Decline in Topsoil Microbial Quotient, Fungal Abundance and C Utilization Efficiency of Rice Paddies under Heavy Metal Pollution across South China

Yongzhuo Liu1, Tong Zhou1, David Crowley2, Lianqing Li1, Dawen Liu1, Jinwei Zheng1, Xinyan Yu1, Genxing Pan1*, Qaiser Hussain1,3, Xuhui Zhang1, Jufeng Zheng1

1 Institute of Resources, Ecosystem and Environment of Agriculture, Nanjing Agricultural University, Jiangsu Nanjing, China, 2 Department of Environmental Sciences, University of California Riverside, Riverside, California, United States of America, 3 Department of Soil Science and Soil Water Conservation, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi, Pakistan

Abstract

Agricultural soils have been increasingly subject to heavy metal pollution worldwide. However, the impacts on soil microbial community structure and activity of field soils have been not yet well characterized. Topsoil samples were collected from heavy metal polluted (PS) and their background (BGS) fields of rice paddies in four sites across South China in 2009. Changes with metal pollution relative to the BGS in the size and community structure of soil microorganisms were examined with multiple microbiological assays of biomass carbon (MBC) and nitrogen (MBN) measurement, plate counting of cultivable colonies and phospholipids fatty acids (PLFAs) analysis along with denaturing gradient gel electrophoresis (DGGE) profile of 16S rRNA and 18S rRNA gene and real-time PCR assay. In addition, a 7-day lab incubation under constantly 25°C was conducted to further track the changes in metabolic activity. While the decrease under metal pollution in MBC and MBN, as well as in cultivable population size, total PLFA contents and DGGE band numbers of bacteria were not significantly and consistently seen, a significant reduction was indeed observed under metal pollution in microbial quotient, in culturable fungal population size and in ratio of fungal to bacterial PLFAs consistently across the sites by an extent ranging from 6% to 74%. Moreover, a consistently significant increase in metabolic quotient was observed by up to 68% under pollution across the sites. These observations supported a shift of microbial community with decline in its abundance, decrease in fungal proportion and in C utilization efficiency under pollution in the soils. In addition, ratios of microbial quotient, of fungal to bacterial and qCO2 are proved better indicative of heavy metal impacts on microbial community structure and activity. The potential effects of these changes on C cycling and CO2 production in the polluted rice paddies deserve further field studies.

Citation: Liu Y, Zhou T, Crowley D, Li L, Liu D, et al. (2012) Decline in Topsoil Microbial Quotient, Fungal Abundance and C Utilization Efficiency of Rice Paddies under Heavy Metal Pollution across South China. PLoS ONE 7(6): e38858. doi:10.1371/journal.pone.0038858

Editor: A. Mark Ibekwe, U. S. Salinity Lab, United States of America

Received February 24, 2012; Accepted May 11, 2012; Published June 11, 2012

Copyright: © 2012 Liu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The present research was funded by the China Natural Science Foundation under grant numbers 40830528 and of 40671180. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: pangenxing@yahoo.com.cn

Introduction

It has been widely accepted that soil plays a key role in sustaining life of Earth ecosystems with the huge abundance of soil microbiota [1]. While crop production in agricultural lands has been increasingly threatened by heavy metal contamination [2], long term impacts of heavy metal pollution on soil microorganisms have been increasingly concerned [3,4]. Microbial biomass has been shown sensitive to increased heavy metal concentrations in soils [3], reduction in microbial diversities and activities of soil microbial community has also been reported either in short-term lab spiked studies [5–7] or under long-term exposure [8–10] to toxic metals in soil.

Furthermore, soil bacteria and fungi are known to have different responses to heavy metal pollution [11]. Khan et al. [7] reported, by plate counting of short term incubated soil, a significant decrease in culturable bacterial colonies but no changes in fungal cells under spiked metal pollution. Likewise, in short term laboratory incubation experiments (ranging from 1 to 18 months) with spiked metals, increase in fungal proportion had been often observed using phosphorus lipid fatty acids analysis (PLFAs) assay [11–15]. In contrast, long term metal polluted agricultural and forest soils often exerted a reduction both in the numbers of culturable fungal cells [16] and in proportion of fungal fatty acids [17,18].

Thus, inconsistent findings had been reported on the effects of metal pollution on soil microbial community with reference to the relative proportion of bacterial and fungal communities between short term incubation with spiked samples and long term polluted field samples and between different assays applied. Nor the potential impact of the pollution-induced microbial changes on the metabolic activity has been well addressed though reduction in total soil microbial abundance had been well recognized for the long term polluted field soils.

China’s croplands at about 2.5 Mha had been increasingly threatened by heavy metal pollution, as reported by the Ministry of Environment Protection [19]. Rice paddies had been concerned
to play a key role in producing cereals for meeting the increasing food demand of China. However, extensive heavy metal pollution had been widely reported in rice paddies from the Yangtze River delta [20,21], from the Pearl River delta [22] as well as from Jiangxi and Guangdong provinces [23,24]. A number of studies had reported toxicities of accumulated heavy metals to the microorganisms in paddy soils under long term pollution in individual single sites of China [25–28]. However, an integrated understanding of the long term impacts of metal pollution on microbial abundance and community structure of rice paddy soil had yet been poorly addressed, nor of the potential changes in microbial activities well reported.

This paper reports a cross site study on the changes with long term heavy metal pollution in microbial abundance, community structure and carbon utilization efficiency of rice paddies across South China. To track any potential consistent changes in microbial abundance and activity, multiple microbiological assays were applied including MBC and MBN measurement, plate counting of cultivable populations, PLFA analysis, DGGE profile and qPCR as well as soil basal respiration under laboratory incubation of field polluted samples in comparison to their background counterparts.

**Materials and Methods**

**Sites**

In a field reconnaissance to the rice paddy region across South China in spring of 2009, a number of rice paddy areas were recognized to be polluted for more than 30 years with heavy metals due to emissions from mining and smelting activities. For a cross site study, 4 sites of rice fields were chosen including Site YX (Yixing Municipality, Jiangsu), Site DX (Dexing County, Jiangxi), Site DY (Dayu County, Jiangxi) and Site DBS (Wenyuan Municipality, Guangdong) from north to south across South China (Figure 1). For comparison, paired soil samples were collected from polluted (PS) and adjacent unpolluted fields (recognized as the background, BGS, by visits to farmers) in a single site. The studied sites are within the area controlled by subtropical monsoon climate with mean annual temperature from 18°C to 25°C, and mean annual rainfall from 1200 mm to 1450 mm from north to south, respectively. These rice paddies have been cultivated normally either with rice-rapeseed/wheat crops or double cropping of rice paddies in a cropping year. The basic geographic information of these sites was described in Table 1. The metal pollution status of these sites and the toxic Cd levels of rice grown in these polluted paddies were previously studied and reported [29-33].

**Topsoil Sampling and Treatment**

Soil sampling was conducted before rice planting in spring of 2009 (No specific permits were required for the sampling). Three composite topsoil samples were randomly collected in depth of 0–15 cm respectively in PS and BGS plots, each of which contained 5 sub-samples collected in a “Z” shaped way with a distance of ~5 m from each other and mixed thoroughly prior to shipping.

After the sampling, samples were sealed and stored in a freeze box for shipping to laboratory. In laboratory, the fresh samples were removed of soil animals and plant fragments if any, and gently crashed to pass a 2 mm sieve before being divided into four portions. The first portion was stored at 4°C for plate counting analysis and soil respiration measurement. The second and third was both stored at ~20°C for PLFA and DNA extraction respectively, but the second frozen-dried before storing. The fourth, however, was air-dried and stored at room temperature for soil property measurement, of which a further portion was ground to pass a 0.15 mm sieve for determination of SOC, total nitrogen and total metal contents.

**Analysis of Soil Properties and Metal Contents**

Measurements of the basic properties and metal contents of the samples were conducted following the protocols described by Lu [34]. Briefly, soil pH was measured with a glass electrode using a 1/2.5 soil/water ratio with a precision pH meter (Mettler Toledo SevenEasy, Switzerland). Soil particle content was determined with hydrometer after dispersion with 0.5 mol L⁻¹ NaOH. SOC content was measured using wet digestion and oxidation with potassium dichromate. Total nitrogen was analyzed with Kjeldahl method. For total and available heavy metal content determination, the samples were digested with a mixed solution of HF/HClO₄/HNO₃ (10/2.5/2.5, v/v/v) and extracted with 1 mol L⁻¹ HCl (1/2.5, v/v), respectively. Content of Cd was determined with graphite furnace atomic absorption spectrometry (GFAAS, SpectrAA220Z, Varian, USA), while those of Pb, Cu and Zn with flame atomic adsorption spectrophotometer (FAAS, TAS-986, China). The data of soil properties and metal contents from the above measurements are organized in Table 2 and 3 respectively.

To evaluate the overall heavy metal pollution degree, the Nemerow pollution index [35] was followed. The index indicates an overall loading of different single polluted metal by highlighting both the maximum and average level of the determined metals. Using the Standards of Soil Environmental Quality (GB15618-1995) as soil quality assessment criteria, the Nemerow pollution index is calculated using the equation as follows:

\[
P_n = \sqrt{\frac{(MaxP_i^2 + AveP_i^2)}{2}}
\]

Where, \(P_n\) is the overall Nemerow pollution index as a sum of \(n\) metal elements analyzed for a soil sample, \(P_i\) is a single pollution intensity index of \(i\)th metal element as its measured concentration (\(C_i\)) divided by the concentration of reference standard (\(R_i\)). And \(MaxP_i\) and \(AveP_i\) is the maximum and average pollution intensity respectively of the analyzed metals in a given soil. The calculated values of Nemerow pollution index of the soils are also given in Table 3.

**Measurements of Soil Microbial Biomass C and N**

The fumigation-extraction procedure [36] was used to determine soil MBC and MBN. The content of K₂SO₄-extracted C from the CHCl₃-treated and untreated soils was determined by an automated TOC Analyzer (Shimazu, TOC-500, Japan) and a K₂CO₃ of 0.45 was used to convert the measured C to MBC. The total N in the extracts was measured by Kjeldahl digestion-distillation procedure and calculated as MBN with the conversion coefficient of 2.22 [37,38].

**Plate Counting of Culturable Colonies**

Plate counting of cultivable microorganisms was performed basically following the procedure described by Zuberer [39]. A portion of a sample (1 g) was suspended in sterilized distilled water to form a decimal dilution up to \(10^{-6}\). An aliquot of 50 or 100 μl of the diluted suspension of \(10^{-4}\) or \(10^{-5}\) was spread on a beef-protein medium plate in Petri dish, and another aliquot of 50 μl at \(10^{-2}\) dilution on a Martin’s medium plate for counting of bacteria and fungi, respectively. Control plates of respective media without soil suspension were also included to check any possible
contamination. The plates were incubated at 28°C until the visible colonies were formed. While the required incubation time was dependent on the media type, the inoculum size and the temperature of incubation [40], the proper length of incubation for colony counting was different between fungal and bacterial in this study. As shown in a pre-experiment respectively for fungi and bacteria, plates carrying 10 to 100 colonies of fungi and those carrying 30 to 200 colonies of bacteria [39] were counted on day 2 and on day 4 after a proper length of incubation with maximum colony size for fungal and bacterial respectively. The colony forming units (CFUs) per gram of a dry sample was then calculated. The plate incubation and counting was done in triplicates of a sample.

Phospholipid Fatty Acid Analysis (PLFA)

1) Extraction and determination. Microbial phospholipid fatty acids were extracted using the modified procedure of Bligh-Dyer [41] as described by Kehrmeyer et al. [42]. Briefly, 5 g of a frozen-dried sample was extracted with a mixture of chloroform/methanol/phosphate (1/2/0.8, v/v/v), and the lipids were separated into neutral lipids, glycolipids, and polar lipids [43,44] on a pre-packed silicon acid column (Ultra-Clean™, 500 mg/4 ml NH-2 SPE columns, Alltech, Inc.). The polar lipid fraction was trans-esterified with a mild alkali solution to recover the PLFA as methyl esters in hexane.

The PLFA obtained was dissolved with a MiDI reagent (Hexane/Methyl tert-Butyl Ether, 1/1, v/v) and analyzed with a gas chromatography (GC, HP 6890 Series, Hewlett Packard, Wilmington, Del.). The fatty acid 19:0 was added as an internal standard.

![Figure 1. A sketch map showing the sampling sites in South China.](image)

The labels of YX, DX, DY and DBS from the north to south represent the sampling sites shown in Table 1. doi:10.1371/journal.pone.0038858.g001

| Site | Location | Plot | GPS location | Pollution source and history |
|------|----------|------|--------------|------------------------------|
| YX   | Xushe Township, Yixing Municipality, Jiangsu | BGS  | 31°24'10"N, 119°41'28"E | No direct pollution upwind |
|      |          | PS   | 31°24'26"N, 119°41'36"E | Emissions from a smelter since late 1960's |
| DX   | Sizhou Village, Dexing Municipality, Jiangxi | BGS  | 29°04'159"N, 117°43'747"E | In-access of emissions in opposite hill slope |
|      |          | PS   | 29°04'159"N, 117°43'761"E | Waste water and particulate from a copper mine since 1950's |
| DY   | Fujiang Village, Dayu County, Jiangxi | BGS  | 25°24'200"N, 114°18'551"E | Opposite hills slope |
|      |          | PS   | 25°24'235"N, 114°18'551"E | Waste water and solid emissions from a waste mining since 1960's |
| DBS  | Dabaoshan, Wengyuan County, Guangdong | BGS  | 24°26'702"N, 113°49'377"E | No direct pollution using upstream water for irrigation |
|      |          | PS   | 24°27'947"N, 113°48'241"E | Intermittent waste water irrigation from a lead and zinc mining since 1960's |

BGS, background soil; PS, polluted soil.
doi:10.1371/journal.pone.0038858.t001
Table 2. Basic physicochemical properties of the studied soil samples.

| Site | Plot | SOC (g kg⁻¹) | TN (g kg⁻¹) | pH (H₂O) | Clay (%) | Silt (%) | Sand (%) |
|------|------|-------------|-------------|---------|---------|---------|---------|
| YX   | BGS  | 28.77±1.11 | 2.69±0.08   | 6.16±0.05| 41.0    | 35.2    | 23.8    |
|      | PS   | 25.27±0.53 | 2.22±0.05   | 6.08±0.05| 42.0    | 37.2    | 20.8    |
| DX   | BGS  | 22.79±1.58 | 1.58±0.05   | 4.87±0.05| 21.0    | 31.2    | 47.8    |
|      | PS   | 22.25±0.35 | 1.96±0.07   | 4.10±0.06| 27.0    | 32.2    | 40.8    |
| DY   | BGS  | 20.40±0.82 | 2.06±0.07   | 5.20±0.05| 22.0    | 31.2    | 46.8    |
|      | PS   | 22.30±1.32 | 1.97±0.13   | 5.01±0.05| 14.0    | 27.2    | 58.8    |
| DBS  | BGS  | 15.23±0.6  | 1.03±0.03   | 5.58±0.13| 22.2    | 23.2    | 54.6    |
|      | PS   | 19.11±0.68 | 1.49±0.08   | 5.45±0.16| 27.0    | 28.8    | 44.2    |

BGS: background soil; PS, polluted soil.

doi:10.1371/journal.pone.0038858.t002

Table 2 (Continued)

Table 3. Total and available contents of heavy metals and the Nemerow pollution index (Means ± S.D.) of the studied soils.

| Sample | Total content (mg kg⁻¹) | Available pool (mg kg⁻¹) | Nemerow index |
|--------|-------------------------|--------------------------|--------------|
|        | Cd  | Pb  | Cu  | Zn  | Cd  | Pb  | Cu  | Zn  |
| YX-B   | 0.45±0.01b                | 59.79±13.28b             | 42.19±0.77b  | 104.89±3.92b | 0.21±0.03b | 12.04±1.31b | 7.23±0.79b | 16.86±1.69b | 1.20±0.04b |
| YX-P   | 6.60±0.27a                | 354.52±87.34a            | 82.56±1.90a  | 172.92±2.67a | 4.65±0.33a | 70.90±2.27a | 24.88±0.22a | 20.81±3.38a | 16.22±0.94a |
| DX-B   | 0.48±0.14b                | 58.95±9.09b              | 640.19±2.98b | 94.57±17.21b | 0.10±0.08b | 18.45±0.75b | 191.44±24.96b | 4.95±1.32b | 9.45±0.03b |
| DX-P   | 1.55±0.14a                | 95.17±7.07a              | 1333.68±129.72a | 163.90±15.41a | 0.18±0.04a | 53.18±0.53a | 472.97±28.26a | 13.36±2.21a | 19.74±1.86a |
| DY-B   | 0.36±0.19b                | 68.89±7.10b              | 36.01±0.17b  | 117.14±5.58b | 0.05±0.06b | 13.69±0.93b | 6.49±0.75b | 3.75±0.33b | 1.20±0.32b |
| DY-P   | 9.60±0.20a                | 329.76±10.60a            | 92.28±4.58a  | 368.45±16.71a | 6.95±0.38a | 85.78±5.67a | 33.57±3.12a | 120.63±1.66a | 24.95±6.22a |
| DBS-B  | 0.29±0.00b                | 33.37±2.08b              | 21.87±1.02b  | 70.40±1.5b | 0.08±0.02b | 8.95±0.58b | 4.84±0.19b | 3.92±0.15b | 0.76±0.01b |
| DBS-P  | 1.49±0.24a                | 133.27±6.67a             | 224.83±5.68a | 248.48±5.87a | 0.31±0.02a | 37.59±1.36a | 77.06±4.01a | 19.98±2.23a | 4.04±0.56a |

Different lowercase characters in a single column indicate significant difference (p<0.05) between the background (B) and polluted (P) soils in a single site.

doi:10.1371/journal.pone.0038858.t003

Real-time PCR (qPCR) Assay

Copy numbers of the bacterial 16S rRNA gene and the fungal internal transcribed spacer (ITS) rRNA gene of all the samples were determined in triplicates using an iCycler IQ5 Thermocycler (Bio-Rad, Hercules, CA). The quantification was based on the fluorescent dye SYBR-green one, which was bound to double stranded DNA during PCR amplification. The primers and the thermal cycling conditions used were basically following those described by Fierer et al. [53]. The DNA concentration of all samples was measured at 260 nm using a UV Spectrophotometer (Bio Photometer, Eppendorf, Germany) and then adjusted to a concentration of 15 ng µl⁻¹. Each reaction was performed in a 25 µl volume containing 15 ng of DNA, 1 µl of 10 µM of each primer and 12.5 µl of SYBR premix EX Taq TM (Takara Shuzo, Shiga, Japan). Melting curve analysis of the PCR products was conducted following each assay to confirm that the fluorescence signal originated from specific PCR products but not from primer-dimers or other artifacts. PCR products were checked for the correct size by comparison to a standardized molecular weight ladder by electrophoresis on 1.5% agarose gel.
A plasmid standard containing the target region was generated for each primer set using total DNA extracted from a sample. The amplified PCR products of the bacterial 16S rRNA gene and the fungal ITS rRNA gene were purified using PCR solution purification kit (Takara, Japan), ligated into pEASY-T3 cloning vector (Promega, Madison, WI) and cloned into Escherichia coli DH5α. Clones containing correct inserts were chosen as the standards for qPCR. Plasmid DNA was isolated using plasmid extraction kit (Takara, Japan) and its concentration was determined with the spectrophotometer as mentioned above. As the sizes of the vector and PCR insert were known, the copies of the 16S rRNA gene and the ITS rRNA gene were directly calculated from the concentration of extracted plasmid DNA. Standard curves were generated using triplicate 10-fold dilutions of plasmid DNA ranging from 3.72×10⁷ to 3.72×10⁹ copies for the bacterial 16S rRNA gene, and 1.09×10⁷ to 1.09×10⁹ copies of template for the ITS rRNA gene per assay. High amplification efficiencies of 95.3% for bacterial 16S rRNA gene and 93.5% for fungal ITS rRNA gene quantification, with a R² value and a slope of 0.992 and of −3.00, of 0.995 and of −3.44, respectively. Thus, a relative fungal-to-bacterial ratio was calculated as the ratio of copy number of all the fungi species to those of bacteria species as amplified with the qPCR assay [33].

**Soil Basal Respiration (BR)**

A sample was moistened to 60% of the water holding capacity (WHC) and conditioned at 25°C for 7 d under aerobic conditions to allow microbial activity stabilized before measurement. Basal respiration (CO₂ evolution) was measured by incubating a sample equivalent to 20 g dry weight at 25°C in a 120 mL airtight jar for 7 days. The moisture of the sample was sustained at 60% of WHC consistently throughout the incubation. A gas sample from the head space of the jar was collected respectively at 2, 6, 12, 24, 36, 48, 60, 72, 96, 120 and 168 h after incubation, and the CO₂ concentration was analyzed by Gas Chromatography (Agilent 4890D, USA) equipped with a stainless steel column (Porapak Q (80/100 mesh)) and a flame-ionization detector (FID) [34]. The basal respiration rate was expressed either on the dry soil base or on the base of soil organic carbon content.

**Data Processing and Statistical Analysis**

The data presentation and treatment was processed with Microsoft Excel 2003, and the results were expressed as Means ± S.D. A paired t-test was used to check the differences between the polluted (PS) and background (BGS) samples in a single site with a significance defined at p<0.05. Principal component analysis (PCA) of DGGE profiles was conducted using the SPSS 16.0 statistical package for Windows to elucidate the microbial community structures based on relative band intensity and positions.

**Results**

**Soil Heavy Metal Pollution**

As listed in Table 2, there was no considerable difference in the basic properties between PS and BGS in a single site. However, there were consistent differences in contents of total and available Cd, Pb, Cu and Zn between the polluted and background soils across the sites though the contents of a single heavy metal element varied with sites (Table 3). As shown by the values of Nemerow pollution index (ranging from 4.0 to 25.0) of the polluted soil from a single site, the degree at which the soil was polluted with heavy metals was in an order of DY > DX > YX > DBS.

**Soil Microbial Biomass C and N, and Microbial Quotient**

As shown in Table 4, soil MBC and MBN ranged from 91.2 mg kg⁻¹ to 769.2 mg kg⁻¹ and from 14.8 mg kg⁻¹ to 69.0 mg kg⁻¹ for BGSs respectively. For PSs, however, MBC and MBN ranged from 87.8 mg kg⁻¹ to 474.1 mg kg⁻¹ and from 11.4 mg kg⁻¹ to 58.4 mg kg⁻¹ respectively. A decrease in MBC under pollution was observed at a degree ranging from 24% in YX and DX to 45% in DY though not significant in DBS. The microbial quotient, a proportion of MBC to SOC, ranged from 0.6% to 3.8% and from 0.5% to 1.9% for BGSs and PSs respectively. This clearly indicated a consistently significant reduction in microbial abundance relative to SOC under metal pollution at a degree of 13% to 50% though varying with sites.

**Culturable Microbial Population Abundance**

Data of culturable colony counting from the plate incubation experiment is shown in Figure 2. Population size of cultivable bacteria ranged from 1.5×10⁸ CFUs g⁻¹ in DY to 6.5×10⁸ CFUs g⁻¹ in YX for BGSs, and from 0.3×10⁸ CFUs g⁻¹ in DX to 7.0×10⁸ CFUs g⁻¹ in YX for PSs. Meanwhile, that of cultivable fungi from 16.0×10⁷ CFUs g⁻¹ in DBS to 18.7×10⁷ CFUs g⁻¹ in DY for BGSs, and from 4.8×10⁷ CFUs g⁻¹ in DX to 13.4×10⁷ CFUs g⁻¹ in DBS for Ps. Here is clearly seen a consistent reduction by 16% to 74% under pollution in the size of cultivable fungal population across the four sites despite of an inconsistent change in that of cultivable bacteria. Nevertheless, the change in the ratio of fungal to bacterial culturable colonies was not consistently seen across the sites.

**Extractable PLFAs**

Data of PLFA analysis is presented in Figure S1. There were totally 36, 35, 30 and 25 single PLFAs detected in BGS and PSs respectively, of which 16 fatty acids were identified common across the sites. Furthermore, the fatty acid 18:0 w3c was detected in all the PSs, while only in the BGS of DY. Whereas, the 18:1 w9c was not detected in the PSs, except in that of YX with a much lower content of PLFA compared to BGSs. The concentrations of PLFAs of the soils studied are organized in Table 5. The extracted total, bacterial and fungal PLFAs of the BGSs ranged from 6.4 nmol g⁻¹ in YX for BGSs, and from 4.8 nmol g⁻¹ in DX to 13.8 nmol g⁻¹ in PSs. Whereas, the extractable PLFAs of DBS, and 18:1 w5c was not detected in the PSs, except in that of YX with a much lower content of PLFA extracted. The concentration of fatty acid 18:1 w9c (one of fungal biomarkers) was shown to reduce in all PSs compared to BGSs. The concentrations of PLFAs of the soils studied are organized in Table 5. The extracted total, bacterial and fungal PLFAs of the BGSs ranged from 11.2 nmol g⁻¹ in YX for BGSs, and from 4.8 nmol g⁻¹ in DX to 13.8 nmol g⁻¹ in YX for PSs. Meanwhile, that of culturable fungi from 16.0×10⁷ CFUs g⁻¹ in DBS to 18.7×10⁷ CFUs g⁻¹ in DY for BGSs, and from 4.8×10⁷ CFUs g⁻¹ in DX to 13.4×10⁷ CFUs g⁻¹ in DBS for Ps. Here is clearly seen a consistent reduction by 16% to 74% under pollution in the size of cultivable fungal population across the four sites despite of an inconsistent change in that of cultivable bacteria. Nevertheless, the change with pollution in the ratio of fungal to bacterial culturable colonies was not consistently seen across the sites.

**Bacterial and Fungal DGGE Bands and Gene Copy Numbers**

Bacterial and fungal DGGE band patterns of all BGSs and PSs from the four studied sites are given in Figure S2A and S2B respectively. The data of bacterial and fungal band numbers of 16S rRNA and 18S rRNA gene retrieved from the DGGE profiles and gene copy numbers were analyzed using qPCR of the studied soils are organized in Table 6. The gene copy number of bacteria and fungi ranged from 0.2×10¹⁰ to 5.5×10¹⁰ and from 0.5×10¹⁰ to 14.3×10¹⁰ for BGSs, and from 0.6×10¹⁰ to 3.8×10¹⁰ and from 0.7×10¹⁰ to 9.5×10¹⁰ for...
Unlike that of bacteria, here the copy number of fungi was seen more or less reduced under pollution in all sites except in DBS. The calculated ratio of fungal/bacterial copy number was seen decreased under pollution slightly in YX site and significantly in the other three sites. Following a similar trend, the band numbers of bacteria from the 16S rRNA gene DGGE profile were consistently higher than those of fungi from 18S rRNA gene profile. However, there was no difference in the bacterial band numbers between PS and BGS in a single site, being 32 \pm 620 on average. Whereas, band numbers of fungi under pollution were seen unchanged in YX and more or less reduced in other three sites. As a result, the calculated ratio of fungal-to-bacterial band numbers was reduced significantly in sites of DX, DY and DBS though no change in YX. These data supported a relative reduction in gene copies and band numbers of fungi rather than of bacteria under heavy metal pollution in the rice paddies.

### Table 4. Soil microbial biomass C, N and microbial quotient, as well as basal respiration and metabolic quotient of the studied soils (Means \( \pm \) S.D.).

| Sample | SMBC (mg kg\(^{-1}\)) | SMBN (mg kg\(^{-1}\)) | Microbial quotient (%) | Basal respiration (mg CO\(_2\)-C kg\(^{-1}\) soil) | Metabolic quotient (mg CO\(_2\)-C g\(^{-1}\) MBC h\(^{-1}\)) |
|--------|----------------------|----------------------|-----------------------|----------------------------------|----------------------------------|
| YX-B   | 623.2 \( \pm \) 35.2a | 40.26 \( \pm \) 1.87a | 2.17 \( \pm \) 0.03a  | 37.10 \( \pm \) 1.17a           | 0.35 \( \pm \) 0.01b              |
| YX-P   | 474.1 \( \pm \) 15.6b | 37.01 \( \pm \) 2.49a | 1.88 \( \pm \) 0.03b  | 30.02 \( \pm \) 2.46b           | 0.40 \( \pm \) 0.01a              |
| DX-B   | 238.59 \( \pm \) 21.35a | 31.61 \( \pm \) 3.23a | 1.05 \( \pm \) 0.09a  | 22.64 \( \pm \) 1.29a           | 0.56 \( \pm \) 0.03b              |
| DX-P   | 181.07 \( \pm \) 12.11b | 11.51 \( \pm \) 0.64b | 0.81 \( \pm \) 0.05b  | 22.61 \( \pm \) 1.08a           | 0.74 \( \pm \) 0.04a              |
| DY-B   | 769.2 \( \pm \) 25.01a | 69.02 \( \pm \) 3.88a | 3.77 \( \pm \) 0.12a  | 24.29 \( \pm \) 2.52a           | 0.19 \( \pm \) 0.02b              |
| DY-P   | 419.47 \( \pm \) 45.00b | 58.35 \( \pm \) 3.66b | 1.88 \( \pm \) 0.20b  | 22.30 \( \pm \) 5.27a           | 0.32 \( \pm \) 0.08a              |
| DBS-B  | 91.56 \( \pm \) 10.58a | 14.78 \( \pm \) 2.89a | 0.60 \( \pm \) 0.07a  | 19.56 \( \pm \) 0.76a           | 1.25 \( \pm \) 0.02b              |
| DBS-P  | 87.78 \( \pm \) 10.07a | 11.41 \( \pm \) 3.15a | 0.46 \( \pm \) 0.06b  | 17.41 \( \pm \) 1.67a           | 1.34 \( \pm \) 0.03a              |

Different lowercase characters indicate significant difference (\( p<0.05 \)) between background (B) and polluted (P) soils in a single site. DOI:10.1371/journal.pone.0038858.t004

Pollution Reduced Microbial Abundance & F/B Ratio

**Figure 2. Culturable population of bacteria (A) and of fungi (B) of the soils studied.** Blank, background soil; Shaded, polluted soil. Different lowercase characters indicate significant difference (\( p<0.05 \)) between the background and polluted soils in a single site. DOI:10.1371/journal.pone.0038858.g002

**Figure 4A and 4B.** Principal component analysis (PCA) of DGGE profiles both of bacterial 16S rRNA and fungal 18S rRNA gene fragments are shown in Figure 4A and 4B, respectively. PCA scores of bacterial DGGE profile showed significant separation (\( p<0.05 \)) along PC1, which accounted for around 50% of the variances, between PS and BGS in DX, DY and DBS sites. For fungal DGGE profiles, the
significant divergence between PS and BGS along PC1 was found in DX and DY not in YX and DBS. Moreover, the significant divergence along PC2 between PS and BGS was found in all sites for both bacterial and fungal profiles. A shift indicated here in bacterial and fungal community structure supplemented the above microbial changes of the rice paddies with heavy metal pollution.

**Basal Respiration**

Data of the basal respiration in the incubation course of the studied soils is shown in Figure S3. Calculated with the cumulic CO₂ production from the whole incubation course, the basal soil respiration rates for the studied soils are given in Table 4. Ranging from 17 mg CO₂·C kg⁻¹ soil to 37 mg CO₂·C kg⁻¹ soil, total basal respiration was not seen different between BGS and PS from a single site except for YX. The basal respiration normalized on the basis of SOC in all sites was not significantly changed under pollution, ranging from 1.0 mg CO₂·C g⁻¹ SOC to 1.5 mg CO₂·C g⁻¹ SOC for BGSs and from 1.0 mg CO₂·C g⁻¹ SOC to 1.1 mg CO₂·C g⁻¹ SOC for PSs. This seemed a similar SOM feature between the BGS and PS in a single site. Whereas, the calculated metabolic quotient, the basal respiration rate on basis of MBC, ranged from 0.2 mg CO₂·C g⁻¹ MBC h⁻¹ to 1.2 mg CO₂·C g⁻¹ MBC h⁻¹ for BGSs, and from 0.3 mg CO₂·C g⁻¹ MBC h⁻¹ to 1.3 mg CO₂·C g⁻¹ MBC h⁻¹ for PSs (Table 4).

There was a clear cross-site trend of increase in the metabolic quotient under pollution at a degree of 7% to 68% (Figure 3).

**Table 5.** Concentrations (nmol g⁻¹) of total, bacterial, fungal PLFAs and the fungal-to-bacterial PLFA ratio (Means ± S.D.) of the studied soils.

| Sample | Total PLFAs (nmol g⁻¹) | Bacterial PLFAs (nmol g⁻¹) | Fungal PLFAs (nmol g⁻¹) | Ratio of fungal to bacterial |
|--------|------------------------|-----------------------------|-------------------------|-----------------------------|
| YX-B   | 46.79±3.06a             | 24.32±1.62a                 | 8.59±0.66a              | 0.35±0.02a                  |
| YX-P   | 32.63±3.49b             | 17.47±1.68b                 | 5.78±0.62b              | 0.33±0.01b                  |
| DX-B   | 18.65±2.04a             | 9.93±1.10a                  | 4.02±0.32a              | 0.40±0.03a                  |
| DX-P   | 20.55±0.92a             | 11.28±0.46a                 | 3.47±0.26b              | 0.31±0.02b                  |
| DY-B   | 49.06±6.10a             | 23.18±2.93a                 | 13.81±1.11a             | 0.60±0.04a                  |
| DY-P   | 22.98±7.27b             | 12.26±0.37b                 | 3.72±0.28b              | 0.30±0.03b                  |
| DBS-B  | 11.23±1.09a             | 6.37±0.47b                  | 2.12±0.29a              | 0.33±0.04a                  |
| DBS-P  | 13.67±1.79a             | 8.01±0.65a                  | 1.91±0.36a              | 0.24±0.03b                  |

Different lowercase characters in a single column indicate significant difference (p<0.05) between the background (B) and polluted (P) soils in a single site. doi:10.1371/journal.pone.0038858.t005

![Figure 3. Overall relative changes in analyzed microbial parameters under metal pollution to the background soils. The upper, middle and lower hollow circle in a vertical bar represents the maximum, average and minimum changes under pollution to the background in a single parameter, 1 and 2, Microbial biomass carbon and nitrogen; 3 and 4, Ratio of microbial biomass C and N to soil organic carbon; 5, Basal respiration; 6, Metabolic quotient (qCO₂); 7 and 8, Bacterial and fungal CFUs; 9, Ratio of fungal to bacterial CFUs; 10, 11 and 12, Total, bacterial and fungal PLFAs; 13, Ratio of fungal to bacterial PLFAs; 14 and 15, Bacterial and fungal DGGE band numbers; 16, Ratio of fungal to bacterial band numbers; 17 and 18, Bacterial and fungal gene copies; 19, Ratio of fungal to bacterial gene copies. doi:10.1371/journal.pone.0038858.g003](image-url)
Table 6. Bacterial and fungal DGGE band numbers and gene copy numbers (Means ± S.D.) of the soils studied.

| Sample | DGGE band number | Gene copy number |
|--------|------------------|-----------------|
|        |                  | Bacteria | Fungi | F/B | Bacteria (×10^6) | Fungi (×10^6) | F/B (×100) |
| YX-B   | 30±1a            | 25±0a    | 0.84±0.02a | 5.46±1.20a | 14.3±4.4a | 2.68±0.10a |
| YX-P   | 30±2a            | 25±1a    | 0.84±0.02a | 3.80±0.44a | 9.50±3.50a | 2.50±0.87a |
| DX-B   | 35±1a            | 20±1a    | 0.59±0.02a | 0.85±0.14b | 1.35±0.29a | 1.60±0.33a |
| DX-P   | 34±1a            | 14±1b    | 0.41±0.01b | 1.46±0.05a | 0.76±0.09b | 0.52±0.07b |
| DY-B   | 32±1a            | 27±2a    | 0.85±0.06a | 1.16±0.09a | 4.20±1.44a | 3.58±0.95a |
| DY-P   | 29±2a            | 20±2b    | 0.66±0.02b | 0.88±0.08b | 0.81±0.61b | 0.91±0.64b |
| DBS-B  | 32±1a            | 22±1a    | 0.69±0.01a | 0.21±0.16a | 0.48±0.19a | 2.43±0.48a |
| DBS-P  | 34±1a            | 21±1a    | 0.62±0.01b | 0.58±0.10a | 0.67±0.08a | 1.16±0.01b |

F/B represents the ratio of fungi to bacteria. Different lowercase characters in a single column indicate significant difference (p<0.05) between the background (B) and polluted (P) soils in a single site.

doi:10.1371/journal.pone.0038858.t006

Figure 4. Principal component analysis of DGGE profiles of bacterial 16S rRNA (A) and fungal 18S rRNA (B) gene fragments. YX-B and YX-P, Background and polluted soil of site YX; DX-B and DX-P, Background and polluted soil of site DX; DY-B and DY-P, Background and polluted soil of site DY; DBS-B and DBS-P, Background and polluted soil of site DBS, respectively.

doi:10.1371/journal.pone.0038858.g004
Consistent Changes in Microbial Community with Heavy Metal Pollution in the Rice Paddies

In literature, either decrease in the size of the microbial biomass [28,35], or no change in MBC [56], or a reduction in culturable bacterial and fungal population size [16], or a decrease in fungal fatty acids [57], as well as a shifted fungal and bacterial communities [38] had been reported for long term metal polluted croplands. A decrease in culturable fungal population was also seen in the study of Nordgren et al. [59] who found the isolated colonies of culturable fungi reduced in a soil close to a brass mill under coniferous forest. Decrease in fungal to bacterial PLFA ratio was detected in an uncultivated soil under metal pollution from acid mining spill [60], and in forest soils along a metal pollution gradient [17].

However, there had been not yet clear and sound understanding of a well established and coherent change in soil microbial community structure and abundance with heavy metal pollution in fields across land use types of soil and metal pollution status. This could be partially because of the varying effects of heavy metal pollution on soil microbial community with pollution status. The changes in microbial populations or processes under field pollution differed from under spiked treatment [61,62], and those with short term pollution [11–13] differed from with long term pollution [17,57–59]. In addition, the effect could be also subject to change with metal toxicity to microorganisms as affected by soil reaction and pollution history of the sites studied [3]. Co-existence of multiple metal elements with varying eco-toxicities to targeted microbial communities [63,64] would have influences in the overall changes in soil microbial community with metal pollution across sites.

An overall comparison of the changes relative to the background in all the analyzed parameters conducted in this study is shown in Figure 3. Among the total 19 parameters analyzed, 3 exerted a consistent and significant decrease across sites under the pollution, including microbial quotient, fungal CFUs and ratio of fungal to bacterial PLFAs. Meanwhile, 1 parameter of the metabolic quotient ($q_{\text{CO}_2}$) showed a consistent and significant increase under the pollution across the sites. The other parameters such as soil MBC and MBN, fungal PLFAs, fungal DGGE band numbers and ratios of fungal to bacterial band numbers and gene copies showed relatively weak trend of decrease under pollution though the others varied widely with sites. Here it is clearly that the parameters showing consistent changes across the sites are mostly those of or associated with fungal abundance and proportion.

Change in Metabolic Activity and the Coherence to Microbial Community Change Under Pollution

It has been well known that microbial community physiologically links to ecosystem C and N cycling and balance [66,67]. Changes in microbial community composition had been well documented to modify ecosystem processes through their changes in physiological processes and thus could exert an important role in terrestrial ecosystems functioning [68,69]. Changes in fungal abundance and species richness due to disturbances would affect the decomposition of soil organic matter [70]. Six et al. [71] noticed that fungal-dominated soils had slow C turnover rates. The role of such a shift of microbial community composition was also argued in a recent work by Zhou et al. [72] as one of the primary feedback mechanisms through which microorganisms regulated soil carbon dynamics. Changes in C utilization efficiency by soil microorganisms under metal pollution had been already proposed as a well measure of microbial response to disturbance and had been considered a sensitive ecophysiological indicator of heavy metal stressed soil [73].

As the soil bacterial and fungal community structure shifted (Figure 4) and the fungal abundance and band numbers showed a reduced tendency under pollution in the present study, it could have further impacts on SOC turnover and the C utilization efficiency of the rice paddies. Here, the reduction in microbial quotient under pollution was significantly related to the decreased fungal-to-bacterial PLFAs ratio (Figure 5). And the increase in $q_{\text{CO}_2}$ was significantly ($p<0.05$) correlated with the reduction in microbial quotient (Figure 6A), and moderately significantly ($p = 0.10$) correlated with the decreased fungal-to-bacterial PLFAs ratio (Figure 6B) under pollution. Thus, a significant increase in microbial metabolic quotient, an indicator of C utilization efficiency of soil, together with the decline in microbial quotient and fungal to bacterial ratio of extracted PLFAs, implicated a potential impact on soil organic carbon protection and CO$_2$ release under heavy metal pollution. The finding here was in agreement with the observation by Chander and Brookes [74] who reported a lower efficiency of C utilization and higher rates of CO$_2$ evolution from a high-metal soil than from a low-metal one after amending with maize or glucose. Such fact could also be seen in the work by Li [75] who reported significant increases by over 20% both in basal respiration under lab incubation and field CO$_2$ evolution from a rice paddy under heavy metal pollution when compared to the non-polluted counterpart. With the same paddy soil as by Li [75], Zhang et al. [76] observed a destruction of water stable coarse micro-aggregates, which had been believed as a physical protection of soil organic matter in soils [77]. Decreased
C utilization efficiency was also reported by Zhou et al. [78] who conducted a laboratory incubation and CO₂ evolution monitoring with different soils from long-term polluted fields in 6 sites across South China though they failed to show a consistent trend of change in qCO₂ with heavy metal loadings. In contrast, increase in fungal abundance and the fungal to bacterial ratio from culturable colony analysis was found in our previous work in good correlation with the soil organic matter accumulation of rice paddy soils from long term experiment sites across South China [79]. Therefore, a consistently higher metabolic quotient by an extent ranging from 7% to 68% over the polluted ones found in this study could indicate a strong potential impact through the shift with reduction in fungal abundance and proportion of heavy metal pollution on C dynamics and CO₂ production from the rice paddies. Of course, the consequence of such impacts and the mechanism behind in relation to the microbial shift deserves further field monitoring studies.

### Intercomparison of Different Assays for Interpreting Heavy Metal Impact on Microbial Community Shift

Many methods had been available for characterizing environmental stress on soil microbial community structure and activity. Plate counting of culturable microorganisms had been shown to be an effective method for depicting heavy metal pollution [80,81]. PLFA analysis, however, provided phenotypic information of the active microbial community composition [12,82] as well as the microbial biomass [83], being used extensively to address environmental stresses [84,85]. More recently, molecular techniques of PCR-DGGE had been increasing employed for monitoring microbial community and diversity changes under environmental stresses [86]. Furthermore, qPCR in combination with microbial activity and biomass indicators has emerged as a promising method to evaluate the impact of environmental factors on microbial community function. The combination of these methods can provide a more comprehensive understanding of the effects of heavy metal pollution on microbial community structure and function.
with PCR-DGGE had been proven as a molecular fingerprinting measure to track the gross differences and changes in microbial population size and structure with a certain kind of stresses in soils [07].

In this study, multiple microbial biological assays ranging from microbial biomass C and N, to molecular fingerprinting techniques were used. Measurements of microbial quotient, fungal and bacterial population size and the ratio of fungal to bacterial PLFAs yielded consistently great difference between the polluted and background soils across sites by up to over 50%. In contrast, molecular fingerprinting yielded less consistent and significant difference between the polluted and the background soils (Figure 3). Again, neither the absolute contents of microbial biomass C and N, nor the abundance and DGGE bands of bacteria with each of the methods employed succeeded to characterize the microbial changes with metal pollution in this study. Whereas, a ratio of fungal to bacterial from almost all the measurements together with qCO2 indicator proved valid to portray the changes in microbial community shift under heavy metal pollution. Therefore, combining the traditional culture-dependent method and the advanced PLFA fingerprinting approach could be employed as a tool for well identifying the changes in soil microbial communities characterized by a reduction in fungal abundance and proportion in soils under heavy metal pollution despite of the inherent bias of each method [08]. It is the authors’ suggestion that use of multiple biological assays by the means of comparison between the polluted and background soils across sites could be a sound methodology for studying microbial community structure shift under environmental stress in croplands.

Conclusions

The present study, by using multiple microbial biological assays including measurement of microbial carbon and nitrogen, plate counting of culturable colonies, PLFA analysis, DGGE profile and qPCR along with soil basal respiration measurement under lab incubation, revealed a consistent change in soil microbial community and metabolic activity under heavy metal pollution of rice paddies across South China. These changes could be characterized by a decline in abundance of overall microbial community and specifically of fungi, in fungal to bacterial ratio as well as an increase in metabolic quotient. Thus, heavy metal pollution could exert significant impact on soil biochemical process related to C metabolism and CO2 evolution from rice paddies. The measurements of soil microbial abundance, population size of culturable colonies as well as extractable PLFAs proved valid to portray the above-mentioned changes under pollution and ratio parameters of microbial quotient, of fungal to bacterial and qCO2 are better indicators for characterizing heavy metal impacts on soil microbial community structure and activity shift with heavy metal pollution in soils. However, the potential impact of heavy metal pollution on soil C cycling would be a critical issue for environmental studies in the near future as the area of heavy metal pollution of rice paddies had been extending in China.

Supporting Information

Figure S1 The PLFA profiles of the soils studied. Blank, background soil; Shaded, polluted soil. (DOC)

Figure S2 DGGE profiles of amplified bacterial 16S rRNA (A) and fungal 18S rRNA (B) gene fragments. YX-B and YX-P, Background and polluted soil of site YX; DX-B and DX-P, Background and polluted soil of site DX; DY-B and DY-P, Background and polluted soil of site DY; DBS-B and DBS-P, Background and polluted soil of site DBS, respectively. M, Marker. White asterisks highlight bands which intensified or only existed in the profiles of background soils; whereas, white rectangles highlight those which intensified or only existed in the polluted counterparts in a single site. (DOC)

Figure S3 Soil basal respiration course under lab incubation at constantly 25°C of the soils studied. Blank, background soil; Shaded, polluted soil. (DOC)

Acknowledgments

The authors are grateful to Woody Smith, Stephen Qi, Rosario Baez Pineda and Sara Jo Dickens for their technical assistance in PLFA analysis at the UC Riverside, USA.

Author Contributions

Conceived and designed the experiments: GP YL DC. Performed the experiments: YL TZ DL QH. Analyzed the data: YL GP DC LL JWZ JFZ QH XZ. Contributed reagents/materials/analysis tools: DC JFZ XY JFZ.

Wrote the paper: YL GP DC.

References

1. Young M, Crawford JW (2004) Interactions and self-organization in the soil-microbe complex. Science 304: 1634–1637.
2. Weber O, Scholz RW, Buhlmann R, Grasmick D (2001) Risk Perception of Heavy Metal Soil Contamination and Attitudes toward Decontamination Strategies. Risk Anal 21: 967–977.
3. Giller KE, Winder E, McGrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol Biochem 30: 1309–1414.
4. Broos K, Martens J, Smolders E (2005) Toxicity of heavy metals in soil assessed with various soil microbial and plant growth assays: A comparative study. Environ Toxicol Chem 24: 634–640.
5. Gao Y, Zhou P, Mao L, Zhi Y, Zhang C, et al. (2010) Effects of plant species coexistence on soil enzyme activities and soil microbial community structure under Cd and Pb combined pollution. J Environ Sci 22: 1040–1048.
6. Harris-Hellal J, Vallaeys T, Garnier-Zarli E, Boissierhine N (2009) Effects of heavy metals on microorganisms and microbial processes in agricultural soils: A review. Soil Biol Biochem 30: 1309–1414.
7. Khan S, Heham AE, Qiao M, Rehman S, He JZ (2010) Effects of Cd and Pb on soil microbial community structure and activities. Environ Sci Pollut Res 17: 289–296.
8. Li Z, Xu J, Tang C, Wu J, Muhammad A, et al. (2006) Application of 16S rDNA-PCR amplification and DGGE fingerprinting for detection of shift in microbial community diversity in Cu-, Zn-, and Cd-contaminated paddy soils. Chemosphere 62: 1374–1380.
9. Srb-Kovács T (2003) Effect of some metal salts on the cultivable part of soil microbial assemblage in a calcareous loam cropland 6 years after contamination. Acta Biologica Szegedienis 52: 201–204.
10. Wakelin S, Chu G, Lardner R, Liang Y, McLaughlin M (2010) A single application of Cu to field soil has long-term effects on bacterial community structure, diversity, and soil processes. Pedobiologia 53: 149–158.
11. Rajapaksha RMCP, Tobor-Kaplon MA, Ba˚a˚th E (1994) Metal toxicity affects fungal and bacterial activities in soil differently. Appl Environ Microbiol 5: 2966–2973.
12. Frostegård Å, Báth és, Tunäid A (1995) Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. Soil Biol Biochem 27: 723–730.
13. Frostegård Å, Tunäid A, Báth és (1996) Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. Soil Biol Biochem 28: 55–63.
14. Kelly J, Haggbloom M, Tate R (1999) Changes in soil microbial communities over time resulting from one time application of zinc: a laboratory microcosm study. Soil Biol Biochem 31: 1455–1465.
15. Sverdrup LE, Linjordet R, Stromman G, Hagen SB, van Gestel CAM, et al. (2006) Functional and community-level soil microbial responses to zinc addition may depend on test system biocomplexity. Chemosphere 65: 1747–1754.
16. Oliveira A, Pampulha EM (2006) Effects of long-term heavy metal contamination on soil microbial characteristics. J Biosoil Bioeng 102: 157–161.

17. Pennanen T, Frostegard A, Fritzle H, Baath E (1996) Phospholipid fatty acid composition and heavy metal tolerance of soil microbial communities along a heavy metal polluted gradient in coniferous forests. Appl Environ Microbiol 62: 420–428.

18. Zhang Y, Dai J, Wang R, Zhang J (2008) Effects of long-term sewage irrigation on agricultural soil microbial structural and functional characteristics in Nanjing, China. Euro J Soil Biol 44: 41–51.

19. China Industrial Economic Information Network. Available: http://www.cinric.org.cn/site951/cjyj/2011-10-27/508741.shtml Accessed 2011 Oct 27 (in Chinese).

20. Wu X, Pan G, Li L (2006) Study on soil quality change in the Yangtze River Delta. Geography Geo-Information Sci 22: 81–91 (in Chinese with English summary).

21. Hang X, Wang H, Zhou J, Ma C, Du C, et al. (2009) Risk assessment of potentially toxic element pollution in soils and rice (Oryza sativa) in a typical area of the Yangtze River Delta. Environ Pollut 157: 2542–2549.

22. Ma J, Pan G, Wan H, Xia Y, Luo W (2004) Investigation on heavy metal pollution in a typical area of the Pearl River Delta. Chin J Soil Sci 35: 636–638 (in Chinese with English summary).

23. Wu Y, Chen T, Kong Q (1986) Heavy metals pollution and control of agricultural soils in China. Chin J Soil Sci 4: 117–119 (in Chinese with English summary).

24. Xu X, Xiu B, Qin J, He S, Li H, et al. (2007) Analysis and evaluation on heavy metal contamination in paddy soils in the lower stream of Dabashan Area, Guangdong Province. J Agro-Enviro Sci 26: 549–553 (in Chinese with English summary).

25. Yao H, Xu J, Huang C (2005) Substrate utilization pattern, biomass and activity of microbial communities in a sequence of heavy metal-polluted paddy soils. Geo-derma 115: 139–146.

26. Yan S, Pan G, Li L (2008) Decline of microbial biomass quotient and change in microbial PLFA community structure of a rice paddy soil under heavy metal pollution - A case study of a polluted rice paddy from southern Jiangsu, China. Environ Geol 17: 1828–1832 (in Chinese with English summary).

27. Wang Y, Shi J, Wang H, Lin Q, Chen X, et al. (2007) The influence of soil heavy metal pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. Ecotoxicol Environ Saf 67: 75–81.

28. Li Y, Rouland C, Benedetti M, Li F, Pando A, et al. (2009) Microbial biomass, enzyme activity and mineralization activity in soils related to soil organic C, N and C:N turnover influenced by acid metal stress. Soil Biol Biochem 41: 969–977.

29. Zhou S, Liao F, Wu S, Ren K, Zhang H, et al. (2008) Farmland soil heavy metal pollution in typical areas of Jiangsu Province based on classification sample plots, Tran CAIE 24: 82–83 (in Chinese with English summary).

30. Xu W, Zhang W (2004) Analysis of acid mine drainage pollution in Daxing Copper Mine, Jiangxi Chemical Industry 1: 81–90 (in Chinese with English summary).

31. Fang H, Liu Z, Yang G, Chen K, Hu X, et al. (2007) Study on natural plants growing on the tailing of Tungsten Mine in Dayu County, Jiangxi Science, 25: 593–597 (in Chinese with English summary).

32. Zhou J, Dang Z, Cai M, Liu C (2007) Soil heavy metal pollution around the Dabaoshan Mine, Guangdong Province, China. Pedosphere 17: 588–594.

33. Zhang LY, Li LQ, Pan GX (2009) Variation of Cd, Zn and Se contents of growing on the tailing of Tungsten Mine in Dayu County. Jiangxi Science, 25: 749–755 (in Chinese with English summary).

34. Lu R (2000) Methods of Soil and Agrochemical Aanalysis. China Agricultural Science and Technology Press, Beijing (in Chinese).

35. Zhang LY, Li LQ, Pan GX (2009) Variation of Cd, Zn and Se contents of growing on the tailing of Tungsten Mine in Dayu County. Jiangxi Science, 25: 749–755 (in Chinese with English summary).

36. Zhou J, Xue K, Xie J, Deng Y, Wu L, et al. (2012) Microbial mediation of Pollution Reduced Microbial Abundance & F/B Ratio.

37. Bremner JM, Mulvaney CS (1982) Nitrogen total. Methods of Soil Analysis. In: Bigham J, ed. Soil Science Society of America, Madison, Wisconsin, 595–624.

38. Brookes PC, Kragt JF, Powlson DS, Jenkinson DS (1985) Chloroform digestion and determination of microbial biomass and turnover from bulk samples and particle size fractions of a typical paddy soil. J Agro-Environ Sci 26: 93–102.

39. Sanguinetti CJ, Dias NE, Simpson AJG (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17: 915–919.

40. Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol 71: 4117–4120.

41. Zheng J, Zhang X, Li L, Zhang P, Pan G (2007) Effect of long-term fertilization on C mineralization and production of CH4 and CO2 under anaerobic incubation from bulk sample particle size fractions of a typical paddy soil. J Agro-Ecology Environ 120: 129–138.

42. Abaye DA, Lawlor K, Hirsch PR, Brookes PC (2005) Changes in the microbial community of an arable soil caused by long-term metal contamination. Eur J Soil Biol 39: 75–81.

43. Muyzer G, Waal ECD, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695–700.

44. MacNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ, et al. (1999) Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 65: 3566–3574.

45. Zelles L, Bau QY, Rackwitz R, Chadwick D, Beece F (1995) Determination of phospholipid- and lipopolysaccharide-derived fatty acids as an estimate of microbial biomass and community structures in soils. Biol Fertil Soils 19: 115–123.

46. Federle T W (1986) Microbial distribution in the soil-new techniques. In: Megusar F, Gantar M (Eds.), Perspectives in Microbial Ecology. Slovene Society for Microbiology, Ljubljana, 493–496.

47. Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol 71: 4117–4120.

48. Sanguinetti CJ, Dias NE, Simpson AJG (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17: 915–919.

49. Fierer N, Jackson JA, Vilgalys R, Jackson RB (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol 71: 4117–4120.

50. Sanguinetti CJ, Dias NE, Simpson AJG (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17: 915–919.

51. Muyzer G, Waal ECD, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59: 695–700.

52. Sanguinetti CJ, Dias NE, Simpson AJG (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17: 915–919.
73. Wardle DA, Ghani A (1995) A critique of the microbial metabolic quotient \((q\text{CO}_2)\) as a bioindicator of disturbance and ecosystem development. Soil Biol Biochem 27: 1601–1610.

74. Chander K, Brookes PC (1991) Microbial biomass dynamics during the decomposition of glucose and maize in metal-contaminated and non-contaminated soils. Soil Biol Biochem 23: 917–925.

75. Li ZP (2009) Impacts of land use change and heavy metal pollution on soil respiration and organic carbon reduction from paddy soil. 29–37. Ph D thesis, Nanjing Agricultural University, Nanjing, China (in Chinese with English summary).

76. Zhang L, Li L, Pan G, Cui L, Li H, et al. (2009) Effects of heavy metals pollution on paddy soil aggregates composition and heavy metals distribution. Chin J Appl Ecol. 20: 2806–2812 (in Chinese with English summary).

77. Six J, Conant RT, Paul EA, Paustian K (2002) Stabilization mechanisms of soil organic matter: Implications for C-saturation of soils. Plant Soil 241: 155–176.

78. Zhou T, Pan G, Li L, Chang S (2009) Effects of Heavy Metals on Soil Respiration and Microbial Indices in Paddy Field of South China. J Agro-Environ Sci 28: 2568–2573 (in Chinese with English summary).

79. Liu D, Liu X, Liu Y, Li L, Pan G, et al. (2011) Soil organic carbon (SOC) accumulation in rice paddy fields under long-term agro-ecosystem experiments in southern China - VI. Changes in microbial community structure and respiratory activity. Biogosciences Discuss 8: 1529–1554.

80. Ellis RJ, Morgan P, Weightman AJ, Fry JC (2003) Cultivation-dependent and independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. Appl Environ Microbiol 69: 3223–3230.

81. Ellis RJ, Neish B, Trett MW, Best JG, Weightman AJ, et al. (2003) Comparison of microbial and meiofaunal community analyses for determining impact of heavy metal contamination. J Microbiol Methods 45: 171–185.

82. Tunlid A, White DC (1992) Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In: Stotzky G, Bollag JM (Eds.), Soil Biochemistry, Vol. 7. Marcel Dekker, Inc., New York, Basel, Hong Kong, 229–262.

83. Joergensen RG, Emmerling C (2006) Human impact on soil organisms and on current measurement methods. J Plant Nutr Soil Sc 169: 293–309.

84. Holland K (2002) Soil microbial community structure in relation to vegetation management on former agricultural land. Soil Biol Biochem 34: 1299–1307.

85. McKinley VL, Peacock AD, White DC (2005) Microbial community PLFA and PHB responses to ecosystem restoration in tallgrass prairie soil. Soil Biol Biochem 37: 946–1958.

86. Mette HN, Neils BR (2002) Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria. J Microbiol Methods 50: 189–203.

87. Ahn JH, Kim YJ, Kim T, Song HG, Kang CH, et al. (2009) Quantitative improvement of 16S rDNA DGGE analysis for soil bacterial community using real-time PCR. J Microbiol Methods 78: 216–222.

88. Gremion F, Chatzinotas A, Kaufmann K, Von Sigler W, Harms H (2004) Impacts of heavy metal contamination and phytoremediation on a microbial community during a twelve-month microcosm experiment. FEMS Microbiol Ecol 48: 273–283.