Manganese-reducing *Pseudomonas fluorescens*-group bacteria control arsenic mobility in gold mining-contaminated groundwater

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ABSTRACT

Previous studies show the importance of iron- and arsenate-reducing bacteria in mobilizing arsenic in groundwater. Here the authors present experimental evidence of arsenic mobilization in connection with bacterially-mediated manganese reduction in groundwater affected by mining activities. Manganese-reducing *Pseudomonas* species were enriched, isolated and identified by 16S rDNA phylogeny from groundwater containing high co-dissolved arsenic (as As^{III}) and manganese. Enrichment cultures dissolved synthetic birnessite and haussmannite efficiently, but Mn reduction by isolates was greatly reduced at upper environmental levels of dissolved As^{III}. Results suggest either a self-limiting release of arsenic coupled to bacterial manganese reduction, in the absence of other electron donors like sulfide, or increased arsenic resistance conferred to Mn-reducing bacteria in consortia.

KEYWORDS: *Pseudomonas*, arsenic mobilization, groundwater contamination, manganese-reducing bacteria, mine tailings

INTRODUCTION

Arsenopyrite found in organic-rich shales and hydrothermal mineralisation associated with gold deposits (Boyle and Jonasson 1973, Rose et al. 1979, Garcia-Sanchez and Alvarez-Ayuso 2003, Wang and Mulligan 2006) undergoes natural weathering exacerbated by the crushing of ore for gold extraction (Villaescusa and Bollinger 2008). Arsenic mobilized from gold mines into groundwater can threaten human and ecological health (Lee et al. 2010). Strategies for remediating arsenic in groundwater vary from costly extraction and off-site treatment to permeable bioreactive barriers or wetlands (Mackay and Cherry 1989, Hoffman 1993, Morrison et al. 2002, Zouboulis and Katsoyiannis 2005). In the latter scenarios, adsorption of As to mineral surfaces and microbially-mediated redox transformations play integral roles (Borch et al. 2010).

Adsorbents like manganese- and iron-oxides or -oxyhydroxides commonly exist in sedimentary aquifers (Kent et al. 2004). Both As(III) and As(V) can adsorb strongly to manganese- and iron-oxides or -oxyhydroxides (Garcia-Sanchez and Alvarez-Ayuso 2003, Dixit and Hering 2003). The high surface area-to-volume ratio of δ-MnO_2 (birnessite) makes it an effective adsorbent for arsenite or arsenate. Previous studies have elucidated the role of birnessite in As(III) oxidation and subsequent adsorption of As(V) to an intermediate $Mn^{III}OOH$ phase (Nesbitt et al. 1998, Manning et al. 2002, Ying et al. 2012). However, under oxygen-depleted conditions, microbially mediated electron transfer, e.g., from organic matter to Fe(III) or Mn(IV), can induce the reductive dissolution of Fe(III)- or Mn(IV)-oxides or -oxyhydroxides, and the destablization or release of adsorbed arsenic (e.g., Tufano et al. 2008). The low crystallinity of δ-MnO_2 that contributes to higher surface area can actually facilitate reductive

dissolution of the mineral structure, and thereby also aid the growth of manganese-reducing bacteria.

Manganese-reducing bacteria have been isolated from a wide variety of environments (e.g., *Geobacter* spp., *Shewanella* spp., *Bacillus* spp., *Pseudomonas* spp.), demonstrating their ubiquity in soils and sediments (e.g., Nealson and Myers 1992, Lovley et al. 2004, Ehrlich and Newman 2009, Cerrato et al. 2010). Some Mn-reducers directly couple oxidation of an organic substrate or hydrogen to the transformation of Mn(IV) to Mn(III) followed by Mn(II) (Lovley and Phillips 1988, Lovley 1991, Aklujkar et al. 2013), with recent evidence revealing this two-step reduction process (Lin et al. 2012). Other Mn-reducers indirectly catalyse Mn reduction by producing redox reactive extracellular metabolites or pigments that donate electrons to Mn(IV) (e.g., Duckworth and Sposito 2007, Brutinel and Gralnick 2012). Furthermore, some Mn-reducing bacteria (e.g., *Pseudomonas*, *Bacillus*) are also capable of Mn(II)-oxidation (Caspi et al. 1998, Rosson and Nealson 1982), thereby completing the redox cycle for this geologically important element.

Reviews of As mobility in contaminated aquifers (e.g., Korte and Fernando 1991, Smedley and Kinniburgh 2002, Stollenwerk 2003, Mukherjee et al. 2006, Fendorf et al. 201*0*) have mainly considered the role of Fe(III)- or As(V)-reducing bacteria in influencing As distribution. Studies have shown that arsenic adsorbed to Fe(III)-ox(yhdrox)ides can be readily remobilized by iron-reducing bacteria in both naturally and anthropogenically contaminated environments (e.g., Cummings et al. 1999, Islam et al. 2004). Direct As(V) reduction can also contribute significantly to overall arsenic mobility in sediments (Ahmann et al. 1997). Notably, although previous studies have examined the role of groundwater bacteria in dissimilatory Mn(IV) reduction (e.g., Lovley and Phillips 1988, Di-Ruggiero and Gounot 1990), no

environmental study has focused on microbial reduction of Mn(IV)-bearing minerals as the primary mechanism for arsenic mobilization in groundwater. This study presents investigation of arsenic mobility linked to the activity of Mn(IV)-reducing bacteria in an aquifer contaminated by gold mine tailings effluent. On the basis of historical groundwater data, it was hypothesized that arsenic-resistant manganese-reducing bacteria were controlling arsenic mobility in groundwater impacted by gold mining. To test this hypothesis, representative groundwaters were sampled and enriched for manganese-reducing bacteria. The enrichment cultures and bacterial isolates derived from these enrichments were then subjected to two types of arsenic tolerance assays to assess the degree of arsenic tolerance and efficiency of Mn reduction under arsenite.

MATERIALS AND METHODS

Field site and historical data

The Stawell gold mine, located in the Western Stawell Zone of the Lachlan Fold Belt, Victoria, Australia, provided the field sites for this study (Fig. 1). The mine sits in a belt of hydrothermally-altered, fault-bounded metasediments (Willman et al. 2010) with gold deposits hosted in quartz-rich turbiditic sands and mudstones overlying thick tholeiitic basalts and intruded by felsic porphyritic dykes and granites (Miller and Wilson 2002). These metasediments comprise a sequence of intercalated aquifers and aquitards. The country rocks contain arsenopyrite, as the primary host mineral for gold, and some of the arsenic present in groundwater is thought to represent a natural chemical weathering product from these rocks (Noble et al. 2010).

Currently, groundwater management by the mine operator involves extractive pumping of contaminated groundwater back to the tailings dam (i.e. "pump back" to the source) to maintain levels below drinking water quality and mine closure standards. Historical groundwater quality data taken from 1995-2009 at Stawell were analysed to look for factors strongly correlated with dissolved arsenic concentrations (Fig. 2). In addition, new data were obtained for selected wells from groundwater sampled during June-July 2010 (for field geochemical parameters and trace metals total concentrations) and May-June 2013 (for arsenic speciation analysis).

Among the sampled wells were four pairs of nested wells screened at roughly 10-12.5 m (SE04A, SE03A, SE05A, SE09A), and 28-30 m (SE04, SE03, SE05, SE09), respectively. SE04A and SE04, along with SE03A and SE03, are located (in order of closest and shallowest to farthest and deepest) along the SW-NE transect away from the mine tailings dam shown in Fig. 1. SE05A and SE05, and SE09A and SE09, form two pairs of nest wells located to the north and east, respectively, of the tailings dam. All of the paired nested wells afforded the opportunity to look for variations in dissolved arsenic concentrations between shallower and deeper regions of the surrounding unconfined aquifer, and to test these data against other geochemical parameters contributing to overall arsenic mobility.

Sampling and site analyses

Groundwater wells were screened for sampling along a NE-SW traverse starting in the mine tailings dam wall and extending approximately 500 meters to the northeast from the dam (Fig. 1), following the principal direction of groundwater flow (URS Corp. 2010). Selected boreholes were pumped out ~24 hours prior to sampling to allow for groundwater recharge. At each

borehole, two 100 mL samples and one 1L sample were collected in acid-washed polyethylene bottles. The 1L samples bottles were filled to the rim with unfiltered groundwater, while the two 100 mL samples were filtered through 0.45 μm pore size nylon filters immediately after bailing. One 100 mL bottle was reserved for anion analysis, while the other was acidified to a pH <2 and reserved for major cation and trace metal analyses. An additional 50 mL groundwater sample was obtained and filtered through a 0.2 μm pore size filter to provide a sterilized control for culturing experiments.

On-site measurements of groundwater pH and electrical conductivity were taken using a Thermo Orion 3-Star portable multi-meter. Samples were filtered on site through cellulose acetate 0.45 μm filters and analysed for major cations (Ca^{2+}, Na^{+}, Mg^{2+}, K^{+}), chloride (Cl^{-}), sulfate (SO_4^{2-}), and trace metals via inductively coupled plasma atomic emission spectrometry (ICP-AES), potentiometric titration, visible-wavelength absorbance spectrophotometry and inductively coupled plasma mass spectrometry (ICP-MS), respectively, as per standard procedures (USEPA 6010C for ICP-AES, APHA 4500-Cl for potentiometric titration, APHA 4500-SO4 for spectrophotometry, and USEPA 6020A for ICP-MS; ALS Environmental Labs, Melbourne). Trace metals were measured as totals by mass to charge ratio, with high mass resolution instrument capability for resolving arsenic peaks from mass interferences. No chromatographic separation was performed prior to trace metals analyses, with the exception of a field speciation method to separate As(III) and As(V) for subsequent ICP-MS analysis (see below). The statistics software package *R* (http://www.R-project.org/) was used to perform correlation testing (with two-tailed significance values) between dissolved arsenic and other geochemical parameters, including dissolved manganese and iron, to look for factors correlating with As mobility.

144

145 **Arsenic speciation analysis**

146 Groundwater sample replicates were preserved and fractionated in the field using an ion
147 exchange (IX) separation method (Karori et al. 2006, Sutton et al. 2009, Kumar and Riyazuddin
148 2010). A chloride form resin (Dowex 1x8) was prepared in a 10mL pipettes plugged with silica
149 and autoclaved cotton wool. The resin was then rinsed with 0.005 NaCl for 20 column volumes.
150 Groundwater from each of the wells was collected in 250 mL polypropylene bottles containing
151 0.5 mM (0.036 g/L) ethylenediaminetetraacetic acid (EDTA) and 0.01 M (0.15 g/L) acetic acid
152 (HAc). Using a syringe, 60mL of EDTA/HAc-amended groundwater was injected through the
153 chloride form resin. The first 15mL of water passing through the pipette was discharged. The last
154 45mL was bottled, placed on ice, and analysed for arsenic via ICP-MS using the methodology
155 identified above (at EML Laboratories, Melbourne). A new pipette with rinsed chloride form
156 resin was used for each well, to ensure no cross-contamination or saturation of the column resin.

157

158 **Cultivation of manganese-reducing bacteria from mine groundwater**

159 Enrichment culturing assays were performed with synthetic manganese-oxide (mixture of
160 birnessite, monoclinic δ-MnO_2; and hausmannite, tetragonal Mn_3O_4; see details below) as an
161 electron acceptor. Manganese-oxide amended broth *Shewanella* medium (Tebo et al. 2007)
162 buffered with piperazine-N, N'-bis(2-ethanesulfonic acid) (PIPES) to pH 7.0-7.2 was inoculated
163 under aseptic conditions with arsenic-contaminated groundwater from bore SE04, which
164 historically exhibited the highest dissolved As levels outside of the tailings dam and dam wall
165 along the dominant groundwater flow path (Fig. 2). All groundwater samples intended for use in
166 culturing experiments remained sealed and refrigerated after collection until inoculations were

167 performed using aseptic techniques in the lab. All broth and solid media cultivation experiments
168 included negative controls either without inoculum or inoculated from the field-filtered
169 groundwater sample. A final concentration of 10 mM of sodium acetate, as the most
170 environmentally ubiquitous substrate, was included as the carbon source, and the sodium
171 chloride concentration was adjusted to represent the average concentration associated with
172 conductivity values measured in SE04 groundwater samples (Table 1). Filter-sterilised vitamin
173 solution and 100% ethanol washed synthetic Mn-oxide, as the sole electron acceptor (final Mn
174 concentration of 15 mM), were added to culture media after autoclaving at 121°C for 15 minutes.
175 For broth cultures, 14 mL of each medium were dispensed into 20 mL foil-capped serum
176 bottles and left overnight in an anaerobic chamber to minimize oxygen content prior to
177 inoculation. Bottles were capped with airtight rubber septa and fastened with aluminum crimp
178 seals within an anaerobic chamber (under N_2 gas). To purge any remaining dissolved oxygen,
179 each bottle was sparged with N_2 gas for ~10 minutes. Each culture was then aseptically
180 inoculated using a sterile syringe with 1 mL of groundwater, and incubated at 30°C to facilitate
181 growth for pre-determined times (Fig. 4A). Quantification of Mn(IV) or Mn(III) conversion
182 (from birnessite or hausmannite, respectively) to dissolved Mn(II) was performed
183 spectrophotometrically using a Hach DR2800 field spectrophotometer and reagents from a
184 periodate-based protocol (Rao 1994).
185 Agar tubes (1.5% w/v) of Mn-oxide amended *Shewanella* medium were also inoculated
186 throughout the agar "plug" (≥ 10 cm deep) to assay for bacterial Mn reduction in a redox
187 gradient. Autoclaved molten agar in glass tubes (cooled to ~50°C in a water bath) was mixed
188 with groundwater from SE04 directly upon removal from the bath, and allowed to set (Fig. 4B).
189 Tubes were capped with autoclaved cotton, loosely fitted with rubber stoppers and incubated at

190 30°C. A redox gradient was allowed to establish within the agar tubes by natural diffusion of air
191 into the upper portion of the agar "plug" within each tube combined with heterotrophic
192 consumption of acetate throughout the semi-solid matrix.

193 Plate culturing assays of Mn-oxide amended *Shewanella* agar medium were conducted to
194 test for bacterial Mn reduction with sodium arsenite added to a final concentration of up to 150
195 ppb (Fig. 5), representative of groundwater values (Fig. 3). Plates were inoculated from nutrient
196 broth cultures enriched from SE04 groundwater, with the inoculum added as a 100 μL aliquot
197 spread evenly across one half of each plate (Fig. 5). The other half of each plate was left
198 uninoculated to provide a negative control (or "blank"). Replicate Mn-oxide amended plate
199 cultures without arsenite were streaked with SE04 inoculum for the purpose of isolating single
200 colonies of Mn-reducers for subsequent growth and identification.

201

202 **Manganese oxide mineral synthesis for culturing experiments**

203 A method for δ-MnO_2 (birnessite) synthesis was adapted from previously published protocols
204 (Tebo et al. 2007, Villalobos et al. 2003). First, the following solutions were prepared and filter
205 sterilised: (A) 75.1 g of $MnCl_2 \cdot 4H_2O$ in 1280 mL of ultrapure laboratory grade water that has
206 been filtered and purified by reverse osmosis (Milli-Q water), (B) 40.0 g of $KMnO_4$ in 1280 mL
207 of Milli Q water, and (C) 28.0 g of NaOH in 1440 mL of Milli Q water. While stirring, solution
208 B was slowly added to solution C, over approximately five minutes. Solution A was then
209 gradually added over approximately 35 minutes to solution B+C while stirring, and a black
210 manganese oxide precipitate formed. Solution A+B+C was left to stand for two hours to allow
211 the precipitate to settle to the bottom, and the supernatant was discarded. The remaining Mn-
212 oxide was then centrifuged at 3800 g, and the supernatant was again discarded. The Mn-oxide

was resuspended in sterile Milli-Q water and shaken for one hour. This washing step was repeated three times before the Mn-oxide was finally resuspended in 100% ethanol for sterilization. The Mn-oxide was heated in an oven a covered sterilized bottle to 50°C overnight to evaporate excess ethanol. The synthetic black precipitate was subsequently identified by x-ray diffraction as a mixture of two synthetic Mn-oxides consisting of MnO_2 (birnessite, monoclinic) and Mn_3O_4 (hausmannite, tetragonal) (Supplementary Figure 1). As either phase was suitable for testing for bacterial Mn reduction, the precipitate was used in culturing experiments.

16S rRNA gene identification

Bacterial DNA from selected cultures was amplified for sequencing analysis by direct lysis polymerase chain reaction (PCR) on a MJ Mini Thermal Cycler (BioRad). The primers used for these reactions were 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). Amplification was performed over 30 cycles (95°C for 2 min, 25 x [98°C for 20 sec, 50°C for 15 sec, 68°C for 1 min], 68°C for 4 min.) using KapaTaq polymerase enzyme (Kapa Biotransformations). To determine DNA concentrations, PCR products were compared on an agarose gel to known standard mass and size ladders. Amplicons from direct lysis of four colony forming units (cfu) grown on agar plates from groundwater extracted from well SE04 were purified and Sanger sequenced (Applied Genetics Diagnostics, University of Melbourne Pathology Department).

RESULTS

Groundwater chemistry

Table 1 presents results from field measurements of pH and electrical conductivity (EC). The highest pH values were located in wells SP102 and SP104, just along the tailings dam wall, indicative of the migration of tailings effluent outward from within the dam (where the pH of fresh tailings sludge is 9.5-10 typically). With the exception of these two wells, generally shallower and deeper wells exhibited lower and higher (i.e. above or below ~6.0) pH values, respectively. With the exception of SE05A, generally conductivity values were comparable among wells and indicative of brackish salinity.

Exceedingly high historical concentrations of total dissolved As in groundwater have been observed in wells located within or near the tailings dam wall, however (e.g., SP104 and 102), leading to the probability that additional As input to the aquifer is sourced from the tailings. As can be seen from Fig. 2, some wells outside of the dam wall (e.g., SE04 and SE03) have exceeded regulatory limits for drinking water (10 ppb) but vary within the range allowed for agricultural use (100-500 ppb). Historical dissolved manganese and arsenic concentration data from wells SE03A, SE03, SE04A, SE04, SE05A, SE05, SE09A and SE09 (Fig. 3) showed a strong positive correlation (Pearson $r^2 = 0.72$, $n = 51$, $p < 0.001$) (Table 2). Higher dissolved manganese and arsenic concentrations were observed in the deeper screened wells SE04, SE03, SE05 and SE09, relative to the more shallow screened "paired mate" wells SE04A, SE03A, SE05A and SE09A. Notably, higher arsenic concentrations were consistently observed in well SE04 for all sampling timepoints (Fig. 2). It is worth noting that no detectable aqueous sulfide has been reported from these wells in the historical groundwater data, and no sulfide odor was noticeable from the samples directly upon recovery in the field.

Results from arsenic speciation analysis are presented in Table 3, where it can be seen that most or all of the dissolved arsenic found in wells along the predominant groundwater flow

path relative to the tailings facility was determined to be as As(III). Two of the wells samples produced results that did not yield mass balance for total arsenic (SD405 and SE04A), possibly due to laboratory error in processing or measuring total arsenic levels in the replicate samples. All other wells sampled yielded mass balance for total arsenic.

Enrichment culturing

Growth of manganese-reducing bacteria was obtained on *Shewanella* broth medium (Tebo et al. 2007) amended with 15 mM Mn-oxides from well SE04 groundwater after 3-4 weeks initial incubation at room temperature (Fig. 4A). Subsequent transfers of positive growth cultures from SE04 to broth medium showed growth after several days to 1 week at 30°C. After 10 days of incubation in agar *Shewanella* medium at 30°C, the positive control culture (a pure culture of *Pseudomonas aeruginosa*) displayed clearing of Mn-oxide near the surface of the solid agar. Tubes inoculated with Mn- and As-enriched groundwater samples began to display this clearing after ~15 days of incubation (Fig. 4B). Figure 4 shows the complete reductive dissolution (clearing of medium to colorless or pale yellow, relative to darker negative control with Mn-oxide suspension) of synthetic Mn-oxide in liquid and semi-solid *Shewanella* media after 3-4 weeks incubation at 30°C.

Isolation of *Pseudomonas fluorescens* group species

Single colony-forming units grew from broth enrichment cultures from well SE04 streaked on to *Shewanella* agar medium amended with 15 mM Mn-oxide. These colonies were identified by the plaque of clear agar surrounding each one and resulting from the localized complete reductive dissolution of synthetic Mn-oxide.

281

282 **Arsenite tolerance assays with enrichment culture**

283 Agar plate experiments were conducted to assay the arsenic tolerance (As[III] as sodium
284 arsenite) of Mn-reducing bacteria enriched previously in *Shewanella* broth medium. Plates
285 showed no manganese reduction on uninoculated agar surfaces ("blanks"), and conversely,
286 showed the complete reductive dissolution of Mn-oxide on inoculated surfaces with no arsenite
287 added. On plates with synthetic Mn-oxide and the arsenite amendments, an intermediate degree
288 of Mn-oxide dissolution was observed. Plates with 50 ppb of sodium arsenite added required
289 twice as long (6 weeks) to exhibit the same degree of reductive Mn-oxide dissolution as did
290 plates with no arsenite (Fig. 5).

291

292 **DNA analysis**

293 PCR amplicons of 16S rDNA extracted from four single colony-forming units found to reduce
294 MnO_2 when grown on agar streak plates were sequenced, aligned into single contigs >1390 base
295 pairs long each, and compared with other environmental 16S rDNA sequences via the NCBI
296 BLAST database (Altschul et al. 1990). All environmental sequences analysed showed a closest
297 16S rDNA sequence similarity (≥99%) to either *Pseudomonas grimontii or Pseudomonas*
298 *veronii*, two closely related members of the *P. fluorescens* group (Elomari et al. 1996, Baïda et
299 al. 2002). A representative sequence has been submitted to GenBank with accession no.
300 JX878497.

301

302 **DISCUSSION**

303 Previous work has found that the ambient arsenic levels in the vicinity of Stawell are
304 significantly above the regional background concentrations (Noble et al. 2010). However, pH
305 values and increasing concentrations of total dissolved arsenic in groundwater with increased
306 proximity to the mine tailings also indicates that the tailings are a point-source for increased
307 arsenic contamination to the aquifer. This input is likely overprinted on ambient arsenic levels
308 and may vary with groundwater recharge and discharge rates. Wells located within or close to
309 the tailings dam wall consistently show elevated As levels (e.g., SE09A, SE09) or pH (e.g.,
310 SP102, SP104) that suggests that some As must also be leaking through the tailings dam wall
311 and therefore also possibly the tailings pond floor. The trend in groundwater As concentrations
312 also reflects the generally northeast-ward direction of groundwater flow, as wells SE05A and
313 SE05 to the east showed the least As of all the sampled nested-pair well sites.

314 Previous work has also shown that As in gold mine process tailings (involving
315 cyanidation like at the Stawell Mine) is present predominantly as As(V) across a range of
316 minerals (Paktunc et al. 2004, Fawcett and Jamieson 2011). Given the evidence that the
317 cyanidized tailings dam is leaking waste to groundwater through the dam walls, therefore it was
318 hypothesised that arsenic adsorbed to Mn-oxides present in the aquifer would predominantly
319 consist of arsenate. Adsorbed As^{5+}, however, may still be subject to reduction to As^{3+} in the
320 groundwater. Conversely, the arsenic speciation data (Table 3) showed predominance of As(III).
321 This result is consistent with the interpretation that any As(V) escaping from the tailings pond is
322 undergoing reduction subsequent to Mn-oxide reductive dissolution in the deeper (28-30 m)
323 aquifer. If the observed Mn(IV) reduction was catalysed by As(III) oxidation, most or all of the
324 speciated As should have been present as As(V), which was not the case. These results suggest

the activity of a consortium of microbes effecting the reduction and remobilization of both Mn(IV) and As(V) into groundwater as Mn(II) and As(III).

The increase in dissolved manganese concentrations in the deeper of nested and paired wells SE03, SE04, SE05 and SE09 is indicative of a transition to suboxic (i.e., < 2 mg O_2/L), reducing conditions in groundwater, an interpretation supported by generally higher pH values in deeper groundwater (Table 1). Thus it is inferred that this transition occurs somewhere below 10-12 m beneath the ground surface in the locations of these boreholes within the aquifer. The exact (time-dependent?) position of this redox transition is currently unknown, however, and requires further investigation. Measurements of pH and EC also suggest substantially different groundwater chemistry between the upper and lower aquifers sampled in this study, which may influence the general speciation of both Mn and As via affecting the extent of microbial activity. The correlation of dissolved arsenic with manganese supports the interpretation that arsenic previously adsorbed to Mn-bearing ox(yhydroxid)es was released as these minerals were reductively dissolved. The observation that dissolved arsenic levels are 5-10 wt% of dissolved manganese suggests that adsorption to Mn-ox(yhydroxid)es is a significant process by which As mobility is mitigated (e.g., Cai et al. 2002, Anawar et al. 2003, Gandy et al. 2007). This finding contrasts with environments in which Fe- and As-reduction play a much greater role than does Mn-reduction in controlling As mobility (e.g., Berg et al. 2008). The data show no correlation between total dissolved As and Fe (Pearson $r^2 = 0.12$, $n = 39$, $p > 0.1$), but a significant correlation with dissolved Mn(II) (Pearson $r^2 = 0.72$, $n = 51$, $p < 0.001$). The order of magnitude or greater difference between dissolved Mn and As concentrations, and lack of correlation between As and dissolved Fe or sulfate (proportionally or inversely, respectively) collectively suggest the groundwater is not sufficiently reducing for Fe(III)- or SO_4^{2-}- reduction. It is

348 therefore hypothesized that, during periods of increased meteoric input and consequently a rising
349 water table with the onset of suboxic or anoxic conditions in the groundwater, the stability of
350 aquifer Mn-ox(yhydroxid)es and immobilized As are most likely to be compromised.
351 Interestingly, Figure 3 suggests the possibility of a "threshold" value of dissolved Mn between
352 0.6-0.8 ppm at which release of As from Mn-oxides becomes significant. Some of the highest
353 observed values of dissolved As (e.g. SE09) likely reflect increased proximity to the tailings
354 facility and the possibility of higher total arsenic concentrations in this well.

355 Manning et al. (2002) found that As(III) oxidation by MnO_2 catalyses reductive
356 dissolution of synthetic birnessite, but also promotes the adsorption of As(V) to the remaining
357 MnO_2 surface sites. Since naturally forming birnessite is generally present in excess of As, this
358 process should contribute to the net sequestration of As (as arsenate) on MnO_2 surfaces.
359 Furthermore, He and Hering (2009*)* found that As(V) adsorbs to synthetic birnessite more
360 efficiently when some Fe(II) is also present, and hypothesized that Fe(III)-oxides or -
361 oxyhydroxides may form in association with birnessite in a way that facilitates the adsorption of
362 As(V). In the absence of Fe(II), As(III) oxidation coupled to Mn(IV) reduction occurred
363 naturally, but the efficiency of As(V) sequestration was not enhanced. If the As present in
364 groundwater at the Stawell Mine is as As(III), it should be reactive with MnO_2 to form As(V)
365 and catalyse release of Mn(II) (Manning et al. 2002). If this process is occurring, relatively low
366 concentrations of Fe-oxides or -oxyhydroxides in the aquifer may explain both the lack of
367 correlation between dissolved As and Fe and the persistence of dissolved As despite the relative
368 abundance of Mn(IV) in the aquifer (He and Hering 2009). Alternatively, the rate of bacterial
369 Mn(IV) reduction would have to be sufficiently fast to maintain the observed concentrations of
370 dissolved As, which seems unlikely given the rapid timescale (hours) of either As(III) oxidation

371 or As(V) adsorption with Mn-oxides (Manning et al. 2002). By implication, therefore, any
372 As(III) present in the Stawell groundwater initially should have been transformed to As(V) in the
373 presence of MnO_2, and would presumably be bioavailable primarily as arsenate to indigenous
374 Mn-reducing bacteria. The observation that most or all arsenic was present as As(III) in deeper
375 groundwater samples supports the interpretation that microbial As-reduction is also occurring,
376 presumably subsequently to Mn(IV) reductive dissolution and release of As(V) to solution.

377 The identification of Mn-reducing *Pseudomonas fluorescens*-related bacteria in
378 groundwater-inoculated cultures supports the interpretation that microbial Mn(IV)-reduction can
379 strongly influence As mobility in groundwater near the mine site. Broth cultures demonstrated
380 the complete reductive dissolution of synthetic Mn-oxide (Fig. 4A) and agar roll tube cultures
381 showed similar reaction efficiency. Interestingly, as the roll tubes incubated aerobically were
382 inoculated throughout, but Mn reduction occurred only near the surface of the solidified agar, it
383 is inferred that a metabolic requirement for oxygen somehow limited the extent of Mn reduction
384 into the agar. This observation would be consistent with the obligately aerobic metabolism of
385 the close relative to the isolate, *P. fluorescens*, although some other related *Pseudomonas* species
386 are capable of facultatively anaerobic growth (coupled directly to nitrate reduction or via cell-to-
387 mineral contact to Mn reduction [Di-Ruggiero and Gounot 1990]). *Pseudomonas* spp. are also
388 among common soil bacteria known to be producers of extracellular electron shuttles (Pak et al.
389 2002, Wang et al. 2010), and reductive dissolution of birnessite by such shuttles has been studied
390 (Duckworth and Sposito 2007). It is hypothesized that the culturing enriched for *P. fluorescens*-
391 group species with the ability to reduce Mn(III or IV) either by contact or extracellular electron
392 transfer. Furthermore, it is inferred from the combined broth medium and agar tube culturing

393 experiments that bacterial reductive dissolution of Mn-oxides or -oxyhydroxides is similarly
394 occurring below the water table at the Stawell Mine.

395 Plate culture experiments with and without sodium arsenite yielded insights into the
396 activity of Mn-reducing bacteria within the context of the groundwater microbial community in
397 the Stawell Mine aquifer. While some *Pseudomonas* spp. exhibit greater tolerance for As(V)
398 than for As(III) (Joshi et al. 2008), at least one strain of *P. fluorescens* also possesses resistance
399 for both arsenate and arsenite (Prithivirajsingh et al. 2001). Mn-reducing *Pseudomonas* spp.
400 with resistance to both As(III) and As(V) could reductively dissolve Mn-oxy(hydroxides) in the
401 presence of arsenate or arsenite. The Mn-reducing *P. fluorescens*-group species isolated in this
402 study, however, exhibited greatly decreased ability to reduce synthetic Mn-oxide under As
403 concentrations representative of site groundwater maxima (150 ppb, Figs. 5 and 6). Although
404 presented as sodium arsenite (As^{III}), a significant portion of this As would have transformed
405 within the experimental time to arsenate (As^{V}), which presumably adsorbed to the Mn-oxide as
406 reported in previous experiments (Ying et al. 2012). The bioavailability of this arsenic as
407 manganese reduction proceeded may have limited the extent to which the bacteria could gain
408 energy from Mn-reduction and/or As-inhibited bacterial enzymes. In this sense, the observed
409 process of synthetic Mn-oxide reduction under arsenite stress could be reflective of a self-
410 limiting subsurface process for As remobilization. This interpretation would be consistent with
411 the observed maxima in historical groundwater data for both manganese and arsenic, and would
412 indicate As is remobilized by the episodic flourishing of Mn-reducers which then decreases and
413 results in incremental transport of As through the aquifer.

414 Previous findings of As sourced from cyanide-processed gold mine tailings being
415 comprised entirely of arsenate (Paktunc et al. 2004) suggest also the possibility that arsenic in the

groundwater is initially present as As(V), and that Mn(III or IV) and As(V) reduction could have been mitigated by different populations of the aquifer microbial community. Further testing of this alternative hypothesis is required, but it is notable that dissolved As levels were correlated only with dissolved Mn, suggesting a direct link between the two elemental cycles. In previous studies wherein microbial As(V)-reduction proceeded separately or in parallel with other reductive dissolution reactions, Fe(III)-reduction was also typically observed (e.g., Berg et al. 2008).

CONCLUSIONS

The results of this study demonstrate a novel case of manganese-reducing bacteria solely controlling the mobility of As in mining-impacted groundwater, and evidence for subsequent microbially-mediated As reduction, in the absence of significant and typically observed Fe-oxide reductive dissolution. The degree of microbial influence over As mobility may be self-limiting, however, as Mn-reducing *Pseudomonas* spp. isolates found in this study displayed high sensitivity to dissolved arsenic (As^{III}) at the upper range of environmentally observed concentrations. The results of our study will aid understanding of the interplay between Mn-cycling and As mobility in As-contaminated aquifers under suboxic or anoxic conditions, with implications for mine site closure and groundwater management planning.

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FIGURE AND TABLE CAPTIONS

FIGURE 1. Google Earth satellite image of Stawell gold mine #2 tailings facility (photo) located on map of Australia (inset line map).

A sampling traverse from tailings dam to northeast (white line) begins at well SP104 and ends at SP109. Wells (with approximate depths in meters below the surface in parentheses) that were studied along this transect, from southwest to northeast, are SP104 (8.1), SP102 (6.4), SD405 (5.5), SD404 (15.0), SP107 (11.4), SE04A (12.5), SE04 (28.0), SE03A (12.5), SE03 (29.5), SP109 (11.7). SE04A and SE04, and SE03A and SE03, are pairs of nested wells located at two different sites, respectively. SE09A (11.5) and SE09 (29), and SE05A (12.5) and SE05 (32.0) are nested wells to the north and east, respectively, of the tailings dam. The road marked "Lavett Rd" no longer exists. On inset line drawing map of Australia, Stawell mine location is represented by filled circle.

FIGURE 2. Groundwater total dissolved arsenic concentrations over time at Stawell gold mine. Historical data from 1994-2009 and 2010-2011 were provided courtesy of Crocodile Gold Corp., Stawell. Data from a sampling trip in June-July 2010 were obtained in this study. Analytical uncertainties lie within the data points. Circled points represent wells located within the tailings, while solid and dashed lines represent points from wells SE04 and SE03, respectively.

FIGURE 3. Graph of groundwater total dissolved arsenic and manganese concentrations in nested pair wells at Stawell gold mine. Concentrations are in ppm for nested shallow (unfilled markers) and deep (filled markers) wells located to the north (SE09A, SE09), northeast (SE03A, SE03, SE04A, SE04) and east (SE05A, SE05) of the tailings storage facility. Data were obtained during 2006-2011. Pearson product-moment correlation coefficient (r^2) = 0.72 , n = 51, $p < 0.001$.

FIGURE 4. Manganese-reduction culturing experiments with Stawell groundwater inoculum. (**A**) Minimal medium amended with synthetic Mn-oxide and inoculated with groundwater from well SE04 (right-hand bottle inoculated with groundwater; leftmost bottle is negative control with no inoculum and middle bottle is negative control with 0.2 μm pore size filtered inoculum). Manganese (IV)-reducing enrichment cultures were incubated at 30°C. After ~3 weeks, all Mn-oxide was completely dissolved (medium was cleared) in inoculated bottle. (**B**) Agar roll tube cultures with synthetic Mn-oxide in minimal medium, incubated aerobically at 30°C and marked periodically at the "interface" between dark-colored birnessite/haussmannite particles in suspension, containing crystal structure-bound Mn(III) or Mn(IV), and the clear agar

containing dissolved Mn(II). SE04-1 and SE04-2 represent duplicate cultures from SE04 groundwater. The positive and negative controls shown were *Pseudomonas aeruginosa* and no cells, respectively. Killed-cell controls (not shown) appeared the same as no cell controls.

FIGURE 5. Mn-oxide-amended plated medium assay for manganese (IV)-reduction in the presence of arsenite. *Shewanella* medium MnO_2-overlay plates were spread with 100 μL of an enrichment culture grown from site SE04 across the left-hand side of each plate. Right-hand side of each plate shows the uninoculated negative control for each condition. As(III) was added as 50 ppb sodium arsenite to right-side plate. Cleared (light colored) portions of plates represent areas of successful bacterial Mn(IV) reduction. Degree of MnO_2 clearing can be compared on each positive growth side of each plate to the respective negative control side of each plate, as well as between plates. The right-hand plate (MnO_2 plus arsenite) required twice as long (6 weeks) to reach the same degree of clearing as the left-hand plate (MnO_2 only).

SUPPLEMENTARY FIGURE 1. X-ray diffraction spectrum of synthetic Mn-oxide used in microbial Mn-reduction culturing experiments. Pattern shows the presence of both birnessite and haussmannite phases.

TABLE 1. Selected groundwater parameters at Stawell gold mine (Victoria, AUS). Field measurements shown are electrical conductivity (as a proxy for salinity) and pH. Data were obtained in June-July 2010 from wells along the sampling traverse northeast of the mine tailings facility. Groundwater temperature was 12°C ± 2°C for all wells.

TABLE 2. Pearson r^2 correlation value with arsenic. Values represent the computed correlation coefficients for trace metal concentrations from the sampled wells, as calculated with the software package *R* (http://www.R-project.org/). Significance values for Mn and Fe are reported in the body of text for a two-tailed distribution.

TABLE 3. Arsenic field-speciation analysis. Data were obtained via ICP-MS after immediate sample processing in the field according to the chromatographic separation method of Karori et al. (2006). Well numbers represent samples taken from wells along primary groundwater flow path on a transect NE of the mine tailings dam. Samples SD405 and SE04A did not produce analytical results that achieved mass balance for total arsenic, and should be regarded as questionable pending further investigation.

TABLE 1: Groundwater parameters at Stawell Gold Mine (Victoria, AUS)

Well	sampling depth (m)	EC (mS)	pH
SD404	14.3	23.8	5.0
SD405	5.6	24.4	3.7
SE03	29.4	13.6	6.6
SE03A	12.5	16.4	4.9
SE04	28.2	21.6	6.8
SE04A	11.4	22.5	5.8
SE05	31.9	15.0	5.5
SE05A	12.3	5.3	4.4
SE09	29.2	21.4	6.2
SE09A	11.3	24.8	5.5
SP102	6.4	13.7	8.1
SP104	8.1	14.9	7.3
SP107	10.9	24.6	5.8
SP109	11.5	17.0	6.1

678

TABLE 2: Pearson r^2 correlation value with arsenic

Mn	0.72
Fe	0.06
Cu	-0.14
Pb	-0.25
Zn	0.01
Al	-0.25
Cr	-0.14
Ni	0.06
Ca	-0.11
Mg	0.26
Na	0.18
K	0.52
HCO_3^-	0.23
SO_4^{2-}	0.2
Cl^-	0.16
NO_2^-	0.16
NO_3^-	-0.16

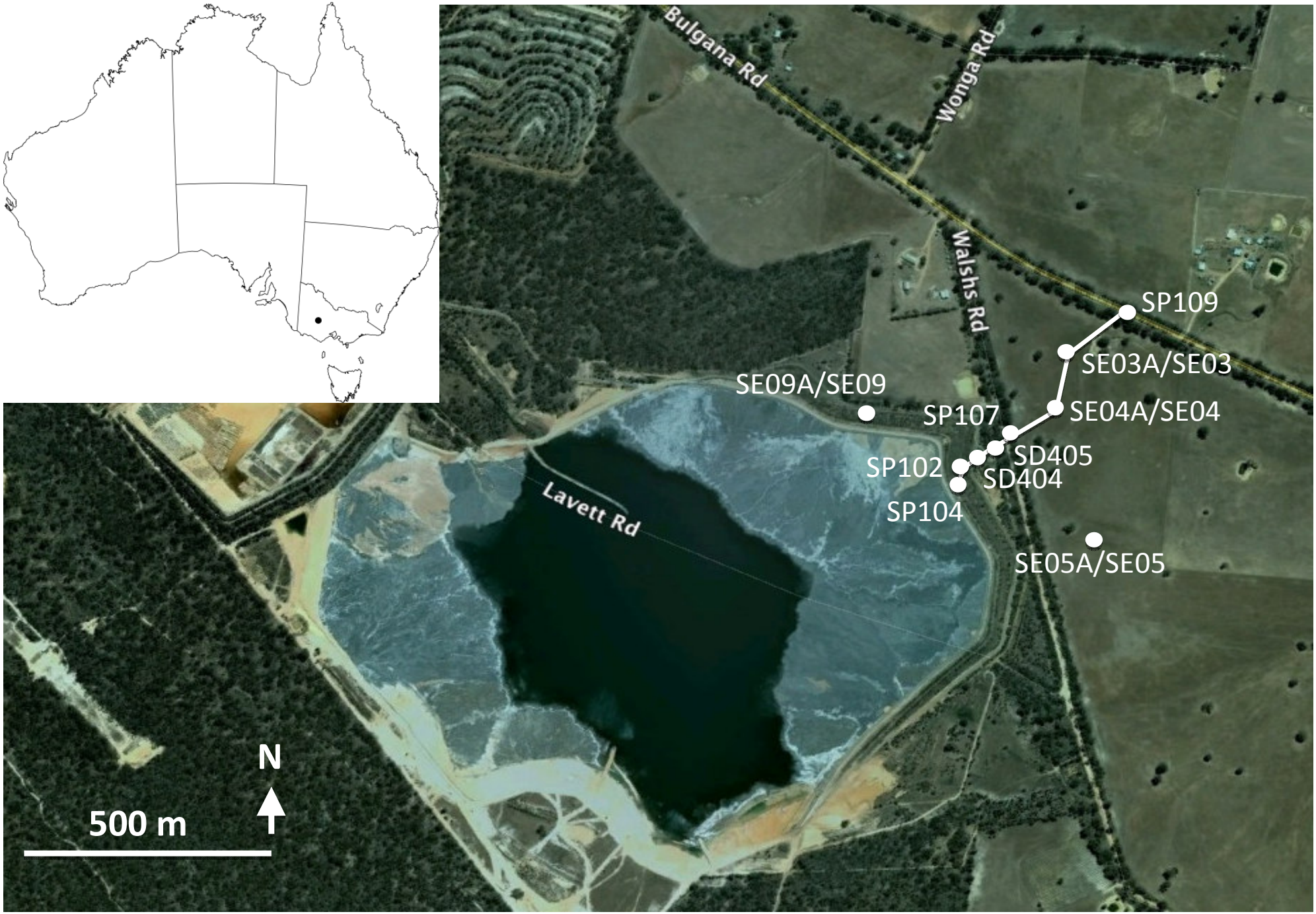
679

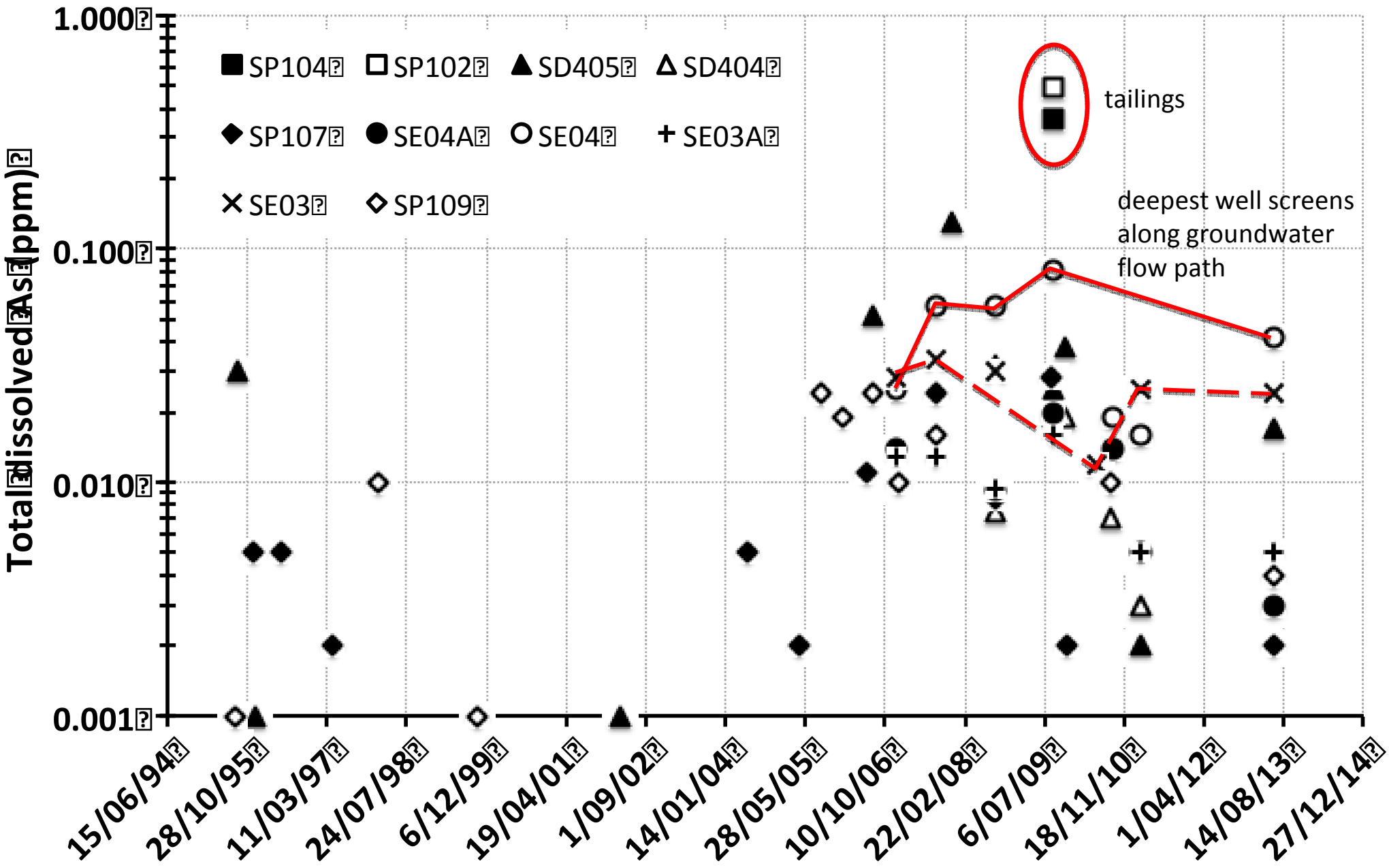
680

681 **TABLE 3. Arsenic field-speciation analysis**

Well ID	Total As (mg/L)	As(III) (mg/L)
SE03	0.024	0.020
SE03A	0.001	0.001
SD405	0.014	0.017
SD404	0.004	0.004
SP109	0.001	0.001
SE04	0.042	0.039
SE04A	0.003	0.010
SP107	0.001	0.001

682


Bulgana Rd
Wonga Rd
Walshs Rd
Lavett Rd
SP109
SE03A/SE03
SE09A/SE09
SP107
SE04A/SE04
SD405
SP102
SD404
SP104
SE05A/SE05
N
500 m


1.000
0.100
0.010
0.001
Total Dissolved As (ppm)
SP104
SP102
SD405
SD404
SP107
SE04A
SE04
SE03A
SE03
SP109
tailings
deepest well screens along groundwater flow path
15/06/94
28/10/95
11/03/97
24/07/98
6/12/99
19/04/01
1/09/02
14/01/04
28/05/05
10/10/06
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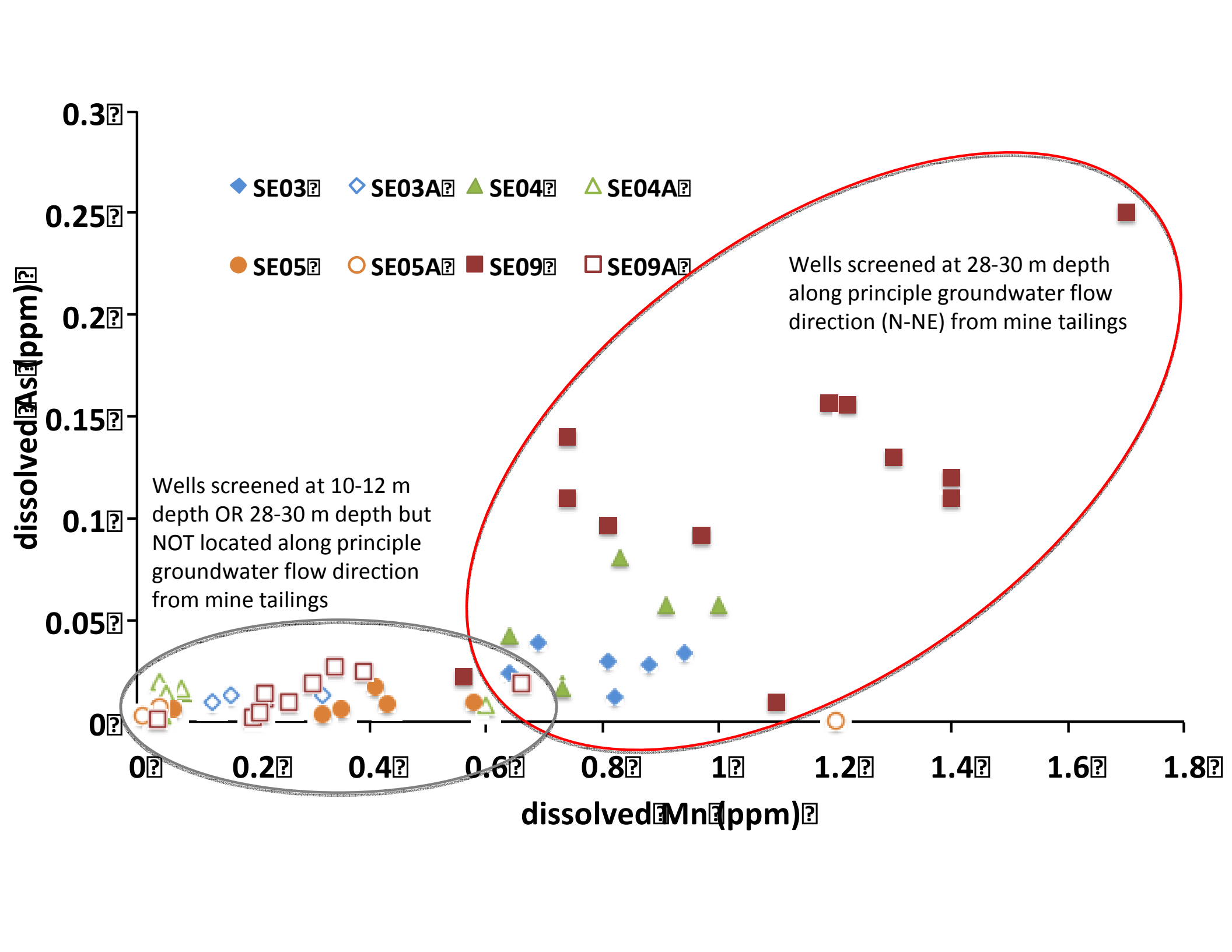
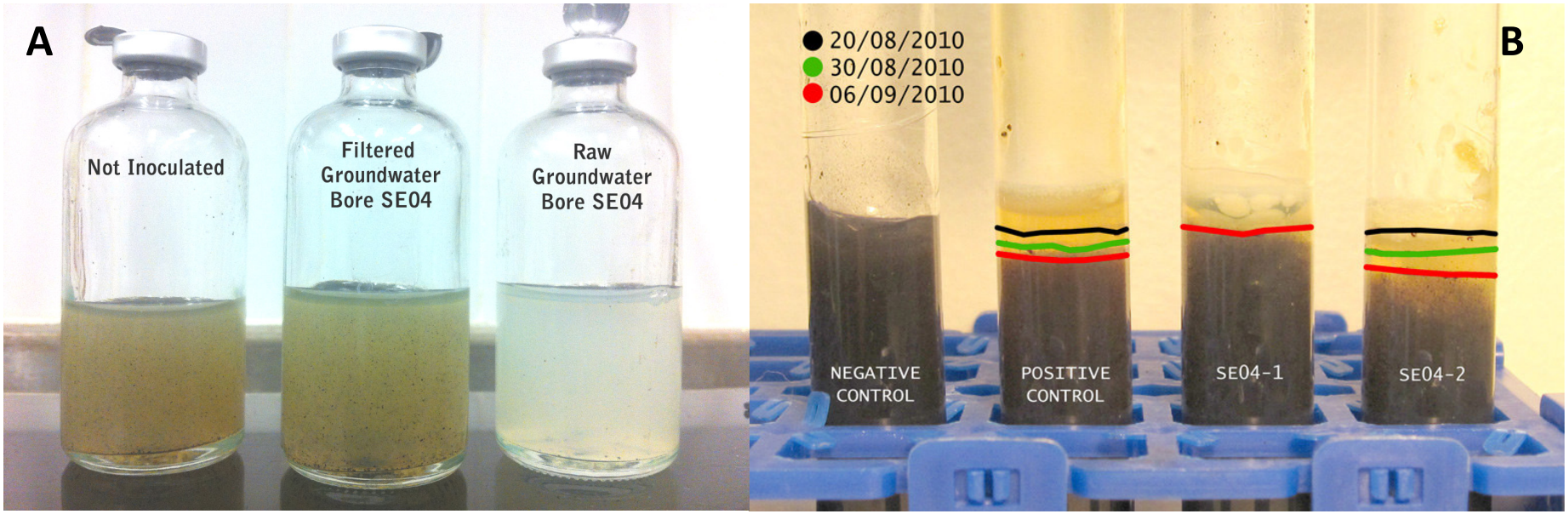
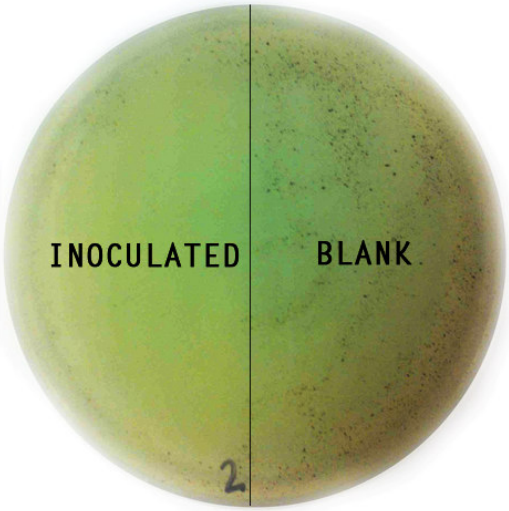
18/11/10
1/04/12
14/08/13
27/12/14


SE03
SE03A
SE04
SE04A
SE05
SE05A
SE09
SE09A
Wells screened at 28-30 m depth along principle groundwater flow direction (N-NE) from mine tailings
Wells screened at 10-12 m depth OR 28-30 m depth but NOT located along principle groundwater flow direction from mine tailings
dissolved As (ppm)
dissolved Mn (ppm)
0.3
0.25
0.2
0.15
0.1
0.05
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8

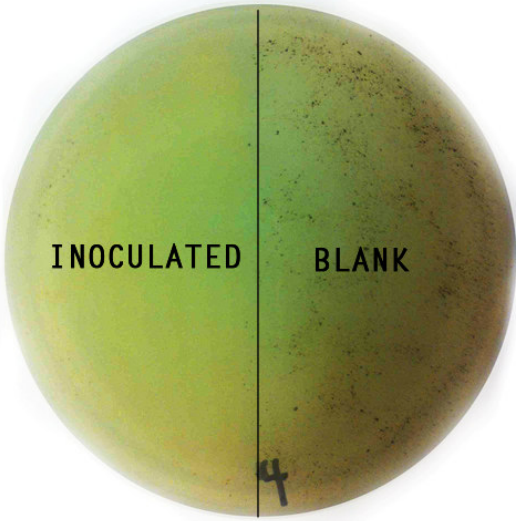

A
Not Inoculated
Filtered Groundwater Bore SE04
Raw Groundwater Bore SE04
20/08/2010
30/08/2010
06/09/2010
B
NEGATIVE CONTROL
POSITIVE CONTROL
SE04-1
SE04-2

15mM MnO_2, No As



3 weeks later

15mM MnO_2, As(III)



6 weeks later

Author/s:
Horvath, AS; Garrick, LV; Moreau, JW

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