

Effects of Heavy Metal Pollution of Apple Orchard Surface Soils Associated with Past Use of Metal-Based Pesticides on Soil Microbial Biomass and Microbial Communities

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ABSTRACT

Apple orchard surface soils in Japan are polluted with copper (Cu), lead (Pb), and arsenic (As) due to long-term use of metal-based pesticides. We investigated the effects of heavy metals accumulated in the surface soils in apple orchards on the microbial biomass and the microbial communities. Soil samples were taken from a chestnut orchard (unpolluted control) and five apple orchards with different degrees of heavy metal pollution. Total concentrations of Cu, Pb, and As in soil ranged from 29 to 931 mg/kg, 35 to 771 mg/kg, and 11 to 198 mg/kg, respectively. The amount of microbial biomass carbon expressed on a soil organic carbon basis decreased with increasing concentrations of heavy metals. Thus, the heavy metals that accumulated in apple orchard surface soils had adverse effects on the soil microbial biomass. The analysis of phospholipid fatty acid (PLFA) composition indicated that the microbial community structure had changed because of the pesticide-derived heavy metals in soil. The relative abundance of gram-positive bacterial marker PLFAs increased and that of fungal marker PLFA decreased with increasing concentrations of heavy metals in soil. Denaturing gradient gel electrophoreses targeting the 16S ribosomal RNA gene of bacteria and the 18S ribosomal RNA gene of fungi also showed shifts in the composition of bacterial and fungal communities induced by soil pollution with heavy metals. However, the diversity of microbial communities was not significantly affected by the heavy metal pollution. This was attributable to the adaptation of the microbial communities in apple orchard surface soils to heavy metals derived from previously used pesticides.

Keywords: Apple Orchard; Denaturing Gradient Gel Electrophoresis; Heavy Metals; Microbial Biomass; Metal-Based Pesticides; Soil Pollution; Phospholipid Fatty Acid

1. Introduction

Soil pollution with heavy metals occurs not only near mines and smelters, but also on agricultural land because of the application of sewage sludge and metal-based pesticides. In Japan, long-term use of Bordeaux mixtures and lead arsenate has led to the pollution of apple orchard surface soils with copper (Cu), lead (Pb), and arsenic (As) [1]. The highest concentrations of Cu, Pb, and As found in alluvial apple orchard surface soils (0 - 10 cm) were 1120, 1810, and 260 mg/kg, respectively [1]. The use of lead arsenate was prohibited in 1978, while Bordeaux mixtures are still utilized in Japan. However, most apple growers stopped the use of Bordeaux mix-

tures by the early 1990s to avoid accumulation of Cu in soil.

Heavy metal pollution exerts toxic effects on soil microbial biomass [2-5] and microbial processes such as soil respiration, nitrogen mineralization, and enzyme activities [6-8]. Therefore, we investigated the effects of heavy metal accumulation on the microbial biomass and microbial activities in apple orchard soils [1,9]. The results indicated that among the heavy metals, the adverse effects were primarily brought about by Cu, which accumulated in apple orchard soils [1,9]. With increasing total concentration of Cu in apple orchard soils, the proportion of microbial biomass in the soil organic matter decreased, and the contribution of fungi to substrate-induced respiration increased [9]. Furthermore, the de-

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hydrogenase activity and soil respiration expressed on a unit of soil organic carbon basis were negatively correlated with the amount of 0.1 M CaCl_2 -extractable Cu [1,9]. Thus, microbial biomass and microbial activities were shown to be adversely affected by the Cu accumulated in apple orchard surface soils. It was also demonstrated by a field-incubation study that the toxicity of Cu to the microbial biomass and microbial activities was largest among the three heavy metals, Cu, Pb, and As [10].

In addition to microbial biomass and microbial activities, the diversity of the microbial communities is an important index of soil quality. Molecular techniques such as phospholipid fatty acid (PLFA) analysis and nucleic acid-based techniques have recently been developed for characterizing soil microbial communities [11,12]. Analysis of the PLFA pattern has been applied to investigate the impact of heavy metal pollution on soil microbial communities [13-19]. In most cases, shifts in the PLFA pattern due to heavy metal pollution were observed. Although there is a good correlation between the total amount of PLFAs and microbial biomass in soil [20], PLFA analysis always has a much stronger focus on bacterial communities than on fungal communities [21]. Therefore, PLFA analysis is more suitable for differentiating between different functional bacterial groups, e.g., actinomycetes, gram-negative bacteria and gram-positive bacteria, than between fungal and bacterial biomass.

Nucleic acid-based techniques have been also used for studying the effects of heavy metal pollution on soil microbial community. Polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE) in particular has been used for the assessment of the bacterial community structure in soils with heavy metal pollution [22-27]. Other PCR-amplified DNA fingerprinting techniques, e.g., amplified ribosomal DNA restriction analysis and terminal-restriction fragment length polymorphism, were used for investigating the bacterial communities in heavy metal polluted soils [15,28-32]. The application of PCR-amplified DNA fingerprinting techniques succeeded to discriminate the community structures between heavy metal-polluted and unpolluted soils for both bacteria and fungi.

Our objective in the present study was to determine the effects of heavy metals accumulated in apple orchard surface soils on microbial biomass and microbial communities. We used PLFA analysis for assessing the diversity of soil microbial communities on the phenotype level and differentiating the microbial biomass into functional subgroups, *i.e.*, gram-negative bacteria, gram-positive bacteria, actinomycetes, and fungi, and PCR-DGGE was used for assessing the diversities of bacterial and fungal communities separately on a genotype level.

2. Materials and Methods

2.1. Soil Sampling

Soil samples A-F were taken from one chestnut orchard and five apple orchards in May 2006. Soils A and B were taken from a chestnut orchard and an apple orchard, respectively, at the Fujisaki Experimental Farm of Hirosaki University, and soils C-F were taken from commercial apple orchards in Fujisaki Town, Aomori Prefecture, Japan. For each orchard, four trees were chosen randomly and soil samples were taken from the 0 - 10 cm layer just beneath the canopy of the trees. All soils were alluvial soils classified as Dystric Fluvisols (FAO/UNESCO). Each moist soil sample was passed through a 2-mm mesh sieve. The moist soil samples were used for the measurement of microbial biomass carbon and PCR-DGGE, and part of the moist soil samples were freeze-dried for PLFA analysis. The residual samples were air-dried for chemical analyses.

2.2. Chemical Analyses

The soil pH was determined with a glass electrode in water using a soil-to-water ratio of 1:2.5. The total carbon and nitrogen contents were simultaneously determined using a Sumigraph NC-90A automatic analyzer (Sumitomo Chemical Co., Ltd., Osaka, Japan). Since carbonates were not present, the total carbon content was equivalent to the organic carbon content. For determining the total amounts of Cu, Pb, and As, the soil samples were digested with a HClO_4 - HNO_3 - H_2SO_4 mixture, and NH_4Cl was subsequently added to the digestion mixture to prevent the co-precipitation of Pb [33]. The total contents of Cu and Pb were determined by atomic absorption spectrometry, and the content of As was determined colorimetrically by using silver diethyldithiocarbamate [33].

2.3. Microbial Biomass Carbon

Moist soil subsamples equivalent to 60 g oven-dry soil were weighed in 200-mL plastic beakers, adjusted to 60% of the maximum water holding capacity with distilled water, and then pre-incubated for 7 d at 25°C. Microbial biomass carbon (C_{mic}) was measured using the fumigation-extraction procedure proposed by Vance *et al.* [34]. Briefly, a portion of the pre-incubated soil sample corresponding to 25 g oven-dry soil was fumigated with alcohol-free chloroform for 24 h. Organic carbon was extracted from fumigated and unfumigated soil samples with 100 mL of 0.5 M K_2SO_4 and quantified using a TOC analyzer (TOC-V_E; Shimadzu Co., Ltd., Kyoto, Japan). A k_{EC} factor of 0.45 was used to estimate C_{mic} from extractable organic carbon [35].

2.4. PLFA

Soil lipids were extracted from 4 g of freeze-dried soil with 10:5:4 volumes of methanol, chloroform, and 0.1 M phosphate buffer (pH 7.0) by using a modified Bligh and Dyer [36] technique as described in Balser and Firestone [37]. PLFAs were separated by silica gel chromatography (Supelco LC-Si SPE cartridges) and methylated by using a methanolic KOH solution. The PLFA-methyl esters were analyzed using both a GC-14B gas chromatograph (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a flame ionization detector and a GCMS-QP2010 gas chromatograph-mass spectrometer (Shimadzu Co., Ltd., Kyoto, Japan). A DB-5 capillary column (0.32 mm \times 30 m; Agilent Technologies Inc., Santa Clara, CA, USA) was used for gas chromatographic separation. Standards containing mixtures of 37 and 24 fatty acid methyl esters (Sigma-Aldrich Japan Co., Tokyo, Japan) were used to help with peak identification. PLFA 19:0 was used as internal standard. The PLFAs were designated in terms of total number of carbon atoms, double bonding and position of the double bonds. The suffixes "c" and "t" indicate cis and trans geometric isomers. The prefixes "i" and "a" refer to iso and anteiso methyl branching. Hydroxy groups are indicated by "OH". Cyclopropyl groups are denoted by "cy", "10 Me" refers to a methyl group on the tenth carbon from the carboxylic end of the fatty acid. The following PLFAs were identified and quantified: i-14:0, a-14:0, 14:0, i-15:0, a-15:0, 15:0, 2-OH 14:0, 3-OH 14:0, i-16:0, 16:1 ω 9c, 16:1 ω 7c, 16:1 ω 7t, 16:1 ω 5c, 16:0, i-17:1 ω 5, 10Me-16:0, i-17:0, a-17:0, 17:1 ω 8c, cy17:0, 17:0, 2-OH 16:0, i-18:0, 18:2 ω 6, 18:1 ω 9c, 18:1 ω 7c, 18:1 ω 7t, 18:1 ω 5c, 18:0, 10 Me-18:0, i-19:0, cy19:0, 20:4, and 20:0.

2.5. PCR-DGGE

DNA was extracted from soil samples (0.25 g) using a MO BIO UltraClean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's specifications. The primer pairs 357F-GC/517R [38], targeting the V3 region in the 16S ribosomal RNA (rRNA) gene for bacteria, and FF390/ FR1-GC [39], targeting the 18S rRNA gene for fungi, were used for PCR amplification. In the PCR reaction, 5 μ L of a 50-fold diluted DNA extract was added to the PCR mix, which was composed of 5 μ L of 10 \times PCR buffer, 4 μ L dNTPs (10 mmol/L each), 0.5 μ L of each primer (25 mmol/L), 0.5 μ L Taq polymerase (5U/ μ L), and ultra pure water to a total volume of 50 μ L. PCR conditions for bacteria were as follows: an initial denaturation at 95°C for 10 min, followed by 35 cycles of 30-sec denaturation at 93°C and 30-sec annealing at 65°C (first 10 cycles), then at 60°C (second 10 cycles), and finally at 55°C (last

15 cycles). Each annealing step was followed by a 1-min extension at 72°C, except for the final extension (at 72°C for 5 min). PCR conditions for fungi were as follows: denaturation for 8 min at 95°C, then 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 45 sec, followed by extension at 72°C for 2 min, and a final extension at 72°C for 10 min. After amplification, PCR products were verified by electrophoresis on a 2% agarose gel followed by staining with SYBR Gold (Invitrogen Corp., Carlsbad, CA, USA).

For bacteria, DGGE was performed using the double gradient technique [40]. PCR products were loaded onto a 6% - 12% polyacrylamide gradient gel with a denaturing gradient ranging from 30% to 60%. For fungi, a 6% polyacrylamide gel with a denaturing gradient ranging from 20% to 50% was used. One hundred percent of denaturant corresponded to 7 M urea and 40% (v/v) formamide. All DGGE analyses were run using a V20-HCDC denaturing gradient gel electrophoresis unit (Sci-Plas Limited, Southam, Warwickshire, UK) at a constant temperature of 60°C. Electrophoresis was carried out for 10 min at 150 V and continued for 16 h after lowering the voltage to 70 V. After electrophoresis, the gels were stained with SYBR Gold and photographed with a digital camera on a blue-light transilluminator (Invitrogen Corp., Carlsbad, CA, USA). Detection and intensity measurement of bands were performed using the Image-J software (NIH-Image, Bethesda, MD, USA).

2.6. Statistics

The data obtained from the PLFA analysis (μ mol/kg) and the DGGE patterns, according to band intensity and position, were analyzed by principal component analysis (PCA) using an Excel add-in for multivariate analysis [41]. Microbial community diversity was calculated using the Shannon index (H'): $H' = -\sum p_i \ln p_i$, with p_i representing the proportional abundance of a given PLFA [42]. For DGGE, p_i represented the percentage of the integrated density of a band, relative to the sum of all bands in a lane.

3. Results

3.1. Properties of Soils

The soils had pH ranging from 4.8 to 6.1, organic carbon content from 1.2% to 4.5%, total nitrogen content from 0.10% to 0.40% and a C/N ratio from 10.8 to 13.2 (Table 1). Total concentrations of Cu, Pb, and As in soil A, which was taken from the chestnut orchard with no history of heavy metal pollution, were 29, 35, and 11 mg/kg, respectively. In soil B, which was taken from the apple orchard adjacent to the unpolluted chestnut orchard, total

concentrations of Cu, Pb, and As were significantly higher than those in soil A. Compared with soil A, soils C and D showed high concentrations for total Cu, but did not for total Pb and As. This fact indicated that Bordeaux mixtures and not lead arsenate had been used in the apple orchards from where soils C and D were taken. In soils E and F, total concentrations of Cu, Pb and As were markedly higher than those in the other soils. This would reflect the long-term use of Bordeaux mixtures and lead arsenate at the apple orchards from where soils E and F were taken.

3.2. Microbial Biomass

The amount of C_{mic} expressed on an oven-dry soil basis was largest in soils A and B and lowest in soil C, indicating that this parameter is not related to the heavy metal concentrations (Table 2). In contrast, the amount of C_{mic} expressed on a total soil organic carbon ($C_{mic}/Org-C$) decreased with increasing heavy metal concentrations (Table 2). $C_{mic}/Org-C$ showed a significant negative correlation with the logarithmic concentrations of total Cu in soil (Figure 1).

3.3. PLFAs

The total amount of PLFAs showed a highly significant positive correlation with the amount of C_{mic} (Figure

2(a)). Furthermore, total PLFAs calculated per gram of soil organic carbon decreased linearly with increasing logarithmic concentrations of total Cu (Figure 2(b)), as observed for $C_{mic}/Org-C$. PCA of the PLFA profiles indicated that there were differences in the PLFA profiles among the soils and the soils were grouped according to the heavy metal concentrations (Figure 3(a)). The soils were mainly separated along PC1, which accounted for 72% of total sample variance. Scores on PC1 had a strong positive correlation with the levels of Cu in the soils (Figure 3(b)).

The relative abundance (as molar percentage of total PLFAs) of marker PLFAs specific to gram-negative bacteria, gram-positive bacteria, actinomycetes, and fungi were calculated to investigate the effects of heavy metal accumulation on the individual microbial subgroups (Figure 4). Gram-positive bacteria were represented by i-14:0, a-14:0, i-15:0, a-15:0, i-16:0, i-17:0, a-17:0, and i-18:0 [43], while gram-negative bacteria were represented by 18:1 ω 7c, cy17:0, and cy19:0 [44], actinomycetes by 10 Me-16:0 and 10 Me-18:0 [45], and fungi by 18:2 ω 6 [43]. The relative abundance of gram-positive bacterial marker PLFAs increased and that of fungal marker PLFA decreased, as the heavy metal concentrations of soil increased (Figure 4).

The Shannon index was significantly lower in soil E

Table 1. Chemical properties of the soils used.

Soil	pH	Total organic carbon (%)	Total nitrogen (%)	C/N ratio	Total Cu (mg/kg)	Total Pb (mg/kg)	Total As (mg/kg)
A	5.75 ± 0.02	2.01 ± 0.18	0.162 ± 0.013	12.4 ± 0.2	28.6 ± 1.2	35.3 ± 3.3	10.6 ± 0.9
B	6.01 ± 0.03	2.54 ± 0.06	0.221 ± 0.011	11.6 ± 0.4	90.9 ± 6.2	79.5 ± 1.7	21.7 ± 0.7
C	6.13 ± 0.01	1.26 ± 0.12	0.097 ± 0.012	13.2 ± 0.6	123.9 ± 25.7	16.7 ± 2.1	5.4 ± 0.4
D	5.52 ± 0.02	2.90 ± 0.28	0.256 ± 0.025	11.3 ± 0.3	464.4 ± 109.0	28.8 ± 0.9	8.1 ± 0.2
E	4.83 ± 0.01	3.48 ± 0.27	0.320 ± 0.024	10.8 ± 0.1	654.9 ± 63.4	535.7 ± 61.2	139.0 ± 17.5
F	4.97 ± 0.02	4.54 ± 0.68	0.396 ± 0.063	11.6 ± 0.3	931.3 ± 103.7	771.0 ± 99.4	197.9 ± 20.2

Values represent means ± SE.

Table 2. Microbial biomass carbon (C_{mic}) expressed on an oven-dry soil basis and on a total soil organic C basis ($C_{mic}/Org-C$).

Soil	C_{mic} (mg/kg)	$C_{mic}/Org-C$ (mg/g soil organic carbon)
A	297 ± 21.6 b	14.9 ± 0.28 c
B	313 ± 44.2 b	12.3 ± 1.50 c
C	132 ± 18.5 a	10.7 ± 1.72 bc
D	191 ± 17.2 ab	6.8 ± 0.90 ab
E	162 ± 20.8 a	4.8 ± 0.79 a
F	231 ± 32.2 ab	5.3 ± 0.83 a

Values represent means ± SE. Values follows by the same letter do not differ significantly according to Tukey's test, $p = 0.05$.

and higher in soil D than in soil A (Table 3). However, the differences were very small.

3.4. PCR-DGGE

A total of 75 different band positions were identified on the DGGE gel for bacteria, and a total of 106 different band positions for fungi. Within one orchard, the band patterns of four replicates were similar but could still be differentiated by the presence of weak bands and changes in band intensities. Some of the bands disappeared in the soils with heavy metal accumulation, while opposite phenomena were also observed. For both bacteria and fungi, the number of bands and the Shannon index in the apple orchard soils (B–F) were not significantly different from those in the unpolluted chestnut orchard soil (A) (Table 3).

For both bacteria and fungi, PCA of the band pattern can separate soils from different orchards (Figure 5). With increasing concentration of total Cu in soil, the scores of PC1 for bacteria increased significantly (Figure 6(a)), while those for fungi decreased significantly (Figure 6(b)). Thus, the composition of bacterial and fungal communities changed because of the heavy metals accumulated in apple orchard surface soils.

Figure 6(b)). Thus, the composition of bacterial and fungal communities changed because of the heavy metals accumulated in apple orchard surface soils.

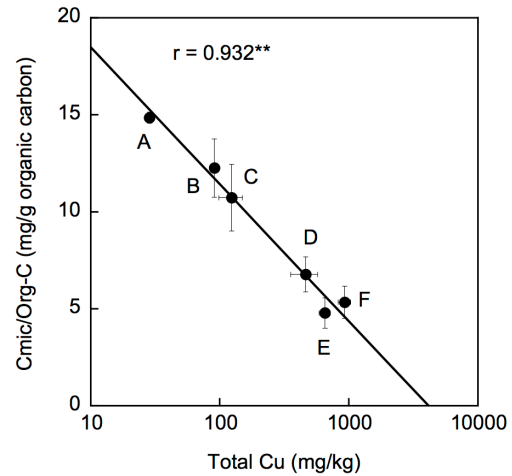


Figure 1. Relationship between the logarithmic concentration of total Cu and the amount of microbial biomass carbon expressed on a soil organic carbon basis ($C_{mic}/Org-C$).

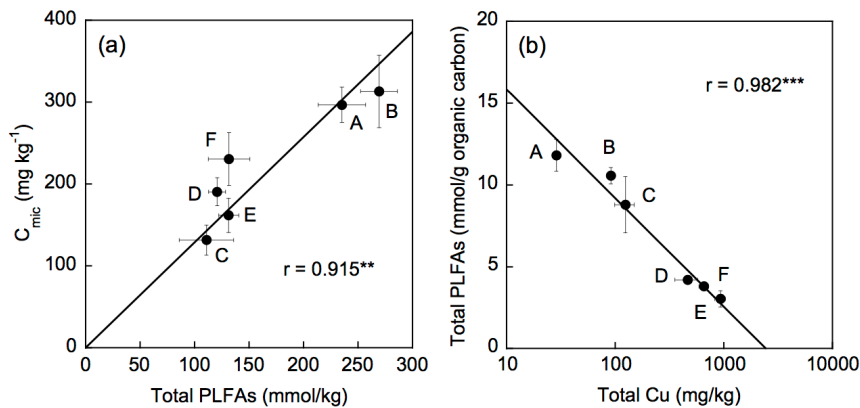


Figure 2. Relationships between the amounts of total PLFAs and microbial biomass carbon (a) and between the logarithmic concentration of total Cu and the amount of total PLFAs expressed on a soil organic carbon basis (b).

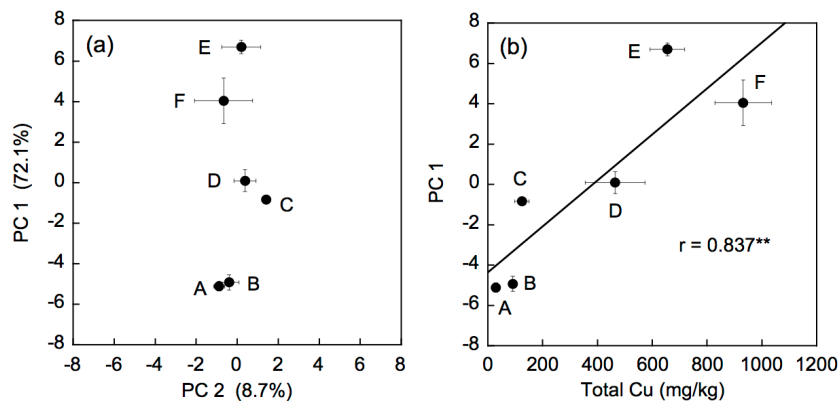


Figure 3. Principal component analysis of PLFA profiles (a) and relationship between total Cu concentrations and scores on PC 1 (b). Values in parentheses denote the variation accounted for by the different components.

4. Discussion

The adverse effects of heavy metals accumulated in apple orchard surface soils on the soil microbial biomass and microbial activities were reported in our previous studies [1,9]. Although 12 years had passed since the soil sampling for the previous studies, significant adverse effects of heavy metals on the soil microbial biomass were still observed. In this period, lead arsenate and Bordeaux mixtures were not used in the apple orchards used in the present study. Thus, the adverse effects on the soil microbial biomass can be attributed to the heavy metals derived from previously used pesticides. Our previous studies have shown that among the heavy metals Cu, Pb, and As, the toxicity of Cu to microbial biomass and microbial activities was largest [1,9,10]. The present results also suggest that Cu accumulated in the apple orchard soils significantly affected the soil microbial biomass.

In the present study, we analyzed the amount and composition of PLFAs to investigate which microbial

groups were affected by the heavy metals accumulated in the soils. The PLFA patterns of soils with different heavy metal concentrations were differentiated from each other by using multivariate PCA. The scores on PC1 showed a

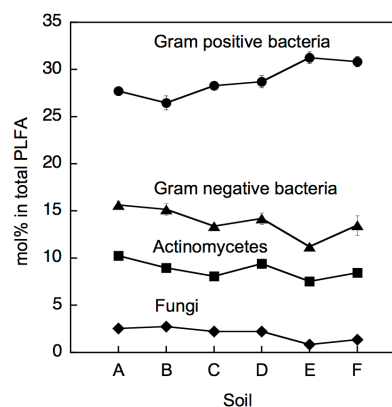


Figure 4. Relative abundance of marker PLFAs specific to gram-negative bacteria, gram-positive bacteria, actinomycetes, and fungi.

Table 3. Shannon indexes for PLFA, bacterial DGGE, and fungal DGGE, and the number of bands observed for DGGE gels.

Soil	PLFA (Shannon index)	DGGE			
		Bacteria		Fungi	
		No. of bands	Shannon index	No. of bands	Shannon index
A	2.98 ± 0.009 bc	30.5 ± 1.50 a	3.02 ± 0.050 a	36.8 ± 1.89 ab	2.92 ± 0.080 ab
B	3.00 ± 0.007 cd	31.3 ± 2.84 a	3.20 ± 0.071 a	44.0 ± 1.29 b	3.20 ± 0.044 b
C	3.02 ± 0.020 cd	21.5 ± 2.25 a	2.58 ± 0.230 a	31.8 ± 3.90 a	3.01 ± 0.150 ab
D	3.07 ± 0.011 d	23.8 ± 0.63 a	2.91 ± 0.013 a	33.0 ± 2.20 ab	2.82 ± 0.074 ab
E	2.91 ± 0.009 a	26.3 ± 3.12 a	2.89 ± 0.192 a	32.0 ± 2.74 a	2.67 ± 0.120 a
F	2.92 ± 0.026 ab	28.8 ± 3.57 a	3.00 ± 0.220 a	30.0 ± 3.08 a	2.72 ± 0.120 a

Values represent means ± SE. Values follows by the same letter do not differ significantly according to Tukey's test, $p = 0.05$.

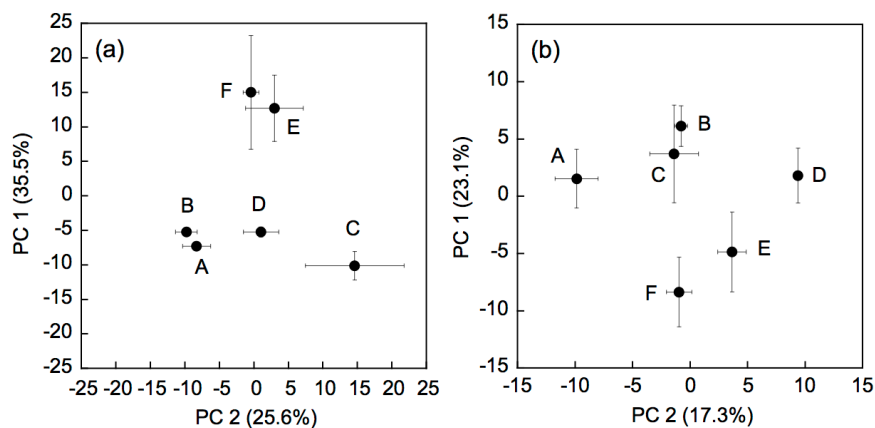


Figure 5. Principal component analyses of DGGE profiles of bacterial community (a) and fungal community (b). Values in parentheses denote the variation accounted for by the different components.

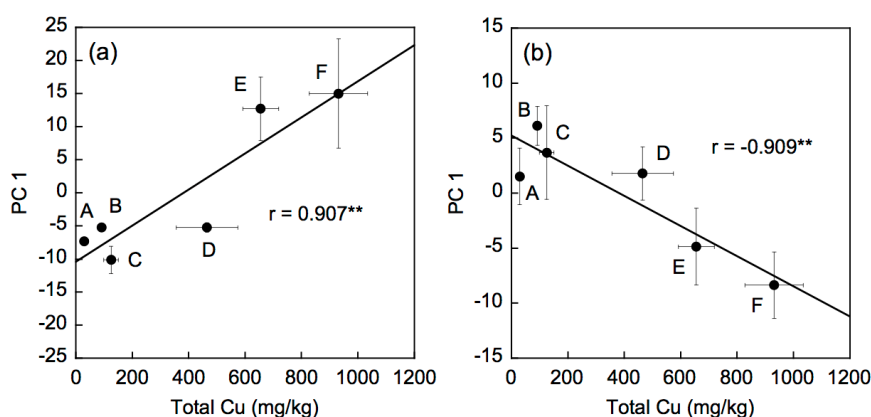


Figure 6. Relationships between total Cu concentration and scores on PC 1; (a) bacterial community, (b) fungal community.

highly positive correlation with the concentration of Cu in the soils. Therefore, the shift in the microbial community composition was ascribed to the pesticides-derived heavy metals accumulated in the apple orchard soils. However, the Shannon index was not appreciably affected by the accumulated heavy metals.

When the percentages of marker PLFAs specific to different microbial groups (relative to the total PLFAs) was used to investigate the effects of heavy metal accumulation on the individual microbial subgroups, the relative abundance of gram-positive bacterial marker PLFAs increased and that of fungal marker PLFA decreased with increasing concentrations of heavy metals in the apple orchard soils. The increase in the relative abundance of gram-positive bacterial marker PLFAs and the decrease in that of fungal marker PLFA have been observed in Scandinavian forest soils polluted with heavy metals, especially with Cu [19], and in soils amended with sewage sludge containing high concentrations of Cu [14]. Frey *et al.* [15] also reported that the relative abundance of fungal marker PLFA decreased by the addition of Cu and zinc (Zn) to soils.

In our previous study [9], by using selective-inhibition with antibiotics, we estimated the contributions of bacteria and fungi to substrate-induced respiration (SIR) in apple orchard soils. The contribution of fungi to SIR increased with increasing concentration of total Cu in soil. This fact suggested that the relative abundance of fungal biomass increased as the total Cu concentration in soil increased, since the SIR is usually correlated with the size of microbial biomass [46]. Fließbach *et al.* [4] also reported an increase in the contribution of fungi to SIR in soils receiving sewage sludge contaminated with heavy metals. However, these findings contradict the present result.

Selective inhibition of SIR is based on the assumption that fungi and bacteria respond similarly to a substrate [47]. Rajapaksha *et al.* [48], who investigated the effects

of Cu and Zn on the activities of fungi and bacteria, reported that fungal activity was less affected by heavy metals than bacterial activity. Therefore, it is suggested that in soils with heavy metal pollution the fungal contribution to SIR is high compared with the bacterial contribution. Consequently, the abundance of fungal marker PLFA is considered to be more suitable as an index of fungal biomass in heavy metal-polluted soils than the fungal contribution to SIR. Thus, the present results indicate that the heavy metals accumulated in apple orchard soils decrease the relative abundance of fungal biomass.

PCA of the PLFA profiles clearly indicated that the composition of the microbial community was affected by the heavy metals accumulated in apple orchard surface soils. However, PLFA analysis always has a much stronger focus on the bacterial community in soil than on the fungal community, because specificity for fungi is only attributed to 18:2 ω 6 [45]. Therefore, we further applied the PCR-DGGE technique targeting the 16S rRNA gene of bacteria and the 18S rRNA gene of fungi for assessing the effects of heavy metals accumulated in apple orchard surface soils on the composition of both the bacterial and fungal communities.

PCA of the band pattern of DGGE clearly indicated that the heavy metals accumulated in apple orchard soils affected the composition of both bacterial and fungal communities. The shift in the composition of the soil bacterial community induced by heavy metals has been reported in several studies that used the PCR-DGGE method [24-26]. Macdonald *et al.* [28] also showed significant effects of Cu on soil fungal communities in a field under pasture using a combination of multiplex-terminal and terminal restriction fragment length polymorphism profiling approaches. These findings are in accordance with the result of our present study.

For both bacteria and fungi, the richness (number of bands) and diversity (Shannon index) were not signifi-

cantly lower by the heavy metals accumulated in the soils. Altimira *et al.* [49] reported for long-term Cu-polluted agricultural soils that the bacterial DGGE profiles were shifted by the pollution but that the values of richness and Shannon index were similar among the soils. They considered that bacterial communities from long-term Cu-polluted soils were well adapted to the high Cu content. Other studies [50,51] also showed that the bacterial diversity did not change significantly after long-term contamination with Cu, Pb and Zn. In contrast, short-term Cu pollution in soil induced significant modifications in the bacterial community structure, but these changes were resilient after a few weeks or months [52]. Therefore, the present result indicates that bacterial and fungal communities in apple orchard surface soils were adapted to heavy metals derived from previously used pesticides.

5. Conclusion

This study clearly indicated the adverse effects of heavy metals derived from previously used pesticides in apple orchard surface soils on the soil microbial biomass. Analysis of the PLFA composition showed that that of the microbial community was affected by pesticide-derived heavy metals in the soils; the relative abundance of gram-positive bacteria increased and that of fungi decreased with increasing concentrations of heavy metals. PCR-DGGE targeting the rRNA genes of bacteria and fungi also demonstrated the shifts in the composition of bacterial and fungal communities induced by soil pollution with heavy metals. However, the diversity of the microbial communities was not significantly affected by heavy metal pollution. This could be explained by the adaptation of microbial communities in apple orchard surface soils to heavy metals derived from previously used pesticides

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