CHAPTER IV

RESULTS

4.1 Soil properties

Two types of agricultural soil samples which have been regularly exposed to pesticides and herbicides; Type I and II soil were collected from the agricultural area at the depth of 2-10 cm. Type I soil was collected from mango orchard in Nakornnayok province, while Type II soil was collected from cassava field in Nakhonratchasima province (Figure 4.1). The soil samples were sieved through 2 mm mesh. Then, they were analyzed for their physical and chemical properties at Department of Soil Science, Faculty of agriculture, Kasetsart University and Soil and Water Group, Agricultural Chemistry Division.

Type I soil Type II soil

Figure 4.1 The agricultural soil Type I and II were collected from mango orchard in Nakornnayok province and cassava field in Nakhonratchasima province.

The soil properties are provided in Table 4.1. Type I soil is classified as loam soil with 40.4% sand, 39.6% silt and 20.0% clay. pH of loam soil was 5.3. Percentage of organic matter, organic carbon and nitrogen were 3.86, 2.24 and 0.19, respectively. C:N ratio was 11.79. Amount of available phosphorus was 81 ppm. Moisture content was 9.18%. Type II soil is silty clay soil with 13.6% sand, 40.4% silt and 46.0% clay. pH of silty clay soil was 7.6. Percentage of organic matter, organic carbon and nitrogen were 3.93, 3.69 and 0.22, respectively. C:N ratio was 16.77. Amount of available phosphorus was 331 ppm. Moisture content was 15.57%.

Table 4.1 Properties of soil samples

Properties	Type I soil (Nakornnayok)	Type II soil (Nakhonratchasima)
Soil texture	Loam	Silty Clay
% sand	40.4	13.6
% silt	39.6	40.4
% clay	20.0	46.0
pH (1:1) in water	5.3	7.6
Organic matter (%)	3.86	3.93
Organic carbon (%)	2.24	3.69
Nitrogen (%)	0.19	0.22
Available phosphorus (ppm)	81	331
C:N ratio	11.79	16.77
Moisture (%)	9.18	15.57

Table 4.1 showed that the properties of two soil samples had different physical and chemical properties. Therefore, two soil sample types were used to compare the soil properties influenced the efficiency of bioremediation treatment for clean up diuron.

4.2 Recovery of diuron from soil

Two type soils were mixed with diuron to the final concentration of 500 and 1,000 ppm and kept for 1 and 15 days. Diuron contaminated in soil was extracted using organic solvent. Various types and concentrations of solvent including methanol (50%, 80% and 100% (v/v)), ethanol (50%, 80% and 100% (v/v)) and acetonitrile (50%, 80% and 100% (v/v)) were used for diuron extraction from soil under the conditions indicated (section 3.6.2.1). The extracted diuron was analyzed by high performance liquid chromatography (HPLC). Percent diuron recovery was shown in Figure 4.2 and 4.3.

80% acetonitrile showed the highest % diuron recovery from two types of soil. Consequently, 80% acetonitrile was used for diuron extraction from soil in the following experiments.

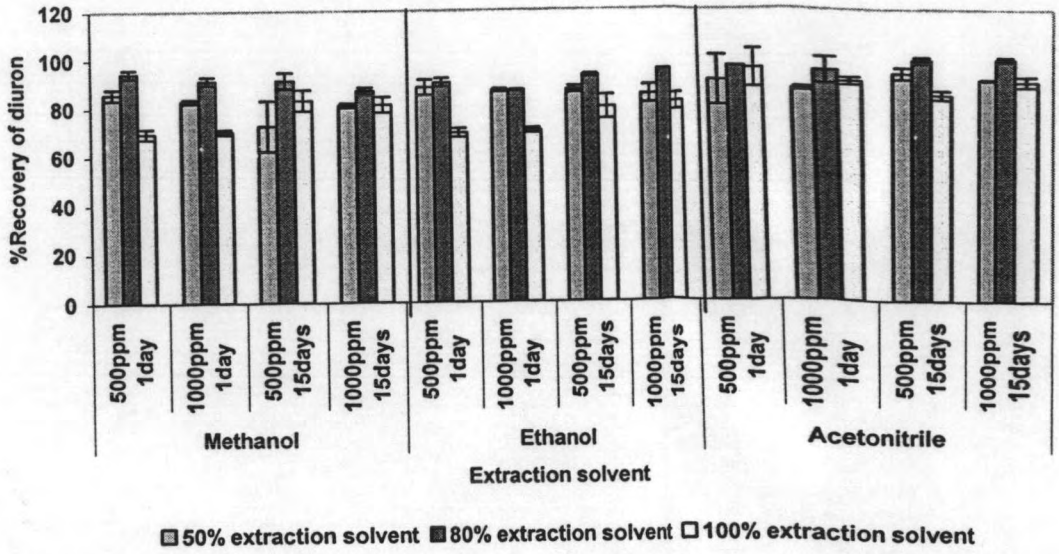


Figure 4.2 Recovery percentage of diuron extracted from loam soil using liquid-liquid extraction.

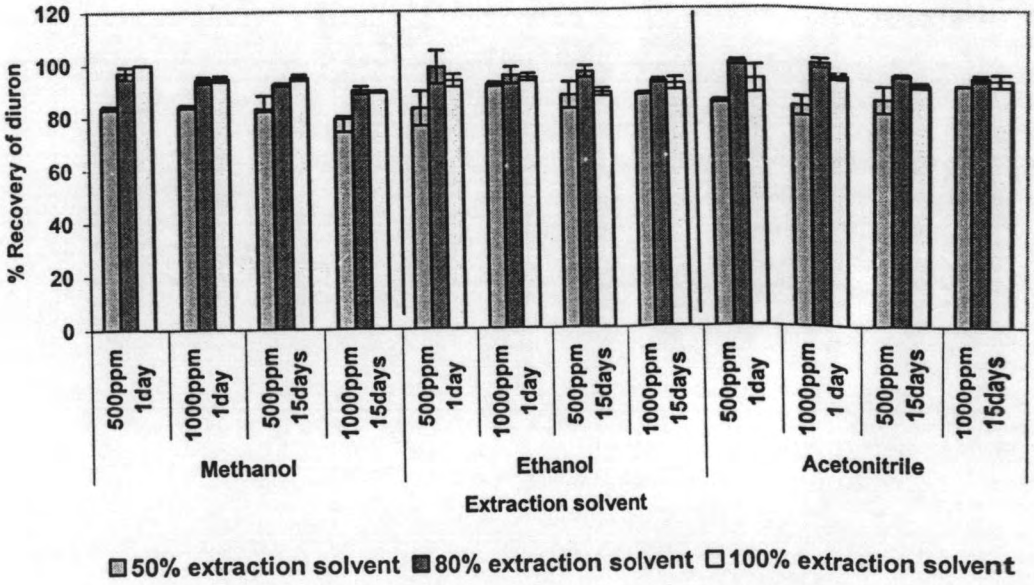
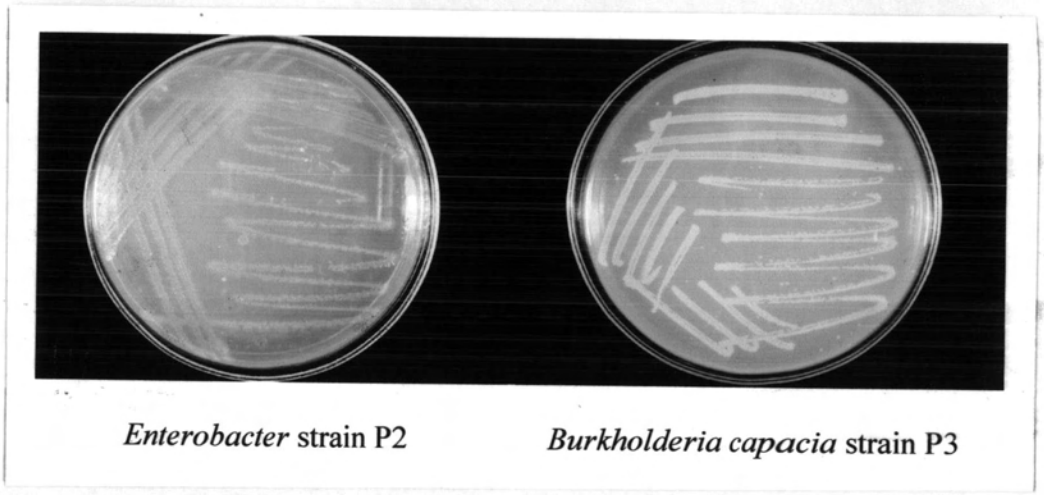


Figure 4.3 Recovery percentage of diuron extracted from silty clay soil using liquid-liquid extraction.

Bacteria used in bioaugmentation treatment are bacterial pure culture and bacterial consortium, which were screened from agricultural soil by our laboratory.

Pure bacterial cultures

The bacterial pure cultures were *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 (Figure 4.4). They degraded diuron differently with degradation efficiency of 59% and 64% of 20-ppm diuron in liquid medium within 17 days, respectively (Figure 4.5).



Enterobacter strain P2 *Burkholderia capacia* strain P3

Figure 4.4 Bacterial pure culture: *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 on LB-agar plate.

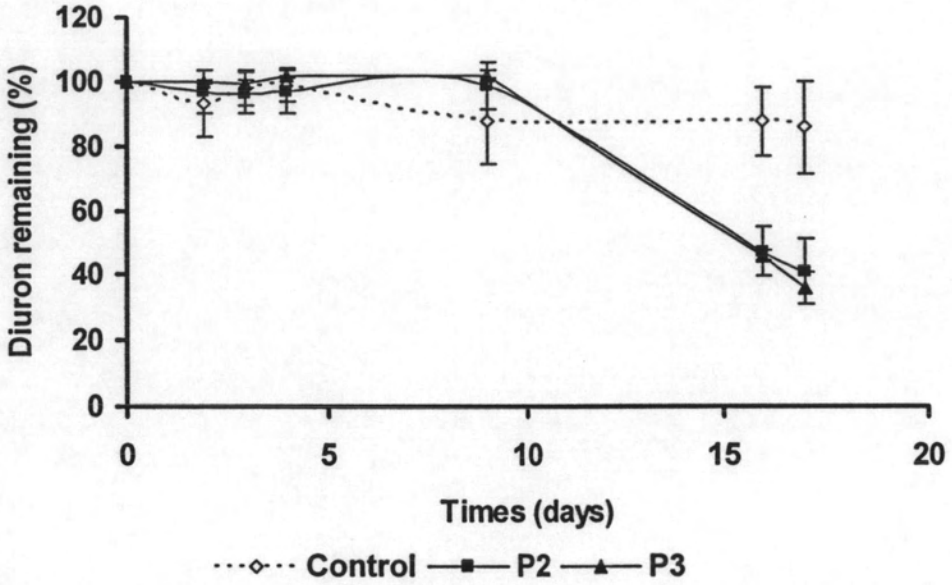


Figure 4.5 Diuron biodegradation in liquid medium of pure bacterial cultures: *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 grown in minimal salt medium containing 0.5% glucose and 20 ppm diuron. The control was the minimal salt medium containing 0.5% glucose and 20 ppm diuron.

Bacterial consortium

Four bacterial consortiums consisted of A1, Y2, R3 and B4 were isolated from agriculture soil by Enrichment technique (Figure 4.6). Bacterial consortium, A1 and R3 could degrade 10 ppm diuron by 74% and 21%, respectively in liquid medium within 8 days of incubation, while Y2 and B4 bacteria could not degrade 10 ppm diuron within 14 days (Figure 4.7).

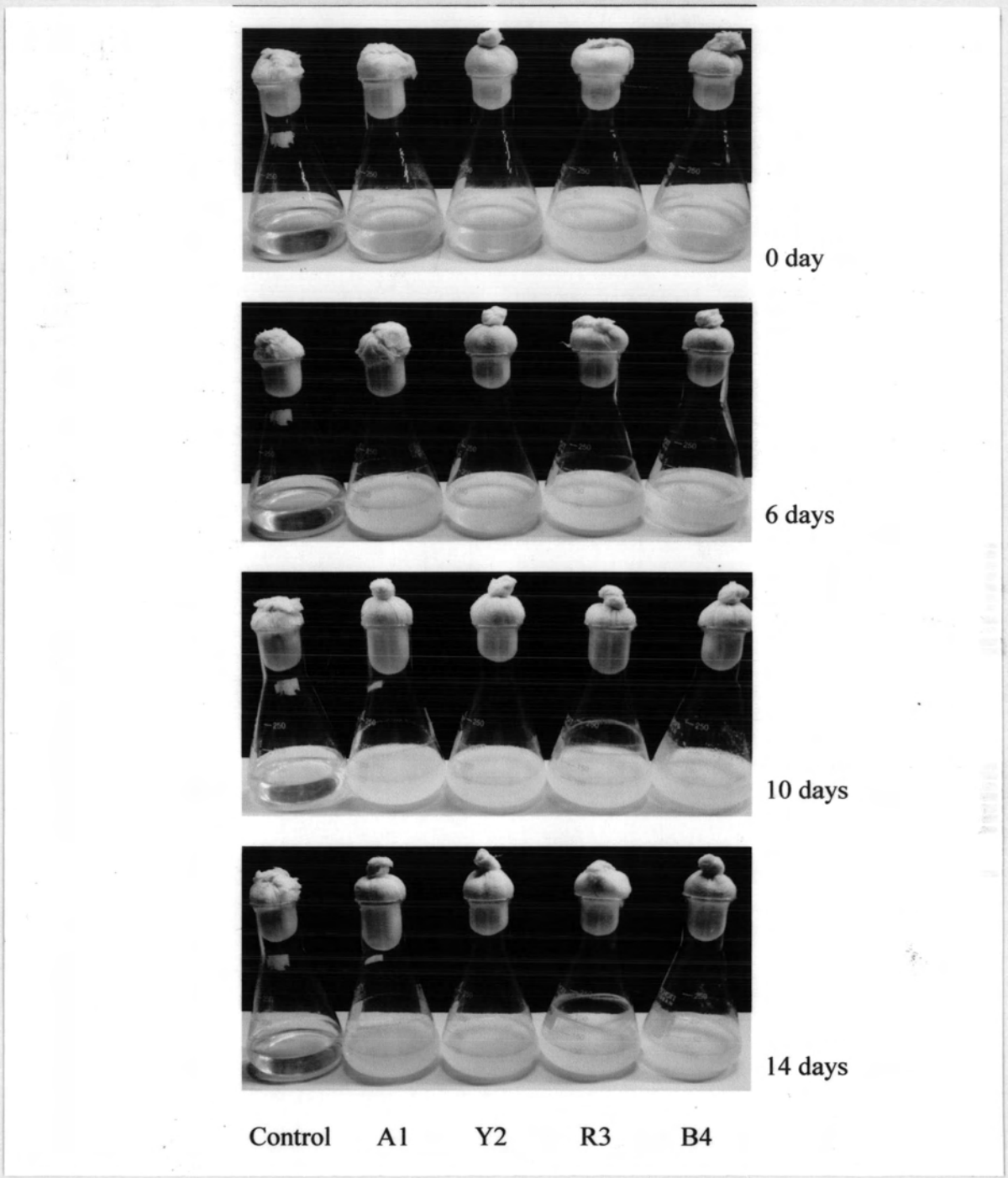


Figure 4.6 Four bacteria consortiums; A1, Y2, R3, and B4 grown in minimal salt medium containing 10 ppm diuron during 14 days. The control was the minimal salt medium containing 10 ppm diuron (abiotic control).

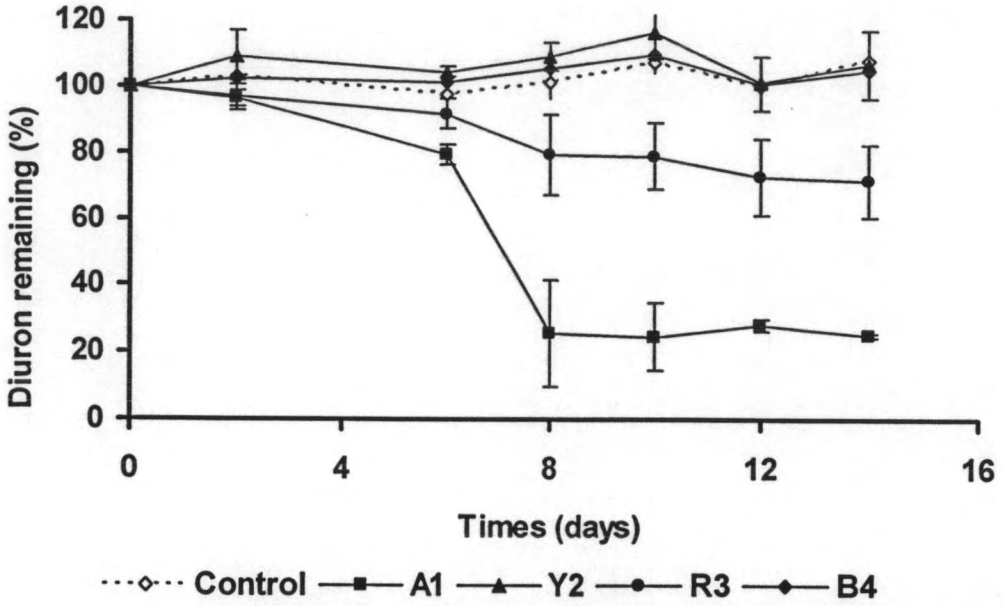


Figure 4.7 Diuron biodegradation of four bacteria consortiums; A1, Y2, R3, and B4 grown in minimal salt medium containing 10 ppm diuron. The control was the minimal salt medium containing 10 ppm diuron (abiotic control).

The morphological and characteristic of bacterial consortium A1

Bacterial consortium A1 containing diuron degradation activity was isolated from soil sample of maize plantation at Chonburi province and identified for their morphological characteristic by Gram's staining, data as shown in Figure 4.8, 4.9, and Table 4.2, respectively. Cells were grown in both rich medium agar plate (LB-agar) for 2 days at 30°C and minimal salt medium agar plate containing 20 ppm diuron (MSM-agar) for 4 days at 30°C and the results indicated that bacterial consortium A1 could grow in both LB-agar and MSM-agar, data as shown in Figure 4.8. These results showed that bacterial consortium A1 could grow under minimal salt medium with diuron as

nutritional source. The LB-agar plate shown 2 types of colony named WA1 and YA1, respectively. Table 4.2 showed characteristic of both white and yellow colonies. The white colony had these characteristic: circular form, convex surface and entire edge, respectively. The yellow colony had these characteristic: circular form, raised surface and undulate edge, respectively. The white and yellow colonies were determined by gram's staining and results were shown in Figure 4.9. The results showed that yellow colonies were cocobacilli and white colonies were cocci; white colony grown in medium including of single gram-positive (arrow a) and gram-negative bacteria (arrow b) and yellow colony including only single gram-negative bacteria.

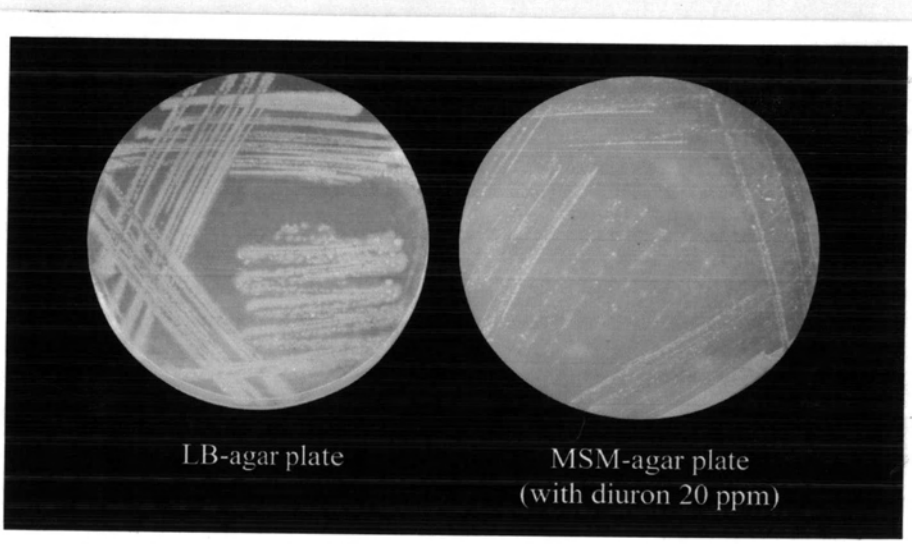


Figure 4.8 Comparison of Bacterial consortium A1 colonies grown on LB-agar and MSM-agar plate containing diuron 20 ppm

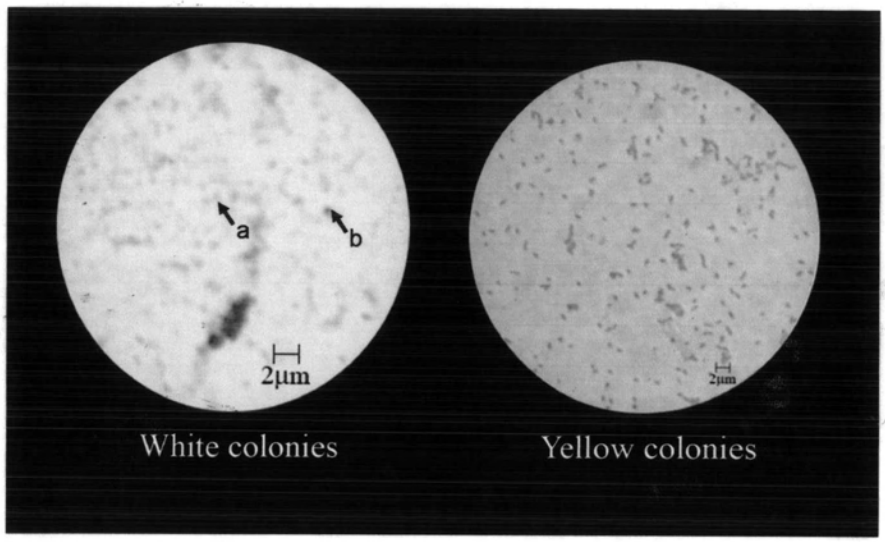


Figure 4.9 Gram's staining of bacterial consortium A1. Left side; white colonies included gram-positive bacteria (arrow a) and gram-negative bacteria (arrow b). Right side; yellow colonies.

Table 4.2 Characteristic of colonies of the bacterial consortium A1 on LB-agar plate

Colony characteristic	**Bacterial consortium**	
	WA1	**YA1**
Color	White	Yellow
Form	Circular	Circular
Surface	Convex	Raised
Edge	Entire	undulate

4.4 Biodegradation of diuron in soil microcosms

The conditions of each bioremediation treatment were explained in section 3.6.1.3 and figure 3.2.

4.4.1 Bioremediation: Condition A

Diuron degradation was conducted at the laboratory scale in which 5 g soil was spiked with various concentrations of diuron (20 ppm and 100 ppm) before treatment. The bioremediation treatment consisted of natural attenuation, biostimulation and bioaugmentation. Natural attenuation is the process that diuron was reduced due to naturally-occurring processes including sorption, chemical reaction as well as degradation by indigenous microorganisms in soil. Biostimulation is the treatment which 20.6, 103 and 206 ppm Triton X-100 (1, 5 and 10 CMC) and 100 ppm *n*-butanol was added as a biostimulating agent of diuron biodegradation. According to Phuempoonsathaporn (2006), *n*-butanol and Triton X-100 exhibited highest leaching efficiency of diuron from soil when compared to other organic solvents and surfactants. Bioaugmentation was carried out in which approximately diuron-degrading bacteria, *Enterobacter* strain P2 and *Burkholderia capacia* strain P3, were provided at 10^8 CFU/g soil. Because *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 could produced biosurfactants. Abiotic control was also carried out.

4.4.1.1 Diuron degradation in loam soil

The results of three bioremediation treatments of 20 ppm diuron-spiked loam soil including natural attenuation, biostimulation and bioaugmentation for diuron analysis were shown in Figure 4.10. By natural attenuation, it degraded 17% diuron in soil within 8 weeks. To assess bioaugmentation treatment (Figure 4.10 a), pure cultures, *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 were added into the soil. Although, *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 were able to degrade 20-ppm diuron with 59-64% efficiency within 17 days in a liquid medium as described in section 4.3, they were able to degrade only 10% diuron in soil within 8 weeks of incubation. Although the various concentrations of Triton X-100 and *n*-butanol were added to the treatment to increase diuron bioavailability, there was only slight degradation within 8 weeks (8.75%, 13.97% and 14.90% with 20.6, 103 and 206 ppm Triton X-100 and 9.06% with 100 ppm *n*-butanol), indicating that diuron was hardly degraded by biostimulation treatment (Figure 4.10 b) under the tested conditions.

Treatment of diuron contaminated in loam soil was studied with a higher concentration, i.e. 100 ppm diuron. At 100 ppm diuron, biodegradation was not detected in all treatments (Figure 4.11).

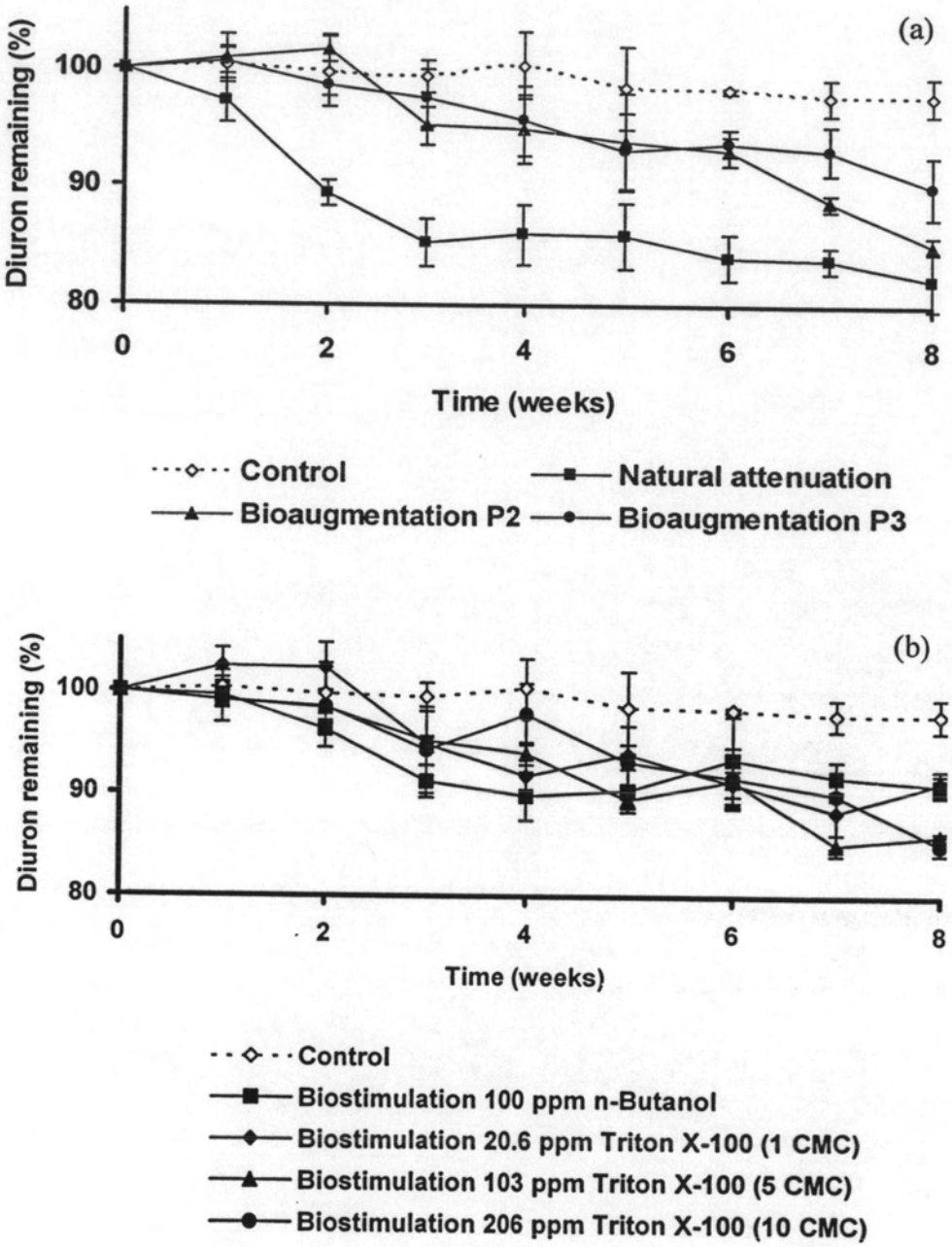


Figure 4.10 Biodegradation of 20-ppm diuron in loam soil. The treatments include natural attenuation, bioaugmentation with pure bacterial cultures; *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 (a) and biostimulation (b). The control was sterilized soil.

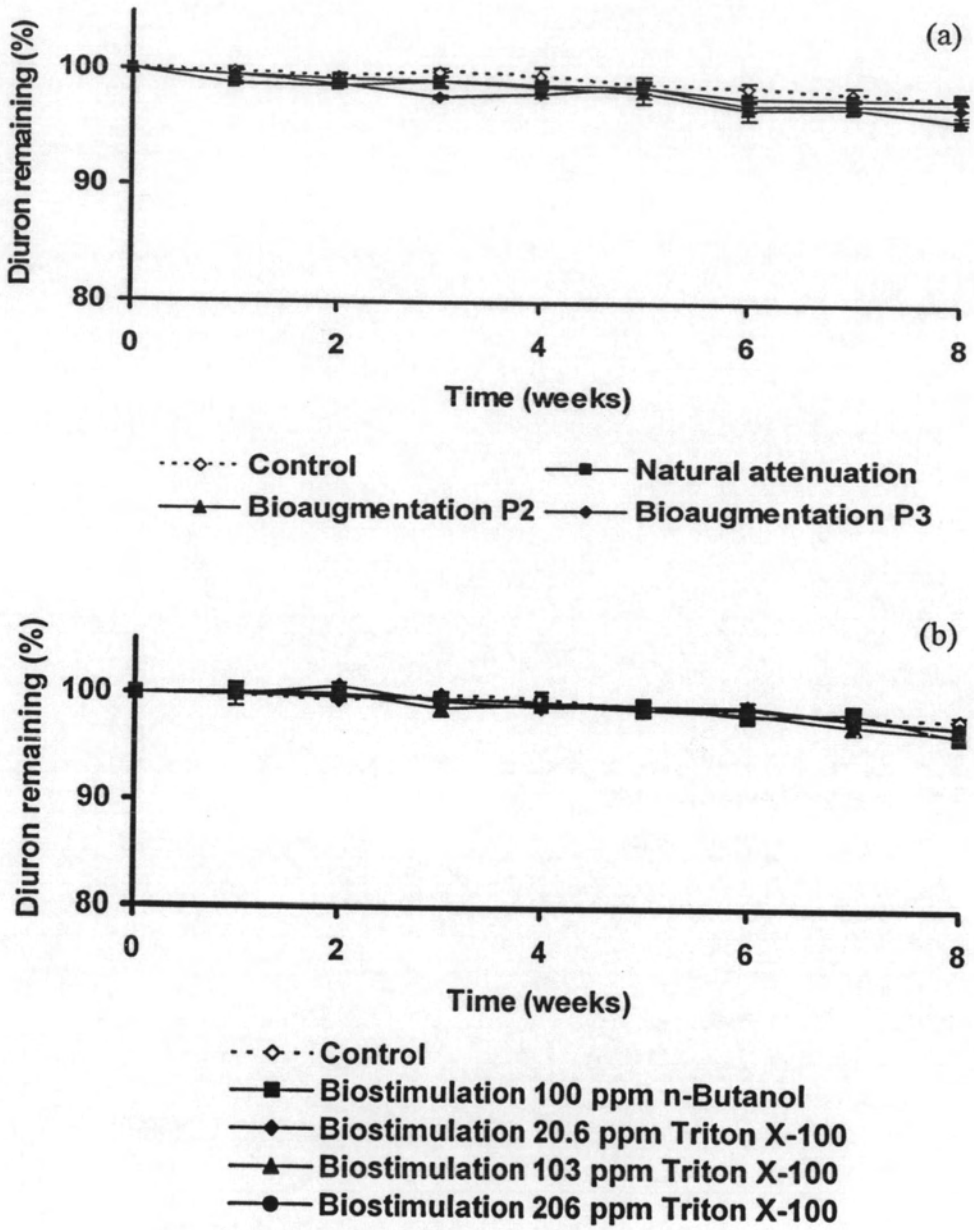


Figure 4.11 Biodegradation of 100-ppm diuron in loam soil. The treatments include natural attenuation, bioaugmentation with pure bacterial cultures; *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 (a) and biostimulation (b). The control was sterilized soil.

Number of diuron-degrading bacteria in loam soil

The populations of diuron-degrading bacteria were determined by plate count technique with the supplementation of 20 ppm of diuron. The number of diuron-degrading bacteria in loam soil with 20-ppm diuron in each treatment was shown in Figure 4.12 (a). By natural attenuation, the numbers of diuron-degrading bacteria were maintaining constantly during 8 weeks of incubation, suggesting that indigenous diuron-degradation bacteria could not significantly grow. By bioaugmentation, diuron-degrading bacteria were provided at 10^8 CFU/g soil into 5 g soil microcosm. The bacterial numbers (for both *Enterobacter* strain P2 and *Burkholderia capacia* strain P3) were observed to be at constant level during the first 4 weeks, suggesting that the bacteria could survive in soil. However, deceasing of bacterial numbers was found after 4 weeks. Similarly, the amount of diuron-degrading bacteria was constant during 8 weeks of biostimulation treatment, suggesting that Triton X-100 and *n*-butanol did not stimulate the growth of these bacteria. These results indicated that diuron-degrading bacteria were in the soil supplemented with 20-ppm diuron.

The number of diuron-degrading bacteria in loam soil with 100-ppm diuron in each treatment was shown in Figure 4.12 (b). The numbers of diuron-degrading bacteria of three bioremediation were constant during 8 weeks of incubation.

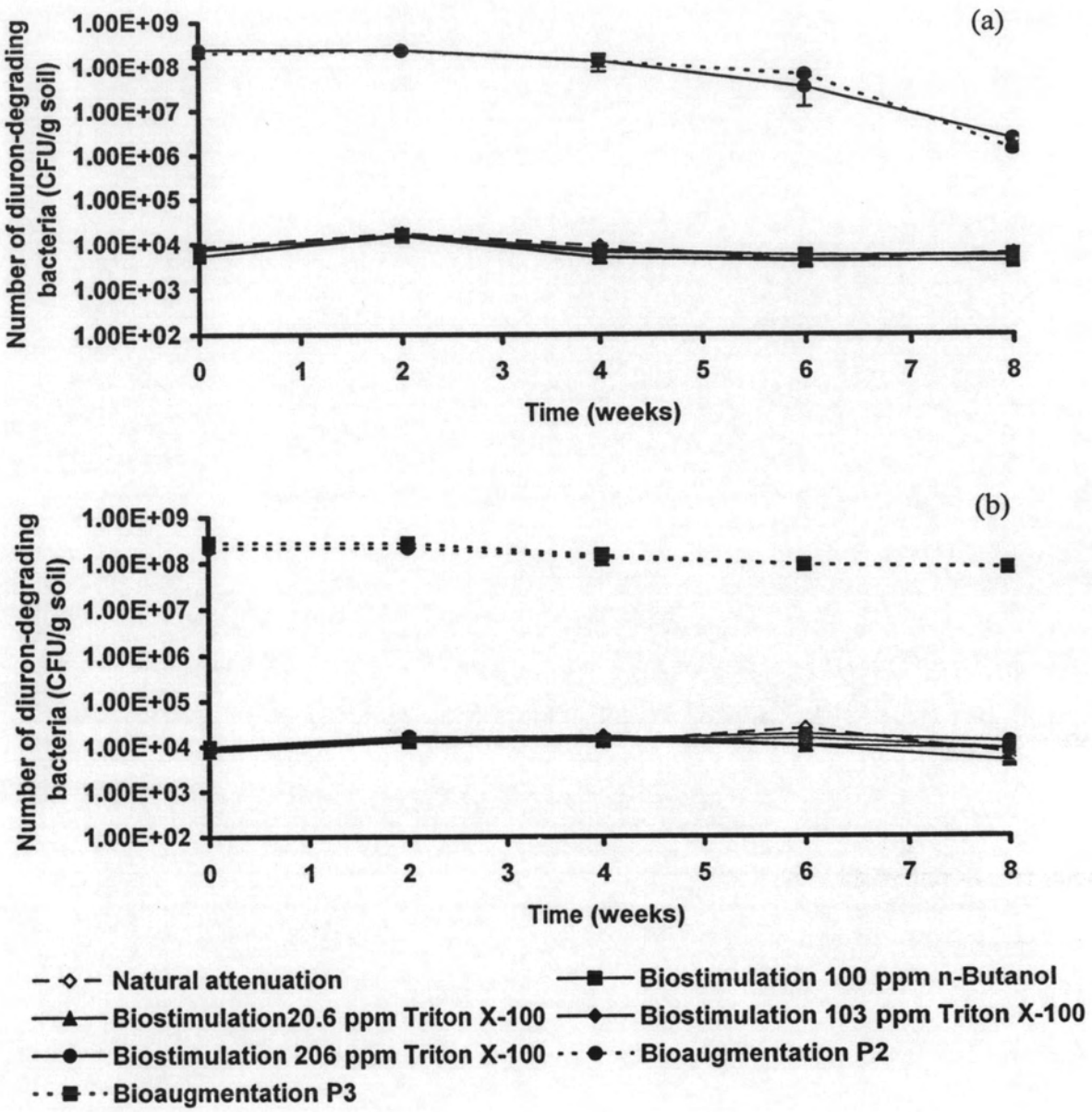


Figure 4.12 Number of diuron-degrading bacteria in loam soil. The number of bacteria was determined, during 8 weeks of incubation, in each treatment which include natural attenuation, biostimulation and bioaugmentation with pure bacterial cultures; *Enterobacter* strain P2 and *Burkholderia capacia* strain P3, (a) 20 ppm diuron and (b) 100 ppm diuron.

Total microbial activity in loam soil

Dehydrogenase activity in soil has been used to monitor total microbial activity as an index for the total oxidative activity (Alef, 1995). Among bioremediation treatments of loam soil treated with 20 ppm diuron as shown in Table 4.2, the addition of 20.6 ppm Triton X-100 (1 CMC) in stimulation treatment increased the dehydrogenase activity 1.99 fold, whereas 103 and 206 ppm Triton X-100 (5 and 10 CMC) resulted in decreasing of the dehydrogenase activity. The addition of bacterial pure culture at 10^8 CFU/g soil with *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 showed slight reduction of dehydrogenase activity. Besides, natural attenuation showed the increasing of dehydrogenase by 1.44 fold at 8 weeks.

Treatment of diuron contaminated in loam soil was attempted with a higher concentration, i.e. 100 ppm diuron. At 100 ppm diuron, the addition of 20.6, 103 and 206 ppm Triton X-100 (1, 5 and 10 CMC) resulted in reducing of the dehydrogenase activity. The addition of bacterial pure culture at 10^8 CFU/g soil with *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 showed slight reduction of dehydrogenase activity, whereas it could not be detected with natural attenuation (Table 4.3).

Table 4.3 Microbial activity estimated by dehydrogenase activity of loam soil treated with 20 or 100-ppm diuron.

Treatments		Dehydrogenase activity at 20 ppm diuron (fold)	Dehydrogenase activity at 100 ppm diuron (fold)
Natural attenuation		1.44 ± 0..19	ND
Biostimulation	100 ppm *n*-Butanol	ND*	ND
	20.6 ppm Triton X-100	1.99 ± 0.21	065 ± 0.24
	103 ppm Triton X-100	0.81 ± 0.13	0.25 ± 0.07
	206 ppm Triton X-100	0.39 ± 0.33	0.39 ± 0.13
Bioaugmentation	*Enterobacter* strain P2	0.81 ± 0.25	0.61 ± 0.11
	Burkholderia capacia strain P3	0.75 ± 0.31	0.62 ± 0.18

* ND: not detected.

** The dehydrogenase activity was compared at 0 and 8 weeks.

4.4.1.2 Diuron degradation in silty clay soil

The degradation of diuron in silty clay soil with 20 or 100-ppm diuron through natural attenuation, biostimulation and bioaugmentation treatment were shown in Figure 4.13 and 4.14. From the result, the samples were collected 4 weeks because diuron concentration was decreased the same as control and samples.

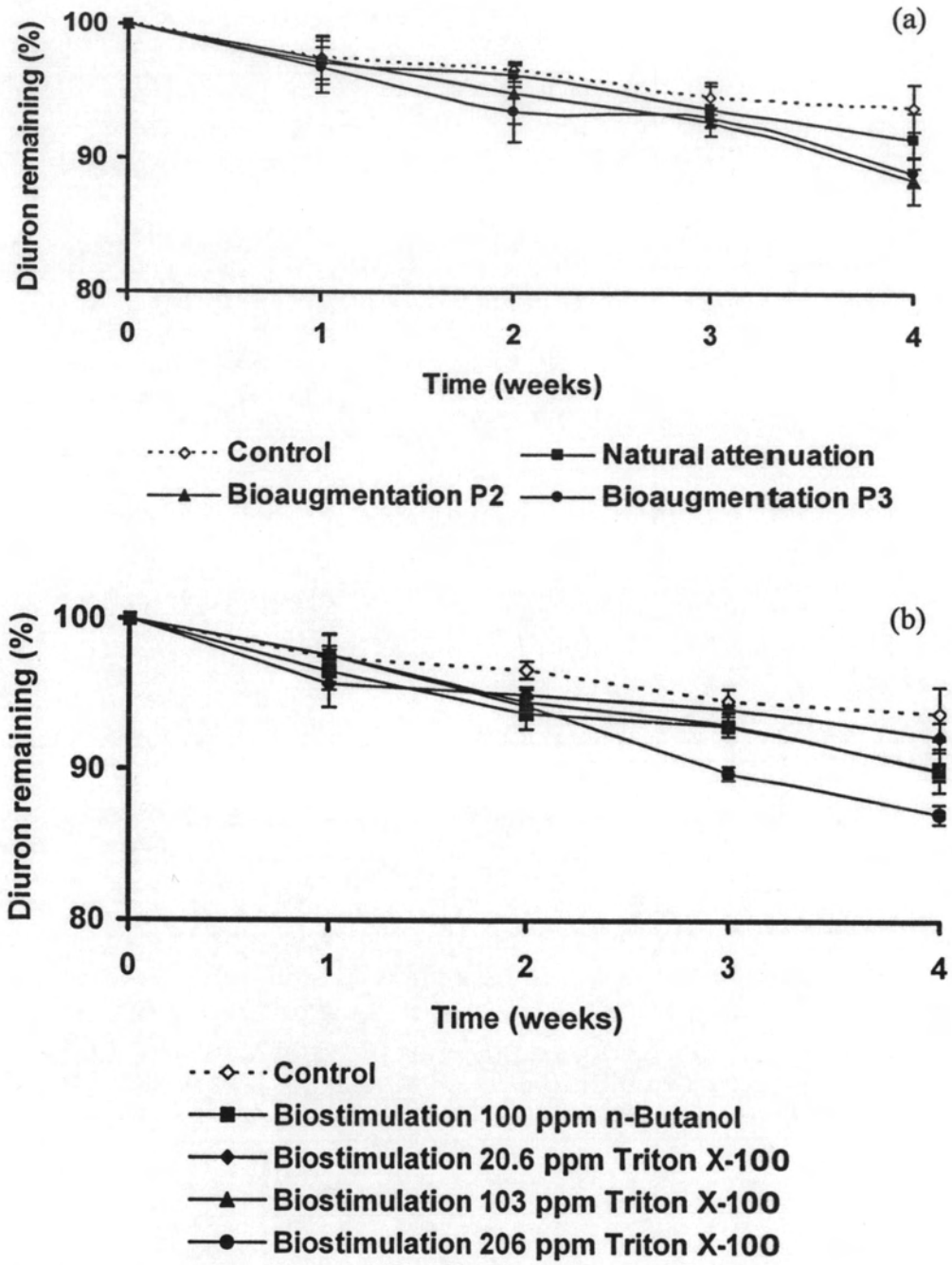


Figure 4.13 Biodegradation of 20-ppm diuron in silty clay soil. The treatments include natural attenuation, bioaugmentation with pure bacterial cultures; *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 (a) and biostimulation (b). The control was sterilized soil.

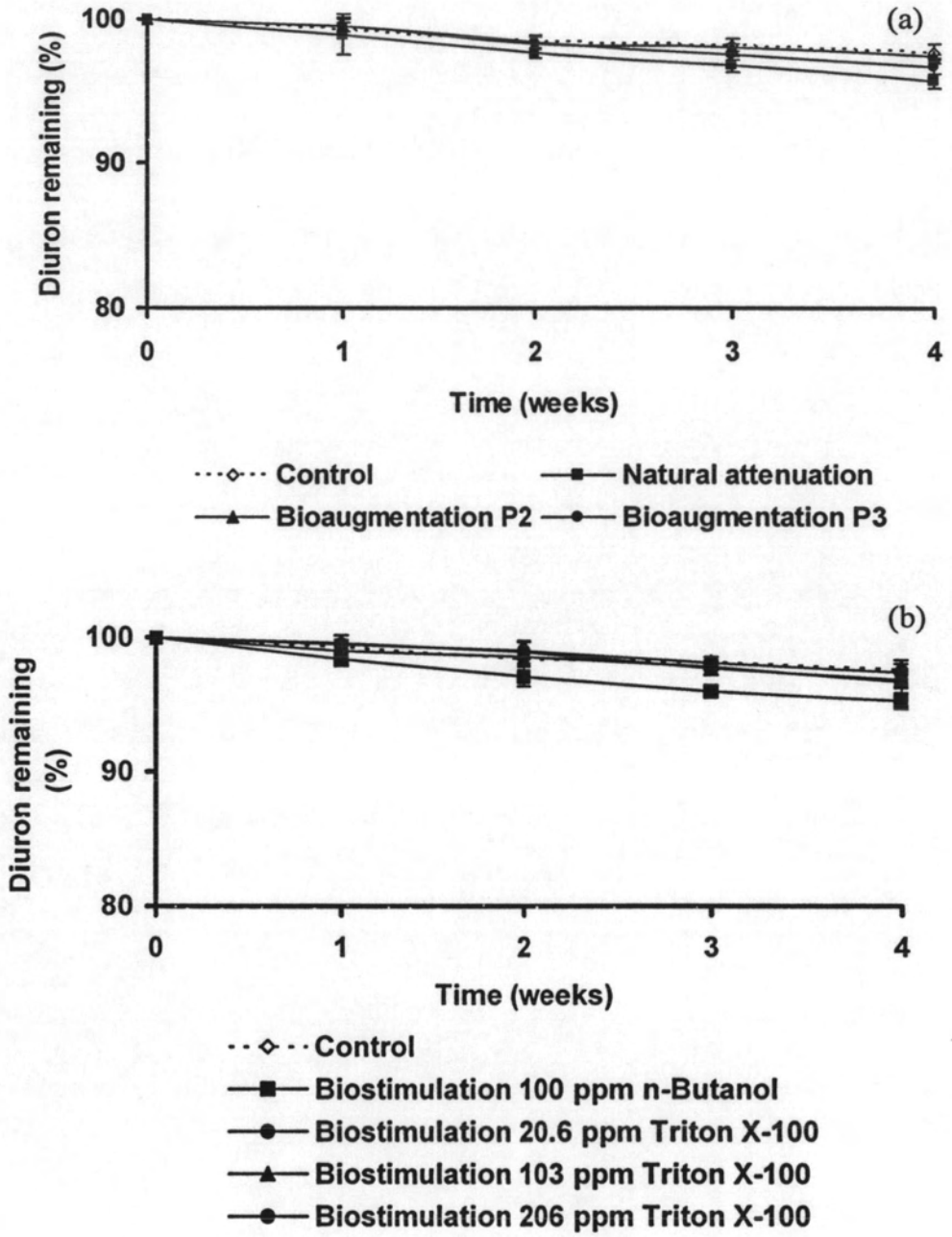


Figure 4.14 Biodegradation of 100-ppm diuron in silty clay soil. The treatments include natural attenuation, bioaugmentation with pure bacterial cultures; *Enterobacter* strain P2 and *Burkholderia capacia* strain P3 (a) and biostmulation (b). The control was sterilized soil.

Number of diuron-degrading bacteria in silty clay soil

The populations of diuron-degrading bacteria were determined by plate count technique with the supplementation of 20 ppm of diuron. The numbers of diuron-degrading bacteria in silty clay soil with 20-ppm diuron slightly deceased within 4 weeks, while the numbers of diuron-degrading bacteria in silty clay soil with 100-ppm diuron were constant during 4 weeks of incubation (Figure 4.15).

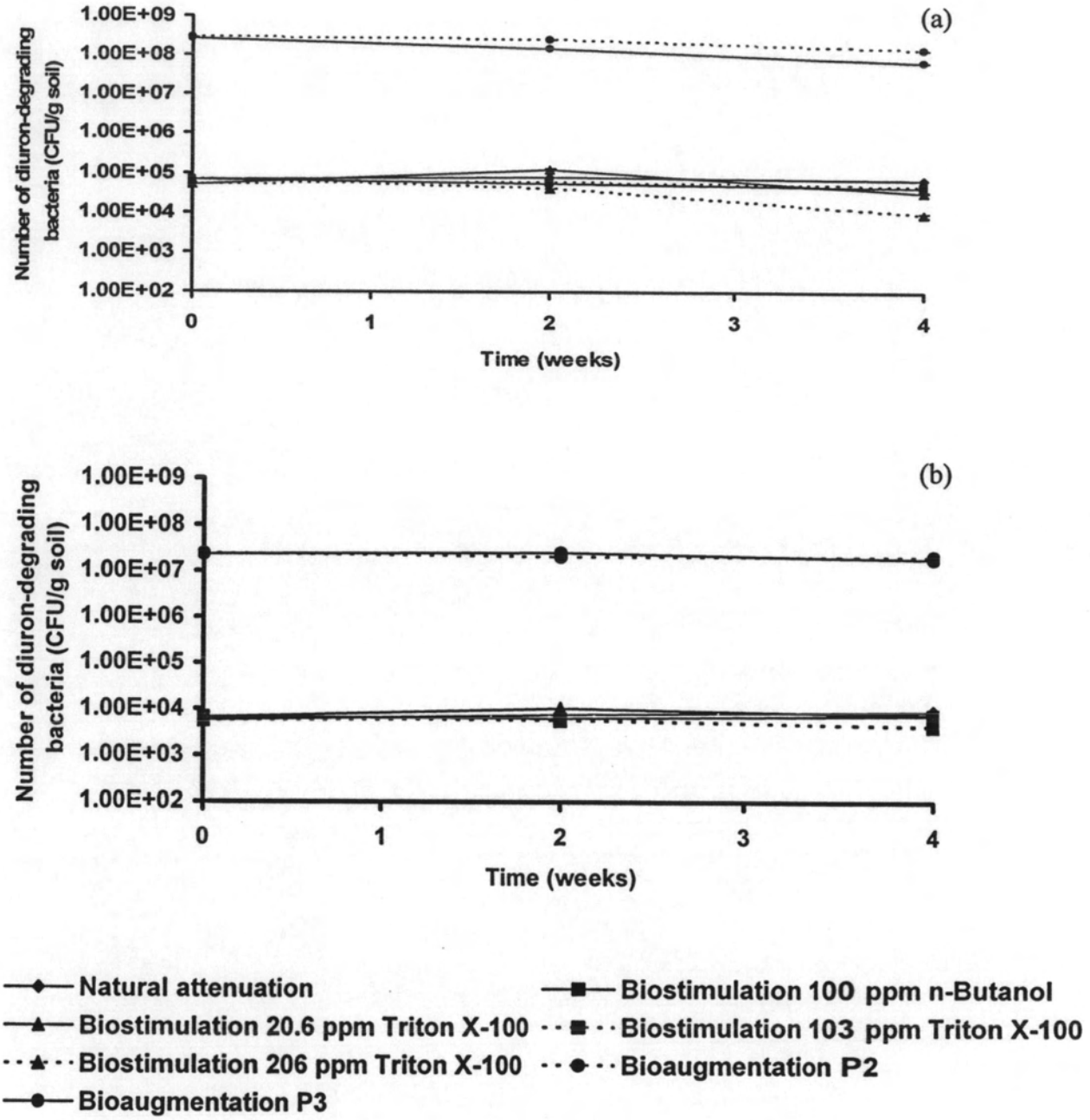


Figure 4.15 Number of diuron-degrading bacteria in silty clay soil. The number of bacteria was determined, during 8 weeks of incubation, in each treatment which include natural attenuation, biostimulation and bioaugmentation with pure bacteria culture; *Enterobacter* strain P2, *Burkholderia capacia* strain P3, (a) 20 ppm diuron and (b) 100 ppm diuron.

Total microbial activity in silty clay soil

Among bioremediation treatments of silty clay soil treated with 20 ppm diuron as shown in Table 4.4, the addition of 20.6, 103 and 206 ppm Triton X-100 in stimulation treatment decreased the dehydrogenase activity with 0.64, 0.02 and 0.16 fold, respectively. The addition of bacterial pure culture at 10^8 CFU/g soil with *Enterobacter* strain P2, *Burkholderia capacia* strain P3 also showed slight reduction of dehydrogenase activity. Besides, natural attenuation showed the decreasing of dehydrogenase by 0.69 fold at 8 weeks. At 100 ppm diuron, the addition of 103 ppm Triton X-100 in stimulation treatment increased the dehydrogenase activity 0.23 fold, whereas 20.6 and 206 ppm Triton X-100 resulted in decreasing of the dehydrogenase activity with 0.31 and 0.09 fold, respectively. The addition of bacterial pure culture at 10^8 CFU/g soil with *Enterobacter* strain P2, *Burkholderia capacia* strain P3 showed slight reduction of dehydrogenase activity. Besides, the reduction of dehydrogenase activity also decreased with natural attenuation (Table 4.4).

Table 4.4 Microbial activity estimated by dehydrogenase activity of silty clay soil treated with 20 or 100-ppm diuron.

Treatments		Dehydrogenase activity at 20 ppm diuron (fold)	Dehydrogenase activity at 100 ppm diuron (fold)
Natural attenuation		0.69 ± 0.08	0.61 ± 0.19
Biostimulation	100 ppm n-Butanol	0.30 ± 0.10	0.54 ± 0.23
	20.6 ppm Triton X-100	0.64 ± 0.22	0.31 ± 0.12
	103 ppm Triton X-100	0.02 ± 0.15	0.23 ± 0.05
	206 ppm Triton X-100	0.16 ± 0.12	0.09 ± 0.02
Bioaugmentation	*Enterobacter* strain P2	0.52 ± 0.34	0.39 ± 0.13
	Burkholderia capacia strain P3	0.47 ± 0.23	0.38 ± 0.15

* The dehydrogenase activity was compared at 0 and 4 weeks.

4.4.2 Bioremediation: Condition B

Diuron degradation was conducted at the laboratory scale in which 5 g soil was spiked with various concentrations of diuron (20 ppm and 100 ppm) before treatment. The bioremediation treatment consisted of natural attenuation, biostimulation and bioaugmentation. Natural attenuation is the process that diuron was reduced due to naturally-occurring processes including sorption, chemical reaction as well as degradation by indigenous microorganisms in soil. Biostimulation is the treatment which 1,000 ppm NH_4Cl was added as a biostimulating agent of diuron biodegradation. Bioaugmentation was carried out in which approximately bacteria consortium A1 were provided at 10^8 CFU/g soil. Abiotic control was also carried out.

4.4.2.1 Diuron degradation in loam soil

The results of three bioremediation treatments of diuron-spiked loam soil including natural attenuation, biostimulation and bioaugmentation for diuron analysis were shown in Figure 4.16. By natural attenuation and bioaugmentation with consortium A1, they were able to degrade 20-ppm diuron 31% within 15 days. By biostimulation with 1,000 ppm NH_4Cl, 20-ppm diuron was degraded by 26% within 15 days as shown in Figure 4.16 (a). In addition to 100-ppm diuron, bioaugmentation treatment was degraded by 22% whereas biostimulation treatment and natural attenuation were degraded 18% and 14%, respectively as shown in Figure 4.16 (b).

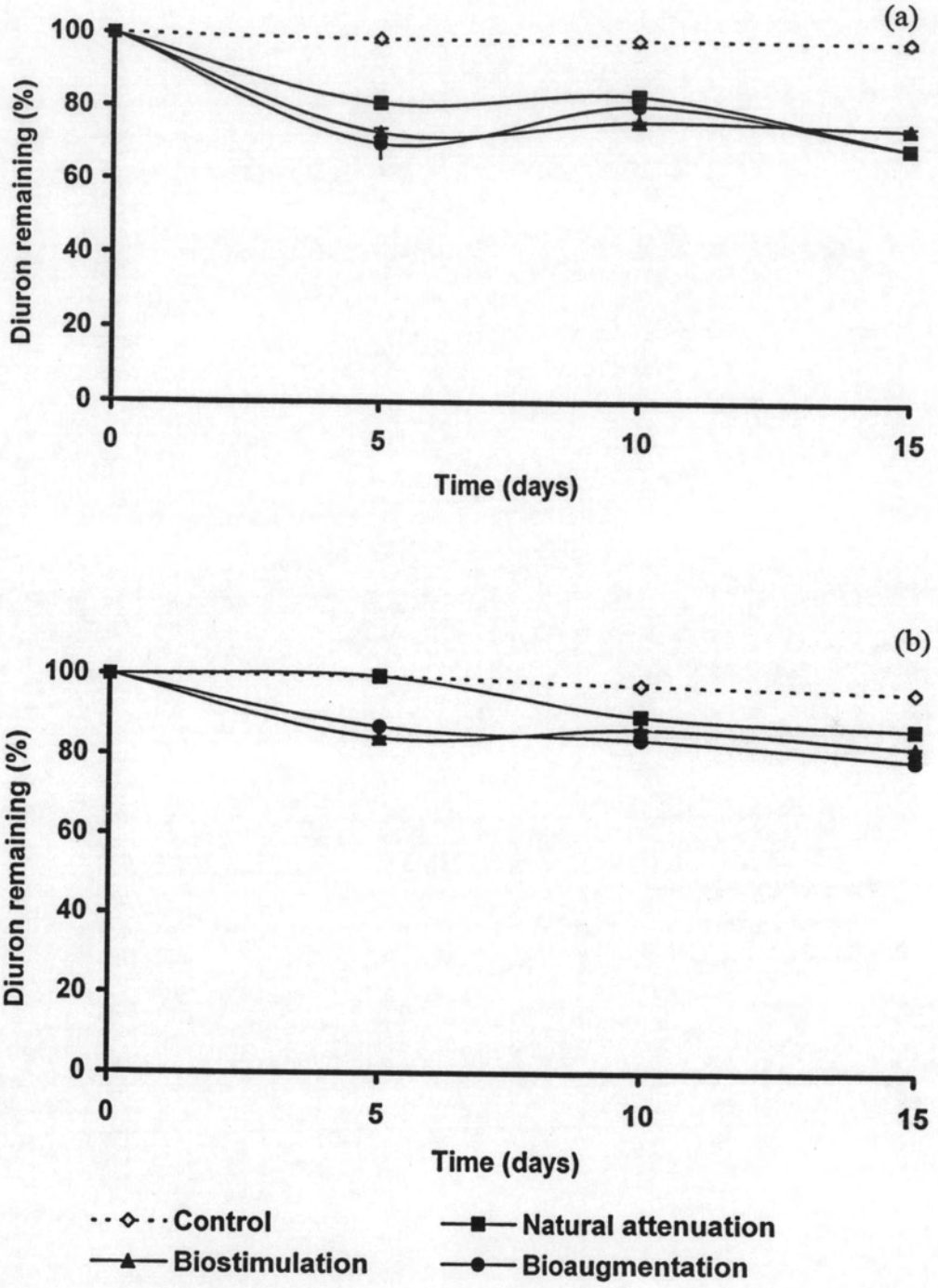


Figure 4.16 Biodegradation of 20 ppm (a) and 100 ppm (b) of diuron in soil loam. The treatments include natural attenuation, biostimulation 1,000 ppm NH_4Cl and bioaugmentation with bacteria consortium A1. The control was sterilized soil

Number of diuron-degrading bacteria in loam soil

The populations of diuron-degrading bacteria were determined by plate count technique with the supplementation of 20 ppm of diuron. The number of diuron-degrading bacteria in each treatment was shown in Figure 4.17. By natural attenuation, the numbers of diuron-degrading bacteria were slightly increased during 15 days as well as biostimulation. By bioaugmentation, it was shown that in the first 5 days the numbers of diuron-degrading bacteria were gradually decreased and gone up again during the next 10 days.

In 100 ppm of diuron, by natural attenuation and biostimulation, the numbers of diuron-degrading bacteria were increased like the result in 20 ppm of diuron. Whereas, by bioaugmentation, the numbers of diuron-degrading bacteria were gradually decreased during the first 5 days and stable during the next 10 days

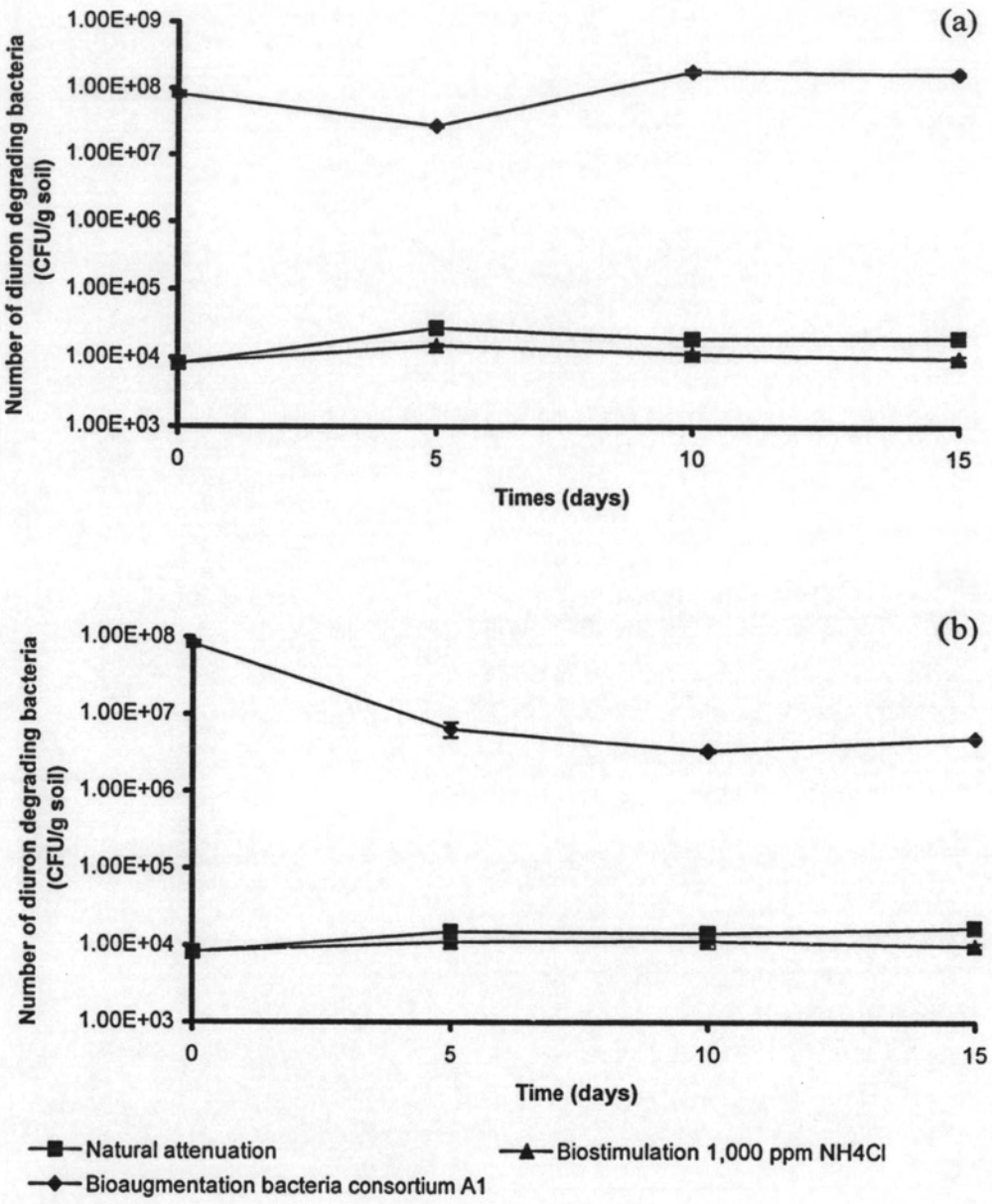


Figure 4.17 Number of diuron-degrading bacteria in loam soil. The number of bacteria was determined, during 15 days of incubation, in each treatment which include natural attenuation, biostimulation and bioaugmentation, (a) 20 ppm diuron and (b) 100 ppm diuron.

Total microbial activity in loam soil

The addition of 1,000 ppm of NH_4Cl by biostimulation treatment resulted in great increasing of dehydrogenase activity by 4.24 fold. The addition of bacterial pure culture at 10^8 CFU/g soil with consortium bacteria A1 showed 1.43-fold of dehydrogenase activity compared with control but less than biostimulation treatment. Similar situation was observed in natural attenuation with 1.47-fold (Table 4.5). To further examine the microbial activity with increasing concentration of diuron, Type-I soil supplemented 100-ppm diuron was assayed. The results as shown in Table 4.5 showed that the addition of 1,000 ppm of NH_4Cl by biostimulation treatment resulted in great increasing of dehydrogenase activity by 4.04 fold. The addition of bacterial pure culture at 10^8 CFU/g soil with consortium bacteria A1 showed 2.38-fold of dehydrogenase activity compared with control. Similar situation was observed to natural attenuation with 1.49-fold.

Furthermore, the results showed that the dehydrogenase activity by bioaugmentation of consortium bacteria A1 exposing to loam soil supplemented with 100-ppm diuron was higher than that 20-ppm diuron about 2-fold, whilst by natural attenuation and biostimulation of 20-ppm and 100-ppm had roughly equal activity.

Table 4.5 Microbial activity estimated by dehydrogenase activity of loam soil treated with 20 or 100-ppm diuron.

Treatments	Dehydrogenase activity at 20 ppm diuron (fold)	Dehydrogenase activity at 100 ppm diuron (fold)
Natural attenuation	1.47 ± 0.25	1.49 ± 0.11
Biostimulation 0.0935 mM NH_4Cl	4.24 ± 0.34	4.04 ± 0.48
Bioaugmentation A1 bacteria consortium	1.43 ± 0.21	2.38 ± 0.32

* The dehydrogenase activity was compared at 0 and 15 days.

4.4.2.2 Diuron degradation in silty clay soil

Diuron biodegradation was conducted through three bioremediation treatments: natural attenuation, biostimulation and bioaugmentation using silty clay soil. The results of three bioremediation treatments including natural attenuation, biostimulation and bioaugmentation for diuron analysis were shown in Figure 4.18.

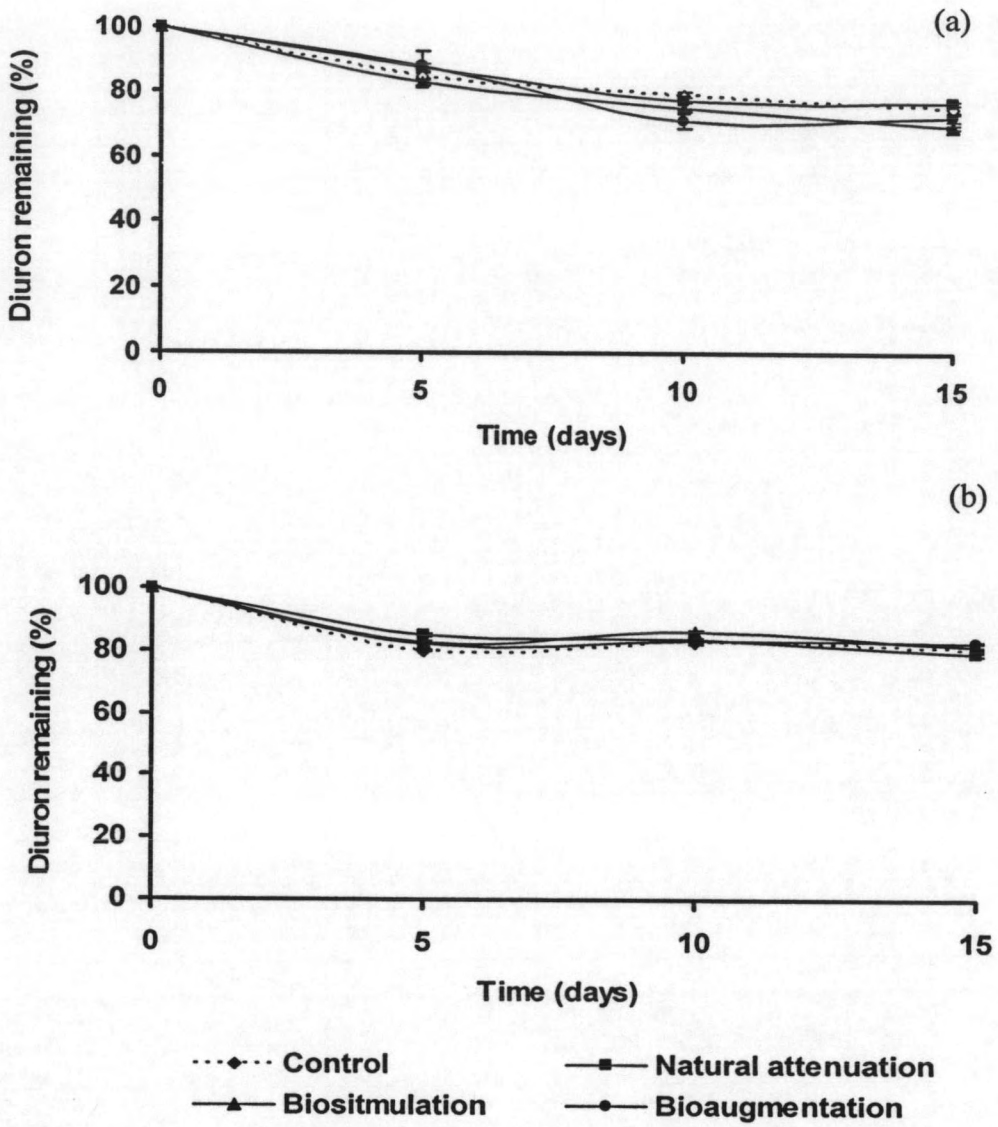


Figure 4.18 Biodegradation of 20 ppm (a) and 100 ppm (b) of diuron in silty clay soil. The treatments include natural attenuation, biostimulation 1,000 ppm NH_4Cl and bioaugmentation with bacteria consortium A1. The control was sterilized soil.

Number of diuron-degrading bacteria in silty clay soil

The populations of diuron-degrading bacteria were determined by plate count technique with the supplementation of 20 ppm of diuron. The number of diuron-degrading bacteria in each treatment was shown in Figure 4.19.

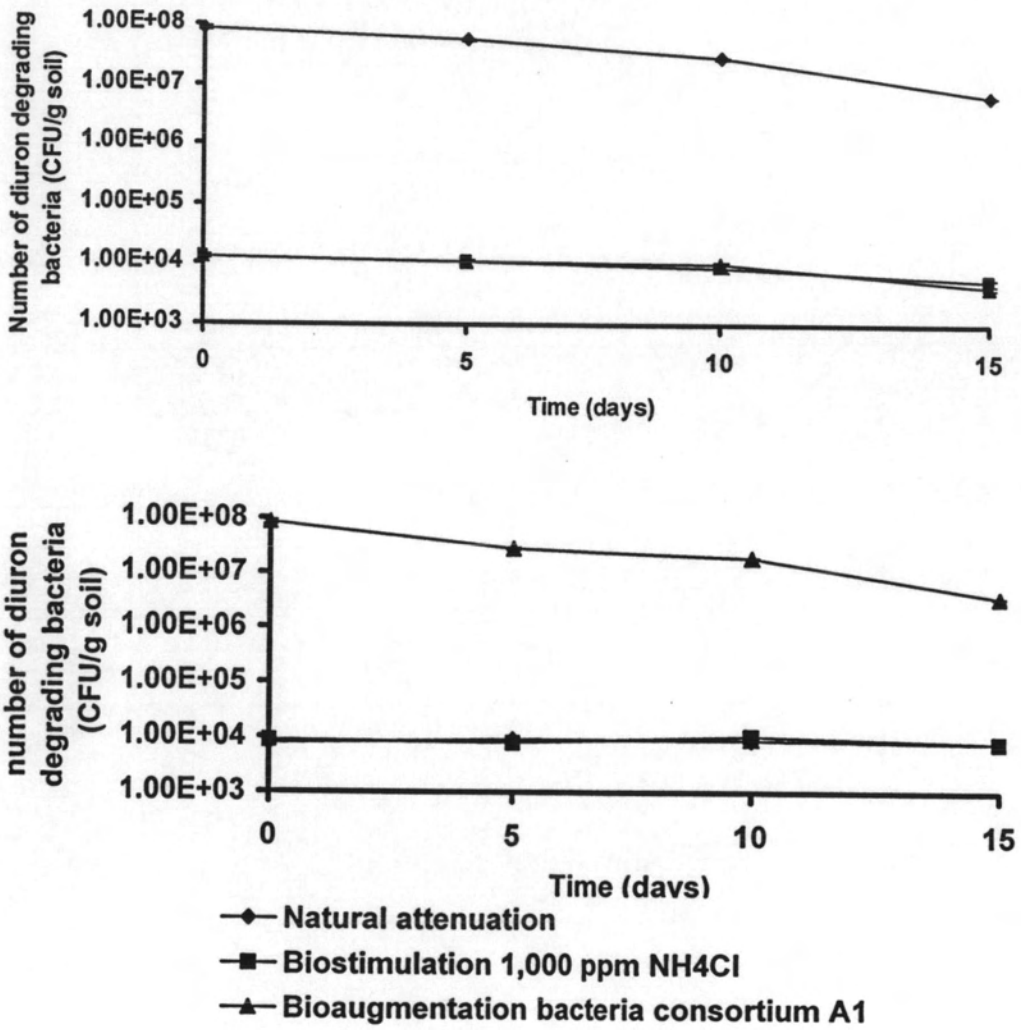


Figure 4.19 Number of diuron-degrading bacteria in silty clay soil. The number of bacteria was determined, during 15 days of incubation, in each treatment which include natural attenuation, biostimulation and bioaugmentation, (a) 20 ppm diuron and (b) 100 ppm diuron.

Total microbial activity in silty clay soil

The total microbial activity of silty clay soil could not be measured from that of the control. It has been reported that the total microbial activity tested in control treatment could be higher than that of the treatment; as a result the data interpretation might be unfeasible (Alef, 1995).

Analysis of microbial community

Soil DNA was isolated from each biological treatment of loam soil under condition B. 200 bp fragment of 16s rDNA was amplified with P338f and P518r primers in PCR reaction as described in Method 3.6.3.3.2. The PCR products were run in SSCP to investigate the bacterial community. The changes among three types of bioremediation treatment were not clearly apparent as DNA bands were smeared, the result suggest that the condition used was not suitable to separate single strand DNA of PCR products and were not able to clarify the bacterial community. Suitable condition is needed for further examination.

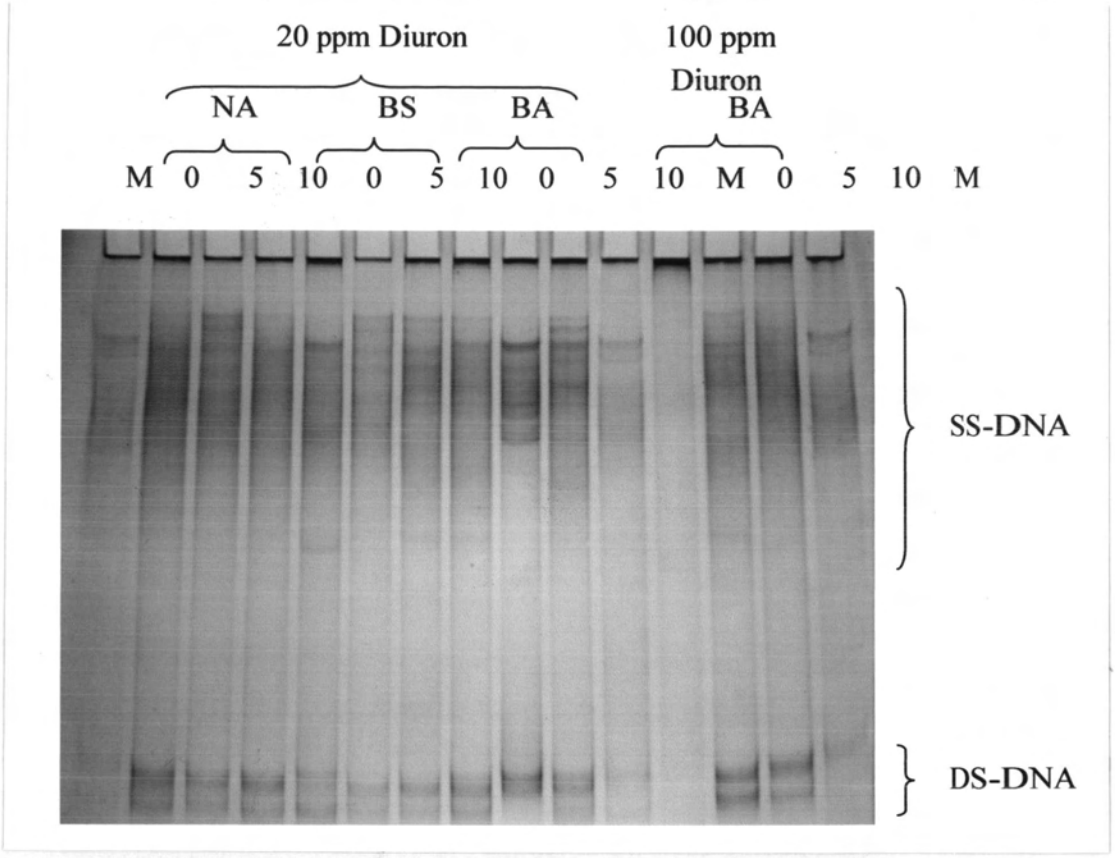


Figure 4.20 SSCP profile of the bioremediation treatment of Condition B: loam soil with 20 ppm diuron for natural attenuation (NA), biostimulation (BS) and bioaugmentation (BA) treatment and with 100 ppm diuron for bioaugmentation in 10 days. Marker (M) is DNA isolated from bacterial consortium A1.