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Original Article

Degradation of 2,4-Dichlorophenol in the Microbial Community of Groundwater Sample by Bacterial Isolate E-6

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A bacterial strain E-6 was isolated from groundwater in which the microbial community failed to degrade 0.1 μg C/ml of 2,4-dichlorophenol (DCP). Pure culture of E-6 degraded 0.1 μg DCP-C/ml in filter-sterilized groundwater, irrespective of inoculum size. However, when inoculated into the groundwater containing a microbial community, E-6 failed to degrade DCP, except when the inoculum size was as large as 10^6 cell/ml. These results suggest that the lack of mineral nutrients, the low concentrations of DCP and the possible presence of toxic substances were not responsible for the failure of DCP degradation by E-6 in the community. The failure may have been attributable both to the cometabolism of DCP by E-6 and to the elimination of uncharacterized dissolved organic carbon (DOC) by the microbial community, *i.e.* the cometabolism of DCP and the elimination of DOC caused E-6 to fail to increase its cell density high enough to make DCP degradation detectable.

INTRODUCTION

Successes of microbial inoculation in enhancement of xenobiotics degradation in soil and aquatic environments have been reported.[1-3] On the other hand, some environmental conditions, such as lack of particular mineral nutrients, low concentrations of xenobiotics in environments, or the presence of toxic substances may cause inoculated microorganisms fail to degrade xenobiotics in natural environments.[4,5]

A 2,4-dichlorophenol (DCP) degrader strain E-6, which is one of the bacterial species isolated from microbial community of a groundwater sample, degraded 0.1 μg C/ml of DCP in filter-sterilized groundwater.[6] However, in spite of the presence of E-6, the sample failed to degrade 0.1 μg DCP-C/ml.[6,7] These results may imply that some factors inhibited E-6 from surviving or expressing its ability to degrade 0.1 μg DCP-C/ml in the microbial community. Such environmental constraints should be elucidated for proper management to enhance degradation of xenobiotics in natural environments.

In this study we investigated possible reasons why E-6 failed to degrade DCP in the microbial community of a groundwater.

MATERIALS AND METHODS

1. *Water Sample*

Water sample was collected by pumping up underwater from the well 200 m deep at the Institute for Rolling Land Research, Hachioji, Tokyo. The pH value of the groundwater sample was 7.3, and the concentration of dissolved organic carbon (DOC) was around 2.0 μg/ml.[7] Experiments were done within 3 hr after collection.

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2. Bacterial Isolate

A bacterial strain E-6,[6] isolated from the groundwater, was used as a test organism. Stock culture of E-6 was maintained on a Mineral Salts (MS) agar slant[6] containing 30 μg DCP-C/ml. As a seed source, E-6 was precultured in the groundwater filtrated with a membrane filter (Sterivex-GS, Millipore Corp., pore size: 0.22 μm) without DCP. The density of E-6 increased to 10^5–10^6 cell/ml within 2–3 days presumably at the expense of DOC in the groundwater.

3. DCP Degradation by Isolate

Degradation of 0.1 μg C/ml of DCP by E-6 in filter-sterilized groundwater was studied in screw-capped Erlenmeyer flasks. Filtration was done as described above. Degradation of DCP by E-6 in the groundwater with a microbial community was also studied as follows: A small amount of unfiltered groundwater was added (1% v/v) into filter-sterilized groundwater. When the density of the microbial community had increased to 10^5–10^6 cell/ml, E-6 and DCP were added. Remaining DCP was analyzed by HPLC.[8]

In some aquatic samples, DCP degradation by other isolate differed depending on the kind of membrane filters used for filter-sterilization.[9] Namely, when a Sterivex-GV filter which was made from hydrophylic polyvinylidene fluoride was used, the degradation of DCP was slower than when Sterivex-GS filter made from mixed esters of cellulose acetate and cellulose nitrate was used. Therefore, the difference in DCP degradation by E-6 was compared by using these two kinds of filters to eliminate microbial community. The pore size of both filters was 0.22 μm. Conditions for incubation and analysis were the same as described above.

4. Enumeration of Density of E-6 and Total Heterotrophic Bacteria

The density of E-6 was estimated by the most probable number (MPN) method using DCP-spiked filter-sterilized groundwater. The DCP concentration was 0.1 μg C/ml and the incubation period was 5 weeks. When DCP in a MPN tube, as measured by HPLC, was degraded more than 50% of the spiked DCP, it was considered as positive indication of the presence of degrader. Details are in the previous article.[7]

Total heterotrophic bacteria were determined by the colony forming unit (CFU) of pour-plate method on MS agar.[6]

All cultures were incubated aerobically at 25°C in the dark.

RESULTS

As shown in Fig. 1, E-6 at inoculum size of 0.3 cell/ml, degraded 0.1 μg C/ml of DCP in the filter-sterilized groundwater without microbial community, but in the presence of microbial community, it failed to degrade DCP.

Within 5 days, the density of E-6 in the filter-sterilized groundwater increased to 4.3×10^5 cell/ml (Table 1), while that in the groundwater with microbial community was 0.3 cell/ml or lower. This indicated that E-6 could not grow in the groundwater with microbial community. The density of total heterotrophic bacteria was in the range between 5.3×10^6 and 1.0×10^7 cell/ml.

Elimination of microbial community either by using a GV-filter or a GS-filter resulted in DCP degradation (Fig. 2). In both flasks, the density of E-6 increased from around 6 cell/ml

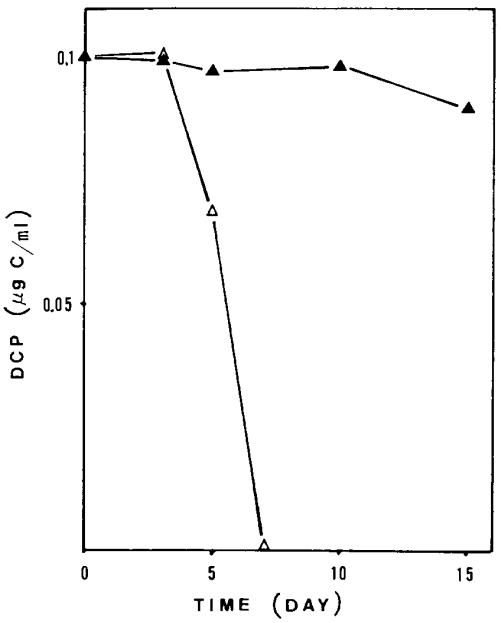

Fig. 1 Degradation of DCP by E-6 in filter-sterilized groundwater with (▲) or a without microbial community (△).

The initial density of E-6 was 0.3 cell/ml.

Table 1 Changes in the density of strain E-6[a] in filter-sterilized groundwater containing 0.1 μg DCP-C/ml with or without a microbial community of 5.3×10^6–1.0×10^7 cell/ml.[b]

Day after incubation	Density of E-6 (cell/ml)	
	with microbial-community	without microbial-community
Day 0	0.3	0.3
Day 5	0.3	4.3×10^5
Day 10	<0.3	9.5×10^5
Day 15	0.3	N.D.

a) Estimated by the MPN method using DCP-spiked filter-sterilized groundwater.
b) Determined by the pour-plate method on MS agar.
N.D.: not determined.

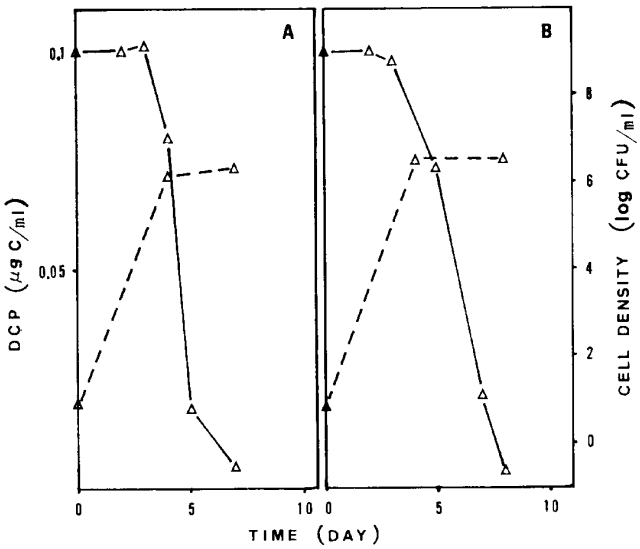

Fig. 2 Degradation of DCP (——) and the density of E-6 (– – –) in GV- (A) or GS-filter-sterilized groundwater (B).
The initial density of E-6 was 6 cell/ml.

to 10^6 cell/ml.

Table 2 shows the increase of E-6 density in filter-sterilized groundwater containing no DCP. This means that E-6 could increase its density at the expense of uncharacterized DOC in the groundwater.

When the inoculum size was as large as 10^6 cell/ml, E-6 degraded DCP even in the presence of a microbial community of 10^6 cell/ml (Fig. 3). In both flasks, there was no significant increase in the density of E-6 and

Table 2 Changes in the density of strain E-6[a] in filter-sterilized groundwater containing no DCP with or without a microbial community of 5.1×10^6–1.0×10^7 cell/ml.[b]

Day after incubation	Density of E-6 (cell/ml)	
	with microbial-community	without microbial-community
Day 0	<0.3	0.3
Day 5	<0.3	9.3×10^4

Notes are the same as described in Table 1.

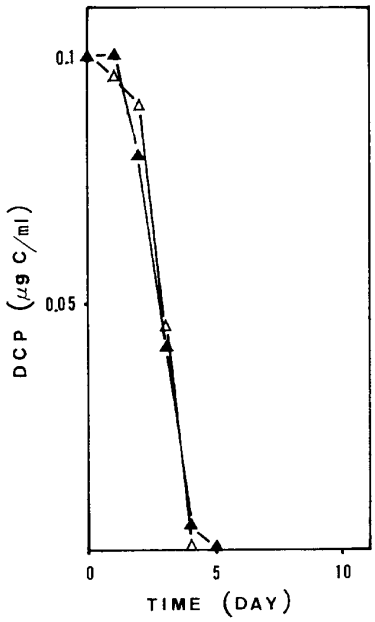

Fig. 3 Degradation of DCP by E-6 in filter-sterilized groundwater with (▲) or without a microbial community (△).
The initial density of E-6 was around 10^6 cell/ml.

Table 3 Changes in the density of strain E-6[a] in filter-sterilized groundwater containing 0.1 μg DCP-C/ml with or without a microbial community of 1.2×10^6–3.0×10^6 cell/ml.[b]

Day after incubation	Density of E-6 (cell/ml)	
	with microbial-community	without microbial-community
Day 0	1.1×10^6	0.9×10^6
Day 4	1.5×10^6	2.0×10^6

Notes are the same as described in Table 1.

total heterotrophic bacteria (Table 3).

Figure 4 shows the degradation of 0.1 and 3.0 μg DCP-C/ml by E-6 in the groundwater with

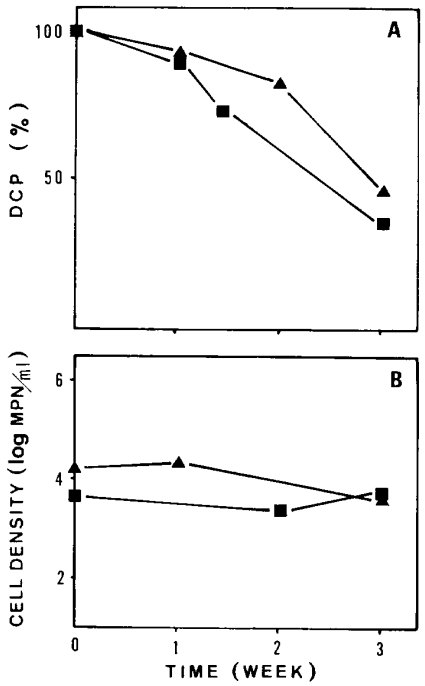

Fig. 4 Degradation of DCP (A) at 0.1 (▲) or 3.0 μg DCP-C/ml (■) and the density of E-6 (B) in groundwater with microbial community.

The initial density of E-6 was around 10^4 cell/ml.

microbial community. E-6 was inoculated at an initial density of around 10^4 cell/ml and at a density of heterotrophic microorganisms of 10^5 cell/ml. At both concentrations DCP was degraded slowly, and the density of E-6 did not change.

DISCUSSION

In the previous paper,[7] we have reported that the density of autochthonous DCP degrader in groundwater at Hachioji, estimated by the MPN-method, was always less than 1 cell/100 ml. This density was considerably low compared with the density of heterotrophic microorganisms, which was hundreds per ml.[7] The density of DCP degrader was ten thousand times lower than that of heterotrophic microorganisms, it was also detected in many aquatic environments.[8] Taking these findings into consideration, we inoculated E-6 at low inoculum size. To study the effect of inoculum size, E-6 was also inoculated at high density.

Degradation of 0.1 μg DCP-C/ml by E-6 was detected after 3 days in the filter-sterilized groundwater without microbial community, when the density of E-6 had increased to 4.3×10^5 cell/ml. This level was considerably higher than observed in our previous study, where the elimination of 0.1 μg DCP-C/ml in aquatic waters was evident when the density of responsible degrader increased to around 10^3–10^4 cell/ml.[8] These data imply that the density should reach a certain level for the degrader to show a detectable degradation of xenobiotics, and that this density varies depending on the activity of degrader.

In the groundwater with microbial community, E-6 failed to degrade DCP when inoculated at a density of 0.3 cell/ml (Fig. 1). The phenomena, where inoculated microorganisms failed to degrade xenobiotics in natural environments, have been related to several environmental stresses.[4,5]

Low concentrations of inorganic nutrients often found in aquatic environments may limit the growth of degrader and the degradation of xenobiotics.[10,11] In the such a case amendment of sample water with certain minerals, phosphate or ammonium nitrate, for example, resulted in the acceleration of xenobiotics degradation.[7,12] Nevertheless, this explanation is not applicable to this study, because E-6 increased its density and degraded DCP in filter-sterilized groundwater without nutrient amendment.

Filtration of groundwater to eliminate the microbial community with either a GS-filter or a GV-filter did not change DCP degradation significantly nor increased the density of E-6 (Fig. 2). This result suggests that toxic substance(s) which may have been adsorbed by the GS-filter,[9] were not responsible for the failure of E-6 to degrade DCP in the microbial community.

A threshold concentration of xenobiotics may exist for growth of microorganisms.[13] Therefore, low concentrations of xenobiotics found in environments may account for the failure of degrader to grow.[4,5] In this study, however, E-6 at high density degraded 0.1 μg DCP-C/ml. This means that the failure of E-6 to increase its density and to degrade DCP in microbial community was not attributable to low concentration of DCP.

As shown in Table 2, E-6 could increase its density at the expense of uncharacterized DOC

in the groundwater. Elimination of DOC by microbial community may cause E-6 to fail to increase its density from initially low to a high enough to show a detectable degradation of DCP. Therefore, E-6 could not degrade DCP in the groundwater with microbial community when E-6 was inoculated at an initial density as low as 0.3 cell/ml.

In an experiment,[14] *Pseudomonas* sp. DP-4, a DCP-assimilating bacterium, was inoculated into a microbial community of non-DCP-degrader and spiked with DCP at various concentrations. DCP was degraded, and a proportional increase in the density of DP-4 was observed with an increase in DCP concentration. In this study, as shown in Fig. 4, in groundwater with microbial community, E-6 could not increase its density although it degraded 3.0 μg DCP-C/ml 30 times higher than 0.1 μg C/ml. The differences between E-6 and DCP-assimilating bacterium DP-4 may imply that E-6 degraded DCP by cometabolism. The phenomenon of cometabolism of xenobiotics at low concentrations in natural environments has been reported elsewhere.[15-17]

Discrepancy was observed: E-6 did not degrade DCP in the flask of groundwater with microbial community when inoculated at an initial density 0.3 cell/ml (Fig. 1). However, in the test tubes used for the MPN, some of them degraded more than 50% of DCP. There were positive codes indicating that E-6 in MPN medium did proliferate and degrade DCP (Table 1 on Days 0, 5 and 15). Possible explanations for this discrepancy: First, DOC concentration in DCP-spiked filter-sterilized groundwater for MPN was higher than that in the flask in which DOC had been eliminated by the microbial community before E-6 was inoculated. This condition allowed E-6 increase its density and to degrade more than 50% of the spiked DCP. Secondly, the incubation period for MPN was 3 weeks longer than for the flask. During this period of incubation slow degradation of DCP gave positive code for MPN.

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REFERENCES

1) D. K. Chatterjee, J. J. Kilbane & A. M. Chakrabarty: *Appl. Environ. Microbiol.* **44**, 514 (1982)
2) J. J. Kilbane, D. K. Chatterjee & A. M. Chakrabarty: *Appl. Environ. Microbiol.* **45**, 1697 (1983)
3) M. Seto & F. Tsurui: *Man Environ.* **15**, 18 (1989) (in Japanese)
4) M. Alexander: "Genetic Control of Environmental Pollutants," ed. by G. S. Omen & A. Hollander, Plenum Press, New York, p. 151, 1984
5) R. M. Goldstein, L. M. Mallory & M. Alexander: *Appl. Environ. Microbiol.* **50**, 977 (1985)
6) E. Martani & M. Seto: *J. Pesticide Sci.* **16**, **1** (1991)
7) E. Martani & M. Seto: *Bull. Jpn. Soc. Microb. Ecol.* **5**, 37 (1990)
8) M. Seto, S. Nakano, E. Martani & J. Makino: *Ecol. Chem.* **9**, 8 (1989) (in Japanese)
9) M. Seto & J. Makino: *Man Environ.* **16**, 70 (1990) (in Japanese)
10) B. A. Wiggins, S. H. Jones & M. Alexander: *Appl. Environ. Microbiol.* **53**, 791 (1987)
11) D. L. Lewis, H. P. Kollig & R. E. Hodson: *Appl. Environ. Microbiol.* **51**, 598 (1985)
12) S. H. Jones & M. Alexander: *FEMS Microb. Lett.* **52**, 121 (1988)
13) S. K. Schmidt, M. Alexander & M. L. Shuler: *J. Theor. Biol.* **114**, 1 (1985)
14) Unpublished
15) R. S. Horvath: *Bacteriol. Rev.* **36**, 146 (1972)
16) W. Brunner, F. H. Sutherland & D. D. Focht: *J. Environ. Qual.* **14**, 324 (1985)
17) R. J. Shimp & F. K. Pfaender: *Appl. Environ. Microbiol.* **49**, 394 (1985)

要　　約

地下試水の微生物群集の中で細菌の分離株 (E-6) による 2,4-ジクロロフェノールの分解

Erni Martani, 瀬戸昌之

地下水から細菌の一種 (E-6) を単離した．この地下水の微生物群集は 0.1 μg 炭素/ml の 2,4-dichlorophenol (DCP) を分解できなかった．濾過滅菌した地下水では E-6 の純粋培養は，その接種密度にかかわらず，0.1 μg 炭素/ml の DCP を分解した．しかしながら，E-6 を微生物群集を含む地下水に接種すると，その接種密度が

10^6 細胞/ml のように高いとき以外は，E-6 は DCP を分解できなかった．これらの結果は，E-6 が微生物群集内で DCP を分解できないのは栄養塩類の欠乏，DCP 濃度の低さ，そして，阻害物質の存在のためではないことを示唆する．DCP を分解できないのは，微生物群集による DCP 以外の溶存有機炭素（DOC）の除去と E-6 が DCP を共役代謝（cometabolism）するためであろう．すなわち，DOC の除去と DCP の共役代謝のために，E-6 は DCP の検出可能な分解を示すのに必要な密度まで増加できないからと考えられた．