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Geochemical and Microbial Stratification in Deep Volcanic Lakes - Implications for Ecohydrological Processes: Insights from Lake Albano (Lazio, Italy)

Faculty of Sciences and Technology

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Declaration of Authorship

I, Bella SAFITRI, declare that this thesis titled, "Geochemical and Microbial Stratification in Deep Volcanic Lakes - Implications for Ecohydrological Processes: Insights from Lake Albano (Lazio, Italy)" and the work presented in it are my own. I confirm that it is original and unpublished. Authors and papers consulted are duly cited in the text and are listed in the included references.

Signed:

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Date:

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Abstract

Over the last three decades, Lake Albano has undergone comprehensive investigations to elucidate the physicochemical mechanisms governing gas outbursts post the catastrophic limnic eruption in Lake Nyos. While the interplay between biogeochemical processes and microbial communities within volcanic lakes is widely recognized, the understanding of these communities remains limited. This study aims to bridge this gap by investigating the intricate interplay among water chemistry, dissolved gases, and microbial community dynamics in Lake Albano, employing an integrated approach of geochemical and microbiological methods, specifically NGS and CARD-FISH. The findings reveal the simultaneous occurrence of diverse functional groups of microbes operating under different physicochemical conditions, suggesting the presence of complex microbial consortia with implications for greenhouse gas generation and consumption. Moreover, the relationship between water depth and the fluctuations in microbial community structure and physiology characteristics within Lake Albano was observed. These findings emphasize the importance of the functional dynamics of microbial communities that consequently shape biogeochemical cycling patterns. Ultimately, comprehending microbial biodiversity and functioning within the ecohydrological processes in Lake Albano contributes essential knowledge for biodiversity conservation, enhancing ecosystem resilience through ecohydrological approaches, and supporting bioprospection for the realization of UN SDGs.

Keywords: Lake Albano, microbial communities, biogeochemical processes, ecohydrological approaches, volcanic lakes, NGS, CARD-FISH, UN SDGs.

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Bella Safitri

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List of Abbreviations

AOM	**A**naerobic **O**xidation of **M**ethane
AWCD	**A**verage **W**ell **C**olor **D**evelopment
CARD-FISH	**CA**talyzed **R**eported **D**eposition **F**luorescence **I**n **S**itu **H**ybridization
CLPP	**C**ommunity **L**evel **P**hysiological **P**rofiles
HNA	**H**igh **N**ucleic **A**cid
LNA	**L**ow **N**ucleic **A**cid
NGS	**N**ext **G**enerations **S**equencing
NMDS	**N**onmetric **M**ulti **D**imensional **S**caling
OTU	**O**perational **T**axonomic **U**nit

1 Introduction

Over the past three decades following the catastrophic limnic eruption in Lake Nyos, Cameroon, in 1986 (Kling et al., 1987; Tanyileke et al., 2019), volcanic lakes were intensively studied, with a particular emphasis on investigating the physicochemical mechanisms regulating the occurrence of the gas bursts and dynamics of CO_2 degassing, and Lake Albano is no exception. Lake Albano, located 20 km southeast of Rome, is the deepest volcanic lake in Europe which is hosted within the youngest crater of the quiescent Colli Albani volcano complex with records of phreatic activity throughout the Holocene (Anzidei et al., 2008; Benedetti et al., 2008).

In 1989-1990, a long seismic swarm occurred beneath Colli Albani, resulting in the injection of a significant amount of CO_2 into the deeper layers of Lake Albano (Amato et al., 1994; Carapezza et al., 2008; Chiodini et al., 2012; Rouwet et al., 2021). This event and the recent limnic eruption at Lake Nyos have raised concerns about the potential geological hazards of Lake Albano. Nevertheless, the CO_2 recharge triggered by the seismic activity in 1989-1990 did not reach a sufficient volume to cause CO_2 supersaturation in the bottom waters of Lake Albano and the consequent sudden release of gas (Ellwood et al., 2009; Rouwet et al., 2021).

Moreover, Lake Albano is located in a temperate climatic condition and is often considered a meromictic lake with occasional complete turnover (Ellwood et al., 2009). Temperate climates prevent volcanic lakes from accumulating CO_2 for prolonged periods, as yearly lake turnover facilitates partial CO_2 release in winter when cold, dense CO_2-free surface waters sink into deeper water layers (Chiodini et al., 2012). In this lake, water overturns from the top (epilimnion) to the bottom (hypolimnion) were occasionally observed in winter periods, possibly due to a substantial temperature decrease (<8.5°C) of the surficial waters (Carapezza et al., 2008). Historically, this turnover phenomenon caused water overflow (Funiciello et al., 2003). In terms of hazard, CO_2 can suddenly be released from Lake Albano during seismic crises that can happen at any time. Hence, it is important to continue monitoring the CO_2 saturation state of deep layers (Rouwet et al., 2021).

The deep layers of Lake Albano are characterized by a significant reservoir of extra-atmospheric dissolved gases, mainly CH_4 and CO_2. Vuong et al. (2022) have

postulated that extreme environments, such as Lake Albano, could potentially host a rich microbial diversity, offering a robust source of versatile bioproducts adaptable to various physicochemical properties. In this system, the biogeochemical processes are intimately related to the microbial population and vary in dependence on the vertical gradient of the physicochemical properties along water columns, as hypothesized by Cabassi et al. (2013). The external inputs of CO_2 indirectly trigger prokaryotic activity, which likely plays a significant role in the development and temporal evolution of the dissolved gases reservoir within the lake. According to Cabassi et al. (2013), the presence of CO_2 in deep lake waters increases water density and promotes the proliferation of microbial communities. These microbes utilize CO_2 to produce CH_4, which undergoes biogenic oxidation in lake water layers where oxygen is available.

The significant importance of microbial communities within ecohydrological processes in Lake Albano is clearly established. Moreover, conducting a microbial survey and conserving microbial biodiversity is critical because ecosystems and their associated genetic reservoirs are key contributors to sustainable development, as emphasized by Vuong et al. (2022). However, the existing studies in Lake Albano have predominantly concentrated on the dynamics of dissolved gases (Carapezza et al., 2008; Chiodini et al., 2012; Cabassi et al., 2013; Rouwet et al., 2021), while the understanding of microbial communities remains limited.

The available research has primarily centered on monitoring E. *coli*, total coliform, and Cyanobacteria concentrations in surface water carried out by the ARPA Lazio State Department of the Environmental and Water Resources Agency for bathing water quality classification (Amorosi and Sangiorgi, 2022). Therefore, the present study aims at bridging the knowledge gap by investigating the complex interplay between the chemistry of water and dissolved gases and microbial community dynamics in Lake Albano. A combined approach that integrates geochemical and microbiological methodologies is essential for a thorough understanding of the underlying ecohydrological processes and their influence on the overall functioning of this biologically active lake. Enhanced insights into these key processes can serve as a foundation for implementing ecohydrological approaches to increase ecosystem resilience, which is being intensively challenged by climate change and human activities.

The main objectives of the study were to investigate the microbial-driven chemical reactions that influence the chemistry of Lake Albano, focusing on the main dissolved gases (CH_4 and CO_2), and assess the influence of these biogeochemical processes, including gas production and consumption, on the mechanisms

governing the evolution of the gas reservoir. Thus, the determination of vertical diversity and distribution of prokaryotes was coupled with the chemical and isotopic composition of waters and dissolved gases measured along the Lake Albano water column and complemented with surface and subsurface microbial community-level physiological profiles (CLPP) analysis by the Biolog Assay. The assessment of bacterial and archaeal assemblages involved different levels of phylogenetic resolution, including the determination of microbial community diversity using next-generation sequencing (NGS) and the analysis of microbial cell abundance and community composition at the single cell level through flow cytometry and catalyzed reported deposition fluorescence in situ hybridization (CARD-FISH).

1.1 Volcanic Lakes and Biogenic Fractions

Volcanic lakes are peculiar natural systems on Earth where magmatic- hydrothermal systems intersect with the surface of the planet. They are considered a common feature of volcanic systems characterized by recent activity, being present in 474 volcanic structures worldwide documented in the VOLADA database (https://vhub.org/tags/voladadatabase). Volcanic lakes provide insights into the volcanic, hydrothermal, and degassing processes of the underlying volcano with its over-time water compositions and color dynamics (Rouwet et al., 2015).

According to Pasternack and Varekamp (1997), volcanic lakes were classified as i) high-activity lakes, affected by the addition of significant amounts of hot and acidic hydrothermal–magmatic fluids, and ii) low-activity lakes (i.e., lakes hosted in quiescent volcanoes), characterized by external fluid inputs at a relatively low rate, low temperature and salinity, and almost-neutral pH allowing a stable vertical stratification and possibly the accumulation of vast amounts of gases, mainly CO_2 and CH_4, in the deep water layers. At these conditions, occasional perturbing events (i.e., earthquakes, landslides, heavy rains) or the progressive attainment of gas saturation conditions may trigger the sudden overturn of lake water, promoting the release of dangerous gas clouds from the dissolved gases reservoir into the atmosphere that also known as "limnic eruptions" (Cabassi et al., 2013).

The chemistry of volcanic lakes changes along the vertical profile as it might get external hydrothermal–magmatic fluids and gases input. According to Rouwet et al. (2015), the thermal and chemical stratification leads to the formation of different zones within a lake (**Figure 1.1**), characterized by distinct chemical-physical features and behavior, as follows:

- The epilimnion: the surface water layer sensitive to external temperature and solar radiation. Epilimnitic water exchanges heat and volatile substances (gases) with the atmosphere. The epilimnion recirculates episodically by wind or by changes in external temperatures.
- The hypolimnion: the deep water layer showing the lowest temperature along the vertical lake profile. Colder water is denser and tends to sink to the bottom of the lake.
- The metalimnion: the water layer between the epilimnion and the hypolimnion, showing a marked thermal gradient. It corresponds to the thermocline, which refers to the plane of the maximum rate of temperature decrease with depth.

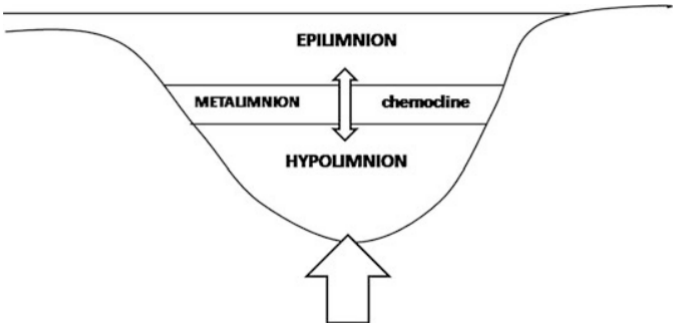

Figure 1.1: Sketch of the lake stratification (Rouwet et al., 2015). The lower arrow indicates eventual heat and fluid input from the aquifer and the double arrow indicates mixing processes.

Gases are the main component of volcanic systems. According to Christenson and Tassi (2015), gases in volcanic lake systems have multiple origins and can be described in the following terms:

1. Deep fluid sources from magma degassing and hydrothermal fluid-rock interactions. The primary components of magmatic gases are H, O, C, S, and Cl, comprising >95% of magmatic volatiles, regardless of the tectonic source environment. In addition, numerous trace-level gases are found in volcanic emissions, including typically a non-reactive N_2 derived from primary component sources; noble gases, which serve as tracers; metal species; and a multitude of trace and ultra-trace species derived from interactions between primary component sources.
2. Atmospheric gases dissolved in ground and lake water sources. The meteoric constituents are comprised of atmospheric gases introduced into the hydrothermal environment by air-saturated rainwater entering into

groundwater systems or by direct equilibration with air in contact with the lake water. The primary components are those of the highest partial pressure in the atmosphere (i.e., N_2, O_2, and Ar), and their equilibrium concentrations in groundwater are proportional to their temperature-dependent Henry's Law solubilities.

3. Metabolic processes of living organisms source (biogenic fraction). Many opportunistic microbial organisms gain metabolic energy from redox reactions involving principally magmatic-hydrothermal C, S, and N, often leaving distinctive isotopic signatures.

Biogenic fractions are potentially a dominant component in volcanic lakes. Irrespective of their primary origin, CO_2, CH_4, N_2, O_2, S gases, and H_2 dissolved in volcanic lakes are involved in biogeochemical processes. Furthermore, the distribution of biomass along the vertical water column of a lake is controlled by the physicochemical properties of the water (i.e., temperature, pH, redox, and dissolved gases). In low-activity lake system, the vertical distribution of these gases and that of microbial populations are closely related (Christenson and Tassi, 2015).

CO_2 is the predominant carbon species in volcanic gases. Dissolved CO_2 in volcanic lakes is typically fed by sub-lacustrine gas discharges. However, the fate of dissolved CO_2 strongly depends on additional consumption processes related to biotic respiration, anaerobic decomposition of organic matter, and microbial oxidation of CH_4, especially in lakes hosted in quiescent volcanoes (Rudd et al., 1974; Rich, 1980; Christenson and Tassi, 2015). In an aerobic environment, i.e., in the epilimnion of crater lakes, microalgae, Cyanobacteria, along with higher plants, are capable of consuming CO_2 through oxygenic photosynthesis, which occurs following two main pathways: light energy conversion to biochemical energy by a photochemical reaction, and CO_2 reduction to organic compounds such as sugar phosphates by Calvin-cycle enzymes (Nelson and Ben-Shem, 2004).

In the hypolimnion, CO_2 biogenic and geogenic inputs are counteracted by microbial reduction processes, mainly by methanogens, a group of microorganisms phylogenetically affiliated to the kingdom Euarchaeota of the domain Archaea (Schoell, 1988). Anaerobic methanotrophy using nitrates as substrates coupled with Fe or Mn reduction can also occur, although most CH_4 oxidation is carried out in the epilimnion (Lopes et al., 2011). The combination of addition and loss of CO_2 related to the various biogeochemical processes occurring at different depths along the vertical water column maintains stable CO_2-rich reservoirs typically characterizing Nyos-type meromictic volcanic lakes (Christenson and Tassi, 2015).

Highly correlated with the presence of CO_2, CH_4 is found in a relatively very low concentration in the oxygenated epilimnion and is produced and stored in the anoxic hypolimnion or is oxidized in the metalimnion. In the presence of free oxygen, particularly at oxic/anoxic boundaries of metalimnion, CH_4 is readily consumed by methanotrophs phylogenetically affiliated to the alpha, beta, and gamma subdivisions of Proteobacteria in the Bacteria kingdom (Hanson and Hanson, 1996). Methanogenic processes proceed through carbonate-reduction and acetate fermentation pathways Schoell (1988) described by the following reactions:

$$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + H_2O \tag{1.1}$$

$$^*CH_3COOH \rightarrow {}^*CH_4 + CO_2 \tag{1.2}$$

where the * indicates the intact transfer of the methyl position to CH_4.

Reaction I.I prevalently occurs in sulfate-free marine sediments, whereas the two processes are competitive in freshwater environments. Methane produced by microbial methyl fermentation is typically ^{13}C-enriched (δ^{13}C-CH_4>-70‰V-PDB) and ^{2}H-depleted (δ^2H-CH_4<-250‰V-SMOW) with respect to that produced by bacterial CO_2 reduction. However, these boundary values may vary depending on the maturity and type of the organic source (Whiticar, 1999).

While methane production in lakes is commonly attributed to anoxic sediments, the phenomenon of CH_4 oversaturation might also occur in surface waters through the vertical and lateral transport of methane from bottom and littoral sediments, particularly observed in small and shallow ponds and lake waters with limited sediment-water interactions (Holgerson and Raymond, 2016). This occurrence is attributed to well-known mechanisms of oxic methanogenesis facilitated by light, nutrients, and salt-dependent microbial processes. In addition, the presence of pelagic micro-anoxic zones has been noted as a contributing factor (Bogard et al., 2014; Tang et al., 2016; Donis et al., 2017; Fazi et al., 2021).

N_2 is the main trace-level gas in volcanic systems, typically in a non-reactive component derived from one of three primary component sources. The triple-bounded N_2 molecule is involved in biological processes to produce reactive N_2-bearing compounds, such as NOx and NHx. Therefore, the distribution of N_2 concentrations in stratified lakes is controlled by biological processes that are able to fix or produce N_2. Biological N_2 fixation depends on light and the presence of bioavailable trace metals (Tison et al., 1977). It is carried out in the water, on the sediment surface, and in sediment pores by heterocyst-forming species such as Cyanobacteria and methane-oxidizing bacteria (Christenson and Tassi, 2015).

Biological N_2 production commonly occurs through the denitrification process in anaerobic conditions. In this process, NO_3^- is used as an electron acceptor during the respiration of bacterial species such as *Pseudomonas* and *Clostridium*. Biological N_2 production may also occur through a process termed anammox (Anaerobic Ammonium Oxidation) in which NH_4 oxidized with NO_2^- as the electron acceptor. Anammox is carried out by phylum Planctomycetes of Bacteria (Jetten et al., 1999).

As previously discussed, biotic respiration influences not only dissolved CO_2 but also dissolved O_2. Dissolved oxygen concentrations in permanently stratified volcanic lakes typically decrease with depth through these oxidative processes. This produces a vertical O_2 profile termed clinograde, where aerobic epilimnion and anaerobic hypolimnion can be clearly distinguished. Oxygen consumption due to biological oxidation of organic matter in the epilimnion is frequently offset by water circulation and photosynthesis, two oxygen renewal mechanisms inactive in the hypolimnion. Pure chemical and photochemical oxidation induced by ultraviolet light may also significantly contribute to oxygen depletion in deep lake waters (Laane et al., 1985).

Hydrogen is one of the main components of volcanic lake gases involved in biogeochemical processes. Molecular H_2 is produced by Cyanobacteria through both photosynthesis and anaerobic fermentation processes (Asada and Miyake, 1999). Hydrogen biogenesis and consumption are related to the metabolic activity of several enzymes, such as nitrogenase, which catalyzes H_2 production concomitantly with the reduction of N_2 to NH_4, and hydrogenase, which is able to both take-up and produce H_2 (Tamagnini et al., 2002). At anaerobic conditions, H_2 is used as an electron donor by methanogenic and sulfate-reducing bacteria in the mineralization processes of organic matter. These processes mainly occur at the water-sediment interface. Thus, H_2 slowly diffuses from the lake bottom sediment toward the surface and is efficiently consumed before arriving at the surface of the lake (Christenson and Tassi, 2015).

1.2 Molecular Techniques for Microbial Communities Assessment

Microbial communities play a crucial role in ecosystem dynamics as the essential drivers of nutrient cycling, particularly in volcanic lake systems, as previously discussed. Microbes might contribute to the stability and resilience of ecosystems and serve as early warning of environmental perturbations or disturbances (Shade et al., 2012; Philippot et al., 2021). Hence, integrating microbial communities

into ecohydrological research would provide valuable insights into ecosystem functioning, conservation, and management.

Before the advent of molecular technologies, the assessment of microbial communities in the environment relied on traditional microbiological methods. These methods were based on culturing isolated microorganisms under laboratory conditions and observing their growth and characteristics. While these traditional methods provided valuable information about cultivable microorganisms and their activity, they had limitations in capturing the full diversity and complexity of microbial communities (Hugenholtz, 2002; Fakruddin and Mannan, 2013). Molecular technologies have revolutionized microbial community analysis by enabling the study of non-cultivable microorganisms and providing a more comprehensive understanding of community composition and dynamics (Rastogi and Sani, 2011). The progression of metagenomics studies and the development of novel bioinformatics approaches have led to the acquisition of complete or nearly complete genome sequences. These genome-based approaches provide information about metabolic potential and a range of phylogenetically informative sequences. These sequences can be used to classify organisms and have made it feasible to construct a complex tree of life, as depicted in **Figure 1.2** (Hug et al., 2016). However, the cost and laboratory and bioinformatic technical requirements of this technique remain important considerations (Usyk et al., 2023).

16S rRNA sequencing is a widely used molecular technique for studying microbial communities and their diversity. It involves sequencing a specific region of the 16S ribosomal RNA gene found in the genomes of Bacteria and Archaea. This gene is highly conserved, meaning it contains similar regions across different microbial species and variable regions that can be used to differentiate between them (Rastogi and Sani, 2011). 16S rRNA sequencing allows the identification and quantification of microbial taxa in a given sample without the need for culture-based techniques. It provides insights into the composition and structure of microbial communities, their relationships with environmental conditions, and their potential functional roles within an ecosystem (Janda and Abbott, 2007).
Traditionally, 16S rRNA gene amplicon sequencing was carried out by cloning and Sanger (capillary electrophoresis) sequencing of PCR amplicons. The advent of next-generation sequencing (NGS) has tremendously simplified and increased the sequencing depth for 16S rRNA gene sequencing (Sanschagrin and Yergeau, 2014). NGS refers to a set of high-throughput DNA sequencing technologies that have revolutionized genomic research, such as the currently most often used Illumina MiSeq/HiSeq platforms and the most recent PacBio and Oxford Nanopore MinION platforms. Unlike traditional Sanger sequencing, which reads DNA fragments

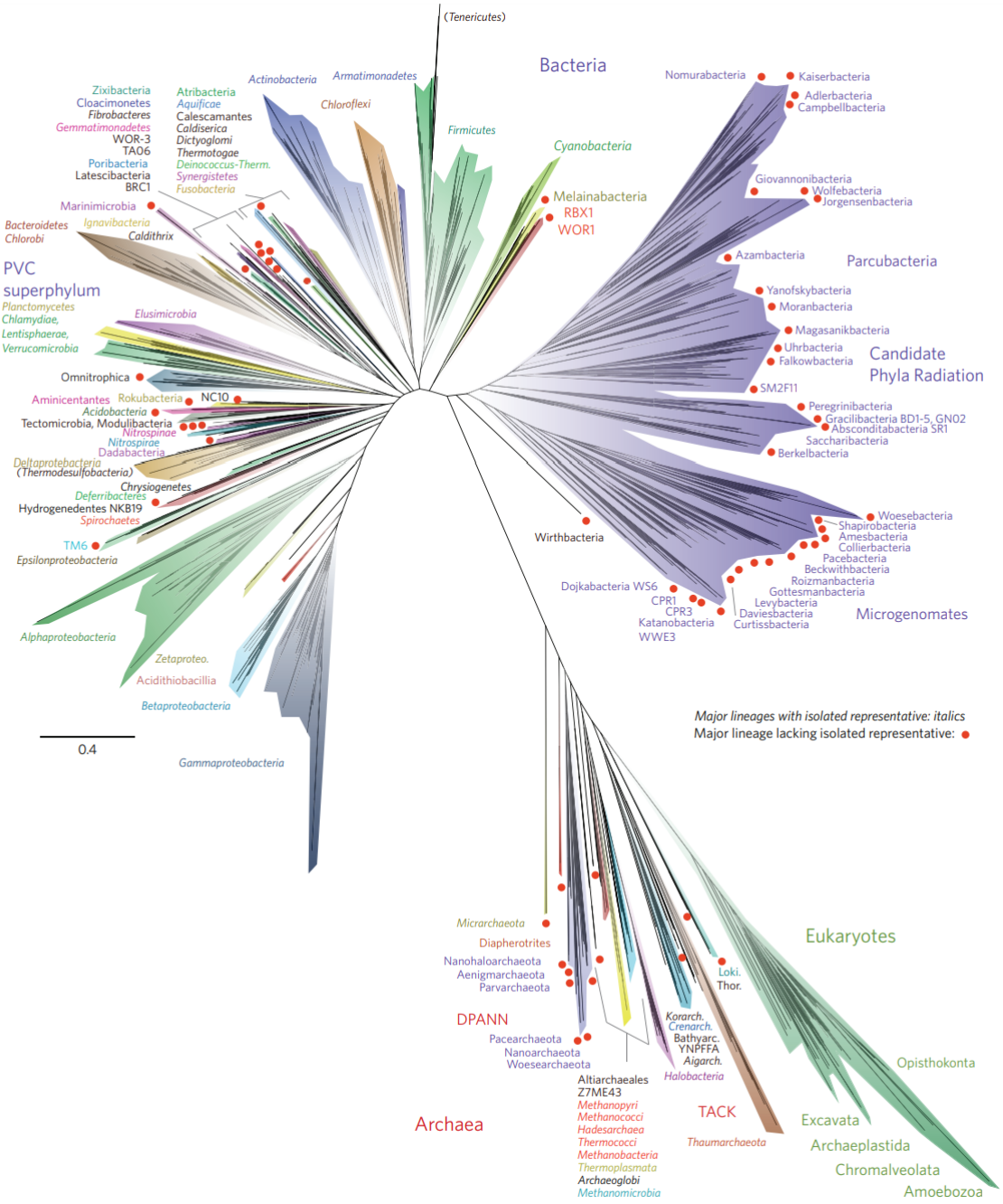

Figure 1.2: A current view of the tree of life, encompassing the total diversity represented by sequenced genomes (Hug et al., 2016). The tree includes 92 named bacterial phyla, 26 archaeal phyla and all five of the Eukaryotic supergroups. Major lineages are assigned arbitrary colors and named in italics, with well-characterized lineage names. Lineages lacking an isolated representative are highlighted with non-italicized names and red dots.

one at a time, NGS allows for massively parallel sequencing of millions to billions of DNA fragments simultaneously. This parallel sequencing enables rapid and cost-effective sequencing of an entire genome or area of interest while providing deeper qualitative information on the composition and functional diversity of microbial communities, even up to the species level (Rastogi and Sani, 2011; Piwosz et al., 2021).

Although sequencing-based studies have revealed the enormous diversity of microbial communities, ecological information on the in situ abundance of specific microbial assemblages can only be reliably obtained by complementary use of microscopy-based molecular methods, such as fluorescence in situ hybridization (FISH) (Amann et al., 1995). FISH provides estimates of relative abundance (a percent contribution to total prokaryotic numbers) of individual microbial assemblage defined by their rRNA gene phylogeny (Not et al., 2002; Piwosz et al., 2021).

The main advantage of FISH over the NGS methods is that the relative abundance of a particular microbial assemblage can be evaluated independently from all other taxa in the samples. Moreover, the FISH procedure can be separately optimized for each target group (probe), which is not possible for PCR with primers targeting many different templates (Piwosz et al., 2021). Furthermore, combining semiquantitative 16S rRNA gene amplicon sequencing with quantitative FISH would enable precise determinations of the abundance of particular taxa.

FISH employs oligonucleotide probes that target short regions (usually 15–25 nucleotides in length) of rRNA genes and bind to a specific sequence of rRNA molecules in intact ribosomes (Piwosz et al., 2021). To date, FISH is one of the most used biomolecular techniques in environmental microbiology. However, the low physiological activity and 16S rRNA content of microorganisms living in oligotrophic environments may result in a low fluorescence signal that is not easily detectable by epifluorescence microscopy analysis (Matturro et al., 2021). In order to overcome this limitation, a more sensitive FISH method called CARD-FISH (catalyzed reporter deposition) has been developed (**Figure 1.3**). Studies have confirmed that CARD-FISH offers a detection limit of approximately 8.9 ± 1.5 16S rRNA molecules per cell, in contrast to the 370 ± 45 16S rRNA molecules per cell reported for conventional FISH. This remarkable difference in detection limits enables a substantial enhancement in cell detectability, particularly in challenging samples. In general, the sensitivity of CARD-FISH surpasses that of conventional FISH by up to 41-fold (Hoshino et al., 2008).

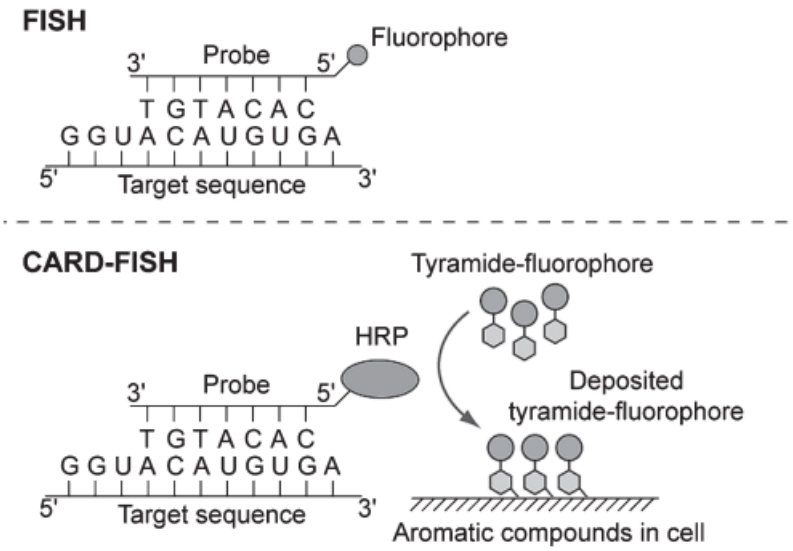

Figure 1.3: Schematic depiction of FISH and CARD-FISH (Kubota, 2013).

CARD-FISH is an enzymatic detection method that involves the deposition of a high number of tyramide molecules (tyramine molecules labeled with a fluorochrome) through the catalytic activity of peroxidase. This method utilizes oligonucleotide probes labeled with Horse Radish Peroxidase (HRP) at the 5′-end. HRP is a nonfluorescent molecule with a molecular weight of around 40 kDa, which is significantly higher than the molecular weights of fluorochromes typically used for FISH probes, ranging from 500 to 1000 Da (Hoshino et al., 2008; Matturro et al., 2021). The probes employed undergo chemical activation upon binding with tyramide molecules, which leads to the generation of a cascade of fluorescence signals and results in improved sensitivity and reduced background noise (Kubota, 2013). CARD-FISH provides up to 200-fold brighter signals than FISH with mono-labeled probes, thus enabling the detection of almost low physiological activity cells with a low ribosome content (Lim et al., 1993). It has become a verified quantitative tool in numerous studies on prokaryotic communities, providing hints on their ecological niche, functions, and interactions (Fazi et al., 2007; Amann and Fuchs, 2008; Matturro et al., 2021; Piwosz et al., 2021).

According to Moter and Gobel (2000), Fazi et al. (2007), Matturro et al. (2021), the protocol for CARD-FISH assay includes the following:

(a) Cell fixation and filtration;
(b) Immobilization of the cells on the filter with low melting-point agarose to prevent cell loss during the CARD-FISH procedure;

(c) Cellular permeabilization with enzymatic treatments;
(d) Inactivation of endogenous peroxidases to reduce potential noise originating from internal peroxidases;
(e) In situ hybridization. It is essential to adhere to the specific stringency requirements of each probe used. Stringency refers to the ability of different probes to bind to their complementary sequences. To facilitate successful pairing, a hybridization buffer is prepared, which is designed to optimize the binding of the probes. This buffer takes into account the stringency characteristics of the probe by adjusting the percentage of Formamide present. By altering the amount of Formamide, the stability of hydrogen bonds within the nucleic acid double helices is affected, creating tension in the duplexes. As a result, any mismatches between the probe and the rRNA are minimized, ensuring that only properly matched probes remain paired;
(f) Washing to remove Formamide and unbound probes and stabilize the pairing of the probes with the target sites;
(g) Deposition of the catalyzed reporter (tyramide-fluorophore complex) and tyramide signal amplification;
(h) DAPI counterstaining; and
(i) Microscopic analysis or visualization under fluorescence microscopy.

2 Methodology

2.1 Study Area

Lake Albano is a polygenetic crater lake approximately 20 kilometers southeast of Rome, central Italy (**Figure 2.1**). The lake has an ellipsoidal shape (3.5×2.3 km), a volume of 4.48×10^8 m^3, a surface area of around 5.7 km^2, and a maximum depth of 167 m, and is the deepest volcanic crater lake in Italy (Anzidei et al., 2008; Chiodini et al., 2012). The major axis of Lake Albano is NW-SE oriented with a relatively low depth ratio (0.45) depending on the large NW flat area occupying almost half of the lake area (Rouwet et al., 2021). In contrast, the SE sector of the lake shows steep walls down to the maximum depth of 167 m, a morphological feature that strongly favors water stratification (Martini et al., 1994; Cabassi et al., 2013). The lake is hosted within five coalescent and partially overlapping coalescent craters formed by hydro magmatic eruptions, being the most active center of the currently quiescent Colli Albani volcanic complex (Carapezza et al., 2008; Anzidei et al., 2008; Rouwet et al., 2021).

2.2 Sampling Strategy

Sampling campaigns were carried out during two separate periods in 2022 and 2023. The first sampling campaign was conducted on 30 August 2022 and took place at the central crater (the deepest point of Lake Albano), indicated as A0 in **Figure 2.1**, to obtain data on the vertical profile of Lake Albano water column. A multi-probe was utilized to measure the physicochemical properties of the lake directly along the depth. Water samples were obtained from a regular interval of 10 m depth using the single hose method for subsequent geochemical and microbial analyses.

The second campaign was conducted on 08 May 2023. Water samples were collected from five sites at increasing distances from the shore of the lake. The sites (**Figure 2.1**), arranged in order, were A1 (at 0 m, 25 m, and 50 m depths), A2 (at 0 m, 25 m, and 50 m depths), A3 (at 0 m and 25 m depths), A4 (at 0 m depth), and A5 (at 0 m depth). The depth of the sites from A1 to A5 were approximately 100 m, 80 m, 25 m, 20 m, and 0 m, respectively. A multi-probe was utilized to measure the

physicochemical properties of each site and depth. Surface and subsurface (up to 50 m depth) water samples were obtained using a Niskin sampler for subsequent microbial community composition and physiological profile analyses.

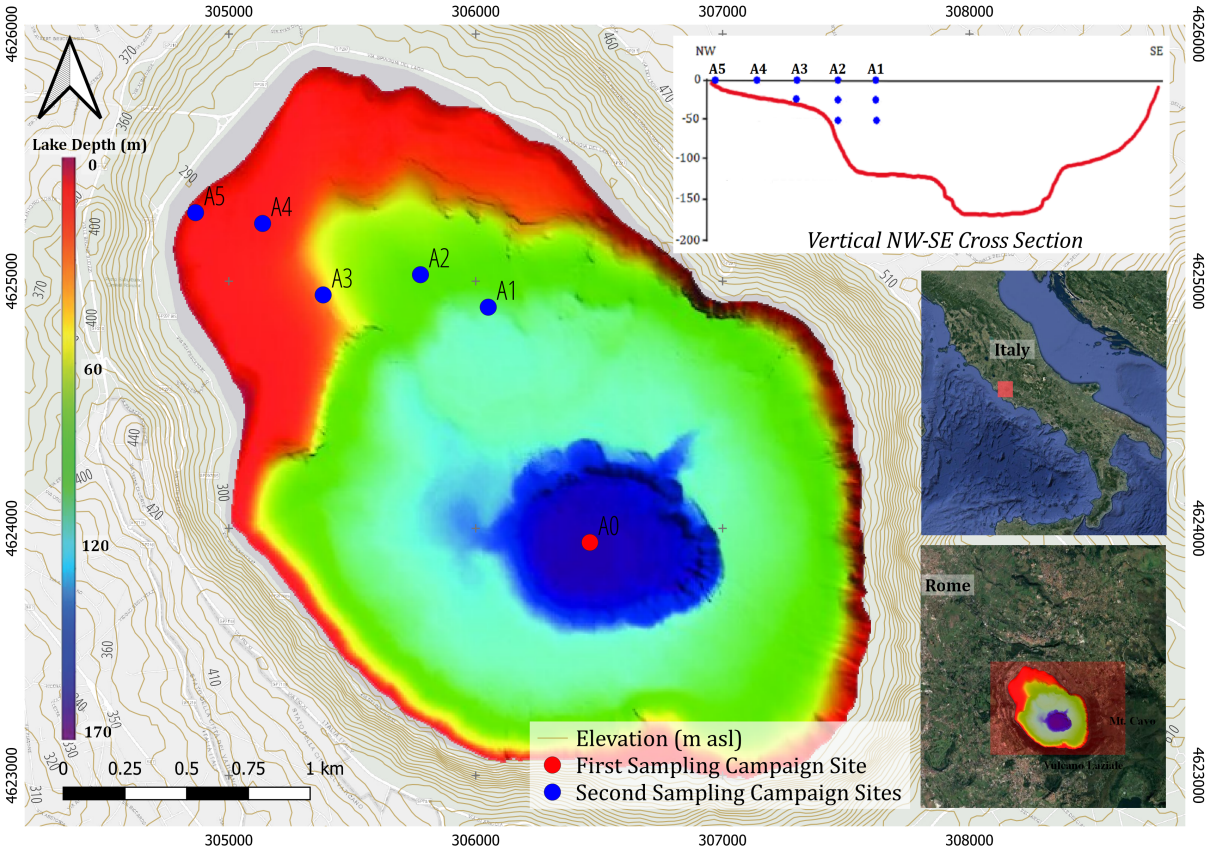

Figure 2.1: Map of the morphobathymetry of Lake Albano modified from Anzidei et al. (2006) with the topography of the area and the sampling sites from lateral and vertical cross-section view. Scale color shows depths ranging between 0 and –167 m.

2.2.1 Field Measurements

Temperature (T), dissolved oxygen (DO), and electrical conductivity (EC) on the initial sampling campaign were measured along the water column down to a depth of 161.5 m using a multiparametric probe Hydrolab IP188A (Hydrolab, USA) equipped with a data logger for data storage. The probe, whose data acquisition frequency was 5 s, was slowly lowered from the surface to the maximum lake depth to obtain measurement intervals <15 cm. The nominal precisions were as follows: depth ±0.05 m; temperature ±0.03°C; DO ±1.56 μmol/L; EC ±0.01 mS/cm, respectively.

During the second campaign, temperature (T), dissolved oxygen (DO), pH, and electrical conductivity (EC) were measured on each site and depth using a multiparametric probe HACH HQ4300 (HACH, USA) to obtain the general physicochemical properties of each sampling site. The probe was carefully lowered from the surface to the specified depth, and the readings were recorded once the value stabilized.

2.2.2 Water and Dissolved Gases Sampling

The sampling of water and dissolved gases was carried out after the field measurements at regular intervals of 10 m using the single hose method (Tassi and Rouwet, 2014). The single hose method is an in situ measurement of the gas/water ratio, and the collection of both the liquid and the gas phase in the lake. This method is particularly employed in meromictic volcanic lakes where the dissolved gas pressure is insufficient to trigger gas self-lifting. This method connects a plastic separator to receive the gas-water mixture spouting from the hose as the intake was lowered to each of the desired sampling depths (Tassi and Rouwet, 2014).

According to the single hose method, a small diameter (6 mm) Rilsan® tube, lowered to the desired sampling depth and connected to a 100 mL syringe equipped with a three-way Teflon valve, was used to pump up the water, as illustrated in **Figure 2.2**. After the displacement of a water volume at least twice the inner volume of the tube, one filtered (0.45 μm) and two filtered–acidified (with ultrapure HCl and HNO_3, respectively) water samples were collected in polyethylene bottles for the analysis of anions and cations, respectively.

The isotope analyses of water (δD-H_2O and δ^{18}O-H_2O) were carried out on samples collected in 40 mL glass bottles. Samples for concentrations and isotopic analyses of dissolved gases were collected using pre-evacuated 250 mL glass vials equipped with a Teflon stopcock. Once the vial was connected to the Rilsan tube through the three-way valve, the stop-cock was opened to allow water to enter up to three-fourths of the vial's inner volume (McNichol et al., 1994; Rouwet et al., 2021). As for the analysis of microbial diversity by NGS and cell abundance by flow cytometry, lake water (500 mL) was collected at nine depths (0 m, -20 m, -60 m, -110 m, -120 m, -130 m, -140 m, -160 m), immediately stored in the cooler for further analysis in the laboratory.

During the second campaign, lake water samples were collected for the analysis of microbial cell abundance with flow cytometry, community composition using CARD-FISH, and functional properties using the Biolog assay. A total of 500 mL of lake water was collected for this purpose. For microbial abundance and

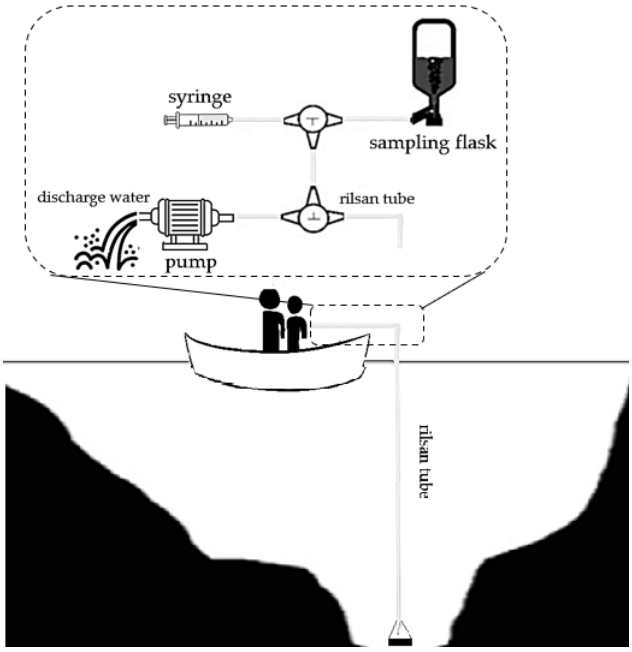

Figure 2.2: Schematic illustration of single hose method. The hose lowered to the desired depth and connected to a pump and a syringe to collect water and dissolved gas samples modified from Tassi and Rouwet (2014).

composition structure analysis, aliquots of 45 mL water sample were fixed for 2 h at 4°C with formaldehyde solution (37% w/v, Sigma Aldrich; final concentration 1%) (Fazi et al., 2007; Fazi et al., 2013). Sub-aliquots of fixed water were filtered onto 0.2 μm pore-size polycarbonate filters (type GTTP; diameter, 47 mm; Millipore, Germany) at low vacuum levels (<0.2 bar). The filters were subsequently stored at -20°C until further processing. For the Biolog assay, aliquots of 100 mL unfixed water samples were stored at 4°C.

2.3 Chemical and Isotopic Analyses

The chemical and isotopic analyses were done for water and dissolved gases in collaboration with the Department of Earth Sciences, University of Florence. Analyses for water include main anions and cations and hydrogen (δD) and oxygen (δ^{18}O) isotopic compositions for water. In contrast, analyses of dissolved gases include inorganic dissolved gases and carbon isotopic compositions (δ^{13}C) for CO_2 and CH_4.

2.3.1 Waters

The main anions (F^-, Cl^-, NO_3^-, SO_4^{2-}, and HCO_3^-) and cations (Na^+, K^+, Mg^{2+}, and Ca^{2+}) were analyzed by ion chromatography (IC) using Metrohm 761 and Metrohm 861 chromatographs (Methrohm AG, CH), respectively. The analytical errors for IC analysis were <5%.

The $D/^1H$ and $^{18}O/^{16}O$ ratios of water (expressed as δD-H_2O and δ^{18}O-H_2O in ‰vs. V-SMOW) were determined using a Finnigan Delta Plus XL mass spectrometer (Thermo Fisher Scientific, USA) according to standard protocols. Oxygen isotopes were analyzed using the CO_2–H_2O equilibration method (Epstein and Mayeda, 1953). Hydrogen isotopes were analyzed on H_2 produced after the reaction of 10 mL of water with metallic zinc at 500°C. Analytical errors for δD-H_2O and δ^{18}O-H_2O analyses were $\pm$0.1‰and $\pm$1‰, respectively.

2.3.2 Dissolved Gases

The compositions of the inorganic dissolved gases in the headspace of the sampling flasks (CO_2, N_2, O_2, Ar, and He) were determined by gas chromatography (GC) using a Shimadzu 15A (Shimadzu, Japan) equipped with a 5 m long stainless steel column packed with Porapak 80/100 mesh and a Thermal Conductivity Detector (TCD), whereas CH_4 was analyzed using a Shimadzu 14A equipped with a 10 m long stainless steel column packed with Chromosorb PAW 80/100 mesh coated with 23% SP 1700 and a Flame Ionization Detector (FID) (Vaselli et al., 2006). The analytical error for GC analysis was $\leq$5%. Assuming that in the sampling flasks the separated gas phase was in equilibrium with the liquid, the number of moles of each gas species in the liquid ($n_{i,l}$) was calculated based on those in the flask headspace ($n_{i,g}$) by means of the Henry's law constants (Wilhelm et al., 1977). The total moles of each gas species in the water sample were given as the sum of $n_{i,l}$ and $n_{i,g}$. The partial pressures of each gas species were then computed based on the total mole values according to the ideal gas law.

The isotopic compositions of carbon (expressed as δ^{13}C for CO_2 and CH_4 in ‰vs. V-PDB) were determined using a Picarro G2201-i CRDS analyzer (Picarro, USA) by analyzing the gas collected in the headspace of the sampling flask (analytical error: 0.16 and 1.15‰, respectively (Rouwet et al., 2021).

2.4 Microbial Community Analyses

Microbial community analyses of this research include microbial cell abundance with flow cytometry, community composition with fluorescence in situ hybridization (CARD-FISH), diversity analysis with 16S rRNA next-generation sequencing (NGS), and microbial functional properties analysis with Biolog EcoPlates assay.

2.4.1 Microbial Abundance Analysis

Flow Cytometry

The microbial cell abundance was analyzed using the portable Flow Cytometer A50-micro (Apogee Flow System, England), equipped with a solid-state laser set at 20 mV and tuned to an excitation wavelength of 488 nm following the protocol optimized by Amalfitano et al. (2018). The volumetric absolute counting was carried out on fixed samples, stained with SYBR Green I (1:10,000 dilution; Molecular Probes, Invitrogen, USA) for 10 min in the dark at room temperature. Light scattering signals (i.e., side and forward scatters) and green fluorescence (530/30 nm) were acquired for cell characterization. Samples were run at low flow rates (38.9 μL/min) to keep the number of detected total events below 1,000 per second. The average acquisition time was >60 seconds per run. The instrumental settings were kept the same for all samples to achieve comparable data, although the total number of detected events differed among samples and replicates. The Apogee Histogram Software (v89.0) was used for data handling and visualization.

DAPI Staining

Total prokaryotic cell abundance quantification was done through the DAPI staining technique. Filter sections were stained with Vectashield® Antifade Mounting Medium with DAPI (Vector Laboratories, USA) at a final concentration of 1 μg/mL. Microbial abundance was estimated using a Leica DM LB 30 epifluorescence microscope (Leica Microsystems GmbH, Germany) at 1000x magnification. At least 10 randomly selected microscopic fields were counted, including a minimum of 300 DAPI-stained cells. Knowing the ratio between the effective filtration surface of the filter (Af = 803 mm^2), the observed portion of the grid (Ac = 0.0009/0.0025 mm^2), the total area of the grid (Ag = 0.01 mm^2), and by calculating the average number of cells per field (N), it was then possible to trace

the number of cells on the entire filter, from which, on the basis of the volume of filtered sample (V), it was possible to determine the concentration of cells in the original sample according to the formula which follows:

$$cells/mL = \frac{[Nx(\frac{Af}{AcxAg})]}{V} \tag{2.1}$$

CARD-FISH

Further microbial community composition was assessed by CARD-FISH following the protocol optimized by Fazi et al. (2007), Fazi et al. (2013), and Matturro et al. (2021). The filters were cut and numbered on the outer part with a regular pencil. Filter sections were then embedded in 0.2% low-melting-point agarose on glass slides and incubated at 37°C for 20 min. The dried agar-coated filters were subsequently dehydrated with 96% ethanol wash for 1 min and air-dried.

Cell permeabilization was done with enzymatic treatments. It was performed by incubation with lysozyme [20 mg/mL, dissolved in 200 μL 0.5M EDTA pH 8, 200 μL 1M Tris-HCl pH 7.4, and 1600 μL MQ water] for 60 min at 37°C, followed by incubation with Proteinase-K working solution [18 μL Proteinase-K stock solution 1 mg/mL and 6 mL Tris-EDTA buffer] for 25 min at 37°C. After each permeabilization process, the solutions were removed by MQ water wash for 1 min. The filters were then treated for inactivation of endogenous peroxidases with incubation in 10 mL 0.01M HCl at room temperature for 10 min. Subsequently, the filters were washed in MQ water for 1 min, dehydrated with 96% ethanol, and air-dried.

Filters were hybridized with rRNA-target Horseradish peroxidase (HRP) labeled oligonucleotidic probes (Biomers, Germany) to target Bacteria (EUB338 I-III) and Archaea (ARCH915) (**Table 2.1**). For EUB 338 I-III probes, the stringency was applied to 55% formamide, while for Archaea, stringency was applied at 35%. 2 μL 50 ng/μL HRP probe was added to 200 μL hybridization buffer [1800 μL 5M NaCl, 200 μL 1M Tris-HCl pH 7.5, 1 g dextran sulfate, 1000 μL 10% blocking reagent, and including Formamide and MQ water depending on probe stringency (**Table 2.1**)] and filter sections were put inside the solutions and incubated at 10 rpm overnight at 35°C.

Filter sections were removed from the hybridization mixture and washed for 10 min at 37°C in prewarmed washing buffer [5M NaCl which depends on probe stringency (**Table 2.1**), 1000 μL 1M Tris-HCl pH 7.5, 1000 μL 0.5M EDTA, 25 μL 20% SDS, and MQ water up to 50 mL]. Filter sections were transferred into a tube with 10 mL of 1xPBS, placed on a medium-speed stirrer for 15 min at room temperature, and dried on blotting paper in a closed box.

Table 2.1: Details of CARD-FISH probes and stringency (Fazi et al., 2007; Greuter et al., 2016; Matturro et al., 2021). HB: Hybridization Buffer; WB: Washing Buffer.

Probe	Target Taxa	Sequence (5′-3′)	Stringency (%)	HB	WB
				Formamide (μL)	5M NaCl (μL)
ARCH 915	Archaea	GTGCTCCCCCGCCAATTCCT	35	3500	420
EUB 338 - I	Bacteria	GCTGCTCCCGTAGGAGT	50	5500	30
EUB 338 - II	Bacteria	GCAGCCACCCGTAGGTGT			
EUB 338 - III	Bacteria	GCTGCCACCCGTAGGTGT			

At this point, HRP-conjugated probes are hybridized to the 16S rRNA within the cells, but no fluorescence signal emission occurs. In order to obtain a fluorescence signal, tyramide is added to the cells and activated by binding with the HRP molecules. From this point forward, all steps must be performed in the dark to prevent dye degradation. Hybridized filters were incubated in Substratum Mix [Solution A: 200 μL 1x PBS + 1 μL 30% H_2O_2; Solution B: 12 μL Solution A + 1200 μL amplification buffer (5 mL 10x PBS, 20 mL 5M NaCl, 5 mL 10% blocking reagent, 2.5 g dextran sulfate, and MQ water up to 50 mL); Substratum Mix: 1000 μL Solution B + 1 μL dye tyramide] at 37°C for 15 min in the dark under slow rotation (10 rpm) for HRP to activate the tyramide and to bind to the wall proteins. For final washing, filters were washed in 1x PBS and placed on a medium-speed shaker for 25 min at room temperature, MQ water for 1 min, and 96% ethanol for 1 min, respectively.

DAPI counterstaining is used to evaluate the relative abundance of the target microorganisms with respect to the total microbial population. The stained filter sections were inspected on a Leica DM LB 30 epifluorescence microscope (Leica Microsystems GmbH, Germany) at 1000x magnification. At least 300 cells were counted in >10 microscopic fields randomly selected across the filter sections. The relative abundance of hybridized cells was estimated as the ratio of hybridized cells to total DAPI-stained cells.

2.4.2 Microbial Diversity Analysis

DNA was extracted from Millipore filter units using the PowerSoil® DNA Isolation Kit (Mo Bio Laboratories Inc., USA) as described for water samples by Chandler et al. (2013) according to the manufacturer's instructions. The concentration of each DNA extract was quantified by NanoDrop Spectrophotometers (Thermo Fisher Scientific, USA), and the DNA was stored at −20°C. DNA was then shipped to a third-party molecular and bioinformatics company for further processing.

The general workflow is shown in **Figure 2.3**. Gene variable region 4 (abV4-C) of 16S rRNA gene sequencing libraries was prepared by a custom protocol based on an Illumina protocol (Illumina, 2015) using 10 ng of extracted DNA as a template for PCR amplification of 16S rRNA gene amplicons. Each PCR reaction (25 μL) contained PCRBIO Ultra mix (12.5 μL) and tailed primermix (400 nM of each forward and reverse). PCR was run with the following program: Initial denaturation at 95°C for 2 min, 30 cycles of amplification (95°C for 15 s, 55°C for 15 s, 72°C for 50 s), and final elongation at 72°C for 5 min. Duplicate PCR reactions were performed for each sample, and the duplicates were pooled after PCR. The forward and reverse primers utilized were: Bacteria/Archaea region 4 (abV4-C): 5'-*GTGYCAGCMGCCGCGGTAA* [515FB] and 5'-*GGACTACNVGGGTWTCTAAT* [806RB] (Apprill et al., 2015; Parada et al., 2016). The amplicon libraries were purified using CleanNGS SPRI beads (CleanNA, NL), following the vendor-recommended protocol, using a sample: bead ratio of 5:4, and the DNA was eluted in 25 μL of nuclease-free water (Qiagen, Germany). DNA concentration was measured using a Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screen tapes (Agilent, USA) were used to validate product size and purity of a subset of sequencing libraries.

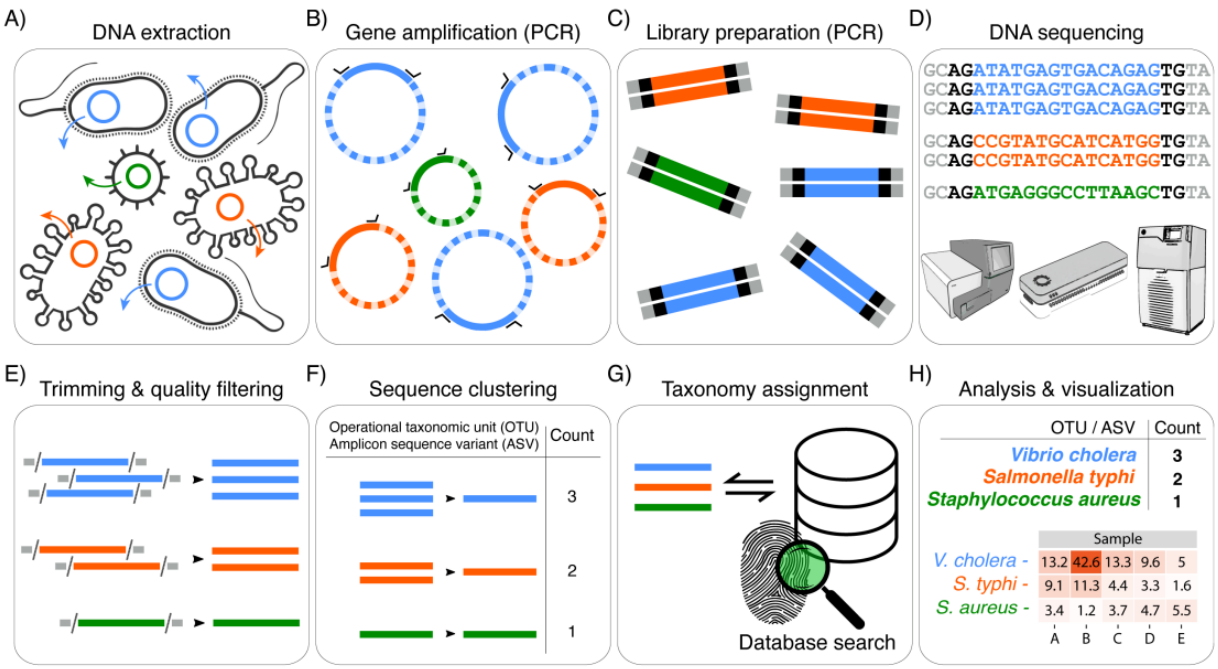

Figure 2.3: Overview of the general workflow from sample to microbial community profile of NGS (from DNAsense internal sequencing report).

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM. The samples were paired-end sequenced (2x300 bp) on a MiSeq (Illumina, USA) using a MiSeq Reagent kit v3, 600 cycles (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. >10% Phix control library was spiked to overcome low complexity issues often observed with amplicon samples.

For 16S rRNA amplicon bioinformatic processing, the generic workflow reported in van Loosdrecht et al. (2016) was followed. abV4-C forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 with the settings SLIDINGWINDOW:5:3 and MINLEN:225 (Bolger et al., 2014). The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 with the settings -m 10 -M 250 (Magoč and Salzberg, 2011). The merged reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered using the usearch v.11.0.667 -cluster_otus command with default settings. Operational taxonomic units (OTUs) abundances were estimated using the usearch v.11.0.667 -usearch_global command with -id 0.97 -maxaccepts 0 -maxrejects 0. Taxonomy was assigned using the uclust classifier as implemented in the assign_taxonomy_.py script in QIIME (Caporaso et al., 2010) and using the updated version of the SILVA database, release 132 (Quast et al., 2013). NGS results were analyzed via RStudio IDE (2022.2.3.492) running the R version 4.2.2 Patched (2022-11-10 r83330) using the ampvis package v.2.7.27.

2.4.3 Microbial Functional Properties Analysis

The microbial functional properties, including metabolic potential (AWCD), Community-level physiological profiles (CLPP), and functional diversity, were analyzed through the Biolog^{TM} assay (Biolog, USA) according to Melita et al. (2022). The surface and subsurface (up to 50 m depth) water samples from the second sampling campaign were analyzed using Biolog^{TM} EcoPlates. The Biolog^{TM} EcoPlates consist of 96 micro-wells containing 31 organic carbon substrates labeled with the respiration-sensitive tetrazolium dye plus one control (each plate contains all substrates and control in three replicates).

All the water samples underwent the same treatment according to the protocol. 120 μL of samples were inoculated into each micro-well of the EcoPlates in triplicate. Victor X3 multi-label reader spectrophotometer (PerkinElmer, USA) to a wavelength of 590 nm was used to detect the intrinsic absorbance signal of the samples (t=0). The plates were covered and incubated at 20°C. After 24 h, the final

color development (t=24) was detected with the same instrument and method as the initials. Data obtained were statistically analyzed.

The values of optical density (OD) at 24 h (t=24) were subtracted by those found at 0 h (t=0) to eliminate the intrinsic absorbance signal (Insam and Goberna, 2004). Negative values were set to zero. Subsequently, the microbial metabolic potential was calculated as the mean degradation activity on all 31 substrates provided and defined as Average Well Color Development (AWCD) (Garland and Mills, 1991).

The metabolic patterns obtained from the degradation of each single substrate present in the Biolog EcoPlates assay can provide relevant information regarding the preferential use of organic compounds to fuel catabolic microbial reactions. This multiparametric analysis is called metabolic fingerprint or community-level physiological profile (CLPP). Prior to the analyses, the $OD_{590\,nm}$ data of each sample were normalized by the respective AWCD value in order to avoid differences related to inoculum density. Furthermore, to simplify the data interpretation, the substrates provided have been grouped into six chemical categories (i.e. carbohydrates, polymers, carboxylic acids, phenolic compounds, amino acids, and amines). The results are expressed as the percentage contribution of each class to the total degradative activity. The functional diversity was assessed through the application of Shannon-Wiener's diversity index (H) (Melita et al., 2019).

2.5 Data Analysis

Statistical analyses and graphical representations were performed using PAST v4.12b open-access statistical software (Hammer et al., 2001). Data in the OTU table were normalized to percent and presented as the 25 most abundant bacterial genera in the samples. Eventually, the abundances of each genus were normalized with respect to the average abundance. Hence, this allowed visualization of the relative increase or decrease in abundance of each genus at the 16 sampling depths, despite the differences in abundance between genera. In addition, the genera were clustered (the x and y axis) to visualize those with similar patterns. Clustering was conducted using the clustering command using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) dendrogram based on unweighted Unifrac distance, and the Venn diagram was applied to cluster sites based on total OTUs distribution. The tests were based on the Bray-Curtis (dis)similarity matrix.

A Nonmetric Multi Dimensional Scaling ordination plot (NMDS), based on the Bray-Curtis (dis)similarity matrix, was used to graphically visualize microbial taxonomic composition (25 most abundant genera) in the samples across sites. The

major chemical characteristics (F^-, Cl^-, NO_3^-, SO_4^{2-}, HCO_3^-, Na^+, K^+, Mg^{2+}, Ca^{2+}, TDS) and dissolved gases (CO_2, N_2, O_2, Ar, and He) were incorporated in the analysis with a vector-fitting procedure. The correlation coefficients between each environmental variable and the NMDS scores were presented as vectors from the origin with the length scaled to make a readable biplot. Stress value indicates the significant concordance between the distance among samples in the NMDS plot and the actual Bray-Curtis distance among samples (Amalfitano et al., 2014).

For the Biolog assay, according to Melita et al. (2022), the multivariate Permutational Multivariate Analysis of Variance)(PERMANOVA test), based on the Gower (dis)similarity matrix, and the univariate non-parametric Kruskal-Wallis test for equal medians were performed to test statistical differences among the sample groups.

3 Results

3.1 Environmental Characteristics

In August 2022, the vertical profiles of Lake Albano were obtained from the surface to the bottom (-161.5 m) (**Figure 3.1** and **Table A.1**). Temperature (T) decreases rapidly with depth and remains relatively constant. A strong thermocline occurred at 10-20 m depth, separating a relatively warm (up to 26.5°C) epilimnion from a cold hypolimnion, the latter being around 9.4°C. The pH values were characterized by relatively high values (>8) and decreased down to the bottom layer, where the minimum value (7.3) was measured. The vertical distribution of the electrical conductivity (EC) values was marked by three haloclines: the shallowest one in correspondence with the thermocline, the main one at 20-95 m depth, and the third one at >100 m depth. Dissolved oxygen (DO) generally showed a clinograde profile, with a slight increase of heterograde oxygen curve at 20 m depth commonly ascribed to oxygen production from photosynthesis of algae population.

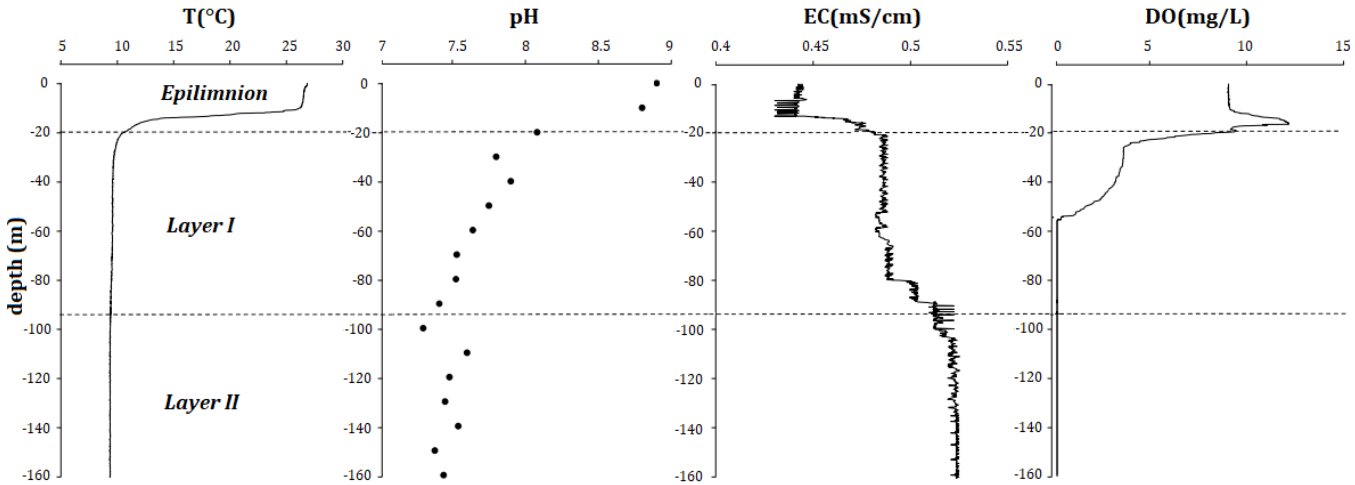

Figure 3.1: Vertical physicochemical profiles along the Lake Albano water column from the first sampling campaign. Water temperature (T in °C), pH, electrical conductivity (EC, in mS/cm), and dissolved oxygen (DO in mg/L). The dotted horizontal lines delimit the various layers.

In detail, atmospherically heated epilimnion was observed with temperatures varying between 26.8°C and 16.2°C, pH values from 8.9 to 8.1, EC from 0.459 mS/cm to 0.487 mS/cm, and DO ranging from 9.04 mg/L (at the lake surface) to 10.5 mg/L (at 20 m depth). An intermediate layer, referred to as Layer I by Chiodini et al. (2012), spanning from -20 m to -95 m, exhibited notable changes. This layer experienced a decline in temperature from 16.2°C to 9.4°C, a drop in pH from 8.1 to 7.4, a slight increase in EC from 0.487 mS/cm to 0.534 mS/cm, and a substantial decrease of DO from 10.5 mg/L (at 20 m depth) to 4.6 mg/L (at 30 m depth) and further to 2.6 mg/L (at 50 m depth). The transition to an anoxic state occurred at a depth of 56 m. A deep layer (-95 m to bottom at -160 m, called Layer II by Chiodini et al. (2012)) was observed with a constant low temperature (9.4°C), relatively constant pH around 7.5, and a slight increase in EC from 0.530 mS/cm to 0.568 mS/cm.

In May 2023, epilimnion (0 m and -25 m) and Layer I (-50 m) physicochemical properties of Lake Albano were obtained, as shown in (**Table 3.1**). Over one year, only the seasonal variations were observed in the upper part of the water column. Compared to the August 2022 survey, the surface water temperature in May 2023 was considerably lower (17.3°C vs. 26.8°C), EC was very slightly higher (0.501 mS/cm vs. 0.459 mS/cm), pH was slightly lower (8.26 vs. 8.9), and DO was slightly higher (9.76 vs. 9.0 mg/L).

Table 3.1: Physicochemical properties of the surface and subsurface water column of Lake Albano from the second sampling campaign. Water temperature (T in °C), pH, electrical conductivity (EC, in mS/cm), and dissolved oxygen (DO in mg/L).

sites		T	pH	EC	DO
A1	0 m	17.5	8.18	0.504	9.81
	-25 m	11.3	7.73	0.505	7.81
	-50 m	11.9	7.46	0.505	4.5
A2	0 m	17.3	8.15	0.505	9.88
	-25 m	11.6	7.65	0.504	8.02
	-50 m	11.3	7.52	0.507	5.21
A3	0 m	17.6	8.44	0.503	9.82
	-25 m	13.4	8.13	0.499	7.96
A4	0 m	18.2	8.23	0.498	9.67
A5	0 m	18.1	8.32	0.497	9.62

3.2 Chemical and Isotopic Composition

3.2.1 Waters

The vertical distribution of major anions and cations in the water column of Lake Albano is shown in **Table 3.2** and **Figure 3.2**. Most of the major anions and cations showed a relatively stable gradient from top to bottom and relatively average values. The chemical composition was dominated by most of the cations (K^+, Ca^{2+}, Na^+; up to 51.36, 35.24, and 28.70 mg/L, respectively) and Cl^- (up to 18.90 mg/L), with alkalinity (HCO_3^-) up to 323.30 mg/L. Lower concentrations were measured for SO_4^{2-}, NO_3^-, and F^- (up to 7.75, 1.00, and 0.738 mg/L, respectively).

Table 3.2: Vertical chemical composition of water in Lake Albano. Sampling depth (m), salinity (expressed as TDS, in mg/L), chemical composition (in mg/L) of the main solutes and isotopic composition of water δD-H_2O and δ^{18}O-H_2O(in ‰vs. V-SMOW) from Lake Albano.

depth	TDS	F^-	Cl^-	NO_3^-	SO_4^{2-}	HCO_3^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	δD-H_2O	δ^{18}O-H_2O
0	421	0.636	18.40	0.344	7.63	280.60	27.20	46.02	15.70	24.10	9.28	2.23
-10	402	0.658	18.90	0.774	7.75	262.30	25.83	45.11	15.60	25.00	9.16	2.10
-20	418	0.624	16.60	0.785	7.22	280.60	24.80	44.55	15.50	27.81	4.86	1.12
-30	401	0.547	16.70	0.645	7.22	268.40	24.40	42.10	14.90	25.70	-0.22	0.26
-40	433	0.559	17.00	0.626	7.33	292.80	26.40	45.80	16.10	26.60	-1.36	0.01
-50	435	0.592	18.20	0.656	7.62	280.60	30.86	51.36	16.50	28.24	0.00	0.30
-60	437	0.738	17.20	0.753	7.01	292.80	25.26	44.77	15.60	32.91	-0.24	0.27
-70	432	0.582	16.80	0.441	7.37	292.80	24.80	43.20	15.30	30.50	-1.00	0.00
-80	449	0.575	17.30	0.989	6.96	305.00	28.70	46.20	16.60	26.50	0.09	0.18
-90	466	0.552	16.40	0.452	5.24	323.30	27.10	44.60	16.90	31.90	-2.55	-0.20
-100	448	0.561	16.90	0.312	4.35	305.00	27.50	44.50	16.20	32.70	-2.65	-0.19
-110	433	0.530	16.10	1.000	4.06	292.80	25.94	44.43	14.90	32.91	-2.26	-0.15
-120	462	0.584	16.50	0.430	3.33	317.20	27.10	45.00	16.20	35.20	-2.16	-0.14
-130	436	0.517	16.50	0.570	3.82	292.80	27.66	46.93	15.70	31.90	-2.63	-0.09
-140	470	0.564	17.50	0.882	4.18	323.30	28.10	45.40	16.90	32.70	-1.30	0.14
-150	461	0.573	18.00	0.817	3.76	311.10	28.23	47.27	16.30	35.24	-2.47	0.05
-160	448	0.583	16.60	0.452	3.58	305.00	26.70	45.30	16.20	33.60	-2.82	-0.05

SO_4 concentrations showed a decreasing trend with depth, with the highest concentration of 7.75 mg/L (at 10 m depth) and the lowest concentration of 3.33 mg/L (at 120 m depth). Conversely, Ca and HCO_3 concentrations exhibited increasing trends with depth. Ca concentration ranged from 24.10 mg/L at the surface to 33.60 mg/L at the bottom, while HCO_3 concentration ranged from 280.60 mg/L at the surface to 305.00 mg/L at the bottom. The increase in HCO_3 concentrations (proxy for alkalinity) reflected lower pH levels and higher dissolved CO_2 concentrations towards the hypolimnion (**Figure 3.4**).

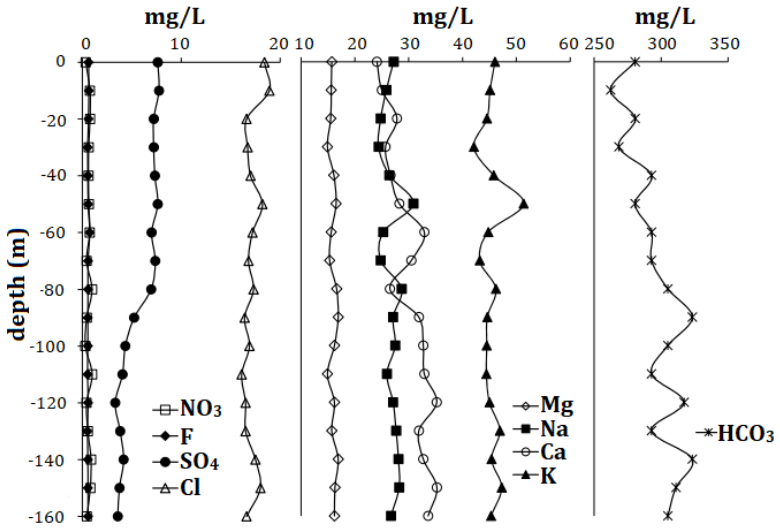

Figure 3.2: Variation in concentration of the main solutes and alkalinity along the vertical profile of Lake Albano.

The isotopic composition of water in Lake Albano is reported in **Table 3.2** and **Figure 3.3**. The values of δD-H_2O and δ^{18}O-H_2O both showed declining trends with depth, ranging from -2.82‰ to 9.28‰ and from -0.20‰ to 2.23‰ vs. V-SMOW, respectively.

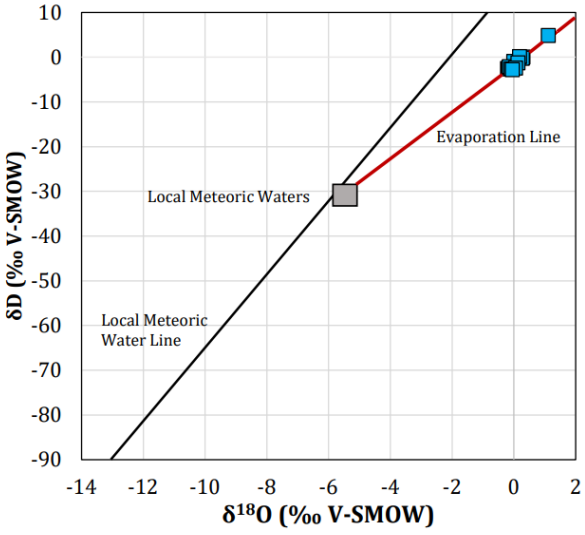

Figure 3.3: Isotopic composition of δ^{18}O vs δD in the water of Lake Albano (blue squares). The local meteoric water data were retrieved from Longinelli and Selmo (2003).

3.2.2 Dissolved Gases

The chemical and isotopic (δ^{13}C-CO_2 and δ^{13}C-CH_4) composition of dissolved gases are reported in **Table 3.3**, **Figure 3.4**, and **Figure 3.5**. Atmospheric-related gases (N_2, O_2, and Ar) were dominating at depths $\leq$20 m (up to 1.11, 0.0125, and 0.36 atm, respectively). The pressure of He was relatively stable along the water column at 0.000022 atm. Deeper waters were anoxic and showed increasing amounts of CO_2 and CH_4 (up to 0.021 and 0.21 atm, respectively) down to the maximum depth, where the total gas pressure was 1.08 atm.

Table 3.3: Vertical chemical composition (in atm) of the main dissolved gases in samples from Lake Albano. Carbon isotopic composition of δ^{13}C-CO_2 and δ^{13}C-CH_4 (in ‰vs. V-PDB) values are reported.

depth	CO_2	N_2	Ar	CH_4	O_2	He	pTOT	δ^{13}C-CO_2	δ^{13}C-CH_4
0	0.00015	1.11	0.0125		0.36	0.000022	1.48	-7.2	
-10	0.00013	1.05	0.0120		0.30	0.000022	1.37	-7.1	
-20	0.00013	0.86	0.0092		0.12	0.000021	0.99	-7.0	
-30	0.00015	0.83	0.0090	0.000000	0.11	0.000021	0.95	-7.4	
-40	0.00015	0.84	0.0096	0.000008	0.09	0.000022	0.94	-7.9	
-50	0.00021	0.86	0.0096	0.000028	0.07	0.000022	0.93	-8.1	
-60	0.00026	0.84	0.0096	0.000208		0.000022	0.85	-8.2	-56
-70	0.00028	0.83	0.0090	0.00056		0.000022	0.84	-7.8	-55
-80	0.00030	0.83	0.0090	0.00278		0.000022	0.84	-7.7	-56
-90	0.00032	0.84	0.0090	0.0061		0.000022	0.86	-7.8	-55
-100	0.00047	0.85	0.0095	0.043		0.000022	0.91	-7.7	-57
-110	0.00066	0.84	0.0095	0.056		0.000022	0.91	-7.6	-58
-120	0.0011	0.85	0.0095	0.096		0.000022	0.96	-7.5	-57
-130	0.0014	0.87	0.0095	0.13		0.000022	1.00	-7.5	-58
-140	0.0017	0.85	0.0095	0.16		0.000022	1.02	-7.4	-57
-150	0.0023	0.87	0.0095	0.18		0.000022	1.06	-6.8	-58
-160	0.0021	0.87	0.0095	0.21		0.000022	1.08	-6.6	-55

In terms of concentrations, N_2 was the dominant component in shallow water, followed by O_2 (which is zero below 60 m depth), Ar, CO_2, He, and CH_4 (**Figure 3.4**). N_2, Ar, and He concentrations were relatively stable around 0.72, 0.017, and 0.00000088 mmol/L, respectively. The CH_4 concentrations were under the detection limit from the surface to 30 m depth and were detected and increased from 40 m depth, ranging from 0.000015 to 0.41 mmol/L at the bottom. The CO_2 concentrations ranged from 0.005 to 0.11 mmol/L from top to bottom, with a clear increase shift in $\geq$90 m depth. Depth reflects at least partly an origin by the decomposition of the organic matter in the anoxic sediments of the lake bottom.

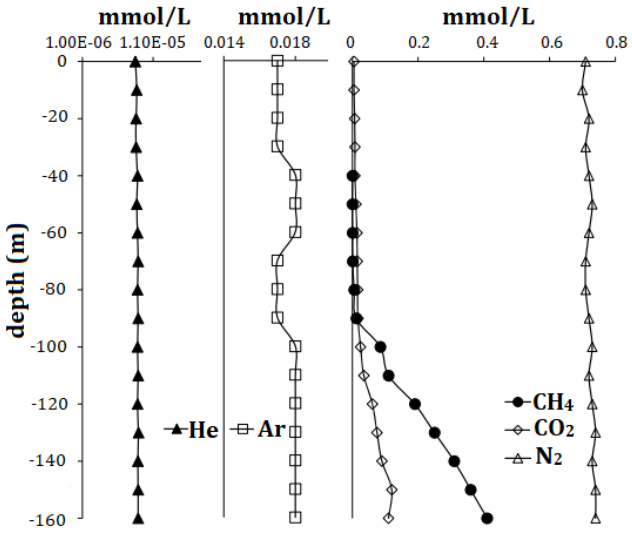

Figure 3.4: Variation in concentration of dissolved gases species along the vertical profile of Lake Albano.

Table 3.3 and **Figure 3.5** shows how the δ^{13}C-CO_2 and δ^{13}C-CH_4 values were relatively increasing from top to bottom. The δ^{13}C-CO_2 values ranged from -8.2‰and -6.6‰vs. V-PDB, whereas δ^{13}C-CH_4 varied from -58‰to -55‰vs. V-PDB.

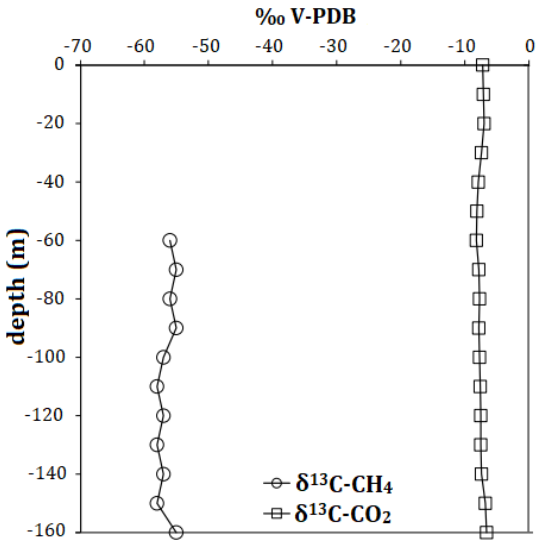

Figure 3.5: Variations in isotopic composition of δ^{13}C-CO_2 and δ^{13}C-CH_4 (in ‰vs. V-PDB) along the vertical profile of Lake Albano.

3.3 Microbial Abundance and Diversity

The microbial cell abundance, analyzed by flow cytometry, showed a declining trend with depth, as depicted in **Figure 3.6**. In the first sampling campaign, the highest value recorded was at the surface with 3.43x10^6 cells/mL, and the lowest values were at 60-120 m range depth with the average of ±1.59x10^6 cells/mL. In regards to the nucleic acid contents, Low Nucleic Acid (LNA) containing prokaryotes predominated throughout the water column with 59.8%-91% of total prokaryotes. In contrast, High Nucleic Acid (HNA) prokaryotes abundances were relatively low and increasing by depth.

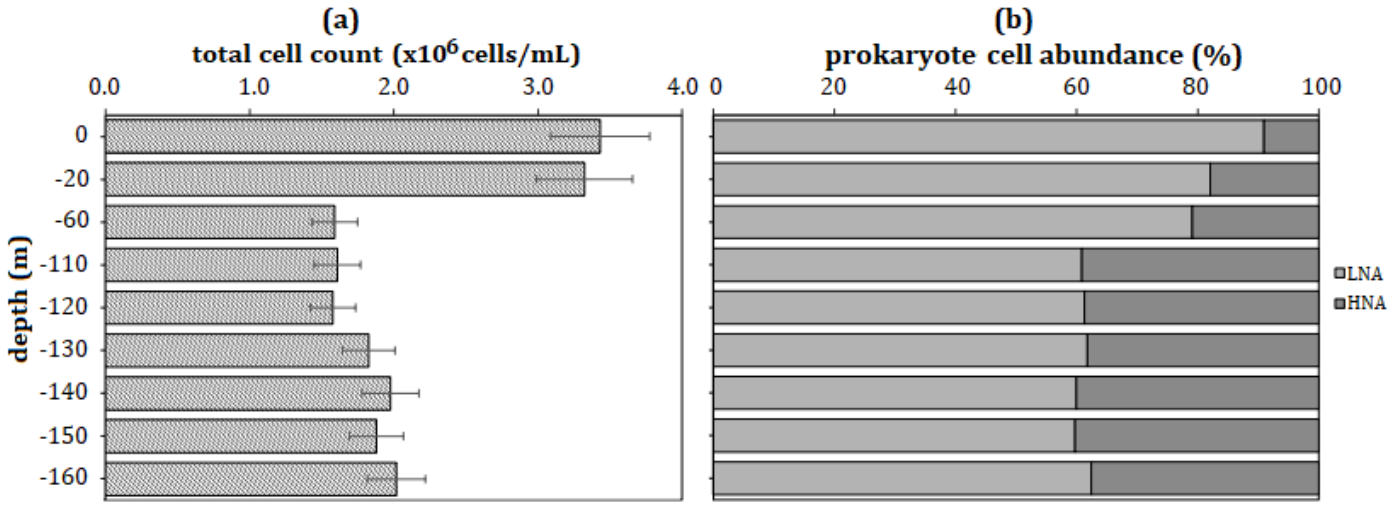

Figure 3.6: Vertical microbial cell abundance profiles along the Lake Albano water column estimated by flow cytometry. (a) vertical profile of cell abundance of the first sampling campaign (expressed as x10^6 cells/mL); (b) relative percentages within the Low Nucleic Acid (LNA) and High Nucleic Acid (HNA) cytometric populations.

NGS analysis retrieved a total of 2,021 OTUs: 2,019 affiliated to 57 known phyla and 2 OTUs to unknown phyla. Shannon index showed an increasing trend with depth [3.46 (0 m), 4.00 (-20 m), 4.20 (-60 m), 3.84 (-110 m), 4.72 (-120 m), 4.24 (-130 m), 4.62 (-140 m), 4.68 (-150 m), 4.54 (-150 m)].

Vertical microbial profiles along the Lake Albano water column estimated by NGS in phylum level are depicted in **Figure 3.7**. In Lake Albano, phyla Proteobacteria and Bacteroidetes were generally dominated throughout the water column, whereas Actinobacteria, Cyanobacteria, and Verrucomicrobia were predominant phyla in epilimnion; Chloroflexi and Planctomycetes were predominant phyla in the intermediate zone of epilimnion-hypolimnion (Layer I); and Epsilonbacteraeota was predominant in the deep layers (Layer II).

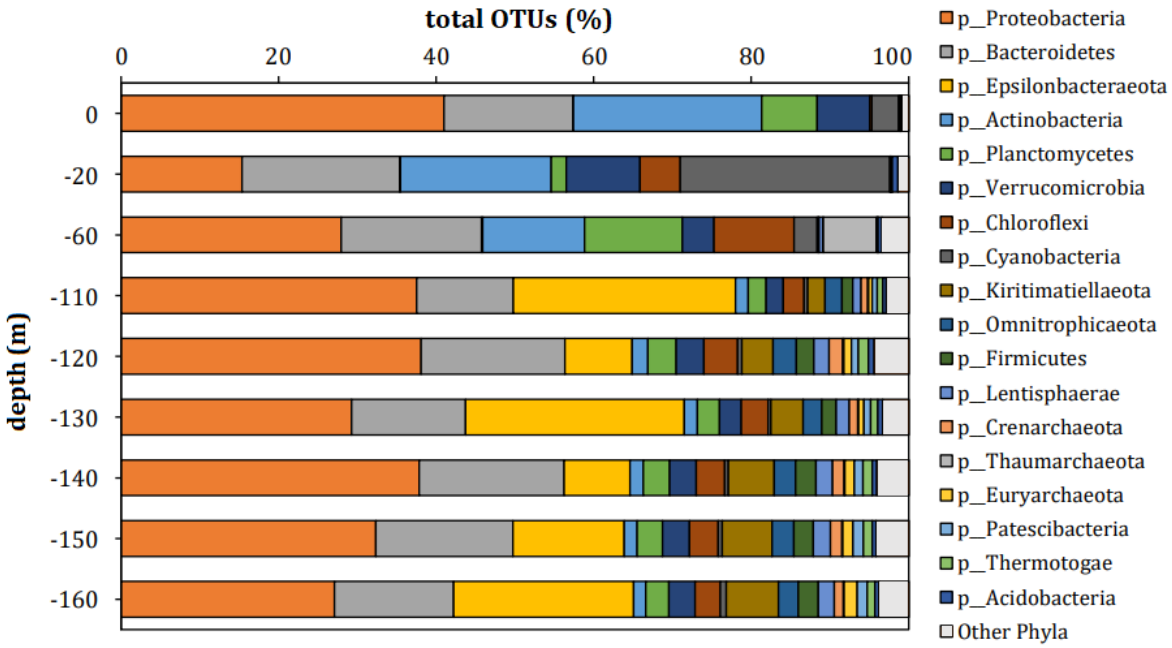

Figure 3.7: Vertical microbial profiles along the Lake Albano water column estimated by NGS in phylum level.

In detail, Proteobacteria was found at the highest percentages across the depth ranging from 15.33% to 40.98% of total OTUs, with the highest at the lake surface, whereas Bacteroidetes was present across the depth with 12.28% to 20.04% range of total OTUs with the highest at 20 m depth. At 20 m depth, the analysis showed the dominance of photosynthetic taxa mostly affiliated with phylum Cyanobacteria that accounted for 26.66% of total OTUs and phylum Actinobacteria that was almost exclusively detected in surface water samples. At 60 m depth, where no free oxygen was detected, a variety of anaerobic microbial functional groups were recognized. Facultative anaerobic microorganisms affiliated with Planctomycetes and Chloroflexi phyla were dominant at 12.39% and 10.17% of total OTUs, respectively. As for the deeper water layers (-110 m to the bottom), a clear shift in terms of microbial community composition was observed. The microbial communities were relatively more diverse yet stable. The most abundant sequences were mainly affiliated with Epsilonbacteraeota, ranging from 8.36% to 28.21%, with the highest abundance at 110 m depth.

Figure 3.8 reported the diversity comparison between Archaea and Bacteria genera across the water column. Bacteria showed a relatively more diverse community rather than Archaea. In terms of Bacteria, the surface was predominated by the hgcl clade genus and Clade III (SAR11) family. The 20 m depth was predominated

with *Planktothrix*, whereas at 60 m depth, hgcl clade, CL500-3, *Rhodoferax* genera, and Anaerolinaceae family were at relatively similar abundances, around 10%. The deep layers (Layer II) have relatively more stable communities predominated with *Methylobacter* and *Sulfuricurvum* genera. In terms of Archaea, the communities were less diverse than Bacteria, with *Candidatus Nitrosopumilus* present across the water column and dominant on the epilimnion and Layer I. In the deep layers (Layer II), the Bathyarchaea class was dominating with 32.03%-50.12% of the total OTUs of Archaea. Woesearchaeia classes, *Methanoregula* and *Methanospirillum* genera were also present in the deep water layers (up to 19.98%, 23.73%, and 4.30% of the total OTUs of Archaea, respectively).

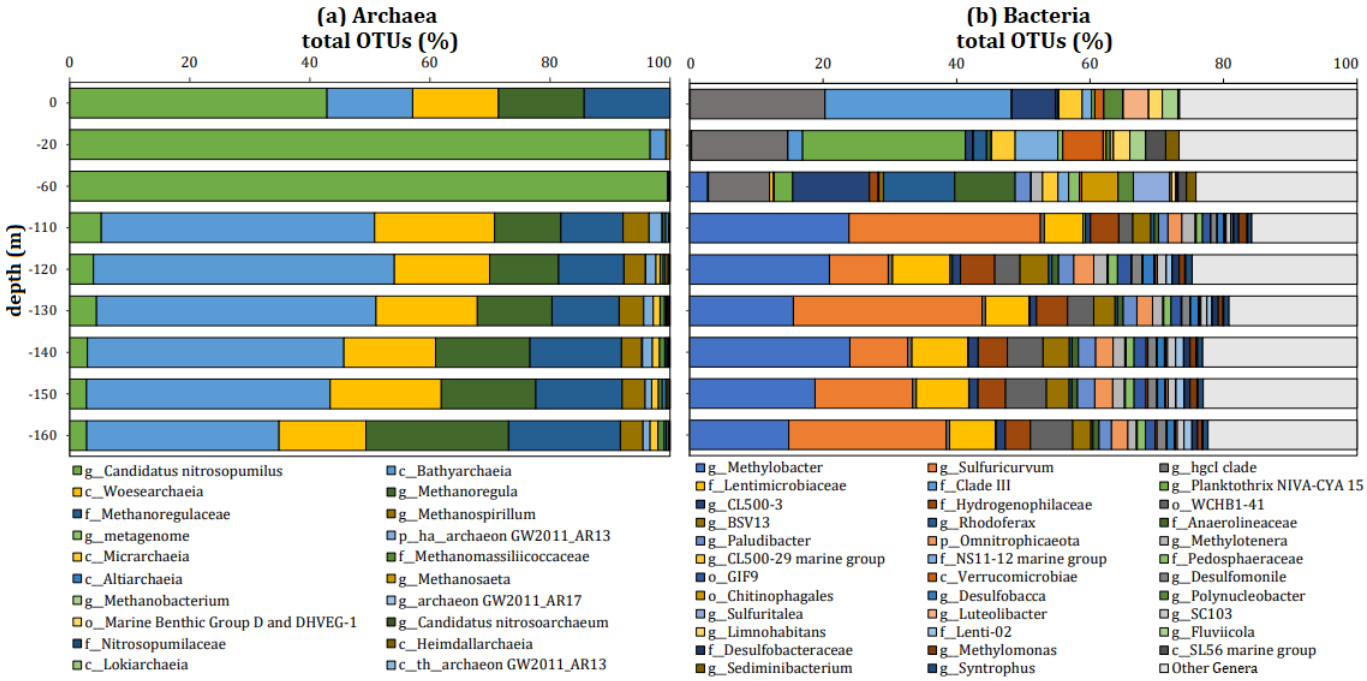

Figure 3.8: Vertical profiles of (a) Archaea and (b) Bacteria genera along the Lake Albano water column estimated by NGS. If no genus name could be assigned, the best assignment is reported.

The 25 most abundant genera and their clusters are shown in **Figure 3.9**. The figure shows two clustered depths based on the 25 most abundance genera. The first cluster was the epilimnion (-0 m and -20 m) and Layer I (-60 m), whereas the second cluster was the deep layers (Layer II; -110 m to the bottom), as hypothesized. The 25 most abundant genera comprised of 23 genera of Bacteria and 2 genera of Archaea (Bathyarchaea class and *Candidatus Nitrosopumilus* genus).

At the surface (0 m depth), OTU_1465 from Clade III family (SAR11), Proteobacteria, and hgcl clade from Actinobacteria were at their highest relative abundances (27.95% and 20.19% of total OTUs, respectively). At 20 m depth, photosynthetic

Planktothrix (Cyanobacteria) dominated at 24.39% of total OTUs. The hgcl clade (Actinobacteria), NS11-12 marine group (Bacteroidetes), and Verrucomicrobiae class (Verrucomicrobia) were also detected at 20 m depth in relatively high abundances (14.40%, 6.38%, and 6.06% of total OTUs, respectively).

At 60 m depth, facultative anaerobic microorganisms CL500-3 (Planctomycetes), *Rhodoferax* (Proteobacteria), hgcl clade (Actinobacteria), and OTU_585 (Chloroflexi) were found at relatively similar abundances (10.72%, 9.96%, 8.51%, 8.40% of total OTUs, respectively). Notably, photosynthetic *Planktothrix* (Cyanobacteria) and members of Thaumarchaeota (*Candidatus Nitrosopumilus*) were also found at this depth at 2.58% and 6.79% of total OTUs, respectively.

At the deep layers, the microbiome that was composed of several anaerobic genera related to methanotroph, such as *Methylobacter* (Proteobacteria) (14.85%-24.01% of total OTUs) and sulfur-oxidizing bacteria *Sulfuricurvum* (Epsilonbacteraeota) were found at high abundance (8.65%-28.68% of total OTUs) genera. OTUs affiliated with the sulfate-reducing bacteria *Desulfomonile* and *Desulfobacca* (Deltaproteobacteria) were also retrieved at the deepest water layer (up to 1.46% and 1.57% of total OTUs, respectively). The Hydrogenophilaceae family was also found with up to 4.97% of total OTUs abundance at this layer II. Overall, as shown by the Nonmetric Multi Dimensional Scaling (NMDS) ordination plot (**Figure 3.10**), the prokaryotic biodiversity of Lake Albano analyzed by NGS showed a vertical stratification of the major microbial groups and a close relation to the gradient of the physicochemical properties.

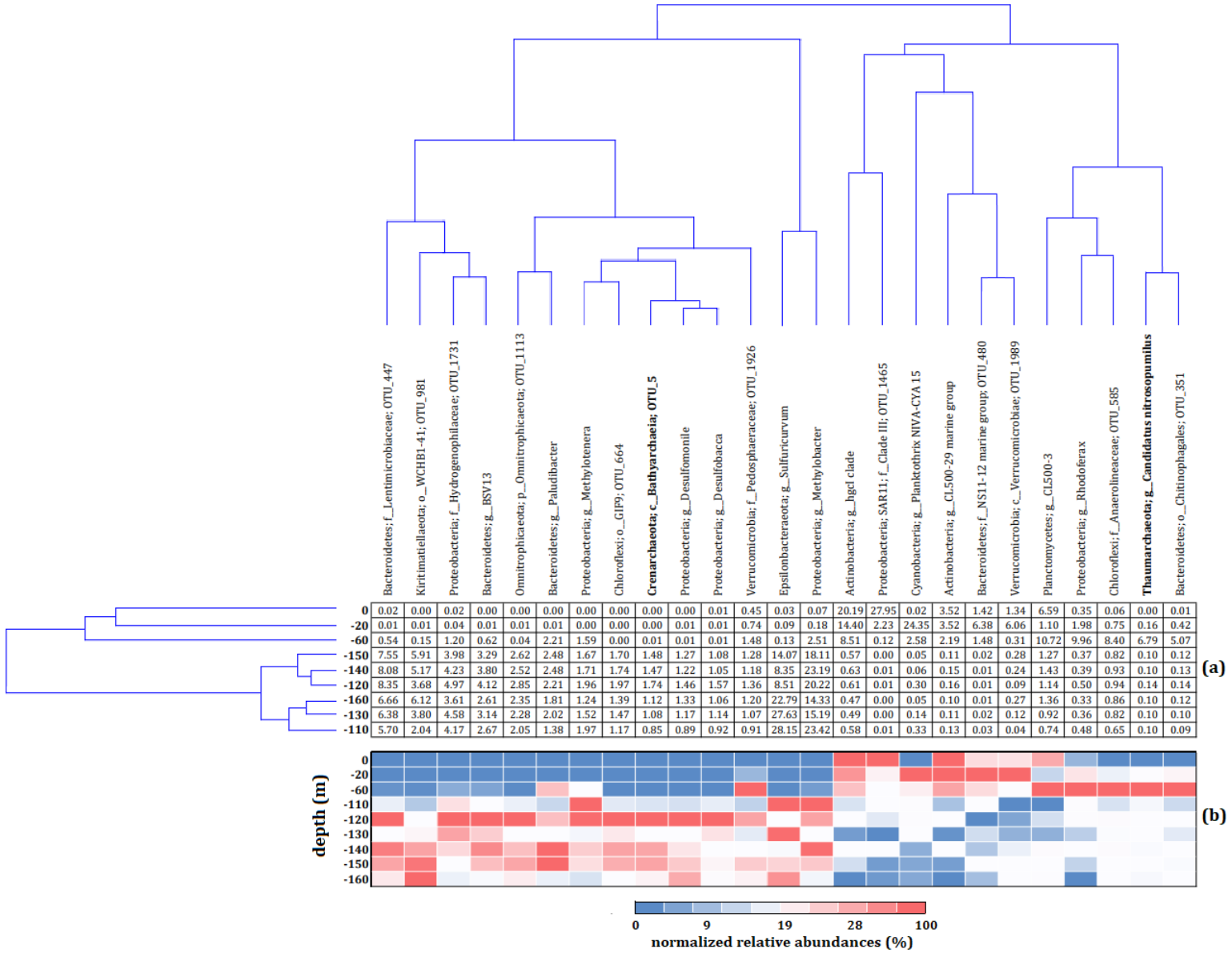

Figure 3.9: Relative abundance of the 25 most prevalent bacterial genera across the vertical water column of Lake Albano. (a) Data are normalized to the percent of total OTUs. (b) Cells in the table are color-coded on a gradient scale from blue to red, representing relative abundances. The abundance of each genus was normalized based on the average abundance, enabling the visualization of abundance fluctuations across the 9 sampling depths, regardless of genus-specific differences in abundance. In addition, the genera were clustered (the y-axis) to visualize those with similar patterns by different shades of color. Each group has both a broad group name (Phylum) and a specific name (Genus).

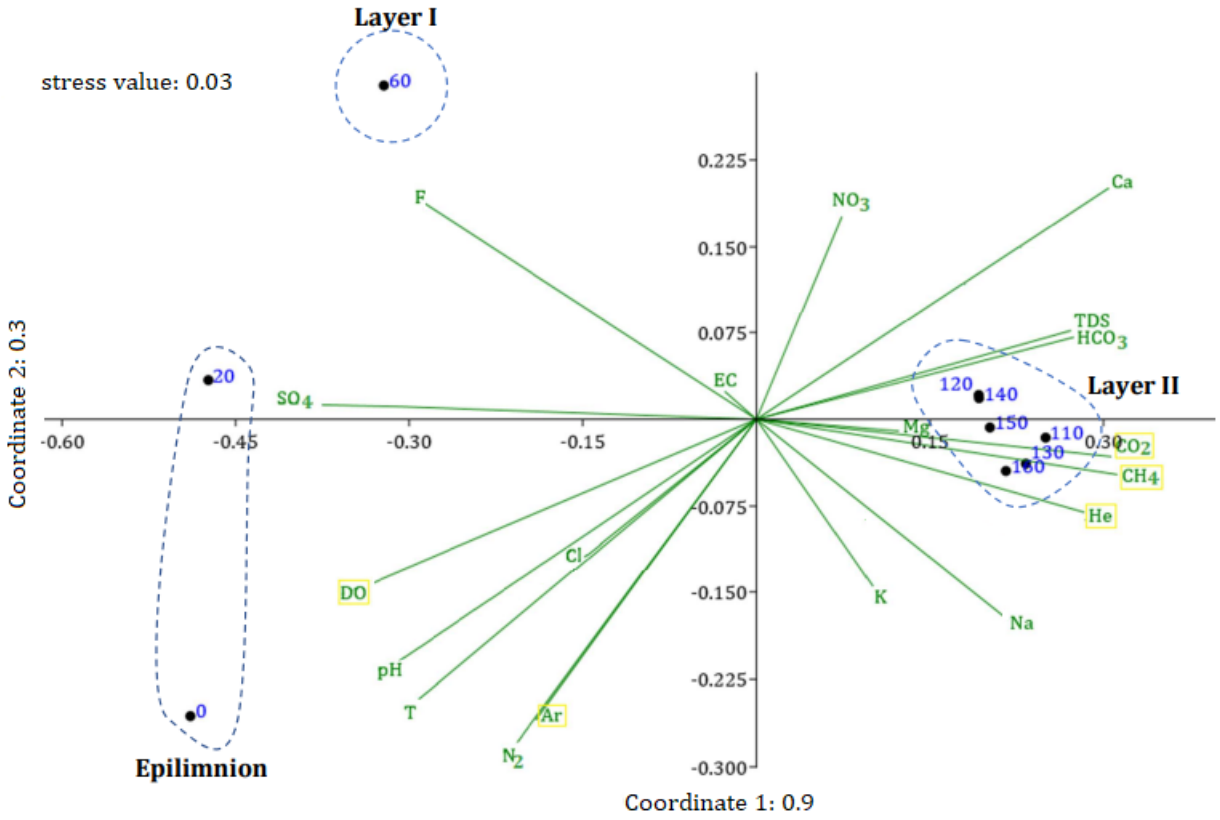

Figure 3.10: Relationships between environmental factors (chemical parameters and dissolved gases) and taxonomic composition, focusing on the 25 most prevalent bacterial genera in the samples taken from various depths. Each data point represents a microbial community at a specific depth. The distance between the data points reflects their similarity, closer points indicate greater compositional resemblance. The chemical parameters were incorporated in the NMDS analysis with a vector-fitting procedure.

3.4 Microbial Community Structural and Physiological Characteristics

In the second sampling campaign, the microbial cell abundance analyzed by flow cytometry, showed a declining trend with depth, as depicted in **Figure 3.11**. It was aligned with the result from the first sampling campaign. The highest value was recorded at the surface of A3 ($4.73x10^6$ cells/mL), and the lowest at 50 m depth of the same sampling station A3 ($7.02x10^5$ cells/mL). Regarding the nucleic acid contents, Low Nucleic Acid (LNA) containing prokaryotes predominated through all sites with a range of 74.4%-86.6% of total prokaryotes. In contrast, High High Nucleic Acid (HNA) prokaryotes abundances were relatively low and increasing by depth.

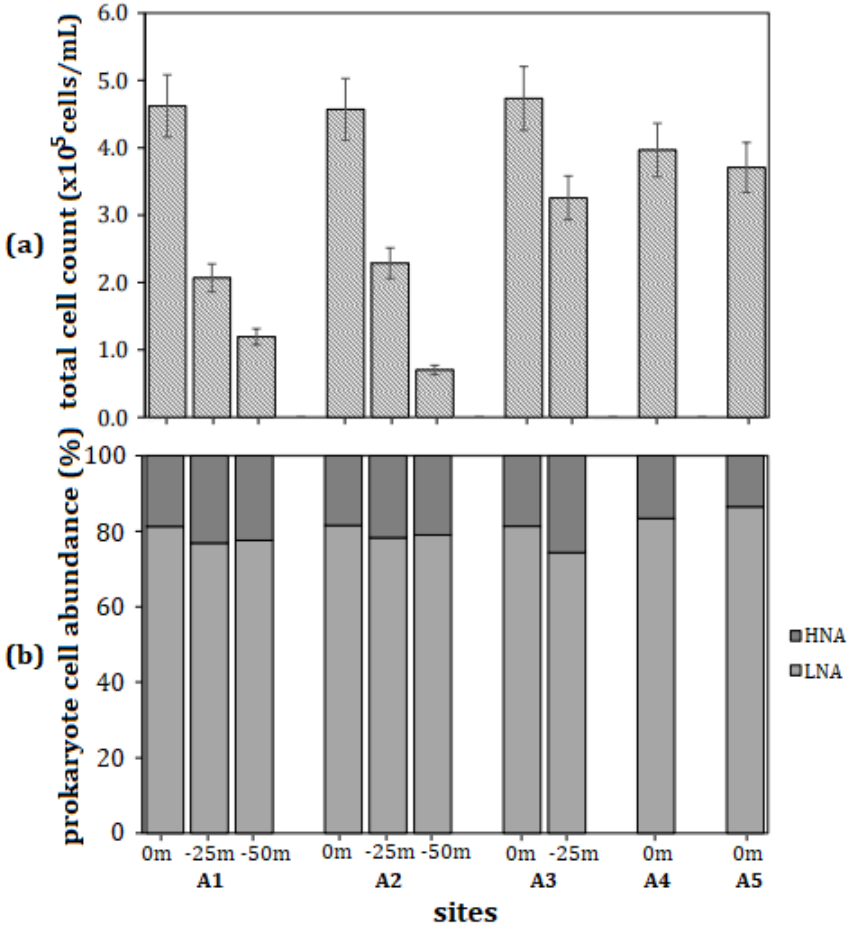

Figure 3.11: Cell abundance of prokaryotes of surface and subsurface water column of Lake Albano estimated by flow cytometry. (a) cell abundance (expressed as $x10^5$ cells/mL); (b) relative percentages within the Low Nucleic Acid (LNA) and High Nucleic Acid (HNA) cytometric populations.

The average prokaryotic abundance, analyzed by epifluorescence microscopy (see **Figure A.1**), corresponding with the flow cytometry results and showed a declining trend with depth. The highest value was recorded at the surface of A5 ($1.79x10^6 \pm 1.13^5$ cells/mL), and the lowest at 50 m depth of A2 ($4.81x10^5 \pm 9.26^3$ cells/mL). Overall, Bacteria (probe EUB338 I-III) represented around 71% of total DAPI-stained cells, whereas Archaea (probe ARCH 915) ranged from 7% to 33%, with the highest abundances at 50 m depth of the A2 site (**Figure 3.12**).

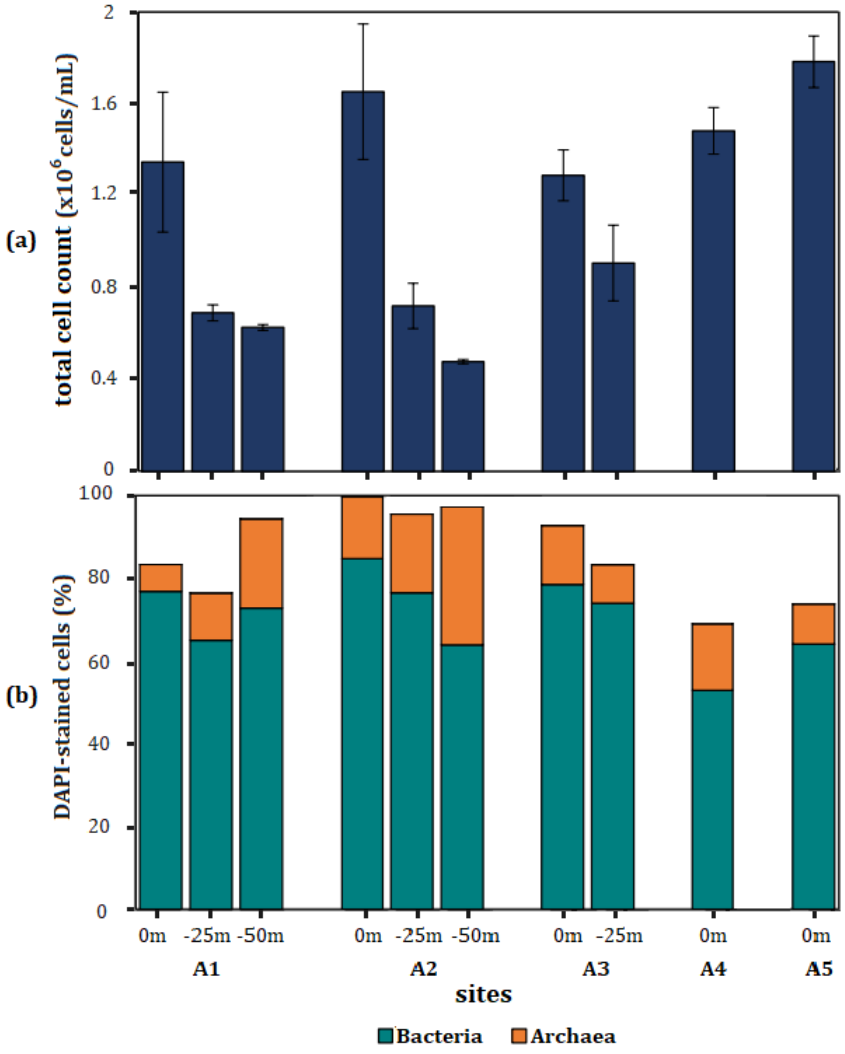

Figure 3.12: Microbial composition of the surface and subsurface water column of Lake Albano estimated by CARD-FISH. (a) total DAPI-stained cells count (cells/mL); (b) bacteria and archaea (expressed as % of DAPI-stained cells).

The multivariate analysis (PERMANOVA) for the Average Well Color Development (AWCD) values of three groups of depths (0 m, 25 m, and 50 m) have identified a statistically significant difference ($P<0.001$) among depths and relatively higher between-group variation compared to the within-group variation ($F = 5.098$;

P=0.0007). The analysis also showed that there were no statistically significant differences (P>0.001) along the coast-broad transect (A1, A2, A3, A4, and A5)(F=1.453; P=0.168). These results aligned with the Kruskal-Wallis test that suggests that there is a significant difference between the medians of the groups being compared (H(chi^2)=104.6; H_c (tie corrected)=108.4; p (same)=8.847E-22).

The microbial community properties of Lake Albano are presented in **Figure 3.13**, illustrating the AWCD values and Shannon-Wiener's diversity index (H). Among the different depths, the microbial community at 25 m displayed the highest AWCD (0.0075) and diversity ($\pm$3.2), followed by the 50 m depth with AWCD of around $\pm$0.0030 and diversity of $\pm$2.7. The microbial community at the surface (0 m depth) showed AWCD of $\pm$0.0022 and diversity of $\pm$2.2.

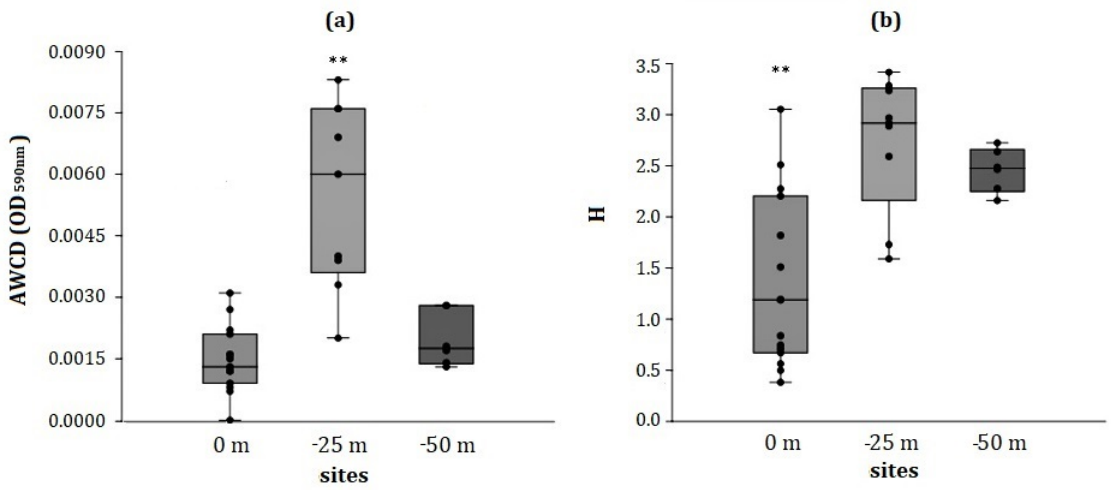

Figure 3.13: Microbial metabolic potential and functional diversity from different depth sites (0 m, 25 m, 50m) of Lake Albano. (a) Average Well Color Development (AWCD) (OD_{590nm}); (b) Shannon–Wiener's diversity index (H).

The microbial utilization patterns of organic substrates (CLPP) are shown in **Table 3.4** and **Figure 3.14**). The microbial community in the 25 m depth showed the highest number of reactive wells. Only two out 31 organic substrates provided by the Biolog assay were not utilized in the 25 m group, whereas four and six were not utilized in the 0 m and 50 m groups, respectively. The utilization patterns of organic substrates also showed that microbial communities from all depth groups were fed preferentially on polymers, which contributed on average for 65.23% of the total absorbance and the highest degradation rate at 0 m depth (up to 72.4% at A2). Inside this class of compounds, tween 40 was the most degraded compound (39.62%), followed by glycogen, α-Cyclodextrin, and tween 80 (6.28%, 4.76%, and 3.33%, respectively).

Table 3.4: Microbial utilization patterns of 31 organic substrates in samples collected from different depth sites (0 m, 25 m, 50m). Values are expressed as a percentage contribution to the total absorbance (OD_{590nm}).

Class of Compounds	Substrates	0 m					25 m			50 m	
		A1	A2	A3	A4	A5	A1	A2	A3	A1	A2
Carbohydrates	β-methyl-D-glucoside	0.5	0.0	0.0	0.7	4.2	10.6	11.2	8.6	1.6	4.8
	D-xylose	0.5	1.8	0.0	7.0	9.6	2.3	0.0	0.5	1.7	6.9
	i-erythritol	0.0	0.0	0.0	0.0	0.3	0.9	0.0	0.5	0.0	2.1
	D-mannitol	1.6	0.0	0.0	0.9	0.0	8.7	7.3	4.5	2.7	0.6
	N-acetyl-D-glucosamine	0.8	0.0	0.0	7.3	5.1	13.4	16.5	12.1	11.8	1.5
	D-cellobiose	0.0	0.0	0.0	1.5	0.8	6.8	5.5	8.0	12.4	13.5
	glucose-1-phosphate	1.3	0.0	1.7	1.4	2.3	2.9	2.0	3.4	0.3	2.8
	α-D-lactose	0.0	16.7	0.0	0.0	1.0	1.1	0.0	1.8	0.0	1.3
	D,L-α-glycerol phosphate	0.0	0.0	0.0	1.7	0.0	3.2	1.0	2.3	0.4	0.1
Carboxylic acids	D-galactonic acid γ-Lactone	7.2	0.0	0.0	5.8	1.0	1.8	4.3	3.6	3.0	2.9
	pyruvic acid methyl ester	0.8	0.0	0.0	1.4	0.0	1.7	1.2	3.4	3.0	3.5
	D-galacturonic acid	0.0	0.0	0.0	1.0	0.6	3.7	0.9	3.5	0.7	1.9
	γ-hydroxybutyric acid	1.1	0.0	0.0	1.7	6.9	2.0	0.7	1.5	0.0	1.8
	D-glucosaminic acid	0.0	0.8	0.0	0.8	0.0	2.1	0.0	1.7	0.0	0.0
	itaconic acid	0.0	0.0	13.4	1.2	1.0	2.5	0.0	0.0	5.2	1.0
	α-ketobutyric acid	0.0	0.3	0.3	2.5	0.0	0.2	0.8	0.7	1.6	4.6
	D-malic acid	0.0	0.0	0.0	0.0	0.0	1.0	0.0	0.1	0.0	0.0
Phenols	2-hydroxy benzoic acid	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	4-hydroxy benzoic acid	0.0	0.0	0.0	4.4	0.0	1.2	0.0	0.3	0.0	0.0
Polymers	tween 40	60.4	72.4	72.8	29.8	46.1	5.4	36.5	21.7	25.2	25.9
	tween 80	8.7	1.7	0.0	2.2	2.5	2.4	2.6	2.0	5.0	6.0
	α-cyclodextrin	12.1	1.5	11.9	3.3	2.6	4.7	2.2	2.1	5.2	2.0
	glycogen	3.6	0.0	0.0	8.1	5.6	8.5	2.3	10.6	14.7	9.5
Amino acids	L-arginine	0.0	0.0	0.0	7.6	8.9	3.9	2.2	1.0	4.5	0.0
	L-asparagine	0.0	4.7	0.0	0.9	0.0	2.0	0.0	0.7	0.0	1.9
	L-phenylalanine	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	L-serine	0.0	0.0	0.0	0.0	0.5	1.2	0.0	0.3	0.0	1.9
	L-threonine	0.0	0.0	0.0	2.1	0.4	1.0	0.0	0.8	0.0	2.2
	glycyl-L-glutamic acid	0.0	0.0	0.0	4.3	0.7	1.4	0.0	2.2	0.9	0.0
Amines	phenylethyl-amine	1.5	0.0	0.0	2.2	0.0	1.5	1.4	2.3	0.0	1.3
	putrescine	0.0	0.0	0.0	0.0	0.0	2.0	1.4	0.0	0.0	0.0

The CLPP in 0 m relied preferentially on polymers, with an average of 78.57%. It was mainly driven by tween 40, ranging from 29.8% to 72.8%. A decreasing contribution to the total metabolic activity was observed for carbohydrates (7.48%), carboxylic acids (5.58%), amino acids (4.85%, phenolic compounds (1.95%), and amines (1.56%), with no reaction found on 2-hydroxy benzoic acid of phenolic compounds and L-phenylalanine of amino acids (**Table 3.4**; **Figure 3.14**).

As for 25 m depth, the CLPP was characterized by a wide utilization of the provided substrates aligned with Shannon-Wienner's diversity index. The site was predominated by the utilization of polymers (46.3%) and carbohydrates (29.25%). Carbohydrate utilizations relied mostly on N-acetyl-D-glucosamine (up to 16.5%) and β-methyl-D-glucoside (up to 10.6%). The carboxylic acids contribution varied in the CLPP with an average of 9.11% and relied mostly on D-galactonic acid

γ-Lactone (up to 4.3%). Amines utilization (average of 8.30%) was driven mainly by phenylethyl-amine degradation (up to 2.3%). A decreasing contribution to the total metabolic activity was observed for amino acids (5.50%) and phenolic compounds (1.56%).

In 50 m depth, CLPP was predominated by the utilization of polymers with an average of 63.09%. Carbohydrates (19.59%) were utilized preferentially, although to a lesser extent with respect to the 25 m depth group conditions. A decreasing contribution to the total metabolic activity was observed for carboxylic acids (10.32%), amino acids (5.27%), and amines (1.73%), with no reaction found on both phenolic compounds.

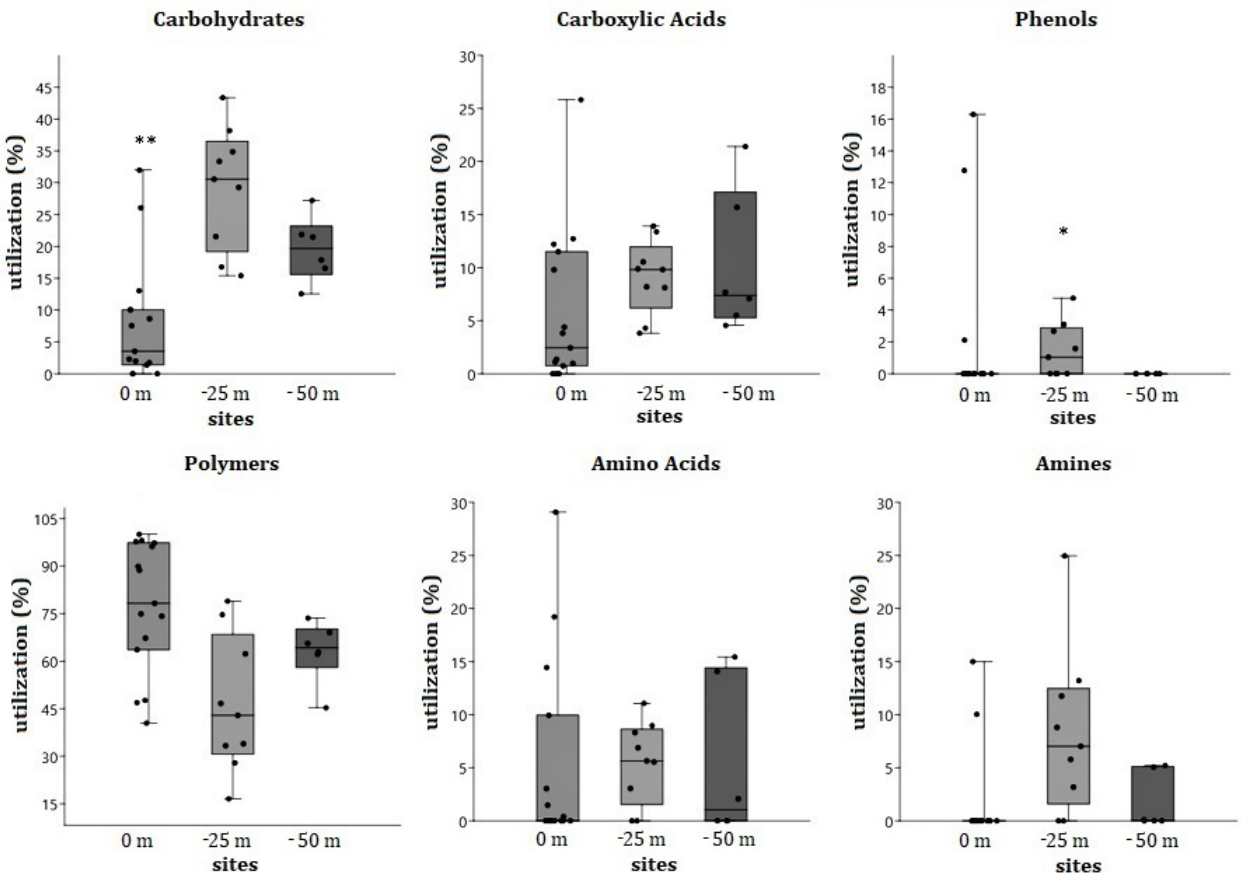

Figure 3.14: Contribution of classes of substrates to the total microbial metabolic potential (OD_{590nm}) in the different depths.

4 Discussion

4.1 Water and Dissolved Gases Sources

The distinct stratification pattern of Lake Albano remained evident as observed in previous studies (Carapezza et al., 2008; Chiodini et al., 2012; Cabassi et al., 2013; Rouwet et al., 2021) and over one year, the physicochemical properties of epilimnion remained substantially unchanged, with only seasonal variations observed. These profiles were similar to the ones recorded by Carapezza et al. (2008) from July 2001 to January 2006. These findings affirm the structure of Lake Albano comprising: (i) an oxygen-rich epilimnion above the 20 m thermocline, (ii) an oxygen-depleted zone down to 60–70 m, the depth of seasonal mixing, and (iii) an anoxic hypolimnion extending to the lake bottom.

In terms of the chemical composition of water and dissolved gases in Lake Albano, the decrease in SO_4 concentrations with depth can be attributed to bacterial activities promoting sulfate reduction in the anoxic hypolimnion. Conversely, the increase in Ca and HCO_3 concentrations with depth can be explained by calcite precipitation processes occurring in shallow waters, followed by dissolution in the hypolimnion (Carapezza et al., 2008). The elevated HCO_3 concentrations (indicative of alkalinity) correlated with lower pH levels and heightened dissolved carbon dioxide concentrations toward the hypolimnion (**Figure 3.4**). Dissolved gas concentrations in Lake Albano also demonstrated that it was the resulted from two processes: organic matter decomposition that affects the CH_4/CO_2 ratio and injection of CO_2-rich gas from the depths (Cioni et al., 2003).

Water isotopic values of Lake Albano demonstrated a coherent relationship with depth, in which $\delta^{18}O$ values were likely to reflect the influence of mixing with surface waters. The δD–$\delta^{18}O$ variations were consistent with a straightforward process of local meteoric water evaporation (Longinelli and Selmo, 2003; Carapezza et al., 2008). Whereas the carbon isotope dynamics of Lake Albano suggest the potential release of isotopically light CO_2 through diffusion, indicating a magmatic source (Chiodini et al., 2012). The highly negative and stable $\delta^{13}C$-CH_4 values indicative of methane production within the lake, were primarily tied to microbial activity (Cabassi et al., 2013).

The outcomes of the August 2022 survey in this study resembled to the findings of the April 2019 survey conducted by Rouwet et al. (2021), albeit with a more pronounced stratification due to seasonal variations. The observations indicate that the equilibrium state of CO_2 degassing has been reached, as evidenced by the almost negligible fluxes observed between different strata. This suggests the presence of a permanent CO_2 reservoir within the deep layers of Lake Albano. Consistent with the study by Rouwet et al. (2021), it was proposed that the gas self-lifting process imposes limitations on the ability of Lake Albano to experience complete degassing. Consequently, the lake is unlikely to evolve into a CO_2-free environment in the future.

In addition, according to Rouwet et al. (2021), Lake Albano has undergone a reduction in lake water level (approximately 6.1 m) and subsequent volume contraction (approximately 3.3×10^7 m^3) over the period 1989-2019. This contraction was the result of extensive well pumping from the Ciampino Plain aquifers. This anthropogenic stressor is likely to exert a destabilizing influence on the lake stratification and the dynamics of CO_2 degassing over the long term.

Furthermore, building on the findings of previous research, it becomes evident that the absorption of CO_2 at the surface of the lake has increased in recent years. This trend might be attributed to the elevated atmospheric CO_2 concentrations linked to global warming, as highlighted by Rouwet et al. (2021). It was suggested that this trend could potentially intensify in the future. The collective impact of external factors such as pumping and global warming on the limnology of the lake and its geogenic CO_2 degassing warrants further investigation, requiring more refined spatial resolutions and frequent temporal geochemical and geophysical surveys.

4.2 Vertical Profiles of Lake Chemistry and Prokaryotic Activity

The 16S rRNA gene sequences analysis in Lake Albano indicated that the phyla Proteobacteria and Bacteroidetes were predominant and widespread throughout the water column. This finding aligns with a previous study by Newton et al. (2011), where these two phyla were also the most abundant in a freshwater bacterial database, and both play crucial roles in various biogeochemical processes.

At the lake surface, 16S rRNA gene sequences affiliated with high relative abundances of Alphaproteobacteria (Clade III, SAR11) and Actinobacteria (hgcl clade) OTUs were retrieved. According to Lanclos et al. (2023), the ubiquitous alpha-proteobacteria of the order Pelagibacterales (SAR11) are the

most prevalent heterotrophic bacterioplankton in global epipelagic waters, with distinct spatiotemporal patterns exhibited by various subclades. Clade III, which dominates Lake Albano, likely belongs to the LD12 subclade (Clade IIIb). LD12 (also referred to as SAR11 clade IIIb) is specifically found in freshwater lake ecosystems and is among the most abundant organisms in freshwater environments (Tsementzi et al., 2019).

Despite being one of the most abundant organisms in freshwater environments, the biology of Clade IIIb bacteria remains very little known due to the lack of available genome representatives (Tsementzi et al., 2019). Nevertheless, Henson et al. (2018) reported that Clade IIIb shares many characteristics with marine SAR11, such as small cell volumes, adaptation to oligotrophic habitats, an obligate aerobic chemoorganoheterotrophic lifestyle with limited metabolic flexibility, and preference for small molecular weight compounds like carboxylic and amino acids as carbon/energy sources. This metabolic adaptation likely contributes to their survival in freshwater environments, where oxygen is abundant and these compounds are more readily available.

Interestingly, LD12 genomes belonging to Clade IIIb possess the EMP pathway, which is not commonly found in most marine SAR11. This metabolic capability enables LD12 organisms to efficiently use glucose as a carbon and energy source (Henson et al., 2018). The ability to effectively metabolize glucose provides LD12 with a competitive advantage in surface water environments where this molecule may be present due to various organic inputs.

The second most abundant phyla in the surface water was assigned to the Actinobacterial Sporichthyaceae family and hgcl clade genus. This observation is consistent with Monard et al. (2016), indicating that Actinobacteria are widely distributed in terrestrial and aquatic ecosystems, whereas Newton et al. (2011) reported that hgcl clade is particularly relevant in freshwater environments.

Numerous studies have highlighted the remarkable capabilities of Actinobacteria to thrive under extreme conditions, including high pH, temperature, and water stress. They are known for their ability to utilize a variety of substrates, including less degradable compounds like chitin, cellulose, and hemicellulose, as well as their resistance to UV radiation (Warnecke et al., 2005; Shivlata and Satyanarayana, 2015). Moreover, hgcl clade is equipped with actinorhodopsin, enabling them to utilize sunlight energy for heterotrophic growth, along with carotenoids that offer protection against high radiation, oxidative stress, and grazing (Pernthaler, 2005;

Sharma et al., 2009; Dwulit-Smith et al., 2018). These distinct characteristics likely explain their occurrence in the photic zone of the lake, where complex carbon substrates can also be available.

Consequently, biological CO_2 consumption and relatively high pH were observed (**Figure 3.1**; **Figure 3.4**). This leads to rapid CO_2 dissolution, resulting in the formation of bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions, which explains the extremely low CO_2 concentrations measured in this water layer (**Figure 3.4**). This insight provides valuable information on carbon dynamics and pH regulation within the surface water of Lake Albano.

Around 20 m depth, where the water remains lighted and oxygenated, phototrophic prokaryotes primarily affiliated with cyanobacterial filamentous microorganisms (*Planktothrix* spp.) dominate. The presence of Cyanobacteria is often linked to significant summer blooms in freshwater lakes and reservoirs (Reynolds et al., 2002). Their preference for CO_2 as the primary carbon source strongly indicates the crucial role of microorganisms in shaping the vertical CO_2 profile in the epilimnion. Moreover, the utilization of CO_2 by these microorganisms results in substantial oxygen production, as evidenced by the increased dissolved oxygen (DO) concentration at this depth (**Figure 3.1**). The hgcl clade of Actinobacteria was also found in relatively high abundance, contributing to CO_2 consumption.

In this shallow water layer, the NS11-12 marine group of Sphingobacteriales (Bacteroidetes) was also detected. While the NS11-12 marine group has been mainly found in marine habitats (Meziti et al., 2015), it was previously detected in Hungarian shallow lakes (Farkas et al., 2020). However, the ecological implications of the NS11-12 marine group remain unclear except for its role in contributing to the stability of microbial co-occurrence in Chesapeake Bay, USA (Wang et al., 2021).

Nevertheless, Sphingobacteriales are known to be chemoorganoheterotrophs, with some species also possessing the capability to degrade recalcitrant organic matter, such as lignin and cellulose, making them important decomposers in freshwater environments. Furthermore, the respiratory metabolism of Sphingobacteriales in freshwater can be facultative anaerobic (Krieg et al., 2010). This flexibility allows them to switch between utilizing oxygen as a terminal electron acceptor under aerobic conditions and employing alternative electron acceptors, such as nitrate, under anaerobic conditions. The study by Dennis et al. (2013) reported that Sphingobacteriales showed a strong chemotactic response to nitrate and phosphate, indicating their ability to detect and utilize nitrate as an alternative

electron acceptor. This adaptability enables them to thrive and compete effectively in changing environmental conditions and variable nutrient availability within freshwater ecosystems.

The Verrucomicrobiae class (Verrucomicrobia) was also detected at 20 m. These Verrucomicrobiae may be associated with typical phototrophic prokaryotes and methanotrophs, similar to what was found in the photic zone of one of the Italian volcanic lakes, Lake Averno, by Tassi et al. (2018). The presence of methanotrophic Verrucomicrobiae could explain the relatively low CH_4 concentrations observed at depths between 0 to 30 m (**Figure 3.4**). As discussed in the study by Tassi et al. (2018), the occurrence of methanotrophic Verrucomicrobiae in the surface waters of lakes with high pH aligns with the recent hypothesis that these bacteria might thrive across a wide range of environmental conditions, not limited to geothermal environments with low pH values. Similar findings have been reported in other meromictic volcanic lakes, where CH_4 oxidation and CO_2 consumption through oxygenic photosynthesis were observed in the epilimnion (Hanson and Hanson, 1996; Nelson and Ben-Shem, 2004).

At approximately 60 m depth, below the oxic-anoxic interface, a diverse microbial community with distinct vertical structure in terms of taxonomy and potential functional composition was observed. This microbial community consisted primarily of chemoorganoheterotrophic bacteria, as evidenced by the presence of CL500-3 (Planctomycetes), *Rhodoferax* (Proteobacteria), Anaerolineaceae (Chloroflexi), the photosynthetic Cyanobacteria *Planktothrix*, and ammonia-oxidizing archaea affiliated with *Nitrosopumilus* or *Candidatus* (Thaumarchaeota). These findings are consistent with a previous study by Okazaki et al. (2017), where they reported a relatively high abundance (ranging from 1.5% to 32.9% of all bacterioplankton) of CL500-3 Planctomycetes, Chloroflexi, and Marine Group I Thaumarchaeota in the oxygenated hypolimnion (oxic-anoxic interface) of the lakes through CARD-FISH analysis.

In freshwater environments, Planctomycetes are predominantly represented by the CL500-3 clade (Phycisphaerae), which forms a well-defined phylogenetic group initially identified in the deep water column of the ultra-oligotrophic Crater Lake (Andrei et al., 2019). Messina-Pacheco (2019) revealed that the CL500-3 Planctomycetes may have significant implications in nitrogen dynamics, as they are believed to be actively involved in dissolved organic nitrogen cycling and nutrient remineralization, distinguishing them from other Planctomycetes genomes. Moreover, several Planctomycetes are known for their unique metabolic capabilities in important nitrogen cycling processes, including anammox and

nitrogen fixation. The process of anammox, occurring under anaerobic conditions, is essential as Planctomycetes convert ammonium and nitrite into nitrogen gas, effectively reducing nitrogen levels in the environment (Fuerst and Sagulenko, 2011).

Rhodoferax bacteria are metabolically versatile and play key roles in various biogeochemical processes, including carbon and nitrogen cycling, iron and manganese reduction, and nitrate reduction under anaerobic conditions (Finneran et al., 2003), whereas Chloroflexi (Anaerolineaceae) are anaerobic bacteria with diverse metabolic capabilities involved in degrading complex organic compounds, nutrient cycling, and decomposition of organic matter in anaerobic environments (Bovio-Winkler et al., 2023). At this depth, conditions are generally inhospitable for most Cyanobacteria, but certain *Planktothrix* species can thrive in anoxic environments due to their low energy requirements for cell metabolism (Rogers et al., 2021). Additionally, the hgcl clade of Actinobacteria was still found at this depth, indicating the occurrence of CO_2 consumption. Overall, the metabolic potential inferred from the 16S rRNA gene sequencing data is primarily related to nitrogen cycling, consistent with the relatively constant NO_3 and N_2 concentrations throughout the depth profile.

At 110-160 m depth, a strong increase of the microbial abundance was observed. NGS revealed that facultative anaerobic *Sulfuricurvum* (Epsilonbacteraeota) dominated this zone, suggesting their potential role in sulfur oxidation. *Sulfuricurvum*, widely distributed in diverse environments, is a significant sulfur-oxidizing bacterium with pivotal contributions to the global sulfur cycle (Li et al., 2019). Notably, this sulfur oxidation was counteracted by the simultaneous presence of sulfate-reducing Deltaproteobacteria (*Desulfomonile* and *Desulfobacca*), which aligns with the pronounced decrease in sulfate (SO_4) concentrations. These particular genera belong to Syntrophobacterales, one of the largest groups of sulfate-reducing bacteria, that undertake anaerobic respiration by utilizing sulfate rather than oxygen (Mandal, 2018). Abundant 16S rRNA gene sequences affiliated with Hydrogenophilaceae were also detected in this depth range, likely associated with the availability of $\sum S^{2-}$. Many members of this family exhibit mixotrophic or chemolithotrophic behavior, utilizing various reduced sulfur compounds or hydrogen as electron donors (Orlygsson and Kristjansson, 2014).

It is worth noting that 16S rRNA gene sequences of aerobic CH_4 oxidizing bacteria *Methylobacter* spp. were found abundant in the anoxic hypolimnion of Lake Albano. *Methylobacter* spp. are commonly known to grow aerobically and ubiquitous in the environment. According to Hogendoorn et al. (2021), *Methylobacter* is

considered an important and often dominant group of aerobic methane-oxidizing bacteria in numerous oxygen-rich environments, where members of this genus contribute mitigating CH_4 emissions. A recent study by Cabrol et al. (2020) revealed that *Methylobacter* and other Methylomonadaceae family might be major methane-oxidizing bacteria (MOB) in the anoxic water. The anaerobic oxidation of methane (AOM) attributed to these taxa has demonstrated effective mitigation of 60–100% of the produced CH_4 within four Northern Siberian stratified lakes. Methanotrophs were also suggested to be co-occurred with denitrifiers and iron-cycling partners for these AOM (Cabrol et al., 2020). This intriguing finding opens new possibilities for investigating the metabolic potentials of such microbes and their role in reducing CH4 emissions in these environments, a crucial component of the global CH_4 cycle. Further research is needed to fully understand the significance of these microbial interactions in the context of CH_4 consumption and anaerobic conditions in the lake ecosystem.

The anaerobic CH_4 consumption was likely counteracted by Archaea, whose presence in water was highlighted in **Figure 3.8**. Methanogenesis carried out by anaerobic archaea represents the largest biogenic source of methane on Earth (Vanwonterghem et al., 2016). The vertical profiles of both the CH_4 concentrations and the δ^{13}C-CH_4 values (**Figure 3.4** and **Figure 3.5**) indicate predominance of CH_4 production over consumption. This pattern aligns with the NGS data, revealing the prevalence of methanogenic microorganisms belonging to the Bathyarchaea and Woesearchaeia classes, and the *Methanoregula* and *Methanospirillum* genera within the deep layers.

The chemical, isotopic, and microbiological findings in Lake Albano consistently demonstrate a significant interplay between the characteristics of the dissolved gas composition and the differentiation of microbial niches across the vertical water column, as depicted in **Figure 4.1**.

4.3 Microbial Community Structural and Physiological Characteristics

The findings of this study indicate a clear association between water depth and the variations in microbial composition and metabolic physiological characteristics within Lake Albano. Regarding cell abundance, both flow cytometry and CARD-FISH analyses indicated a gradual decline in cell abundance from 0 m to 50 m depths. This decline aligns with the general trend of bacterial abundance decreasing with water depth in freshwater systems, as highlighted by Liu et al.

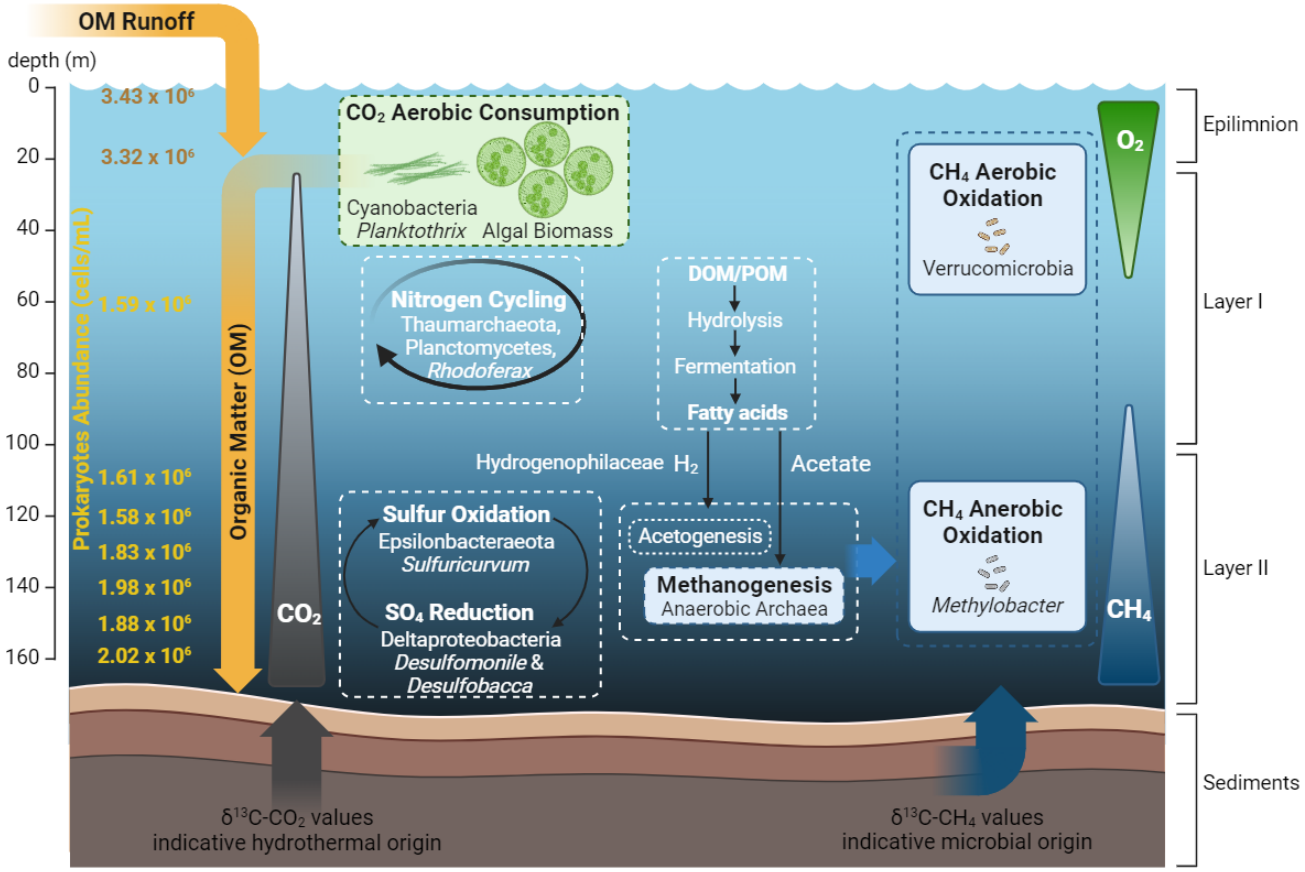

Figure 4.1: Schematic conceptual model of the interactions between microbial communities and geochemical properties at different depths in Lake Albano (created with BioRender).

(2016). Concerning microbial compositions, the increasing trend in diversity with depth could be linked to the augmentation of ecosystem functions. It is noted that ecosystem functions and multifunctionality commonly exhibit an increasing trend toward deep water, as demonstrated by Zhang et al. (2021).

In Lake Albano, the prevalence of low nucleic acid content (LNA) prokaryotic cells was observed across all sampling sites and water layers from both sampling campaigns. Correspondingly, Hu et al. (2022) highlighted the widespread nature of LNA bacteria, estimating their presence at levels ranging from 20% to 90% of the total bacterial community in marine and freshwater environments. LNA bacteria are characterized by their small cell size and genomes, remarkably high genetic diversity, and vital role in material cycling within nutrient-limited environments (Hu et al., 2022).

In terms of microbial metabolic physiological characteristics, there was an ascending trend in substrate utilization capability, transitioning from 0 m (the surface layer) to 50 m (the oxic/anoxic interface zone), and finally, the highest

utilization in 25 m (the deeper epilimnion). Despite certain limitations inherent to laboratory-based experimental methods, the Biolog assay offered a means to evaluate the latent metabolic potential of both surface and subsurface microbial communities in Lake Albano. This investigation provided insight to comprehend better the effects of potential inputs of allochthonous organic substrates on the volcanic lake ecosystems. The Biolog assay is dependent on the diverse ability of aquatic microorganisms to acclimate to the spatial and temporal fluctuations in both the quantity and quality of the dissolved organic matter (DOM) pool present within the aquatic environment (Kothawala et al., 2014). Notably, this methodology has been consistently employed in diverse aquatic contexts (Christian and Lind, 2007; Tiquia, 2010; Melita et al., 2019).

The predominant utilization of polymers was observed across all depth groups, with the highest utilization occurring at the surface (0 m). The proficient capacity to utilize complex polymers, particularly tween 40, could potentially indicate the presence of organic pollutants within the lake waters. This functional trait has been associated with environments impacted by pollution (Sala et al., 2006; Oest et al., 2018; Melita et al., 2019). Polymers are complex carbon substrates that can be classified as natural or synthetic based on their origin (Verma et al., 2023). In particular, tween 40 is nonionic surfactants utilized as emulsifiers for oil-in-water mixtures in industries such as food, pharmaceuticals, cosmetics, animal feed, and plastic products (Bešter-Rogač, 2007).

The metabolic pathway of tween 40 involves its decomposition into simpler molecules, which microorganisms can employ for energy production and growth. Microbes capable of utilizing tween 40 typically possess specific enzymes like lipases, which can cleave the ester bonds present in the molecule. Lipases are the endo enzymes that randomly break the ester bonds into fatty acids, glycerol, and other alcohols (Pathak and Navneet, 2017). Moreover, the microbial utilization of tween 40 contributes to nutrient cycling within ecosystems by releasing simpler compounds that other members of the microbial food web can assimilate. By transforming complex organic compounds into simpler forms, microbes actively participate in the carbon cycling of ecosystems, potentially influencing carbon sequestration and release dynamics. These microbial adaptations suggest an evolutionary response to environments where compounds like tween 40 are present. This adaptation can enhance their competitiveness in those environments.

The potential of microbes to metabolize complex organic compounds like tween 40 could be harnessed for pollution control and bioremediation purposes. Such microbes could aid in the degradation of these compounds within contaminated

environments, contributing to environmental remediation efforts. As Pathak and Navneet (2017) suggest, the utilization of microbial enzymes for biodegradation is a proficient approach for environmental remediation, contributing to the development of an eco-friendly environment.

At the 25 m depth group, the microbial communities displayed relatively enhanced efficiency in metabolizing the majority of the provided substrates. Even though the degradation activity on Biolog carbon sources did not directly mirror the presence of these specific compounds in the lake water, the observed higher uniformity in substrate utilization and a relatively greater affinity for organic substrates suggest the presence of a broader array of metabolic pathways. This expanded metabolic versatility might enable microbial communities to harness a diverse range of carbon sources (Cho and Kim, 2000; Melita et al., 2019).

At 25 m and 50 m depth groups, the dominant metabolic profiles after polymers were primarily centered around carbohydrates and carboxylic acids. Notably, within these depths, microbial communities exhibited a preference for glycogen as the second most utilized polymer. This observation aligns with the presence of photosynthetic Cyanobacteria at depths of 20 m and 60 m, as evidenced by the data from the August 2022 survey (refer to **Figure 3.9**). The initial metabolic products of photosynthesis in Cyanobacteria are sugar phosphates. Cyanobacteria tend to store excess photosynthates as polysaccharides (primarily glycogen) and may constitute up to 60% of the biomass (Frigaard, 2018). These compounds have the potential to serve as a nutrient source for other microorganisms within the ecosystem. This suggests that the elevated abundance of Cyanobacteria could lead to increased sugar phosphates and glycogen levels in the environment, fostering the co-occurrence and potentially synergistic interactions among microbial communities capable of utilizing these products.

Furthermore, Clade IIIb SAR11 alpha-proteobacteria, previously highlighted as abundant in the epilimnion (**Figure 3.9**), might be one of the microbial assemblages that co-occur with Cyanobacteria. As previously discussed, these SAR11 bacteria have a preference for small molecular weight compounds like carboxylic and amino acids as their carbon/energy sources, with some members having the metabolic capability to efficiently utilize glucose for both carbon and energy acquisition (Henson et al., 2018).

Glycogen, acting as a readily available glucose source and reserve of energy for various organisms, from bacteria to humans, is a complex polysaccharide consisting of glucose units interconnected in a linear structure. The accumulation of glycogen in bacterial cells typically occurs during the stationary phase or when

there is a limitation of inorganic nutrients, conditions that are likely to have been present in the deeper epilimnion (Roach, 2004). Moreover, the presence of specific functional abilities within these groups can be inferred, considering that the degradation of glycogen involves a set of extracellular enzymes (such as glycogen phosphorylases and α-glycosidases) possessed exclusively by bacteria capable of completing carbohydrate degradation processes (Henrissat et al., 2002). Glycogen phosphorylase releases a glucose unit from the polymer as a metabolite, named glucose-1-P, that can be used by the cell, whereas α-glucosidase is present in the lysosome to break glycogen down to glucose (Roach, 2004).

Carbohydrates hold significant importance as energy-rich carbon sources and function as storage molecules within aquatic bacterial metabolism (Arnosti et al., 2014). They are swiftly and preferentially processed through the catabolic heterotrophic pathway, involving the oxidation of both simple and complex carbohydrates with oxygen serving as the terminal electron acceptor (Keith and Arnosti, 2001; Rosenstock and Simon, 2003). Concurrently, carboxylic acids constitute a part of the labile organic matter pool in the aquatic environment. Carboxylic acids are a crucial carbon source for aquatic microorganisms (Obernosterer and Benner, 2004; Sala et al., 2006).

5 Conclusions

This study contributes to the essential knowledge of biodiversity conservation, basis for the implementation of nature-based solutions for ecohydrological approaches to augment ecosystem resilience, support the growth of microbial-based biotechnology for sustainable industrial applications, and ultimately, to encourage investments from environmental, social, and corporate governance initiatives towards the realization of the UN Sustainable Development Goals (primarily SDG 13: Climate Action and SDG 14: Life Below Water). Based on the findings presented in this study, the following conclusions can be drawn:

1. The composition and isotopic features of CO_2 and CH_4 forming gas reservoirs within Lake Albano are governed by different biogeochemical processes and hold particular importance for potential rollover events. These biogeochemical processes are intimately related to the development of coexisting and competitive microbial populations that depend on physicochemical conditions along the vertical water columns.
2. In the epilimnion, both CO_2 and CH_4 diffusing from the deeper water layers are rapidly consumed by photosynthesis and oxidation processes, and dispersed into the atmosphere by periodic water circulation. Moreover, the balance between CO_2 and CH_4 production and consumption, driven by contrasting microbial activities, governs the gradient of metabolic diversity within anaerobic water layers. This concept highlights that inputs of CO_2 from hydrothermal/magmatic systems do not correspond to equivalent CO_2 outputs from the lake surface, as this gas is primarily utilized by microbiota and involved in physicochemical reactions within the lake. Lake Albano, and potentially similar bio-active volcanic lakes globally, functions as a CO_2 sink, significantly influencing local carbon budgets.
3. The CO_2 and CH_4 dynamics within the lake also indicate that biological processes tend to stabilize lake stratification. However, during volcanic unrest, deep-origin gases could oversaturate the hypolimnion, leading to gas outbursts tied to volcanic events. Continuous anthropogenic stressors might also destabilize stratification and CO_2 degassing dynamics over the long term.

4. The study observed that water depth is closely linked to the fluctuations in microbial community structure and physiology characteristics within Lake Albano, influencing the functional aspects of microbial communities and consequently affecting biogeochemical cycling patterns.
5. The study emphasizes the need for more extensive research on the nuances of global environmental microbial ecology and how changes in microbial community structures affect ecosystem functions.
6. For future research, it is suggested to do microbial surveys in extreme environments, regions where ecological interactions have been underexplored, or where massive shifts are expected to occur to get a better grasp of the status of global microbial populations.

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A Appendix A

A.1 Vertical Physicochemical Profiles of Lake Albano

Table A.1: Vertical physicochemical profiles along the Lake Albano water column from the first sampling campaign. Water temperature (T in °C), pH, electrical conductivity (EC, in mS/cm), and dissolved oxygen (DO in mg/L).

depth	T	pH	EC	DO
0	26.8	8.9	0.443	9.04
-10	26.5	8.8	0.442	9.1
-20	16.2	8.1	0.460	10.5
-30	10.0	7.8	0.486	4.6
-40	9.6	7.9	0.486	3.5
-50	9.6	7.8	0.486	2.6
-60	9.6	7.6	0.484	0.7
-70	9.6	7.5	0.488	0.0
-80	9.5	7.5	0.489	0.0
-90	9.4	7.4	0.504	0.0
-100	9.4	7.3	0.513	0.0
-110	9.4	7.6	0.520	0.0
-120	9.4	7.5	0.522	0.0
-130	9.4	7.5	0.523	0.0
-140	9.4	7.5	0.524	0.0
-150	9.4	7.4	0.524	0.0
-160	9.4	7.4	0.524	0.0

A.2 DAPI-staining and CARD-FISH Visualization

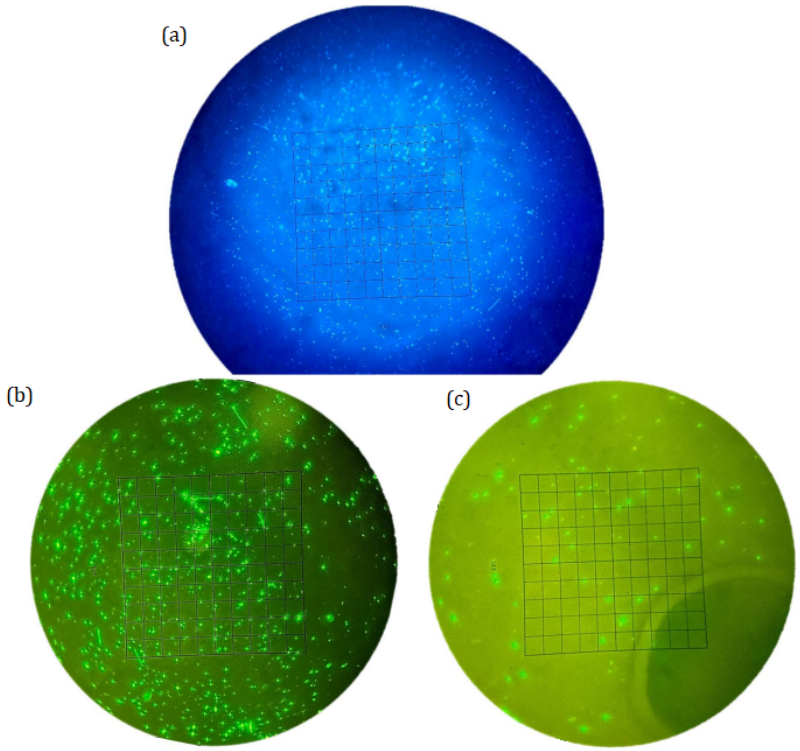

Figure A.1: Microbial cell visualization using DAPI staining and CARD-FISH observed under a microscope at 1000x magnification (grid size = 0.1 x 0.1 mm). a) microbial cells stained with DAPI; b) bacterial cells targeted by CARD-FISH probe EUBI-III; and c) archaeal cells targeted by CARD-FISH probe ARCH 915.