CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Physical and chemical properties of soil

The physical and chemical properties of soil were shown in Table 4.1. Soil sample was classified as sandy loam with pH 6.9. Background concentration of carbofuran in soil was 1.28 mg/kg soil. This soil sample was collected from a rice field that has a known history of carbofuran application.

Table 4.1 Physical and chemical properties of soil sample

Property	Value
EC (ms/cm)	0.28
CEC (cmol/kg)	8.49
Organic carbon (%)	0.8904
Total nitrogen (%)	0.10
Exchangeable K (mg/kg soil)	648.20
Available P (mg/kg soil)	24.37
Sulfur (mg/kg soil)	68.71
Sand, silt, clay (%)	55, 28.6, 16.4
Soil texture	Sandy loam

4.2 Carbon and nitrogen contents of organic amendment

Analysis of C and N contents indicated that CS had the highest C-content while CP had the lowest C-content (Table 4.2). The highest N-content was found in CM and the lowest N-content was found in GL (Table 4.2).

Table 4.2 Carbon and Nitrogen contents in organic amendments

Content	RS	CS	CC	CM	CP	ML	GL
Organic carbon (%)	41.45	47.58	43.66	29.78	8.88	33.56	39.96
Total nitrogen (%)	0.43	0.28	0.81	1.58	0.99	0.28	0.00

Table 4.3 C/N ratio of soil sample after amended with organic amendments

Samples	Load (%)	C/N ratio
RS	0.5	10.75
	1.0	12.78
	1.5	14.21
	5.0	24.40
CS	0.5	11.13
	1.0	13.29
	1.5	15.39
	5.0	28.68
CC	0.5	10.65
	1.0	12.27
	1.5	13.77
	5.0	21.86
CM	0.5	9.63
	1.0	10.26
	1.5	10.81
	5.0	13.30
CP	0.5	8.91
	1.0	8.91
	1.5	8.92
	5.0	8.93
ML	0.5	10.44
	1.0	11.92
	1.5	13.37
	5.0	22.51
GL	0.5	9.30
	1.0	10.50
	1.5	12.50
	5.0	48.90

4.3 Effects of organic and inorganic amendments on carbofuran degradation

This experiment investigated the effects of organic and inorganic amendments on stimulation of the indigenous microorganisms to degrade carbofuran in soils. Figure 4.1 depicted the degradation profiles of carbofuran in microcosms amended with RS. The corresponding kinetic data of carbofuran degradation in soil added with each of organic and inorganic amendments fitted to a modified first-order kinetic model was tabulated in Table 4.4. The coefficients of determination, r^2, ranged between 0.92-0.99 indicating a good fit of the data to the first-order kinetic model (Table 4.4). Results indicated that among the amendments used in this study, RS, and CC at all loads showed a stimulation effect on carbofuran degradation with a short $t_{1/2}$ of 7-11 days compared to control i.e., soil without amendment ($t_{1/2}$ 16 days) ($p < 0.05$).
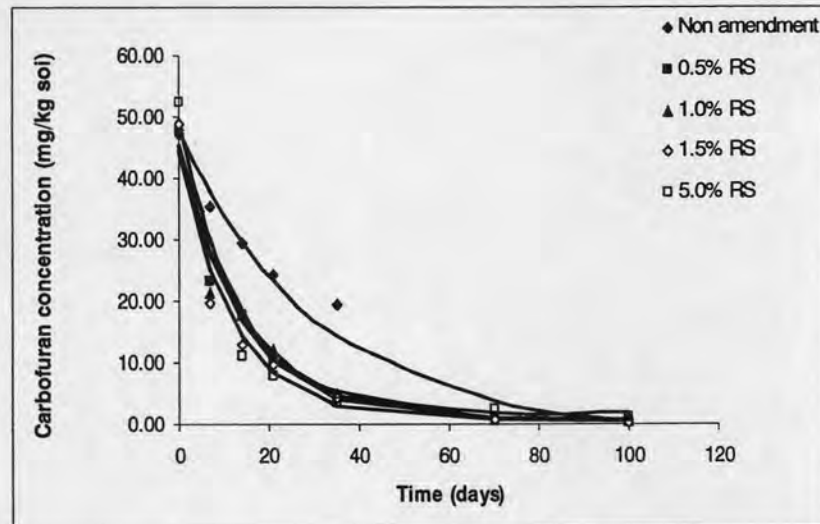
Figure 4.1 Degradation of carbofuran in soil amended with RS (Lines = Carbofuran

concentration fitted to the Modified First order Kinetic Model)

Superior result was obtained at 1.5% RS indicating by the shortest $t_{1/2}$ of 7 days. RS and CC might be used as energy source i.e., C source and/or electron donor by indigenous microorganisms in the soil (Zhang et al., 2002). When compared RS and CC to the other agricultural residues used in this study, it was observed that RS and CC were more bulky and light in weight than others leading to an increase in the soil porosity which in turn improving air ventilation an oxygen diffusion in soil, which are more favorable environmental condition for microorganisms (Venkateswarlu and Sethunathan, 1979).

The addition of CS at the low load of 0.5-1.5% (w/w) stimulated carbofuran degradation in soil indicated by a shorter $t_{1/2}$ of carbofuran, 11 days, than in unamended soil (p<0.05) (Table 4.4). Increasing the load of CS added to the soil from 1.5% to 5% resulted in a decrease in carbofuran degradation in which $t_{1/2}$ of carbofuran increased from 11 days to 17 days which was not significantly different from control ($t_{1/2}$ of 16 days). Addition of organic amendment at the high load might increase organic content in the soil which can absorb the contaminant thus obstruct an attachment between microorganisms and contaminant in the soil resulting in a decrease in degradation ability of microorganisms in soil (Rahman et al., 2003).

CP and CM at all loads showed a negative effect on carbofuran degradation in soil. Half-lives of carbofuran were significantly longer (p<0.05) than in soil without amendment i.e., 20-23 days (Table 4.4). CP and CM used in this study had low organic

Table 4.4 Degradation rate constant (k) and half-lives ($t_{1/2}$) of carbofuran in soil, autoclave soil amended with organic amendments and soil mended with autoclaved amendments

Amendment	Load (%)	Soil			Autoclaved soil			Soil with autoclaved amendments		
		k_1 (1/day)	$t_{1/2}$ (1/day)[1]	r^{2} [2]	k_1 (1/day)	$t_{1/2}$ (1/day)[1]	r^{2} [2]	k_1 (1/day)	$t_{1/2}$ (1/day)[1]	r^{2} [2]
None	-	0.0476	16	0.92	0.0110	64	0.98	0.0476	15	0.92
RS	0.5	0.0730	10 *	0.99	0.0180	39 *	0.98	-	-	-
	1.0	0.0685	10 *	0.97	0.0160	44 *	0.98	-	-	-
	1.5	0.1000	7 *	0.96	0.0150	47 *	0.97	0.0780	9 *	0.97
	5.0	0.0720	9 *	0.99	0.0110	64	0.99	-	-	-
CS	0.5	0.0630	11 *	0.99	0.0180	39 *	0.98	-	-	-
	1.0	0.0630	11 *	0.99	0.0110	64	0.93	-	-	-
	1.5	0.0610	11 *	0.98	0.0190	36 *	0.96	0.0570	12	0.99
	5.0	0.0400	17	0.93	0.0200	35 *	0.94	-	-	-
CC	0.5	0.0620	11 *	0.99	0.0009	80 *	0.99	-	-	-
	1.0	· 0.0650	10 *	0.99	0.0130	52 *	0.90	-	-	-
	1.5	0.0630	11 *	0.99	0.0110	64	0.97	0.0550	13	0.99
	5.0	0.0635	11 *	0.97	0.0170	42 *	0.95	-	-	-
CM	0.5	0.0310	22 *	0.97	0.0140	48 *	0.97	-	-	-
	1.0	0.0305	23 *	0.97	0.0110	65	0.94	-	-	-
	1.5	0.0295	23 *	0.97	0.0090	74 *	0.96	0.0380	18	0.98
	5.0	0.0320	22 *	0.99	0.0210	33 *	0.98	-	-	-
CP	0.5	0.0350	20 *	0.99	0.0130	54 *	0.95	-	-	-
	1.0	0.0.305	23 *	0.99	0.0110	64	0.92	-	-	-
	1.5	0.0345	20 *	0.98	0.0130	53 *	0.94	0.0370	19	0.96
	5.0	0.0346	20 *	0.96	0.0100	68 *	0.99	-	-	-
ML	0.5	0.0705	9 *	0.98	0.0140	49 *	0.94	-	-	-
	1.0	0.0320	22 *	0.96	0.0210	33 *	0.99	-	-	-
	1.5	0.0320	22 *	0.99	0.0220	31 *	0.90	0.0544	13	0.99
	5.0	0.0345	20 *	0.98	0.0190	37 *	0.97	-	-	-
GL	0.5	0.0626	11 *	0.99	0.0210	33 *	0.94	-	-	-
	1.0	0.0280	25 *	0.95	0.0230	30 *	0.97	-	-	-
	1.5	0.0315	22 *	0.99	0.0140	43 *	0.97	0.0220	31 *	0.92
	5.0	0.0340	20 *	0.99	0.0160	44 *	0.98	-	-	-

[1] Comparison between treatments and none-amended soil are significantly different (LSD, P<0.05) if marked with *
[2] Coefficient of determination for non linear regressions

carbon of 8.8% and 29.78%, respectively, (Table 4.2) implying that the supply of C in soil amended with CP and CM might not be sufficient resulting in a low degradation of carbofuran. Sorption of carbofuran to CP and CM added to the soil might reduce a bioavailability of carbofuran to microorganisms (Rahman et al., 2003). High concentration of CP and CM components such as lignin, and salts could inhibit the indigenous microorganisms in the soil and put the microbial population under stress environment (Gibert et al., 2004; Scelza et al., 2007). In addition, CP and CM contains high N of 0.99% and 1.58% (Table 4.4), thus N present in these amendments might be used by microorganisms capable of degrading carbofuran as a N source instead of N in carbofuran structure resulting in a decrease of carbofuran degradation.

A negative effect of adding organic amendments on biostimulating degradation of contaminant have been reported. Degradation of atrazine decreased when the amount of CP and CM added to the soil increased in the study of Moorman et al. (2001) and Alvey and Crowley (1995). They explained that these amendments consist of N which may repress some atrazine-degrading microorganisms that utilize atrazine as a N source or these amendment may contain some toxic compound that inhibit indigenous atrazine degraders. The degradation of phenanthrene in soil at day 280 of incubation decreased from 30% to 20% when amount of CP was increased from 0.83% to 22.7% (Scelza et al., 2007). Heavy metal and salts containing in CP might inhibit the indigenous and/or introduced soil microbial population as well as a degradation of phenanthrene. A study by Gibert et al. (2004) found that CP did not improve sulfate degradation in soil because of a high amount lignin present in their compost might inhibit microbial activity and also sulphate degradation ability of microorganisms in soil.

GL and ML at a low load of 0.5% improved carbofuran degradation in soil with a shorter $t_{1/2}$ of 9 and 11 days, respectively, than in unamended soil (p<0.05) (Table 4.4). However, at high loads of 1.0% to 5.0% of GL and ML inhibited the degradation of carbofuran in soil indicating by a long $t_{1/2}$ of carbofuran in the range of 20-25 days (Tabel 4.4). These results implied that microorganisms in the soil used GL and ML at low load of 0.5% as their C-source while high load of GL and ML provided more favorable C-source than carbofuran for microorganisms resulting in a decrease in carbofuran degradation. GL and ML mainly contained 30-40% sucrose, 4-9% glucose and 5-12% fructose (Sopade et al., 2007) which microorganisms could use, as C-source, easier than carbofuran resulting in a low degradation of carbofuran in soil

amended with GL and ML. When the environment contains two or more sources of C, microorganisms will use the most assimilable and the most concentrate C-source. When every sources of C are equal, all of sources will be then simultaneously utilized (Schmidt and Alexander, 1995). An inhibitory effect on xenobiotic degradation by GL was reported in various studies. Pentachlorophenol (PCP) degradation by *Pseudomonas* sp. stopped when glucose concentration in culture medium reached 100 mg/L. GL at this concentration might be sufficient as an energy source for this strain without using PCP as its energy source (Kaufman, 1978). Similar results were reported by Vroumsia et al. (1999). They found that the degradation of 2,4-D and 2,4-DCP decreased from 37% to 15% and 56% to 33%, respectively, when glucose concentration in culture medium was increased from 5 g/L to 10 g/L.

Inhibitory effect of inorganic amendments on carbofuran degradation was observed. Half-lives of 27-30 days were found in soil amended with inorganic amendments in comparison to control with $t_{1/2}$ of 16 days (Table 4.5) ($p<0.05$). Soil sample might already has enough trace elements for indigenous microorganisms. Therefore, an addition of inorganic amendment to the soil might increase concentration of the inorganic elements up to the inhibitory level thus decrease carbofuran degradation ability of the microorganisms in the soil. Previous data reported the inhibitory effects of trace elements in biostimulation. Trindade et al. (2002) reported a negative effect of nitrogen in biostimulaiton of total petroleum hydrocarbon. Nitrogen can be toxic to the microorganisms due to the possibility of ammonia generation in the soil, which can be lethal at high concentrations. Moreover, the ammonium ion promotes the increase of the oxygen demand which can cause the lack of oxygen for the ecosystem of microorganisms (Walworth et al., 1997; Steffensen and Alexander, 1995). An addition of phosphate in phosphate and ortho-phosphates forms was reported to increase the precipitation of calcium and iron phosphates resulting in P which was unavailable to biosynthesis of nucleic acids, ATP, and cellular component i.e., phospholipids of the microbe. This resulted in a decrease of the microbial activities as well as a degradation of contaminant (U.S. Environmental Protection Agency, 1985).

Metabolites observed were carbofuran phenol and 3-keto carbofuran (Tables 4.6, 4.7) suggesting that the pathway of carbofuran degradation is hydrolysis. Hydrolysis

Table 4.5 Degradation rate constants (k) and half-Lives ($t_{1/2}$) of carbofuran in soil and autoclaved soil amended with inorganic amendment

Amendment	Load (mL)	Soil			Autoclaved soil		
		k_1 (1/day)	$t_{1/2}$ (1/day)[1]	$r^{2[2]}$	k_1 (1/day)	$t_{1/2}$ (1/day)[1]	$r^{2[2]}$
None	-	0.0476	16	0.92	0.0110	64	0.98
Inorganic	1.0	0.0340	20 *	0.99	0.0195	36 *	0.98
	2.0	0.0330	21 *	0.97	0.0245	28 *	0.98

[1] Comparison between treatments and none-amended soil are significantly different (LSD, P<0.05) if marked with *

[2] Coefficient of determination for non linear regression

of carbofuran yielded carbofuran and methylamine as degradation products (Chapalamadugu and Chuadry 1992). Methylamine could be further used by microorganisms for their growth. Carbofuran was reported to be metabolized to carbofuran phenol by *Pseudomonas, Arthrobacter* and *Bacillus* sp., which was then being degraded to an undetectable compound (Cain and Head, 1991). Carbofuran phenol could be degraded further via ring cleavage yielding CO_2 and H_2O by carbofuran degraders (Trabue et al., 1997). 3-Keto carbofuran concentrations in soil samples were shown in Table 4.7. Since carbofuran phenol and 3-keto carbofuran were reported as the metabolites of carbofuran resulted from the activities of hydrolase (Tomasek and Karns, 1989) and oxidase enzyme (Evert, 2002), respectively, therefore, we speculated that the indigenous microorganisms in the soil samples produced enzyme hydrolase and oxidase responsible for degrading carbofuran. The study on the activities of these two enzymes in the soil microcosms should be conducted to confirm the conclusions.

A half-life of carbofuran of 9 days in soil amended with 1.5% autoclaved RS was not significantly from soil amended with 1.5% RS i.e., 7 days ($p<0.05$) (Table 4.4) indicating that microorganisms in RS did not play an important role in carbofuran degradation in soil but indigenous microorganisms did.

Results from abiotic control experiment revealed that carbofuran degradation was a biological process indicating by approximately 4-times longer half-life of carbofuran observed in autoclaved soil than in non-autoclaved soil (Table 4.4).

Findings from this study indicated that an approach to use biostimulation technique should concern about types and load of the amendments. In our study, we selected RS at a load of 1.5% to biostimulate carbofuran degradation in aged soil due to the shortest $t_{1/2}$ of carbofuran in soil amended with this amendment.

4.4 Effects of organic and inorganic amendments on number of carbofuran degraders

This experiment determined the effects of organic and inorganic amendments on number of carbofuran degraders in the soil. Results indicated that number of carbofuan degrading microorganisms varied among types and loads of amendment which was summarized as the means over 7 sampling times in Table 4.8. Means of each individual treatment at each sampling time were shown in Appendix A

Table 4.6 Concentrations of carbofuran phenol in soil amended with RS

Sampling date	Carbofuran phenol concentration (mg/kg soil) [1]				
	CF only	0.5% RS	1.0 % RS	1.5% RS	5% RS
0	0.14 ± 0.00	0.39 ± 0.00	0.89 ± 0.36	1.30 ± 0.65	0.00 ± 0.00
7	0.04 ± 0.01	1.04 ± 0.36	0.27 ± 0.90	0.27 ± 0.90	0.04 ± 0.16
14	0.14 ± 0.05	0.04 ± 0.01	0.05 ± 0.01	0.01 ± 0.03	0.31 ± 0.18
21	0.02 ± 0.01	0.37 ± 0.13	0.02 ± 0.37	0.08 ± 0.33	0.35 ± 0.23
35	0.02 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.01
70	0.02 ± 0.01	0.03 ± 0.01	0.06 ± 0.01	0.04 ± 0.01	0.00 ± 0.00
100	0.14 ± 0.05	0.11 ± 0.04	0.14 ± 0.02	0.10 ± 0.01	0.00 ± 0.01

[1] Data expressed as means and standard deviation of three replicates samples

Table 4.7 Concentrations of 3-keto carbofuran in soil amended with RS

Sampling date	3-keto carbofuran concentration (mg/kg soil) [1]				
	CF only	0.5% RS	1.0 % RS	1.5% RS	5% RS
0	0.09 ± 0.07	0.54 ± 0.25	0.78 ± 0.46	0.00 ± 0.00	0.00 ± 0.00
7	0.21 ± 0.11	0.16 ± 0.02	0.13 ± 0.02	0.29 ± 0.15	0.08 ± 0.05
14	0.08 ± 0.03	0.11 ± 0.00	0.10 ± 0.00	0.08 ± 0.20	0.36 ± 0.12
21	0.01 ± 0.00	0.65 ± 0.36	0.15 ± 0.36	0.00 ± 0.05	0.07 ± 0.01
35	0.01 ± 0.03	0.22 ± 0.06	0.29 ± 0.06	0.33 ± 0.23	0.00 ± 0.16
70	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01	0.00 ± 0.01
100	0.03 ± 0.03	0.01 ± 0.00	0.02 ± 0.00	0.02 ± 0.01	0.00 ± 0.01

[1]Data expressed as means and standard deviation of three replicates samples

Additional of RS, CS, CC, CM, and CP at a low load of 0.5, 1.0, 1.5% (w/w) did not increase number of carbofuran degraders in soil indicating by a non-significantly different ($p<0.05$) between number of carbofuran degraders in amended soil and control (Table 4.8). However, the high load of these amendments of 5% improved the growth of carbofuran degraders in soil demonstrating by a significantly higher number of carbofuran degraders in all microcosms treated with 5% amendments (Table 4.8) ($p<0.05$). These results suggested that carbofuran degraders could use organic amendment added to the soil as energy source for their growth resulting in an increase in number of degraders. Addition of RS and CS at the low loads of 0.5%-1.5% enhanced carbofuran degradation (Table 4.4) in soil but did not improve growth of carbofuran degraders (Table 4.8) indicating that RS and CS might stimulate carbofuran degradation activities of indigenous carbofuran degraders. However, RS and CS might not be used for their growth. High load of RS and CS, 5% w/w, resulted in 10 times higher number of carbofuran degraders in soil comparing to 0.5-1.5% loads. It was not surprised to see that there were carbofuran degraders in soil without an addition of carbofuran. This was because carbofuran degraders could use various C-sources for their growth, not limited to only carbofuran. Results revealed that in microcosms amended with the 5.0% load of RS and CS, carbofuran degraders mainly used organic amendments in the soil for stimulating their growth. High amount of C and N added to the carbofuran contaminated soil are more favorable energy sources for indigenous microorganisms than C and N containing in the ring of carbofuran resulting in an increase in number of carbofuran degraders without stimulating carbofuran degradation (Schmidt and Alexander, 1985).

Number of carbofuran degraders tend to increase at a high load of 5% CM and CP added (Table 4.8) while carbofuran degradation was inhibited in soil microcosms treated with all loads of these two amendments (Table 4.4) implying that an increase in number of carbofuran degraders did not enhance carbofuran degradation in soil. CM and CP added might be used as energy source for indigenous carbofuran degraders, however, these two amendments might absorb carbofuran in the soil resulting in an improvement of microbial growth and a reduction in bioavailability of carbofuran to microorganisms.

Number of carbofuran degrader was obviously higher in the soil amended with GL and ML than in non-amended soil at all loads (Table 4.8) ($p<0.05$), while low carbofuran degradation in these treatments was observed (Table 4.4). These results

confirmed an explanation that microorganisms prefer to use GL and ML for their growth instead of carbofuran, resulted in reduction of carbofuran degradation in these soils. A high dose of glucose inhibited microbial activity and contaminant degradation by increasing in the number of microbial antagonisms for carbon source from glucose and contaminants (Duquenne et al., 1996).

These results are in contrast with the finding of Barahona et al. (2004) who found that an addition of 3% (w/w) corn residues increased the number of hydrocarbon-degrading microorganisms as well as increased diesel removal from 40 g/kg soil to 13.2 g/kg soil. Stimulation of total petroleum hydrocarbon (THP) degradation by using compost as a soil amendment improved both microbial activity and hydrocarbon degradation (Riffaldi et al., 2005). Microorganisms in the soil sample might use both THP and these organic amendments for their growth resulting in a stimulation effect on both microbial growth and degradation activity.

Additional of 1 and 2 ml of inorganic amendments significantly decreased the number of carbofuran degraders compared to control (Table 4.9) ($p < 0.05$). Level of inorganic elements added to soil sample might be too high and then caused an adverse effect to indigenous microorganisms. Reasons to explain this evident included (i) level of trace elements in soil sample might be already sufficient for microorganisms thus an addition of inorganic amendment might increase inorganic concentration up to the inhibitory level (Moorman et al., 2001); (ii) nutrients added to the soil might have low solubility which in turn unbioavailable to microorganisms (Bento et al., 2005); and (iii) inorganic amendments increased only population of heterotrophic microorganisms but did not increase population of degrading microorganisms (Carmichael et al., 1997).

Number of microorganisms in soil spiked with carbofuran and amended with each amendments were lower than in non-spiked soil, which indicated that carbofuran might be toxic to microorganisms present in (Table 4.8).

Table 4.8 Average number of carbofuran degraders and microbial dehydrogenase activity in soil amended with organic amendments

Amendment	Load (%)	Soil with carbofuran		Soil without carbofuran	
		Carbofuran degrader [1][2] (Log CFU/g soil)	Dehydrogenase [1][2] (mg INTF/g soil h)	Carbofuran degrader [1][2] (Log CFU/g soil)	Dehydrogenase [1][2] (mg INTF/g soil h)
None	-	8.05	14.33	7.58	10.04
RS	0.5	7.89 *	11.68 *	7.75	9.10
	1.0	8.08	18.14 *	7.96	9.67
	1.5	8.18	18.02 *	8.03 *	10.44
	5.0	8.99 *	18.87 *	8.80 *	20.53 *
CS	0.5	7.98	10.20 *	7.89	5.64 *
	1.0	8.10	15.93	7.97	7.73 *
	1.5	8.02	16.77 *	8.13 *	9.54
	5.0	9.07 *	16.91 *	8.64 *	12.72
CC	0.5	8.06	13.69	8.01 *	4.20 *
	1.0	8.02	15.60	7.99 *	7.20 *
	1.5	8.12	17.70 *	8.10 *	9.32
	5.0	9.12 *	20.36 *	8.73 *	21.03 *
CM	0.5	8.21	8.18 *	8.16 *	2.39 *
	1.0	8.08	12.40	8.02 *	3.84 *
	1.5	8.16	10.40 *	8.13 *	5.76 *
	5.0	9.20 *	19.27 *	8.77 *	23.28 *
CP	0.5	8.00	7.45 *	7.96	1.44 *
	1.0	7.84	9.45 *	7.81	2.43 *
	1.5	8.03	9.21 *	7.96	1.80 *
	5.0	9.05 *	8.61 *	8.60 *	10.93
ML	0.5	8.42 *	14.02	8.15 *	12.77
	1.0	8.32 *	16.61	8.26 *	16.75 *
	1.5	8.32 *	14.91	8.23 *	20.05 *
	5.0	8.94 *	16.78 *	8.27 *	17.85 *
GL	0.5	8.38 *	14.58	8.09 *	10.95
	1.0	8.18	16.72	8.13 *	13.92
	1.5	8.34 *	15.28	8.28 *	14.71 *
	5.0	9.03 *	11.99	8.27 *	13.33

[1] Data are expressed as the means of three replicates

[2] LSD test for multiple comparison versus a control group (p<0.05) was run in SPSS program between non-amended soil and the other treatments. Those treatments with * indicated statistically different at the 95% confidence level.

Table 4.9 Average number of carbofuran degraders and microbial dehydrogenase activity in soil amended with inorganic solution

Amendment	Load (mL)	Soil with carbofuran		Soil without carbofuran	
		Carbofuran degrader [1][2] (Log CFU/g soil)	Dehydrogenase [1][2] (mg INTF/g soil h)	Carbofuran degrader [1][2] (Log CFU/g soil)	Dehydrogenase [1][2] (mg INTF/g soil h)
None	-	8.05	14.33	7.58	10.04
Inorganic	1	7.12 *	3.20 *	7.02 *	1.84 *
	2	7.16	2.08 *	7.13 *	1.55 *

[1] Data are expressed as the means of three replicate samples

[2] LSD test for multiple comparison versus a control group ($p < 0.05$) was run in SPSS program between non-amended soil and the other treatments. Those treatments with * indicate statistically different at the 95% confidence level.

4.5 Effects of organic and inorganic amendments on soil microbial activity

Biological activity in soil can be determined by dehydrogenase activity. Results revealed that dehydrogenase activity in the soil microcosms varied among 7 sampling times and load of amendment summarized as the means in Table 4.8. Means of each individual treatment at each sampling time were shown in Appendix A.

Addition of RS, CS, and CC at a low load of 0.5% was not sufficient to stimulate microbial activity in the soil (Table 4.8) as well as growth of carbofuran degraders (Table 4.8) while addition of these amendments at the high load of 1.0 to 5.0% markedly stimulated microbial activity in which dehydrogenase activity was found to be significantly higher than control (Table 4.8) ($p<0.05$). This result was in correlation with a high degradation of carbofuran found in RS, CS, and CC amended soil (Table 4.8) except of 5% CS. However, number of carbofuran degraders in microcosms added with 1.0-5.0% of these amendments was not increased with the increase in dehydrogenase activity. Indigenous carbofuran degraders might use RS, CS, and CC for stimulating their activities but not for growth. Dehydrogenase activity dose not only represent the activity of carbofuran degraders but also the other types of microorganisms in the soil, resulting in a non-correlation results between number of carbofuran degraders and dehydrogenase activity in soil.

High loads of organic amendment reflected a high load of C-source which could be used as energy source for microorganisms resulting in an increase in the microbial activity. Organic amendments might improve oxygen diffusion by increase soil porosity (Venkateswaru and Sethunathan, 1979) and nutrient availability to microorganisms (Barahona et al., 2004). Organic matter was known as one of factors affecting microorganisms in soil. It can be either an activator or inhibitor when it presences in optimum or excessive concentration, respectively (Zhang et al., 2002; Bento et al., 2005). Plant residues such as tobacco residue and rice straw were reported to have a stimulation effect on soil microbial activities revealed by an increase in dehydrogenase activity up to 3 to 5 times than soil without amendment (Lin et al., 2005).

CP was found to inhibit microbial activity indicated by a decrease of dehydrogenase activity in the soil amended with this material in comparison to control (Table 4.8) indicating that their might be some inhibitors containing in CP suppressed soil microbial activity. These might be the cause of decrease in carbofuran degradation efficiency in soil microcosms treated with CP (Table 4.4). Similar finding

was reported by Crecchio et al. (2004). Microbial activity was inhibited after soil contaminated with atrazine was amended with compost at a high concentration of 24 ton/ha which indicated by a reduction of dehydrogenase activity and a suppressive of atrazine mineralization. High nitrogen content of compost was responsible to this evident due to a nitrogen in atrazine ring was less subject to mineralization in the presence of nitrogenous amendments (Alvey and Crowley, 1995). Dehydrogenase activity was decreased when 0.5-1.5% CM was added to the soil (Table 4.8). In contrast CM at the load of 5.0% significantly promoted the activity of microorganisms in the soil but did not enhance carbofuran degradation activity.

An additional of GL and ML at all loads did not affect microbial activity. Dehydrogenase activity in soil added with these amendment was not significantly different from control (p<0.05) (Tables 4.8). This result was not correlated with an increase in the number of carbofuran degraders (Table 4.8) when GL and ML were used as soil amendments. Kulkarni et al. (2005) reported that on additional of GL inhibited the growth of *Pseudomonas putida* and microbial activity in aerobic condition due to the metabolites of glucose led to an acidic condition which was toxic to the microorganisms.

Inorganic amendment was found to inhibit microbial activity demonstrated by a significantly lower of dehydrogenase activity than control (Table 4.9) (p<0.05). Results related with the decrease of carbofuran degradation efficiency (Table 4.4) and number of carbofuran degraders (Table 4.8) found in soil treated with inorganic amendments, confirming that our soil sample has sufficient inorganic nutrient for microorganisms. An addition of excess inorganic source increase inorganic level equal to inhibitory level resulting in a decrease in degradation ability of microorganisms and decreased in microbial population (Moorman et al., 2001).

4.6 Effects of organic and inorganic amendments on soil microbial respiration

This experiment investigated the effects of organic and inorganic amendment on soil microbial respiration which was measured by determining total CO_2 generated from the soil by titration method. Figure 4.2 depicted total CO_2 generation profiles in microcosms treated with organic and inorganic amendments. Results indicated that addition of RS CS, and CC at all loads enhanced total CO_2 generation in the range of 8-10 mg CO_2/kg days compared to soil without amendment i.e., 3 mg CO_2/kg days (Figure 4.2a-4.2c). In general, as a load of organic amendment increased, total CO_2

generated from the soil increased (Figure 4.2a-4.2c). This might due to a high organic carbon of each amendment could result in high organic matter in soil which in turn contributed to higher total CO_2 generated from the soil. RS and tobacco residue added to the soil were found to stimulate and improve soil microbial respiration with an approximately 6 times increasing of CO_2 production comparing to non-amended soil (Kara, 2000). Additional of corn and sugar cane residues to the soil stimulate the microbial respiration by increased of CO_2 emitted with approximately 25 times compared to non-stimulated soil. CO_2 emitted increased significantly with the increasing of a load of corn residues (Barahona et al., 2004).

Additional of CM at the loads of 0.5-1.5% did not markedly increase total CO_2 generation from CM-amended soil in comparison to soil without amendment (Figure 4.2d), while 5.0 % CM improved the total CO_2 generation. CM contains low content of organic carbon of 29.78% (Table 4.2) compared to RS, CS, and CC of 41.45%-47.58%, therefore large amount of CM was required in order to stimulate microbial activity and consequently increase total CO_2 generation.

Inhibitory effect on soil respiration was evident in soil microcosms treated with CP at all loads indicating by a lower of CO_2 generation than in control after 35 days of incubation (Figure 4.2e). CP consists of the lowest content of organic C, 8.88%, (Table 4.2) among other amendments which might not be suitable for stimulating microbial activity leading to a low CO_2 generation from the soil. In addition, microbial activity might be inhibited by some compounds such as lignin, heavy metal, and salts containing in CP (Gibert et al., 2004; Scelza et al., 2007).

Soil microcosms added with ML and GL obviously showed a higher amount of CO_2 generation among the treatments in this study (Figure 4.2f-4.2g). This might due to the fact that GL and ML consisted of high C-containing sugars i.e., sucrose, glucose, and fructose (Sopade et al., 2007), thus microorganisms in the soil can easily access these sugars as their C-source to stimulate their growth as well as their activities resulting in large amount of CO_2 emitted from the soil. Soil microbial respiration represents activity of all types of indigenous microorganisms, not only carbofuran degraders, therefore a non-correlation between CO_2 generation and number of carbofuran degraders in soil was observed (Table 4.8, Figure 4.2).

Inorganic amendments inhibited soil microbial respiration indicated by a low dehydrogenase activity, low number of carbofuran degraders (Table 4.9) and total CO_2 generated from soil amended with inorganic solution in comparison to soil without amendment (Figure 4.2h). Results indicated that inorganic nutrients in our sample are sufficient for microorganisms in soil. Negative effects of inorganic nutrients added to soil might be resulted from following reason: (i) high concentration of nitrogen can be lethal to microorganisms at high concentrations (Steffensen and Alexander, 1995); (ii) ammonium ion promote the increase of oxygen demand which can cause the lack of oxygen in microbial ecosystem (Steffensen et al., 1995); and (iii) high concentration of K_2HPO_4 caused osmotic pressure due to a high concentration of soluble salts.

Though soil microcosms treated with 5% GL showed the highest amount of CO_2 generation, but carbofuran degradation in this soil microcosm was inhibited. Therefore, it was not selected to be used in biostimulation of carbofuran degradation in aged soil. RS soil amended with 1.5% RS had the $t_{1/2}$ of carbofuran of 7 days without inhibitory effects on microbial population and activities. Inorganic amendment at the volume of 1 mL could stimulate soil microcosms better than 2 mL elucidated by a shorter $t_{1/2}$ of carbofuran and higher microbial population and activities than soil amended with 1 mL of inorganic solution (Table 4.5, 4.9). Therefore, we selected 1.5% RS and 1 mL of inorganic amendment to biostimulate carbofuran degradation in aged soil in the next experiment. An increase in total CO_2 generation at day 70 in soil amended with 5% of RS, CS, CC, CM, ML, and GL might due to a high remaining organic carbon from each amendment in the microcosm resulted in a high organic matter in soil.
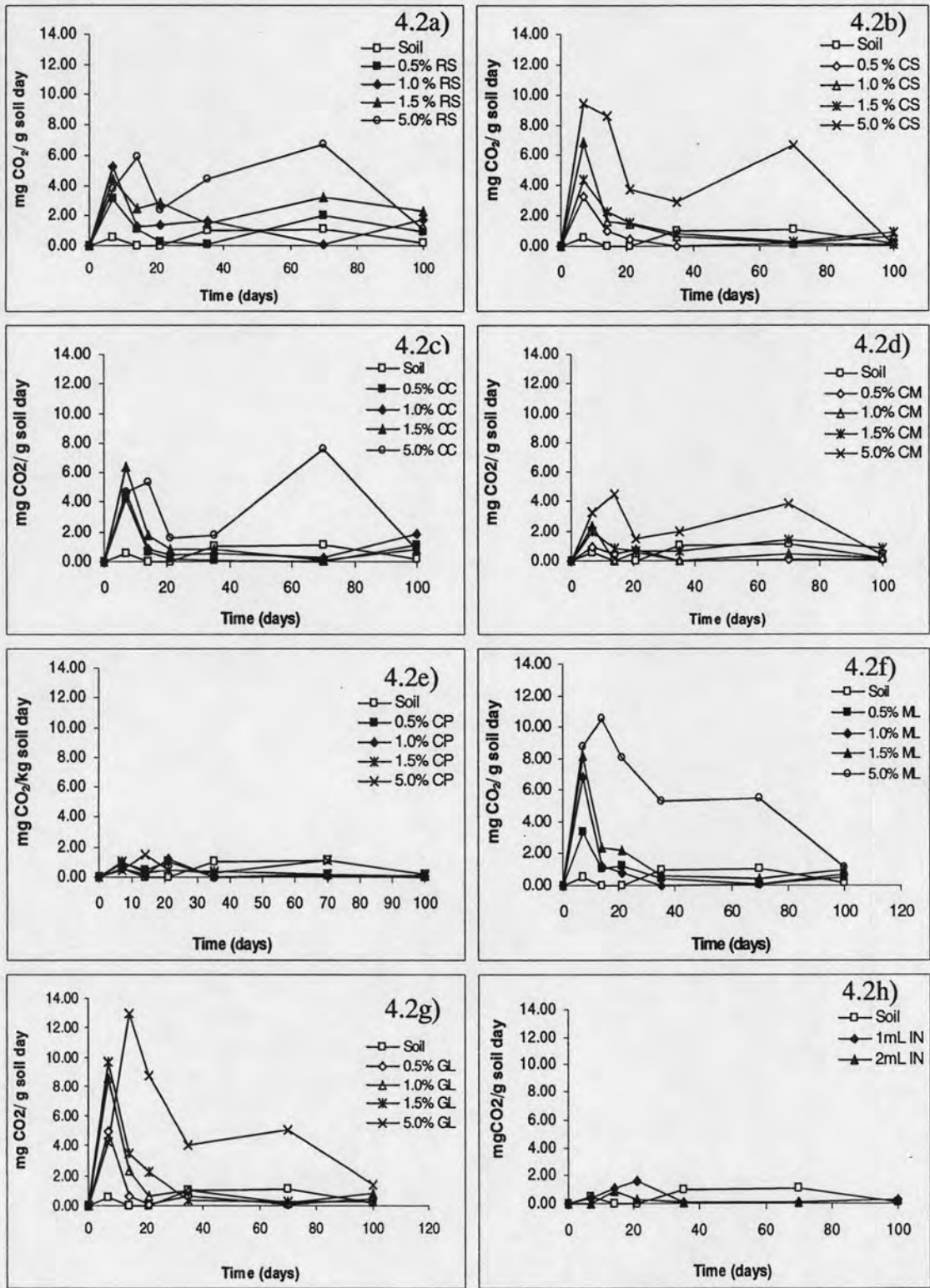
Figure 4.2 Effects of amendments on total CO_2 generation from soil of carbofuran

4.7 Effects of organic and inorganic amendments on carbofuran degradation in aged soil

This experiment investigated the effects of organic and inorganic amendments on stimulation of the indigenous microorganisms to degrade carbofuran in aged soils. Aged contaminated soil received our attention because aging process made the contaminants become increasingly resistant with the time to extraction and difficult to remove. The carbofuran concentration in aged soil after 60 days of aged period is approximately 4.36 mg/kg soil. Figure 4.3 depicted the degradation profiles of carbofuran in microcosms incubated with 1.5% RS and 1 mL inorganic amendment. The corresponding kinetic data of carbofuran degradation in aged soil added with each of organic and inorganic amendments fitted to a modified first order kinetic model were tabulated in Table 4.10. The coefficients of determination, r^2, ranged between 0.97-0.99 indicating a good fit of the data to the first order kinetic model (Table 4.10). Results indicated that aged process reduced carbofuran bioavailability indicating by longer half-life of carbofuran in non-amended aging soil (34 days) (Table 4.10) than in non-amended recently contaminated soil (16 days) (Table 4.4). Aging process might increase the sorption of carbofuran into soil matrices.
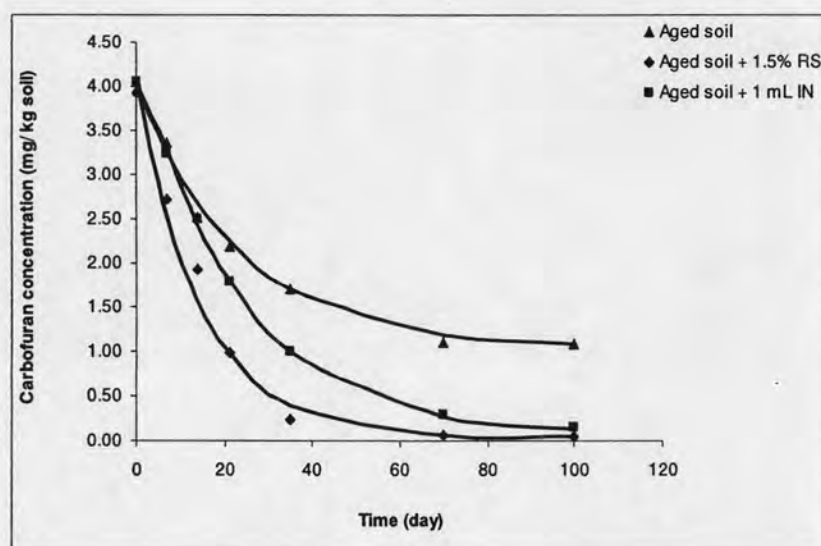

Figure 4.3 Degradation of carbofuran in aged soil (Lines = Carbofuran concentration fitted to the Modified First order Kinetic Model)

Table 4.10 Degradation rate constants (k) and half-Lives ($t_{1/2}$) of carbofuran in aged soil and aged soil amended with organic or inorganic amendments

Sample	k_1 (1/day)	$t_{1/2}$ day[1]	$r^{2[2]}$
Aged soil	0.0205	34	0.99
Aged soil + RS 1.5%	0.0700	9 *	0.97
Aged soil + IN 1 mL	0.0475	15 *	0.99

[1] LSD test for multiple comparison versus a control group (p<0.05) was run in SPSS program. Those treatments with * indicate statistically different at the 95% confidence level

[2] Coefficient of determination for non linear regressions

Additional of 1.5% RS as well as 1 ml inorganic amendment improved carbofuran degradation in aged soil indicating by a significantly shorter half-life of carbofuran of 9 days, (Table 4.10) and 15 days, (Table 4.10) respectively, than 34 days in control (p<0.05). When the new source of nutrient was added to aged soil, microorganisms would prefer to use freshly added organic and inorganic amendments for their growth which in turn activate their activities as indicated by an increase in number of carbofuran degraders, dehydrogenase activity (Table 4.11), and soil respiration (Figure 4.4).

Table 4.11 Average number of carbofuran degraders and dehydrogenase activity amended with 1.5% RS or 1mL inorganic nutrients

Sample	Carbofuran degrader [1] [2] (Log CFU/g soil)	Dehydrogenase activity [1] [2] (mg INTF/kg soil h)
Aged soil	8.47 ± 0.07	13.47 ± 0.15
Aged soil + 1.5%RS	8.70 ± 0.01 *	17.80 ± 1.18 *
Aged soil + 1 mL IN	8.62 ± 0.02 *	10.93 ± 1.30

[1] Data are expressed as the mean of three replicate samples with standard deviation

[2] LSD test for multiple comparisons versus a control group (p<0.05) was run in SPSS program. Those treatments with * indicate statistically different at the 95% confidence level.
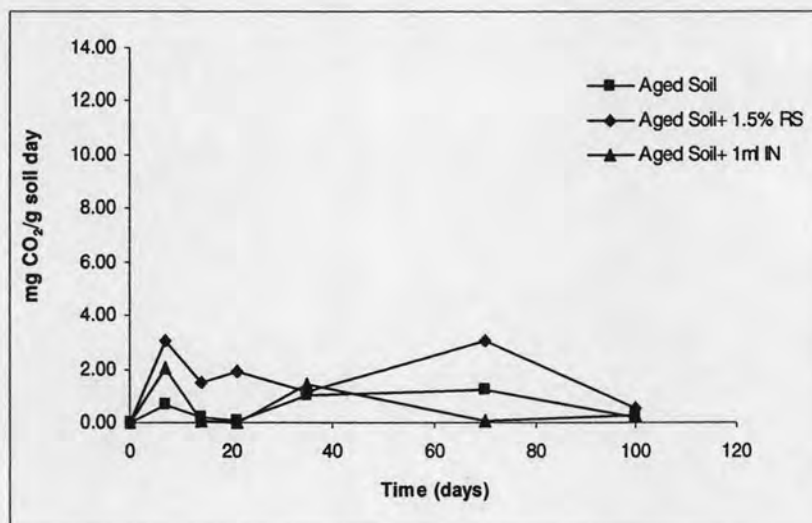
Figure 4.4 Effects of organic and inorganic amendments on total CO_2 generation in aged soil microcosms amended with 1.5% RS and 1 ml of inorganic solution

4.8 Kinetic analysis of carbofuran degraders obtained from 1.5% RS stimulated soil and non-stimulated soil in batch culture

This experiment was conducted to discover the responses of microbial to a substrate concentration. Six groups of carbofuran degraders from 1.5% RS stimulated soil and non-stimulated soil were obtained by enrichment technique. These microorganisms are capable of using carbofuran as their sole C-source (C-RS), sole N-source (N-RS) and sole C and N sources (C,N-RS) and non-stimulated degraders utilizing carbofuran as their sole C-source (C-NS), sole N-source (N-NS), and sole C and N source (C,N-NS). Carbofuran degradation abilities and growth kinetic analysis of these carbofuran degraders in BSM were investigated in the batch cultures with the assumption that carbofuran is an only one substrate limiting the growth of carbofuran degraders in BSM.

4.8.1 Biodegradation of carbofuran by non-stimulated and stimulated carbofuran degraders

Biodegradations of carbofuran in C-limited BSM containing initial carbofuran concentration of 50 mg/L by C-NS and C-RS were shown in Figure 4.5. Carbofuran degradations by other carbofuran degraders in BSM with each initial carbofuran concentrations were depicted in Appendix D. Degradation of carbofuran in

BSM was described by a modified first order kinetic model with r^2 ranged between 0.95-0.99 (Table 4.12) indicating a good fit data to the model. Half-lives of carbofuran in BSM were tabulated in Table 4.12.

Results indicated that stimulated degraders were more effective in degrading carbofuran than non-stimulated degraders with the $t_{1/2}$ of 3-12 days and 8-19 days, respectively (Table 4.12). These may due to the fact that degradation activities of the degraders were stimulated by amendment resulting in more rapid degradation of carbofuran by stimulated degraders than non-stimulated degraders. Use of carbofuran as C- N-, or C/N-source by degraders did not affect the degradation of carbofuran in BSM indicated by non-markedly different between $t_{1/2}$ of carbofuran in BSM inoculated with each type of degraders. These results suggested that all types of degraders played a significant role in degrading carbofuran.
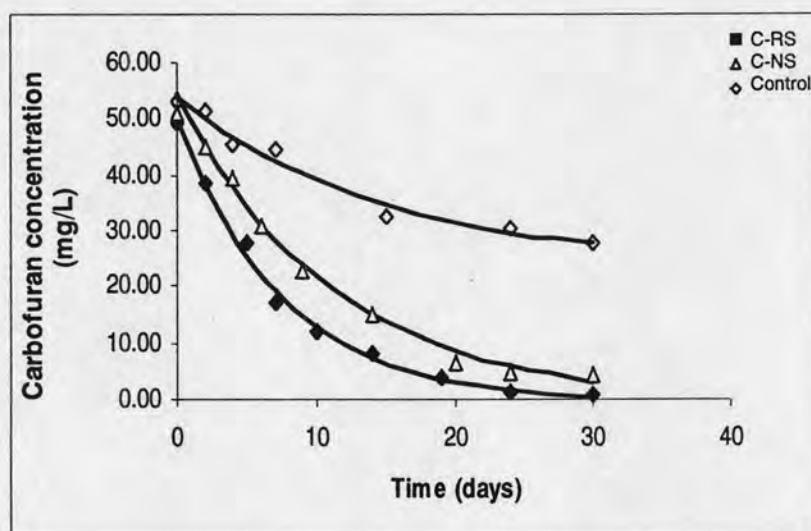

Figure 4.5 Degradation of carbofuran by carbofuran degraders in C-limited BSM containing carbofuran at the initial concentration of 50 mg/L (Lines = Carbofuran concentration fitted to the Modified First order Kinetic Model)

Half-lives of carbofuran in BSM inoculated with each type of carbofuran degraders at the highest concentration of 150 mg/L were in the range of 11-19 days which was higher than that in BSM at the low concentrations of 5-100 mg/L ($t_{1/2}$ 4-13 days) (Table 4.12). Results revealed that carbofuran at high concentration might inhibit activities of/toxic to carbofuran degraders. It is well documented that degradation ability of microorganisms was low at a high concentration of contaminant

(Chen et al., 2006; Montiel et al., 2006). Growth kinetic study with various contaminant concentrations will provide the information on the inhibitory level of the contaminant for each group of microorganisms.

A longer $t_{1/2}$ of 21-46 days (Table 4.12) observed in control i.e., BSM without inoculation confirming that degradation of carbofuran in BSM was a biological process.

4.8.2 Growth kinetic analysis of carbofuran degraders

This study was conducted to investigate growth kinetics of carbofuran degraders obtained from non-stimulated and stimulated soil in batch culture at various initial carbofuran concentrations in BSM. Figure 4.6 depicted the typical microbial growth curves of non-stimulated and stimulated carbofuran degraders in C-limited BSM. Microbial growth curves of non-stimulated and stimulated carbofuran degraders in N-limited BSM and C,N-limited BSM were shown in Appendix D. Results elucidated that growths of carbofuran degraders in all treatments could be described by the first order growth kinetic equation with r^2 ranged between 0.84-0.99 indicating a good fit of the data to the equation (Table 4.13). Specific growth rates (μ) and growth yields of each type of carbofuran degraders in BSM obtained from each concentration were tabulated in Table 4.13.

As for non-stimulated carbofuran degraders, an increase in carbofuran concentration led to an increase in μ of carbofuran degraders when the initial carbofuran concentrations were in the range of 5-150 mg/L. μ of carbofuran degraders were stable when the initial carbofuran concentration in BSM was increased from 100 mg/L to 150 mg/L (Table 4.13) indicating that there was no substrate inhibition on the growth of carbofuran degraders. Thus, the initial carbofuran concentration corresponds to the specific growth rates of non-stimulated carbofuran degraders were fitted to Monod equation. The kinetic parameters obtained from fitting the data to the model had r^2 ranged between 0.82-0.99 as shown in Table 4.14. C,N-NS showed the highest maximum specific growth rate, μ_{max}, of 1.88 1/day in comparison to N-NS and C-NS of 1.80 and 1.40 1/day, respectively, (Table 4.14) implying the highest growth of C,N-NS among non- stimulated carbofuran degraders. In addition, C,N-NS had the lowest halfsaturation concentration, Ks, of 26.03 mg/L which was lower than C-NS and N-NS of 44.21 and 71.55 mg/L, respectively, revealing that C,N-NS had the highest capability to use carbofuran as their energy sources than others.

Table 4.12 Degradation rate constant (k) and half-lives ($t_{1/2}$) of carbofuran in BSM

Carbofuran degraders	Carbofuran concentration (mg/L)	k_1 (1/day)	$t_{1/2}$ (day)	r^2 [1]
C-RS	5	0.072	9	0.98
	50	0.136	5	0.99
	100	0.070	9	0.98
	150	0.055	12	0.95
N-RS	5	0.181	4	0.97
	50	0.241	3	0.95
	100	0.077	9	0.99
	150	0.068	10	0.97
C,N-RS	5	0.091	8	0.98
	50	0.100	7	0.95
	100	0.090	8	0.99
	150	0.061	11	0.99
C-NS	5	0.097	7	0.99
	50	0.089	8	0.98
	100	0.096	7	0.96
	150	0.050	14	0.96
N-NS	5	0.054	13	0.99
	50	0.082	8	0.99
	100	0.085	8	0.98
	150	0.037	19	0.98
C,N-NS	5	0.015	46	0.96
	50	0.022	32	0.98
	100	0.031	22	0.96
	150	0.019	36	0.95
Control C-limited BSM	5	0.015	46	0.96
	50	0.022	32	0.98
	100	0.031	22	0.96
	150	0.019	36	0.95
Control N-limited BSM	5	0.017	40	0.94
	50	0.030	23	0.98
	100	0.025	28	0.94
	150	0.023	30	0.95
Control C&N-limited BSM	5	0.017	40	0.95
	50	0.027	25	0.98
	100	0.023	30	0.96
	150	0.033	21	0.99

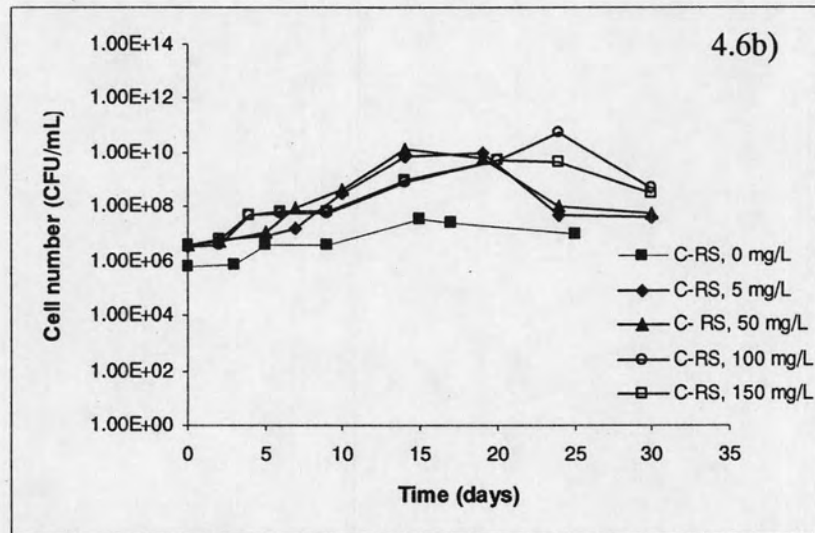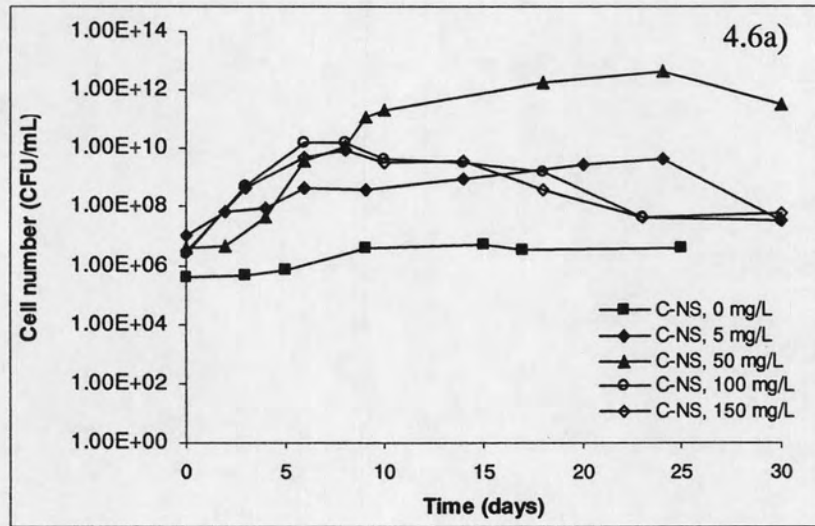[1] Coefficient of determination for non linear regression

Figure 4.6 Growth curves of 4.6a) C-NS and 4.6b) C-RS in C-limited BSM at each initial concentration.

Table 4.13 Specific growth rate of stimulated and non-stimulated carbofuran degraders fitted to the first-order kinetic equation

carbofuran degraders	Concentration (mg/L)	Specific growth rate (μ) (1/day)	$r^{2[1]}$
C-RS	0	0.272	0.92
	5	0.484	0.95
	50	0.592	0.97
	100	0.387	0.94
	150	0.312	0.98
N-RS	0	0.247	0.82
	5	0.977	0.99
	50	1.018	0.90
	100	1.029	0.83
	150	0.344	0.91
C,N-RS	0	0.139	0.81
	5	0.173	0.96
	50	0.613	0.88
	100	0.956	0.90
	150	0.186	0.98
C-NS	0	0.197	0.88
	5	0.220	0.88
	50	0.636	0.87
	100	1.121	0.91
	150	1.001	0.92
N-NS	0	0.224	0.94
	5	0.294	0.84
	50	0.602	0.84
	100	1.188	0.91
	150	1.159	0.86
C,N-NS	0	0.156	0.99
	5	0.288	0.92
	50	1.225	0.85
	100	1.558	0.99
	150	1.551	0.94

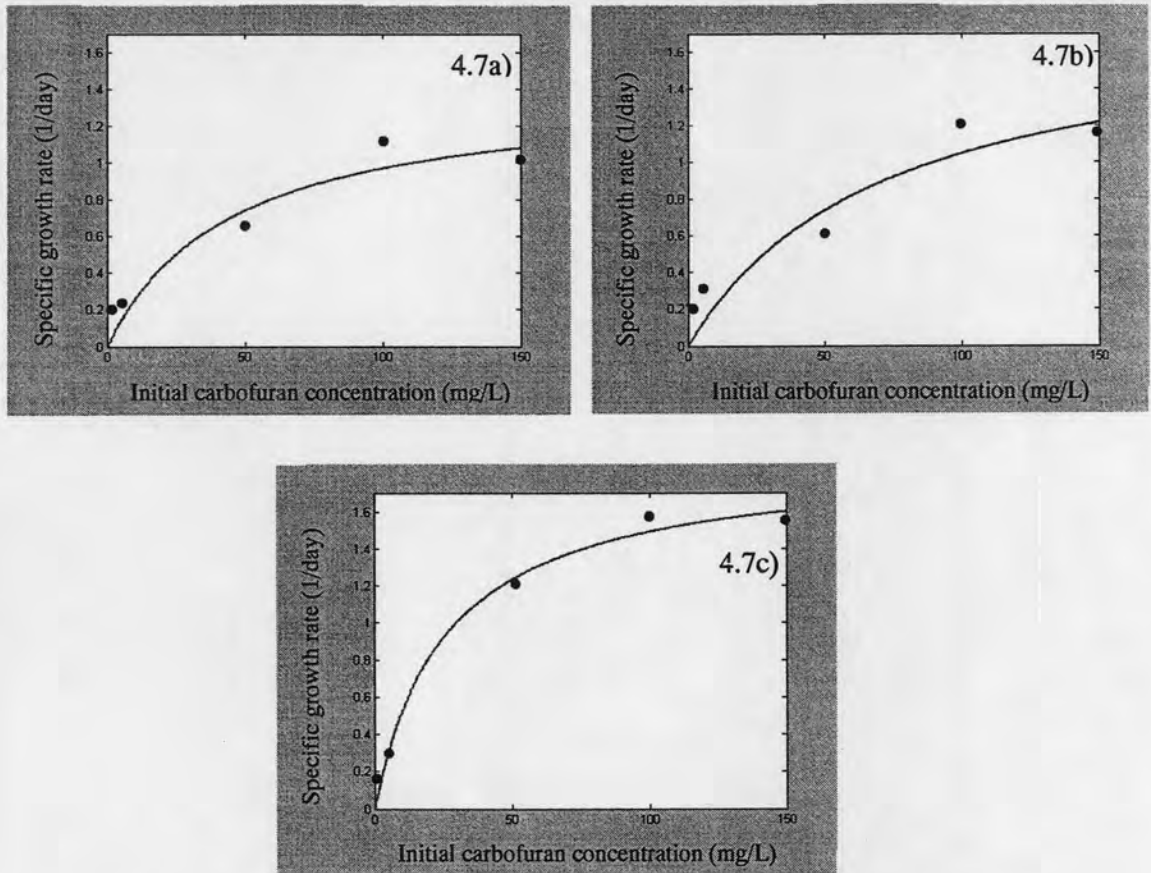[1] Coefficient of determination for non linear regression

Figure 4.7 The initial carbofuran concentration corresponds to the specific growth
rates of non-stimulated carbofuran degraders fitting to Monod model using
the non-linear least squares technique from MATLAB program with 95%
confidence 4.7a) in C-limited BSM, 4.7b) in N-limited BSM , 4.7c) in
C,N-limited BSM (• = observed data; line = fitted data)

Table 4.14 Kinetic parameter of non-stimulated microcosms fitted to Monod equation.

Kinetic parameters	C-BSM	N-BSM	C,N-BSM
μ_{max} (1/day)	1.40	1.80	1.88
K_s (mg/L)	44.21	71.55	26.03
r^2	0.80	0.92	0.98

Results with stimulated carbofuran degraders found that at the low initial carbofuran concentrations in BSM of 0-50 mg/L, μ of carbofuran degraders increased with an increase in an initial carbofuran concentration. However, when the initial carbofuran concentration in BSM was increased up to 150 mg/L, a decrease in μ of stimulated carbofuran degraders was found. These correlations between initial substrate concentrations and μ demonstrated that there is a substrate inhibition on growth of carbofuran degraders which could be described by Haldane model. Kinetic parameters of stimulated carbofuran degraders obtained from Haldane model with r^2 ranged between 0.70-0.92 were shown in Table 4.15. N-RS had the highest μ_{max} of 2.29 1/day in comparison to C-RS and C,N-RS, respectively (Table 4.15) indicating a higher growth of N-RS among the stimulated carbofuran degraders. In addition, N-RS showed the lowest Ks of 6.26 comparing to C-RS and C,N-RS of 9.00 and 24.82 mg/L (Table 4.15), respectively, implying a higher capability of utilizing carbofuran by N-RS than the others stimulated carbofuran degraders. The inhibitory effects of high carbofuran concentration on growth of carbofuran degraders were found after the degraders were stimulated with 1.5% RS. Substrate inhibition coefficient (Ki) of C-RS, N-RS, and C,N-RS were 41, 51, and 40.78 mg/L, respectively. The lowest carbofuran concentration which caused the dead of C-RS, N-RS, and C,N-RS were 19.21, 17.88, and 32.00 mg/L. From these results, we suggested that carbofuran concentration in the contaminated site should be lower than 17.88 mg/L which corresponded to 13.23 L/kg soil base upon K_d value of 0.74 in our soil sample in order to obtain an effective biostimulation process by these isolated carbofuran degraders.

μ_{max} of degraders obtained from stimulated soil were higher than non-stimulated soil might due to the fact that amendments added to the soil were incorporated to cells leading to high growth rate of degraders in BSM.
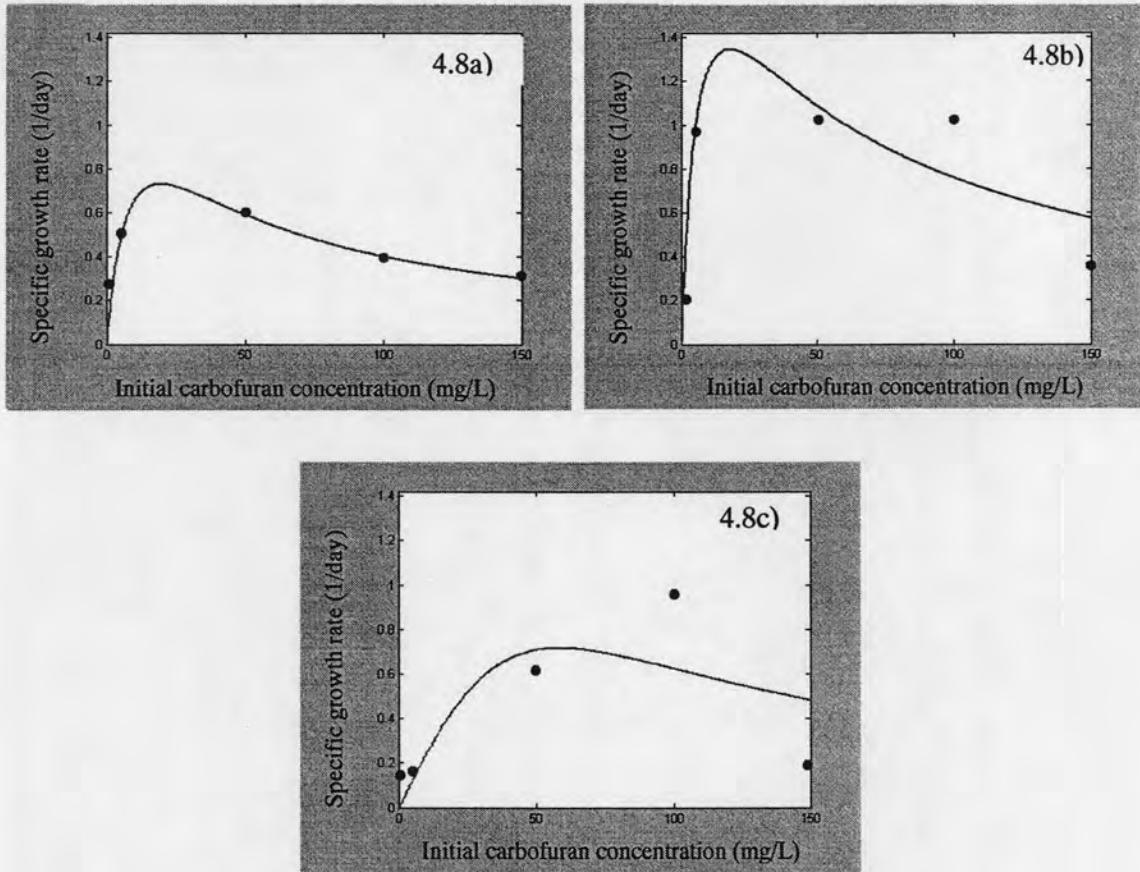
Figure 4.8 The initial carbofuran concentrations corresponds to the specific growth rates of stimulated carbofuran degraders fitting to Haldance model using the non-linear least squares technique from MATLAB program with 95% confidence 4.8a) in C-limited BSM, 4.8b) in N-limited BSM , 4.8c) in C,N-limited BSM. (• = observed data; line = fitted data)

Table 4.15 Kinetic parameters of stimulated carbofuran degraders fitted to Haldance equation

Kinetic parameters	C-BSM	N-BSM	C,N-BSM
μ_{max} (1/day)	1.42	2.29	1.03
K_s (mg/L)	9.00	6.26	24.82
Ki (mg/L)	41.00	51.10	40.78
S* (mg/L)	19.21	17.88	32.00
r^2	0.92	0.90	0.70