ISOLATION AND IDENTIFICATION OF 2,4-DICHLOROPHENOL DEGRADING BACTERIA FROM A GROUNDWATER SAMPLE*)

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Ringkasan

Sampel air tanah dipompa dari kedalaman 200 meter di daerah Hachioji, Tokyo. Komunitas mikrobia dalam sampel air tersebut mampu merombak 2,4-dichlorophenol (DCP) pada konsentrasi 1.0 μ g-C/ml. Penambahan ulang DCP pada 1.0 μ g-C/ml mempercepat phase aklimasi perombakan DCP. DCP tidak dapat terombak apabila DCP ditambahkan pada konsentrasi 0.1 μ g-C/ml; bahkan penambahan ulang pada konsentrasi ini juga tidak meningkatkan perombakan 0.1 μ g DCP-C/ml secara nyata.

Lima strain bakteri perombak DCP telah dapat diisolasi dari sampel air tersebut di atas setelah penambahan ulang 1.0 μ g-C/ml. Perlakuan penambahan ulang 0.1 μ g DCP-C/ml tidak ada satupun bakteri perombak DCP yang dapat diisolasi. Identifikasi dari kelima isolat bakteri di atas menunjukkan bahwa kelima isolat bakteri tersebut termasuk dalam genus *Pseudomonas* meskipun grupnya berbeda.

Abstract

Sample water was collected from 200 meter deep at Institute of Rolling Land Research, Tokyo University of Agriculture and Technology, Hachioji, Tokyo. 2,4-dichlorophenol (DCP) at concentration of 1.0 μ g-C/ml was degraded by microbial community of this sample water. Repeated spike of DCP at this concentration reduced the acclimation period of DCP degradation. However, 0.1 μ g DCP-C/ml was not degraded in the same groundwater sample. Moreover, repeated spike of 0.1 μ g-C/ml did not enhance DCP degradation at initial concentration of 0.1 μ g-C/ml.

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Five strains of DCP-degrading bacteria were isolated from the water sample after repeated spike of DCP at concentration 1.0 μ g-C/ml. None of DCP degrader was isolated from repeated spike 0.1 μ g DCP-C/ml. Identification of these isolates showed that although they are the member of different group, all of these isolates were identified as *Pseudomonas* sp.

Introduction

Although groundwater is a vital water resource, the groundwater requirement in many countries is served by rural domestic wells which is particularly vulnerable to undetected contamination (Wilson *et al.*, 1983b). Additionally, microbiology of terrestrial subsurface was ignored for some reasons such as : a). indications that number of microorganisms decreased sharply; b). the low concentration of organic compunds in recharge water and aquifer is too low for supporting the growth of microorganisms; c). groundwater was considered to be pure and wholesome (Wilson *et al.*, 1983a).

However, interest concerning microbial and xenobiotics pollution in groundwater was awakened by the findings of chlorophenols, pesticides and herbicides in groundwater (Kool *et al.*, 1989; Patrick *et al.*, 1987). Furthermore, microbial activity was still detected in groundwater (Swindoll *et al.*, 1988).

Natural aquatic environments were contaminated by xenobiotics at less than ppb level (Carey *et al.*, 1988). Moreover, some xenobiotics were reported persist for a long period at low concentration in natural environments (Alexander, 1981). Although low concentration was presumed to be non-toxic, a blue-green algae (*Agmenellum quadruplicatum*) was sensitive to 0.05 ng/ml of aniline (Batterton *et al.*, 1978), and several xenobiotics and herbicides were biomagnified into microorganisms and animals (Watanabe *et al.*, 1983; Winger *et al.*, 1990).

Natural microbial communities sometime failed to degrade low concentration of xenobiotics. The possible reasons for this failure have been proposed, such as the complexity of chemical structure of xenobiotics, the threshold concentration of xenobiotics, or scarcity of responsible degrader (Hoover *et al.*, 1986; Martani & Seto, 1990; Spain *et al.*, 1980; Wiggins *et al.*, 1987. Microbial inoculation has been done for enhancement of xenobiotics degradation natural environments. However, the success of inoculation is highly depend on the activity of degrader, ability of degrader in the adaptation to new environment, or the limiting factors found in natural environments (Goldstein *et al.*, 1985; Martani & Seto, 1991; Zaidi *et al.*, 1988).

This research was conducted to isolate and identify 2,4-dichlorophenol (DCP) degrading bacteria from a groundwater sample. The

DCP degrader can be used as comparison study for the biodegradation of DCP by natural microbial community (Martani & Seto, 1990), and also as an additional culture collection of xenobiotics degrader. DCP was chosen as a model compound of xenobiotics because it is one of the basic chemical structure of herbicides widely used in the world. By this compound, it was considered that results from this study is not limited only for specific compound, but can be applied for general xenobiotics.

Materials and Methods

Water sample

Water sample was collected by pumping from 200 meters underground at Institute for Rolling Land Research, Tokyo University of Agriculture and Technology. This area is located on a small forest in a hill of Hachioji, western Tokyo. The pH value was around 7.2. Nitrated and Phosphate concentrations were 14 — 17 ng N/ml and 55 — 90 ng P/ml, respectively. Total organic carbon and dissolved organic carbon of this sample water was 2.3 and 2.0 μ g/ml, respectively. DCP was not detected in this groundwater sample (Martani & Seto, 1990).

Isolation of DCP-degrading bacteria

Two 500 ml-screw-capped Erlenmeyer flasks containing 400 ml of groundwater sample were prepared. This first and the second flask received DCP at 1.0 μ g-C/ml and 0.1 μ g DCP-C/ml, respectively; they were incubated aerobically at 25° C. During incubation time, repeated spike DCP were conducted to the both flasks. Remaining DCP was measured using high performance liquid chromatography (HPLC) (Table 1). Detail analysis were described previously (Martani & Seto, 1990).

Subsamples from these flasks were diluted using a buffer solution (Martani & Seto, 1990) and pour-plated on Mineral Salts (MS) agar (Table 2). The MS agar was spiked with DCP, so that isolates will meet the same concentration with DCP in the flasks of interest. Each isolate from both flasks was transferred on MS agar slants containing 1.0 gr 0.1 μ g DCP-C/ml. To examine the survival of isolates during transfering on MS agar medium, the colonies were transfered several times on the same agar slants. The survive isolates were examined their ability to degrade DCP in MS medium (Martani & Seto, 1991).

Isolates which can degrade DCP in MS solution were maintained on MS agar containing 30 μ g FCP-C/ml.

Identification of selected isolates

DCP-degrading isolates were identified based on the characteristics of cell morphology and biochemical activities, such as: oxidase, catalase, oxidation-fermentation test (O-F test), formation of fluorescent-pigment, aromatic ring cleavage (Gerhardt *et al.*, 1981), quinone system (Kuraishi *et al.*, 1985), and vitamin requirements (Katayama & Kuraishi, 1989).

Results and Discusion

DCP degradation at 1.0 and 0.1 μ g-C/ml

In the isolation experiments, DCP was degrade by natural microbial community in the groundwater sample only when it was spiked at concentration of 1.0 μ g-C/ml after around 10 day-incubation period (Fig. 1 B). Acclimation period of 1.0 μ g-C/ml of DCP spiked at the second and third times was reduced to 1 — 2 days.

No degradation of 0.1 μ g DCP-C/ml was observed even until 3 weeks incubation period. Although there was no complete degradation of DCP in the flask spiked with 0.1 μ g DCP-C/ml, DCP was spiked repeatedly to a final concentration of 0.1 μ g-C/ml (Fig. 1).

The failure of degradation of DCP at low concentration in the groundwater was consistent with other study. It may be attributable to the failure of induction of enzyme(s) responsible to the xenobiotics degradation (Button & Robertson, 1985; Martani & Seto, 1990); or to the accumulation of toxic substances (Hoover *et al.*, 1986).

Isolation of DCP degrading microbes

27 bacterial strains were isolated from the repeated spike of 1.0 μ g DCP-C/ml, and 25 strains were isolated from 0.1 μ g-C/ml of DCP. Each of these isolates was transferred 5 times on MS agar slants containing 1.0 or 0.1 μ g DCP-C/ml. Only 18 and 15 isolates from 1.0 or 0.1 μ g-C/ml, respectively, survived during repeated transfer on MS agar slants.

Screening of 33 survived-isolates showed that 5 isolates degraded 1.0 and/or 0.1 μ g DCP-C/ml in MS medium (Martani & Seto, 1990). All of these DCP degrader were isolated from repeated spike

DCP at 1.0 μ g-C/ml. None of the degrader was originated from 0.1 μ g-C/ml.

Identification of DCP degrader

The electron micrographs of the DCP-degrading microbes were showed in Fig. 2 to Fig. 6. Morphological characteristics indicated that all of the degraders have rod shape and polar flagella. Complete results of the identification (table 3), showed that the 5-strains DCP degrader have similar characters in gram reaction, oxidase, catalase, and aromatic-ring cleavage. These data may imply that these isolates are belonged to the same genus, that is *Pseudomonas*. Morphological characteristics of these isolates were coincide with those by Wilson *et al.* (1983 a). Although they were not identified until genus level, they also isolated large number of gram negative-rod bacteria from various depth of subsurface water samples.

Furher examination showed that they have different characters in Quinone system and ability to form fluorrescent pigment. Except of strain E-6 which has ubiquinone with 10 perethrenoid (ubiquinone-10, Q-10), all of DCP degrading isolates have ubiquinone-9 (Q-9). Only strain E-4 produces fluorescent pigment of King A-B medium. These last data indicated that although the 5 DCP degrader are belong to *Pseydomonas*, they came from different group (Buchanan & Gibbon, 1974).

Based on the Quinone system and formation of fluorescent pigment, it was concluded that strain E-4 is *Pseudomonas* sp. group I which can produce fluorescent pigment; whereas strain Et-5, Et-13 and Et-14 are non-forming fluorescent pigment of *Pseudomonas* sp. group I.

In case of strain E-6, based on its ubiquinone -10, E-6 belongs to the *Pseudomonas* sp. group V (Kuraishi *et al.*, 1985). One of the characteristics of group V is the requirement of vitamins for their growth (Buchanan & Gibbon, 1974), but still there is possibility that some strains are able to adapt to their invironments so that they can grow in media containing no vitamin after some adaptation period (Katayama & Kuraishi, 1980). In this study strain E-6 has Ubiquinone-10 but it does not require vitamin. Therefore, we suggested that E-6 is non-requiring vitamin of *Pseudomonas* sp. group V.

Table 1. **HPLC analysis for 2,4-dichlorophenol (DCP)**

Instruments	:	Yanaco L-4000 W (Yanagimoto Co., Ltd.) Shodex M-315 (Showa Denko K.K.)
Colums	:	Shodex RS pak DS-613 & DS-613 (p) (Showa Denko K.K.)
Column temperature	:	50°C
Eluent	:	Water: Methanol (3.5 : 6.5, v/v), pH 11.2 (Na_2HPO_4 -NaOH buffer)
Flow rate	:	1.0 ml/min
Sample size	:	100 μ l
Detector & Integrator	:	Chromatocorder 12 (System Instruments Co., Ltd.)

Tabel 2. **Composition of Mineral Salts (MS) solution (in μ g/ml of deionized water)**

Solution A	:	Na_2HPO_4	298.0
		KH_2PO_4	122.0
Solution B	:	NH_2NO_4	160.0
Solution C	:	$MgSO_4 \cdot 7H_20$	24.6
		$CaCl_2 \cdot 2H_2O$	14.8
		Na_2SiO_3	3.7
Solution D	:	$MnCl_3 \cdot 4H_2O$	1.98
		H_3BO_3	0.62
		$Na_2MoO_4 \cdot 2H_2O$	2.42
Solution E	:	$FeCl_3 \cdot 6H_2O$	0.54
		$CuSO_4 \cdot 5H_2O$	0.0124
		$ZnSO_4 \cdot 7H_2O$	0.00144
		$CoSO_4 \cdot 7H_2O$	0.00140

The pH value is 7.2
Sterilized at 121°C for 15 min.

*) MS agar is MS solution added with nutrient agar (Eiken) at 0.1 mg/ml, and purified agar (Kyokuto) at 8.0 mg/ml.

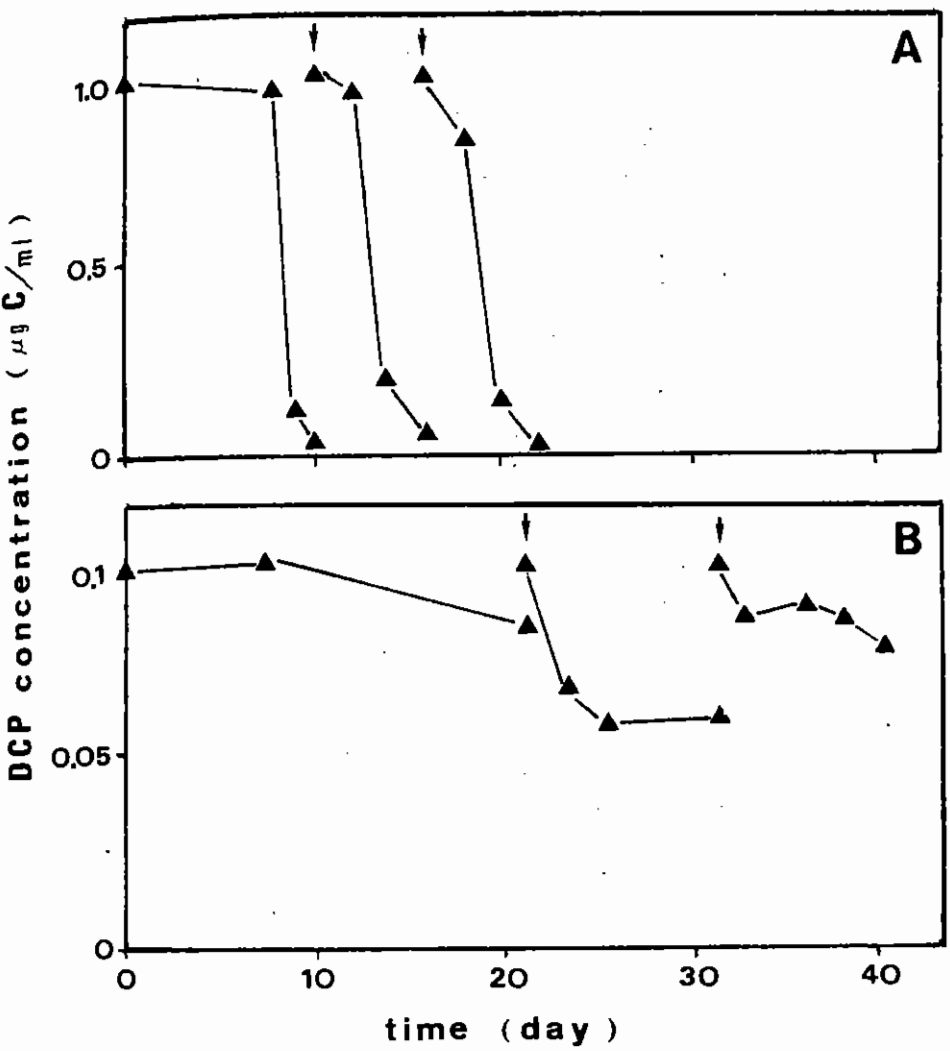

Fig. 1. Degradation of DCP repeatedly spiked at 1.0 (A) or 0.1 μ g-C/ml (B) in the groundwater sample.
Arrows indicate the time of repeated spike of DCP

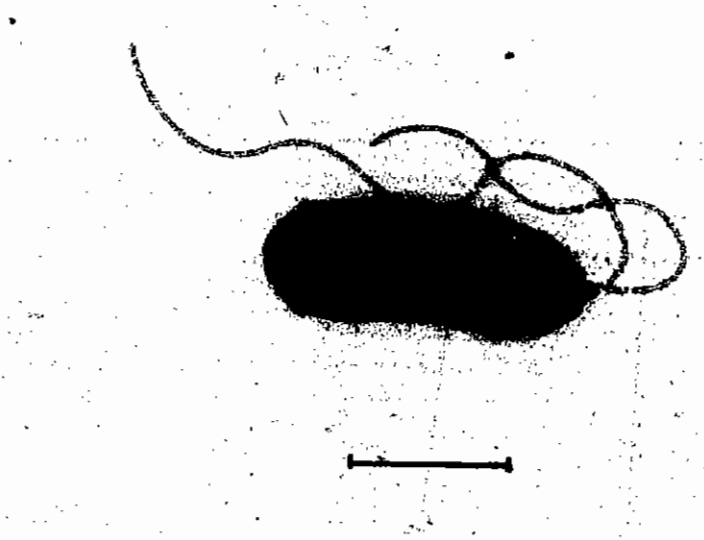

Fig. 2. **Electron micrograph of strain E-4**
The bar represents 1 μ m

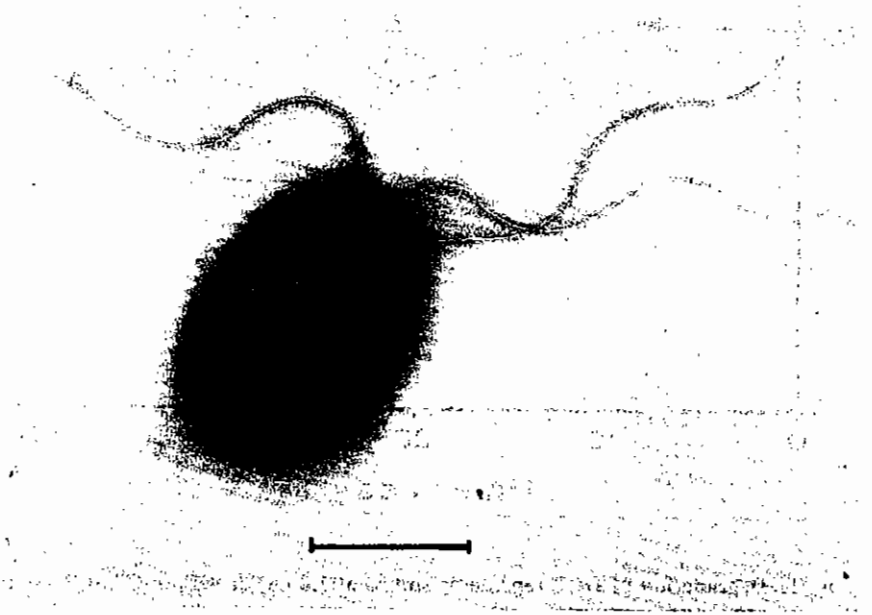

Fig. 3. **Electron micrograph of strain E-6**
The bar represents 1 μ m

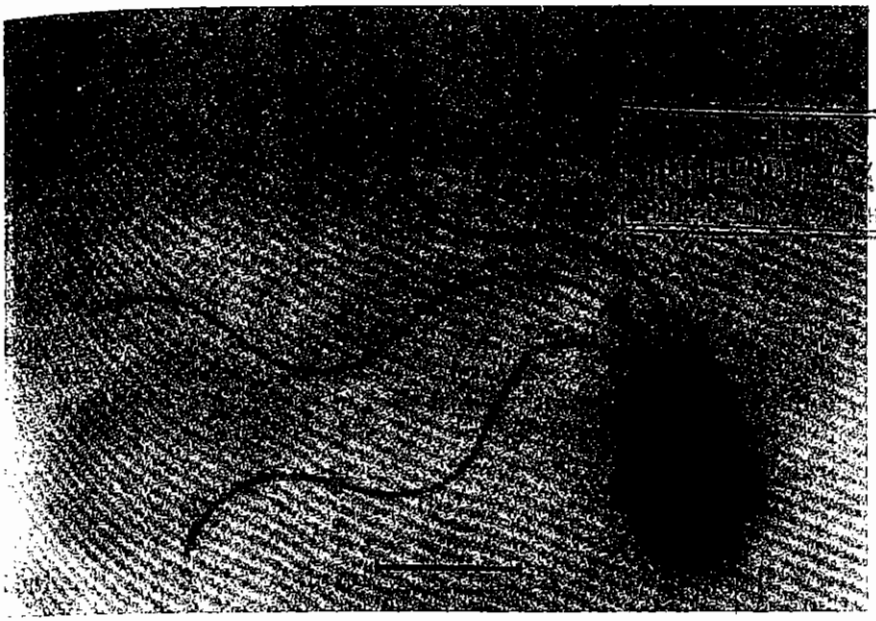

Fig. 4. Electron micrograph of strain Et-5.
The bar represents 1 μ m

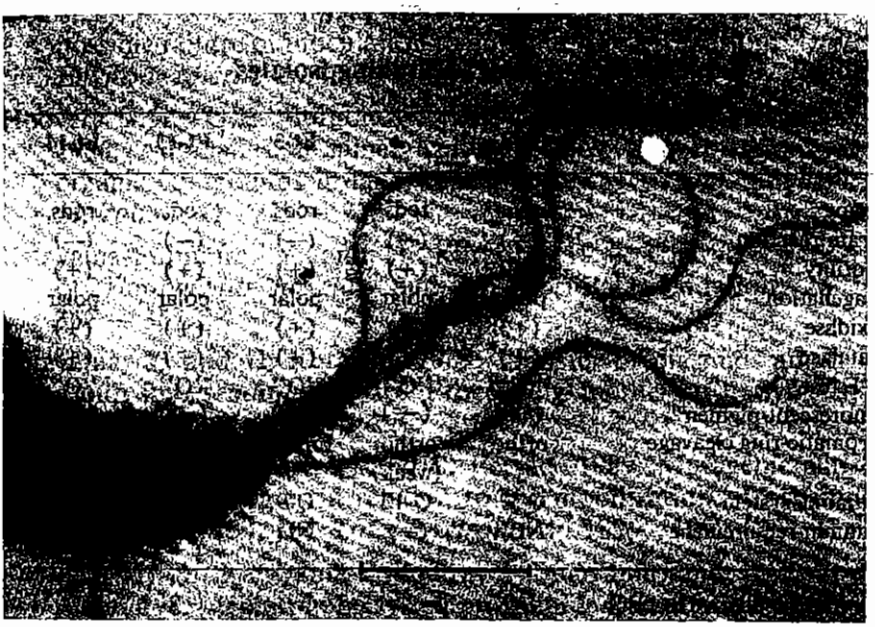

Fig. 5. Electron micrograph of strain Et-13.
The bar represents 1 μ m

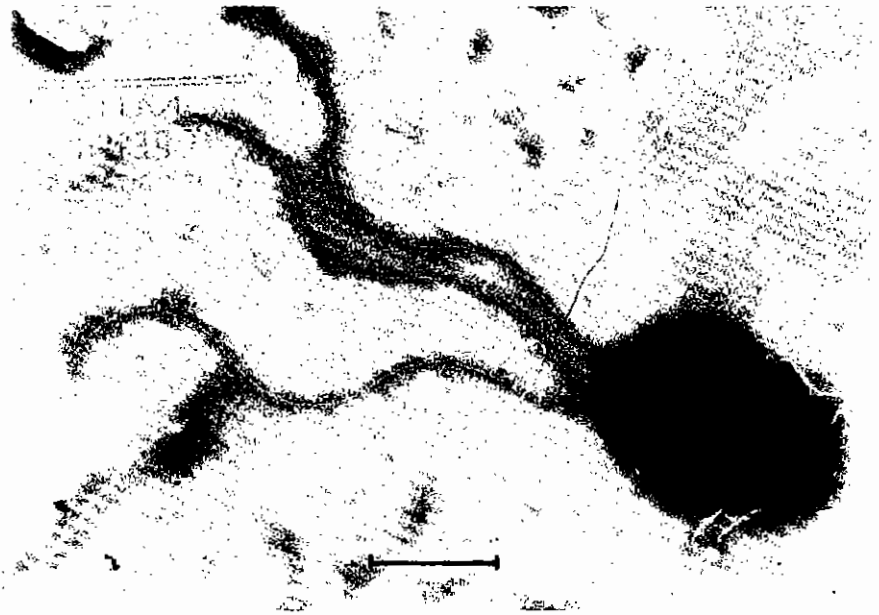

Fig. 6. **Electron micrograph of strain Et-14.**
The bar represents 1 μ m

Table 2. **Characteristics of DCP-degrading isolates**

	E-4	E-6	Et-5	Et-13	Et-14
Shape	rods	rods	rods	rods	rods
Gram reaction	(−)	(−)	(−)	(−)	(−)
Motility	(+)	(+)	(+)	(+)	(+)
Flagellation	polar	polar	polar	polar	polar
Oxidase	(+)	(+)	(+)	(+)	(+)
Catalase	(+)	(+)	(+)	(+)	(+)
O-F test	"O"[a]	"O"	"O"	"O"	"O"
Fluorescent-pigmen[b]	(+)	(−)	(−)	(−)	(−)
Aromatic-ring cleavage	ortho[c]	ortho (weak)	ortho	ortho (weak)	ortho
Quinone system	Q-9[d]	Q-10	Q-9	Q-9	Q-9
Vitamin-requirement	ND[e]	(−)	ND	ND	ND

a) "O", oxidative reaction
b) Examined in King A-B medium
c) ortho pathway
d) Q, Ubiquinone
e) ND, Not determined

References

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. *Science*. 211 : 132 — 138.

Batterton, J., K. Winters and C. Van Baalen. 1978. Anilines : Selective toxicity to blue green algae. *Science*. 199 : 1068 — 1070.

Buchanan, R.E. and N.E. Gibbon. 1974. *Bergey's manual of determinative bacteriology*. 8th ed. The Williams & Wilkins, Baltimore. pp. 141 — 199.

Button, D.K. and B.R. Robertson. 1985. Effect of toluene exposure time and concentration on introduction of high affinity values for toluene oxidation by bacteria of estuarine seawater sample. *Mar. Ecol. Prog. Ser.* 26 : 187 — 193.

Carey, J.H., M.E. Fox and J.H. Hart. 1988. Identity and distribution of chlorophenols in the north arm of the Fraser river estuary. *Water Poll.Res.J. Canada*. 1 : 31 — 44.

Gerhardt, P., R.G.E. Murray, R.N. Costillow, E.W. Nester, W.A. Wood, N.R.Kireg and G.B. Phillips. 1981. *Manual of methods for general microbiology*. American Society for Microbiology. Washington DC. pp. 409 — 443.

Goldstein, P., R.G.E. Murray and M. Alexander. 1985. Reasons for possible failure of inoculation to enhance biodegradation. *Appl. Environ. Microbiol.* 50 : 977 — 983.

Hoover, D.G., G.E. Borgonovi, S.H. Jones and M. Alexander. 1986. Anomalies in mineralization of low concentrations of organic compounds in lake water and sewage. *Appl. Environ. Microbiol.* 51 : 226 — 232.

Katayama-Fujimura, Y. and H. Kuraishi. 1980. Characterization of *Thiobacillus novellus* and its thiosulfate oxidation. 26 : 357 — 367.

Katayama, Y. and H. Kuraishi, 1989. Transfer of plasmids among group in *Pseudomonas* sp. Proceeding in the Int'l. Sym. in Microbial Ecology, Kyoto.

Kool, H.J., C.F. Van Kreyl and S. Persad. 1989. Mutagenic activity in groundwater in relation to the mobilization of organic mutagens in soil. *Sci. Total Environ.* 84 : 185 — 199.

Kuraishi, H., Y. Katayama-Fujimura, J. Sugiyama amd T. Yokoyama. 1985. Ubiquinone system in fungi I: Distribution of ubiquinones in the major families of ascomycetes, basidiomycetes and deuteromycetes and their taxonomic implications. *Trans. Mycol. Soc. Japan*. 26 : 383 — 395.

Martani, E. and M. Seto. 1990. Possible reasons for the failure of natural microbial community to degrade low concentration of 2,4-dichlorophenol. *Bull. Jpn. Soc. Micr. Ecol.* 5 : 37 — 44.

Martani, E. and M. Seto. 1991. Effects of mineral nutrients on the biodegradation of 2,4-dichlorophenol at low concentration by microbial community and bacterial isolates. *J. Pesticide Sci.* 16 : 1 — 7.

Patrick, R., E. Ford and J. Quarles. 1987. *Groundwater contamination in United States*. Univ. Pennsylvania Press, Blockley Hall, Philadelphia, P.A. 518 p.

Spain, J.C., D.H. Pritchard and A.W. Bourquin. 1980. Effect of adaptation on biodegradation rates in sediment/water cores from estuarine and freshwater environments. *Appl. Environ. Microbiol.* 40 : 726 — 734.

Swindoll, C.M., C.M. Aelion, D.C. Dobbins, O. Jiang, S.C. Long and F.K. Pfaender. 1988. Aerobic biodegradation of natural and xenobiotic organic compounds by sub-surface microbial communities. *Environ. Toxicol. Chem.* 7 : 291 — 299.

Watanabe, S., S. Watanabe and M. Ito. 1983. Investigation on the contamination of freshwater fish with herbicides (CNP, chlorometoxynil, benthiocarb and molinate). *J. Pesticide Sci.* 8 : 47 — 53.

Wiggins, B.A., S.H. Jones and M. Alexander. 1987. Explanation for the acclimation period preceding the mineralization of organic chemicals. *Appl. Environ. Microbiol.* 21 : 793 — 796.

Wilson, J.T., J.F. Mc Nabb, D.L. Balkwill and W.C. Chiorse. 1983a. Enumeration and characterization of bacteria indigenous to a shallow watertable aquifer. *Groundwater.* 21 : 134 — 142.

Wilson, J.T., J.F.Mc. Nabb, B.H. Wilson and M.J. Noonan. 1983b. Biotransformation of selected organic pollutants in groundwater. *Dev. Ind. Microbiol.* 24 : 225 — 233.

Winger, P.V., D.P. Schultz and W.W. Johnson. 1990. Environmental contaminant concentration in the biota from the lower Savanah river, Georgia and South Carolina. *Arch. Environ. Contam. Toxicol.* 19 : 101 — 117.

Zaidi, B.R., G. Stucki and M. Alexander. 1988. Low chemical concentration and pH as factors limiting the success of inoculation to enhance biodegradation. *Environ. Toxicol. Chem.* 7 : 143 — 151.