

Halotolerant bacteria from saline soils in Ballesteros, Cagayan, Philippines

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ABSTRACT

The coastal soils of Ballesteros, Cagayan, Philippines have gotten more saline in recent years due to anthropogenic factors like aquifer over-pumping leading to saltwater intrusion. While detrimental to agriculture in the province, this presented an opportunity to explore the possibility of bioprospecting beneficial halotolerant soil bacteria in the area that could be used for salt-tolerant crop varietal improvement. For two climactic seasons in 2019, composite soil samples (0-30 cm depth) were collected from twenty (20) sampling points, five (5) from each salinity level (Severely Saline, Moderately Saline, Slightly Saline, and Non-Saline) as determined by the 2017 BSWM Soil Salinity Map of Ballesteros, Cagayan. The soil samples were subjected to plate counts and 16S metagenomic sequencing, alongside soil textural and field pH and EC analyses. The bacterial population counts cultured in R2A-9% NaCl medium showed that the Severely Saline soil samples had counts significantly lower than the other three Salinity Levels. Meanwhile, counts in R2A-15% NaCl medium were significantly lower in Severely Saline soils collected during the wet season. Statistically higher Shannon Diversity Indices (H') were recorded during the Dry Season. Further, the sampled soils from the non-Saline level had significantly higher H' values compared to the sampled soils of the Moderately and Severely Saline levels, while having similar H' values with the Slightly Saline level, resultant of much lower salt levels making it more

hospitable to more bacterial species. Five potential halotolerant bacteria of interest were detected: *Planococcus maritimus*, *Pseudomonas fluorescens*, *Pseudomonas azotoformans*, *Bellilinea caldifistulae*, and *Ectothiorhodospira haloalkaliphila*.

INTRODUCTION

Coastal soils are characterized by their generally saline nature, the main hindrance of crop growth and productivity in those areas (Tripathi et al., 2006). Around 400,000 hectares of such soils are in the Philippines, which, while relatively small compared to other regions in the world, still has a negative effect on agricultural production of the country, but much more so to the people that depend on such areas for food and livelihood (DA-PhilRice, 2001). The coastal municipality of Ballesteros, located in the province of Cagayan, Philippines, is one such place where saline soils hinder agricultural productivity. The Bureau of Soils and Water Management (BSWM), a staff bureau of the Philippines' Department of Agriculture (DA) and the nationally mandated agency on soil and water resources management has mapped in 1999 and identified the soil salinity in the coastal province of Cagayan (FAO, 1999). The DA-BSWM (2017) has also determined increased salinity of soils due to the intrusion of saltwater, over-pumping of aquifers, and seepage of seawater in the rivers. The agency has documented changes on soil salinity in span of 18 years and noted increased extent of severity in the municipality of Ballesteros covering less than 10 ha (1999) to 823 ha (2017) of its surface soils having salinity levels greater than 8 dS/m. It has been previously documented in literature that rice can only resist at maximum of

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KEYWORDS

soil microbiology; microbial ecology; halotolerant bacteria; saline soils; bacterial community

3.0 dS/m EC in soil (Islam et al., 2020). Since Ballesteros' main land use is paddy rice, salinity has become one of the leading causes of its decreasing rice yield, ranging from 33.0% in slightly saline soils up to 58.3% on severely saline ones on average for irrigated paddies and up to 50% for non-irrigated ones during the 2015–2016 cropping season. An increasing number of studies have verified that certain halophilic (extremely salt-loving) and halotolerant (salt-tolerant) microbes can either remediate, restore, or enhance saline soils, especially in areas where common crops such as rice are planted (Arora et al., 2012; Mindari et al., 2015). Moreover, since such beneficial halophilic and halotolerant microbes are most likely found in saline soils with at least 0.2 M of soluble salts (Kushner, 1993), it is highly possible for them to be found in saline soils (Rodriguez-Valera, 1988) like in the coastal province of Ballesteros. The detection of saline-tolerant microbes in the area can improve crop varieties with higher tolerance of salt stress and productivity in coastal and saline areas. Internationally, such studies have been conducted which led to the discovery of certain strains helpful in the growth of certain crops in saline conditions (Ahmad et al., 2013; Phour & Sindhu, 2020).

The main objective of this study was to quantify, characterize, and evaluate the halotolerant bacterial community in the coastal soils of Ballesteros, Cagayan in terms of its relative abundance and diversity. This main objective attempted to fill the current research gap in pinpointing potential sites in the municipality where agriculturally beneficial soil halotolerant bacteria may be found. Specifically, the halotolerant bacterial population and community diversity was determined using both traditional plate-counting techniques and specialized molecular genetic techniques, and qualitatively correlated with the differing soil salinity levels of the area, alongside both field physical (soil texture) and chemical measurements like field pH and Electrical Conductivity (EC).

MATERIALS AND METHODS

A preliminary sampling regime was conducted during the 2018 wet season (November 5-8) to select the sampling points that can be practically traversed, to identify each selected sampling point's soil texture, to measure their initial field EC and pH, and to establish the dilutions needed for the conduct of plate count. The sampling points were determined with aid from Figure 1, which shows the 2017 DA-BSWM Soil Salinity map for the municipality of Ballesteros, Cagayan, as generated by the Geomatics and Soil Information Technology Division (GSITD) of the DA-BSWM (2017). The DA-BSWM classified the saline areas in the map based on the FAO (n.d.) criterion (Table 1). As seen in the figure, five (5) sampling points were designated from each salinity level, for a total of twenty (20) points. The areas were sampled twice in the year 2019, once during the dry season (July 2-5) and once during the wet season (October 8-11). From each sampling point, a square-shaped one (1) hectare (ha) area was identified, where composite soil samples were taken from nine (9) equidistant points at the 0-30 cm depth. The field pH/EC of the sample was determined by mixing at least 50 g of composited soil with an equivalent volume (at least 50 ml) of deionized water, making a 1:1 soil/water mixture ratio, allowed to settle for a minute, and then measured using a calibrated handheld pH/EC meter. This step was conducted only during the 2018 wet season preliminary sampling and in the 2019 dry season sampling, as drastic change in soil pH, and EC during the short-term (<10 years), especially in paddies, are not expected, regardless of its management (Behera et al., 2009; Minasny et al., 2016). The rest of the composite soil was then subsampled

for the analyses stated in Table 2. The subsamples for soil textural analysis were stored in polypropylene (PP) bags and transported to the DA-BSWM Greenhouse, Quezon City, at ambient temperature. Afterwards, the soils were air-dried and passed through a 2 mm sieve until at least a 50-g sample was collected, placed in plastic containers, and then submitted to the Soil Physics and Mineralogy Section – Soil and Water Resources Research Division (SPMS-SWRRD) Laboratory, also in DA-BSWM, Quezon City, for the particle size analysis. Much like the field soil pH/EC, this step was performed only during the preliminary sampling and in the 2019 dry season sampling because soil texture is an inherent soil property that does not change in a decade or century (Sanchez et al., 2003).

The subsamples for halotolerant bacterial plate count and 16S Metagenomic Sequencing were collected in separate freezer bags and transported in a cooler (about 10°C) to the DA-BSWM SBRMS-SWRRD Laboratory in Quezon City and stored at a -20°C freezer until analysis, following literature recommendations (ISO, 2007; Rubin et al., 2013; Akpan & Ilyasu, 2015; ISO, 2017). Subsamples for halotolerant bacteria plate count analysis were analyzed as received, with no air-drying, sieving, or any other pre-treatment. Five (5) g of soil sample was mixed with 45 ml sterile Phosphate-MgCl buffer via a rotary shaker set at 200 rpm for 10 min to make the 10^{-1} dilution. From that dilution, 5 ml was aliquoted to another 45 ml sterile Phosphate-MgCl buffer and mixed by hand for 60 seconds to make a 10^{-2} dilution. This process was repeated until a final dilution of 10^{-5} was attained, for a total of 5 separate dilution bottles. From each dilution bottle, 1 ml was pour plated into R2A agar plates amended with differing percentages of NaCl, as documented by Irshad et al. (2014): 3%, 5%, 9%, 15%, 20%, and 30%. The plates were then incubated at 25°C for 7 days and the grown bacterial colonies counted under a manual colony counter. The counts were computed using the standard formula for colony-forming units per gram dry weight (cfu/g) of soil (Zuberer, 1994). Sites that have registered no colony counts in any NaCl concentration were removed from the analysis.

Lastly, the subsamples for 16S Metagenomic Sequencing were submitted to the DNA Sequencing Core Facility Laboratory, University of the Philippines – Philippine Genome Center (UP-PGC) for 16S Metagenomic Analysis via Next Gen Sequencing (Illumina). The laboratory documented the following steps. Bacterial DNA extraction was conducted using Quick-DNA Fecal/Soil Microbe Miniprep (Zymo Research) following manufacturer's protocol. Samples that failed this extraction procedure were re-extracted using Dneasy Powersoil Pro Kit (QIAGEN), also following manufacturer recommendations. Samples that still failed this extraction process were reextracted once more using the UP-PGC in-house CTAB protocol for soil samples. All samples were eluted to 30 µl of nuclease-free water. After the DNA extraction procedures, the samples were processed following the 16S Metagenomics Sequencing Library Preparation using the following kits: 2x KAPA HiFi HotStart Ready Mix (Cat. No. KK2601), Nextera XT Indexes (Cat. No. FC-131-1001 and FC-131-1002), and Illumina custom primers with overhang targeting the 16S rRNA v3-v4 gene regions. The libraries obtained were quantified using Qubit dsDNA HS assay (Cat. No. Q32851), while the library sizes were validated using TapeStation 2200 D1000 assay kit (Cat Nos. 5067-5582, 5067-5583). The libraries obtained were normalized, pooled, denatured, and diluted to the final loading concentration of 12 pM following the standard workflow and spiked with PhiX (Cat No. FC-110-3001). Afterwards, it was loaded to Illumina MySeq using paired end v3 600 cycles sequencing reagent (Cat. No. MS-102-3001). The data was analyzed using the MiSeq

Reporter 16S Metagenomics Analysis Module version 2.6.2.3, which taxonomically classified it using the ClassifyReads algorithm (Wang et al., 2007) using the Illumina-curated version of the May 2013 Greengenes Consortium Database as reference. The ClassifyReads algorithm has the advantage of conducting the taxonomic classification with no bootstrapping, resulting in improved performance over the traditional Ribosomal Database Project (RDP) classifier (Illumina, 2014). The obtained sequence files are not yet deposited in any public archive. The relative abundances of the top 20 most abundant species were

derived by taking the total number of identified species-level reads and multiplying it to the percentage of identified reads recorded per species. For instance, if there are 1000 identified species-level reads, and Species A composes 10% of it, then Species A has 100 reads. The derived relative abundances were then collated and used as a proxy for the number of species for the sampling site's Shannon-Weaver Diversity Index (H') (Ortiz-Burgos, 2016).



Figure 1: The DA-BSWM (2017) Soil Salinity Map (0-30 cm depth) of the Cagayan Province and the designated soil sampling sites in Ballesteros, Cagayan based on the DA-BSWM (2017) Soil Salinity Map. (Legend: A – Severely Saline, B – Moderately Saline, C – Slightly Saline, D – Non-Saline) © Mapped using QGIS 3.28.6-Firenze with Google Satellite Imagery.

Table 1: Soil Salinity Classes, BSWM (2017) and FAO (n.d.)

Salt concentration of the soil water (dS/m)	Salinity Classes (DA-BSWM, 2017)	Salinity Classes (FAO, n. d.)	Effects on Crop Plants (FAO, n. d.)
0–2	Non-Saline	Non-Saline	Negligible
2–4	Slightly Saline	Slightly Saline	Yields of sensitive crops may be restricted
4–8	Moderately Saline	Moderately Saline	Yields of many crops restricted
8–16	Severely Saline	Strongly Saline	Only tolerant crops yield satisfactorily
>16		Very Strongly Saline	Only a few very tolerant crops yield satisfactorily

Table 2: Sampling Weight for each Analyses

Analysis conducted	Subsample Weight
Particle Size Analysis (done only in preliminary sampling)	≥100 g
Field EC/pH (done only in preliminary sampling and 2019 dry season)	≥50 g
Halotolerant Bacteria Plate Count	≥10 g
16S Metagenomic Sequencing (not done during preliminary sampling)	≥10 g

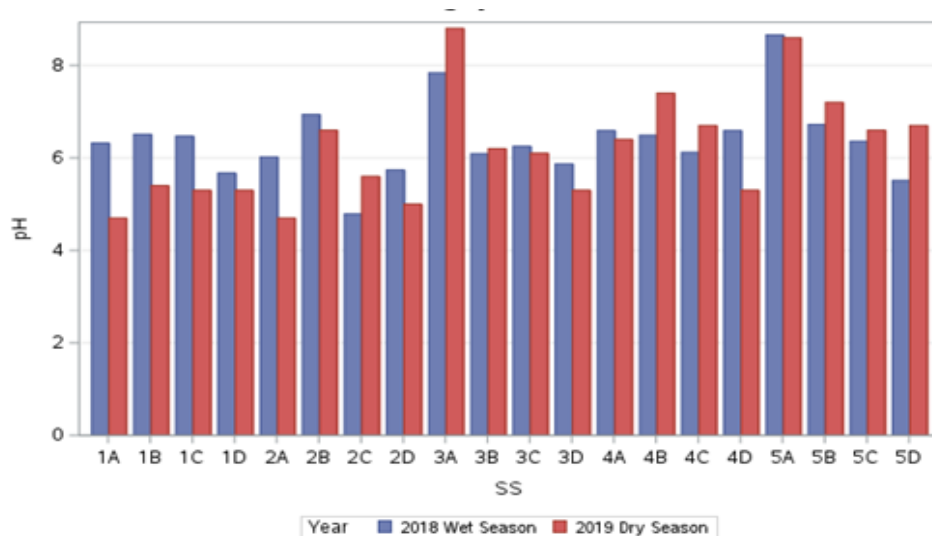
All quantitative data obtained (Field Soil pH, Field Soil EC, cfu/g, H⁺) were subjected to Two-Way ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc test. The two factors for the inferential analyses are season and salinity level. The statistical program used was the cloud-based SAS OnDemand for Academics. The cfu/g data were log-transformed to ensure the homogeneity of variances. Additionally, the Reads data obtained from the 16S Metagenomic Sequencing were also subjected to Non-Metric Multidimensional Scaling (NMS) based on Bray-Curtis Distance using the software PCORD version 7.

RESULT

Soil Particle Size and Field pH/EC

The forty (40) initially sampled Ballesteros soils (20 from 2018 wet season, 20 from 2019 dry season), were found to be predominantly clay in texture (45%), which is followed by sandy loam (25%). Other soil textures also found in the sampled areas include sand (10%), silty loam (5%), sandy clay loam (5%), loamy sand (5%), and silty loam (5%). The field pH of the

sampled soils in the municipality (Figure 2) ranged from 4.8 to 7.8 during the 2018 wet season and 4.7 to 8.8 during the 2019 dry season. No statistically significant interactions were found between season and salinity level ($p = 0.9128$) nor were there significant differences across seasons ($p = 0.5530$) and within salinity levels ($p = 0.0497$) after post-hoc testing. The field EC (Figure 3), on the other hand, ranged from 0.95 to 12.80 dS/m during the 2018 wet season and 0.02 to 0.28 dS/m during the 2019 dry season. The EC results from the field sampling at Ballesteros reflect the need for more detailed soil sampling following FAO (FAO, n.d.) soil salinity classes to indicate the “strongly saline” and “very strongly saline” areas as these highly affect crops (Table 1). There was no statistically significant interaction between the salinity level and season ($p = 0.9128$). There are also no statistically significant differences across season ($p = 0.5553$) and salinity level ($p = 0.0497$) after post-hoc testing. In terms of EC, while there was also no statistically significant interaction between season and salinity level ($p = 0.9047$), there was a highly significant difference in EC levels across seasons ($p < 0.0001$). Finally, no statistically significant differences in EC were found within salinity levels ($p = 0.9015$).

**Figure 2:** Field soil pH levels recorded for each sampling site. Figure generated by SAS OnDemand for Academics version 9.04.01M6P11072018

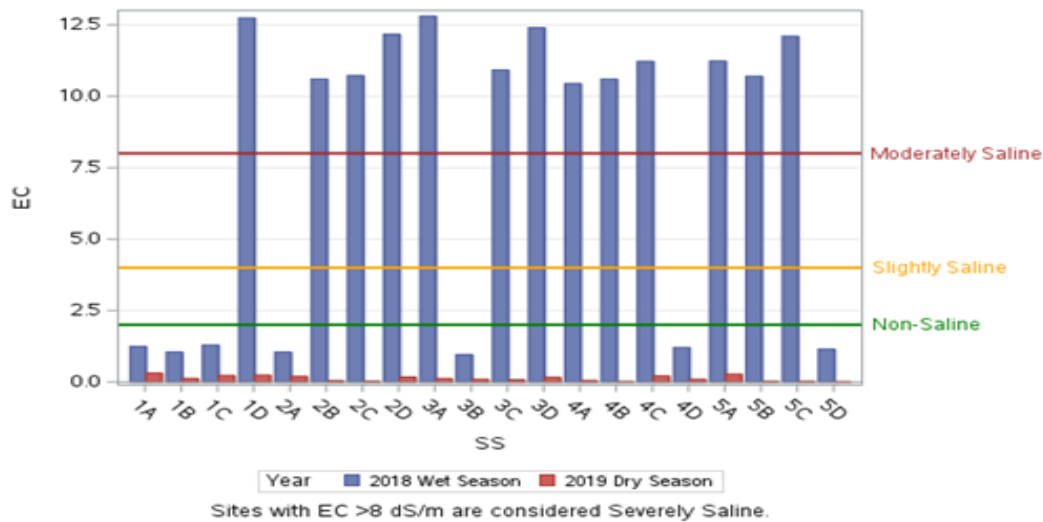


Figure 3: Field soil EC levels recorded for each sampling site in Ballesteros, Cagayan. Figure generated by SAS OnDemand for Academics version 9.04.01M6P11072018

Plate Counts and Metagenomic Analysis

Forty (40) samples (20 from 2019 wet season, 20 from 2019 dry season) were collected for plate counts and metagenomic analysis. Figure 4 shows the log colony counts of halotolerant bacteria cultured from the sampled soils from each salinity level per season. Only the counts from the 15% NaCl-R2A media had a statistically significant interaction between season and salinity level ($p = 0.1592$ for 3% $p = 0.1109$ for 5%, $p = 0.8124$ for 9%, $p = 0.0475$ for 15%, $p = 0.6669$ for 20%, and $p = 0.2717$ for 30%) as detailed in Figure 5, therefore, the p -values for season and

salinity level were no longer interpreted separately. Additionally, log cfu/g counts from the differing NaCl concentrations do not seem to differ significantly across seasons ($p = 0.7612$ for 3%, $p = 0.8511$ for 5%, $p = 0.8598$ for 9%, $p = 0.1886$ for 20%, and $p = 0.6422$ for 30%). Finally, only the 9% NaCl-R2A media registered statistically significant differences within salinity levels ($p = 0.4952$ for 3%, $p = 0.8505$ for 5%, $p = 0.0003$ for 9%, $p = 0.6061$ for 20%, and $p = 0.7397$ for 30%), as shown in Figure 6.

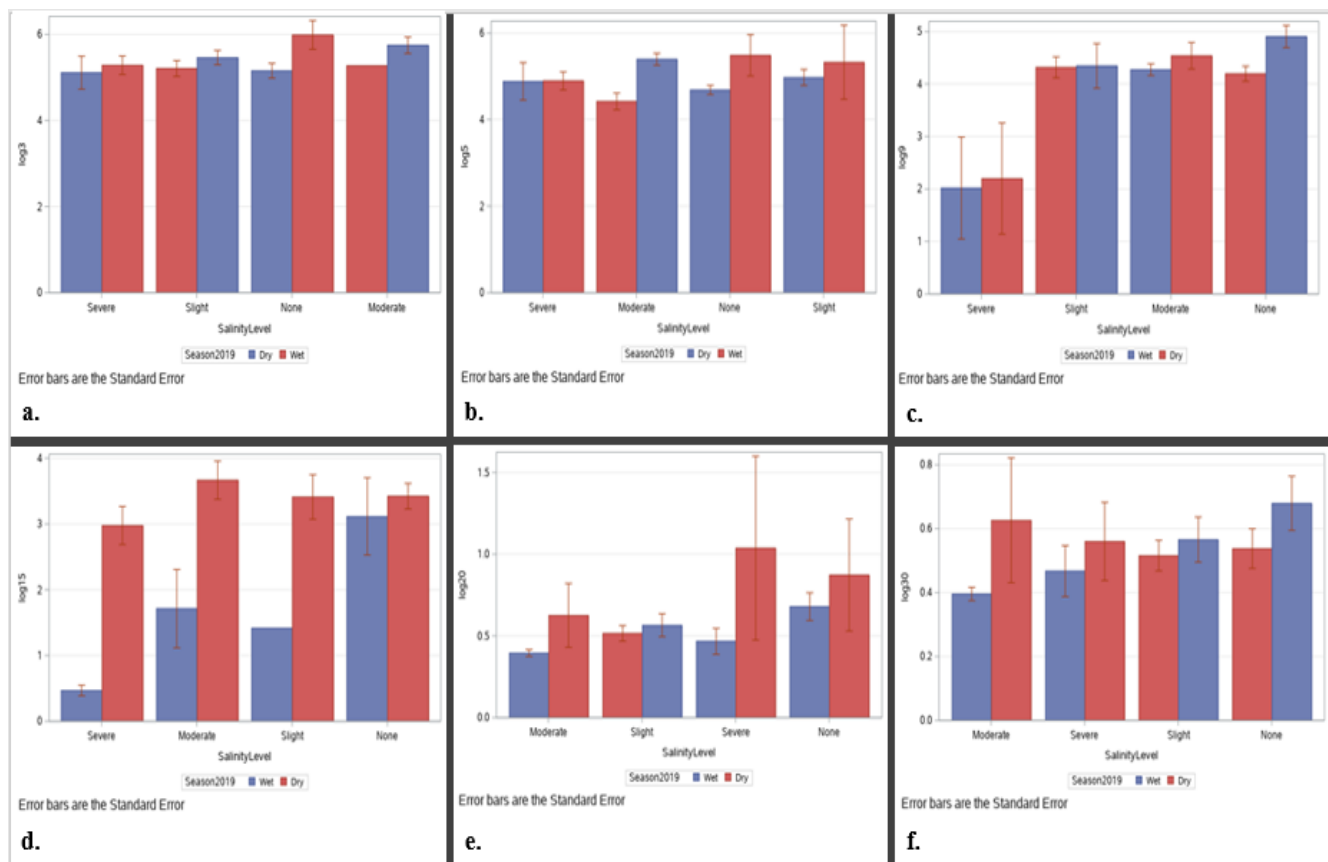


Figure 4: Log halotolerant bacterial counts of composited soil samples plated in a.) 3% NaCl-R2A, b.) 5% NaCl-R2A, c.) 9% NaCl-R2A, d.) 15% NaCl-R2A, e.) 20% NaCl-R2A, and f.) 30% NaCl-R2A. Figure generated by SAS OnDemand for Academics version 9.04.01M6P11072018

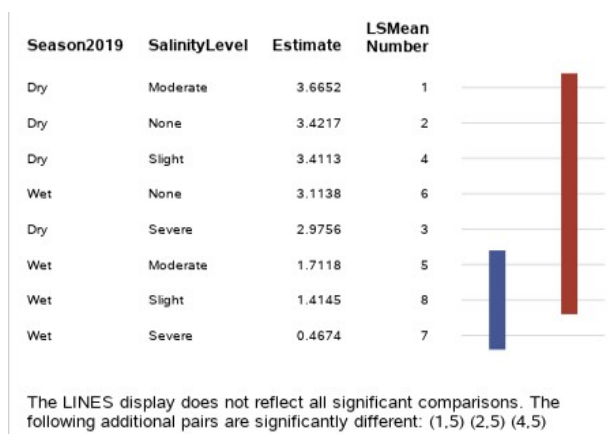


Figure 5: Post-hoc result of the season-salinity level interaction for the 15% NaCl-R2A log cfu/g counts. Figure generated by SAS OnDemand for Academics version 9.04.01M6P11072018

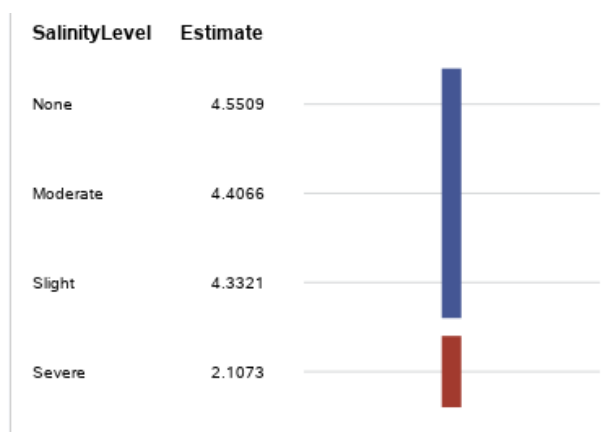


Figure 6: Post-hoc result of the salinity level differences for the 9% NaCl-R2A log cfu/g counts. Figure generated by SAS OnDemand for Academics version 9.04.01M6P11072018

The H' values (Figure 7) were derived from 4,042,093 high quality Reads detected by the UP-PGC's DNA Illumina Sequencing Software and Database. This equates to 211 bacterial database hits, 145 of which were identified up to the species level, with the rest up to the genus level only. No statistically significant interaction was found between Season and Salinity Level ($p = 0.4780$), but a statistically higher H' was

found in the dry season compared to the wet season ($p = 0.0133$). While within Salinity Levels, the non-Saline level has a significantly higher H' compared to the Moderately Saline and Severely Saline levels, with the Slightly Saline level having comparable values with all groups ($p = 0.0106$) after post-hoc testing (Figure 8). Among the numerous NMS ordination plots generated by PCORD version 7, the two-dimensional solution with a final stress score of 12.622 (randomization test p -value = 0.0040) after removing outliers was chosen after verifying consistency of interpretation after three repeated runs, as it has the lowest dimensionality with a stress level less than 20.000 (Figure 9). From the NMS Ordination Plot, five Contour Overlay plots were produced, one for each of the following halotolerant bacteria of interest found in the area: *Planococcus maritimus*, *Pseudomonas azotoformans*, *Pseudomonas fluorescens*, *Bellilinea caldifistulae*, and *Ectothiorhodospira haloalkaliphila* (Figures 10a-e).

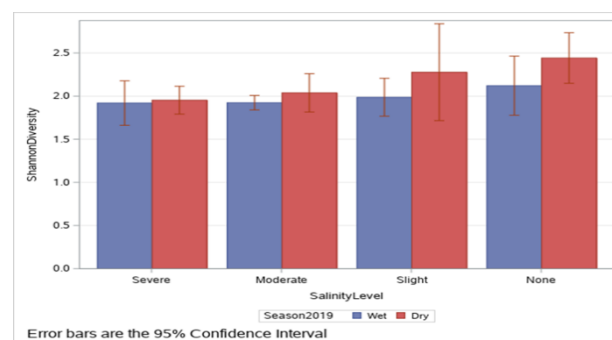


Figure 7: Computed H' values for each sampling site. Figure generated by SAS OnDemand for Academics version 9.04.01M6P11072018

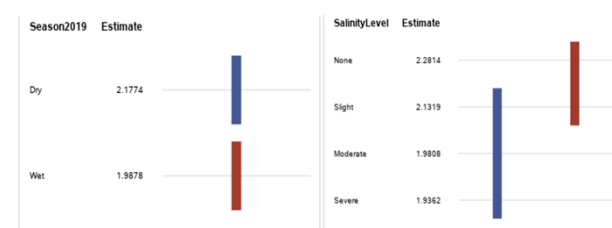


Figure 8: Post-hoc test results of the computed H' values for Season (left) and Salinity Level (right). Figure generated by SAS OnDemand for Academics version 9.04.01M6P11072018

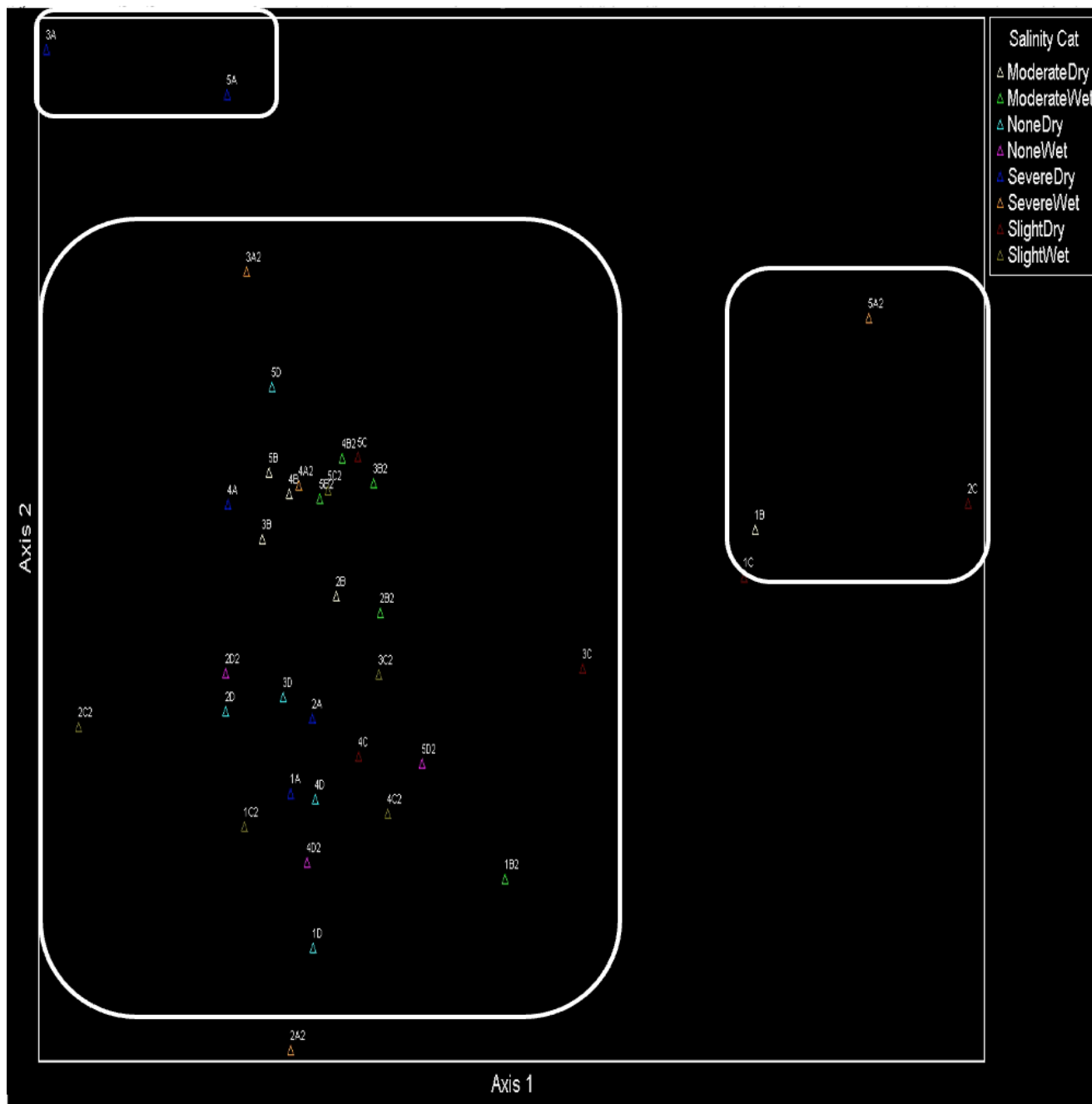


Figure 9: NMS Ordination Plot of the Ballesteros Soil Samples (Final Stress = 12.622, randomization test p-value = 0.0040). Legend: x - site number, xA – Severe Salinity Level, Dry Season 2019; xA2 – Severe Salinity Level, Wet Season 2019; xB – Moderate Salinity Level, Dry Season 2019; xB2 - Moderate Salinity Level, Wet Season 2019; xC – Slight Salinity Level, Dry Season 2019; xC2 - Slight Salinity Level, Wet Season 2019; xD – Non-Saline Salinity Level, Dry Season 2019; xD2 – Non-Saline Salinity Level, Wet Season 2019. The shapes are the patterns of note found in the ordination space. Figure generated by PC-ORD Version 7.11.

DISCUSSION

Relationship of Ballesteros Soil Texture Distribution and its Field pH/EC

The sampled areas were predominantly clayey, representative of the municipality's soils, which were devoted mainly to paddy rice. (DA-BSWM, 2017). Other land uses, like pasture grasslands for cattle grazing and orchards growing mostly coconut trees, were also found, accounting for the prevalence of loamy and silty soils in differing ratios. Sand also composed some sampled areas, as they were the prevalent texture near the sea. Clay is known to have the highest available water holding capacity among the soil types, and thus has the highest potential to retain the salts from saline water (Adamu & Aliyu, 2012). Results have shown that while the field soil pH is not greatly affected by seasonality, which is consistent with previous studies (Behera et al., 2009; Minasny et al., 2016), the inverse is

true for its field soil EC, as the wet season readings were significantly higher than the dry season readings across all Salinity Levels. This suggests temporal variability considering the 2017 BSWM Soil Salinity Map of Ballesteros, Cagayan, this can be attributed to rainfall being one of the main drivers of soil salinity, as increased water availability can lead to poor drainage of water from the soil, which aids in the retention of salts for a longer period than expected (Eswar et al., 2021). Ballesteros follows the typical Type III Modified Corona Climate Classification, as its distinct wet season was observed during the months of September to November, as shown by the data provided by the National Aeronautics and Space Administration (NASA) - Langley Research Center (LARC) via the Prediction of Worldwide Energy Resources (POWER) project (Figure 10). As evidenced by the figure, the wet season samples were collected during the midst of the municipality's high rainfall. Therefore, the predominance of clay soils in the sampled areas,

coupled with the increased rainfall and the ongoing saltwater intrusion phenomenon in the province (DA-BSWM, 2017), means that the predominantly clayey soils had more salt-infused

water than usual, leading to higher EC readings.

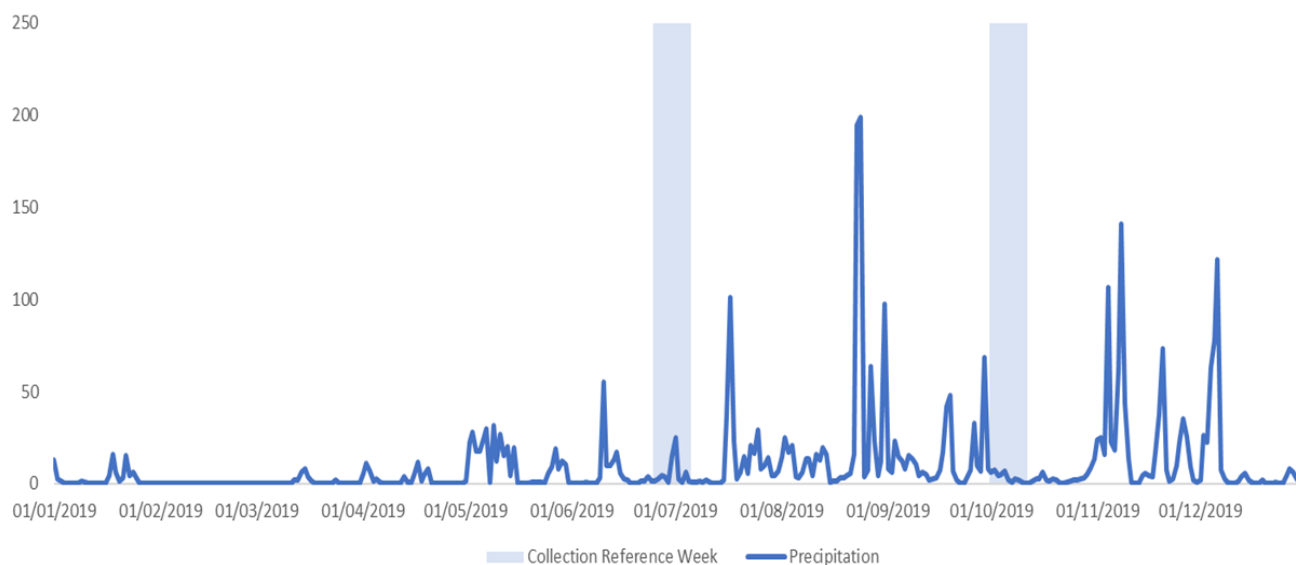


Figure 10: Daily Corrected Precipitation Rates in Ballesteros Cagayan in 2019 (Source: <https://power.larc.nasa.gov/data-access-viewer/>). The Collection Reference Week included the 7 days before the four-day sampling regime. Figure generated by Microsoft® Excel® for Microsoft 365 MSO (Version 2411 Build 16.0.18227.20082) 32-bit.

Cultivability of Halotolerant Bacteria from Ballesteros soils.

An observable decrease of culturable halotolerant bacteria was seen as the NaCl concentration of the R2A media increased, which coincided with previously cited results (Irshad et al., 2014). This is attributed to EC sharing a negative exponential relationship with microbial biomass C, microbial biomass N, and basal soil respiration, among other microbial activity indicators (Iwai et al., 2012). This means that as soil salinity increases, microbial—and by extension bacterial—diversity exponentially decreases. However, as illustrated by Figure 4, it was also observed that in the sampled soils, bacterial counts were observed in all tested NaCl-R2A media concentrations, regardless of their supposed salinity level in the field. This not only concurs with the initial claims of massive diversity of untapped soil halotolerant bacteria (Oren, 2002), but also implies the existence of obligate halophiles in all the sampled soils, as they are known in literature to survive NaCl levels higher than about 3.5% (Corral et al., 2020), though functional analyses must be conducted to verify this claim. Furthermore, there is also a potential overlap of abundance of halotolerant bacteria regardless of salinity level in general because some species of bacteria are found across differing salinity levels, as detailed in the metagenomic analyses. For example, one of the determined halotolerant bacteria of interest, *Pseudomonas azotoformans*, was detected in both Moderate (1B) and Slightly Saline (1C, 2C, 3C) soil samples in the dry season, and in a Severely Saline (1A2) soil sample in the wet season. Another identified halotolerant bacterium of interest, *Bellilinea caldifistulae*, was detected in both Slightly Saline (4C) and Non-Saline (1D) soils in the wet season, and in the Severely Saline (2A2) and Slightly Saline (4C2) soils in the wet season. This concurs with previous literature that there is a similarity in physiological requirements in the bacterial communities (Oren, 2002; Irshad et al., 2014) of the sampled soils. (The data further indicates that Ballesteros soils are more suitable for halotolerant bacteria that can survive at least 9% and up to 15% NaCl concentration, especially during the dry season for the latter, where the statistically highest counts were recorded (Figures 5 and 6). The laboratory has attempted to isolate the cultured halotolerant bacteria for identification using the same R2A-NaCl media they were taken

from, to no success. This may be due to the media not containing the requisite nutrients coming from the initial soil habitat crucial to their survival, growth, and reproduction (Vieira & Nahas, 2005; Delgado-Garcia et al., 2018).

Detected Halotolerant Bacteria of Interest and their Tentative Locations

The generated NMS Ordination plot (Figure 9) seems to show that a separate cluster composing of sites 3A and 5A was apparent, alongside another unique cluster composed of sites 1B, 1C and 2C, and 5A2. The rest of the sites also formed a singular group separate from the first two. This result implies that the soil microbial communities in the sampled areas of Ballesteros are not solely determined by their salinity level, meaning the majority of the sampled soils have very similar microbial communities. This is consistent with previous findings on halotolerant bacterial communities have generally similar or overlapping physiological requirements (Oren, 2002; Irshad et al., 2014). Saline soils are often deficient in nutrients required for crop growth (Arora & Vanza, 2017). This fact further contributed to the decreasing rice yield of not just the municipality of Ballesteros, but also the Cagayan province (DA-BSWM, 2017), but at the same time allowed for halotolerant bacteria to establish distinct communities, as they are either able to utilize less common soil compounds like hydrocarbons or polyols (Delgado-Garcia et al., 2018), they possess salinity survival mechanisms like the “compatible solute” or the “salt-in” strategies (Kapadia et al., 2022), or both.

Among the numerous species of bacteria identified in the soils of this area via 16S Metagenomic Sequencing, several bacteria of interest were listed. Figure 11a displayed the Contour Overlay plot of one such species, *Planococcus maritimus*, wherein a certain strain of which has been noted to have properties that promote plant growth of wheat in saline media and soil irrigated with saline water (Arora & Vanza, 2017; Waghmode et al., 2019). The probable main mechanism of growth promotion in saline soil contributed by this species is the removal of salts in the surrounding soil, allowing the plant to survive and thrive. The bacterial species, and by extension, the strain, has a high

probability of being detected in site 3A, as represented by the redder gradient in the Contour Overlay Plot. This is consistent with the Reads data, where indeed only site 3A has registered Reads for the organism. Another halotolerant bacterium of interest found in the sites was *Pseudomonas fluorescens*. The species was documented to be used with *Rhizobium* strains to increase the survival and growth of mung bean (*Vigna radiata*) in salt-stressed soils, for it dilutes adverse effects of salinity on the mung bean's relative water content and assimilation rate of CO₂, while also improving its photosynthetic rate, chlorophyll content, and water use efficiency (Ahmad et al., 2013). For this species, it is highly probable that it would be detected in the 2C and 5A2 sites according to its Contour Overlay Plot (Figure 11b), but in cross-checking with the Reads data, not only was it detected in the two sites, but the species was also detected in site 1B. Another member of the genus *Pseudomonas*, *P. azotoformans*, was also noted as a bacterium of interest in the region. The species, according to the Reads data, was found in sites 1B, 1C, 2C, 3C, and 5A2, which is consistent with its generated Contour Overlay Plot (Figure 11c). A specific strain of these bacteria was touted as a promising rhizobacterial isolate that allows for mustard (*Brassica juncea*) growth in salt-stressed conditions (Phour & Sindhu, 2013). More specifically, the strain

managed to increase mustard root and shoot dry weight by 291% even after 90 days of sowing, while also stimulating the plant's growth in saline soils as high as 12 ds/m when combined with *Pseudomonas argentensis*. The Contour Overlay Plot for this bacterium is also like the one generated for *P. fluorescens*, indicating that they may have the same exact niche and requirements in the environment. Other bacteria of interest detected which can hold potential include the following: *Bellilinea caldifistulae*, a strictly anaerobic bacterium first isolated in thermophilic anaerobic sludge which can tolerate up to 3% NaCl (Yamada et al., 2007) and *Ectothiorhodospira haloalkaliphila*, a mesophile first isolated in a shallow water soda lake known to tolerate a salt range of 2.5-15% (Imhoff & Suling., 1996). Their respective Contour Overlay Plots are detailed in Figures 10d and 10e, which shows that the former has a high probability of being found in site 2A2, while the latter can most likely be found in site 3A. In terms of the Reads data, *Bellilinea caldifistulae* was found on sites 4C, 1D, 2A2, 4C2, and 5D2, while *Ectothiorhodospira haloalkaliphila* was found in Sites 3A and 2D2.

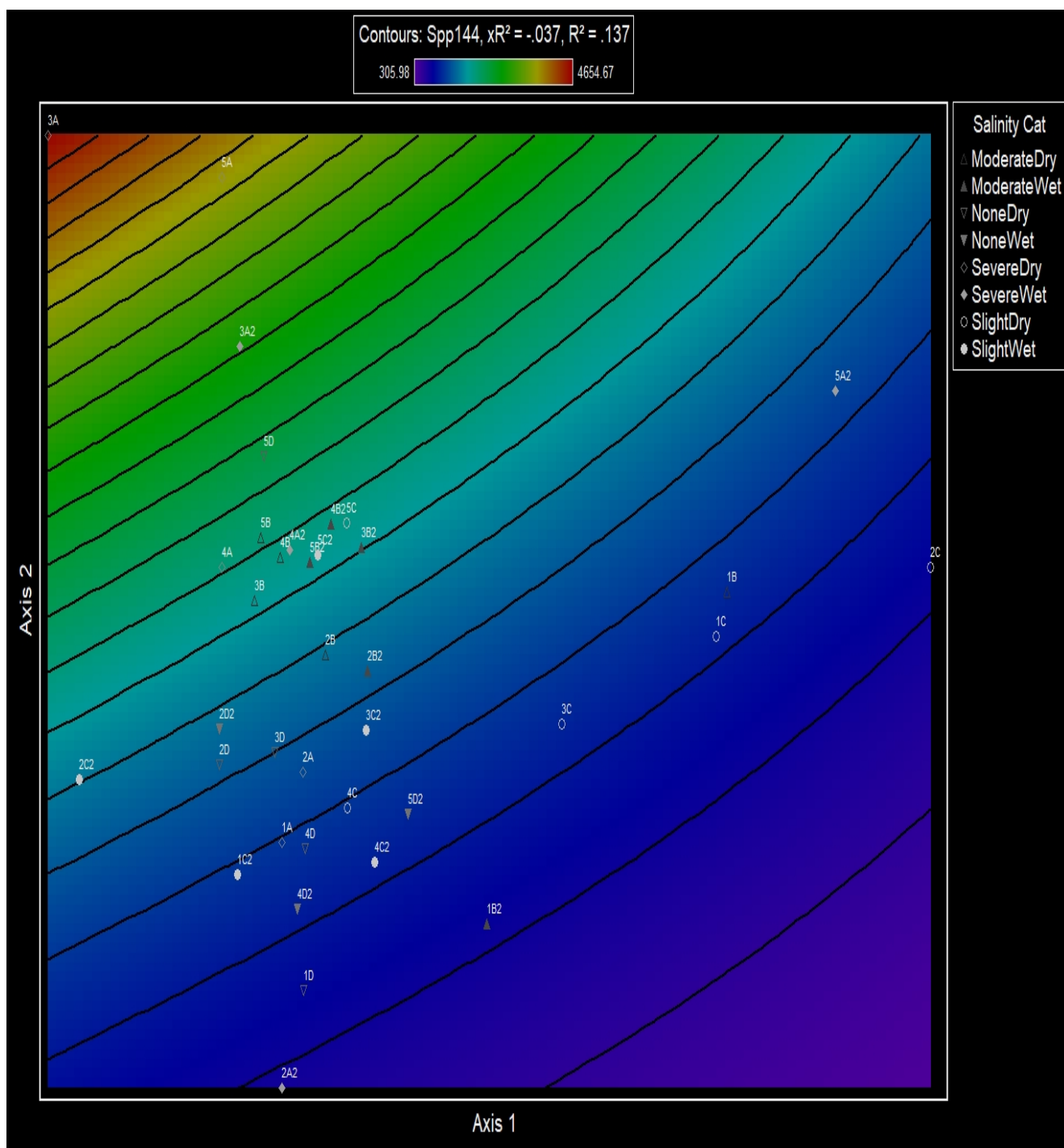


Figure 11a: Contour Overlay plot for *Planococcus maritimus*. Figure generated by PC-ORD Version 7.11

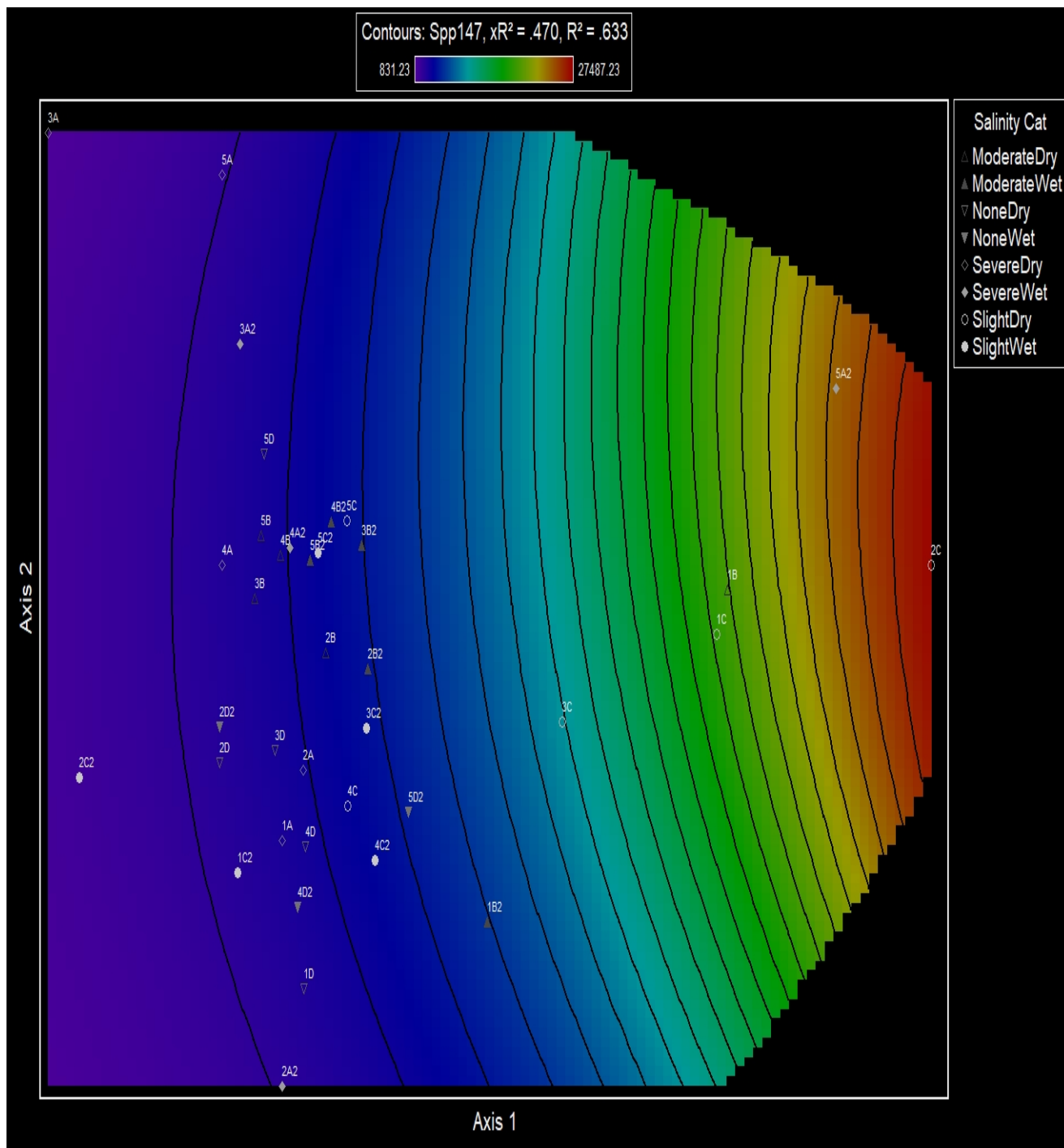


Figure 11b: Contour Overlay plot for *Pseudomonas fluorescens*. Figure generated by PC-ORD Version 7.11

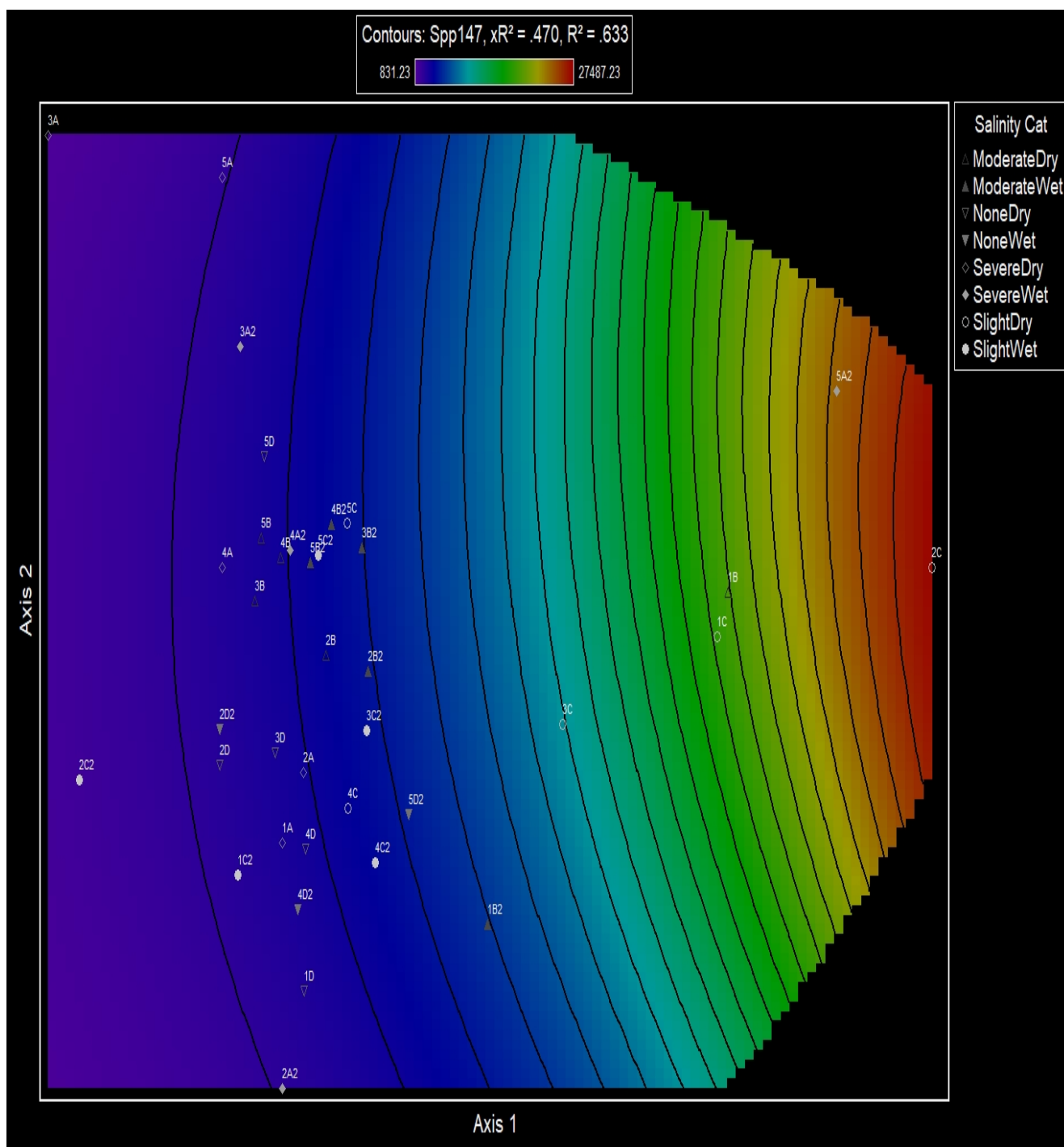


Figure 11c: Contour Overlay plot of *Pseudomonas azotoformans*. Figure generated by PC-ORD Version 7.11

While it can be argued that the Contour Overlay Plots do not exactly reflect the Reads data, it can still be a useful guide in determining the potential mining locations of the detected bacteria of interest. Also, it is still possible that the detected bacteria can be found in the sites situated in the non-red gradients of the Contour Overlay Plots, as that could be attributed to a more rigid sequencing process (e.g. increase of sample size, more specific targeting of hypervariable regions of the 16S gene). This can also address the limitation that most of the Reads (more than 50%) obtained by the Sequencer did not lead to species “hits” in its database. Lastly, it should be noted

that microbial diversity in terms of H' was found to be higher in the dry season, coinciding with previous literature that high temperatures and moistures stimulate soil microbial activity and nutrient cycling (Žiřčáková et al., 2015; Gałazka et al., 2017). This may also apply to halotolerant bacteria as well, as some known halotolerant bacteria like *Planococcus maritimus*, *Pseudomonas azotoformans*, *Pseudomonas fluorescens*, *Bellilinea caldifistulae*, and *Ectothiorhodospira haloalkaliphila* were more likely found during the dry season, evidenced by their contour overlay plots (Fig. 11).

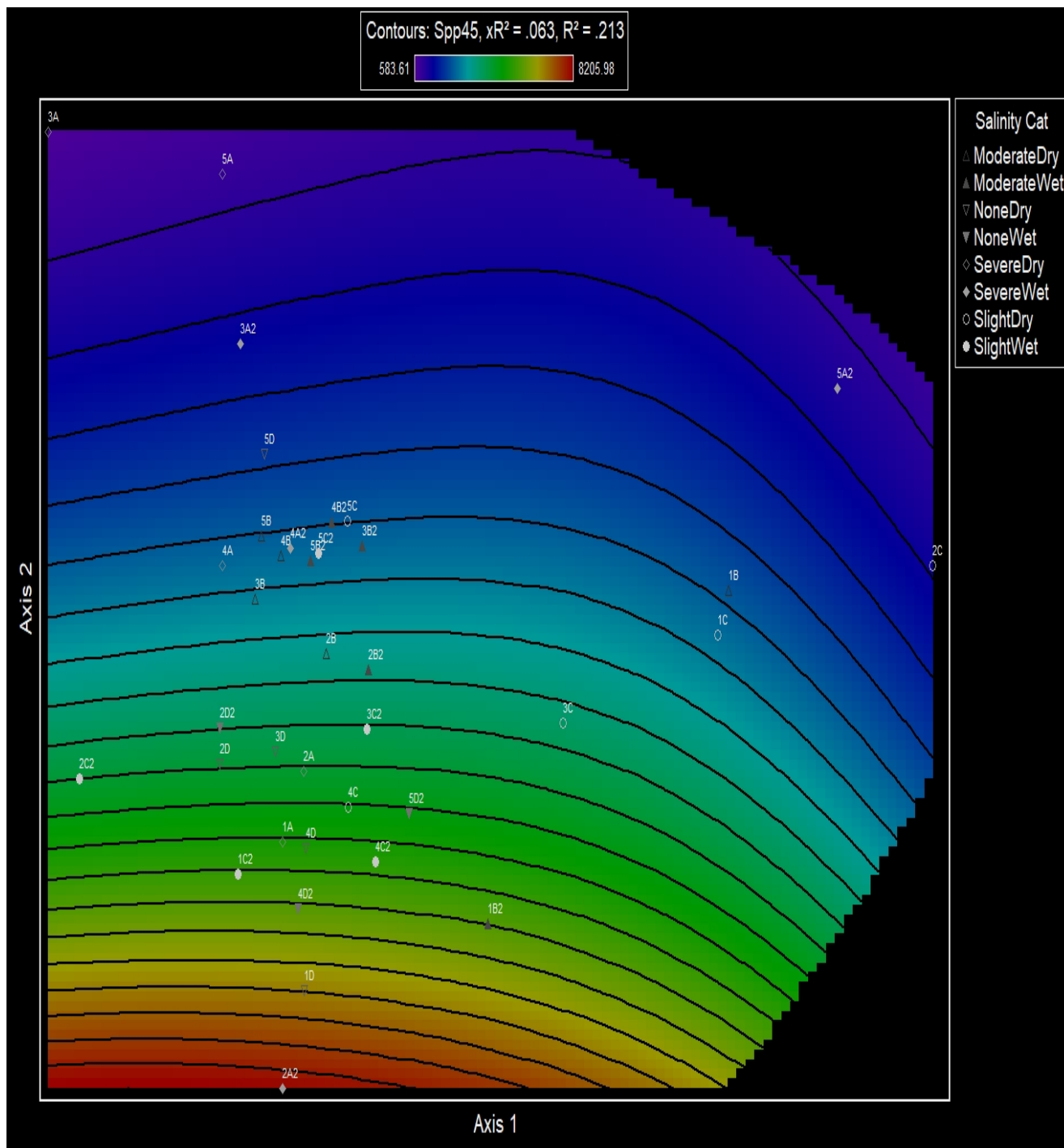
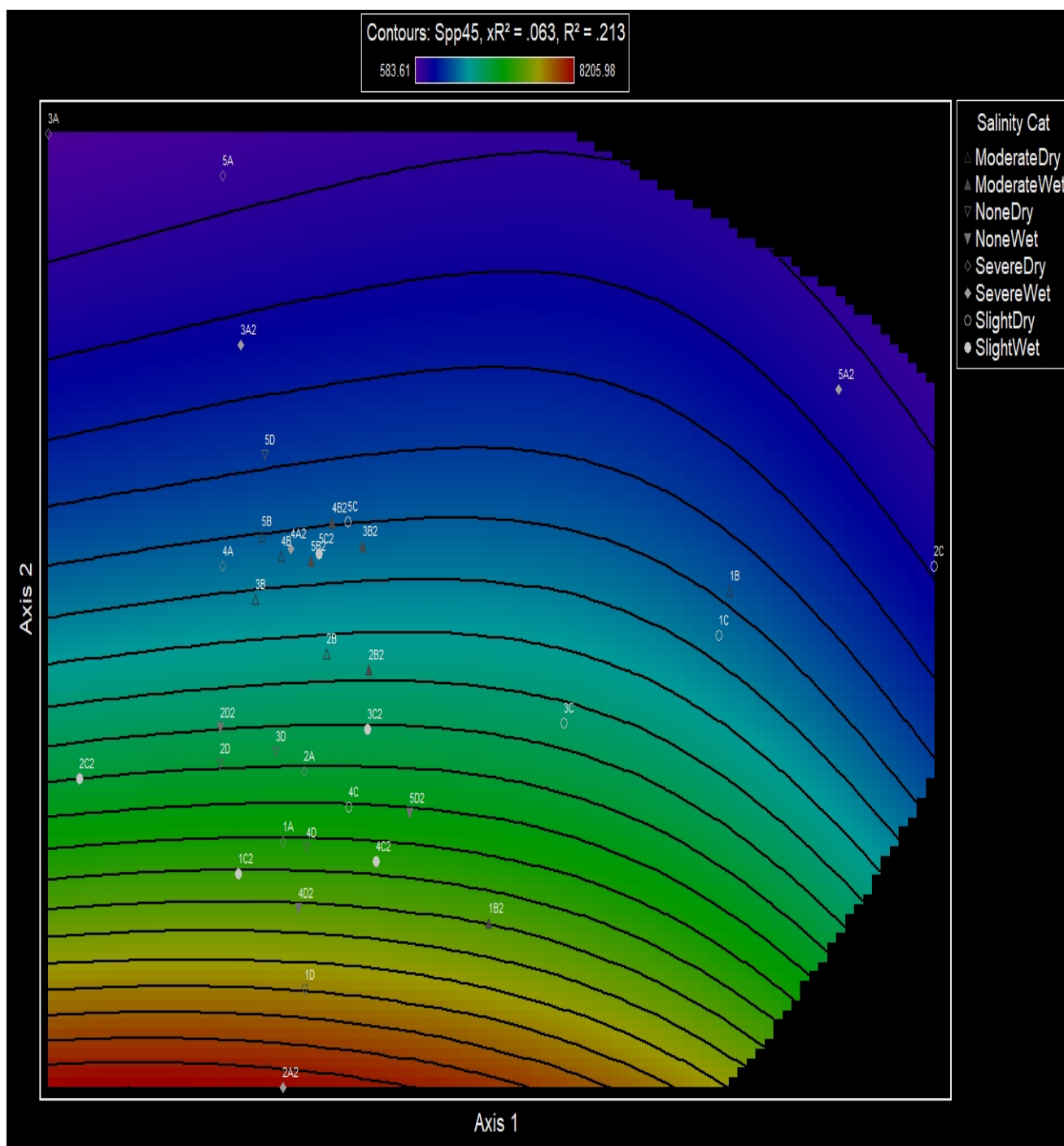


Figure 11d: Contour Overlay plot of *Bellilinea caldifistulae*. Figure generated by PC-ORD Version 7.11



the detected halotolerant bacteria of interest are readily available in the areas in which they were found. Hence, one recommendation is the resampling of soil in those areas, this time considering inter-annual variations in precipitation, temperature, salinity, and soil nutrients, as a form of verification. This may also involve rhizospheric sampling, for halotolerant bacteria may have thriving associations with the roots of the local flora. To aid these endeavors, isolation using more specialized media is recommended, preferably using soil extracts from the site as a component to such media to improve success rates. Once pure isolates have been obtained, polyphasic taxonomy is suggested to properly identify the isolates, coupled with *in silico* analyses to clarify their taxonomic positions. A more rigid 16S Metagenomic Sequencing process is also important to undertake (e.g. usage of specific hypervariable regions of the 16S gene), to improve the detection and identification of other potential bacteria in the soils of the municipality. Usage of other molecular markers, which can include genetic markers for halotolerant bacteria, which can be performed in tandem with functional analyses of the bacterial sequences are also highly suggested, as not only do these enable more precise identification of the species within the target bacterial group, they also confirm whether the isolates really do exhibit halotolerant (or even halophilic) traits, alongside other functions of note like plant growth and yield improvement. Finally, after isolation, cultivation, identification using more precise molecular and genetic markers, and functional analyses of the bacteria of interest have been completed, testing of their capabilities in remediating saline soil or improving crop growth in saline soils is recommended in both the greenhouse and the field setting.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

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