EFFECTS OF MICROBIAL INHIBITORS ON ANAEROBIC DEGRADATION OF DDT

Yei-Shung Wang[1], Tzu-Chuan Chiu[1], Jui-Hung Yen[1]

[1]National Taiwan University, Taipei

Introduction

Chlorinated insecticide DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] was extensively used for controlling pests in the agricultural field and human-being living environments in the past several decades. Due to the chemical stability, DDT was extremely persistent and recalcitrant in soils and sediments and it was banned by nations. Microorganisms usually play important roles in reducing organochlorine compounds in the environments[1,2,3]. Under low-oxygen conditions, microbial dechlorination is thought as the onset of highly chlorinated compounds. Methanogenic and sulfate-reducing bacteria participate in microbial dechlorination under anaerobic condition has been reported[4,5]. In this study, a mixed anaerobic culture enabling to dechlorinate DDT was obtained from river sediment in Taiwan. In order to understand the effect of these microorganisms on DDT dechlorination, microbial inhibitors BESA (2-bromoethanesulfonate) and molybdate, for inhibiting methanogenic and sulfate-reducing bacteria, respectively, were chosen to investigate the interaction between specific microbial communities and their degradation activities. Besides, a molecular technique, denaturing gradient gel electrophoresis[6] (DGGE), based on analyzing the 16S rDNA of bacteria, was used for monitoring the bacterial community structure in this study.

Methods and Materials

Chemicals: *p, p*'-DDT [1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane] with 98% purity was purchased from Riedel-deHaën Co, Germany. HPLC-graded solvents used in this experiment, including *n*-hexane and acetone were purchased from E. Merck Co, Germany. The *p,p*'-DDT stock solutions (1 mg/mL) were prepared in acetone and then stored at 4 .

Culturure: Sediment was collected from Er-Jen River, a serious contaminated river located at southern Taiwan. After collecting, sediment was immediately stored in a jar, and then kept in cold before using. Anaerobic mixed culture was prepared by mixing sediment (100g) and culture medium (400 mL) in a 1-L serum bottle to make a slurry-like culture under a modular atmosphere controller system (dwscientific Co, England) filling with N_2, H_2, and CO_2 gases (85:10:5). The culture medium consists of (in g/L): NH_4Cl (2.7), $MgCl_2$ $6H_2O$ (0.1), $CaCl_2$ $2H_2O$ (0.1), $FeCl_2$ $4H_2O$ (0.02), K_2HPO_4 (0.27), KH_2PO_4 (0.35), yeast extract (1.0) and resazurin (0.001), was conducted from Chang et al[7].

Batch procedures: The batch degradation of DDT were performed by adding 5 mL of anaerobic mixed culture to a 125-mL serum bottle containing 45 mL of culture medium, and then 10 µg/mL of *p,p'*-DDT was spiked to serum bottles. To avoid oxygen involved and possible photolysis, serum bottle was sealed with a butyl rubber stopper capped with an aluminum top and incubated in darkness. In the inhibitor study, BESA or molybdate was additional added in final concentrations of 5 mM and or 50 mM, respectively.

Residue analysis: Residue of DDT in sample culture was extracted by *n*-hexane, and then analyzed by gas chromatography (Agilent technologies 6890N network series GC system, Agilent technologies Co., USA), which was equipped with a ^{63}Ni electron capture detector (ECD) and a HP-1 fused silica capillary column (film thickness, 0.33 mm; inner diameter, 0.25 mm; length, 30 m, Hewlett Packard Co., USA). High purity of nitrogen was used as both carrier and make-up gas. The flow rate of carrier gas was 3.5 mL / min (20:1 split ratio). The column temperature program was set at 170 in initial for 2 min, and then increased to 210 by 2.5 / min, held for 2 minutes, and then increased to 250 by 10 / min, and held for 5 minutes. Injection port and detector temperatures were set at 250 and 300 , respectively.

DNA extraction and purification: At regular intervals, 3 mL of anaerobic mixed cultures was taken and extracted by using an UltraClean Soil DNA kit (MO BIO Laboratories, Inc). After extraction, the total genomic DNA was further purified for removal of humic acids by DNA extracts electrophoresis in a 1% agarose gel. After staining with ethidium bromide (EtBr), the genomic DNA was excised from the gel and collected with a QIAquick gel extraction kit (QIAGEN GmbH, Hilden, Germany).

PCR-DGGE: Polymerase chain reaction (PCR) was carried out with a thermal cycler 9700 (Applied Biosystem, USA). The PCR reaction mixture (50 µL) contained 0.2 mM of each dNTP, 4.0 mM $MgCl_2$, 1X reaction buffer solution, 1 unit of ABgene DNA polymerase, 1 µL of total genomic DNA, and 0.4 µM of each primer. Bacterial 16S rDNA was specifically amplified by using the primer pairs as described by Gelsomino et al.[8] (1999). The primers 968f and 1401r roughly spanned the region of V6 through V8 of 16S ribosomal DNA. A GC-clamp of 40 bases, as described by Muyzer et al.[6] (1993), was attached to the 5'-end of the forward primer. The sample was amplified as follows: 94°C for 2 minutes, followed by 35 cycles of 94°C for 30 sec, 64°C for 1 minute, 72°C for 1 minute and with a final extension at 72°C for 10 minutes. The PCR product was confirmed by electrophoresis through a 1% (w/v) agarose gel in 1X TAE buffer, followed by staining with EtBr.

DGGE analysis was performed using a D-Code universal mutation detection system (Bio-Rad, Hercules, Calif.). PCR product (20 µL) was loaded onto a 7% (w/v) denaturing gradient polyacrylamide gel consists of 40% to 60% of formamide and urea from up side through bottom. The electrophoresis was run at 60°C in 1X TAE for 12 hours at a constant voltage of 75V. After the electrophoresis, gel was stained with SYBR Green I nucleic acid gel stain, visualized on an UV transilluminator, and photographed with a digital camera.

Results and discussion

The anaerobic microbial degradation of *p,p'*-DDT was studied by adding microbial inhibitors BESA and molybdate. BESA, a structure analog of coenzyme M which is a cofactor involved in methane biosynthesis[9], has been regarded as a methanogenetic bacteria inhibitor, and molybdate, an inhibitor which impedes the synthesis of ATP surfurylase[10], has been thought as an inhibitor of sufate-reducing bacteria. Addition of BESA and molybdate would specifically inhibit methanogenetic bacteria and sulfate-reducing bacteria, respectively. In this study, the half-life ($t_{1/2}$) of *p,p'*-DDT was calculated by fitting to the first order kinetics equation, and the result was presented in Table 1. According to the consequence, although the addition of BESA or molybdate could not completely inhibit the occurrence of degradation, our results showed the dechlorination of *p,p'*-DDT was delayed while microbial inhibitors added, and 50mM of both BESA and molybdate added lead a higher degree of inhibition than any other treatment in DDT-dechlorination. This result implied that dechlorination of *p,p'*-DDT was involved in a complicated interaction between microbial communities.

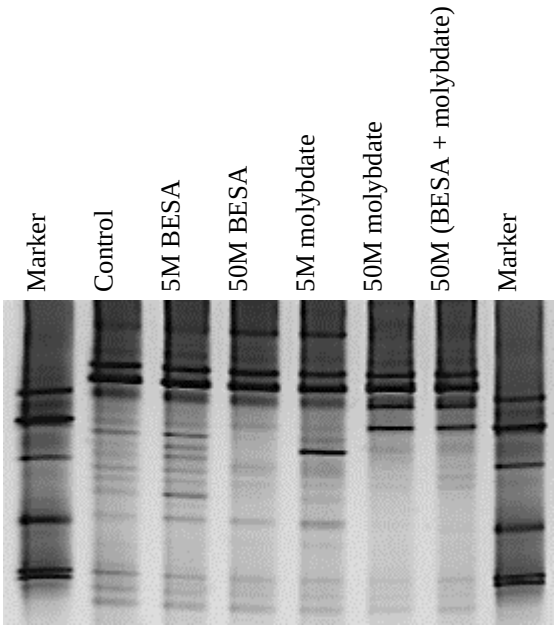
Table 1: The half-life of *p,p'*-DDT in the addition of inhibitors under anaerobic conditions.

Treatment		*p,p'*-DDT	
		k	$t_{1/2}$(days)
Control		0.13	5.15
5 mM	BESA	0.09	7.69
50 mM	BESA	0.10	7.10
5 mM	Molybdate	0.10	6.80
50 mM	Molybdate	0.09	7.40
50 mM	BESA+Molybdate	0.07	10.40

k: rate constant
$t_{1/2}$: half-life

Bacterial community structures in the DDT-degrading anaerobic mixed culture incubated with or without microbial inhibitors were also investigated. In this research, we attempt to evaluate the feasibility of using PCR-DGGE to describe the effect of BESA and molybdate on bacterial dynamics under anaerobic condition in sediment. The result of PCR-DGGE analysis was shown in Figure 1. Each band observed on DGGE pattern represents a dominant bacterial group. The marker lanes were used to compare the relative parallel positions with those on other

Figure 1: PCR-DGGE analysis of 16S rDNA sequence fragments obtained from the DDT-degrading culture incubated with microbial inhibitors (BESA or molybdate) for 15 days.



lanes. Several bands could be clearly observed on the DGGE fingerprint implied some of DDT-degrading bacteria were proliferated. There were fifteen distinct bands could be observed on control lane, but fewer band was showed in the others that treated with inhibitors. Addition of 5 mM of BESA did not affect the DDT-degrading bacterial community structure, but the bacterial community structure was slightly changed by adding 50 mM of BESA. This result implied that the inhibition of microbial DDT-degrading ability by BESA was due to the suppression of methanogenic bacteria. The supplementation of 5 or 50 mM of sulfate-reducing bacteria inhibitor molybdate caused in changing of bacterial community structures. Comparison of bacterial community structures of treatment without inhibitor (control) with those incubated with molybdate, many bands disappeared and some newly predominant bands appeared. It is very difficult to infer whether these disappeared bands are involved in the dechlorination of *p,p'*-DDT. But our results indicated both methanogenic and sulfate-reducing bacteria were involved in the anaerobic dechlorination of *p,p'*-DDT.

Acknowledgement

The research was supported in part with funds provided by the National Science Council of the Republic of China (NSC -90-2317-B-002-012).

References

1. Siddique T., Okeke B. C., Arshad M. and Frankenberger W. T. Jr. (2003) J. Environ. Qual. 32, 47-54.
2. Maule A., Plyte S. and Quirk A. V. (1987) Pest. Biochem. Physiol. 27, 229-236.
3. Bidlan R. and Manonmani H. K. (2002) Process Biochem. 38, 49-56.
4. Ye D. Quensen III J. F., Tiedje J. M. and Boyd S. A. (1995) Appl. Environ. Microbiol. 61(6), 2166-2171.
5. Gupta M., Suidan M. T. and Sayles G. D. (1996) Wat. Sci. Tech. 34(5-6), 403-410.
6. Muyzer G., De waal E.C., Uitterlinden A.G. (1993) Appl. Environ. Microbiol. 59, 695-700.
7. Chang B. V., Liu W. G. and Yuan S. Y. (2001) Chemosphere. 45, 849-856.
8. Gelsomino A., Keijzer-Wolters A.C., Cacco G., van Elsas J.D. (1999) J. Microbiol. Method. 38, 1-15.
9. Gunsalus R. P., Romesser J. A and Wolfe R. S. (1978) Biochem. 17, 1274-2377.
10. Mohn, W. W. and Tiedje J. M. (1992) Microbiol. Rev. 56, 482-507.