. Sci Tot Environ 10:219-230

Plimmer JR, Klingebiel UI (1976) Photolysis of hexachlorobenzene. J Agric Food Chem 24:721-723

Tabak HH, Quave SA, Mashni CI, Barth EF (1981) Biodegradability studies with organic priority pollutant compounds. J Water Poll Control Fed 50:1503-1518

Received November 3, 1988; Accepted May 5, 1988.