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Comparative Toxicity of Chlorophenols, Nitrophenols, and Phenoxyalkanoic Acids to Freshwater Bacteria

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Since the discovery of their growth-regulating properties, in the 1940s, phenoxyalkanoic acids have found widespread agricultural and horticultural uses (Sly 1985). These herbicides enter watercourses through run-off, or by direct addition for control of aquatic macrophytes (Hellawell and Bryan 1982). Release to rivers of manufacturing wastewater containing finished chemical product and associated phenolic raw materials also increases the quantity of these compounds in the aquatic environment. Phenolic and phenoxy organic compounds, unlike inorganic pollutants, e.g. heavy metals, can act as sources of carbon and energy for bacteria. Their biodegradation may thus occur in freshwater habitats (Watson 1977; Spain and Van Veld 1983; Birmingham and Colman 1985) and is dependent on environmental conditions (Baker et al. 1980; Nesbitt and Watson 1980 a,b). However, phenolic and phenoxy compounds are also potentially toxic to bacteria as they act as general metabolic inhibitors (Buikema et al. 1979). This toxicity is potentially important because of the key role of bacteria in biological sewage-treatment and natural-water purification. The present paper describes the use of a rapid technique to assess the toxicity of chlorophenols, nitrophenols and phenoxyalkanoic acids to bacteria isolated from a phenolic-polluted and an unpolluted stream. Toxicity was found to be related to bacterial genus and to the structure of the test compounds; pseudomonads were amongst the most resistant isolates, while phenoxyalkanoic acids were the least toxic compounds.

MATERIALS AND METHODS

Bacterial isolates were obtained during a study of the microbial ecology of two streams in West Yorkshire, Northern England; one stream (Sugden Beck) was polluted with chlorophenols, nitrophenols and phenoxyalkanoic acids, while the adjacent stream (Stubbs Beck) was unpolluted. Surface sediment was collected aseptically from the bed of both streams. Initial isolation of bacteria from the sediments, on casein-peptone-starch agar, was followed by identification to group or genus level (Milner and Goulder 1986). Pure cultures were maintained on nutrient agar (Oxoid) slopes at 4°C.

Toxicity towards bacteria was determined by measuring inhibition of growth on agar plates, using the method of Liu and Kwasniewska

(1981) but with a modified growth medium. Isolates were first grown in a liquid medium, containing 10% nutrient broth No.2 (Oxoid), 0.2 g/L glucose and 0.2 g/L sodium acetate, for 24 h at 20°C with shaking. Cell concentration was adjusted to optical density = 0.1 (650 nm), using fresh growth medium, and 0.25 mL aliquots were then uniformly spread on pre-dried agar plates (15 g agar/L of growth medium).

Firstly, the toxicity of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,6-trinitrophenol (picric acid), to 166 bacterial isolates, was determined by spotting 0.01 mL of 25, 50, 100 and 250 mM solutions, in 50% v/v acetone/distilled water, onto each of three freshly-seeded agar plates per bacterial isolate. A volume (0.01 mL) of solvent (50% v/v acetone/distilled water) was also spotted onto each agar plate as a control. After incubation at 20°C for 18 h, the diameter of the clear (inhibition) zone in the bacterial lawn, around the locus of each spot, was measured.

Secondly, the toxicity of the following compounds; 2-chlorophenol (2-MCP), 3-chlorophenol (3-MCP), 4-chlorophenol (4-MCP), 2,4-dichlorophenol (2,4-DCP), 4-chloro-2-methylphenol (PCOC), 2,4-dinitrophenol (2,4-DNP), picric acid, 2-methyl-4,6-dinitrophenol (DNOC), 2-sec-butyl-4,6-dinitrophenol (DNBP), 2,4-D, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 2-methyl-4-chlorophenoxyacetic acid (MCPA), 2-(2,4-dichlorophenoxy)-proprionic acid (2,4-DP), 2-(2-methyl-4-chlorophenoxy)-proprionic acid (CMPP), 4-(2,4-dichlorophenoxy)-butyric acid (2,4-DB) and 4-(2-methyl-4-chlorophenoxy)-butyric acid (MCPB), to randomly selected isolates representing the major genera from Sugden Beck and Stubbs Beck was determined. These sixteen chlorophenols, nitrophenols and phenoxyalkanoic acids were chosen because all, except 2-MCP and 2,4,5-T, were present in Sugden Beck (Milner and Goulder 1986). The following genera or groups of bacteria were used to prepare seeded agar plates; from Sugden Beck, Pseudomonas (1 isolate); from Stubbs Beck, Pseudomonas (2 isolates), Aeromonas (1 isolate), Flavobacterium (1 isolate) and Gram-positive bacteria (1 isolate). Toxicant solutions (250 mM) were prepared using 50% v/v acetone in distilled water as diluent. A volume of 0.01 mL was then spotted onto the seeded agar surface together with a 0.01 mL control spot (maximum five spots per plate). Each compound was tested thirty times against each of the six bacterial isolates; the diameters of inhibition zones were measured after incubation at 20°C for 18 h.

RESULTS AND DISCUSSION

The results from testing 2,4-D and picric acid against all 166 isolates are summarized in Table 1. All isolates were inhibited at all toxicant concentrations but there were no inhibition zones with the control solvent. The isolates showed a straight-line relationship between the diameter of the inhibition zone and $\log_e$ (concentration + 1) of test compound. GLIM (Baker and Nelder 1978) was used to calculate mean slope, which increases with increase in toxicity, for each genus or group of isolates from each stream. Statistically significant differences in toxicity

Table 1. Toxicity of 2,4-D and picric acid to freshwater bacteria isolated from Sugden Beck and Stubbs Beck.

Genus or group of bacteria	n of isolates	2,4-D Mean slope	2,4-D SE	Picric acid Mean slope	Picric acid SE
Sugden Beck isolates					
Pseudomonas	86	2.32	0.05	1.90	0.04
Gram-positive group	4	2.55	0.05	2.27	0.04
Stubbs Beck isolates					
Pseudomonas	25	2.50	0.03	2.06	0.05
Aeromonas	14	2.66	0.03	2.23	0.06
Flavobacterium	9	2.49	0.04	3.35	0.05
Acinetobacter	4	2.70	0.05	2.27	0.08
Enterobacteriaceae	3	2.53	0.06	1.97	0.09
Chromobacterium	1	2.57	0.10	2.02	0.15
Gram-positive group	20	2.73	0.02	2.29	0.06

The slope values, which indicate relative toxicity, were obtained from straight-line plots of diameter of inhibition zone (mm) against $\log_e$ (concentration in mM + 1), an increase in slope thus indicates increase in toxicity.

were identified by comparing changes in deviance (residual sum of squares) with the chi-square distribution, after fitting or weighting-out different genera or groups from the overall model. Taken together, the isolates from phenolic-polluted Sugden Beck were more resistant (P less than 0.01) to 2,4-D and picric acid than were the Stubbs Beck isolates. With Sugden Beck isolates, both test compounds were more toxic (P less than 0.01) to Gram-positive bacteria than to *Pseudomonas*. With Stubbs Beck isolates the situation was more complex. Both compounds were more toxic (P less than 0.05) to Gram-positive bacteria, *Aeromonas* and *Acinetobacter* than to *Pseudomonas* and *Chromobacterium*. There was, however, marked difference in the toxicity of 2,4-D and picric acid to *Flavobacterium*, this genus was the most 2,4-D resistant but the most picric acid sensitive.

The greater resistance of the Sugden Beck isolates presumably reflected a response to pollution by the bacterial community of that stream. The observation (Table 1) that *Pseudomonas* isolates were generally more resistant than other genera which are frequent amongst freshwater isolates (e.g. *Aeromonas*, *Acinetobacter*, *Flavobacterium*: Baker and Farr 1977; Inniss and Mayfield 1979; Nuttall 1982) may, in part, explain the dominance of *Pseudomonas* in Sugden Beck which was described by Milner and Goulder (1986). The relative resistance of different genera obtained in this study, may not, however, be constant for all sites. The resistance of a particular genus may change through acclimation; also the response of bacteria to haloaromatic compounds is, to some extent, plasmid mediated (Reineke 1984; Amy et al. 1985) and there may be inter-generic transfer of plasmids.

When all sixteen chlorophenols, nitrophenols and phenoxyalkanoic acids were tested against selected isolates, it was found that all compounds inhibited bacterial growth. Mean diameters of

Table 2. Toxicity of chlorophenols, nitrophenols and phenoxyalkanoic acids to representative bacterial isolates.

Chlorophenols					
Compound	2-MCP	3-MCP	4-MCP	PCOC	2,4-DCP
Diameter (mm)	13.6	17.3	18.6	20.2	25.7

Nitrophenols				
Compound	Picric acid	DNBP	DNOC	2,4-DNP
Diameter (mm)	15.3	16.2	17.7	21.7

Phenoxyalkanoic acids							
Compound	2,4-DB	MCPB	2,4-D	2,4,5-T	CMPP	2,4-DP	MCPA
Diameter (mm)	13.7	15.4	15.9	16.4	17.0	17.1	17.3

Values are mean diameters of inhibition zones, n for each compound = 180 (six isolates times 30 replicates), SE = 0.38 to 0.54. See text for key to abbreviations.

inhibition zones are given in Table 2. Significant differences were identified by a chi-square test after adding or weighting-out compounds from a GLIM model constructed using a single toxin concentration. The compounds may be arranged in order of increasing toxicity as follows: (2-MCP 2,4-DB) (picric acid MCPB) (2,4-D) (DNBP 2,4,5-T) (CMPP 2,4-DP MCPA 3-MCP) (DNOC) (4-MCP) (PCOC) (2,4-DNP) (2,4-DCP). Compounds in different parentheses had significantly different toxicity (P less than 0.05). In general, but with some exceptions, chlorophenols and nitrophenols appeared to be more toxic than phenoxyalkanoic acids which perhaps supports the suggestion of Milner and Goulder (1986), based on multiple-regression analysis, that nitrophenols may have inhibited viability and heterotrophic activity of bacteria in Sugden Beck.

Relationships found between relative toxicity to bacteria (Table 2) and chemical structure can be summarized as follows. (1) Toxicity of chlorophenols increased with increase in degree of chlorination, but was also related to the position of chlorine substitution on the phenol molecule, hence the three isomers of mono-chlorophenol exhibited marked differences in toxicity; these observations support those made by Liu and Kwasniewska (1981) and Liu et al. (1982) using mixed bacterial cultures and a Bacillus sp. (2) Toxicity of nitrophenols decreased with increase in degree of nitro-substitution. (3) Toxicity of phenoxyalkanoic acids increased with decrease in phenoxy side-chain length.

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REFERENCES

Amy PS, Schulke JW, Frazier LM, Seidler RH (1985) Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. Appl

Environ Microbiol 49:1237-1245
Baker JH, Farr IS (1977) Origins, characterization and dynamics of suspended bacteria in two chalk streams. Arch Hydrobiol 80:308-326
Baker MD, Mayfield CI, Inniss WE (1980) Degradation of chlorophenols in soil, sediment and water at low temperature. Wat Res 14:1765-1771
Baker RJ, Nelder JA (1978) Generalised linear interactive modelling, Release 3. Numerical Algorithms Group, 7 Banbury Road, Oxford
Birmingham BC, Colman B (1985) Persistence and fate of 2,4-D butoxyethanol ester in artificial ponds. J Environ Qual 14: 100-104
Buikema AL, McGinniss MJ, Cairns J (1979) Phenolics in aquatic ecosystems : a selected review of recent literature. Mar Environ Res 2:87-181
Hellawell JM, Bryan KA (1982) The use of herbicides for aquatic weed control in water supply catchments. J Inst Wat Engr Sci 36:221-233
Inniss WE, Mayfield CI (1979) Seasonal variation of psychrotrophic bacteria in sediment from Lake Ontario. Wat Res 13: 481-484
Liu D, Kwasniewska K (1981) An improved agar plate method for rapid assessment of chemical inhibition to microbial populations. Bull Environ Contam Toxicol 27:289-294
Liu D, Thomson K, Kaiser KLE (1982) Quantitative structure-toxicity relationship of halogenated phenols on bacteria. Bull Environ Contam Toxicol 29:130-136
Milner CR, Goulder R (1986) The abundance, heterotrophic activity and taxonomy of bacteria in a stream subject to pollution by chlorophenols, nitrophenols and phenoxyalkanoic acids. Wat Res 20:85-90
Nesbitt HJ, Watson JR (1980a) Degradation of the herbicide 2,4-D in river water - I. Description of study area and survey of rate determining factors. Wat Res 14:1683-1688
Nesbitt HJ, Watson JR (1980b) Degradation of the herbicide 2,4-D in river water - II. The role of suspended sediment, nutrients and water temperature. Wat Res 14:1689-1694
Nuttall D (1982) The populations, characterization and activity of suspended bacteria in the Welsh River Dee. J Appl Bact 53: 49-59
Reineke W (1984) Microbial degradation of halogenated aromatic compounds. In: Gibson DT (ed) Microbial degradation of organic compounds. Marcel Dekker, Inc., New York, pp 319-360
Sly JMA (1985) Review of usage of pesticides in agriculture and horticulture in England and Wales 1980-1983. Preliminary report 41. Ministry of Agriculture Fisheries and Food, London
Spain JC, Van Veld PA (1983) Adaptation of natural microbial communities to degradation of xenobiotic compounds: effects of concentration, exposure time, inoculum, and chemical structure. Appl Environ Microbiol 45:428-435
Watson JR (1977) Seasonal variation in the biodegradation of 2,4-D in river water. Wat Res 11:153-157

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