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Contrasting Arsenic biogeochemical cycling in two Moroccan alkaline pit lakes

Marina Héry[1*], Angélique Desoeuvre[1], El Mehdi Benyassine[2], Odile Bruneel[1], Sophie Delpoux[1], Eléonore Resongles[1], Abdelilah Dekayir[2], Corinne Casiot[1].

[1] HydroSciences Montpellier, University of Montpellier, CNRS, IRD, Montpellier, France.

[2] Equipe Géoexplorations & Géotechniques, University Moulay Ismail, BP.11201, Zitoune, Meknes, Morocco.

marina.hery@umontpellier.fr *Correspondence and reprints

angelique.desoeuvre@umontpellier.fr

benyassine.elmehdi@gmail.com

odile.bruneel@ird.fr

eleonore.resongles@umontpellier.fr

sophie.delpoux@umontpellier.fr

dekayir@yahoo.fr

corinne.casiot-marouani@umontpellier.fr

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Abstract

Pit lakes resulting from the flooding of abandoned mines represent a valuable freshwater reserve. However, water contamination by toxic elements, including arsenic, compromises their use for freshwater supply. For a better management of these reserves, our aim was to gain insight into arsenic cycling in two Moroccan alkaline pit lakes. We first showed that dimethylarsenic dominated in stratified lake ZA whereas in lake ZL1, As(V) was prevailing. Because microbially mediated processes largely contribute to arsenic cycling, the diversity of arsenic-methylating and -oxidizing bacteria was determined through the sequencing of *arsM* and *aioA* genes. Diverse *arsM*-carrying bacteria were thriving in ZA while a low diversity of *aioA* genes was detected in ZL1. We also determined the structure of the total bacterial communities by fingerprinting (ARISA). Contrasting arsenic speciation and bacterial communities in the two lakes were associated with differences of conductivity, Total Organic Carbon and temperature. In ZA, dissolved oxygen and redox potential were the main factors driving the total bacterial community structure and the ArsM diversity. In ZL1, stable bacterial communities were associated with limited water physico-chemistry variations. Our study provides new insights into the biogeochemical behavior of arsenic and the role of arsenic transforming bacteria in alkaline pit lakes.

Keywords: arsenic; biotransformation; alkaline pit lake; methylation; oxidation.

1. Introduction

Mining of metalliferous deposits leads to excavation of large amounts of rocks resulting in open pits where mineralized rocks are exposed to weathering. As rainwater accumulates and groundwater rebounds, a lake may form inside the mine pit. Lake waters

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represent precious freshwater supply, especially in areas suffering from deficient water resources as in semi-arid area in southern Mediterranean countries. However, the potential release of metals and metalloids from mineralized rocks seriously jeopardizes the safe use of this freshwater resource. Among these toxic elements, arsenic represents a threat for environmental and public health. Arsenic is widespread in mining areas where it is frequently associated with the exploited metal sulfides [1].

In Morocco, opencast mining operations of metal sulfides left a number of pit lakes which are currently used for irrigation and livestock watering [2-4]. These lakes exhibit near-neutral to alkaline pH due to the predominance of carbonate rocks [5]. Arsenic concentrations exceeding Moroccan standards for irrigation water have been measured in these lakes [2-4]. The release of arsenic from mine pit wall is favored at alkaline pH because arsenic oxyanions, arsenite ($H_2AsO_3^-$) and arsenate ($HAsO_4^{2-}$), hardly sorb to the surfaces of negatively charged iron oxyhydroxides [6,7].

Microorganisms have evolved diverse strategies to transform arsenic for detoxification or metabolism purpose) [8,9]. Arsenic microbially mediated biotransformations are highly relevant to the global arsenic cycling in the environment [10]. Many bacteria have the ability to oxidize arsenite into less toxic and less mobile arsenate [11-14], for detoxification purposes or energy generation. This function is conferred by an arsenite oxidase (encoded by *aio* gene) [15-17]. Another type of As(III) oxidase was identified in 2010 in haloalkaliphile *Alkalilimnicola ehrlichii* MLHE-1. In this strain isolated from Mono Lake, the oxidation of arsenite is coupled to the reduction of nitrate and carried out by a new gene family called *arxA* [18]. Some microorganisms have the capacity to transform arsenite into methylated forms [19]. This biomethylation process involves the S-adenosylmethionine methyltransferase encoded by arsM genes [20]. Methylated As species, both volatile (e.g. trimethylarsenic (TMA) and trimethylarsine (TMAO) and non-volatile (e.g. monomethylarsenic (MMA) and

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dimethylarsenic (DMA)), are less toxic than their inorganic forms [21]. Thus, biomethylation is generally considered as one of the main detoxification pathways for As in the environment [22]. However, because some intermediate products are highly toxic, arsenic biomethylation as a detoxification process has been questioned [21, 23-24]. Finally, under reducing conditions, dissimilatory arsenate-reducing prokaryotes use As(V) as final electron acceptor for anaerobic respiration [25].

So far, attention has been mainly focused on arsenic biogeochemistry in paddy soils [26], marine waters [27-28], groundwater used as drinking water (for a recent review see [29]), or in extreme environments such as soda lakes located in geothermal regions [30-31]. In comparison, the question of the microbially mediated transformations of arsenic in alkaline mine pit lakes, characterized by a moderate salinity (conductivity) compared to soda lakes (6.1-15.5 mS/cm^{-1} in ZA or ZL1 compared to 42-86 mS/cm^{-1} in Mono Lake,) has received little attention. Such alkaline pit lakes are common along the southern margin of the Mediterranean Sea, where metal deposits occur within carbonated layers of Mesozoic and Tertiary sediments [5,32].

In the present study, we investigated the spatial and seasonal dynamics of arsenic concentrations (total dissolved As and its species arsenite, arsenate, monomethylarsenic (MMA) and dimethylarsenic (DMA)) in two Moroccan alkaline pit lakes used for irrigation and livestock watering. The dynamic of the whole bacterial community thriving in these lakes was assessed by an automated fingerprinting approach (Automated Ribosomal Intergenic Spacer Analysis [33]). ARISA has a similar capacity as metabarcoding to discriminate environmental samples and to correlate bacterial community structure with environmental variables [34]. To gain insight into the biogeochemical processes controlling arsenic speciation in these environments, and based on arsenic speciation results, we specifically

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targeted microbial populations involved in arsenic oxidation and biomethylation through the study of their respective marker genes *aioA* and *arsM*.

2. Materials and methods

2.1.Site description

The Zeida mining area is located in the High Moulouya basin. It is bounded by the High Atlas on the southeast and by the Middle Atlas on the northwest (Fig. 1A). The geology of the area has been described in [4] and [35]. The climatic conditions in the upper Moulouya region are semi-arid, with annual precipitation of 100–400 mm and mean annual temperatures of 12–14°C. The region faces a rainfall deficit caused by recurrent droughts. Lead has been exploited until 1985, from lead–barite ores [2]. In Zeida, the exploited orebody consisted mainly of cerussite [$PbCO_3$] (70%) and galena [PbS] (30%) [36]. After mining activity ceased, the region shifted into livestock breeding and orchards (apple trees).

The two pit lakes under study were lake ZA (32°50.112’ N, 04°57.223’ W), with a volume of 1 Mm^3, and lake ZL1 (32°47.517’ N, 04°58.815’ W), with a volume of 2 Mm^3. They are located respectively 2 km north-east and 5 km south-west from the small town of Zeida (Fig. 1A). ZA is located next to the waste pile of the ore treatment facility of Zeida which treated the ore by mechanical (crushing at 250 mm, grinding at 0.3 mm) and chemical processes (flotation with the use of sulfydrate sodium amylxanthate, sodium silicate and pine tree oil) (Fig. 1B). ZL1 is surrounded by tailings material from ore excavation (Fig. 1C).

2.2.Sampling

In October 2012 and in July 2013, water samples (1 L) were collected from a boat in lake ZA and lake ZL1, at the surface and the bottom of the lakes (5 m depth for lake ZA, 12.5 m depth for lake ZL1), using a Merkos sampler (Hydro-bios, Germany). The main physico-

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chemical parameters (pH, temperature, redox potential, conductivity and dissolved oxygen concentration) were measured *in situ* as soon as the sampler was taken out of the water using an HQ40d portable multi-parameter (HACH Co., Loveland, CO, USA) equipped with a refillable standard pH liquid electrode (pHC30101), a standard conductivity electrode (CDC40101), a gel filled ORP electrode (MTC10101) and a standard LDO electrode (LDO10101).

2.3. Sample treatment and chemical analyses

Fifty milliliters of unfiltered water was used for the analysis of bicarbonate and carbonate by titration with standardized HCl 0,1 N. For total organic carbon, samples (60 ml) were taken in amber glass bottles previously decontaminated by heating at 550 °C, preserved by adding H_3PO_4 1:1000 (v:v) and analyzed within 2 weeks. Another 200 ml of water were filtered in the field through 0.22 µm Millipore membranes fitted on Sartorius polycarbonate filter holders. Subsamples were preserved for the analysis of major anions (Cl^-, SO_4^{2-}) and cations (Na^+, K^+, Mg^{2+}, Ca^{2+}), trace elements and arsenic speciation according to the routine procedures described in [37] and analyzed within a few days after collection.

Inorganic and methylated arsenic species (As(III), As(V), MMA and DMA) were analyzed using anion-exchange chromatography (25 cm × 4.1 mm i.d. Hamilton PRP-X100 column and SpectraSystem P4000 ThermoScientific pump) coupled to ICP-MS (Thermo X7 Series), in gradient elution mode, with an ammonium phosphate eluent, as described in [38]. Analysis of major cations and anions was carried out using ion chromatography (Dionex ICS-1000). Total organic carbon concentration was determined using a high-temperature catalytic oxidation method (HTCO), with a Shimadzu TOC-VCSH total organic carbon analyzer. Analysis of trace elements (Mn, Al, Zn, Pb, and Fe) was carried out using ICP-MS (Thermo X7 Series), as described elsewhere [37].

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To estimate the photosynthetic microbial biomass, Chlorophyll a (Chla) was quantified on 300 ml samples of lake waters previously filtrated on glass fiber filters according to the method NF T 90 -117 (Centre d'analyses Mediterrannée Pyrénées, Perpignan, France).

2.4 Sample treatment and microbial analysis

Microbial analyses were performed on water samples from the surface and the bottom of each lake, for the two sampling campaigns. For each water sample, six aliquots of 250 ml were filtrated in the field through 0.22 µm cellulose acetate filter (Sartorius stedim biotech, Aubagne, France) to obtain six replicates. Each filter was immediately transferred to a cryotube, kept at 4°C in a container with ice packs before reaching the laboratory and frozen at -80°C until DNA extraction. This protocol was applied in October 2012 for ZL1 lake and in July 2013 for ZA and ZL1 lakes. For ZA lake in October 2012, an alternative treatment was used to collect the microbial biomass; indeed, the high suspended matter content prevented the filtration of ZA lake waters *in situ*. Water samples (300 ml, in triplicate) were centrifuged (6,000 g, 20 min) back to the laboratory to pellet the microbial cells. The cell pellets were stored at -20°C until DNA extraction. Samples were named as follow: ZAsO12 for ZA lake sampled at the surface in October 2012, ZAbO12 for ZA lake sampled at the bottom in October 2012, ZAsJ13 and ZAbJ13 for ZA in July 2013, ZL1sO12 and ZL1bO12 for ZL1 in October 2012, ZL1sJ13 and ZL1bJ13 for ZL1 in July 2013.

2.4.1. Microbial DNA isolation and Automated Ribosomal Intergenic Spacer Analysis (ARISA)

Total DNA was extracted from the filters (ZL1 October 2012 and July 2013, and ZA July 2013) or from cell pellets obtained by centrifugation (ZA October 2012) using the

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PowerWater® DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Yield of DNA was determined using the QuBit® fluorometer (Invitrogen, Carlsbad, CA, United States) and the high sensitivity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). For each sample, triplicate DNA extractions were performed from three of the six filters or pellets (the three other replicates were kept at -80°C as backup). The bacterial intergenic spacer regions between the small (16S) and the large (23S) subunits of ribosomal sequences were amplified from extracted DNA by PCR, using primers ITSF (5′-GTCGTAACAAGGTAGCCGTA-3′) and ITSR (5′-GCCAAGGCATCCACC-3′) [39]. For each of the eight samples, three independent PCR were performed on triplicate DNA extracts. PCR mix was prepared as follows: around 1 ng of DNA extract, 12 µl of amplitaq Gold 360 master mix (Thermo Fisher Scientific), 2.5 µg of bovine serum albumin, and 0.5 µM of each primer, in a total volume of 25 µl. Cycling conditions were as previously described in [39]. PCR products were checked on a 2% agarose gel. Then PCR products were analyzed on a microfluidic lab-on-a-chip (Agilent technologies, Santa Clara, United States). The lab-on-a-chip device is a fully automated electrophoresis system consisting of circuits of tiny closed channels and wells, etched onto a plastic microchip. Samples (PCR products) passed through selected pathways in a controlled manner. The DNA fragments (ranging between 263 and 1500 bp depending on the bacterial taxa) were separated using the Agilent 2100 bioanalyser and the High Sensitivity DNA Analysis Kit. The fluorescence intensity was plotted versus the DNA fragment size and electropherograms corresponding to the band profiles characteristic of each bacterial community were edited with the Agilent 2100 bioanalyser software (including background noise removal). Then, three reproducible ARISA profiles were obtained for each water sample.

Data (expressed as peak size and peak area) were exported into Excel. One peak corresponds to one band on the profile (characterized by its size), and thus theoretically to one

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bacterial phylotype present in the community; the area of the peak reflects the relative abundance of the corresponding phylotype inside the whole community. To take into account the variability of the technique, peak sizes (band sizes) were classified inside 10 pb-interval classes. A peak table was generated using the Agilent software for each triplicate.

Statistical analyses were performed using the free software R 3.4.3 (http://www.r-project.org/, 2017). Nonmetric multidimensional scaling (NMDS) was used to graphically depict differences between the structures of the bacterial communities from the different samples (based on the normalized ARISA data expressed as the area of the peaks). The significance of the observed repartition of samples on the ordination plot was assessed by an ANalysis Of SIMilarity (ANOSIM, 999 permutations). To examine the relationships between bacterial structure communities and environmental variables, the main physico-chemical variables (temperature, pH, conductivity, redox potential, dissolved oxygen concentration and Total Organic Carbon concentration) were fitted onto NMDS. The environmental fitting (ef) model was tested with Monte Carlo permutation tests (999 randomized runs) to determine its significance. Plotting was limited to the most significant variables with argument p.max = 0.05 (after adjustment of the p values with the Banferroni correction). The length of the arrow is proportional to the correlation between the ordination of bacterial community structures and the environmental variable, called the strength of the gradient.

2.4.2. arsM and aioA amplification, cloning and sequencing

For each of the eight samples, triplicate DNA extracts were pooled in equimolar proportions and PCR were performed on the eight DNA pools. Reactions, cycling conditions and primers for *arsM* gene amplification were previously described in [40]. For *aioA* gene amplification, primers aoxBM1-2F (5'-CCACTTCTGCATCGTGGGNTGYGGNTA-3') and

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aoxBM3-2R (5'-TGTCGTTGCCCCAGATGADNCCYTTYT C-3') were used to amplify a 1100 bp fragment [17]. The reaction contained 0.32 µM of each primer, 10 ng of DNA extract, 1.5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 2.5 U of BIOTAQ™ DNA polymerase (Eurobio, France) and 1X PCR buffer. Water was added to a final volume of 50 µl. The PCR conditions included a 5 min denaturation step at 94°C, followed by 35 cycles of a 45 s denaturation at 94°C, primer annealing of 45 s at 55°C, a 50 s extension at 72°C, and a final extension at 72°C for 5 min. The *arsM* and *aioA* PCR products were visualized and purified after migration on an agarose gel using the Gel Band Purification Kit (GE-Healthcare, Munich, Germany).

arsM and *aioA* purified PCR products were cloned into a pCR® 2.1 vector and transformed into One Shot® TOP10 chemically Competent *Escherichia coli* TOP10 (Life Technologies, Carlsbad, CA) according to the protocols of the manufacturer. Twenty-seven to 45 positive clones per library were sequenced by GATC biotech (Konstanz, Germany) with the M13 reverse primer. Nucleotide sequences were aligned with Muscle [41]. Conversion in protein sequences and phylogenetic analysis were carried out with the software MEGA6.06 [42]. Amino acid sequences were compared with the GenBank database (NCBI) using BLAST [43]. Operational taxonomic units (OTUs) were determined using similarity levels between sequences of at least 80% of nucleotide similarity for *arsM* and 95% for *aioA* using Mothur v.1.33.2 [44]. Amino acid phylogenetic trees were constructed with a sequence representative of each OTU using the Maximum Likelihood method based on the LG model with 1000 bootstraps replicates. Initial tree(s) for the heuristic search were obtained by applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT model. A discrete Gamma distribution was used. The OTU-based DNA clustering data were also used to calculate rarefaction curves with R 3.6.0 to assess OTU richness from the different samplings.

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240 *2.4.3. Sequence accession numbers*

241 The nucleotide and amino acid sequences reported in this article have been deposited
242 in the GenBank database under the accession numbers API83135to API83142.1 for *aioA* and
243 API83143.1 to API83165.1for *arsM*.

244

245 **3. Results**

246 *3.1.Physico-chemical characterization of the lake waters*

247 The two lakes exhibited a water temperature in the range 19.8 – 25.7 °C (Table 1). The
248 highest temperature was recorded at the surface of lake ZA in July 2013. The pH was slightly
249 alkaline, from 8.5 to 8.9 in ZA and from 9.0 to 9.1 in ZL1. The two lakes were characterized
250 by very high conductivity, particularly ZL1 (6.1 – 7.0 mS/cm in ZA; 14.5 – 15.5 mS/cm in
251 ZL1). In lake ZA, redox potential (Eh) and dissolved oxygen concentration values (DO)
252 revealed a redox gradient from the surface (DO = 9-11.5 mg/L, exceeded saturation in July
253 2013; Eh = 360-400 mV) to the bottom (DO = 4-5 mg/L; Eh = 60-150 mV). Conversely,
254 minor variations of DO and Eh occurred in lake ZL1 between the surface (DO=7.9-8.2 mg/L;
255 Eh=312-336 mV) and the bottom (DO=8.3-9.2 mg/L; Eh=320-331 mV) whatever the
256 sampling date. Total Organic Carbon concentration (TOC) was higher in ZA (~ 30-40 µg/L)
257 than in ZL1 (~ 2-5 µg/L). The Chla concentration, measured in July 2013 at the surface, was
258 higher in ZA (7 µg/L) than in ZL1 (< 1 µg/L).

259 Major ion concentrations revealed the Na-Cl-SO_4 facies of the lake waters (Table 1).
260 The concentrations of these elements largely exceeded the regulation values for irrigation
261 waters in the Moroccan legislation ($[Na^+]$ > 69 mg/L; $[Cl^-]$ > 350 mg/L; $[SO_4^{2-}]$ > 250 mg/L).
262 Maximum arsenic concentrations reached 42 µg/L in ZA and 147 µg/L in ZL1. The values in
263 ZL1 also exceeded Moroccan water quality standards for irrigation (100 µg/L). ZA was

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characterized by the predominance of dimethylarsenic and As(V) (July 2013) or dimethylarsenic and As(III) (October 2012). However, DMA was not detected at the bottom of lake ZA in October 2012. In ZL1, As(V) was the only species detected (Table 1). Among other trace elements regulated for irrigation water, Li and Mo also exceeded Moroccan standards in ZA ([Mo] > 10 µg/L) and ZL1 ([Mo] > 10 µg/L; [Li] > 2500 µg/L) (Table S1).

3.2. Whole bacterial community analysis

Whole bacterial community structure and its spatio-temporal variations were assessed by ARISA. The data, expressed as peak tables (deduced from ARISA electrophoregrams generated for each sample in triplicate), were used for NMDS analysis. The NMDS ordination shows that distinct bacterial communities thrived in waters from lakes ZL1 and ZA (ANOSIM r2 = 0.5845, p = 0.001; Fig. 2A). In lake ZL1, a limited spatio-temporal variability was observed among bacterial communities. In October 2012, the structures of the bacterial communities at the surface and at the bottom were undistinguishable. In contrast, marked temporal and spatial variations were observed in lake ZA. In particular, the bacterial communities sampled in October 2012 appeared widely different between surface and bottom. Vector fitting of possible explanatory environmental parameters revealed that conductivity (Cond), temperature (T°) and TOC contributed to the structuration of the bacterial communities in lake ZL1 and lake ZA. In ZL1, bacterial community structures were associated with a higher conductivity (r^2 = 0.7382, p = 0.006). In ZA, the structure of the communities was mainly associated to higher temperature (r^2 = 0.4322, p = 0.006) and TOC concentration (r^2 = 0.5364, p = 0.012) (Fig. 2A). On NMDS analysis based on data from lake ZA only, Eh (r^2 = 0.9768, p =0.006), DO (r^2 = 0.9335, p = 0.006) and conductivity (r^2 =0.9934, p = 0.006) emerged as the main factors structuring the communities (Fig. 2B). Other

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288 factors correlated with spatio-temporal changes in bacterial community structure were
289 temperature (r^2 = 0.9316, p = 0.006) and TOC (r^2 = 0.9535, p = 0.018).

290

291 *3.3. Arsenic methylating populations*

292 No *arsM* amplification was obtained with DNA extracted from ZL1 water samples.
293 *arsM* gene could be amplified from all the ZA samples except those collected at the bottom in
294 July 2013. The *arsM* diversity was not entirely covered (rarefaction curves did not reach an
295 asymptote), reflecting the important diversity of methylating bacteria in lake ZA (Fig. S1). A
296 total of 22 OTUs were identified. In October 2012, the richness was slightly higher at the
297 surface (13 OTUs) than at the bottom of the lake (8 OTUs). In July 2013, twelve OTUs were
298 identified at the surface of the lake.

299 Phylogenetic analysis (performed with one representative sequence of each of the 22
300 OTUs) showed a wide phylogenetic distribution of methylating bacteria in ZA (Fig. 3).
301 Amino acid derived sequences were affiliated with ArsM sequences from Acidobacteria,
302 Chloroflexi, Proteobacteria, Firmicutes, Bacteroidetes, Nitrospirales, Planctomycetes as well
303 as uncultured environmental clones. Interestingly, the large majority (83 %) of the ArsM
304 sequences from ZAbO12 grouped into five specific OTUs (containing no sequences from
305 other samples). On the same way, 37% of the clones from ZAsO12 grouped into five specific
306 OTUs. These observations suggest that distinct and specific methylating populations are
307 thriving at the surface and at the bottom of this lake. No clear seasonal variations were
308 observed for the distribution of *arsM* gene at the surface of the lake, suggesting these
309 populations were stable over time. The most dominant OTU (represented by sequence
310 ZAsJ13.23) contained sequences from all the ZA water samples (11% from ZAsO12, 3%
311 from ZAbO12 and 58% from ZAsJ13). This OTU was affiliated (87% of amino acid

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312 similarity) with the aerobic chemo-organotroph Acidobacteria *Bryobacter aggregatus* isolated
313 from an acid peat soil [45]. The second most dominant and specific OTU (represented by
314 sequence ZAbO12.22) contained 49% of the ZAbO12 sequences affiliated with a clone from
315 a Chinese paddy soil contaminated with arsenic (90% of amino acid similarity) [46]. The third
316 main OTU (represented by sequence ZAbO12.36) was also specific of the bottom of the lake
317 (25% of the ZAbO12 library). This OTU was related to *Thermoflexus hugenholtzii* (80% of
318 amino acid similarity), a thermophilic, heterotrophic microaerophilic and facultatively
319 anaerobic bacterium isolated from sediment of Great Boiling Spring in Nevada [47]. Overall,
320 few OTUs were closely related to any ArsM sequence from an isolated microorganism (Fig.
321 3).

322

323 *3.4. Arsenite oxidizing populations*

324 The *aioA* gene could be amplified only from ZL1sO12 and ZL1sJ13. The whole *aioA*
325 diversity was covered (rarefaction curves reached an asymptote) for sample ZL1sO12 (Fig.
326 S2), reflecting a low diversity of oxidizing microbial populations (4 OTUs). A higher richness
327 (8 OTUs) was observed for sample ZL1sJ13.

328 The sequences from ZL1sO12 grouped into four OTUs that also contained sequences
329 from July 2013. Other sequences from July 2013 grouped into four specific OTUs suggesting
330 possible seasonal structuration of arsenite-oxidizing bacterial communities. The majority of
331 the AioA sequences could not be related with confidence to any known organism, suggesting
332 the presence in ZL1 of As(III) oxidizers not described yet. The two dominant OTUs were
333 related to the putative AioA of the facultatively phototrophic *Rhodobacter capsulatus* (97%
334 and 98% of amino acid similarity respectively) (Fig. 4).

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4. Discussion

4.1. Arsenic speciation in the mine pit lakes

In lake ZL1, arsenic was present as inorganic As(V), whatever the season and the depth. This is consistent with the fully oxygenated conditions observed. Indeed, As(V) is the thermodynamically stable species in oxic conditions [48]. Arsenic speciation in ZL1 is consistent with other As-rich alkaline pit lakes in Canada and California where the shallow water depth favors oxygenated conditions and rapid oxidation of arsenite near the bottom sediment-water interface [49, 50]. In the present study, As(III) was not detected in lake ZL1. Thus, bacterially mediated reduction based on the *ars* detoxification system does not seem to contribute significantly to arsenic cycling in this lake. If dissimilarity As(V) reduction is not prone to occur in the oxygenated waters, such reductive process is not excluded in the anoxic sediments of the lake. Further characterization of the lake sediments would be required to determine if arsenic cycling in these pit lakes includes arsenate respiration as previously observed in sediments from a Mongolian soda lake [31].

In lake ZA, arsenic speciation was dominated by DMA. Several studies associated arsenic methylation in aquatic environments with phytoplankton development, linked to nutrient enrichment or seasonality (i.e light and temperature increase) [48, 51-52]. In the present study, ZA lake was characterized by a ten-fold higher TOC concentration as compared to lake ZL1. Moreover, the Chla concentration (July 2013) was seven-fold higher in lake ZA (7 µg/L) than in lake ZL1 (1 µg/L). This suggests phytoplankton development and accumulation of organic matter in lake ZA. Tang et al. [53] recently demonstrated that anoxia induced by the degradation of algal biomass in freshwater promoted reduction and methylation of arsenic at the sediment/water interface, owing to the significant increase in arsenate reductase genes (*arrA* and *arsC*), and arsenite methyltransferase genes (*arsM*).

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Although anoxia was not detected at the bottom of lake ZA, the DO decline from surface to bottom suggests that anoxia may occur in sediments, promoting microbial processes similar to those described by Tang et al. [53]. Thus, the presence of DMA in ZA might be linked to the eutrophic character of this lake. The high productivity of lake ZA may be favored by the erosion and leaching of nutrients from the ore flotation residues originating from the Zeida ore treatment facility, stored next to the lake. Indeed, the phosphorus content of these residues (1.77 ‰, [3]) is higher than the average content in soil (0.6 ‰) and they contain fine particles (50 % of particles with size lower than 0.2 mm, [3]), propitious for phosphorous leaching. DMA co-existed either with As(III) (October 2012) or with As(V) (July 2013) in ZA lake. However, DMA was not detected at the bottom of ZA in July 2013. Seasonal differences observed in arsenic speciation in ZA lake remains unexplained and a more comprehensive view of arsenic cycling in these pit lakes would require further investigations.

4.2. Microbial contribution to arsenic biogeochemical cycling in Moroccan pit lakes

A recent study investigated bacterial diversity in a meromictic pit lake where arsenic was present under the forms As(III) and As(V) depending on the redox conditions. Bacterial genera including potential As(III) oxidizers and As(V) reducers were identified although functional genes involved in arsenic biotransformation were not investigated [54]. Arsenic biogeochemistry has been studied in highly saline soda lakes originating from geothermal water input in California [55, 56] and Mongolia [31]. Soda lakes exhibit considerably higher As concentrations (~ 15 mg As/L) and salinity (70 to 90 g/L) compared to lakes ZL1 and ZA, but similar pH (pH 9.8). Arsenic speciation in soda lakes is dominated by inorganic As(III) and As(V) species [55]. Arsenic oxidation in Mono Lake is mediated by bacteria and is coupled to the reduction of nitrate rather than oxygen. Although both *aio*A and *arx*A transcripts were detected in Mono Lake transcriptome, As(III) oxidation appeared to be

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predominantly catalysed by ArxA under anoxic conditions [57]. In the present study, the detection of *aio*A gene in ZL1, together with the presence of As(V), suggests that aerobic bacterial arsenite oxidation occurred in this pit lake. However, arsenic might be released from the lake walls in the form of As(V). Furthermore, considering the oxygenated conditions of lake ZL1 whatever the season and depth, abiotic As(III) oxidation, although being a slow process, may not be excluded. Further investigations based on the expression of *aioA* would be necessary to conclude if bacterial oxidation actually occurs in ZL1. Because it is not possible to infer phylogenetic relationships based on *aioA* gene, the identity of the potential arsenite oxidizers remains unknown. The fact that *aio*A amplification failed for ZA lake samples doesn't necessarily reflects the absence of arsenite oxidizers. They may be present in very low proportions or they may not be targeted by the primers used in this study.

Contrary to ZL1, arsenic in ZA was predominantly in the methylated form (DMA). A wide range of prokaryotic and eukaryotic microorganisms (bacteria, archaea, algae, and fungi) has the capacity to methylate arsenic [19], including photosynthetic organisms [58]. Furthermore, complex organoarsenic compounds such as arsenosugars are produced by diverse organisms including algae, bacteria, and fungi [59]. Methyl arsenic species may originate from the degradation of such organic compounds by bacterial activity (as shown for seawater bacteria degrading arsenobetaine, [60]). However, our results clearly showed that the genetic potential for bacterially mediated arsenic methylation (*arsM* gene) is phylogenetically diverse and widely distributed in ZA waters. This strongly suggests that bacterial activity plays a direct role in the production of DMA in this lake. Environmental diversity surveys of *arsM* gene on aquatic environments are scarce. An unexpected diversity of bacteria with the genetic potential for arsenic methylation was also evidenced in a river impacted by acid mine drainage [40]. Conversely, arsenic methylation activity was not evidenced in Mono Lake, contrary to other arsenic-related functions [57]. Recently, Tang et al. [53] demonstrated in

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laboratory experiments that organic carbon enriched anoxic environments promoted the growth of arsenite methylating bacteria, leading to enhanced formation of methylated arsenic at the sediment/water interface. To our knowledge, the present work is the first report of methylated forms of arsenic associated with the presence of methylating bacteria in an alkaline pit lake. Similarly to *aioA*, *arsM* phylogeny does not allow to infer taxonomic identification of the corresponding bacteria. However, our findings strengthen the idea that bacterially mediated arsenic methylation may contribute to the global As biogeochemical cycle in freshwater ecosystems [40, 61]. A prominent role of biomethylation in soils was also recently suggested, based on the unexpected abundance of *arsM* in soil metagenomes [62].

4.3. Physico-chemical drivers of bacterial communities

ARISA fingerprinting showed that the structure of the whole bacterial community differed between the two lakes. The main parameters that shaped these communities were conductivity, TOC and temperature. Salinity (i.e. conductivity) can indeed strongly control microbial community composition [63, 64]. In freshwater reservoirs, organic carbon concentration appeared an important driver of the bacterial community composition, besides pH and alkalinity [65]. Thus, the contrasting values of conductivity, total organic carbon concentration and temperature in lakes ZA and ZL1 probably selected distinct bacterial populations. The study of the marker genes involved in As biotransformations points to the same conclusion. Indeed, a high diversity of bacteria with the *arsM* gene was recovered in lake ZA while this gene was not detected in lake ZL1. Conversely, *aioA* gene was amplified only in lake ZL1. This suggests that distinct bacterial groups were involved in the As cycle in ZA and in ZL1.

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In lake ZL1, the spatio-temporal variations of the whole bacterial community as well as of the arsenite oxidizing populations were limited. This is in agreement with the narrow range of variations highlighted for the water physico-chemical conditions. On the contrary, in the stratified ZA lake, pronounced changes in bacterial community structure and ArsM diversity were associated with marked spatio-temporal variations of the water physico-chemistry. Eighty three percent of ArsM sequences recovered at the bottom of ZA lake were not recovered in the surface waters (Fig.3). This may be explained by the drastic change in DO and Eh between surface (DO = 8.7-11.5 mg/L, Eh = 361-399 mV) and bottom (DO = 4.1-4.8 mg/L, Eh = 61-153 mV) waters. Reducing conditions generated near the sediment/water interface may have selected specific bacterial populations involved in As methylation [53]. Moreover, the dominant OTU containing 58% of the *arsM* sequences retrieved from surface waters in July 2013 represented only 10% of the whole *arsM* sequences in October 2012 (Fig. 3). This suggests a seasonal dynamic of the bacterial populations involved in As methylation in lake ZA. This dynamic can be related to seasonal change of TOC, temperature or DO (Table 1). The physico-chemical drivers structuring the bacterial community carrying As transforming genes are poorly documented in the literature. In soils contaminated with arsenic [46, 66], the most important factors shaping As transformation functional genes were soil pH, phosphate-extractable As, and amorphous Fe content [66]. Total C and N also drove the variation in gene abundance and microbial community associated with As biotransformation [46]. Further work is necessary to decipher the physico-chemical parameters shaping the bacterial diversity involved in As biotransformation (methylation, oxidation) in alkaline pit lakes.

4.4. Environmental significance

Arsenic biotransformations have been overlooked so far for moderately saline alkaline pit lakes which are potentially important freshwater resources in semi-arid Mediterranean areas.

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In the present study, there is a body of evidence that the bacterial communities inhabiting such pit lakes are contributing to As biogeochemical cycling. The eutrophic or oligotrophic status of the pit lakes and the salinity appeared to be important drivers of the As-oxidizing and As-methylating bacterial populations. Arsenic biomethylation evidenced in lake ZA may contribute to attenuate the toxicity of arsenic compared to lake ZL1 where no methylation occurs [21]. However, the possible presence of toxic volatile forms should be assessed since they may represent a threat for public health [53]. Furthermore, other elements such as Mo and Li compromise the use of this resource for irrigation [3].

5. Conclusion

Here we report for the first time arsenic speciation and As-related bacterial genes diversity in Moroccan alkaline mine pit lakes. The first outcome is the contrasting arsenic speciation in the two pit lakes that may be explained by the different content in organic carbon. Indeed, high organic carbon concentration in ZA was associated with a redox and DO gradient while lake ZL1, characterized by lower organic carbon content, showed no stratification. In lake ZA, the high TOC content is probably an important factor associated with biomethylation activity and the presence of diverse *arsM*-carrying bacteria. In lake ZL1, fully oxygenated conditions involved the predominance of As(V), in agreement with thermodynamic predictions. Bacterial populations carrying the *aioA* gene were evidenced in lake ZL1, suggesting that biotic As(III) oxidation may occur. For a more comprehensive view of arsenic cycling in these lakes, further work should focus on arsenic speciation and bacterial diversity at the sediment/water interface of the lake where reductive conditions are prone to favor As(V) reduction and biomethylation.

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Conflict of interest

Authors declare no conflict of interest.

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686 **Legends to figures**

687 **Figure 1.** General map of the study area (A) showing mine pit lakes ZA and ZL1, located
688 north and south from the town of Zeida (or Zaida), closed to the Moulouya River (B).
689 Situation of lake ZA closed to ore treatment residues (C) and lake ZL1 surrounded by tailings
690 piles (D). Water sampling stations are indicated by a red star. Adapted from Google Maps and
691 Google Earth.

692

693 **Figure 2.** Nonmetric multidimensional scaling (NMDS) ordination plots derived from
694 weighted pairwise Unifrac distances for bacterial communities structure (ARISA) for all
695 samples (A) or samples from lake ZA only (B). Stress values for ordination plot are < 0.2
696 which indicates that these data are well-represented by the two-dimensional representation.
697 Environmental fitting is based on the main physico-chemical parameters (T°: temperature;
698 pH; Cond: conductivity; Eh: redox potential; DO: dissolved oxygen concentration; TOC: total
699 organic carbon concentration). The full and dotted arrows represent significant and non-
700 significant fittings, respectively (Bonferroni corrected, $p < 0.05$).

701 **Figure 3**: ArsM phylogenetic tree based on 112 to 157 AA sequences using the Maximum
702 Likelihood method. ArsM sequences (in bold) are representative sequences of each OTUs
703 based on a 80% nucleotide sequence similarity cut-off. For each of the three clone libraries,
704 the percentage of clones contained in each OTU is indicated in brackets.

705

706 **Figure 4:** AioA phylogenetic tree based on 242 to 265 AA sequences using the Maximum
707 Likelihood method. AioA sequences (in bold) are representative sequences of each OTUs
708 based on a 95% nucleotide sequence similarity cut-off. For each clone libraries, the
709 percentage of clones contained in each OTU is indicated in brackets

710

Supplementary material

711 **Table S1** - Concentrations of trace elements at the surface and at the bottom of lakes ZA and
712 ZL1 in October 2012 and July 2013

Parameter (µg/L)	ZA lake				ZL1 Lake			
	October-2012		July-2013		October-2012		July-2013	
	surface	bottom (5 m)	surface	bottom (5 m)	surface	bottom (12.5 m)	surface	bottom (12.5 m)
Li	924	922	2296	2282	4502	4450	11400	10870
B	985	1009	980	997	2586	2539	2600	2479
Al	15.2	39.6	25.0	32.1	<DL	<DL	37.0	25.0
Si	<DL	<DL	2025	3017	<DL	<DL	16420	16120
Ti	<DL	<DL	1.6	2.1	<DL	<DL	7.1	6.0
V	5.1	5.3	2.8	2.5	15.7	15.5	17.3	16.6
Cr	<DL	<DL	0.2	0.2	<DL	<DL	0.8	0.9
Mn	66.8	78.6	6.6	46.2	5.3	4.1	7.9	8.1
Fe	22.2	18.8	32.4	40.9	<DL	<DL	31.2	32.0
Co	0.6	0.6	0.5	0.6	<DL	0.3	0.2	0.2
Ni	1.6	1.5	2.2	2.1	<DL	<DL	1.3	1.3
Cu	1.2	<DL	3.0	2.6	<DL	<DL	2.9	<DL
Zn	11.5	7.7	5.6	61.6	<DL	26.9	28.9	<DL
Se	0.5	n.d.	0.4	0.2	4.6	9.1	6.4	3.8
Rb	34.5	36.5	33.8	35.4	248.9	248.3	256.3	246.4
Sr	1300	1362	3961	4061	1672	1663	4730	4564
Mo	21.0	18.7	28.2	26.8	260.7	263.4	262.5	251.5
Ag	<DL	<DL	5.5	<DL	<DL	<DL	<DL	<DL
Cd	0.1	0.1	0.1	0.1	0.7	0.6	0.6	0.7
Sb	3.1	2.5	2.0	1.5	2.4	2.2	2.4	2.0
Cs	0.3	0.3	0.3	0.3	4.1	4.1	3.5	3.5
Ba	41.5	40.3	29.9	30.6	83.3	82.7	86.9	81.2
Tl	0.0	0.0	0.5	0.0	0.1	0.0	0.0	0.0
Pb	16.1	9.2	8.1	6.1	10.9	3.7	11.6	6.4
U	31.9	30.9	29.0	27.8	321.2	319.0	308.9	300.1

713
714 n.d. = not determined; <DL = below detection limit

715

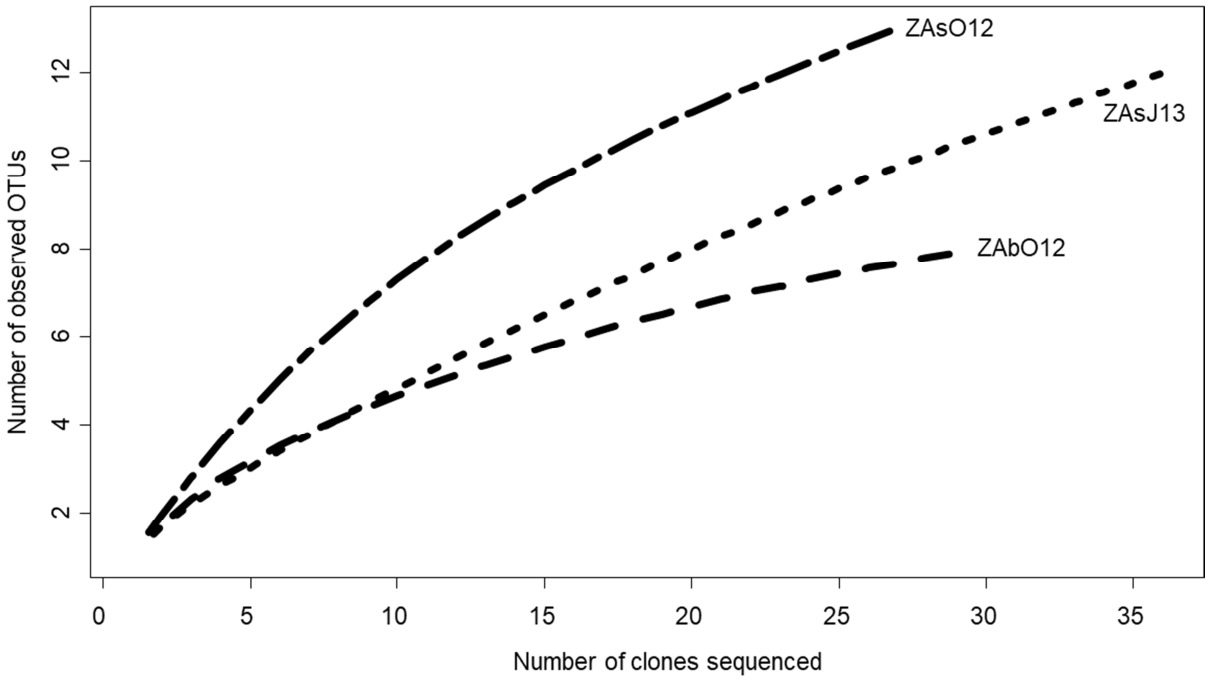

Figure S1: Rarefaction curves of *arsM* gene for the ZA libraries. The total number of sequences analyzed is plotted against the number of OTUs observed in the same library.

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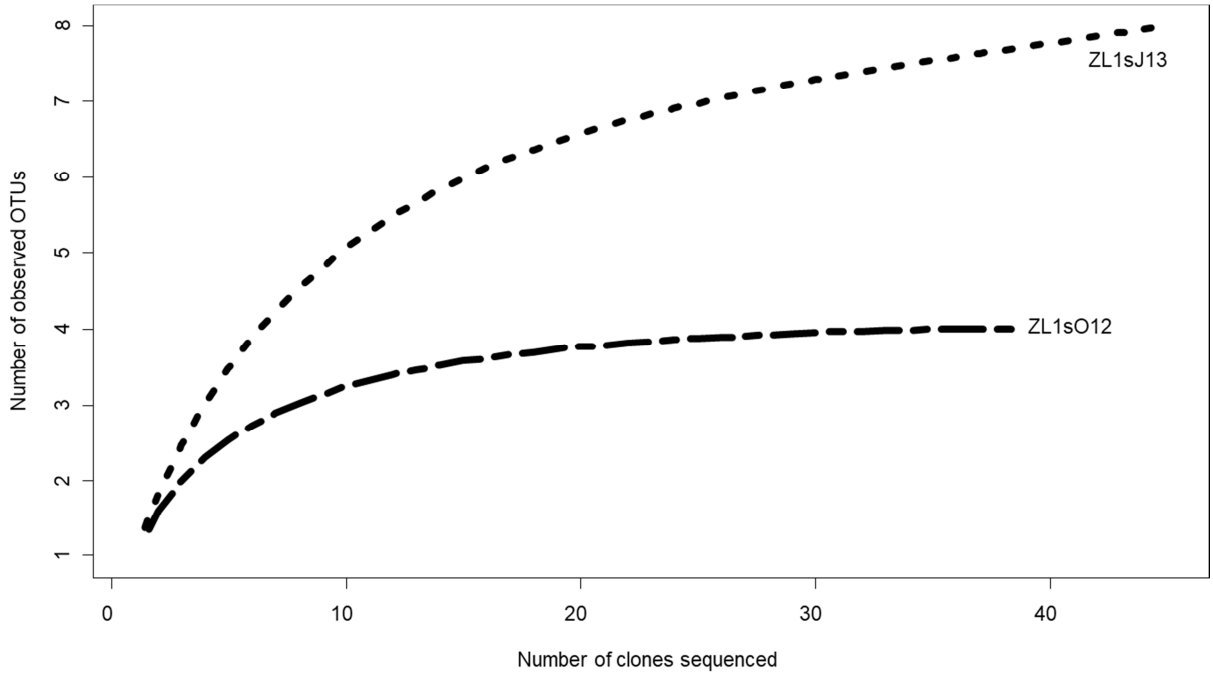

723

724 **Figure S2:** Rarefaction curves of *aioA* gene for the ZL1 libraries. The total number of

725 sequences analyzed is plotted against the number of OTUs observed in the same library.

726

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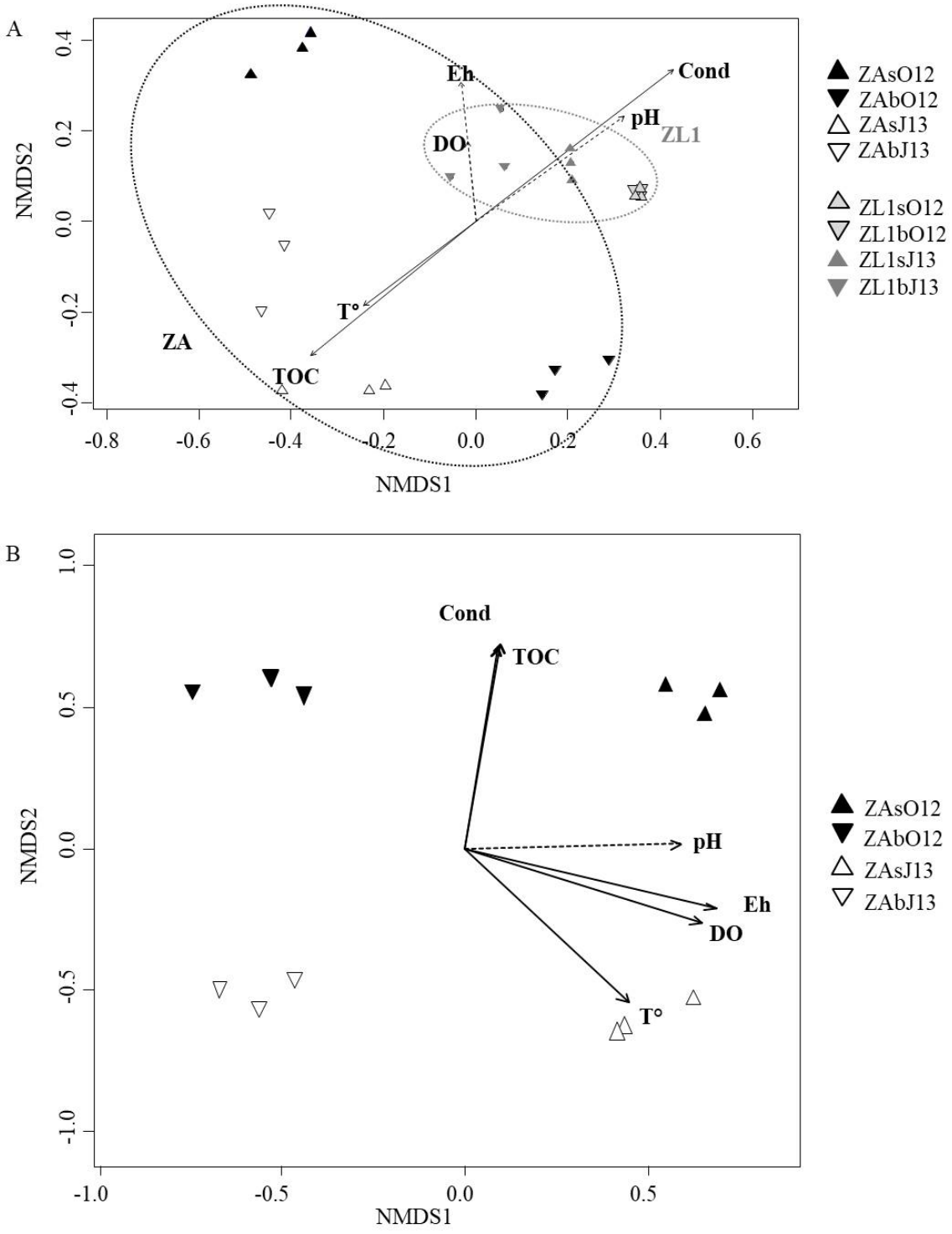
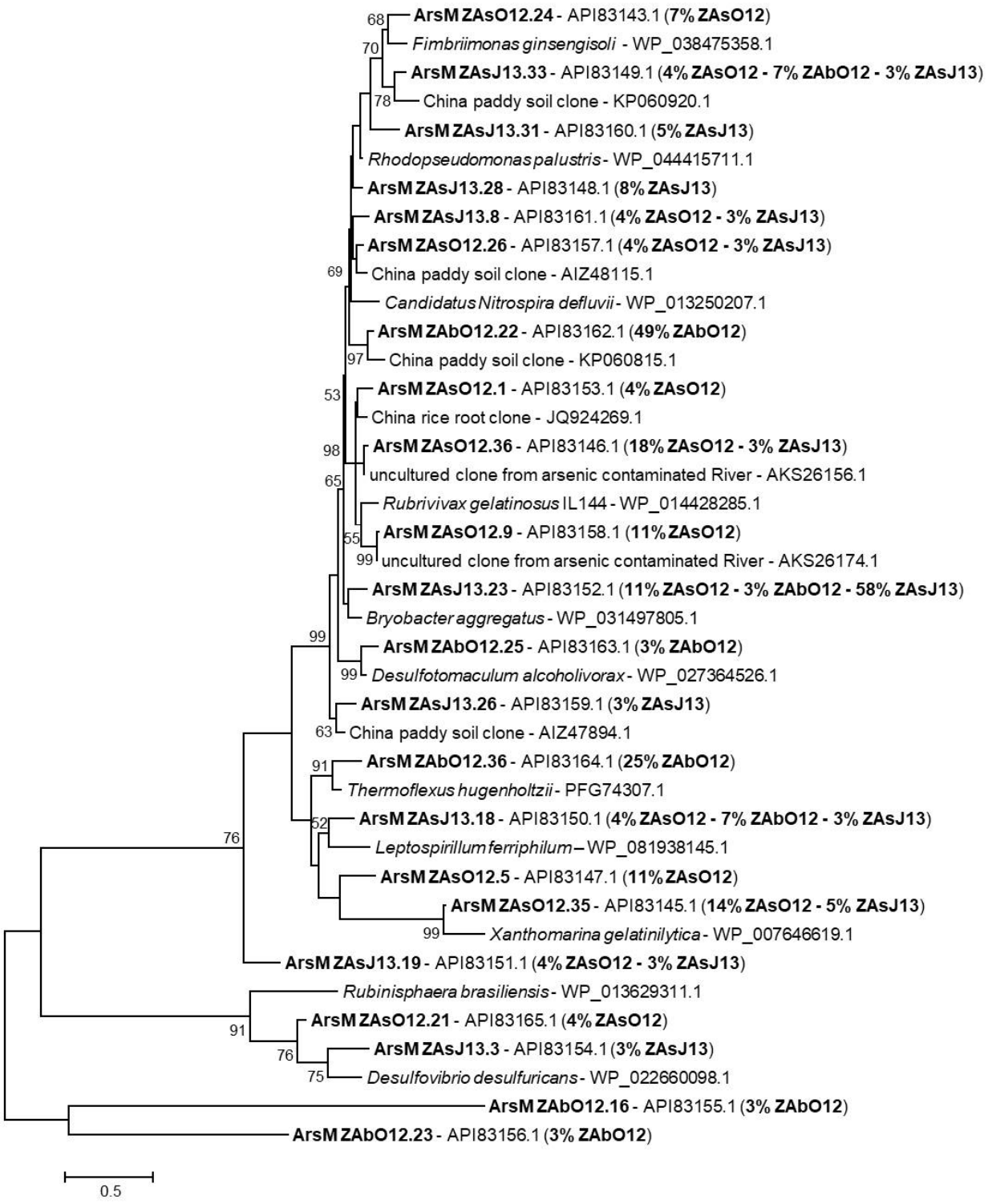
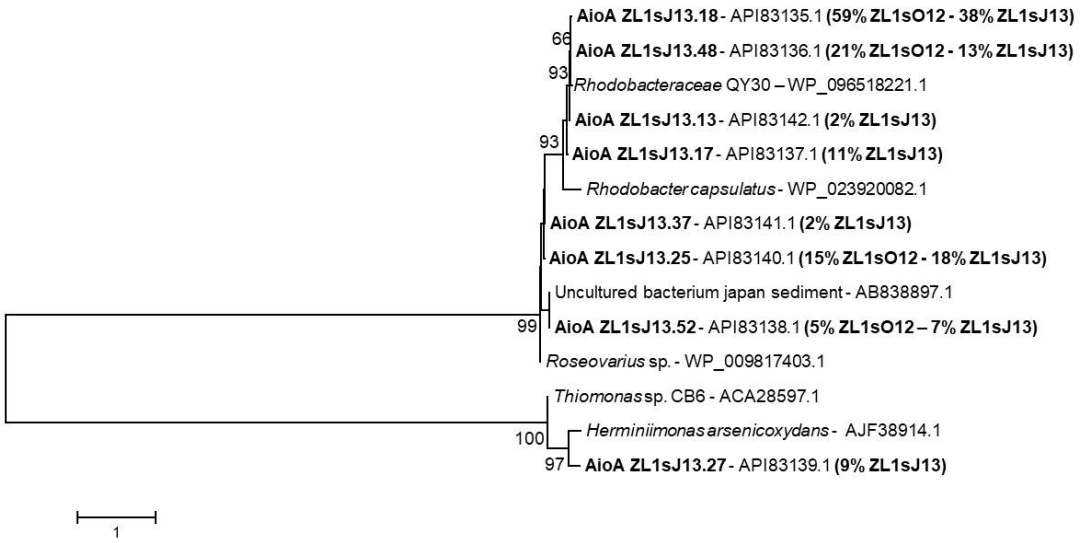
Table 1 - Main physico-chemical parameters (temperature, pH, conductivity, redox potential, dissolved oxygen concentration), concentration of Total Organic Carbon (TOC), chlorophyll a (Chla), major anions and cations and arsenic species at the surface and at the bottom of lakes ZA and ZL1 in October 2012 and July 2013. n.d. = not determined; <DL = below detection limit

	Parameter	Unit	ZA lake				ZL1 Lake			
			October-2012		July-2013		October-2012		July-2013	
			surface	bottom (5 m)	surface	bottom (5 m)	surface	bottom (12.5 m)	surface	bottom (12.5 m)
Physico-chemical parameters	T	°C	21.8	19.8	25.7	21.8	21.5	21.1	22.3	21
	pH		8.8	8.7	8.9	8.5	9.0	9.0	9.1	9.1
	Cond.	µS/cm	7020	6930	6150	6100	15470	15490	14530	14460
	Eh	mV	361	61	399	153	312	331	336	320
	DO	mg/L	8.7	4.1	11.5	4.8	8.2	8.3	7.9	9.2
Chlorophyll	Chl.a	µg/L	n.d.	n.d.	7.0	n.d.	n.d.	n.d.	<1	n.d.
Total organic carbon	TOC	mg/L	39.7	40.6	30.7	28.0	4.3	5.0	2.3	2.8
Major anions and cations	CO_3^{2-}	mg/L	38	65	96	67	568	522	612	600
	HCO_3^-	mg/L	719	638	472	590	2147	2287	1873	1829
	Cl^-	mg/L	1122	1125	1038	1022	3278	3347	3114	3046
	NO_3^-	mg/L	n.d.	n.d.	n.d.	n.d.	n.d.	35	n.d.	n.d.
	SO_4^{--}	mg/L	1543	1541	1466	1434	1721	1763	1742	1702
	Ca^{++}	mg/L	50	45	38	49	n.d.	n.d.	n.d.	n.d.
	Mg^{++}	mg/L	161	162	194	191	168	192	266	266
	Na^+	mg/L	1317	1322	1321	1275	3970	4166	4085	4009
	K^+	mg/L	34	38	41	35	133	115	104	103
Arsenic species	As(III)	µg/L	2.5	1.1	<DL	<DL	<DL	<DL	<DL	<DL
	DMA	µg/L	34.8	<DL	21	23	<DL	<DL	<DL	<DL
	As(V)	µg/L	<DL	<DL	14	19	115	114	142	147


A
NMDS2
NMDS1
Eh
Cond
pH
ZL1
DO
T°
ZA
TOC
ZAsO12
ZAbO12
ZAsJ13
ZAbJ13
ZL1sO12
ZL1bO12
ZL1sJ13
ZL1bJ13
B
NMDS2
NMDS1
Cond
TOC
pH
Eh
DO
T°
ZAsO12
ZAbO12
ZAsJ13
ZAbJ13


68
ArsM ZAsO12.24 - API83143.1 (7% ZAsO12)
70
Fimbriimonas ginsengisoli - WP_038475358.1
ArsM ZAsJ13.33 - API83149.1 (4% ZAsO12 - 7% ZAbO12 - 3% ZAsJ13)
78
China paddy soil clone - KP060920.1
ArsM ZAsJ13.31 - API83160.1 (5% ZAsJ13)
Rhodopseudomonas palustris - WP_044415711.1
ArsM ZAsJ13.28 - API83148.1 (8% ZAsJ13)
ArsM ZAsJ13.8 - API83161.1 (4% ZAsO12 - 3% ZAsJ13)
ArsM ZAsO12.26 - API83157.1 (4% ZAsO12 - 3% ZAsJ13)
69
China paddy soil clone - AIZ48115.1
Candidatus Nitrospira defluvii - WP_013250207.1
ArsM ZAbO12.22 - API83162.1 (49% ZAbO12)
97
China paddy soil clone - KP060815.1
53
ArsM ZAsO12.1 - API83153.1 (4% ZAsO12)
China rice root clone - JQ924269.1
98
ArsM ZAsO12.36 - API83146.1 (18% ZAsO12 - 3% ZAsJ13)
65
uncultured clone from arsenic contaminated River - AKS26156.1
Rubrivivax gelatinosus IL144 - WP_014428285.1
55
ArsM ZAsO12.9 - API83158.1 (11% ZAsO12)
99
uncultured clone from arsenic contaminated River - AKS26174.1
ArsM ZAsJ13.23 - API83152.1 (11% ZAsO12 - 3% ZAbO12 - 58% ZAsJ13)
Bryobacter aggregatus - WP_031497805.1
99
ArsM ZAbO12.25 - API83163.1 (3% ZAbO12)
99
Desulfotomaculum alcoholivorax - WP_027364526.1
ArsM ZAsJ13.26 - API83159.1 (3% ZAsJ13)
63
China paddy soil clone - AIZ47894.1
91
ArsM ZAbO12.36 - API83164.1 (25% ZAbO12)
Thermoflexus hugenholtzii - PFG74307.1
52
ArsM ZAsJ13.18 - API83150.1 (4% ZAsO12 - 7% ZAbO12 - 3% ZAsJ13)
76
Leptospirillum ferriphilum – WP_081938145.1
ArsM ZAsO12.5 - API83147.1 (11% ZAsO12)
ArsM ZAsO12.35 - API83145.1 (14% ZAsO12 - 5% ZAsJ13)
99
Xanthomarina gelatinilytica - WP_007646619.1
ArsM ZAsJ13.19 - API83151.1 (4% ZAsO12 - 3% ZAsJ13)
Rubinisphaera brasiliensis - WP_013629311.1
91
ArsM ZAsO12.21 - API83165.1 (4% ZAsO12)
76
ArsM ZAsJ13.3 - API83154.1 (3% ZAsJ13)
75
Desulfovibrio desulfuricans - WP_022660098.1
ArsM ZAbO12.16 - API83155.1 (3% ZAbO12)
ArsM ZAbO12.23 - API83156.1 (3% ZAbO12)
0.5



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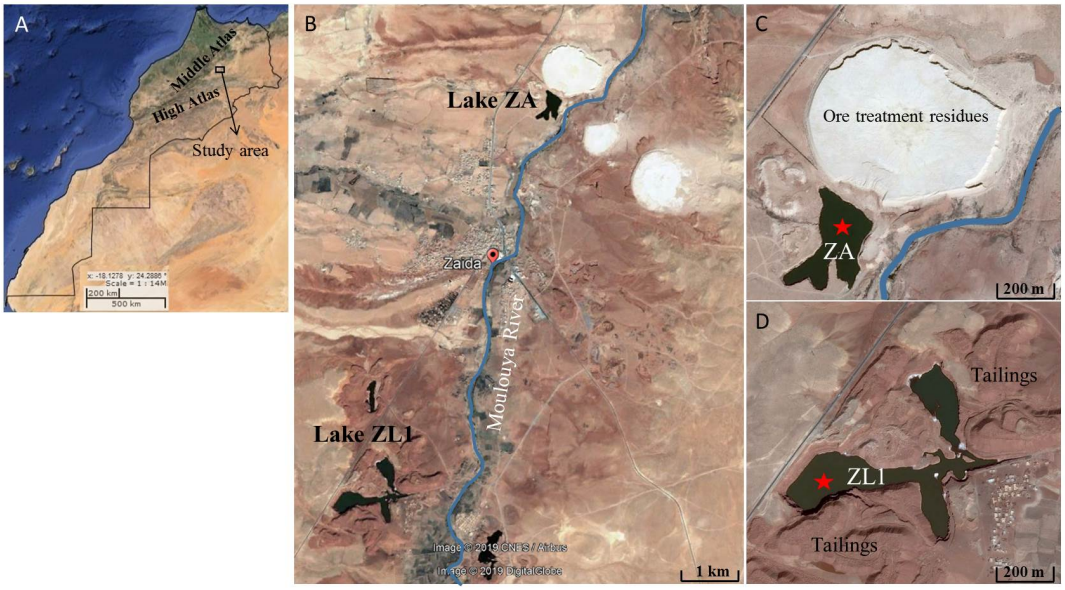

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