An Investigation of the Accumulation Capabilities of *Timonius* spp. at Sibuyan Islands Ultramafic Sites in the Philippines

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In Partial Fulfillment
of the Requirements for the Degree of Bachelor of Science in Biology major in Medical Biology

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CERTIFICATION

This is to certify that the thesis proposal entitled:

An Investigation of the Accumulation Capabilities of *Timonius* spp. at Sibuyan Islands Ultramafic Sites in the Philippines

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I. BASIC INFORMATION

PROJECT TITLE:
An Investigation to the Accumulation Capabilities of *Timonius* spp. at Sibuyan Islands Ultramafic Sites in the Philippines

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TARGET UNITED NATIONS SUSTAINABLE DEVELOPMENT GOALS *(refer to https://sdgs.un.org/goals)*

SDG 11: Sustainable Cities and Communities

Plants exhibiting the capacity to accumulate trace metals in their physiological systems have shown potential in assisting phytoremediation efforts. This study aims to determine whether *Timonius* spp. possess the capability to engage in this restorative technique for specific trace metals. Phytoremediation involves the use of plants to rehabilitate environments affected by water, soil, and air pollution. It can be particularly applicable in urban settings, where plants with accumulative properties can be employed to cleanse contaminated lands, especially those burdened with heavy metals. Soil and land contamination by heavy metals renders them toxic to plants, animals, and humans, thereby posing significant environmental and health hazards. In addition to its environmental benefits, phytoremediation represents a cost-effective alternative to conventional methods of environmental remediation, such as soil disposal. Overall, the utilization of accumulator plants in phytoremediation holds promise as a sustainable and economically viable approach to the restoration of heavy metal-contaminated lands.

SDG 13: Climate Action

Ultramafic soils are renowned for their high concentrations of certain metals that possess phytotoxic properties, adversely affecting plant growth, particularly in non-adapted species, and contributing to climate change. The inclusion of plants capable of metal accumulation can play a pivotal role in mitigating climate change, as they possess the capacity to sequester and store carbon within their above-ground tissues and the surrounding soil. The carbon can then react with the accumulated heavy metals, forming more stable carbonates, thereby effectively removing carbon from the atmosphere. Moreover, their remedial capabilities can restore heavily contaminated soils, enhancing soil health and reducing metal content, resulting in improved water retention capacity. This, in turn, aids in diminishing the risks associated with soil erosion and drought, both of which are consequences of climate change.

SDG 15: Life on Land

The contamination of soil with heavy metals has detrimental effects on plant growth and biodiversity, thereby impacting wildlife habitats. Plants that exhibit accumulation abilities hold the potential to remediate such soil and contribute to its restoration. This study aims to contribute to the existing body of research by investigating whether *Timonius* spp. possess the capacity to fulfill this role. Utilizing accumulator plants for phytoremediation presents an environmentally friendly alternative to conventional remediation methods, eliminating the need for chemicals and other harmful substances that can further disrupt the ecosystem. Furthermore, the utilization of accumulator plants promotes biodiversity conservation by providing habitats for various wildlife species, while concurrently improving soil fertility and fostering plant growth. In essence, this research endeavors to support and sustain both wildlife and plant populations.

II. TECHNICAL DESCRIPTION

A. ABSTRACT

The accumulation of heavy metals is considered an adaptive feature for plants, with the family Rubiaceae being a very well-represented family. They have the unique ability to accumulate heavy metals in varying degrees and thus thrive in ultramafic and metalliferous soils, which are otherwise toxic to other plants and animals. One group classified as such is the *Timonius* species, a subject of ongoing research, particularly in the field of systematics in the Philippines. Sibuyan, most especially Mt. Guiting-Guiting is known to be an ultramafic area, with a recorded presence of *Timonius* species in the area. This study aims to analyze the concentration of metals in collected plant and soil samples of *Timonius* species in Sibuyan, Philippines using Flame Atomic Absorption Spectroscopy (FAAS). The results of the analysis will be compared to previously established threshold levels to determine the species' accumulator ability, their capacity to accumulate metals within their tissues (Bioconcentration Factor), and their tolerance to these soils (Tolerance Factor). Phytoremediation, a natural alternative to the rehabilitation of lands polluted with heavy metals by anthropogenic activities, is one of, if not the primary, applications of accumulator species, which is essential in combating the negative effects of anthropogenic activities to the environment.

Keywords: accumulation, heavy metal, Philippines, phytoremediation, *Timonius*, ultramafic

B. RATIONALE

The vegetation naturally occurring in ultramafic soils exhibits distinct morphological and physiological characteristics compared to plants found in non-ultramafic soils (Proctor et al., 1998). Reeves et al. (2018) characterize these plants as having the ability to accumulate exceptionally high concentrations of heavy metals in their foliar tissue, exceeding accepted threshold values, when found in their natural habitat. While not all flora in ultramafic soils possess the specialized mechanism of (hyper)accumulation, these plant populations are known to adopt tolerance mechanisms to mitigate the adverse effects of heavy metal toxicity (van der Ent & Reeves, 2015; van der Ent et al., 2014). Ultramafic soils are similarly distinguishable in various regions worldwide. Although ultramafic regoliths are sparsely distributed globally, they are notable for their acidic and depauperate nature, containing high concentrations of "potentially phytotoxic trace elements," such as iron and magnesium, among others (Vithanage et al., 2019).

There are currently no studies describing the accumulation of heavy metal capabilities of *Timonius* spp.. Studies circumbscribing heavy metal accumulation in select species is lacking, attributable to “the lack of systematic screening of plant species,” according to Reeves et al. (2017). The efforts of the scientific community in discovering accumulator plant species are further hindered by a biased focus on Ni hyperaccumulators, given their widespread occurrence in renowned Ni-rich ultramafic and metalliferous soils, as well as the need to exercise caution in order to avoid erroneous and inflated findings resulting from airborne heavy metal contamination (e.g., in the case of copper) of specimens collected in ultramafic sites near smelting and mining areas (Reeves et al., 2018).

While plants possess remarkable adaptability to stressful conditions, only a limited number of species are capable of tolerating heavy metal stress. Therefore, it is essential to investigate the accumulation abilities of any known plant species that naturally occur in ultramafic soils. Such research contributes to existing databases cataloging hyperaccumulator plant species and enhances our understanding of trace metal regulation and the evolution of tolerance mechanisms in plants. These findings, coupled with the recommendation of conducting genetic and molecular assays, can assist in assessing phylogenetic relationships within the taxa. Moreover, the identification of accumulator species can facilitate the conservation of these plants and support applied biotechnological endeavors related to phytoremediation, mining, and extraction, particularly in light of the imminent threat posed by heavy metal contamination in natural habitats, which can occur through airborne dust, soil leachates, and sewage sludge (Castanares & Bohdan, 2020; van der Ent, et al., 2014).

C. OBJECTIVES

The study aims to determine and evaluate the accumulating capabilities of *Timonius* spp. in Sibuyan, Philippines, where there have been identified presence of *Timonius* spp.

1. Determine the concentrations of heavy metals in the soil samples collected from Mt. Guiting-Guiting in Sibuyan, Philippines.
2. Examine the plant specimens gathered from Mt. Guiting-Guiting by employing digestion and extraction techniques to determine their ability to amass heavy metals (Cu, Ni, Mn) present in the area.
3. Assess the potential of collected *Timonius* species as metal accumulator plants and its ability to contribute to phytoremediation efforts by comparing concentrations of heavy metal in the specimens to known heavy metal threshold levels.

D. SIGNIFICANCE OF THE STUDY

Heavy metal accumulation represents only a small fraction of known plant species extensively studied and used in phytoremediation, a method employed to restore areas impacted by anthropogenic activities (Raffa et al., 2021). Thus, identifying potential and novel species capable of accumulating heavy metals in ultramafic outcrops in the Philippines is crucial for devising region-specific phytoremediation strategies, given its inherent sustainable and cost-effective nature. As these plants naturally accumulate such metals within their tissues, minimal intervention is needed (Dixit et al., 2015). Overall, phytoremediation is a technique that holds great promise for mitigating environmental pollution and delivering numerous advantages to both the environment and human health (Cunningham & Ow, 1996).

The investigation of accumulation as a property in plant species holds significance due to their ability to adapt in otherwise phytotoxic environments. Research circumscribing such facilities contributes to our understanding of tolerance mechanisms in plants and likewise has profound implications in various fields. Such research can aid in the assessment of phylogenetic relationships, allowing for a deeper analysis of genetic diversity and evolutionary history. Additionally, these findings can support conservation efforts by advocating for species that can contribute to ecosystem rehabilitation (Castanares & Bohdan, 2020). However, extensive research on this topic is lacking, underscoring the need for further investigations on hyperaccumulator plants, particularly within the *Timonius* genus in the Philippines. By highlighting the abundance of hyperaccumulator species in this genus, this research may establish new grounds, which could aid in phylogenetic assessments and add to the economic significance of the genus.

E. RELATED LITERATURE AND STUDIES

Currently, there have been limited studies investigating the accumulating capabilities of *Timonius*, and phylogenetic analyses have posed challenges to such research due to evolving classifications. In a recent study by Fernando (2013), *Timonius arboreus* was examined for its heavy metal accumulation potential, revealing its ability to accumulate nickel predominantly in its leaves. However, the unstable taxonomic position of *Timonius arboreus* within the genus necessitates a reassessment of its nickel-accumulating capabilities. The dynamic nature of *Timonius* as a genus presents difficulties for studies, but relying on up-to-date taxonomic studies can provide a solution to this issue.

Although there is a possibility that *Timonius* spp. may possess heavy metal accumulating capabilities, it is noteworthy that several members of the Rubiaceae family, to which *Timonius* belongs, are recognized as nickel hyperaccumulators, particularly within the genus *Psychotria*, encompassing species such as "*Psychotria gabriellae* (formerly *P. douarrei*) from New Caledonia, *P. grandis* from Puerto Rico" (van der Ent et al., 2015), among others. Furthermore, van der Ent et al. (2015) identified a sub-hyperaccumulator, *Timonius cf. eskerianu*s, within the *Timonius* family. Thus, while the accumulating capabilities may be present in *Timonius*, it is inconclusive to assume their presence in all members, especially those found in the Philippines. There remains a possibility of such abilities, particularly on Mt. Guiting-Guiting, where a rainforest similar to that of Mount Silam in Sabah, Malaysia, has been described. The composition of the soil in these two mountains is also similar (Proctor et al., 1988).

Floral Biodiversity in Ultramafic Areas

Floral Biodiversity

The Philippines is known to have a diverse range of forest formations that occur over different substrates, which allows for distinct vegetation (Fernando et al., 2008). However, it is important to know that the influence of edaphic factors on biodiversity is unknown since the geoecology of ultramafic areas in South and Southeast Asia remains understudied (van der Ent et al., 2015; Proctor 1992, 2003). Ultramafic areas are said to cover only one percent of the Earth's land surface, but they promote high levels of endemism and biodiversity despite low soil fertility and a drier environment (Garnier et al., 2009). To add to this, ultramafic areas are highly prioritized for their biodiversity conservation since these areas contain vast amounts of endemic species, ecotypes, and rare species (Boyd et al., 2009) Ultramafic soils are known to contain high levels of trace metals such as chromium (Cr), nickel (Ni), manganese (Mn), and cobalt (Co), to name a few. They also have a low Calcium: Magnesium ratio and are deficient in essential macronutrients that are required for plant growth (Vithanage et al., 2019). Trace metals are known to be environmental contaminants with a relatively high density but are toxic to living organisms at low concentrations (Jaishankar et al., 2014; Sungur et al., 2021). Additionally, a study conducted by Kierczaak et al. (2020) stated that ultramafic soils and rocks are typically site-specific and diverse but similarly have elevated Ni, Cr, and Co concentrations that are transferred from bedrock to soil and then to accumulator plants. A complex interaction of variables, such as soil chemistry, nutrient availability, and competition with other plant species, is expected to have an impact on the growth of the *Timonius* genus in ultramafic soils. To completely comprehend the principles underpinning this genus' success in these difficult habitats, more research is required.

The majority of nickel hyperaccumulators in tropical regions, such as the countries present in Southeast Asia, are known to come from ultramafic soils (Bouman et al., 2018). The Philippines is a country that is filled with plenty of ultramafic soils from locations where various studies have been conducted. A study conducted by Fernando et al. (2018) described locations in the Dinagat and Mindanao Islands as ultramafic, from which they discovered a new species of *Medinillathereseae*, though the said species was not mentioned to have hyperaccumulating tendencies. Another study led by Ata et al. (2016) recognized Zambales and Surigao del Norte as locations with ultramafic complexes in the Philippines, wherein they classified the said locations into Belt I and Belt III, respectively, according to Yumul's four proposed belts of ophiolite formations in 2007. Lastly, Sibuyan Island, the target location of this study, is also considered to be ultramafic, specifically Mount Guiting-Guiting which had acidic soil (pH 4.4-5.5) as well as a relatively low Mg/Ca quotient and an exchangeable nickel value of 1.0-23.8μg g^{-1} (Proctor et al., 1998). For this study, the group will mainly try to focus on *Timonius*, which is under the Tribe *Guettardeae*. From the study by Chavez et al. (2020), *Timonius* spp. can be found in ultramafic sites but it is still understudied whether or not these species have hyperaccumulating capabilities. However, there have been studies made that the Family Rubiaceae has been studied as a nickel and aluminum hyperaccumulator in different countries (Jansen et al., 2000; van der Ent et al., 2015).

Novel species that are found in ultramafic areas

Ramos & Manangkil (2022) study in Zambales, Philippines identified six plant species in the area that showed a potential in phytoremediation due to the sequestration of heavy metals in their shoots and roots, a characteristic of hyperaccumulator plants. These plants include *Cyperus difformis*, *Scirpus juncoides*, *Fimbristylis miliacea*, *Centella asiatica*, *Sphagneticola trilobata*, and *Monochoria vaginalis,* which showed cadmium in either their shoots, roots, or both. Similarly, Fernando et al. (2014) reported *Rinorea niccolifera* to be a newly-discovered Ni hyperaccumulator also found in Zambales, Luzon, Phlippines.

In 2019, Claveria et al. studied a possible hyperaccumulator, *Pteris melanocaulon* which is said to thrive in mines in Cebu and Surigao. The researchers found that the said plant was greatly similar to *Pteris vittata* and *Pityrogramma calomelanos* in terms of its bioaccumulation factor, translocation factor, and uptake mechanisms. Specifically, *P. melanocaulon* showed high amounts of arsenic in its above ground tissues and was deemed an arsenic hyperaccumulator.

Sites and Sightings

Floral structures refer to the various parts of the flower, including the petals, sepals, stamens, and pistils, while fruit structures can vary greatly, including differences in shape, size, color, and texture. These structures provide important diagnostic features for identifying different plant species (Espinosa & Pinedo, 2018). They noted that differences in corolla tubes, numbers of corolla lobes, and fruit morphology, such as shape and indumentum, can be used to identify different species. For instance, a study by Razak et al. (2016) used floral and fruit characteristics, as well as molecular data, to differentiate between *Timonius* species in Southeast Asia. The authors observed that the shape, size, and color of the flowers were useful for distinguishing between different species, as were differences in the structure of the fruit. A study on the taxonomy of *Timonius* in South America by Michelangeli et al. (2013) highlighted the importance of floral and fruit structures in distinguishing between species. The authors noted that differences in the shape and size of the fruit, as well as the presence or absence of certain structures, were useful diagnostic features. Furthermore, Davidson et al. (2002) noted that differences in chloroplast DNA sequence data can also be used to distinguish *Timonius* species.

According to Chavez et al. (2020), the characteristics used to differentiate between *T. lanceolatus,* and *T. alejandroanus* includes the expansion and elongation of the disk during fruit development, the shape and texture of the fruits, and the type of stipular aestivation, among other features. The authors noted that *T. lanceolatus* has staminate inflorescences and ellipsoid to obovoid fruits that are moderately strigose, while *T. alejandroanus* has globose to oblate, glabrous fruits. Additionally, *T. lanceolatus* has coriaceous leaves and staminate flowers with corolla tubes, while T. alejandroanus has chartaceous leaves and lacks corolla tubes in staminate flowers. The stipules of *T. lanceolatus* are also imbricated with a glabrous outer surface, while those of *T. alejandroanus* are valvate. Furthermore, the pistillate flowers and fruits of *T. alejandroanus* are densely strigose, while those of *T. lanceolatus* are not. Another comparison was the disk of *T. lanceolatus* and its fruit expand horizontally during development, while the disk of *T. alejandroanus* does not expand or elongate during fruit development.

The flowering season of *Timonius* species is influenced by a variety of factors, including climate, geography, and the specific species' biology. In general, *Timonius* species tend to flower during the rainy season, although some species may produce flowers throughout the year. The flowering season of *Timonius* species varies depending on the region and the species itself. In the Philippines, for example, *Timonius* species tend to flower during the rainy season from June to November (Matsumoto et al., 2020). In summary, when collecting *Timonius* species, it is best to do so during the flowering and fruiting seasons in order to properly distinguish between species based on their floral and fruit structures.

Timonius is a genus of flowering plants in the family Rubiaceae. The genus is endemic to the Philippines, and various species can be found throughout the country, including Luzon, Visayas, and Mindanao. In Luzon, for example, several species of *Timonius* spp. can be found, and some of these are *Timonius alejandroanus, Timonius appendiculatus, Timonius arboreus, Timonius auriculatus, Timonius confertiflorus, Timonius dumagat, Timonius eremiticus, Timonius lindleyanus, and Timonius pachyphyllus.* In the Visayas region, for example, there are several species of *Timonius* that can be found. One of these is *Timonius dumagat*. *Timonius confertiflorus* is also found in Guimaras and Panay in the Visayas region, as well as in Camarines Sur in Luzon and Surigao in Mindanao. These species of *Timonius* are unique to the Philippines and are important components of the country's biodiversity.

Table 1. List of *Timonius* spp. found in the Philippines. The highlighted rows are the species found in Sibuyan.

***Timonius* spp.**	**Location**
T. alejandroanus	Taft, Mt. Calbiga, Samar
T. appendiculatus	Aurora, Batangas, Camarines, Rizal, Sorsogon, Davao Oriental, and Surigao del Norte

T. arboreus	Guimaras, Leyte, Bataan, Camarines, Isabela, Laguna, Nueva Ecija, Rizal, Sorsogon, Zambales, Agusan del Norte, Surigao, Mindoro, Negros, Palawan, Panay, Sibuyan, and Ticao
T. auriculatus	Dinagat, Quezon, and Surigao del Norte
T. caudatifolius	Agusan del Norte, Bukidnon and Davao Oriental
T. confertiflorus	Guimaras, Camarines Sur, Surigao, Panay, Samar
T. dumagat	Divilacan, Aubarede Peninsula, west side facing Bicobian, Salniwan Spring, Isabela
T. epiphyticus	Basilan, Biliran, Agusan del Norte, Bukidnon, Davao, Davao del Sur, Lanao del Sur, Samar
T. eremiticus	Mt. Pulgar, Puerto Princesa City, Palawan
T. ferrugineus	Palawan and Taytay
T. finlaysonianus	Bukas Grande, Dinagat Surigao del Norte and Samar
T. gammillii	Mt. Guiting Guiting, Sibuyan Island, Romblon Palawan
T. gracilipes	Buacao, Cebu
T. lanceolatus	Dinagat, Surigao del Norte
T. longiflorus	Zamboanga
T. longistipulus	Leyte
T. noli-tangere	Brgy. Sta. Rosa, Balangiga, Eastern Samar
T. obovatus	Aurora, Zambales, Sibuyan
T. oligophlebius	Mt. Kililibong, Sorsogon Isabela
T. pachyphyllus	Mt. Irig, Tanay, Rizal
T. palawanensis	Brookes Point, Palawan, Borneos, Negros
T. pseudoarboreus	Brgy. Amot, Sitio Igad, Burdeos, Quezon Polilio, Quezon Cagayan, Laguna, and Isabela
T. pulgarensis	Mt. Pulgar, Puerto Princesa, Palawan

T. quinqueflorus	Guinyangan, Quezon Isabela
T. ridsdalei	Diguyo, Palanan, Isabela
T. rotundus	Dinagat, Surigao del Norte
T. samarensis	Catbalogan, Western Samar
T. spes-vitarum	Palawan
T. stevendarwinii	Brooke's Point, Brgy. Malis, Magagong settlement, Palawan Quezon and Rizal
T. sulitii	Taft Mt. Calbiga, Eastern Samar
T. ternifolius	Babuyan Island Busuanga, Camiguin, Culion, Ilocos Norte, Palawan, PanayBucas Grande
T. trichophorus	Aurora, Agusan del Norte, Bukidnon, Surigao del Norte, Samar
T. urdanetensis	Agusan del Norte
T. valetonii	Surigao, Panay, Sibuyan

Timonius species have been definitively observed in ultramafic areas, establishing their confirmed presence in such environments. As investigated in a study by Do et al. (2020), some *Timonius* species were found to be hyperaccumulators, specifically for Nickel, namely *T. bougainvillensis* and *T. pubistipulus* var. *pubescens* in Papua New Guinea. However, the classification of *Timonius* spp. in the Philippines as a hyperaccumulator and the identification of the specific species involved remain subjects of ongoing scientific investigation. In addition, other species of *Timonius* have not been reported to exhibit hyperaccumulation or to grow exclusively in ultramafic soils.

For this study, the groups intend to collect samples in Sibuyan Island as the said location meets the criteria required for this study which include the presence of *Timonius* spp. as well as the presence of ultramafic soil. Sibuyan Island, found among the islands in Romblon, is also dubbed as the "Galapagos of Asia" due to its high number of endemic species that has been recorded. One of the most famous landmarks of Sibuyan is Mt. Guiting-Guiting is the highest point of Sibuyan Island, with an elevation of 2,058 meters above sea level, and is composed of high levels of biodiversity, hence its protected status (Afable, 2022). According to Proctor et al. (1998) Mt. Guiting-Guiting is an ultramafic mountain whose soil is acidic with low concentrations of exchangeable potassium, as well as, relatively low Mg/Ca quotient and exchangeable nickel that is a common characteristic for ultramafic soils. Along with Mt. Bloomfield in Palawan, Mt. Guiting-Guiting in Sibuyan Island was also part of the first well studied ultramafic forests in the Philippines. These studies provided basic information on the unique vegetation as well as the stunted growth of sclerophyllous trees, presence of open patches that are often dominated by grasses, and lastly the absence of woody plants which are often distinct features of the

Philippine ultramafics (Ellenita et al., 2020). Sibuyan Island, like Romblon, has a Type III climate wherein there is a relatively dry period around February to March and a wet period during the rest of the year and its average temperatures play an important role in phytoremediation due to its impact on both the plants biomass and heavy metal accumulation (Proctor et al., 1998; Tumaneng et al., 2015; Kudo et al., 2023). Due to its distinctive geographic location and a variety of ecosystems, the island has a diverse range of floral species. The island's proximity to the deep sea has helped to keep its plant life distinct from that of neighboring islands. The island is home to a variety of habitats, including mangrove forests, lowland forests, and montane forests.

Physiological Effects of Heavy Metal Accumulation to Seed Germination in Non-hyperaccumulating Plants

The term "heavy metals" in the soil refers to the presence of "elements that have a density at least 5 times higher than that of water. " (Smiljanić et al., 2019). Examples of such heavy metals would be nickel, cadmium, copper, cobalt, lead, chromium, zinc, and mercury. Although these metals can be naturally found in the environment, plants require that only a low concentration of them is present at a time. An unusually high concentration of it can become toxic to the physiological state of plants, or phytotoxic, that are not naturally adapted to its presence. Plants have varying threshold or tolerance levels when exposed to heavy metals, based on multiple factors. The threshold concentration of heavy metals is defined as the concentration of these heavy metals above what the plant can handle that then causes toxic effects on the plant's health. The type of heavy metal, exposure time, and plant species affects this, as such it is important to monitor and make sure that there is no prolonged exposure for plants that are not hyperaccumulators.

Mt. Guiting Guiting in Sibuyan Island soil has been studied previously to contain Cu, Ni, and Mn (Proctor et al., 1998). As such the metals that have to be determined if there is a high accumulation of in the plant would be based on the presence of metals that are currently present in the area itself.

Table 2. WHO Permissible Limits for chosen heavy metals within plants (Ogundele et al., 2015).

Element	**Permissible value of Plant (ug/kg^{-1})**
Cu	10
Ni	10
Mn	20

Some areas naturally have a high concentration of heavy metals within their soil, and as such, there are plants that can either tolerate the presence of these metals or are dependent on their presence for their physiological processes. There are also areas that do not naturally have such heavy metals due to increased pollution by human activities that produce a high concentration of these metals (Smiljanić et al., 2019). The plants within these areas that are suddenly introduced to heavy metals are not adapted to the presence of these heavy metals and tend to experience negative effects. Various heavy metals are known to cause toxicity in plants, which affects plant productivity. These would be considered environmental pollutants that threaten the agro-ecosystem. These specifically include lead, nickel, cadmium, copper, cobalt, chromium, and mercury (Ghosh & Sethy, 2013).

Most of these metals in ultramafic areas are micronutrients, and are needed by the plant for its processes, yet can be harmful/toxic to the plant in high concentrations, which is the case in areas that have heavy metal pollution. Nickel is used by plants as a cofactor to convert urea into ammonium, a compound important in plant growth (Liu et al. 2011). Yet it can become harmful when a large amount of it is absorbed by the plant. It is reported to be toxic to most plant species, affecting amylase, protease, and ribonuclease enzyme activity, thus retarding seed germination and the growth of many crops. It has been reported to affect the digestion and mobilization of food reserves like proteins and carbohydrates in germinating seeds, reducing plant height, root length, fresh and dry weight, chlorophyll content, enzyme carbonic anhydrase activity, and increasing malondialdehyde content (MDA) and electrolyte leakage (Ghosh & Sethy, 2013), while for high concentrations of copper (Cu), "Cu stress leads to reduced germination rate and induces biomass mobilization by release of glucose and fructose, thereby inhibiting the breakdown of starch and sucrose in reserve tissue by inhibition in the activities of alpha-amylase and invertase isoenzymes" (Ghosh & Sethy, 2013). Other than in the actual plants, it also affects seed germination by "down-regulating activity of alpha-amylase or enolase, which has been reported to affect overall metabolism, water uptake, and failure to mobilize reserve food" (Ghosh & Sethy, 2013).

Metal Accumulation Mechanism: Principle Behind Phytoremediation

Environmental contamination, particularly the presence of heavy metals, poses significant challenges which require effective remediation techniques. Phytoremediation is a technique that utilizes plants in rehabilitating contaminated land, air, or water. Specifically, the use of metal-accumulating plants for this technique is said to have great advantages including low cost, recyclable metal-rich tissue, minimal environmental disturbance, among others (Memon et al., 2001). This approach encompasses strategies including phytoextraction, rhizofiltration, phytostabilization, and plant assisted bioremediation. Phytoremediation could be a sustainable and efficient method for addressing metal contamination in the environment, thus the mechanisms and principles of metal accumulation in plants are to be discussed.

Heavy metals in high concentrations are toxic to plants, possibly altering their physiological makeup. Some plant species are said to show different strategies when growing in the presence of heavy metals in order to survive, specifically, the basic responses were outlined by Baker & Walker (1990) as a) metal excluders, b) metal indicator, and c) metal accumulators. Excluders would act by limiting metals from translocation into their above-ground tissues, while still showing significantly large amounts in their roots. Indicators reflect the amount of metals in the soil as it accumulates metals in its above-ground tissues. Accumulators would also accumulate metals in their above-ground tissue. Specifically, once a plant can store certain metals above 0.1% (Ni, Co, Cu, Cr, Pb) or 1% (Zn) in their leaves, these would be considered as hyperaccumulators (Baker & Walker, 1990).

Plants are said to accumulate metals in their tissues in two ways, active or inactive accumulation (Naila et al., 2019). Active accumulation refers to plants accumulating airborne metals and metalloids into their leaves, whereas inactive accumulation is when plants uptake metals from the soil through their roots, and sequester the metas in their above-ground tissues, the leaves, stems and shoots. Moreover, Memon et al. (2001) states plants may either concentrate or localize certain metals in their roots and stems, or metals in their nontoxic form are accumulated and stored for use and distribution later on. Naila et al. (2019) explored metal accumulators, noting the multiple mechanisms done by the plant, some strategies noted by the author and colleagues included:

Root Uptake: Plants first take up metals available in the soil through their roots, however the amount and type of metal differs depending on the plant. The metals must be in its free form to be absorbed by the plant. The process of metal absorption is facilitated by the reduction of metals through the activation of a specific plasma membrane-bound metal reductase enzyme. This would convert the metals into a less toxic and more accessible form available to the plant. Once absorbed, the metals are translocated from its roots towards its shoots in the xylem by a transportation stream. Moreover, Naila et al. (2019) stated that fungi and metal-chelating agents aid in metal uptake by aiding in transforming metals in soil into their free form for uptake and mobilization, allowing the metals to be more available for the plant's root uptake.

Chelating Agents: Plants are able to detoxify heavy metals in various parts, specifically in the epidermis, trichomes, and cuticle, allowing them to accumulate and tolerate the heavy metals they absorb. This detoxification is done through chelating agents wherein the uptaken metal would react with ligands forming complex compounds, this allows the metal to be present in an inactive form. The detoxified metals would then be removed through the stomata subsidiary and guard cells. However, the metals which were not detoxified would accumulate in other tissues of the plant, this would lead to oxidative stress and toxicity, thus the eventual death of the plant. This can be prevented through the next mechanism. Chelation uses ligands to reduce the toxicity of metals in plants, specifically these would include phytochelatins, organic acids, metallothioneins, and the likes. Complexes formed from chelation would be transported into vacuoles

Metal Tolerance: Through biodegradation or biotransformation, a plant is able to tolerate the metals it absorbed as these processes change metals into their inert form. For a plant to tolerate metal, its cell metabolism is modified and tolerable metal ion concentrations are maintained. This is possible through the previously mentioned chelation.

Metal toxicity in plants primarily occurs in the plasma membrane (Naila et al., 2019), if the plasma membrane is not protected, the metal toxicity would lead to various effects leading to impaired cellular function and plant function. Besides tolerating the metals absorbed and accumulated in their tissues, controlling the entry of metal is important to avoid the said toxic effects, this can be achieved through metal ions binding to polygalacturonic acid (Naila et al., 2019). According to Dalvi & Bhalerao (2013), polygalacturonic acid is a cation exchanger found in cell wall pectins which is said to restrict plant uptake.

Plants with the ability to accumulate heavy metals may also uptake said metals in large amounts. From regular accumulator plants, would then be classified as hyperaccumulator plants, depending on how much of a certain metal it can accumulate, as previously mentioned. It is said that the genes of hyperaccumulator plants that are involved with their hyperaccumulation and hypertolerance are not species-specific; rather, the way these genes are expressed differs from that of non-hyperaccumulating plants, and there is currently no available full genome sequence of hyperaccumulator plants (Verbruggen et al., 2009). According to Verbruggen et al. (2009), there are 5 key steps in plant's hyperaccumulation: 1) uptake of metals from the soil to its roots, 2) translocation from root to shoot, 3) sequestration on the shoot vacuoles, 4) detoxification by chelation of trace metals, and 5) other adaptive processes in hyperaccumulators.

There are certain plants, called hyperaccumulators, that are able to tolerate and absorb heavy metals greatly with no toxicity effects on the plant (Verbruggen et al., 2009). Hyperaccumulators comprise only a minuscule number of all the plant species known, this is further emphasized in a study by Verbruggen et al. (2009), wherein it states that among plant species there are only about 450 that are known hyperaccumulators of trace metals (Zn, Ni, Mn, Cu, Co, and Cd), metalloids (As), and nonmetals (Se), specifically, which is said to comprise only 0.2% of angiosperms at the time. Most of the Ni hyperaccumulators in tropical areas are represented by " Euphorbiaceae, Phyllanthaceae, Salicaceae, Buxaceae, and Rubiaceae." (Reeves et al., 2018). Rubiaceae have a large number of members that are hyperaccumulators of many different metals such as Al, Ni, etc., with species such as *Psychotria gabriellae* and *Phyllomelia coronata* (Reeves et al., 2018).

Out of all the trace elements that are commonly seen, the majority of the taxa that have been known to be hyperaccumulators mainly extract nickel and copper. Nickel has the highest number of taxa that act as its hyperaccumulators, followed by copper. These may be due to plants using these two as micronutrients. While the rarest trace element known to have a hyperaccumulator would be thallium, attributed to how severely toxic it is as an environmental pollutant. Unlike non-hyperaccumulators, hyperaccumulating plants have adaptive intrinsic regulatory mechanisms, which is what allows the heavy metals to accumulate in their above-ground tissues (e.g., leaves) rather than their roots. (Sytar et al., 2021). They are also able to detoxify these as well (Verbruggen et al., 2009).

Ultramafic areas in the Philippines contain unique floral biodiversity and high endemism, which means species that have unique abilities are able to withstand and thrive in the presence of high concentrations of metals within the soil. Yet for areas that do not naturally have these high concentrations of metal naturally and would suddenly be introduced due to the pollution brought on by anthropogenic activities, plants in those areas are not able to withstand such high concentrations within their systems, ultimately leading to abnormal physiological conditions. These have slowly manifested themselves as threats to agriculture and health due to elevated levels of certain minerals. *Timonius* spp. is a genus of plants that is wherein species have been found to thrive on ultramafic substrates with elevated heavy metal contents, suggesting potential for phytoremediation and other green technologies, including phytomining. There are currently few studies on its capabilities, due to constant revisions of its members, although there is now a start on the investigation of such capabilities. In determining the extent of the accumulation capabilities of *Timonius* species collected in the area, they would then be able to be determined for their potential in contributing to phytoremediation efforts. Phytoremediation still has a high potential as a method of remediation and as a field of study to be used as a model for future technologies.

F. METHODOLOGY

The methodology of this study will mainly be based on the original research, as the objective of this study will be the analysis of the hyperaccumulation abilities of *Timonius* spp. found in Mt. Guiting-guiting, Philippines.

Sampling — Plant collection for this study will be conducted on Sibuyan Island, Philippines, an ultramafic site that was not included in the original study. An initial analysis of the soil will be done before the extraction of *Timonius* spp.. Once soil samples are confirmed to be ultramafic, the plant samples collected will also be analyzed molecularly to confirm the identity of *Timonius* spp. since these plants are very diverse. The sampling procedure will be further elaborated in the following sections.

Sites and Permit Application. Once the thesis proposal has been approved, the group will start to collect the forms and permits for collection, as well as the laboratory services that will be used for this study. The target site for this study would be Mt. Guiting-guiting, Sibuyan, due to the confirmed presence of *Timonius* species in the area. The group will ask for the guidance of Prof. Cecilia Moran for the copies of the permits that they had used in prior studies at the said location. As for the laboratory services, the group will contact Dr. Albano from the Department of Chemistry to inquire whether the needed bulbs for the Flame Atomic Absorption Spectroscopy are available; a back-up laboratory facility will also be contacted if the required bulbs are not available.

Soil Collection. The soil samples, where the plant species are found, that will be collected will be at least 1 kilogram, which will be stored in plastic containers before they are subjected to soil digestion. These samples will be done in triplicates. For this study, the group opts to test the pH prior to collection to determine whether or not the soil sample would be acidic, a common characteristic of ultramafic soil. Once confirmed acidic, the samples that will be taken must be collected at least 10-60 cm in depth with loose organic materials removed.

Plant Collection. *Timonius* spp. will be collected by the group at the selected site on Sibuyan Island mentioned above. At least 0.5 grams of plant parts (including leaves, stems, and roots) will be collected, and triplicates will also be collected for each individual sample to ensure reliability and accuracy for the statistics that will be conducted. Plant samples will be collected from September 2023 - October 2023. Once the plant samples have been collected, these will then be replaced by the researchers by replanting seeds of the species in the same area, to ensure that the population would remain the same or proliferate even further in the future.

Extraction — The mineral analysis will be performed by first extracting the plant tissues (leaves, stems, and roots) and soil samples using dry ashing and wet digestion methods (Tokalıoğlu & Kartal, 2004). The extracted samples will then be subjected to inductively coupled plasma optical emission spectrometry (ICP-OES) to determine the concentration of three minerals of interest, namely copper (Cu), nickel (Ni), and manganese (Mn) (Yener, 2019). Prior to digestion, glassware and equipment will be cleaned and rinsed with a 10% nitric acid solution to minimize contamination. All experiments will be carried out in triplicates to ensure the accuracy of the results.

0.5 grams of leaves, stems, and roots from selected *Timonius* samples will be ground into powder to produce a homogenous mixture and will be measured using a digital analytical balance (Van Der Ent et al., 2015). The mass of the grounded samples will be transferred into 30 mL silica crucibles and placed in a muffled place and at an increased temperature of 4708°C within 1 to 16 hours. The residue sample will be dissolved in a 5 mL solution of 20% HCL acid, then filtered through a Whatman filter paper into a 25 mL volumetric flask, with the addition of deionized water to create the solution (Kalra, 1998). Subsequently, the contents will be heated on a hot plate to facilitate the dissolution of the ash (Mills, 1996).

In the process of analyzing plant, foliar, and leaf litter samples, a fraction of the sample was crushed and ground prior to undergoing wet digestion. The specific volume of HNO3 (65%) used for digestion was determined based on the sample mass, following a similar methodology outlined by Ishak et al. (2015). The digestion process involved gentle boiling of the mixture over a hot plate at 90˚C for 1-2 hours, with intermittent swirling and covering using a watch glass. Additional HNO_3 (65%) was added until digestion was complete. The resulting digested sample was transferred to a 50-mL volumetric flask using a funnel equipped with filter paper, and the volume was adjusted to 50 mL with deionized water. The digested samples were then sealed and stored in the refrigerator for subsequent analysis using flame atomic absorption spectrometry (FAAS). It should be noted that complete avoidance of foliar sample contamination with soil particulates, especially in the case of ground herbs, is challenging. Elevated concentrations of Fe and Cr in foliar samples may serve as indicators of potential soil contamination, as these elements are prominent constituents of ultramafic soils (Van Der Ent et al., 2015).

In soil sample analysis, the collected samples are prepared in several steps. First, the samples are dried at 100°C for 2 hours in an electric oven. Then, the dried samples are ground and sieved using a fine mesh with a 75 µm pore size. Next, a mixture of 150 mL HCl and 5 mL HNO_3 is added per 1 g of sample in a container, which is heated in a sand bath for 60 minutes. After cooling, a solution of 5 mL HCl and 50 mL distilled water is used to wash the container's sides, followed by boiling the mixture for 3 minutes. The resulting mixture is filtered and transferred to 100 mL containers. These prepared samples are stored at 4°C until further analysis using Flame Atomic Absorption Spectroscopy (Al-Hamzawi & Al-Gharabi, 2019).

Flame Atomic Absorption Spectroscopy – In quantifying the amount of heavy metal present in the sample, Flame Atomic Absorption Spectroscopy (FAAS) will be used. The concentration of metal ions in a sample can be determined using the flame atomic absorption spectroscopy method. FAAS would be the best method of choice for plant samples that will be collected, as "flame atomization is the method of choice if our samples contain 1–10 mg Zn^{2+}/L" (Harvey, 2015). For the sample preparation, the sample will be dissolved in an appropriate solvent. After that, the sample solution is filtered to get rid of any contaminants present.

Before performing FAAS on collected samples, a calibration curve must be made using standardized solutions with known concentrations of the element is focused, and the results of the standardized solutions will be collected to be used as a reference for the samples. The next phase would be the atomization of the unknown samples, as described by Moldovan (2018), the process would involve drawing the sample solution into a narrow tube, transferring it to the nebulizer wherein the solution is broken down to a smaller form of mist or aerosol. Subsequently, the aerosolized sample is transported to the flame through the assistance of a carrier gas, breaking up the aerosolized sample into separate atoms.

For the light source, FAAS makes use of a hollow cathode lamp. The element being studied is present in the lamp, which also emits light at a particular wavelength that is unique to that element. The light that has been released is subsequently directed through a monochromator. Only the wavelength of interest can pass through the monochromator and reach the detector, which will then be measured. The output of the detector is proportional to the element's concentration in the sample. The results will be placed into MS Excel along with the results of the standard solutions. Data analysis is the last phase, which is the analysis of the results of the detector. To determine the concentration of the element within the sample, the results can be compared to the calibration curve. Results are expressed in parts per million (ppm) or parts per billion (ppb). This process will be repeated for each of the elements to be detected within the sample (Cu, Ni, and Mn), although mercury will not be analyzed due to health risks. The results will be tabulated per species, comparing the heavy metal concentrations found in each part of the plant (leaves, shoot, and root). The results of the FAAS will be inputted into MS Excel for statistical analysis.

Bioconcentration Factor and Tolerance Factor – The Bioconcentration Factor (BCF) and the Tolerance Factor (TF) represent the compatibility of the plant and the metal it was able to uptake from its surroundings. BCF measures the ability of a substance, such as heavy metals, to accumulate in the tissues of organisms such as plants. It is the ratio of the concentration of a chemical in the tissue of an organism to the concentration of the chemical in the surrounding environment. It can be measured by each plant part, and it can be measured with the following equation (Mishra & Vimal Chandra Pandey, 2019):

$$BCF = \frac{Metal(plantpart)}{Metal(substrate)}$$

Equation 1. Bioconcentration Factor

A high BCF value indicates that the substance has a high potential to accumulate in the tissues of the organism, which may be attributed to either how well the substance accumulates or how well the organism allows the substance to accumulate within it.

The Tolerance Factor is indicated by the Translocation Factor, as the translocation value determines how well the plant is able to tolerate the metal it has within the body. The faster it is able to be translocated within the body, the better the tolerance which is important to determine the plant's ability to move heavy metals within its tissues. Most especially it is to locate where the heavy metals accumulate the most within the plant's tissues, based on where specifically the concentration is high in the different tissues. In this study, it will be the comparison of the sample's leaves, shoots, and roots. It is calculated as the ratio of the heavy metal concentration of the plant's shoots or leaves to the heavy metal concentration in the plant's soil or roots (Singh et al., 2011).

$$TF = \frac{Concentration\ (plant\ part)}{Concentration\ (root)}$$

Equation 2. Translocation Factor

The TF compares the concentration of heavy metals in the different parts of the plant to the heavy metal concentration found in the roots. Determining where the plant translocates/accumulates the heavy metals absorbed, differentiating hyperaccumulators from non-hyperaccumulators. (Zakaria et al., 2021). The value will be calculated for root-stem and root-leaf for each species collected, and will then be tabulated in Excel, to be statistically analyzed.

Data Analysis — Statistical analysis would be performed at the end of the study, specifically posthoc one-way ANOVA, as used in the original study. Graphs for each species collected based on the parameters of heavy metal concentration, BCF, and TF will be done, for each part of the plant. The programs to be used will be R Studio and SPSS.

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III. GANTT CHART

Major Activity/ Tasks	2023							2024		
	JUN	JUL	AUG	SEP	OCT	NOV	DEC	JAN	FEB	MAR
Finalizing of Thesis Proposal	■									
Permit Application	■	■								
Permit Approval		■	■							
Sample Collection			■	■	■					
Sample Digestion					■	■				
Sample Analysis						■	■	■		
Statistical Analysis								■	■	
Finalizing Thesis Paper									■	■

IV. BUDGETARY REQUIREMENTS

	REQUIREMENTS	**BUDGET**
Permits	Application & Processing	P10,000
Sample Collection/ Travel Fees	Transportation	P20,000
	Accommodation	P15,000
	Materials	P10,000
Laboratory	Materials	P10,000
TOTAL		P65,000