Geosphere-Biosphere Interactions in *Bio-Activity* Volcanic Lakes: Evidences from Hule and Río Cuarto (Costa Rica)

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**Abstract**

Hule and Río Cuarto are maar lakes located 11 and 18 km N of Poás volcano along a 27 km long fracture zone, in the Central Volcanic Range of Costa Rica. Both lakes are characterized by a stable thermic and chemical stratification and recently they were affected by fish killing events likely related to the uprising of deep anoxic waters to the surface caused by rollover phenomena. The vertical profiles of temperature, pH, redox potential, chemical and isotopic compositions of water and dissolved gases, as well as prokaryotic diversity estimated by DNA fingerprinting and massive 16S rRNA pyrosequencing along the water column of the two lakes, have highlighted that different bio-geochemical processes occur in these meromictic lakes. Although the two lakes host different bacterial and archaeal phylogenetic groups, water and gas chemistry in both lakes is controlled by the same prokaryotic functions, especially regarding the CO₂-CH₄ cycle. Addition of hydrothermal CO₂ through the bottom of the lakes plays a fundamental priming role in developing a stable water stratification and fueling anoxic bacterial and archaeal populations. Methanogens and methane oxidizers as well as autotrophic and heterotrophic aerobic bacteria responsible of organic carbon recycling resulted to be stratified with depth and strictly related to the chemical-physical conditions and availability of free oxygen, affecting both the CO₂ and CH₄ chemical concentrations and their isotopic compositions along the water column. Hule and Río Cuarto lakes were demonstrated to contain a CO₂ (CH₄, N₂)-rich gas reservoir mainly controlled by the interactions occurring between geosphere and biosphere. Thus, we introduced the term of *bio-activity* volcanic lakes to distinguish these lakes, which have analogues worldwide (e.g. Kivu: D.R.C.-Rwanda; Albano, Monticchio and Averno: Italy; Pavin: France) from volcanic lakes only characterized by geogenic CO₂ reservoir such as Nyos and Monoun (Cameroon).

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**Introduction**

Volcanic lakes are peculiar natural systems on Earth, although they are a common feature of volcanic systems characterized by recent activity, being present in 476 volcanic structures worldwide (VHub, CVL Group page; [1]). A volcanic lake simultaneously acts as both a calorimeter and a condenser for acidic volatiles from magmatic and hydrothermal degassing [2–6]. Thus, its existence and durability strictly depends on the balance between i) inputs of meteoric water and hydrothermal-magmatic fluids and ii) losses related to evaporation, permeation through sediments and streaming [7]. Volcanic lakes were basically classified, as follows [1,4]: i) “high-activity” lakes affected by the addition of significant amounts of hot and hyperacidic hydrothermal–magmatic fluids; ii) “low-activity” lakes, characterized by CO₂-dominated fluid inputs at a relatively low rate from sub-lacustrine fluids discharges, favoring the establishment of a stable vertical stratification and possibly the accumulation of high amounts of dissolved gases in the deep water layers. At these conditions, a lake overturn triggered by either i) external events, such as earthquakes, landslides or extreme
weather conditions or ii) the progressive attainment of gas saturation conditions may cause the abrupt release of toxic gas clouds in the atmosphere. This phenomenon, also known as “limnic eruption”, was firstly documented at Monoun and Nyos lakes (Cameroon) in 1964 and 1966, respectively [8–15]. Accordingly, low activity lakes are commonly indicated as “Nyos-type” lakes.

In Costa Rica, volcanic lakes are found in quiescent systems (Congo and Barva), as well as in volcanoes characterized by moderate hydrothermal activity (Irazú and Tenorio) and strong magmatic fluid emissions (Rincón de la Vieja and Poás) [16,17]. Hule and Río Cuarto are low-activity, Nyos-type, maar lakes located at 11 and 18 km N of Poás volcano (Fig. 1), respectively, in relation of a 27 km long fracture zone passing through the Sabana Redonda cinder cones, the Poás summit craters (Botos, Active Crater and Von Frantzius) and the Congo stratocone [18]. In these two lakes, changes in the water color and fish death events were repeatedly reported, suggesting the occurrence of rollover episodes related to inputs of deep-originated gases [18]. To the best of our knowledge, no information is available on these lakes for chemical and isotopic compositions of dissolved gases deriving from geogenic sources and the structure of prokaryotic communities. The latter are expected to play pivotal ecological functions, encompassing nutrient remineralization and carbon cycling, which is firmly linked to the fate of dissolved C1 gases, i.e. CH4 and CO2.

This paper presents the geochemical (water and dissolved gas chemistry) and microbiological results obtained from samples collected in 2010 during the 7th Workshop of the Commission on Volcanic Lakes (CVL; Costa Rica 10–21 March 2010), which is part of the International Association of Volcanology and Chemistry of the Earth’s Interior (IAVCEI), by a group of geochemists, limnologists, biologists and volcanologists from different universities and scientific institutions. The aim of this multidisciplinary research was to unravel the bio-geochemical processes controlling the physical-chemical features of Hule and Río Cuarto lakes along the vertical profiles, showing their implications for lake stratification and stability, and proposing evidences for a new classification system.

**Morphological and Limnological Outlines**

### 2.1 Morphological features

Lake Hule (10°17′42″N, 84°12′37″W) lies within the 2.3 × 1.8 km wide Hule basin, a volcanic depression also hosting Lake Congo to the north, which is separated from Lake Hule by a volcanic cone, and Lake Bosque Alegre (unofficial name) [18–20]. Lake Hule has a half-moon shape, a surface area of about 5.5 × 10^6 m², an estimated water volume of 6.9 × 10^6 m³, and a maximum depth of ~23 m [17,18,21,22] (Fig. 2). The northern shoreline of the lake shows three tributaries, whereas an emissary (Rio Hule) is located to the NE [18,23,24].

Río Cuarto maar (10°21′23″N, 84°13′00″W) has a rim whose maximum elevation is ~52 m a.s.l. Lake Río Cuarto shows steep sided walls and a flat bottom, a morphology typical of maar lakes. The lake has an E-W axis of 738 m, a mean width of 581 m, a surface of 3.3 × 10^6 m² and a water volume of 15 × 10^6 m³ [18,23] (Fig. 3). Río Cuarto is the deepest (~67 m) natural lake in Costa Rica [19]. A small tributary is located on the eastern shore, whereas no emissaries were recognized [25].

The main morphological features of Hule and Río Cuarto lakes can be summarized using the “depth-ratio” [26], which is a dimensionless parameter equal to the ratio between the average depth (the volume divided by the surface area of the lake) and the maximum depth of the lake. The obtained results are 0.55 and 0.68, respectively, for Lake Hule and Lake Río Cuarto, corresponding to an average depth of 12.6 and 45.5 m. According to Carpenter’s heuristic classification [26], the depth-ratio values are consistent with the so-called ellipsoid shape (typical values comprised between 0.5 and 0.66), considered a common feature for volcanic lake basins, even though Río Cuarto morphometry tends to approximate a steep-sided frustum model, corresponding to steep sides and flat bottom [27]. Such morphological features tend to prevent water vertical mixing, favoring meromictic conditions [28]. Thus, these physical parameters have a strong influence on the vertical distribution of chemical species, especially approaching the lake bottom where bio-geochemical processes have their maximum efficiency [29].

### 2.2 Limnological features and rollover events

At Lake Hule, the limit between epilimnion and hypolimnion, marked by a very weak thermocline and the complete depletion of O2, was reported to occur at a depth ranging between ~10 and ~12 m [23,24]. As reported by [22], this lake shows a persistent vertical stratification and the presence of CO2 in the deepest water strata. Occurrence of fish death episodes, associated with sudden changes of water color from dark blue to red and strong smell in the lake surroundings, were reported by the local population in the last years (4 to 5 events from 1989 to 2002). These events, which took place during the cool, rainy and windy season (i.e. from December to February), were interpreted as caused by rollover phenomena [16,17,18,30].

The transition between epilimnion and hypolimnion in the meromictic Lake Río Cuarto was measured at 20 and 25 m depth in May-June and January-February, respectively [18,25]. Rollover events, testified by fish killing and color changes of lake water from green to yellow-reddish, were observed in 1920 [31], between 1978 and 1991 [22], in January 1997 [16] and in February 2010 [18], just one month before our sampling. These events were possibly triggered by cooling of the shallow water layer caused by an anomalous weather characterized by low air temperature and strong winds [18,25,32].

**Materials and Methods**

### 3.1 Sampling of water and dissolved gases

Water and dissolved gas sampling was carried out in March 2010 along vertical profiles from the lake surface to the bottom at regular intervals of 5 m (Lake Hule) and 10 m (Lake Río Cuarto), in sites corresponding to the deepest points. Permission to sample in both lakes was guaranteed by Red Sismológica Nacional and Universidad de Costa Rica. According to the *single hose* method [33–35], water and dissolved gas samples were collected using a sampling line consisting of 10 m long Rilsan tubes (*Φ* = 6 mm) connected among them by steel connectors. Once the tube end was lowered to the chosen depth, water was pumped up to the surface through the sampling line using a 150 mL glass syringe equipped with a three-way tellon valve and transferred into plastic bottles after the displacement of a water volume double than the inner volume of the tube. One filtered (0.45 µm) and two filtered-acidified (with ultrapure HCl and HNO3, respectively) water samples were collected in polyethylene bottles for the analysis of anions, cations and trace species, respectively. A fourth water aliquot was collected in glass bottles with the addition of HgCl2 for the analysis of prokaryotic populations. The filters were stored at...
−20°C in RNA later solution (Quiagen, Italy), to prevent nucleic acid degradation. Dissolved gases were sampled using pre-evacuated 250 mL glass vials equipped with a Teflon stopcock and connected to the sampling line used to collect water samples. Sampling flasks were filled with water up to ¾ of the inner volume [36–38].

3.2 Field measurements

Water depth (m), temperature (°C), pH, Eh and electrical conductivity (EC; µS cm⁻¹) along the lake vertical profiles were measured using a Hydrolab MiniSonde 5 equipped with a data logger for data storage. The nominal precisions were: depth ±0.05 m; T±0.1°C; pH±0.2; Eh±20 mV; EC±1 µS cm⁻¹. Alkalinity was measured in situ by acidimetric titration using 0.01 N HCl. The analytical error for alkalinity analysis was ±5%.

3.3 Chemical and isotopic analysis of water and dissolved gases

Main anions (Cl⁻, SO₄²⁻, NO₃⁻, Br⁻ and F⁻) and cations (Na⁺, K⁺, Ca²⁺, Mg²⁺, NH₄⁺ and Li⁺) were analyzed by ion-chromatography (IC) using Metrohm 761 and Metrohm 861 chromatographs, respectively. The analytical error for major water constituents was ±5%. Trace elements at selected depths were analyzed at the INGV of Palermo by Inductively Coupled Plasma Mass spectrometry (ICP-MS, Agilent 7500-ce). For most of the elements the analytical uncertainty was in the order of 5-10% [39].

The ¹⁸O/¹⁶O and ²H/¹H isotopic ratios of water (expressed as δ¹⁸O-H₂O and δD-H₂O % vs. V-SMOW, respectively) from selected depths were analyzed using a Finnigan Delta plusXP continuous-flow mass spectrometer (MS) coupled with a GasbenchII gas-chromatographic device (GBII), according to equilibration techniques with CO₂ for oxygen [40], and with H₂ for
Figure 2. Panoramic view and bathymetric map of Lake Hule (modified after Göcke [24]).
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Figure 3. Panoramic view and bathymetric map of Lake Río Cuarto (modified after Göcke et al. [25]).

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| Lake          | depth | date    | T   | pH | Eh | HCO$_3^-$ | F$^-$ | Cl$^-$ | NO$_3^-$ | SO$_4^{2-}$ | Ca$^{2+}$ | Mg$^{2+}$ | Na$^+$ | K$^+$ | NH$_4^+$ | Fe$_{tot}$ | Mn | TDS | δD-H$_2$O | δ$^{18}$O-H$_2$O | δ$^{13}$C$_{DIC}$ | δ$^{13}$C$_{DICcalc}$ |
|--------------|-------|---------|-----|----|----|-----------|-------|-------|----------|------------|----------|--------|-------|------|--------|-----------|----|-----|----------|----------------|----------------|----------------|
| Hule         | 0     | March-10| 24.1| 7.0| 11 | 42        | 0.04  | 1.2   | 0.05     | 2.0        | 7.0      | 2.3     | 2.8   | 1.5  | 0.01  | 0.09 | 0.003 | 60       | −20.3        | −3.8             | n.a.            |
|              | 5     | March-10| 21.8| 6.5| 33 | 42        | 0.04  | 1.9   | 0.04     | 2.1        | 7.4      | 2.6     | 3.1   | 1.7  | 0.01  | n.a. | n.a.  | 61       | n.a.         | n.a.            | n.a.            |
|              | 10    | March-10| 21.1| 6.3| 23 | 46        | 0.04  | 1.2   | 0.09     | 2.2        | 7.2      | 2.5     | 3.5   | 1.5  | 0.2   | 0.03 | 0.92  | 66       | −19.8        | −3.7             | −11.8           |
|              | 15    | March-10| 20.9| 6.5| −170| 60       | 0.03  | 1.8   | 0.07     | 1.4        | 7.5      | 2.8     | 3.4   | 1.5  | 0.4   | n.a. | n.a.  | 79       | −n.a.        | n.a.            | n.a.            |
|              | 23    | March-10| 20.8| 6.6| −227| 61       | 0.04  | 1.2   | 0.07     | 1.9        | 8.2      | 2.7     | 3.6   | 1.5  | 0.3   | 0.07 | 0.78  | 90       | −22.5        | −3.9             | −14.3           |
| Rio Cuarto   | 0     | March-10| 27.9| 7.5| 166| 85       | 0.05  | 1.8   | 0.4      | 1.1        | 12       | 5.1     | 5.7   | 2.7  | 1.9   | 0.02 | 0.04  | 116      | −20.3        | −3.3             | −8.3            |
|              | 10    | March-10| 24.7| 6.8| −191| 92       | 0.04  | 1.9   | 0.6      | 0.88       | 13       | 5.1     | 5.5   | 2.7  | 2.0   | 3.1   | 0.27  | 127      | −19.7        | −3.4             | −7.9            |
|              | 20    | March-10| 24.7| 6.8| −215| 93       | 0.04  | 1.7   | 0.1      | 0.91       | 13       | 5.1     | 5.5   | 2.7  | 2.1   | n.a. | n.a.  | 124      | −78          | −8.8             | n.a.            |
|              | 30    | March-10| 24.7| 6.8| −230| 93       | 0.04  | 1.9   | 0.03     | 1.1        | 13       | 4.9     | 5.5   | 2.7  | 2.1   | n.a. | n.a.  | 124      | −86          | −8.8             | n.a.            |
|              | 40    | March-10| 24.6| 6.8| −243| 103      | 0.05  | 2.1   | 0.03     | 0.95       | 14       | 5.0     | 5.5   | 2.8  | 2.4   | 3.6   | 0.27  | 140      | −22.2        | −3.4             | −8.4            |
|              | 50    | March-10| 24.7| 6.6| −239| 105      | 0.05  | 1.8   | 0.03     | 0.67       | 13       | 5.1     | 5.6   | 2.8  | 3.3   | 5.4   | 0.36  | 143      | −22.6        | −3.5             | −5.1            |
|              | 60    | March-10| 24.7| 6.5| −245| 163      | 0.06  | 1.8   | 0.08     | 0.51       | 14       | 5.6     | 5.9   | 3.3  | 9.0   | 15.6  | 0.63  | 219      | −24.5        | −3.7             | −3.7            |
|              | 67    | March-10| 24.7| 6.6| −246| 179      | 0.05  | 1.9   | 0.09     | 0.42       | 15       | 6.0     | 6.1   | 3.5  | 11    | 22.6  | 0.66  | 246      | −23.6        | −3.6             | −5.2            |

Ion contents and TDS are in mg L$^{-1}$. n.a.: not analyzed; n.d.: not detected.
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hydrogen [41]. The analytical uncertainties were ±0.08% and ±1% for δ18O and δD values, respectively.

The 13C/12C ratios of TDIC (expressed as δ13C_{TDIC} % vs. V-PDB) at selected depths were determined on CO2 produced by reaction of 3 mL of water with 2 mL of anhydrous phosphoric acid in vacuum [42] using a Finningan Delta Plus XL mass spectrometer. The recovered CO2 was analyzed after a two-step extraction and purification procedures of the gas mixtures by using liquid N2 and a solid-liquid mixture of liquid N 2 and trichloroethylene [43,44]. The analytical uncertainty was ±0.05 %.

Dissolved gas composition was calculated using i) the composition of the gas phase stored in the headspace of the sampling glass flasks, ii) the gas pressure in the flask headspace, iii) the headspace volume, and iv) the solubility coefficients in water of each gas compound [45]. The inorganic gas compounds hosted in the flask headspace (CO2, N2, CH4, Ar, O2, Ne, H2 and He) were determined using a gas-chromatograph (Shimadzu 15a) equipped with a Thermal Conductivity Detector (TCD). Methane was analyzed with a Shimadzu 14a gas-chromatograph equipped with a Flame Ionization Detector (FID). The analytical error for dissolved gas analysis was ±5%.

The analysis of the 13C/12C ratios of CO2 (expressed as δ13C-CO2 % vs. V-PDB) stored in the flask headspace (δ13C-CO2_{STRIP}) of selected samples was carried out with a Finningan Delta S mass spectrometer after standard extraction and purification procedures of the gas mixtures [43,44]. Internal (Carrara and San Vincenzo marbles) and international (NBS18 and NBS19) standards were used for the estimation of external precision. The analytical uncertainty was ±0.05%. The 13C/12C ratio of dissolved CO2 (δ13C-CO2) was calculated from the δ13C-CO2_{STRIP} values using the ε1 factor for gas-water isotope equilibrium proposed by Zhang et al. [46], as follows:

\[ \varepsilon_1 = \delta^{13}C_{CO_2} - \delta^{13}C_{CO_2_{STRIP}} = (0.0049 \times T) - 1.31 \]  

The analysis of the 13C/12C and 2H/1H ratios of dissolved CH4 (expressed as δ13C-CH4 % vs. V-PDB and δD-CH4 % vs. V-SMOW, respectively) of selected samples was carried out by mass spectrometry (Varian MAT 250) according to the procedure and the sample preparation described by Schoell [47]. The analytical uncertainty was ±0.15%.
The $^3\text{He}/^4\text{He}$ ratios, expressed as $R/\text{Ra}$ values, where $R$ is the $^3\text{He}/^4\text{He}$ isotopic ratio in gas samples and $\text{Ra}$ is that of the air equal to $1.39 \times 10^{-6}$ [48,49], were determined in selected gas samples stored in the sampling flask headspace at the INGV laboratories of Palermo, using the method described in Inguaggiato and Rizzo [50]. The $R/\text{Ra}$ values were corrected for air contamination on the basis of measured He/Ne ratios. The analytical uncertainty was $\pm 0.3\%$.

3.4 Microbiological analysis

DNA extraction for the analysis of microbial populations was performed according to the protocol reported by Mapelli et al. [51] and quantified by NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). 16S rRNA gene was amplified in PCR reactions using universal primers for bacteria with GC-clamp as described in Marasco et al. [52]. Denaturing Gradient Gel Electrophoresis (DGGE), applied to the bacterial 16S rRNA gene amplified from the total water metagenome, was performed by loading DGGE-PCR products (~150 ng) in a 0.5 mm polyacrylamide gel (7% w/v) acrylamide-bisacrylamide, 37.5:1) containing 40 to 55% urea-formamide denaturing gradient, where 100% denaturant corresponds to 7 M urea and 40% [vol/vol] formamide [52]. DGGE profiles were analyzed by using Image J software (available at http://rsb.info.nih.gov/ij/) and cluster analysis was performed using Microsoft Excel XLSTAT software (Addinsoft Inc., New York, NY, USA). DGGE bands were excised from the gel, eluted in water, PCR amplified and sequenced as previously described [52]. The partial 16S rRNA gene sequences obtained from the excised DGGE bands were edited in Chromas lite 2.01 (http://www.technelysium.com.au) and subjected to BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The nucleotide sequences were deposited in the EMBL public database under the accession numbers HF930552-HF930593. To test the presence of bacteria involved in anaerobic ammonium oxidation (anammox), the functional gene $\text{hzaA}$ was amplified using primers $\text{hzaA}_{526\text{F}}$ and $\text{hzaA}_{1857\text{R}}$ as previously reported [53].

454 pyrosequencing assays were performed by using universal-bacterial primers targeting the variable regions of the 16S rRNA, V1-V3 (27 F mod 5' – AGRGGTTTGTACMTGGCTCAG – 3'; 519 R mod bio 5' – GTNTTACNGCGCAGKCCTG - 3'), amplifying a fragment of approximately 400 bp, and 16S rRNA archaeal primers arch344F (5' – ACGGGGCGACGAGGCAGCG - 3') and arch915R (5' – GTGCCCTCCCGCAATTCCCT - 3'). The amplified 16S rRNA regions contained enough nucleotide variability to be useful in identification of bacterial and archaeal species [54,55]. PCR reactions and next generation 454 pyrosequencing were performed at MR DNA laboratories (Shallowater, TX – U.S.A.). A first quality filtering was applied, removing all the sequences that were shorter than 300 bp, longer than 500 bp or with an average quality score under 25. All original and non-chimeric 454 sequences are archived at EBI European Read Archive. The high-quality 16S rRNA gene sequences obtained by 454 pyrosequencing were analysed using QIIME [56]. The sequences were clustered into operational taxonomic units based on a threshold of 97% (OTU97) sequence identity, using uclust [57] and one sequence for each OTU97, as representative, was aligned to Greengenes (http://greengenes.lbl.gov/) using PyNast [56]. Sequence identification was conducted using Ribosomal Database Project classifier [58], with default parameters. For each sample rarefaction curves of the observed species and of Shannon index were estimated in order to analyse the species sampling coverage. The OTU97 diversity within and between sample/s (respectively alpha and beta diversity) was estimated using QIME workflow script alpha_rarefaction.py. Shannon diversity index was calculated by PAST software [59]. Library coverage was calculated for each library using the equation $C = [1 - (n1/N)] \times 100$, where $n1$ is the number of singleton OTU97, and $N$ is the total number of reads in the library. To remove noise from the data, including potential rare contaminants, OTU97 not meeting the criterion of being present at least 0.1% of the total number of reads were removed.

Figure 5. Vertical profiles (in mg L$^{-1}$) of HCO$_3^-$, NO$_3^-$, SO$_4^{2-}$, NH$_4^+$, Fe$_{tot}$ and Mn in Lake Hule (a) and Lake Rio Cuarto (b).

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Results

4.1 Vertical profiles of temperature, EC, pH and Eh

Temperature, EC, pH, and Eh along the vertical profiles of the lakes are shown in Tab. 1 and Fig. 4. Both Hule and Río Cuarto lakes showed relatively high temperature at the surface (24.1 and 27.9°C, respectively), and a thermocline at shallow depths (starting from −2.5 and −5 m, respectively), with minimum temperatures of 20.5 and 24.6°C, respectively, at the lake bottom (Fig. 4a). The temperature profiles were consistent with those reported in previous studies [17, 18, 23, 24, 32, 36], except those of the epilimnion, likely because present and past measurements were carried out in different periods of the year. Lake Hule did not show a clear chemocline, as shown by the EC values that almost constantly increased (from 84 to 140 µS cm⁻¹) with depth (Fig. 4b). Conversely, Lake Río Cuarto showed two chemoclines: the first one (from 159 to 186 µS cm⁻¹) near the surface and the second one (from 190 to 378 µS cm⁻¹) between −40 and −67 m depth. The vertical profile of pH values at Lake Hule exhibited a sharp decrease from 6.9 to 6.3 between the depths of 0 m and 10 m, and an opposite trend below this depth, where pH rose from 6.3 to 6.6 (Fig. 4c). At Lake Río Cuarto the pH values decreased in the shallower water strata (from 7.5 to 6.8) and from −40 to −60 m depth (from 6.8 to 6.5), and slightly increased (up to 6.6) at the lake bottom (Fig. 4c). Eh values at Lake Hule (Fig. 4d) showed a sharp decrease between −10 and −15 m (from 35 to −163 mV) and reached the minimum values at lake bottom (−200 mV), whereas at Lake Río Cuarto it strongly decreased (from +166 at surface to −191 mV) at the depth of 10 m displaying the lowest value (−246 mV) at the lake bottom.

4.2 Chemical and isotopic composition of water samples

Both lakes showed low TDS values (up to 90 and 246 mg L⁻¹, respectively, at lakes bottom) and a Ca^{2+}-HCO₃⁻ composition (Tab. 1). Concentrations of HCO₃⁻, NH₄⁺, Fetot and Mn (Fig. 5a–b) tended to increase towards the two lakes bottom (up to 61 and 179 mg L⁻¹, 0.3 and 11 mg L⁻¹, 0.3 and 22 mg L⁻¹, 0.9 and 0.7 mg L⁻¹ in Hule and Río Cuarto, respectively), whilst oxidized nutrients NO₃⁻ and SO₄²⁻, typical electron acceptors in anaerobic environments, showed an opposite behaviour in Lake Río Cuarto, decreasing to 0.03 and 0.4 mg L⁻¹, respectively (Fig. 5b). On the contrary, F⁻, Cl⁻, Ca^{2+}, Mg²⁺, Na⁺, K⁺ and, only in Lake Hule, NO₂⁻ and SO₄²⁻, did not display specific vertical trends along the lakes water column.

The δD-H₂O values in Hule, in Río Cuarto, and in Lake Río Cuarto ranged from −26.5 to −20.5 % and −24.4 to −19.7 % V-SMOW, respectively, while those of δ¹⁸O-H₂O varied from −4.7 to −4.6 % and from −4.5 to −4.0 % V-SMOW, respectively (Tab. 1). The δ¹³CNDIR, values were between −14.5 and −11.9 % and −8.6 to −5.7 % V-PDB, in Hule and Río Cuarto, respectively.

Trace element composition did not differ significantly between the two lakes. The most abundant trace elements (>4 µg L⁻¹) along Hule and Río Cuarto vertical profiles were Al, B, Ba, Rh, Sr and Zn. The maximum concentrations of Co, Cu, Ni, Ti and V (< 2.2 µg L⁻¹) were observed at the bottom layer of Lake Río Cuarto (−67 m) and the other measured trace elements (As, Cd, Cr, Cs, Li, Mo, Pb, Sb, Se, Th, U) were all <1 µg L⁻¹ (Tab. 2). In terms of vertical distribution, those trace elements that clearly increased towards both lakes bottom were Al, As, Ba, Co, Ni, Sr, Ti and V (Tab. 2), whilst Mo concentrations showed a decrease with depth only in Lake Río Cuarto.
Table 3. Chemical composition (µmol L\(^{-1}\)) and total pressure (pTOT; in atm) of dissolved gases (CO\(_2\), N\(_2\), CH\(_4\), Ar, O\(_2\), Ne, H\(_2\) and He) and \(\delta^{13}\)C-CO\(_2\) (expressed as \%' V-PDB), \(\delta^{15}\)C-CH\(_4\) (expressed as \%' V-PDB), \(\delta^D\)CH\(_4\) (expressed as \%' V-SMOW) and R/Ra values of gas samples collected.

| lake         | depth | CO\(_2\) | N\(_2\) | CH\(_4\) | Ar  | O\(_2\) | Ne  | H\(_2\) | He  | pTOT | \(\delta^{13}\)C-CO\(_2\) | \(\delta^{15}\)C-CH\(_4\) | \(\delta^D\)CH\(_4\) | R/Ra | He/Ne |
|--------------|-------|----------|--------|--------|-----|--------|-----|--------|-----|------|------------------|------------------|----------------|------|-------|
| Hule         | 0     | n.a.     | n.a.   | n.a.   | n.a.| n.a.   | n.a.| n.a.   | n.a.| n.a. | n.a.             | n.a.             | n.a.           | n.a. | n.a.  |
|              | 5     | 29       | 520    | n.d.   | 13  | 160    | 0.06| 0.007  | n.d.| 0.88 | n.a.             | n.a.             | n.a.           | n.a. | n.a.  |
|              | 10    | 191      | 537    | 1.7    | 12  | n.d.   | 0.005| 0.005  | 0.005| 0.79 | n.a.             | n.a.             | n.a.           | n.a. | 1.0   |
|              | 15    | 257      | 552    | 66     | 13  | n.d.   | 0.006| 0.001  | 0.008| 0.85 | n.a.             | n.a.             | n.a.           | n.a. | 1.4   |
|              | 23    | 1090     | 618    | 232    | 15  | n.d.   | 0.008| 0.03   | 1.1  | \(-16.2\) | \(-62.5\)            | \(-159\)          | 0.95           | 4.1  |
| Rio Cuarto   | 0     | n.a.     | n.a.   | n.a.   | n.a.| n.a.   | n.a.| n.a.   | n.a.| n.a. | n.a.             | n.a.             | n.a.           | n.a. | n.a.  |
|              | 10    | 239      | 528    | 73     | 12  | 0.33   | 0.006| 0.01   | n.d.| 0.87 | n.a.             | n.a.             | n.a.           | n.a. | n.a.  |
|              | 20    | 357      | 524    | 206    | 13  | n.d.   | 0.007| 0.02   | n.d.| 0.96 | \(-14.3\)          | \(-60.7\)         | \(-233\)        | n.a. | n.d.  |
|              | 30    | 751      | 559    | 546    | 13  | n.d.   | 0.007| 0.02   | 0.31| 1.3  | \(-14.2\)          | \(-61.9\)         | \(-239\)        | n.a. | 45    |
|              | 40    | 1045     | 576    | 746    | 14  | n.d.   | 0.007| 0.02   | 0.05| 1.4  | \(-13.9\)          | \(-63.8\)         | \(-241\)        | n.a. | 7.6   |
|              | 50    | 1450     | 522    | 1080   | 13  | n.d.   | 0.007| 0.03   | 0.09| 1.6  | \(-11.6\)          | \(-72.3\)         | \(-250\)        | 1.15 | 13    |
|              | 60    | 2090     | 538    | 2435   | 13  | n.d.   | 0.006| 0.05   | 0.25| 2.6  | \(-6.5\)           | \(-74.8\)         | \(-248\)        | n.a. | 39    |
|              | 67    | 1790     | 532    | 2830   | 13  | n.d.   | 0.007| 0.04   | 0.34| 2.9  | \(-6.6\)           | \(-77.2\)         | \(-251\)        | 1.09 | 49    |

Dissolved gas concentrations are in µmol L\(^{-1}\). n.a.: not analyzed; n.d.: not detected.

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4.3 Chemical and isotopic composition of dissolved gases

Molecular nitrogen was the most abundant dissolved gas in the shallow portion of the two lakes (down to the depths of 15 m and 20 m at Lake Hule and Lake Rio Cuarto, respectively; Tab. 3). At lower depths CO₂ dominated the gas composition (up to 1090 and 2090 mol L⁻¹ at Lake Hule and Lake Rio Cuarto, respectively), except at the bottom of Lake Rio Cuarto (Fig. 6a–b) where CH₄ concentrations up to 2830 mol L⁻¹ were measured. O₂ is not present below 10 m depth at Hule and Rio Cuarto, defining a clear anaerobic zone (Fig. 6a–b). Ar and Ne did not vary significantly with depth, whereas H₂ and He concentrations increased with depth in both lakes (up to 0.01 and 0.03 mol L⁻¹ and to 0.04 and 0.3 mol L⁻¹ in Hule and Rio Cuarto, respectively; Tab. 3). It is noteworthy to point out that He was an order of magnitude more abundant at Rio Cuarto than at Hule. The maximum total pressure (pTOT; Tab. 3) value of dissolved gases was measured at the bottom of Lake Rio Cuarto (2.9 atm), whereas pTOT in Lake Hule ranged from 0.79 to 1.1 atm.

The δ¹³C-CO₂ value at the bottom of Lake Hule was −16.2 % V-PDB (Tab. 3). At Lake Rio Cuarto, the δ¹³C-CO₂ values showed an increase with depth, ranging from −14.3 at −20 m to −6.5 % V-PDB at the lake bottom. No specific trends were recognized in the epilimnion (Fig. 7). The δ¹³C-CH₄ values, basically characterized by the same interval (from −77.2 to −60.7 % V-PDB) in both lakes, showed a rapid decrease in the Lake Rio Cuarto hypolimnion. The δD-CH₄ values of Lake Rio Cuarto were significantly more negative (from −251 to −233 % V-SMOW) when compared to that of Lake Hule bottom (−159 % V-SMOW; Tab. 3). The R/Ra values, corrected for the presence of atmospheric helium [61], were 0.95 in Lake Hule (lake bottom) and 1.15 and 1.09 in Lake Rio Cuarto (at −50 and −67 m depth, respectively; Tab. 3).

4.4 Prokaryotic diversity along the water column

Phylogenetic analyses of 16S rRNA DGGE derived sequences (Fig. 8a–b) allowed to detect 7 phyla within the bacterial communities and to identify the prevalent taxonomic groups colonizing the Hule and Rio Cuarto lakes at different depths (Tab. 4). Overall, the sequences were related to uncultured unclassified bacteria previously described in aquatic environments, mainly represented by freshwater of lacustrine origin.

At Lake Hule a clear shift in taxa distribution was evaluated, corresponding to the transition at 10 m depth of the redox potential from positive to negative. The lake epilimnion was mainly colonized by aerobic heterotrophic Bacteroidetes and Betaproteobacteric while deeper anoxic layers (>10 m depth; Fig. 4d) were inhabited by bacteria belonging to the phylum Chlorobi, comprising anaerobic photoautotrophic bacteria (Chlorobium clathratiforme and Ignavibacterium album). Bacteroidetes and Betaproteobacteria phyla were also the main components of the bacterial community in Lake Rio Cuarto. In this lake the shallower portion (down to the depth of 40 m) was colonized by Cyanobacteria affiliated to the genera Synechococcus, Merismopedia and Cyanobium. Differently from Lake Hule, the more uniform composition of the bacterial community in Lake Rio Cuarto can be related to the homogeneity of the redox conditions along the water column, which is negative in all the analyzed layers except at the lake surface (Fig. 4d).

The results of DGGE analysis were taken into account to select a sub-set of samples to gain a deeper insight into the microbiome structure by massive pyrosequencing of bacterial and archaeal 16S rRNA libraries. This high-throughput analysis was applied to 3 samples for each lake (0, 10, 15 m depth from Lake Hule, named H0, H10 and H15, and 30, 50, 60 m depth from Lake Rio Cuarto, named RC30, RC50 and RC60). Unfortunately, any archaeal library could not be obtained from sample H0. The number of final reads varied among the samples, similarly to the OTU number, nonetheless a significant coverage of bacterial and archaeal diversity was reached in all the samples (Tab. 5).
number of OTU97 present in the archaeal communities was constant along the water column of Lake Rio Cuarto, while in Lake Hule a significant increase was observed with depth (Tab. 5). In all the samples, Proteobacteria were the most abundant bacterial phylum, with the exception of the water samples collected from Lake Rio Cuarto at 50 and 60 m depths (RC50 and RC60) where Cyanobacteria and Chloroflexi were the prevalent phyla, respectively (Tab. 6). Cyanobacteria were also present at high percentage (29.4%) in the oxic surface water sample in lake Hule (Tab. 6). The phylum Chlorobi was widespread in both the lakes in all the samples characterized by negative Eh values, with significant prevalence at 10 and 15 m depth in Lake Hule (10.5 and 17.6%, respectively). Among Proteobacteria, the Epsilon-subgroup was a minor community component in both lakes and Deltaproteobacteria were more abundant in Río Cuarto, especially in the deeper layers (Tab. 6). Among Proteobacteria, the Epsilon-subgroup was a minor community component in both lakes and Deltaproteobacteria were more abundant in Río Cuarto, especially in the deeper layers (Tab. 6). Alpha- and Gamma-proteobacteria were differently distributed in the two lakes. The latter were particularly abundant in shallower Hule layers (H10 and H15), while the former were present at high percentages throughout the whole Hule water column (Tab. 6). The class Betaproteobacteria, mainly represented by the Comamonadaceae and Methylophilaceae families, was abundant at all depths in both the lakes (Tab. 6). In Lake Hule between 12.9 and 22.8% of the bacterial community was represented by sequences belonging to the ACK-M1 cluster of the order Actinomycetales, whose presence in lacustrine habitats was previously reported (Tab. 6) [62]. At the oxic-anoxic interfaces, anaerobic ammonium oxidation (anammox) was indicated as an autotrophic denitrification metabolism co-responsible of nitrogen loss from water environments [63]. The research of bacterial taxa known to be responsible of anammox reaction was performed by amplifying with specific primers the functional gene hzsA, encoding for hydrazine synthase and recently proposed as an anammox phylomarker [53]. The PCR amplification showed negative results, confirming that anammox populations are absent at Hule and Río Cuarto lakes.

As far as the archaeal community is concerned, Euryarchaeota were the most abundant phylum in Lake Rio Cuarto (up to 99%). Methanomicrobia were the most abundant class within this phylum, encompassing in particular the orders Methanomicrobiales and Methanosarcinales (Tab. 7). Lake Hule showed a different archaeal community, being dominated by Parvarchae and Micrarchae, with significant concentrations of Crenarchaeota (8.1 and 13.7% at 10 and 15 m depth, respectively), and a minor percentage of Methanomicrobia and unknown taxa (Tab. 7).

![Figure 7. Vertical distribution of $\delta^{13}$C-CO$_2$ and $\delta^{13}$C-CH$_4$ of Lake Río Cuarto.](https://www.plosone.org/doi/10.1371/journal.pone.0102456.g007)

Figure 7. Vertical distribution of $\delta^{13}$C-CO$_2$ and $\delta^{13}$C-CH$_4$ of Lake Río Cuarto. See the text for further details. doi:10.1371/journal.pone.0102456.g007
Discussion

5.1 Processes controlling the water chemistry

Water isotopes can provide notable information on physical-chemical processes affecting the chemistry of volcanic lakes, such as evaporation, water-rock interaction and hydrothermal/meteoric inputs [64]. As shown in Fig. 9, water samples plot near the Global Meteoric Water Line (GMWL) [65] and the Costa Rica Surface Water Line [66], indicated that in both lakes the main water source is meteoric, consistently with their Ca²⁺(Mg²⁺)-HCO₃⁻ composition, which is typical for superficial waters and shallow aquifers worldwide [67]. Both lakes show a slight D- and ¹⁸O- depletion at increasing depth, likely related to evaporation affecting epilimnetic waters [64,68,69].

The parallel increases of HCO₃⁻ (Fig. 5) and dissolved CO₂ (Fig. 6) along the vertical profiles suggest that the behaviour of these two chemical species is controlled by the following reactions:

\[ CO_2 + H_2O \rightarrow H_2CO_3 \quad (2) \]

and

\[ H_2CO_3 + H_2O \rightarrow H_3O^+ + HCO_3^- \quad (3) \]

The observed weak decreases of SO₄²⁻ and NO₃⁻ concentrations (Fig. 5) with depth possibly result from microbial activity occurring at anoxic conditions. The lack of free oxygen in the hypolimnion is favorable for nitrate reduction by microbial denitrification, a typical process in anoxic water bodies [70–74]. The genus *Pseudomonas* is known to include denitrifier species [75] and was retrieved at high abundance in pyrosequencing libraries in the anoxic layers of Lake Hule, constituting up to 16% of the total bacterial community (Tab. 6). In the Hule anoxic layers, 16S rRNA pyrosequencing allowed to detect additional denitrifying genera like *Sulfuricurvum, Opitutus* and *Geothrix* (Tab. 6). Sulfate reducing bacteria (SRB) of the genus *Geothrix* (Fig. 5b). Nevertheless, the relatively low SO₄²⁻ and NO₃⁻ concentrations, typical of meteoric-sourced lakes, implies that sulfate reduction and denitrification have a minor impact on the chemistry of the two lakes. The increase of NH₄⁺ concentrations with depth (Fig. 5) is apparently suggesting direct NH₄⁺ production within the hypolimnion via ammonification processes [76].

The increase of Fe and Mn contents in the deepest water layers can be attributed to direct production inside the bottom sediments by minerogenic processes [77–79], although their presence as solutes is limited by the formation of insoluble Fe- and Mn-hydroxides. Gocke [24] suggested that the high concentration of Fe in Lake Hule is also caused by the addiction of yellow/brownish Fe(OH)₃ material through the southern brooklets, which subsequently precipitates in the hypolimnion and iron is reduced to the ferrous state, as also supported by the relatively low Eh values (Fig. 4). Oxidation of hypolimnetic Fe²⁺ in the epilimnion would explain the yellow-reddish color of the shallow water layer that was occasionally observed in these lakes as a consequence of water rollover [18,25]. Nevertheless, the red coloration observed at Lake Hule in February 1991 was likely caused by the presence of dense purple clumps or masses floating of *Merismopedia* [18], a genus belonging to the phylum Cyanobacteria that were observed by DGGE at −30 and −40 m depth in Lake Río Cuarto (Tab. 4).

As shown in the spider-diagrams of Fig. 10, where concentrations of Al, Ba, Cr, Cu, Ni, Rh, Sr, Ti and V at maximum depths for both lakes are normalized to those measured in basalt rock samples collected from the young intra-caldera cone at Laguna Hule (the only one available) [80], water-rock interactions efficiently mobilized soluble elements such as Ba, Rb and Sr, whereas Al and Ti were basically retained in the rock matrix. In particular, Cr and Ni, as well as As and Co, are possibly related to the dissolution of Mn-and Fe-oxide particles that settled through the chemocline [78,81,82]. The concentrations of dissolved V are strongly correlated with those of Fe, similarly to what observed for Mo and Mn [29,83], likely because they belong to the same mineralogical paragenesis. For what concerns the other trace elements, Cu and Zn may be related to dissolution of stable organic complexes buried in the bottom sediments [29]. Cs, Rb and B, which are strongly correlated with Li (Tab. 2), can be considered as conservative elements, likely due to the strong affinity of alkali ions and boric acid for the aqueous phase [82]. The relatively low Mo concentrations at increasing depth in Lake Río Cuarto (Tab. 2) may be related to its consumption during microbial nitrate reduction [29].

5.2 Processes governing chemical and isotopic composition of dissolved gases

5.2.1 Noble gases, N₂, O₂ and H₂. Dissolved gas species in volcanic lakes basically originate from i) external sources (e.g. atmosphere, volcanic-hydrothermal fluids) and/or ii) microbial activity occurring both in lake water and at water-sediment interface [4,14,28,84,85,86].

Dissolved Ar and Ne in lakes are related to air dissolution through the lake surface, a process that is mainly controlled by atmospheric pressure and the water temperature [87]. The inert noble gases behave inertly in any bio-geochemical process and thus along the lake water column they are affected by advection and diffusion. Accordingly, Ar and Ne concentrations in the two investigated lakes did not show significant variations with depth (Tab. 3). Conversely, O₂, which is typically consumed by aerobic microbial populations for oxidation of organic matter and reduced ionic species, rapidly decreases with depth, and was virtually absent at depths ≥5 and 10 m, in Hule and Río Cuarto lakes, respectively. It is worth noting that the N₂/Ar ratios were slightly higher than that of air saturated water (~40), suggesting the addition of N₂ from an extra-atmospheric source. This hypothesis is expected to be confirmed by δ¹⁵N values that are presently not available, although the relatively high N₂/Ar ratios are apparently consistent with nitrate depletion with depth and microbial denitrification in both lakes. Consistently with the N₂ excess, the distribution of N₂ concentrations in both lakes showed significant variations with depth (Tab. 3), probably related to N₂ production and consumption by denitrifiers and nitrogen fixing prokaryotes, respectively. Microbial N₂ fixation, depending on light [88] and the presence of bio-available trace metals [89], can be carried out by heterocyst-forming species in water and in sediment pores [90–92]. *Cyanobacteria* were indeed retrieved by both DGGE and
Table 4. Phylogenetic identification of the bacterial sequences retrieved from 16S rRNA DGGE gel.

| Sample          | Phylum            | Band   | Closest relative | Acc.n* | % Environments          | Closest described specie                   | Acc. n* | %    |
|-----------------|-------------------|--------|------------------|--------|--------------------------|--------------------------------------------|---------|------|
| Hule 0 m        | Actinobacteria    | 1      | Unc. bact.       | GU127259 | 99 Anoxic plant reservoir | Planktophila limnetica                     | FJ428831 | 95   |
| 0 m             | Betaproteobacteria| 2      | Massilia sp.     | FJ477729 | 99 Soil                   | Massilia aerilata                         | EF688526 | 96   |
| 5 m             | Betaproteobacteria| 7      | Unc. Betaproteobacterium | HM153624 | 99 Freshwater sample      | Limnobacter thiooxidans                    | AJ289885 | 94   |
| 5 m             | Betaproteobacteria| 8      | Unc. Methylophilaceae bact. | HM855563 | 100 Yellowstone Lake water | Methylocrobium mobilis                    | CP001672 | 95   |
| 5 m             | Bacteroidetes     | 5      | Unc. Flexibacter sp. | FN686188 | 98 Lake Zurich water      | Lihzhenia tanjinensis                      | EU183317 | 93   |
| 5 m             | Bacteroidetes     | 3      | Unc. bact.       | EU033667 | 99 Lake Gatun water       | Musculinibacter daejeonensis              | AB267717 | 83   |
| 5 m             | Bacteroidetes     | 4      | Unc. bact.       | JF295800 | 97 Soil                   | Pedobacter terricola                      | EF46147  | 83   |
| 5 m             | Bacteroidetes     | 6      | Unc. bact.       | HM129930 | 98 Freshwater             | Lihzhenia tanjinensis                      | EU183317 | 92   |
| 10 m            | Bacteria          | 9      | Unc. bact.       | DQ642387 | 99 Anoxic freshwater      | Chlorobium phaeovibrioides                | Y08105  | 82   |
| 10 m            | Betaproteobacteria| 11     | Unc. bact.       | HQ53799  | 99 Water sample           | Undibacterium pirigum                     | AM307630 | 96   |
| 10 m            | Betaproteobacteria| 10     | Unc. Undibacterium sp. | GU074044 | 99 Water sample           | Massilia aerilata                         | EF688526 | 97   |
| 15 m            | Chlorobi          | 12     | Unc. Chlorobi bact. | FJ902335 | 99 Limestone sinkholes    | Chlorobium clathratiforme                 | CP002110 | 96   |
| 15 m            | Chlorobi          | 13     | Unc. Chlorobi bact. | FJ902335 | 99 Limestone sinkholes    | Chlorobium clathratiforme                 | CP002110 | 96   |
| 15 m            | Chlorobi          | 14     | Unc. bact.       | HM285636 | 99 Riverine alluvial aquifers | Ignavibacterium album                     | AB478415 | 88   |
| 15 m            | Chlorobi          | 15     | Unc. bact.       | HM285636 | 98 Riverine alluvial aquifers | Ignavibacterium album                     | AB478415 | 88   |
| 23 m            | Chlorobi          | 17     | Unc. bact.       | HM285636 | 92 Riverine alluvial aquifers | Ignavibacterium album                     | AB478415 | 86   |
| Rio Cuarto 0 m  | Betaproteobacteria| 19     | Unc. Proteobacterium | GU074082 | 99 Freshwater             | Burkholderia andropogonis                 | AB021422 | 95   |
| 0 m             | Cyanobacteria     | 18     | Unc. bact.       | GQ001946 | 99 Freshwater             | Synechococcus rubescens                    | AF317076 | 98   |
| 10 m            | Betaproteobacteria| 21     | Unc. bact.       | DQ060410 | 98 Soil enrichment culture | Methylovorax glaciatus                    | FR737205 | 97   |
| 10 m            | Betaproteobacteria| 22     | Unc. bact.       | GU291353 | 98 Tropical lakes         | Sulfitalea hydrogenivorans                | AB552842 | 94   |
| 10 m            | Bacteroidetes     | 20     | Unc. bact.       | AM490988 | 98 Profundal lake sediments | Owenweeksia hongkongensis                | AB125062 | 88   |
| 10 m            | Bacteria          | 23     | Unc. Chloroflexi bact. | AB116427 | 93 Coastal marine sediment | Ignavibacterium album                     | AB478415 | 82   |
| 30 m            | Cyanobacteria     | 24     | Unc. Cyanobacterium | FJ844063 | 99 High mountain lake      | Merismopedia tenusisima                   | AJ839891 | 97   |
| 30 m            | Bacteroidetes     | 25     | Unc. bact.       | FJ473720 | 97 Green Lake water       | Owenweeksia hongkongensis                | AB125062 | 88   |
| 40 m            | Bacteroidetes     | 29     | Unc. bact.       | AM490988 | 98 Profundal lake sediment | Solitalea koreensis                      | EU787448 | 88   |
| 40 m            | Cyanobacteria     | 26     | Unc. bact.       | HO653660 | 97 Shallow freshwater lake | Cyanobium gracile                         | AF001477 | 96   |
| 40 m            | Cyanobacteria     | 27     | Unc. bact.       | GU305729 | 99 Oligotrophic lakes     | Cyanobium gracile                         | AF001477 | 98   |
| 40 m            | Cyanobacteria     | 28     | Unc. bact.       | FJ262922 | 99 Freshwater             | Merismopedia tenusisima                   | AJ839891 | 97   |
| 40 m            | Deltaproteobacteria| 31     | Unc. bact.       | EF515611 | 97 Anaerobic bioreactor sludge | Synthrophobacter pfeffnerii              | X82875  | 82   |
| 40 m            | Bacteria          | 33     | Unc. Chloroflexi bact. | AB116427 | 93 Coastal marine sediment | Ignavibacterium album                     | AB478415 | 82   |
| 40 m            | Bacteria          | 32     | Unc. Chlorobi bact. | GO390242 | 98 Low-sulphate lake       | Ignavibacterium album                     | AB478415 | 82   |
| 40 m            | Bacteroidetes     | 30     | Unc. Haliscomenobacter sp. | HM028523 | 99 sediment resuspension | Candidatus Aquisris caliphila            | AJ786341 | 99   |
| 50 m            | Betaproteobacteria| 37     | Unc. bact.       | GU291353 | 98 Tropical lakes         | Sulfitalea hydrogenivorans                | AB552842 | 95   |
| 50 m            | Bacteroidetes     | 34     | Unc. bact.       | AM490988 | 98 Profundal lake sediment | Owenweeksia hongkongensis                | AB125062 | 88   |
| 50 m            | Bacteroidetes     | 36     | Unc. bact.       | FJ612364 | 99 Dongping Lake Ecosystems | Sphingobacterium alimentarium             | FN908502 | 88   |
Table 4. Cont.

| Sample | Phylum | Band | Closest described specie | Accession number | Closest relative species in GenBank | % identity between DGGE band sequence and closest relative sequence |
|--------|--------|------|--------------------------|-----------------|-----------------------------------|-------------------------------------------------------------------|
| 50 m   | Bacteroidetes | 35   | Unc. bact. FJ612364      | 99 Dongping Lake Ecosystems | Sphingobacterium alimentarium | 99%
| 60 m   | Chloroflexi | 38   | Unc. bact. JF305756      | 86 Mature fine tailings   | Dehalogenimonas lykanthroporepellens | 86%
| 60 m   | Bacteria     | 39   | Unc. bact. GQ860063      | 99 PCB-Spiked sediments | Owenweeksia hongkongensis | 99%
| 60 m   | Bacteroidetes | 40   | Unc. bact. FM956124      | 98 Rice field soil        | Methylovorus glucosotrophus | 98%
| 60 m   | Betaproteobacteria | 41  | Unc. bact. DQ060410      | 98 Soil enrichment culture  | Methanobrevibacter wolinii | 98%

The table reports the identification of the dominant bands in the PCR-DGGE fingerprinting profiles marked in Fig. 8. %: percent of identity between the DGGE band sequence and closest relative sequence in GenBank. Acc. N.: Accession number of the closest relative sequence in Genbank. Environment: environment of origin of the closest relative sequence.

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through the bottom, as supported by the presence of CO₂-rich bubbling pools and caverns or boreholes with high CO₂ concentrations deriving from oxidation of previously produced hydrocarbons. Lake Río Cuarto (Fig. 7) were thus produced by the combination of different processes occurring at various depths in the lake: (i) CO₂ reduction and (ii) degradation of organic pyrosequencing in surface layers of Río Cuarto and Hule lakes (where they constitute 26% of the total bacterial community in the oxic layer H0, Tab. 6), supporting the occurrence of N₂ fixation in both the lakes.

H₂ increase with depth in the hypolimnion at Hule and Río Cuarto (Tab. 3) suggests a production of H₂ likely related to fermentation of organic matter under anaerobic conditions at the water-sediment interface. Additionally, photoreactions carried out by Cyanobacteria, abundantly present in the Río Cuarto deep layers and in the upper layer of the Hule water columns (Tab. 6), could be responsible of H₂ production [93–97]. Once produced at the lake bottom, H₂ can be consumed acting as electron donor for hydrogenotrophic methanogenic archaea and SRB [98–100], detected in Río Cuarto pyrosequencing libraries. Moreover, it slowly diffuses up to shallower, oxygenated layers where it can be consumed by hydrogen-oxidizing bacteria [101–104].

The presence of an extra-atmospheric source for helium can be recognized on the basis of the R/Ra values (Tab. 3), which are relatively high (up to 20 or more) for mantle gases, and as low as 0.01 in fluids from crustal sources [61]. Dissolved gas samples from Hule and Río Cuarto lakes showed R/Ra values ~1 that, coupled with the relatively high He/Ne ratios (49 and 4.1 at Lake Río Cuarto and Lake Hule, respectively), indicate a significant fraction of mantle He, whose uprising is likely favored by the fault system characterizing this area [18].

5.2.2 CO₂ and CH₄. CO₂ and CH₄ are the most abundant extra-atmospheric dissolved gases present in Hule and Río Cuarto lakes. As already mentioned, dissolved CO₂ controls pH values and HCO₃⁻ concentrations. Previous studies [17,18,20,22,23] have hypothesized that these lakes are affected by CO₂ inputs from a deep source, possibly from a CO₂-rich bubbling pool or borehole. High CO₂ concentrations characterizing this area [18,105]. A significant contribution of mantle CO₂ is indicated by the δ¹³C-CO₂ value of the dissolved gas sample collected at the maximum depth of Lake Río Cuarto (−6.6% vs. V-PDB; Tab. 3), which is in the range of mantle gases (from −8 to −4 % vs. V-PDB) [106]. Although not confirmed by the δ¹³C-CO₂ values, the CO₂/CH₄ ratio measured in the dissolved gas at the bottom of Lake Hule (4.7) is too high, even higher than that of Río Cuarto bottom sample (0.63), to be ascribable to microbiological processes. This would imply that even at Lake Hule a strongly negative isotopic signature of CO₂ is externally added to the bottom waters, possibly from a CO₂-rich source deriving from oxidation of previously produced hydrocarbons.

The δ¹³C-CO₂ values at the bottom of Lake Hule (−16.2 % vs. V-PDB) and at depths between −20 and −50 m in Lake Río Cuarto (as low as −14.3 % vs. V-PDB; Tab. 3) were intermediate between those generated by organic matter degradation [24] and mantle degassing [107–109], indicating that along the vertical profiles of both lakes, excluding the bottom layers, biogenic processes are the most important sources of CO₂.

According to the classification proposed by Whiticar [110], the δ¹³C-CH₄ and δD-CH₄ values of the Hule and Río Cuarto lakes indicate that CH₄ has a biogenic origin (Fig. 11). The vertical profiles of the concentrations and δ¹³C values of CO₂ and CH₄ of Lake Río Cuarto (Fig. 7) were thus produced by the combination of different processes occurring at various depths in the lake:

1) At the bottom of the lake, CO₂ inputs from a deep source likely related to the hydrothermal fluid circulation [18,111] promote methanogenic processes that have their maximum efficiency within the sediments. Methanogenesis takes place through i) CO₂ reduction and ii) degradation of organic
matter through acetate fermentation [47,110,112–115]. These processes can be described by the following reactions:

\[ \text{CO}_2 + 8H^+ + 8e^- \rightarrow \text{CH}_4 + \text{H}_2\text{O} \]  

(4)

and

\[ \ast \text{CH}_3\text{COOH} \rightarrow \ast \text{CH}_4 + \text{CO}_2 \]  

(5)

where the \( \ast \) indicates the intact transfer of the methyl position to CH4.

2) In the hypolimnion, microbial CH4 production is still active, although this process is accompanied by CO2 dissolution, CH4 oxidation, and vertical diffusion of both the gas species. Moreover, in correspondence of aerobic/anoxic boundaries, anaerobic decomposition of organic matter [116–118], and CH4 oxidation carried out by methanotrophic bacteria can efficiently produce CO2 in lakes [86,119–121].

3) In the epilimnion, photosynthetic microorganisms (e.g. *Cyanobacteria*) convert light into biochemical energy through oxygenic photochemical reactions combined with CO2 assimilative reduction. Vertical water circulation favors the activity of photosynthetic and methanotrophic bacterial populations, as well as the continuous addition of atmospheric gases from the lake surface.

These hypotheses were confirmed by the 16S rRNA pyrosequencing of samples collected along the water column of Lake Rio Cuarto, demonstrating that archaeal communities encompass almost exclusively methanogenic populations (Tab. 7) typical of freshwater ecosystems, namely *Methanomicrobiales* and *Methanosarcinales* [122–124], as also observed in freshwater meromictic lake sediments [125]. *Methanosarcinales* included solely the acetate-utilizing methanogen *Methanoseta*, the most abundant archaelic genus along the Rio Cuarto water column. Within the H2-CO2 utilizing methanogens of the order *Methanomicrobiales*, *Methanoregula* was the prevalent genus, but unclassified *Methanomicrobiales* and *Methanoregulaceae* sequences were also detected (Tab. 7).

The lack of isotopic data along the vertical profile of Lake Hule did not allow to investigate in detail the (bio)-geochemical processes controlling the vertical profiles of CO2 and CH4. In this lake the majority of the archaeal 16S rRNA sequences were affiliated with unclassified Euryarchaeota, showing high similarity with the Candidate divisions Micrarchae and Parvarchae (Tab. 7) previously described by metagenomic studies of an acidic ecosystem by Baker *et al.* [126,127]. These archaeal sequences belong to the ARMAN (Archaeal Richmond Mine Acidophilic Nanoorganisms) lineages, which are among the smallest cellular life forms known [126], still poorly described from an ecological perspective. The presence of novel uncultivated lineages in the Lake Hule water is linked to neither specific metabolism nor the influence on the water and dissolved gas chemistry. However, besides a minor fraction of known acetotrophic methanogenic *Methanosarcinales* (Tab. 7), the archaeal community of Lake Hule included also the Miscellaneous Crenarchaeota Group (MCG), within the phylum Crenarchaeota (Tab. 7). MCG is a cosmopolitan clade that was previously detected in both freshwater [128] and marine ecosystems [129], where it had been hypothesized to have a significant role in dissimilatory methane oxidation [129]. This hypothesis leads to the speculation that MCG could have the same ecological function also in the Lake Hule. It is worth noting that the minor percentage of known methanogenic archaea in Lake Hule compared to that of Lake Rio Cuarto corresponds to the differences between the lakes in CH4 concentrations (Tab. 3).

16S rRNA pyrosequencing of bacterial communities showed that type I and type II methanotrophic bacteria, belonging to the Gamma- (*Methylocaldum, Methylophilos, Crenothrix*) and Alpha-subgroup of proteobacteria (*Methylcystecae*) [123,130], respectively, were abundant in the anoxic layers of Hule and Rio Cuarto (Tab. 6), suggesting a key role in the carbon cycle. Within the Beta-proteobacteria, additional families that encompass methyliotrophic bacteria, namely *Methylphilicaeae, Rhodocyclaceaee, and Comamonadaeae* [131,132], were retrieved by deep sequencing in the same water layers both in Lake Hule and Lake Rio Cuarto, the latter hosting up to 36% of *Methylphilicaeae* at 30 m depth (Tab. 6). Within the family *Comamonadaeae*, relevant in Lake Hule, 5.2% of the bacterial sequences from the surface layer were affiliated to the *Limnobis* genus, which was reported to play a functional key role in freshwater habitats and showing high ecological diversification [133]. Moreover, 6.3% of the bacterial sequences were affiliated to the genus *Rubrivinax* that includes, among the few characterized

| Sample   | N. reads/sample | N. OTU97 | % Coverage* | Shannon index** |
|----------|-----------------|----------|-------------|-----------------|
| H0 Bacteria | 9384           | 586      | 0.98        | 4.21            |
| H10 Bacteria | 11115          | 615      | 0.98        | 4.21            |
| H15 Bacteria | 15872          | 1260     | 0.97        | 4.98            |
| RC30 Bacteria | 32932          | 3017     | 0.95        | 4.60            |
| RC50 Bacteria | 13291          | 1609     | 0.94        | 4.37            |
| RC60 Bacteria | 13530          | 1882     | 0.93        | 5.08            |
| H10 Archaea  | 1405           | 68       | 0.99        | 2.76            |
| H15 Archaea  | 5889           | 289      | 0.98        | 2.08            |
| RC30 Archaea | 2429           | 177      | 0.96        | 2.66            |
| RC50 Archaea | 2005           | 172      | 0.95        | 2.81            |
| RC60 Archaea | 2937           | 178      | 0.97        | 2.34            |

*Library coverage was calculated as C = 1-n/N, where n is the number of OTU97 without a replicate, and N is the total number of sequences.

**Shannon diversity index calculated using PAST.

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| PHYLUM/CLASS | FAMILY | GENUS | H0 | H10 | H15 | RC30 | RC50 | RC60 |
|-------------|--------|-------|----|-----|-----|------|------|------|
| Actinobacteria | Uncl.Actinobacteria | Other | 0.00 | 0.01 | 0.01 | 2.79 | 3.87 | 0.87 |
| Bacteroidetes | Uncl.Bacteroidetes | Other | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 |
| Bacteroidetes | Flavobacteriales | Other | 0.00 | 0.00 | 0.00 | 3.78 | 1.89 | 4.29 |
| Bacteroidetes | Sphingobacteriales | Other | 0.00 | 0.04 | 0.00 | 3.78 | 1.89 | 4.29 |
| Chlorobi | Chitinophagaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Chlorobi | Chlorobiaceae | Other | 0.00 | 0.00 | 0.00 | 3.78 | 1.89 | 4.29 |
| Chloroflexi | Dehalococcoidetes | Other | 0.00 | 0.00 | 0.00 | 4.24 | 1.20 | 39.52 |
| Cyanobacteria | Synechococcales | Other | 29.4 | 2.56 | 1.45 | 19.19 | 40.20 | 1.47 |
| Planctomycetes | Pirellulales | Other | 0.00 | 0.39 | 0.72 | 0.02 | 0.03 | 0.00 |
| Alphaproteobacteria | Rhizobiales | Methylosinus | 0.07 | 0.12 | 0.47 | 0.02 | 0.03 | 0.00 |
| Alphaproteobacteria | Rhodospirillales | Uncl. Rhodospirillaceae | 0.04 | 0.97 | 1.14 | 0.08 | 0.04 | 0.01 |
| Betaproteobacteria | Burkholderiales | Uncl. Burkholderiaceae | 0.73 | 0.08 | 0.04 | 0.16 | 0.26 | 0.00 |
| Betaproteobacteria | Comamonadaceae | Uncl. Comamonadaceae | 0.57 | 0.05 | 0.05 | 1.38 | 0.38 | 1.55 |
| Betaproteobacteria | Uncl. Betaproteobacteria | Other | 0.00 | 0.00 | 0.00 | 0.27 | 0.09 | 0.56 |
| Betaproteobacteria | Burkholderiales | Uncl. Burkholderiaceae | 0.73 | 0.08 | 0.05 | 0.15 | 0.14 | 0.01 |
| Betaproteobacteria | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.38 | 0.23 | 0.36 |
| Betaproteobacteria | Burkholderiales | Uncl. Burkholderiaceae | 0.16 | 0.05 | 0.05 | 1.38 | 0.38 | 1.55 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Uncl. Burkholderiaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Burkholderiales | Comamonadaceae | Other | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| PHYLUM/CLASS | ORDER       | FAMILY       | GENUS             | H0   | H10  | H15  | RC30 | RC50  | RC60 |
|-------------|-------------|--------------|-------------------|------|------|------|------|------|------|
| Betaproteobacteria | Burkholderiales | Oxalobacteraceae | Uncl. Oxalobacteraceae | 2.52 | 0.94 | 0.86 | 0.01 | 0.00  | 0.00 |
| Betaproteobacteria | Burkholderiales | Oxalobacteraceae | Polynucleobacter     | 0.67 | 0.25 | 0.14 | 0.07 | 0.15  | 0.00 |
| Betaproteobacteria | Methylophilales | Uncl. Methylophilales | Uncl. Methylophilales | 0.70 | 1.01 | 0.32 | 0.00 | 0.00  | 0.00 |
| Betaproteobacteria | Methylophilales | Methylophilaceae | Uncl. Methylophilaceae | 0.03 | 4.21 | 2.72 | 35.99| 11.75 | 20.57|
| Betaproteobacteria | Rhodocyclales  | Rhodocyclaceae  | Uncl. Rhodocyclaceae | 0.00 | 1.05 | 0.36 | 0.91 | 2.13  | 0.09 |
| Deltaproteobacteria | Desulfuromonadales | Geobacteraceae | Geobacter           | 0.00 | 0.00 | 0.01 | 0.24 | 0.04  | 0.66 |
| Deltaproteobacteria | Myxococcales   | Uncl. Myxococcales | Uncl. Myxococcales | 0.00 | 0.00 | 0.98 | 0.07 | 0.03  | 0.06 |
| Deltaproteobacteria | Spirobacillales | Uncl. Spirobacillales | Uncl. Spirobacillales | 0.10 | 0.01 | 0.04 | 1.65 | 3.55  | 0.67 |
| Deltaproteobacteria | Syntrophobacterales | Syntrophobacterales | Syntrophobacter      | 0.00 | 0.00 | 0.00 | 0.96 | 0.62  | 6.96 |
| Epsilonproteobacteria | Campylobacterales | Helicobacteraceae | Sulfurcurvum        | 0.00 | 0.00 | 1.86 | 1.15 | 1.91  | 0.07 |
| Gammaproteobacteria | Uncl. Gammaproteobacteria | Uncl. Gammaproteobacteria | Uncl. Gammaproteobacteria | 0.00 | 0.00 | 0.00 | 0.44 | 1.72  | 0.10 |
| Gammaproteobacteria | Enterobacteriales | Enterobacteriaceae | Uncl. Enterobacteriaceae | 0.17 | 0.64 | 0.05 | 0.11 | 0.00  | 0.02 |
| Gammaproteobacteria | Legionellales   | Legionellaceae  | Uncl. Legionellaceae | 0.00 | 0.05 | 0.02 | 0.45 | 0.07  | 0.05 |
| Gammaproteobacteria | Methylococcales | Methylococcales | Crenothrichaceae     | 0.00 | 17.25| 3.37 | 0.00 | 0.00  | 0.00 |
| Gammaproteobacteria | Methylococcales | Methylococcales | Methylocaldum        | 0.07 | 1.24 | 2.76 | 2.31 | 0.67  | 4.38 |
| Gammaproteobacteria | Methylococcales | Methylococcales | Methylocaldum        | 0.01 | 0.24 | 1.12 | 0.00 | 0.00  | 0.00 |
| Gammaproteobacteria | Pseudomonadales | Pseudomonadales | Pseudomonas          | 0.01 | 16.31| 16.47| 0.00 | 0.02  | 0.02 |
| Gammaproteobacteria | Xanthomonadales | Sinobacteraceae | Uncl. Sinobacteraceae | 0.83 | 0.39 | 0.02 | 0.02 | 0.03  | 0.01 |
| Verrucomicrobia | Optitutae     | Optitutae     | Optitutus           | 0.89 | 2.09 | 2.50 | 0.00 | 0.01  | 0.00 |
| Verrucomicrobia | Uncl. Optitutae | Uncl. Optitutae | Uncl. Optitutae     | 2.61 | 0.89 | 0.06 | 0.00 | 0.04  | 0.00 |
| Verrucomicrobia | Uncl. Verrucomicrobia | Uncl. Verrucomicrobia | Uncl. Verrucomicrobia | 0.00 | 0.00 | 0.03 | 1.52 | 0.72  | 0.69 |
| WS3          | Uncl. WS3     | Uncl. WS3     | Uncl. WS3           | 0.00 | 0.00 | 0.00 | 0.64 | 0.22  | 0.47 |

Uncl: unclassified. Results are expressed as % of the sequences.
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Table 7. List of the taxonomic groups, identified according to the results of the 16S rRNA pyrosequencing, composing the archaeal communities in the freshwater samples collected along the depth profiles of the Hule and Río Cuarto lakes.

| GENUS            | FAMILY          | ORDER                  | CLASS            | PHYLUM       |
|------------------|-----------------|------------------------|------------------|--------------|
|                  |                 |                        |                  |              |
| **Unknown seq.** | **Unknown seq.**| **Unknown seq.**       | **Unknown seq.** |              |
|                  | **Uncl. Archaea**| **Uncl. Archaea**       | **Uncl. Archaea**|              |
| **Crenarchaeota**| **MCG**         | **Uncl. MCG**           | **Uncl. MCG**    |              |
| **Uncl. MCG**    | **pGrfC26**     | **Uncl. pGrfC27**       | **Uncl. pGrfC27**|              |
| **Euryarchaeota**| **Uncl. Euryarchaeota** | **Uncl. Euryarchaeota** | **Uncl. Euryarchaeota** |              |
| **Methanomicrobia** | **Methanomicrobia** | **Methanomicrobia**    | **Methanomicrobia** |              |
| **Methanoregulaceae** | **Methanoregulaceae** | **Methanoregulaceae** | **Methanoregulaceae** |              |
| **Methanosarcinales** | **Methanosarcinales** | **Methanosarcinales** | **Methanosarcinales** |              |
| **Cand. Parvarchaea** | **Cand. Parvarchaea** | **Cand. Parvarchaea** | **Cand. Parvarchaea** |              |

than this external CO₂ was not in equilibrium with HCO₃⁻ responsible of the positive shift of the d13C-CO₂ with respect to that already present in the lake, are likely significant between measured and calculated d13C-TDIC values, depending on both microbial activity and inputs of hydrothermal CO₂, was documented in other meromictic lakes hosted in volcanic environments, such as Lake Kivu, D.R.C. [34] and the Italian lakes of Albano, Averno and Monteclico [86].

As shown in Fig. 12, water samples from the shallower strata (down to 40 m depth) of Lake Río Cuarto displayed d13C-TDIC and d13C-TDICcalc values basically consistent. On the contrary, samples from depth >40 m showed a strong difference between the two sets of values: at ~50 m depth, d13C-TDICcalc were more negative than d13C-TDIC, whereas an opposite behavior was observed in the deeper water layer, as well as at the maximum depth of Lake Hule (Tab. 1). At the lake bottoms, continuous inputs of hydrothermal CO₂, characterized by d13C-CO₂ values significantly less negative with respect to that already present in the lake, are likely responsible of the positive shift of the d13C-TDICcalc values, since this external CO₂ was not in equilibrium with HCO₃⁻. In the shallower layers, especially at the depth of ~60 m, addition of non-equilibrated biogenic CO₂ played an opposite role (Fig. 12), whereas at depth ≤40 m CO₂ concentrations were too low to significantly affect the d13C-TDICcalc values, which were consistent with the d13C-TDIC ones. The disagreement between measured and calculated d13C-TDIC values, depending on both microbial activity and inputs of hydrothermal CO₂, was documented in other meromictic lakes hosted in volcanic environments, such as Lake Kivu, D.R.C. [34] and the Italian lakes of Albano, Averno and Monteclico [86].
Figure 9. $\delta^{18}O$–$\delta D$ diagram for the water samples from Lake Hule (blue squares) and Lake Río Cuarto (red squares). See the text for details.

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Figure 10. Spider-diagrams, where concentrations of selected trace elements in Lake Hule (a) and Lake Río Cuarto (b) maximum depths are normalized to those measured in basalt rock samples collected from the young intra-caldera cone at Laguna Hule [80].

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ecological function of certain prokaryotes in these intriguing ecosystems, particularly in the case of Lake Hule, remains cryptic. In particular, among the bacterial community, Lake Hule hosted the Actinomycetales ACK-M1 cluster [140], whose phenotypic and metabolic traits have not yet been described. The ACK-M1 cluster was one of the most abundant bacterial taxonomic groups in Lake Hule, reaching up to 22.8% in the oxic water layer H0 (Tab. 6). Moreover, in the Lake Hule waters, the Alphaproteobacterial order Rickettsiales showed relatively high concentrations (18.3% of the total bacterial community in the oxic layer H0; Tab. 6). This order comprises intracellular organisms, pinpointing the importance of symbiotic relationships in these lakes. In this context, the impact of the associations between bacteria and algae [141] or phytoplankton [142] on nutrients re-mineralization was recently discussed showing the crucial role of trophic levels interaction on the food web of lacustrine habitats, possibly relevant also in volcanic lakes.

Conclusions

Hule and Río Cuarto are meromictic maar lakes mainly fed by meteoric water, and characterized by significant amounts of dissolved gases, partially consisting of CO2 having a hydrothermal-magmatic origin, in their hypolimnion. They are currently classified as low activity or, alternatively, “Nyos-type” lakes [4], implying that a limnic eruption could be expected to occur from these lakes, as confirmed by the rollover events they have experienced. However, gases stored in the deep layers of Hule and Río Cuarto are fundamentally different with respect to those of Nyos and Monoun lakes, a difference that must be considered for evaluating the eruption risk. The gas reservoirs of the two

Figure 11. δ¹³C-CH₄ vs. δD-CH₄ plot (modified after Whiticar [110]) of Lake Hule (blue square) and Lake Río Cuarto (red squares). See the text for further details.
doi:10.1371/journal.pone.0102456.g011
Cameroonian killer lakes are composed of almost pure CO₂ and basically their temporal evolution only depends on a high magmatic gas input rate [12,13]. At Nyos, the risk of gas bursts was successfully mitigated artificially by discharging the deep-seated gases at the lake surface [35,143]. On the contrary, the gas reservoirs of Hule and Río Cuarto lakes consist of CO₂, CH₄ and N₂ in comparable amounts, mainly controlled by the activity of a microbial network governed by CO₂ and CH₄ metabolism, thus the possible occurrence of a lake rollover that may pose a local risk is not directly related to the input rate of external CO₂.

Despite geographic separation, Lake Río Cuarto and Lake Hule showed similar physical-chemical settings, though hosting phylogenetically distinct bacterial and archaeal communities. Phylogenetic difference apart, however, both lakes have revealed the presence of the same prokaryotic ecological functions deeply involved in affecting water and gas chemistry.

On the whole, Lake Hule and Lake Río Cuarto host a CO₂(CH₄,N₂)-rich gas reservoir which is mainly controlled by the complex and delicate interactions occurring between geosphere and biosphere and whose monitoring can appropriately be carried out by coupling the conventional geochemical approach with studies about prokaryotic colonization. Consequently, for these lakes we can introduce the new definition of bio-activity lakes. This term can be extended to several other volcanic lakes which show similar compositional features of water and dissolved gases, e.g. Kivu (D.R.C.-Rwanda) [34,144], Monticchio, Albano and Averno (Italy) [37,86,145–147], Pavin (France) [121,148].

Figure 12. Vertical distribution of measured and calculated δ¹³C TDIC for the water samples from Lake Río Cuarto. See the text for further details.
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