ABSTRACT

Title of Dissertation: **ASSESSING BEST PRACTICES FOR REFORESTATION OF AREAS DEGRADED BY ARTISANAL AND SMALL-SCALE GOLD MINING IN THE PERUVIAN AMAZONIAN REGION OF MADRE DE DIOS**

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Artisanal and Small-Scale Gold Mining (ASGM) have pressured the Peruvian Southern Amazon rainforest, causing deforestation, soil degradation, and mercury (Hg) emissions in large areas. The Peruvian government and NGOs have performed reforestation projects in areas degraded by ASGM in this region using native plant species with an economic value. Previous research has studied the restoration of areas degraded by ASGM in this region. However, there is

lack of information about the Hg's distribution, accumulation, and the effects of Hg exposure in native plants in this region. Additionally, few studies have investigated the recovery of soil fertility in these degraded areas during restoration.

In this dissertation, the distribution and predictors of Hg accumulation in soil and native plant species from artisanal mining sites and the primary forest near these sites were studied. The highest Hg concentrations in soil were found in the intact primary forest topsoil and the plant rhizosphere area. The highest Hg levels in plants were found in the foliage of the intact primary forests. The Hg levels found in the plant leaves of the primary forest are the highest ever recorded in this region, exceeding values found in forests impacted by Hg pollution worldwide and raising concerns about the extent of the ASGM impact in this ecosystem.

The effects of Hg exposure on the survival, growth, health, and rhizosphere microbial communities of three Amazonian agricultural plant species, aji dulce (Capsicum Chinense), sacha culantro (Eryngium foetidum), and uncucha (Xanthosoma sagittifolium L. Schott) were also investigated. The lowest observable concentration of Hg affecting the plant's health was 2 mg kg^{-1} dw. This Hg concentration was three times lower than the soil screening level (SSL) for Hg in agricultural soil established in the Peruvian regulation. This suggests that a review of this SSL may be necessary before developing restoration projects in Amazonian areas impacted by ASGM.

The soil physic-chemical characteristics, such as soil organic matter (SOM), electrical conductivity (EC), pH, nutrients, and bacterial communities, in the rhizosphere soil of plant species growing in naturally regenerated and reforested areas impacted by ASGM and in the primary forest near these areas were assessed. The results suggest that the plant species *Acacia loretensis* and *Inga sp.* (*Inga sp*. when planted with biochar as a soil amendment) might be good

candidates for restoring areas degraded by ASGM, due to the level of nutrients and SOM in their rhizosphere.

Finally, the integration of Indigenous and scientific knowledge to monitor and manage land degradation in regions impacted by ASGM was also studied. The findings emphasize the need to use the Indigenous communities' knowledge of their territory for the early detection of soil degradation and facilitate a dialogue about land degradation and restoration of areas affected by ASGM between local communities, researchers, and policymakers to develop more sustainable and successful restoration projects in Indigenous communities affected by ASGM.

ASSESSING BEST PRACTICES FOR REFORESTATION OF AREAS DEGRADED BY ARTISANAL AND SMALL-SCALE GOLD MINING IN THE PERUVIAN AMAZONIAN REGION OF MADRE DE DIOS

by

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Chapter 1: Introduction

1.1 Artisanal and Small-Scale Gold Mining and Mercury

Artisanal and Small-Scale Gold mining (ASGM) is gold mining conducted by individual miners or small enterprises with limited capital investment and production (WHO, 2016). This activity is sometimes illegal or informal and is mainly carried out in developing countries, lacking regulation in this sector (TGIATOC 2016). Because of the methods used and the lack of regulations and/or enforcement, ASGM has several human and environmental health impacts, such as mercury emissions and deforestation (Caballero Espejo et al., 2018). Mercury (Hg) is used in ASGM to extract the metal from soil due to its ability to form a stable complex with the gold called amalgam. The import of Hg was restricted in Peru in 2015. Currently, artisanal miners buy Hg in the illegal market. Hg is exported from Mexico to Bolivia and then is transported to Peru as smuggling (WWF, 2019)

According to the United Nations Environment Programme (AMAP/UN Environment, 2019), ASGM is the primary source of anthropogenic Hg emissions worldwide. Other important mercury emissions sources are fossil fuel combustion and non-ferrous metal production (Fig. 1-1). Mercury mining is also a source of mercury emissions (Wang et al., 2004). Mercury mines produce waste containing inorganic mercury in different mineral phases, such as mercuric sulfide and mercuric chloride (Navarro, 2008).

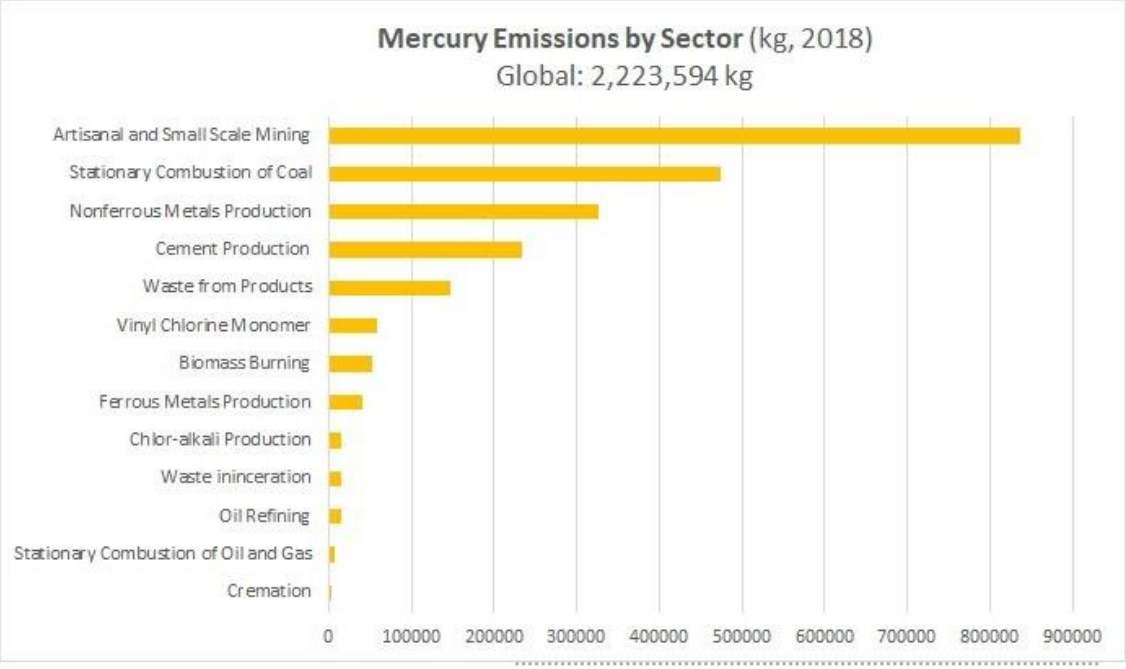

Fig. 1-1. 2018 Mercury emissions by sector (AMAP/UN Environment, 2019)

1.2 Mercury in the Environment

Mercury (Hg) is a heavy metal found in the environment in three forms: elemental (metallic), inorganic, and organic. Elemental or metallic mercury, Hg (0), is a liquid at room temperature (22°C) but can evaporate quickly, forming a toxic vapor. Its vapor pressure is 1.22 x 10-3 mmHg at 20°C, and due to this high vapor pressure, metallic Hg is volatile and can be found in the environment, mainly in the air in its gaseous form (Beckers & Rinklebe, 2017). This form of Hg has been used in the manufacture of thermometers, electric bulbs, and switches because of its high coefficient of expansion, which allows it to expand and contract with small

temperature changes, and its high vapor pressure, which allows it to vaporize at the operating temperature of the bulbs, and its ability to conduct electricity (US EPA, 2015).

Inorganic mercury, Hg (II), can be found naturally in the environment in rocks and fossil fuels. This form is soluble in water and can combine with other chemical elements, like chlorine and sulfur, forming salts, such as mercuric chloride and mercuric sulfide (Fiorentino et al., 2011). These salts can enter the environment through volcanic emissions, dust, and weathering of the rocks containing them (Tchounwou et al., 2003). Inorganic Hg can also enter the environment through anthropogenic activities, such as the manufacture of personal care products where mercuric chloride and mercuric oxide are used as preservatives, and paints where phenylmercuric nitrate or acetate are used as paint preservatives (Goldman et al., 2001). Fungicides are another source of Hg. In these products, mercuric chloride, phenylmercuric acetate, and phenylmercuric oxiquinolate, among other mercury compounds, are used because of their toxicity to fungi (Prasad et al., 1986; Guntenmann & Lisk, 1960). Other sources include emissions from coal and oil-fired power plants that burn coal and oil containing Hg as an impurity. The combustion process forms elemental mercury and mercuric chloride emitted into the atmosphere (Senior et al., 2000). Waste incinerators that burn municipal and industrial waste with Hg products are another source of Hg emissions to the atmosphere. The Hg species mainly emitted by these incinerators are elemental Hg and mercuric chloride (van Velzen et al., 2002).

Organic Hg is produced from inorganic Hg by anaerobic microorganisms, mainly in aquatic ecosystems (Hong et al., 2012; US EPA, 2015). One of the organic Hg compounds, methylmercury, is the most toxic and bioavailable organic species of Hg (Mason & Lawrence, 1999).

The three Hg species are involved in ASGM emissions. Elemental Hg is used to extract gold from soil and form an amalgam. This amalgam is burned to release Hg and get the metal, emitting Hg vapors (elemental and mercuric chloride) into the atmosphere (Esdaile & Chalker, 2018). Part of the Hg is also discharged into water and soil in the amalgamation process because this artisanal mining activity is performed mainly by hand without proper safety measurements (Lacerda, 1997).

Elemental Hg can be transported long distances in the atmosphere by wind and is usually oxidized to inorganic Hg (Durnford et al., 2010). Inorganic Hg can also be transported long distances, mainly attached to particles in air and water. It can deposit over surface waters and soil from the air through dry or wet deposition (with rainwater) (Pirrone et al., 2013). Once in aquatic ecosystems, inorganic Hg can be transformed into organic Hg by some anaerobic microorganisms. It can enter the food chain when macroinvertebrates and fish take it up. It can also biomagnify (concentrate in organisms in higher levels of the food chain that eat contaminated organisms in lower levels of the chain) (Mergler et al., 2007) (Fig. 1-2). Also, organic Hg (methylmercury) can cause liver and gill damage to fish exposed to this substance through direct contact and dietary exposure (de Oliveira Ribeiro et al., 2002).

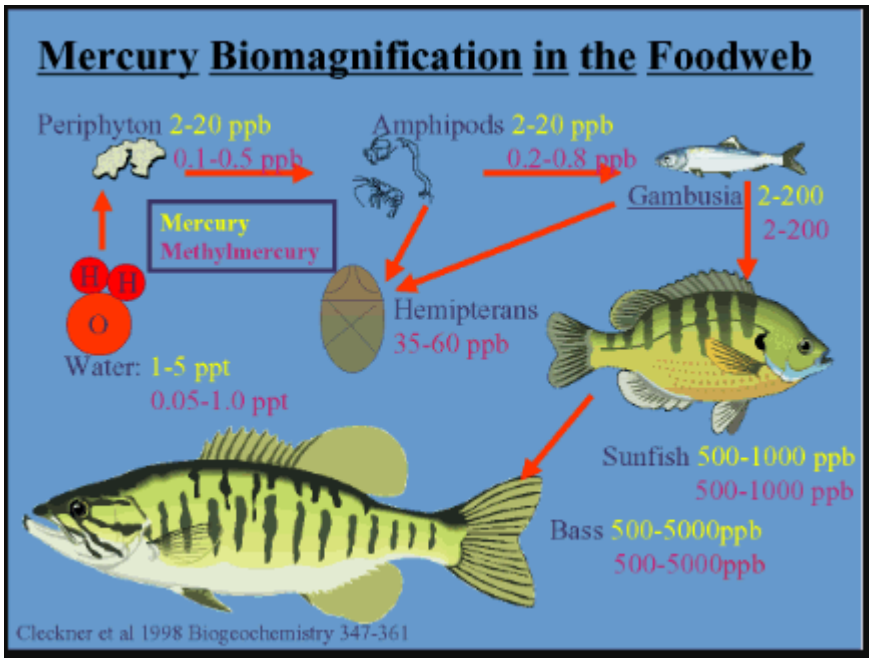

Fig. 1-2. An example of Hg biomagnification in the food web (Cleckner et al., 1998)

The most common human exposure pathway to Hg is consuming fish contaminated with Hg (mainly in its organic form). Other exposure pathways are consuming vegetables, meat, or dairy contaminated with Hg (inorganic and organic form) and inhaling Hg vapors (Tchounwou et al., 2003). After oral exposure to Hg above certain levels (1.6 μg/Kg body weight of methylmercury and four μg/Kg body weight of inorganic Hg), humans can develop adverse health effects, such as brain, heart, kidney, lung, neurological, developmental, and immune system damage (WHO, 2011; Santos et al., 2018; Dórea & Marques, 2016). Exposure to methylmercury can affect the nervous system, especially in babies and young children, because methylmercury is a neurotoxin (Castoldi et al., 2003).

Hg exposure can also have adverse effects on plants and wildlife. Some studies reported adverse health effects in plants after Hg exposure, such as inhibition of shoot and root length (Siegel et al., 1984; Patra & Sharma, 2000); inhibition of photosynthesis (Clijsters & Van Assche, 1985; Küpper et al., 1998); stunting of seedlings and roots, leaf chlorosis and browning of leaf points (Kabata-Pendias & Pendias, 2001); reduction in chlorophyll content in leaves (Cho & Park, 2000); and genotoxic effects (Panda et al., 1992). Other studies found adverse health effects in animals exposed to Hg, such as renal necrosis, brain damage, reduction in enzyme activity, and increase in mortality (Potter & Matrone, 1974; Calabrese et al., 1977; Neathery & Miller, 1975; Patra & Sharma, 2000; US EPA, 2015).

1.3 Artisanal and Small-Scale Gold Mining in the Amazon

One of the ecosystems affected by the Hg emissions from ASGM is the Amazon rainforest (Crespo-Lopez et al., 2021, 1998). The health of the Amazonian Forest has been of concern in the last century as it is today. The international community now recognizes the adverse effects of climate change in that region with profound economic, social, and geopolitical implications. Artisanal and small-scale gold mining (ASGM) is one of the many issues the Amazon Forest faces and has been growing in the last decades (Lobo et al., 2016).

The Peruvian Amazonian region of Madre de Dios is one of the most biodiverse regions on the planet, providing diverse ecosystem services, such as water supply and climate change mitigation (Fisher et al., 2018). However, ASGM has extensively impacted this region's environment. One of the main impacts of ASGM in this region is the deforestation of one-third of the buffer zones in the protected forest from 1984 to 2017 (Caballero Espejo et al., 2018). These buffer zones in the Amazon region are vital because they prevent habitat loss in the protected areas surrounded by them (DeFries et al., 2005). Another impact found was that nine of the 15 most consumed fish species in Madre de Dios, such as mota, chambira, and bagre, had mercury levels exceeding the safe limit set by the US EPA (EPA, 2019). Additionally, 78% of residents of the region's capital, Puerto Maldonado, had high levels of Hg in their bodies. The average Hg concentration in this population’s hair was 2.7 ppm, almost three times the US EPA Mercury Reference Concentration in human hair (1 ppm) (US EPA, 2001) (Fernández, 2013), which presents a significant impact on the local population. The study reporting these impacts was shared with the Peruvian Government and published in the media, leading to the development of campaigns by several NGOs asking for the declaration of an environmental emergency in Madre de Dios (SERVINDI, 2013; OCMAL, 2013). The Peruvian Government

then launched military operations to stop illegal mining activity. However, the lack of success of these operations caused a change in the government strategy (SERVINDI, 2017).

Recently, the government launched a multi-sectoral program, including military action and reforestation of public areas degraded by ASGM using indigenous plant species that provide ecosystem services (Andina Noticias, 2019). This program has received criticism due to the lack of coordination with local scientists who have experience with mercury contamination and soil degradation caused by ASGM in the region (SPDA, 2019). Some of the scientists' worries are related to the lack of research on the effect of mercury exposure on the Amazonian plant species (Mongabay Latam, 2019). To optimize the efforts of the Peruvian Government and reestablish the local environmental ecosystem that was impacted by mining, there is a need to understand better how mining pollution and soil degradation would affect the health of indigenous plant species. As mentioned, Hg exposure can reduce plant growth and inhibit photosynthesis, among other adverse health effects. Thus, plants growing in an environment with soil or air polluted by Hg, as the areas impacted by ASGM, would not be able to provide the ecosystem services expected (food, medicinal plants, timber).

Some local scientists have developed reforestation experiments in areas degraded by ASGM using indigenous trees (Roman-Dañobeytia et al., 2020). These experiments and the government reforestation program do not consider soil remediation before reforestation because the mercury levels in the degraded soil are below the soil screening levels (SSL) included in the Peruvian regulation (6 mg kg^{-1} in agricultural soil). The SSL of mercury is the maximum level of mercury that can be present in soils before starting to present a significant risk to the environment and human health. Higher mercury concentrations were still below the SSL in the reference forest (non-degraded) near the degraded areas (CINCIA, 2019; Velasquez et al., 2020).

The same studies characterized the degraded soils as poor, with low fertility and low organic matter content. Metals, such as Hg, have an affinity for humic acids, organo-clays, and oxides coated with organic matter (Elliot et al., 1986; Connell & Miller, 1984), so Hg would not be attached to degraded soils and would leach. However, once the soil was recovered through reforestation, and because the ASGM activities continue in the region, Hg could deposit over the restored soil and plants, being attached to the organic matter and posing an environmental and health risk, even entering the food chain.

To improve the reforestation programs, it would be essential to find indigenous plant species resistant to mercury and with the potential to prevent mercury entry into the food chain. The use of plants to reduce the concentration or toxic effects of contaminants in the environment is called phytoremediation (Arthur et al., 2005; Ghosh & Singh, 2005). The main techniques of phytoremediation of heavy metals include phytostabilization, the immobilization of metals in soils through sorption by roots, precipitation, complexation, or reduction by rhizosphere, and phytoextraction, the uptake of metals from soil by plant roots and their translocation and accumulation in aboveground biomass (Ali et al., 2013; Tangahu et al., 2011). The most appropriate plant species for phytoremediation are those called hyperaccumulators, plants capable of accumulating large metal concentrations and tolerant to the toxic effects of these metals (Barcelo & Poschenrieder, 2003; van der Ent et al., 2013). Four hundred plant species of 45 families have been considered hyperaccumulators of metals such as cobalt, copper, chromium, lead or nickel, zinc, or manganese (Baker & Brooks, 1989). A hyperaccumulator of Hg has not been identified yet, so research is needed to screen indigenous plant species for the potential to hyperaccumulate mercury in contaminated soils.

1.4 Soil Screening Levels of Mercury

Another initiative of the Peruvian Government within its multi-sectorial program is the review of environmental regulations related to mercury, such as the SSL of mercury in agricultural soils. This review is due to the interest of the Peruvian Government in reforesting the areas degraded by ASGM using agricultural plant species. This value (6 mg/Kg dry weight) (Table S3-3) is mandatory in the design of regulations and public policies. It is also mandatory to design and apply environmental management instruments, such as environmental impact assessments and environmental and human health risk assessments (MINAM, 2013). Risk assessments are required by the Peruvian regulation for the remediation of contaminated sites (MINAM, 2017), following a similar approach to the U.S. EPA guidelines (US EPA, 1993).

To determine a robust value of SSL of a contaminant in soil, relevant and adequate toxicity data for local species is required (European Commission, 2003). It is crucial to consider the physical and chemical characteristics of the local soil, the ecologically important species that could be affected after exposure to the contaminant, and the environmentally relevant contaminant levels in local soils. Peruvian mercury SSL in agricultural soil regulation was established in 2013 (MINAM, 2013), adopting the Canadian regulation[1]. This international regulation used to set mercury SSL in Peru was established in a country with quite a different geography, climate, and ecosystem characteristics. To set the Peruvian regulations, local research with indigenous species and characteristics of local soils in the three Peruvian ecological systems (coast, mountains, and forest) were not considered. Other factors not contemplated were local land uses (activities considered in the category of agricultural soil, such as forestry, cattle raising,

[1] Personal communication with the German Society for International Cooperation (GiZ) on July 16, 2020.

etc.) and environmentally relevant levels of contaminants in local sites. A comparison between mercury SSL in agricultural soil of different countries is shown in Table S3-3.

A high variability between mercury SSL exists in different countries and different regions of the world. The main reasons for this variability are political and scientific (different scientific and technical approaches) (Provoost et al., 2006)—for example, the differences in potential exposure pathways to contaminated soil for human and ecological receptors considered. Different pathways consider different potential receptors of concern with different sensitivity to contaminants. Some countries decided not to consider specific pathways due to the lack of literature or data or the predominance of specific land uses in their territory (Carlon, 2007). Another difference is the consideration of soil characteristics (correction for organic matter, clay content, pH, etc.) Some soil characteristics, such as the organic matter content, can influence the mobility and bioavailability of metals in soil. An increase in the organic matter content in soil can reduce the mobility of some metals, such as Pb, Zn, Cu, and Hg, reducing their uptake by plants (Hashimoto et al., 2009; Branzini et al., 2010; Cozzolino et al., 2016). pH can also influence the mobility of metals in soil. Mobility of metals like Cu, Ni, Cd, Hg, and Zn in the soil increases at low pH (Temminghoff et al., 1997; Richards et al., 1999; Yin et al., 1996). Less mobility in the soil means a lower likelihood of the metals being taken up by plants and other organisms living in this soil so that the SSL would be higher. Some countries decided to consider standard soil (homogeneous soil) due to the lack of significant differences between soil characteristics in their territory (Carlon, 2007).

Only Brazil, Ecuador, and Peru have established mercury SSL in agricultural soils among the South American countries in the Amazonian region affected by artisanal mining. The values in these countries vary between 0.8 and 12 mg kg^{-1} dry weight (dw) and were set based on

international methodologies. The Peruvian Government compared the Canadian, US EPA, and European methodologies for deriving mercury SSL before establishing its value in adopting the Canadian SSL. In Brazil, São Paulo State first adopted the mercury SSV from the Netherlands before developing its own methodology based on the US EPA methodology (US EPA, 1996).

Some of the main differences between the Canadian, US EPA, and European methodologies are related to the protected receptors, exposure pathways, soil type dependence, data requirements, and data analysis. Regarding the protected receptors, the European and Canadian methodologies consider the protection of human health, terrestrial and aquatic ecosystems (Carlon, 2007; CCME, 2006), while the US EPA methodology considers the protection of human health and groundwater (USEPA, 1996). US EPA has developed a guideline for deriving SSL to protect terrestrial ecosystems from 10 contaminants, but this guideline does not include mercury (USEPA, 2000).

Regarding exposure pathways, the European methodologies consider soil outdoor and indoor exposure, soil-derived diet exposure, and water pathways. Some European countries do not consider different exposure pathways and different protection levels (target, trigger, and intervention values) for different land uses (agricultural, residential, industrial) (Carlon, 2007). The Canadian methodology considers general pathways similar to European methodologies, including a nutrient and energy cycling pathway. It considers different exposure pathways and different protection levels for different land uses (CCME, 2006). The US EPA methodology considers similar general pathways to European and Canadian methodologies but only for human exposure and residential land use. In soil-derived diet exposure, it only considers exposure through plant ingestion but does not consider exposure through meat, fish, or dairy ingestion (US EPA, 1996).

Regarding soil type, the European methodologies consider a standard soil type for calculating the SSL (organic matter content 2-3.4%, clay content 10%). Some European countries consider a correction of the SSV for clay and organic matter content of the soil in site-specific risk assessments (Carlon, 2007). The Canadian methodology considers one standard type of soil (0.5% organic carbon content and 8-11% clay content) for the derivation of the SSL for inorganic substances and two types of standard soils (organic carbon content of 0.5% in both standard types of soil and clay content 8-11% for coarse soils and 15-20% for fine soils) for the calculation of SSL for organic substances. It does not consider any correction of the SSL for clay or organic matter content of the soil for site-specific assessments (CCME, 2006). The US EPA methodology considers a standard type of soil (0.6% organic carbon content and 8-28% clay content) for calculating the SSL without correction for the soil's clay and organic matter content for site-specific assessments (USEPA, 1996).

Regarding data requirements and data analysis for the derivation of the SSL, the European and Canadian methodologies use similar general methods for data analysis with some variations: when little toxicity data for different species is available (less than three taxonomic groups), they select the lowest reported toxicity value (Canada) or the lethal concentration for 50% of the test organisms (Europe) and divide it by an assessment (safety) factor to calculate the SSL. When sufficient toxicity data for different species is available, they array all reported values in a statistical distribution and select a particular percentile to calculate the SSL (5th percentile in European methodology and 25th percentile in Canadian methodology for target value) (Carlon, 2007; CCME, 2006). The Canadian methodology additionally uses a "weight-of-evidence" approach to select the data. The available toxicity data is examined to determine

whether it is biased by excessive numbers of chronic data (non-observable adverse effect concentration - NOAEC or lowest observable adverse effect concentration- LOAEC) or by acute data (effect concentration 50- EC50 or lethal concentration 50- LC50). The statistical distribution is not used if one type of data is over-represented (based on expert judgment). The US EPA methodology is different because it only considers one species (humans). It uses default conservative assumptions to calculate the SSL for different human exposure pathways. Exposure equations and pathway models are run in reverse to calculate SSLs corresponding to a specific level of risk. The mercury SSLs are based on a hazard quotient of 1. For groundwater pathways, SSLs are based on nonzero maximum contaminant level goals (MCLGs) or maximum contaminant levels (MCLs) (US EPA, 1996).

As mentioned before, relevant and adequate toxicity data for different species from different trophic levels is required to determine the SSL of a contaminant in soil. If there is no published data for local indigenous species, international literature can be used, but the values obtained would not be as robust (European Commission, 2003). Peruvian mercury SSL regulation was set without considering local toxicity data. The current Peruvian Government has expressed interest in revising mercury SSL in agricultural soil using data from Peruvian indigenous species, but this data is lacking. The work presented here aims to generate adverse health effects after Hg exposure data for these indigenous plant species. The Peruvian authorities could use the data to revise mercury SSL in agricultural soil and develop environmental and human health risk assessments in areas degraded by ASGM.

1.5. Project Overview

The main goal of this dissertation is to assess the accumulation and effects of Hg contamination due to ASGM on Amazonian plant species. The plants chosen for this study are

used for reforestation and conservation purposes and for providing ecosystem services (food, medicinal plants, timber) in the Peruvian Amazonian region of Madre de Dios. Specifically, the project will help close the knowledge gap on the threshold of harmful exposure to mercury of three Amazonian indigenous species of plants. This study will also determine for the first time the impact of Hg on the survival, growth, and health of these species after exposure to different concentrations of Hg in soil. It will also evaluate the bioaccumulation of mercury by plant species growing in ASGM sites when exposed to mercury through soil and air and their potential for being used in phytoremediation of Hg-contaminated soils. Moreover, this study will examine the influence of soil properties and soil amendments on Hg bioaccumulation by these plant species.

Finally, the study will examine for the first time soil physicochemical characteristics in the rhizosphere of plant species growing in naturally regenerated and reforested areas impacted by ASGM and in the primary forest near these areas. It will measure the recovery of the primary forest levels of these soil properties in the rhizosphere and bulk soil in areas with different natural regeneration ages and areas reforested with various methods. It will also identify native plant species that can be good candidates for restoring areas degraded by ASGM in the Amazon rainforest.

Integrating Indigenous and scientific knowledge to monitor land degradation in areas impacted by ASGM will also be evaluated. The study will collect information about indicators of soil degradation used by Indigenous people, proposals for restoration, incorporating the Indigenous needs, and challenges to overcome to design sustainable restoration projects in three Indigenous communities affected by ASGM in the Madre de Dios region. The governments in the Amazon region can use the study's results to improve the design and implementation of

restoration programs in areas degraded by ASGM in Indigenous communities. Indigenous communities can also use the results to make decisions about restoring the areas degraded by ASGM in their territory.

Chapter 2: Hg distribution and accumulation in soil and vegetation in areas impacted by artisanal gold mining in the Southern Amazonian region of Madre de Dios, Peru

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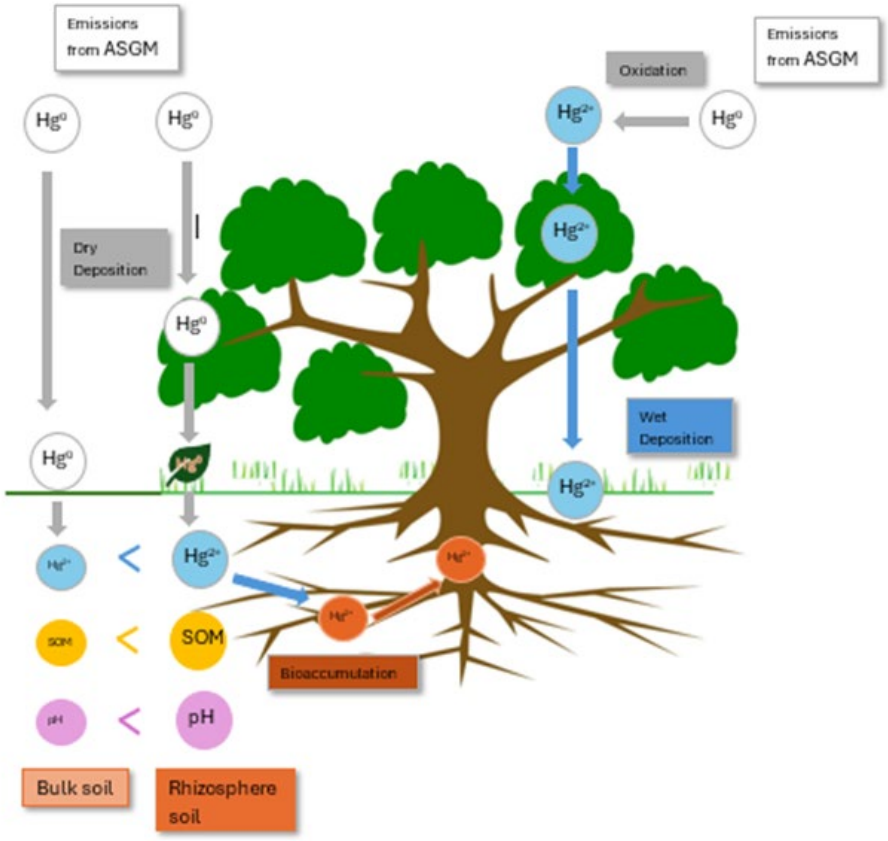
Graphical Abstract



Abstract

Artisanal and small-scale gold mining (ASGM) is the primary global source of anthropogenic mercury (Hg) emissions. It has impacted the Amazon rainforest in the Peruvian region of Madre de Dios. However, few studies have investigated Hg's distribution in terrestrial ecosystems in this region. We studied Hg's distribution and its predictors in soil and native plant species from artisanal mining sites. Total Hg concentrations were determined in soil samples collected at different depths (0-5 cm and 5-30 cm) and plant samples (roots, shoots, leaves) from 19 native

plant species collected in different land cover categories: L1 naked soil (L1), gravel piles (L2), natural regeneration (L3), reforestation (L4), and primary forest (L5) in the mining sites. Hg levels in air were also studied using passive air samplers. The highest Hg concentrations in soil (average 0.276 and 0.210 mg kg^{-1} dw.) were found in the intact primary forest (L5) at 0-5 cm depth and in the plant rooting zones at 5-30 cm depth, respectively.

Moreover, the highest Hg levels in plants (average 0.64 mg kg^{-1} dw) were found in foliage of intact primary forest (L5). The results suggest that the forest in these sites receives Hg from the atmosphere through leaf deposition and that Hg accumulates in the soil surrounding the roots. The Hg levels found in the plant leaves of the primary forest are the highest ever recorded in this region, exceeding values found in forests impacted by Hg pollution worldwide and raising concerns about the extent of the ASGM impact in this ecosystem. Correlations between Hg concentrations in soil, bioaccumulation in plant roots, and soil physical-chemical characteristics were determined. Linear regression models showed that the soil organic matter content (SOM), pH, and electrical conductivity (EC) predict the Hg distribution and accumulation in soil and bioaccumulation in root plants.

1. Introduction

Artisanal and Small-Scale Gold Mining (ASGM) is typically conducted by individual miners or small enterprises with limited capital investment and production (WHO, 2016). This activity is often illegal and informal and is mainly carried out in countries with limited regulation in this sector (TGIATOC, 2016). ASGM can result in several human and environmental health impacts, such as mercury (Hg) pollution and deforestation (Caballero Espejo et al., 2018; Gerson et al., 2020; Gerson et al., 2022). According to the United Nations Environment Programme (UNEP), South America is the world's second-largest producer of anthropogenic Hg emissions, being

ASGM the primary source of these emissions. ASGM is also the main contributor to Hg pollution worldwide (AMAP/UNEP, 2019). In the Southern Peruvian Amazon, it is estimated that 180 metric tons of Hg are released into the environment annually, 35% of which are emitted into the atmosphere (Cardo & Vargas, 2017; Telmer & Veiga, 2008). Liquid elemental Hg is used in ASGM to extract the gold from the soil, forming an amalgam that is burned, emitting Hg vapors (elemental and mercuric chloride) into the atmosphere (Esdaile & Chalker, 2018). The wind can transport gaseous elemental mercury (GEM) into the atmosphere. The gaseous elemental mercury GEM can be oxidized to Hg^{2+} (Durnford et al., 2010) and deposited over surface waters and soil through dry deposition or wet deposition (Pirrone et al., 2013). Hg^{2+} can be transformed into methylmercury, which is a toxic form of organic Hg, by anaerobic microorganisms and enter the food chain mainly in aquatic ecosystems (Mergler et al., 2007; Lodenius & Malm, 2019).

Previous studies have proved that vegetation has a key role in the Hg cycle. When GEM is in the atmosphere, it can deposit over the canopy and be absorbed by leaves stomata, where it is oxidized to Hg^{2+} (Laacouri et al., 2013), then enters the forest soils via litter deposition and throughfall (dry deposition) (Driscoll et al., 2013). This Hg pathway is especially important in the Amazon rainforest, where some studies have shown that litter deposition and throughfall account for 94% of the Hg input flux (Gerson et al., 2022). Once in the soil, Hg^{2+} can be transformed into methylmercury, and both species can also enter the food chain in terrestrial ecosystems. (Li et al., 2017; Rothenberg et al., 2016; Yung et al., 2019; Rieder et al., 2011).

The Peruvian Amazonian region of Madre de Dios is one of Earth's most biodiverse regions (Tarazona & Miyashiro-Lopez, 2020), providing diverse ecosystem services, such as water supply and climate change mitigation (Fisher et al., 2018). However, this region has been

extensively impacted by ASGM causing deforestation of 100,000 ha of forest from 1984 to 2017 (Caballero Espejo et al., 2018). Nine of the 15 most consumed fish species in Madre de Dios had Hg levels exceeding the safe limit set by the US EPA (CAMEP, 2013). Additionally, Hg levels two times the US EPA Mercury Reference Concentration in human hair (1 mg kg^{-1}d.w.) were found in residents of 46 communities of the region (Feingold et al., 2020), which may present a health risk to the local population.

The Peruvian government and local NGOs have started restoration projects in areas degraded by ASGM using native plant species that can provide ecosystem services including food for humans and wildlife (Román et al., 2020). However, there are very few published studies and thus a lack of understanding about Hg's accumulation, and effects on the Amazonian soil and plants, especially on native vegetation. Here we examine for the first time Hg distribution and accumulation in different parts of native plant species in areas impacted by ASGM in the Peruvian Amazon. We studied how physic-chemical characteristics of soil (soil organic matter (SOM), pH, and EC) influence this Hg accumulation in native plants. We measured GEM in the atmosphere and total Hg in plant leaves, shoots, roots, and soil in two ASGM sites. We determined bioaccumulation factors (BCF) and predicted Hg accumulation by indigenous plant species in these sites using soil Hg concentrations and physic-chemical characteristics as co-factors. We hypothesized that SOM, soil pH, and EC would drive Hg bioaccumulation from soil by Amazonian plant species. We also expected that Hg levels in plant leaves correlate with Hg levels in soil without translocation from roots to leaves, evidencing litter deposition as a key pathway for Hg in the Amazonian soil.

2. Materials and methods

2.1 Study area

We performed field work in the Southern Peruvian Amazonian region of Madre de Dios. We selected two ASGM sites using some of the most common artisanal mining techniques, called minimally mechanized techniques (Caballero-Espejo et al., 2018) to extract and transport the soil and gold material. One of the selected sites is a mining concession in Laberinto district, Tambopata province, 50 km from Puerto Maldonado, capital of Madre de Dios region, along the Madre de Dios River, 200 meters above sea level (masl) at UTM coordinates 19 L 433050/8597900. The second site is a mining concession in Inambari district, Tambopata province, 100 km from Puerto Maldonado, in the "La Pampa" area. La Pampa is in the Tambopata National Reserve buffer zone, 220 masl, at UTM coordinates 19 L 397039/8560472 (). Both sites are formalized mining concessions that have opted to eliminate the use of mercury. However, it is worth noting that in both areas, there are nearby illegal activities that still use mercury for the amalgamation process.

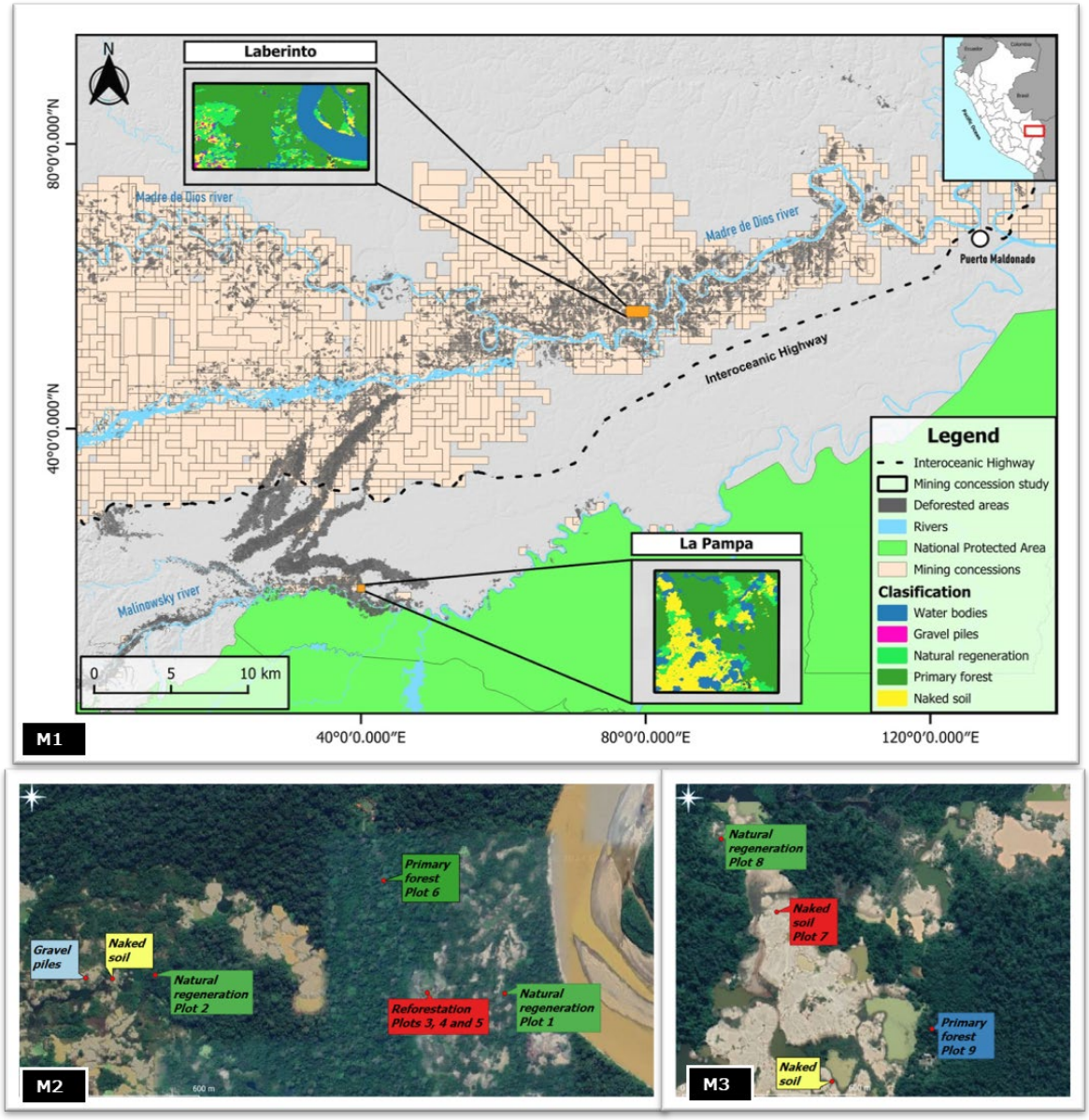


Fig. 2-1. **M1.** Map of the two sampling sites in Laberinto and La Pampa districts in the Madre de Dios region, Peru. **M2**. Map of the land cover category plots in Laberinto site. **M3**. Map of the land cover category plots in La Pampa site.

At each mining concession, we studied different land cover categories according to the levels of degradation left by the mining activity. We also considered a category for the primary forest not impacted by this activity at each study site (Fig. 2-2). During the mining process, the miners

deforest the area and then excavate the soil with high-pressure water hoses (Caballero & Espejo, 2018), separate and pile up gravel, and process the old deep sediments where the gold is located (Salinas, 2007). This mining method breaks and washes the topsoil, leaving behind highly degraded and poor in nutrients sandy and gravelly subsoils (Timsina et al., 2022). In this study, we considered the following land cover categories (Fig. 2-2): L1- naked soil; in this category, the soil consists mainly of sand with no vegetation cover; L2 - gravel piles; this category consists of piles of cobbles and pebbles left by the mining activity with no vegetation cover; L3 - natural regeneration; in this category, the soil was once composed of sand or pebbles over which vegetation has grown naturally, changing the soil characteristics.; L4 - reforestation; in this category, the soil was once composed of sand or pebbles over which plants have been reforested by human intervention, changing the soil characteristics. The plant species in this category are different from the ones in the natural regeneration and primary forest, L5 - primary forest; this category represents the intact forest non-deforested by ASGM.

The Laberinto concession has an extension of 449 ha. From the total area, 5.4% is formed of naked soil, 0.94% is gravel piles, 16.4% consists of natural regeneration, 2.3% is reforestation, and 54.7% is primary forest. The rest of the extension, 20.2%, are ponds. The La Pampa concession has an extension of 100 ha. Of the total area, 25.7% is formed of naked soil, 13.1% consists of natural regeneration, 5% is reforestation, and 47.6% is primary forest. The rest of the extension, 8.7%, are ponds (Fig.2- 1. M1).

We selected one plot of 50 x 20 m per land cover category at each mining site with some exceptions as follows. At the Laberinto site, we chose two natural regeneration plots instead of one due to the diversity of plant species in the natural regeneration at this site (Fig. 2-1. M2). The age of the plants in these plots is unknown, but according to the miners, the mining activity in the

plots stopped eight years before our study, and the plants started to grow after the abandonment date. In the first natural regeneration plot, we found 29 different plant species of trees and shrubs, and in the second plot, 59 different plant species of trees and shrubs. In Laberinto, we chose three reforestation plots instead of one to account for the three different soil amendments applied during the reforestation in this area. In these reforestation plots, 30 different tree species were planted five years before our study. We selected one plot per soil amendment, one plot where biochar was applied, and one plot where biochar enriched with NPK was applied. We also selected one plot where no soil amendments were applied. These plots were sampled to study the influence of different soil amendments in the Hg distribution in soil and in the Hg accumulation by plants. One primary forest plot with 125 different plant species of trees and shrubs was also chosen. The age of the primary forest is unknown, but according to the local population, this forest was never disturbed by mining activity, and the region was first inhabited 120 years ago, so we assume that the forest is at least that old. At La Pampa site, we chose one natural regeneration plot with 24 different plant species of trees and shrubs (Fig. 2-1. M3). The age of the plants in the plot is unknown, but according to the miners, the mining activity in the plot stopped three years before our study and the plants started to grow after the abandonment date. In this site, we also chose one reforestation plot where 13 different tree species were planted 3 years before our study, but the plants were very poorly developed, and the soil still had sand characteristics at the time of the sampling. Thus, we decided to consider this plot as naked soil. Finally, we chose one primary forest plot with different plant species of trees and shrubs. The number of plant species in this plot is unknown. The age of the primary forest is also unknown, but according to the local population, this forest was never disturbed by mining activity, so we assume that this forest is the same age as the primary forest in Laberinto.

The plots in both mining concessions were sampled in 2022 in the rain season (March-April) and the dry season (August-September), except the primary forest in Laberinto that could not be sampled in the rain season due to flooding in that area. Similarly, only the reforestation plot where no soil amendments were applied was sampled in the rain season. Additionally, due to the poor development of the plants in the naked soil in La Pampa, they were only sampled in the rain season to prevent any damage.



Fig. 2-2. Land cover categories at the sampling sites. **L1.** Naked soil (top left). **L2.** Gravel piles (top right). **L3**. Natural regeneration (bottom left). **L4.** Reforestation (bottom center). **L5**. Primary forest (bottom right).

2.2 Sample collection

2.2.1 Plant Sampling

At the forested land cover categories, natural regeneration (L3), reforestation (L4), and primary forest (L5) in each site, we collected samples of plant tissue (roots, shoots, and leaves) from 19 of the most abundant plant species in 50 x 20 m plots in each land cover category grouped into ten families (Table 2-1 and 2Table S1-1). To ensure the representativeness of the plant collection, at least three of the most abundant plant species per land cover category were selected, and at least four individuals of each plant species, when possible, were collected (Rautio et al., 2020).

Four fresh foliage subsamples were collected from the outside crown of each individual plant at 1.5-2 m height in the four orientations (north, south, east, and west) and mixed to form a composite sample. The same procedure was followed to collect shoot samples. To study the influence of leaf age on Hg levels, deciduous and perennial plant species were sampled (Table S2-1). The selected plant species were not superficially rooted. Thus, root samples were collected from each individual plant at 5-30 cm depth. Under each plant, a sampling point was selected, surface litter was removed, and a hole of 30 cm deep was excavated near the plant roots with a stainless-steel trowel. Three subsamples of the first three orders of roots (0 -5 mm diameter) were cut with stainless steel scissors collected at 5-30 cm depth, and mixed to form a composite sample. These types of roots were chosen because they are responsible for nutrient uptake (Freschet & Roumet, 2017). The root sampling was performed similarly for all the selected plant species. Plant samples were collected using stainless-steel scissors and clean disposable gloves, placed into individual paper bags, and cold-stored in labeled sealed plastic bags until transportation to the laboratory.

In total, two hundred thirteen plant tissue samples were collected in the rainy season of 2022 (35 root, 35 shoot, and 35 leaf samples in Laberinto, and 36 root, 36 shoot, and 36 leaf samples in La Pampa) and 267 samples in the dry season of 2022 (65 root, 65 shoot, and 65 leaf samples in Laberinto, and 24 root, 24 shoot, and 24 leaf samples in La Pampa). One composite sample of roots, one composite sample of shoots, and one composite sample of leaves were collected per individual plant.

Table 2-1. Plant species collected in the two sampling sites.

Sampling site	Land cover category	Plant species	n	Family
Laberinto	Natural regeneration (L3)	*Acacia loretensis*	8	*Fabaceae*
		Cecropia polystachya	6	*Urticaceae*
		Chromolaena laevigata	9	*Asteraceae*
		Ficus insipida	4	*Moraceae*
		Inga ruiziana	6	*Fabaceae*
		Ochroma pyramidale	3	*Asteraceae*
		Zanthoxylum aff sprucei	7	*Rutaceae*
	Reforestation (L4)	*Calliandra angustifolia*	16	*Fabaceae*
		Hura crepitans	17	*Euphorbiaceae*
		Inga spp	15	*Fabaceae*
	Primary forest (L5)	*Quararibea wittii*	3	*Malvaceae*
		Pseudolmedia laevis	3	*Moraceae*

		Psychotria sp10	3	*Rubiaceae*
La Pampa	Naked soil (L1)	*Tabebuia serratifolia*	4	*Bignoniaceae*
		Inga spp	4	*Fabaceae*
		Dipteryx micrantha	4	*Fabaceae*
	Natural regeneration (L3)	*Cecropia engleriana*	5	*Urticaceae*
		Cecropia membranacea	3	*Urticaceae*
		Chromolaena laevigata	8	*Asteraceae*
		Tibouchina longifolia	8	*Melastomataceae*
	Primary forest (L5)	*Psychotria sp10*	8	*Rubiaceae*
		Inga alba	4	*Fabaceae*
		Inga ruiziana	4	*Fabaceae*
		Quararibea wittii	8	*Malvaceae*

2.2.2 Soil Sampling

We collected soil samples at each site through a stratified systematic sampling of the different land cover categories. At each land cover category, plots of 50 m x 20 m of easy access were selected, and soil samples were taken in 5 sampling points per plot, when possible: one point at each vertex and one point in the center, at different depths (0-5 cm, 5-30 cm). In the reforestation category (L4) at the Laberinto site, a plot per soil amendment applied and a plot where no soil amendments were applied were sampled. Soil samples were collected using the clean hands-dirty

hands protocol (EPA Method 1669) using a stainless-steel hand shovel. At each sampling point, the surface litter was removed, and a 50 cm-deep, square-shaped hole was excavated. Soil from the four walls of the hole was taken and mixed to form a composite sample at each depth (0-5 cm and 5-30 cm), then sieved to < 2 mm to remove stones and roots, and cold-stored in labeled sealed plastic bags until transportation to the Mercury and Environmental Chemistry Laboratory (LAMQA), managed by the Instituto de Investigaciones de la Amazonia Peruana (IIAP) and CINCIA in Madre de Dios, Peru.

At the forested land cover categories, rhizosphere soil was collected at the same points where plant roots were sampled (see section 2.2.1). At each point, surface litter was removed, and a hole 30 cm deep was excavated near the plant roots. Three subsamples of soil surrounding the roots at 5-30 cm depth were collected and mixed to form a composite sample using the clean hands-dirty hands protocol (EPA Method 1669) with a stainless-steel hand shovel and cold-stored in labeled sealed plastic bags until transportation to the LAMQA laboratory.

Soil subsamples were transported to the United States under USDA soil permit number 525-23-163-97136, and analyzed for physic-chemical characteristics (SOM, pH, EC) at the Environmental Engineering Laboratory, Department of Civil and Environmental Engineering, University of Maryland, College Park.

In total, 86 non-rhizosphere soil samples were collected in the rainy season of 2022, in March and April (46 samples in Laberinto and 40 samples in La Pampa), and 111 non-rhizosphere soil samples were collected in the dry season of 2022, in August and September (71 samples in Laberinto and 40 samples in La Pampa). Seventy-one rhizosphere soil samples were collected in the rainy season of 2022 (35 samples in Laberinto and 36 samples in La Pampa), and 89

rhizosphere soil samples were collected in the dry season of 2022 (65 samples in Laberinto and 24 samples in La Pampa).

2.2.3 Air sampling

We used passive air samplers (PAS) developed by MacLagan et al. (2016) to sample GEM. The PAS used sulfur-impregnated activated carbon to collect GEM that passes through a Radiello© diffusive to prevent the sampling of particulate-bound Hg. We deployed the PAS in duplicate at each site during the 2022 rain season for 55 days (March–May 2022) and the 2022 dry season for 92 days (August 2022-November 2022). Duplicates of the PAS were deployed in one forested and one deforested area in each site (4 PAS total per site per season). The PAS were attached to aluminum posts in deforested areas and tree branches in forested areas 1-2 m above the ground using nylon cable ties. The PAS were stored in double resealable plastic bags before deployment and post-deployment. Trip and storage blanks were collected to assess contamination during sampling, transport, and storage to the LAMQA Laboratory, in Puerto Maldonado, Madre de Dios, Peru.

2.3 Sample analysis

2.3.1 Atmospheric Mercury Analysis

Total Hg concentrations of GEM collected by PAS were determined by thermal desorption, amalgamation, and atomic absorption spectroscopy (US EPA Method 7473) using a DMA-80 instrument (Milestone Inc.) following the method from McLagan et al. (2016). We performed quality control using certified reference material, NIST SRM 2692c (bituminous coal, 0.1790 µg g^{-1}) and SRM IAEA-436A (4.26 mg kg^{-1}), and liquids standards. Values were considered

acceptable when the relative percent difference measured for continuous calibration verification and quality control standards was within 10% of the accepted values.

We calculated GEM concentrations using the total mass of Hg sorbed by the PAS divided by the deployment time and sampling rate (0.135 m^3 day^{-1}) (McLagan et al., 2016; McLagan et al., 2018) adjusted for temperature and wind according to the average temperature and wind measurements for the Madre de Dios region reported by World Weather Online (McLagan et al., 2018).

2.3.2 Soil and Plant Mercury Analysis

All the samples were freeze-dried for 48 hours; soil samples were sieved to < 1mm and plant shoot and leaf samples were cut to < 1 mm for homogenization before analysis. Root samples were brushed to remove soil particles attached to the root surface and cut to < 1 mm for homogenization before analysis. All the tissues samples from individual plants were processed and analyzed separately.

The samples were analyzed for total Hg on a Milestone Direct Mercury Analyzer (DMA-80) via thermal decomposition atomic absorption spectroscopy (EPA Method 7473). For QA/QC, strict blanks control was performed at the beginning of each run. Reference materials IAEA-456 (0.077 mg kg^{-1}), SRM 2711a (7.42 mg kg^{-1}), SRM-1575a (0.0399 mg kg^{-1}), and ERM-CC141 (0.083 mg kg^{-1}) and liquid standards with a recovery rate of 90-110% were used for quality control.

The detection limit for total Hg was 0.001 μg g−1 dw, calculated as three times the standard deviation (SD) of the blank. Two to three replicates per sample were analyzed, and values were accepted when the relative percentage difference between the two samples was within 10%. All reported concentrations are in dry weight.

2.3.3 Soil Organic Matter Content Analysis

The organic matter content was determined as a percentage of the loss by ignition of 10.0 g soil in a furnace at 450 °C (Thermo Scientific muffle furnace Thermolyne) for three hours (ASTM, 2014).

2.3.4 Electrical Conductivity and pH Analysis

The pH and EC of the soil were measured in soil distilled water solution (1:2) using a Thermo Scientific Versa Star meter at the Environmental Engineering laboratory at the University of Maryland, USA.

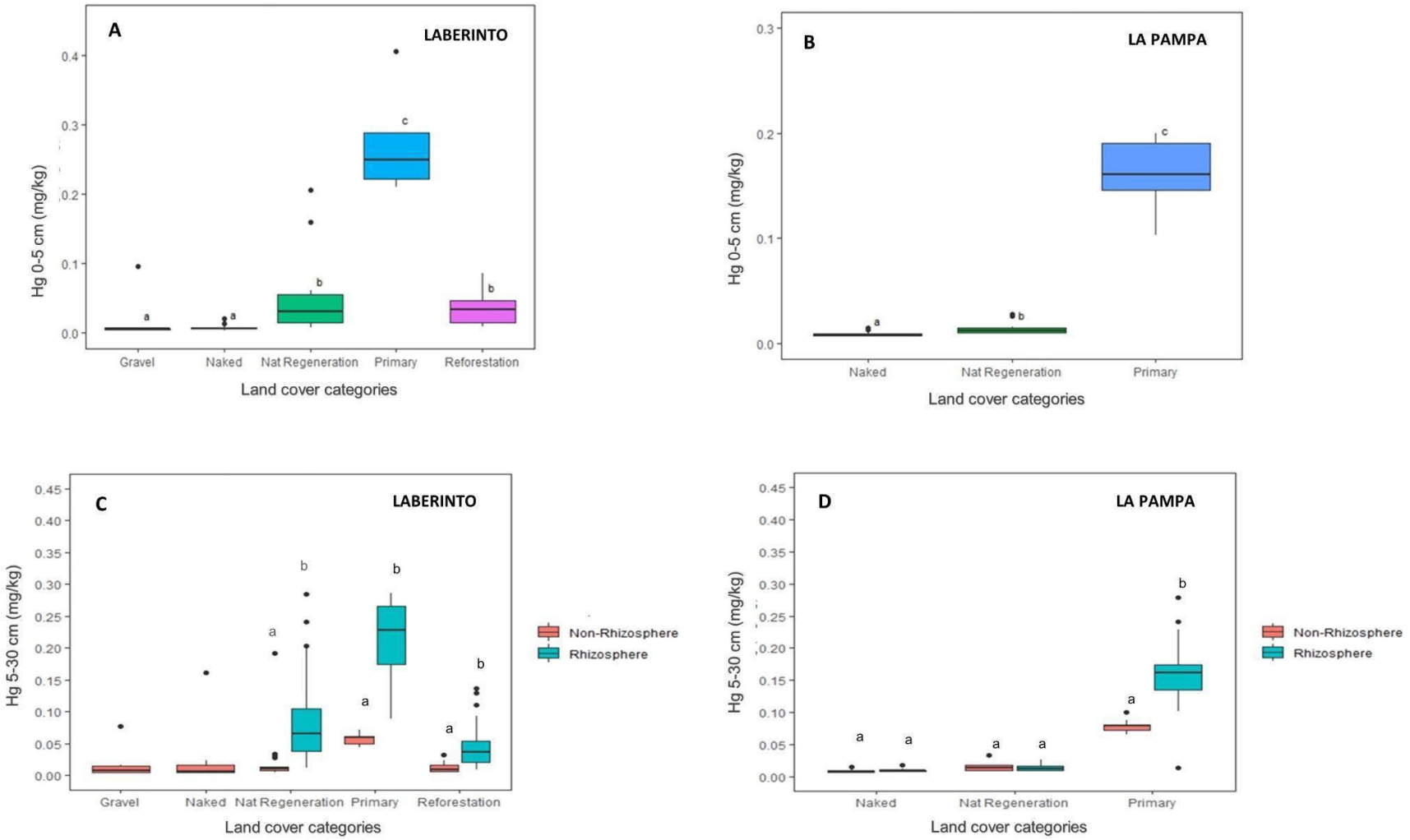
2.4 Data analysis and statistical analysis

Total Hg concentrations in tissues and soils, SOM, pH, and EC in soils were reported as the mean ± standard deviation. We performed statistical tests using an alpha of 0.05. Data were log-transformed, and linearity, homoscedasticity (Levene), normality (Shapiro-Wilk), and independence tests (Durbin-Watson) were performed. Multicollinearity between variables was also tested. Relationships between variables were evaluated using Pearson correlations, and general linear models were developed to predict Hg levels in soil and plants. Akaike information criterion (AIC) was used to choose the models that better fit our data. Comparisons of Hg, SOM, pH, and EC levels between land cover categories and soil depths were performed using the non-parametric Kruskal-Wallis's test, followed by Wilcoxon as a post-hoc test. The bioaccumulation factor (BCF) was estimated using the measured Hg concentrations and expressed as the ratio of the metal concentration in the roots to that in rhizosphere soil (Yoon et al., 2006). All statistical analyses were performed using R version 4.3.2 statistical software, and the ggplot2 package was used for visualizations.

3. Results and discussion

3.1 Total Hg concentrations in soils

Among the studied land cover categories, the primary forest (L5) had the highest Hg levels in soil with a mean ± standard deviation of 0.276 ± 0.079 mg kg^{-1} and 0.162 ± 0.032 mg kg^{-1} in the top layer 0-5 cm in Laberinto and La Pampa, respectively) followed by natural regeneration (L3) (mean 0.047 ± 0.053 mg kg^{-1} and 0.015 ± 0.007 mg kg^{-1} in the top layer 0-5 cm in Laberinto and La Pampa, respectively) (Fig. 2-3). We found significantly higher total Hg concentrations in soil at 0-5 cm depth in the Laberinto site compared to the La Pampa site, and no significant difference in Hg levels in soil at this depth between seasons in either site (Table S2-2)


A
LABERINTO
Hg 0-5 cm (mg/kg)
Gravel
Naked
Nat Regeneration
Primary
Reforestation
Land cover categories
B
LA PAMPA
Hg 0-5 cm (mg/kg)
Naked
Nat Regeneration
Primary
Land cover categories
C
LABERINTO
Hg 5-30 cm (mg/kg)
Non-Rhizosphere
Rhizosphere
Land cover categories
D
LA PAMPA
Hg 5-30 cm (mg/kg)
Non-Rhizosphere
Rhizosphere
Land cover categories

1

Fig. 2-3. Distribution of Hg in soil in the two sampling sites. **A**. Hg concentrations in soil at 0-5 cm in the Laberinto site. **B.** Hg concentrations in soil at 0-5 cm in La Pampa site. **C**. Hg concentrations in soil at 5-30 cm in the Laberinto site. **D**. Hg concentrations in soil at 5-30 cm in La Pampa site. Different lower-case letters above the boxes indicate significant differences ($p \leq 0.05$) between Hg concentrations within land cover categories and in rhizosphere and non-rhizosphere soil. In graphs C and D, the significance letters are different for each land cover category.

The non-forested land cover categories, naked soil (L1) and gravel piles (L2), had significantly lower Hg concentrations than the forested ones, natural regeneration (L3), reforestation (L4), and primary forest(L5). These levels in impacted sites are similar to concentrations reported by other studies in sites impacted by ASGM in Madre de Dios (Velasquez Ramírez et al., 2021). However, in the primary forest (L5), soil Hg concentrations found in Laberinto were higher than the ones previously reported for primary forest in this region (previously reported values are between 0.10 -0.14 mg kg^{-1}) (Gerson et al., 2022; Velásquez Ramírez et al., 2021; Mandros, 2019). Significant differences between Hg concentrations throughout the two depths evaluated were only found in the primary forest (L5) compared to the other land cover categories, with levels decreasing with depth (range of 0.276 - 0.057 mg kg^{-1}, n = 10, and 0.162 - 0.080 mg kg^{-1}, n = 20, from 0 - 30 cm in Laberinto and La Pampa, respectively; Table 2-2). We hypothesized that this is due to the SOM contents in the soil profile, and this difference is stronger in the primary forest profile (L5) (Table S2-3). Also, the top layer of soil can receive Hg deposition via throughfall and litterfall (Wright et al., 2016; Gerson et al., 2022). This Hg distribution is consistent with reports from tropical forests impacted by ASGM in Asia and Brazil (Tomiyasu et al., 2020; Ma et al., 2022; Lima et al., 2022). Even though, the Hg levels in soil found in this

study are lower than the soil quality criteria for Hg in agricultural soils established in the Peruvian regulation (6.6 mg kg^{-1}) (MINAM, 2013) the values are in the range of the preliminary critical limits to prevent ecological effects due to Hg in soil set by the United Nations Environment Programme (0.07 – 0.30 mg kg^{-1}) (UNEP, 2002), which may pose a threat to environmental health in these sites.

Table 2-2. Hg concentrations in soil (mg kg^{-1}) in different land cover categories in the two sampling sites.

Sampling site	Land cover category	Depth (cm)	n	Hg (mg kg^{-1})
Laberinto	Naked soil	0 – 5	10	0.0085 ± 0.005
		5 – 30	10	0.024 ± 0.045
	Gravel piles	0 – 5	8	0.017 ± 0.032
		5 – 30	8	0.017 ± 0.025
	Natural regeneration	0 – 5	18	0.047 ± 0.053
		5 – 30	18	0.023 ± 0.043
		Rhizosphere (5 – 30)	43	0.103 ± 0.116
	Reforestation (average concentration of the plots with no-soil amendments, plot with biochar, and	0 – 5	20	0.036 ± 0.022
		5 – 30	20	0.013 ± 0.007
		Rhizosphere (5 – 30)	48	0.043 ± 0.030

	plot with biochar + NPK)			
	Reforestation without soil amendments	0 - 5	10	0.037 ± 0.022
		5 - 30	10	0.014 ± 0.007
		Rhizosphere (5 – 30)	25	0.040 ± 0.017
	Reforestation with biochar	0 - 5	5	0.034 ± 0.027
		5 - 30	5	0.015 ± 0.010
		Rhizosphere (5 – 30)	12	0.054 ± 0.041
	Reforestation with biochar + NPK	0 - 5	5	0.036 ± 0.022
		5 - 30	5	0.009 ± 0.006
		Rhizosphere (5 – 30)	11	0.036 ± 0.036
	Primary forest	0 – 5	5	0.276 ± 0.0790
		5 – 30	5	0.057 ± 0.011
		Rhizosphere (5 – 30)	9	0.210 ± 0.064
La Pampa	Naked soil	0 – 5	20	0.008 ± 0.002
		5 – 30	20	0.008 ± 0.002
		Rhizosphere (5 – 30)	12	0.009 ± 0.003
	Natural regeneration	0 – 5	10	0.015 ± 0.007
		5 – 30	10	0.016 ± 0.009
		Rhizosphere (5 – 30)	24	0.013 ± 0.005

	Primary forest	0 – 5	10	0.162 ± 0.032
		5 – 30	10	0.080 ± 0.010
		Rhizosphere (5 – 30)	24	0.160 ± 0.050

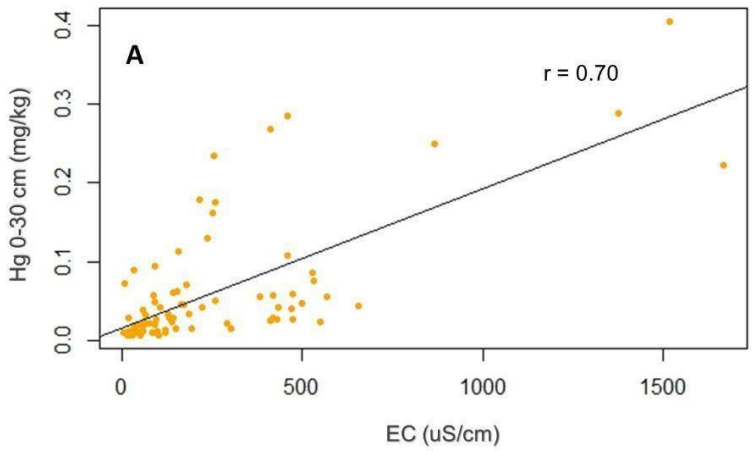
Soils in Madre de Dios are classified as oxisols and ultisols according to the US Soil Taxonomy (Powers et al., 2004; Quesada et al., 2011). Hg accumulation in soils depends on soil components; Hg has an affinity for SOM, iron, clay, and hydraulic conductivity. In areas impacted by ASGM, atmospheric Hg deposits over the soil surface and is carried down through the soil profile by water, where the Hg is complexed with some elements like dissolved organic matter (Ravichandran, 2004). In soils, like ultisols, the water penetration is limited, favoring the Hg accumulation in the top layers, as the pattern found in our study (Table 2-2). In contrast, in oxisol soils, water penetration is favored, explaining the accumulation of Hg at depth in these soils (Grimaldi et al., 2008).

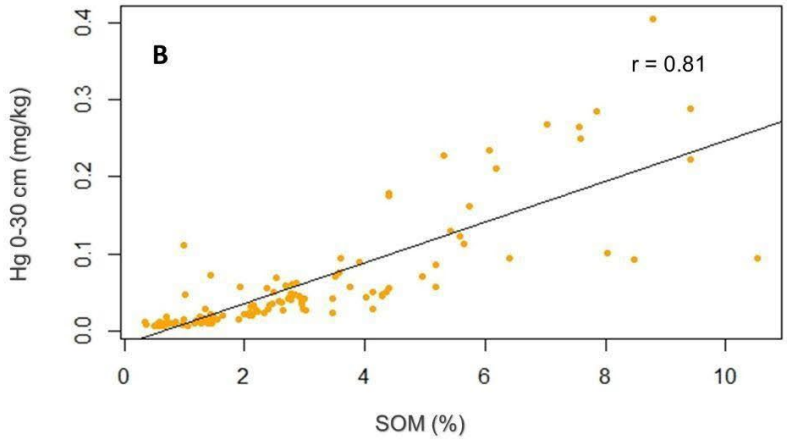
We also found statistically significant higher Hg concentrations in the soil surrounding plant roots at 5-30 cm depth compared to the bulk soil at the same depth in all the forested land cover categories (L3, L4, L5) in Laberinto site and the primary forest (L5) in La Pampa site (Table 2-2 and Fig. 2-3). Similar Hg distribution in rhizosphere soil was found in sites impacted by Hg pollution in Europe and China (Ghezzi et al., 2022; Ma et al., 2019). We hypothesized that Hg is accumulated in soil with higher SOM due to its affinity for humic acids, organo-clays, and oxides coated with SOM (Elliot et al., 1986; Connell & Miller, 1984). We found significantly higher SOM in forested areas compared to non-forested areas and in rhizosphere soil compared to bulk soil and a strong correlation ($r = 0.81$, $p < 0.05$) between average total Hg concentrations and SOM supporting this hypothesis (Fig. 2-4.B and Table S2-3). Another explanation for the

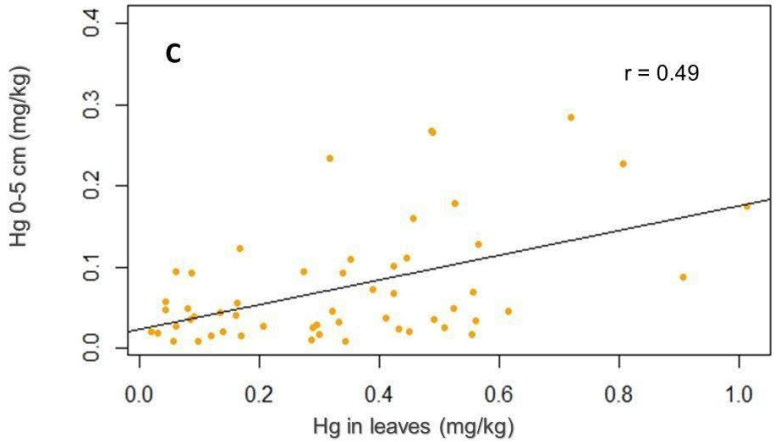
difference in Hg levels between forested and non-forested land cover categories is the role of vegetation on the Hg input from the atmosphere to the soil, being throughfall and litterfall major pathways for Hg entry to the soil in areas impacted by Hg pollution with variables like leaf age and leaf area index influencing these pathways (Gerson et al, 2021; Witt et al., 2009). A moderate correlation ($r=0.48$, $p<0.05$) between total Hg concentration in plant leaves and soil and very little translocation of Hg from plant roots to leaves supported this explanation (Fig. 2-4.D and Table 2-3). Previous studies have reported higher Hg inputs from the atmosphere to the soil in the primary forest compared to the secondary forest (natural regeneration) in the same region due to a higher leaf area index and greater litterfall mass (Gerson et al., 2021). These factors might also explain the higher Hg levels found in soil in the primary forest in our study.

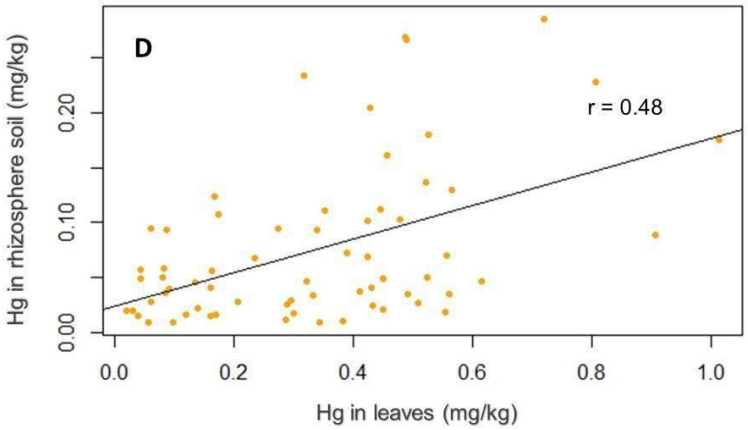
Table 2-3. Hg concentrations in plant tissues (mg kg^{-1}) in different land cover categories in the two sampling sites. The Hg concentrations shown are the average concentrations from different plant species from each land cover category and sampling site.

Sampling site	Land cover category	n	Hg (mg kg^{-1})		
			Root	Shoot	Leaf
Laberinto	Natural regeneration (L3)	43	0.018 ± 0.013	0.016 ± 0.013	0.227 ± 0.147
	Reforestation (L4)	48	0.011 ± 0.010	0.020 ± 0.014	0.267 ± 0.165
	Primary forest (L5)	9	0.060 ± 0.032	0.137 ± 0.123	0.636 ± 0.235
La Pampa	Naked soil (L1)	12	0.016 ± 0.012	0.003 ± 0.001	0.046 ± 0.030
	Natural regeneration (L3)	24	0.014 ± 0.012	0.005 ± 0.002	0.045 ± 0.023
	Primary forest (L5)	24	0.076 ± 0.045	0.057 ± 0.045	0.274 ± 0.088


A
r = 0.70
Hg 0-30 cm (mg/kg)
EC (uS/cm)
0.0
0.1
0.2
0.3
0.4
0
500
1000
1500


B
r = 0.81
Hg 0-30 cm (mg/kg)
SOM (%)
0.0
0.1
0.2
0.3
0.4
0
2
4
6
8
10


C
r = 0.49
Hg 0-5 cm (mg/kg)
Hg in leaves (mg/kg)
0.0
0.1
0.2
0.3
0.4
0.0
0.2
0.4
0.6
0.8
1.0


D
r = 0.48
Hg in rhizosphere soil (mg/kg)
Hg in leaves (mg/kg)
0.00
0.10
0.20
0.0
0.2
0.4
0.6
0.8
1.0

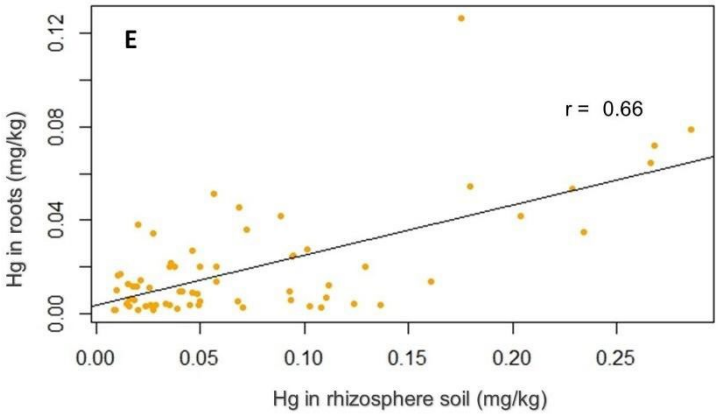
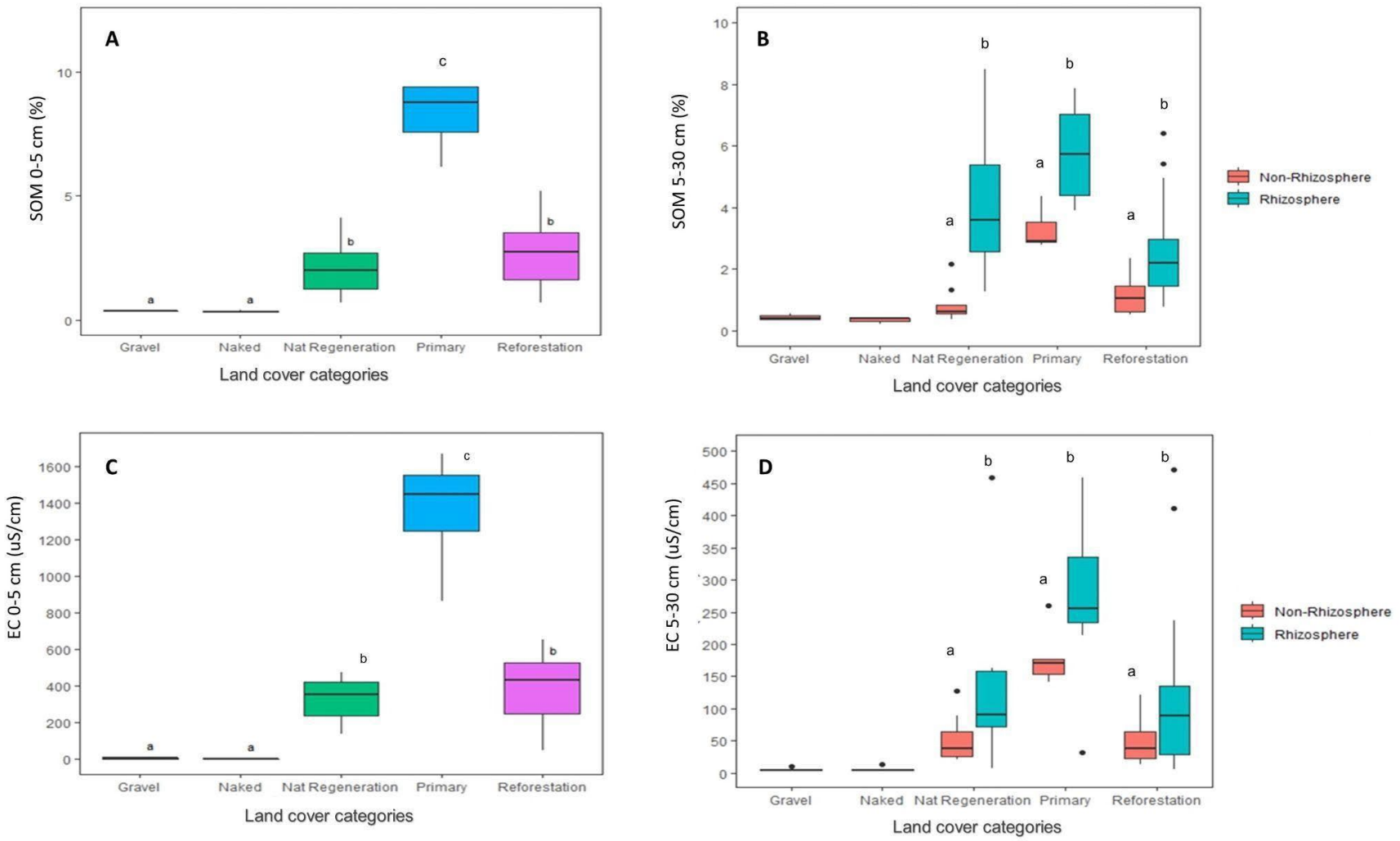
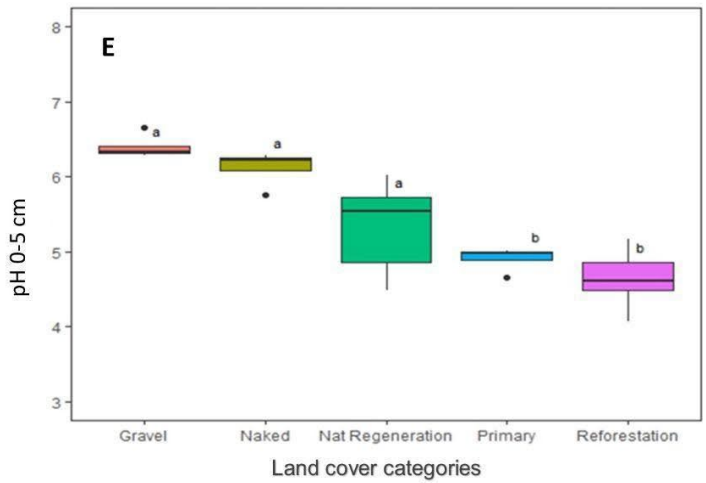

E
r = 0.66
Hg in roots (mg/kg)
0.00
0.04
0.08
0.12
0.00
0.05
0.10
0.15
0.20
0.25
Hg in rhizosphere soil (mg/kg)

Fig. 2-4. **Correlations between Hg levels and soil characteristics at p < 0.05 in Laberinto site. A.** Correlation between Hg concentration in soil at 0-30 cm and EC. **B**. Correlation between Hg concentration in soil at 0-30 cm and SOM. **C**. Correlation between Hg concentration in soil at 0-5 cm and in plant leaves. **D.** Correlation between Hg concentration in rhizosphere soil and Hg concentration in plant leaves. **E.** Correlation between Hg concentration in plant roots and rhizosphere soil.

3.2 Soil physic-chemical characteristics and total Hg levels.

SOM, soil pH, and EC were studied among all the selected land cover categories in the Laberinto site. SOM and EC levels were significantly higher in the primary forest (L5) (average 8.27 % and 1358 uS cm^{-1} in the top layer 0-5 cm, respectively, n =5), followed by natural regeneration (L3) and reforested soil (L4) (average 2.05 -2.70 % and 328 - 389 uS cm^{-1} in the top layer 0-5 cm, n = 9 and n = 15, respectively) (Table S2-3 and Fig. 2-5). The non-forested land cover categories had significantly lower SOM and EC levels than the forested ones. The SOM levels found in our study are similar to levels previously reported in areas impacted by ASGM in Madre de Dios (0.15 - 0.90 and 1.06 -3.21 % in non-forested and forested areas, respectively) (Román et al., 2020; Velasquez et al., 2020; 2021)


A
SOM 0-5 cm (%)
Gravel
Naked
Nat Regeneration
Primary
Reforestation
Land cover categories
B
SOM 5-30 cm (%)
Non-Rhizosphere
Rhizosphere
C
EC 0-5 cm (uS/cm)
D
EC 5-30 cm (uS/cm)


E
pH 0-5 cm
8
7
6
5
4
3
a
a
a
b
b
Gravel
Naked
Nat Regeneration
Primary
Reforestation
Land cover categories

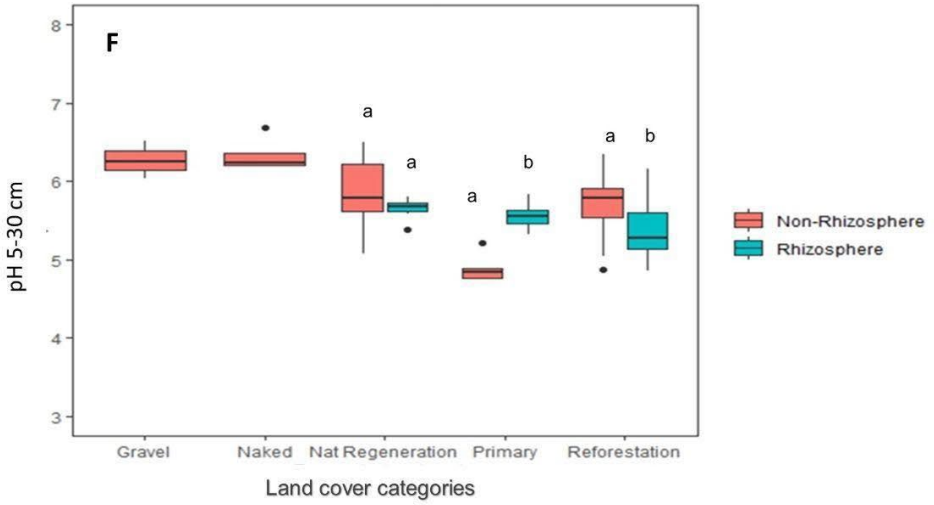

F
pH 5-30 cm
8
7
6
5
4
3
a
a
a
b
a
b
Gravel
Naked
Nat Regeneration
Primary
Reforestation
Land cover categories
Non-Rhizosphere
Rhizosphere

Fig. 2-5. **Soil physic-chemical characteristics in Laberinto site**. **A.** SOM at 0-5 cm. **B**. SOM at 5-30 cm. **C**. EC in soil at 0-5 cm. **D**. EC in soil at 5-30 cm. **E**. pH in soil at 0 – 5 cm. **F**. pH in soil at 6 – 30 cm. Significant differences between SOM and pH among different soil land cover categories and in the rhizosphere and non-rhizosphere soil at $p < 0.05$, and differences between EC within land cover categories and in the rhizosphere and non-rhizosphere soil at $p < 0.1$ are indicated by different lower-case letters above the boxes. Due to the small sample size, the difference in EC was not significant ($p > 0.05$), but the rhizosphere soil tended to have a higher EC value than the non-rhizosphere soil. In graphs B, D, and F, the significance letters differ for each land cover category.

EC measures the ability of soil to conduct electricity. It is positively related to the clay content and cation exchange capacity of soil and is often used as an indicator of the availability of nutrients in soil (Khongnawang et al, 2019, Smith and Doran, 1997). Optimum EC and SOM levels for fertile soil are 1700 – 5100 uS cm^{-1} and 3 -5 % respectively (Smith and Doran, 1997; Wilson, 2003). Comparing the SOM and EC levels of primary forest (L5) with the land cover categories degraded by mining activity, naked soil (L1) and gravel piles (L2), we found that the ASGM activity reduces the SOM and EC by 96 and 99 %, respectively, likely due to the use of high-pressure water cannons to dislodge and wash the soil. Thus, soil degradation from ASGM, by drastically decreasing the SOM and EC to levels much lower than the optimum, reduces the soil's capacity of supporting plant growth (Timsina et al., 2022).

pH levels were significantly lower in the primary forest (L5) and reforested soil (L4) compared to the other land cover categories (average 4.91 ± 0.17 and 4.66 ± 0.30 in the top layer 0-5 cm, n = 5 and n = 15, respectively; Fig. 2-5 and Table S2-3). These pH levels in the primary forest are similar to those reported by other studies in primary forest near ASGM sites in Madre de Dios (4.76 - 4.8) (Román et al., 2020; Velasquez et al., 2020; 2021). pH values are lower in the forested land cover categories with higher SOM due to its decomposition and the production of organic acids that lower the soil pH (Hong et al., 2019). Differences between SOM and EC

throughout the vertical soil profile were found in all the studied land cover categories, with levels decreasing with depth in the forested land cover categories (Fig. 2-5 and Table S2-3). These observations are consistent with reports from tropical forests impacted by ASGM in Madre de Dios and other world regions (Velasquez et al., 2020; Tomiyasu et al., 2020). In forests, litter accumulates, decomposes, and is integrated in the top layers of the soil, and these top organic layers contain more SOM than the mineral layers immediately below (Magdoff & Van Es, 2021). Also, EC in the soil is related to clay content, which decreases with depth, as previously reported in areas impacted by ASGM in the region (Velasquez et al., 2020). Similar to other studies (Ma et al., 2109; Herre et al., 2022), our results illustrate that SOM and EC levels in the soil surrounding plant roots at 5-30 cm depth are significantly higher compared to the bulk soil at the same depth in all the forested land cover categories in both sites ($p < 0.05$ for SOM and $p < 0.1$ for EC) (Fig. 2-5). This is due to the contribution of dead plant roots to SOM and plant root exudates to EC (Magdoff & Van Es, 2021; Garcia et al., 2005).

The SOM and EC levels showed distribution characteristics similar to those of Hg, while the pH distribution was the opposite (Table S2-3). These observations are generally consistent with the results reported in previous studies (Velasquez et al., 2020; Du et al., 2019; Gong et al., 2014; Obrist et al., 2011). The geochemical affinity of organic matter for Hg explains the critical influence of SOM in the Hg distribution in the soil (Gong et al., 2014; Obrist et al., 2011; Wang et al., 2016; Zhou et al., 2015). Also, clay content (thus EC) is positively related to Hg sorption in tropical soils due to its interaction with the minerals in the clay fraction. In contrast, pH is negatively related to Hg due to the hydrolysis of Hg cations at low pH (Soares et al., 2015).

The correlation between Hg and SOM, pH, and EC in the soil in forested land cover categories in the Laberinto site was analyzed. Significant positive correlations between Hg and SOM (r =

0.81, $p < 0.05$) and EC ($r = 0.70$, $p < 0.05$) were found (Fig. 2-4). Based on these relationships, linear regression models were developed to explain the distribution of Hg in soil in the forested land cover categories in the Laberinto site and the influence of soil characteristics like SOM, soil pH, EC, land cover categories, and soil depth in this distribution. Based on the Akaike information criterion, we found a model that explains 88% of the variance in Hg in the soil in the forested land cover categories in the Laberinto site (Table 2-4 and Supplementary information Table S2-7). Our results confirm the findings shown by linear mixed models previously developed to explain Hg levels in soil in areas impacted by ASGM in the Madre de Dios region using vegetation cover, SOM, and soil pH as explanatory variables (Velasquez et al., 2021). The distance to the point source of Hg emission was not included as a variable in our model since it is difficult to establish precise points in areas where the amalgamation process might take place in different unknown points at different times. Similarly to our research, other authors found higher Hg levels in soil in the primary forest in the vicinity of ASGM sites than in the impacted sites. This was explained by the fact that SOM is one of the most important variables explaining the Hg fate in soils in ASGM sites and by the role of the forest canopy as a driver of Hg input in the Amazon forest impacted by ASGM (Velasquez et al., 2021; Gerson et al., 2022).

Table 2-4. Linear regressions of Hg concentrations in soil, roots, and BCF against soil properties, land cover categories, and soil depths.

Dependent variable	Intercept	Coefficients	R^2
$\log Hg_{soil\ 0\text{-}30\ cm}$	-1.233	0.183 SOM – 0.0003 EC – 0.828 log pH – 0.178 $Depth_2$ + 0.009 $Depth_3$ + 0.140 $LC_{Primary}$ – 0.153 $LC_{Reforestation}$	0.88
$\log Hg_{roots}$	1.232	6.466 $Hg\ rhizosphere_{soil}$ – 0.0018 EC – 0.612 pH	0.61

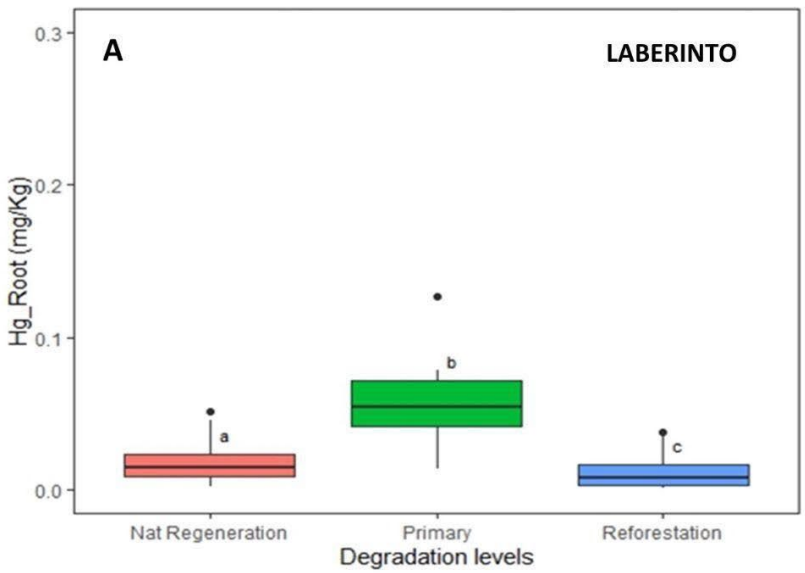
log BCF	2.627	-0.513 log Hg rhizosphere $_{soil}$ – 0.0015 EC – 0.684 pH + 0.617 LC $_{Primary}$ – 0.100 LC $_{Reforestation}$	0.50

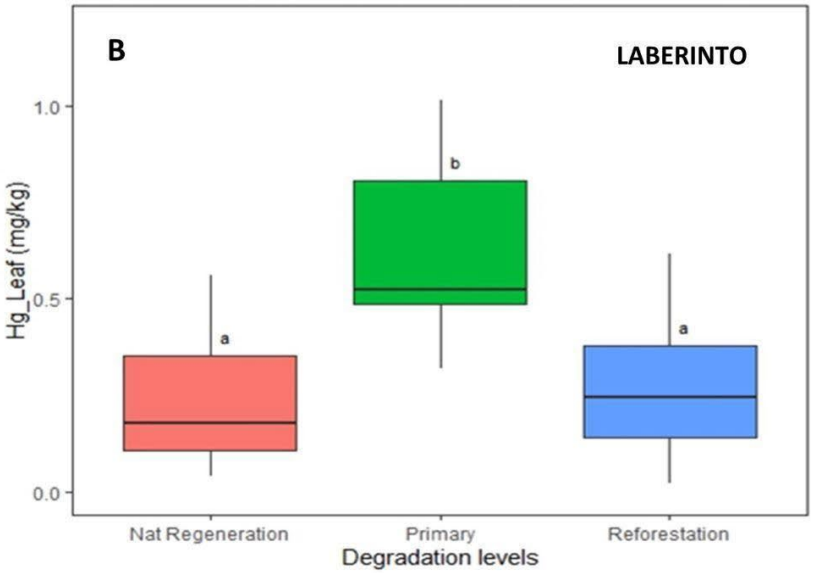
Note: Depth $_2$ is a categorical variable representing the non-rhizosphere soil at 5-30 cm depth. Depth $_3$ is a categorical variable representing the rhizosphere soil at 5-30 cm depth. $LC_{Primary}$ is a categorical variable representing the land cover category of primary forest. $LCR_{eforestation}$ is a categorical variable representing the land cover category of reforestation.

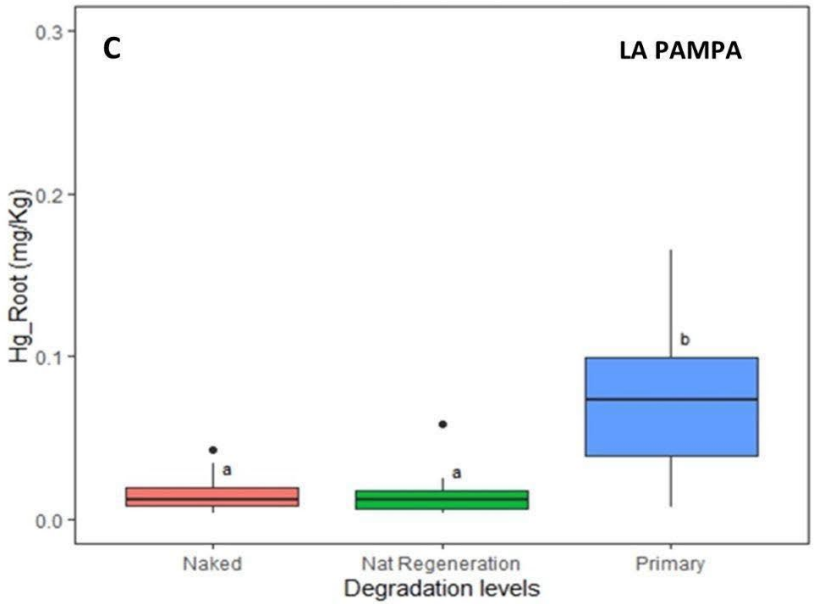
3.3 Total Hg concentration in plants

Concentrations of total Hg in plant tissues in the different land cover categories are shown in Table 2-3. For all species, the Hg levels were highest in the leaves, followed by the roots, and the lowest levels were found in the shoots, which agrees with the results reported in other studies (Lindberg et al., 1979; Frescholtz et al., 2003). We did not find a significant difference in Hg concentrations in the plant tissues among the different plant species within the land cover categories (Table S2-4). However, we found a significant difference in Hg concentrations in the plant tissues among the land cover categories (Fig. 2-6). For instance, the Hg concentrations in the roots in our study were higher in the land cover categories with higher soil Hg levels (Fig. 2-6). We found a moderate correlation between the Hg concentration in the soil and the plant roots ($r = 0.66$, $p < 0.05$) (Fig. 2-4.E). That may be because the Hg can be absorbed by the roots in direct contact with the contaminated soil through the pores in the root cell walls (Beauford et al., 1977). However, other factors, such as the SOM and soil pH, affect this absorption. At low pH, Hg is more available for plant uptake because it is present as free radicals, and precipitation and complexation with other ions decreases (Yu et al., 2018; Hussain et al., 2023). On the contrary, SOM forms stable complexes with Hg reducing its availability for plant uptake (Wang et al., 1997). In line with that, some authors reported that the addition of soil amendments, such as

biochar and NPK, aimed to increase the SOM and EC, decreases the Hg uptake by plants (Addai et al., 2023). However, we did not find significant differences in the soil properties or in the Hg levels in soil and roots of the plants sampled in the reforestation plots where different amendments were applied in Laberinto site (Table S2-5), most likely due to the lower application rate of the amendments, 1.1 t ha-1, compared to previous studies, 5 t ha-1 (Addai et al., 2023).


A
LABERINTO
Hg_Root (mg/Kg)
0.3
0.2
0.1
0.0
a
b
c
Nat Regeneration
Primary
Reforestation
Degradation levels


B
LABERINTO
Hg_Leaf (mg/kg)
1.0
0.5
0.0
a
b
a
Nat Regeneration
Primary
Reforestation
Degradation levels


C
LA PAMPA
Hg_Root (mg/Kg)
0.3
0.2
0.1
0.0
a
a
b
Naked
Nat Regeneration
Primary
Degradation levels

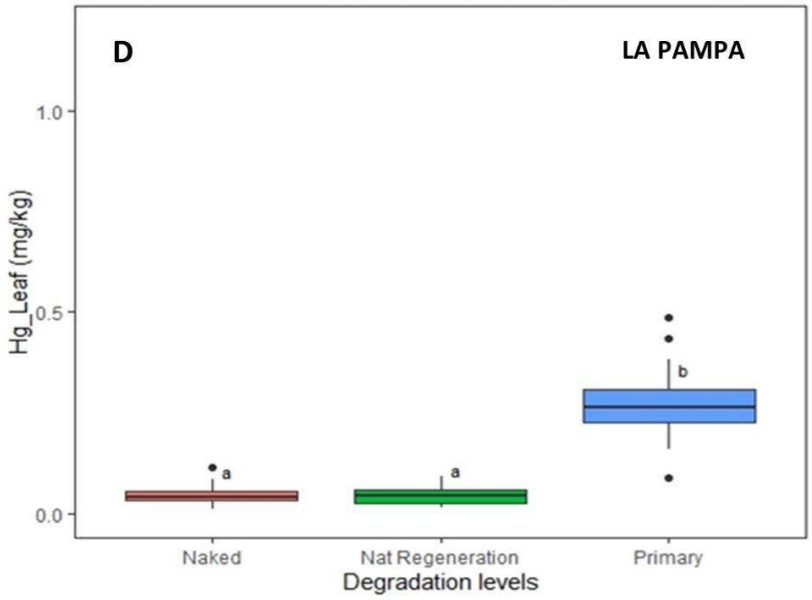

D
LA PAMPA
Hg_Leaf (mg/kg)
1.0
0.5
0.0
a
a
b
Naked
Nat Regeneration
Primary
Degradation levels

Fig. 2-6. **Distribution of Hg in plant tissues in the two sampling sites**. **A.** Hg concentrations in plant roots in the Laberinto site. **B**. Hg concentrations in plant leaves in the Laberinto site. **C**. Hg concentrations in plant roots in La Pampa site. **D.** Hg concentrations in plant leaves in La Pampa site. Different lower-case letters above the boxes indicate significant differences ($p \leq 0.05$) between Hg concentrations within land cover categories.

To study the Hg uptake by plant roots, we used the bioaccumulation factor (BCF). Some authors proposed this factor as an indicator of the capacity of plants to hyperaccumulate metals when the factor is higher than one (Yoon et al., 2006). Hyperaccumulator plants can be used to remediate soils contaminated with Hg through phytoremediation (Barceló & Poschenrieder, 2003). We did not find significant differences in BCF among the plant species collected in the sampling sites due to the high variability of the data (0.063 -2.370, n = 160 (Supplementary information Table S2-6), and as such, we could not identify plant species with hyperaccumulation potential.

We observed very low Hg translocation from the roots to the shoots and leaves (Table 2-3). Other researchers reported the same and hypothesized this is a defensive mechanism used by plants to prevent contaminants from entering the cytoplasm (Marrugo-Negrete et al., 2015; Wang & Greger, 2004). We hypothesize that the high Hg levels in leaves found in our study do not result from translocation from the roots but from atmospheric deposition, as suggested by other authors (Frescholtz et al., 2003; Cui et al., 2014; Fu et al., 2009; Gerson et al., 2022). Litter deposition has been proven to be a main pathway for Hg in soil, especially in the Amazon rainforest (Gerson et al., 2022). This pathway is likely to drive the Hg patterns in soils found in our study. As previously mentioned, we found a moderate correlation between average total Hg concentrations in superficial and rhizosphere soil and total Hg concentrations in plant leaves (Fig. 2-4).

Our study shows the highest Hg levels in fresh foliage from trees and shrubs ever reported in the Amazon region in the primary forest (L5) at the Laberinto site, surpassing levels found in tree leaves in other sites of the region (Gerson et al., 2022) and in other regions of the world impacted by ASGM (Melieres et al., 2003) and Hg industrial emissions (Pleijel et al., 2021; Zhou et al., 2016). Similar Hg levels in fresh foliage in shrubs in the Amazon were only found in the secondary forest at an ASGM site in Colombia (0.581 ± 0.738 mg kg^{-1}) (Marrugo-Negrete et al., 2015). Higher Hg levels were found in trees, shrubs, and herbs near Hg mines in Europe (0.13 - 2695 mg kg^{-1}) (Molina et al., 2006) and in ferns in ASGM sites in Asia (2.8 – 6.2 mg kg-1) (Kono et al., 2012). In our study, higher Hg levels in leaves were found in the primary forest (L5) compared to the other land cover categories (Fig. 2-6). The differences in Hg levels in plant leaves can be explained by different GEM levels, with higher GEM concentrations increasing potential Hg deposition (Gerson et al., 2022; Driscoll et al., 2013). Also, the leaf stomata, leaf placement, area, and age can influence Hg accumulation in foliage. Leaves with a higher stomatal conductance, such as the ones from broadleaved trees, have a higher air-leaf GEM exchange, and trees with higher leaf area and older leaves accumulate higher GEM concentrations (Graydon et al., 2006; Laacouri et al., 2013; Wang et al., 2016; Pleijel et al., 2021). These factors may also explain the differences found in the land cover categories in our study. We collected the plant leaves at the same height and placement (1.5 – 2 m height, outside crown) in all the plant species. We only studied the influence of age on the Hg levels in leaves, although we did not find significant differences in Hg levels in deciduous leaves compared to perennial leaves (Table S4); thus, the leaf age does not seem to explain the higher Hg levels found in leaves in the primary forest.

Higher GEM concentrations were found in the Laberinto site (5.14 ± 0.55 ng m^{-3}, n = 6) compared to the La Pampa site (2.56 ± 0.65 ng m^{-3}, n = 4), following the same pattern as Hg levels in leaves (Table 2-3). Hg was not actually used at the mining concessions we sampled, but ASGM activities are carried out in nearby areas. The number and location of the point sources of those Hg emissions in these areas were not known. The GEM concentrations observed in both sites were above the global southern hemisphere average background concentration of approximately one ng m^{-3} (Sprovieri et al., 2016). They were comparable to concentrations previously reported in areas near ASGM sites in Madre de Dios (2.-5 ng m^{-3}) and in some urban and industrial areas of the United States (2.1 - 4.4 ng m^{-3}) and Asia (4.47 - 5.34 ng m^{-3}) (Gerson et al., 2022; Szponar, 2022; Sprovieri et al., 2010). Adverse health effects in plants exposed to Hg through the air, like reduction in growth and necrosis, have been found in laboratory settings (Siegel et al., 1983; Kabata-Pendias & Pendias, 2001) and a quality criterion of 0.035 – 0.6 mg kg^{-1} of Hg in plant tissue has been proposed by United Nations to protect environmental health (Vries et al., 2003). The Hg levels found in plants in the primary forest (L5) in our study are above that range, which may pose a risk to environmental health. However, there is a lack of information about the toxicity of Hg in Amazonian plants exposed to Hg through air and in the animals feeding in the plant tissues accumulating Hg, like the leaves.

The correlations between Hg in plant roots, BCF and Hg in soil, SOM, pH, and EC in the forested land cover categories in the Laberinto site were analyzed. A moderate positive correlation between Hg in roots and Hg in soil ($r = 0.66$, $p < 0.05$) was found, as previously mentioned (Fig. 2-4.E). Linear regression models were developed to explain the distribution and accumulation of Hg in plant roots in the forested land cover categories in the Laberinto site considering Hg in soil, SOM, soil pH, EC, and land cover categories as independent variables.

Based on the Akaike information criterion, we found models explaining 61% and 50% of the variance in Hg in roots and BCF, respectively, in the forested land cover categories in the Laberinto site (Table 2-4 ; Supplementary information Table S2-8 and Table S2-9). Linear regression models were previously developed to explain Hg accumulation in plant tissues in areas impacted by Hg pollution in China using Hg in soil, SOM, clay content, soil pH, and concentration of mineral oxides in the soil as explanatory variables (Hussain et al., 2023). Factors like the content of selenium, sulfur, and oxides such as manganese, iron and aluminum oxides can influence Hg uptake by plants because Hg can form insoluble complexes with these compounds in the rhizosphere soil, reducing its bioavailability (Zhang et al., 2012; Wang et al., 2019; Ma et al., 2022; Hussain et al., 2023) but these variables were not considered in this study. This is the first study developing regression models to explain Hg levels in plant roots and the Hg bioaccumulation from soil to plant roots in areas degraded by ASGM in Madre de Dios region. However, more research, including the levels of minerals capable of forming complexes with Hg in soil and a higher sample size, is needed to develop more robust models to explain a higher percentage of the Hg accumulation in Amazonian plant species in these areas impacted by ASGM.

4. Conclusions

This study is the first to investigate the distribution of Hg in soil and plants in land cover categories with different degradation levels from ASGM in the Amazon rainforest. We found higher Hg concentrations in the intact primary forest than the other land cover categories, with some levels in soil and plants above the reference level proposed by UNEP to protect

environmental health. In addition, we showed that the ASGM activity drastically reduces the SOM and EC in soil, decreasing the soil's capacity to support plant growth.

Hg accumulates in rhizosphere soil, plant roots, and leaves, with a positive correlation between Hg levels in soil and plant roots and little translocation from the roots to the shoots and leaves. We also found a moderate correlation between Hg in soil and Hg in leaves and strong correlations between Hg concentrations in soil, SOM, and EC. These results show the vital role of the Amazonian forest in Hg capture through foliage, deposition to the soil, and accumulation and stabilization in the rhizosphere, where it forms stable complexes with SOM and minerals. This stabilization may reduce the risk of Hg being transported to other nearby areas, such as aquatic ecosystems, where it can enter the food chain through fish and pose a human health hazard to local communities. Our findings reinforce the necessity of conserving the Amazonian forest and reforesting the areas degraded by ASGM and exposed to Hg pollution. We found that soil characteristics like SOM, pH, and EC are essential for understanding Hg fate in areas impacted by ASGM in the Amazon rainforest. This work is vital to determine the risk of exposure to Hg in this environment. However, more research is needed to consider other variables, like the concentration of minerals, to better explain Hg accumulation in plants in these sites.

Chapter 3: Effects of mercury exposure in three Amazonian agricultural plant species and soil microbiome

(This chapter is under preparation for *Chemosphere*)

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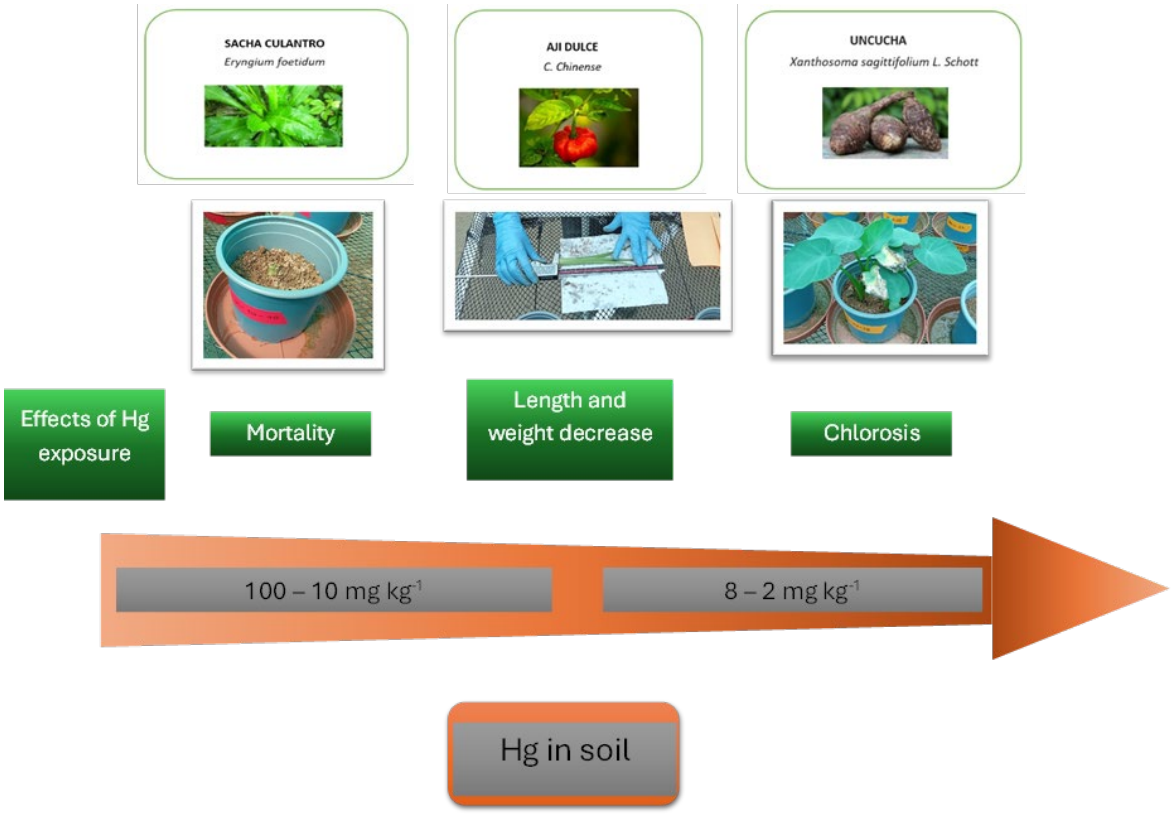
Graphical Abstract



Abstract

The Amazonian region of Madre de Dios in Peru is highly impacted by Hg emissions from ASGM in the Amazon rainforest. Peru adopted SSL for Hg in agricultural soil to protect the environment and human health from Hg exposure based on Canadian regulations (6.6 mg kg-1) without considering research on native species and local soil. We studied the impact of Hg exposure on the survival, growth, and health of three Amazonian agricultural plant species, aji

dulce *(Capsicum Chinense)*, sacha culantro *(Eryngium foetidum)*, and uncucha (*Xanthosoma sagittifolium L. Schott)*. We also examined the effect of this exposure on their rhizosphere microbial communities. Two experiments were performed following US EPA 850.4100 and OECD-208 guidelines for toxicity tests with plants. In each experiment, soil collected from a farm non-impacted by ASGM (control soil) was spiked with Hg to the following concentrations: 10, 50, and 100 mg kg^{-1} dw for the first experiment and 2, 4, 6, and 8 mg kg^{-1} of dry soil (dw) for the second experiment. Plants were grown in the Hg-spiked soil in 10 and 40 pots per Hg concentration tested and per plant species in the first and second experiments. At the end of the experiments, survival, growth, chlorosis in leaves, and total bacterial population in the rhizosphere soil were evaluated. Adverse health effects, such as mortality, reduction in root length, and above-ground weight, were found in aji dulce (Capsicum Chinense) and uncucha (Xanthosoma sagittifolium L. Schott) plants after exposure to every Hg concentration tested and mortality was found in sacha culantro *(Eryngium foetidum)* plants after exposure to 50 and 100 mg kg^{-1} of Hg. The lowest observable concentration of Hg affecting the plant's health was 2 mg kg^{-1} dw. The exposure to that Hg concentration caused chlorosis in the leaves of the plant species aji dulce (*Capsicum Chinense)* and uncucha (*Xanthosoma sagittifolium L. Schott).* This Hg concentration was three times lower than the SSL for Hg in agricultural soil established in the Peruvian regulation, suggesting that a review of this SSL may be necessary for developing environmental and human health risk assessments in areas impacted by ASGM in the Amazonian region.

1. Introduction

Mercury (Hg) is a heavy metal that can be found naturally in the environment in three different forms: Hg^0 (vapor), Hg^{+2}, and organic mercury (Driscoll et al., 2013). Hg can enter the

environment through anthropogenic activities like Artisanal Gold Mining (ASGM) (Esdaile & Chalker, 2018). According to the United Nations Environment Programme (UNEP), ASGM is the main contributor to global Hg emissions and the primary source of Hg pollution in South America (AMAP / UN Environment, 2019). In Peru, the Southern Amazonian region of Madre de Dios is one of the most impacted by this mining activity in the Amazon rainforest, with an estimation of 180 metric tons of Hg emitted into the environment annually (Cardo & Vargas, 2017) and high Hg levels in humans reported (Gonzalez et al., 2019; Feingold et al., 2020; Silman et al., 2022). Exposure to Hg, especially in its organic form, can adversely affect human and environmental health. Humans can develop adverse health effects such as neurological, developmental, and immune system damage (Joint FAO/WHO, 2011; Santos Serrão de Castro & de Oliveira Lima, 2018; Dórea & Marques, 2016). On the other hand, plants can suffer inhibition of growth (Siegel et al., 1984; Patra & Sharma, 2000), photosynthesis (Clijsters & Van Assche, 1985; Küpper et al., 1998), and genotoxic effects (Panda et al., 1992), among others. Soil bacteria can also be impacted by exposure to Hg. Some of the effects reported in the literature are a decrease in soil basal respiration and microbial biomass carbon (Lima et al., 2019), a reduction in bacterial diversity (Ma et al., 2019), and a shift in microbial community composition toward Hg-resistant bacteria (Sas-Nowosielska et al., 2008).

To protect the environment and human health from exposure to pollutants, environmental quality criteria such as Soil Screening Levels (SSL) have been developed worldwide (Carlon, 2007; USEPA, 1996). In Peru, SSLs were established in 2013 (MINAM, 2013), and their use is mandatory in designing and applying environmental management instruments, such as environmental impact assessments and environmental and human health risk assessments (MINAM, 2013). These risk assessments are required by the Peruvian regulation for the

remediation of contaminated sites (MINAM, 2017), following a similar approach to the US EPA guidelines (US EPA, 1993). To determine a robust value of SSL of a contaminant, relevant and adequate toxicity data for local species is required. It is also essential to consider the physical and chemical characteristics of the local soil, the ecologically important species that could be affected after exposure to the contaminant, and the environmentally relevant contaminant levels in local soils (European Commission, 2003). However, the SSL for Hg in agricultural soil (including forest soil) established in the Peruvian regulation (6.6 mg kg^{-1}) was adopted from the Canadian regulation[2], a country with a different geography, climate, and ecosystem characteristics. To set the Peruvian SSLs, research with indigenous species and characteristics of local soils were not considered. Other factors that were also not contemplated were local land uses (activities considered in the category of agricultural soil, such as forestry, cattle raising, etc.) and environmentally relevant levels of contaminants in local sites.

A high variability between SSL for Hg exists in different countries and regions of the world (Table S3-3). The main reasons for this variability are political decisions and different scientific and technical approaches (Provoost et al., 2006). For example, variations may arise from the consideration of protected receptors, potential pathways of exposure to contaminated soil for these receptors, soil characteristics (correction for organic matter, clay content, pH, etc.), and data analysis methods (the use of the lowest reported toxicity value or a percentile of the available toxicity data) (Carlon, 2007). Among the South American countries in the Amazonian region affected by ASGM, only Brazil, Ecuador, and Peru have established SSL for Hg in agricultural soils. The values in these countries varied between 0.8 and 12 mg/Kg dry weight (dw) and were set based on international regulations (CONAMA, 2009; República de Ecuador,

[2] Personal communication with the German Society for International Cooperation (GiZ) on July 16, 2020.

2003; MINAM, 2013). One of the main obstacles to developing SSL for Hg in the Amazonian countries is the lack of published studies about Hg toxicity on Amazonian species and soil.

The Peruvian government and local NGOs have started restoration projects in areas degraded by ASGM using native plant species that can provide ecosystem services including food for humans and wildlife (Román-Dañobeytia et al., 2020). However, there are very few published studies and thus a lack of understanding about Hg effects on the Amazonian plants.

Here, we study for the first time the impact of Hg exposure on the survival, growth, and health of three Amazonian agricultural plant species: aji dulce *(Capsicum Chinense)*, sacha culantro *(Eryngium foetidum)*, and uncucha *(Xanthosoma sagittifolium L. Schott)*. We also examined the effect of this exposure on the abundance of their rhizosphere microbial communities. The selected plant species are traditional crops in the Madre de Dios region, part of the diet and economy of the local population and have been included in the Peruvian regulations for the protection of the native biological diversity (MINAM, 2012).

This study aims to generate data about adverse health effects after Hg exposure in Amazonian plants that can be integrated into a review and revision of the SSL for Hg in agricultural soil in Peru and other Amazonian countries and furthermore develop environmental and human health risk assessments necessary before developing restoration projects in areas degraded by ASGM in the Amazonian region.

2. Materials and methods

2.1 Design of effects of Hg exposure studies

The study was developed in a nursery implemented at the Instituto de Investigaciones de la Amazonia Peruana (IIAP) in Madre de Dios, Peru. We selected three Amazonian agricultural

plant species: aji dulce *(Capsicum Chinense)*, sacha culantro *(Eryngium foetidum)*, and uncucha *(Xanthosoma sagittifolium L. Schott)* (Fig. 3-1 and Table 3-2) because they are part of the diet and economy of the local population, and their seeds are commercially available.

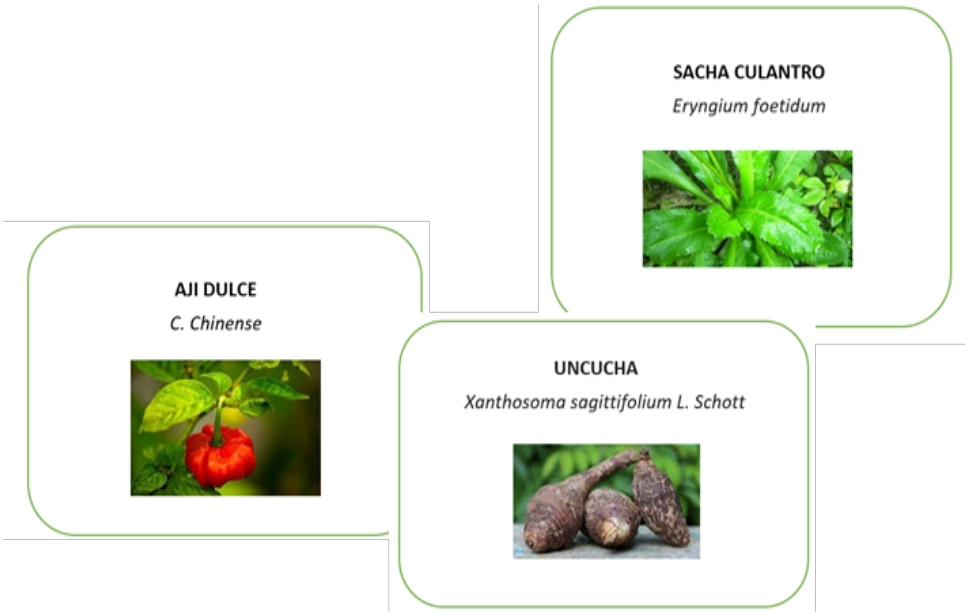


Fig. 3-1. Plant species used in effects of Hg exposure study.

Table 3-2. Characteristics of the plant species studied.

Plant species	Family	Growth habit	Type of root	Use
Capsicum chinense	*Solanaceae*	Sub-shurb	Tap	Food
Eryngium foetidum	*Apiaceae*	Herb	Tap	Food, medicine
Xanthosoma sagittifolium L. Schott	*Araceae*	Forb	Fibrous	Food, medicine

The soil used in the study was collected in Madre de Dios, in an agricultural field not impacted by ASGM, at UTM coordinates 19L 458420.42 / 8591638.40, at 0-30 cm depth and transported in plastic bags to the nursery facility. The total Hg concentration in the control soil was analyzed at the Mercury and Environmental Chemistry Laboratory (LAMQA), managed by the Instituto de Investigaciones de la Amazonia Peruana (IIAP) and Centro de Innovacion Cientifica Amazonica (CINCIA) in Madre de Dios, Peru. Soil subsamples were transported to the United States under USDA soil permit number 525-23-163-97136 and analyzed for physic-chemical characteristics including Soil organic matter content (SOM), pH, electrical conductivity (EC), and total recoverable metals (other than Hg) at the Environmental Engineering Laboratory, Department of Civil and Environmental Engineering, University of Maryland, College Park. The results are shown in Table 3-3.

Table 3-3. Physic-chemical characteristics of the control soil used in the study.

Characteristics	
pH	4.57 ± 0.82
EC (uS cm^{-1})	309.20 ± 55.65
SOM (%)	3.70 ± 0.67
Hg (mg kg^{-1})	0.078 ± 0.007
Al (mg g^{-1})	12.43 ± 1.50
Ca (mg g^{-1})	0.18 ± 0.010
Cr (mg g^{-1})	0.01 ± 0.003
Cu (mg g^{-1})	0.01 ± 0.003
Fe (mg g^{-1})	20.87 ± 2.80
K (mg g^{-1})	0.97 ± 0.060
P (mg g^{-1})	0.18 0.023

2.2 Spiking of soil with Hg

We performed two experiments following the standardized and internationally accepted guidelines for toxicity tests with plants OCDE-208 (ODCE, 2006), US EPA - OCSPP 850.4000, and OCSPP 850.4100 (US EPA, 2012a; 2012b). In each experiment, soil was spiked, adding aliquots of $HgCl_2$ solutions up to 50% of its water-holding capacity to get the desired Hg concentrations (Ghani, 2002) (0.078 mg kg^{-1} (control), 10 mg kg^{-1}, 50 mg kg^{-1}, and 100 mg kg^{-1} dw for the first experiment and 0.078 mg kg-^{1} (control), 2 mg kg^{-1}, 4 mg kg^{-1}, 6 mg kg^{-1} and 8 mg kg^{-1} dw for the second experiment, respectively). The Hg concentrations were selected following the US EPA guidelines for toxicity tests with plants and Hg toxicity literature (Patra & Sharma, 2000). The $HgCl_2$ solutions and soil were mixed thoroughly for 9 minutes at 100 rpm using a mixer (Kitchen Center, Chile) and left equilibrated for two weeks to increase the Hg adsorption in the soil, mimicking the bioavailability of Hg in field soils (Ghani, 2002; Crout et al., 2006). The total Hg concentration of the spiked soils was analyzed for Mercury m immediately after spiking and after the equilibration period, all final Hg concentrations were within 90 – 110 % of the target concentrations and that Hg was not volatilized during equilibration. No fertilizer was used to prevent interactions of its components with Hg.

2.3 Effects of Hg exposure in plants assessments

In both experiments, aji dulce seeds were germinated in a germination tray with control soil (0.078 mg kg^{-1} dw) and transplanted to 400 cm3 plastic pots with Hg-spiked soil after emergence. Sacha culantro shoots and uncucha tubers were propagated directly in 400 cm^3 plastic pots with Hg-spiked soil. The experiments were performed in a nursery (non-closed system). The temperature and humidity during the experiments were not controlled but

monitored thoughout. The first experiment consisted of 10 pots per Hg concentration tested (10 mg kg^{-1} to 100 mg kg^{-1}) and per plant species, with one plant per pot, in a randomized complete block design. The duration of the Hg exposure in this experiment was 21 days (June-July 2022) in total for the aji dulce plants and 21 days after the emergence of 50% of the control plants for the sacha culantro and uncucha plant species. T The temperature during the experiment was 28.84 ± 4.98 °C, and the humidity was 58.77 ± 15.82 %. The plants were irrigated with filtered water. At the end of the experiment, survival and growth were assessed. The whole plants were removed, and their length was measured. The plants were freeze-dried for 48 hours. Their mass before and after drying was determined to constant weight. This experiment aimed to find the range of Hg concentrations that affected the plant´s health (US EPA, 2012a). As every Hg concentration tested in the first experiment affected the plant´s health, we developed a second experiment with lower Hg concentrations (2 mg kg^{-1} to 8 mg kg^{-1}). This second experiment consisted of 40 pots per Hg concentration tested and per plant species, with a plant per pot, in a randomized complete block design, with eight replicates of five pots each per Hg concentration tested. The duration of the Hg exposure in this experiment was 32 days in total for the aji dulce plants, 21 and 32 days after the emergence of 50% of the control plants for the uncucha and sacha culantro plants, respectively. The experiment was carried in October-November 2022. The temperature during the experiment was 34.99 ± 3.57 °C, and the humidity was 61.24 ± 12.61 %. The plants were irrigated with filtered water. At the end of the experiment, survival, growth, and chlorosis in leaves were evaluated.

2.4 Assessment of Hg effects on microbial abundance

Rhizosphere soil samples were collected from the pots of the second experiment and kept frozen at -20°C until analysis. The samples were transported to the United States under USDA soil

permit number 525-23-163-97136, and the abundance of their total bacterial population was determined at the Environmental Engineering Laboratory, Department of Civil and Environmental Engineering, University of Maryland, College Park. DNA was extracted from 0.25 g of soil sieved to < 2 mm using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The concentration of nucleic acids in the samples was measured using a NanoDrop 2000 UV vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA) and ranged from 5 to 14 ng/µL. The abundance of the bacterial 16S rRNA gene in the samples was determined by quantitative real-time PCR (qPCR). The primer and cycling conditions used are shown in Table S3-1. PCR products of 16S rRNA gene amplification obtained from Escherichia coli strain HB101 were purified Wizard SV gel and PCR clean-up kit (Promega, Madison, Wisconsin) following the manufacturer's instructions. Standard curves were prepared with 10-fold serial dilutions of purified DNA in triplicate. The efficiency and R2 of all assays were above 0.985 and ranged between 90-105%, respectively. These reactions were conducted by BioRad CFX Connect Realtime Systems (Bio-Rad Laboratories, Hercules, CA, USA), using 8.5 µL RT-PCR Grade Water (Invitrogen by Thermo Fischer Scientific, TX, USA), 0.5 µL of template, 12.5 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), to a final concentration of 0.2 µM of each of the forward and reverse primers, with a total of 25 µL reaction volume. In the qPCR runs, 3 µL of DNA template was replaced with PCR Grade Water as the negative control. DNA from *Escherichia coli* strain HB101 was used as positive control. Data were converted to represent the average copy number of targets per g dry weight using Equation 1 in supplementary information.

2.5 Soil Mercury Analysis

Soil samples were freeze-dried for 48 hours and later sieved to < 1 mm for homogenization before analysis. The samples were analyzed for total Hg on a Milestone Direct Mercury Analyzer (DMA-80) via thermal decomposition atomic absorption spectroscopy (EPA Method 7473). For QA/QC, blanks were run at the beginning of each run. Reference materials IAEA-456 (0.077 mg kg^{-1}), S.R.M. 2711a (7.42 mg kg^{-1}), SRM-1575a (0.0399 mg kg^{-1}), and ERM-CC141 (0.083 mg kg^{-1}) and liquid standards with a recovery rate of 90-110% were used for quality control.

The detection limit for total Hg was 0.001 $\mu g\ g^{-1}$ dw, calculated as three times the standard deviation (SD) of the blank. Three replicates per sample were analyzed, and values were accepted when the relative percentage difference between the two samples was within 10%. All reported concentrations are in dry weight.

2.6 Soil Organic Matter Content, Electrical Conductivity, pH, and Metals Analysis

The organic matter content was determined as a percentage of the loss by ignition of 10.0 g soil in a furnace at 450 °C (Thermo Scientific muffle furnace Thermolyne) for three hours (ASTM, 2014). The pH and EC of the soil were measured in soil distilled water solution (1:2) using a Thermo Scientific Versa Star meter. The concentration of total recoverable metals (other than Hg) was measured following EPA Method 200.7 using an ICP-OES (Shimadzu Model ICPE-9820). The quantification limit of the instrument was 1.39 mg L^{-1} for K and 0.14 mg L^{-1} for the other elements analyzed. For quality control, 3% nitric acid blanks were run at the beginning of each run, and liquid standards with a recovery rate of 90-110% were used for quality control.

2.7 Data analysis and statistical analysis

Total Hg concentrations in soils, plant length, dry weight, leaf chlorosis, and total number of bacteria were reported as the mean ± standard deviation. We performed statistical tests using an alpha of 0.05. Comparisons of plant length, dry weight, chlorosis, and total number of bacteria between the Hg concentrations tested were performed using the non-parametric Kruskal-Wallis test, followed by Wilcoxon as a post hoc test. This test also determined the lowest observable effect concentration (LOEC) values.

All statistical analyses were performed using R version 4.3.2 statistical software, and the ggplot2 package was used for visualizations.

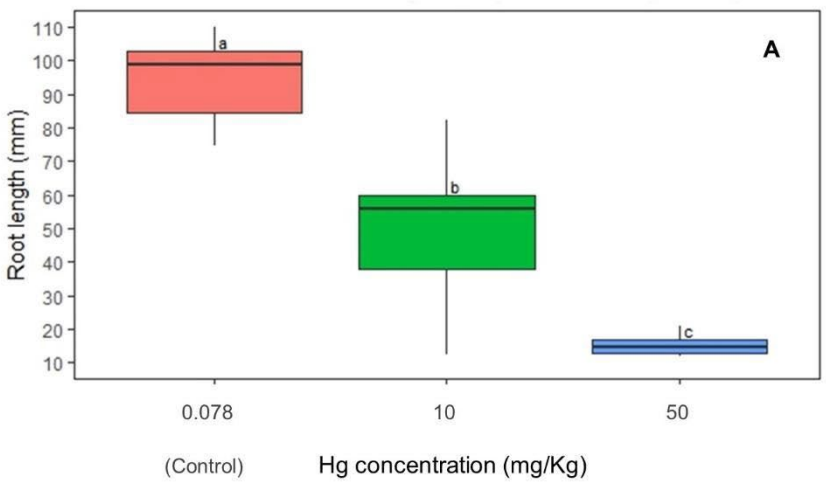
3 Results and discussion

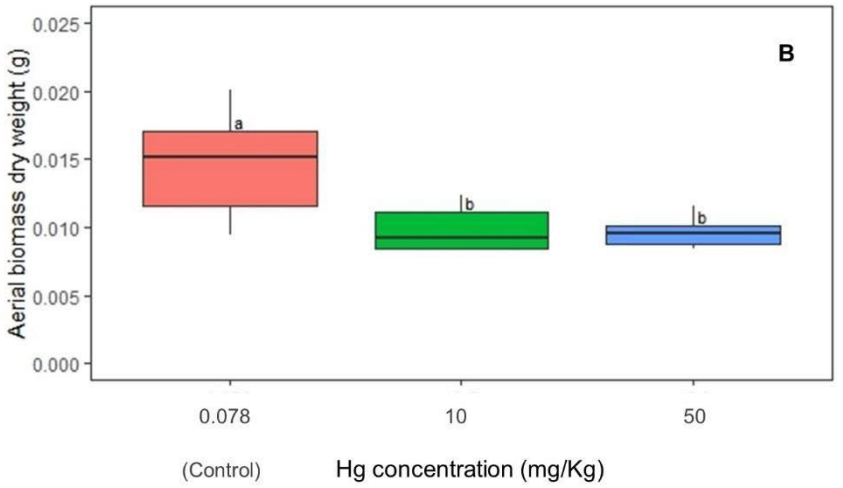
3.1 Effect of Hg on plant survival and growth

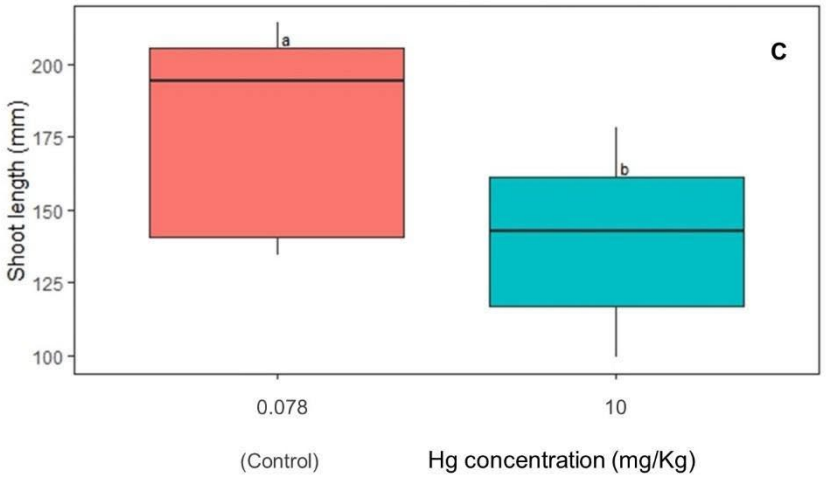
The selected plants are all common agricultural plants in the area and therefore it is important to assess the impact of Hg on their survival and growth. The plants have different types of roots and that can impact the effects of Hg exposure on them.

In the effects of Hg exposure experiments, the sensitivity of the studied plant species to Hg was shown to be different. In the first experiment, after 21 days of exposure, all the individuals from the uncucha and sacha culantro plant species growing in soil with Hg concentrations of 50 and 100 mg kg^{-1} died. Regarding the aji dulce plants, all the individuals exposed to 100 mg kg^{-1} of Hg died, and the mortality of the plants growing in soil spiked with 50 mg kg^{-1} of Hg was 40% at the end of the first experiment. The aji dulce plants started to show signs of distress after three days of exposure to Hg, and the uncucha and sacha culantro showed signs of distress after ten days of exposure to Hg.

Additionally, the exposure to Hg in aji dulce and uncucha plants reduced root length, shoot length, and above-ground biomass. The exposure to 10 and 50 mg kg^{-1} of Hg caused a 48% and 84% decrease in root length and a 32% and 33% decrease in above-ground dry weight, respectively, in aji dulce (Fig. 3-2.A and 3-2.B). Hg concentration of 10 mg kg^{-1} caused a 22% reduction in shoot length and a 43% reduction of above-ground dry weight in uncucha (Fig. 3-2.C and 3-2.D). No significant differences ($p < 0.05$) in length or above-ground biomass were found in the sacha culantro plants growing in soil spiked with Hg compared to the plants growing in the control soil (data not shown).


A
Root length (mm)
110
100
90
80
70
60
50
40
30
20
10
a
b
c
0.078
(Control)
10
50
Hg concentration (mg/Kg)


B
Aerial biomass dry weight (g)
0.025
0.020
0.015
0.010
0.005
0.000
a
b
b
0.078
(Control)
10
50
Hg concentration (mg/Kg)


C
Shoot length (mm)
200
175
150
125
100
a
b
0.078
(Control)
10
Hg concentration (mg/Kg)

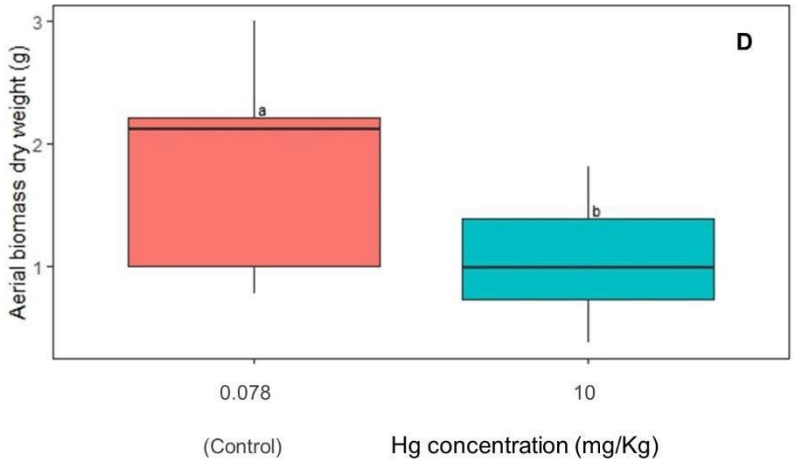

D
Aerial biomass dry weight (g)
3
2
1
a
b
0.078
(Control)
10
Hg concentration (mg/Kg)

Fig. 3-2. Effect of Hg exposure on root length **(A)** and aerial (above-ground) biomass dry weight **(B)** of aji dulce (*Capsicum Chinense)* after 21 days of Hg treatment. Effect of Hg exposure on shoot length **(C)** and aerial (above-ground) biomass dry weight **(D)** of uncucha (*Xanthosoma sagittifolium L. Schott)* after 21 days of Hg treatment. Different lower-case letters above the boxes indicate significant differences ($p < 0.05$) between mean ± SD ($n = 10$) of Hg concentrations and control.

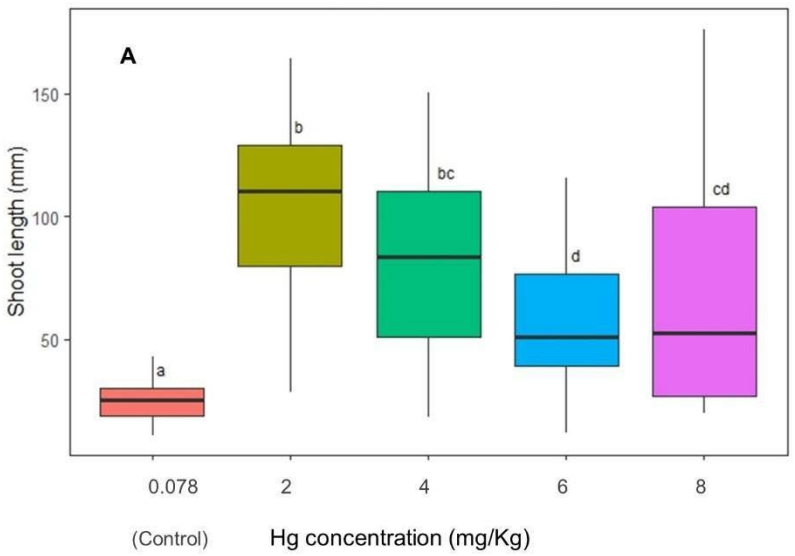
Plant length is an important characteristic and is related to plant growth and biomass yield (Wang et al., 2018). Meanwhile, dry weight estimates the plant's capacity to convert solar energy into stored carbon and the accumulation of nutrients like nitrogen and phosphorous (Tracey et al., 2016). The reduction in dry weight and length found in our first experiment is consistent with the results from previous studies at similar Hg concentrations in agricultural plant species such as spruce (Godbold, 1991), tobacco (Suszcynsky and Shann, 1995), tomato (Cho and Park, 2000), alfalfa (Zhou et al., 2007; El-Shehawi et al., 2022), Indian mustard (Shiyab et al., 2009), maize (Ghani, 2010), mustard greens (Wang et al., 2018), oat and bean (Lima et al., 2019). Mortality was not reported in any of these studies, even after exposure to higher Hg concentrations than the ones tested in our experiment. Some of these studies were developed in soil setups where Hg was added to the soil and others in hydroponic setups where Hg was added to liquid solutions. The bioavailability of Hg for the plants in the hydroponic experiments might be higher than in our study, where Hg was added to soil, due to the interaction between Hg and soil components, such as organic matter, which reduces its bioavailability (Di Salvatore et al., 2012; Boszke et al., 2008). That might illustrate that the studied Amazonian plant species are more sensitive to Hg than other agricultural plant species because they showed mortality even when they were only exposed to the available fraction of the Hg added to the soil.

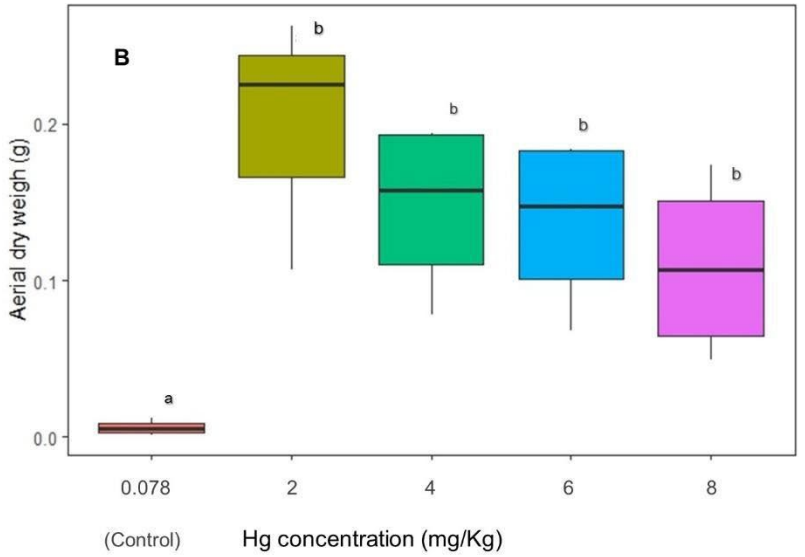
Several authors have reported that Hg reduces plant growth by inhibiting chlorophyll biosynthesis and damaging leaf cell structure (Cargnelutti et al., 2006; Shiyab et al., 2009). Other studies have shown that Hg exposure reduces the uptake of Fe and S by decreasing Fe absorption by roots and translocation to other parts of the plants and downregulating the sulfate transporters in roots, which affects plant growth and photosynthesis (El-Shehawi et al., 2022). In the first experiment, we did not find visual signs of chlorophyll inhibition, such as the yellowing of leaves, but we found a reduction in root length in aji dulce plants. Our results agree with studies concluding that the inhibition of plant growth caused by Hg exposure at the concentrations tested in the first experiment was due to a decrease in root growth and root damage, which prevents the uptake of nutrients (Godbold & Huttermann, 1988; Godbold, 1991). It has also been reported that heavy metal accumulation in roots blocks the entry of nutrient ions essential for plant growth, such as Ca, Mg, and K (Burzynski, 1987), which could also explain our findings.

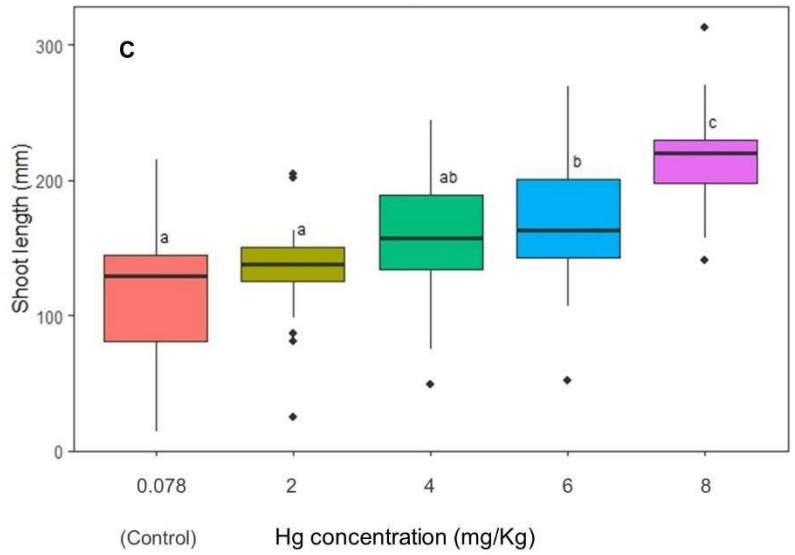
At the end of the second experiment, we observed an increase in the length of the aji dulce plants growing in the soil spiked with Hg compared to those growing in the control soil (Fig. 3-3.A). The exposure to Hg increased the shoot length of aji dulce plants from 25.1 ± 8.61 mm (control plants) to 105 ± 33.5 mm (2 mg kg^{-1} of Hg), 84.6 ± 37.9 mm (4 mg kg^{-1} of Hg), 56.7 ± 27.2mm (6 mg kg^{-1} of Hg), and 69.9 ± 47.4 mm (8mg kg^{-1} of Hg). Likewise, we found significantly higher length ($p < 0.05$) in the uncucha plants growing in soil with 6 and 8 mg kg^{-1} of Hg compared to the control plants (Fig. 3-3.C). The exposure to these Hg concentrations caused a 44% and 85% increase in shoot length, respectively. No significant difference ($p < 0.05$) in plant length was found among the sacha culantro plants exposed to different Hg treatments and the control (data not shown). Previous studies have reported an increase in plant length after exposure to low Hg concentrations, similar to and lower than the ones tested in our second

experiment, in tomato (Shekar et al., 2011), oat (Thimann & Bonner, 1949), cucumber (Cargnelutti et al., 2006), and onion plants (Subhadra et al., 1991) due to hormesis. This is a phenomenon where stimulation of a parameter occurs after exposure to low concentrations of a substance, and inhibition of the same parameter occurs after exposure to higher concentrations of the same substance (Southam and Chrlich, 1943). Hormesis has been found in plants exposed to metals such as cadmium, mercury, zinc, arsenic, lead, and selenium, affecting several endpoints, such as length, biomass, enzyme activities, and reproduction (Calabrese and Baldwin, 2003). It has been explained as stimulation of defense mechanisms resulting in activation of the plant metabolism (Barcelo & Poschenrieder, 2002). We hypothesize that hormesis is also responsible for the increase in shoot length in the uncucha plants observed in our second experiment because we observed a decrease in shoot length in this plant species after exposure to higher Hg concentrations in the first experiment and an increase in the same parameter after exposure to lower Hg concentrations in the second experiment with the same exposure time in both experiments.

Despite the differences in plant length found in these plants, there was no significant difference ($p < 0.05$) in the aerial dry weight of aji dulce plants exposed to the Hg treatments (Fig. 3-3.B), and no significant difference ($p < 0.05$) in the aerial dry weight of the uncucha plants growing in soil spiked with Hg compared to the control plants (Fig. 3-3.D).


A
Shoot length (mm)
150
100
50
a
b
bc
d
cd
0.078
(Control)
2
4
6
8
Hg concentration (mg/Kg)


B
Aerial dry weigh (g)
0.2
0.1
0.0
a
b
b
b
b
0.078
(Control)
2
4
6
8
Hg concentration (mg/Kg)


C
Shoot length (mm)
300
200
100
0
a
a
ab
b
c
0.078
(Control)
2
4
6
8
Hg concentration (mg/Kg)

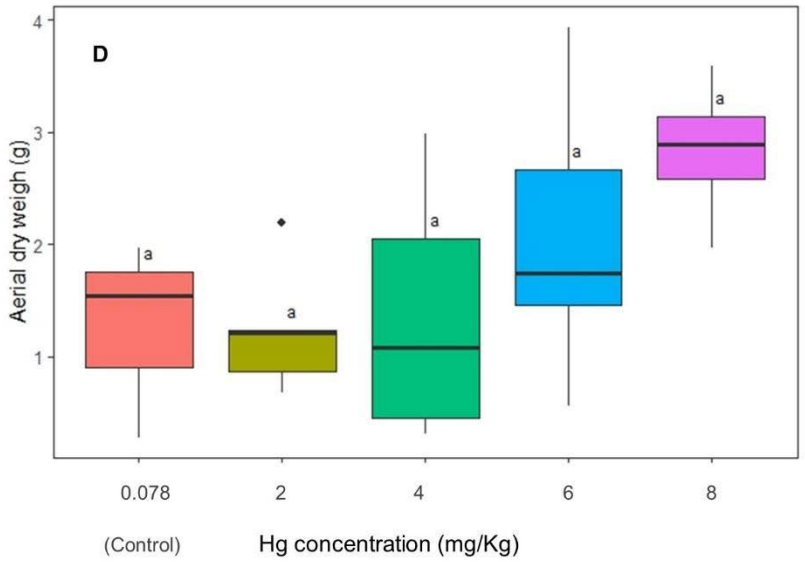

D
Aerial dry weigh (g)
4
3
2
1
a
a
a
a
a
0.078
(Control)
2
4
6
8
Hg concentration (mg/Kg)

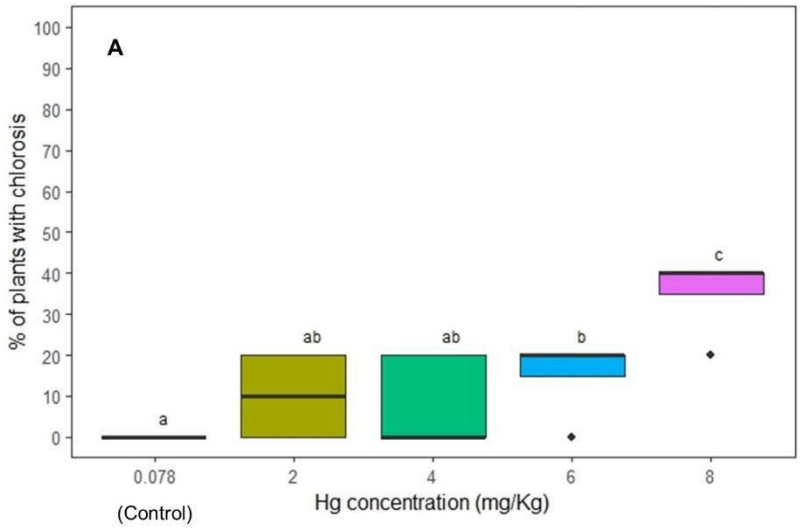
Fig. 3-3. Effect of Hg exposure on shoot length **(A)** and aerial (above-ground) biomass dry weight **(B)** of aji dulce (*Capsicum Chinense)* after 32 days of Hg treatment. Effect of Hg exposure on shoot length **(C)** and aerial (above-ground) biomass dry weight **(D)** of uncucha (*Xanthosoma sagittifolium L. Schott*) after 21 days of Hg treatment. Different lower-case letters above the boxes indicate significant differences ($p < 0.05$) between mean ± SD ($n = 40$) of Hg concentrations and control.

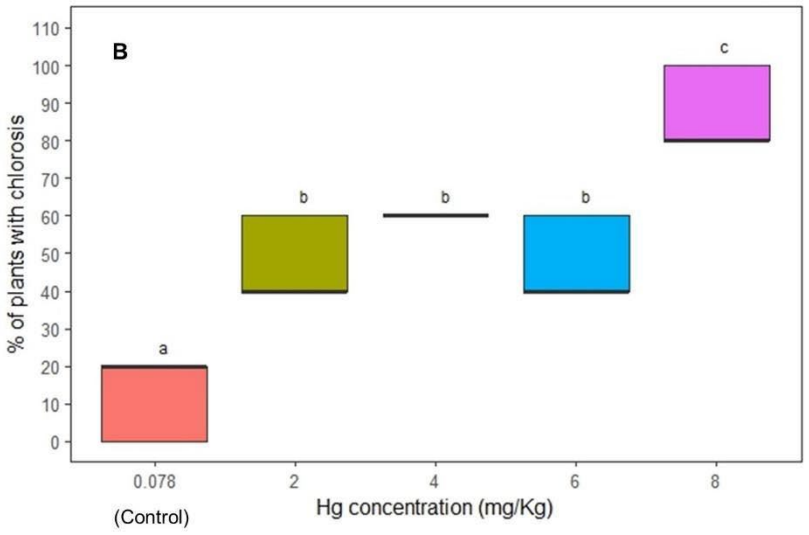
Plant growth is often expressed by the increase in length and mass, with a linear relationship between both variables (Tracey et al., 2016; Prouxl, 2021; Franco & Kelly, 1998). An increase in plant length without increasing biomass can happen when plants are under some stress (Proulx, 2021). Some studies showed that nutrient deficiency affects plants' linear relationship between length and weight (Mandak & Pysek, 1999; Weiner, 2004). For instance, plants grow more weight in their roots at the expense of shoot weight in a low-nutrient environment to increase their capacity to access the most limiting resource (Bloom et al., 1985). Other authors have reported that plants gaining length without weight increase perform photosynthesis at a lower rate than those gaining length and dry weight (Nagashima & Terashima, 1995). The increase in plant length without an increase in weight has not been reported as a toxic effect of Hg in plants before. However, signs of photosynthesis disturbance, like the yellowing of leaves, were observed in our second experiment. Thus, this might explain the increase in shoot length without biomass gain in the aji dulce and uncucha plants exposed to Hg in that experiment. Root length and weight were not evaluated in the second experiment due to the entanglement of the roots, which made it difficult to extract the whole root from the pots. More research about the effects on root length, root damage, and blocking of ions uptake by low Hg concentrations similar to the ones tested in this experiment is needed to find if the reduction in nutrient uptake, as

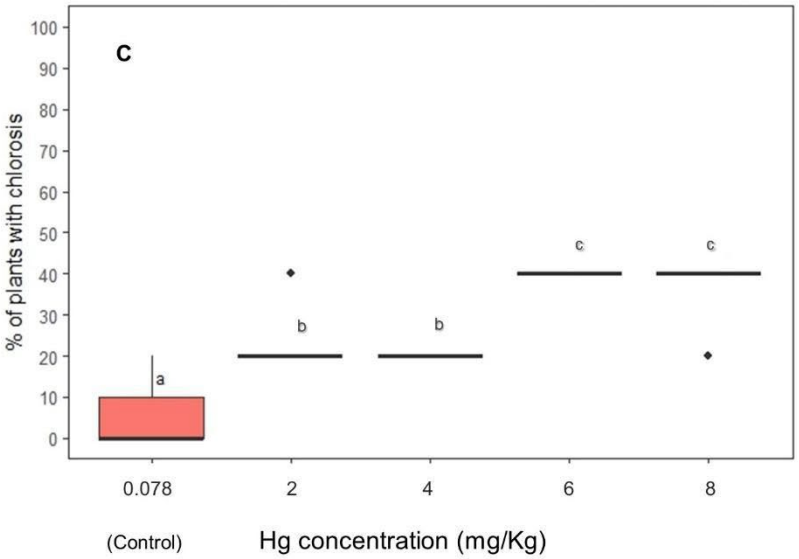
hypothesized in our first experiment, could also cause the nonlinear relationship between shoot length and mass observed.

3.2 Effect of Hg on plant leaves

The effects of Hg exposure on plant leaves were observed during the second experiment. The aji dulce and uncucha plants exposed to Hg showed yellowing (chlorosis) and death (necrosis) of foliar tissue. The percentage of plants suffering from these injuries in leaves increased with increasing Hg concentrations (Fig.3-4). The aji plants did not show any signs of toxicity after 21 days of exposure. Thus, we decided to extend the experiment's duration to assess the effects changes with time. The first signs of chlorosis appeared after 27 days of Hg exposure. At the end of the experiment, after 32 days of Hg exposure, the Hg concentrations of 2 and 4 mg kg^{-1} caused an increase in chlorosis in leaves by 272%. The Hg concentrations of 6 and 8 mg kg^{-1} caused an increase in the same parameter by 578% (Fig.3-4.D). Regarding the uncucha plants, the signs of chlorosis and necrosis appeared after 7 days of exposure to the higher Hg concentrations. After 14 days of exposure, the Hg concentrations of 6 and 8 mg kg^{-1} caused an increase in chlorosis and necrosis from 0% in the control plants to 15% and 35% in the plants exposed to these Hg treatments, respectively (Fig.3-4.A). After 21 days of exposure, Hg concentrations of 2, 4, and 6 mg kg^{-1} increased the chlorosis and necrosis in leaves by 313.3 ± 57.7%, and Hg concentration of 8 mg kg^{-1} increased the same parameter by 600% (Fig.3-4.B).


A
% of plants with chlorosis
100
90
80
70
60
50
40
30
20
10
0
a
ab
ab
b
c
0.078
(Control)
2
4
6
8
Hg concentration (mg/Kg)


B
% of plants with chlorosis
110
100
90
80
70
60
50
40
30
20
10
0
a
b
b
b
c
0.078
(Control)
2
4
6
8
Hg concentration (mg/Kg)


C
% of plants with chlorosis
100
90
80
70
60
50
40
30
20
10
0
a
b
b
c
c
0.078
(Control)
2
4
6
8
Hg concentration (mg/Kg)


D

Fig. 3-4. Effect of Hg exposure on chlorosis in leaves after 14 days of exposure **(A)** and 21 days of exposure **(B)** in uncucha plants (*Xanthosoma sagittifolium L. Schott*). Different lower-case letters above the boxes indicate significant differences ($p < 0.05$) between mean ± SD ($n = 40$) of Hg concentrations and control. Effect of Hg exposure on chlorosis in leaves after 32 days of exposure in aji dulce plants (*Capsicum Chinense)* **(C)**. Different lower-case letters above the boxes indicate significant differences ($p < 0.1$) between mean ± SD ($n = 40$) of Hg concentrations and control. Chlorosis and necrosis in leaves of uncucha plants after 21 days of Hg exposure **(D).**

Chlorosis, or the yellowing of leaves, is a symptom of a deficiency of nutrients, like iron, magnesium, manganese, and nitrogen, needed to produce chlorophyll. This deficiency reduces the photosynthesis rate and plant growth (Miller et al., 1984; Ye et al., 2019; Ivanov et al., 2022; Shao et al., 2020). It can also be a symptom of photosynthesis inefficiency. Exposure to Hg can downregulate the genes related to iron uptake in roots, causing the inefficiency of photosynthesis (El-Shehawi et al., 2022). Hg can also reduce the uptake of nutrients by the roots, which alters some biochemical processes (Calgaroto et al., 2015). It can also replace some nutrients that produce chlorophyll, inhibiting the photosynthetic electron transport chain (Patra et al., 2004). Our results are consistent with previous studies reporting chlorosis as an effect of Hg exposure to concentrations similar and higher to the ones tested in our second experiment in agricultural plant species, like alfalfa (El-Shehawi et al., 2022) and tobacco (Suszcynsky & Shann, 1995), and non-agricultural plant species, such as Jatropha curcas (Gao et al., 2010), and Cyrtomium macrophyllum (Xun et al., 2017).

Although the uptake of nutrients was not measured in this study, it could explain the chlorosis in leaves found in our second experiment. Nutrient uptake in Hg toxicity studies with these plant species should be measured to prove this hypothesis.

In our first experiment, we did not observe chlorosis in the plants despite using higher Hg concentrations. This difference may be explained by a longer exposure time in the second experiment for the aji dulce plants. For the uncucha plants, however, the chlorosis appeared at a shorter exposure time to lower Hg concentrations than the ones tested in the first experiment.

It has been reported that heat stress exacerbates chlorosis in leaves in agricultural plants like soybean (Djanaguiraman et al., 2011), sorghum (Djanaguiraman et al., 2014), and wheat (Akter & Rafiqul Islam, 2017), after air temperature increases of 6-10°C from an initial temperature of 28°C, and at air temperatures higher than 40°C. The heat stress affects the capability of repairing photosynthetic machinery, thus reducing the chlorophyll content. The average air temperature in our second experiment was 35°C, 6°C higher than the average temperature in the first experiment. There are no published studies about the influence of temperature in the effects of Hg exposure in plants, but apart from the Hg concentrations tested, air temperature was the main difference between both experiments, so we hypothesized that the increase in temperature was responsible for the appearance of chlorosis in the uncucha plants in this experiment. However, toxicity tests in a temperature-controlled environment would be needed to confirm this hypothesis.

Some climate change studies have reported increased maximum and minimum temperatures in the Madre de Dios region, up to 0.2°C and 0.3°C per decade, respectively, since 1964 (Vicente-Serrano et al., 2018). Future estimations predict an increase in the average temperature in the region of 2.8-3.2°C and more frequent extreme events, like heat waves and flooding (Llacza et al., 2021). In this climate change scenario, toxic effects of Hg exposure in agricultural plant species, like chlorosis, might be aggravated, affecting the local communities' livelihood and health.

3.3 Effect of Hg on rhizosphere soil bacterial population

To evaluate the effect of exposure to different Hg concentrations on the rhizosphere soil bacterial population of the plant species studied, the total number of bacteria in the rhizosphere soil samples collected at the end of the second experiment, after 21 days of Hg exposure for the uncucha plants, and 32 days of exposure for the aji dulce plants, was quantified. Only samples of the uncucha and aji dulce plants were analyzed because they showed toxic effects after Hg exposure in the second experiment. A reduction in the total number of bacteria in the rhizosphere soil from $1.90 \times 10^{11} \pm 2.24 \times 10^{11}$ number of gene copies per grams of dry weight to $6.66 \times 10^{10} \pm 1.19 \times 10^{9}$ number of gene copies per grams of dry weight was found in the uncucha plants exposed to 6 mg kg^{-1} of Hg compared to the control plants (Fig.3-5.A). No significant difference ($p < 0.05$) in the total bacterial abundance in the rhizosphere soil between the plants growing in the other Hg treatments and the control plants was found in this plant species (Fig.3-5.A). No significant difference ($p < 0.05$) in the total number of bacteria in the rhizosphere soil between the plants growing in any of the Hg treatments and the control plants was found in the aji plants (Fig.3-5.B).

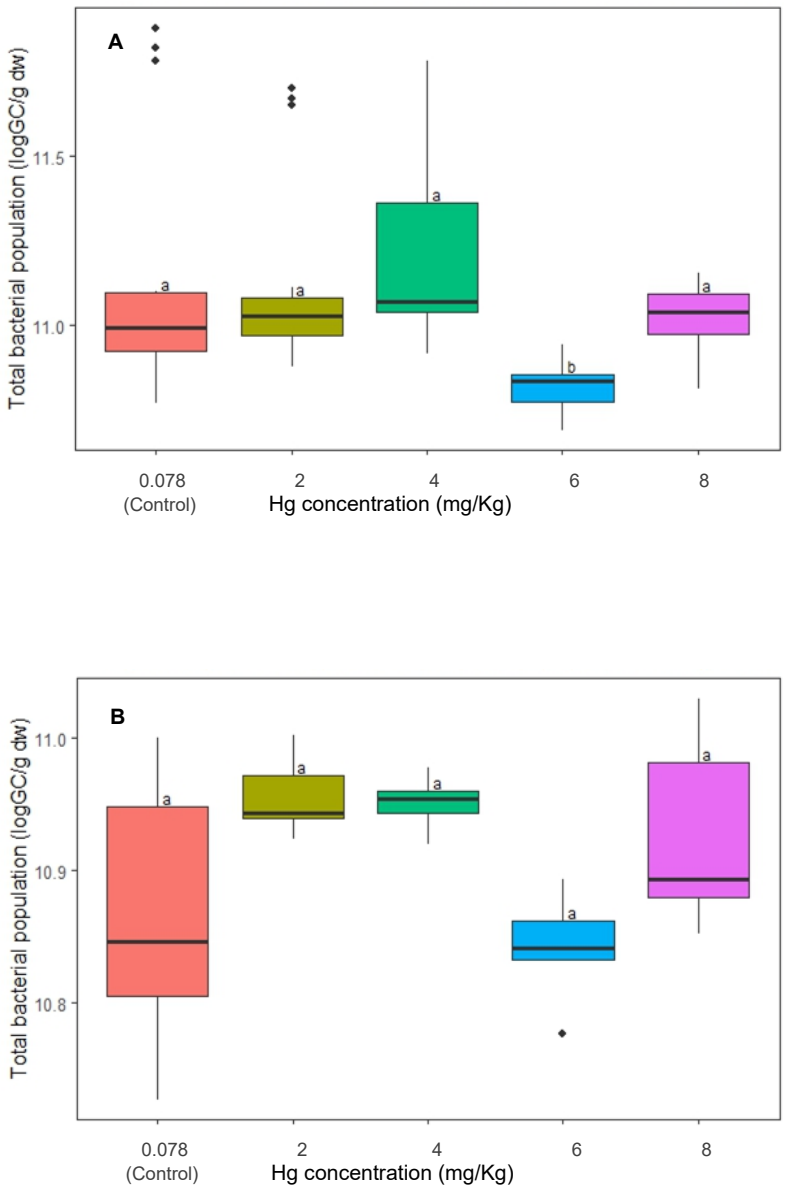


Fig. 3- 5. Effect of Hg exposure on the total number of bacteria (number of gene copies/gram dry weight) in rhizosphere soil in uncucha plants (*Xanthosoma sagittifolium L. Schott*) after 21 days of exposure **(A)** and in aji dulce plants (*Capsicum Chinense)* after 32 days of exposure **(B).** Different lower-case letters above the boxes indicate significant differences ($p < 0.05$) between mean ± SD ($n = 40$) of Hg concentrations and control.

Rhizobacteria plays an important role in plant health. For example, they help with nutrient acquisition and reduce the adverse effects of plant pathogens and exposure to pollutants (Ahemad and Kribet, 2014). Previous studies have reported no significant differences in the total number of culturable bacteria in the rhizosphere of grasses exposed to Hg concentrations higher than those tested in our experiment (Sas-Nowosielska et al., 2008). However, it has been established that less than 1% of soil microorganisms are cultivable on growth media (Rappe & Giovannoni, 2003), so these studies do not show the Hg effects on the total number of bacteria in the rhizosphere.

Other studies reported a decrease in total bacterial abundance (including culturable and non-culturable bacteria) in the rhizosphere of clover and rice plants growing in soil with higher and similar Hg concentrations than in this study (Zhu et al., 2021; Hu et al., 2022). That would prove that Hg exposure does not affect the culturable bacteria but affects the non-culturable bacteria in the rhizosphere soil. Still, more studies assessing both types of bacteria separately in plant rhizosphere should be performed to prove this hypothesis.

All these studies found changes in the composition of the rhizosphere microbial communities due to the Hg exposure. The abundance of specific types of bacteria sensitive to Hg, such as nitrifying, iron-reducing, and sulfur-aminoacid decomposing bacteria, decreased, and the abundance of types of bacteria resistant to Hg, like protein-decomposing and nitrogen-fixing bacteria, increased.

From our knowledge, this study is the first to assess the effects of Hg exposure on the total number of bacteria in the rhizosphere of Amazonian agricultural plant species. Other studies have evaluated the effects of Hg exposure on microbial activity, measured as soil basal respiration, in the rhizosphere of non-native agricultural plants, oat and bean, growing in

Amazonian soil and found a reduction of this activity after exposure to Hg concentrations similar and higher to the ones in this study (Lima et al., 2019). Our findings may suggest that the total number of bacteria in the rhizosphere soil of the plant species evaluated is not affected by the exposure to the Hg concentrations tested in our experiment. Still, our assays are based on total DNA, so we may have quantified alive and dead bacteria. Also, for the calculation of the total number of bacteria one gene copy per bacterial cell was considered. Still, the number of 16S rRNA copies in the bacterial cells can vary from 1-25, so statistically significant differences may be missed. More research is needed to determine if these plant species' rhizosphere microbial community composition and activity changes due to Hg exposure.

4 Conclusions

Here, we study for the first time the effects of Hg exposure on three Amazonian native agricultural plant species, aji dulce *(Capsicum Chinense)*, sacha culantro *(Eryngium foetidum)*, and uncucha (*Xanthosoma sagittifolium L. Schott).* The Hg concentration of 100 mg kg^{-1} caused the death of all the plants evaluated, and the Hg concentration of 50 mg kg^{-1} caused the death of all the uncucha and sacha culantro plants. We found a decrease in length and weight in aji dulce plants after exposure to 10 and 50 mg kg^{-1} of Hg and a decrease in the same parameters in the uncucha plants after exposure to 10 mg kg^{-1} of Hg. Hg concentrations from 2 to 8 mg kg^{-1} caused chlorosis in leaves of the aji dulce and uncucha plant species, with an increased percentage of affected plants by increased Hg concentrations. The lowest Hg concentration producing toxic effects in these plant species was 2 mg kg^{-1}, three times lower than the SSL for Hg in agricultural soil established in the Peruvian regulation. Our results also suggest that heat stress exacerbates the appearance of chlorosis caused by Hg exposure in the uncucha plant species. These findings support the need to review and revise the soil screening levels for Hg in Peru and other

Amazonian countries and develop toxicity levels that consider Amazonian organisms and soil, to better understand the potential impacts of Hg in plant development, especially in the Amazonian countries affected by ASGM and climate change, where environmental policies are based on data from international regulations without considering the characteristics of local ecosystems.

Chapter 4: Integrating scientific and Indigenous knowledge about soil degradation and restoration in areas impacted by artisanal gold mining in the Southern Peruvian Amazon

(This chapter is under preparation for *Land Degradation and Development*)

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Abstract

Artisanal and Small-Scale Gold Mining (ASGM) have pressured the Amazon rainforest, causing deforestation and soil degradation in large areas. Previous research has studied the restoration of areas degraded by ASGM in this region, focusing on recovering the composition of primary

forest plant species through natural regeneration. Reforestation of these degraded areas using plant species with an economic value is also being evaluated as an alternative to natural regeneration. There is a lack of information about the recovery of soil fertility in these areas. This study examines physico-chemical characteristics, such as soil organic matter (SOM), electrical conductivity (EC), pH, nutrients, and bacterial communities, in the rhizosphere soil of plant species growing in naturally regenerated and reforested areas impacted by ASGM and in the primary forest near these areas. Rhizosphere SOM, EC, pH, total recoverable calcium (Ca), potassium (K), magnesium (Mg), and phosphorous (P) levels were determined in rhizosphere soil samples collected at 5-30 cm depth under nine plant species in different land cover categories: natural regeneration with different ages, reforestation with varying amendments of soil, and primary forest at two mining sites. The highest levels of SOM, EC, and nutrients were found in the primary forest, and the lowest levels of these parameters were found in the 3-year-old naturally regenerated areas. The results suggest that in rhizosphere soil, SOM, pH, EC, and total recoverable Ca return to primary forest conditions after eight years of natural regeneration and five years of reforestation for pH and EC. Among the studied plant species, Acacia *loretensis* and *Inga sp*. (*Inga sp.* when planted with biochar as a soil amendment) might be good candidates for restoring areas degraded by ASGM. This study also examines the integration of Indigenous and scientific knowledge to monitor and manage land degradation in areas impacted by ASGM. Indicators of soil degradation used by Indigenous communities and proposals for restoration were identified, incorporating the communities needs. The findings emphasize the need to facilitate a dialogue about land degradation and restoration of areas impacted by ASGM between local communities, researchers, and policy-makers to develop more sustainable and successful restoration projects in Indigenous communities affected by ASGM.

1. Introduction

The Amazon is the largest tropical forest in the world and plays an important role in regulating nutrient cycling and global climate (Buscardo et al., 2016; Soares-Filho et al., 2010). However, it has been under pressure from anthropogenic activities, such as agriculture, logging, and artisanal and Small-Scale Gold Mining (ASGM) over the past several decades (McGrath et al., 2001; Alarcón Aguirre et al., 2021; Alarcón Aguirre et al., 2023). The Peruvian Amazonian region of Madre de Dios is a part of the Tropical Andes Biodiversity Hotspot (Myers et al., 2000). It provides ecosystem services, such as water supply and climate change mitigation (Fisher et al., 2018). Still, this region has been largely impacted by ASGM. This activity is often performed illegally or informally in countries with limited environmental regulations (The Global Initiative Against Transnational Organized Crime, 2017). It can produce human and environmental health impacts, such as mercury (Hg) pollution, deforestation, and soil degradation (Caballero Espejo et al., 2018; Gerson et al., 2020; Gerson et al., 2022), and threaten the food supply of vulnerable populations like Indigenous communities (Diringer et al., 2015).

During the ASGM process with minimally mechanized techniques, miners deforest the area and excavate the soil using high-pressure water (Caballero Espejo et al., 2018), separate and pile up the rocks, and process soil sediments where the gold is located (Salinas, 2007). The mining process breaks up soil components and washes away its nutrients, leaving soil mainly composed of sand (Timsina et al., 2022). ASGM in the Madre de Dios region has caused the deforestation of approximately100,000 ha of forest from 1984 to 2017 (Caballero Espejo et al., 2018) and has reduced soil organic matter (SOM) and electrical conductivity (EC) by 96 and 99 %, respectively (Rodriguez-Pascual et al., 2024).

Multiple efforts have been made to restore degraded lands in the Amazon rainforest in the last decades using different methods, such as reforestation with native and non-native plant species, soil amendment application, and the management of naturally regenerated forests (Garate-Quispe et al. 2021a, 2021b; Román-Dañobeytia et al., 2021; Lefebvre et al., 2021; Velásquez Ramírez et al., 2021). Natural forest recovery depends on the degree of degradation (Chazdon, 2008). In tropical areas impacted by agriculture, the forests can naturally recover some characteristics within a few decades (Guariguata & Ostertag, 2001; Chazdon et al., 2007; Feldpausch et al., 2007; Norden et al., 2015). However, natural regeneration in areas impacted by ASGM it might be slower and more complex because the soil is highly degraded. Also, natural regeneration in these areas has not been widely studied (Peterson & Heemskerk, 2001; Lamb et al., 2005; Ren et al., 2007; Kalamandeen et al., 2020). Reforestation and soil amendment applications are costly, especially in remote areas like the ones impacted by ASGM in the Amazon, and have not been extensively systematized (Román-Dañobeytia et al., 2021; Meza et al., 2006).

Trees have been reported to increase soil fertility over time, as indicated by soil total carbon and nutrients (Sanchez et al., 1985; Bayala et al., 2019), and the rhizosphere is an important area where these elements are produced (McCormack et al., 2015). However, soil characteristics vary due to plant species and management (Malysz & Overbeck, 2018; Wang et al., 2001). Studies on tree rhizospheres can help predict the long-term nutrient supply needed for forest growth (Hummes et al., 2024). When considering the need to restore areas degraded by ASGM in the Amazon rainforest, it is important to understand how different plant species and forest management practices change soil characteristics and how they can be used for sustainable restoration.

Additionally, In the Madre de Dios region, 10 Indigenous communities have been affected by ASGM in the last decades. Some of these communities are located in protected areas like the Amarakeri Communal Reserve, co-managed by the Servicio Nacional de Areas Protegidas (National Service of Areas Protected by the State) (SERNANP) and the Indigenous organization Ejecutor del Contrato de Administracion de la Reserva Comunal Amarakaeri (Executor of the Amarakaeri Communal Reserve administration contract) (ECA Amarakaeri. In this reserve, around 2,500 hectares have been deforested by mining activity in the last three years (Finer & Mamani, 2024), and long-term environmental impacts caused by ASGM in this reserve are well known and have been reported by the Indigenous communities (Finer & Novoa, 2017) However, only deforestation is currently monitored by the government and Indigenous organizations, but no studies on soil degradation or restoration projects of the impacted land have been done in this reserve.

Local Indigenous peoples and populations that have long inhabited regions have developed an extraordinary knowledge of the environment needed for their survival and thriving in this environment (Kliskey et al., 2009; Krupnik & Jolly, 2002; Usher 2000; Marino E., 2015; Gutierrez et al., 2019). This knowledge makes them capable of observing environmental changes with a comprehension of the variability of the environment with time that other people cannot observe (Alessa et al., 2016). Indigenous knowledge has been integrated into systems to monitor environmental threats, like natural disasters and land degradation in several countries, for instance, by collecting and analyzing information that enables warning messages (Stringer & Reed, 2007; Alessa et al., 2016; Macherera & Chimbari, 2016). With the same spirit, the Peruvian government through SERNANP uses Indigenous knowledge to monitor early signs of deforestation in some protected areas through communal guards' programs. These communal

guards are members of the Indigenous communities who patrol the protected areas to identify deforestation due to illegal activities in their territories (Sarmiento Barletti & Rolando, 2024). Still, the government is not using Indigenous knowledge to identify other environmental impacts, like soil degradation.

The scientific monitoring of land degradation has been based substantially on remote sensing. However, some land degradation indicators, such as changes in plant species composition, cannot be captured with these techniques (Herrmann et al., 2014). Local land users, such as Indigenous people, have much to contribute to understanding land degradation and ensuring that scientific studies capture relevant variables for them. The need to include local knowledge in land degradation evaluations has been acknowledged by previous studies (Cowie et al., 2018; IBPES, 2018). However, how local land users could be involved and their knowledge included has been studied less (Herrmann et al., 2020).

Here, we examine for the first time soil physico-chemical characteristics, such as SOM, EC, pH, nutrients, and bacterial communities, in the rhizosphere of plant species growing in naturally regenerated and reforested areas impacted by ASGM and in the primary forest near these impacted areas. We compared properties in the rhizosphere and bulk soil in the primary forest and in areas with different natural regeneration ages and areas reforested with various methods. We also identified native plant species that can be good candidates for restoring areas degraded by ASGM in the Amazon rainforest. Finally, we assessed how Indigenous and scientific knowledge can be combined to monitor land degradation in areas impacted by ASGM. We identified indicators of soil degradation used by Indigenous people, as well as proposals for restoration, incorporating the Indigenous needs and challenges to overcome for the design of sustainable restoration projects in these communities.

2. Materials and methods

2.1 Study area

We performed our rhizosphere study in the Southern Peruvian Amazonian region of Madre de Dios in two ASGM sites where minimally mechanized mining techniques are used to extract and transport the soil and gold material (Caballero-Espejo et al., 2018). One of the selected sites is a mining concession in Laberinto district, Tambopata province, 50 km from Puerto Maldonado, capital of Madre de Dios region, along the Madre de Dios River, 200 meters above sea level (masl) at UTM coordinates 19 L 433050/8597900. The second site is a mining concession in Inambari district, Tambopata province, 100 km from Puerto Maldonado, in the "La Pampa" area. La Pampa is in the Tambopata National Reserve buffer zone, 220 masl, at UTM coordinates 19 L 397039/8560472.

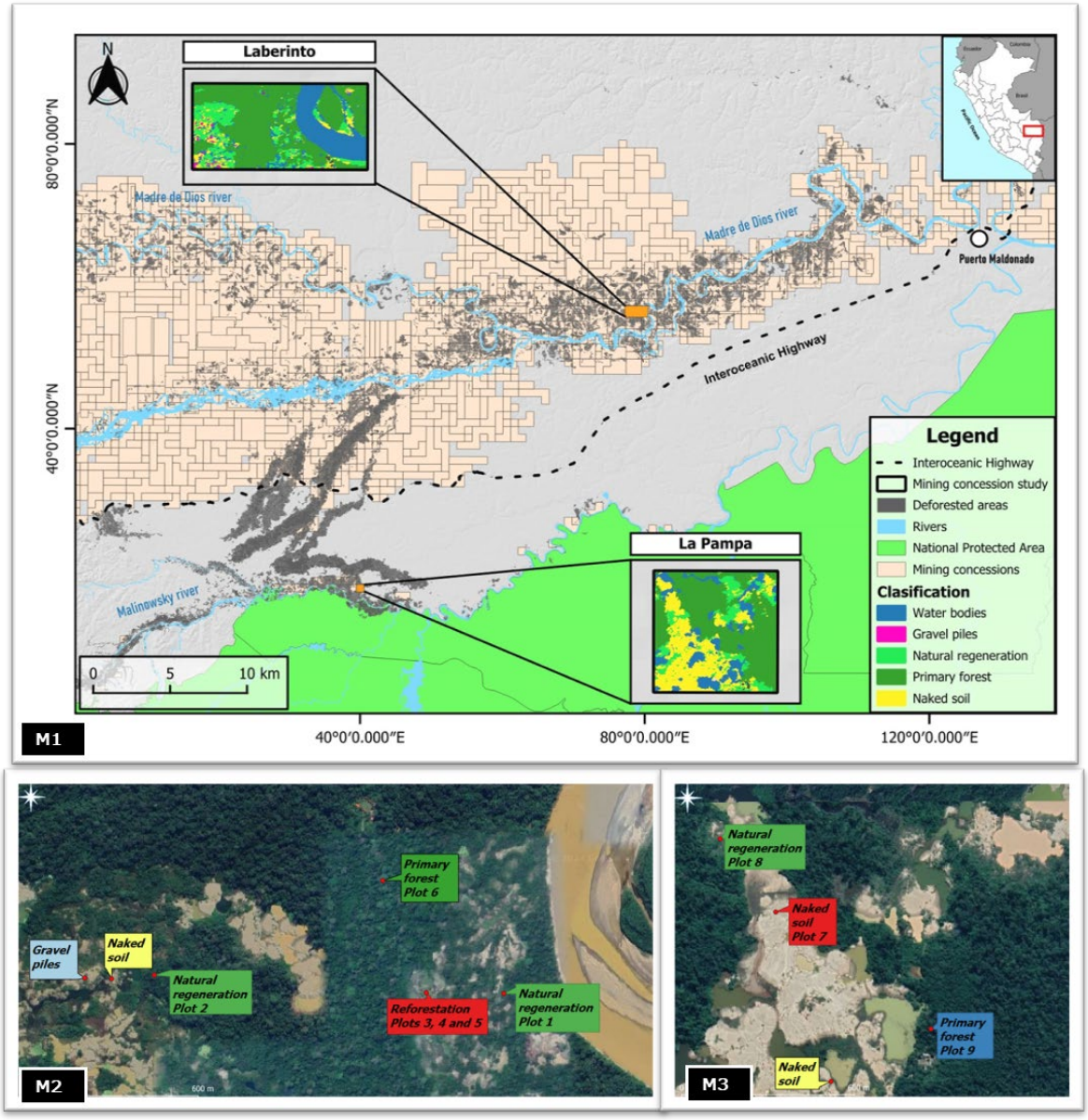


Fig. 4-1. **M1.** Map of the two sampling sites in Laberinto and La Pampa districts in the Madre de Dios region, Peru. **M2**. Map of the land cover category plots in Laberinto site. **M3**. Map of the land cover category plots in La Pampa site (Rodriguez-Pascual et al., 2024).

At each mining concession, we studied land cover categories with different levels of restoration after the mining activity had been abandoned and on the control category in which primary forest was not impacted by mining (Fig. 4-2). We considered some of the land cover categories

from Rodriguez-Pascual et al., 2024 (Fig. 4-2): L3 - natural regeneration; in this category, the vegetation has grown naturally over a soil once composed of sand and/or rocks; L4 - reforestation; in this category, plants have been reforested by human intervention over a soil once composed of sand and/or rocks. The plant species in this category differ from those in the natural regeneration and primary forest, L5 - primary forest; this category represents the intact forest that is non-deforested by ASGM.

The Laberinto concession has an extension of 449 ha. From the total area, 73.4% is occupied by the land cover categories we studied, 16.4% consists of natural regeneration, 2.3% is reforestation, and 54.7% is primary forest. The rest of the concession's area consists of ponds, and degraded soil without plants. The La Pampa concession has an extension of 100 ha. Of the total area, 60.7% is occupied by the land cover categories we studied, 13.1% consists of natural regeneration, and 47.6% is primary forest (Fig. 4-1. M1). The rest of the concession's area consists of ponds, and degraded soil without plants.

We selected plots of 50 x 20 m per land cover category at each mining site (Fig. 4-1. M2). At the Laberinto mining site, we selected a natural regeneration plot where we found 29 different plant species of trees and shrubs. The age of the plants in this plot is unknown, but according to the miners, the mining activity stopped eight years before our study (2015), and the plants started to grow after that date. We also chose three reforestation plots at this mining site. No soil amendments were used in one of the plots (reforestation control plot), and different soil amendments were applied in the other two reforestation plots (reforestation with biochar in one plot and reforestation with biochar enriched with NPK in the other). In these reforestation plots, 30 native and non-native tree species with economic value were planted five years before our study (2017) (Roman-Dañobeytia et al., 2020). One primary forest plot with 125 plant species of

trees and shrubs was also selected in Laberinto. The age of the primary forest is unknown, so we assume it is 120 years old because the region was first inhabited 120 years ago.

At the La Pampa site, we selected one natural regeneration plot with 24 plant species of trees and shrubs (Fig. 4-1. M3). The age of the plants in the plot is unknown, but according to the miners, the mining activity stopped three years before our study (2019), and the plants started to grow after that date. We also chose one primary forest plot with an unknown number of tree and shrub plant species. The age of the primary forest is also unknown, so we assume that the age of this forest is the same as the primary forest in Laberinto. The plots in both mining concessions were sampled in the dry season 2022 (August-September) (Table 4-1)



Fig. 4-2. Land cover categories at the sampling sites. **L3**. Natural regeneration (bottom left). **L4.** Reforestation (bottom center). **L5**. Primary forest (bottom right) (Rodriguez-Pascual et al., 2024).

In addition to the rhizosphere study, we developed a social study with focus groups in the Amarakeri Communal Reserve (Fig.4-3). This reserve, which extends 402,335.62 hectares, is located southwest of the Madre de Dios region, in the Manu province, 191 km from Puerto Maldonado, capital of the Madre de Dios region, at 183-500 meters above sea level (masl). The

Reserve is co-managed by the Peruvian Government through SERNANP and the Indigenous organization ECA-Amarakaeri. Ten native communities from the Harakbut, Yine, and Matsiguenka people live in its buffer zone, and three of these communities are affected by the ASGM. The focus groups were performed in these three communities.

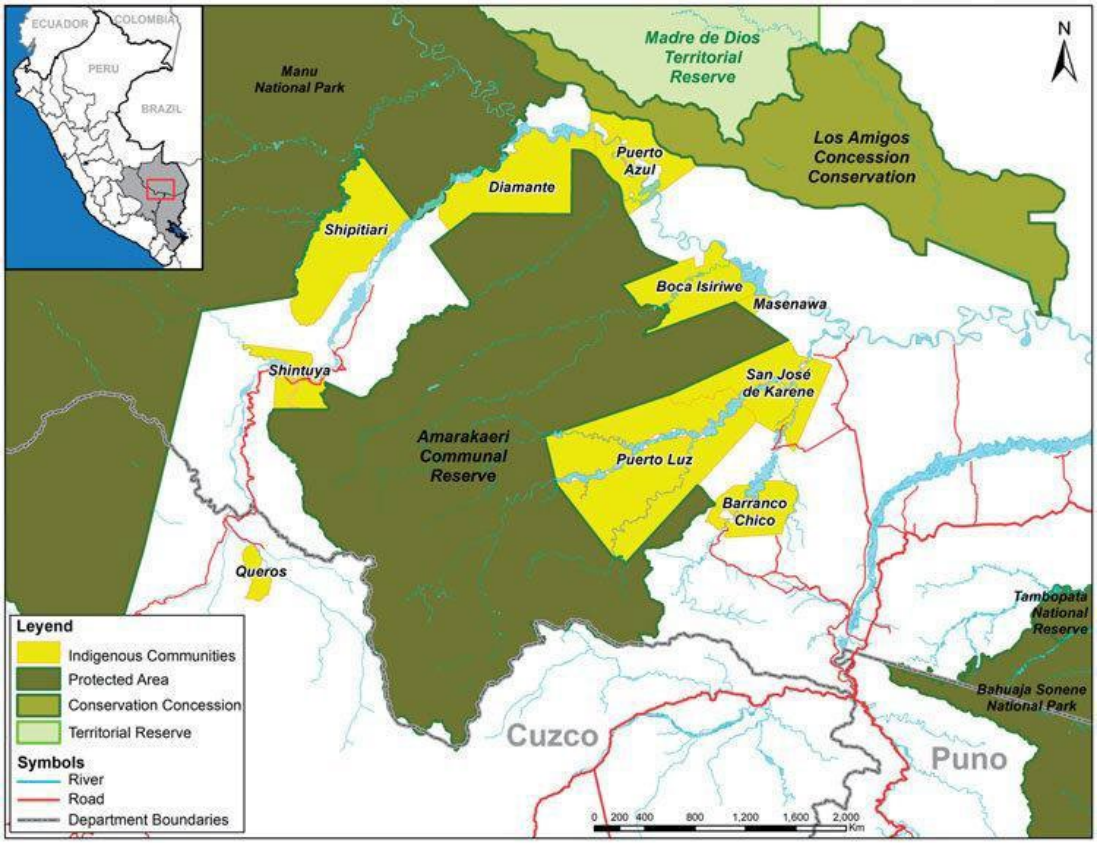


Fig. 4-3. Amarakaeri Communal Reserve, Madre de Dios, Peru. (from Fisher et al., 2020).

2.2 Sample collection

2.2.1 Rhizosphere Soil Sampling

In the selected land cover categories, we collected rhizosphere soil samples in 50 x 20 m plots in each category under nine of the most abundant plant species grouped into seven families (Table 4-1). To ensure the representativeness of the plant collection, two of the most abundant plant species and three individuals of each plant species per land cover category were selected (Rautio et al., 2020).

Table 4-1. Plant species selected in the two mining sites.

Sampling site	Land cover category	Age of restoration	Plot number	Plant species	N	Family	Growth habit	Leaf type
Laberinto	Natural regeneration	8 years	1	*Cecropia polystachya*	3	Urticaceae	Tree	Perennial
				Acacia loretensis	3	Fabaceae	Tree	Perennial
	Reforestation	5 years	3	*Hura crepitans*	3	Euphorbiaceae	Tree	Perennial
				Inga spp	3	Fabaceae	Tree	Perennial
			4	*Hura crepitans*	3	Euphorbiaceae	Tree	Perennial
				Inga spp	3	Fabaceae	Tree	Perennial
			5	*Hura crepitans*	3	Euphorbiaceae	Tree	Perennial
				Inga spp	3	Fabaceae	Tree	Perennial
	Primary forest	N/A	6	*Quararibea wittii*	3	Malvaceae	Tree	Deciduous

				Pseudolmedia laevis	3	Moraceae	Tree	Perennial
La Pampa	Natural regeneration	3 years	8	*Chromolaena laevigata*	3	Asteraceae	Shrub	Perennial
				Cecropia engleriana	3	Urticaceae	Tree	Perennial
	Primary forest	N/A	9	*Psychotria sp10*	3	Rubiaceae	Shrub	Perennial
				Quararibea wittii	3	Malvaceae	Tree	Deciduous

Rhizosphere soil was collected under the selected plants at each land cover category. At each point, surface litter was removed, and a hole 30 cm deep was excavated near the plant roots. Three subsamples of soil surrounding the roots at 5-30 cm depth were collected and mixed to form a composite sample using the clean hands-dirty hands protocol (EPA Method 1669) with a stainless-steel hand shovel. In total, forty-two rhizosphere soil samples were collected in the dry season of 2022 (30 samples in Laberinto and 12 samples in La Pampa).

The samples were cold-stored in labeled sealed plastic bags until transportation to the laboratory. Soil subsamples were transported to the United States under USDA soil permit number 525-23-163-97136 and analyzed for physico-chemical characteristics (SOM, pH, EC, nutrients) and total number of bacteria at the Environmental Engineering Laboratory, Department of Civil and Environmental Engineering, University of Maryland, College Park.

2.3 Sample analysis

2.3.1 Soil Organic Matter Content Analysis

Soil samples were sieved to < 2 mm and oven-dried at 105°C. The organic matter content was determined as a percentage of the loss by ignition of 10.0 g soil in a furnace at 450 °C (Thermo Scientific muffle furnace Thermolyne) for three hours (ASTM, 2014).

2.3.2 Electrical Conductivity and pH Analysis

The pH and EC of the soil were measured in soil distilled water solution (1:2) using a Thermo Scientific Versa Star meter at the Environmental Engineering laboratory at the University of Maryland, USA.

2.3.3 Nutrient Analysis

The concentration of total recoverable nutrients important for plant growth and development (Ca, K, Mg, and P) was measured following EPA Method 200.7 with an ICP-OES (Shimadzu Model ICPE-9820). The quantification limit of the method was 1.39 mg L^{-1} for K and 0.14 mg L^{-1} for Ca, Mg, and P. For quality control, 3% nitric acid blanks were run at the beginning of each run, and liquid standards with a recovery rate of 90-110% were used for quality control.

2.3.4 Microbial tests

DNA was extracted from 0.25 g of dried and sieved soil using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The concentration of nucleic acids in the samples was measured using a NanoDrop 2000 UV vis spectrophotometer (Thermo Fischer Scientific, Waltham, MA). The abundance of the bacterial 16S rRNA gene in the samples was measured by quantitative real-time PCR (qPCR). The primer and cycling conditions used are shown in Table S4-1. PCR products with the positive control template DNA from *Escherichia coli*, strain HB101, were cleaned with Wizard SV gel and PCR clean-up kit (Promega, Madison, Wisconsin), then tested with NanoDrop and used as the stock for a standard series of eight dilution points (10-fold dilution) for the qPCR reactions. These reactions were conducted by BioRad CFX Connect Realtime Systems (Bio-Rad Laboratories, Hercules, CA, USA), using 8.5 µL RT-PCR Grade Water (Invitrogen by Thermo Fischer Scientific, TX, USA), 0.5 µL of template, 12.5 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), to a final concentration of 0.2 µM of each of the forward and reverse primers, with a total of 25 µL reaction volume. In the qPCR runs, 3 µL of DNA template was replaced with RT-PCR Grade Water as the negative control. To ensure the quality of the qPCR runs, R^2 was above 0.99; efficiency was between 90% and 105%. DNA from *Escherichia coli* strain HB101 was used as

positive control. Data were converted to represent the average copy number of targets per g dry weight using Equation 1 in the supplementary information.

2.4 Data analysis and statistical analysis

SOM, pH, EC, nutrients, and total number of bacteria in rhizosphere soils are reported as the mean ± standard deviation. We performed statistical tests using an alpha of 0.05. Comparisons of SOM, pH, EC, nutrients, and total number of bacteria between land cover categories and plant species were performed using the non-parametric Kruskal-Wallis's test, followed by Wilcoxon as a post-hoc test. All statistical analyses were performed using R version 4.3.2 statistical software, and the ggplot2 package was used for visualizations.

2.5 Focus groups

We conducted nine focus groups between 17 October 2022 and 20 October 2022 in three Indigenous communities affected by ASGM in the Amarakaeri Communal Reserve. The smallest community is comprised of 40 families and the biggest one is comprised of 110 families. The focus groups involved a total of 54 people, with an average of 53% being women. Each group consisted of six people. We sought heterogeneous groups including different genders and ages and promoted the participation of individuals who do not usually dominate the conversation. We chose heterogeneous groups to capture how the output changes with group dynamics. We prompted the conversation using guiding questions and paused to discuss any issue the participants identified as interesting or controversial. The respondents were recruited through the Indigenous organization ECA-Amarakaeri and local chiefs. (Supplementary information, focus group methodology). Participation was voluntary, and oral informed consent was obtained at the beginning of the sessions. Human research oversight and approval was provided by the

University of Maryland, College Park (IRB #1728819). Our sample size for this community was small and might be biased towards individuals with a pre-existent interest in the topic or supporters of the chiefs due to the recruitment method used.

3. Results and discussion

3.1 Rhizosphere soil SOM, pH, and EC in land cover categories

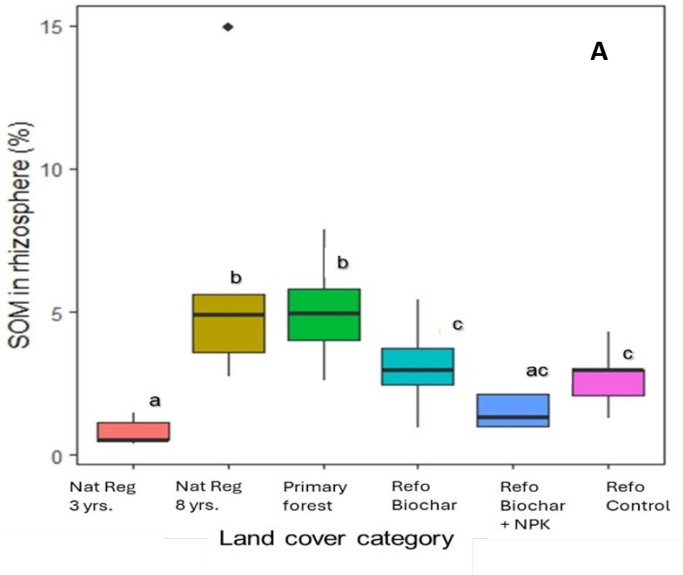
Rhizosphere soil organic matter (SOM), pH, and EC were studied among the selected land cover categories at the Laberinto and La Pampa sites. SOM and EC levels were significantly lower ($p < 0.05$) and pH levels were significantly higher ($p < 0.05$) in the naturally regenerated plot three years old in the La Pampa site (average 0.82 % and 11.4 uS cm^{-1}, $n = 6$, respectively) than in the naturally regenerated plot eight years old in Laberinto and in the other land cover categories. No significant difference in SOM and EC was found between the eight-year-old naturally regenerated plot and the reforested and primary forest land cover categories (Fig.4). From our knowledge, this is the first study examining rhizosphere soil physico-chemical properties in areas naturally regenerated and reforested after ASGM degradation in the Amazon rainforest.

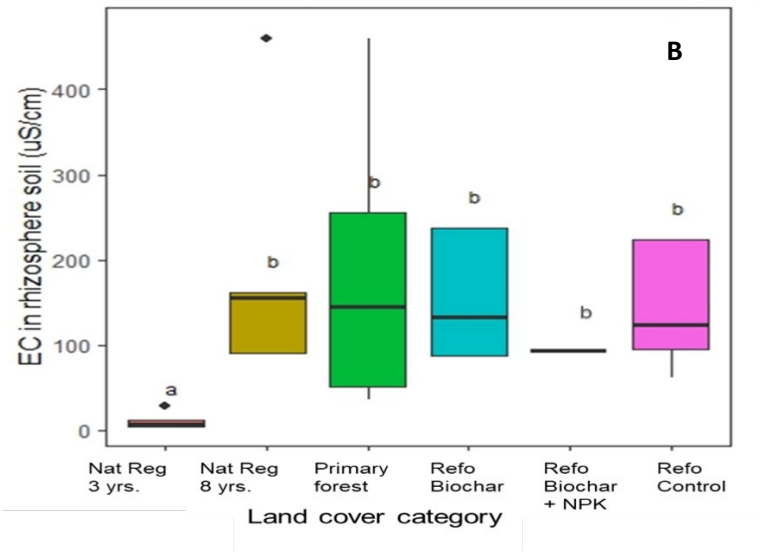
3.1.1 Rhizosphere Soil Organic Matter and electrical conductivity

Studies in naturally regenerated and reforested forests after anthropogenic degradation (other than ASGM) found higher SOM and EC levels in the rhizosphere of naturally regenerated forests, which had higher species diversity than reforested ones. Both categories had no difference in pH levels (Hummes et al., 2024). The findings related to SOM are consistent with our results as our study's 8-year-old naturally regenerated areas had higher SOM levels and a higher species diversity than the reforested areas. However, the influence of the regeneration age

was not assessed in previous studies. Still, this factor seems relevant to the differences found in our study, showing a recovery of rhizosphere SOM to the levels of the primary forest after eight years of natural regeneration and a recovery of rhizosphere EC after eight years of natural regeneration and after five years of reforestation.

The influence of age has been assessed in the reforestation of temperate forests in Asia. Liu et al. (2018) reported a higher SOM level in 25-year-old reforested forests compared to five-year-old reforested ones. Our study did not assess the influence of the age of reforestation, but the influence of soil amendments in reforestation was evaluated. We did not find significant differences ($p < 0.05$) in soil properties due to the soil amendments (data not shown), most likely due to the lower application rate of the amendments, 1.1 t ha^{-1}, compared to previous studies, 5 t ha^{-1} (Addai et al., 2023)


A
SOM in rhizosphere (%)
15
10
5
0
a
b
b
c
ac
c
Nat Reg 3 yrs.
Nat Reg 8 yrs.
Primary forest
Refo Biochar
Refo Biochar + NPK
Refo Control
Land cover category


B
EC in rhizosphere soil (uS/cm)
400
300
200
100
0
a
b
b
b
b
b
Nat Reg 3 yrs.
Nat Reg 8 yrs.
Primary forest
Refo Biochar
Refo Biochar + NPK
Refo Control
Land cover category

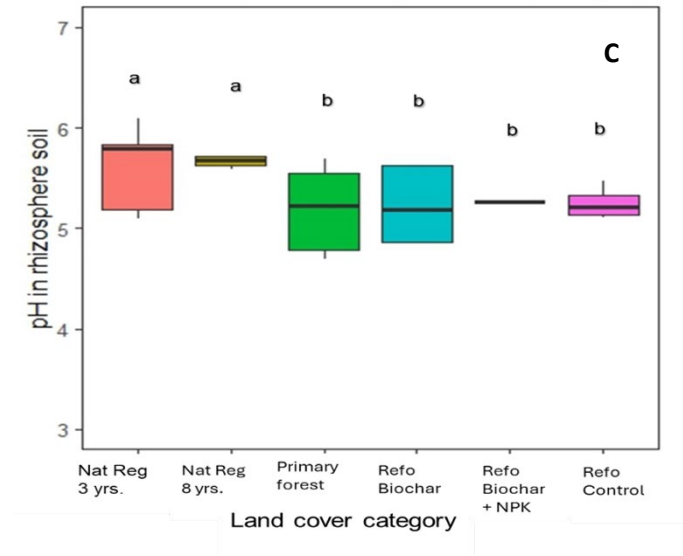

C
pH in rhizosphere soil
7
6
5
4
3
a
a
b
b
b
b
Nat Reg 3 yrs.
Nat Reg 8 yrs.
Primary forest
Refo Biochar
Refo Biochar + NPK
Refo Control
Land cover category

Fig. 4-4. **Rhizosphere soil SOM, pH, and EC in Laberinto and La Pampa sites**. **A.** SOM in rhizosphere soil at 5-30 cm. (n = 12 for primary forest, n = 6 for the other land cover categories). **B**. EC in rhizosphere soil at 5-30 cm. (n = 12 for primary forest, n = 6 for the other land cover categories). **C**. pH in rhizosphere soil at 5 – 30 cm. (n = 12 for primary forest, n = 6 for the land cover categories). Different lower-case letters above the boxes indicate significant differences between SOM, EC, and pH among different land cover categories at $p < 0.05$.

Various studies have shown that deforestation affects the soil's carbon content (C) and reduces acidity (Martins et al. 1991; Cravo and Smyth 1997; Fernandes 1999).

Cerri et al. (1996) reported that the concentration of C in different places in the Amazon region varies slightly. In tropical forests, high temperatures and rainfall accelerate the loss of soil nutrients; thus, a greater stock of nutrients is found in the biomass and made available through decomposition (Luizao, 1989). That is why most soils in the Amazon have low fertility. Because of the region's fragile ecosystem, deforestation can break the cycle of C and nutrients in the soil. Consistent with these findings, our study shows a reduction of rhizosphere SOM and EC of 83.7% and 94%, respectively, between the primary forest and the most degraded soil (3-year-old naturally regenerated soil after ASGM degradation) (Fig. 4-4).

EC measures the soil's ability to conduct electricity. It is related to the soil's cation exchange capacity and is often used as an indicator of the availability of nutrients in the soil (Khongnawang et al., 2019; Smith and Doran, 1997).

Vegetation can optimize nutrient cycling and positively affect soil chemical and physical properties. This is especially important in tropical soils, which lack nutrients (Ricklefs, 1996). Plants add organic matter to the soil in various ways, whether as roots, litterfall, or root exudates in the rhizosphere (Bertin et al., 2003). However, the main part of C sequestration occurs belowground, involving roots, other plant parts, and soil organisms (Nair et al., 2010). They can

also retrieve nutrients from soil deeper layers through their roots and increase nutrient availability by exuding organic acids in the rhizosphere area (Feldpauch et al., 2007). The amount of root exudates depends on the tree species (Neumann and Romheld, 1999). The quality and quantity of litterfall, roots, and root exudates are essential in driving the changes in rhizosphere SOM and nutrients (Garkoti and Singh, 1999; Kumar et al., 2021; Dhyani et al., 2019).

Our findings prove that plant species in the naturally regenerated areas can return to the rhizosphere SOM and EC levels of the primary forest after eight years of regeneration and that the plant species used in the reforested areas increase the rhizosphere SOM levels by 72.3% compared to the most degraded soil (3-year-old naturally regenerated areas) and recover the rhizosphere soil EC levels of the primary forest after five years of reforestation (Fig. 4-4) most likely due to the litterfall and root exudates as reported in the previous studies mentioned.

3.1.2 Rhizosphere Soil pH

Regarding soil pH, it has been found that the degradation of Amazonian forests by agricultural activities and pasture causes an increase in the soil pH by the traditional practice of burning the soil for fertilization. These burn-offs add ashes to the soil, increasing the pH. Soil pH is important for nutrient availability, soil microorganism establishment, and plant development (Rocha et al., 2023). The optimum pH for nutrient availability ranges from 4.5 to 6.5 (Taiz et al., 2017).

Some studies found that even 81 years after planting pasture in Amazonian areas degraded by agriculture, the soil pH at 0-30 cm depth was higher than in primary forest areas (Moraes et al., 1996).

Our study shows an increase in rhizosphere soil pH of 7.9 % between the 3-year-old naturally regenerated soil after ASGM degradation (5.60 ± 0.40) and the primary forest (5.19 ± 0.35) (Fig. 4-4).

Amazonian soil is naturally acidic due to several factors, such as the content of organic acids (Quesada et al., 2011). In ASGM in the Amazon, the miners cut trees and burn the soil before excavating an area to extract gold (Salinas, 2007). Then, high-pressure water is used to break and wash the soil, leaving soil mostly formed of sand (Timsina et al., 2022). The increase of rhizosphere soil pH in ASGM degraded areas is probably not likely due to the addition of ash from the burnoffs before the mining activity because the soil is later washed with high-pressure water. The lack of organic acids in this degraded soil due to the soil washing performed during the mining activity might be the reason for our study's higher pH observed in the 3-year-old naturally regenerated soil. However, an analysis of the organic acids in the rhizosphere of this area should be conducted to prove this hypothesis.

3.1.3 Rhizosphere Effect

The rhizosphere soil is influenced by rhizodeposition, nutrients, and the microbial community that lives there (Massaccesi et al., 2015). These factors cause differences in the rhizosphere soil's physico-chemical and biological properties compared to the non-rhizosphere soil (bulk soil). This is called the rhizosphere effect (Kumar & Garkoti, 2022).

Plant roots' horizontal and vertical size affect soil properties, such as the soil organic carbon (SOM content beyond the rhizosphere area. Some studies found higher SOM below tree canopies than in the area outside the tree canopy and a positive correlation between tree presence and SOM up to 8 m away from trees in Mediterranean forests, with most of their roots located in the topsoil at 0-30 cm depth (Howlett et al., 2011; Simón et al., 2013). Yuan et al. (2020) found that

plant rhizosphere had lower pH and higher nutrient content than the bulk soil and that the rhizosphere effect is larger in trees than in shrubs in subtropical forests in Asia. This is consistent with the results of our study, where significantly higher SOM and lower pH ($p < 0.05$) were found in the rhizosphere soil compared to the bulk soil in all the land cover categories studied (Table 4-2).

The root systems in the Amazon rainforest can be extensive, with 2-4 km of roots per square meter of forest area. These roots are located in the topsoil (Metcalfe et al., 2008). Positive relations between soil properties in the bulk soil, such as organic matter and nutrients, and the presence of tree species have been previously reported in the Amazon rainforest (Olival et al., 2021; Rodriguez- Pascual et al., 2024)

Physico-chemical characteristics of the bulk soil were previously measured in the same plots assessed in our study (Table 4-2. Adapted from Rodriguez-Pascual et al., 2024) showing an increase in SOM and EC and a decrease in pH with restoration age. Still, the recovery of these soil characteristics to primary forest levels in the bulk soil was not achieved after 5 years of reforestation and 8 years of natural regeneration (Table 4-2). These results are consistent with previous studies, which reported that SOM, pH, and EC at 0-30 cm depth did not recover after 10 and 15 years of natural regeneration in areas degraded by ASGM in the Madre de Dios region (Alarcón-Aguirre et al., 2023; Chambi-Legoas et al., 2021). The restoration of areas degraded by ASGM seems slower than areas degraded by other anthropogenic activities. For example, some authors found that SOM at 0-40 cm depth recovers after seven years of natural regeneration in an area degraded by bauxite mining in the Brazilian Amazon (Brasil Neto et al. 2021).

Our findings prove that even SOM, pH, and EC in rhizosphere soil return to primary forest conditions after eight years of natural regeneration and five years of reforestation for pH and EC;

the same levels are not reached in the bulk soil. More research is needed to assess the restoration age needed to recover the original soil physico-chemical properties in the bulk soil in areas degraded by ASGM and restored with different methods. This information is critical for designing sustainable restoration programs in these areas in the Amazon rainforest.

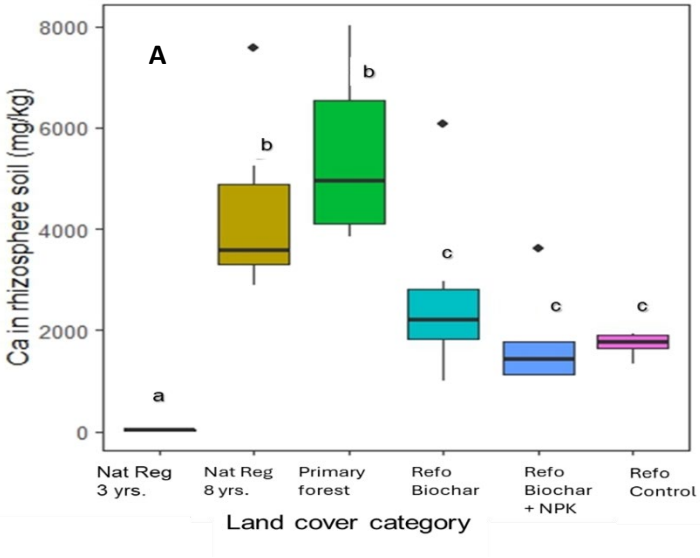
Table 4-2. Soil physico-chemical characteristics in different land cover categories in the two mining sites

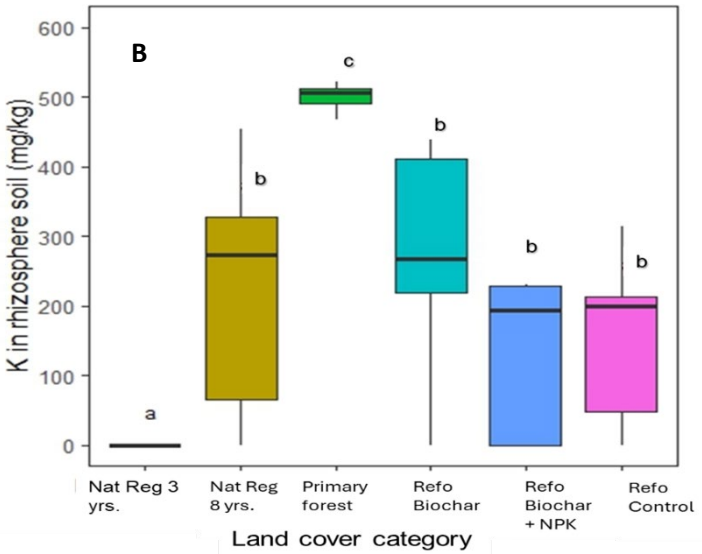
Land cover category / Age of restoration	Type of soil (Depth in cm)	N	SOM (%)	EC (uS cm^{-1})	pH
Natural regeneration / 3 years old	Bulk soil (5 – 30)	5	0.43 ± 0.20	4.1 ± 0.68	5.80 ± 0.50
	Rhizosphere (5 – 30)	6	0.815 ± 0.427	11.4 ± 9.09	5.60 ± 0.40
Natural regeneration / 8 years old	Bulk soil (5 – 30)	9	0.835 ± 0.573	53.10 ± 37.40	5.86 ± 0.50
	Rhizosphere (5 – 30)	6	6.14 ± 4.19	191 ± 142	5.67 ± 0.05
Reforestation without soil amendments / 5 years old	Bulk soil (5 – 30)	5	1.242 ± 0.692	51.18 ± 23.58	5.55 ± 0.33
	Rhizosphere (5 – 30)	6	2.77 ± 0.958	195 ± 169	5.25 ± 0.15
Reforestation with biochar / 5 years old	Bulk soil (5 – 30)	5	1.371 ± 0.460	52.34 ± 43.43	5.78 ± 0.56
	Rhizosphere (5 – 30)	6	3.12 ± 1.51	153 ± 66.5	5.22 ± 0.34
Reforestation with biochar + NPK / 5 years old	Bulk soil (5 – 30)	5	0.735 ± 0.422	42.37 ± 34.64	5.77 ± 0.25
	Rhizosphere (5 – 30)	6	1.64 ± 0.50	93.3 ± 45	5.26 ± 0.15
Primary forest	Bulk soil (5 – 30)	5	3.290 ± 0.663	181 ± 46.50	4.89 ± 0.19
	Rhizosphere (5 – 30)	6	4.99 ± 1.50	188 ± 141	5.19 ± 0.35

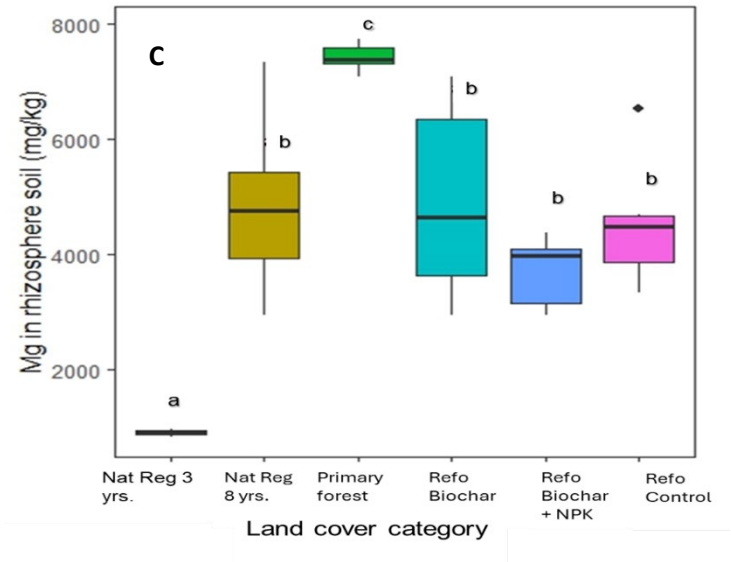
Note: Bulk soil values from Rodriguez-Pascual et al., 2024.

3.1.4 Nutrients in rhizosphere soil

Nutrient levels in the rhizosphere soil, specifically total recoverable calcium (Ca), total recoverable potassium (K), total recoverable magnesium (Mg), and total recoverable phosphorous (P), were studied among the selected land cover categories at the Laberinto and La Pampa sites. The highest levels of total recoverable Ca, K, Mg, and P were found in the primary forest (5448 ± 1696 mg kg^{-1} dry weight (dw), 501 ± 19.8 mg kg^{-1} dw, 7457 ± 299 mg kg^{-1} dw, 162 ± 172 mg kg^{-1} dw, respectively (n = 12)). The lowest levels were found in the naturally regenerated plot three years old in the La Pampa site (32.6 ± 12.6 mg kg^{-1} dw of Ca, under the quantification limit for K, 907 ± 54.8 mg kg^{-1} dw of Mg, 18.4 ± 20.2 mg kg^{-1} dw of P (n = 6)) (Fig. 4-5). No significant difference in nutrient levels was found between the naturally regenerated plot eight years old in Laberinto site and the reforested plots except for Ca, with higher levels in the naturally regenerated plot eight years old. No significant difference in nutrient levels was found between the reforestation plots with different soil amendments, most likely due to the lower application rate of the amendments, 1.1 t ha^{-1}, compared to previous studies, 5 t ha^{-1} (Addai et al., 2023). From our knowledge, this is the first study examining nutrient levels in rhizosphere soil in areas naturally regenerated and reforested after ASGM degradation in the Amazon rainforest.


A
Ca in rhizosphere soil (mg/kg)
8000
6000
4000
2000
0
a
b
b
c
c
c
Nat Reg 3 yrs.
Nat Reg 8 yrs.
Primary forest
Refo Biochar
Refo Biochar + NPK
Refo Control
Land cover category


B
K in rhizosphere soil (mg/kg)
600
500
400
300
200
100
0
a
b
c
b
b
b
Nat Reg 3 yrs.
Nat Reg 8 yrs.
Primary forest
Refo Biochar
Refo Biochar + NPK
Refo Control
Land cover category


C
Mg in rhizosphere soil (mg/kg)
8000
6000
4000
2000
a
b
c
b
b
b
Nat Reg 3 yrs.
Nat Reg 8 yrs.
Primary forest
Refo Biochar
Refo Biochar + NPK
Refo Control
Land cover category

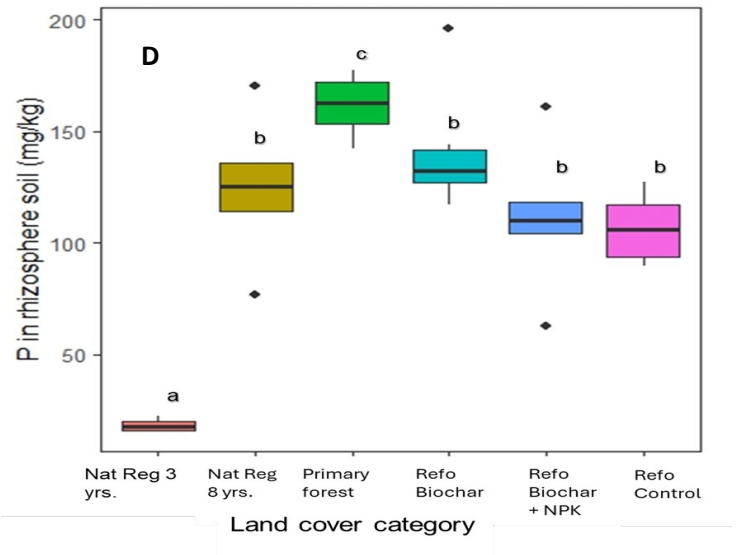

D
P in rhizosphere soil (mg/kg)
200
150
100
50
a
b
c
b
b
b
Nat Reg 3 yrs.
Nat Reg 8 yrs.
Primary forest
Refo Biochar
Refo Biochar + NPK
Refo Control
Land cover category

Fig. 4-5. **Rhizosphere soil Total recoverable Ca, K, Mg, and P in Laberinto and La Pampa sites**. **A.** Total recoverable Ca in rhizosphere soil at 5-30 cm. (n = 12 for primary forest, n = 6 for the other land cover categories). **B**. Total recoverable K in rhizosphere soil at 5-30 cm. (n = 12 for primary forest, n = 6 for the other land cover categories). **C**. Total recoverable Mg in rhizosphere soil at 5 – 30 cm. (n = 12 for primary forest, n = 6 for the other land cover categories). **D**. Total recoverable P in rhizosphere soil at 5 – 30 cm. (n = 12 for primary forest, n = 6 for the other land cover categories). The lower-case letters above the boxes indicate significant differences between total recoverable Ca and total recoverable P among land cover categories at $p < 0.05$, between total recoverable K among land cover categories at $p < 0.075$, and between total recoverable Mg among land cover categories at $p < 0.065$.

In Amazonian forests, the SOM is primarily formed by plant litter decomposition. This decomposition also contributes to the cation exchange capacity (CEC) and provides nutrients such as P, K, Ca, and Mg (Duxbury et al., 1989; Pinho et al., 2012).

This is consistent with our study's results. We found the highest levels of nutrients in the rhizosphere soil in the land cover categories with higher SOM.

As mentioned before, the quality and quantity of plant litter and root exudates are important factors driving the changes in rhizosphere SOM and nutrients (Garkoti and Singh, 1999; Kumar et al., 2021; Dhyani et al., 2019).Our findings show that plant species in the naturally regenerated areas can return to the rhizosphere soil total recoverable Ca levels of the primary forest after eight years of regeneration and that these plant species and the ones used in the reforested areas increase the rhizosphere soil total recoverable K, Mg, and P levels by 224%, 434.7%, and 574%, respectively, compared to the most degraded soil (3-year-old naturally regenerated areas) (Fig. 4-5).

Ca, K, P, and Mg are important nutrients for plant growth and development. Ca plays an important role in the formation of plant tissues and acts as a structural component of plant cells,

such as pectin (Verma et al., 2022). Potassium is related to the activation of enzymes involved in energy metabolism, such as energy production (Cornut et al. 2021). Magnesium is important for activating proteins involved in photosynthetic processes because it is the central atom of the chlorophyll molecule (Mao et al., 2022). Phosphorus plays an important role in reactions involving NADPH and ATP and is related to the enzymes involved in photosynthesis (Meng et al., 2021).

Previous studies have assessed K and P levels in rhizosphere soil in naturally regenerated and reforested areas degraded by human activities in other regions of the world, finding higher levels of K and available P in the rhizosphere of naturally regenerated areas compared to reforested areas due to the higher plant diversity of the first ones (Hummes et al., 2024). These differences were not found in our study despite the differences in plant diversity between naturally regenerated and reforested areas. The influence of the natural regeneration age was not assessed in previous studies. Still, this factor seems relevant to the results of our study, showing a significant difference in nutrient levels between the three-year-old naturally regenerated areas and the eight-year-old naturally regenerated areas (Fig. 4-5).

Our study did not assess the levels of nutrients in the bulk soil in different land cover categories. However, previous studies in areas degraded by ASGM in the Madre de Dios region reported that the levels of P and K, available Ca and Mg in the bulk soil at 0-30 cm depth did not recover after ten years of natural regeneration (Alarcón-Aguirre et al., 2023; Chambi-Legoas et al., 2021).The recovery of nutrient levels in the bulk soil in areas degraded by ASGM seems slower than in areas degraded by other anthropogenic activities. For example, some studies reported that the levels of nutrients, such as available Ca and Mg, in the bulk soil at 0-30 cm were recovered

after five years of natural regeneration in areas degraded by agriculture in the Brazilian Amazon (Soares, 2006).

Our findings supports that the levels of Ca in rhizosphere soil return to primary forest conditions after eight years of natural regeneration. However, neither eight years of natural regeneration nor five years of reforestation achieve those primary forest levels for K, Mg, and P. Additional studies are needed to assess the restoration age needed to recover the original rhizosphere soil nutrient levels in areas degraded by ASGM and restored with different methods. This information would be critical for designing sustainable restoration programs in these areas in the Amazon rainforest.

3.2 Soil properties per plant species

Soil characteristics may vary depending on the plant species (Malysz & Overbeck 2018). SOM, pH, EC, and nutrient levels in the rhizosphere soil, such as total recoverable Ca, total recoverable K, total recoverable Mg, and total recoverable P, were studied among the most abundant plant species in the selected land cover categories at the Laberinto and La Pampa sites. The highest levels of SOM in rhizosphere soil were found in the primary forest and the eight-year-old natural regeneration, and the lowest in the three-year-old natural regeneration ($p<0.05$). Comparing the species in the natural regeneration and reforestation plots, *Acacia loretensis* (found in the eight years old natural regeneration) has a higher SOM level than most of the plant species in the reforestation plots (8.27 ± 5.04 %), except *Inga sp*. with biochar and *Hura crepitans* without soil amendments (3.12 ± 1.92 % and 3.12 ± 0.968 %, respectively) (Fig.4-6) The lowest levels of EC in rhizosphere soil were found in the three-year-old natural regeneration ($p<0.05$). Comparing the species in the natural regeneration and reforestation plots, *Acacia l*

oretensis, *Inga sp*. with biochar, and *Inga sp*. without soil amendments have the highest EC levels (311 ± 162 uS cm^{-1}, 185 ± 57.5 uS cm^{-1}, 289 ± 200 uS cm^{-1}, respectively) (Fig.4-6)

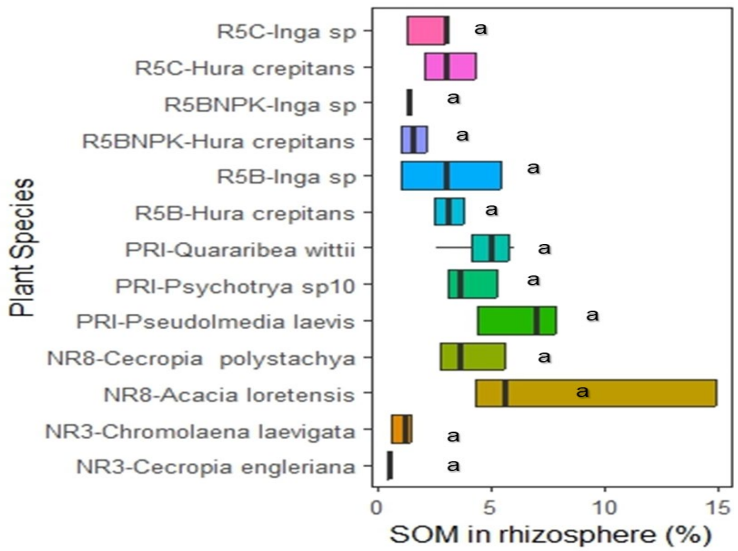
No significant differences in pH in the rhizosphere soil among plant species were found ($p<0.05$) (data not shown).

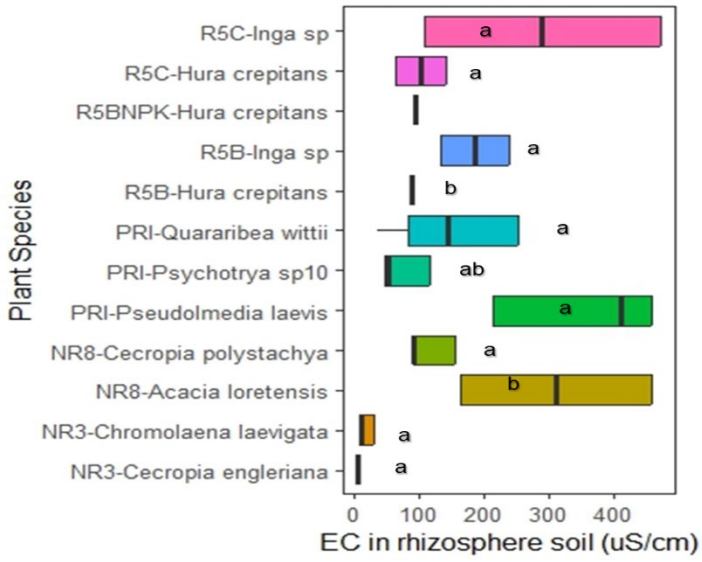
The highest levels of total recoverable Ca in rhizosphere soil were found in the plant species in the primary forest and the eight-year-old natural regeneration, and the lowest levels were found in the three-year-old natural regeneration ($p<0.05$). Comparing the species in the natural regeneration and reforestation plots, *Acacia loretensis* (found in the eight years old natural regeneration) has a higher Ca level than most of the plant species in the reforestation plots (4750 ± 2452 mg kg^{-1}), except *Inga sp*. with biochar and with biochar plus NPK (3317 ± 2395 mg kg^{-1}, 2177 ± 1282 mg kg^{-1}) (Fig.4-6).

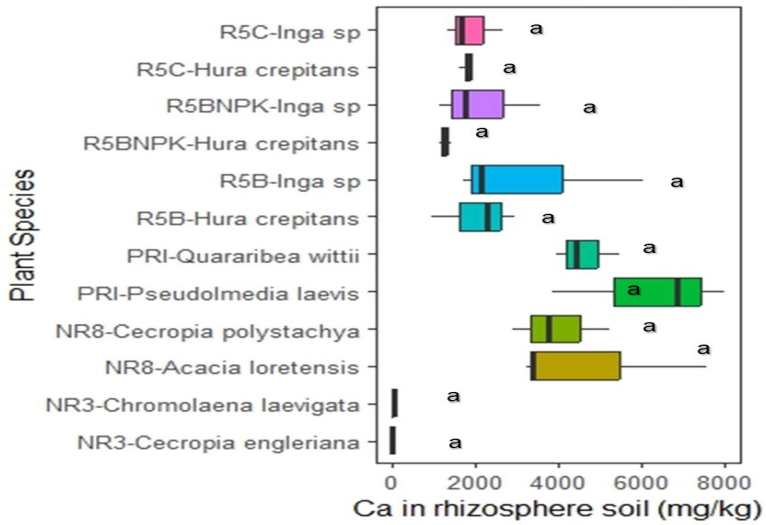
The highest total recoverable K and Mg levels in rhizosphere soil were found in the plant species in the primary forest and the eight-year-old natural regeneration ($p<0.05$). The lowest levels of total recoverable Mg were found in the three-year-old natural regeneration ($p<0.05$). The levels of total recoverable K in the rhizosphere soil in the three-year-old natural regeneration were under the detection limit. Due to the small sample size, the difference in total recoverable K and Mg in the rhizosphere soil among the plant species in the 8-year-old natural regeneration and reforestation plots was not significant ($p > 0.05$). Still, the *Inga sp*. with biochar tended to have higher K and Mg values than the other plant species (376 ± 138 mg kg^{-1}, 5960 ± 1647 mg kg^{-1}, respectively) (Fig.4-6).

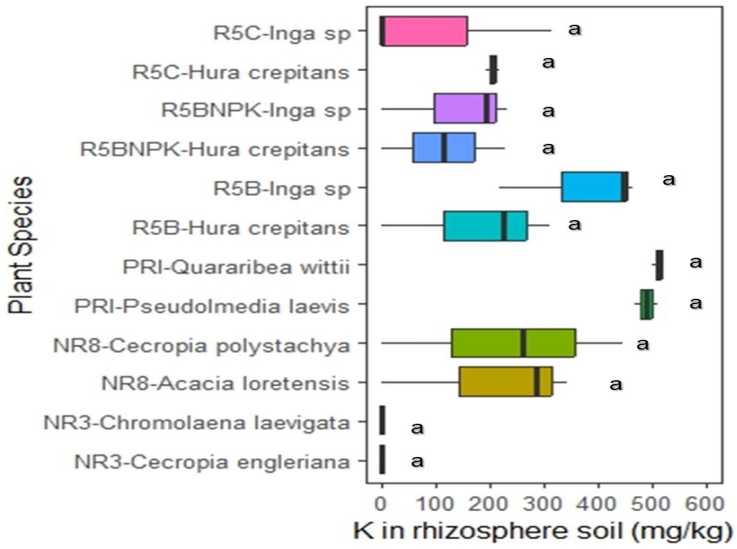
The highest levels of total recoverable P in rhizosphere soil were found in the primary forest plant species and the lowest in the three-year-old natural regeneration ($p<0.05$). Comparing the species in the natural regeneration and reforestation plots, *Cecropia polystachya* (found in the

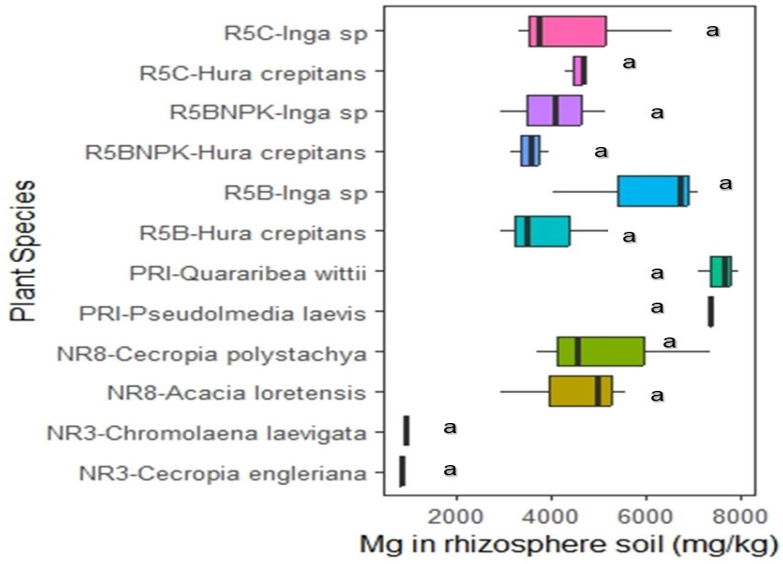
eight years old natural regeneration) has a higher P level than most of the plant species in the reforestation plots (140 ± 27.8 mg kg^{-1}), except *Inga sp*. with biochar and *Hura crepitans* with biochar and NPK (148 ± 42.2 mg kg^{-1}, 136 ± 36.1 mg kg^{-1}) (Fig.4-6).


Plant Species
R5C-Inga sp
R5C-Hura crepitans
R5BNPK-Inga sp
R5BNPK-Hura crepitans
R5B-Inga sp
R5B-Hura crepitans
PRI-Quararibea wittii
PRI-Psychotrya sp10
PRI-Pseudolmedia laevis
NR8-Cecropia polystachya
NR8-Acacia loretensis
NR3-Chromolaena laevigata
NR3-Cecropia engleriana
a
0
5
10
15
SOM in rhizosphere (%)


Plant Species
R5C-Inga sp
R5C-Hura crepitans
R5BNPK-Hura crepitans
R5B-Inga sp
R5B-Hura crepitans
PRI-Quararibea wittii
PRI-Psychotrya sp10
PRI-Pseudolmedia laevis
NR8-Cecropia polystachya
NR8-Acacia loretensis
NR3-Chromolaena laevigata
NR3-Cecropia engleriana
a
b
ab
0
100
200
300
400
EC in rhizosphere soil (uS/cm)


Plant Species
R5C-Inga sp
R5C-Hura crepitans
R5BNPK-Inga sp
R5BNPK-Hura crepitans
R5B-Inga sp
R5B-Hura crepitans
PRI-Quararibea wittii
PRI-Pseudolmedia laevis
NR8-Cecropia polystachya
NR8-Acacia loretensis
NR3-Chromolaena laevigata
NR3-Cecropia engleriana
a
0
2000
4000
6000
8000
Ca in rhizosphere soil (mg/kg)


Plant Species
R5C-Inga sp
R5C-Hura crepitans
R5BNPK-Inga sp
R5BNPK-Hura crepitans
R5B-Inga sp
R5B-Hura crepitans
PRI-Quararibea wittii
PRI-Pseudolmedia laevis
NR8-Cecropia polystachya
NR8-Acacia loretensis
NR3-Chromolaena laevigata
NR3-Cecropia engleriana
a
0
100
200
300
400
500
600
K in rhizosphere soil (mg/kg)


Plant Species
R5C-Inga sp
R5C-Hura crepitans
R5BNPK-Inga sp
R5BNPK-Hura crepitans
R5B-Inga sp
R5B-Hura crepitans
PRI-Quararibea wittii
PRI-Pseudolmedia laevis
NR8-Cecropia polystachya
NR8-Acacia loretensis
NR3-Chromolaena laevigata
NR3-Cecropia engleriana
a
2000
4000
6000
8000
Mg in rhizosphere soil (mg/kg)

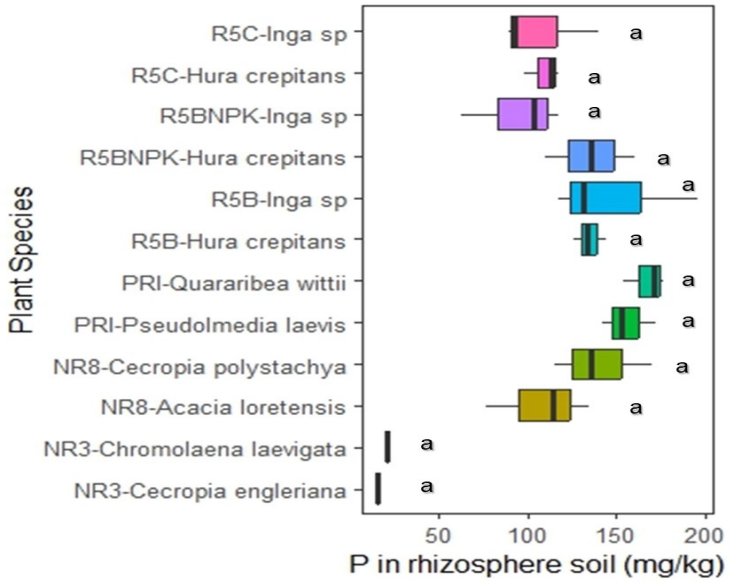

Plant Species
R5C-Inga sp
R5C-Hura crepitans
R5BNPK-Inga sp
R5BNPK-Hura crepitans
R5B-Inga sp
R5B-Hura crepitans
PRI-Quararibea wittii
PRI-Pseudolmedia laevis
NR8-Cecropia polystachya
NR8-Acacia loretensis
NR3-Chromolaena laevigata
NR3-Cecropia engleriana
a
50
100
150
200
P in rhizosphere soil (mg/kg)

Fig. 4-6. **Rhizosphere soil SOM, EC, total recoverable Ca, K, Mg, and P in different plant species in Laberinto and La Pampa sites**. **A.** SOM in rhizosphere soil at 5-30 cm. (n = 6 for *Quararibea wittii*, n = 3 for the other plant species). **B**. EC in rhizosphere soil at 5-30 cm. (n = 6 for *Quararibea wittii*, n = 3 for the other plant species). **C.** Total recoverable Ca in rhizosphere soil at 5-30 cm. (n = 6 for *Quararibea wittii*, n = 3 for the other plant species). **D**. Total recoverable K in rhizosphere soil at 5-30 cm. (n = 6 for *Quararibea wittii*, n = 3 for the other plant species). **E.** Total recoverable Mg in rhizosphere soil at 5 – 30 cm. (n = 6 for *Quararibea wittii*, n = 3 for the other plant species). **F**. Total recoverable P in rhizosphere soil at 5 – 30 cm. (n = 6 for *Quararibea wittii*, n = 3 for the other plant species). The lower-case letters next to the boxes indicate significant differences between SOM, EC, total recoverable Ca, K, Mg, and P among plant species within different land cover categories at $p < 0.05$. The significance letters are different for each land cover category.

Note: R5C = reforestation five years old without soil amendments (control). R5BNPK = reforestation five years old with biochar and NPK. R5B = reforestation five years old with biochar. PRI = primary forest. NR8 = natural regeneration eight years old. NR3 = natural regeneration three years old.

From our knowledge, this is the first study examining SOM, pH, EC, and nutrient levels in rhizosphere soil in different plant species in areas naturally regenerated and reforested after ASGM degradation in the Amazon rainforest. Previous studies in rhizosphere soil in naturally regenerated and reforested areas degraded by human activities in other regions of the world found bigger changes in rhizosphere soil with the presence of coniferous trees compared to broadleaved trees. For example, they found higher K levels in coniferous trees' rhizosphere soil and even higher levels when these trees were in naturally regenerated areas compared to reforested areas with lower plant diversity (Hummes et al., 2024).

The plant species assessed in our study are broadleaved. Coniferous trees were not among the most abundant species in the land cover categories studied. There is a lack of research on the influence of tropical tree species on rhizosphere soil characteristics, especially in areas degraded by ASGM.

In natural regeneration, pioneer plant species are the first ones growing in degraded areas. These species provide nutrients to the soil that allow species' growth from other ecological orders in a process called succession (MacArthur & Wilson, 2001; Maia et al., 2020; Campos and Martins, 2016; Rodrigues et al., 2004). Natural regeneration has been used as a restoration process used in the Amazon for decades, but its success depends on the degree of degradation and the available sources of seeds or spores (Poorter et al. 2016; Chazdon and Uriarte 2016; De Andrade et al. 2020). This t process involves recovering ecosystem functions, such as the nutrient cycle, without human intervention (Chazdon and Uriarte 2016). A case study oin areas degraded by bauxite mining in the Amazon showed the recovery of some soil characteristics after seven years of regeneration (Brasil Neto et al. 2021). However, after mining, deficient physical, chemical, and biological soil conditions or isolation from seed sources may stop or slow down the succession (Ash et al., 1994; Bradshaw, 1997). In these cases, restoration treatments can accelerate succession toward the recovery of the original forest (Andres & Mateos, 2006). Some of these treatments are using soil amendments and planting tree species that provide nutrients to the soil.

The results of our study suggest that the species Acacia *loretensis* and *Inga sp*. (*Inga sp*. when planted with biochar as a soil amendment), might be good candidates for restoring areas degraded by ASGM due to the levels of SOM, EC, and nutrients in their rhizosphere soil. Using soil amendments can be expensive, especially in remote areas like the ones degraded by ASGM in the Amazon. However, *Inga sp.* is a pioneer species capable of fixing nitrogen in the soil (Romero-Alvarado et al., 2002); hence, the benefits of using this species could compensate for the soil amendment cost. *Acacia loretensis* can be used in woodwork, so it would be a good candidate for restoration in areas where the economic value of the forest is sought.

This study did not evaluate how different plant species influence the recovery of the primary forest levels of SOM, pH, EC, and nutrients in the bulk soil. More research about this influence and the evaluation of more Amazonian plant species are needed to find more native plant species suitable for restoring areas degraded by ASGM in the Amazon. This information would be critical for designing sustainable restoration programs in these areas of the Amazon rainforest.

3.3 Rhizosphere soil bacterial population in land cover categories

Rhizobacteria are important for plant health. They help with nutrient acquisition and reduce the adverse effects of plant pathogens (Ahemad and Kribet, 2014). Soil physic-chemical characteristics influence root exudation patterns and, thus, affect the rhizosphere microbial communities (Berg and Smalla, 2009; Philippot et al., 2013)

In our study, the rhizosphere soil bacterial population was quantified in different land cover categories in Laberinto and La Pampa sites to assess the influence of soil degradation and regeneration in rhizosphere microbial population in areas impacted by ASGM.

The total number of bacteria in the rhizosphere soil was significantly lower ($p < 0.05$) in the naturally regenerated plot three years old in the La Pampa site (11.2 ± 0.315 log GC/grams of dry weight) than in the naturally regenerated plot eight years old in Laberinto and the other land cover categories. No significant difference in the total number of bacteria was found between the eight-year-old naturally regenerated plot and the reforestation and primary forest land cover categories (Fig. 4-7).

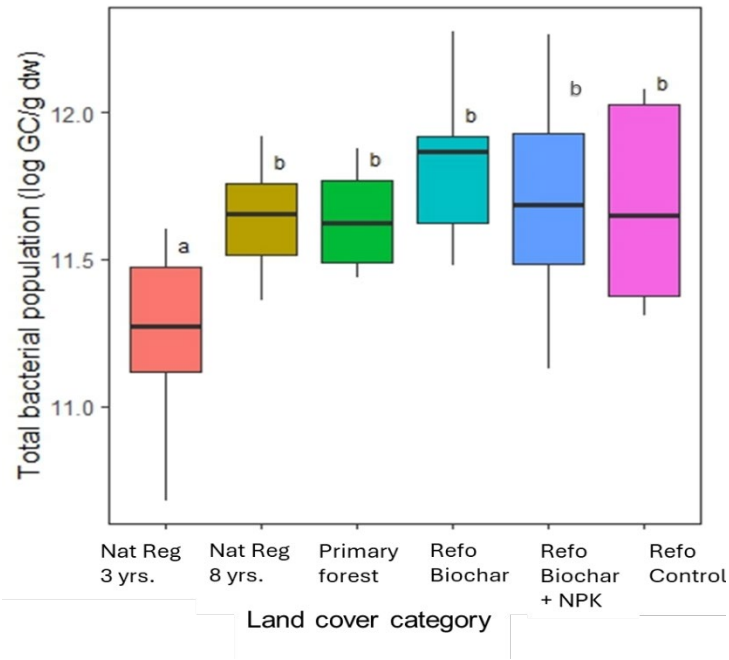


Fig. 4-7. Total number of bacteria in rhizosphere soil (number of gene copies/gram dry weight) in land cover categories in Laberinto and La Pampa sites. (n = 12 for primary forest, n = 6 for the rest of land cover categories). Different lower-case letters above the boxes indicate significant differences between mean ± SD among different soil land cover categories at $p < 0.05$.

From our knowledge, this is the first study examining the microbial communities in the rhizosphere in areas naturally regenerated and reforested after ASGM degradation in the Southern Amazon rainforest. Previous studies have reported shifts in rhizosphere microbial community composition and increased bacterial diversity after 25 years of reforestation in forests degraded by human activities in Asia (Liu et al., 2018)

In the Amazon, some studies found that microbial functions associated with phosphorous transformation in rhizosphere soil returned to the levels of the primary forest after 18 years of natural regeneration in areas degraded by agriculture in Brazil (Pedrinho et al., 2023). Other studies reported increased microbial biomass and soil enzymatic activities in the bulk soil at 0-10

cm depth after three years of reforestation in areas degraded by ASGM in Guyana. Still, they did not compare these parameters with primary forest levels (Couic et al., 2021).

Our findings may suggest that the total number of bacteria in the rhizosphere soil recovers the levels of the primary forest after five years of reforestation and eight years of natural regeneration. Still, our assays are based on total DNA, so we may have quantified alive and dead bacteria. Also, one gene copy per bacterial cell was considered to calculate the total number of bacteria. Still, the number of 16S rRNA copies in the bacterial cells can vary from 1-25, so statistically significant differences may be missed. For the same reason, no clear conclusions can be made about the differences in the total number of bacteria in the rhizosphere of the different plant species in the land cover categories assessed. More research is needed to determine if the rhizosphere microbial community composition changes due to the restoration age, soil characteristics, or the plant species in the different land cover categories evaluated.

3.4 Indigenous perspectives on land degradation and restoration in areas impacted by ASGM

3.4.1 Perceptions of land degradation

Focus groups were used to collect information about perceptions of land degradation by ASGM in three Indigenous communities in the Amarakaeri Communal Reserve. These communities have been impacted by ASGM, suffering from deforestation of 10% of their territory in average due to this activity from 2000-2019 (Global Forest Watch, 2019).

The participants of the focus groups were asked to indicate the environmental, economic, cultural, and social impacts of ASGM in their communities. Many of the effects identified were related to land degradation and management. This is an important concern for the communities

because they are land resources-based societies. The participants were asked to mention their livelihood activities and identify the areas where they developed these activities over a community map. They indicated artisanal mining (ASGM) and subsistence farming as their main activities. Hunting, fishing, and logging are other traditional activities common among the group of participants. The three studied communities have legal rights to their territory, which helped reduce the number of illegal miners invading their land. Still, some miners settled there before the communities had their rights over the land, and the government gave them mining permits. The regional government also gave some mining permits after the communities had the right over the land. One of the communities tried to make agreements with the miners to contain the areas where ASGM is performed with some success and some of the participants in the focus group mentioned that the extent of the ASGM in the community has decreased. However, the contrary was stated in the other two communities. In these communities, a communal guards' program has been developed in collaboration with the SERNANP and international NGOs to prevent illegal miners from entering their territory with limited success (Gonzales & Maldonado, 2016). The ASGM in the communities is performed chiefly with minimally mechanized techniques; however, some miners use heavy machinery.

Land degradation by ASGM occurs in the riverbanks and forests. The degradation of the riverbanks increases the risk of flooding in the communities, especially in one of the studied communities that was relocated due to this issue. The other communities have also been relocated due to soil degradation and conflicts with the miners for the land.

Some Indigenous people also perform mining activities or rent their land to miners. This situation has provoked disagreements and division in the communities between the Indigenous miners and those against mining. They mentioned that Indigenous people perform mining

because agriculture is not profitable (low prices of crops, high prices of oil, climate change affecting crops). An Indigenous leader participating in the focus group expressed the following:

> "Mining communities have started with artisanal mining…the invasions encourage us to do mining. The government grants concessions to guests. The native does not sell his territory; necessity forces him to do so."

The indicators of land degradation used by the Indigenous communities and mentioned in the focus groups were:

- Reduction of forest cover (reduction of "shade").
- Reduction of diversity of plant species in naturally regenerated areas.
- Reduction of wild animals they hunt.
- Mine tailings covering the riverbanks and aguajales (palm swamps).
- Soil with a lot of sand and gravel left by mining.
- Reduction of fertile land for agriculture.

Most of these indicators are related to indicators of land degradation developed by scientists. Table 4.3 compares both.

Table 4-3. Comparison of Indigenous indicators and Scientific indicators of land degradation

Indigenous indicators of land degradation	Scientific indicators	References
Soil with sand and gravel in degraded areas	Land cover categories (naked soil and gravel piles)	Rodriguez-Pascual et al., 2024
Only a few plant species in natural regeneration	Reduction of diversity indexes of plant species in natural regeneration	Chambi-Legoas et al., 2021
Mine tailings covering riverbanks	Changes in hydrology in rivers	Dethier et al., 2019
Crops do not grow in degraded areas	Reduction of soil fertility (nutrients and SOM)	Rodriguez-Pascual et al., 2024

Indigenous residents have observed similar land cover categories after ASGM degradation to those reported in other areas of the region where ASGM with minimally mechanized techniques is performed, such as gravel piles and sandy naked soil (Rodriguez-Pascual et al., 2024; Velásquez Ramírez et al., 2021). They also have observed natural regeneration in the areas degraded by ASGM, with a low diversity of plant species, mostly *Ochroma pyramidale*, some species of *Cecropia,* and *Gynerium sagittatum*. This is consistent with studies on natural regeneration in other areas after ASGM degradation in the Madre de Dios region (Alarcón-Aguirre et al., 2023; Chambi-Legoas et al., 2021).

Before ASGM activity, the land is "prepared" by cutting the trees and burning the soil (Velásquez Ramírez et al., 2021). Not all the "prepared" land is mined in these Indigenous communities. Indigenous people, mostly Indigenous women, use the non-mined land for subsistence agriculture. After the ASGM stopped and the mining areas were abandoned, they tried to use the degraded land for agriculture, but the crops did not grow. The Indigenous residents think this is likely due to the low fertility of these degraded areas. Studies on soil fertility have not been performed in areas degraded by ASGM in the Amarakaeri Communal Reserve. Still, the observations of the Indigenous people seem to be consistent with studies in areas degraded by ASGM with minimally mechanized techniques in the Madre de Dios region reporting reductions of soil SOM and EC by 96 and 99 % after the ASGM activity (Rodriguez-Pascual et al., 2024).

3.4.2 Proposals for restoration of areas degraded by ASGM

The participants of the focus groups were also asked to propose solutions for land degradation. The focus groups in all three communities proposed reforestation with plant species with an economic value, such as species used for timber and crops, and plants with a cultural value, such as a species of *Cecropia*, from which they extract fibers for traditional handicrafts. These species have disappeared in some communities due to an excess of use. Some species of *Cecropia* have been observed growing in the naturally regenerated areas after ASGM degradation in the communities. Still, they are not the same species used for traditional handicrafts. The communities also proposed the reforestation of riverbanks degraded by ASGM to reduce the risk of flooding. The plant species proposed for reforestation were mainly *Theobroma cacao, Bertholletia excelsa, Swietenia macrophylla, Cedrela odorata, Cedrelinga cateniformis*. The first

two species are crops, and the others are used for timber. However, none of these species have been reported growing in naturally regenerated areas after ASGM degradation in the Madre de Dios region (Alarcón-Aguirre et al., 2023; Chambi-Legoas et al., 2021; Rodriguez-Pascual et al., 2024). The focus group results highlighted need to perform scientific studies about soil degradation and restoration to find suitable plant species to be used in these communities, considering the Indigenous needs.

Focus group participants stated that the communities do not have the material resources to develop the reforestation of the degraded areas. They also indicated that their traditional knowledge is insufficient to restore the soil fertility in the areas degraded by ASGM. They are willing to work with the government and NGOs in the restoration of these areas, although they lack trust in the government due to a perception of corruption. The communities have participated in non-subsistence economic projects supported by the government, mainly cocoa and banana farming, and fish farming projects. They are aware of the environmental impacts of the ASGM and want to move to more sustainable activities such as non-subsistence agriculture, fish farming, and tourism. However, they also indicated that these projects have not been successful so far because of bad management by the government, divisions, and social conflicts in the communities due to ASGM. Previous studies have identified these conflicts as a cause of the abandonment of traditional practices such as communal management of resources and territory (Gonzales & Maldonado, 2016).

One of the participants expressed their frustration with the divisions and the lack of success of the agriculture projects in the communities:

“Projects are not welcome in this community. They are all focused on mining activity and say that the projects prevent them from mining. Mining is not a long-term activity. You have to think

of alternatives. I must disappear from this world so that another generation can conduct the projects well."

Another issue identified in the focus groups was Indigenous women's lack of participation in decision-making. However, they have an important traditional role in agriculture and the conservation of native plant species. This lack of involvement has been aggravated because of the settlement of non-indigenous miners in the communities where they have formed mixed families with Indigenous women. The female participants in the focus groups in one of the communities stated that Indigenous women in mixed marriages are very discriminated against and even stripped of their rights as members of the community.

Due to the complexity and interrelations of ASGM's environmental and social impacts in these communities, the restoration of degraded land needs to be multidimensional and incorporate expertise from several disciplines, such as social science, anthropology, ecology, soil science, engineering, and agronomy. These interventions would need to be done in close consultation with Indigenous people to ensure that they incorporate their needs and increase the likelihood of appropriateness and acceptance. Restoration programs could also take advantage of Indigenous knowledge of their territory to identify degraded areas and plant species growing in natural regeneration areas that can be tested for the restoration of these degraded areas.

4. Conclusions

This study is the first to investigate the SOM, EC, pH, and nutrients in the rhizosphere of plants in naturally regenerated and reforested areas after ASGM degradation in the Amazon rainforest. We showed that the ASGM activity drastically reduces the SOM and EC in rhizosphere soil, decreasing the soil's capacity to support plant growth. However, we found that in rhizosphere

soil, SOM, pH, and EC return to primary forest conditions after eight years of natural regeneration and five years of reforestation for pH and EC. Still, the same levels are not reached in the bulk soil. We also proved that the levels of nutrients, such as total recoverable Ca, in rhizosphere soil return to primary forest conditions after eight years of natural regeneration. However, that is not true for total recoverable K, Mg, and P.

Among the studied plant species in naturally regenerated and reforested areas after ASGM degradation, we found that Acacia *loretensis* and *Inga sp*. (*Inga sp*. when planted with biochar as a soil amendment) might be good candidates for restoring areas degraded by ASGM due to the levels of SOM, EC, and nutrients in their rhizosphere soil. This work is vital and much-needed first step for the design of restoration projects with a focus on soil fertility in areas degraded by ASGM in the Amazon rainforest. However, more research about the restoration age to recover the primary forest soil physic-chemical properties in areas degraded by ASGM and the influence of more Amazonian plant species in this recovery is needed. This information would be critical for designing sustainable restoration programs in these areas in the Amazon rainforest.

Our results show that Indigenous people have an in-depth knowledge of soil degradation in their territory. They identify several visible indicators based on observation and practical knowledge. Still, these indicators coincided with scientific studies of soils degraded by ASGM in many aspects. Our findings emphasize the value of local knowledge in identifying land degradation by ASGM. They also emphasize the need to integrate Indigenous knowledge with scientific knowledge and facilitate an open dialogue about land degradation and restoration of areas impacted by ASGM between local communities, researchers, and policy-makers. Combining the findings of participatory research with the ones from scientific studies can help develop more sustainable and successful restoration projects than either approach could achieve alone.

Chapter 5: Conclusions

In this dissertation, we studied the distribution of Hg in soil and plant species and the effects of Hg exposure in Amazonian plants in areas impacted by ASGM. We also identified native plant species that can be good candidates for restoring these areas in Peru's Madre de Dios region. We finally evaluated the integration of Indigenous and scientific knowledge to monitor and manage land degradation in regions impacted by ASGM.

In the second chapter, we found higher Hg concentrations in intact primary forests than in areas degraded by ASGM, with some levels in soil and plants above the reference level proposed by UNEP to protect environmental health. In addition, we showed that ASGM activity drastically reduces the SOM and EC in soil, decreasing the soil's capacity to support plant growth.

Hg accumulates in rhizosphere soil, plant roots, and leaves. We found strong correlations between Hg concentrations in soil, SOM, and EC. These results show the vital role of the Amazonian forest in Hg capture through foliage, deposition to the soil, and accumulation and stabilization in the rhizosphere, where it forms stable complexes with SOM and minerals. This stabilization may reduce the risk of Hg being transported to nearby areas, such as aquatic ecosystems, where it can enter the food chain through fish and pose a human health hazard to local communities. Our findings reinforce the necessity of conserving the Amazonian forest and reforesting the areas degraded by ASGM and exposed to Hg pollution.

In the third chapter, we study for the first time the effects of Hg exposure on three Amazonian native agricultural plant species, aji dulce (Capsicum Chinense), sacha culantro (Eryngium foetidum), and uncucha (Xanthosoma sagittifolium L. Schott). We found a decrease in length and weight in aji dulce plants after exposure to 10 and 50 mg kg^{-1} of Hg and a decrease

in the same parameters in the uncucha plants after exposure to 10 mg kg^{-1} of Hg. Hg concentrations from 2 to 8 mg kg^{-1} caused chlorosis in leaves of the aji dulce and uncucha plant species, with an increased percentage of affected plants by increased Hg concentrations. The lowest Hg concentration producing toxic effects in these plant species was 2 mg kg^{-1}, three times lower than the SSL for Hg in agricultural soil established in the Peruvian regulation. Our results also suggest that heat stress exacerbates the appearance of chlorosis caused by Hg exposure in the uncucha plant species. These findings support the need to review and revise the soil screening levels for Hg in Peru and other Amazonian countries and develop toxicity levels that consider Amazonian organisms and soil to better understand the potential impacts of Hg in plant development, especially in the Amazonian countries affected by ASGM and climate change, where environmental policies are based on data from international regulations without considering the characteristics of local ecosystems.

In the fourth chapter, we investigate the SOM, EC, pH, and nutrients in the rhizosphere of plants in naturally regenerated and reforested areas after ASGM degradation in the Amazon rainforest. We found that in rhizosphere soil, SOM, pH, and EC return to primary forest conditions after eight years of natural regeneration and five years of reforestation for pH and EC. Still, the same levels are not reached in the bulk soil. We also proved that the levels of nutrients, such as total recoverable Ca, in rhizosphere soil return to primary forest conditions after eight years of natural regeneration. However, that is not true for total recoverable K, Mg, and P.

Among the studied plant species in naturally regenerated and reforested areas after ASGM degradation, we found that *Acacia loretensis* and *Inga sp*. (*Inga sp*. when planted with biochar as a soil amendment) might be good candidates for restoring areas degraded by ASGM due to the levels of SOM, EC, and nutrients in their rhizosphere soil. This work is a vital and

much-needed first step for designing restoration projects focusing on soil fertility in areas degraded by ASGM in the Amazon rainforest.

Our results also show that Indigenous people have an in-depth knowledge of soil degradation in their territory. They identify several visible indicators based on observation and practical knowledge. Our findings emphasize the value of local knowledge in identifying land degradation by ASGM and the need to integrate Indigenous knowledge with scientific knowledge and facilitate an open dialogue about land degradation and restoration of areas impacted by ASGM between local communities, researchers, and policy-makers.

Appendices A: SI of Chapter 2

Table S2-1. Details about plant species collected in the two sampling sites.

Sampling site	Land cover category	Season	Plot number	Plant species	N	Family	Growth habit	Leaf type
Laberinto	Natural regeneration	Rain	1	*Acacia loretensis*	4	Fabaceae	Tree	Perennial
				Cecropia polystachya	2	Urticaceae	Tree	Perennial
				Chromolaena laevigata	4	Asteraceae	Shrub	Perennial
				Ochroma pyramidale	3	Asteraceae	Tree	Perennial
		Rain	2	*Inga ruiziana*	3	Fabaceae	Tree	Deciduous
				Chromolaena laevigata	3	Asteraceae	Shrub	Perennial
				Zanthoxylum aff sprucei	3	Rutaceae	Tree	Deciduous
	Natural regeneration	Dry	1	*Ficus insipida*	4	Moraceae	Tree	Perennial
				Cecropia polystachya	4	Urticaceae	Tree	Perennial
				Acacia loretensis	4	Fabaceae	Tree	Perennial
		Dry	2	*Inga ruiziana*	3	Fabaceae	Tree	Deciduous

				Chromolaena laevigata	2	Asteraceae	Shrub	Perennial
				Zanthoxylum aff sprucei	4	Rutaceae	Tree	Deciduous
	Reforestation	Rain	3	*Calliandra angustifolia*	4	Fabaceae	Tree	Perennial
				Hura crepitans	5	Euphorbiacea e	Tree	Perennial
				Inga spp	4	Fabaceae	Tree	Perennial
		Dry	3	*Calliandra angustifolia*	4	Fabaceae	Tree	Perennial
				Hura crepitans	4	Euphorbiacea e	Tree	Perennial
				Inga spp	4	Fabaceae	Tree	Perennial
			4	*Calliandra angustifolia*	4	Fabaceae	Tree	Perennial
				Hura crepitans	4	Euphorbiacea e	Tree	Perennial
				Inga spp	4	Fabaceae	Tree	Perennial
			5	*Calliandra angustifolia*	4	Fabaceae	Tree	Perennial
				Hura crepitans	4	Euphorbiacea e	Tree	Perennial
				Inga spp	3	Fabaceae	Tree	Perennial

	Primary forest	Dry	6	*Quararibea wittii*	3	Malvaceae	Tree	Deciduous
				Pseudolmedia laevis	3	Moraceae	Tree	Perennial
				Psychotria sp10	3	Rubiaceae	Shrub	Perennial
La Pampa	Naked soil	Rain	7	*Tabebuia serratifolia*	4	Bignoniaceae	Tree	Deciduous
				Inga spp	4	Fabaceae	Tree	Perennial
				Dipteryx micrantha	4	Fabaceae	Tree	Perennial
	Natural regeneration	Rain	8	*Cecropia engleriana*	1	Urticaceae	Tree	Perennial
				Cecropia membranacea	3	Urticaceae	Tree	Perennial
				Chromolaena laevigata	4	Asteraceae	Shrub	Perennial
				Tibouchina longifolia	4	Melastomataceae	Shrub	Perennial
		Dry	8	*Chromolaena laevigata*	4	Asteraceae	Shrub	Perennial
				Tibouchina longifolia	4	Melastomataceae	Shrub	Perennial
				Cecropia engleriana	4	Urticaceae	Tree	Perennial
	Primary forest	Rain	9	*Psychotria sp10*	4	Rubiaceae	Shrub	Perennial

				Inga ruiziana	4	Fabaceae	Tree	Deciduous
				Quararibea wittii	4	Malvaceae	Tree	Deciduous
		Dry	9	*Inga alba*	4	Fabaceae	Tree	Perennial
				Psychotria sp10	4	Rubiaceae	Shrub	Perennial
				Quararibea wittii	4	Malvaceae	Tree	Deciduous

Table S2-2. Hg concentrations in soil (mg kg^{-1}) at 0-5 cm depth in different land cover categories and different seasons in the two sampling sites.

Sampling site	Land cover category	Depth (cm)	Season	n	Hg (mg kg^{-1})
Laberinto	Naked soil	0 – 5	Rain	5	0.007 ± 0.004
			Dry	5	0.097 ± 0.007
	Gravel piles	0 – 5	Rain	4	0.028 ± 0.046
			Dry	4	0.006 ± 0.001
	Natural regeneration	0 – 5	Rain	9	0.048 ± 0.062
			Dry	9	0.047 ± 0.046
	Reforestation	0 – 5	Rain	5	0.032 ± 0.016
			Dry	15	0.037 ± 0.024
	Primary forest	0 – 5	Dry	5	0.276 ± 0.079
La Pampa	Naked soil	0 – 5	Rain	10	0.008 ± 0.002
			Dry	10	0.009 ± 0.003
	Natural regeneration	0 – 5	Rain	5	0.014 ± 0.007
			Dry	5	0.015 ± 0.008
	Primary forest	0 - 5	Rain	5	0.170 ± 0.043
			Dry	5	0.153 ± 0.016

Table S2-3. Soil physicochemical characteristics in different land cover categories in the Laberinto site

Land cover category	Depth (cm)	N	SOM (%)	EC (uS cm^{-1})	pH
Naked soil	0 – 5	5	0.348 ± 0.050	3.96 ± 0.68	6.12 ± 0.25
	5 – 30	5	0.332 ± 0.101	6.11 ± 4.93	6.34 ± 0.24
Grave piles	0 – 5	4	0.365 ± 0.025	5.97 ± 2.97	6.40 ± 0.18
	5 – 30	4	0.430 ± 0.096	5.99 ± 2.80	6.27 ± 0.21
Natural regeneration	0 -5	9	2.050 ± 1.150	328.00 ± 132.00	5.33 ± 0.54
	5 – 30	9	0.835 ± 0.573	53.10 ± 37.40	5.86 ± 0.50
	Rhizosphere (5 – 30)	21	4.460 ± 2.650	145.00 ± 149.00	5.65 ± 0.14
Reforestation (average levels of the plots with no-soil amendments, biochar, and biochar + NPK)	0 -5	15	2.700 ± 1.360	389.00 ± 188.00	4.66 ± 0.30
	5 – 30	15	1.120 ± 0.573	48.60 ± 32.60	5.70 ± 0.39
	Rhizosphere (5 – 30)	35	2.490 ± 1.390	119.00 ± 126.00	5.39 ± 0.36
Reforestation without soil amendments	0 - 5	5	3.121 ± 1.581	454.88 ± 169.07	4.54 ± 0.10
	5 - 30	5	1.242 ± 0.692	51.18 ± 23.58	5.55 ± 0.33
	Rhizosphere (5 – 30)	12	2.435 ± 0.651	143.23 ± 141.70	5.34 ± 0.31
Reforestation with biochar	0 - 5	5	2.503 ± 1.189	386.44 ± 213.97	4.69 ± 0.44
	5 - 30	5	1.371 ± 0.460	52.34 ± 43.43	5.78 ± 0.56
	Rhizosphere (5 – 30)	12	2.711 ± 1.703	82.15 ± 82.67	5.33 ± 0.35

Reforestation with biochar + NPK	0 - 5	5	2.468 ± 1.504	326.60 ± 198.39	4.76 ± 0.28
	5 - 30	5	0.735 ± 0.422	42.37 ± 34.64	5.77 ± 0.25
	Rhizosphere (5 – 30)	11	1.801 ± 0.679	130.72 ± 163.87	5.54 ± 0.49
Primary forest	0 -5	5	8.270 ± 1.390	1358 ± 348	4.91 ± 0.17
	5 – 30	5	3.290 ± 0.663	181 ± 46.50	4.89 ± 0.19
	Rhizosphere (5 – 30)	9	5.810 ± 1.440	269 ± 139	5.56 ± 0.17

Table S2-4. Hg concentrations in plant tissues (mg kg^{-1}) in the plant species studied.

Plant species	Family	n	Hg (mg kg-1)	Hg (mg kg-1)	Hg (mg kg-1)
			Root	Shoot	Leaf
Acacia loretensis	Fabaceae	8	0.013 ± 0.013	0.007 ± 0.003	0.173 ± 0.066
Calliandra angustifolia	Fabaceae	16	0.006 ± 0.005	0.028 ± 0.018	0.239 ± 0.163
Cecropia engleriana	Urticaceae	5	0.018 ± 0.007	0.004 ± 0.001	0.054 ± 0.016
Cecropia membranacea	Urticaceae	3	0.009 ± 0.007	0.007 ± 0.003	0.064 ± 0.024
Cecropia polystachya	Urticaceae	6	0.023 ± 0.015	0.014 ± 0.007	0.150 ± 0.154
Chromolaena laevigata	Asteraceae	17	0.017 ± 0.013	0.007 ± 0.006	0.101 ± 0.073

Dipteryx micrantha	Fabaceae	4	0.010 ± 0.003	0.004 ± 0.002	0.026 ± 0.013
Ficus insipida	Moraceae	4	0.026 ± 0.013	0.021 ± 0.005	0.367 ± 0.074
Hura crepitans	Euphorbiaceae	17	0.020 ± 0.015	0.021 ± 0.010	0.291 ± 0.182
Inga alba	Fabaceae	4	0.044 ± 0.015	0.053 ± 0.018	0.327 ± 0.066
Inga ruiziana	Fabaceae	10	0.047 ± 0.055	0.022 ± 0.016	0.288 ± 0.146
Inga spp	Fabaceae	19	0.009 ± 0.008	0.007 ± 0.004	0.221 ± 0.166
Ochroma pyramidale	Malvaceae	3	0.028 ± 0.013	0.015 ± 0.011	0.095 ± 0.018
Pseudolmedia laevis	Moraceae	3	0.068 ± 0.013	0.235 ± 0.187	0.578 ± 0.125
Psychotria sp10	Rubiaceae	11	0.093 ± 0.044	0.098 ± 0.057	0.450 ± 0.272
Quararibea wittii	Malvaceae	11	0.050 ± 0.035	0.041 ± 0.022	0.313 ± 0.181
Tabebuia serratifolia	Bignoniaceae	4	0.022 ± 0.016	0.004 ± 0.001	0.074 ± 0.034
Tibouchina longifolia	Melastomataceae	8	0.011 ± 0.006	0.005 ± 0.001	0.020 ± 0.006
Zanthoxylum aff sprucei	Rutaceae	7	0.020 ± 0.015	0.038 ± 0.011	0.364 ± 0.137

Table S2-5. Hg concentrations in plant roots in reforestation plots with different soil amendments in Laberinto site.

Plot number	Soil amendment	Plant species	n	Hg in root (mg kg-1)

3	No amendment	Calliandra angustifolia	4	0.004 ± 0.002
		Hura crepitans	4	0.030 ± 0.008
		Inga spp	4	0.007 ± 0.003
4	Biochar	Calliandra angustifolia	4	0.010 ± 0.007
		Hura crepitans	4	0.015 ± 0.008
		Inga spp	4	0.011 ± 0.008
5	Biochar + NPK	Calliandra angustifolia	4	0.005 ± 0.005
		Hura crepitans	4	0.011 ± 0.001
		Inga spp	3	0.004 ± 0.001

Table S2-6. BCF of Hg for plant species collected in the two sampling sites.

Plant species	Family	n	BCF
Acacia loretensis	Fabaceae	8	0.063 ± 0.034
Calliandra angustifolia	Fabaceae	16	0.302 ± 0.391
Cecropia engleriana	Urticaceae	5	1.380 ± 0.874
Cecropia membranacea	Urticaceae	3	0.699 ± 0.601
Cecropia polystachya	Urticaceae	6	0.336 ± 0.320
Chromolaena laevigata	Asteraceae	17	0.752 ± 0.763
Dipteryx micrantha	Fabaceae	4	0.899 ± 0.336
Ficus insipida	Moraceae	4	0.210 ± 0.076
Hura crepitans	Euphorbiaceae	17	0.659 ± 0.503
Inga alba	Fabaceae	4	0.263 ± 0.098

Inga ruiziana	Fabaceae	10	0.436 ± 0.275
Inga spp	Fabaceae	19	0.571 ± 1.060
Ochroma pyramidale	Malvaceae	3	0.775 ± 0.863
Pseudolmedia laevis	Moraceae	3	0.282 ± 0.018
Psychotria sp10	Rubiaceae	11	0.603 ± 0.357
Quararibea wittii	Malvaceae	11	0.304 ± 0.200
Tabebuia serratifolia	Bignoniaceae	4	2.370 ± 1.700
Tibouchina longifolia	Melastomataceae	8	1.110 ± 0.693
Zanthoxylum aff sprucei	Rutaceae	7	0.361 ± 0.249

Table S2-7. Estimates, standard deviation, and significance of coefficients of the linear regression model for Hg levels in soil in the forested strata in the Laberinto site.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	-1.2330934	0.2917205	-4.227	6.24e-05
SOM	0.1826367	0.0215420	8.478	9.32e-13
EC	-0.0002904	0.0001328	-2.187	0.031629
log pH	-0.8275609	0.3940916	-2.100	0.038887
Depth $_{2}$	-0.1776561	0.0600545	-2.958	0.004067
Depth $_{3}$	0.0092437	0.0654156	0.141	0.887982
LC $_{\text{Primary}}$	0.1402442	0.0762681	1.839	0.069651
LC $_{\text{Reforestation}}$	-0.1530679	0.0435848	-3.512	0.000734

Table S2-8. Estimates, standard deviation, and significance of coefficients of the linear regression model for Hg levels in roots in the forested strata in the Laberinto site.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	1.232359	1.122297	1.098	0.28121
Hg $_{\text{rhizosphere soil}}$	6.466384	1.021180	6.332	6.43e-07
EC	-0.001853	0.000570	-3.250	0.00292
pH	-0.612122	0.202843	-3.018	0.00526

Table S2-9. Estimates, standard deviation, and significance of coefficients of the linear regression model for Hg BCF in the forested strata in the Laberinto site.

	Estimate	Std. Error	t value	Pr(>|t|)
Intercept	2.6279056	1.3362920	1.967	0.05959
log $Hg_{\text{rhizosphere soil}}$	-0.5132122	0.2728353	-1.881	0.07079
EC	-0.0014576	0.0005649	-2.580	0.01563
pH	-0.6835976	0.2293555	-2.981	0.00603
LC_{Primary}	0.6166062	0.2294685	2.687	0.01219
$LC_{\text{Reforestation}}$	-0.1004621	0.2141491	-0.469	0.64275

Appendices B: SI of Chapter 3

Table S3-1. Primer used in qPCR in this study.

Targeting gene	Primer	Sequence	Thermo cycler program	Reference
16S rRNA universal	357F 520R	5′ - CCTACGGGAGGCAGCAG - 3′ 5′ - GTATTACCGCGGCTGCTGG - 3′	2 min at 98°C, 10 s at 98°C, 10s at 59.9°C, 30 s at 68°C, 40 cycles	Wei et al., 2015

Equation 1. Formula to calculate gene copy number per gram of dry weight

$$Number\ of\ gene\ copies\ per\ gram\ of\ dry\ weight\ = \frac{\frac{SQ\ \times\ 25\ \mu L\ \times\ Avog}{bp\ \times\ 660\ \times\ 1 \times 10^{9}}}{3\ \mu L\ \times\ dw}$$

Where:

SQ = Starting quantity of qPCR

Avog = Avogrado number = 6.0221 x 10^{23} molecules /mol

bp = length of DNA primer

660 = average mass of 1 bp

dw = dry weight of soil sample from which DNA was extracted

Table S3-3. Comparison between soil Hg SSL in international regulations.

	Mercury SSL, agricultural soil (mg kg^{-1} dry weight (dw))			Mercury SSL, residential soil (mg kg^{-1} dry weight (dw))				
Country	Target value[a]	Trigger value[b]	Intervention value[c]	Target value	Trigger value	Intervention value	Observations	References
Brazil (Federal)	0.5	NA	12	0.5	NA	36	The target value is for soil in general, regardless of the land use.	CONAMA, 2009
Brazil (Sao Paulo State)	0.5	NA	1.2	0.5	NA	0.9	The target value is for soil in general, regardless of the land use.	CETESB, 2014
Peru	6.6	NA	NA	6.6	NA	NA		MINAM, 2013
Ecuador	NA	NA	0.8[d]	NA	NA	1		Republica de Ecuador, 2003
Chile	1-1.5[e]	NA	NA	NA	NA	NA	Maximum allowed concentration in soil before sewage sludge application. Different values for different regions and pHs.	INN, 2004
Netherland	0.3	NA	10	0.3	NA	10	Values are for soil in general, regardless of land use. Intervention value is to be corrected by organic matter and soil clay content.	Carlon, 2007

Belgium (Wallon region)	NA	0.5	5	NA	9	56	Intervention value is to be corrected by organic matter and soil clay content.	Carlon, 2007
Austria	NA	1	NA	NA	2	10	For topsoil (0-30 cm soil depth) in agricultural soil.	Carlon, 2007
France	NA	3.5	7	NA	3.5	7		Carlon, 2007
Germany	0.1-1	5	NA	0.1-1	20	NA	Target value is for soil in general, regardless of land use. It varies for different types of soil (sand, clay, loam). Trigger value for 0-30 cm soil depth. For 30-60 cm soil depth, the value has to be multiplied by 1.5.	Carlon, 2007
Poland	NA	NA	2-10	NA	NA	2-10	Intervention value varies with soil depth and saturated hydraulic conductivity.	Carlon, 2007
Slovakia	NA	NA	0.15-0.75	NA	2	10	Values are for soil in general, regardless of land use. Intervention value varies for different types of soil (sand, clay, loam).	Carlon, 2007
Canada	6.6[f]	NA	NA	6.6[f]	NA	NA	The value for environmental health	CCME, 2006

							protection considering soil contact as a limiting pathway is 12 mg kg^{-1}. The value for human health risk protection considering soil ingestion as a limiting pathway is 6.6 mg kg^{-1}. For the protection of both, the lowest value is considered. Site-specific conditions should be considered when applying these values.	
Australia	NA	NA	NA	NA	1-15	NA	Trigger values for residential soil, ingestion of homegrown produce pathway. Values are for inorganic mercury only. 1 mg kg^{-1} for ecological risk assessment and 15 mg kg^{-1} for human health risk assessment.	Department of Environment and Conservation. The Government of Western Australia, 2010
China	NA	0.5-3.4	2-6	NA	8	33	Values vary for different soil pH and type of crop in agricultural soil.	Chen et al., 2018; Li et al., 2019
USA	NA[g]	NA[g]	NA[g]	NA	23	NA	For residential soil, ingestion of	USEPA, 1996

							homegrown produce pathway.	

NA: not applicable

a) Target value: below this value, soil quality is assured (clean soil).

b) Trigger value: Above this value, further investigation is needed, such as a site-specific risk assessment.

c) Intervention value: above this value, remediation is advisable.

Note: Some countries have different terminologies for the protection levels. For the construction of this table, the terminologies were adapted to the most common one (target, trigger, intervention).

d) Ecuador considers a value like the old Canadian target value from 1991 as remediation value for agricultural, commercial, and industrial soil but not for residential soil (CCME, 1999).

e) The Chilean Northern region considers 1.5 mg kg^{-1} for pH > 6.5 and 1 mg kg^{-1} for pH < 6.5 as target values for agricultural soil. According to the US EPA, pH < 6.5 promotes the leaching of heavy metals. Chilean Southern region considers 1 mg kg^{-1} as the target value for agricultural soil regardless of pH. These values were adapted from European regulations for the application of sewage sludge in agriculture (Council Directive 86/278/EEC).

f) Canada has updated its SSL in different opportunities over time, increasing the value from 0.8 mg kg^{-1} to 2mg kg^{-1} and the last update to 6.6 mg kg^{-1}. In every update, they changed the methodology for the derivation of the SSL, considering different endpoints (in the last update, they excluded the toxicity of mercury in microbial processes); they also eliminated the use of safety factors when there is no sufficient published data (Gaudet et al., 1995; CCME, 1999).

g) Reference: personal communication with EPA Office of Chemical Safety and Pollution Prevention on May 28, 2020.

Appendices C: SI of Chapter 4

Focus group methodology

Objectives

1. Participatory identification of existing problems in the communities in relation to environmental impacts of mining.
2. Participatory identification of real, accessible, and achievable solutions to the problems identified.
3. Participatory mapping of areas impacted by mining in the communities and future uses of these areas, based on information already existing in the current Life Plans.
4. Identification of different attitudes, social norms and intergenerational and intergender power dynamics in relation to environmental issues in the community.

Activities

Session 1: Participatory identification of problems and solutions

- Time: 60 minutes

Methodology: Mixed random groups (men and women, different ages), maximum 6 people per group. Identify environmental problems related to mining in the community and actions the community could do to mitigate these problems. List the problems in a big paper

sheet and write the solutions in colored cards. Each group will paste their cards on a big piece of paper with a Wanamei tree drawn on it (the Wanamei tree is part of an important Harakbut myth, symbol of the community union to overcome the adversity).

Guiding questions:

- What environmental problems has the community faced in the last 5 years?
- Are these problems related to the mining activity?
- Is the deforestation caused by the mining activity an important problem in the community?
- Have you noticed changes in climate patterns? Are these changes related to the environmental impacts of mining?
- What actions could the community do to mitigate the environmental problems identified?
- Should the community restore the areas impacted by the mining activity?
- What stakeholders/allies (government, NGOs, etc.) should the community have to mitigate the environmental problems?
- Should the community participate in public programs, like “Programa Bosques” to conserve the forest?

Session 2: Participatory mapping

- Time: 60 minutes focus groups

Methodology: Mixed random groups (men and women, different ages), maximum 6 people per group. Locate areas where mining activities are performed in the community and areas impacted by these mining activities. Use colored stickers to mark these areas on a community map. Compare with the zoning map included in the current Life Plan of the community. Propose future uses for the impacted areas and write them on the map.

Guiding questions:

- Where are the mining activities performed in the community's territory?
- Has the location of these activities changed in the last five years?
- What future locations does the community want for these activities?
- Where are the areas impacted by the mining activities in the community's territory?
- Has the location of these areas changed in the last five years?
- What future land uses does the community want for these impacted areas?

Table S4-1. Primer used in qPCR in this study.

Targeting gene	Primer	Sequence	Thermo cycler program	Reference
16S rRNA universal	357F 520R	5′ - CCTACGGGAGGCAGCAG - 3′ 5′ - GTATTACCGCGGCTGCTGG - 3′	2 min at 98°C, 10 s at 98°C, 10s at 59.9°C, 30 s at 68°C, 40 cycles	Wei et al., 2015

Equation 1. Formula to calculate gene copy number per gram of dry weight

$$Number\ of\ gene\ copies\ per\ gram\ of\ dry\ weight = \frac{\frac{SQ\ \times 25\ \mu L\ \times Avog}{bp\ \times 660\ \times 1\times\ 10^{9}}}{3\ \mu L\ \times dw}$$

Where:

SQ = Starting quantity of qPCR

Avog = Avogrado number = 6.0221 x 10^{23} molecules /mol

bp = length of DNA primer

660 = average mass of 1 bp

dw = dry weight of soil sample from which DNA was extracted

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