Philippine Journal of Science
146 (3): 331-338, September 2017
ISSN 0031 - 7683
Date Received: 20 Oct 2016

Bioavailability and Accumulation Assessment of Copper in *Pityrogramma calomelanos*

Joshua Karl A. Dahilan and **Juliet Q. Dalagan**

Xavier University-Ateneo de Cagayan
Corrales Avenue, Cagayan de Oro City, 9000 Philippines

Bioavailability and hyperaccumulation of copper (Cu) in *Pityrogramma calomelanos* was evaluated using sequential extraction technique (SET) and atomic absorption spectrophotometry (AAS). Bioaccumulation factor (BAF) was found to be greater than 1 which means that *P. calomelanos* is a metallophyte, a plant capable of accumulating metals into its roots and shoots. Translocation factor (TF) which was less than 1 signified that *P. calomelanos* is a possible excluder, a plant that prevents metal transport to the plant shoots. The highest Cu uptake in the fronds was 821.60 $mg_{Cu}/kg_{\text{dry weight}}$ indicating that the plant is not a hyperaccumulator. Fourier transform infrared (FTIR) spectrum of the soil, above and below ground parts of the plant revealed shifting of the absorption bands which is indicative of the interaction of Cu with the functional groups present in the plant and soil. FTIR spectra of above and below ground parts of the plant showed the interaction of Cu with the O-H group of the carboxylic acid at 2973 cm^{-1}, Cu with C=O group at about 1639 cm^{-1} and Cu with C-H group at 1162 cm^{-1}. FTIR spectra of the soil illustrated the attachment of Cu to soil minerals by the emergence of the 1033 cm^{-1} peak. Bioavailable Cu through SET analysis revealed 430.70 mg_{Cu}/kg_{soil} of soluble and exchangeable Cu, 380.67 mg_{Cu}/kg_{soil} of Cu bound to carbonates and 425.97 mg_{Cu}/kg_{soil} of Cu bound to iron and manganese oxides.

Key words: accumulation assessment, bioaccumulation factor, bioavailability, copper, hyperaccumulator, *Pityrogramma calomelanos,* translocation factor

INTRODUCTION

Copper (Cu) is a metal that has many applications in human society from power generation and transmission to electronic product manufacturing, and to the production of industrial machinery and transportation vehicles. Copper is also an essential trace mineral and enzyme co-factor for oxidases such as cytochrome c oxidase, superoxide dismutase and tyrosinases and is important for both physical and mental health of organisms, but it can be toxic at high levels. Cu accumulates in the environment in its free state, eventually reaching concentrations hazardous to health as it generates reactive oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radicals that damage proteins, lipids and DNA (Das et al. 2013; Rascio and Navari-izzo 2011).

Most copper is mined and its high concentration in mining sites is a pressing concern today. It is abundantly mined in the Philippines from the mining sites located in Surigao and Manila City. The Mines and Geosciences Bureau of the Philippines, Philippine Metallic Mineral Production Statistics as of March 2014 showed that 376,106 dry metric tons (dmt) of copper (Cu) concentrate were produced in 2013 (Republic of the Philippines Department of Environment and Natural Resources - Mines and

*Corresponding author: jdalagan@xu.edu.ph

Geosciences Bureau). Copper is a metal that usually leaches off from mining sites into households and other infrastructures through wastewaters and contamination. High Cu soil contamination can result in health hazards and environmental destruction attributed to its high toxicity (Yang et al. 2002).

The solubility of Cu in the soil environment is mainly contributed by Cu^{2+} ion. Copper bound to organic matter (OM) are known to significantly reduce bioavailability and mobility in organic soils hindering a plants' ability to take up Cu due to the formation of stable Cu-OM chelates using carbon and nitrogen groups like –COOH and other aliphatic and aromatic electron-rich groups found in organic matter structures (Artiola, 2005). The bioavailability and toxicity of the metals in polluted soils are influenced by the total metal content and the metal ion speciation in the soil. Although fractionation is typically operationally defined, the bioavailability of copper to soil fauna and plants can often be closely related to the distribution of metal fractions in the soil. For example, exchangeable copper, which can be extracted from soil matrix using salt, is believed to be the most important, if not the only, bioavailable fraction for plant root accumulation. The theory behind sequential extraction technique (SET) is that the most mobile metals are removed in the first fraction and continue in order of decreasing mobility. All SETs facilitate fractionation. Tessier and co-workers (1979) named these fractions exchangeable, carbonate bound, Fe and Mn oxide bound, organic matter bound, and residual. These are also often referred to in the literature as exchangeable, weakly absorbed, hydrous-oxide bound, organic bound, and lattice material components, respectively (Ratuzny et al. 2009). Typically, metals of anthropogenic inputs tend to reside in the first four fractions and metals found in the residual fraction are of natural occurrence in the parent rock (Hall et al. 1996).

Phytoremediation, a term applied to a group of technologies that use plants to reduce environmental toxins primarily those with anthropogenic origin, is a developing technique that provides low cost and environmentally-sustainable process in removing Cu from soil (Peer et al. 2005). Phytoremediation includes phytoextraction, phytodegradation, phytovolatilization, rhizosphere degradation, rhizofiltration, phytostabilization, and phytorestoration (Long et al. 2002). Only 24 known species of plants are hyperaccumulators of copper, indicating that such plants accumulate 1000 *mg* of copper per *kg* dry leaf tissue (Baker, 1989). *Pityrogramma calomelanos*, commonly known as pakong-gubat or fern, belongs to the family Pteridaceae and is a wild and exotic fern known as an arsenic hyperaccumulator. It can be found in tropical and sub-tropical regions of the world. It is a known hyperaccumulator of arsenic. Accumulation of the heavy metal is up to 8,350 µg As/g dry weight (mostly in the fronds), while the rhizoids contain 88 to 310 µg As/g dry weight (Francesconi et al. 2002).

P. calomelanos has been reported to grow abundantly in copper mining sites found in Surigao and Manila City and its potential for phytoremediation was observed by field experiments conducted by Ateneo de Manila University and funded by the Department of Science and Technology. Bansah and Addo (2016) reported the phytoremediation potential of plants including *P. calomelanos*. They investigated *P. calomelanos* for the remediation of mine degraded soils in Ghana and results revealed that the plant accumulated significant amount of heavy metals including copper. Perlatti et al. (2016) evaluated the biogeochemical processes in the rhizosphere of different native plants including *P. calomelanos* in an abandoned mining site in Brazil. Results indicated that the plants under study promote phytostabilization of copper.

Thus this study primarily aims to assess the bioavailability of Cu in *P. calomelanos,* to determine the amount of copper uptake accumulated by the fronds (above ground) and roots (below ground) of *P. calomelanos* at various concentrations of copper solutions using atomic absorption spectrophotometry (AAS) and to provide an analysis for the potential use of *P. calomelanos* as means of phytoremediation in Cu-contaminated mining sites through assessing its accumulation of Cu and the bioavailability of Cu in its soil for further applications and analysis. Hence, further investigation for its potential as Cu hyperaccumulator using pot experiments under controlled conditions was proposed.

MATERIALS AND METHODS

Mature *P. calomelanos* ferns were provided by Manresa Farms in Cagayan de Oro City. The plants were maintained under greenhouse conditions. They were placed in pots, fenced in, distanced equally with each other, and provided with sufficient sunlight. A roof served as protection against rain contamination. Soil was kept moist by adding distilled water to pots every day during the experimental period. No nutrients were given to the plants in the course of the experiment.

Copper Treatments, Transplantation, Sampling of Mature *P. calomelanos* and Soil Sampling

Plants in untreated soil were utilized as control set-up with 3 replicates. Seven kg of soil for each Cu treatment was soaked and mixed with 2.1 L of Cu solutions. For each treatment, the following Cu concentrations were used: 500, 1000, 2000, 3000, 4000, and 5000 mg_{Cu}/kg_{soil} added as $CuSO_4 \cdot 5H_2O$ (99.999% trace metals Sigma-Aldrich).

Transplantation of mature plant was conducted 10 days after Cu treatment of the soil. Sampling was conducted 61 days after transplantation.

For soil sampling, samples were taken from 0 cm to 15 cm depth. Soil samples passed through a 40 mesh sieve before they were air-dried at room temperature. The residual moisture was removed by heating at 100˚C for 3 h. Oven-dried samples were pulverized to a fine powder and sieved through a <1 µm sieve.

Atomic Absorption Spectrophotometry Analysis of Cu in Plant and Soil

Analysis of copper in soil was based on the US EPA Method 3050B. In brief, oven dried soil sample was mixed thoroughly and subjected to a mechanical shaker to homogenize. Particles were separated by using a 40 mesh sieve. Soil sample was added with 10 mL of 1:1 (v/v) HNO_3 : H_2O solution, heated at 95°C for 10-15 min without boiling and cooled. Additional mL of concentrated HNO_3 was added and it was heated for another 30 min. The solution was allowed to evaporate and 2 mL of reagent water and 3 mL of 30% H_2O_2 were added to the solution and warmed. Ten mL concentrated HCl was added to the sample and heated again on a hot plate and refluxed at 95°C for 15 min, cooled, and filtered. The filtrate was collected in a 100-mL volumetric flask and diluted to the mark with distilled water. The sample was analyzed by AAS (AAnalyst 400 Perkin Elmer).

Copper in plant samples were analyzed based on the AOAC Official Method 957.03. Plant samples were separated using plant cutters into above ground biomass, consisting of the leaves and stems, and below ground biomass, consisting of the roots. Samples were washed and allowed to dry in oven at 65°C for 72 h. Plant sample was weighed and ashed in a muffle furnace at 500°C for 2 h and cooled. Ash was wetted with 10 drops water and added with 3 mL of HNO_3. Excess HNO_3 was evaporated on a hot plate set at 100°C. The plant sample was transferred quantitatively in 100-mL volumetric flask and 10 mL 5% lanthanum solution was added to the mixture and diluted to volume. Lanthanum was used to act as a releasing agent that prevents interference from phosphate which might be present as a soil nutrient. The supernatant was decanted and transferred in a 100-mL volumetric flask and diluted to the mark using 10% HCl and sample was subjected to AAS analysis.

Fourier Transform Infrared Spectrophotometry Analysis

Plant samples, above and below ground, were homogenized using a mortar and pestle and dried at 65°C until constant weight to remove water in the plant. Treated and untreated plant samples, roots and fronds, were then subjected to FTIR spectrophotometer analysis-Attenuated Total Reflectance (ATR) (Perkin Elmer Spectrum 100). Soil samples were homogenized and dried at 85°C until constant weight to remove excess water. A very small amount of soil was placed in the ATR crystal area and IR spectra were collected from 500 cm^{-1} to 4000 cm^{-1}.

Sequential Extraction Technique Analysis.

Bioavailability of Cu for the uptake into *P. calomelanos* was measured using SET analysis as described by Tessier et al. (1979). Figure 1 shows the schematic diagram of the procedure.

Statistical Analysis

The difference in Cu uptake for each interval treatments and each fraction in the sequential extraction analysis were established by t-test.

RESULTS

Soil Parameter Analysis

The soil conditions prior to Cu treatments are shown in Table 1. Nitrogen, phosphorus, and potassium are known as the primary plant nutrients because they generally promote plant growth. Cation exchange capacity (CEC) is the total capacity of a soil to hold exchangeable ions. It influences the soil's ability to hold onto essential nutrients like N, P, and K (Nam et al. 2006). These soil parameters (NPK and CEC) showed no significant effect on bioavailability of metal as indicated by Degryse and co-workers (2009). High organic matter content (17.27%) in the soil indicates that the Cu present in the soil is non-bioavailable since the organic matter would likely interact through complexation reaction with Cu making it difficult for Cu to be translocated (Degryse et al. 2009). The soil pH of 6.64 was a little beyond the acceptable range. An increased soil pH would eventually lead to a decreased bioavailability of Cu. This is due to the precipitation of metals in an alkaline environment (Takáč et al. 2009).

P. calomelanos has been found to take up copper either in its roots and fronds as reported by Perlatti and co-workers (2015) in their study on the analysis of Cu on several plants including *P. calomelanos* from abandoned mining sites in Northeastern Brazil (Perlatti et al. 2015). In this current study, mature *P. calomelanos* plants were exposed to different concentrations of Cu in a controlled environment in Manresa farm. Fronds are the components of the above-ground part of the plant, and the roots are considered as the below-ground part of the plant. Results indicating the uptake of Cu in *P. calomelanos* are shown in Figure 2.

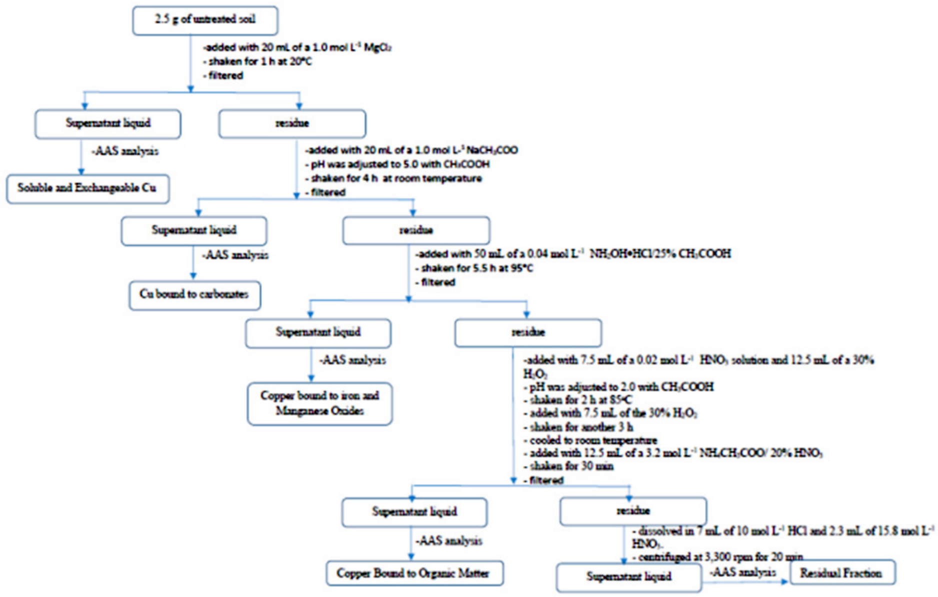

Figure 1. Schematic diagram of SET analysis

Table 1. Profile of Soil Sample Used in the Study.

Soil Analysis	Result	Acceptable Range	Reference
pH	6.64	6.3-6.5	Wuana and Okieimen, 2011
Nitrogen (%)	0.41	0.15-0.50	Astera, 2015
Phosphorus (mg_P/kg_{soil})	97.6	85-100	Astera, 2015
Potassium (mg_K/kg_{soil})	1,110	1,000-1,500	Astera, 2015
Organic Matter (%)	17.27	3.0-6.0	Schoenholtz, et al, 2000
Cation Exchange Capacity ($m.eq/100g_{soil}$)	20.55	1-100	Reganold and Harsh, 1985
Texture	Sandy Loam	Loam	
Initial Cu Content (mg_{cu}/kg_{soil})	188.00	100-300	

Copper Uptake of *P. calomelanos* in 61 days

In order for a plant to be considered as a hyperaccumulator, all these criteria should be met: (a) the bioaccumulation factor (BAF), which is defined as the ratio of metal in plant dry mass to those in soil, is greater than or equal to 1 (Masarovicova et al. 2010); (b) the translocation factor (TF), which is defined as the total metal content in plant shoot to that in the roots, must also be greater than or equal to 1 (Masarovicova et al. 2010); and (c) the Cu uptake is greater than 1000 mg of Cu per kg of the above ground part of the plant. Figure 3 shows the results of the calculated bioaccumulation factors and translocation factors of *P. calomelanos*.

Bioavailability is the proportion of total metals that are available for incorporation into the living organism, in this case *P. calomelanos*. SET was employed for the measurement of the bioavailability of Cu in the soil. Bioavailability of Cu for the uptake into *P. calomelanos* was measured using SET analysis. Figure 4 shows the summary of the SET analysis of the soil treated with 4000 mg_{Cu}/kg_{soil}.

Fourier Transform Infrared (FTIR) Spectrophotometry Analysis

FTIR analysis was conducted to study the interaction of Cu with functional groups present in the soil, above ground and below ground parts of the plant. FTIR spectrum of plant and soil revealed shifting of absorption bands of functional groups in the presence of Cu at 500 mg_{Cu}/kg_{soil} and 4000 mg_{Cu}/kg_{soil} treatment. This signifies that there is interaction between Cu and the functional groups present in the plant and soil.

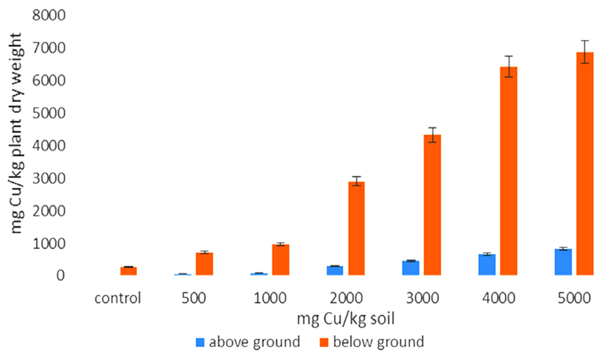

Figure 2. Summary of copper uptake in different copper treatments in 61 days.

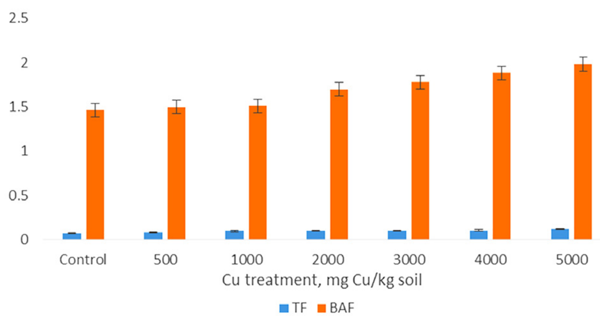

Figure 3. Bioaccumulation and translocation factors of P. calomelanos.

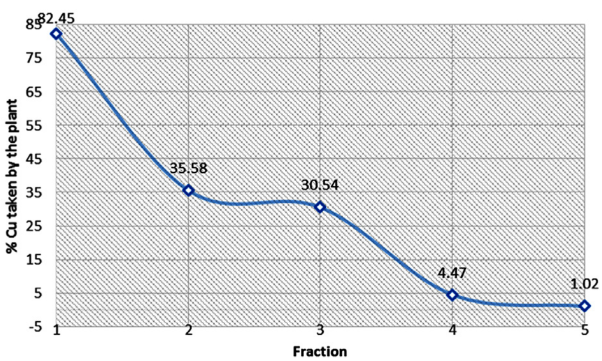

Figure 4. SET analysis of soil.

DISCUSSION

The data in Figure 2 revealed that Cu in *P. calomelanos* above-ground has lower concentrations with a range of 19.58 to 821.18 $mg_{Cu}/kg_{\text{dry weight}}$ than the uptake of Cu below-ground, with a range of 262.93 to 6883.32 $mg_{Cu}/kg_{\text{dry weight}}$. This difference in Cu uptake in the above ground and below ground is attributed to the inability of the plant to translocate Cu from the roots to the shoots. Based on the above results, *P. calomelanos* has a tendency to sequestrate more heavy metals in the roots than in the shoots. This is a characteristic of a non-hyperaccumulating plant (Degryse et al. 2009).

Furthermore, it was observed that the ratio of *P. calomelanos* in the above ground to the below ground remains consistent at 0.1 even if there is an increasing trend of Cu uptake in both parts of the plant. This could mean that *P. calomelanos* could exhibit the same ratio even at concentrations beyond 5000 ppm of Cu treatments making it a non-hyperaccumulating plant. Based on the criteria of a hyperaccumulator, it is implied that *P. calomelanos* is not a hyperaccumulator. These results in Figure 3 illustrated a higher Cu uptake in the below ground than the above ground part of the plant. Furthermore, the highest uptake in the above ground was only 821.18 $mg_{Cu}/kg_{\text{dry weight}}$ (at 5000 mg_{Cu}/kg_{soil} treatment) showing that *P. calomelanos* is not a hyperaccumulator although an increasing trend of Cu uptake in the above ground part of the plant was observed as Cu treatment was increased.

Based on the TF values, *P. calomelanos* can be classified as an excluder having TF values ranging 0.074-0.119. An excluder is a plant that excludes metal uptake to the shoot and keeps it in the roots instead. On the other hand, based on the BAF values of greater than 1, *P. calomelanos* can be considered as a metallophyte. A metallophyte is a plant that uptakes Cu from soil to the plant organ, both above ground and below ground parts of the plant.

The amount of bioavailable Cu taken by the plant per fraction is the difference between the Cu concentration in the soil without plant and soil with plant divided by the amount of Cu initially present per fraction. SET analysis of the treated soil with plants was analyzed after 61 days to assess which chemical form of Cu did *P. calomelanos* took up from the soil. Results showed that a significant amount of bioavailable Cu was lost in the soil with plant after 61 days. The highest % of Cu that was taken by the plant was 82.45% in the first fraction which was attributed as extractable Cu. This is followed by fraction 2 which is the Cu bound to carbonates, and the Cu bound to iron-manganese oxides. These 3 fractions contained the bioavailable fractions of Cu since fraction 1, the extractable Cu, is considered as the free metal form. Fractions 2 and 3 – Cu bound to carbonates and Cu bound to iron-manganese oxides, respectively – are ligand-free as indicated by Degryse et al. (2009). Hence, these fractions were the forms of Cu that was taken up by *P. calomelanos*. On the other hand, the amounts of Cu in fractions 4 and 5 were way below those of fractions 1, 2 and 3. This could be explained by the chemical form of Cu present in fractions 4 and 5. Cu bound to the organic matter in fraction 4 and Cu bound to the soil matrix in fraction 5 contained the non-bioavailable fractions of Cu due to the strong adhesive forces of the complexation reaction between Cu and organic matter and Cu and the soil matrix (Degryse et al. 2009).

Figure 5 illustrates a slight shift in the 1595 cm^{-1} absorption band of the above ground part of the plant. This band is attributed to the binding of Cu at the amide C=O group that can be found in the phytochelatin compound of the plant (Merlin et al. 2014). Moreover, the absorption band at 2973 cm^{-1} which has shifted to 2918 cm^{-1} can be associated to the binding of Cu with the carboxylic acid O-H group that can also be found in the phytochelatin compound of the plant (Peer et al. 2005). A significant shifting of the absorption band at 2973 cm^{-1} and at 1036 cm^{-1} was also observed. This is due to the binding of Cu with C-O group. These shifts in absorption bands confirm the presence of Cu in the above ground part of the plant. The addition of Cu increases the absorption band intensities of the amide, carboxylic acid, and alkane groups because addition of Cu favors phytochelatin biosynthesis due to the increase production of glutathione, a major component found in phytochelatin (Peer et al. 2005; Javaid et al. 2011). Furthermore, FTIR analysis of the below ground part of the plant showed shifting of absorption bands at 2938 cm^{-1} to 2927 cm^{-1} and 909 cm^{-1} to 911 cm^{-1}.These are indicative of the binding of Cu at the carboxylic acids O-H group and C-O groups, as shown in Figure 6 (Peer et al. 2005; Javaid et al. 2011). FTIR spectra of the untreated soil and treated soil with 500 mg_{Cu}/kg_{soil} and 5000 mg_{Cu}/kg_{soil} showed a shift in the absorption band at 1033 cm^{-1} as shown in Figure 7**.** These are indicative of Cu bound to minerals like iron and manganese (Robertson et al. 2016).

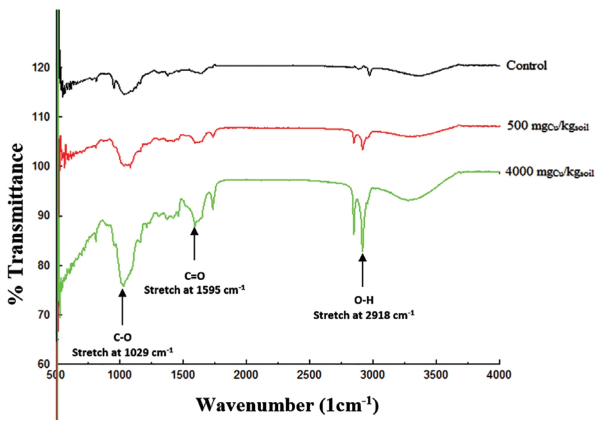

Figure 5. FTIR spectra of the above-ground part of untreated *P. calomelanos* and treated *P. calomelanos* with 500 mg_{Cu}/kg_{soi} and 4000 mg_{Cu}/kg_{soil}.

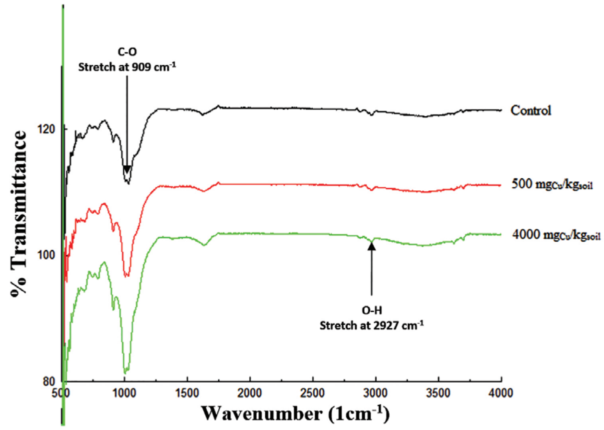

Figure 6. FTIR spectra of the below-ground part of untreated *P.calomelanos* and treated *P.calomelanos* with 500 mg_{Cu}/kg_{soil} and 4000 mg_{Cu}/kg_{soil}.

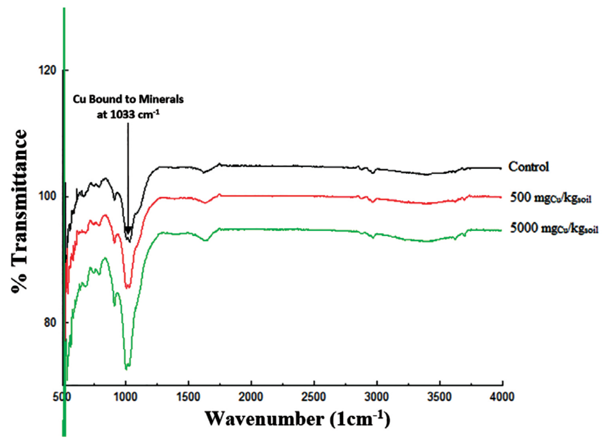

Figure 7. FTIR spectra of untreated soil and treated soil with 500 mg_{Cu}/kg_{soil} and 5000 mg_{Cu}/kg_{soil}.

Statistical Analysis

T-test revealed that there was a significant difference in the Cu uptake for each interval treatments in the above ground and below ground parts of *P calomelanos.* There was also a significant difference in the amount of bioavailable copper for each fraction in the sequential extraction analysis.

CONCLUSION

Copper is a metal known for its numerous uses. It is commonly mined which has contributed to the contamination of mining sites. One of the ways to rehabilitate copper mining sites is by phytoremediation using *P. calomelanos*.

P. calomelanos was investigated for its ability to accumulate copper by evaluating its BAF and TF values. Results revealed that BAF values ranged 1.462-1.983 which suggested that the plant is a metallophyte. TF values ranging 0.074-0.119 showed that it is an excluder. *P. calomelanos* accumulated 821.18 mg of Cu per kg of the above ground part of the plant making it a nonhyperaccumulator. Using sequential extraction technique (SET), Cu was found to be bioavailable as extractable Cu, Cu bound to carbonates, and Cu bound to iron-manganese oxides. Non-bioavailable forms of Cu were Cu bound to organic matter and Cu bound to the soil matrix.

FTIR spectra analysis of the *P. calomelanos* revealed that Cu attaches to the C-O, C=O and O-H groups that are found in the phytochelatin compound in the above ground part of the plant. Cu was also observed to bind with minerals like iron and manganese in the soil.

ACKNOWLEDGMENT

To the IPH Project team headed by Dr. Teresita Perez and Dr. Rene Juna Claveria of Ateneo de Manila University-Environmental Science Department for not just funding this thesis but for also imparting knowledge and perspective through trainings, reports, and updates.

CONFLICT OF INTEREST

The authors declare no conflict of interests in this study.

LITERATURE CITED

ARTIOLA JF. 2005. Speciation of Copper in the Environment. Handbook of Elemental Speciation II: Species in the Environment, Food, Medicine & Occupational Health, p. 175-177.

ASTERA, M. 2015. The Ideal Soil v2.0: A Handbook for New Agriculture.

BAKER A. JM. 1989. Terrestrial higher plants which hyperaccumulate metallic elements– a review of their distribution, ecology and phytochemistry. Biorecovery 1:81-126.

BANSAH KJ, ADDO WK. 2016. Phytoremediation Potential of Plants Grown on Reclaimed Spoil Lands, Ghana Mining Journal 16(1):68-75

DAS S, GOSWAMI S, DAS TA. 2013. Copper hyperaccumulating plants from Barak Valley, South Assam, India for phytoremediation. International Journal of Toxicological and Pharmacological Research 5(1), 30-32.

DEGRYSE F, SMOLDERS E, PARKER DR. 2009. Partitioning of metals (Cd, Co, Cu, Ni, Pb, Zn) in soils: concepts, methodologies, prediction and applications - a review. European Journal of Soil Science, 60:590-612.

FRANCESCONI K, VISOOTTIVISETH P, SRIDOKCHAN W, GOESSLER W. 2002. Arsenic species in an arsenic hyperaccumulating fern, *Pityrogramma calomelanos*: A potential phytoremediator of arsenic-contaminated soils. The Science of the Total Environment 284:27-35.

HALL GEM, GAUTHIER G, PELCHAT JC, PELCHAT P, VAIVE JE. 1996. Application of a sequential extraction scheme to ten geological certified reference materials for the determination of 20 elements. Journal of Analytical Atomic Spectrometry 11(9):787-796.

JAVAID A, BAJWA R, SHAFIQUE U, ANWAR J. 2011. Removal of heavy metals by adsorption on *Pleurotus ostreatus*, Biomass and Bioenergy 35:1675-1682.

LONG XX, YANG XE, NI WZ. 2002. Current situation and prospect on the remediation of soils contaminated by heavy metals. Chinese Journal of Applied Ecology 13(6):757-762.

MASAROVICOVA E, KRA´L ˇOVA K, KUMMEROVA M. 2010. Principles of classification of medicinal plants as hyperaccumulators or excluders. Acta Physiol Plant 32:823-829.

MERLIN N, NOGUEIRA BA, DE LIMA VA, DOS SANTOS LM. 2014. Application of Fourier Transform Infrared Spectroscopy, Chemical and Chemometrics Analyses to the Characterization of Agro-Industrial Waste. Quim. Nova 37(10):1584-1588.

NAM MH, JEONG SK, LEE YS, CHOI JM AND KIM HG. 2006. Effects of nitrogen, phosphorus, potassium and calcium nutrition on strawberry anthracnose, Plant Pathology 55:(246-249).

PEER W, BAXTER IR, RICHARDS EL, FREEMAN JL, MURPHY AS. 2005. Phytoremediation and Hyperaccumulator Plants. Molecular Biology of Metal Homeostasis and Detoxification, Volume 14 of the series Topics in Current Genetics, p. 299-340.

PERLATTI F, FERREIRA, T, ROMERO, R, COSTA M, OTERO X. 2015. Copper accumulation and changes in soil physical–chemical properties promoted by native plants in an abandoned mine site in northeastern Brazil: Implications for restoration of mine sites, Ecological Engineering 82:103-111.

PERLATTI F, FERREIRA TO, SARTOR LR, OTERO XL. 2016. Copper Biogeochemistry in Response to Rhizosphere Soil Processes Under Four Native Plant Species Growing Spontaneously in an Abandoned Mine Site in NE Brazil. Water Air Soil Pollut 227:142.

RASCIO N, NAVARI-IZZO F. 2011. Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting. Plant Science 180:169-181.

RATUZNY T, GONG Z, WILKE B-M. 2009. Total concentrations and speciation of heavy metals in soils of the Shenyang Zhangshi Irrigation Area, China. Environmental Monitoring and Assessment, 156(1-4):171-180.

REGANOLD JP and HARSH JB. 1985. Expressing cation exchange capacity in milliequivalents per 100 grams and in SI units. Journal of Agronomic Education, Vol. 14, No. 2, Fall 1985.

Republic of the Philippines Department of Environment and Natural Resources - Mines and Geosciences Bureau. (March 2014). Philippine Metallic Mineral Production [Data File]. Retrieved from http://www.mgb.gov.ph/Files/Statistics/MetallicProduction.pdf

ROBERTSON J, SHAND C, FERNANDEZ EP. 2016. The application of Fourier transform infrared, near infrared and Xray fluorescence spectroscopy to soil analysis. Spectroscopy Europe. 28(4):9-13.

SCHOENHOLTZ SH, VAN MIEGROET H and BURGER JA. 2000. A review of chemical and physical properties as indicator of forest soil quality: challenges and opportunities. Forest Ecology and Management 138:335-356.

TAKÁČ P, SZABOVÁ T, KOZÁKOVÁ L, BENKOVÁ M. 2009. Heavy metals and their bioavailability from soils in the long-term polluted Central Spis region of SR. Plant Soil Environment, 55, 167-172.

TESSIER A, CAMPBELL PGC, BLSSON M. 1979. Sequential extraction procedure for the speciation of particulate trace metals. Analytical Chemistry, 51(7):844-851.

WUANA RA and OKIEIMEN FE. 2011. Heavy Metals in Contaminated Soils: A Review of Sources, Chemistry, Risks and Best Available Strategies for Remediation. International Scholarly Research Netwrok ISRN Ecology Volume 1-20.

YANG XE, LONG XX, NI WZ, HE ZL, STOFFELLA PJ, CALVERT DV. 2002. Assessing copper thresholds for phytotoxicity and potential toxicity in selected crops. Journal of Environment, Science and Health B, 37:625-635.