DOCTORAL THESIS

ANALYSES OF PROKARYOTIC COMMUNITIES IN LAKES NYOS AND MONOUN (CAMEROON) AND BACTERIOLOGICAL ASSESSMENT OF GROUNDWATER QUALITY IN THE VICINITIES OF BOTH LAKES

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Dedicated

To the memory of the victims of the Lakes Nyos and Monoun gas disasters

“The role of the infinitely small in nature is infinitely great.”

-Louis Pasteur-

View of Lake Nyos, 2013. Photo courtesy of Yutaka Yoshida

CONTENTS

STATEMENT

This thesis is based on three scientific papers, which were published and/or are under preparation. They are referred to in the text by Roman numerals (I, II and III).

I planned the three works, advised by Akira Ueda and Shogo Nakamura. I did all samplings, assisted by Wilson Fantong Yetoh, Gregory Tanyileke, Victor Hell, Minoru Kusakabe, Takeshi Ohba and Kamtchueng Tchakam. I did the molecular analyses work for papers I and II in the Biosphere function laboratory III of University of Toyama, assisted by Akihiro Nakamura, Akihiro Sakatoku and Daisuke Tanaka. The physico-chemical parameters analyses of lake water were done by Takeshi Ohba of the Laboratory of Volcanology and Geochemistry of University of Tokai. Bacteriological analyses of drinking water were conducted by the Laboratory of soil and environmental chemistry of University of Dschang. I wrote all papers, then finalized together with all the co-authors.

The journal papers generated, conferences and workshops attended in the framework of this study are listed at the end of the thesis.

Abstract

ABSTRACT

Lakes Nyos and Monoun intriguing ecosystems have been monitored for the last three decades following their CO_2 gas explosions. Despite the important role that prokaryotes play in lakes' nutrient cycles, their community composition have not been investigated in both lakes. Equally, the microbial quality of groundwater, which is a very important commodity for people living in the vicinities of both lakes have not been assessed heretofore. The lack of knowledge regarding the two latter fields weakens the understanding of the lakes' biogeochemistry and the awareness of the general issues related to water quality.

This work consists of three parts. Parts one and two focus on the study of bacterial and archaeal community composition of Lakes Nyos (I) and Monoun (II). The study used Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE), a profiling technique, as well as cloning/sequencing of 16S rRNA genes, on both lakes samples. Furthermore, quantitative analysis (qPCR) was performed on Lake Monoun's samples to assess the prokaryotic abundance. The results of prokaryotes community studies on both lakes revealed that bacterial and archaeal communities are distributed along the water column following a stratified pattern in both lakes, with a rich microbial diversity likely to include several novel microorganisms. Physico-chemical data and microbial sequences suggested a close correspondence of the potential microbial functions to the physico-chemical pattern of the lakes. However, it appeared that prokaryotic communities markedly differed between both Lakes.

Community members in Lake Nyos were represented by the potential bacterial phyla Actinobacteria (28.3%), Firmicutes (19.6%), Proteobacteria (15.2%), Candidate divisions (TM7, OP8, DO1) (10.9%), Bacteroidetes (8.7%), Chlorobi and Caldiserica (6.5% each) and

Fusobacteria and Cyanobacteria (2.2% each). The phyla Thaumacheota was the only (100%) potential representant of archaeal communities.

For Lake Monoun, the retrieved sequences were distributed into six phyla for bacteria. Sequences matching with bacteria of the Proteobacteria group dominated (63.7%), followed by Chloroflexi and Spirochaetes (12.1% each), then Firmicutes (6.0%), and finally Bacteroidetes (3.0%) and Nitrospirae (3.0%). As for sequences obtained with the archaeal primer, they were distributed into the phyla Euryarchaeota (23.3%) and Thaumarchaeota (20.0%). The highest (56.7%) proportion did not match with known archaea and was termed as unclassified. Absolute quantitative PCR of the 16S rRNA gene for bacteria and archaea showed that bacteria were numerically more important than archaea in all the samples and that bacterial abundance decreases while archaeal abundance increases with depth.

In the third part (III), the study focused on identifying biological indicators of pollution in groundwater sources in the vicinities of Lakes Nyos and Monoun. Culture methods were used to detect and count total coliforms, fecal coliforms and fecal streptococci in water samples. Results revealed the presence of the three bacterial indicators in nearly all the water sources. In the Lake Monoun area, all the nineteen analysed samples contained total coliforms. Then, 16 (84.2%) and 17 (89.5%) samples contained faecal coliforms and faecal streptococci, respectively. All the seventeen samples collected around Lake Nyos contained total and faecal coliforms, and 16 (94.1%) samples contained faecal streptococci. The high bacterial counts raise a concern on the suitability of those water sources which are used for drinking and other domestic purposes. This study provides evidence of fecal contamination in most of the water sources in both areas, implying health threat to consumers and suggesting the necessity to improve attention to environmental sanitation control.

The work presented in this thesis is a first approach in studying the microbial diversity in Lakes Nyos and Monoun and in assessing bacterial quality of groundwater in the vicinities of both lakes. It brings light on two important aspects, namely: on one hand, the knowledge of community composition and diversity, which increase our understanding of the plausible biogeochemical functioning of both lakes, and which can be used for monitoring and predicting environmental change and guide for further studies in microbial ecology. On the other hand, the detected bacterial indicators give a snapshot of the status of the water resource in the vicinities of the lakes. Snapshot which can be used as a guide to water management, and to predicting the presence of certain pathogens, and therefore, protecting local people's health.

Keywords: CO_2-rich lake, bacterial and archaeal communities, microbial diversity, biogeochemistry, bacteriological pollution indicators

要 旨 (ABSTRACT IN JAPANESE)

ニオス湖とマヌーン湖は, 興味深い生態系を有している湖で, 二酸化炭素のガス爆発から約30年間, モニタリングがされている。原核生物は, 湖の栄養塩の循環において重要な役割を果たしているにもかかわらず, 両湖水中のそれらの群集構造は研究調査されていない。同様に, 両湖の近隣で生活する人々にとって大変重要な生活必需品である地下水の微生物学的質についても, これまでに評価されていない。これら2つの情報の欠乏は, 両湖の生物地球化学的な理解や水質に関する一般的な問題の認識を低下させる。

本研究は3つのパートから構成されている。パート1と2では, ニオス湖とマヌーン湖における細菌と古細菌の群集構造についての研究に焦点を合わせた。その研究では, 両湖の試料について, プロファイリング技術であるPolymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE) 法と, 16S rRNA遺伝子のクローニングとシークエンス方法を用いた。さらに, 原核生物の存在量を評価するために, マヌーン湖の試料の定量解析 (qPCR) を行った。両湖の原核生物の群集構造を調べた結果は, 両湖の層状パターンに応じた水柱に沿って細菌と古細菌が分布していることを示し, いくつかの新規な微生物を含みながら微生物の多様性に富んでいることが明らかとなった。物理化学的データと微生物の塩基配列の解析は, 微生物の潜在的機能と湖の物理化学的パターンとが密に対応していることを示唆した。しかしながら, 両湖水間の原核生物の群集構造は著しく異なっていることが示された。

ニオス湖の群集において推定された細菌門は, Actinobacteria (28.3%), Firmicutes (19.6%), Proteobacteria (15.2%), Candidate divisions (10.9%), Bacteroidetes (8.7%) とChlorobi

とCaldiserica (それぞれ6.5%), Fusobacteria と Cyanobacteria (それぞれ2.2%) だった。Thaumacheota門が古細菌群集の唯一 (100%) の推定代表者であった。

マヌーン湖では, 読みだされた塩基配列は, バクテリアに関する6つの門に分布した。合致した細菌は, Proteobacteriaが優勢で (38.5%), 次いでChloroflexiとSpirochaetes (それぞれ12.1%), Firmicutes (6.0%), そしてBacteroidetesとNitrospirae (それぞれ3.0%) であった。古細菌のプライマーで得られた塩基配列に関しては, Euryarchaeota (23.3%), Thaumarchaeota (20.0%) に分布した。既知の古細菌と一致しないものが最も多く (56.7%), unclassifiedと名付けた。細菌と古細菌の16S rRNA遺伝子の絶対定量PCRでは, 全試料において細菌の方が古細菌に比べて数的に多く, 深さに応じて細菌の存在量は減少していく一方で, 古細菌の存在量は増加していくことが示された。

パート3は, ニオス湖とマヌーン湖周辺の地下水源中の汚染の生物学的指標の同定に焦点を合わせた研究である。試料水中の全大腸菌群数と糞便大腸菌群数, 糞便連鎖球菌数を計測するために, 培養法が用いられた。結果は, ほとんど全ての水試料にこれらの3種の指標細菌の存在が示された。マヌーン湖地域では, 19の分析された全ての試料で全大腸菌群を含んでいた。そして, 16試料 (84.2%) と17試料 (89.5%) で糞便大腸菌群と糞便連鎖球菌をそれぞれ含んでいた。ニオス湖周辺で採集した17試料全てで全大腸菌群と糞便性大腸菌群を, 16試料 (94.1%) で糞便性連鎖球菌を含んでいた。高い細菌計数は, それらの水源を飲料水や他の家庭生活用水に適用することに懸念を生じる。本研究は, 両地域におけるほとんどの水源の糞便汚染の証拠を提供するとともに, 消費者の健康を脅かす

恐れがあることを暗示しており, 環境衛生管理への意識改革が必要であることを示唆している。

本博士論文において示された研究は, ニオス湖とマヌーン湖における微生物の多様性の研究と両湖の周辺地下水の細菌学的質の評価において最初の取り組みである。それは, 2つの重要な面に光を投げかけている, すなわち1つは, 微生物の群集構造と多様性についての知見で, それは両湖の妥当と思われる生物地球化学的機能について我々の理解を強めることと, そして, 環境変化のモニタリングと予測に使用でき, 微生物生態学の今後の研究の指針となることである。一方, 検出された指標細菌は, 両湖周辺の水源の状況を評価できる。それは, 水管理の指針や病原菌の存在予測に利用でき, さらには, 地域の人々の健康管理に繋がるだろう。

ABBREVIATIONS

bp: base pair

c.f.u.: Colony Forming Unit

DGGE: Denaturing Gradient Gel Electrophoresis

DNA: Deoxyribo Nucleic Acid

EDTA: Ethylene Diamine Tetraacetic Acid

FC: Fecal Coliform

OTU: Operational Taxonomic Unit

PCR: Polymerase Chain Reaction

qPCR: quantitative Polymerase Chain Reaction

rRNA: ribosomal Ribo Nucleic Acid

TC: Total Coliform

TS: Total Streptococci

UNICEF: United Nations Children's Fund

WHO: World Healh Organisation

GLOSSARY OF TERMS

Archaea (Archaebacteria): Single-celled microorganisms, having no nucleus, distinguished from bacteria on molecular phylogenetic grounds and often found in hostile environments

Bacteria (Eubacteria): Microorganisms, having no nucleus that can exist either as independent (free-living) organisms or as parasites (dependent on another organism for life). They are comprised of either beneficial, spoilage or disease-causing members

Biogeochemistry: Scientific discipline that involves the study of the chemical, physical, geological, and biological processes and reactions that govern the composition of the natural environment

Fingerpinting technique: Technique used for comparing the nucleotide sequences of fragments of DNA from different sources

Gel electrophoresis: Method used to separate mixtures of DNA, RNA, or proteins

Indicator organism: Microorganism which fulfill a set of criteria that suggest the presence of other organisms especially pathogens

Metabolism: Set of life-sustaining chemical transformations within the cells of living organisms

Microbial diversity: Spectrum of variability among all types of microorganism in a given group

Pathogen: Microorganism that causes disease

Potable water: Drinking water that is safe and wholesome for human consumption

Prokaryote: Microorganisms (unicellular or group of cells) that lack a distinct nucleus and membrane-bound organelles and that are classified into two domains (Bacteria and Archaea)

The three-domain system: Biological classification that divides cellular life forms into three groups. Each domain constitutes a taxonomic rank

Chapter 1

1. INTRODUCTION

1.1. Meromictic lakes and prokaryotes

Lakes which the water body does not mix up throughout the entire water column are called meromictic lakes (Hutchinson, 1975; Wetzel, 2001), as a divergence to the holomictic lakes, which mix to the bottom at some time during the year. Meromictic lakes are usually separated into layers, therefore exhibiting a stratified pattern due to high density difference. The stratification is set up by chemical or physical gradients as discovered and described for the first time by Findenegg (1935) in Austria, Yoshimura (1936) in Japan and Hutchinson (1937) in the USA. Circulation can however occur in a restricted layer when the mixing forces are greater than the stabilizing forces. The bottom layer is known as the monimolimnion, where the waters circulate little. The uppermost layer is known as the mixolimnion, that can be divided into three parts (epilimnion, metalimnion and hypolimnion) and that acts as an indivual lake. The transition zone between layers is referred to as the chemocline (Hutchinson 1937, Walker, 1974). Meromictic lakes are widely distributed throughout the world, but remain

comparatively rare based on the actual estimation that only one lake in 800 is meromictic (Hakala et al., 2004). Moreover, few can be termed as the "unusual among the unusual". The high CO_2 concentration and strongly stratified water columns of Lakes Nyos and Monoun make them some of those unusual and noteworthy lakes. Hence, they have captivated researchers for decades. Numerous works enhanced our understanding of their physico-chemical aspects which led to mitigate other hazards associated with the lakes. Such unique lacustrine environments deserve special attention in terms of microbial ecology, as they represent useful biogeochemical models.

Microbes are essential drivers of environmental processes which are interlinked in a way that they affect environmental conditions and allow the continuous survival of ecosystems. Hence, they are believed to be of fundamental importance for the understanding of processes regulating biogeochemical cycles in various environments. The recent increased interest in microbes led to exciting progress in the development of new technologies for studying them. Nowadays, the methods used in microbial ecology have progressed from standard culture techniques to modern molecular biological techniques. These molecular biological methods are based on the 16S (18S) rRNA genes and are needed to study several candidate divisions. Indeed, barely 1% of the total number of prokaryotic species present in a given sample can be cultivated *in vitro* (Rastogi et al., 2011). Hence, molecular techniques are crucial for an intensive characterisation of microbial communities in a given environment. DGGE is one of the numerous fingerprinting approaches that have been designed to study microbial communities (Muyzer et al., 1993). The advent of molecular taxonomy (based on the 16S rRNA) has shed a light on the comprehension of the domains of life. The living world was then divided into three domains (Figure 1), Eukarya, Bacteria and Archaea. Bacteria and Archaea constitute the Prokaryotes group, whose members possess remarkable metabolic abilities. They

are the main players in the biogeochemical carbon cycling in aquatic ecosystems, *via* various metabolic mechanisms among which CO_2 fixation.

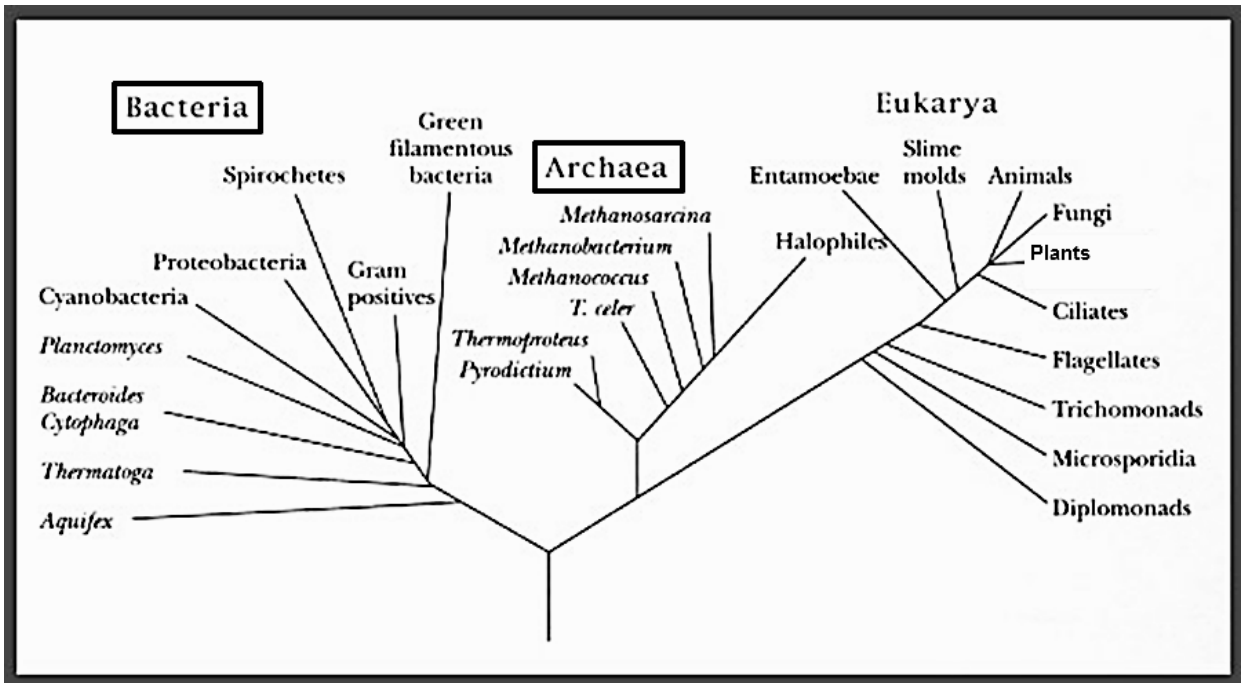

Figure 1: The three domains of life illustrated in a speculative tree for rRNA genes, showing major branches Bacteria, Archaea, and Eukarya (Woese, 1977).

Bacteria drive the transformations and cycling of most biological elements in freshwater ecosystems (Newton et al., 2011) where they contribute to the cycles of nutrients and carbon in two major ways: by producing new bacterial biomass and by re-mineralizing organic carbon and nutrients (del Giorgio and Cole, 1998). All these mechanisms are driven under the life phenomenon of bacterial respiration, resulting in profound effects on the overall carbon and gas balance in aquatic ecosystems (del Giorgio and Cole 1998; Peura et al., 2012). In fact, a combination of biochemistry and genome sequence analysis is revealing that respiration amongst the Bacteria and Archaea can be found at its most extreme flexibility. They are able to utilise a

diverse range of electron acceptors including substrates such as sulfur, nitrogen, metals such as Fe (III) and Mn (IV), among others (Richardson, 1999). They undergo oxic and anoxic respiration. In anoxic conditions of the hypolimnion and monimolimnion, one of the end products of microbial metabolism is CH_4 along with CO_2. The produced CH_4 may then be oxidised by methane-oxidising bacteria (methanotrophs) or emitted to the atmosphere (Peura et al., 2012). On the other hand, methanogenic archaea can utilise CO_2, acetate or methanol as electron acceptors, with H_2 as the electron donor, in the anaerobic process of methanogenesis (Richardson, 1999). It therefore comes out that the field of bacterial respiration is challenging to scientists working in the environmental field. However, experiments to assess and characterise the prokaryotic community in the unique environments of Lakes Nyos and Monoun have not been carried heretofore.

1.2. Bacterial pollutants in drinking water

Bacterial pollutants in water are a constant threat to humans. The WHO estimates that about 1.1 billion people globally drink unsafe water (Kindhauser, 2003), which is often contaminated with faecal material. Swallowing faecal material contaminated water results in an increased risk of transmission of disease. It is estimated that unsafe water is the cause of 88% cases of diarrhoeal diseases in the world (WHO, 2003a). This issue is particularly significant in developing countries where there is a higher rate of endemic gastrointestinal diseases and pathogens concentrations in wastewater (Martins et al., 1983; Jimenez et al., 2002). Sub-Saharan Africa is ranked as the slowest part of the world in achieving improved sanitation given the low proportion of resident (31% in 2006) having access to improved sanitation (UNICEF-WHO 2008). Cameroon has been fully engaged with the Millennium Development Goals (MDGs) and the proportion of its population

having access to drinking water increased from 50.5% in 2001 to 70% (mostly in urban areas) in 2006 (Ako et al., 2010). This indicates not only an improvement of the sanitary conditions, but dependence of a significant proportion (about 30%) of the population of urban and rural areas on doubtful water sources such as rivers, springs and wells (Njiné et al., 2001; Katte et al., 2003; Ndjama et al., 2008; Ako et al., 2009; Temgoua, 2011; Ateba et al., 2012; Wirmvem et al., 2013) for their multipurpose daily uses. In fact, tap water in Cameroon is a luxury that only few people can afford when available, and that does not exist at all in numerous rural and suburban areas. Availability of drinking water therefore remains a great challenge, in spite of the abundant water resource in Cameroon (Ndah and Xue, 2010).

In Lakes Nyos and Monoun vicinities (as in many other areas of Cameroon), access to a reliable drinking water source is still a matter of serious concern. Such concern has not been ignored by the SATREPS-NyMo project (http://www.jst.go.jp/global/english/kadai/h2214_cameroun.html), which main objective is to make the Lakes Nyos and Monoun areas safe for populations through degassing. Beyond protecting the surrounding populations from gas exhalations, it also aims at achieving better living conditions, which include a better access to potable water. Fulfilling such goals entails assessing the quality of water sources used by people.

1.3. Aims

The main objectives of this thesis were to characterise the microbial communities in Lakes Nyos and Monoun water columns at different depths using PCR-DGGE, and assess the bacteriological quality of drinking water in the vicinities of both lakes. Specifically, it involved the following aspects:

1- Characterisation of the prokaryotic community pattern and diversity, using the PCR-DGGE of the 16S rRNA gene and sequencing techniques

2- Analysis of the prokaryotes DNA sequences in order to predict their plausible functions and their putative effect on the geochemistry of the lakes, with a focus on methane related species

3- Assessment of water quality in the vicinities of Lakes Nyos and Monoun using bacterial indicators of pollution.

Chapter 2

2 MATERIALS AND METHODS

2.1 Description of sampling sites

2.1.1 Description of Lakes Nyos (I) and Monoun (II)

Lakes Nyos and Monoun are volcanic crater lakes located on the Cameroon volcanic line (CVL) at N06°26'23 in the Northwest and E10°18'02.3 in the West, respectively. The CVL extends for nearly 1500 km from the Gulf of Guinea islands through southwestern Cameroon and into northern Nigeria and northern Cameroun (Freetz and rex, 2000). Both lakes are CO_2-rich meromictic lakes. That is, they have CO_2-rich bottoms and stratified water columns. The stratification is due to differences in physico-chemical parameters, mainly the CO_2 concentrations. Toxic gas was released from the water of both lakes on August 15, 1984 for Lake Monoun and August 21, 1986 for Lake Nyos. Both gas disasters claimed numerous lives estimated at about 37 people at Lake Monoun, 1800 people and 3000 cattle at Lake Nyos (Sigdursson et al. 1987; Sigvaldason et al. 1989). Post disasters monitoring of lakes indicated that CO_2 in the lakes was increasing speedily. Scientists therefore came to the agreement that the toxic gas was the accumulated CO_2 that have been released at the surface upon overturning of the bottom waters. Indeed, CO_2 continuously seeps from a pool of magma that resides beneath the lakes and accumulates in their bottom waters.

However, the mechanisms that triggered the water's overturn are still a matter of debate. Three possible theories have been suggested to explain the overturn: the theory of Kanari (1989) hypothesising a small confined area of the lake that released the gas allowing a permanent stratification of Lake Nyos; the theory of Kling (1989) describing a heat influx into the system causing instability; and the theory of a landslide within the lake that would have caused the displacement of the bottom CO_2-saturated waters. In order to prevent recurrence of gas explosions, degassing pipes were installed to suck out the CO_2 from the bottom water layers to the surface. This was done under the Nyos-Monoun Degassing Program launched in 1996 (Minoru, 2015).

Lake Nyos is a circular maar, approximately 210 m deep (Figure 2A) with a surface area of 1.58 km^2 (Nagao et al., 2010). Its column can be divided into three sections separated from each other by an upper and a lower chemocline (Figure 3A). The first layer (epilimnion) extends between 0 and -55 m where water is convectively mixed annually during the dry season. This layer has a low CO_2 concentration and conductivity, due to the exchange with atmosphere and the dilution by rainwater. The second layer (metalimnion) extends from -55 m to -180 m and the third layer (hypolimnion) from -180 m to -200 m. A fourth layer (monimolimnion) is found from -200 m to the bottom. Initially one pipe was installed in Lake Nyos in 2001, and two additional pipes in 2011. Tilapia was introduced in the lake in the years 1990, but its culture did not thrive.

Lake Monoun has a surface area of 0.31 km^2 and a maximum depth of 100 m (Kling et al., 2005; Kusakabe et al., 2008). Based on physico-chemical parameters, three basins were identified, of which two (main and west basins) are shown on the bathymetry (Figure 2B). The Panke River inflowing from the north recharges the upper layer at -15 m. The basins depths are -98.5 and -55 m for the main and central basins, respectively. The highest amount of CO_2 is contained in the

main basin and was estimated to be approximately 330 megamoles soon after the 1984 gas eruptions

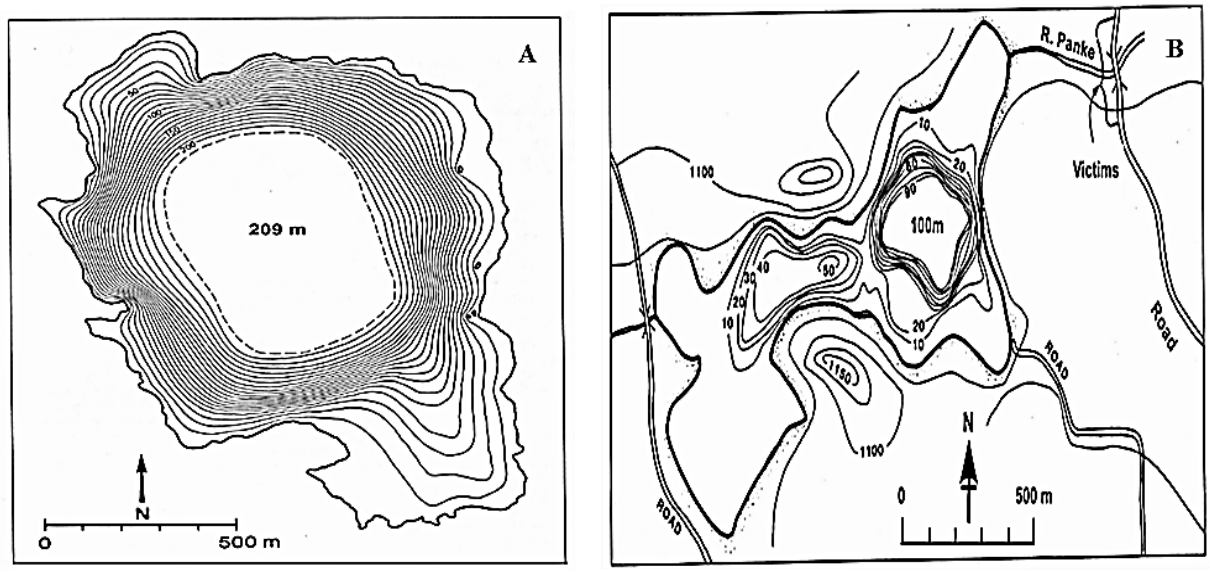

Figure 2: Bathymetry of Lakes Nyos (A) and Monoun (B).

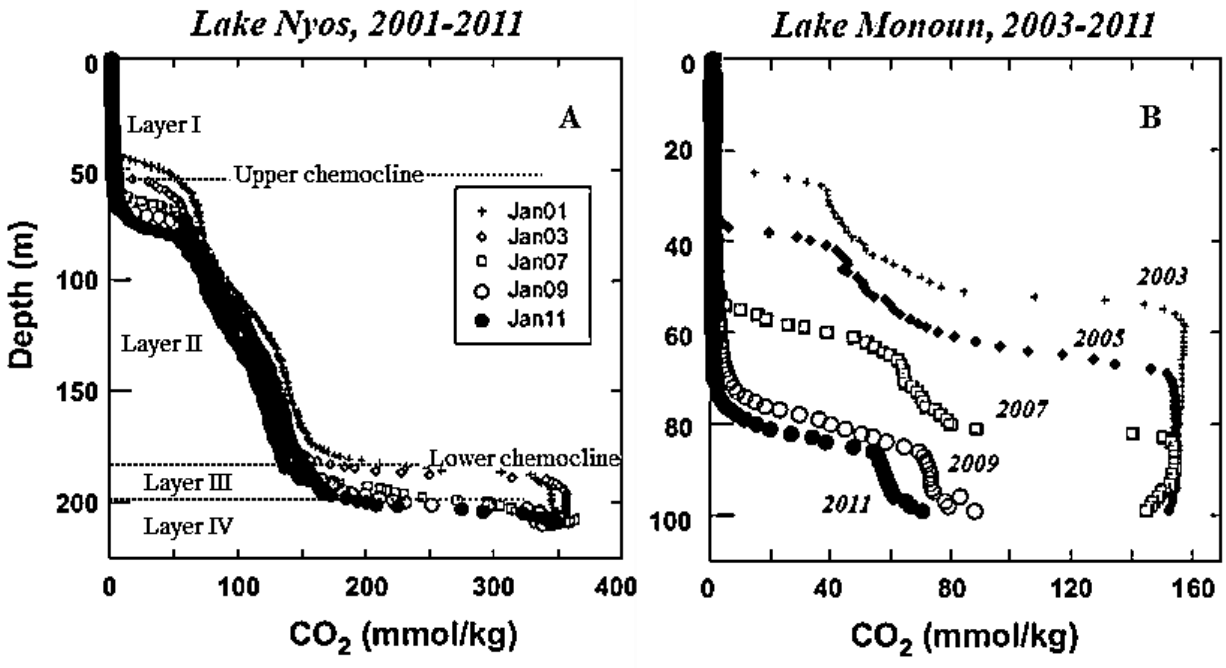

Figure 3: Lakes Nyos (A) and Monoun (B) stratification pattern evolution until 2011 (Minoru, 2015).

A degassing pipe was installed in the main basin in February 2003, then two additional pipes installed in February 2006 to increase the gas removal rate. As it can be seen on Figure 3B, the upper chemocline has shifted from -25 m (in 2003) to -180 m (in 2011). Methane is also found in the lake in a marginal proportion (~2.2 mol %) of the total gas. However, its concentrations were reported to increase with time and may account for up to 37% and 23% to the total dissolved gas pressure and saturation level, respectively (Issa et al., 2013). The population around the lake has increased and own croplands very close to the lake shore. A local fishery is currently carried out at shallow depths of the lake and could importantly affect the lake's internal food web. Regrettably, no data are available on the nutrients content of the lake. Though, such important anthropogenic activities might contribute to increasing the organic carbon budget through internal organic deposits from fishery and catchment runoff.

Degassing was effective in both lakes. Degassing in Lake Nyos is still going on and 33% of the maximum amount of CO_2 (observed in 2003) has been removed (Minoru, 2015). In Lake Monoun, more than 90% of the maximum CO_2 value observed in 2003 was removed by 2011. The drastic reduction of CO_2 was materialized by the loss of the pipes' self-lifting capacity. However, based on the previous observations suggesting a permanent magmatic CO_2 recharge of the lakes, it is essential to keep monitoring them and removing the CO_2 to prevent gas re-buildup.

2.1.2 Groundwater sampling sites in the vicinities of Lakes Nyos and Monoun (III)

Water sources for various purposes, including drinking were sampled from wells, springs, and streams.

The Nyos area is located in the north-west region of Cameroon (Figure 4A), covered by a grassy vegetation and a humid tropical equatorial climate. Climate is close to that of the Ndop plain, the nearest area to Nyos with available climate data. It has a long rainy season (mid-March to mid-November) and a short dry season (mid-November to mid-March) (Molua and Lambi, 2006; Wirmvem et al., 2013). Annual temperature and rainfall ranges are from 21.7 to 22.5°C and 1,000 to 2,000 mm, respectively (Ndzeidze, 2008; Wirmvem et al., 2013). The Basement Complex around Nyos consists of slightly foliated granitic rocks (Freeth and Rex, 2000). Survivors of Lake Nyos catastrophe were housed in refugees' camps just after the disaster, while the government of Cameroon is preparing their resettlement. However, some of the survivors have chosen to join their homeland after the disaster. The majority of the population (82.0%) depends solely on smallholder subsistence agriculture for their livelihoods (Balgah and Buchenrieder, 2014). Among them, 55.4% are farmers, followed by grazers (33.8%) (Bang, 2008). Animals are not penned and are voided directly onto pasture for grazing or streams for watering. They therefore defecate directly on those sites or in streams and rivers. The deposited faeces probably pollute the water, and cause disease following consequent use by inhabitants. In fact, gastro-intestinal diseases are recurrent in the area. Statistics obtained from the health center at Nyos indicated a high prevalence of intestinal parasitic diseases. Out of 15 patients, about 4 (26.7%) complain of a gastro-intestinal disease, including acute intestinal amebiasis, intestinal parasitosis, salmonellosis, acute enteritis, intestinal helminthiasis, gastroenteritis and abdominal pains accompanied by diarrhea (Health Center's records).

The Monoun area is located in the west region of Cameroon (Figure 4B), a mountainous area with a shrub land and an equatorial climate. The mean temperature is around 23°C. It has an annual average rainfall of about 1,900 mm (from March to November). Ferralitic soils cover the

area. Basaltic and gabbroic lavas and pyroclastic materials overlay the crystalline basement that is mainly made of granite and gneiss. Several streams of the plateau discharge into the Noun River that flows substantially North-South and drains the area (Segalen, 1967). Three decades after Lake Monoun disaster, its vicinities have evolved to a sub-urban area, with a large population. The Foumbot town alone had ca 80,000 inhabitants in 2001 (Uwizeyimana and Uginet, 2003). Farming represents the main activity with an important production of vegetable crops in Foumbot (close to Monoun) supplying the major cities of Cameroon and some neighbouring countries (Fotio et al., 2013). The Foumbot town is well known to have recurrent epidemics of cholera since the first reported outbreak in 1997. The important number of infected persons in Foumbot during the 2004, 2010, 2011 epidemics and the recurrence of outbreaks made the town one of the foci of cholera in Cameroon (Red Cross information Bulletin, 2004; International Federation of Red Cross and Red Crescent Societies, 2012).

2.2 Sampling

2.2.1 Lake water sampling and processing (I, II)

A 1.6 l Niskin bottle was used to collect water samples along a vertical transect at the end of the dry season (March 2013) for both lakes. At Lake Nyos, water samples were collected at the center of the water column at depths: 0, 10, 25, 35, 45, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200 and 210 m. A sediment sample was also collected at -210 m. At Lake Monoun, they were collected at the center of the central basin at depths 0, 15, 30, 45, 55 m and main basin at depths 0, 15, 30, 40, 75, 98.5 m. Each sample was transferred to a 1 l sterile polypropylene bottle and immediately kept in an ice-cooled box. Subsequently, they were vacuum filtered with a 0.22 µm membrane

filter, then stored frozen until DNA extraction. Simultaneously with the water sampling, a profile of the water column was done using a conductivity-temperature-depth (CTD, Ocean Seven 316, Idronaut, Italy) profiler fitted with sensors to measure the conductivity, temperature, pressure (which is later converted to depth), pH, dissolved oxygen and redox potential.

2.2.2 Groundwater sampling (III)

Sampling of the water sources took place during the rainy season, on late October 2013. The sampling points, including springs, streams, shallow and deep wells around Lakes Nyos and Monoun were selected prospectively on assistance of a residents' representative and are shown on Figures 4A and 4B. Seventeen points were sampled around Lake Nyos (in Nyos, Cha, and Bwabwa) and nineteen points were sampled around Lake Monoun (in Njindou, Nkoup, Kouomboum and Foumbot town). The samples were collected in sterile polypropylene bottles, kept ice-cooled during the sampling, transported to the laboratory within18 hours after the sample collection and bacterial culture done immediately. Maps showing the sampling locations were generated using Adobe Illustrator CS2 Software from 1/50,000 Topographic Maps Bafoussam 4a (Institut Geographique national 1970) for Lake Monoun, and 1/50,000 Cameroon special edition map (Defense Mapping Agency 1986) for Lake Nyos.

2.3 Analysis of prokaryotic community composition

2.3.1 DNA extraction and PCR-DGGE (I, II)

Total DNA was extracted from the membrane filters using a soil sample DNA extraction kit (UltraClean® Soil DNA Isolation Kit; Mo Bio Laboratories, CA, USA) and following the manufacturer's instructions. The genomic DNA was then quantified with a spectrophotometer (Nanodrop 1000 Spectrophotometer; Thermo Fisher Scientific K.K., Kanagawa, Japan) and stored at -20°C for subsequent use.

For Lake Nyos, bacterial 16S rRNA gene's amplicon were generated using the primers GC-341f and 518r (Muyzer et al., 1993) in a simple PCR (Tables A1 and A2). Archaeal 16S rRNA was amplified in a nested nested PCR, which first round used the primers 20f and 958r and second round the inner primers GC-340f and 519r (Nam et al., 2008) (Tables A1 and A2).

For Lake Monoun samples, a nested PCR was used to amplify both bacterial and archaeal 16S rRNA genes. For bacteria, the primers set GM3f and GM4r (Muyzer et al., 1995) was used in the first round of PCR and, the inner primers GC-341f and 518r in the second round (Tables A1 and A2). Archaeal 16S rRNA gene's amplicon were generated using the primers set 109f and Uni1492r in the first round of PCR and the inner primers 109(T)f and GC-515r (Sousa et al., 2007) in the second round (Tables A1 and A2).

All the PCR were ran in a thermal Cycler Dice (Takara PCR Thermal Cycler Dice, Takara Bio Inc.) following the programs indicated in Table A2. Visualization of the amplified DNA was performed by electrophoresis of 2 µl of PCR products in 2% agarose gel in TAE buffer (40 mM

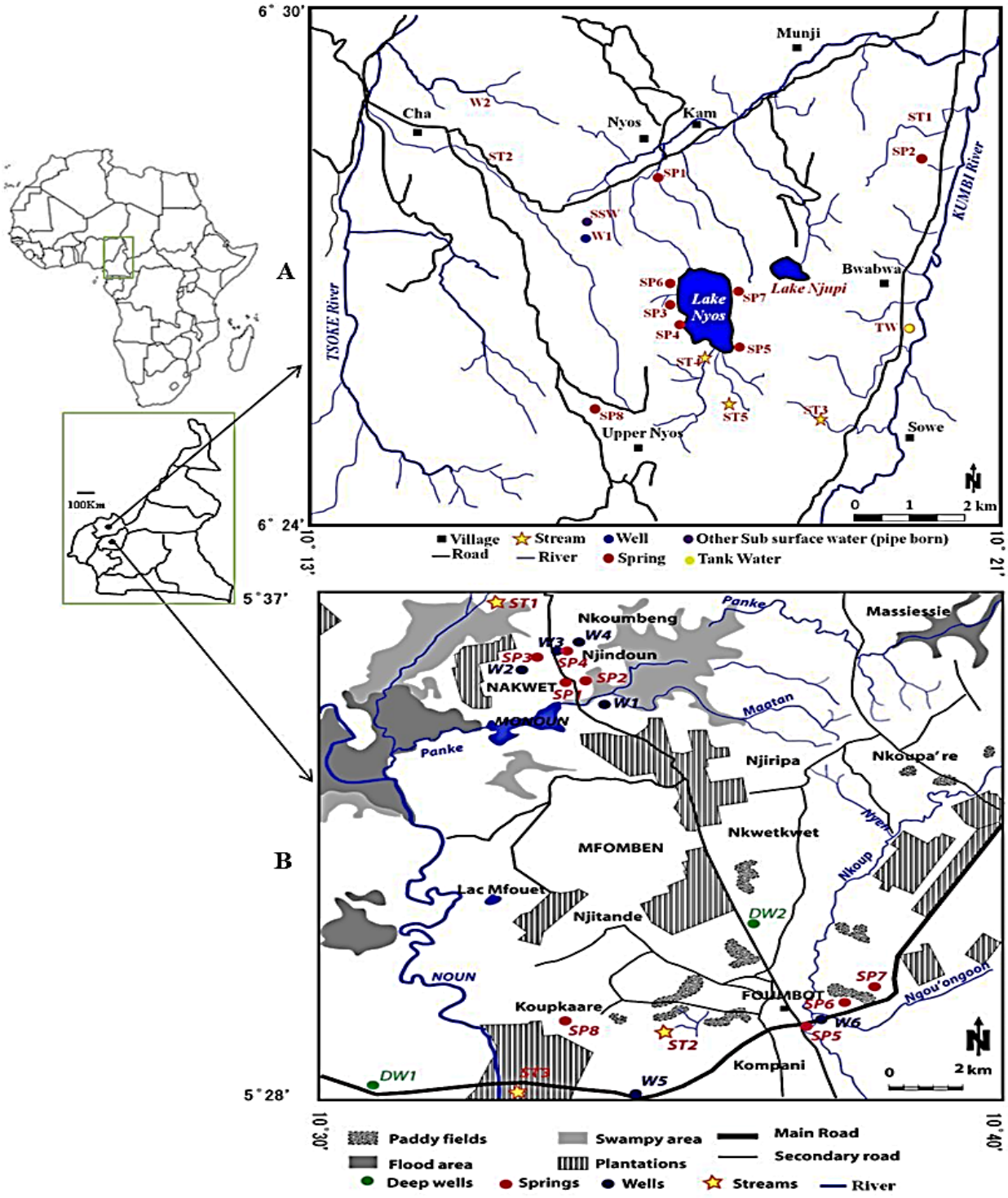

Figure 4: Location of the study sites around Lake Nyos (A) and Lake Monoun (B). Stars and dots indicate the sampling points named as follows: DW (Deep well), SP (spring), ST (Stream), W (Well). Names of localities are written on the map. The main rivers are represented by blue lines and the main roads by black lines. Land use and flood or swampy areas are indicated by shapes fills.

Tris-acetate, 1 mM EDTA, pH 8) with a DNA Mass Ladder Standard (Nippon Gene, Tokyo, Japan).

The samples were analyzed by DGGE with a Dcode^{TM} Population Base System (Bio-Rad, Laboratories, CA, USA). All DGGE analyses were performed in 0.5XTAE buffer as previously described (Muyzer et al., 1993) with little modifications (Table A3). According to how the band profile looked like, the PCR volume was chosen. Thus, 40 to 100 µl of PCR product was used for DGGE as indicated in Table A3. Samples were analyzed simultaneously with a molecular marker in the gel's extreme right and left wells. Following the electrophoresis, the gel was stained with 0.5 mg l^{-1} ethidium bromide for 30 minutes and destained with 0.5XTAE buffer for 20 minutes. Pictures were captured and documented using a C-5060 Wide Zoom imaging systems (Olympus, Tokyo, Japan) and a UV-transilluminator (MultiDoc-It^{TM} Digital Imaging System; UVP, CA, USA).

2.3.2 DGGE bands extraction, cloning of 16S rDNA fragments and sequencing (I, II)

Representative bands on all DGGE gels were stabbed with a sterile pipette tip and placed in 100 µl sterile water. DNA was extracted from the gel piece by overnight incubation at 4°C, and the supernatant used as template in a PCR with the inner primer. The resulting amplicon was ran on a DGGE gel to check its original position. This operation was repeated until the band appeared to be single. Amplicons corresponding to a single and well-positioned band were purified on Qiaquick columns (Qiagen, Tokyo, Japan). Subsequently, the purified bands were directly sequenced with BigDye Terminator v3.1 cycling kit (Applied Biosystems, CA, USA) using the

corresponding PCR primer. The sequencing primer was either just the reverse primer (Lake Nyos sequences) or both the reverse and forward primers of the second PCR (Lake Monoun sequences) (Tables A1 and A2).

Following the BigDye sequencing, the PCR reaction was analyzed on a 3130 Genetic Analyzer (ABI-Prism sequencer, Applied Biosystems). For the bands that failed to be sequenced directly, showing many ambiguous peaks, 8.3 ng of their purified PCR was cloned using the TA-cloning kit (Promega, Madison, Wi, USA) and a clone library was constructed. Screening of the clone libraries by PCR and DGGE was carried out as described by Schabereiter-Gurtner and co-workers (2001). To confirm the presence of inserts, white colonies of each sample were selected, re-suspended in 100 µl DDW, then cells were lysed by boiling for 5 min, followed by 5 min centrifugation at 12000 rpm. The supernatant was used as template to amplify the insert. Consequently, the PCR product from each positive clone was ran on a DGGE gel and for each band, one insert matching the band patterns for the original samples was selected for sequencing. Then, 18 µl of PCR product generated with the primers M13F and M13R were purified on Qiaquick columns, a BigDye PCR performed using M13R primer, and the sequence read on an ABI-Prism sequencer (Applied Biosystems).

2.3.3 Sequence editing, analysis and Taxonomic affiliation (I, II)

The two sequences obtained from each band with the forward and reverse primers were assembled into a contiguous sequence and manually edited in the ATGC assembly software (Genetyx, Tokyo, Japan). The resulting sequences were checked for chimera artifacts using DECIPHER (Wright et al., 2012) and chimeric sequences removed.

Taxonomic affiliations were done through a multiple sequence alignment using the SILVA alignment tool (Pruesse et al., 2012) at a confidence threshold of 85%.

2.3.4 Ecological indices and statistical analyses (I, II)

The positions and relative signal intensities of bands in the gel tracks were determined with the FP Quest software (Bio-Rad, Laboratories, CA, USA). Bands data were exported to the Primer software (Version 2, Primer-E Ltd, Plymouth, UK) where a similarity index and clustering of the samples were calculated based on Bray-Curtis similarity and complete linkage algorithms. DGGE bands signals were used as well to calculate the Shannon indices for taxon richness and diversity estimation. *H'* was calculated using the following equation (Andreoni et al., 2004):

$$H' = \sum_{i=0}^{R} Pi \ln Pi$$

Where, *pi* represents the relative signal intensities of bands in a track and *R*, the Richness. All the detected bands were used for the calculation of diversity indices.

Heatmaps and relational dendrograms were generated based on Bray–Curtis distances using the heatmap 2 function from the gplot package in R version 2.12.0.

2.3.5 Standard DNA and quantitative PCR

Abundance of bacterial and archaeal 16S rRNA genes was estimated by absolute qPCR. In order to generate standard DNA, representative full-length bacterial and archaeal 16S rRNA gene sequences were amplified from *E. coli* strain K-12 W3110 and the sample MB30 of Lake Monoun,

respectively. Bacterial standard DNA was obtained from previous study (Tanaka et al., 2015). Archaeal standards were prepared as follows. The sample MB30 was chosen as its DGGE profile showed many bands matching with known archaea. Archaeal 16S rRNA gene in the sample was amplified using the primers Ar3F (TTCCGGTTGATCCTGCCGGA) (Nehmé et al., 2009) and Uni1492r to amplify nearly full-length archaeal 16S rRNA gene fragments at 1495 bp. A clone library was constructed using the pGEM-T Easy Vector of the TA-cloning kit (Promega, Madison, Wi, USA) following the manufacturer's instructions. A BigDye PCR was performed using M13 forward and reverse primers and the archaeal primers ARC787F and ARC1059R, and the DNA sequence read on an ABI-Prism sequencer (Applied Biosystems). Glycerol stocks were generated for the archaeal 16S rRNA gene clone with the best alignment criteria that were 100% coverage and 95% identity to the organism Uncultured *Methanospirillum* sp., the clone whose sequence was released into the GenBank/EMBL/DDBJ databases under the accession number LC015090. Plasmid DNA was purified from 6 ml cultures using the Miniprep System (Promega, Madison, Wi, USA) and quantified using a spectrophotometer (Nanodrop 1000 Spectrophotometer). Plasmid lengths were 4518 bp and 4488 bp for bacteria and archaea, respectively. Number of 16S rRNA gene copies was calculated assuming that: the average molecular mass of a dsDNA is $6.6X10^{11}$ ng mol^{-1}, Avogadro's number of copies mol^{-1} is $6.02X10^{23}$ (Agrawal and Lal., 2009; Smith et al., 2006):

$$\text{Number of copies (per µl)} = \frac{\text{Concentration (ng per µl)X 6.02 X } 10^{23} \text{ (copies per mol)}}{\text{length(bp)X6.6X}10^{11} \text{ (ng per mol)}} \text{ (Agrawal and Lal, 2009)}$$

A standard was then prepared in triplicates by serial 10-fold dilutions spanning from 10^8 to 10^1 copies μl^{-1} for bacteria and archaea and used to generate the standard curve for quantification of each group of microorganisms. Bacterial 16S rRNA gene fragments was amplified using universal

primers Uni1392r (5′-ACGGGCGGTGTGTAC-3′), 1055f (5′-ATGGCTGTCGTCAGCT-3′) (Baek et al., 2009) and the probe 16STaq1115 (5'-(6-FAM)-CAACGAGCGCAACCC-(TAMRA)-3') (Harms et al., 2003). For the amplification of archaeal 16S rRNA gene fragment, universal primers ARC787F (5′-ATTAGATACCCSBGTAGTCC-3′), ARC1059R (5′-GCCATGCACCWCCTCT-3′) and the TaqMan probe ARC915F ((FAM)-AGGAATTGGCGGGGGAGCAC-(TAMRA)) (Nehmé et al., 2009) were used. The reaction was carried out in a Thermal Cycler Dice Real Time System (TP-850 Takara Bio). Data were obtained from samples and non-template controls in a triplicate amplification. Each reaction mixture had a volume of 25 µl and consisted of 12.5 µl of premix Ex Taq (Probe qPCR) (2X), 0.2 µM of each primer, 0.25 µmol l^{-1} of TaqMan probe and approximately 10 ng DNA template. The qPCR amplification conditions were as follows: one hold at 95°C for 30 s, followed by 40 cycles of denaturation for 5 s at 95 °C and simultaneous annealing and extension for 30 s at 60 °C. Following the qPCR, data were analyzed using the TP850 software v4.02 B. The efficiencies of the qPCR were 104.4% (R^2 = 0.999) and 96.2% (R^2= 0.997) for bacteria and archaea, respectively.

2.4 Bacterial analyses in groundwater samples (III)

Total coliforms (TC), fecal coliforms (FC) and fecal streptococci (FS) were detected and enumerated using the membrane filtration technique (APHA, 1992; Djuikom et al., 2006). The process involved vacuum filtration of the water samples through a 47 mm diameter, 0.45 µm pore size cellulose membrane (Sartorius Stedim).

Appropriate dilution was performed for each sample (initially, all were 1,000 X diluted, and the dilution factor adjusted after the culture if needed). Then, 10 ml was filtered from the diluted samples for total and fecal coliforms; and 50 ml for fecal streptococci. Upon filtration, the membrane filter was placed on a specific culture medium contained in sterile petri dishes. Then, petri dishes were inverted and incubated at appropriate temperature for each bacterial group. Simultaneously, a blank was incubated as control for contamination. Between two samples, funnels were sterilised by flaming.

Total and fecal coliforms were identified using Lactose TTC Agar with Tergitol 7 culture medium (Merck, France) on which yellow-orange colonies were detected and enumerated after 24 h incubation at 37°C for total coliforms, and 24 h at 44°C for fecal coliforms. Red-brown colonies of fecal streptococci were identified and enumerated on Slanetz and Bartley agar (Merck, France) upon 48 h incubation at 37°C.

The FC bacteria count from both study areas, were used to group the water sources into risk categories: 0 colony forming unit 100ml (c.f.u. 100 ml^{-1}) (conformity); 1-10 c.f.u. 100 ml^{-1} (low risk); 10-100 c.f.u. 100 ml^{-1} (intermediate risk); 100-1,000 c.f.u. 100 ml^{-1} (high risk); and >1,000 c.f.u. 100 ml^{-1} (very high risk) (WHO, 1997).

The FC to FS (FC:FS) ratio was used to differentiate human from non-human sources of fecal contamination for samples having FS count greater than or equal to 100.

Chapter 3

3 RESULTS

3.1 Analysis of prokaryotic community composition

3.1.1 Prokaryotic communities pattern and ecological indices in different water layers

Based on the assumptions that the bands observed in each DGGE profile represent different Operational Taxonomic Units (OTUs), and the intensity of each band provides insight into the relative abundance of each OTU, the number of dominant OTUs and the Shannon Diversity Indexes were determined for each sample. Dendrograms showing the clustering pattern of the samples were constructed from the resemblance matrix of the DGGE band profile.

3.1.1.1 Lake Nyos (I)

The number of detectable bands varied from 6 to 23 per track for bacteria (Figure 5A). Sequenced bands (46) are indicated with an arrow. For archaea (Figure 5B), fewer bands were observed and varied from 2 to 15 per track, among which 15 indicated with an arrow were sequenced. The

similarities of all gel tracks were calculated to determine the information content of the banding patterns in terms of the structural diversity of the samples.

The sample clusters can be observed on the dendrograms for the bacterial (Figure 6A) and archaeal (Figure 6B) communities. The bacterial banding pattern was differentiated into 4 clusters as a function of depth as follows: 2 upper clusters from 0 to -10 m, -25 to -80 m and 2 lower clusters from -90 to -160 m and -180 to -210 m. The archaeal banding pattern showed 3 clusters: distributed from -25 to -80 m, -90 to -180 m and -200 to -210 m.

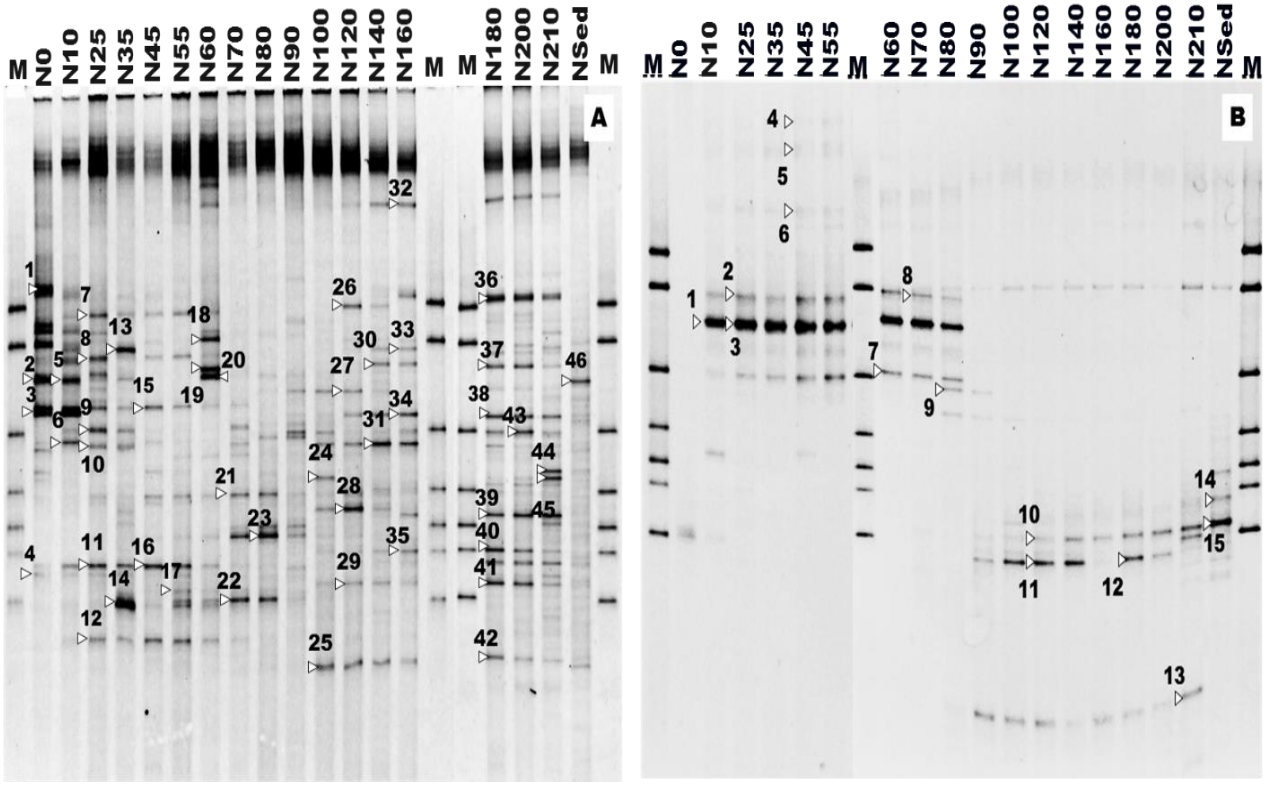

Figure 5: Denaturing gradient gel electrophoresis (DGGE) profiles of bacterial (A) and archaeal (B) communities at different depths in Lake Nyos. **Arabic numerals**: Sequenced bands for bacteria and archaea. **M:** Mass ladder standards. The samples were named as N for Lake Nyos and the number in the sample name refers to the sampling depth.

The Shannon Diversity Indexes indicated for the bacterial community (Figure 7A) that the sediment samples had the lowest diversity, followed by the sample from -80 m and -70 m. All the other samples showed a diversity index between 2 and 2.5 For the archaeal community (Figure 7B), The surface sample had the lowest H', followed by the -40 m. It can be observed that, in a general way, all the samples from -80 m to the bottom had a shannon index higher than that of the samples from 0 to -70 m.

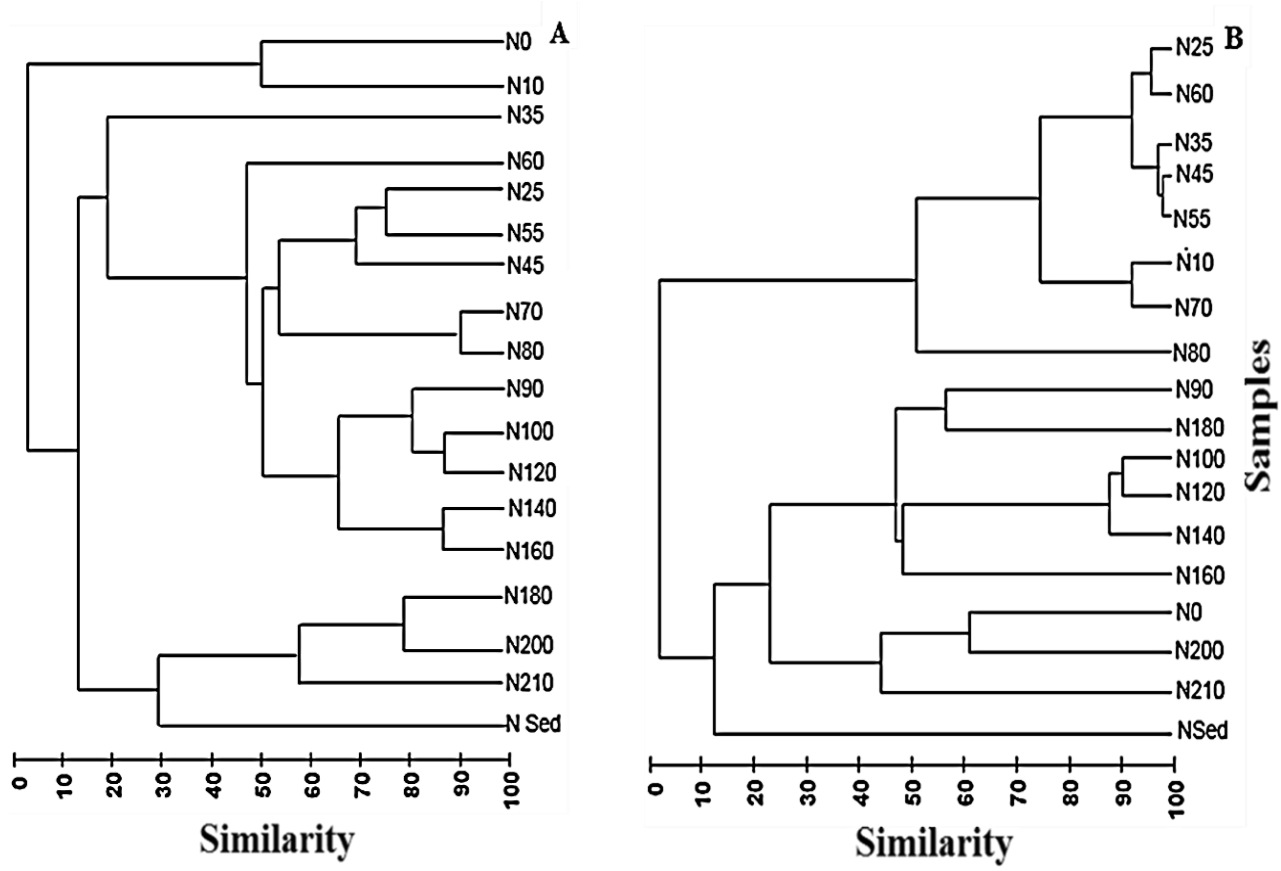

Figure 6: Dendrograms based on the Bray-Curtis similarity index calculated using the OTUs abundance, and hierarchical clustering calculated using complete linkage algorithms in PRIMER 6 for bacteria (A) and archaea (B) in Lake Nyos samples.

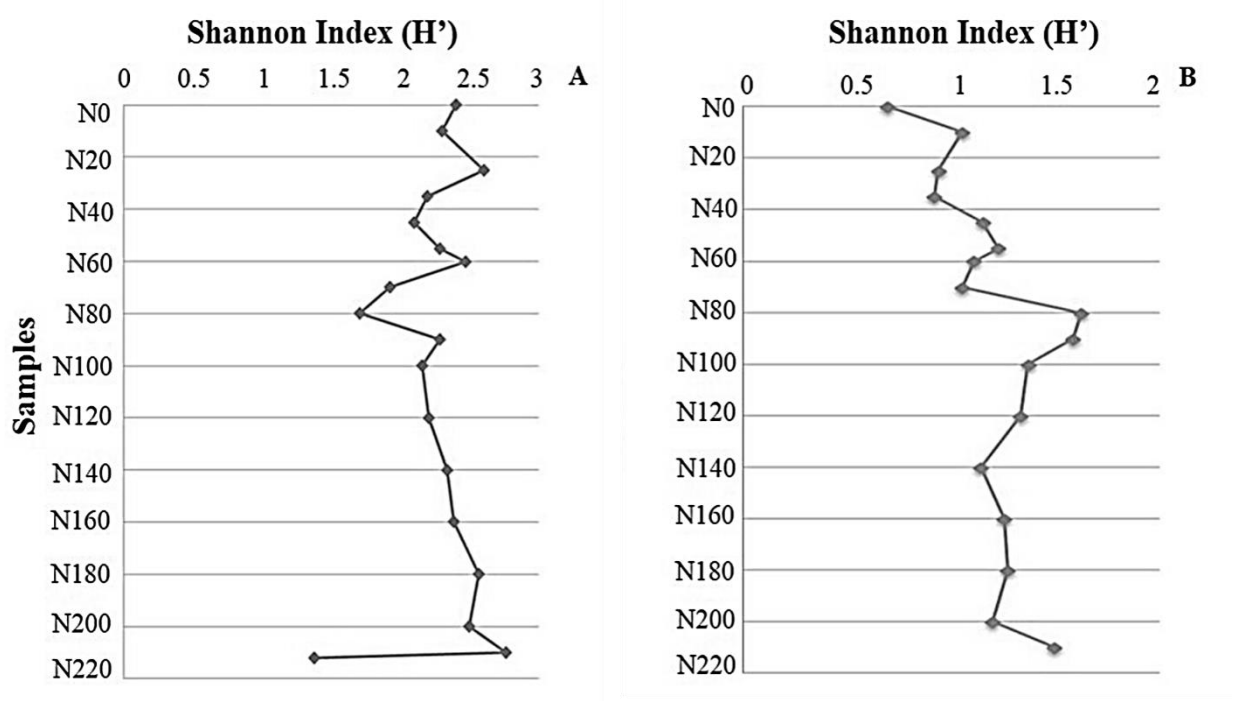

Figure 7: Vertical changes of Shannon-Weaver index of diversity (H') in Lake Nyos based on the number and relative intensities of the bands from bacterial (A) and archaeal (B) DGGE analyses of PCR-amplified 16S rRNA gene.

3.1.1.2 Lake Monoun (II)

The DGGE profiles of bacterial and archaeal communities associated with the samples from both basins of Lake Monoun are shown in Figure 8 A and B. The number of detectable bands on the gel tracks for bacteria (Figure 8A) varied from 18 to 38. In total, 33 bands indicated with an arrow on the gel's picture were sequenced, and named as from B1 to B33. For the archaeal community DGGE pattern (Figure 8B), the band count on each gel track varied widely from 3 to 36 with the lowest counts observed in the surface samples. Thirty bands indicated with an arrow on the gel's picture were sequenced and named as from A1 to A30.

On the dendrogram of the bacteria DGGE (Figure 9A), 3 main clusters can be observed. The archaea DGGE dendrogram (Figure 9B) shows 2 main clusters, separating the surface (0 m) samples from other samples. Both dendrograms unanimously raise a similarity between the samples from 0 and -30 m of both basins, and the samples from -40, -45 and -55 m from the main and central basins, respectively, indicating a similarity between the samples from the same depth, no matter the basin.

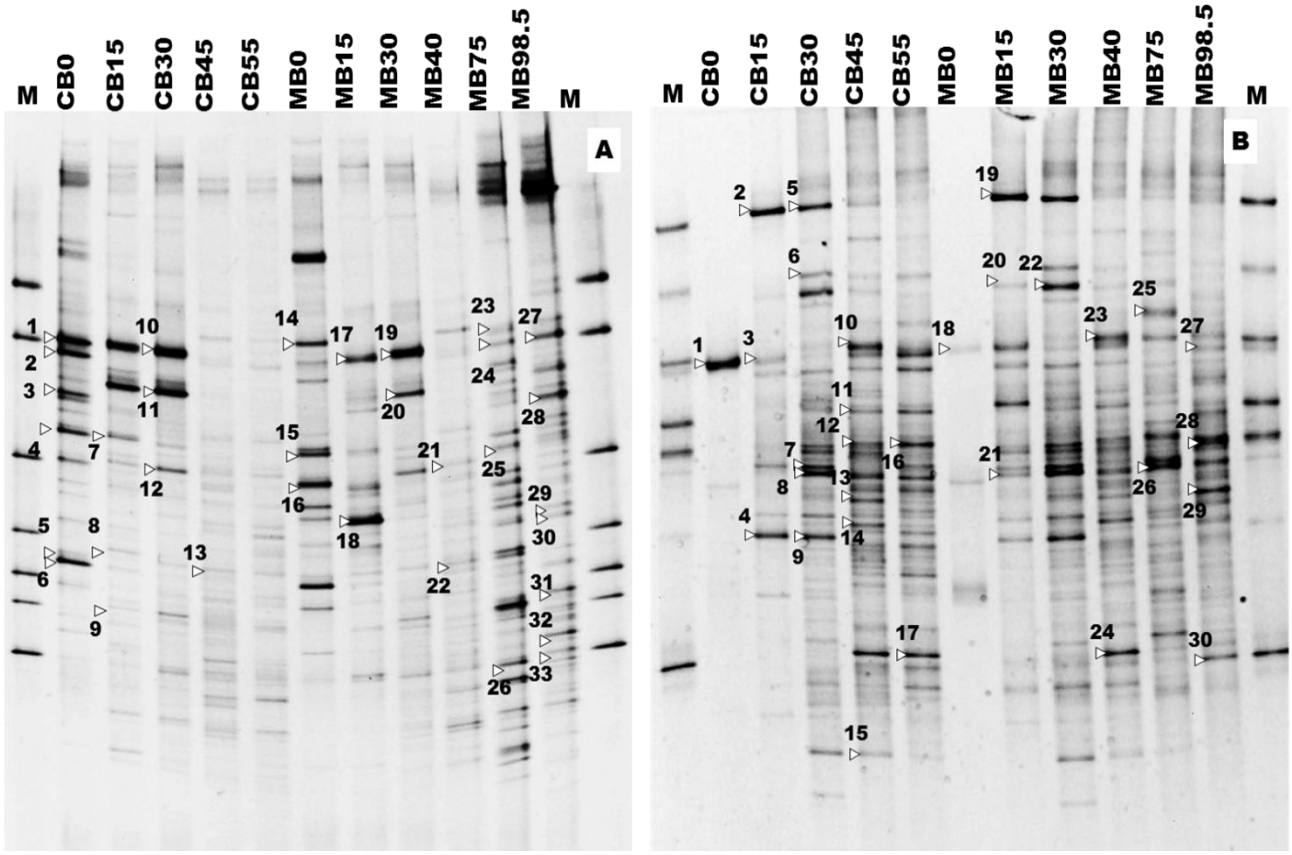

Figure 8: Denaturing gradient gel electrophoresis (DGGE) profiles of bacterial (A) and archaeal (B) communities at different depths in Lake Monoun. **Arabic numerals:** Sequenced bands for bacteria and archaea. **M:** Mass ladder standards. The samples were named as CB for central basin and MB for main basin and the number in the sample name refers to the sampling depth.

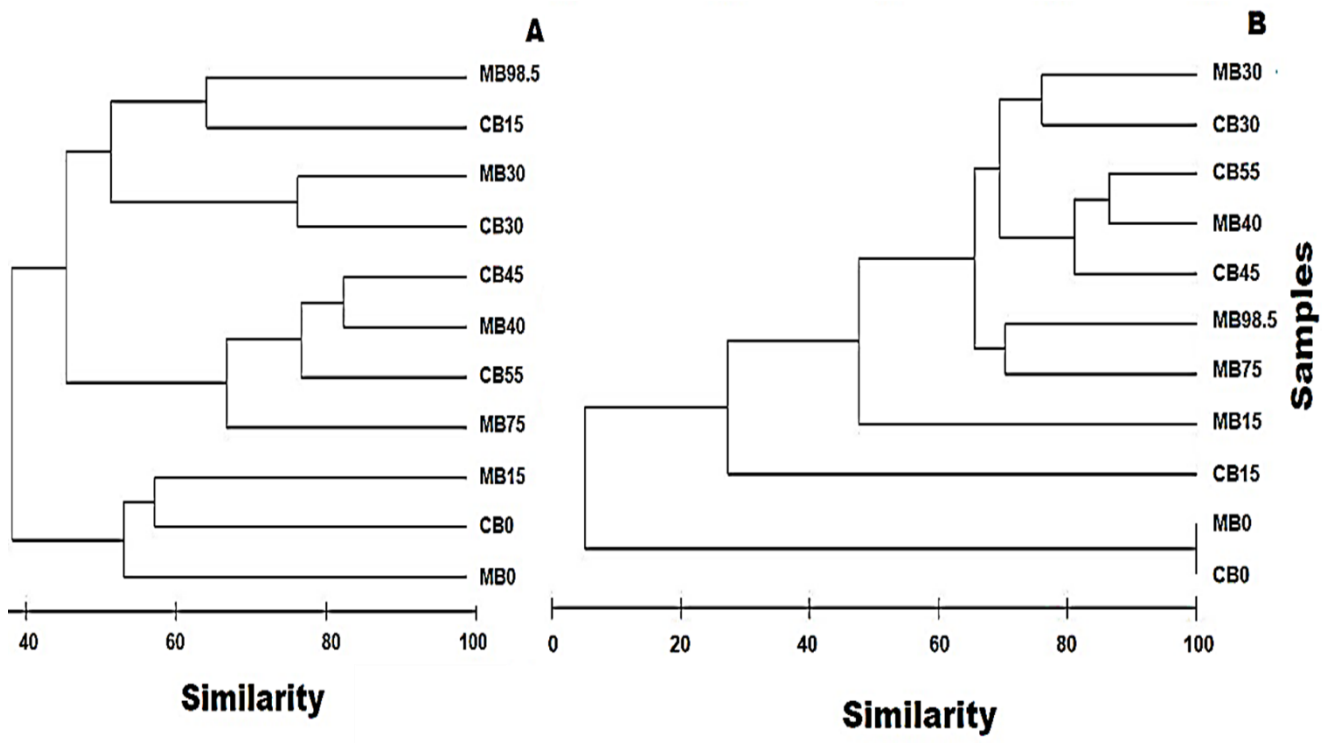

Figure 9: Dendrograms based on the Bray-Curtis similarity index calculated using the OTUs abundance, and hierarchical clustering calculated using complete linkage algorithms in PRIMER 6 for bacteria (A) and archaea (B) in Lake Monoun samples.

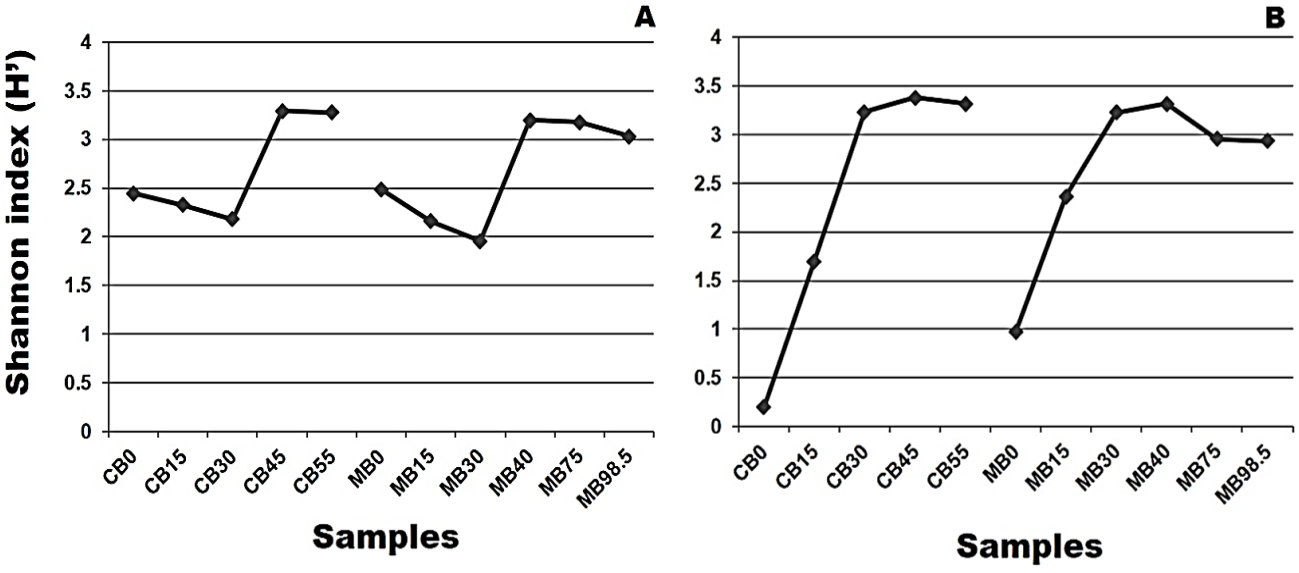

Figure 10: Vertical changes of Shannon-Weaver index of diversity (H') in Lake Monoun based on the number and relative intensities of the bands from bacterial (A) and archaeal (B) DGGE analysis of PCR-amplified 16S rRNA gene.

As for archaea (Figures 10B), the highest diversities were obtained from the samples at -45, -55 and -30 m of the central basin, followed by the samples 30, 40, 75 and 98.5 m of the main basin.

3.1.2 Community composition and taxonomic assignments

3.1.2.1 Lake Nyos (I)

Sequence distribution in the phylogenetic orders according to the sample is displayed in Figure 11A for bacteria and Figure 11B for archaea.The nearest relatives were identified for bacterial (Table 1) and archaeal (Table 2) sequences. Both bacteria and archaea domains exhibited distinct communities at different depths of the lake's water column and their dendrograms indicated a stratified pattern along the water column (Figures 6A and 6B) with the largest difference occuring around 80-90 m depth. The bulk of the matches of bacterial sequences belonged to the phyla Actinobacteria and Firmicutes that accounted for 28.3% and 19.6%, respectively. Both phyla were abundant in the samples from 0 to -55 m, while fewer representatives were found at deeper sites. Then followed representatives matching with the phyla Proteobacteria (15.2%), of which only a Gamma member was found in the -30 m sample. All the other Proteobacteria were found from -60 m. The Candidate divisions (10.9%) and Bacteroidetes (8.7%) matches were found in the samples from -120 m to the bottom. The putative Chlorobi and Caldiserica (6.5% each) were detected only from -120 m and -180 m, respectively. The fewer proportions were represented by members of the phyla Cyanobacteria and Fusobacteria (2.2% each), and were found only in the samples from -160 m and -210 m, respectively.

For the archaeal sequences, the closest relatives all belonged to the phylum Thaumarcheota (100%). To a finer taxonimic level, they were distributed as follows: Marine group I (60%) found in the samples from -10 to -80 m, Miscellaneous Crenarchaeotic Group (13%) and AK59 group (27%) found in the sample from -120 m.

3.1.2.2 Lake Monoun (II)

The closest relatives and taxonomic affiliation of sequences are shown in Table 3 and Table 4 for bacteria and archaea, respectively. The distribution of orders according to the sample is displayed in Figure 9A for bacteria and Figure 9B for archaea. The prokaryotic community was different according to the sampling layer. The retrieved sequences were distributed into six phyla for bacteria as follows: Proteobacteria (63.7%), Chloroflexi and Spirochaetes (12.1% each), Firmicutes (6%) and Bacteroidetes (3.0%), and Nitrospirae (3.0%). The distribution of orders which is displayed on Figure 12A indicates that relatives of Caulobacterales, Enterobacteriales and Pseudomonadales were detected in the 0 m sample of the central basin. Pseudomonadales-related sequences were also detected in the 0 m sample of the main basin and the -15 m sample of both basins. Methylococcales-related sequences were found in the samples from -15 m and -30 m of both basins and at -75 m of the main basin. Sphingobacteriales-related sequences were found in the sample from -15 m and -45 m of the central basin. In the samples from -75 and -98.5 m of the main basin, sequences close to Spirochaetales were detected. Those close to Nitrospirales were found in the -75 m sample. Dehalococcoidiales relatives were found in the -30 m sample of the central basin then, in the -40 and -98.5 m samples of the main basin. Methylophilales were found in the -30 m sample of the central basin and Syntrophobacterales relatives in those from -40 and

-75m of the main basin. Finally, Sphingomonadales and Clostridiales relatives were found in the -15 m sample of the central basin and -98.5 m sample of the main basin, respectively.

As for sequences obtained with the archaeal primer, they were distributed into the phyla Euryarchaeota (23.3%) and Thaumarchaeota (20.0%). The highest proportion (56.7%) did not match with known archaea and was termed as unclassified. As indicated on Figure 12B, the Methanomicrobiales sequences were found in the -30 m sample of the central basin and the samples from -15, -75 and -98.5 m of the main basin. The Halobacteria relatives were detected in the samples from -15 and -30 m of the main basin. The Miscellaneous Crenarchaeotal Group (MCG) relatives were detected at -15, -30, -45, -55 m of the central basin and at -40 m of the main basin.

Table 1: Taxonomic affiliation of the 16S rRNA gene sequences for Lake Nyos samples generated with bacterial universal primers in comparison with taxa from the SILVA database.

Band No	Sampling depth	Sequence name	Base pairs	Genbank accession No	Similarity (%)	Closest relative (Accession No)	Taxonomic affiliation* (Phylum/Class/Order)
1	0	NyosB1 genotype 1_1	141	AB907636	100	Uncultured *Exiguobacterium* sp. (AB476620)	Firmicutes; Bacilli; Bacillales
2	0	NyosB2 genotype 1_2	148	AB907637	100	*Exiguobacterium undae* (FN870072)	Firmicutes; Bacilli; Bacillales
3	0	NyosB3 genotype 1_3	141	AB907638	100	Uncultured *Exiguobacterium* sp. (AB476620)	Firmicutes; Bacilli; Bacillales
4	0	NyosB4 genotype 2	141	AB907639	94	Unidentified marine bacterioplankton (KC000230)	Actinobacteria; Actinobacteria; Gaiellales
5	10	NyosB5 genotype 1_4	141	AB907640	100	Uncultured *Exiguobacterium* sp. (AB476620)	Firmicutes; Bacilli; Bacillales
6	10	NyosB6 genotype 1_5	131	AB907641	99	*Exiguobacterium undae* ((FN870072)	Firmicutes; Bacilli; Bacillales
7	25	NyosB7 genotype 3_1	122	AB907642	98	Marine metagenome (AACY020486889)	Actinobacteria; Actinobacteria; Acidimicrobiales
8	25	NyosB8 genotype 4_1	142	AB907643	97	Uncultured bacterium (JF830189)	Firmicutes; Bacilli; Bacillales
9	25	NyosB9 genotype 4_2	155	AB907644	97	*Exiguobacterium acetylicum* (AB680246)	Firmicutes; Bacilli; Bacillales
10	25	NyosB10 genotype 5	144	AB907645	100	*Exiguobacterium indicum* (AJ846291)	Firmicutes; Bacilli; Bacillales
11	25	NyosB11 genotype 6	115	AB907646	97	Uncultured bacterium (HQ661326)	Actinobacteria; Actinobacteria; Acidimicrobiales
12	25	NyosB12 genotype 3_2	122	AB907647	100	Uncultured bacterium (JX406220)	Actinobacteria; Actinobacteria; Acidimicrobiales
13	35	NyosB13 genotype 7	159	AB907648	100	*Acinetobacter radioresistens* (AY568478)	Proteobacteria; Gammaproteobacteria; Pseudomonadales
14	35	NyosB14 genotype 8	126	AB907649	100	Uncultured bacterium (KC554646)	Actinobacteria; Actinobacteria; Micrococcales
15	45	NyosB15 genotype 9	140	AB907650	99	Uncultured bacterium (EU803396)	Actinobacteria; Actinobacteria; Frankiales
16	45	NyosB16 genotype 10_1	130	AB907651	98	Uncultured bacterium (EU803908)	Actinobacteria; Actinobacteria; Acidimicrobiales
17	55	NyosB17 genotype 11	128	AB907652	95	Uncultured bacterium (GU305756)	Actinobacteria; Actinobacteria; Acidimicrobiales
18	60	NyosB18 genotype 12	158	AB907653	98	Uncultured bacterium (HQ218511)	Proteobacteria; Gammaproteobacteria; Pseudomonadales
19	60	NyosB19 genotype 13	154	AB907654	99	Uncultured bacterium (EU828423)	Proteobacteria;; Betaproteobacteria; Burkholderiales
20	60	NyosB20 genotype 14	144	AB907655	95	*Collimonas* sp. CT_MP11E8 (GQ160908)	Proteobacteria; Betaproteobacteria; Burkholderiales
21	70	NyosB21 genotype 15	135	AB907656	95	Uncultured bacterium (GU127273)	Actinobacteria; Actinobacteria; Frankiales
22	70	NyosB22 genotype 16	130	AB907657	98	Uncultured bacterium (AB630437)	Proteobacteria; Alphaproteobacteria; Rhizobiales
23	80	NyosB23 genotype 17	138	AB907658	91	Uncultured bacterium (AB930731)	Proteobacteria; Betaproteobacteria; Methylophilales
24	100	NyosB24 genotype 18	136	AB907659	83	Uncultured bacterium (GU305756)	Actinobacteria; Actinobacteria; Frankiales

Table 1 (continued)

Band No	Sampling depth	Sequence name	Base pairs	Genbank accession No	Similarity (%)	Closest relative (accession No)	Taxonomic affiliation* (Phylum/Class/Order)
25	100	NyosB25 genotype 19	149	AB907660	89	Uncultured bacterium (HQ218511)	Proteobacteria; Gammaproteobacteria; Pseudomonadales
26	120	NyosB26 genotype 20	111	AB907661	84	Uncultured bacterium (KC424712)	Bacteroidetes; (vadinHA17)
27	120	NyosB27 genotype 21	125	AB907662	86	Uncultured prokaryote (GU208276)	Chlorobi; Ignavibacteria; Ignavibacteriales
28	120	NyosB28 genotype 22_1	145	AB907663	93	Uncultured bacterium (HQ330597)	Bacteroidetes; Sphingobacteriia; Sphingobacteriales
29	120	NyosB29 genotype 10_2	125	AB907664	95	Uncultured bacterium (HE804605)	Actinobacteria; Actinobacteria; Coriobacteriales
30	140	NyosB30 genotype 23	111	AB907665	89	Uncultured bacterium (AJ306788)	Candidate division OD1
31	140	NyosB31 genotype 24	114	AB907666	83	Uncultured *Parcubacteria* bacterium (GQ354955)	Candidate division OD1
32	160	NyosB32 genotype 25	99	AB907667	92	Uncultured bacterium (GU363013)	Actinobacteria; Actinobacteria; Acidimicrobiales
33	160	NyosB33 genotype 10_3	131	AB907668	83	Uncultured bacterium (KC527501)	Cyanobacteria (ML635J-21)
34	160	NyosB34 genotype 26_1	119	AB907669	92	Uncultured bacterium (AB364876)	Chlorobi; Ignavibacteria; Ignavibacteriales
35	160	NyosB35 genotype 22_2	149	AB907670	92	Uncultured bacterium (HQ330597)	Bacteroidetes; Sphingobacteria; Sphingobacteriales
36	180	NyosB36 genotype 27_1	144	AB907671	88	*Caldisericum exile* AZM16c01 (AP012051)	Caldiserica; Caldisericia; Caldisericales
37	180	NyosB37 genotype 27_2	129	AB907672	82	Uncultured bacterium (FJ535510)	Caldiserica; Caldisericia; Caldisericales
38	180	NyosB38 genotype 26_2	129	AB907673	89	Uncultured bacterium (AB364876)	Chlorobi; Ignavibacteria; Ignavibacteriales
39	180	NyosB39 genotype 28	155	AB907674	82	Uncultured bacterium (EU050690)	Caldiserica; Caldisericia; Caldisericales
40	180	NyosB40 genotype 22_3	147	AB907675	95	Uncultured bacterium (HQ330597)	Bacteroidetes; Sphingobacteria; Sphingobacteriales
41	180	NyosB41 genotype 10_4	132	AB907676	96	Uncultured bacterium (HE804605)	Actinobacteria; Actinobacteria; Coriobacteriales
42	180	NyosB42 genotype 29	136	AB907677	86	Uncultured bacterium (EU488149)	Candidate division OP8
43	200	NyosB43 genotype 30	121	AB907678	91	Uncultured bacterium (JX223334)	Candidate division OD1
44	210	NyosB44 genotype 31	137	AB907679	88	Uncultured bacterium (EU009820)	Firmicutes; Erysipelotrichia ; Erysipelotrichales
45	210 (Sed)	NyosB45 genotype 32	117	AB907680	97	Uncultured bacterium (GU214131)	Candidate division TM7
46	210 (Sed)	NyosB46 genotype 33	134	AB907681	100	*Cetobacterium* (DQ814855)	Fusobacteria; Fusobacteria; Fusobacteriales

* SILVA taxonomic classification was determined using 85% confidence threshold as cut-off for taxonomic assignment. Per cent homology between query sequences and closest relatives were searched using the sequence match tool of the SILVA database.

Table 2: Taxonomic affiliation of the 16S rRNA gene sequences for Lake Nyos samples generated with archaeal universal primers in comparison with taxa from the SILVA database.

Band No	Sampling depth	Sequence name	Base pairs	Genbank accession No	Similarity (%)	Closest relative (accession No)	Taxonomic affiliation* (Phylum/Class)
1	10	NyosA1 genotype 1_1	117	AB907765	92	Uncultured archaeon (AY534150)	Thaumarchaeota Marine Group I
2	25	NyosA2 genotype 1_2	115	AB907766	91	Uncultured archaeon (JX427546)	Thaumarchaeota Marine Group I
3	25	NyosA3 genotype 1_3	112	AB907767	93	Uncultured archaeon (AB550817)	Thaumarchaeota Marine Group I
4	45	NyosA4 genotype 1_4	93	AB907768	95	Thaumarchaeota archaeon MY2 (AVSQ01000019)	Thaumarchaeota Marine Group I
5	45	NyosA5 genotype 1_5	87	AB907769	94	Thaumarchaeota archaeon MY2 (AVSQ01000019)	Thaumarchaeota Marine Group I
6	45	NyosA6 genotype 1_6	112	AB907770	93	Uncultured archaeon (AB550817)	Thaumarchaeota Marine Group I
7	60	NyosA7 genotype 1_7	108	AB907771	93	Marine metagenome (AACY020175098)	Thaumarchaeota Marine Group I
8	70	NyosA8 genotype 1_8	111	AB907772	94	Thaumarchaeota archaeon MY2 (AVSQ01000019)	Thaumarchaeota Marine Group I
9	80	NyosA9 genotype 2	103	AB907773	73	Uncultured archaeon (AY534150)	Thaumarchaeota Marine Group I
10	120	NyosA11 genotype 3_1	113	AB917141	79	Uncultured archaeon (AB364920)	Thaumarchaeota AK59
11	120	NyosA12 genotype 3_2	84	AB917142	81	Uncultured archaeon (EU155999)	Thaumarchaeota AK59
12	180	NyosA10 genotype 3	100	AB907774	77	Uncultured archaeon (EU155999)	Thaumarchaeota AK59
13	210	NyosA13 genotype 4_1	101	AB917143	99	Uncultured archaeon (KJ424515)	Thaumarchaeota Miscellaneous Crenarchaeotic Group
14	210 (Sed)	NyosA14 genotype 4_2	102	AB917144	97	Uncultured archaeon (GU127871)	Thaumarchaeota Miscellaneous Crenarchaeotic Group
15	210 (Sed)	NyosA15 genotype 5	101	AB917145	79	Uncultured archaeon (AB364920)	Thaumarchaeota AK59

*SILVA taxonomic classification was determined using 85% confidence threshold as cut-off for taxonomic assignment. Per cent homology between query sequences and closest relatives were searched using the sequence match tool of the SILVA database.

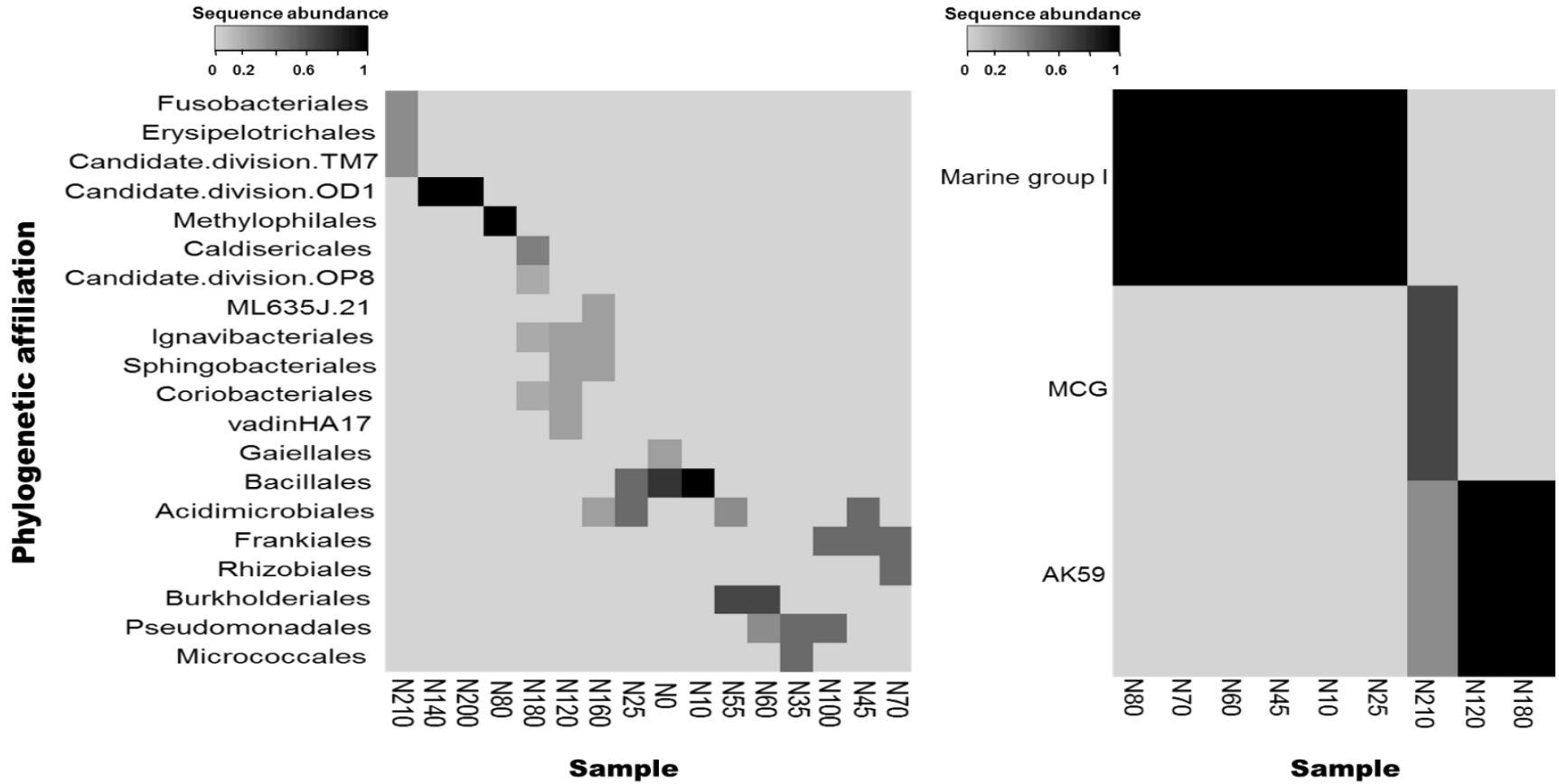

Figure 11: Heat map and relational dendogram based on Bray-Curtis distances between samples from Lake Nyos and the detected sequences for bacteria (A) and archaea (B). The diagram was generated using the count (abundance) of sequenced bands in each sample. The color intensity (light to dark grey) in each panel shows the percentage of an order in a sample, referred to as the abundance per sample at the upper left.

Table 3: Taxonomic affiliation of the 16S rRNA gene sequences for Lake Monoun samples generated with bacterial universal primers in comparison with taxa from the SILVA database.

Band name	Sampling site-depth	Sequence name	Base pairs	Genbank accession No	Similarity (%)	Closest relative (accession No)	Taxonomic affiliation* (Class/Order)
B1	CB0	Monoun_B_OTU1	161	LC005669	100	*Acinetobacter* sp.(AB719400)	Gammaproteobacteria; Pseudomonadales
B2	CB0	Monoun_B_OTU2	161	LC005670	100	*Acinetobacter radioresistens* (AY568478)	Gammaproteobacteria; Pseudomonadales
B3	CB0	Monoun_B_OTU3	161	LC005671	99	Uncultured bacterium (JF137496)	Gammaproteobacteria; Pseudomonadales
B4	CB0	Monoun_B_OTU4	161	LC005672	98	Uncultured acinetobacter (AY568488)	Gammaproteobacteria; Pseudomonadales
B5	CB0	Monoun_B_OTU5	134	LC005673	100	Uncultured Caulobacteraceae (EF019710)	Alphaproteobacteria; Caulobacterales
B6	CB0	Monoun_B_OTU6	160	LC005674	99	Endophytic bacterium LW6 (JX966385)	Gammaproteobacteria; Enterobacteriales
B7	CB15	Monoun_B_OTU7	160	LC005675	99	*Acinetobacter lwoffii* CIP 70.31 (APQT01000052)	Gammaproteobacteria; Pseudomonadales
B8	CB15	Monoun_B_OTU8	135	LC005676	100	Uncultured Sphingomonadaceae (HM438320)	Alphaproteobacteria; Sphingomonadales
B9	CB15	Monoun_B_OTU9	160	LC005677	95	Uncultured bacterium (JN032880)	Gammaproteobacteria; Methylococcales
B10	CB30	Monoun_B_OTU10	160	LC005678	97	Uncultured bacterium (HQ828043)	Betaproteobacteria; Methylophilales
B11	CB30	Monoun_B_OTU11	159	LC005679	100	Uncultured bacterium (AB930641)	Gammaproteobacteria; Methylococcales
B12	CB30	Monoun_B_OTU12	152	LC005680	99	Uncultured bacterium (AF407200)	Dehalococcoidia; Dehalococcoidiales
B13	CB45	Monoun_B_OTU13	156	LC005681	94	Uncultured bacterium (HM481321)	Bacteroidetes; Sphingobacteriales
B14	MB0	Monoun_B_OTU14	160	LC005682	100	Pseudomonadales bacterium CH8 (JN571052)	Gammaproteobacteria; Pseudomonadales
B15	MB0	Monoun_B_OTU15	161	LC005683	97	*Acinetobacter* sp. Seah-As2w (FJ607348)	Gammaproteobacteria; Pseudomonadales
B16	MB0	Monoun_B_OTU16	161	LC005684	100	*Acinetobacter* sp. Hg4-05 (EU304251)	Gammaproteobacteria; Pseudomonadales
B17	MB15	Monoun_B_OTU17	159	LC005685	98	*Methylomonas methanica* MC09 (CP002738)	Gammaproteobacteria; Methylococcales
B18	MB15	Monoun_B_OTU18	161	LC005686	95	Uncultured bacterium (AB286491)	Gammaproteobacteria; Pseudomonadales
B19	MB30	Monoun_B_OTU19	159	LC005687	100	Uncultured bacterium (AB930641)	Gammaproteobacteria; Methylococcales
B20	MB30	Monoun_B_OTU20	159	LC005688	99	*Methylomonas methanica* MC09 (CP002738)	Gammaproteobacteria; Methylococcales
B21	MB40	Monoun_B_OTU21	132	LC005689	99	Uncultured bacterium (AF407200)	Dehalococcoidia; Dehalococcoidiales
B22	MB40	Monoun_B_OTU22	160	LC005690	84	Uncultured Syntrophaceae bacterium (HQ003558)	Deltaproteobacteria; Syntrophobacterales
B23	MB75	Monoun_B_OTU23	135	LC005691	99	Uncultured bacterium (GQ402751)	Nitrospira; Nitrospirales
B24	MB75	Monoun_B_OTU24	160	LC005692	100	*Treponema zuelzerae* (FR749928)	Spirochaetes; Spirochaetales
B25	MB75	Monoun_B_OTU25	160	LC005693	94	Uncultured bacterium (HG529082)	Gammaproteobacteria; Methylococcales
B26	MB75	Monoun_B_OTU26	180	LC005694	93	Uncultured bacterium (AB364726)	Deltaproteobacteria; Syntrophobacterales
B27	MB98.5	Monoun_B_OTU27	160	LC005695	100	*Treponema zuelzerae* (FR749928)	Spirochaetes; Spirochaetales
B28	MB98.5	Monoun_B_OTU28	136	LC005696	92	Uncultured Chloroflexales bacterium (AF355054)	Dehalococcoidia; Dehalococcoidiales
B29	MB98.5	Monoun_B_OTU29	158	LC005697	96	Uncultured bacterium (JQ245548)	Dehalococcoidia; Dehalococcoidiales
B30	MB98.5	Monoun_B_OTU30	160	LC005698	96	Bacterium enrichment culture KWE30-49 (JQ670721)	Firmicutes; Clostridiales
B31	MB98.5	Monoun_B_OTU31	160	LC005699	97	Uncultured bacterium (JX225542)	Spirochaetes; Spirochaetales
B32	MB98.5	Monoun_B_OTU32	134	LC005700	100	Uncultured bacterium (EU777712)	Firmicutes; Clostridiales
B33	MB98.5	Monoun_B_OTU33	160	LC005701	100	Uncultured bacterium SJA-69 (AJ009476)	Spirochaetes; Spirochaetales

*SILVA taxonomic classification was determined using 85% confidence threshold as cut-off for taxonomic assignment. Percent homology between query sequences and closest relatives were searched using the sequence match tool of the SILVA database

Table 4: Taxonomic affiliation of the 16S rRNA gene sequences for Lake Monoun samples generated with archaeal universal primers in comparison with taxa from the SILVA database.

Band name	Sampling site-depth	Sequence name	Base pairs	Genbank accession No	Similarity (%)	Closest relative (accession No)	Taxonomic affiliation* (Class/Order)
A1	CB0	Monoun_A_OTU1	330	LC005634		None	Unclassified
A2	CB15	Monoun_A_OTU2	373	LC005635		None	Unclassified
A3	CB15	Monoun_A_OTU3	325	LC005636		None	Unclassified
A4	CB15	Monoun_A_OTU4	370	LC005637	93	Uncultured archaeon (HQ269182)	Thaumarchaeota; Miscellaneous Crenarchaeotic Group
A5	CB30	Monoun_A_OTU5	373	LC005638		None	Unclassified
A6	CB30	Monoun_A_OTU6	356	LC005639		None	Unclassified
A7	CB30	Monoun_A_OTU7	354	LC005641	98	Uncultured archaeon (DQ785304)	Euryarchaeota; Methanomicrobiales
A8	CB30	Monoun_A_OTU8	354	LC005642	98	Uncultured archaeon (DQ785304)	Euryarchaeota; Methanomicrobiales
A9	CB30	Monoun_A_OTU9	401	LC005643	95	Uncultured archaeon (HQ269182)	Thaumarchaeota; Miscellaneous Crenarchaeotic Group
A10	CB45	Monoun_A_OTU10	373	LC005644		None	Unclassified
A11	CB45	Monoun_A_OTU11	331	LC005645		None	Unclassified
A12	CB45	Monoun_A_OTU12	303	LC005646		None	Unclassified
A13	CB45	Monoun_A_OTU13	401	LC005648	91	Uncultured archaeon (JQ245926)	Thaumarchaeota; Miscellaneous Crenarchaeotic Group
A14	CB45	Monoun_A_OTU14	377	LC005649	84	Uncultured archaeon (JX494430)	Thaumarchaeota; Miscellaneous Crenarchaeotic Group
A15	CB45	Monoun_A_OTU15	349	LC005650		None	Unclassified
A16	CB55	Monoun_A_OTU16	377	LC005652	86	Uncultured archaeon (JX494442)	Thaumarchaeota; Miscellaneous Crenarchaeotic Group
A17	CB55	Monoun_A_OTU17	341	LC005653		None	Unclassified
A18	MB0	Monoun_A_OTU18	326	LC005654		None	Unclassified
A19	MB15	Monoun_A_OTU19	373	LC005656		None	Unclassified
A20	MB15	Monoun_A_OTU20	355	LC005657	80	Uncultured archaeon (JX426877)	Euryarchaeota; Halobacteriales
A21	MB15	Monoun_A_OTU21	350	LC005659	97	Uncultured archaeon (DQ785304)	Euryarchaeota; Methanomicrobiales
A22	MB30	Monoun_A_OTU22	381	LC005660	80	Uncultured archaeon (DQ785304)	Euryarchaeota; Halobacteriales
A23	MB40	Monoun_A_OTU23	373	LC005661		None	Unclassified
A24	MB40	Monoun_A_OTU24	374	LC005662	96	Uncultured archaeon (KJ424454)	Thaumarchaeota; Miscellaneous Crenarchaeotic Group
A25	MB75	Monoun_A_OTU25	327	LC005664		None	Unclassified
A26	MB98.5	Monoun_A_OTU26	367	LC005665		None	Unclassified
A27	MB98.5	Monoun_A_OTU27	384	LC005666	98	Uncultured archaeon (GU135465)	Euryarchaeota; Methanomicrobiales
A28	MB98.5	Monoun_A_OTU28	368	LC005667		None	Unclassified
A29	MB98.5	Monoun_A_OTU29	341	LC005668		None	Unclassified
A30	MB75	Monoun_A_OTU30	386	LC015089	98	Uncultured archaeon (DQ785304)	Euryarchaeota; Methanomicrobiales

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*SILVA taxonomic classification was determined using 85% confidence threshold as cut-off for taxonomic assignment. Percent homology between query sequences and closest relatives were searched using the sequence match tool of the SILVA database

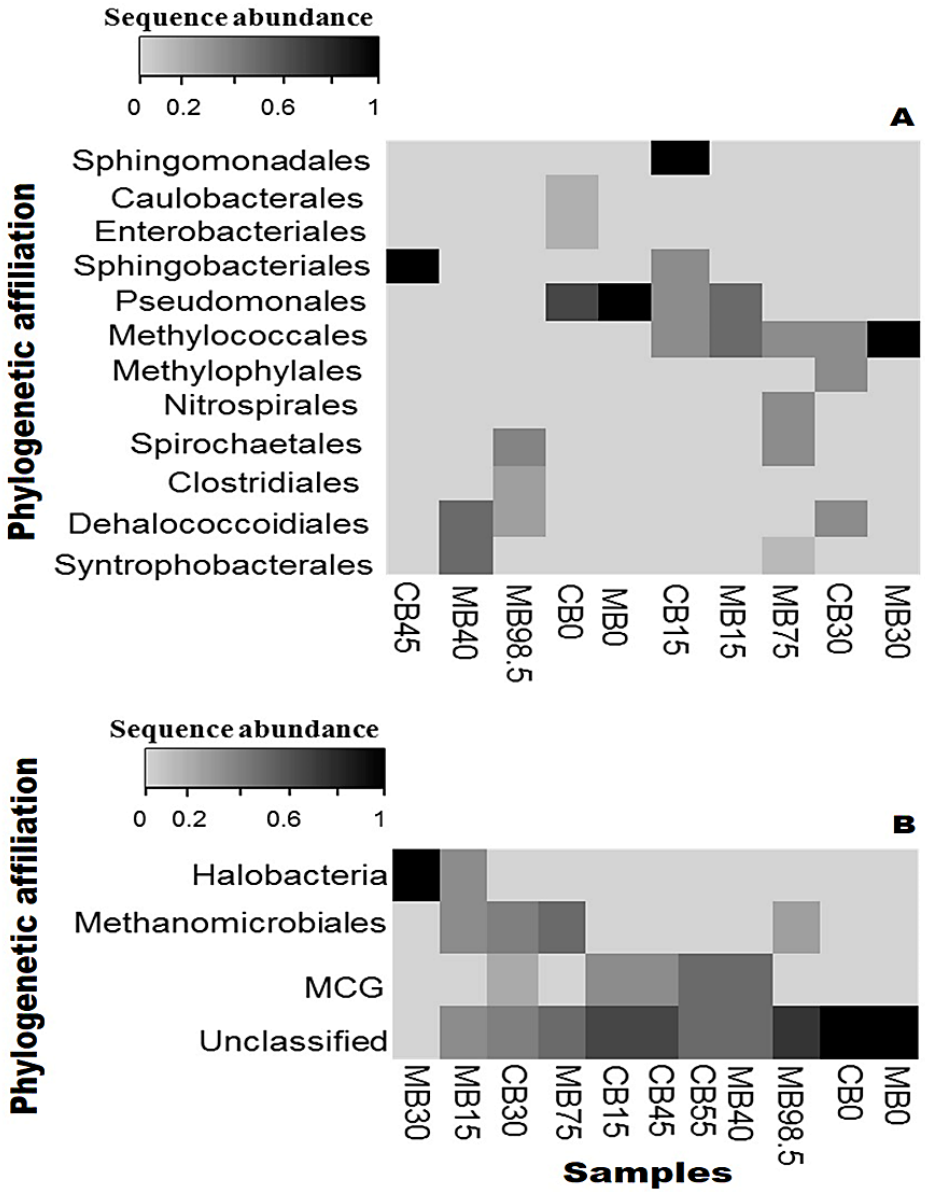

Figure 12: Heat map and relational dendogram based on Bray-Curtis distances between samples from Lake Monoun and the detected sequences for bacteria (A) and archaea (B). The diagram was generated using the count (abundance) of sequenced bands in each sample. The color intensity (light to dark grey) in each panel shows the percentage of an order in a sample, referred to as the abundance per sample at the upper left.

3.1.3 Bacterial and archaeal abundance along the water columns (II)

Results for quantification of communities (Table A4 and Figure 13) revealed that surface samples had high bacterial and low archaeal count while, bottom samples had higher archaeal and lower bacterial counts.

The gene copies numbers for bacteria were determined to vary between $3.6X10^6$ and $7.2X10^7$ in the central basin. In the main basin, they varied between $5.8X10^5$ and $1.6X10^7$ copies ml^{-1}. For archaea, they varied between copies $8.4X10^0$ and $1.2X10^3$ copies ml^{-1} in the central basin. The range was from $1.3X10^1$ to $1.7X10^5$ copies ml^{-1} in the main basin.

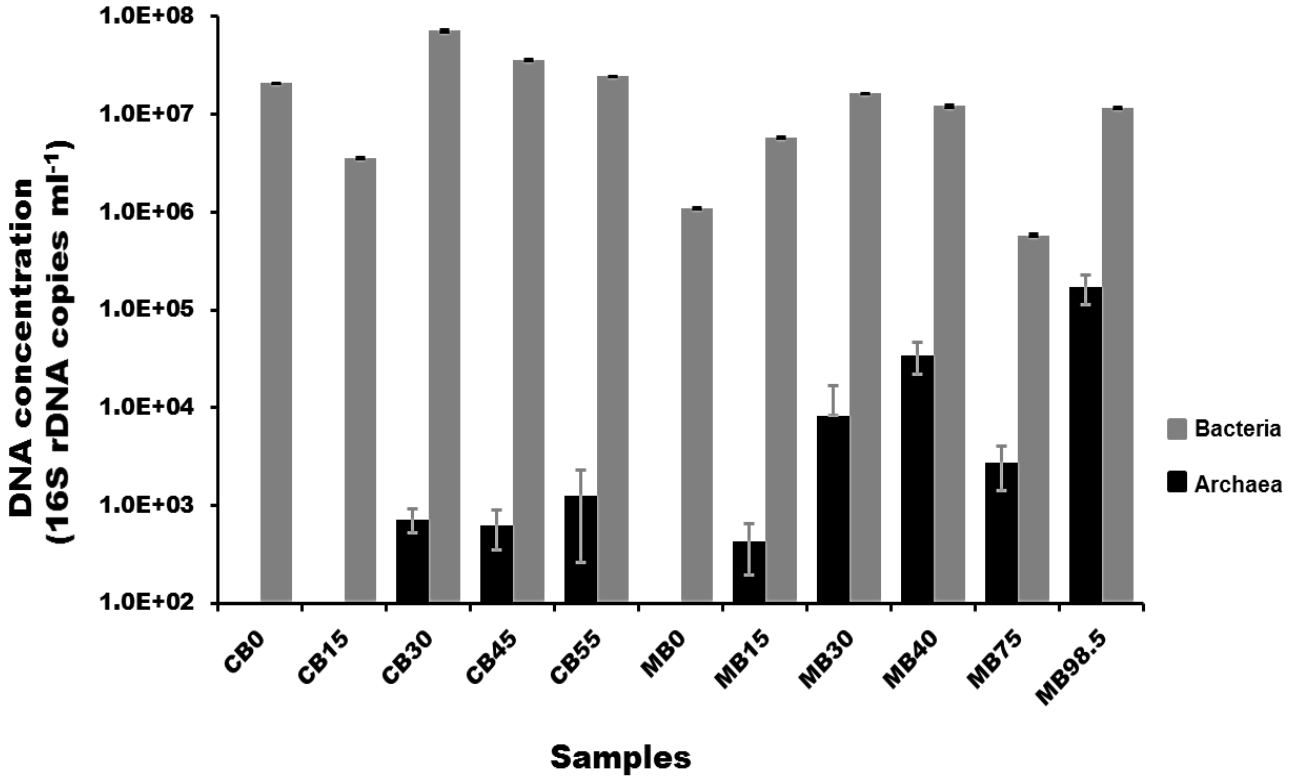

Figure 13: Abundance of total Bacterial and Archaeal 16S rRNA genes in Lake Monoun samples estimated by absolute real-time PCR.

3.1.4 Physico-chemical parameters of the water columns

3.1.4.1 Lake Nyos (I)

The physico-chemical parameters (Table A4) of the lake in March 2013 plotted on Figure 14 show the overwhelming domination of CO_2 (aq), HCO_3^- and Fe^{2+} species. The CO_2 concentrations increased from 0.2 to 153 mmol kg^{-1}. Similar to the C25 profile, bicarbonates increased from 2.4 to 38.9 mmol kg^{-1} with a slight decrease (2 to 1.8 mmol kg^{-1}) from -25 m to -70 m. The Fe^{2+} concentration was low from 0 to -80 m, then increased considerably from -90 m to -210 m. The pH slightly decreased while the temperature slightly increased from about -70 m to the bottom of the lake. Dissolved oxygen (DO) was not detected.

3.1.4.2 Lake Monoun (II)

The physico-chemical parameters (Table A5) of Lake Monoun in March 2013 are shown on Figure 15, the temperature varied from 20ºC to 25ºC, and the pH decreased towards the bottom from 7.5 to 5.6. CO_2 concentration varied from 1.5 to 98 mmol kg^{-1} from the surface to the bottom. A significant shift is observed at ~ -73 m, corresponding to the chemocline. Dissolved oxygen varied from 13 ppm to 35 ppm above -5 m, dropped to 7.9 ppm and 5.6 ppm at -6 m and -7 m, and then, fell to 4.5 ppm from ~ -8 m. Around -20 m, DO amount reduced (about 2.3 ppm), and the anoxia appeared to get more severe with DO values close to 0 ppm towards the bottom in both CB and MB. The conductivity and bicarbonates trends were similar. Iron is dominant among the parameters and an important increasing trend is observed towards the bottom.

3.2 Bacterial analyses in groundwater samples (III)

3.2.1 Total coliforms in water sources around Lakes Nyos and Monoun (III)

The values of the TC counts shown in Table 5 indicate that all the 17 samples contained TC (Figure 14A) with counts ranging from 3 x 10^5 to 18 x 10^5 c.f.u. 100 ml^{-1} (Figure 14B).

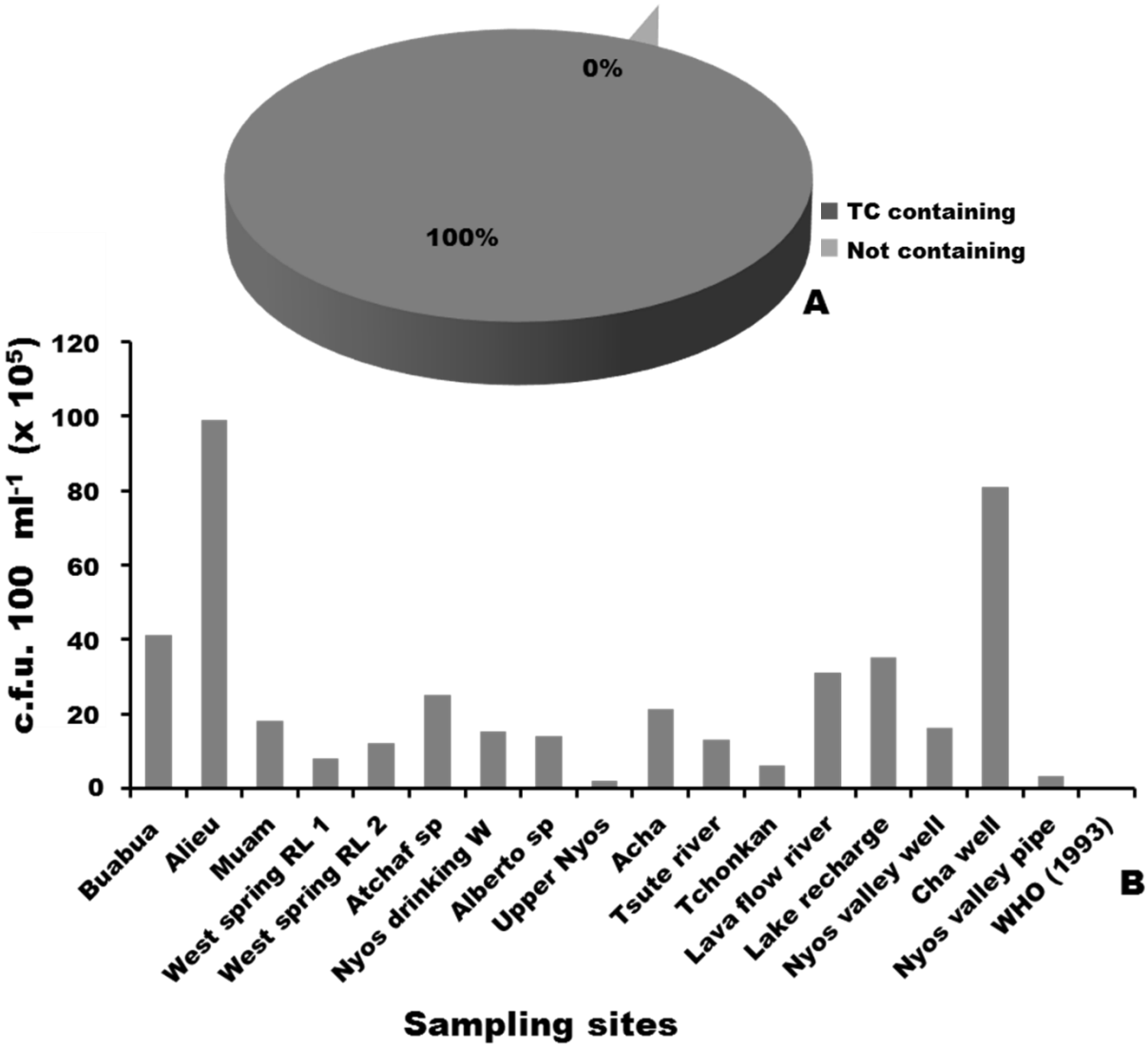

Figure 14: Total Coliform containing samples percentage (A) and Total Coliform counts in the water samples around Lake Nyos (B).

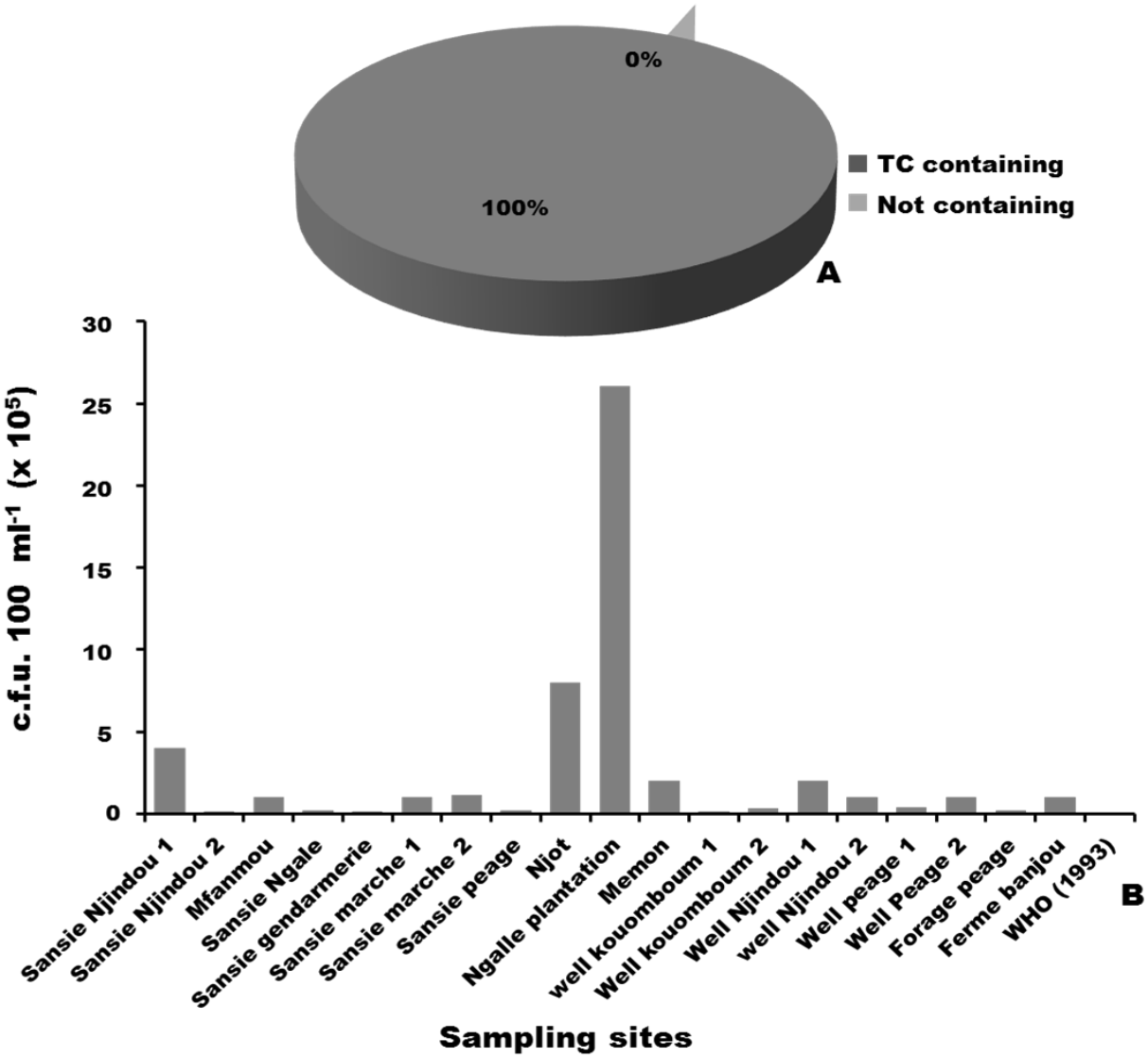

Figure 15: Total Coliform containing samples percentage (A) and Total Coliform counts in the water samples around Lake Monoun (B).

In Lake Monoun area samples, TC counts (Table 6) indicate that all the 19 samples collected in the Monoun area contained TC (Fıgure 15A), with counts ranging from 0.1 x 10^5 to 26 x 10^5 c.f.u. 100 ml^{-1} (Fıgure 15B).

3.2.2 Fecal coliforms and streptococci in water sources around Lakes Nyos and Monoun (III)

Counts for FC and FS around the Nyos area are shown in Table 5. All were contaminated with FC (Figure 16A) with counts ranging from 40 to 1,260 c.f.u. 100 ml^{-1} (Figure 16C). Sixteen of the samples (94.1%) were contaminated with FS (Figure 16B) within a count range of 5 to 964 c.f.u. 100 ml^{-1} (Figure 16C).

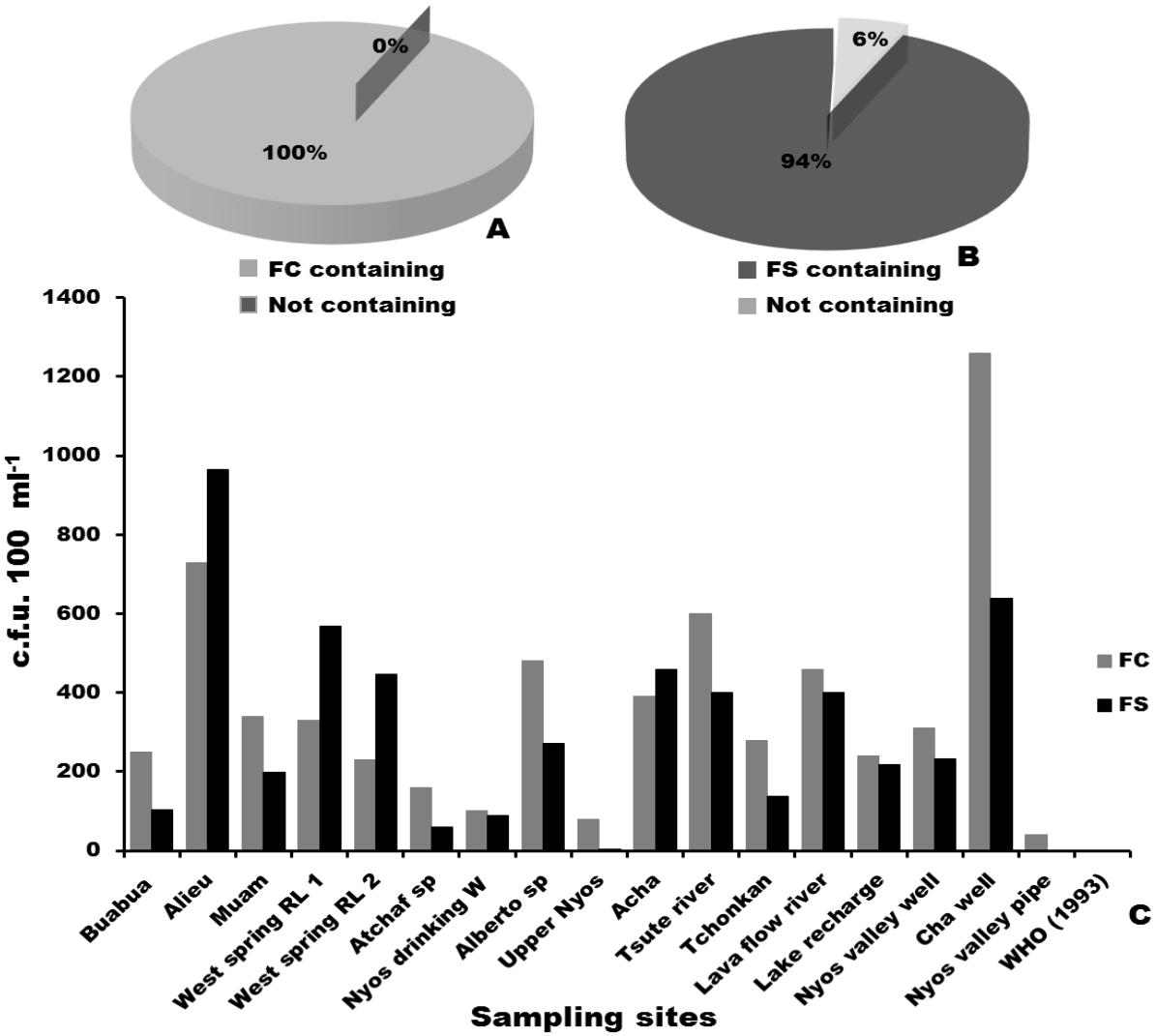

Figure 16: FC containing samples percentage (A), FS containing samples percentage (B) and FC and FS counts in the water samples around Lake Nyos (C).

The samples around Lake Monoun, are shown in Table 6. Sixteen (84.2%) contained FC (Figure 17A), with counts ranging from 10 to 340 c.f.u. 100 ml^{-1} ((Figure 17C); and 17 (89.5% Figure 17B), with counts ranging from 2 to 348 c.f.u. 100 ml^{-1} (Figure 17C). The bulk of the samples appeared to represent a risk (low, intermediate or high). Only 1 sample (Nfanmou) complied with the WHO drinking water guidelines regarding FC and FS.

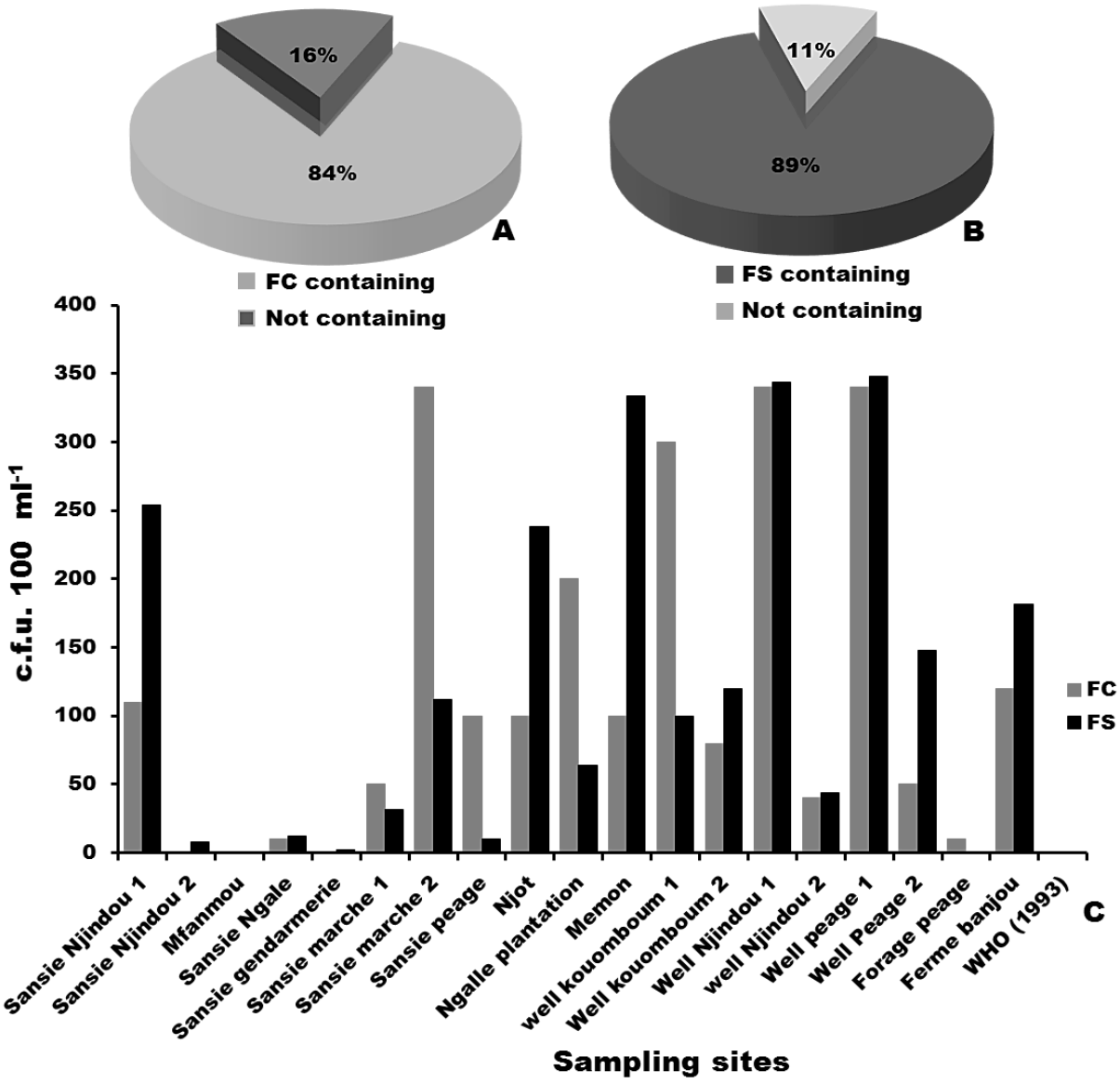

Figure 17: FC containing samples percentage (A), FS containing samples percentage (B) and FC and FS counts in the water samples around Lake Monoun (C).

Table 5: Values of the bacterial counts in samples around Lake Nyos.

Site	Code	Class	Factor	Use	TC ($X10^5$)	FC	FS	FC:FS
						c.f.u. 100 ml^{-1}		
Buabua	TW	Tank water	Livestock	Drinking, washing up, cooking	41	250	104	2.40
Alieu	SP1	Spring	Livestock	Drinking	99	730	964	0.76
Muam	SP2	Spring	Livestock	Drinking	18	340	199	1.71
West spring RL 1	SP3	Spring	Livestock	Drinking	8	330	568	0.58
West spring RL 2	SP4	Spring	Livestock	Drinking	12	230	446	0.52
Atchaf sp	SP5	Spring	Livestock	Drinking	25	160	59	-
Nyos drinking W	SP6	Spring	Livestock	Drinking	15	100	89	-
Alberto sp	SP7	Spring	Livestock	Drinking	14	480	272	1.76
Upper Nyos	SP8	Spring	Livestock	Drinking	2	80	5	-
Acha	ST1	Stream	Livestock	Drinking, washing up, cooking	21	390	458	0.85
Tsute river	ST2	stream	Livestock	Drinking, washing up, cooking	13	600	400	1.50
Tchonkan	ST3	Stream	Livestock	Drinking, washing up, cooking	6	280	138	2.03
Lava flow river	ST4	Stream	Livestock	Drinking	31	460	400	1.15
Lake recharge	ST5	Stream	Livestock	Drinking	35	240	218	1.10
Nyos valley well	W1	Well	Livestock	Drinking, washing up, cooking	16	310	232	1.34
Cha well	W2	Well	Livestock	Washing up, cooking	81	1260	640	1.97
Nyos valley tap	SSW	SSW	Livestock	Drinking	3	40	0	-
WHO (1993)					0	0	0	

Table 6: Values of the bacterial counts in samples around Lake Monoun**.**

Site	Code	Class	Factor	Use	TC	FC	FS	FC:FS
					c.f.u. 100 ml^{-1}			
Sansie Njindou 1	SP1	Spring		Drinking	$4X10^5$	110	254	0.43
Sansie Njindou 2	SP2	Spring		Drinking	$0.1X10^5$	0	8	-
Nfanmou	SP3	Spring		Drinking	$1X10^5$	0	0	-
Sansie Ngale	SP4	Spring		Drinking	$0.2X10^5$	10	12	-
Sansie gendarmerie	SP5	Spring		Drinking	$0.1X10^5$	0	2	-
Sansie marché 1	SP6	Spring		Drinking	$1X10^5$	50	32	-
Sansie marché 2	SP7	Spring	Latrine upstream	Drinking	$1.1X10^5$	340	112	3.04
Sansie peage	SP8	Spring		Drinking	$0.2X10^5$	100	10	-
Njot	ST1	Stream		Drinking, washing up, cooking	$8X10^5$	100	238	0.42
Ngalle plantation	ST2	Stream	Farming	Drinking, watering	$26X10^5$	200	64	-
Memon	ST3	Stream	Laundry	Washing up, cooking	$2X10^5$	100	334	0.30
well kouomboum 1	W1	Well		Washing up, cooking	$0.1X10^5$	300	100	3.00
Well kouomboum 2	W2	Well		Washing up, cooking	$0.3X10^5$	80	120	0.67
Well Njindou 1	W3	Well		Washing up, cooking	$2\ X10^5$	340	344	0.99
well Njindou 2	W4	Well		Cooking , washing up	$1X10^5$	40	44	-
Well peage 1	W5	Well		Cooking, washing up	$0.4X10^5$	340	348	0.98
Well Peage 2	W6	Well		Cooking, washing up	$0.1\ X10^5$	50	148	0.34
Forage peage	DW1	Bore hole	24 m to latrine	Drinking	$0.2X10^5$	10	0	-
Ferme banjou	DW2	Bore hole	chicken husbandry	Drinking	$1X10^5$	120	182	0.66
WHO (1993)	WHO				0	0	0	

Note: TC (total coliforms), FC (fecal coliforms), FS (fecal streptococci), CFU (colony forming units), DW (deep well), TW (tank water), SSW (Sub Surface Water), PW (pipe water), C (conformity), L (low risk), I (intermediate risk), H (high risk). WHO (World Health organization).

Chapter 4

4 DISCUSSION

4.1 Microbial communities distribution and diversity

4.1.1 Communities pattern in lakes Nyos and Monoun

Bacterial and archaeal communities detected in both lakes showed a stratified distribution in the water column agreeing with previous studies based on physico-chemical approaches. Communities as determined by DGGE and sequencing analyses suggested that prokaryotic communities are different between both lakes. Several genera belonging to diverse functional groups were detected. This diversity of microbial communities likely reflects the uniqueness of Lakes Nyos and Monoun. However, it is worth mentioning that artifacts may have been introduced in the detection of microbial assemblages during DNA extraction, PCR or DGGE and therefore, could alter the actual diversity of microbial communities (Casamayor et al., 2002). Consequently, the microbial community composition as detected by DGGE in this study may mainly represent the most abundant phylotypes. If functionally active, they could considerably impact the geochemical cycles taking place in the lake.

Altogether, the dendrograms showed clusters of the water stratification following the physicochemical pattern indicated by Kusakabe et al. (2008); Microorganisms require different

metabolic pathways to survive and differences in concentrations of the chemical species may, to a large extent, explain differences in the bacterial and archaeal communities between the water layers. The shallowest and deepest sampling sites are well separated as shown by lake Nyos dendrograms. The same separation is observed with lake Monoun's samples, with a clustering of samples from approximately same depth, no matter the basin. This indicates the structuring role of the physicochemical parameters that have likely influenced the microbial community distribution.

4.1.2 Bacterial diversity in Lakes Nyos and Monoun

Highest proportions of the recovered bacterial sequences from both lakes samples matched with Proteobacteria, Actinobacteria and Bacteroidetes which are known to be among the 5 dominant phyla in freshwater ecosystems (Newton et al., 2001). More precisely, Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria were mostly found in the epilimnion samples (0 to -30 m) of Lake Monoun and between 0 to -100 m in Lake Nyos samples. Meanwhile, the Deltaproteobacteria known to occur mainly in benthic environments (Hideyuki, 2005; Schwarz et al., 2007) were found in the hypolimnion samples of lake Monoun as also detected in Lake Tanganyika (Nold et al., 1998; de Wever et al., 2005).

Enterobacterales tribes represented by relative sequences detected in the 0 m sample of Lake Monoun are referred to as transient members of lake communities, brought in from the surrounding environment (Stoeckel et al., 2007). They have been detected at the surface of many lakes (Wiśniewska et al., 2007) and are indicators of pollution (Stoeckel et al., 2007).

Acidimicrobiales counted with the most represented sequences in Lake Nyos samples. They could contribute to iron species metabolism (Hideyuki et al., 2005; Newton et al., 2011) or

to CO_2 fixation (Johnson et al., 2009; Esparza et al., 2010). Surprisingly, acidimicrobiales relatives were not detected in Lake Monoun samples, despite that the lake contains higher amount of ferrous iron than Lake Nyos (Kusakabe et al., 2008). The non-detection of those species does not feature their inexistence in Lake Monoun, rather, it could be due to limitations of PCR-based fingerprinting and cloning techniques using universal primers that most easily amplify microbes which constitute at least 1% of the total ecosystem community (Casamayor et al., 2002; Lueders et al., 2003).

The second most represented phyla among the bacteria detected in Lake Nyos was Firmicutes, dominated by Bacillales relatives. This high prevalence suggests that they could constitute one of the major active microbial fractions and play important roles in biogeochemical dynamics and diverse degradation processes.

Then followed the Caldiserica which are known to metabolise sulfur compounds, Thiosulfate, sulfite and elemental sulfur (Mori et al., 2009); the Fusobacteria which are mostly known as parasitic and pathogenic bacteria (spectrum of human pathogens in human (Benett and Eley, 2003); the Chlorobi which are obligate anaerobic sulfur oxidizers (Hell et al., 2008) and Cyanobacteria, whose species inhabit or occasionally dominate, both near-surface epilimnic and deep, and hypolimnic waters of lakes. Cyanobacteria oxidize sulfide as their outmost important photosynthetic electron donor (Whitton, 1973). The later phyla could therefore be part of the sulfur cycling microorganisms of Lake Nyos.

Several candidate division matches were recovered among which OD1, OP8 and TM7 relatives. OD1 division members are not much known because they have never been cultivated and only partial genome have been recovered. They have been mostly found in anoxic environments, where they may play a role in sulfur and methane oxidation (Elshahed et al., 2005,

Peura et al., 2012). Other candidate divisions representatives, which are widely spread in the environment but their function is still unclarified were TM7 (Hugenholtz et al., 2001) and OP8 (Dhillon et al., 2003).

The occurrence of syntrophobacterales relatives in the -75 m sample of Lake Monoun is in accordance with the hypolimnic tribe representation of freshwater lakes (Hideyuki et al., 2005). They are metabolically versatile tribes whose many members are sulfate reducing bacteria. Some are unable to use sulfate as electron acceptor, but rather reduce protons or form formate. For this reason, they require H_2 utilizing organisms (methanogens or sulfate reducers) as syntrophic partners (Kuever et al., 2005). In the case of Lake Monoun, formate formation would be the most plausible pathway due to very low sulfate content (Kusakabe et al., 2008). Their trend to co-exist with methanotrophs is also featured in this study with the simultaneous detection with methylococcales in the -75 m sample.

4.1.3 Archaeal diversity in Lakes Nyos and Monoun

Archaeal communities in Lake Nyos appeared to be homogen as all the sequences matched with Thaumarchaeota tribes. However, the very low similarities to the closest match of some sequences suggest that they could represent other organisms than Thaumarchaeota or new species under this phyla. This is the case of, the NyosA9 genotype 2, matching with Marine Group I Thaumarchaeota (in the SILVA database), which rather matched with Methanolobus psychophilus R15, a methanogen species of the Euryarchaeota phyla in the NCBI database for bacteria and archaea. The variation with database was equally observed with the sequences of NyosA11 genotype 3_1, NyosA12 genotype 3_2, NyosA10 genotype 3 and NyosA15

genotype 5. Thaumarchaeota relatives were also found among Lake Monoun sequences. They were discovered to be ammonia oxidizers and their activity could also be linked to methane metabolism in the deep water sediments (Bar or et al., 2014).

The remaining archaeal sequences from Lake Monoun samples included Euryarchaeta relatives' Methanomicrobia with a very high similarity (97-98%), MCG and Halobacteria. MCG members, representing a considerable proportion of our archaeal libraries have been described as a highly diverse (seventeen subgroups) group. They have not been cultivated yet, hence the limited knowledge of any precise function. However, their widespread distribution and activity have suggested important role in marine sedimentary process *via* metabolic pathways using electrons acceptors other than sulfate (Kubo et al., 2012). They may, therefore, participate in carbon cycling in Lake Monoun. Halobacteriales are members of communities in hypersaline environments (Zvyagintseva et al, 1987).

4.1.4 Methane metabolism related genes in Lakes Nyos and Monoun

Methane gas which concentrations have been reported to increase over time in the lakes may contribute to increasing their total dissolved gas pressure (Issa et al., 2013). This implies a potential role of methane in triggering the overturn of the water columns. The stability and safety of Lakes Nyos and Monoun are therefore closely associated with methane. Hence, the interest to look closer at the diversity in the potential methane-related population in the lakes.

A very few proportion of the sequences recovered from Lake Nyos samples was related to methane metabolism. Here represented by the potential Methylophylale (NyosB23 genotype 17),

which are also known to play a role in methanol, nitrogen and sulfate metabolisms (Beck et al., 2014). The NyosB22 genotype16 could be another potential methanogen. It matched with Rhizobiales tribes in the SILVA database. However, a finer taxonomic affiliation obtained from the NCBI database indicates a high similarity (98%) with a Methylocystis echinoides strain. The latter organism is a type II methanotrophic bacterium (Dedysh et al., 2001). As for methanogenesis, the SILVA matches did not suggest any methanogen. However, the Thaumarchaeotal match of NyosA10 genotype 3 (Table 2) sequence could be a potential methanogen as it matches with Methanolobus psychophilus R15, a psychrophilic methanogen (Zhang et al., 2008b) in the NCBI DNA database for bacteria and archaea.

The putative methanogens from Lake Monoun samples were obtained from the bands A7, A8, A21, A26 and A28 and all matched with Methanomicrobiales. Dominance of Methanomicrobiales among methanogens has been reported in several meromictic lakes (Biderre-Petit et al., 2011; Nettmann et al., 2008; Selig et al., 1994). It can be noticed on the gel that a pattern similar to the pair A7/A8 is equally found in the lanes MB15, MB30 and MB75. Assuming that bands migrating to the same position in different lanes in a DGGE gel may have the same sequence, we could expect more other bands to represent potential methanogenic communities. Accordingly, they could account for the synthesis of methane in the lake, subject to their metabolic activity.

Recovery of methanogens-related sequences in samples from micro-aerated layers (2.9 ppm and 1.2 ppm DO at -15 and -30 m, respectively) are not in accordance with the strictly anaerobic metabolism featured in previous research (Yuan et al., 2009). Although methanogenesis is thought to be an exclusive anaerobic function, some microbial ecology research have reported their existence under aerobic conditions (Fetzer, 1993; Erkel et al., 2006). It has also been

demonstrated that methanogenesis could occur in aerated soils (Bauer et al., 2008; Peters et al., 1995) and its yield increase in microaerated digesters (Lim et al., 2013). Sequences related to methanogens were also detected in aerated layers of Lake Kivu (Lliros et al., 2010). The potential existence of methanogens in the microaerated layers of Lake Monoun infers the occurrence of water-mixing processes transporting the putative methanogenic microorganisms from the deeper anoxic layers to upper layers. Indeed, the low oxygen contents of the water column under ~ -8m could result from the double-diffusive convection (Schmid et al., 2004; Schmid et al., 2010) of ferrous iron from the bottom to the surface layers, where it combines with oxygen, hence creating anoxic conditions. CO_2 could, therefore, be transported in micro-scales to the upper water layers as well, and constitute a substrate for methanogenesis. Although this study suggests the presence of such species in oxygenated lake layers, their metabolic functioning remains a matter to be addressed.

It can be inferred from sequence analyses that the potential methanotrophs in Lake Monoun comprised Methanococcales. The latter tribes are known to oxidize dissolved CH_4 to CO_2, acting as a dynamic biofilter and undertaking a major role in greenhouse gas exchanges (Kessler et al., 2011; Rossi et al., 2013). They were detected simultaneously with Methylophylales. Their simultaneous occurrence has been reported in various environments (Qiu et al., 2009; Beck et al., 2013; Cabassi et al., 2014). However, Methylophylales may play no role in methane oxidation and were rather reported as denitrifiers (Ginige et al., 2004). The relevant coexistence of Methylococcales and Methylophylales suggest that they may be involved in cooperative function, the nature of which is still unknown (Beck et al., 2013). Likewise, both tribes play an important role in methanotrophy (Nercessian, 2005; Kalyuzhnaya et al., 2008; Chistoserdova et al., 2011).

4.2 Prokaryotes abundance along the water column (II)

The quantitative analysis was done only for Lake Monoun, due to insufficient samples for a similar analysis on Lake Nyos. qPCR showed the co-existence of communities of both domains in Lake Monoun samples, and revealed a pronounced discrepancy between both. Bacteria were numerically dominant over archaea in all the samples. The general decreasing trend of number of bacterial gene copies and increasing trend of number of archaeal gene copies with depth is in line with previous results obtained from other meromictic lakes (Lentini et al., 2012; Lliros et al., 2010). The high bacterial count in the 0 m sample of the central basin, could be related to the fact that surface water is subject to much fluctuation due to its contact with the external environment. Furthermore, the central basin is most used by fishermen. Interestingly, the increase in abundance materialized by peaks (-30 m in MB and CB for bacteria) on the qPCR plots (Figure 6) occurs at depths of important change in basic environmental parameters such as oxygen. It also corresponds to the oxic/anoxic zone of the lake from 2003-2005. Variations in communities' quantity could also be related to nutrients distribution along the water column (Nam et al., 2008; Steger et al., 2011). However, data on nutrients and previous microbial community studies on Lake Monoun are inexistent, making it very difficult to establish a causal relationship between the size of communities and environmental factors. Such increase in communities in the oxic/anoxic zones were observed in studies on Lakes Cadagno (Tonolla et al., 2005) and Kivu (Lliros et al., 2010). Further analyses are necessary to decipher whether the increase is related to all the detected communities or to specific tribes.

4.3 Biological pollution indicators in groundwater (III)

4.3.1 Total coliforms in water sources

The high coliform counts indicate that water supplies are unchlorinated, and may be of limited sanitary significance (WHO, 1997). In fact, TC bacteria are good indicators of treatment efficiency because of their sensitivity to chlorine (Gavini et al., 1985; Tallon et al., 2005) and inevitably grow where water supplies are not treated with chlorine (WHO, 1997). However, in some of the samples collected from wells around Lake Monoun, the high TC counts may rather indicate the inefficiency of chlorine treatment. During the sampling in this area, the owners often reported to treat their well with chlorine. The inefficiency may be due to the type and dose of chlorine, as well as the frequency of the treatment. Similarly, high TC counts were observed in urban and sub-urban areas in Cameroon (Katte et al., 2003; Ako et al., 2009; Kuitcha et al., 2010; Temgoua, 2011; Ateba et al., 2012; Wirmvem et al., 2013). According to the WHO guidelines, permissible counts in drinking water are 1 to 10 c.f.u. 100 ml^{-1} for TC (WHO, 1993). Although TC bacteria are generally thought not to cause illness, their presence in a water body indicates that the water supply may be vulnerable to contamination by harmful microorganisms.

4.3.2 Fecal coliforms and streptococci in water sources around Lakes Monoun and Nyos

The FC and FS bacterial indicators are evidence of a recent contamination by fecal material originating from humans or other warm-blooded animals, which contaminating material may contain disease-causing microorganisms such as certain bacteria, viruses, or other parasites

(Kravitz et al., 1999). The majority of the water samples analysed from both areas contained both FC and FS, thus failing to comply with the drinking water standards indicated by WHO. Water contamination with FC and FS was also reported in other areas of Cameroon (Djuikom et al., 2006). Poor protection, poor sanitation conditions and practices could be the source of contamination. As evidence among the samples of the Nyos area, "Nyos valley tap", a sub-surface water source appeared to have the less count of FC and no FS; meanwhile, "Nyos valley well" (an open well) located at about 25 meters from the "Nyos valley tap", showed higher counts of FC and FS. The difference of counts between the two water points may results from unclean material which probably entered the well from its opening, or via the container used to collect water. Another site that had relatively few count of bacterial indicator is the "Upper Nyos" spring located at a hilltop; this location likely contributes to receiving less runoff. The counts observed here infer that all the 17 samples represent a potential risk to human health.

4.3.3 Potential contamination sources in water around Lakes Monoun and Nyos

Among the water sources sampled around Lake Monoun, only the spring "Nfanmou" complied with the conformity guidelines of the WHO concerning FC and FS. Other sites such as "Forage peage", "Sansie gendarmerie" and "Sansie Njindou" showed relatively low counts of FC and FS, but still not complying with the water quality standards of the WHO. The variation observed in the microbial counts of water samples might be indicative of wide variation in hygiene behaviors. As observed during the sampling, people wash their clothes near or inside the water sources; others immerse containers in opened sources such as streams or wells to collect water, while some of the sources were channeled through pipes. It should be noted that some of the wells and streams

sampled for bacterial analysis in this study are not used by people for drinking but, for other purposes such as watering or rinsing vegetables that are eaten uncooked. Irrigating crops (raw eaten crops) with such water represent a route of infection by the parasites (Ntangmo et al., 2012). Likewise, using it in washing up dishes for immediate use constitutes a risk of contamination. Some of the water sources such as "Sansie marché 2" are located downstream of pit latrines; contamination of this site is evidenced by fecal bacteria contamination (FC: 340 c.f.u. 100 ml^{-1}, FS: 112 c.f.u. 100 ml^{-1}) and the FC:FS ratio of 3.04. In fact, a ratio of less than 0.7 strongly suggests warm blood animals waste other than human; a ratio between 0.7 and 1, would indicate the predominance of livestock waste; FC:FS values between 1 and 2 are considered as area of uncertainty; a ratio between 2 and 4 suggests the presence of human wastes mixed with other material (Olivieri, 1982; Edwards et al., 1997; Donderski and Wilk 2002; Djuikom et al., 2006). With respect to its FC count, the stream "Ngalle plantation" can be classified as a high risk water source, meaning that it represents a potential danger to human health. It also contained high TC and FS. This could be predictable from the disposal of farm products wastes in the stream during the various activities in the plantation. Samples from the two deep wells, "Ferme Banjou" and "Forage peage" contained both FC and FS, and only FC, respectively. Such bacterial contamination of underground water has been reported in several other studies (Nola et al., 2002; Erah et al., 2002; Obiri-Danso et al., 2009). The FC:FS ratio of "Ferme Banjou", suggests a contamination with animal wastes, which can be explained by the poultry farming on the site. As for "Forage peage", it was contaminated with FC. This suggests a contact of the water with fecal material; the pit latrine located at 24 m (horizontally) may represent a potential factor of contamination. Contamination of groundwater as studied by Caldwell and Parr (1937) defined varying bacterial transport distances (from 3 to 25 m). According to their study, a greater distance

reduces the extent to which microbes from pit latrine wastes may be transported and contaminate groundwater. Another risk could be the contamination by viruses that is likely to occur when latrines are found within a radius of 50 m in the vicinity of pumps or wells (Verheyen et al., 2009). Apart from the possible point sources of contamination pointed out in the previous paragraphs, faeces and urine deposited on the top and slopes of the hills by livestock or deposited by farmers as manure (Champaud et al., 1983; Tonfack et al., 2009) could constitute another important pollution factor via rainwater runoff.

Despite the relative cleanliness of the Nyos area, counts of the bacterial indicators in the water samples were high. Hygienic habits were identified as a potential factor of contamination of the wells. Whereas livestock faeces could be an important source of contamination for the other water sources, given the pastoral activities of the Nyos area inhabitants. In fact, livestock agriculture is considered one of the primary causes of bacterial contamination of surface and ground waters. This occurs during transport of pathogens with subsurface drainage water to surface water systems (O'Connor 2002; Rufete et al., 2006). Consistent with this, the FC:FS ratios of open surface water sources like Alieu, West spring RL 1 and 2 and Acha (ranging from 0.52 to 0.85) (Table 5) suggests a contamination by warm blooded animals wastes.

Chapter 5

5 CONCLUSIONS AND PROSPECTS

This first study of microbial ecology of Lakes Nyos and Monoun gives insights into the microbial community in both lakes. The results indicate a unique, rich and stratified niche whose distribution along the water column appeared to be dictated by environmental parameters in both lakes. The analyses of bacterial DNA sequences indicate a high proportion of new division candidates.

Lake Nyos eubacterial sequences were highly diverse, while all the archaeal sequences matched with Thaumarchaeota. Bacterial and archaeal sequences detected in Lake Monoun samples all belonged to various groups of microorganisms. All are susceptible to play important role on the lakes' biogeochemistry. This study also provides evidence that putative methanogenesis and methanotrophy genes account for a significant proportion of the metagenome of Lake Monoun. Analyses of Lake Nyos did not suggest the same. The methane related genes could, therefore, have important implications for the methane budget in Lake Monoun, subject to their functionality. It can be inferred that: on one hand, putative methanogenic archaea may use the geogenic CO_2, to produce methane in the anoxic or microaerated layers; existence of putative methanogenic genes using other pathways (for instance, the aceticlastic pathway) would be

clarified by studies using specific primers for methanogens. On the other hand, methane oxidation would likely be proceeded by methanotrophic bacteria in the aerated and anoxic layers of the lake. Functional and quantitative analyses of methane genes are required to get a better insight into the lakes' methane metabolism.

Furthermore, our results highlighted that PCR-DGGE fingerprinting could potentially represent an operable bio-monitoring approach to assess the lakes water microbial communities. Hence, characteristic bands could be used to monitor the evolution of important chemical species in the lakes ecosystem, thereby complementing the traditional physicochemical analyses. Moreover, biotechnological use of microbes in the mitigation of CO_2 (bio-mitigation) in the 2 lakes could be one of the future interests.

We obviously have got the bacterial community in both lakes samples as determined by DGGE, but this does not insure the disclosure of the whole bacterial and archaeal communities. Still, further studies addressing important issues such as the shifts in communities' structure and functional variations linked to the seasons' spatio-temporal changes, targeting specific genes in various biogeochemical cycles, and microorganisms culture would be more illuminating on the biogeochemistry of the lakes. Microbial diversity knowledge will be broader using high-throughput technologies.

The bacteriological assessment of drinking water quality led to the conclusion that the bulk of the water sources in the vicinities of Lakes Nyos and Monoun contained coliforms and streptococci. The presence of significant populations of coliform and non-coliform bacteria in water, identified as potential indicators of pollution may then impact the overall quality of untreated drinking water. The use of molecular methods for a higher sensitivity and specific culturable and/or non-culturable pathogens detection would be helpful in furthering research on

biological water quality in the study areas. Regular chlorine treatment of drinking water and wells, and boiling of water before drinking are recommended. The council should be aware of the hazard of waste accumulation on water quality. The government should fulfill the basic complacence of providing safe drinking water to the community.

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I- **Rosine E. Tiodjio**, Akihiro Sakatoku, Akihiro Nakamura, Daisuke Tanaka, Wilson Y. Fantong, Kamtchueng B. Tchakam, Gregory Tanyileke, Takeshi Ohba, Victor J. Hell, Minoru Kusakabe, Shogo Nakamura, and Akira Ueda. 2014. Bacterial and archaeal communities in Lake Nyos (Cameroon, Central Africa). *Scientific Reports (Nature Publishing Group)* doi:10.1038/srep06151.

II- **Rosine E. Tiodjio**, Wilson Y. Fantong, Kamtchueng B. Tchakam, Gregory Tanyileke, Takeshi Ohba, Victor J. Hell, Minoru Kusakabe, Shogo Nakamura, and Akira Ueda. 2015. Bacteriological assessment of drinking water sources in the vicinities of Lakes Nyos and Monoun (Cameroon, Central Africa). *Journal of Environmental Science and Water Resources (Wudpecker journals) 4(3) pp 060-070.*

III- Brice T. Kamtchueng,Wilson Y. Fantong, Akira Ueda, **Rosine E. Tiodjio**, Katsuro Anazawa, Mengnjo J. Wirmvem, Joseph O. Mvondo, Luc S. Nkamdjou, Minoru Kusakabe, Takeshi Ohba, Gregory Tanyileke, Joseph V. Hell. 2014. Assessment of shallow groundwater in Lake Nyos catchment (Cameroon, Central-Africa): implications for hydrogeochemical controls and uses. *Environ. Earth Sci. 72(9) pp 3663–3678.*

IV- Brice Tchakam Kamtchueng, Wilson Yetoh Fantong, Mengnjo Jude Wirmvem, **Rosine E. Tiodjio**, Alain Fouépé Takounjou, Kazuyoshi Asai, Serges L Bopda Djomou,

Minoru Kusakabe, Takeshi Ohba, Gregory Tanyileke, Joseph Victor Hell, Akira Ueda. 2015. A multi-tracer approach for assessing the origin, apparent age and recharge mechanism of shallow groundwater in the Lake Nyos catchment, Northwest, Cameroon. *Journal of Hydrology 523 pp 790–803.*

V- Mika Tawara, Akihiro Sakatoku, **Rosine E Tiodjio**, Daisuke Tanaka and Shogo Nakamura. 2015. Cloning and Characterization of a Novel Agarase from a Newly Isolated Bacterium *Simiduia* sp. Strain TM-2 Able to Degrade Various Seaweeds. *Applied Biochemistry and Biotechnology*. *In press*.

VI- **Rosine E. Tiodjio**, Akihiro Sakatoku, Issa, Wilson Y. Fantong, Daisuke D. Komatsu, Urumu Tsunogai, Kamtchueng B. Tchakam, Gregory Tanyileke, Takeshi Ohba, Victor J. Hell, Minoru Kusakabe, Daisuke Tanaka, Shogo Nakamura, and Akira Ueda. 2015. PCR-DGGE-based analysis of the prokaryotic communities of Lake Monoun, Cameroon. ***(Under preparation).***

LIST OF CONFERENCES/SYMPOSIUMS

I- **Rosine E. Tiodjio**, Akihiro Sakatoku, Akihiro Nakamura, Daisuke Tanaka, Wilson Y. Fantong, Kamtchueng B. Tchakam, Gregory Tanyileke, Takeshi Ohba, Victor J. Hell, Minoru Kusakabe, Shogo Nakamura, and Akira Ueda. 2013. "Bacterial community composition in the Nyos watershed (Cameroon): preliminary results." International Association of Volcanology and Chemistry of the Earth's Interior (IAVCEI), Commission of Volcanic Lakes, 8th Workshop on volcanic lakes, Kagoshima, p. 40., Kagoshima, Japan, July 2013.

II- **Rosine E. Tiodjio**, Akihiro Sakatoku, Akihiro Nakamura, Daisuke Tanaka, Wilson Y. Fantong, Kamtchueng B. Tchakam, Gregory Tanyileke, Takeshi Ohba, Victor J. Hell, Minoru Kusakabe, Shogo Nakamura, and Akira Ueda. 2013. "Microbial community diversity in Lake Nyos. *Volcanic lakes Symposium*, Tokai, Japan, September 2013.

III- **Rosine E. Tiodjio**, Akihiro Sakatoku, Akihiro Nakamura, Daisuke Tanaka, Wilson Y. Fantong, Kamtchueng B. Tchakam, Gregory Tanyileke, Takeshi Ohba, Victor J. Hell, Minoru Kusakabe, Shogo Nakamura, and Akira Ueda. PCR-DGGE-based assessment of the prokaryotes (bacteria and archaea) diversity in the water column of Lake Monoun, with a focus on the methane pathways. *Volcanic lakes Symposium*, Tokai, Japan, October 2014.

IV- Daisuke Tanaka, Kei Sato, **Rosine E. Tiodjio**, Akihiro Sakatoku and Shogo Nakamura. 2015. Characterization of airborne microbial communities at suburban and high-

altitude sites in Toyama, Japan. *9th Asian Aerosol Conference*, Kanazawa, Japan, June 2015.

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APPENDICES

Table A1: Primers and probes used for the study of prokaryotic communities in Lakes Nyos and Monoun.

Target gene	PCR Primers (forward/reverse)	Sequence (5' →3')	Program*	Reference
		Simple PCR Primers		
16S bacterial rDNA	GM3f /GM4r	AGAGTTTGATCMTGGC / TACCTTGTTACGACTT	1	Muyzer et al., 1995
	GC-341f /518r	CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-CCTACGGGAGGCAGCAG/ ATTACCGCGGCTGCTGG	2	Muyzer et al., 1993
16S archaeal rDNA	20f/958r	TTCCGGTTGATCCYGCCGGA/ATTACCGCGGCTGCTGG	3	Nam et al., 2008
	GC-340f /519r	CGCCCGCCGCGCGGCGGGCGGGGCGGGGGCGGCACGGG-CCCTACGGGGYGCASCAG/ TTACCGCGGCKGCTG	4	Nam et al., 2008
	109f /Uni1492r	ACTGCTCAGTAACACGT / CGGCTACCTTGTTACGAC	5	Sousa et al., 2007
	109(T)f /GC-515r	ACKGCTCAGTAACACGT/ CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGATCGTATTACCGCGGCTGCTGGCA	6	Sousa et al., 2007
	Ar3F/Uni1492r	TTCCGGTTGATCCTGCCGGA / CGGCTACCTTGTTACGAC	7	Nehmé et al., 2009
		qPCR primers		
16S bacterial rDNA	1055f/Uni1392r	ATGGCTGTCGTCAGCT / ACGGGCGGTGTGTAC	8	Baek et al., 2009
	16STaq1115	(FAM)-CAACGAGCGCAACCC-(TAMRA)		Harms et al., 2003
16S archaeal rDNA	ARC787F/ ARC1059R	ATTAGATACCCSBGTAGTCC / GCCATGCACCWCCTCT	9	Nehmé et al., 2009
	ARC915F	(FAM)-AGGAATTGGCGGGGGAGCAC-(TAMRA)	-	Nehmé et al., 2009
		Cloning primers		
	M13F/M13R	GTTTTCCCAGTCACGACGTT / GGAAACAGCTATGACCATGA	10	-
BigDye PCR			11	-

***** Details for PCR programs are indicated in table A2

Table A2: Programs for the PCR reactions in this study.

Program	Step	Temperature	Time	No. cycles
1	initial denaturation	94°C	3 min	1
	Denaturation	94°C	1 min	30
	Annealing	65°C	30 sec	
	Elongation	72°C	30 sec	
	Final elongation	72°C	3 min	1
	Hold	4°C		1
2	initial denaturation	94°C	3 min	1
	Denaturation	94°C	10 sec	20
	Annealing	65°C down 0.5/cycle	30 sec	
	Elongation	72°C	30 sec	
	Denaturation	94°C	10 sec	10
	Annealing	55°C	30 sec	
	Elongation	72°C	30 sec	
	Final elongation	72°C	3 min	1
	Hold	4°C		
3, 4	initial denaturation	94°C	3 min	1
	Denaturation	94°C	1 mi n	10
	Annealing	53°C down 1/cycle	1 min	
	Elongation	72°C	3 min	
	Denaturation	94°C	1 min	20
	Annealing	43°C	1 min	
	Elongation	72°C	3 min	
	Hold	4°C		1
5, 6	initial denaturation	94°C	3 min	1
	Denaturation	94°C	1 mi n	10
	Annealing	65°C down 1/cycle	40 sec	
	Elongation	72°C	1 min	
	Denaturation	94°C	1 min	10
	Annealing	55°C	30 sec	
	Elongation	75°C	1 min	
	Final elongation	72°C	5 min	1
	Hold	4°C		1

Table A2 (continued)

Program	Step	Temperature	Time	No. cycles
7	initial denaturation	95°C	5 min	1
	Denaturation	95°C	1 min	30
	Annealing	55°C	1 min	
	Elongation	72°C	1 min	
	Final elongation	72°C	5 min	1
	Hold	4°C		1
8,9	Hold	95°C	30 sec	1
		95°C	5 sec	40
		60°C	30 sec	
10	initial denaturation	94°C	3 min	1
	Denaturation	94°C	1 min	35
	Annealing	60°C	1 min	
	Elongation	72°C	1 min	
	Final elongation	72°C	3 min	1
	Hold	15°C		1
11	Hold	96°C	1 min	1
	Denaturation	96°C	10 sec	25
	Annealing	50°C	5 sec	
	Elongation	60°C	4 min	
	Hold	15°C		1

Table A3: DGGE parameters.

Sample	DNA volume (µl)	Acrylamide concentration	Denaturing gradient	Run time (h)	Voltage (V)	Temperature (°C)
Nyos (bacteria)	40	8%	25%-60%	14	70	60
Nyos (archaea)	40	8%	25%-60%	15	70	60
Monoun (bacteria)	100	8%	30%-60%	14	70	60
Monoun (archaea)	40	6%	25%-65%	17	70	60

Table A4: Physico-chemical parameters of the Lake Nyos water column in March 2013.

Depth (m)	Temp. (℃)	pH	C25 (μScm^{-1})	HCO_3^- ($mmolkg^{-1}$)	CO_2 ($mmolkg^{-1}$)	Fe^{2+} (mgl^{-1})
0.0	24.8	7.3	252.4	2.4	0.2	1.6
10.0	22.0	6.9	200.7	2.03	0.5	0.6
25.0	22.0	6.9	196.5	2.01	0.5	1.5
35.0	22.0	6.8	193.2	2.0	0.6	0.6
45.0	21.9	6.8	186.8	1.9	0.6	1.7
55.0	21.8	6.6	170.9	1.8	0.9	0.9
60.0	21.8	6.5	161.4	1.8	1.1	0.2
70.0	21.8	6.3	157.1	1.8	1.7	0.3
80.0	21.8	6.1	176.0	1.9	3.3	0.5
90.0	22.0	5.7	299.2	2.7	10.1	14.8
100.0	22.3	5.5	626.1	6.8	40.7	52.9
120.0	22.7	5.4	762.8	9.1	63.9	73.8
140.0	23.0	5.4	830.7	10.4	79.6	82.6
160.0	23.3	5.3	922.0	12.2	101.9	95.3
180.0	23.5	5.3	991.7	13.6	121.6	90.1
200.0	23.8	5.3	1056.1	15.0	135.5	102.5
210.0	24.2	5.6	2379.5	38.9	153.5	214.5

Table A5: Physico-chemical parameters of the Lake Monoun water column on March 2013.

Depth (m)	Temp. (℃)	pH	C25 (μScm^{-1})	HCO_3^- ($mmolkg^{-1}$)	CO_2 ($mmolkg^{-1}$)	Fe^{2+} (mgl^{-1})	O_2 (mgl^{-1})
0.0	25.4	7.5	130.4	157.4	1.5	2.2	16.185
10.0	20.5	6.7	147.0	175.3	2.4	2.8	3.61
20.0	20.1	6.6	154.8	179.7	2.5	5.5	2.346
30.0	19.8	6.7	190.5	230.3	3.0	12.4	1.26091
50.0	19.8	6.6	215.4	247.6	3.8	17.6	0.654
60.0	20.1	6.4	345.7	396.3	7.5	47.0	0.420769
70.0	20.5	6.2	471.7	555.5	11.8	81.9	0.356923
75.0	20.8	6.1	610.8	806.1	18.5	136.6	0.390909
85.0	22.1	5.6	1411.3	2139.6	84.1	392.9	0.655714
87.0	22.1	5.6	1417.3	2089.0	85.0	432.7	0.602
90.0	22.2	5.6	1428.3	2121.5	85.0	432.2	0.534
93.0	22.2	5.6	1439.7	2127.2	85.7	439.5	0.503158
95.0	22.7	5.7	1724.6	2546.7	92.5	541.6	0.597059
97.0	23.3	5.7	1979.1	3055.9	95.7	668.4	0.580476
98.0	23.5	5.7	2110.2	3287.7	97.8	725.4	0.562105