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# Changes in the abundance of Actinobacteria and Proteobacteria in soils impacted by mining activities

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**ABSTRACT**

Data on soil bacterial abundances can provide early signs of soil alterations and can be used in the rehabilitation programs of former mining sites. In this study, we used catalyzed reporter deposition-fluorescence *in-situ* hybridization (CARD-FISH) to assess specific bacterial abundances in soils collected from sites with different levels of mining disturbance. Using specific oligonucleotide probes, more than 70% of the microbial biomass in forest sites belonged to the Domain Bacteria but less than 53% in the mined-out and quarry sites. Phylum Actinobacteria and Classes Gamma- and Delta-Proteobacteria counts were also shown to be three (3) to six (6) times higher in the forest than in the mined-out and quarry site with Phylum Actinobacteria showing higher abundance in the forest sites while Delta-Proteobacteria dominated in the mining-disturbed areas. All bacterial variables are positively correlated with organic matter, nitrogen and phosphorus, suggesting control of bacterial abundance by nutrients; but is negatively correlated with soil pH. Given the large spatial and temporal variations in soil microbial diversity our results provided an insight into the dynamics of soil bacteria in undisturbed and mining-disturbed soil that can be used in the proper design and management of rehabilitation programs for mining-disturbed areas.

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## INTRODUCTION

Bacteria are numerically and functionally important components of soil ecosystem, mediating various processes such as the mineralization of organic compounds (Gahan and Schmalenberger 2014), soil aggregation (Forster 1990), plant growth promotion (Hayat et al. 2010) and plant disease suppression (Köberl et al. 2017). The important roles of microorganisms in soil processes emphasized the need to understand how their diversity and abundance are impacted by environmental and anthropogenic disturbances. The ability to predict microbial responses to disturbances will help in the formulation of proper design and implementation of soil rehabilitation and restoration programs in post-mining environments.

A variety of biotic and abiotic factors, both natural and anthropogenic, are known to influence bacterial abundance and

**KEYWORDS**

CARD-FISH, mining impacts, Actinobacteria, soil bacteria, Province of Dinagat Islands, soil rehabilitation

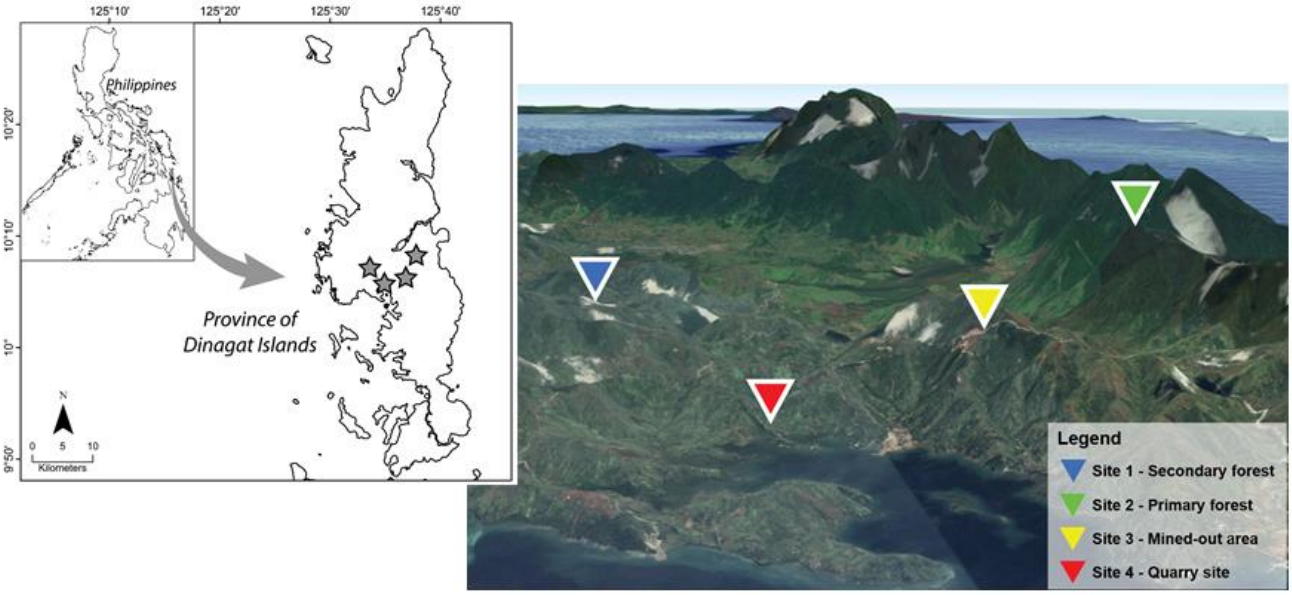



**Figure 1: Sampling sites in the Province of Dinagat Islands, Philippines**

diversity. The physico-chemical characteristics of soil, particularly pH, commonly controls microbial counts (Kuramae et al. 2012) but other factors are also significant including co-existing plants species (Nwuche and Ugoji 2010, Llamado et al. 2013), the presence of other microorganisms such as fungi and other bacteria (Boer et al. 2005, Seneviratne et al. 2015) and soil tillage conditions (Mangalassery et al. 2015, Nivelle et al. 2016). Similarly, pests and diseases are important causes of widespread death of forest vegetation although abiotic factors, such as forest fires or passage of strong typhoons, have greater impact in microbial diversity and abundance. Studies have shown that abiotic events can cause large scale disturbance in soil and removal of organic carbon resulting to significant changes in the microbial biomass and diversity (Smith et al. 2008, Holden and Treseder 2013).

The extraction of minerals from the land is one of the human activities that cause large-scale disruption of the environment because of the sheer volume of earth materials that are involved in the operation. After the removal of vegetation, top soil is extracted which are then stockpiled for future backfill and reclamation of the site (Haldar 2013, Wildman 2015). Drastic changes occur on the physico-chemical and biological conditions of the stockpiled soil and the mined area, the magnitude of which is dependent on the time that lapsed from removal to reclamation (Abdul-Kareem and McRae 1984). A negative relationship between the microbial abundance and the age of the stockpiled soil was seen in studies including lower concentrations of inorganic nutrient and organic carbon and lower microbial population (Ghose 2004). The decline in microbial diversity and abundance in post-mined sites were also found to be irreversible (Quadros et al. 2016).

But even with the recognized role of bacteria in soil processes, its study is limited by the difficulty of culturing these microorganisms in the laboratory. The recent development of deoxyribonucleic acid (DNA) based methods of analysis, such as polymerase chain reaction (PCR) and gene sequencing, circumvented the problem and allowed a more comprehensive study of the soil bacterial diversity. Studies using these methods revealed high microbial diversity in soil with the dominant bacterial groups differing among diverse soils types and at several spatial scales (O'Brien et al. 2016). Phylum Proteobacteria with its classes, Alpha-Gamma-and Delta-Proteobacteria dominated in tall grass prairies (Spain et al. 2009, Ushio et al. 2013) and in grassland of different land type use (Will et al. 2010). In forest ecosystems, Acidobacteria, Proteobacteria (Alpha-, Beta-and Gamma-Proteobacteria), Actinobacteria and Bacteroidetes are the dominant phyla with phylotypes richness significantly correlated with pH (Lauber et al. 2009). It was also revealed that the bacterial groups were highly correlated with specific soil factors explaining the differences in dominant bacterial communities among land use types and farming conditions (Gömöryová et al. 2019, Suzuki et al. 2019). The need to study the bacterial diversity in specific habitats is therefore necessary for the complete understanding of ecosystem responses to human activities.

In this study, we used catalyzed-reporter deposition fluorescence *in situ* hybridization (CARD-FISH), to determine the abundances of specific groups of bacteria in soils that are disturbed by mining activities. Although PCR-based analysis offers a more comprehensive study of the bacterial diversity, CARD-FISH allow the detection of individual bacterial group, to investigate their responses to environmental controls (Pernthaler et al. 2002; Austria et al. 2019). Oligonucleotide probes that specifically target the Domain Bacteria (EUB 338), Class Gamma-Proteobacteria (GAM42a), Class Delta-Proteobacteria (DEL) and Phylum Actinobacteria (HGC69A) were used to compare the abundances of these microorganisms in soils subjected to varying levels of human disturbance (secondary forest, primary forest, decommissioned mine site and active quarry site). The aim is to identify the influence of mining activities on the physico-chemical characteristics of the soil and consequently on the dynamics of soil microorganisms. Findings from this study can provide preliminary data on the impacts on mining on bacterial abundances in the study area which can be used in the design of rehabilitation programs for abandoned mining sites.

## MATERIALS AND METHODS

### Site Description and Sampling Protocol

This study was conducted in the Province of Dinagat Islands located in the southern part of the Philippines (Figure 1; 10.1282° N, 125.6095° E). The area is classified as climatic Type II where there is no dry season and with a very pronounced maximum rain period from December to February. Soil sampling was done in October 2017, February and April, 2018 which are the periods when weather conditions allowed field collection. The province has a total land area of 139.9 km² and

is characterized by a rolling and mountainous terrain. Temperature in the study area ranged from 21.1 to 35˚C with highest temperature seen in August which is also the driest month. The soil environment in Dinagat Islands is characterized by an ultramafic outcrop that is rich in chromite deposits with soil pH ranging from 4.8 to 6.9, average organic matter (OM) content of 2.25% and phosphorus (P) content ranging from 1 to 37 ppm (Baker et al. 1992; Lillo et al. 2019). Nickel and chromite mining is a main industry in the area.

**Sampling and Laboratory Analysis of Soil**

Sample collection followed the stipulations in the sampling permit (Wildlife Gratuitous Permit No. R13-2018-18) issued by the Department of Environment and Natural Resources (DENR) Regional Office XIII. Four (4) sites were chosen from the study area which represented different levels of disturbance. Site 1 (10º 07' 0.58" N; 125°33'22.03" E) is a secondary forest dominated by ferns, grasses and small trees and is located on the edge of a former mining area. Site 2 (10°06' 00.5"N; 125° 34' 35.0"E) is a primary forest that is generally undisturbed by mining activities but with a few minor logging activities. Site 3 (10° 07' 26.6"N; 125° 37' 15.9"E) is a former mine site that has been inactive for 2 years while Site 4 (10º 5' 22.37"N; 125º 34' 24.18"E) is an active quarry site. Soil samples were collected for the analysis of physico-chemical characteristics (moisture, pH, % OM, nitrogen or N, P, potassium or K) and bacterial abundance. Three sampling plots that were around 20 to 50 m apart were chosen randomly for Sites 1, 2 and 3, In Site 4, samples were taken from 4 plots with different levels of disturbance. Plot 1 is located in an undisturbed area of the quarry site with its existing vegetation. Plot 2 is located in the exposed side wall of the quarry site while Plot 3 is from the area of the quarry where the topsoil has been removed. Soils from Plot 4 were taken from the topsoil that has been removed (less than a year) and stockpiled along the side of the quarry site and still bearing some of its vegetation.

From each of the sampling plots (a total of 13 plots from the 4 sampling sites), triplicate soil samples (around 1 kg each) were collected from the area 10 cm below the surface, cleaned of large plant materials and rocks, pooled together before it is placed in zip lock freezer bags and stored on ice for transport. All samples were processed and preserved in the field laboratory within the day of collection. The preserved soil samples were transported to the Biology Research Laboratory of Adamson University, Manila, Philippines for processing and analysis of bacterial abundance. At the same time, adequate amount of the same soil samples was brought to the Department of Agriculture Caraga Region, Philippines for the analysis of physico-chemical parameters, nitrogen (N, Kjeldahl method), phosphorus (P; Vanadomolybdate method) potassium (K; Microwave Plasma-Atomic Emission Spectrometer) and organic matter (OM) content. Other physico-chemical parameters such as pH, moisture and aggregate stability were measured at the Materials Science and Polymer Chemistry Laboratory in Caraga State University, Butuan City, Philippines. It must be noted that soil samples from Site 4 were not analyzed for percent OM and percent moisture due to some logistical reasons.

**Analysis of Bacterial Abundance**

Analysis of soil samples for bacterial abundance using CARD-FISH followed the procedure by Ushio et al. (2013) with some modifications. Table 1 shows the description of the oligonucleotide probes used in this study, including the bacterial groups covered by the probe and the gene sequence (Alm et al. 1996). Briefly, 0.5 g of the pooled soil samples was transferred in a 2 ml centrifuge tube and 400 µl 20 % paraformaldehyde (PFA; final concentration 2-4%) was added to fix the sample. Afterwards, the tube was filled with phosphate buffer solution

**Table 1: Description of the oligonucleotide probes used in the study. Probe designation according to *Alm et al. (1996)*.**

| Probe | Coverage | Oligonucleotide sequence |
|---|---|---|
| EUB 338 | Domain Bacteria except *Planctomycetales* and *Verrucomicrobia* | *GCTGCCTCCCGTAGGAGT* |
| GAM42A | Gamma-Proteobacteria | *GCCTTCCCACATCGTTT* |
| DELTA495a | Delta-Proteobacteria | *AGTTAGCCGGTGCTTCCT* |
| HGC69A | mostly Actinobacteria | *TATAGTTACCACCGCCGT* |

(PBS) up to 2 ml, homogenized completely and stored overnight at 4 °C. After the overnight fixation. the samples were washed twice with 1 x PBS, centrifuged at 10,000 rpm for 5 min at 4°C and washed with 1:1 PBS/Ethanol. The samples are now ready for processing or storage at -20˚C. During the analysis, one hundred (100) µl of the fixed sample is diluted with 900 µl of PBS/ethanol and dispersed by ultrasonic dispersion (sonicator) at minimum power for 30 s. Twenty (20 µl) of the dispersed sample is diluted in 10 ml of sterilized distilled water and the suspension was slowly filtered onto a Nucleopore filter (0.2 µm pore size, 25 mm in diameter) using vacuum filtration. After filtration, the filters were dipped in 0.2 % low melting point agarose and dried in an incubator at 37˚ C. The agarose-embedded filters were placed in a sealed Petri dish and incubated in a lysozyme solution (10 mg lysozyme, 100 µl 0.5 M ethylenediaminetetraacetic acid (EDTA; pH 8.0), 100 µl 1 M Tris-hydrochloride (pH8.0), 800 of sterilized water) at 37℃ for 1 h. After washing with alcohol, filters were dried and processed or stored at -20 °C for further processing. The filters were divided into sections and placed in a hybridization buffer (3.6 ml 5 M NaCl, 0.4ml 0.1 M Tris-HCl, 20 µl 20% (w/v) SDS, *x* ml deionized H2O, *x* ml 30-50% formamide concentrations, 2.0 mL 10% (w/v) blocking reagent). Add 2.0 g of dextran sulphate which contains the 15 µl horse radish peroxidase-labelled probe (concentration at 50 ng $\mu l^{-1}$). Hybridization was carried out by incubating the filters at 35 ˚C for 12 – 15 h. Signal was amplified by placing the filters in a mixture of amplification buffer (2 mL 20X PBS, 0.4 mL 10% (w/v) blocking reagent, 16 mL 5 M NaCl, 4 g dextran sulphate, sterile deionized H2O) and tyramine hydrochloride conjugates (100 µl $Alexa_{488}$, 25.2 µl tyramine hydrochloride, 874.8 absolute ethanol) for 10 to 45 min at room temperature (30-35 ºC). After washing with deionized water to remove the excess buffer, filters were mounted onto glass slides with 4′, 6-diamidino-2-phenylindole (DAPI) mix and observed under epifluorescence microscope (Zeiss Axio Scope A; 100x magnification). Total microbial abundance was determined by counting the cells that took up the DAPI stain (blue signals) while specific bacterial abundances are determined by counting the cells that took up the probes (green signals). All samples were analyzed in triplicates.

**Data Analysis**

Bacterial counts were log transformed before statistical analysis to minimize the variation in the data. The differences in the bacterial counts and in environmental parameters among study sites were determined using Analysis of Variance (ANOVA; PASW version 18). On the other hand, the correlations among the bacterial and environmental variables were determined by Canonical Correlations Analysis using XLSTAT 2019.

## RESULTS AND DISCUSSIONS

**A. Soil characteristics:**

All soil samples are of clay nature and all have stable soil aggregates. The soil moisture content of the three sites ranged from 4.25 to 6.80 % and no significant difference among sites were found. However, sampling sites differ on some of the soil

**Table 2: Soil properties of the four sampling sites with different levels of disturbances.** The data is presented as mean of 4 to 5 replicates ± standard deviation. Units are shown in parentheses. (*) indicates significant differences among sites (ANOVA, p <0.05). NE is not examined. Same superscript indicate no significant difference between values.

| Sampling site | Moisture Content (%) | pH* | OM* (%) | N* (g $kg^{-1}$) | P* (g $kg^{-1}$) | K (g $kg^{-1}$) |
|---|---|---|---|---|---|---|
| Site 1 | 5.00 ± 1.02 | 5.46[a] | 3.2 ± 0.59 | 2.42 ± 1.31 | 0.28 ± 0.08[c] | 0.26 ± 0.07 |
| Site 2 | 4.71 ± 0.46 | 5.20[a] | 3.14 ± 1.74 | 1.90 ± 0.95 | 0.45 ± 0.12 | 0.37 ± 0.05 |
| Site 3 | 4.25 ± 0.40 | 6.66[b] | 1.97 ± 0.64 | 0.35 ± 0.19 | 0.22 ± 0.02[c] | 0.39 ± 0.08 |
| Site 4 | NE | 6.32[b] | NE | 1.00 ± 0.91 | 0.24 ± 0.10[c] | 0.51 ± 0.29 |

physico-chemical characteristics (Table 2). Soil pH was variable, ranging from 4.13 to 7.11, with the lowest pH recorded from the primary forest site and the highest from the quarry site. Statistical analysis revealed significant differences in the average pH with the more acidic pH seen in forested areas (5.46 and 5.20 mean pH, Site 1 and Site 2, respectively). The lower pH detected in the forested sites compared with the disturbed sites is consistent with observed acidic nature of soils in other forest ecosystems which was due to the removal of base cations from the surface soil secondary to leaching and uptake by trees (Fujii et al. 2018).

Analysis of the OM content (% OM), a parameter generally used to characterize soil quality, revealed that the values are within those reported for forest soils, that is, generally at 1 to 5 % OM (Osman 2013). The highest % OM was detected in the primary forest area (6.0%), while the other sites contain moderate levels of organic matter and were near the value of 2.25% that was previously reported in the study area (Lillo et al. 2019). The forested sites showed significantly higher soil OM content ($p < 0.05$; mean ± SD: 3.20 ± 0.59 and 3.14 ± 1.74 % OM, Site 1 and Site 2 respectively) than the mined-out site (Site 3; mean ± SD: 1.97 ± 0.64 % OM). Nitrogen, phosphorus and potassium contents (NPK) were detected in all sites (detection limit: 0.01 g $kg^{-1}$) but significant differences were detected only in the total nitrogen and phosphorus content of the soil. The forested sites (Site 1 and 2) have significantly higher total N content ($p < 0.01$) than sites 3 and 4. Phosphorus concentration is low (range: 0.20 to 0.45 g $kg^{-1}$) but the primary forest contained a significantly higher phosphorus than the other study sites ($p < 0.01$). There are no significant differences in the potassium levels among the sites.

**B. Differences in the total microbial and bacterial abundance among sites**

Significant differences were detected in the total microbial abundance and the dominant bacterial group in soils with different levels of disturbances by mining activities. Changes in the soil microbial biomass as a consequence of disturbance have been shown in previous studies using other DNA based methods (Daniel 2005), but this is the first study in the Philippines to use the nucleic acid probe-based method CARD-FISH to determine the impact of mining activities on the abundances of Domain Bacteria, Phylum Actinobacteria, Class Gamma-Proteobacteria and Class Delta-Proteobacteria.

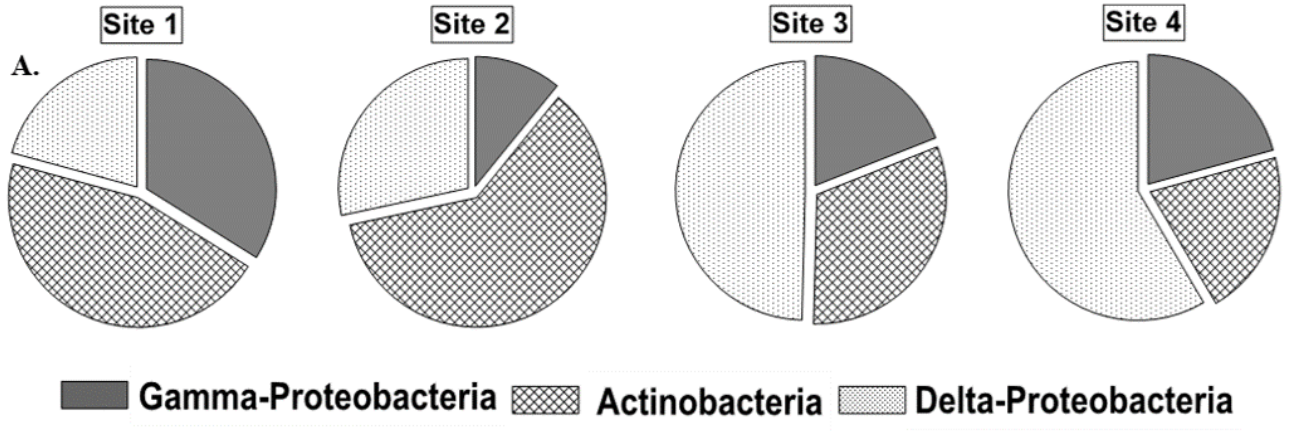
Total microbial abundance (Table 3), revealed by DAPI staining, was significantly different among sites (ANOVA; p value < 0.01). Microbial abundances in soil samples taken from mined-out site (mean ± SD = 10.9 ± 0.55 x $10^9$ cells $g^{-1}$) is lower by 57% than the microbial abundance in soils collected from the primary forest (mean ± SD = 25.60 ± 4.20 x $10^9$ cells $g^{-1}$). The total microbial count declined as the level of disturbance increased, that is, there is decreasing microbial abundance as one goes from secondary forest to primary forest to active quarry site to mined-out site. It is noteworthy that the inverse relationship between the total microbial abundance and disturbance was also observed in soils samples collected from the active quarry site (Site 4). Microbial counts (ANOVA, $p < 0.05$) were significantly lower in the more disturbed plots and there is decreasing microbial abundance as one goes from Plot 4 (stockpiled soil that still carries the original vegetation; mean ± SD = 21.01 ± 1.71 x $10^9$ cells $g^{-1}$) to Plot 1 (undisturbed portion of the quarry site; mean ± SD = 17.85 ± 2.26 x $10^9$ cells $g^{-1}$) to Plot 2 (exposed side of the mountain; mean ± SD = 11.45 ± 0.33 x $10^9$ cells $g^{-1}$) to Plot 3 (quarry site where rocks are being mined; mean ± SD = 8.16 ± 1.15 x $10^9$ cells $g^{-1}$).

Total bacterial abundance, revealed by the EUB338 count, ranges from 52 to 79 % of the DAPI count suggesting that bacteria is a significant component of the microbial biomass in the study area (Table 2). The mined-out site showed the lowest percentage of bacterial abundance (52 %), followed by the quarry site (53 %) while the forested sites, Site 1 and 2 showed the highest percentages, 79 and 70 % the total microbial abundance, respectively. Results suggest that bacteria are the dominant microorganism in undisturbed areas and the lower bacterial contribution to the total microbial population in disturbed areas suggests a higher contribution of other organisms. As bacteria are known to be more susceptible to environmental stressors than other microbial communities (de Vries et al. 2006; Sun et al. 2017) shifts in the dominant microorganism may have occurred after the disturbance (Kane et al, 2020). Shifts in fungal and bacterial importance has been observed along soil pH gradient (Rousk et al, 2010), while nitrogen fertilization reduces the fungi-bacterial ratio due to the lower nutrient demand of fungi than bacteria (Demoling et al. 2008, Zechmeister-Boltenstern et al, 2016). In forest ecosystems the decrease in the relative abundance of fungi with increasing soil fertility was shown to be due to the reduction the amount of ectomycorrhizal hyphae caused by high nitrogen concentration (Pennanen et al, 1999; Nillson et al, 2007).

The shifts in the dominant microorganisms, however may have important consequences to the soil fertility. While fungi and bacteria are the main decomposers of organic compounds in soil, changes in fungal-to-bacterial biomass ratio can have important implications to the energy flow because bacteria are shown to

**Table 3: Total microbial abundances (revealed by DAPI staining) of soil samples taken from the four sampling sites.** Microbial abundance of the 4 plots in Site 4 are enumerated and the average abundance is indicated. % Microbial population that took up EUB338 Probe was computed as EUB 338-positive cells/DAPI-stained cells x 100. Abundance is at x$10^9$ cells $g^{-1}$

| | DAPI stained cells | EUB338-positive cells | % Microbial Population that took up EUB338 Probe |
|---|---|---|---|
| Site 1 | 20.88 ± 2.6 | 14.40 ± 0.35 | 70 |
| Site 2 | 25.60 ± 4.2 | 20.22 ± 0.34 | 79 |
| Site 3 | 10.90 ± 0.6 | 5.67 ± 0.88 | 52 |
| Site 4 | 14.60 ± 4.9 | 7.74 ± 0.51 | 53 |




| B. | Site 1* | Site 2* | Site 3 | Site 4* |
|---|---|---|---|---|
| Gamma-Proteobacteria | 1.77 ± 0.19 | 0.49 ± 0.03 | 0.35 ± 0.13 | 0.32 ± 0.05 |
| Actinobacteria | **2.66 ± 0.70** | **2.72 ± 1.29** | 0.57 ± 0.13 | 0.48 ± 0.13 |
| Delta-Proteobacteria | 1.22 ± 0.23 | 1.27 ± 0.31 | 0.90 ± 0.22 | **0.89 ± 0.13** |

**Figure 2: A) Percent contribution of each bacterial group to the total number of bacteria that hybridized with the three (3) probes. B) Per site abundances (x $10^9$cells $g^{-1}$) of bacteria that bind with specific oligonucleotide probes. (*) indicates significant differences in the abundances of bacteria (ANOVA, p < 0.05). Numbers in bold are significantly higher than the other counts (Post-Hoc; Tukey's b)**

contribute more to energy flow in the terrestrial systems (Wang et al. 2019). In addition, bacteria (and some Archaea), and not fungi can fix nitrogen from the atmosphere further emphasizing the important role of bacteria in soil nutrient cycling. Within bacterial groups, ecological function varies necessitating the identification of major bacterial group in the specific soil environment.

**C. Changes in the dominant bacterial group**

The abundances of the specific bacterial groups investigated in this study differ among sampling sites (Figure 2A). Phylum Actinobacteria is significantly more abundant than Gamma- and Delta-Proteobacteria in the two forested sites (Figure 2B; mean ± SD: 2.66 ± 0.70 and 2.72 ± 1.29 x $10^9$ cells $g^{-1}$ of soil, Site 1 and Site 2, respectively) but has lower counts than the two bacterial groups in the more disturbed sites. The counts represented 47 and 61% (secondary and primary forest, respectively) of the total number of bacteria that hybridized with the three (3) RNA probes. On the other hand, Class Delta-Proteobacteria (mean ± SD: 0.90 ± 0.22 and 0.89 ± 0.13 x $10^9$ cells $g^{-1}$ of soil, mined-out and quarry site, respectively) dominated over Phylum Actinobacteria and Class Gamma-Proteobacteria in the more disturbed sites. The counts represented 50 and 58 % of the total probe-positive bacteria in the mined-out and quarry site, respectively.

Among the many bacterial groups that are detected by previous studies using PCR and gene sequencing of soil bacteria, Phylum Actinobacteria and Proteobacteria are among the most frequently detected bacteria in soil (Axelrood et al. 2002; Rojas et al. 2016). As an important mediator of plant litter decay, the highly diverse Phylum Actinobacteria are detected in a range of soils and land uses including uncultivated forests, cultivated soils, pasture areas and even street dust. The detection of this bacteria from a wide variety of media led to the common knowledge that these microorganisms are controlled and encouraged by human and animal activities (Hill et al. 2011). The presence of vegetation and animals in the forested areas may have allowed the dominance of Actinobacteria in undisturbed sites. It must also be noted that compared with the disturbed areas, forested areas have high organic matter content which were previously shown to determine the actinobacterial community structure (Hill et al. 2011; Singh et al. 2013).

Delta-Proteobacteria was detected in all the study sites, but while Actinobacteria have significantly lower counts in the mined-out and quarry sites, Delta-Proteobacteria appeared to be have a more consistent numbers in the four study sites (Figure 2B). The higher counts of Delta-Proteobacteria in disturbed areas may be due to the ability of this group of bacteria to thrive in conditions that supported limited metabolic reaction (Baker and Banfield 2003). As known sulfate and iron reducers, they are among the microorganisms commonly encountered in acid mine drainage habitats (Méndez-García et al. 2015) where they transform sulfuric acid to hydrogen sulfide and generate alkalinity (Ayangbenro et al. 2018). Their use as a bioindicator of degraded soils or as bioremediation agent for contaminated soils therefore, warrants further investigation.

Canonical correlations analysis of bacterial and environmental variables revealed that the environmental variables explain 66.67 % of the variability in the bacterial variables. The microbial abundances were found to be positively correlated with nitrogen and organic matter, but is negatively correlated with pH and potassium (Figure 3) suggesting that these factors are the significant determinants of microbial abundances in the area. Soil pH tend to increase after mining activity (Prematuri et al, 2020), mining therefore can have indirect effect on microbial abundances by its effect on the soil pH. The results of this study, however, is in contrast with other studies, where soil pH is positively correlated with bacterial abundances (Rousk et al, 2009). This may be due to the individual response of each taxa as several studies have shown that bacterial groups respond differently to soil pH (Wu et al, 2017). Soil pH is positively correlated with the abundance of Actinobacteria but is negatively correlated with Proteobacteria (Wang et al, 2019). This was also shown in a continental scale study, where the relative abundances of Actinobacteria and Bacteroides are positively correlated with soil pH while Acidobacteria is negatively correlated with soil pH (Lauber et al, 2009). The

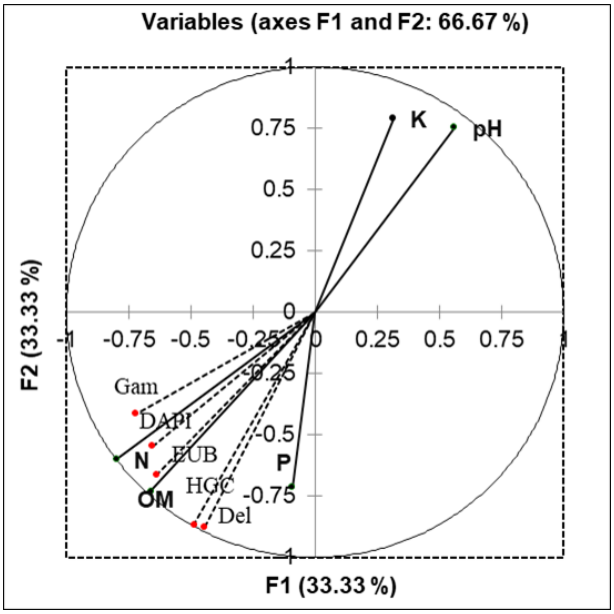



**Figure 3: Canonical correlation analysis of the bacterial abundances and environmental parameters showing the relationship between the bacterial variables (DAPI: total microbial abundance; EUB: total bacterial abundance; Gam: Gamma-Proteobacteria; eHGC: Actinobacteria; DEL: Delta-Proteobacteria) and the environmental variables (N: Nitrogen, OM: Organic Matter, P: Phosphorus, K: Potassium)**

inconsistent result was also proposed to be due to co-variation of soil pH with other environ3mental variables including carbon content of the soil and moisture (Rousk et al, a2010).

It appears from the results of this study that the use of CARD-FISH method can be used to determine the dynamics of major bacterial group in soil environment and can have important use in the planning of rehabilitation programs for former mine sites. At present several methods are currently being investigated to increase the chance of success of any rehabilitation effort including increasing soil productivity by the use of bio-conditioner (Pantoja-Guerra et al, 2019), revegetation of the area (Sheoran et al, 2010; Buta et al, 2019) and fertilization (Cao et al, 2020), all with the purpose of stimulating microbial activity which provides nutrients and organic C to the soil (Sheoran et al, 2010).

In conjunction with these methods, inoculation of microbes to improve plant colonization and growth in former mining sites (Aggangan and Anarna 2019, Moreira-Grez et al. 2019) are being investigated. In these studies, the use of native soil microbiome or custom-made microbial inoculant is suggested to prevent incompatible interactions with exogenous microbes (Moreira-Grez et al. 2019). Identification of the most abundant and ubiquitous microbiota in the area that will be subjected to rehabilitation is needed because these are the microbes that are needed in the early plant colonization of nutrient-limited and post-mining areas (Wubs et al, 2016).

## CONCLUSION

The use of CARD-FISH method and specific oligonucleotide probes in our investigation revealed significant differences in the microbial biomass of disturbed and undisturbed areas which were found to be correlated with the organic matter nitrogen phosphorus concentration and pH of the soil. Another important finding of this study is the determination that disturbed and undisturbed areas are dominated by different groups of bacteria. Actinobacteria are more numerous in the forested areas while Delta-Proteobacteria is more dominant in the soils taken from mining disturbed area. The results of this study have emphasized the need to study soil microbial community to aid in the design of rehabilitation programs for former mining sites. However, because of the limited coverage of the oligonucleotide probes used in this study, it is suggested that other culture independent method such as PCR and gene sequencing should be used in conjunction with CARD-FISH to determine the total bacterial diversity of the soils impacted by mining activities.

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## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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