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Research article

Stimulation of soil microorganisms in pesticide-contaminated soil using organic materials

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Abstract: Agrochemicals such as pesticides have contributed to significant increases in crop yields; however, they can also be linked to adverse effects on human health and soil microorganisms. For efficient bioremediation of pesticides accumulated in agricultural fields, stimulation of microorganisms is necessary. In this study, we investigated the relationships between bacterial biomass and total carbon (TC) and total nitrogen (TN) in 427 agricultural soils. The soil bacterial biomass was generally positively correlated with TC and TN contents in the soil, but some soils had a low bacterial biomass despite containing high amounts of TC and TN. Soils of two fields (fields A and B) with low bacterial biomass but high TC and TN contents were investigated. Long-term pesticide use (dichloropropane-dichloropropene and fosthiazate in field A and chloropicrin in field B) appeared to have contributed to the low bacterial biomass observed in these soils. Soil from field A was treated with different organic materials and incubated for 1 month under laboratory conditions. The bacterial biomass in field A soil was enhanced in treatments containing organic materials rich in TN. Application of organic materials stimulated the growth of microorganisms with the potential to bioremediate pesticide-polluted soils.

Keywords: soil microorganisms; pesticides; dichloropropane-dichloropropene; fosthiazate; chloropicrin; bioremediation; organic materials

1. Introduction

Soil microorganisms are one of the most important indicators of soil fertility. Microorganisms play important roles in the decomposition of organic materials and the cycling of carbon, nitrogen, phosphorus, sulfur and several other nutrients in soil [1–3]. Microorganisms can also degrade xenobiotic compounds such as petroleum hydrocarbons and pesticides [4–8].

In the last century, agrochemicals such as chemical fertilizers and pesticides have been developed to enhance agricultural productivity [8]. Food and vegetable yields have been significantly enhanced but at the cost of risks to human health and the environment. Chemical fertilizers and pesticides have the potential to cause significant environmental hazards, including reductions in the number and activities of soil microorganisms [9,10].

To protect soil microorganisms from the harmful effects of agrochemicals, it is necessary to either minimize the use of agrochemicals or increase the abundance and activities of soil microorganisms to accelerate the biodegradation process. In our previous study, we developed a method to stimulate soil microorganisms and microbial nutrient cycling activities through the control of total carbon (TC), total nitrogen (TN), and the C/N ratio in petroleum hydrocarbon-polluted soils [11]. Microbial stimulation also led to improvements in the bioremediation of polluted soils.

In this study, the relationship between bacterial biomass and organic materials in agricultural soils was investigated. The aim of the study was to stimulate soil microorganisms in pesticide-contaminated soil by manipulating soil organic materials.

2. Materials and Methods

2.1. Analysis of agricultural soil samples for chemical properties

Soils were collected from various agricultural fields from Hokkaido to Okinawa in Japan. A composite sample from about five randomly selected points in a field was taken from the top 10-cm soil layer after removing a few centimeters of the surface crust. The TC in the soil samples was determined using the total organic carbon analyzer (TOC-V_{CPH}; Shimadzu, Kyoto, Japan) and the solid sample combustion unit (SSM-5000A; Shimadzu, Kyoto, Japan). To analyze TN, total phosphorus (TP), and total potassium (TK), soil was digested in a Kjeldahl digestion unit (Gerhardt, Königswinter, Germany) and filtered (ADVANTEC No. 6; Toyo Roshi Co. Ltd., Tokyo, Japan). TN, TP, and TK in the filtrate were determined by the indophenol blue method [13], the molybdenum blue method [14], and atomic absorption spectrophotometry (Hitachi, Tokyo, Japan), respectively. Soil pH (1:2.5 soil-to-water suspension, w/v) was analyzed using a pH meter (LAQUA F-72, Horiba, Kyoto, Japan).

2.2. Estimation of bacterial biomass in agricultural soils

Bacterial biomass in the soils was measured by quantification of the environmental DNA (eDNA) using the slow-stirring method [15]. To extract the eDNA from the soils, a 1.0-g soil sample was mixed with 8.0 mL of DNA extraction buffer (100 mM tris(hydroxymethyl)aminomethane, 100 mM sodium EDTA, 100 mM sodium dihydrogen orthophosphate, 1.5 M sodium chloride, and 1%

(w/v) hexadecyltrimethylammonium bromide) and 1.0 mL of 20% (w/v) sodium dodecyl sulfate solution; the suspension was agitated with a propeller for 20 min. The suspension was centrifuged at 5,000 × *g* for 10 min, then about 700 μL of supernatant was transferred into a 1.5 mL micro tube and 700 μL of chloroform-isoamyl alcohol was slowly added. The mixture was centrifuged at 18,000 × *g* for 10 min followed by a further addition of 300 μL of isopropanol and precipitation by centrifugation at 18,000 × *g* for 20 min. The pellet of crude nucleic acid was dissolved in TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) after drying. The extracted eDNA was quantified based on the intensity of the eDNA bands after electrophoresis on an agarose gel using Kodak 1D 3.6 Image Analysis Software (Kodak, CT, USA). The bacterial biomass in the soil was estimated by using the equation $Y = 1.70 \times 10^8 X$ ($r^2 = 0.96$), where Y and X are the bacterial biomass g^{-1} soil and the amount of eDNA, respectively [15].

2.3. Agricultural practices in the fields rich in TC and TN but low in bacterial biomass

Two representative fields rich in TC and TN but low in bacterial biomass (field A and B) were investigated for agricultural practices (Table 1). In field A, watermelon–tomato cropping system was practiced for more than 30 years, while only solanaceous crops were planted for more than 40 years in field B. Both of the fields were treated with chemical fertilizers and pesticides. Pesticides were used in both fields almost every year since they were first cultivated. In field A, dichloropropane-dichloropropene (D-D) (250 kg/ha) and fosthiazate (200 kg/ha) were used for controlling root-knot nematodes in watermelon and tomato. Field B was fumigated with chloropicrin (220 L/ha) to protect solanaceous crops from bacterial wilt.

Table 1. Agricultural practices in fields A and B.

Field	Cropping system	Cropping history	Target pest	Pesticide(s)	
				Name	Rate
A	Watermelon-tomato	>30 years	Root-knot nematode	1. DD* 2. Fosthiazate	250 kg/ha 200 L/ha
B	Solanaceous crops	>40 years	Broad spectrum	1. Chloropicrin	220 L/ha

* Dichloropropane-dichloropropene

Table 2. Properties of the pesticides used in fields A and B.

Pesticide	Log P_{ow}*	Toxicity in rat		Reference
		Oral, LD_{50} (mg/kg)	Dermal, LD_{50} (mg/kg)	
Dichloropropane-dichloropropene	1.4–2.28	140	2,100	[16]
Fosthiazate	1.752	51–73	861–2,396	[17]
Chloropicrin	2.09	250	-	[16]

* n-octanol/water partition coefficient

Properties of the pesticides used in field A and B are shown in Table 2. Among the three pesticides, fosthiazate seems most toxic followed by dichloropropane-dichloropropene and chloropicrin.

2.4. Effect of organic materials on bacterial biomass in pesticide-contaminated soil

To investigate the effect of treatment with organic materials on bacterial biomass in pesticide-contaminated soil, soil from field A was sampled, filled into sterile pots (200 g/pot, 25% moisture content) and incubated with the following five treatments: 1) untreated control; 2) pig manure (1% w/w); 3) soybean meal (0.5%); 4) peat moss (1% w/w); and 5) soybean meal (0.5%) + peat moss (1%). Soybean meal and peat moss were autoclaved and dried at 60 °C for 3 days before treatment, while non-autoclaved pig manure was used for the treatment. The TC, TN, C/N ratio and bacterial biomass in each organic material are shown in Table 3. Sterile distilled water was added frequently to maintain the moisture content throughout the incubation period. After 1 month of incubation of the treated soils at 22 °C (12 h) / 18 °C (12 h), soil bacterial biomass was analyzed by the slow-stirring method [15].

Table 3. Total carbon (TC), total nitrogen (TN) and carbon/nitrogen (C/N) ratio of the organic materials used in this study.

Organic material(s)	TC (mg/kg)	TN (mg/kg)	C/N ratio	Bacterial biomass (× 10^8 cells/g)
Pig manure	287,000	24,100	11.9	11.9
Soybean meal	446,800	77,000	5.8	ND
Peat moss	504,200	5,020	100.4	ND
Soybean meal + peat moss (0.27:0.73, w/w)	488,700	24,400	20.0	ND

3. Results

3.1. Analysis of bacterial biomass, TC, and TN in agricultural soils

Bacterial biomass, TC and TN in 427 agricultural soil samples were analyzed for the construction of an agricultural soil database (Table 4). Levels of bacterial biomass were categorized into the following six groups: 1) extremely low (<1.0×10^8 cells/g); 2) very low (1.0×10^8 to 2.9×10^8 cells/g); 3) low (3.0×10^8 to 4.4×10^8 cells/g); 4) medium (4.5×10^8 to 5.9×10^8 cells/g); 5) high (6.0×10^8 to 9.9×10^8 cells/g); and 6) very high (≥10.0×10^8 cells/g). Bacterial biomass varied in accordance to TC and TN contents in the soil. In the soils with very high bacterial biomass, average TC and TN were 35,200 mg/kg and 1,550 mg/kg, respectively, while average TC and TN in the soils with low bacterial biomass were 17,470 mg/kg and 890 mg/kg, respectively. These results suggest that TC and TN are closely related to bacterial biomass in the soil.

Table 4. Relationship between bacterial biomass and soil organic matter level in agricultural soils.

Bacterial level		Organic matter		Number of sample
Category	Bacterial biomass ($\times 10^8$ cells/g)	TC (mg/kg)	TN (mg/kg)	
Extremely low	<1.0	23,540	880	35
Very low	1.0–2.9	17,470	890	30
Low	3.0–4.4	23,110	1,040	41
Medium	4.5–5.9	23,450	1,170	38
High	6.0–9.9	27,480	1,160	91
Very high	>10.0	35,200	1,550	192

3.2. Investigation of soils with low bacterial biomass in soils

Thirty-five soil samples with extremely low bacterial biomass (<1.0 × 10^8 cells/g) were analyzed for TC, TN, TP, TK, and pH (Table 5). Seven samples (samples 1 to 7) contained TC less than 10,000 mg/kg, and 15 samples (samples 21 to 35) contained TC above 20,000 mg/kg. Average values of TC, TN, TP, and TK in 35 samples were 23,540, 880, 1,971, and 6,375 mg/kg, respectively. Similarly, average soil pH was 6.34. Although TC, TN, C/N ratio, TP, TK, and pH in most of the samples seem at suitable levels, an extremely low bacterial biomass indicate the possibility of pollution in the soil.

Table 5. Chemical properties of the soils with extremely low level of bacterial biomass (<1.0 × 10^8 cells/g).

Sample number	TC (mg/kg)	TN (mg/kg)	C/N ratio	TP (mg/kg)	TK (mg/kg)	pH	Bacterial biomass ($\times 10^8$ cells/g)
1	1,230	270	4.6	370	3,680	6.65	ND
2	1,580	200	7.9	220	3,980	7.60	ND
3	1,802	350	5.1	1,040	1,560	6.69	ND
4	3,584	240	14.9	290	4,300	7.35	0.8
5	3,992	290	13.8	2,330	16,220	6.86	ND
6	7,673	530	14.5	370	5,670	6.50	ND
7	8,462	410	20.6	610	860	6.51	0.9
8	13,640	810	16.8	870	10,940	6.42	ND
9	15,010	840	17.9	2,380	5,760	7.77	0.5
10	15,720	630	25.0	600	11,560	6.71	0.8
11	17,110	600	28.5	1,070	4,060	5.95	0.1
12	17,410	640	27.2	2,460	6,990	5.72	0.9
13	17,560	500	35.1	6,940	4,790	5.67	ND
14	18,280	850	21.5	2,220	3,530	6.10	0.1

15	18,480	580	31.9	980	3,430	5.50	0.1
16	18,530	1,560	11.9	3,120	6,560	5.44	ND
17	19,000	990	19.2	880	4,820	5.50	0.9
18	19,490	960	20.3	3,310	5,540	6.67	ND
19	19,570	960	20.4	2,250	10,200	5.82	ND
20	19,620	380	51.6	1,940	4,830	6.22	ND
21	20,230	670	30.2	1,100	4,010	6.12	0.1
22	20,980	1,180	17.8	3,900	6,890	6.62	0.4
23	21,590	930	23.2	1,690	4,880	5.93	0.7
24	22,740	1,120	20.3	2,740	6,480	5.53	ND
25	23,680	840	28.2	1,990	10,230	6.27	0.8
26	23,710	880	26.9	1,980	7,720	5.56	0.3
27	26,520	960	27.6	2,200	6,300	7.28	ND
28	32,400	930	34.8	3,140	3,610	6.25	ND
29	35,000	890	39.3	1,870	10,540	7.10	ND
30	35,460	950	37.3	1,740	17,370	6.90	ND
31	41,650	1,420	29.3	4,890	3,190	6.77	ND
32	42, 000	1,270	33.1	3,040	2,600	6.80	ND
33	46,860	2,880	16.3	2,520	1,750	6.30	ND
34	72,750	2,180	33.4	460	14,110	5.49	0.7
35	99,710	1,020	97.8	1,520	4,180	5.46	ND
Average	23,540	880	25.8	1,970	6,375	6.34	-

*ND=not detected (<6.6×10^6 cells/g)

3.3. Investigation of soils with extremely low levels of bacterial biomass but high TC and TN

Average values of TC, TN, C/N ratio, and bacterial biomass in two representative fields (fields A and B) with extremely low bacterial biomass (<6.6×10^6 cells/g-soil) but high TC (≥22,500 mg/kg) and TN (≥990 mg/kg) are shown in Table 6. In both fields TC was ≥22,500 mg/kg and TN was ≥990 mg/kg. C/N ratio in field A and B was 15 and 21, respectively.

Table 6. Soil properties in two agricultural fields used in this study (n = 2).

Field	Value	TC (mg/kg)	TN (mg/kg)	C/N ratio	Bacterial biomass*
A	Average	22,500	1,500	15	ND
	SD	3,535	141	1.4	-
B	Average	23,000	990	21	ND
	SD	2,828	127	0	-

*ND=not detected (<6.6×10^6 cells/g)

Data suggest that long-term use of nematicidal and bactericidal pesticides significantly reduced bacterial biomass in soils, even with high levels of TC and TN and optimum C/N ratio.

3.4. Stimulation of bacterial biomass in pesticide contaminated soils through the control of TC and TN

To increase the bacterial biomass and improve bioremediation processes in pesticide-polluted soils, soil sampled from field A was treated with different organic materials. After 1 month of incubation under laboratory conditions, bacterial biomass in soil treated with pig manure, soybean meal, and a mixture of soybean meal and peat moss increased from below the detection limit to 2.8×10^8, 3.8×10^8 and 3.3×10^8 cells/g, respectively (Figure 1). Bacterial biomass did not increase in the soil treated with peat moss only and in the untreated soil (control). The results suggested that microorganisms in pesticide-polluted soils were stimulated by the application of nitrogen-rich organic materials.

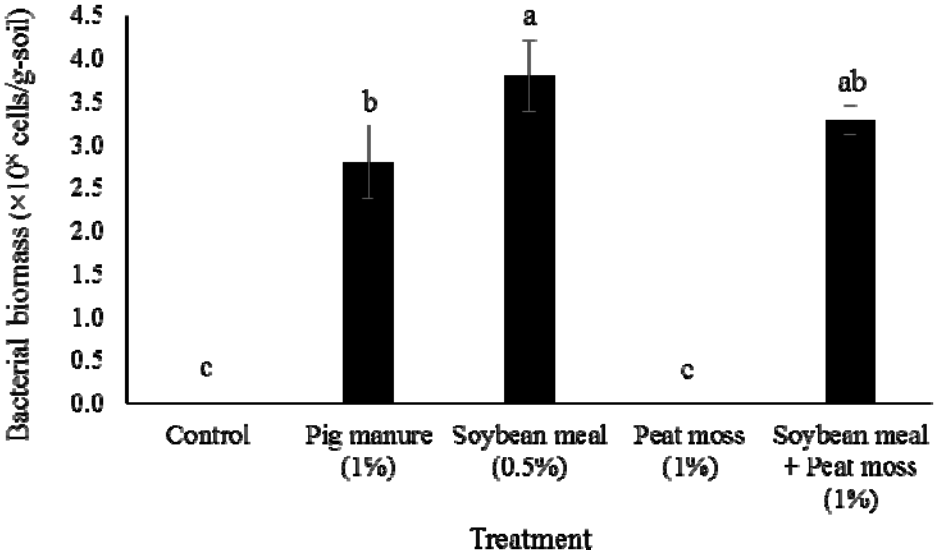

Figure 1. Effect of different organic materials on bacterial biomass in a pesticide-polluted soil after 1 month of incubation, (n = 2). Values with the same letters do not differ significantly at $p > 0.05$ (least significant difference test).

4. Discussion and Conclusion

Microorganisms are in intimate contact with the soil environment and as such are considered ideal monitors of pollution [18,19]. The results reported here indicate that long-term use of a broad spectrum fumigant (chloropicrin) and nematicidal pesticides (DD and fosthiazate) reduced the abundance of soil bacteria. Detrimental effects of chloropicrin on bacteria have been reported previously, such as mutagenesis [20] and reduction in biomass and activity [21]. Similarly, nematicides can cause suppression and alteration of the soil microflora [22]. Usually, the harmful effects of pesticides on soil microorganisms are manifested only at high concentrations [23,24]. However, a significant reduction in microbial dehydrogenase activity by DD was observed even

when applied at the usual field rate [25]. Therefore, the use of these pesticides over long time periods appears detrimental for both the abundance and activities of soil microorganisms.

TC, TN, and C/N ratio are closely related to the number and activities of microorganisms in soil [12]. In this study, bacterial biomass were positively related to TC and TN, but some soil samples with relatively lower TC also contained high amount of bacterial biomass (data not shown). Higher bacterial biomass even in low TC might be due to a suitable C/N ratio in those soils.

In this study, bacterial biomass below the detection level ($<6.6 \times 10^6$ cells/g) was observed in two pesticide-polluted soils, which was substantially lower than the average bacterial biomass in agricultural soils (~ 6.0×10^8 cells/g) [11]. High bacterial biomass ($>1.0 \times 10^9$ cells/g) has been reported in petroleum hydrocarbon-polluted agricultural soils [4,5,15]. Therefore, the environmental impact of pesticide pollution is more severe than that of petroleum hydrocarbon pollution.

In this study, bacterial biomass was enhanced by applying some organic materials. Although, the bacterial biomass of 2.8×10^8 cells/g in the pig manure treated soil may include both introduced and stimulated microorganisms, a significant enhancement of the bacterial biomass in the soils applied with autoclaved organic materials (soybean meal and the mixture of soybean meal and peat moss) clearly indicated that indigenous microorganisms in pesticide polluted soils can be stimulated by using nitrogen-rich organic materials.

Stimulation of soil microorganisms through the control of soil organic matter (TC, TN, and C/N ratio) has been demonstrated as an efficient method to enhance bioremediation of agricultural soils polluted by petroleum hydrocarbons [4]. In this study, treatment of a pesticide-polluted soil with nitrogen-rich organic materials led to an increase in the soil bacterial biomass by up to 3.8×10^8 cells/g. The efficiency of organic material treatment in increasing abundance of microorganisms was lower when compared with that in petroleum hydrocarbon-polluted soils. Manipulation of soil properties (TC, TN, and C/N ratio) using organic materials has the potential to accelerate bioremediation processes in pesticide-polluted agricultural soils.

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Conflict of Interest

The authors have no conflict of interest to disclose.

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