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1 Contrasting Arsenic biogeochemical cycling in two Moroccan alkaline pit lakes

2

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19

20 Abstract

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

37

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

39 1. Introduction

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

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

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

56 Microorganisms have evolved diverse strategies to transform arsenic for detoxification
57 or metabolism purpose) [8,9]. Arsenic microbially mediated biotransformations are highly
58 relevant to the global arsenic cycling in the environment [10]. Many bacteria have the ability
59 to oxidize arsenite into less toxic and less mobile arsenate [11-14], for detoxification purposes
60 or energy generation. This function is conferred by an arsenite oxidase (encoded by *aio* gene)
61 [15-17]. Another type of As(III) oxidase was identified in 2010 in haloalkaliphile
62 *Alkalilimnicola ehrlichii* MLHE-1. In this strain isolated from Mono Lake, the oxidation of
63 arsenite is coupled to the reduction of nitrate and carried out by a new gene family called *arxA*
64 [18]. Some microorganisms have the capacity to transform arsenite into methylated forms
65 [19]. This biomethylation process involves the S-adenosylmethionine methyltransferase
66 encoded by *arsM* genes [20]. Methylated As species, both volatile (e.g. trimethylarsenic
67 (TMA) and trimethylarsine (TMAO) and non-volatile (e.g. monomethylarsenic (MMA) and

68 dimethylarsenic (DMA)), are less toxic than their inorganic forms [21]. Thus, biomethylation
69 is generally considered as one of the main detoxification pathways for As in the environment
70 [22]. However, because some intermediate products are highly toxic, arsenic biomethylation
71 as a detoxification process has been questioned [21, 23-24]. Finally, under reducing
72 conditions, dissimilatory arsenate-reducing prokaryotes use As(V) as final electron acceptor
73 for anaerobic respiration [25].

74 So far, attention has been mainly focused on arsenic biogeochemistry in paddy soils
75 [26], marine waters [27-28], groundwater used as drinking water (for a recent review see
76 [29]), or in extreme environments such as soda lakes located in geothermal regions [30-31]. In
77 comparison, the question of the microbially mediated transformations of arsenic in alkaline
78 mine pit lakes, characterized by a moderate salinity (conductivity) compared to soda lakes
79 ($6.1-15.5 \text{ mS/cm}^{-1}$ in ZA or ZL1 compared to $42-86 \text{ mS/cm}^{-1}$ in Mono Lake,) has received
80 little attention. Such alkaline pit lakes are common along the southern margin of the
81 Mediterranean Sea, where metal deposits occur within carbonated layers of Mesozoic and
82 Tertiary sediments [5,32].

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

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

94

95 **2. Materials and methods**

96 *2.1. Site description*

97 The Zeida mining area is located in the High Moulouya basin. It is bounded by the High Atlas
98 on the southeast and by the Middle Atlas on the northwest (Fig. 1A). The geology of the area
99 has been described in [4] and [35]. The climatic conditions in the upper Moulouya region are
100 semi-arid, with annual precipitation of 100–400 mm and mean annual temperatures of 12–
101 14°C. The region faces a rainfall deficit caused by recurrent droughts. Lead has been
102 exploited until 1985, from lead–barite ores [2]. In Zeida, the exploited orebody consisted
103 mainly of cerussite [PbCO_3] (70%) and galena [PbS] (30%) [36]. After mining activity
104 ceased, the region shifted into livestock breeding and orchards (apple trees).

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

112

113 *2.2. Sampling*

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

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

123 2.3. Sample treatment and chemical analyses

124 Fifty milliliters of unfiltered water was used for the analysis of bicarbonate and
125 carbonate by titration with standardized HCl 0,1 N. For total organic carbon, samples (60 ml)
126 were taken in amber glass bottles previously decontaminated by heating at 550 °C, preserved
127 by adding H₃PO₄ 1:1000 (v:v) and analyzed within 2 weeks. Another 200 ml of water were
128 filtered in the field through 0.22 µm Millipore membranes fitted on Sartorius polycarbonate
129 filter holders. Subsamples were preserved for the analysis of major anions (Cl⁻, SO₄²⁻) and
130 cations (Na⁺, K⁺, Mg²⁺, Ca²⁺), trace elements and arsenic speciation according to the routine
131 procedures described in [37] and analyzed within a few days after collection.

132 Inorganic and methylated arsenic species (As(III), As(V), MMA and DMA) were
133 analyzed using anion-exchange chromatography (25 cm × 4.1 mm i.d. Hamilton PRP-X100
134 column and SpectraSystem P4000 ThermoScientific pump) coupled to ICP-MS (Thermo X7
135 Series), in gradient elution mode, with an ammonium phosphate eluent, as described in [38].
136 Analysis of major cations and anions was carried out using ion chromatography (Dionex ICS-
137 1000). Total organic carbon concentration was determined using a high-temperature catalytic
138 oxidation method (HTCO), with a Shimadzu TOC-VCSH total organic carbon analyzer.
139 Analysis of trace elements (Mn, Al, Zn, Pb, and Fe) was carried out using ICP-MS (Thermo
140 X7 Series), as described elsewhere [37].

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

145

146 *2.4 Sample treatment and microbial analysis*

147 Microbial analyses were performed on water samples from the surface and the bottom
148 of each lake, for the two sampling campaigns. For each water sample, six aliquots of 250 ml
149 were filtrated in the field through 0.22 µm cellulose acetate filter (Sartorius stedim biotech,
150 Aubagne, France) to obtain six replicates. Each filter was immediately transferred to a
151 cryotube, kept at 4°C in a container with ice packs before reaching the laboratory and frozen
152 at -80°C until DNA extraction. This protocol was applied in October 2012 for ZL1 lake and in
153 July 2013 for ZA and ZL1 lakes. For ZA lake in October 2012, an alternative treatment was
154 used to collect the microbial biomass; indeed, the high suspended matter content prevented
155 the filtration of ZA lake waters *in situ*. Water samples (300 ml, in triplicate) were centrifuged
156 (6,000 g, 20 min) back to the laboratory to pellet the microbial cells. The cell pellets were
157 stored at -20°C until DNA extraction. Samples were named as follow: ZAsO12 for ZA lake
158 sampled at the surface in October 2012, ZAbO12 for ZA lake sampled at the bottom in
159 October 2012, ZAsJ13 and ZAbJ13 for ZA in July 2013, ZL1sO12 and ZL1bO12 for ZL1 in
160 October 2012, ZL1sJ13 and ZL1bJ13 for ZL1 in July 2013.

161

162 *2.4.1. Microbial DNA isolation and Automated Ribosomal Intergenic Spacer Analysis* 163 *(ARISA)*

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

166 PowerWater® DNA Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's
167 instructions. Yield of DNA was determined using the QuBit® fluorometer (Invitrogen,
168 Carlsbad, CA, United States) and the high sensitivity Assay Kit (Thermo Fisher Scientific,
169 Waltham, MA, USA). For each sample, triplicate DNA extractions were performed from
170 three of the six filters or pellets (the three other replicates were kept at -80°C as backup). The
171 bacterial intergenic spacer regions between the small (16S) and the large (23S) subunits of
172 ribosomal sequences were amplified from extracted DNA by PCR, using primers ITSF (5'-
173 GTCGTAACAAGGTAGCCGTA-3') and ITSr (5'-GCCAAGGCATCCACC-3') [39]. For
174 each of the eight samples, three independent PCR were performed on triplicate DNA extracts.
175 PCR mix was prepared as follows: around 1 ng of DNA extract, 12 µl of amplitaq Gold 360
176 master mix (Thermo Fisher Scientific), 2.5 µg of bovine serum albumin, and 0.5 µM of each
177 primer, in a total volume of 25 µl. Cycling conditions were as previously described in [39].
178 PCR products were checked on a 2% agarose gel. Then PCR products were analyzed on a
179 microfluidic lab-on-a-chip (Agilent technologies, Santa Clara, United States). The lab-on-a-
180 chip device is a fully automated electrophoresis system consisting of circuits of tiny closed
181 channels and wells, etched onto a plastic microchip. Samples (PCR products) passed through
182 selected pathways in a controlled manner. The DNA fragments (ranging between 263 and
183 1500 bp depending on the bacterial taxa) were separated using the Agilent 2100 bioanalyser
184 and the High Sensitivity DNA Analysis Kit. The fluorescence intensity was plotted versus the
185 DNA fragment size and electropherograms corresponding to the band profiles characteristic
186 of each bacterial community were edited with the Agilent 2100 bioanalyser software
187 (including background noise removal). Then, three reproducible ARISA profiles were
188 obtained for each water sample.

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

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

195 Statistical analyses were performed using the free software R 3.4.3 ([http://www.r-](http://www.r-project.org/)
196 [project.org/](http://www.r-project.org/), 2017). Nonmetric multidimensional scaling (NMDS) was used to graphically
197 depict differences between the structures of the bacterial communities from the different
198 samples (based on the normalized ARISA data expressed as the area of the peaks). The
199 significance of the observed repartition of samples on the ordination plot was assessed by an
200 ANalysis Of SIMilarity (ANOSIM, 999 permutations). To examine the relationships between
201 bacterial structure communities and environmental variables, the main physico-chemical
202 variables (temperature, pH, conductivity, redox potential, dissolved oxygen concentration and
203 Total Organic Carbon concentration) were fitted onto NMDS. The environmental fitting (ef)
204 model was tested with Monte Carlo permutation tests (999 randomized runs) to determine its
205 significance. Plotting was limited to the most significant variables with argument $p.max =$
206 0.05 (after adjustment of the p values with the Banferroni correction). The length of the arrow
207 is proportional to the correlation between the ordination of bacterial community structures and
208 the environmental variable, called the strength of the gradient.

209

210 2.4.2. *arsM* and *aioA* amplification, cloning and sequencing

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

215 aoxBM3-2R (5'-TGTCGTTGCCCCAGATGADNCCYTTYT C-3') were used to amplify a
216 1100 bp fragment [17]. The reaction contained 0.32 μ M of each primer, 10 ng of DNA
217 extract, 1.5 mM MgCl₂, 200 μ M of each deoxynucleoside triphosphate, 2.5 U of BIOTAQ™
218 DNA polymerase (Eurobio, France) and 1X PCR buffer. Water was added to a final volume
219 of 50 μ l. The PCR conditions included a 5 min denaturation step at 94°C, followed by 35
220 cycles of a 45 s denaturation at 94°C, primer annealing of 45 s at 55°C, a 50 s extension at
221 72°C, and a final extension at 72°C for 5 min. The *arsM* and *aioA* PCR products were
222 visualized and purified after migration on an agarose gel using the Gel Band Purification Kit
223 (GE-Healthcare, Munich, Germany).

224 *arsM* and *aioA* purified PCR products were cloned into a pCR® 2.1 vector and
225 transformed into One Shot® TOP10 chemically Competent *Escherichia coli* TOP10 (Life
226 Technologies, Carlsbad, CA) according to the protocols of the manufacturer. Twenty-seven to
227 45 positive clones per library were sequenced by GATC biotech (Konstanz, Germany) with
228 the M13 reverse primer. Nucleotide sequences were aligned with Muscle [41]. Conversion in
229 protein sequences and phylogenetic analysis were carried out with the software MEGA6.06
230 [42]. Amino acid sequences were compared with the GenBank database (NCBI) using
231 BLAST [43]. Operational taxonomic units (OTUs) were determined using similarity levels
232 between sequences of at least 80% of nucleotide similarity for *arsM* and 95% for *aioA* using
233 Mothur v.1.33.2 [44]. Amino acid phylogenetic trees were constructed with a sequence
234 representative of each OTU using the Maximum Likelihood method based on the LG model
235 with 1000 bootstraps replicates. Initial tree(s) for the heuristic search were obtained by
236 applying the Neighbor-Joining method to a matrix of pairwise distances estimated using a JTT
237 model. A discrete Gamma distribution was used. The OTU-based DNA clustering data were
238 also used to calculate rarefaction curves with R 3.6.0 to assess OTU richness from the
239 different samplings.

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 API83135 to API83142.1 for *aioA* and
243 API83143.1 to API83165.1 for *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-SO₄ 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

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

269

270 3.2. Whole bacterial community analysis

271 Whole bacterial community structure and its spatio-temporal variations were assessed
272 by ARISA. The data, expressed as peak tables (deduced from ARISA electrophoregrams
273 generated for each sample in triplicate), were used for NMDS analysis. The NMDS ordination
274 shows that distinct bacterial communities thrived in waters from lakes ZL1 and ZA (ANOSIM
275 $r^2 = 0.5845$, $p = 0.001$; Fig. 2A). In lake ZL1, a limited spatio-temporal variability was
276 observed among bacterial communities. In October 2012, the structures of the bacterial
277 communities at the surface and at the bottom were undistinguishable. In contrast, marked
278 temporal and spatial variations were observed in lake ZA. In particular, the bacterial
279 communities sampled in October 2012 appeared widely different between surface and bottom.
280 Vector fitting of possible explanatory environmental parameters revealed that conductivity
281 (Cond), temperature (T°) and TOC contributed to the structuration of the bacterial
282 communities in lake ZL1 and lake ZA. In ZL1, bacterial community structures were
283 associated with a higher conductivity ($r^2 = 0.7382$, $p = 0.006$). In ZA, the structure of the
284 communities was mainly associated to higher temperature ($r^2 = 0.4322$, $p = 0.006$) and TOC
285 concentration ($r^2 = 0.5364$, $p = 0.012$) (Fig. 2A). On NMDS analysis based on data from lake
286 ZA only, Eh ($r^2 = 0.9768$, $p = 0.006$), DO ($r^2 = 0.9335$, $p = 0.006$) and conductivity (r^2
287 $= 0.9934$, $p = 0.006$) emerged as the main factors structuring the communities (Fig. 2B). Other

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

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).

335

336 4. Discussion

337 4.1. Arsenic speciation in the mine pit lakes

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

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

360 Although anoxia was not detected at the bottom of lake ZA, the DO decline from surface to
361 bottom suggests that anoxia may occur in sediments, promoting microbial processes similar to
362 those described by Tang et al. [53]. Thus, the presence of DMA in ZA might be linked to the
363 eutrophic character of this lake. The high productivity of lake ZA may be favored by the
364 erosion and leaching of nutrients from the ore flotation residues originating from the Zeida
365 ore treatment facility, stored next to the lake. Indeed, the phosphorus content of these residues
366 (1.77 %, [3]) is higher than the average content in soil (0.6 %) and they contain fine particles
367 (50 % of particles with size lower than 0.2 mm, [3]), propitious for phosphorous leaching.
368 DMA co-existed either with As(III) (October 2012) or with As(V) (July 2013) in ZA lake.
369 However, DMA was not detected at the bottom of ZA in July 2013. Seasonal differences
370 observed in arsenic speciation in ZA lake remains unexplained and a more comprehensive
371 view of arsenic cycling in these pit lakes would require further investigations.

372

373 *4.2. Microbial contribution to arsenic biogeochemical cycling in Moroccan pit lakes*

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

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

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

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].

419

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.

433 In lake ZL1, the spatio-temporal variations of the whole bacterial community as well
434 as of the arsenite oxidizing populations were limited. This is in agreement with the narrow
435 range of variations highlighted for the water physico-chemical conditions. On the contrary, in
436 the stratified ZA lake, pronounced changes in bacterial community structure and *ArsM*
437 diversity were associated with marked spatio-temporal variations of the water physico-
438 chemistry. Eighty three percent of *ArsM* sequences recovered at the bottom of ZA lake were
439 not recovered in the surface waters (Fig.3). This may be explained by the drastic change in
440 DO and Eh between surface (DO = 8.7-11.5 mg/L, Eh = 361-399 mV) and bottom (DO = 4.1-
441 4.8 mg/L, Eh = 61-153 mV) waters. Reducing conditions generated near the sediment/water
442 interface may have selected specific bacterial populations involved in As methylation [53].
443 Moreover, the dominant OTU containing 58% of the *arsM* sequences retrieved from surface
444 waters in July 2013 represented only 10% of the whole *arsM* sequences in October 2012 (Fig.
445 3). This suggests a seasonal dynamic of the bacterial populations involved in As methylation
446 in lake ZA. This dynamic can be related to seasonal change of TOC, temperature or DO
447 (Table 1). The physico-chemical drivers structuring the bacterial community carrying As
448 transforming genes are poorly documented in the literature. In soils contaminated with arsenic
449 [46, 66], the most important factors shaping As transformation functional genes were soil pH,
450 phosphate-extractable As, and amorphous Fe content [66]. Total C and N also drove the
451 variation in gene abundance and microbial community associated with As biotransformation
452 [46]. Further work is necessary to decipher the physico-chemical parameters shaping the
453 bacterial diversity involved in As biotransformation (methylation, oxidation) in alkaline pit
454 lakes.

455 4.4. Environmental significance

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

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

466

467 5. Conclusion

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

481

482 **Conflict of interest**

483 Authors declare no conflict of interest.

484

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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

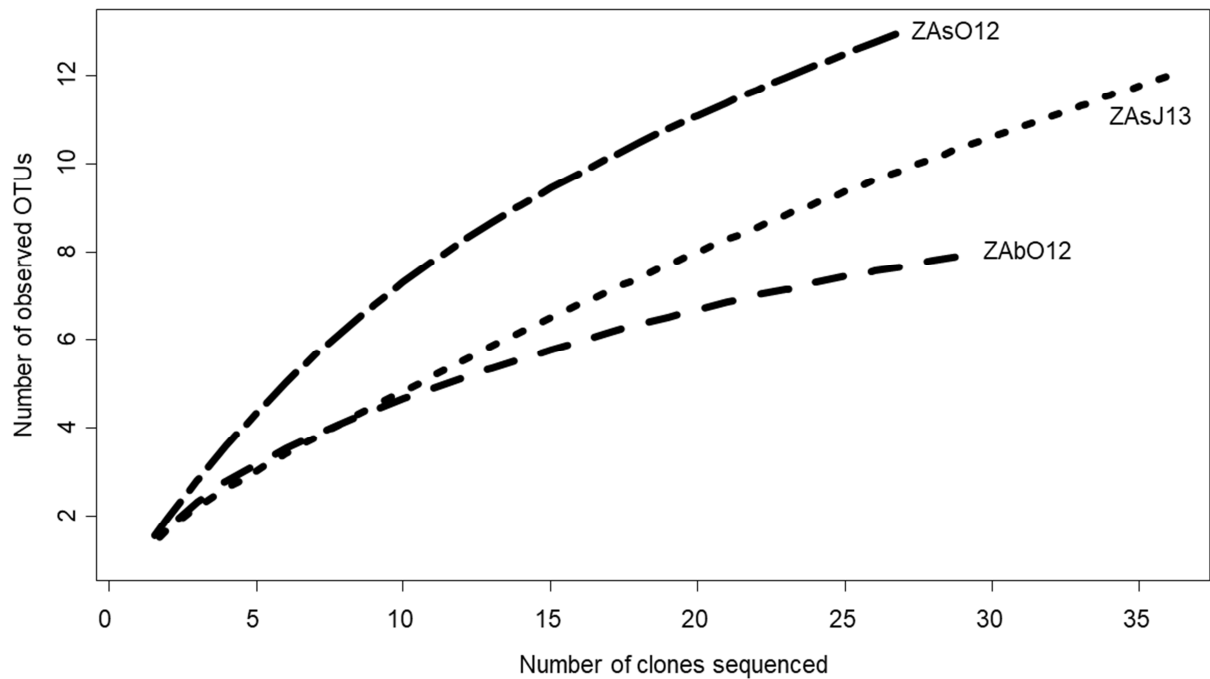
711 **Table S1** - Concentrations of trace elements at the surface and at the bottom of lakes ZA and
 712 ZL1 in October 2012 and July 2013

Parameter ($\mu\text{g/L}$)	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

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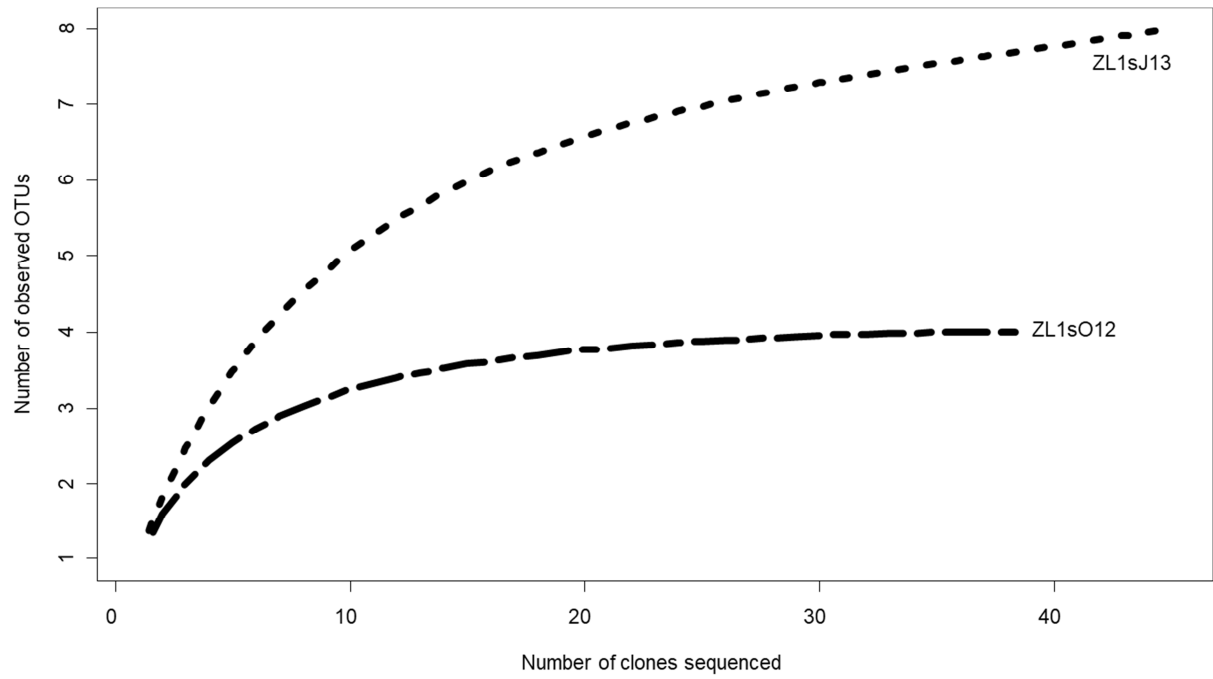
718 **Figure S1:** Rarefaction curves of *arsM* gene for the ZA libraries. The total number of

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

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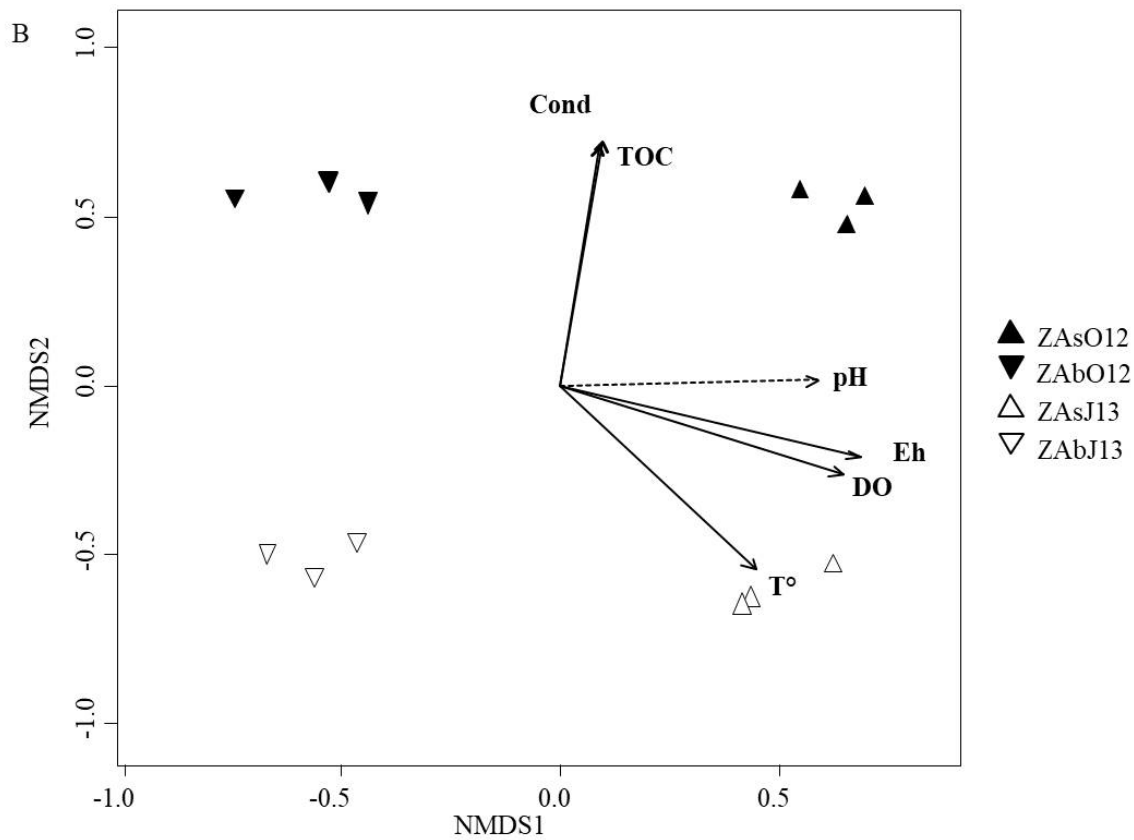
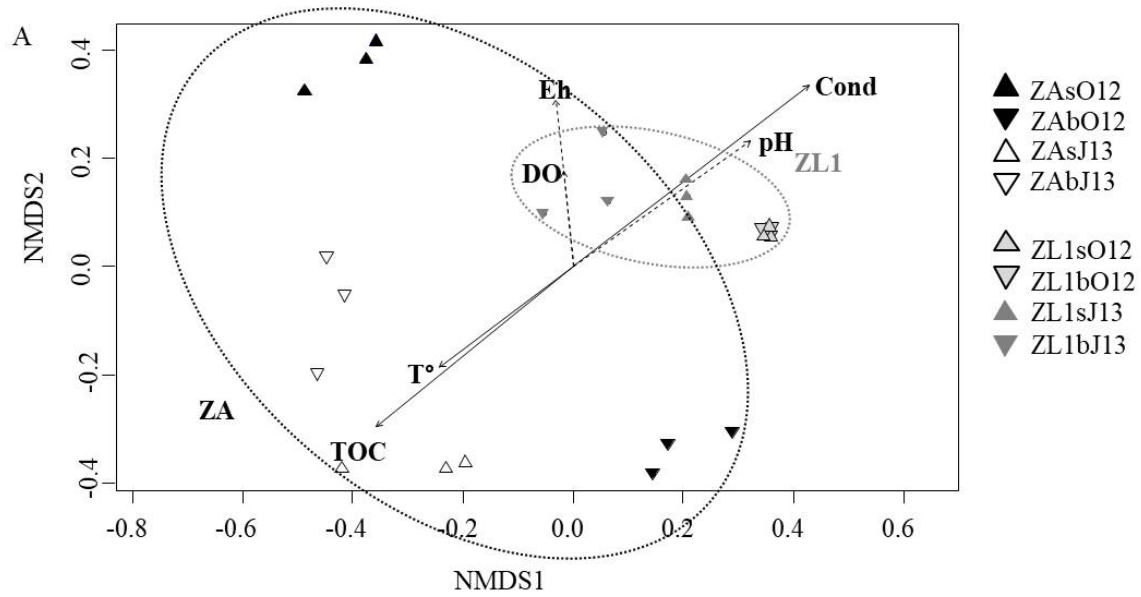
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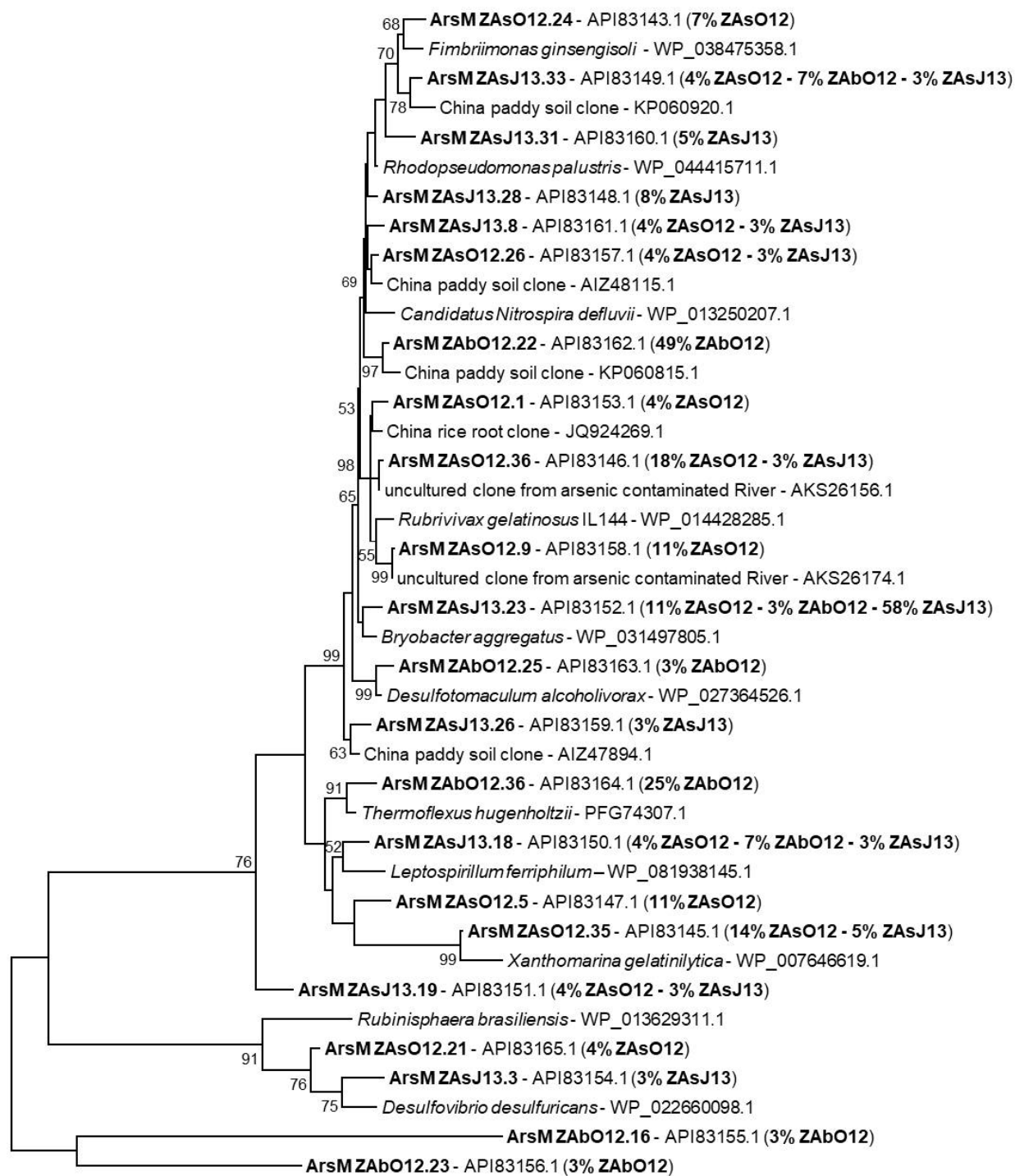
724 **Figure S2:** Rarefaction curves of *aioA* gene for the ZL1 libraries. The total number of
725 sequences analyzed is plotted against the number of OTUs observed in the same library.

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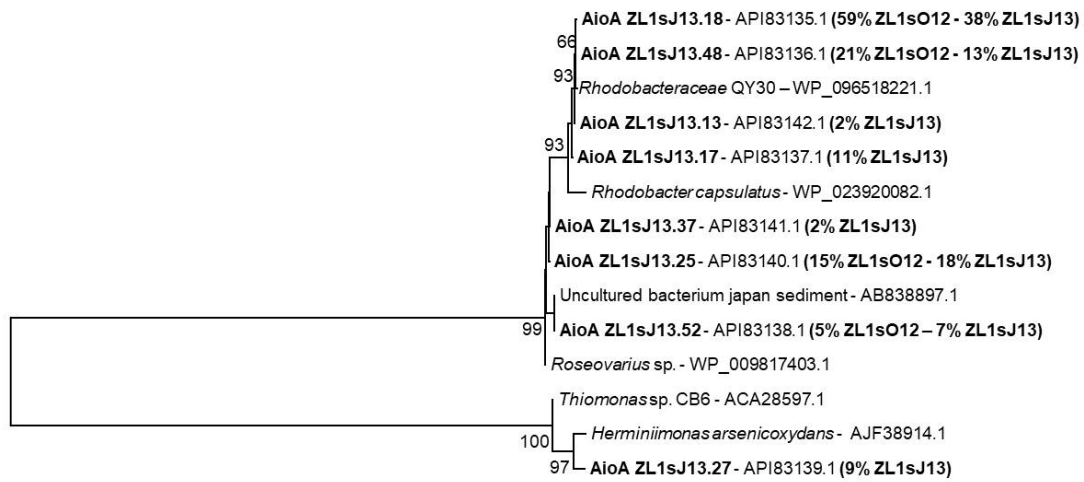
Table 1 - Main physico-chemical parameters (temperature, pH, conductivity, redox potential, dissolved oxygen concentration), concentration of Total Organic Carbon (TOC), chlorophyll a (Chla), major anions and cations and arsenic species at the surface and at the bottom of lakes ZA and ZL1 in October 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	Chla	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



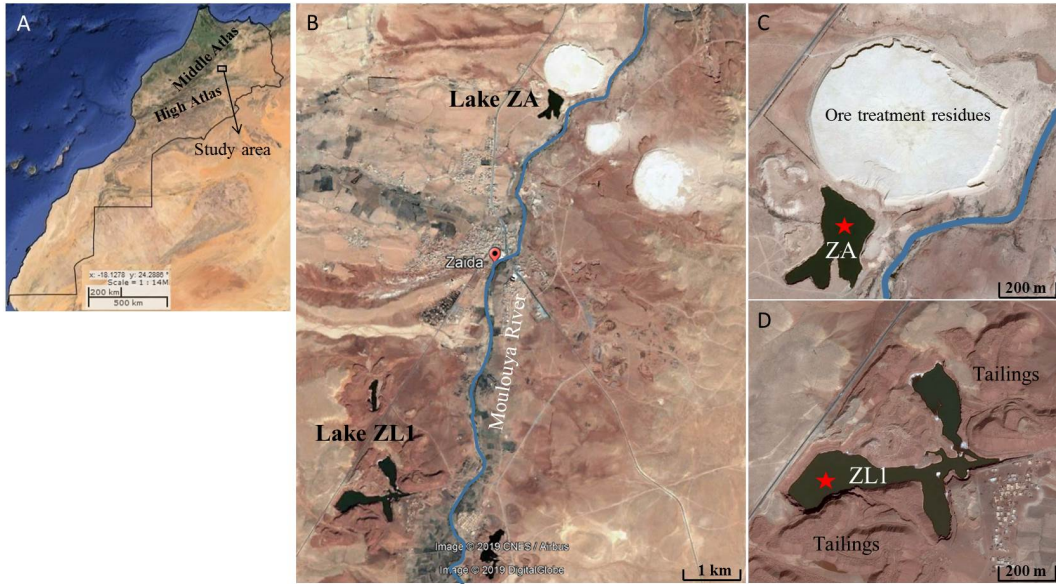


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