Bioremediation of lead-contaminated paddy field by using ramie, agrobacterium and organic matter

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Abstract. Lead (Pb) contamination is one of environmental issue because of its great influence on human health and ecological system. Paddy fields in Karanganyar, Indonesia are indicated to be contaminated by Pb, due to many local industries that dispose their effluents in local water ways. This research aimed to investigate the influence of combined application of Ramie (Boehmeria nivea)-Chelator-Inorganic Fertilizers on the change of Pb level in soil. The experiment was arranged in experimental factorial design using Randomized Complete Block Design as the based design, consist of three factors and three replications. First factor was Inorganic fertilizer consist of without inorganic fertilizers (p0) and with inorganic fertilizers (p1); second factor was chelator (B) consist of without chelator (b0), chelator of Agrobacterium sp. 1; (b1) and chelator of compost (b2); the last factor was Ramie (T) consist of without ramie (t0) and with ramie application (t1). The main parameter observed in this research was effectivity of phytoremediation and Pb levels in soil and in plant shoot and root. Data was analyzed by statistical analysis using ANOVA continued by DMRT, T-Test and Correlation Test. The results showed that interaction between inorganic fertilizers, chelator and ramie clearly enhanced total bacterial colonies. Ramie was able to decreased Pb level in soils up to 29.51%. Agrobacterium sp. 1; increased effectivity of Ramie in decreasing Pb level in soil. The phytoremediation effectivity increased up to 41.82% by increasing roots Pb uptake (9.78 µg) and shoots Pb uptake (25.97 µg). Compost decreased roots Pb uptake (1.74 µg) and shoots Pb uptake (14.85 µg).

1. Introduction
Karanganyar has considerable potential for agriculture with almost 30% of the area is paddy fields which produce more than 350.000 tons per year [1]. However, due to many local industries, mostly textile, that dispose effluents to rivers or other water bodies, this agricultural area indicated to be contaminated by heavy metals [2]. According to Jaishree and Khan [3], textile industry waste contains Lead (Pb), Fe, Cu, Cd, Zn, Ni and Cr. Lead may induce neurological damage, lower IQ and kidney disease for human being [4]. According to Indonesian Government Regulation No 101 in 2014, the maximum permitted level of Pb allowed for agricultural area is 3 mg/L. Lead in plants could limit photosynthesis, disturb mineral nutrition and water balance, change hormonal status, and influence membrane structure and permeability [5]. Maximum concentration of Pb level in some substances is vary, depend to the type. For example, maximum Pb level in leafy vegetables, legumes vegetables, cereal grains, natural mineral waters are 0.3 mg/kg, 0.2 mg/kg, 0.2 mg/kg, and 0.01 mg/kg respectively [6].

Phyto remediation appeared to have great potential to remove Pb either soil or water by using plants to absorb it. [7]. Phytoremediator plants with high economic value, will give benefits, both for environment and farmer. One of phytoremediator plant with high economic value is Ramie
(Boehmerianivea). Wang [8] said that ramie also has high economic value because the fiber can be used as raw material for woven crafts and it was known that the quality of fiber did not decrease when the heavy metal content increases in its plant tissue. Ramie could be useful for various things, such as raw material for textile, paper, compost, and fodder [9].

There have been several attempts to improve the effectiveness of phytoremediation. One of the efforts made is using chelator and inorganic fertilizers. Chelators that have been used were inoculum from Agrobacterium sp. I3 and compost. Agrobacterium sp. I3 is known to be able to reduce chromium metal pollutant from soil [10]. Agrobacterium sp. I3 released sideroforhydroxamate which could increase metal mobility which could enhance the absorption of Pb by the plant.

Fertilizer could improve the effectiveness of phytoremediation by supporting plant's growth. This research aimed to verify the influence of combined phytoremediation (Ramie-Chelator-Inorganic Fertilizers) to contaminated paddy fields on changes of Pb level in soil and ramie.

2. Materials and Methods

The field experiment was conducted in the paddy field in Waru, Kebakkramat, Karanganyar, Central Java, Indonesia, from June to August 2016. The experiment site was located in tropical region at 7°30’36.4” S and 110°54’21.4” E. Laboratory analysis were conducted at Faculty of Agriculture, Sebelas Maret University, Surakarta, and Integrated Laboratory of Indonesian Environmental Agriculture Research Institute in Pati, Central Java.

2.1. Materials

Materials used were 5 weeks old ramie, inoculum Agrobacterium sp. I3 isolated from contaminated soil by the first author, compost (relatively small amount of macro and micro mineral nutrient such as N, P, K, Ca, Mg, Zn, Cu, B, Mo and Si), NPK fertilizer, Luria Bertani (LB) broth and agar, carrier for Agrobacterium sp. I3 (compost + rice bran + effective microorganism), medium and chemicals for laboratory analysis.

2.2. Methods

The field experiment was arranged in factorial Randomized Completely Block Design. Consist of three factors and three replications. First factor was inorganic fertilizers (P): without inorganic fertilizers (p0) and with inorganic fertilizers (p1); second factor was chelator (B): without chelator (b0), Agrobacterium sp. I3 (b1) and compost (b2); the last factor was ramie (T): without ramie (t0) and with ramie (t1). The parameters in this research was Pb levels in soils, phytoremediation effectiveness, Pb levels in plants shoot and root and Pb uptake in shoots and root, soil acidity (pH), total organic carbon, cation exchange capacity (CEC), total bacteria in rhizosphere and ramie biomass were also conducted.

In one plot it will contain 25 sub-plots. Each sub-plot was planted with 2 ramie seedlings. Upkeep was done by regulating water irrigation, weeding the grass. 5 Samples were randomly selected in each plot.

Application of Agrobacterium sp. I3 that was conducted 2 weeks after planting, harvesting that was conducted 30 days after application of chelator of Agrobacterium sp I3, and laboratory analysis.

The scaling up of Agrobacterium sp. I3 was carried by using LB Broth (g of yeast extract, 10 g of proteose peptone, 5 g of NaCl, and 1000 mL of distilled water) until it reached density 10⁹ cfu/mL. Carrier was made by mixing 15kg compost, 7,5kg rice bran, 750ml effective microorganism and 15 L water. Application Agrobacterium sp. I3 on carrier was by spraying and mixing 600 mL of Agrobacterium sp. I3 for every 2 kg carrier and incubated for 3 days. Application of Agrobacterium sp. I3 was carried out on after 2 weeks of planting by spreading around top soil.

Inorganic fertilizer used was NPK fertilizer. The dosage of fertilizer used was based on Sudjindro et al., (2005) 130,4 kg/ha of urea, 55,5 kg/ha of super phosphate SP-36, 50 kg/ha of potassium chloride (KCl). Dosage for compost was 10 ton/ha [9]. The application of NPK fertilizer was done a day before planting.
Soil characteristics including soil pH (potentiometric method), Cation Capacity Exchange / CEC (ammonium treatment method), Soil Organic Matter (Walkley and Black method), Population of bacteria *Rhizobium* sp. *I3* (Plate Count method) and the level of lead (wet destruction method followed by reading with AAS).

Plant characteristic including plant height and dry weight (including root and shoot), lead content and lead absorption in plant (wet destruction method followed by reading the metal content using AAS).

Data was analyzed with ANOVA at 95% confidence levels, followed by Duncan's Multiple Range Test or/and Independent Sample T Test if the F-test was significant. Relationship between the variables was tested with correlation test.

3. Result

### Table 1. Soil Characteristics

| pH       | Organic-C (%) | CEC (me/100 g) | Total bacterial colonies (Log 10 CFU/g) | Pb level (mg/kg) | Phytoremediation effectiveness (%) |
|----------|---------------|----------------|----------------------------------------|------------------|-------------------------------------|
| Before Treatments | 7.55 | 3.31 | 19.61 | 12.62 | 12.20 | - |
| Treatments | | | | | | |
| P0B0T0 | 6.96 | 2.91a | 30.22 | 12.65ab | 9.13 | 25.20 |
| P0B0T1 | 6.82 | 3.52b | 30.94 | 12.75abc | 8.60 | 29.51 |
| P0B1T0 | 6.76 | 3.28a | 32.25 | 15.59d | 7.20 | 40.97 |
| P0B1T1 | 6.76 | 3.93b | 22.32 | 19.03e | 7.10 | 41.82 |
| P0B2T0 | 6.88 | 3.11a | 22.40 | 11.82a | 9.05 | 25.85 |
| P0B2T1 | 6.88 | 4.57b | 30.16 | 13.06abc | 9.69 | 20.60 |
| P1B0T0 | 6.59 | 2.85a | 29.98 | 14.54bcd | 9.36 | 23.34 |
| P1B0T1 | 6.93 | 4.25b | 20.29 | 14.98cd | 7.96 | 34.81 |
| P1B1T0 | 6.88 | 3.47a | 25.73 | 13.80abcd | 7.20 | 41.02 |
| P1B1T1 | 6.75 | 3.28b | 27.48 | 12.70abc | 8.36 | 31.46 |
| P1B2T0 | 6.69 | 3.23a | 26.79 | 12.59ab | 8.28 | 32.12 |
| P1B2T1 | 6.66 | 3.34b | 24.65 | 15.69d | 9.13 | 25.21 |

Note: Numbers followed by the same letter at the same column show no significant different in DMRT test at 5% level; *Tabel 1 showed that pH decrease after treatment. The highest decrease of pH was in the treatment with inorganic fertilizer. Soil C-organic changed after treatment with the highest C-organic in soil without inorganic fertilizer, with compost and with ramie (P0B2T1). Soil CEC increased after treatment; the highest CEC was in soil without inorganic fertilizer, with *Agrobacterium* sp. *I3* and without ramie (P0B1T0). Most of total bacterial colonies increased after treatment. The highest total bacterial, the combination of without inorganic fertilizer, with *Agrobacterium* sp. *I3* and with ramie (P0B1T1). Soil Pb level decreased in all treatment with the highest decrease in soil without inorganic fertilizer, with *Agrobacterium* sp. *I3* and with ramie (P0B1T1) with 41.82%.
Table 2. The result of plant analysis

|                | Biomass (gr) | Pb Levels (µg/g) | Pb Uptake (µg) |
|----------------|--------------|-----------------|----------------|
|                | Roots | Shoots | Total | Roots | Shoots | Total | Roots | Shoots | Total |
| P0B0T1         | 0.19   | 3.45   | 3.65a | 6.25  | 3.39   | 3.53  | 1.20  | 11.70  | 12.90 |
| P0B1T1         | 1.55   | 7.85b  | 9.40c | 6.12  | 3.36   | 3.83  | 9.65  | 25.99  | 35.64 |
| P0B2T1         | 0.20   | 3.85a  | 4.05a | 2.53  | 2.68   | 2.68  | 0.52  | 10.37  | 10.89 |
| P1B0T1         | 0.32   | 5.91b  | 6.22b | 6.30  | 3.30   | 3.50  | 2.37  | 19.47  | 21.84 |
| P1B1T1         | 1.35   | 7.04b  | 8.39bc| 7.45  | 3.69   | 4.30  | 9.91  | 25.95  | 35.86 |
| P1B2T1         | 0.54   | 6.40b  | 6.94bc| 5.48  | 3.02   | 3.21  | 2.97  | 19.33  | 22.30 |

Source: Author research (primer)
Note: Numbers followed by the same letter at the same column show no significant different in DMRT test at 5% level

Table 3. The influence of chelator to Pb level and Pb uptake

|                | Pb level (µg/g) | Pb uptake (µg) |
|----------------|-----------------|----------------|
|                | Root | Shoot | Root | Shoot |
| B0             | 6.28 b  | 3.34 b | 1.79 a | 15.59 a |
| B1             | 6.78 b  | 3.53 b | 9.78 b | 25.97 b |
| B2             | 4.01 a  | 2.85 a | 1.74 a | 14.85 a |

Source: Author research (primer)
Note: Numbers followed by the same letter at the same column show no significant different in DMRT test at 5% level

From Duncan’s Multiple Range Test was found that Pb level in plant and Pb uptake by plant affected by chelator (Table 3). Pb level both in root and shoot was found highest with Agrobacterium sp. I3 (B1) treatment. This also happened with Pb uptake by plant. The highest Pb uptake both in root and shoot also found with Agrobacterium sp. I3(B1) treatment.

4. Discussion

4.1. Soil Pb and Soil Properties
The research sites (before the experiment conducted) had alkaline condition, caused by pollutant from industrial textile waste. The wastewater from the textile industry has a pH ranging from 7.45-9.43 [3]. Soil pH is an important parameter to be analyzed since it determines the solubility of soil minerals such as heavy metals and nutrients [11]. According to [12] high pH soil allows the dominant metal ion exchange due to rainfall so that rain significantly affects the process of displacement or decrease in metal concentration.

There is a treatment that can reduce soil pH, soil pH decreases from 7.55 to 6. According to Vašák et al., [13] the addition of NPK fertilizer decreased soil pH up to 0.89 because of Nitrogen contained fertilizers cause soil acidification [14]. The result showed that the treatment did not significantly affect soil pH (p> 0.05) – However, this experiment showed that soil alkalinity was neutralized. NPK application might decreased soil pH. In the intensive used of NPK, the soil pH will be lowered and induce the metal uptake.
Organic-C level before treatments were high (3.31%) since after harvesting the paddy, organic matter such as residual crops was left on or amended into soil. Fertilizer application by combining inorganic fertilizer and organic fertilizer significantly increased the Organic-C soil [8] and [15].

Cation Exchange Capacity (CEC) illustrated the exchange of positively charged cation or ions on the negatively charged colloidal surfaces of the soil [16]. P0B1T0 (without inorganic fertilizer + Agrobacterium + Without Ramie) had the highest CEC. According to Eruola et al., [17], CEC will increase along with the decreasing of Pb levels in soils. Results indicated that the treatments did not show significantly change the CEC (p> 0.05).

Analysis of total bacterial count reflected the abundance of indigenous bacteria in soil. The result showed the total number of bacterial was 0.6 x 10^{12} CFU/g. According to Lenart and Koladka [18] the presence of heavy metal contamination in soil did not decrease the amount of bacteria, Actinomycetes, and fungi. Lenart and Koladka [18] reported that in soils contaminated by Zn, Cd and Pb still found mesophylic bacteria and fungus and Actinomycetes.

The highest total bacterial count was shown in P0B1T1 (without inorganic fertilizer + Agrobacterium + Ramie) (0.3 x 10^{10} CFU/g). Miura et al., [19] described that bacteria show a response to the application of nitrogen fertilizer since the nutrients needed by bacteria and plant to grow are same. Based on the analysis of total bacterial colonies in the initial soil analysis, it is known that the population of bacteria on soil in the experimental site is quite high (i.e. 12.62 Log 10 CFU/g). The high population of bacteria on the soil indicates adequate nutrition of the bacteria, in other words the nutrient content on the soil is high enough so that the basic fertilizer in the experiment did not clearly influence the total number of bacteria in soil. Inoculation of Agrobacterium sp. I increased the population of bacteria in the soil and ramie increased bacterial population because its plant roots produce exudate in the form of organic acid as the source of carbon and nitrogen needed by bacteria [20].

Contamination of heavy metals such as Pb is accumulative that continuous exposure of wastewater containing Pb will increase Pb level in soil if there is no efforts to reduce it. Results showed that the chelator has a significant effect (p<0.05) on the soil Pb level. The lowest soil Pb level was in the inoculum Agrobacterium sp. I. It means that inoculation of Agrobacterium sp. I has the highest effectiveness in reducing Pb in soil.

According to Rosariastuti et al., [10], Agrobacterium sp. I can significantly reduce heavy metal such as Cr. Plant combined with bacterial showed a great potential in phytoremediation than by using plants only [21] [22] [23]. According to correlation test, there was significantly negative correlation between soil Pb levels and total bacterial colonies (r=-0.455).

The highest decreased of soil Pb level was P0B1T1 since soil with those treatments had high of either population of bacteria or organic-C. High organic carbon means that soil has high CEC which retain (via ionic bonding) the metal in the surface of organic colloid. This mechanism lowering metal uptake. The high level of organic-C may lead to organic-metals complex forms so that Pb is more easily absorbed by plants. While, the P0B1T1 treatment showed the neutral pH and CEC that was moderate sufficiently supported phytoremediation.

4.2. Lead Level in Plant Analysis

The greatest plant biomass, 9.4 g, was shown by the treatment of ramie without addition of inorganic fertilizers and chelator of Agrobacterium sp. I (P1B1T1) while ramie biomass in control treatment (P0B0T1) was the lowest 3.65g. Inoculation of Agrobacterium sp. I could decrease heavy metals in soil so that supporting plant growth [10]. In this study, inoculation of Agrobacterium sp. I had the highest effectiveness in reducing soil Pb This acid can oxidize Pb^{2+} (not available) becomes Pb^{4+} (available for plants). Ramie even without the combination of inorganic fertilizer and chelator can still uptake lead metal in lead contaminated soil. Plants can release phytochelatin substances, so far phytochelatin is only found if an environment around the plant experiences excess heavy metals. Phytocelatin can help detoxify heavy metals and increase their uptake into plants [24].

Lead level in shoots was higher than Pb levels in roots (Table 2). Ramie roots was able to uptake Pb up to 12,90 µg per plant. Pb uptake of Ramie in control treatment was greater than in Ramie with chelator
of compost treatment (P0B2T1). Lead uptake in shoots was higher than roots. It means that Ramie able to translocate Pb from roots to shoots. The ANOVA showed that chelator was significantly affect (p<0.05) Pb level and Pb uptake in plants.

Table 3 showed that the chelator of compost treatment showed the lowest of Pb level in plants either in roots or shoots. Compost precisely inhibit the Pb absorption by the plants, so that it was also impacted on Pb uptake in plants. Based on the Table 3, the lowest Pb uptake was shown by the chelator of compost.

According to Huang and Schnitzer (1997), organic matter contains a negative charge that binds heavy metals, compost contains fulvic acid consisting of phenolic OH and COOH group which could bind metals and form a stable organic-metal bond. A stable organic-metal bond caused the metal to become unavailable to the plant. Agrobacterium sp. I3 released sideroforhydroxamate which could increase metal mobility which could enhance the absorption of Pb by the plant.

5. Conclusion
Inorganic fertilizer, Agrobacterium sp. I3 or compost and ramie increased Organic-C level, CEC and total bacteria colony in soil, but decreased soil pH and Pb. Application of organic matter decrease Pb soil level. Soil Pb in this experiment include Pb in organic matter (chelator), the important role of organic matter in phytoremediation is preventing heavy metal uptake by roots and not decreasing soil Pb since Pb is still in soil after “chelated” by organic matter.

Inorganic fertilizer, Agrobacterium sp. I3 and ramie could increase plant biomass, Pb level in plant and Pb uptake by plant. Application of chelator could increase Pb level in plant and Pb uptake by plant, especially Agrobacterium sp. I3.

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