Tracing sulfate recycling in the hypersaline Pétrola Lake (SE Spain): A combined isotopic and microbiological approach

N. Valiente a, R. Carrey b, N. Otero b,c, M.A. Gutiérrez-Villanueva a, A. Soler b, D. Sanz a, S. Castaño a, and J.J. Gómez-Alday a

a Biotechnology and Natural Resources Section, Institute for Regional Development (IDR), University of Castilla–La Mancha (UCLM), Campus Universitario s/n, 02071 Albacete, Spain
b Grup d’Mineralogia Aplicada i Medi Ambient, Dep. Mineralogia, Petrologia i Geologia Aplicada, Facultat de Ciències de la Terra, Universitat de Barcelona (UB), C/ Martí i Franquès s/n, 08028, Barcelona, Spain
c Serra Hunter Fellowship, Generalitat de Catalunya, Spain

Abstract

Sulfur (S) plays a significant role in saline environments, and sulfate (SO₄²⁻) is an important component of the biogeochemical S-cycle since it acts as the main electron acceptor in anoxic sediments. The purpose of this paper is to evaluate the fate of S, its origin, and processes affecting sulfate outcome in the hypersaline Pétrola Lake in the Castilla-La Mancha region (High Segura Basin, SE Spain). The lake is the terminal discharge zone of an endorheic basin with considerable anthropogenic pressures. Anthropogenic activities (mainly agricultural inputs and wastewater discharge), together with bedrock leaching of sulfate and sulfide-rich sediments, increase dissolved SO₄²⁻ in surface and groundwater up to 123,000 mg/L. The source and fate of sulfate in this environment was investigated coupling hydrochemistry, including hydrogen sulfide (H₂S) microprofiles, isotopic analyses (δ³⁴S, δ¹⁸O₅Ο₄, δ²H₂O, δ¹⁸O₅H₂O, and tritium), mineralogical determinations, and molecular biology tools (16SrDNA amplification and sequencing). The origin of dissolved SO₄²⁻ in water is related to pyrite oxidation from Lower Cretaceous sediments, and secondary gypsum dissolution. Under the lake, dissolved SO₄²⁻ decreases with depth, controlled by three main processes: (1) seasonal evaporation cycles, (2)
hydrodynamic instability caused by the different density-driven groundwater
flow, and (3) sulfate-reduction processes, i.e. dissimilatory bacterial sulfate
reduction (BSR). These processes control the continuous recycling of sulfur in
the system. Lake water and groundwater are in hydraulic connection, and a
density-driven flow (DDF) is able to transport reactive organic matter and
dissolved \( \text{SO}_4^{2-} \) towards the underlying aquifer. Hydrochemical evolution in
depth, \( \text{H}_2\text{S} \) production (up to 0.024 nmol/cm\(^3\)·s) and the presence of sulfate-
reducing bacteria suggest the existence of BSR processes. However, isotope
techniques are insufficient to elucidate BSR processes since their isotopic effect
is masked by low isotope fractionation and high \( \text{SO}_4^{2-} \) concentrations. The
pattern here described may be found in other saline basins worldwide.

**Highlights**

Dissolved sulfate is derived from pyrite oxidation and secondary gypsum
dissolution in Lower Cretaceous sediments.

Dissolved sulfate concentrations are controlled by evaporation, mixing, and
bacterial sulfate reduction.

Mixing is driven by a density-driven flow towards the underlying aquifer.

The isotopic effect of bacterial sulfate reduction is masked.

Hydrogen sulfide microprofiles and molecular techniques suggested the
occurrence of bacterial sulfate reduction processes.

**Keywords**

Hypersaline lake; Sulfur recycling; Stable isotopes; Bacterial sulfate reduction;
Density-driven flow.
Sulfur (S) is the fourteenth most abundant element in the Earth’s crust, with an average abundance of 260 µg/g (Schlesinger, 2005). Sulfur exists in sediments as organic and inorganic forms such as metal sulfides (e.g. pyrite, marcasite), sulfate-bearing minerals (e.g. gypsum or anhydrite), and dissolved sulfate. There is evidence that sulfur plays a crucial role in biogeochemical processes since it is a significant electron donor and acceptor in many bacterial metabolisms (Jørgensen, 1988; Holmer and Storkholm, 2001; Glombitza et al., 2013). Sulfur transformations carried by microbes are closely linked with the carbon cycle, particularly sulfate ($SO_4^{2-}$) reduction coupled to organic carbon oxidation in anoxic environments. Dissimilatory Bacterial Sulfate Reduction (BSR) is a significant mineralization pathway. Almost half of sedimentary organic matter in marine sediments is metabolized with sulfate, which is subsequently reduced to sulfide (Jørgensen, 1982). The main product of BSR processes is hydrogen sulfide ($H_2S$). However, dissolved sulfide also exists as a bisulfide ion ($HS^-$) and a sulfide ion ($S^{2-}$), depending on pH conditions. Only 10% of $H_2S$ derived from sulfate reduction is buried in sediment as sulfides (e.g. pyrite), with most of the $H_2S$ re-oxidized again to $SO_4^{2-}$. Jørgensen (1982) estimated that up to half of the oxygen uptake in sediments could be consumed for sulfide re-oxidation. Sulfide may be oxidized to sulfate through several intermediates (sulfite, thiosulfate, elemental sulfur), which can be dismutated to produce sulfate and sulfide by microbial disproportionation (Bak and Cypionka, 1987; Canfield and Thamdrup, 1994). Large amounts of sulfate are released either from re-oxidation of sulfides during the desiccation of wetlands or by chemolithotrophic denitrification in nitrate-polluted areas (Lamers et al., 2001; and references therein). Several risks can be linked to sulfide oxidation, such as the release of heavy metals and metalloids to the environment (Corkhill et al., 2008).

Sulfur cycling is of major interest in the field of marine, estuarine, and lake ecosystems for various reasons: (1) gypsum dissolution is an important source of sulfate for microorganisms in evaporitic deposits (Machel, 2001); (2) $SO_4^{2-}$...
concentrations can exceed 2,600 mg/L in marine waters and can be even higher in continental saline systems (70,000 mg/L, Kulp et al., 2006); and (3) sulfur derives from human activities such as mining (sulfide ores), fossil fuel emissions and agriculture (fertilizers) (Brimblecombe et al., 1989). Since sulfate is a common constituent of agricultural fertilizers (Vitòria et al., 2004; Oren et al., 2004), human practices in agricultural regions have increased the amount of $\text{SO}_4^{2-}$ in aquatic systems (Zak et al., 2009; Baldwin and Mitchell, 2012).

Over the past century, stable sulfur isotopes have been used to determine the sources and processes affecting the speciation of sulfur compounds (Thode et al., 1949). There is a large variation of $\delta^{34}S$ in nature: for instance, sulfides usually range from $-5\%$ to $+10\%$, terrestrial evaporites usually range from $-15\%$ to $+10\%$, and marine evaporites have higher values of $+10\%$ to $+35\%$ (Krouse and Grinenko, 1991; Clark and Fritz, 1997; Nordstrom et al., 2007). Due to that variation, the sulfur isotope composition ($\delta^{34}S$) of dissolved $\text{SO}_4^{2-}$ has been used as an environmental tracer in hydrological systems. More recently, coupling the isotope analyses of sulfur and oxygen of dissolved $\text{SO}_4^{2-}$ has allowed a better identification and quantification of sulfate sources (Krouse and Mayer, 2000; Tichomirowa et al., 2010; Li et al., 2011). Isotope techniques have also been applied to trace processes in the biogeochemical sulfur cycle.

The natural distribution of sulfur isotopes is controlled mainly by the fractionation imparted by dissimilatory BSR (Chambers and Trudinger, 1979; Canfield, 2001). BSR processes cause fractionation in both the S and O isotopes of dissolved $\text{SO}_4^{2-}$, increasing the isotope composition of S and O in the remaining sulfate at known $\epsilon^{34}\text{S}/\epsilon^{18}\text{O}$ ratios between about 0.6 and 4.3 (Böttcher et al., 1998; Brunner et al., 2005). Fractionation of sulfur isotopes can be also caused by microbial sulfur disproportionation: depletions down to $37\%$ for sulfide, and enrichments up to $35\%$ for sulfate (Habicht et al., 1998; Böttcher et al., 2005). Sulfur isotope fractionation is also observed during gypsum precipitation with enrichments up to $2.0\%$ (Van Driessche et al., 2016). Conversely, sulfide oxidation causes insignificant isotope fractionation for $\delta^{34}\text{S}_{\text{SO}_4}$ (Habicht et al., 1998), as well as other abiotic processes such as the dissolution of sulfate minerals from evaporites (Claypool et al., 1980). These
pathways can be observed in the sedimentary sulfur cycle (Jørgensen, 1990; Werne et al., 2004), as well as in the pelagial sulfur cycle in systems dominated by phototrophic bacteria (Overmann et al., 1996). The use of oxygen isotopes not only in dissolved $\text{SO}_4^{2-}$, but also in water, helps to understand the biogeochemical processes affecting sulfate fate (Craig, 1961).

This paper aims to provide insights on the sulfur cycle in a hypersaline lake-aquifer system affected by significant anthropogenic activities. Pétrola Lake is one of the most representative saline wetlands in the Castilla-La Mancha Region (High Segura Basin, SE Spain). However, the lake is disturbed by agricultural activities and urban wastewater discharges, which empty directly into the body of water. These practices are among the most frequently stated problems leading to the degradation of inland wetlands since they can alter the natural saline system by modifying the mass balance and the intensity of biogeochemical processes. Therefore, studying the processes controlling sulfur recycling can help to understand the saline lake-aquifer relationship and the biogeochemical processes involved, improving the characterization of water resources. Preliminary results using hydro-chemical and isotopic tools suggested that S cycling in Pétrola basin depended on the relationship between BSR kinetics and the hydrodynamic instability driven by a density-driven flow (DDF) (Valiente et al., 2017). This approach showed limited results in elucidating S recycling in this hypersaline system. Omorieghe et al., (2013) showed the usefulness of combining geochemical, stable isotopes and microbiological data in an acid mine drainage-derived lake. A similar approach was adopted in the present paper, including hydro-chemical and isotopic tools on surface and groundwater samples and on sediment samples as well as the addition of molecular biology tools, in order to identify sulfate sources and the processes taking place in the system.

2. Study Area

The hydrogeological boundary of the Pétrola Lake–aquifer system extends over 43 km$^2$ (Fig. 1). The lake itself occupies the terminal discharge zone of an
endorheic basin in a semiarid area, with various small streams discharging into the lake in a radial pattern. The region is characterized by irregular rainfall, long episodes of drought, and torrential precipitation. Pétrola Lake is located in the southeastern Castilla-La Mancha region, and is part of the Segura River Basin. Farming (cultivation, raising livestock) is the main economic activity in the area. According to the European database Corine Land Cover 2000 (European Environment Agency, Copenhagen), irrigation and dry land occupy about 17 km², which represents 40% of the total basin surface. Crops are fertilized mainly using inorganic fertilizers. Urban wastewater (from a population of 850 inhabitants) is discharged, untreated, directly to the lake.

The lake occupies about 1.76 km² and is shallow, its depth nowhere exceeding 2 meters (in Spanish: Confederación Hidrográfica del Segura, unpublished data). In 2007, the total volume of groundwater withdrawals from the Pétrola hydrogeological boundary was 2 Mm³/year. Water volume in the Pétrola Lake shows oscillations mainly depending on the climatic events. Seasonal fluctuations show a maximum water volume at early spring (reaching about 0.90 hm³ in February), concurring with abundant precipitation and low evaporation rates, and a minimum volume (or completely dryness) at the end of summer, related to few precipitation events and maximum evaporation rates (López-Donate et al., 2004). The work of Vicente et al. (1998) has shown that chemical hydrofacies changes slightly between early spring (Mg-Cl-SO₄) and early fall (Mg-Na-Cl-SO₄). The same chemical composition (Mg-Na-Cl-SO₄) was found in summer by Ordóñez et al. (1973).

The basin geology comprises mainly Mesozoic materials (Utrilla et al., 1992). The bottom of the sequence is formed of oolitic carbonate Jurassic rocks. The base of the Lower Cretaceous unit corresponds to the Weald Facies and consists of argillaceous sediments overlain by sands and sandy-conglomerate sediments with intergranular porosity, which reaches the Barremian. Albian deposits (Utrillas Facies) consist of siliciclastic sands, sandy-conglomerates, and reddish to dark-grey clay to argillaceous sediments deposited over Aptian sediments. The Utrillas Facies comprises sandy-conglomerate sediments interstratified by grey-to-black argillaceous sediments with organic matter and
sulfides (mainly pyrite). The main aquifer is formed of Lower Cretaceous sediments (Utrillas Facies) comprising siliciclastic sands, conglomerates, and siltstones with a thickness that can exceed 60 metres. Groundwater flow in this aquifer is radial and centripetal from the recharge areas (basin margins) to the lake (Gómez-Alday et al., 2014). In a saline lake–aquifer interface, the difference in density between the brine from the lake and fresh groundwater can produce a density-driven flow (DDF) from saline lake water towards the underlying aquifer (Zimmermann et al., 2006). In previous studies using electrical resistivity tomography in Pétrola Lake, Gomez-Alday et al. (2014) observed the existence of a dense brine perched on less dense fresh groundwater as a result of DDF, caused by the instability of the saline boundary layer.

Fig. 1 near here

3. Materials and Methods

3.1 Sampling

Four PVC piezometers were installed in the lake in September 2008 following a radial pattern from the eastern lake margin (SE, GW-34) towards the lake center (NW, GW-26) (Fig. 2). The depth profiles and screened intervals of each piezometer are detailed in Figure 2. The piezometers are 5 cm in inner diameter, and were installed at different depths: 12.1 m (GW-12), 25.8 m (GW-26), 34.1 m (GW-34), and 37.9 m (GW-38). Screen lengths are 4 m (GW-12), 9 m (GW-26), 5 m (GW-34), and 3 m (GW-38). Screening zones were isolated by internal bentonite seals. A total of 37 water samples were collected between September 2008 and October 2011 from five control points: four piezometers and surface water (SW-0). Simultaneous measurements of groundwater level were performed for piezometers GW-12, GW-26, and GW-34 using a ceramic CTD-Diver stand-alone sensor (piezometer GW-38 is an artesian well, so it was not measured). Data were collected from February 2010 to October 2011 at 24-hour intervals (n=609 daily measurements). Precipitation data for the study
period were acquired from meteorological station AB07 (Ministry of Agriculture and Fisheries, Food and Environment of Spain) in Pozo Cañada, about 16 km from Pétrola Lake.

Sediment samples (n=13) were taken from the cores recovered during piezometer construction (Fig. 2, samples ANNA-1 to ANNA-22). The sediment cores were collected at eight different depths from 3.4 to 37.3 metres below ground surface (mbgs). All the samples were preserved in frozen polypropylene tubes with dry ice at -80°C, stored in polyethylene bags, closed, and shipped to the laboratory. Simultaneously, another 12 samples from an evaporitic crust were collected following a linear transect from the depocenter (sample PE MIN 1, next to a breakwater) to the eastern margin of the lake (sample PE MIN 12) at intervals of 15 meters (Fig. 1C). Furthermore, seven samples of secondary gypsum were collected from several silt deposits belonging to Lower Cretaceous sediments, cropping out in the study area (Fig. 1C). Three cores from the upper 20 cm of recent organic-rich sediments deposited on the lake bottom were collected for DNA extraction and microprofiling using Plexiglass coring tubes (5 cm inner diameter and 20 cm long) in January 2016. Each coring tube was capped at top and bottom with silicone rubber, cooled, and immediately taken to the laboratory following the methodology of Kondo et al. (2004).

3.2 Chemical analyses

Physical-chemical parameters were measured in situ with portable electrodes (n=32). The control parameters were water temperature (T), pH, electrical conductivity (EC), redox potential (Eh), and dissolved oxygen (DO). In surface water, measurements were performed directly, whereas in the four piezometers measurements were made using a flow-through chamber to minimize the effect of air exchange. Water samples were stored at 4°C in darkness prior to further analysis following official standard methods (APHA-AWWA-WEF, 1998). Water samples for major ions were filtered with a 0.45 μm nylon Millipore® filter. For
the determination of dissolved organic carbon (DOC), Fe and Mn, samples were filtered with a 0.20 μm nylon Millipore® filter. Alkalinity determinations were carried out in the laboratory by acid–base titration. HCO$_3^-$, SO$_4^{2-}$, and Cl$^-$ contents were measured by ion chromatography (DX120, Vertex). Na$^+$, K$,^+$, Ca$^{2+}$, Mg$^{2+}$, total Fe, and Mn concentrations were measured in an atomic absorption spectrophotometer equipped with an air-acetylene burner. DOC concentrations were determined using a Shimadzu Analyzer in the research services at the University of A Coruña.

### 3.3 Isotope analyses

Isotope analyses were performed for 34 water samples. Isotope ratios of sulfur ($^{34}$S/$^{32}$S) and oxygen ($^{18}$O/$^{16}$O) from dissolved SO$_4^{2-}$ were measured on BaSO$_4$ precipitated by the addition of 5% BaCl$_2$-2H$_2$O and HCl, boiling it to prevent BaCO$_3$ precipitation (Dogramaci et al., 2001). The δ$^{34}$S$_{SO4}$ was analysed in a Carlo Erba Elemental Analyzer (EA) coupled in continuous flow to a Finnigan Delta C IRMS at the “Centres Científics i Tecnològis” at the Universitat de Barcelona (CCiT-UB). The δ$^{18}$O$_{SO4}$ values were analysed in duplicate with a ThermoQuest high-temperature conversion analyzer (TC/EA) unit with a Finnigan MAT Delta C IRMS at the CCiT-UB. $^{18}$O/$^{16}$O ratios from H$_2$O were measured by the CO$_2$ equilibration method using a Multiflow device coupled in line to a continuous flow Isoprime Mass Spectrometer. The δ$^2$H$_{H2O}$ values were obtained by measuring H$_2$ in the reduction of Cr with an elemental analyzer (EuroVector) with a continuous flow mass spectrometer (Isoprime Mass Spectrometer) at the LIE-US. δ$^{18}$O$_{H2O}$ and δ$^2$H$_{H2O}$ determinations were carried out at the Stable Isotopes Laboratory at the University of Salamanca (LIE-US). Radioactive isotopes of H$^3$ (tritium) in groundwater were determined by liquid scintillation (n=5) counting in $^{14}$C and the Tritium Dating Service at the Autonomous University of Barcelona.

Furthermore, isotopes were analysed in 37 solid samples. The δ$^{34}$S values from gypsum and sulfide were determined on Ag$_2$S as described by Canfield et al. (1986) and Hall et al. (1988). These analyses were performed in a SIRA-II dual
inlet spectrometer at the LIE-US. The $\delta^{18}$O values from gypsum samples were also analysed in duplicate with a ThermoQuest high-temperature conversion analyzer (TC/EA). Results are reported in $\delta$ values relative to international standards (Vienna Canyon Diablo Troilite (VCDT) for $\delta^{34}$S and Vienna Standard Mean Ocean Water (VSMOW) for $\delta^{18}$O and $\delta^{2}$H. Analytical reproducibility by repeated analyses of both international and internal reference samples of known isotopic composition is determined to be about $\pm 0.2\%o$ for $\delta^{34}$S, $\pm 0.5\%o$ for $\delta^{18}$O of $\text{SO}_4^{2-}$, $\pm 0.3\%o$ for $\delta^{18}$O in water, $\pm 1\%o$ for $\delta^{2}$H in water, and $\pm 0.4$ for Tritium Units (TU).

### 3.4 Mineral identification

Sediment samples from piezometer cores (n=8) and recent evaporitic crusts from the lake bottom (n=12) were analysed using electronic microscopy (ZEISS DSM 940) and X-ray diffraction (Bruker-AXS D5005). For this purpose, thin films were obtained from the solid samples. The microscopy analyses were performed at the Rock Preparation Service of the University of Salamanca. The X-ray diffraction analyses were performed at the Jaume Almera Earth Sciences Institute at the Spanish National Research Council (ICTJA-CSIC). Saturation indices (SI) of minerals were calculated using the PHREEQC thermodynamic software (Parkhurst and Appelo, 1999). The SI was calculated for halite (NaCl), gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), hexahydrite ($\text{CaSO}_4 \cdot 6\text{H}_2\text{O}$), and epsomite ($\text{CaSO}_4 \cdot 7\text{H}_2\text{O}$) using the Pitzer database (recommended for saline environments; Wolery et al., 2004).

### 3.5 Hydrogen sulfide microprofiles

Hydrogen sulfide activity in recent organic matter-rich sediments was evaluated by means of $\text{H}_2\text{S}$ microprofiles. Microprofiles were measured using an $\text{H}_2\text{S}$ amperometric microelectrode (Unisense A/S, Denmark) with a tip diameter of 100 $\mu$m. Measurements were taken on individual fresh sediment cores (n=3) collected during January 2016 as described above. Cores were stabilized using
lake water for a period not longer than 24 hours. All profiles were carried out at
50 μm intervals to reach a depth of 1 centimeter. Microprofile measurements
and H₂S activity calculations were performed using dedicated software
(SensorTrace Pro v.2.8.2, Unisense A/S, Denmark). To calculate H₂S
consumption and production, the method published by Berg et al. (1998) was
used assuming steady-state conditions where transport of solutes occurs by
diffusion.

3.6 DNA extraction, 16S rDNA amplification, sequencing and phylogenetic
analysis

Molecular techniques were used for bacterial identification. Aliquots of 50 g
(n=3) of recent organic sediment were collected from the top 2 cm of the cores
when microprofiling was finished. Then, aliquots of sediment were
homogenized. For enrichment, a sulfate-reducing bacteria culture medium was
prepared (Pfennig et al., 1981) and supplemented with 7% NaCl and 1%
MgCl₂·6H₂O (Caumette et al., 1991). Bacteria enrichments (n=3) were
inoculated with 1 g of each aliquot of homogenized sediment and incubated at
30ºC for 14 days. DNA extraction from enriched cultures was performed using a
Bacterial Genomic DNA Isolation Kit (Canvax). Nucleic acid purity was
determined by scanning spectrophotometry (Sambrook et al., 1989).

DNA extracts were mixed into a single sample and used to amplify fragments of
16S rRNA gene (16S rDNA) by polymerase chain reaction (PCR). In a first step,
16S rDNA fragments were amplified via PCR using a set of specific
oligonucleotide primers DSV435F and DSV1430R for sulfate-reducing bacteria
from the Desulfovibrionaceae family, following Tanaka et al. (2002).
Amplifications were performed in 30 μL volumes with 1.75–2.50 μg of purified
DNA template, 1 X PCR buffer (Canvax), 2.5 mM MgCl₂, 0.8 mM concentrations
of each deoxynucleoside triphosphate, 0.75 mM concentrations of each primer
(IDT), and 1 U of Horse-Power Taq DNA polymerase (Canvax). In addition,
10% of polyvinylpyrrolidone was added to the PCR mixture to enhance PCR
performance (Koonjul et al., 1999). Reactions were carried out using a GenePro
(Bioer) cycler in order to control the following PCR conditions: one cycle at 95°C for 15 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min for 35 cycles, and a final extension step at 72°C for 10 min. The resulting PCR product was sequenced (seq1).

A second PCR was performed using DNA extracts and a primer set designed by the authors. The second set of primers (DHF and DHR) was designed based on an alignment of 16S rDNA from various strains belonging to Desulfovibrio genus from saline environments. Forward primer DHF (5'-TTATGGGGAAGGTGCGCT-3'; Escherichia coli positions 158-177) was used in combination with reverse primer DHR (5'-CCTGTTTGCTACCCACGCTT-3'; E. coli positions 761-742). The specificity of DHF and DHR primers was tested before use using the BLAST software (Altschul et al., 1997) at GenBank to ensure no matches were found with other bacterial 16S rDNA. PCR contained composition described above. Second PCR was performed as follows: a first step at 90°C for 5 min, denaturation at 95°C for 30 s, annealing at 54°C for 40 s, extension at 72°C for 1 min for 35 cycles, and a final extension step at 72°C for 10 min. Finally, aliquots (1, 2, and 3 µL) of the second PCR product were analysed on a 1% (w/v) denaturing agarose gel electrophoresis, visualized with 1X RedSafe™ (iNtRON Biotechnology) and compared to a 2 µL sub sample of 1 Kb molecular DNA ladder (Invitrogen, USA). The remaining volume of second PCR product was used for sequencing (seq2). The sampling preparation and analytical procedure was performed at the Institute for Regional Development (University of Castilla-La Mancha). Sequencing was carried out using an Applied Biosystems 3730xl DNA Analyzer with 3730xl DNA Analyzer 96-Capillary Array (50 cm) by Macrogen Inc.

Each 16S rDNA sequence was compared using Blast from NCBI (Camacho et al., 2009). The closest 16S rDNA sequences were aligned using Clustal Omega (Sievers et al., 2011). Phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei distance with the program MEGA7 (Kumar et al., 2016). Robustness of the phylogenetic results was checked by bootstrap (1000 replicates). Sequences have been submitted to GenBank under accession numbers MF682361 and MF682362.
4. Results

4.1 Hydrogeology

Groundwater level in the piezometers is affected by precipitation events and regional groundwater potential. In GW-12, the level is generally influenced by variations in the lake, mainly related to local precipitation patterns (Fig. 3). The levels also reflect variations in the regional groundwater flow (RGF) potential for February 2010 to October 2011. In piezometer GW-12, groundwater levels noticeably decreased from end of June 2010 to mid-November 2010, coinciding with the end of the dry months. Then, levels dramatically increased from November 2010 to June 2011, coinciding with a period of regular precipitation. Levels fell again during the last months of 2011. The evolution of water levels in GW-26 shows an increasing trend from the end of June 2010 to mid-November 2010. In contrast to shallow piezometers, the groundwater evolution in GW-34 largely reflects variations in the regional groundwater potential.

Tritium levels (Table 1) in water samples range from 0.2 ±0.4 TU to 4.6 ±0.3 TU (n=5), with the highest values in surface lake water (SW-0), reaching 4.6 ±0.3 TU. The shallowest piezometer (GW-12) shows the highest tritium levels in groundwater (1.5 ±0.3 TU), whereas GW-34 yields the lowest (0.2 ±0.4 TU).

4.2 Hydrochemistry

Table 2 shows the mean values of the chemical analyses for each control point. The detailed results of the chemical analyses for each sample (n=36) are presented as Supplementary Information (Appendix A). Average pH values range from 7.0 (in piezometer GW-34) to 8.1 (in surface water samples, SW-0). The average EC ranges from 2,743 μS/cm (in the deepest piezometer, GW-38),
to 83,300 μS/cm (SW-0). Average Eh values are between +3 mV (in the shallowest piezometer, GW-12) and +148 mV (SW-0). The average DO ranges from 0.2 mg/L (in piezometer GW-34) to 3.6 mg/L (SW-0). The average DOC concentration ranges from 3.2 mg/L (in the deepest piezometer, GW-38) to 305 mg/L (SW-0).

All major ions except Ca\textsuperscript{2+} show minimum average concentrations in the deepest piezometer (GW-38), with maximum concentrations found in surface water (SW-0). Surface water also shows the highest standard deviations in all the major ions. The average SO\textsubscript{4}\textsuperscript{2−} concentration ranges from 1,140 mg/L to 69,300 mg/L, whereas average Cl\textsuperscript{−} is between 240 mg/L and 50,800 mg/L. The HCO\textsubscript{3}− contents show a minimum average value of 411 mg/L and a maximum average value of 1,074 mg/L. The average Na\textsuperscript{+} contents range from 122 mg/L to 34,800 mg/L, whereas K\textsuperscript{+} contents range from 12.9 mg/L to 5,130 mg/L. Mg\textsuperscript{2+} ranges between 165 mg/L and 38,500 mg/L, both in SW-0 samples. Ca\textsuperscript{2+} shows a different depth pattern from Mg\textsuperscript{2+}, with minimum average values of 183 mg/L in the deepest piezometer (GW-38), but maximum values (637 mg/L) at depth (GW-26) instead of at the surface. Mn concentrations do not follow the same trend as the major ions; samples are below the detection limit in surface water and the highest average contents (Mn up to 11.8 μg/L and Fe up to 263 μg/L) are in the deepest piezometer (GW-38). These data were used to determine sample water types. As shown in Figure 4, water type varies between Mg-Ca-SO\textsubscript{4}-HCO\textsubscript{3} water (GW-38) and Mg-Na-SO\textsubscript{4}-Cl water (SW-0).

4.3 Isotope data

The isotope analyses of water samples are presented in Table 3. δ\textsuperscript{34}S\textsubscript{SO\textsubscript{4}} in the water samples ranges from −25.9‰ (in the deepest piezometer, GW-38) to −16.2‰ (in piezometer GW-34), with an average value of −21.7‰ (n=15). The average δ\textsuperscript{34}S\textsubscript{SO\textsubscript{4}} shows little variation with depth, with only the deepest piezometer having significantly lower values. δ\textsuperscript{18}O\textsubscript{SO\textsubscript{4}} in water samples ranges
between +6.2‰ and +17.5‰, with an average of +12.5‰ (n=15). A decrease in δ¹⁸O₅O₄ is observed with depth. δ¹⁸O₅H₂O in water samples ranges from -8.8‰ to +4.2‰, with an average of -4.5‰ (n=33), and δ²H₅H₂O ranges from -52.4‰ to +15.1‰, with an average of -35.2‰ (n=29). Both δ¹⁸O₅H₂O and δ²H₅H₂O show the same pattern with depth as δ¹⁸O₅O₄, with decreasing values from SW-0 to GW-38. These values are shown in Figure 5, which also includes the Global Meteoric Water Line (GMWL; Craig, 1961) and the weighted average precipitation from Madrid. Madrid was selected as the reference station because most of the precipitation at Pétrola Lake is influenced by European Atlantic fronts. The weighted average precipitation was calculated with data from the International Atomic Energy Agency (IAEA) for the period of 1970 to 2006, using the values corresponding to dates with precipitation of over 20 mm (n=79) (Schotterer et al., 1996).

Isotope data from sulfate-bearing minerals and sulfides are shown in Table 4. The δ³⁴S₅gypsum ranges from -30.3‰ (at 27 mbgs) to -9.9‰ (at 3.4 mbgs), with a mean of -21.2‰ (n=20). The δ¹⁸O₅gypsum varies from -2.8‰, in secondary gypsum samples, collected from Lower Cretaceous sediments, to +18.2‰ in sulfate-bearing minerals from the evaporitic crust, with a mean of +10.6‰ (n=25). The δ³⁴S₅sulfide values from piezometers range from -40.5‰ (at 37.3 mbgs) to -11.7‰ (at 11 mbgs), with a mean of -30.4‰ (n=12).
4.4 Mineral identification

Mineral identification analyses corresponding to recent evaporitic crusts from the lake bottom show sulfate minerals of calcium (gypsum, CaSO$_4$·2H$_2$O) and/or magnesium (hexahydrite, MgSO$_4$·6H$_2$O) in all samples. Moreover, epsomite (MgSO$_4$·7H$_2$O) is present in four of the twelve samples; halite occurs in all samples, and quartz is observed in most of the surface sediment samples. No sulfides are found in these sediments.

Core sediments from the piezometers are characterized by high organic matter contents. All the organic sediments are rich in sulfides, mostly pyrite, but sphalerite can also appear, at around 11 mbgs (ANNA-8 and ANNA-12b). Most of the cores have sulfate-bearing minerals such as gypsum, hexahydrite, and starkeyite (MgSO$_4$·4H$_2$O), as well as iron oxides (except sample ANNA-8, at 11 mbgs, where sulfur is only present as a sulfide).

Mean SI values for each sample point are shown in Table 5, which evidences that only gypsum precipitates. The SI$_{\text{Gypsum}}$ values calculated from water samples range between -1.03 and 0.67 (both in SW-0). SI$_{\text{Epsomite}}$ values range between -3.08 (GW-38) and -0.10 (SW-0). SI$_{\text{Halite}}$ values vary between -6.62 (GW-38) and -0.01 (SW-0), and SI$_{\text{Hexahydrite}}$ values range between -3.42 (GW-38) and -0.29 (SW-0).

4.5 Hydrogen sulfide rates

Complete H$_2$S microprofiles and detailed zones of production and consumption in the water-sediment interface are shown in Supplementary Information (Appendix B). Concentration of H$_2$S reaches values up to 3.8 mmol/L at 8 mm depth (Profile 3). Production of H$_2$S is observed from the first 3 mm, 5 mm and 1 mm for profiles 1, 2 and 3, respectively. Maximum H$_2$S production reaches values up to 0.024 nmol/cm$^3$·s (Profile 2), similar to maximum production measured in the rest of sediment cores (0.022 nmol/cm$^3$·s). Nevertheless, H$_2$S production is not observed through the whole profile in the studied sediment
cores. Consumption of H$_2$S is noticed at narrow zones interspersed with sulfide production areas.

### 4.6 16S rDNA amplification and phylogenetic affiliation

The products of the PCR performed with the second primer set (DHF and DHR) were analysed by denaturing agarose gel electrophoresis. Patterns are shown in the Supplementary Information (Appendix C, Figure C1). 16S rDNA amplification using the designed primers for *Desulfovibrio* sp. resulted in products of predicted size (604 bp). Phylogenetic trees are included in Supplementary Information (Appendix C). The determined sequences seq1 and seq2 are closely related to *Desulfovibrio senezii* (accession no. NR_024887.1), a gram-negative bacterium isolated from a solar saltern in California (Tsu et al., 1998). The results reveal that sulfate-reducing bacteria from the *Desulfovibrionaceae* family are present in hypersaline sediments from Pétrola Lake.

### 5. Discussion

#### 5.1. Hydrochemical changes across the salt water-fresh water interface

The variability in surface water and groundwater chemistry can be influenced by climate, anthropogenic factors, biogeochemical (water-rock interaction) processes, as well as fluctuations in water level. Commonly, during wet periods, precipitation can dilute surface water, whereas in dry periods, surface water salinity can increase by evaporation. Seasonality causes chemical variations, which are observed in surface water (SW-0) samples. Those samples from hydrological year 2010 show minimum concentrations of SO$_4^{2-}$ (43,227 mg/L), Cl$^-$ (21,768 mg/L) and DOC (140 mg/L) during high water-level season (February 2010). Maximum concentrations in SW-0 are found during low water-level stage (October 2010): SO$_4^{2-}$ (122,957 mg/L), Cl$^-$ (102,814 mg/L) and DOC (646 mg/L). Regarding groundwater samples, SO$_4^{2-}$, Cl$^-$ and DOC
concentration decrease from GW-12 to GW-38, but seasonal variations are not observed. Seasonality is also reflected in the $\delta^{18}$O$_{H2O}$ and $\delta^{2}$H$_{H2O}$. Kinetic fractionation processes affect oxygen and hydrogen isotopes differently during evaporation. In arid regions with low relative humidity, the evaporation rate is faster and the deuterium excess of the meteoric water line is higher (Clark and Fritz, 1997). The $\delta^{18}$O$_{H2O}$ and $\delta^{2}$H$_{H2O}$ results in the study area show a slope (5.2) consistent with evaporation processes (Fig. 5). Samples from Pétrola basin intercept the GMWL close to the local weighted average precipitation value. Water samples close to the local weighted precipitation value belong to regional fresh groundwater samples (GW-38). Samples from SW-0, GW-12 and GW-26 show a positive shift from the GMWL (Fig. 5). These results indicate that the $\delta^{18}$O$_{H2O}$ and $\delta^{2}$H$_{H2O}$ observed in surface water are due to the evaporation of rainwater with a similar isotopic composition as the local weighted average precipitation. Groundwater isotopes ($\delta^{18}$O$_{H2O}$ and $\delta^{2}$H$_{H2O}$) from GW-12 and GW-26 show a mixture between evaporated lake saltwater and regional freshwater (GW-38). Hydrochemical evolution across the saltwater-freshwater interface has been studied. Cl$^{-}$ evolution has been employed as conservative tracer (Fig. 6). The Cl$^{-}$ concentrations were higher in GW-12 than in surface water for samples taken in February 2010, April 2010, and October 2011. These samples were collected during wet periods due to precipitation events that increase freshwater input into the lake and thereby dilute surface waters. Other processes influencing the trend in Cl$^{-}$ concentration is the precipitation of Cl$^{-}$-bearing minerals, supported by the identification of halite in lake sediments. On the other hand, samples taken in July, October, and November 2010 show higher EC and Cl$^{-}$ contents in surface water than in groundwater (GW-12). These results are in agreement with the absence of significant precipitation events in the summer of 2010, in which evaporation processes determined high salt concentrations in the surface water. The intense evaporation processes were also evidenced in July 2010 by means of $\delta^{18}$O$_{H2O}$ and $\delta^{2}$H$_{H2O}$. Then, despite precipitation in autumn 2010 (Fig. 3), Cl$^{-}$ concentrations measured in October and November 2010 increased up to 103,000 mg/L (Fig. 6). This increase is
most likely related to the dissolution of the surficial salts previously precipitated in the lake during previous dry seasons.

The decrease in Cl\(^-\) with depth can be explained by a mixing process between lake saltwater (due to DDF) and regional freshwater (RGF represented by GW-38). These Cl\(^-\) variations measured in piezometers are also consistent with the isotope composition of \(\delta^{18}O_{H_2O}\) and \(\delta^{2}H_{H_2O}\), where the highest Cl\(^-\) concentrations match up with the heaviest isotope values. Despite the significant variations in surface water salinity, and the observed differences in water density (SW-0 has values up to 1.29 g/cm\(^3\), and GW-38 has values around 1.01 g/cm\(^3\)), groundwater chemistry remains almost constant suggesting quasi-equilibrium between DDF and RGF during the studied period. The buoyancy of the RGF upwards limits the impact of DDF in solute transport.

Sulfate and DOC concentrations are also represented in depth (Fig. 6). The trend of SO\(_4^{2-}\) concentrations is similar to that observed for Cl\(^-\). In surface water, SO\(_4^{2-}\) concentration can be affected by evaporation, external inputs (streams), precipitation and dissolution of S-bearing minerals, as well as sulfate-reduction processes. In groundwater, the decrease in SO\(_4^{2-}\) concentration with depth could be the result of a mixing process between lake saltwater and freshwater from RGF (GW-38), as inferred for Cl\(^-\). However, decrease in SO\(_4^{2-}\) is slightly higher than Cl\(^-\) decrease in depth in samples from July 2010, October 2010 and November 2010. A possible explanation may be a combined effect of mixing and other processes that decrease the amount of dissolved SO\(_4^{2-}\), such as mineral precipitation or BSR processes. Sulfate reduction can occur in environments with low oxygen and nitrate concentrations, low Eh, and the presence of reactive organic matter (Hem, 1985; Miao et al., 2012). Concerning to organic matter, the highest DOC levels are found in surface water samples. Moreover, the sediment from the bottom of the lake has shown itself to be an important source of organic C (Carrey et al., 2014). DOC concentrations dramatically decrease from surface water to GW-12, mainly related to organic carbon consumption and the availability of organic substrate. DOC contents in GW-12 are still significant and can be derived from the organic matter not
consumed in the sediment-water interface from the lake. In the deepest piezometers (GW-34 and GW-38), DOC seems to be related to the organic matter present in the Lower Cretaceous sediments from the Utrillas Facies (Carrey et al., 2013).

5.2 Source of sulfate

Potential sources of sulfate in Pétrola Lake are mainly related with sulfides and sulfates (Fig. 7). In general, $\delta^{34}$S in all S-bearing species (dissolved $\text{SO}_4^{2-}$, sulfide and sulfate-bearing minerals) show negative values (-40‰ to -9.9‰). This range, and the absence of relevant sedimentary evaporite formations in the study area, imply that the main source of sulfur in Pétrola basin is disseminated pyrite in the organic-rich sediments interbedded in the Utrillas Facies (Gómez-Alday et al., 2014). Disseminated secondary gypsum derives from disseminated pyrite oxidation showing $\delta^{34}$S values (-22.1‰ to -15.7‰) consistent with those observed in the sulfate-bearing minerals from the lake evaporitic crusts (-20.5‰ to -18.3‰).

Sulfide oxidation should theoretically impart negligible isotope fractionation for $\delta^{34}$S$_{\text{SO}_4}$ (Habicht et al., 1998), and $\delta^{18}$O$_{\text{SO}_4}$ should be derived from oxygen in water and/or air. The sulfide oxidation box in Figure 7 was drawn using maximum and minimum values of $\delta^{34}$S$_{\text{Sulfide}}$, whereas $\delta^{18}$O$_{\text{SO}_4}$ was calculated following the equations defined by Van Stempvoort and Krouse (1994) for the theoretical value of dissolved $\text{SO}_4^{2-}$ derived from sulfide oxidation. The authors defined a lower value in which all the oxygen in dissolved $\text{SO}_4^{2-}$ is derived from water oxygen ($\delta^{18}$O$_{\text{SO}_4} = \delta^{18}$O$_{\text{H}_2\text{O}}$), and an upper limit where part of the sulfate oxygen is derived from water and part from air, and where fractionation in the incorporation of oxygen and water has been considered ($\delta^{18}$O$_{\text{SO}_4} = 0.62 \cdot \delta^{18}$O$_{\text{H}_2\text{O}} + 9$, Eq. 1). The $\delta^{18}$O$_{\text{H}_2\text{O}}$ used in Eq. 1 is the average value measured at the corresponding depth throughout the sampling period (2008-2011). The lowest $\delta^{34}$S$_{\text{Sulfide}}$ value (-40.5‰) is found in the deepest sample at 37.3 mbgs. Most of the sulfide samples range from -40‰ to -33‰, except three
samples collected between 8 mbgs and 11 mbgs (samples ANNA-11b, ANNA-12b and ANNA-22c) that show higher $\delta^{34}\text{S}_{\text{Sulfide}}$ values, from -19‰ to -11‰.

Sulfate-bearing minerals (gypsum) present as secondary mineral in lower Cretaceous sediments and in evaporitic crust (gypsum, hexahydrite, and epsomite) deposited seasonally in the lake are another potential source of dissolved sulfate in the system. Isotopically, samples corresponding to sulfate-bearing minerals show significant variability in $\delta^{34}\text{S}_{\text{SO}_4}$ and $\delta^{18}\text{O}_{\text{SO}_4}$ values.

Samples collected from the lake evaporitic crust show a low variance in $\delta^{34}\text{S}_{\text{SO}_4}$ (mean value of -20.1‰ ± 0.8‰), whereas $\delta^{18}\text{O}_{\text{SO}_4}$ ranges from +14.3‰ to +18.2‰. The high values of $\delta^{18}\text{O}_{\text{SO}_4}$ in the lake evaporites compared to sulfates derived from sulfate oxidation can be explained by sulfate recycling. Dissolved $\text{SO}_4^{2-}$ can undergo sulfate reduction processes, generating $\text{H}_2\text{S}$, which can be re-oxidized. Then, dissolved $\text{SO}_4^{2-}$ may precipitate during evaporation producing enrichments up to +2.0‰ and +3.6‰ for $\delta^{34}\text{S}_{\text{SO}_4}$ and $\delta^{18}\text{O}_{\text{SO}_4}$, respectively (Lloyd, 1968; Van Driessche et al., 2016). $\delta^{18}\text{O}$ values found in the sulfate-bearing minerals deposited in lake evaporites are higher than the expected due to precipitation. These higher values of $\delta^{18}\text{O}_{\text{SO}_4}$ may be explained by variations in the degree of evaporation of surface water and the incorporation of $\delta^{18}\text{O}_{\text{H}_2\text{O}}$ signal of evaporated water in $\text{SO}_4^{2-}$ during $\text{H}_2\text{S}$ re-oxidization. This is supported by the $\delta^{18}\text{O}_{\text{SO}_4}$ values of the evaporites sampled in the linear transect (PE MIN-1 to PE MIN-12) of the lake, which show slightly lower oxygen values in samples from the lake margin, indicating less evaporated water (PE MIN-10, PE MIN-11) and slightly higher values in evaporites collected closer to the depocenter (PE MIN-1, PE MIN-2), indicating more evaporated water.

The isotope composition of sulfate-bearing minerals sampled in the piezometer cores shows more variability. Samples of sulfate-bearing minerals ANNA-17, ANNA-18, and ANNA-20, collected from 27 mbgs to 34 mbgs, show $\delta^{34}\text{S}_{\text{SO}_4}$ values between -30‰ and to -20‰, similar to the sulfides sampled at the same depth (Fig. 7). Furthermore, their $\delta^{18}\text{O}_{\text{SO}_4}$ values are in agreement with the precipitation of sulfate derived from sulfide oxidation. Samples ANNA-1 (10.8 mbgs) and ANNA-21 (8.4 mbgs) show similar $\delta^{34}\text{S}_{\text{SO}_4}$ values, but their higher $\delta^{18}\text{O}_{\text{SO}_4}$ values cannot be explained from sulfide oxidation in equilibrium with the
groundwater. These samples of secondary gypsum were collected at intermediate depths and δ\(^{18}\)O\(_{SO_4}\) should be interpreted as a mixture between sulfate derived from sulfide oxidation and from sulfate from the dissolved evaporitic crust minerals from the lake, which have a higher δ\(^{18}\)O\(_{SO_4}\) values. Finally, sample ANNA-22, obtained at 3.4 mbgs, has a δ\(^{34}\)S\(_{SO_4}\) of -9.9‰ and a δ\(^{18}\)O\(_{SO_4}\) of +16‰. Since gypsum dissolution has little or negligible isotope fractionation for sulfur and oxygen isotopes (Claypool et al., 1980), the values for sample ANNA-22 cannot be explained by precipitation or dissolution processes. An alternative explanation could be pyrite oxidation with an isotope composition similar to those found at 10 mbgs (δ\(^{34}\)S\(_{Sulfide}\) of -11‰, Fig. 7) and a highly water evaporated oxygen signature (δ\(^{18}\)O\(_{SO_4}\) of +16.4‰).

Secondary gypsum sampled from Lower Cretaceous outcrops (PE ARMO samples) show δ\(^{18}\)O\(_{SO_4}\) values much lower than sulfate-bearing minerals from the evaporitic crust from the lake, but similar to mineral samples collected from deep points in the piezometers (ANNA-17, ANNA-18, and ANNA-20). These δ\(^{18}\)O\(_{SO_4}\) values from PE ARMO samples (between -2.8‰ and +9.0‰) are close to those calculated for sulfate derived from sulfide oxidation in equilibrium with groundwater according to equation 1 (Van Stempvoort and Krouse, 1994). Moreover, samples PE ARMO-4a and PE ARMO-5 show the highest δ\(^{34}\)S\(_{SO_4}\) and δ\(^{18}\)O\(_{SO_4}\) of the group. These samples fall close to groundwater samples collected in GW-38, in the area of sulfate derived from sulfide oxidation (Fig. 7). Surface water (SW-0) and groundwater samples GW-12 and GW-26 show δ\(^{34}\)S\(_{SO_4}\) and δ\(^{18}\)O\(_{SO_4}\) values inside the area defined by the lake evaporites analysed. No significant differences are observed in the isotope composition of dissolved SO\(_4^{2-}\) between sampling dates, suggesting seasonal stability on both sulfate sources and recycling processes.

5.3 Sulfate recycling

For a better understanding of the processes involved in sulfate fate, the isotope composition of S and O from dissolved SO\(_4^{2-}\) was compared to sulfate-bearing minerals, and sulfides at different depths (Fig. 8). Additionally, values of δ\(^{18}\)O\(_{SO_4}\) which would be derived from sulfide oxidation following Eq. 1 are also
compared. For this purpose, the average $\delta^{18}O_{H2O}$ values of water for each sampling depth are used for the calculation. Surface water samples (SW-0), show the same $\delta^{34}S_{SO_4}$ and $\delta^{18}O_{SO_4}$ as the sulfate-bearing minerals from the lake’s evaportic crust, and similar $\delta^{34}S_{SO_4}$ values to secondary gypsum from Lower Cretaceous sediments (Fig. 8). Despite the changes in depth of the dissolved $SO_4^{2-}$ concentration, no noticeable variation is observed in the isotope composition at SW-0, GW-12, GW-26 and GW-34 (although isotope data is limited). The high $SO_4^{2-}$ concentration buffers the isotope composition and may mask sulfate-reduction processes. In the lake surface, precipitation and dissolution of S-bearing minerals must be also considered. During dry periods, evaporite mineral precipitation occurs, but also the high $SO_4^{2-}$ concentration would mask the slight isotope fractionation of gypsum precipitation. Sulfur evolution in the lake is dominated by the seasonal cycles of mineral precipitation and dissolution, which are common in hypersaline lakes (Cartwright et al., 2009). Then, in wet periods, evaporites are redissolved, increasing $SO_4^{2-}$ concentrations with no significant changes in the isotope values because dissolution produces insignificant isotope fractionation for both $\delta^{34}S_{SO_4}$ and $\delta^{18}O_{SO_4}$.

In GW-12, both the $SO_4^{2-}$ concentrations as well as the S and O isotope composition of dissolved $SO_4^{2-}$ become stable. The $\delta^{34}S_{SO_4}$ is similar to the isotope composition of the sulfate-bearing minerals from piezometer cores, which are also within the range of sulfide minerals collected at this depth (Fig. 8). However, $\delta^{18}O_{SO_4}$ is clearly higher than the value of sulfate-bearing minerals from the piezometer cores and the theoretical value of sulfate calculated for sulfides at this depth. Sulfate reduction processes cannot explain this high $\delta^{18}O_{SO_4}$ values since the $\delta^{34}S_{SO_4}$ did not show to be affected by this reaction. S-recycling incorporating higher $\delta^{18}O_{H2O}$ from evaporated water during mixing processes between lake water and fresh water, as suggested by hydrochemical and tritium data (Table 1) could explain this high $\delta^{18}O_{SO_4}$ values. The huge $SO_4^{2-}$ concentration at this depth (12.1 mbgs) supports this idea. Thus, the more likely hypothesis is that its origin is related to lake water recharge by DDF. The
δ³⁴S_{SO₄} and δ¹⁸O_{SO₄} in GW-12 is similar to dissolved SO₄²⁻ in the lake (Fig. 8), supporting this hypothesis. Currently, the system seems to have reached a hydrodynamic equilibrium between surface water, with the associated DDF, and the hydraulic potential of RGF. This assumption is also in agreement with water isotopic data (δ¹⁸O_{H₂O} and δ²H_{H₂O}) measured in the piezometers (Table 3).

Groundwater samples obtained from GW-26 and GW-34 show δ³⁴S_{SO₄} and δ¹⁸O_{SO₄} values indicating a mixing trend between GW-12 and GW-38. These results further support the idea of a mixing process between DDF and RGF. In the deepest well (GW-38), δ³⁴S_{SO₄} and δ¹⁸O_{SO₄} values are slightly higher than the expected values from sulfide oxidation and close to the ones from secondary gypsum from Lower Cretaceous outcrops. The dissolved SO₄²⁻ found in GW-38 could be derived mainly from secondary gypsum dissolution, which is mixed with the recharge water from the lake. This mixing process is in agreement with the water isotopic values of the samples. Hence, it could conceivably be hypothesized that sulfate derived from sulfide oxidation and secondary gypsum dissolution constantly accumulates in the lake. Seasonal variations affect precipitation and dissolution of S-bearing minerals in lake surface. Dissolved SO₄²⁻ can be also transported to the underlying aquifer by means of DDF.

5.4 Sulfate reduction processes

Evidence of BSR processes is provided by the microprofiling data, which show that H₂S is produced in the first cm of recent organic-rich sediments. Calculated rates of H₂S production (mean of 0.023 nmol/cm³·s) are similar to those described previously in Pétrola Lake (up to 0.025 nmol/cm³·s; Menchen et al., 2016), and are also in the range of other lake sediments (about 0.017 nmol/cm³·s; Smith and Klug, 1981), and slightly below saltmarsh sediments (up to 0.033 nmol/cm³·s; Roychoudhury et al., 2003) and estuarine sediments (up to 0.047 nmol/cm³·s in closed state system, Richards and Pallud, 2016). Microprofiles of H₂S show well-defined zones for sulfate reduction processes. The limiting factor for these processes are neither the organic matter nor the dissolved SO₄²⁻. Thus, the shape of the H₂S production curve suggests the re-
oxidation of H$_2$S to SO$_4^{2-}$ in the suboxic zone below the water-sediment interface. Furthermore, as BSR processes take place in the water-sediment interface, a significant part of the produced H$_2$S is feasible to be re-oxidized again to dissolved SO$_4^{2-}$. Besides the processes cited above, the formation of sulfur intermediates and further microbial disproportionation must be considered. Microbes responsible for BSR, such as Desulfobulbus spp. and Desulfovibrio spp., are able to perform disproportionation reactions (Widdel and Pfennig, 1982; Krekeler et al., 1997). Disproportionation of sulfur intermediates would lead to larger isotope fractionations in dissolved SO$_4^{2-}$ with $\delta^{34}$S$_{SO_4}$ enrichment up to +35‰ (Böttcher et al., 2005). Moreover, a depletion in $\delta^{34}$S from H$_2$S should be expected (up to -37‰; Habicht et al., 1998). Nevertheless, measurements of H$_2$S and sulfur intermediates were not performed. Thus, data is insufficient to make any assumption about the presence of microbial disproportionation in recent organic-rich sediments from Pétrola Lake.

Assuming the existence of sulfate-reducing processes, values of $\delta^{34}$S and $\delta^{18}$O$_{SO_4}$ from water samples (SW-0, GW-12, and GW-26) and mineral samples (lake evaporites) can be explained. In dissimilatory BSR, DOC acts as an electron donor, reducing sulfate under anaerobic conditions (Nordstrom et al., 2007), and the rate of sulfate reduction is typically C-limited (Madigan, 1997). Anaerobic conditions and high DOC concentrations are found in surface and piezometer water samples (Appendix A). Dissolved oxygen values show anoxic conditions, with minimum values of 0.26 mg/L in surface water, 0.03 mg/L in the shallowest piezometer (GW-12), and 0.08 mg/L in the one immediately below (GW-26). The main sources of DOC in lake water, with values up to 646 mg/L in surface water (October 2010), are the degradation of autochthonous (primary productivity of the lake) and allochthonous (wastewater) sources (Gómez-Alday et al., 2014). Organic matter deposition has shown a high capacity to promote reducing conditions in batch and column experiments (Carrey et al., 2014). In fact, the amount of organic C present in the sediment can be as high as 23% (Valiente et al., 2016). Previous laboratory experiments have also shown the high capacity of organic-rich sediments to promote reducing conditions (Carrey et al., 2013). In the sampled surface water and groundwater, NO$_3^{-}$
concentration are negligible and oxygen is limited, so the main electron acceptor is dissolved $\mathrm{SO}_4^{2-}$. These conditions are favourable for sulfate reduction (Champ et al., 1979).

Isotopically, BSR results in an increase in both the $\delta^{34}\mathrm{S}_{\mathrm{SO}_4}$ and $\delta^{18}\mathrm{O}_{\mathrm{SO}_4}$. Assuming that this reaction follows a Rayleigh distillation in a closed system, a negative relationship between $\ln[\mathrm{SO}_4^{2-}]$ and $\delta^{34}\mathrm{S}_{\mathrm{SO}_4}$ and $\delta^{18}\mathrm{O}_{\mathrm{SO}_4}$ would be expected (Spence et al., 2001). However, the samples show the opposite trend: an increase in $\delta^{34}\mathrm{S}_{\mathrm{SO}_4}$ and $\delta^{18}\mathrm{O}_{\mathrm{SO}_4}$ as $\ln[\mathrm{SO}_4^{2-}]$ increases. One feasible hypothesis to explain the lack of correlation, even when chemical data indicate favourable conditions for sulfate reduction, is that the large amount of $\mathrm{SO}_4^{2-}$ in surface and groundwater could mask the isotope shift associated to BSR. Furthermore, traces of sulfide and a faint odour of $\mathrm{H}_2\mathrm{S}$ were occasionally noted during surface water sampling, suggesting sulfate reduction, that was confirmed by microprofiling.

Biologically, 16S rDNA amplification was successful using two set of 16S rDNA-targeted primers of sulfate-reducing bacteria. Sequencing confirms the presence of sulfate-reducing bacteria belonging to Desulfovibrionaceae family (seq-1) and to Desulfovibrio genus (seq-2). However, the results do not discard the presence of other bacterial communities able to reduce sulfate in lake sediments. These results complement the hypothesis of BSR processes in the hypersaline sediments of Pétrola Lake. Prior studies that have noted the low isotope fractionation for $\delta^{34}\mathrm{S}$ ($\varepsilon=-2.0\%_o$) produced by bacteria from genus Desulfovibrio in hypersaline mats ($D.\ halophilus$; Detmers et al., 2001). Therefore, the low variability in the isotope composition observed in the samples can also be attributed to the low isotope fractionation.

Based on the surface water and groundwater samples (SW-0, GW-12, and GW-26) with the highest $\delta^{34}\mathrm{S}_{\mathrm{SO}_4}$ and $\delta^{18}\mathrm{O}_{\mathrm{SO}_4}$ values, a simplified model has been calculated for the evolution of $\mathrm{SO}_4^{2-}$ concentrations and $\delta^{34}\mathrm{S}$ isotope composition during BSR (Fig. 9). No data was found in the literature about isotope fractionation for $\delta^{34}\mathrm{S}$ produced by the closest microorganism reported by phylogenetic analyses ($D.\ senezii$). Thus, the reported isotope fractionation for $D.\ halophilus$ was used for calculation: a 40% complete sulfate reduction is
needed to produce a 1‰ shift in $\delta^{34}$S$_{SO_4}$. Consequently, taking into account the high SO$_4^{2-}$ concentration of the samples, the isotope effect of BSR is masked. The evolution of $\delta^{18}$O$_{SO_4}$ does not agree with a BSR model, and a negative correlation between $\delta^{18}$O$_{SO_4}$ and $1/[SO_4^{2-}]$ is observed ($R^2=0.94$), suggesting that mixing rather than other processes is controlling $\delta^{18}$O$_{SO_4}$. This may be influenced by the constant cycles of evaporation and dissolution in surface water that also mask the relationship between isotope composition and SO$_4^{2-}$ concentration.

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6. Conclusions

In the Pétrola Lake-aquifer system, the continuous recycling of sulfur is controlled by three main processes: (1) seasonal evaporation cycles, (2) hydrodynamic instability driven by the different density-driven groundwater flow, and (3) sulfate-reduction processes (BSR). Sulfate distribution is controlled by evaporation (due to seasonal drought) and mixing processes (from a DDF towards the underlying aquifer). Hydrochemical and isotopic evolution in depth indicate that lake water and groundwater are in hydraulic connection, and DDF is able to transport reactive organic matter and other solutes (e.g. SO$_4^{2-}$) from the lake to the underlying aquifer. Despite seasonal variations found in surface water, SO$_4^{2-}$ concentrations and $\delta^{34}$S$_{SO_4}$ become almost constant during the study period in the piezometers, suggesting quasi-equilibrium between DDF and RGF. Sulfate in Pétrola Lake is originally derived from: (1) oxidation of disseminated pyrite present in the Lower Cretaceous sediments, and (2) dissolution of secondary gypsum derived from sulfide oxidation in Lower Cretaceous sediments. The presence of sulfate-reducing bacteria belonging to Desulfovibrio family and the detected production of H$_2$S in the first cm of sediment, confirm the occurrence of BSR processes. Nevertheless, the isotope effect of BSR is masked due to low isotope fractionation and the large amount of dissolved SO$_4^{2-}$ in surface water and groundwater. The analysis of sulfur dynamics undertaken here has extended our knowledge of the sulfur cycle in a hypersaline system. Further work could focus on the interaction between S-
cycle and pollutants attenuation (e.g. nitrate), as well as other potential biotic
processes (e.g. microbial disproportionation) in Pétrola hypersaline lake-aquifer
system.

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Tracing sulfate recycling in the hypersaline Pétrola Lake (SE Spain): A combined isotopic and microbiological approach

N. Valiente\textsuperscript{a}, R. Carrey\textsuperscript{b}, N. Otero\textsuperscript{b, c}, M.A. Gutiérrez-Villanueva\textsuperscript{a}, A. Soler\textsuperscript{b}, D. Sanz\textsuperscript{a}, S. Castaño\textsuperscript{a}, and J.J. Gómez-Alday\textsuperscript{a}

\textsuperscript{a} Biotechnology and Natural Resources Section, Institute for Regional Development (IDR), University of Castilla–La Mancha (UCLM), Campus Universitario s/n, 02071 Albacete, Spain

\textsuperscript{b} Grup d’Mineralogia Aplicada i Medi Ambient, Dep. Mineralogia, Petrologia i Geologia Aplicada, Facultat de Ciències de la Terra, Universitat de Barcelona (UB), C/ Martí i Franquès s/n, 08028, Barcelona, Spain

\textsuperscript{c} Serra Hunter Fellowship, Generalitat de Catalunya, Spain

Figure captions

<table>
<thead>
<tr>
<th>Figure</th>
<th>Caption</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>A) Location of Castilla-La Mancha region. B) Pétrola endorheic basin and Pétrola Lake. C) Geological map of Pétrola Basin and location of sample points: 1. SW-0; 2. Transect of PE MIN solid samples; 3. Piezometers; 4. PE ARMO 2; 5. PE ARMO 3; 6. PE ARMO 4 a and PE ARMO 4 b; 7. PE ARMO 5; 8. PE ARMO 6; 9. PE ARMO 7.</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Map with the location of SW-0 and piezometers in Pétrola Lake. Piezometers follow a radial pattern from the lake margin (SE, GW-34, x: 624943.5, y: 4299678.2) towards the lake center (NW, GW-26, x: 624921.9, y: 4299674.6), separated at 6 meters’ equidistance. The simplified hydrogeological cross-section I–I’ shows the geology below the lake and the location of the piezometers. Mineral samples (ANNA-1 to ANNA-22) from the cores are indicated in the piezometers map. AB: anoxic brine. masl: meters above sea level. mbgs: meters below ground surface.</td>
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<td>Figure 3.</td>
<td>Groundwater depth profiles in GW-12, GW-26, and GW-34 piezometer (lines). Precipitation (P) between February-2010 and October-2011 (bars). Electrical conductivity (EC) profiles in the piezometers in mS/cm are shown for the studied period.</td>
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<td>Figure 4.</td>
<td>Piper diagram showing the chemical composition of the water samples during the studied period.</td>
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<tr>
<td>Figure 5.</td>
<td>Regression line for $\delta^{18}$O$\textsubscript{H_2O}$ and $\delta^{2}$H$\textsubscript{H_2O}$ values in water samples (n=29) collected from piezometers and surface water. GMWL: Global Meteoric Water Line (Craig, 1961). WP: weighted precipitation from Madrid.</td>
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</table>
Figure 6. Hydrochemical profiles: sulfate (SO$_4^{2-}$), chloride (Cl$^-$), and dissolved organic carbon (DOC), from samples collected in the piezometers and surface water: a) February 2010; b) April 2010; c) July 2010; d) October 2010; e) November 2010; f) October 2011.

Figure 7. $\delta^{34}$S$_{SO_4}$ versus $\delta^{18}$O$_{SO_4}$ plot. The evaporite lake area is defined by the maximum and minimum analysed values for $\delta^{34}$S and $\delta^{18}$O in sulfate-bearing mineral samples from the lake. The sulfate oxidation area is defined by the maximum and minimum analysed values for $\delta^{34}$S, as well as the maximum and minimum calculated values for $\delta^{18}$O derived from sulfide oxidation (Van Stempvoort and Krouse, 1994). The fertilizer area is adapted from Vitòria et al. (2004). Atmospheric $\delta^{18}$O value is defined in the literature (Kroopnick and Craig, 1972). Sulfate-bearing minerals from: e.c.: evaporite crust; p.c.: piezometer cores; s.c.: secondary gypsum.

Figure 8. A) $\delta^{34}$S vs depth of dissolved sulfate, sulfides, and sulfate-bearing minerals from lake, piezometers, and basin outcrops. B) $\delta^{18}$O vs depth of dissolved sulfate, sulfate-bearing minerals from lake, piezometers, and basin outcrops, and $\delta^{18}$O values calculated following the upper limit equations (Eq. 1) of Van Stempvoort and Krouse (1994) using the higher $\delta^{18}$O$_{H_2O}$ measured at the corresponding depth throughout the sampling period.

Figure 9. $\delta^{34}$S$_{SO_4}$ vs SO$_4^{2-}$ concentration for SW-0, GW-12, and GW-26 sampling points. The graph shows simplified models of the evolution of sulfate concentration and $\delta^{34}$S values during BSR using isotope fractionation of -2‰.
Figure 9

The graph illustrates the relationship between sulfate concentration ($\text{SO}_4^2$) and the sulfur isotope ratio ($\delta^{34}\text{S}_{\text{SO}_4}$) for different models. The models are colored differently:

- Model GW-26 (red cross)
- Model GW-12 (orange cross)
- Model SW-0 (gray cross)

The graph shows data points representing different percentages: 100%, 80%, 60%, 40%, and 20%. The x-axis represents the sulfur isotope ratio, while the y-axis represents the sulfate concentration in milligrams per liter (mg/L).
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Tables

Table 1. Tritium activity (TU) in water samples. GW: groundwater. SW: surface water.

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<th>Sample Point</th>
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<td>0.8 ± 0.4</td>
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<td>GW-34</td>
<td>30/04/2010</td>
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<td>GW-26</td>
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<td>SW-0</td>
<td>18/06/2010</td>
<td>4.6 ± 0.3</td>
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Table 2. Mean values for sample point of the chemical analyses in water samples (mg/L, except Fe and Mn in μg/L). EC: electric conductivity (μS/cm). Eh: redox potential (mV). DO: dissolved oxygen (mg/L). T: temperature (ºC). DOC: dissolved organic carbon (mg/L). BLD: below limit of detection. GW: groundwater. SW: surface water.

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<th>Eh</th>
<th>DO</th>
<th>T</th>
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<th>HCO₃</th>
<th>Cl</th>
<th>Na</th>
<th>K</th>
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<td>BLD</td>
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Table 3. Isotope values (‰) in water samples. GW: groundwater. SW: surface water. nd: no determined.

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<td>nd</td>
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Table 4. Isotope values (‰) of δ\(^{34}\)S and δ\(^{18}\)O in S-bearing mineral samples. Depth in metres below ground surface (mbgs). nd: no determined.

<table>
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<th>Sample Point</th>
<th>Description</th>
<th>Mineral</th>
<th>Depth (mbgs)</th>
<th>δ(^{34})S</th>
<th>δ(^{18})O</th>
<th>δ(^{18})O(_{\text{SO}_4})</th>
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Table 5. Mean saturation indices for gypsum, epsomite, halite, and hexahydrite. GW: groundwater. SW: surface water.

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<th>Sample Point</th>
<th>SI_{Gypsum}</th>
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<th>SI_{Halite}</th>
<th>SI_{Hexahydrite}</th>
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<td>-1.10</td>
<td>-1.60</td>
<td>-1.38</td>
</tr>
</tbody>
</table>
Supplementary Information - Appendix A

Click here to download Background dataset for online publication only: AppendixA_Valiente_CHEMGE-S-17-00184.pdf
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