

## Microbial Mineral Weathering for Nutrient Acquisition Releases Arsenic<sup>∇</sup>

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**Tens of millions of people in Southeast Asia drink groundwater contaminated with naturally occurring arsenic. How arsenic is released from the sediment into the water remains poorly understood. Here, we show in laboratory experiments that phosphate-limited cells of *Burkholderia fungorum* mobilize ancillary arsenic from apatite. We hypothesize that arsenic mobilization is a by-product of mineral weathering for nutrient acquisition. The released arsenic does not undergo a redox transformation but appears to be solubilized from the apatite mineral lattice during weathering. Analysis of apatite from the source area in the Himalayan basin indicates the presence of elevated levels of arsenic, with an average concentration of 210 mg/kg. The rate of arsenic release is independent of the initial dissolved arsenic concentration and occurs at phosphate levels observed in Bangladesh aquifers. We also demonstrate the presence of the microbial phenotype that releases arsenic from apatite in Bangladesh aquifer sediments and groundwater. These results suggest that microbial mineral weathering for nutrient acquisition could be an important mechanism for arsenic mobilization.**

Tens of millions of people in Southeast Asia are drinking groundwater containing elevated levels of arsenic that is naturally released from the sediment into the groundwater (22, 49). Multiple mechanisms have been proposed to explain this phenomenon, including arsenic mobilization during reduction of Fe(III) (hydr)oxides (14, 22, 27), arsenate reduction and mobilization (25, 28, 51), and redox cycling and release from arsenic-rich sulfides (30). Each mechanism probably plays a role under certain conditions but cannot fully explain the prevalence of arsenic or all the features of its distribution in groundwater (39, 42).

It is well known that bacteria can obtain nutrients from solid-phase minerals (1, 4, 5, 36, 47). Subsurface environments are oligotrophic, and bacteria have been shown to preferentially weather minerals that contain phosphate and ammonia, both required for growth (36, 37). Microbial production of organic acids, ligands, and extracellular polysaccharides could all promote dissolution of minerals (35, 44–46). This is different than microbes using solid-phase minerals in redox processes such as Fe(III) reduction, resulting in the release of toxic metals (2, 24, 28). During redox reactions, microbes use the solid-phase minerals for energy. During nutrient acquisition, microbes access trace nutrients from the minerals for cellular growth (5), and redox alterations may not be occurring.

Despite the importance of bacterially promoted mineral weathering for nutrient acquisition, the release of potentially dangerous ancillary elements such as arsenic from minerals to groundwater has not been investigated.

We propose a novel mechanism for arsenic mobilization in aquifer systems: microorganisms weather and dissolve minerals to obtain nutrients and, during this process, cause an ancillary release of arsenic into the groundwater. Microcosm experiments were conducted with *Burkholderia fungorum*, phosphate-free artificial groundwater (AGW), and natural apatite. Apatite from the Himalayas was also analyzed for arsenic. Finally, bacteria extracted from Bangladesh sediments and water samples were incubated on mineral phosphate-solubilizing (MPS) plates to determine if this phenotype is present in arsenic-rich deltaic environments. Results indicate that ancillary arsenic release may mobilize arsenic in aquifer systems.

The generalized formula for apatite is  $\text{Ca}_5(\text{PO}_4)_3(\text{F},\text{Cl},\text{OH})$ . Purified apatite was obtained from the Himalayas (18) and was digested with hot nitric acid. Digestions were performed to determine the total amount of arsenic associated with the apatite. Approximately 0.5 g of apatite was digested in 5 ml of nitric acid at 90°C for 1 h and then brought to 20 ml with deionized water (8). The total amount of arsenic was measured by inductively coupled plasma mass spectrometry. For microcosm experiments, apatite was purchased from Ward's Scientific and ground with mortar and pestle to a fine powder. It contained 470 mg/kg arsenic. The percentages of As(V) and As(III) in the solid-phase arsenic from the Ward's Scientific apatite and one Himalayan sample were determined by X-

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ray absorption near-edge structure spectroscopic analyses at the Stanford Synchrotron Radiation Laboratory on beam line 11-2 (13, 31, 34).

*Burkholderia fungorum* was utilized because of its ability to weather rocks for nutrient acquisition. Specifically, experiments with basalt and granite have shown that it can weather these rocks and release phosphate into the environment (47, 48). Members of the *Burkholderia* genus have been isolated from granitic soils (48) and have been shown to be efficient at mineral weathering (40). Unfortunately, little work has been done to characterize microbial populations in the aquifers and soils of Southeast Asia, and isolates were not available (9, 19, 20). The metabolic function of *Burkholderia fungorum* is indistinguishable from those of other heterotrophic bacteria and therefore was chosen as a model organism (12).

*Burkholderia fungorum* (ATCC BAA-463) was grown overnight at 30°C while being shaken in tryptic soy broth, washed, and resuspended in P-limited AGW. Approximately  $5 \times 10^5$  cells/ml were added to each microcosm. In abiotic controls, cells were killed by being autoclaved and then added to the microcosm. The P-limited AGW is based on the recipe of Wu et al. (47) and is similar to low-ionic-strength groundwater; per liter of solution, the major components are glucose (0.2 g),  $\text{NH}_4\text{Cl}$  (0.04 g),  $\text{KCl}$  ( $5.0 \times 10^{-4}$  g), and  $\text{MgSO}_4$  ( $5.0 \times 10^{-4}$  g) (added as  $1 \times 10^{-3}$  g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ). Glucose was added as the carbon source. Each 500-ml Wheaton bottle contained 200 ml of P-limited AGW and 2 g of apatite. Bottles were shaken at 30°C throughout the course of the experiment. The microcosm experiments were performed under oxic conditions. Experiments included addition of (i) 2 ml of live *B. fungorum* cells, (ii) 2 ml of killed *B. fungorum* cells, (iii) a no-cell control, (iv) 2 ml of live *B. fungorum* cells and an arsenate spike, and (v) 2 ml of live *B. fungorum* cells with a pH 7 phosphate spike. Samples were spiked with arsenic because recent field studies have shown that arsenic release is independent of the initial arsenic concentration (39). Therefore, a field-relevant method should indicate release in the presence of increasing initial arsenic concentrations. A phosphate spike was included to determine if release occurred under field-relevant phosphate conditions. Abiotic experiments were performed with gluconic acid and the Ward's Scientific apatite. Eight incubations with 0.2 g of apatite and 20 ml of gluconic acid were performed at concentrations ranging from 314 nM to 3.14 M. Samples were shaken at 200 rpm for 24 h at 37°C.

Aliquots were removed from each microcosm at the sampling points for analysis of pH, gluconic acid, glucose, cations, and trace metals. pH was analyzed immediately with an Accumet pH electrode (Fisher Scientific). Glucose and gluconic acid samples were filtered with a Millex-GP 0.22- $\mu\text{m}$  filter (Millipore) and frozen. Glucose was analyzed by the Amplex red glucose kit (Invitrogen) using a Stratagene Mx3005P (La Jolla, CA) system in plate reader mode. Gluconic acid was measured by ion chromatography on a Dionex ICS-2000 (Sunnyvale, CA) with an AS11HC column in gradient mode. Trace metal and cation samples were filtered with a Millex-GP 0.22- $\mu\text{m}$  filter (Millipore) and stored in 1% hydrochloric acid at 4°C. Samples were analyzed by inductively coupled plasma mass spectrometry with a high-resolution Axiom single collector instrument (21). Arsenic species, i.e., As(III) and As(V),

were determined by hydride generation atomic fluorescence spectrometry (32).

The amount of phosphate remaining in the apatite was not examined because only a small percentage of the apatite was solubilized and it would not be possible to detect these small variations. Previous microcosm experiments with phosphate-limited AGW and basalt or granite have shown that little to no cell growth occurs in the absence of a mineral phosphate source and that apatite was most likely the source of phosphate (47, 48). Plating and flow cytometer analyses during the initial experiments indicated that without phosphate or apatite, significantly less growth occurred. However, the fine-grained apatite in the microcosms made flow cytometer counting and plate counting difficult. Therefore, the mineral solids at the end of one round of incubation were examined by scanning electron microscopy (SEM) to better understand microbe-mineral interactions (7). Samples were prepared inside an anaerobic glove bag. To prepare samples for SEM observations, cell-mineral suspensions were fixed in 2.5% glutaraldehyde in a 0.05 M Na cacodylate buffer solution of pH 7. One droplet of fixed cell-mineral gel was placed on the surface of a glass coverslip that was cleaned with 1 mg/ml polylysine solution prior to use. The sample was allowed to settle down onto the coverslip for 20 min. The sample-coated coverslips were sequentially dehydrated using various proportions of ethanol and distilled water, followed by critical point drying (7). The coverslips were mounted onto SEM stubs and Au coated for observations with a Zeiss Supra 35 VP FEG scanning electron microscope. The only time that the samples were exposed to air was during critical point drying and Au coating. The scanning electron microscope was operated at the accelerating voltage of 15 kV. Short working distance (6 to 10 mm) and low beam current (30 to 40 mA) were used to achieve maximum image resolution.

MPS phenotype plates were prepared in two layers (10). The bottom layer consisted of 10 ml of 1% purified agar. The top layer consisted of a nutrient medium with 2% purified agar, 1% glucose, 0.1%  $\text{MgSO}_4$  (0.21%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ), 0.1%  $\text{NaCl}$ , and 0.5%  $\text{NH}_4\text{Cl}$ . After being autoclaved, 0.5 g sterile hydroxyapatite (Sigma), vitamins, and minerals were added to the top layer of media. The hydroxyapatite was washed 10 times in deionized water to remove sorbed phosphate. The vitamin and mineral mix was altered (23), and no phosphate was present in the MPS phenotype plates except for in the solid-phase hydroxyapatite. Plates were inoculated with cells and incubated aerobically at 30°C. When poured, the plate contents are cloudy because of the presence of solid-phase hydroxyapatite in the agar. The only mechanism causing the plates to become clear is the solubilization of hydroxyapatite. Previous studies have shown that clearings on the plates occur only in the presence of the MPS phenotype (10).

Four sediment samples and six water samples from Bangladesh were tested for the presence of the MPS phenotype. All water and sediment samples were from Arahazar Upazila, located 30 km east of Dhaka in a transition zone between the uplifted, low-arsenic Pleistocene Madhupur terrace to the northwest and a high-arsenic region on the opposite bank of the Meghna River to the southeast (50). Groundwater arsenic concentrations in the region range from less than 5  $\mu\text{g/liter}$  to 900  $\mu\text{g/liter}$  (43). Sediments were extracted by combining 10 g

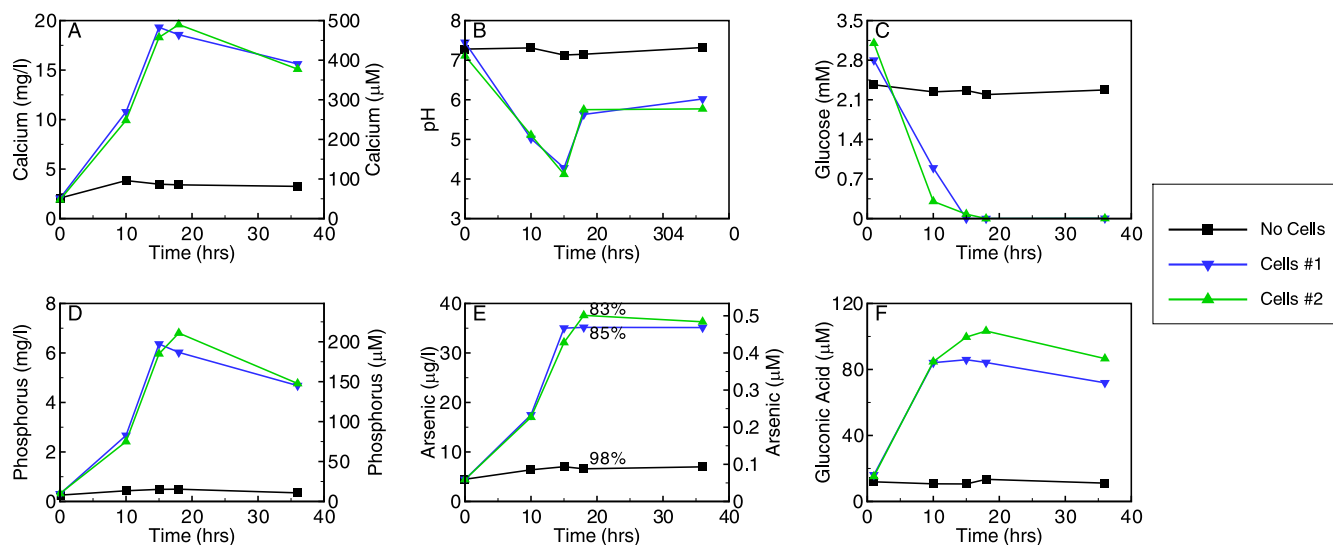


FIG. 1. Change in concentrations over time during microcosm experiments examining arsenic release from apatite. Results are shown for two duplicate microcosms and for the no-cell control. The killed control is shown in Fig. 4, and the killed control and no-cell control were always nearly identical. (A) Change in calcium concentrations over time. (B) Change in pH over time. (C) Change in glucose concentrations over time. (D) Change in phosphorus concentrations over time. (E) Change in arsenic concentrations over time. The proportion of arsenate As(V) relative to As(V) plus As(III) is indicated at the fourth time point and is presented as a percentage. The arsenic was released as oxidized arsenate in all incubations tested, with an average of  $87\% \pm 5\%$  As(V) and  $13\% \pm 5\%$  As(III) in the aqueous phases of the microcosms. (F) Change in gluconic acid concentrations over time.

of sediment with 20 ml of AGW augmented with 1% Tween 80 and shaken overnight at  $30^{\circ}\text{C}$  (3). The sediment was allowed to settle by gravity, two volumes were plated, 50  $\mu\text{l}$  and 100  $\mu\text{l}$ , and the plates were sealed with Parafilm and incubated at  $30^{\circ}\text{C}$ . Collecting microbially pristine sediment samples from Bangladesh aquifers is difficult and requires novel techniques (33, 41) compared to those traditionally utilized (16, 29). Traditional drilling techniques are not available, and extensive work has been undertaken with the local drillers to obtain pristine samples that are not contaminated by drilling fluid (33, 41). Briefly, during drilling a core or evacuated cylinder is lowered through the drill casing to collect sediment from below the casing. Sediment is therefore collected below the casing from undisturbed sediment. The sediment was stored anaerobically at room temperature until use. These sediments are considered to be pristine with unaltered microbial populations and are our best method for assessing in situ microbial processes (42).

Groundwater samples obtained by sterile techniques were also examined. Groundwater from six wells was concentrated by ultrafiltration (15), and the concentrated retentate was utilized to inoculate plates. Briefly, 100 liters of water was concentrated to 250 ml using a Rexeed 25S dialysis filter (Dial Medical Supply, Chester Springs, PA), and 100  $\mu\text{l}$  of this retentate was used to inoculate the plates. The plates were then stored at room temperature in the dark, and growth was observed over the first week. This method was performed with a sterile technique at the wellhead in Bangladesh and greatly decreased the chance of having problems with contamination or long-term storage. Standard MPS plates plus three controls were included in the experiment. The four-plate procedure included (i) standard MPS plates, (ii) MPS plates with no phosphate and no hydroxyapatite, (iii) MPS plates with phos-

phate but no hydroxyapatite, and (iv) MPS plates with phosphate and hydroxyapatite. The goal of using the additional plates was to better understand under what conditions hydroxyapatite is mobilized and further constrain the phenotype in Bangladesh. Good photographs of the plates could not be obtained at the field clinic, but consistent patterns were observed and reported.

Apatite,  $\text{Ca}_5(\text{PO}_4)_3(\text{F},\text{Cl},\text{OH})$ , is an abundant source of phosphate and is present in the aquifers and rivers of Bangladesh (6, 22). Dissolution of 13 purified apatite samples from the Himalayas resulted in an average arsenic concentration of 210 mg/kg (all values are 1, 3, 10, 11, 15, 24, 51, 78, 110, 200, 220, 250, and 1,800 mg/kg). These samples are not from Bangladesh but were upgradient within the drainage basin and may represent source material for the delta. These apatite samples were not used in the incubations, as we used the standard mineral, but indicate that apatite is a potential source of phosphate and also arsenic for the shallow aquifers in Bangladesh. Arsenic X-ray absorption near-edge structure analysis on beam line 11-2 at the Stanford Synchrotron Radiation Laboratory of the Ward's Scientific apatite used in the microcosms and the 51-mg/kg sample from the Himalayas indicated that both samples had approximately  $84\% \pm 6\%$  arsenate and  $16\% \pm 6\%$  arsenite. We assume that the arsenic is replacing the phosphorus in the mineral structure and not just sorbed on the surface, since washes with water do not release the arsenic from the mineral.

Aqueous samples were collected from a series of microcosm incubations over time and analyzed. Control incubations with killed cells and without cells did not release phosphorus, calcium, or arsenic, and glucose levels remained unchanged (Fig. 1). In live incubations, phosphorus and calcium concentrations increased over time, indicating dissolution of apatite, and glu-

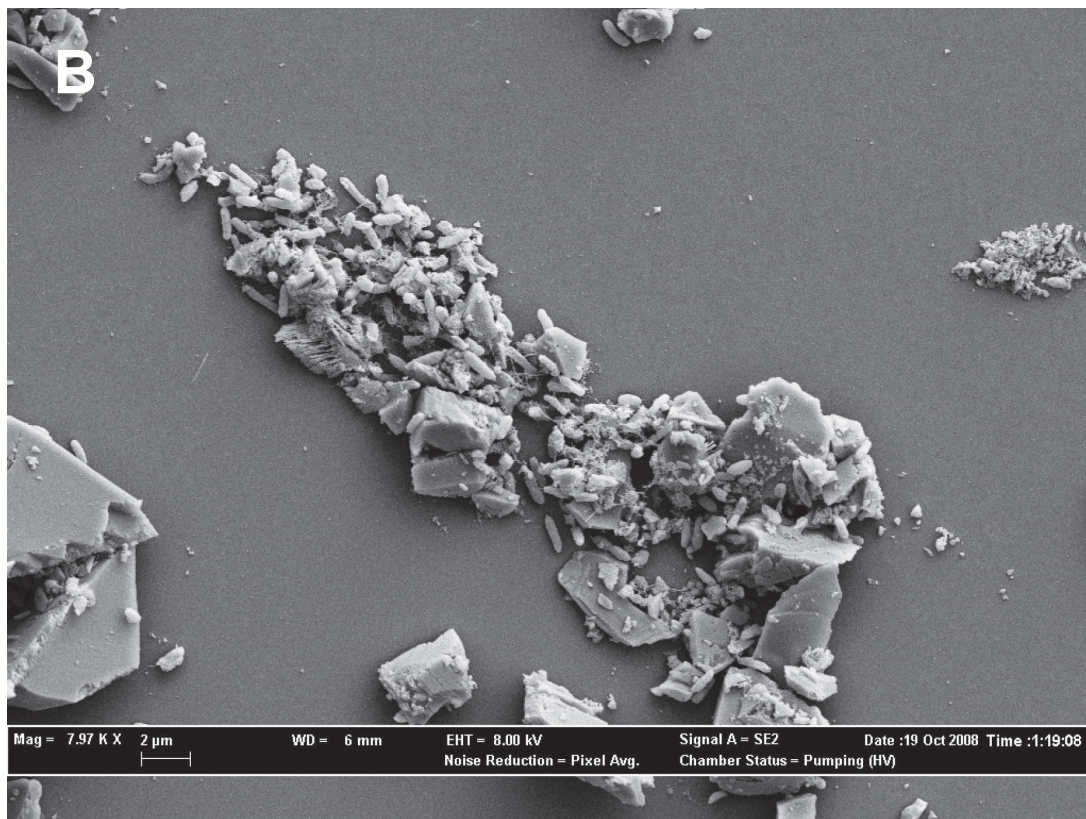
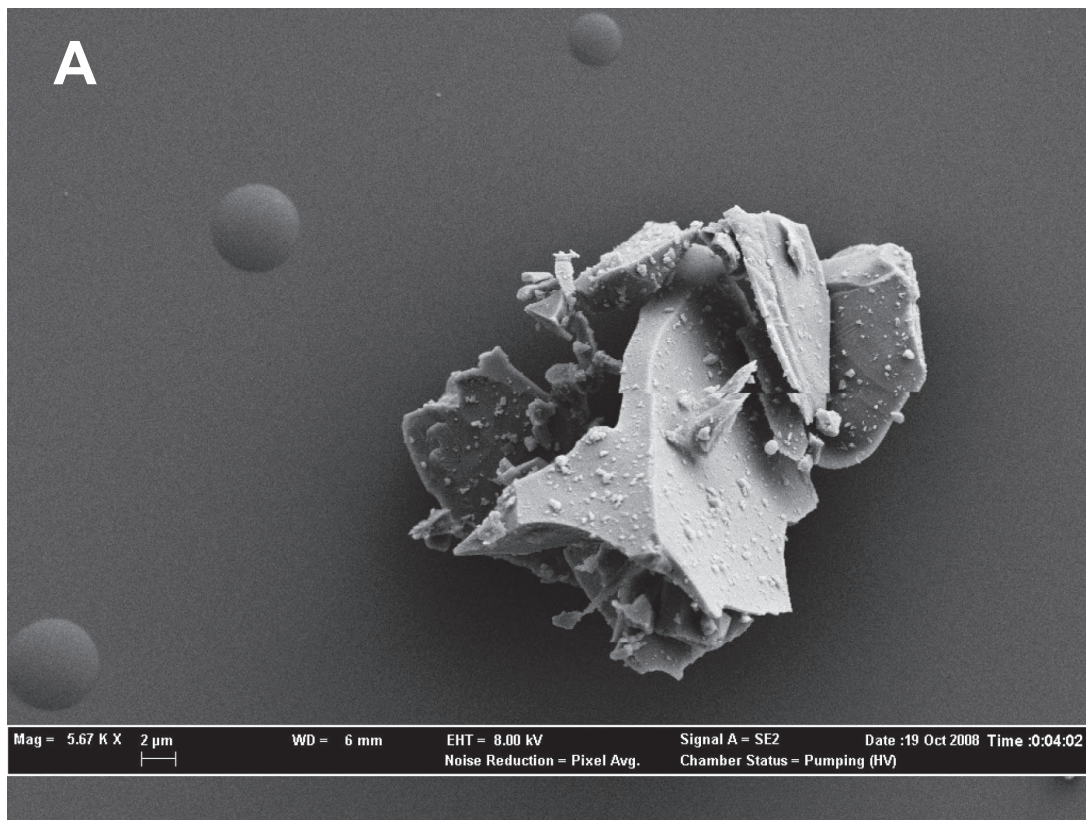


FIG. 2. Secondary electron images from microcosm sediments at the end of 24 h of incubation. (A) Image of apatite grains from the killed control. Microbes were not observed in association with the minerals, and the minerals appear unaltered. (B) Image of apatite grains from a live microcosm amended with *B. fungorum* and glucose. Rod-shaped cells are observed in close contact with the apatite minerals, and dissolution features are present on the minerals.

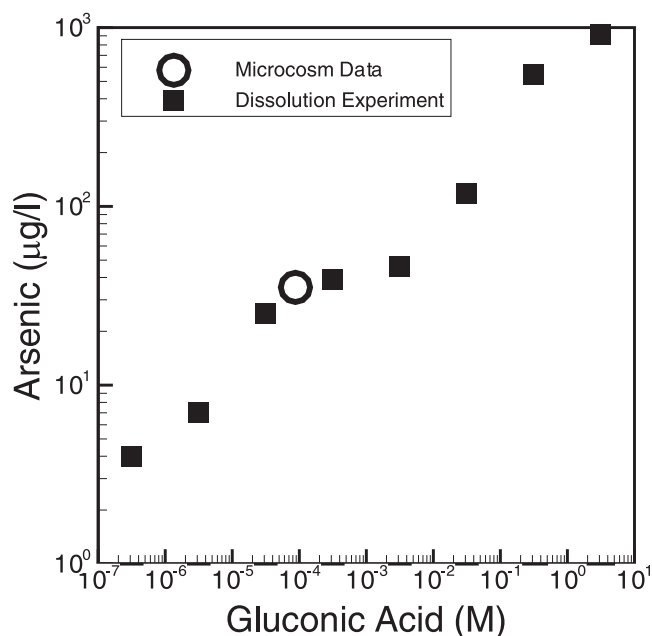


FIG. 3. Gluconic acid concentration versus arsenic concentration from abiotic experiments, along with the average gluconic acid and arsenic concentrations from the live microcosms in Fig. 1. A log-log relationship was observed between gluconic acid concentrations and the amount of arsenic released from the apatite. In addition, the amount of arsenic released during microcosm experiments was consistent with that of the abiotic experiments.

cose levels decreased, indicating microbial respiration (Fig. 1). More phosphorus is released than can be consumed by the bacteria, and the concentrations increase over time. More importantly, arsenic levels systematically increased with calcium and phosphorus from 4 to 35 µg/liter arsenic. The increase was consistent in duplicate bottles and experiments (Fig. 1). The arsenic was released as oxidized arsenate in all incubations tested, with an average of 87% ± 5% As(V) and 13% ± 5% As(III) in the aqueous phases of the microcosms (Fig. 1E). The percentages of As(V) and As(III) in the aqueous phase were nearly identical to those in the mineral. Therefore, no arsenic redox chemistry was occurring. This indicates that microbes catalyzed the release of arsenic from the apatite. We hypothesize that microbes preferentially weather minerals for nutrient acquisition, and during this process, arsenic contained in the mineral will be released into the water.

SEM was performed to better understand mineral dissolution and potential microbe-mineral interactions. In the killed control (Fig. 2A), apatite grains are observed with no cells and no dissolution features. In the live incubations with cells, glucose, and apatite, there is an intimate relationship between the apatite minerals and the cells (Fig. 2B). Cells are observed on the surfaces of the apatite, and dissolution textures are present on the surfaces of the apatite. These observations are consistent with the idea that apatite was weathered by *B. fungorum* and that during this weathering process, arsenic was released.

Further measurements and experiments were conducted to better understand the mechanisms of apatite dissolution and arsenic release (Fig. 3). Control incubations with killed cells and without cells did not show a change in pH over the course

of the incubations (Fig. 1 and 4). In live incubations, the pH level systematically decreased (Fig. 1). In the no-cell control, gluconic acid levels were constant, whereas in the live incubations, gluconic acid levels increased (Fig. 1E). The production of gluconic acid from glucose during respiration has previously been observed with *Burkholderia fungorum* (11, 46, 48). Gluconic acid is typically further oxidized by bacteria but can accumulate under stress (12). In order to compare microbial metabolism to abiotic controls, apatite was shaken for 24 h at 30°C in increasing strengths of gluconic acid (Fig. 3). The amount of arsenic release was approximately linear on a log-log scale of gluconic acid and arsenic concentrations. In addition, the amount of arsenic released during the live microcosms due to microbial production of gluconic acid was consistent with the abiotic experiments (Fig. 3). This indicates that microbial oxidative degradation of glucose to gluconic acid by microbes in intimate contact with apatite is a likely cause of arsenic release from the mineral structure into the water.

Additional incubations were conducted to examine the effect of increasing initial arsenic and phosphate concentrations and to better constrain field observations. The amount of arsenic released as a function of initial arsenic concentration was examined because field observations indicate that the release of arsenic occurs at a constant rate and is independent of arsenic concentration (39). Results indicate that during ancillary arsenic release, the amount of arsenic released is independent of the initial arsenic concentration (Fig. 4A). In addition, the pH decreases consistently at all arsenic levels consistent with the mechanism of gluconic acid production (Fig. 4B). We examined the amount of arsenic released as a function of phosphate concentrations to determine if release occurs under field-relevant conditions. The amount of arsenic released decreases as the initial aqueous phosphate concentration increases above 0.1 mM (Fig. 4C and E). The arsenic is released when the pH decreases but remains steady when 10 and 100 mM phosphate are present. The limiting phosphate concentrations are higher than concentrations observed in Bangladesh groundwater (Fig. 4E). Microbially mediated arsenic release from a commonly occurring mineral was documented in the presence of increasing arsenic concentrations and at in situ phosphate levels, indicating that the mechanism is consistent with field observations.

In separate experiments, we checked for the presence of the MPS phenotype in bacteria collected from sediments of shallow Bangladesh aquifers. Cells extracted from Bangladesh sediment were incubated on MPS phenotype plates that contained only solid-phase phosphate. Each sample gave rise to colonies surrounded by easily observed clearings (Fig. 5). These clearings can be present only if the hydroxyapatite is solubilized. Plates had, on average, about 5 to 10 colonies with clearings, which corresponds to about 100 to 200 colonies per gram. After performing initial sediment incubations, we returned to Bangladesh and incubated MPS plates with groundwater that was concentrated by ultrafiltration using sterile techniques. Four types of plates were incubated as follows: (i) standard MPS plates, (ii) MPS plates with no phosphate and no hydroxyapatite, (iii) MPS plates with phosphate but no hydroxyapatite, and (iv) MPS plates with phosphate and hydroxyapatite. A consistent pattern emerged with the plates (data not

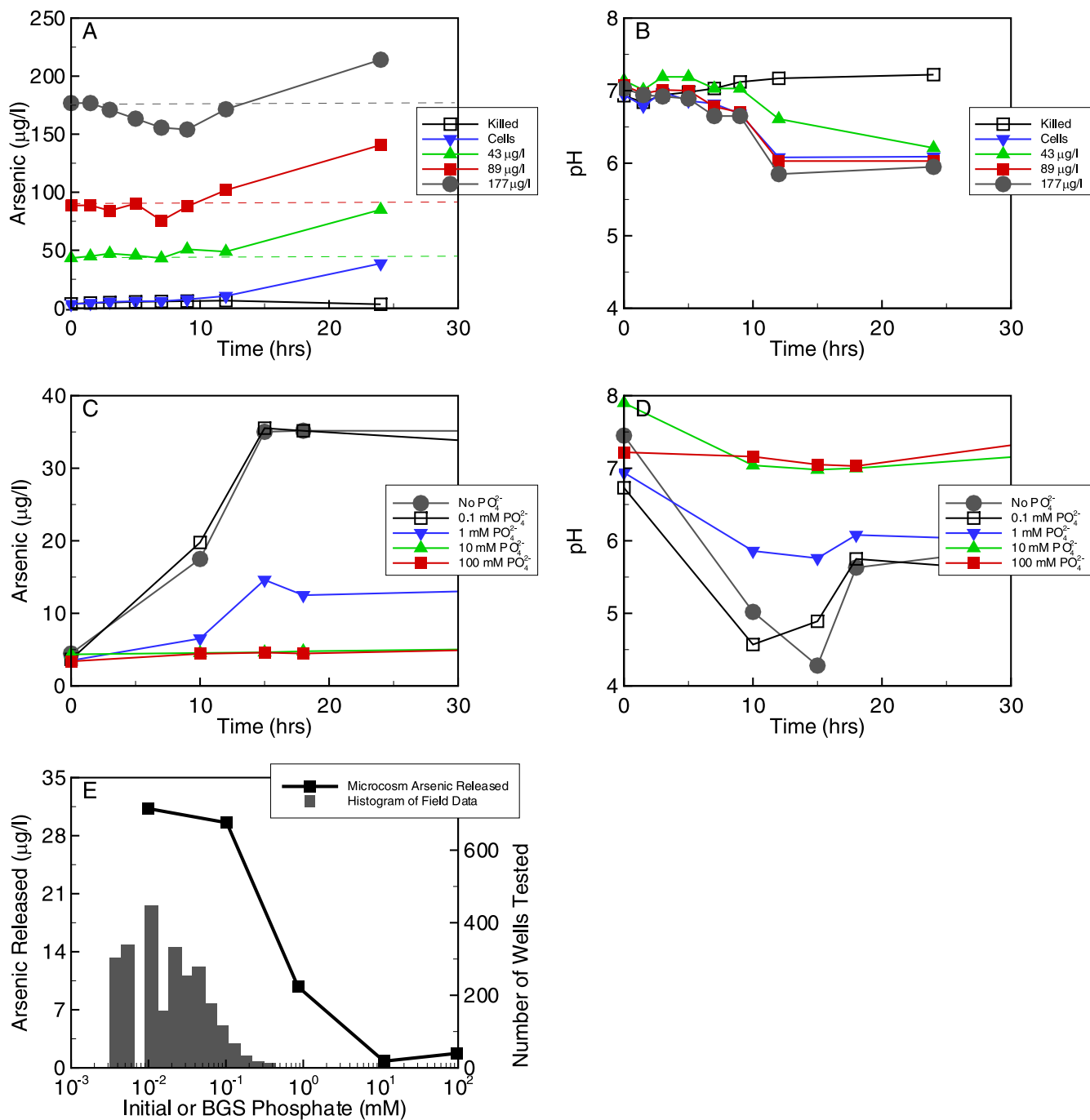


FIG. 4. (A) Release of arsenic over time at various initial arsenic concentrations. These experiments were conducted to determine if the release of arsenic occurs at all initial arsenic concentrations, as observed in the field. Microcosms were spiked with arsenate. The killed control is shown for reference, which did not release arsenic. (B) Change in pH over time for the arsenic spike experiments. (C) Change in concentration of arsenic over time at different initial phosphate concentrations. All microcosms contained *B. fungorum*, and the phosphate was spiked at pH 7. (D) pH changes during the phosphate spike experiments. (E) Release of arsenic as a function of initial phosphate concentrations. The gray bars indicate the distribution of detectable phosphate levels observed in Bangladesh aquifers, as determined by the British Geological Survey (BGS) (22).

shown). The standard MPS plates had colonies and abundant clearings (~100 colonies). The MPS plates with no phosphate and no hydroxyapatite had colonies but significantly fewer colonies (~20), indicating that phosphate may have been limited. The MPS plates with phosphate but no hydroxyapatite had significant colonies (~100). The MPS plates with phosphate

and hydroxyapatite had colonies (~100) but no clearings, indicating that cells were using the soluble phosphate. When phosphate was present in either the solid or aqueous phase, significantly more colonies were observed. This would correspond to about 3 colonies per ml of groundwater. When only solid-phase phosphate as hydroxyapatite was present, clearings

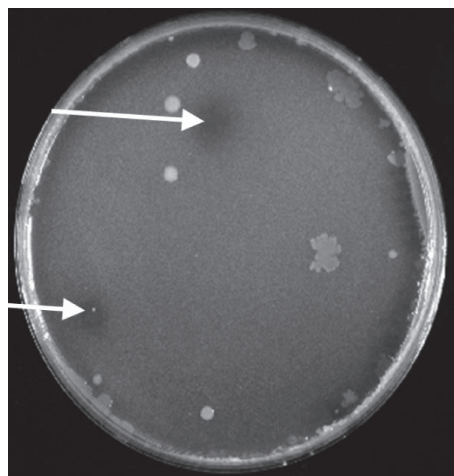


FIG. 5. MPS phenotype plate containing only solid-phase phosphate as hydroxyapatite was incubated with cells extracted from shallow Bangladesh sediment. The clearings are highlighted by arrows and indicate that phosphate-solubilizing bacteria were extracted from the sediment and are present in the shallow aquifers of Bangladesh. Small clearings are observed around many of the other colonies but do not appear in the photographs.

were observed. This was corroborating evidence that the MPS phenotype is present in Bangladesh, and under low phosphate conditions, hydroxyapatite is solubilized. This indicates that bacteria capable of solubilizing phosphate from solid-phase hydroxyapatite are present in the shallow aquifers of Bangladesh.

We hypothesize that the required conditions for ancillary arsenic release during microbial mineral weathering appear to include a nutrient limitation, the presence of poorly weathered minerals such as apatite, a supply of organic carbon, and an active microbial population. Each of these conditions is present for Bangladesh aquifers. The MPS phenotype is present in Bangladesh, and the in situ phosphate levels should promote its expression and, hence, mineral weathering with the associated arsenic release. Bangladesh aquifers are organic carbon rich, and organic carbon has been correlated with arsenic (26). Ancillary arsenic release may also occur during weathering of feldspars, biotite, and hornblende. These minerals are common in Bangladesh aquifers (6, 31, 38), and they have been shown to be potential sites of microbial weathering (5, 36, 37, 40).

We postulate that nutrient acquisition and ancillary arsenic release are independent of redox conditions and will also occur during fermentation, Fe(III) reduction, and methanogenesis. Importantly, we believe that ancillary arsenic release has been obscured by the overprint of iron cycling. Laboratory and field experiments are showing that Fe and As release are decoupled (17, 33, 42) and that arsenic release occurs at a constant rate across various depositional environments (39). These observations are consistent with our novel mechanism that can occur under all redox conditions. Therefore, we consider ancillary arsenic release to potentially be a more important factor than Fe(III) reduction for controlling arsenic release from the minerals into the groundwater of Bangladesh. This novel mechanism may clarify some of the unexplained geochemical patterns

related to the presence of arsenic in groundwater and its worldwide impact on human health.

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