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REVIEW

Recent Technology on Bio-remediation of POPs and Persistent Pesticides

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Abstract

Clean-up technology for contaminated soil and water with persistent organic pollutants (POPs) and other pesticides is required. A new aerobic pentachloronitrobenzene (PCNB)-degrading bacterium, *Nocardioides* sp. strain PD653, was isolated from an enrichment culture in an original soil-charcoal perfusion system. Strain PD653 also degraded hexachlorobenzene (HCB) with a liberation of chloride ions to CO_2 under aerobic conditions. It is the first aerobic bacteria capable of mineralizing HCB. Moreover, an aerobic dieldrin-degrading fungus, *Mucor* sp. strain DDF, was isolated from soil to which endosulfan had been applied annually for many years. Strain DDF degraded dieldrin to 1.01 μM from 14.3 μM during 10-day incubation at 25°C. On the other hand, the application technology remains inadequate for remediating contaminated sites. Therefore, we developed a method to introduce the degrading-bacterial consortium into contaminated soil using a special charcoal material that enriched the soil with a methylthio-*s*-triazine degrading bacterium and the chloro-*s*-triazine degrading bacterial consortium CD7. For *in situ* bioremediation study, the enriched charcoal with CD7 was used at a contaminated site with simazine. The material was effective for preventing penetration of simazine into subsoils and nearby aquatic environments for approximately two years.

Discipline: Agricultural environment
Additional key words: hexachlorobenzene, dieldrin, *s*-triazine, bioremediation

Introduction

Organochlorine pesticides, e.g., hexachlorobenzene (HCB; C_6Cl_6) and dieldrin ($C_{12}H_8Cl_6O$), are persistent synthetic chemicals. HCB and dieldrin has been extensively used for controlling fungal disease and insect pests, respectively, but they cause great damage to agricultural crops and vegetables. Their use has been prohibited in many countries since the early 1970s because of their susceptibility to biological magnification, their high toxicity, and their long persistence in the environment. Although reports differ on the half-lives of HCB and dieldrin in soil differ to some extent, the average half-life of HCB and dieldrin in soil is approximately more than nine and seven years, respectively[2,4]. Therefore, HCB and dieldrin were listed as persistent organic pollutants (POPs) by the Stockholm convention in 2001.

HCB and dieldrin are still found in environments such as uplands or/and paddy fields even more than 30 years since their prohibition[12,6]. Moreover, dieldrin at residual concentrations exceeding the limit set by the Food Sanitation Law of Japan (dieldrin: <0.02 ppm, fresh weight basis) has been detected in cucumbers produced in some agricultural areas in Japan[6]. Thus, contamination with organochlorine pesticides is still a serious environmental problem and an efficient method for remediation is required.

Meanwhile, *s*-triazines are recognized as a major class of herbicides and are widely used in agriculture for controlling various weeds. They are classified into three groups: chloro-, methylthio-, and methoxy-*s*-triazines. In the *s*-triazine family, chloro-*s*-triazines such as atrazine and simazine are the most popular. In particular, atrazine is used globally to control annual grasses and broadleaf weeds in fields of major crops, such as corn, sorghum,

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and sugar cane. They have been detected in ground and surface water[3,11]. Methylthio-*s*-triazines such as simetryn and dimethametryn are used in rice paddy fields, and have been frequently detected in river water[11], a lake basin[13], and river sediment[9]. Chloro- and methylthio-*s*-triazines were often detected in river water[11] and river sediment[9].

Among the removal technologies for such residual pesticides, bioremediation is considered to be the most cost-effective because residues of HCB, dieldrin, or *s*-triazines are low in concentration and wide in range. Microbial degradation is a promising, effective technique to remediate environmental pollutants.

Here we describe the aerobic biodegradation of organochlorine pesticides (HCB, dieldrin, and triazine) by isolated soil microorganisms. Moreover, we developed a cleanup technology using a special charcoal enriched with a degrading-bacterial consortium.

1. Aerobic biodegradation of HCB by isolated soil bacteria

(1) Aerobic Mineralization of Hexachlorobenzene by *Nocardioides* sp. PD653

The enrichment of PCNB-degrading bacteria was isolated using the original soil-charcoal perfusion method[14,7,15,18]. The isolated strain, PD653, was characterized on the basis of comparative morphology, physiology, and comparison of the 16S rRNA sequences. The isolated strain PD653 belongs to a species of gram-negative, catalase-positive, oxidase-negative, non-spore-forming, and non-motile rods (0.7-0.8 × 1.0-1.2 μm in size), which form pale-yellow circular colonies. The GC content of the strain was 70.8%. The 16S rRNA sequence of strain PD653 (1,487 nucleotides) was compared with that of the bacterial sequences in the GenBank. Strain PD653 exhibited a high sequence similarity with that of bacteria classified as *Nocardioides*. The highest sequence similarity (97.1%) of the 16S rRNA gene was found in *Nocardioides* sp. OS4[10]. Thus, strain PD653 is assigned to a novel species in the genus *Nocardiodes*.

(2) HCB degradation

On aerobically culturing strain PD653 in mineral salt medium (MM) containing HCB, the initial concentration of 8.0 μM of HCB decreased to 1.5 μM during nine days of cultivation, and accumulation of chloride ions up to 34.0 μM was observed (Fig. 1). The apparent increase of OD_{600} was not obtained after nine days of cultivation (Fig. 1).

(3) Mineralization of HCB

Strain PD653 grown on R2A agar plate was inoculated into glass jars containing 20 mL of MM supplemented with 3.6 μM ^{14}C-HCB. The presence or absence of ^{14}C-HCB in the specimen was determined using HPLC equipped with a radioactivity flow-through detector. In the 14-day culture fluid, the radioactivity decreased by 39.5% of its initial theoretical value. Radioactive HCB was not detected in the culture fluid, and the radioactivity was predominantly found in unknown water-soluble metabolites after one day of cultivation. The adsorbed ^{14}C-HCB residue on the jar walls was only 2.2%. The PUF column captured 13.1% of the residue, which was ascertained as HCB volatilized during cultivation. The NaOH traps recovered 36.8%, and 84.7% of the trapped radioactivity was precipitated as $Ba^{14}CO_3$ by adding $BaCl_2$, indicating that the radioactivity was attributed to $^{14}CO_2$ (Fig.2).

(4) Biotransformation experiment by using the resting cells

In order to identify the intermediate of HCB catabolism, the resting cells of strain PD653 were used in a degradation experiment. By incubating HCB with the resting cells, the decreased HCB levels and increased levels of a metabolite were observed (Fig. 3). This metabolite was identified as PCP by its HPLC retention time and UV spectrum; they were found to be identical to those of an authentic sample. To detect other minor metabolites, PCP was incubated with the culture of the resting cells for a short period (2 h). Though the decrease of PCP was very little, tetrachlorohydroquinone and 2,6-dichlorohydroquinone were detected as acetylated derivatives (Fig. 4). These intermediates were identified by the comparison of their GC retention times and MS spectra with au-

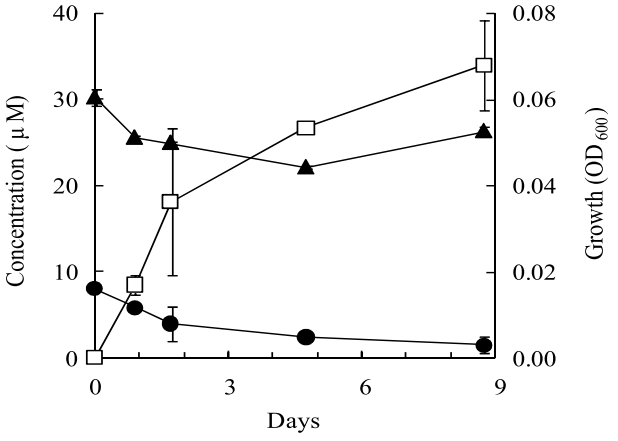

Fig. 1. Aerobic degradation of HCB by *Nocardioides* sp. strain PD653 in the mineral salt medium (MM)

The changes in OD_{600} of strain PD653 (▲) and evolution of chloride ions (□) in accordance with the HCB degradation (●) were demonstrated. OD_{600} and concentrations of the materials were mean values of the duplicate experiments. Error bars indicate S.D.[18]

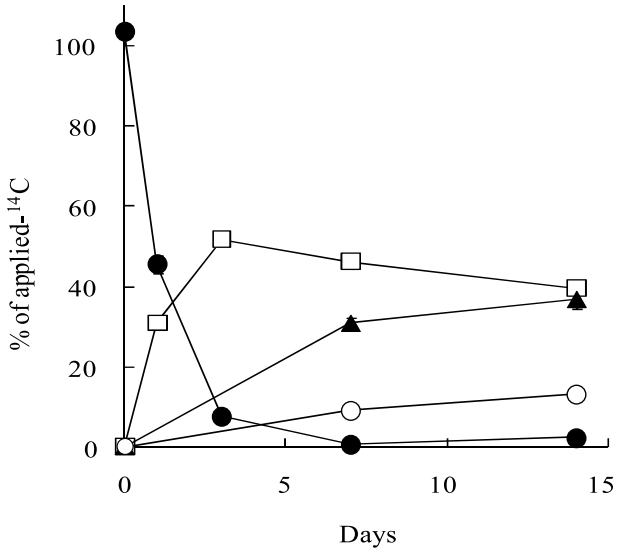

Fig. 2. Mineralization of [U-ring-^{14}C] HCB by *Nocardioides* sp. strain PD653

Disappearance of ^{14}C-HCB (●) and evolution of ^{14}C-labelled unidentified water soluble metabolites (□) and $^{14}CO_2$ (▲) were demonstrated. Volatile ^{14}C-HCB (○) captured on the PUF column is also shown. Radioactivity of the materials was mean value of the duplicate experiments, and was expressed as the percentage of that of the initial applied ^{14}C. Error bars indicate S.D. [18]

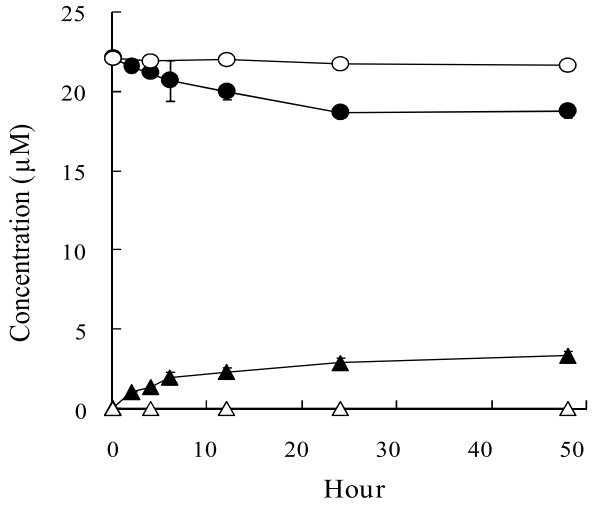

Fig. 3. Generation of PCP accompanied by degradation of HCB by resting cells of *Nocardioides* sp. strain PD653

The time courses of disappearance of HCB (●) and generation of PCP (▲) are demonstrated. The concentrations of HCB (○) and PCP (△) in heat-killed control cultures were also demonstrated. Mean values (n = 3) and S.D. of concentrations of the materials are shown[18].

thentic samples. Degradation of HCB and the appearance of these metabolites were not observed in the control culture with autoclaved resting cells. According to the results of resting-cell study, the putative metabolic pathway of HCB and PCNB by strain PD653 is proposed in Fig. 5.

2. Aerobic biodegradation of dieldrin by isolated soil fungi

(1) Degradation experiment

The degradation experiment was performed using 35 strains of *Trichoderma* spp. and 36 strains that were isolated from a soil with annual endosulfan applications for more than 10 years until 2008. After pre-incubation for seven days, 50 μl of dieldrin in acetone was added to each inoculated flask (final concentration: 13.2 μM). Among *Trichoderma* spp., *Trichoderma* sp. strain 93155 was capable of degrading dieldrin with 19.7% (2.6 μM) degradation after 14 days of incubation. However, a fungus, isolated from soil contaminated with endosulfan, degraded dieldrin by 95.8% compared with 13.2 μM in initial concentration. It was named strain DDF. Another fungal strain with the ability to degrade dieldrin but having a lower degradation capability of 43.3% (5.7 μM) was also isolated. A further experiment was performed using strain DDF without pre-incubation. In the time course degradation experiment, strain DDF degraded dieldrin to

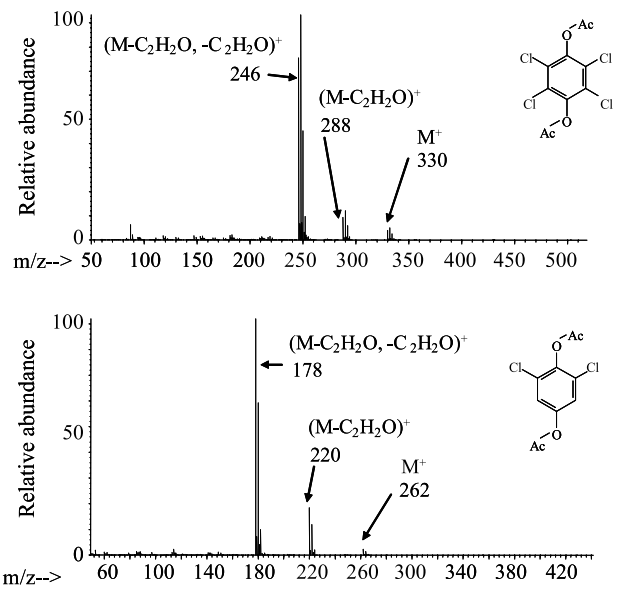

Fig. 4. GC/MS analysis of metabolites obtained from degradation of PCP by resting cells of *Nocardioides* sp. strain PD653

Acetylated derivatives of the metabolites were analyzed. The scanning was carried out at mass range of 50–600 (m/z)[18].

1.01 μM from 14.3 μM during 10-day incubation at 25°C (Fig. 6).

(2) Identification of dieldrin-degrading fungus

The ITS rRNA sequence of strain DDF (471 nucleotides, GenBank accession no. AB536702) was compared with those of the fungal sequences in the GenBank. Strain DDF exhibited a high sequence similarity to those of *Mucoraceae* fungi as shown by the constructed phylogenetic dendrogram. The highest sequence identity (100%) was found with *Mucor racemosus* f. *racemosus* (GenBank accession no. AY213659)[8]. Strain DDF was designated as *Mucor racemosus* strain DDF. To date, *Mucor alternans* has been reported to degrade dieldrin and DDT by Anderson *et al.*[1]. However, the isolated *M. alternans* degraded only 20% of dieldrin. The degradation of strain DDF is superior to *M. alternans*. Therefore, strain DDF could be a candidate for the bioremediation of sites contaminated with dieldrin.

3. Clean-up technology of *s*-triazines using a bacterial consortium

(1) Simultaneous biodegradation of chloro and methylthio-*s*-triazines with a newly bacterial consortium

Bacterial consortium CD7, which can mineralize simazine, was obtained by using the soil-charcoal perfusion method[14], and a degrading bacterium *β*-Proteobacteria CDB21 was isolated from CD7 by Iwasaki *et al.*[7]. Bacterial consortium CD7 was more effective for bioremediation than strain CDB21 because CD7 could utilize simazine as the sole carbon and nitrogen sources in mineral salt medium[14,7]. Moreover, Charcoal A100 enriched with CD7 was confirmed to be an effective material for bioremediation[14,16]. However, CD7 and strain CDB21 could not degrade methylthio-*s*-triazines, while *Rhodococcus* sp. FJ1117YT[5] could transform methylthio-*s*-triazines to their hydroxyl analogues via sulfur oxidation, and accumulate 2-hydroxy-triazines. The expected metabolic pathways of chloro- and methylthio-*s*-triazines in the mixed culture are shown in Fig. 7.

(2) Simultaneous degradation of chloro- and methylthio-*s*-triazines using Charcoal A100 enriched with strain FJ1117YT and CD7

Simultaneous degradation of chloro- and methylthio-*s*-triazines was conducted using Charcoal A100 enriched with strain FJ1117YT and CD7. Charcoal A100 enriched with both strain FJ1117YT and CD7 or CD7 alone degraded simazine and atrazine completely for 15

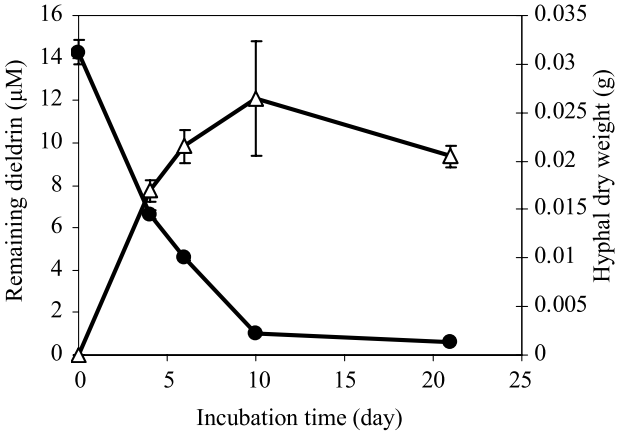

Fig. 6. Time course of degradation of dieldrin by strain DDF. Solid circles indicate the concentration of remaining dieldrin and open triangles indicate the hyphal growth as dry weight

Error bars indicate ±SE for triplicate samples[8].

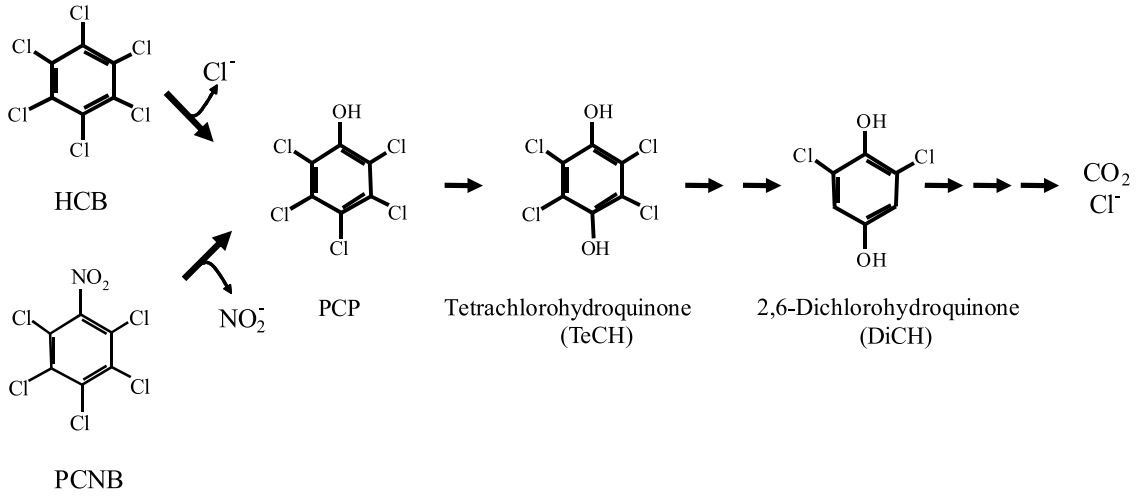

Fig. 5. Possible metabolic pathway of HCB and PCNB by *Nocardioides* sp. strain PD653 under strict aerobic conditions[17, 18]

days (Fig. 8A, B). On the other hand, it degraded simetryn and dimethametryn over 80% in sulfur-free medium, but not in the presence of sulfate (in MM-S) (Fig. 8C, D). Thus, Charcoal A100 enriched with strain FJ1117YT and CD7 could be a promising model to construct a multifunctional material enriched with bacterial consortium for *in situ* bioremediation.

4. *In situ* bioremediation of simazine-contaminated site using Charcoal A100 enriched with bacterial consortium CD7

A 1-cm-thick layer of Charcoal A100 enriched with CD7 was placed under the subsoil at a 15-cm depth at a treatment plot in a golf course. As the control plot, Charcoal A100 without CD7 was set in the same manner. Porous glass cups were inserted at four locations in each plot to collect the soil solution directly beneath the charcoal layer. After the simazine application, we periodically examined changes in the simazine concentration in the soil solution and the soil and charcoal layers. The simazine was applied twice a year for two years.

(1) Simazine in soil solution

In the control plot, more than 0.01 mg/L of simazine was detected at all locations until the seventh week after the first application. In the treated plot, the simazine was 0.005 mg/L or less at all the sampling locations until the sixth week after the first application. After six months, simazine was not detected at all the sampling locations. The simazine-degradation of soil solution in the treatment plot reached 92% at six months after the first application compared with the control plot. However, this degradation was slightly retarded to 70% after the second application compared with the control plot. After the third and fourth application, the degradation in the soil solution of the treated plot was still more than 60% (Fig. 9).

(2) Simazine in the charcoal layer

The simazine in the charcoal layer was maintained at 5 to 8 mg/kg dry matter until six months after the first application in the control plot, owing to the adsorption of simazine. In the treated plot, simazine reached a maximum (3 mg/kg dry mater) at one month after the first application, and then decreased by 95% of the residual amount in the control plot at 5.5 months after the first application. A similar trend with the first trial was observed also after the second application[15].

(3) Long-term monitoring of simazine-degrading bacteria in charcoals

Changes in simazine-degrading bacteria in charcoals during the long-term field scale study were examined by means of the most probable number (MPN) method. The simazine-degrading activities of serially diluted

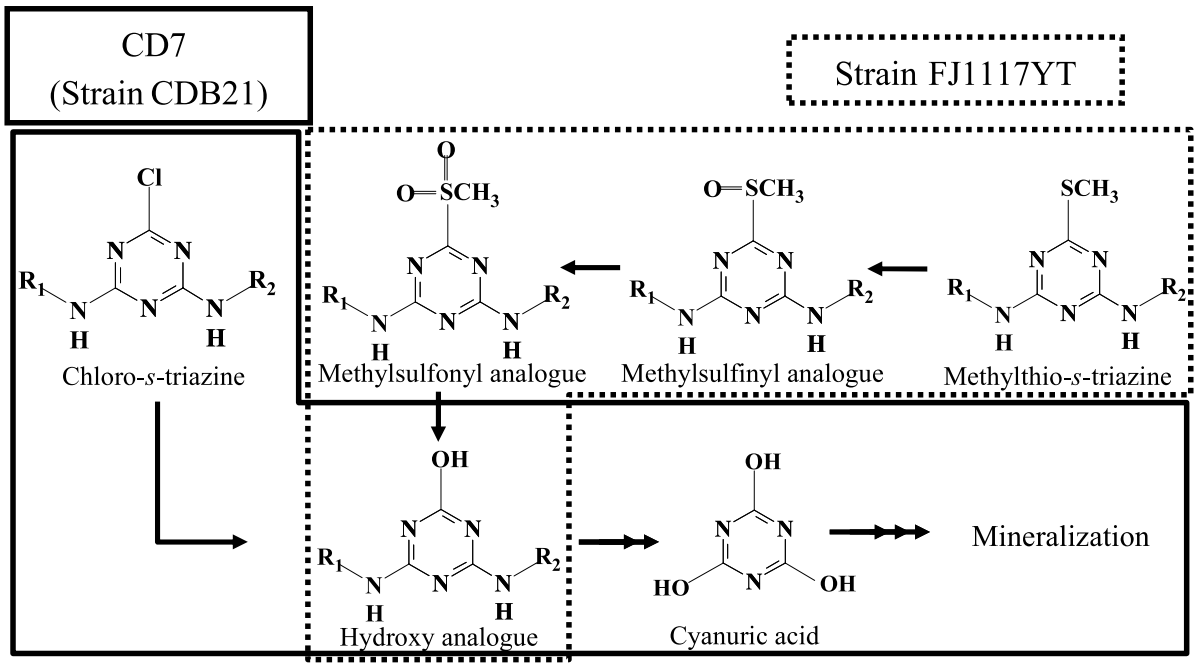

Fig. 7. The expected metabolic pathways of chloro- and methylthio-*s*-triazines degraded by CD7 (strain CDB21) and strain FJ1117YT

Simazine (R_1, R_2= C_2H_5) and atrazine [R_1= C_2H_5, R_2=$CH(CH_3)_2$] were selected as chloro-*s*-triazines, and simetryn (R_1, R_2= C_2H_5) and dimethametryn [R_1= C_2H_5, R_2= $CH(CH_3)CH(CH_3)_2$] were used as methylthio-*s*-triazines[19].

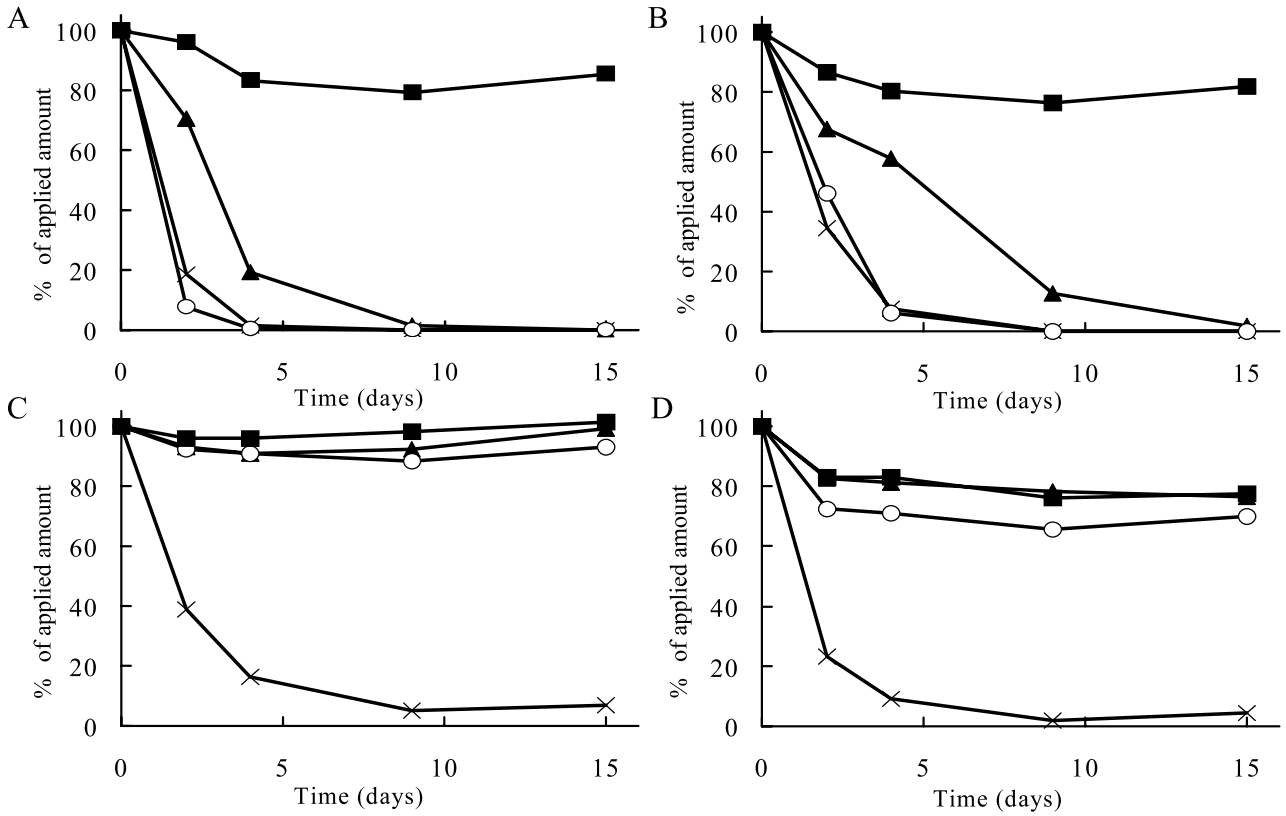

Fig. 8. Time course of simultaneous degradation of chloro- and methylthio-*s*-triazines with Charcoal A100 enriched with strain FJ1117YT and CD7

Degradation of simazine (A), atrazine (B), simetryn (C), and dimethametryn (D) by strain FJ1117YT and CD7 with (▲) or without (×) sulfate, with CD7 alone (○), and using non-enriched Charcoal A100 as a control (■) are shown[19].

charcoal samples rapidly decreased from initially inoculated 10^8 MPN/g charcoal to 10^5 MPN/g charcoal within four months. After that, 10^5 MPN/g charcoal level of simazine-degrading bacteria survived and retained their degrading capacities during nearly two years of the study. Thus, by laying charcoal enriched with simazine-degrading bacteria consortia under the subsoil in contaminated fields, we were able to minimize simazine pollution of the subsoil, river water, and groundwater for at least several years (Fig. 9).

Conclusion

Nocardioides sp. PD653 and *Mucor* sp. DDF were isolated from contaminated soil as a HCB- and dieldrin-degrader, respectively. These strains can be applied to *in situ* bioremediation. In order to rapidly enrich and isolate organochlorine pesticides-degrading bacteria, we have developed a soil-charcoal perfusion method using special charcoal (Charcoal A100) as a microhabitat and adsorbent of organic chemicals. Furthermore, we have developed Charcoal A100 enriched with a degrading-bacterial consortium as a new material for bioremediation. We were successful in degrading simazine in the subsoil and aquatic environments for two years by laying Charcoal A100 enriched with a simazine-degrading bacterial consortium (CD7) under the subsoil of contaminated sites.

Acknowledgments

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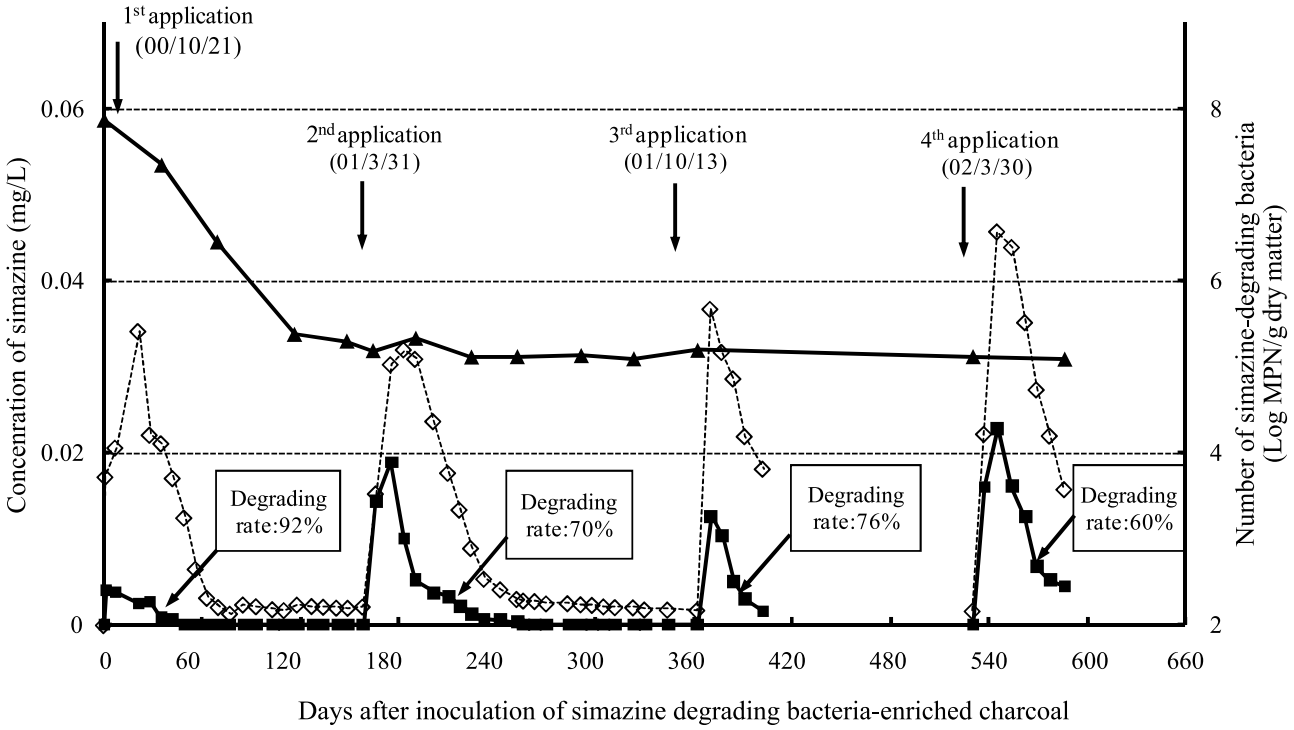

Fig. 9. Change in concentration of simazine in soil water (15-20 cm) at both plots (treatment (■), control (◇)) and number of simazine-degrading bacteria in charcoal (▲) after introducing into subsoil at treatment plot

In the control plot, a charcoal material without bacterial enrichment was laid in the same manner as in the treatment plot. Concentration of simazine in soil water was mean values of the quadruplicate experiments. Simazine application was performed 4 times (see arrows) during the experiment[15, 16].

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