

eDNA metabarcoding reveals spatial structuring of bacterial communities in the volcanic crater Lake Holon, Philippines

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ABSTRACT. Crater lakes of volcanic origin represent special cases of freshwater ecosystems where the morphology of the basin, hydrological isolation, and spatial gradients of physicochemical parameters might influence the microbial community structuring. To date, Lake Holon is an understudied volcanic crater lake in Southern Philippines at the microbial level. Here we report the first assessment of bacteria using environmental DNA (eDNA) metabarcoding of Lake Holon with respect to spatial differences of the central and peripheral regions of the lake. Samples of near-surface waters from six sites of the lake were taken *in situ* for physicochemical characterization and eDNA isolation. Total genomic DNA was extracted, and the V3-V4 regions of the 16S rRNA genes were sequenced using high-throughput Illumina MiSeq100 Plus platform. Sequence reads (total = 944,206 ASVs) were processed by the EasyAmplicon, and DADA2 pipelines; community diversity was analyzed in R software. Functional profiles were predicted using PICRUSt in Parallel Meta-suite (PMS). Bacteria showed significant spatial differences in the studied crater lake. Central sites harbored distinct and very diverse communities including unclassified bacterial lineages potentially associated with acidic conditions including pelagic cyanobacterium *Synechococcus rubescens*, suggestive of possible selective pressure by localized physicochemical conditions and water column processes. In contrast, less acidic and better-oxygenated peripheral locations contained copiotrophic heterotrophs such as *Pseudomonas aeruginosa*, potentially due to greater connectivity with the littoral zone and terrestrial environment or possibly anthropogenic effects. Alpha and beta diversity analysis also showed heterogeneous community structure at central locations and relatively homogeneous community structure at peripheral locations of the lake, in agreement with environmental filtering at different locations. This research gives the first insight into the bacterial diversity in Lake Holon and previously unknown fine-scale biogeography of microbes in a volcanic crater lake basin. The research demonstrates eDNA metabarcoding and serves as a basis for future studies and limnological monitoring of tropical crater lakes.

Keywords: 16S rRNA gene, bacterial community, environmental DNA, microbial biogeography, volcanic crater lake

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1. Introduction

Crater lake ecosystems are unique types of freshwater ecosystems created through the interaction of geological events, basin morphology, hydrologic connectivity, watershed processes, and physicochemical processes occurring within the system. The enclosed or semi-enclosed nature of the basins creates an opportunity for the development of significant heterogene-

ity in terms of chemistry, nutrients, oxygenation, and biological communities (Umaña, 2010). These are some of the reasons why crater lakes are important natural settings for studies on the influence of environmental gradients on the composition of microbial communities in aquatic systems. There is sufficient evidence from research studies carried out in volcanic lakes to show that microbial communities can vary depending on water chemistry, habitat, depth, and environmental

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gradients (Löhr et al., 2006; Hou et al., 2019; Nana et al., 2020). However, the organization of microbial communities has been relatively understudied in tropical crater lakes and especially with molecular techniques.

Lake Holon is a volcanic crater lake at high elevation within the caldera of Mount Melibengoy, or Mount Parker, in T'boli, South Cotabato, Southern Philippines. Its position within a volcanic caldera with steep walls and an isolated upland terrain results in a geographically restricted crater-basin system with low accessibility. The surrounding area's vegetation includes a wide array of flora and fauna, thereby enhancing the ecological and conservation importance of the lake and its drainage basin. Besides its ecological importance, Lake Holon is very valuable culturally and economically for the local community, especially the Indigenous T'boli, whose involvement in community-based stewardship and ecotourism management has become an essential component of the lake's governance. The lake and its drainage basin provide the local population with ecosystem values, recreational facilities, and an emerging ecotourism industry, stressing the importance of finding the right balance between conservation and human exploitation (Olivar et al., 2023; Alampay, 2024). Given the increasing human activity around the lake, ecological baseline studies are increasingly essential for identifying changes in the lake's ecosystem and making management decisions based on scientific evidence.

Beyond these ecological and cultural values, Lake Holon offers ecosystem services that benefit the surrounding communities and promote sustainable management of the crater lake environment. These ecosystem services include freshwater resources, biodiversity conservation, cultural values, recreation activities, and ecotourism. Thus, there is a need for balancing the efforts aimed at protecting the environment and ensuring sustainable human activity. These services have implications for several United Nations Sustainable Development Goals (SDGs), including SDG 6 (Clean Water and Sanitation) due to the conservation of freshwater resources, and SDG 15 (Life on Land) because of inland freshwater ecosystem conservation and associated biodiversity conservation. Furthermore, community-based stewardship and involvement of Indigenous people in the management of lake and landscape resources are consistent with principles of SDG 11 (Sustainable Cities and Communities) and SDG 13 (Climate Action). Nevertheless, although the ecological importance, cultural significance, and growing importance of ecotourism of Lake Holon are obvious, scientific knowledge necessary for the development of an effective and sustainable lake management strategy is scarce. One example of this problem is the absence of basic data on microbial biodiversity and its distribution within the lake and its surroundings that could help assess the effect of environmental variability and human impact.

Water body ecological status assessments have typically been based upon physicochemical characteristics such as pH, temperature, dissolved oxygen, conductivity, and nutrient content. Physicochemical analyses are important for describing water bodies' quality;

however, such assessments represent only a static state of environmental parameters and might not include biological reaction to environmental variability. Microorganisms are key elements of lakes' ecosystems and participate in decomposition of organic substances, nutrient recycling, production of organic material, and biogeochemical cycles (Azam et al., 1983; Falkowski et al., 2008). Thus, bacterial community structure represents additional information about ecosystem function and environmental changes since microorganisms are highly responsive to physicochemical variations, nutrient availability, terrigenous loadings, and human influence (Newton et al., 2011).

Microbial community assembly in crater lakes can be dependent on the presence of spatial environmental gradients, since the differences in physicochemical heterogeneity could cause different selective pressures inside a comparatively small basin. Discrepancies between open-water central parts and peripheral areas affected by littoral or peripheral inputs could be caused by dissimilarities in terrestrial inputs, organic matter loadings, water circulation, oxygen levels, pH, nutrient regime, and local human activities. Peripheral waters could be influenced by catchment-derived inputs, littoral processes, and terrestrial runoff while central waters might be determined by pelagic and local physicochemical factors. Previous studies indicated that bacterial community composition may vary across pelagic, littoral, and catchment-influenced habitats, emphasizing the vital role of local environmental conditions in structuring freshwater microbial assemblages (Lindström et al., 2005; Logue and Lindström, 2010; Zhang et al., 2019; Marois et al., 2022). Also, it was established that there are spatial zones of bacterioplankton composition in the lakes located at a high elevation level (Aguilar et al., 2022). Nonetheless, the degree to which bacteria exhibit spatial structure within many tropical crater lakes is still unresolved, such as in Lake Holon. Establishing whether different parts of the lake house unique assemblages of bacteria is crucial for elucidating how microbial communities assemble in space and for developing spatial sampling schemes in Lake Holon for future studies on tropical lakes as reference.

The development of molecular ecology techniques has increased the ability to describe the biodiversity of aquatic systems, not only using traditional culture-based and microscope-based techniques. The use of eDNA can provide information about the genetic content of environmental samples, such as water, without invasive procedures (Taberlet et al., 2012). Combined with next-generation sequencing, eDNA metabarcoding enables the study of complex community structures by identifying various bacteria, both abundant and rare, unculturable, and unidentifiable by other methods (Thomsen and Willerslev, 2015). Sequencing of 16S rRNA genes can be used to identify taxonomic structure and diversity of bacterial communities in freshwater ecosystems. This type of analysis is especially beneficial when applied to poorly studied ecosystems where no microbial baseline has been created.

Marker gene databases not only help in classifying the microbial communities but can even help in forming hypotheses about their possible functional properties. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) makes it possible to make predictions about functional profiles using 16S ribosomal RNA gene data through the phylogenetic comparison of reference genomes. While functional profiling does not directly estimate the levels of gene expression or metabolic activities and hence should be taken cautiously, it may help to form initial thoughts about any differences in the functional capabilities of microbes existing in different environments (Langille et al., 2013).

In spite of the extensive usage of eDNA metabarcoding and next-generation sequencing in aquatic systems, the bacterial diversity and spatial distribution pattern in Lake Holon have yet to be explored. To the best of our knowledge, there is no published work that employed 16S rRNA eDNA metabarcoding to assess the bacterial community structure of Lake Holon. This is quite a significant lack of information due to the special features of the lake including its closed basin form, hydrologically isolated nature, and possible physical/chemical separation of the central area from the surrounding one.

Thus, eDNA metabarcoding of the V3-V4 regions of the bacterial 16S rRNA gene was performed in the present study to investigate bacterial communities in six sites in Lake Holon.

This research determines the composition of the bacterial community in the crater lake through 16S rRNA metabarcoding, look into diversity and community composition of the bacteria spatially, associate bacterial composition of communities to some physicochemical parameters of the water, and predict possible functional potential of bacterial communities using PICRUSt. It was hypothesized that bacterial communi-

ties would be spatially differentiated within the crater lake, with separate bacterial assemblages being present in the central and peripheral parts of the lake.

2. Materials and Methods

2.1. Study Area and Sampling Locations

Lake Holon, a pristine crater lake often referred to as the “Crowning Jewel of the South”, located within a dormant stratovolcano Mt. Parker in T’boli, South Cotabato, Philippines (Fig. 1) (Pepito et al., 2020). Recognized as one of the cleanest inland water bodies in the country, Lake Holon is found to have minimal human disturbance, oligotrophic conditions, and high ecological value as a freshwater environment and ecotourism site (Olivar et al., 2023). Sampling sites were established to sample a range of microbial community composition. Sites S19-S21 were designated in the lake center (pelagic zone, minimal direct terrestrial influence). Whereas sites S22-S24 were designated in the outskirts area (the littoral zone affected by adjacent vegetation and environmental runoff). Prior informed consent and permits were obtained before the sampling period. During the sampling period, coordination with local authorities and relevant custodians was conducted to ensure proper access and compliance with community and local environmental regulations, as well as ethical sampling practices. The geographic coordinates of each sampling site were recorded using a handheld GPS device (Table 1).

2.2. Sample Collection and Physico-chemical Parameters

Water collection was done within the one-month (May 2025) sampling period. *In situ* physico-chemical parameters, including water temperature, salinity,

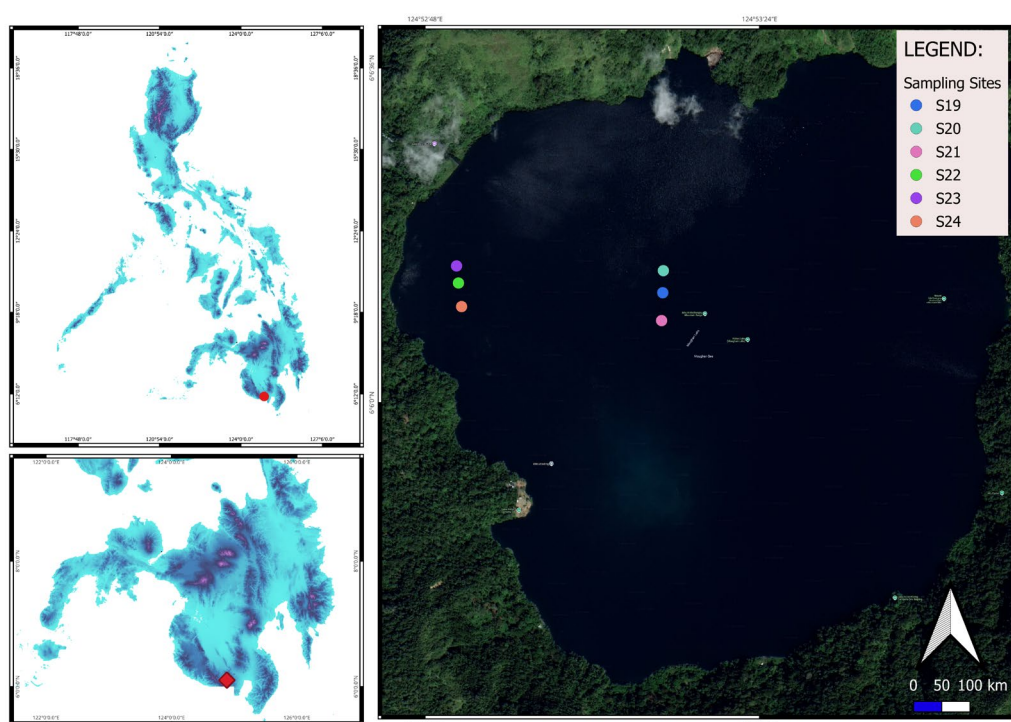


Fig. 1. Map of the Philippines (A); Map of Mindanao (B); Map of Lake Holon showing the sampling sites (C).

Table 1. GPS coordinates of sampling sites in Lake Holon, Philippines.

Site	Location	Coordinates	Description
S19	Center 1	6.103100° N, 124.887084° E	Deep pelagic zone; minimal shore-line influence; open water condition
S20	Center 2	6.103325° N, 124.887084° E	
S21	Center 3	6.102875° N, 124.887084° E	
S22	Outskirt area 1	6.103863° N, 124.880943° E	Littoral zone; proximity to vegetation and shoreline inputs
S23	Outskirt area 2	6.104088° N, 124.880943° E	
S24	Outskirt area 3	6.103638° N, 124.880943° E	

and pH, were measured at each sampling sites using a YIERYI BLE-C600 multi-parameter water quality tester, while dissolved oxygen (DO) was measured using a YIERYI DO9100 dissolved oxygen meter. For each parameter, measurements were taken in triplicate readings at each sampling site prior to water collection. For the eDNA collection, a total of 10L of near-surface waters (max. depth of 1m) was collected from each site. Representing three independent biological replicates in the lake center (pelagic zone, minimal direct terrestrial influence) and another three in the outskirts area (the littoral zone affected by adjacent vegetation and environmental runoff), that totals to six libraries (S19-S24) all in all. The sampling containers were cleaned and sterilized with 10% bleach and rinsed with distilled water before use to minimize contamination. Water samples were filtered through a sterile 60 mm Buchner funnel system equipped with 50 mm polyethersulfone (PES) membrane filters (0.22 µm pore diameter size). All the membranes were placed in an ice box at approximately 0-4°C for transport. They were placed in sterile capped containers and brought to the Molecular Systematics and Conservation Genomics Laboratory, Center for Biodiversity Studies and Conservation (CBSC) at the Premier Research Institute of Science and Mathematics (PRISM), Mindanao State University-Iligan Institute of Technology, for DNA extraction.

2.3. eDNA Extraction and Sequencing

Total genomic DNA was extracted according to the manufacturer’s protocol using the HiPurA™ DNA Extraction Kit (Vadhani Industrial Estate, Mumbai, India). For bacterial 16S rRNA gene V3-V4 region, it was amplified with universal primers Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC). Samples were outsourced to Macrogen Inc., Seoul, South Korea for PCR, library construction and sequencing on Illumina MiSeqi100 Plus platform. There were six consensus amplicon libraries corresponding to the central sites (S19, S20, S21) and peripheral sites (S22, S23, S24).

2.4. Microbial Community and Bioinformatics Analysis

Raw reads were generated and quality check was done under FastQC. FLASH (Fast Length Adjustment of Short reads) was utilized to merge the paired-end reads, a rapid and precise tool for genome assembly and analysis. The resulting sequences were deposited

to NCBI GenBank and made publicly available under the accession nos. SRX34212210-SRX34212215. The resulting raw sequences were uploaded for subsequent bioinformatics analyses of amplicon sequence variants (ASVs). Downstream bioinformatics analysis was performed in the Easyamplicon pipeline, a freely available and reproducible amplicon-based microbiome study workflow that incorporates multiple tools to analyze raw sequencing reads, taxonomic classification, and diversity (Yousuf et al., 2024). Genus based taxonomic assignments were used to first summarise bacterial community profiles at genus levels based on reference. The pipeline allows flexible use of reference databases such as Greengenes and SILVA, depending on analytical requirements. For higher taxonomic resolution, sequence data were further processed using the DADA2 pipeline algorithm to infer amplicon sequence variants (ASVs), enabling species-level resolution where sequence identity permitted. Alpha diversity indices (Shannon, Simpson, and Chao1) and beta diversity analyses (principal component analysis and heat-maps) were computed using R statistical software (version 4.5.3). PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) was performed using the Parallel-Meta Suite (PMS) package to systematically assign KEGG Orthology. The KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses gene functions and links genomic information with higher-order functional information. The metabolic pathways were annotated using the KEGG BRITE hierarchy where, prediction accuracy of functional annotations was assessed using the Nearest Sequenced Taxonomy Index, which measures the phylogenetic similarity between operational taxonomic units and their closest individually sequenced relatives (Chen et al., 2022; Yang et al., 2023). Moreover, statistical analysis, data visualization, including diversity plots and ordination figures, was performed in R using standard graphical packages (Callahan et al., 2016).

3. Results and Discussion
3.1. Baseline Bacterial Community Composition and Amplicon Sequence Variants (ASVs) Distribution

High-throughput sequencing for eDNA metabarcoding provides a high-resolution lens into the microbial landscape of Lake Holon. Bacterial community structure serves as a biological integrator of ecosystem health that reflects both historical stability and current environmental perturbations (Newton et al., 2011).

This study represents the first molecular characterization of Lake Holon's microbiome, bridging the gap between its status as a pristine "protected area" and the functional biological processes that maintain its oligotrophic state. After post-quality control, it revealed a total of 944,206 ASVs reads for all sites (S19 = 157,140; S20 = 140,609; S21 = 166,966; S22 = 177,626; S23 = 148,290; S24 = 153,575) with S19-S21 designated as central sites while S22-S24 as peripheral sites and assigned to a total of 222 bacterial families.

The amplicon sequence variants (ASVs) associated with the 16S rRNA gene from Lake Holon, Philippines, showed that the microbial community structure had a small number of hyper-abundant taxa (Fig. 2). With an ASV surpassing 3.0×10^5 total reads, the unidentified Proteobacteria is the largest taxonomic group. Overall, *Pseudomonas aeruginosa* (HE978271) with a sequence read of 1.6×10^5 was considered to be the most abundant organism, followed by *Acinetobacter johnsonii* (Z93440) with sequence reads approximately 1.2×10^5 . Beyond these baseline taxa, a sudden, exponential drop in sequence yield was seen across the rest of the microbial communities. *Bacillus cereus* (AE016877) exhibited a low dominance and clear profile, with approximately 3.0×10^4 total reads. Following this was the specialized aquatic/autotrophic taxa namely *Synechococcus rubescens* (NR_125841.1) and *Rheinheimera aquatica* (GQ168584) producing $< 2.0 \times 10^4$ reads. These numbers made the remaining sequence a large assemblage that consisted of rare taxa clustered below the 1.0×10^4 reads threshold containing tiny pieces of several satellite relatives of *Acinetobacter* (*A. indicus*, *A. junii*, *A. variabilis*, *A. schindleri*, *A. haemolyticus*, and *A. gyllenbergii*), *Ilumatobacter fluminis*, *Salmonella enterica*, *Chitinibacter tainanensis*, *Algisphaera agarilytica*, and green microalga *Chlorella vulgaris*.

ASVs characterizing Lake Holon reflect community assembly driven by specific niches and opportunistic heterotrophs continued with the autotrophic and oligotrophic taxa. The high abundance of unknown Proteobacteria demonstrates the continuous role of uncultured microbial dark matter in environmental metagenomic datasets. Many of these lineages tend to predominate in the pristine environment, where a

myriad of uncharacterized metabolic pathways seems to have evolved to benefit from transient pools of raw material and to adjust to minor environmental changes (Gatinho et al., 2023; Ngesom et al., 2025). Considering that Lake Holon is a remote volcanic crater lake, these lineages likely represent locally derived, highly adapted microbial dark matter that is unique for this high-altitudinal area (Gies et al., 2014; Mapelli et al., 2015; Garcia-Lopez et al., 2022; Henríquez et al., 2025).

The co-dominance of *P. aeruginosa* with the presence of *A. johnsonii* could be used as a bioindicator for the ecological status of Lake Holon (Lazar et al., 2017; Crone et al., 2020). Though a semi-arid region without arable farming sites, like fish harvesters at the end of the day (Hernández-Méndez et al., 2026), Lake Holon is a popular tourism destination. The occurrence of these taxa implies a microbiological intrusion produced by local organic colonization and possible tourist-induced perturbations (Crone et al., 2020; Rezapouraghdam et al., 2024; Fadilah et al., 2025; Cordero et al., 2026). Genomic versatility, metabolic adaptability, and a complex regulatory network of two-component systems in *P. aeruginosa* allow rapid transition from saprophytic survival in the environment to opportunistic growth (Klockgether et al., 2011; Francis et al., 2017; Ambreetha et al., 2024; Gupta et al., 2025). This species creates robust extracellular polymeric substances (EPS) that contribute to the formation of stationary biofilms to protect it from shear and nutrient fluctuations and from human effects (e.g., personal care products or camping graywater) (De Alexandre Sebastião et al., 2013; Mahto et al., 2022; Yang et al., 2025). In addition, *A. johnsonii* acts as a major functional group in regions where localized nutrient enrichment is limited and organic decomposition is complicated (Lu et al., 2021; Ren et al., 2025; Wang et al., 2025a). Annual closure of Lake Holon allows the ecosystem to recover from human footprint (Francas, 2025). The development of these kinds of heterotrophs therefore reflects a complex microbial community that oscillates between oligotrophic purity and microbial influx spurred by tourism (Cirri and Pohnert, 2019; Díaz-Torres et al., 2021; Li et al., 2025).

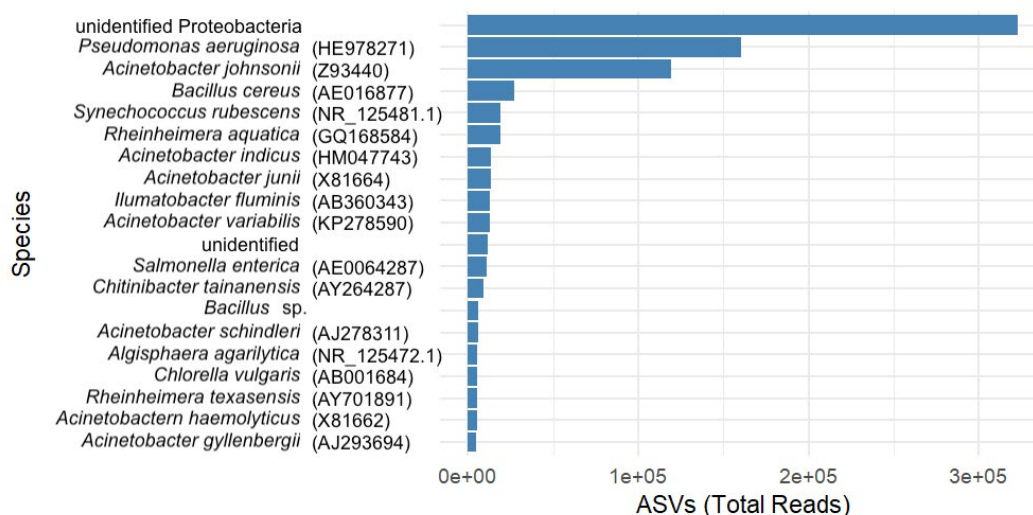


Fig. 2. Total Amplicon sequence variants (ASV) count in Lake Holon, Philippines.

The sudden decrease to a long tail of rare taxa indicates that resilient heterotrophs in particular may temporarily outdominate these predominant functional niches, while a diverse field of background taxa stays and contributes to sustaining the lake's baseline state (Forster et al., 2021; Qiu et al., 2024). The low sequence abundance of photoautotrophic cyanobacterium *S. rubescens* highlights that heterotrophic activity is dominant in this area over primary production (Matthews et al., 2023; Caro et al., 2025). Such a pattern is common in deep, clear or stratified crater lakes on which the cycling of nutrients is limited to very individual thermal strata. *B. cereus* retention illustrates the structural strength of the community. By virtue of its spore-forming Firmicutes properties, it evades low nutrient or tourism times by means of metabolic dormancy, returning when resources are replenished (Caro et al., 2025). Furthermore, the numerous rare low abundant *Acinetobacter* relative taxa, *A. indicus*, *A. junii*, and *A. variabilis* indicate a high microbial diversity in the genus. This pattern also shows that relatively well-dwelling *A. johnsonii* species is adapted to those of the microbial seed bank, with its relatives remaining in low extent at all (Shade et al., 2014; Mikkelsen et al., 2016; Forster et al., 2021; Mills et al., 2025). That redundancy can help stabilize community function if selective pressures change (Fu et al., 2025).

3.2. Spatial Heterogeneity and Site-Specific Relative Abundances

The relative abundance of bacterial communities varied considerably between the center (S19-S21) and outskirt (S22-S24) sampling sites of Lake Holon (Fig. 3). The bacterial assemblages across all sites were primarily composed of *Pseudomonas aeruginosa*, unknown taxa, *Synechococcus rubescens*, *Acinetobacter* spp., and a number of *Bacillus*, *Rheinheimera*, and *Chitinibacter*. The total ASV profile presented in Fig. 3 revealed that the unidentified Proteobacteria showed the highest overall reads, with *Pseudomonas aeruginosa* followed by

Acinetobacter johnsonii, indicating that Proteobacteria continue to be one of the most prominent microbiota in the lake environment. It agrees with the previous freshwater metabarcoding studies reporting predominance of Proteobacteria in oligotrophic and moderately disturbed freshwater systems as they are metabolically versatile and adapt to varying environmental conditions (Newton et al., 2011; Xie et al., 2024; Feng et al., 2024; Lezcano et al., 2024; Wu et al., 2024).

The pH in the center of the lake was lower (4.39-4.48) than in the periphery (5.30-5.60) (Table 2). Acidic environments exert a significant impact on the structure of bacterial communities, by promoting the survival of acidic taxa, while inhibiting acidic sensitive bacterial groups (Lauber et al., 2009). Acidic freshwater is rich in Proteobacteria and Acidobacteria which maintain physiological organisms for survival within a low pH ecosystem (Jones et al., 2009). The large number of unknown Proteobacteria in these center sites may therefore reflect localization to an acidic environment such as the one found within the lake. Additionally, the dissolved oxygen (DO) ratio in the center sites was slightly lower (6.20-6.30 mg/L) than in the outskirts (6.50-6.70 mg/L), which may indicate changes in bacterial metabolism and composition.

Another notable observation in the center sites was the relatively high abundance of *Synechococcus rubescens*, particularly in the S20 and S21 region. *Synechococcus* is a photosynthetic cyanobacterium frequently found in oligotrophic freshwater systems and an important contributor to primary productivity and carbon cycling (Callieri, 2008; Lucius and Hagemann, 2024; Laila et al., 2026). The presence of the species in the center could reflect stable pelagic conditions with intermediate nutrients and stable multiples in relatively permanent environmental conditions with intermediate nutrients and stable thermal conditions (Paerl and Otten, 2013). It was observed that the water temperatures in the center sites ranged from 23.60-24.13°C thus, providing favorable conditions for cyanobacterial persistence.

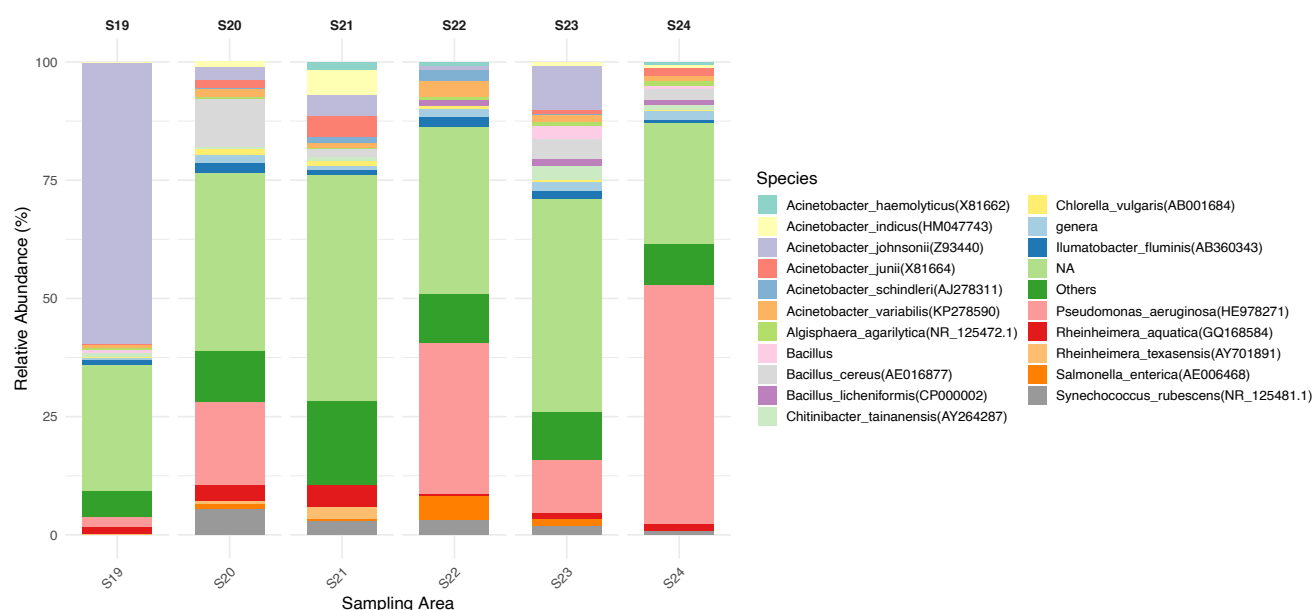


Fig. 3. Relative abundance of bacterial species across sites S19-S24 based on 16S rRNA gene amplicon sequencing; taxa with <1% relative abundance were grouped as “Others”; “NA” denotes sequences that could not be assigned at the species level.

Table 2. Physico-chemical parameters in Lake Holon.

Sample	Location	pH	Temperature (°C)	Salinity (ppm)	Dissolved Oxygen (mg/L)
S19	center	4.41	24.13	80	6.3
S20	center	4.39	23.6	85	6.3
S21	center	4.48	24	95	6.2
S22	outskirt	5.3	24.5	80	6.6
S23	outskirt	5.5	24.7	100	6.5
S24	outskirt	5.6	24.9	110	6.7

Note: *parameters were measured in triplicates and average values are recorded

The outskirts sites (S22-S24) had been notably enriched by *Pseudomonas aeruginosa*, particularly at S24, which accounted for over 50% of the total bacterial abundance. *P. aeruginosa* was upregulated as pH, salinity and dissolved oxygen were rising in S22-S24 (Table 2). Outskirt areas had an increased salinity value (80-110 ppm), higher DO concentration (6.50-6.70 mg/L), and slightly warmer temperature (24.50-24.90°C). *Pseudomonas* are metabolically flexible bacteria that can prosper in both enriched and disturbed waters (Mena and Gerba, 2009). The predominant occurrence of these organisms at outskirts sites may be suggestive of increased organic matter production or the presence of more environmental exposure at lake margins. *P. aeruginosa* is known as an opportunistic pathogen species and as an environmentally robust bacterium, capable of biofilm formation and degradation of xenobiotics (Moradali et al., 2017). Their local abundance in the periphery of this lake may reflect growing anthropogenic influence or terrestrial runoff through its periphery. Previous studies suggest that in shoreline and littoral zones, nutrient and organic matter inputs from surrounding vegetation, soil erosion and human activities are often higher and therefore support copiotrophic bacterial lineages such as *Pseudomonas* (Crump et al., 2007). Moreover, the recorded higher pH levels in the outskirts sites might also support the diversity of *Pseudomonas*, as many as this species can be found under near-neutral or slightly acidic conditions.

The presence of numerous species of *Acinetobacter* (*A. johnsonii*, *A. junii*, *A. schindleri*, *A. indicus* and *A. variabilis*) in both center and outskirts sites are likely contributing to the ecological significance of *Acinetobacter* in lake Holon. *Acinetobacter* species are found in freshwater systems in abundance and have been reported to carry out nutrient cycling, degradation of organic matter, and environmental stress resistance (Doughari et al., 2011). The fact that they persisted at so many sites indicate a wide ecological tolerance and survival for different environmental physicochemical conditions. The presence of *A. johnsonii* in particular agrees with observations of freshwater lakes for which this species generally predominates in oligotrophic to mesotrophic environments (Bergogne-Bérézin and Towner, 1996). Active decomposition on the lake may also suggest the existence of *Bacillus cereus* and other *Bacillus* species at the center and outskirts areas. Specialist species of *Bacillus* are producers of extracellular enzymes that

breakdown complex organic materials and contribute to recycling of nutrients from an aquatic water system (Mandic-Mulec et al., 2016; Emmanuel et al., 2025; Punnorkodu et al., 2026). Their association may also be linked to sediment resuspension and terrestrial runoff, especially in shorelines. *Chitinibacter tainanensis* and *Algisphaera agarilytica* were also found across multiple sites in Lake Holon. The presence of these bacteria indicates active microbial decomposition within the lake, as both groups are known to actively participate in the breakdown of complex aquatic polysaccharides. These bacteria play a major role in nutrient remineralization especially in oligotrophic volcanic lakes with poor nutrient availability (Azam et al., 1983; Falkowski et al., 2008; Aguilar et al., 2022).

The observed differences in bacterial composition between the center and outskirts sites indicate that physicochemical gradients play a substantial role in the microbial composition at Lake Holon. pH appeared to be one of the key environmental variables mediating bacterial communities. Fierer and Jackson (2006) found similar results, with pH being one of the strongest predictors of bacterial diversity in aquatic and terrestrial environments. The center sites were generally more acidic, thus producing more unknown and acid-tolerant taxa, while the slightly less acidic margin favors metabolically versatile genera, such as *Pseudomonas* and *Acinetobacter*. Dissolved oxygen (DO) also has contributed to spatial imbalances detected, where higher DO concentrations in the outskirts likely supported aerobic heterotrophic bacteria capable of rapid organic matter degradation. Aerobic bacterial communities are prevalent in oxygen-rich environments, where oxygen improves metabolic performance and nutrient utilization (Wetzel, 2001). Additionally, the slightly higher temperatures in the outskirts could have increased microbial metabolic activity, promoting higher bacterial growth rates. Similarly, the gradual increase in salinity from the center toward the outskirts areas may also affect the composition of bacteria. Although the salinity is still low, slight ionic variations of freshwater systems could influence different concentrations of microbes by affecting osmotic equilibrium and the solubility of nutrients (Logares et al., 2014). Certain taxa such as *Pseudomonas* and *Acinetobacter* possess adaptive mechanisms allowing tolerance to changing ionic conditions, which may explain their increased abundance in the outskirts areas.

The fact that a significant number of sequences were NAs regardless of the location sampled emphasizes the taxonomic inadequacies associated with sequence annotation. The NAs may correspond to sequences of unknown taxa that could not be successfully mapped against any known reference sequences in the databases, and may include novel microbial taxa from Lake Holon that are currently undocumented. In addition, the high number of NAs could be attributed to the poor coverage of the current microbial reference libraries, especially with regard to tropical freshwater lakes, which have been relatively underrepresented in the global sequence database. However, despite the limitations, the presence of a large number of unassigned taxa has ecological significance since this could mean the presence of an important fraction of microbial diversity that remains unresolved within this volcanic crater lake. Based on the restricted nature of the crater basin habitat of Lake Holon and the unique physical and chemical characteristics of its environment, some of these unclassified taxa could be microbes that have evolved under specific environmental conditions; this, however, needs further verification. The high fraction of unassigned taxa makes future analyses concerning the ecological characterization of the microbial community of this lake difficult, especially concerning community characterization and environment-taxon associations, as well as the determination of the metabolic profile of the microbial species of this lake. For future studies, there should be consideration of incorporating methods that would increase taxonomic and functional resolution, such as long read amplicon sequencing, compre-

hensive reference databases, shotgun metagenomics, and, where possible, genome-resolved metagenomics.

Noteworthy, a number of species identified in Lake Holon, such as *Synechococcus rubescens*, *Rheinheimera aquatica*, *Ilumatobacter fluminis*, *Chitinibacter tainanensis*, and *Algispheera agarilytica*, seem to have little to no presence in any of the literature in relation to freshwater bodies in the Philippines. Hence, the identification of such may be regarded as possible new molecular records for the Philippines, or at the very least, as first detections in a volcanic crater lake in the Philippines. This is, however, subject to verification since species-level assignment using only the V3–V4 16S rRNA amplicons may not be sufficient.

3.3. Diversity indices in Lake Holon

The alpha diversity indices revealed marked variation in microbial community structure among the six sampling sites in Lake Holon (Fig. 4). Species richness and Chao1 estimates showed identical trends, indicating that observed richness closely reflected the estimated total richness across sites. Site S20 recorded the highest richness and Chao1 values ($S=138$), suggesting the presence of the greatest number of taxa and indicating a highly diverse community with minimal undetected species. This was followed by S23 ($S=128$) and S24 ($S=126$), while S22 ($S=100$) and S21 ($S=94$) exhibited moderate richness levels. In contrast, S19 recorded the lowest richness and Chao1 values ($S=66$), reflecting comparatively reduced taxonomic composition.

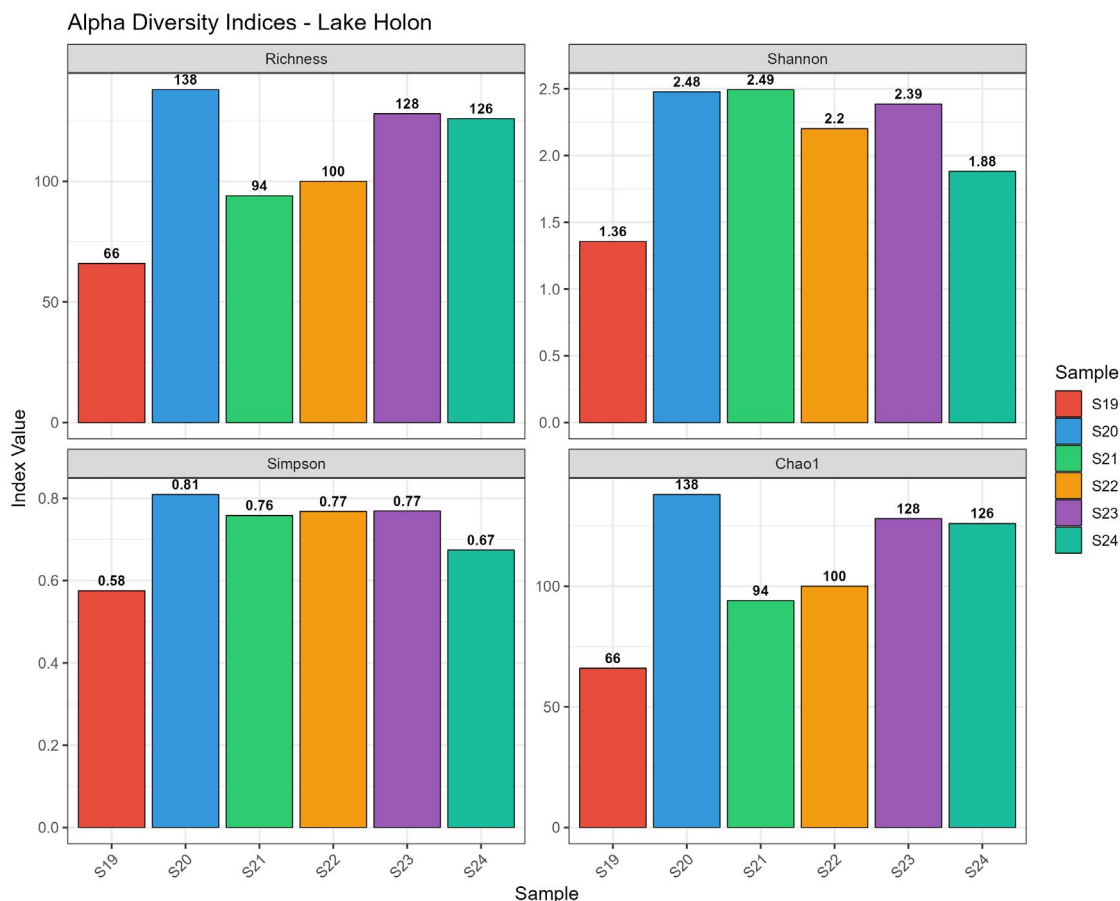


Fig. 4. Bar plot of alpha diversity metrics, including Shannon diversity, Species richness, Simpson diversity, and Chao1 estimator, across six sampling sites in Lake Holon, Philippines.

The Shannon diversity index, which incorporates both species richness and evenness, showed that S21 exhibited the highest diversity value ($H' = 2.49$), closely followed by S20 ($H' = 2.48$). This indicates that although S20 contained the highest number of taxa, S21 supported a more proportionally distributed community structure with greater ecological balance. Sites S23 ($H' = 2.39$) and S22 ($H' = 2.20$) also demonstrated relatively high diversity, suggesting moderately complex and stable assemblages. Meanwhile, S24 showed a lower Shannon value ($H' = 1.88$), and S19 exhibited the lowest value ($H' = 1.36$). The markedly low diversity in S19 suggests reduced community complexity, lower species richness, and a more uneven distribution of taxa within the assemblage.

A similar trend was observed in the Simpson diversity index, which emphasizes dominance and community evenness. Site S20 demonstrated the highest dominance-adjusted diversity, indicating lower dominance and greater evenness among taxa depicting a more stable and evenly distributed species assemblage. Sites S22 and S23 shared nearly identical Simpson values ($D = 0.77$), followed closely by S21 ($D = 0.76$), reflecting relatively stable and balanced community structures across these sampling areas. In contrast, S24 consistently displayed lower diversity relative to the aforementioned sites, although it remained considerably higher than S19 which again recorded the lowest value ($D = 0.58$). The persistently low Simpson diversity value in S19 further indicates the probable dominance of a few taxa and reduced ecological stability within the site. The diversity indices collectively suggest that S20 and S21 represent the most biologically diverse and ecologically balanced sites in Lake Holon, whereas S19 appears to harbor a less complex and less evenly distributed microbial community. Sites such as S20 and S21 may harbor a more complex community structure in which species abundance is more proportionally distributed, representing greater ecological balance and community heterogeneity compared to the other sampling sites. Furthermore, the observed differences among sites may reflect variations in environmental conditions, habitat heterogeneity, nutrient availability, and physicochemical characteristics influencing microbial assemblage structure and distribution (Deng et al., 2024; Hou et al., 2025).

The observed variation in species richness and diversity in Lake Holon sampling sites may be attributed to the differences in habitat conditions, ecological stability, resource availability, environmental disturbances across the lake. Biological and water physicochemical factors both likely influenced the distribution, abundance, and evenness of species in each site (Therriault and Kolasa, 1999; Dai et al., 2023; Loucif and Chenchouni, 2024; Salami et al., 2025). The high species richness recorded in S20 suggests that this site may provide favorable environmental conditions (Langenheder and Ragnarsson, 2007; Deng et al., 2017), such as suitable substrate composition, adequate nutrient availability (Deng et al., 2017), and optimal water quality parameters (Cardinale, 2011; Desrosiers et al., 2013; Deng et al., 2017). Areas especially with

structurally complex habitats often support more ecological niches and allow more or greater numbers of species to coexist (Giller, 2012; Meerhoff and de los Angeles González-Sagrario, 2022; Soukup et al., 2022). However, although S20 had the highest richness, its diversity is lower than S21, which indicates that some species may have been numerically dominant, reducing community evenness. On the other hand, S21 exhibited the highest diversity indices despite having lower species richness than S20 which could suggest a more balanced distribution of individuals among species, where no single species strongly dominated the community. High diversity with moderate richness is commonly associated with healthy ecosystems showing strong ecological interactions and balanced competition (Mougi, 2024).

3.4. Spatial Variation in Microbial Community Composition in Lake Holon

The rarefaction graphs show a definite variation in taxonomic richness observed in all six locations of Lake Holon, even as it reveals the adequacy of sequencing depth in sampling most of the diversity observed (Fig. 5). The rarefaction curves increase sharply initially and gradually reach asymptotic levels, implying that there is a decrease in the recovery of new species with the increasing sequencing efforts. The observation shows that the bacterial community was well sampled in the process and any variation in richness cannot be due to the inadequate sequencing depth.

The highest level of richness was seen in Sample S20, which achieved approximately 138 taxa, while S23 and S24 both achieved approximately 128 and 126 taxa respectively. Hence, these samples contained the most taxonomically diverse bacterial community. S22 showed medium richness at around 100 taxa, while S21 showed a lower richness of about 94 taxa. Lastly, sample S19 had the least number of taxa at about 66, representing a much lower taxonomic diversity compared to other samples. Hence, the richness for the five samples follows this order: $S20 > S23 > S24 > S22 > S21 > S19$. While there is a strong resemblance between S23 and S24 in terms of bacterial richness, there is an evident disparity between S19 and all the other samples, showing a very high spatial heterogeneity of microbial diversity in Lake Holon. Notably, most curves tend to plateau before or just about the vertical dashed line, which represents the read number of 118,000. This is an indication that the major diversity patterns have been successfully captured at a certain sequencing depth. However, S20 is increasing slightly at high read numbers (Fig. 5).

The low richness in S19 may indicate stronger environmental filtering or localized disturbance at the site, while the higher richness in the S20, S23, and S24 suggest a higher niche and lower environmental stress (Lauro et al., 2009). This is consistent with oligotrophic freshwater lakes, where the predominant taxa dominate the reads while the rare biosphere has a secondary role in contributing the overall richness (Newton et al., 2011). In such systems even minor changes in

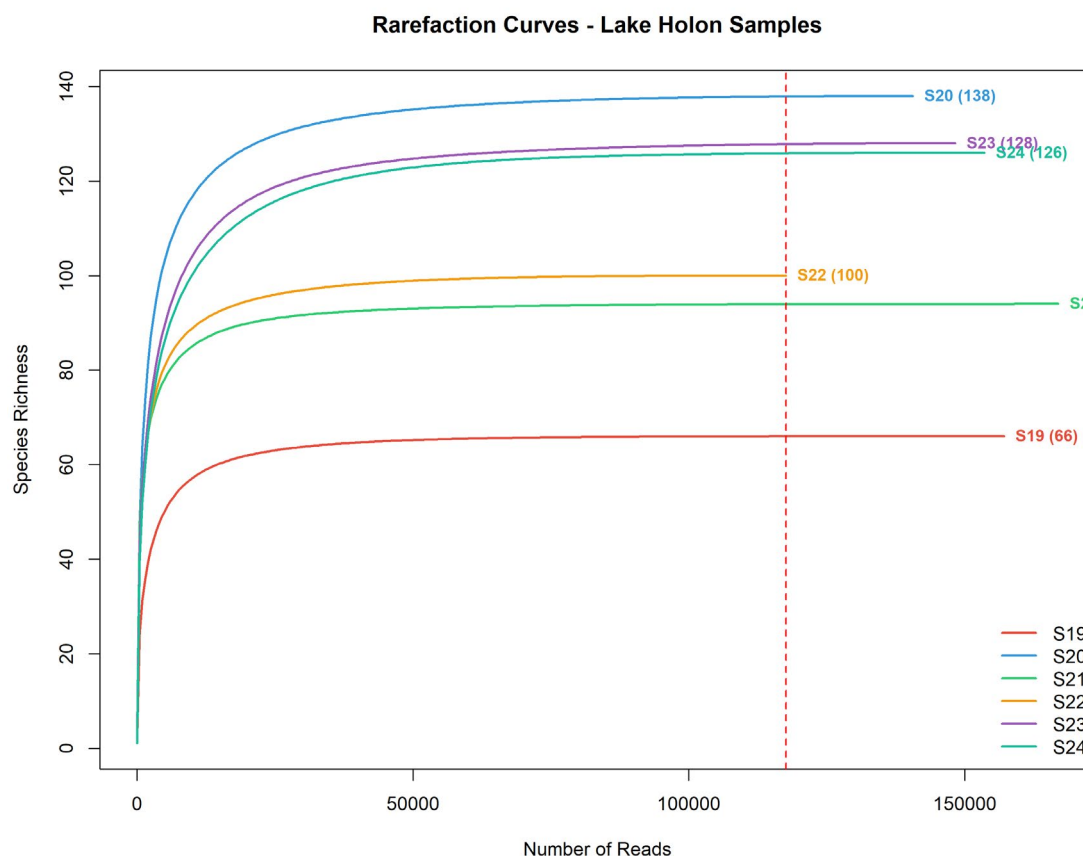


Fig. 5. Rarefaction curves showing observed species richness as a function of sequencing depth. Curves approach an asymptote, indicating sufficient sequencing depth.

nutrient supply and disturbance results in perceptible changes in richness because copiotrophic taxa rapidly colonize recently-developed resources while oligotrophic specialists recede (Lauro et al., 2009). Richness increased at the outskirts sites, indicating that nutrient inputs might relax resource constraints, increasing the space for opportunistic heterotrophs to compete at an expanded niche, without replacing native oligotrophs (Lauro et al., 2009; Newton et al., 2011).

Beta dispersion analysis revealed that outskirts sites had smaller and tighter clusters around their

centroid and center sites had dispersion (Fig. 6). The median distance to centroid was ~ 0.36 for outskirts samples versus ~ 0.44 for center samples. The narrower the region, the more homogeneous the communities, as expected from the deterministic filtering by constant and consistent environment, or similar disturbance (Chase and Myers, 2011). A higher dispersion of center sites indicates a more heterogeneous distribution and is more susceptible to stochastic factors like ecological drift and variable colonization (Zhou and Ning, 2017).

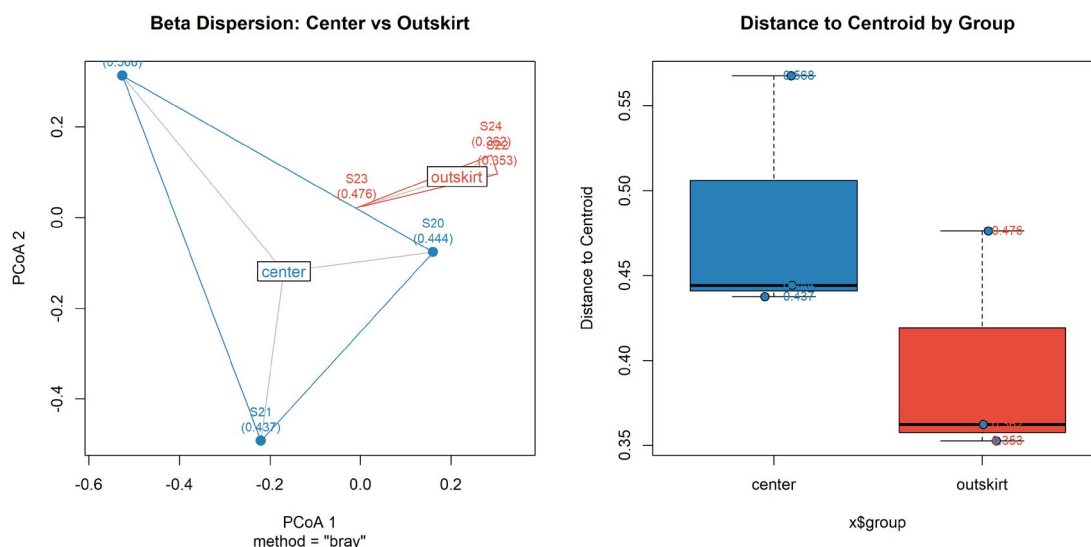


Fig. 6. Left: Principal Coordinate Analysis (PCoA) of Bray-Curtis distances illustrating the dispersion of samples around group centroids. Right: Boxplot of distances to centroid by group.

The beta dispersion analysis based on Bray–Curtis distances is illustrated (Fig. 6), showing the variability of microbial community composition within the defined groups (“center” and “outskirt”). In the PCoA ordination plot, samples belonging to the center group (S19, S20, and S21) exhibited a wider spatial dispersion around their centroid compared to the outskirts group (S22, S23, and S24). This indicates greater heterogeneity and compositional variability among microbial communities in the center sites. In particular, S19 and S21 were positioned farther from the centroid, suggesting that these samples differed substantially in community composition relative to the other center samples.

In contrast, the outskirts samples formed a relatively tighter clustering around their centroid, indicating lower within-group variability and more similar microbial assemblages among these sites. Although S24 appeared slightly separated from S22 and S23, the overall dispersion of the outskirts group remained comparatively smaller than that of the center group. The relatively tighter clustering of the outskirts samples suggests that these sites likely experience more similar environmental conditions, which could lead to a more homogeneous microbial community composition (Marois et al., 2022; Wang et al., 2025b). Ecologically, lower within-group variability often indicates that the microbial assemblages are being shaped by comparable physicochemical factors such as temperature, pH, dissolved oxygen, nutrient availability, light penetration, sediment characteristics, or hydrological conditions (Marois et al., 2022; Tran et al., 2025; Velasco, 2026; Wissmueller, 2025; Ma et al., 2026). When environmental conditions are relatively uniform across sites, ecological selection pressures become more consistent, favoring similar groups of microorganisms and leading to reduced community dissimilarity (Ning et al., 2024; Duque-Castaño et al., 2025; Vimercati et al., 2026).

The boxplot of distances to centroid further supported these observations. The center group exhibited higher median distances and a broader range of dispersion values, indicating greater variability in microbial community structure among center sites. Conversely, the outskirts group showed lower median distances and narrower dispersion, showing a more homogeneous community composition. The results suggest that microbial assemblages in the center sites were more compositionally heterogeneous, whereas outskirts sites harbored relatively more stable and similar microbial communities.

Bray–Curtis dissimilarity detected highly variable compositional turnover in sites (ranging between 0.62 to 0.92) (Fig. 7). S19 was highly dissimilar to all other sites, especially S22 (0.92), with S22 and S24 presenting the lowest degree of dissimilarity (0.62) and showed similar communities. This structure was supported by ordination analyses. NMDS with a stress value of 0 (Fig. 8) and a Shepard plot with a non-metric $R^2=1$ (Fig. 10) confirmed that the two-dimensional ordination accurately showed the original Bray–Curtis dissimilarities. PCoA accounted for 58.6% of total variance on the first two axes and distinguished S19, S21, and S20 from S22, S23 and S24 (Fig. 9). This discrepancy

is consistent with our previous findings of “center” and “outskirt” samples. The clustering of S22, S23, and S24 indicates an environmental homogenization in the outskirts zone likely influenced by shoreline processes, wind mixing and human effects related to ecotourism (Eiler et al., 2011). In similar spatial structures, other crater lakes have also been reported, where microhabitats induced by depth, light and nutrient gradients promote beta diversity (Lindström, 2000). For smaller, shallow lakes, even slight changes in their exposure to wind or organic input create unique microbial habitats because bacterial generation times are very short and communities respond quickly according to environmental changes (Shade et al., 2011; Lindström, 2000). The distinctiveness of S19, which was the most underdetermined and least abundant (both richness and dissimilarity), might be due to a reference condition where anthropogenic influence is low, serving as a standard for assessing human impact across the lake. Beta dispersion as well as ANOSIM shows different assembly processes between center and outskirts zones. PCoA1 explained 34.2% of the total variance, while PCoA2 accounted for 24.4%, collectively representing 58.6% of the variation in microbial assemblages across the lake (Fig. 9).

The ordination plot revealed clear spatial separation among several sampling sites, indicating distinct microbial community structures. S19 was positioned far from the other samples along the positive axis of PCoA1 and PCoA2, suggesting that it possesses a highly distinct microbial assemblage compared to the other sites. Likewise, S21 was separated along the negative axis of PCoA2, indicating another unique microbial composition. The distinct positioning of these samples suggests that environmental conditions at these locations may strongly influence microbial diversity and community structure (Liu et al., 2023; Wang et al., 2023; Zhang et al., 2024).

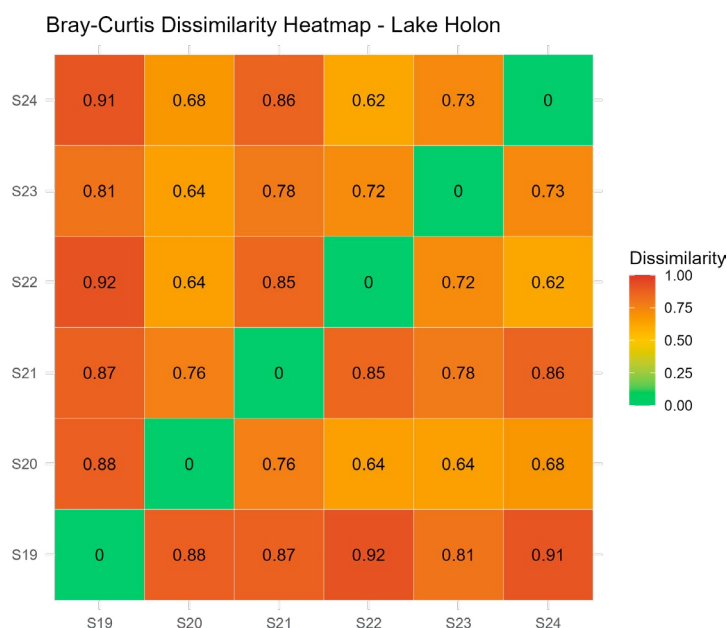


Fig. 7. Pairwise dissimilarity matrix visualized as a heatmap. Values ranged from 0 (identical) to 1 (completely dissimilar).

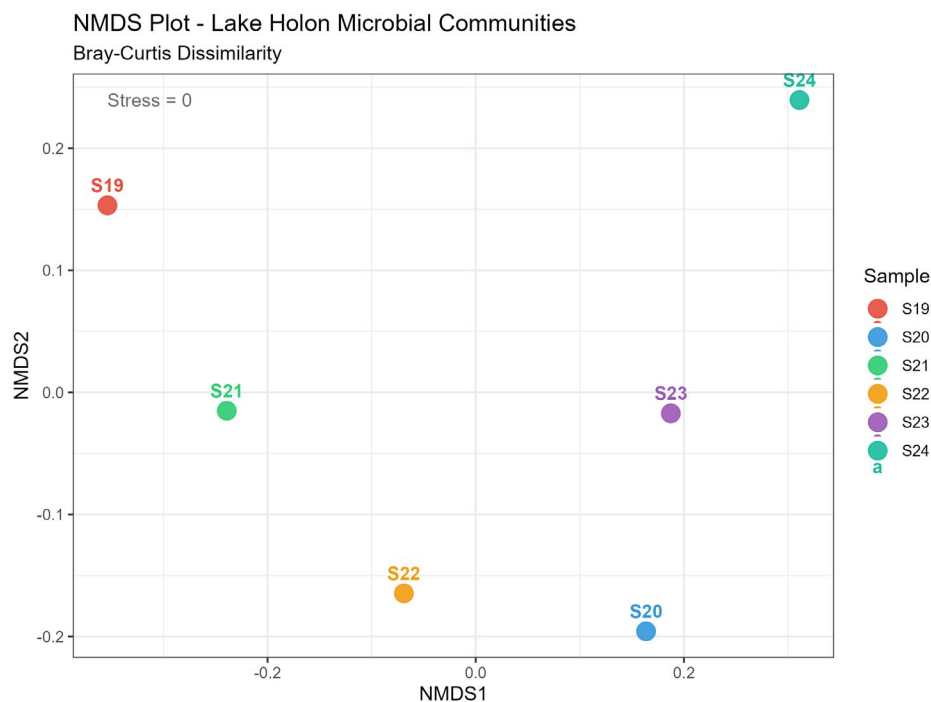


Fig. 8. Non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity (stress = 0).

Conversely, S22 and S24 clustered closely together, indicating relatively similar microbial communities and lower compositional variation between these sites. S20 and S23 occupied intermediate positions within the ordination space, suggesting that there is moderate similarity with neighboring samples but still maintaining distinct community characteristics (Fig. 9). Thus, the observed clustering and separation patterns of microbial communities in Lake Holon are spatially structured rather than uniformly distributed which is evidently demonstrated by the analysis that microbial community composition varies significantly across spatial locations (Zhang et al., 2023; Loucif and Chenchouni, 2024).

3.5. Functional Prediction of the Microbiome Composition

The functional potential of the bacterial communities in Lake Holon was predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) based on KEGG pathway annotations at Level 2 (Fig. 11). The predicted functional profile revealed the presence of different pathways that corresponded to amino acid metabolism, carbohydrate metabolism, lipid metabolism, metabolism of cofactors and vitamins, signal transduction, cellular processes, xenobiotic biodegradation and metabolism, and protein families. These pathways are

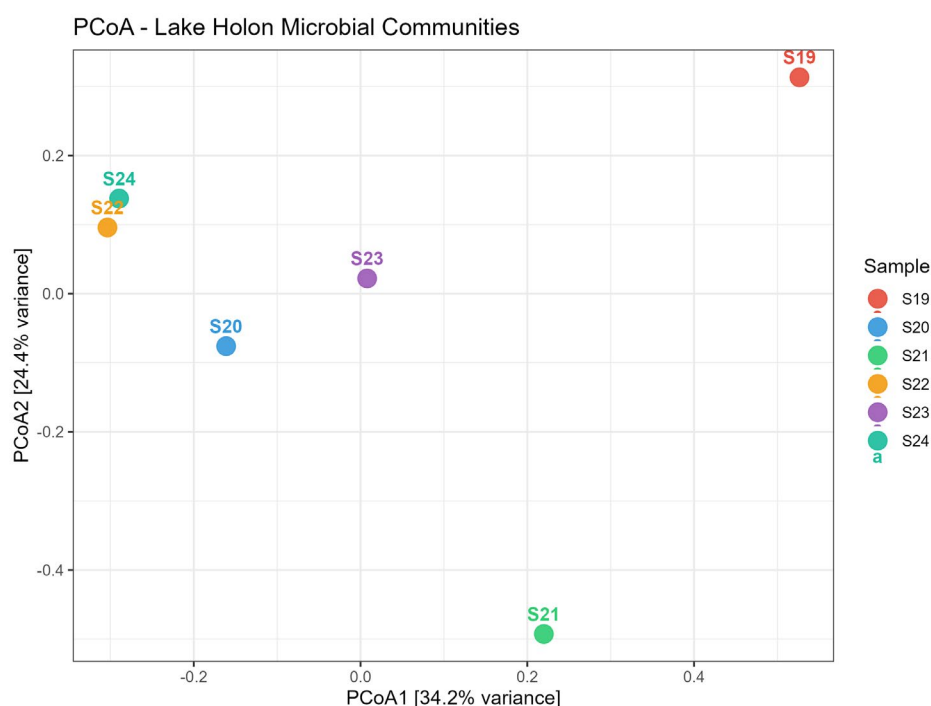


Fig. 9. Principal coordinates analysis based on Bray-Curtis distances. PCoA1 explains 34.2% and PCoA2 explains 24.4% of variance, separating samples by spatial location.

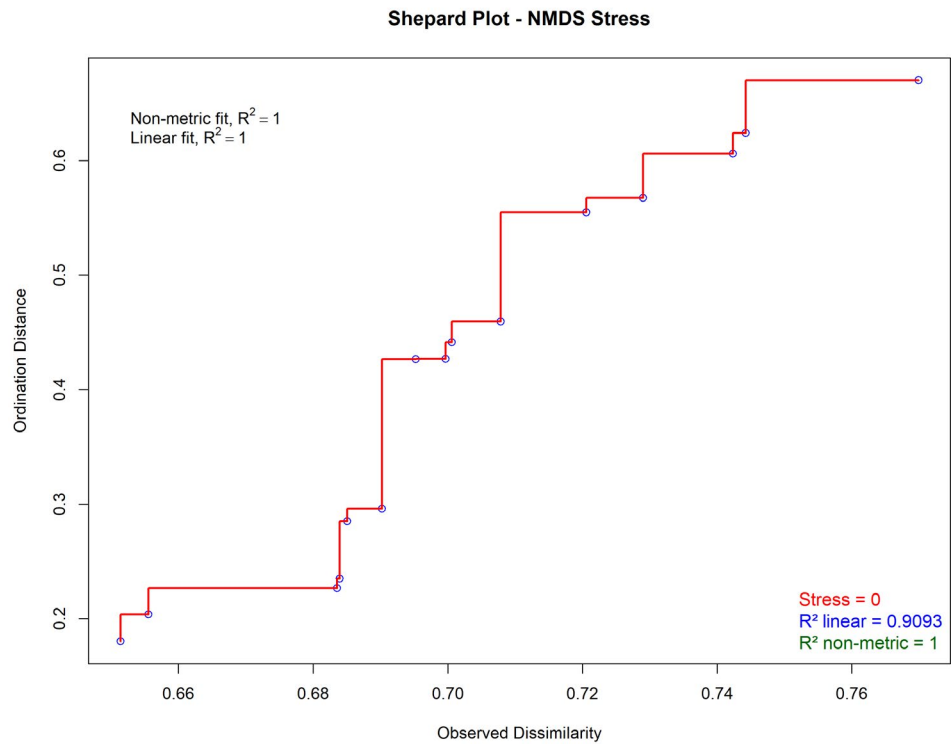


Fig. 10. Shepard plot showing the relationship between observed Bray-Curtis dissimilarities and ordination distances obtained from NMDS. Non-metric fit $R^2 = 1$ and liner fit $R^2 = 0.9093$ indicate excellent ordination quality.

essential metabolic functions commonly found in fresh-water bacterial communities and indicate broad functional capabilities related to nutrient cycling, energy production, and environmental adaptation (Langille et al., 2013; Douglas et al., 2020). The dominant carbohydrate and amino acid metabolism pathways indicate that bacterial communities play an important role in carbon decomposition, organic matter turnover and

nitrogen recycling in the oligotrophic lake ecosystem (Azam et al., 1983; Newton et al., 2011). Similarly, the abundance of energy metabolism and signal transduction pathways indicate that the microbial community is well adapted to local environmental changes such as pH, dissolved oxygen, and nutrient availability (Stock et al., 2000).

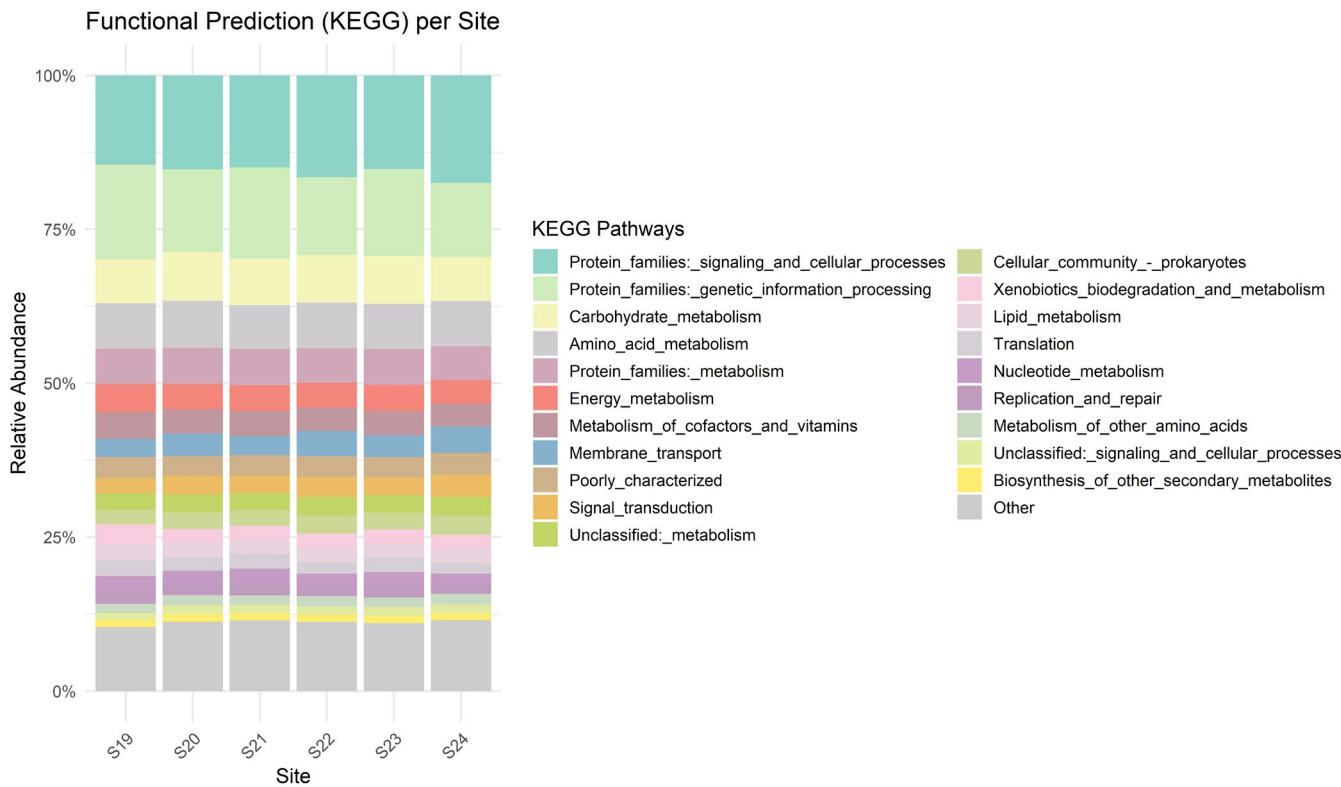


Fig. 11. PICRUSt predictions of the functional composition of the respective microbiome samples as represented at KEGG pathway.

The presence of xenobiotic biodegradation pathways implies the metabolic versatility of dominant bacterial genera such as *Pseudomonas* and *Acinetobacter*, which are known to degrade numerous natural and anthropogenic organic compounds (Mena and Gerba, 2009; Moradali et al., 2017). Although Lake Holon is considered a relatively clean ecosystem, these pathways represent the ability of resident microbes to digest organic compounds from the surrounding vegetation and human activities rather than pollution. Nevertheless, due to the nature of this method, which predicts the functional potential based on the analysis of the taxonomy profile of 16S rRNA gene sequences, the conclusions need to be further validated by shotgun metagenomics or metatranscriptomics (Douglas et al., 2020).

3.6. Ecological implications for Lake Holon

Collectively these data suggest a balancing act of spatial diversity and homogenization in the microbial ecosystem of Lake Holon. Low dispersion and high microbial diversity and the high richness in the outskirt indicates that tourism-induced pollutants may be selecting for a fraction of copiotrophic taxa at outskirt sites, thus reducing heterogeneous data across samples (Eiler et al., 2011). The abundance of opportunistic heterotrophs of *Pseudomonas* and *Acinetobacter* in outskirt sites confirm a local organic loading and nutrient supplementation interpretation (De Victorica and Galván, 2001). They show a high rate of adaptation of labile carbon sources and are closely related to anthropogenic-determined freshwater systems (De Victorica and Galván, 2001; Newton et al., 2011). The middle zone still has greater variation and diversity and may be a less disturbed reference condition mainly influenced by stochastic neutral dynamics (Zhou and Ning, 2017). The presence of high proportions of unclassified Proteobacteria across all sites also emphasizes the persistence of microbial dark matter in isolated aquatic systems, similar to other oligotrophic lakes at high altitudes (Sommaruga, 2001). These unclassified lineages frequently harbor unique metabolic pathways to benefit from transient resource pulses and small environmental variations, which serve to reinforce the ability of ecosystems to maintain stability (Sommaruga, 2001). Future studies comprising seasonal sampling and visitor counts would help distinguish if the observed patterns are dominated by tourism, natural stratification or their synergy.

4. Conclusion

This study presents the first eDNA metabarcoding-based assessment of bacterial communities in Lake Holon, providing a baseline for microbial ecosystem monitoring. The community was dominated by unidentified Proteobacteria, with notable spatial differences between center and outskirt sites driven by environmental gradients and possible anthropogenic influence. *Rheinheimera aquatica* and *Salmonella enterica* are

particularly abundant in S21 and S22, respectively. *Pseudomonas aeruginosa* is primarily dominating across all sites along with other bacterial assemblages, such as *Synechococcus rubescens*, *Acinetobacter* spp., and a number of *Bacillus*, *Rheinheimera*, and *Chitinibacter*.

Various taxa found in Lake Holon, specifically *Synechococcus rubescens*, *Rheinheimera aquatica*, *Ilumatobacter fluminis*, *Chitinibacter tainanensis*, and *Algisphaera agarilytica*, seem to have little presence in any research publications that pertain to the freshwater bodies in the Philippines. Such findings may thus imply potential new molecular records for the Philippines, and even more cautiously, for a Philippine volcanic crater lake. Nevertheless, the significance of this finding is further emphasized by the capability of eDNA metabarcoding to uncover the hidden diversity of microorganisms in less-studied tropical freshwater habitats. However, such biogeographical information should be viewed as preliminary, since species identifications based only on 16S rRNA gene amplicons in V3-V4 region need to be further verified. Nevertheless, this taxonomic information for any occurrence may serve as first record claims after verification.

Overall, the lake remains a relatively healthy oligotrophic system, although peripheral areas show early signs of ecological disturbance. These findings highlight the use of eDNA metabarcoding for freshwater monitoring and support SDG 6 (Clean Water and Sanitation), SDG 11 (Sustainable Cities and Communities), and SDG 15 (Life on Land), emphasizing its value in guiding conservation and sustainable management of Lake Holon.

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Conflict of Interest

The authors declare no conflict of interest.

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