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Morphological and Metabolic Responses to Starvation by the Dissimilatory Metal-Reducing Bacterium *Shewanella alga* BrY

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The response of the dissimilatory metal-reducing bacterium *Shewanella alga* BrY to carbon and nitrogen starvation was examined. Starvation resulted in a gradual decrease in the mean cell volume from 0.48 to 0.2 μm^3 and a dramatic decrease in Fe(III) reductase activity. Growth of starved cultures was initiated with O_2, ferric oxyhydroxide, Co(III)-EDTA, or Fe(III)-bearing subsurface materials as the sole electron acceptor. Microbially reduced subsurface materials reduced CrO_4^{2-}. Starvation of dissimilatory metal-reducing bacteria may provide a means of delivering this metabolism to contaminated subsurface environments for in situ bioremediation.

Contamination of subsurface environments with radionuclides, metals, aromatic hydrocarbons, and/or halogenated solvents poses a global threat to the quality of our drinking water. Traditional remediation efforts in contaminated environments have relied on expensive pump-and-treat methods. An attractive alternative technology may be in situ remediation, in which contaminants are degraded either chemically or biologically by "redox reactive walls" (46, 57, 58).

The metabolism of dissimilatory metal-reducing bacteria (DMRB) may provide a mechanism for creating permeable bioremediation barriers in contaminated subsurface environments. DMRB gain energy to support anaerobic growth by coupling the oxidation of H_2 or organic matter to the reduction of a variety of multivalent metals (32). This metabolism can lead to the complete mineralization of organic contaminants or to the precipitation and immobilization of metal contaminants under anaerobic conditions. For example, the mineralization of aromatic hydrocarbon contaminants in a shallow aquifer located in Bemidji, Minn., was mediated by the metabolism of DMRB (3, 33). Microbial U(VI) and Co(III)-EDTA reduction have been recognized as a means of immobilizing radioactive uranium (9, 36) or cobalt (10) isotopes and thus represent a potential method for the bioremediation of environments contaminated with these radionuclides. The fate of halogenated organic contaminants in sediments, soils, and aquifers may also be influenced by DMRB because microbially reduced iron minerals have been used to reductively dehalogenate carbon tetrachloride (22), monochlorinated aromatic compounds (24, 27), and nitroaromatic compounds (23).

In situ bioremediation strategies using DMRB would rely on either stimulating naturally occurring DMRB populations or inoculating preadapted or genetically engineered DMRB into contaminated environments. Recent studies have demonstrated that the mineralization of aromatic hydrocarbons in a contaminated aquifer by indigenous populations of DMRB could be stimulated by the addition of synthetic iron chelators (37–39) or natural humic acids (39). However, it has also been shown that the addition of specifically adapted bacteria, or genetically engineered bacteria, can enhance the degradation rates of organic contaminants (12, 15, 19, 25, 54, 59). The primary limitation of this latter approach is the ability to effectively deliver bacterial inocula to subsurface contaminant plumes, since vegetative bacteria tend to bind rapidly to the substratum and are thus rarely found far downstream from injection wells (50).

Starvation can provide a means of preparing bacterial inocula for in situ bioremediation (29, 30). In order to survive the oligotrophic conditions of certain natural environments, many non-spore-forming bacteria are thought to exhibit a starvation-survival response, under which cell size and susceptibility to harsh conditions are reduced drastically (1, 16, 26, 28, 48, 56, 59). Although these cells are metabolically dormant, they maintain their full genetic capacity (7, 8, 41, 44, 51) and they are resuscitated to the vegetative state when exposed to nutrients. The use of starved cells in bioremediation scenarios may offer a unique advantage, as the reduced cell size may facilitate transport of bacteria through the substratum (20, 30). Starvation of a subsurface isolate resulted in a decrease in cell size and in a greater penetration of the starved isolate into sandstone cores than was found for vegetative cells (18, 31, 40). Starvation has also been used to prepare a bacterial inoculum, which, upon resuscitation, created a biobarrier that was impermeable to aqueous contaminants (6, 8). Similar scenarios have been used to selectively plug oil reservoirs for secondary oil recovery (8, 13, 14).

The purpose of this study was to examine how a model DMRB, *Shewanella alga* BrY, responds to starvation. We are examining the feasibility of using starvation as a means of preparing DMRB inocula which would be introduced into contaminated subsurface environments, readily delivered to a contaminant plume, and then resuscitated and grown on the contaminants in situ.

Preparation of carbon- and nitrogen-starved cells. *S. alga* BrY (9, 47) is an obligately respiratory, facultatively anaerobic bacterium that was isolated from anaerobic sediments of the Great Bay estuary, New Hampshire. It can grow anaerobically by coupling the oxidation of organic acids or H_2 to the dissimilatory reduction of Fe(III), Mn(VI), or Co(III)-EDTA (9). Triplicate cultures of *S. alga* BrY were grown aerobically in 100 ml of tryptic soy broth (30 g/liter) at 25°C on a rotary shaker at 150 rpm for 15 h. Cells were harvested by centrifugation

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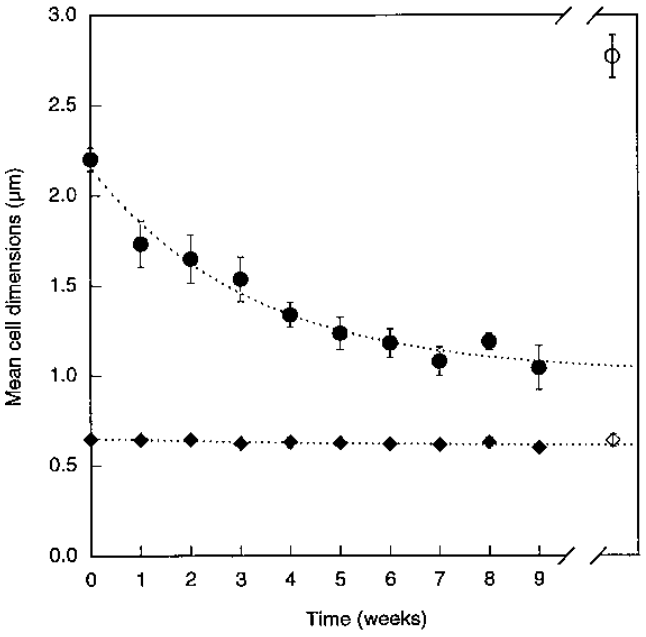

FIG. 1. Mean cell length (●) and mean cell width (◆) of starved *S. alga* BrY cells and mean cell length (○) and mean cell width (◇) of resuscitated *S. alga* BrY cells after 30 h of aerobic growth. Correlation coefficients for the shown curves were 0.74 and 0.97, respectively (see text). Error bars for figures represent the 95% confidence intervals.

(6,000 × *g*, 4°C, 30 min) during the late exponential/early stationary growth phase and washed twice in sterile phosphate-buffered saline (PBS) (pH 7.0) (31). Optimal Fe(III) reductase activity is expressed at this stage of growth (21). Washed cells of each culture were resuspended in 100 ml of sterile PBS. The cell suspensions were stirred aseptically with a magnetic stir bar at room temperature for 9 weeks. Cell viability was determined by inoculating serially diluted samples onto tryptic soy agar plates (30 g/liter). Serial dilutions were made in sterile PBS. Viable plate counts were determined after 1 to 5 days of incubation at 30°C. The optical density of the starving cell suspensions was determined by absorbance at 600 nm.

Cell size determination. Samples (0.1 ml) of the starving cell suspensions or of aerobically resuscitated cultures were removed at various times and fixed in 4.9 ml of glutaraldehyde (2.5% final concentration). A subsample (1 ml) of the fixed cells was concentrated by centrifugation (13,000 × *g*, 25°C, 10 min), and the cell pellet was resuspended in 0.1 ml of sterile PBS. Resuspended samples were mildly sonicated in an FS5 sonicator (Fisher Scientific, Pittsburgh, Pa.) for 2 min to break apart cell clumps. The morphological integrity of the sonicated cells was confirmed by phase-contrast microscopy. An aliquot (20 μl) of the sonicated cell suspension was placed on a gelatin-coated slide (0.075% gelatin), allowed to air dry, stained with acridine orange (0.02%), and washed with sterile distilled H_2O. An Axioskop epifluorescence microscope (Zeiss, Oberkochen, Germany) with a 100× plan NEOFLUAR objective and the Zeiss filterset 10 (blue excitation, green emission) equipped with a Zeiss camera were used to obtain digital images for subsequent analysis. The images were acquired by the program NIH Image (version 1.57) which was also used for the subsequent quantitative analysis of average cell morphology.

Image analysis. Digital image analysis was used to obtain exact quantitative information about changes in average cell morphology. The cells were detected by a two-step procedure as described previously (45).

Three pictures of each of the three batch cultures were analyzed at all time points. Only focused, clearly delineated, isolated cells larger than 0.225 μm^2 (corresponding to 10 pixels) were analyzed. Each picture contained from 20 to 600 such cells, so that a total of 6,869 individual cells were measured in 99 pictures.

Growth induction of starved cells. Aerobic resuscitation experiments were conducted in 100-ml aliquots of tryptic soy broth medium (30 g/liter), at 25°C on a rotary shaker at 150 rpm. Anaerobic resuscitation experiments were conducted in anaerobic basal medium. The anaerobic basal medium contained (in grams per liter of deionized H_2O): $NaHCO_3$, 2.5; NH_4Cl, 1.5; KH_2PO_4, 0.6; KCl, 0.1; vitamins, 10 ml, and trace minerals, 10 ml (4, 35); and sodium lactate, 20 mM. Standard anaerobic techniques were used throughout the anoxic resuscitation regimen (4, 5, 8). All anoxic media were boiled and cooled under a constant stream of 80% N_2–20% CO_2, dispensed into aluminum-sealed culture tubes under the same gas phase, capped with butyl rubber stoppers, and sterilized by autoclaving (121°C, 20 min.). Additions to sterile media, inoculation, and sampling were done using sterile syringes and needles (5). All incubations were at room temperature in the dark. All resuscitation experiments were conducted with a 10% (vol/vol) inoculum of the starved cell suspension (giving a final concentration of $1.1 \pm 0.09 \times 10^6$ CFU ml^{-1}). All experiments were performed in triplicate.

The basal medium was modified to test for resuscitation with different electron acceptors. Amorphous ferric oxyhydroxide was prepared as described previously (35) and provided at a concentration of ca. 100 mM. Co(III)-EDTA was prepared as described previously (10, 17) and provided at a concentration of 1 mM. The iron-bearing subsurface material was obtained from Hanford, Wash. The material was sieved, and all fractions of <500 μm were combined. A fraction of this material (1.0 g) was added to 9 ml of basal medium, giving a final Fe(III) concentration of 1.92 ± 0.52 mM. Negative controls for resuscitation with different electron acceptors did not contain lactate.

Analytical techniques. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time. The amount of Fe(II) solubilized after 15 min in 0.5 N HCl was determined with ferrozine as previously described (35). The concentration of Fe(III) was determined with hydroxlyamine hydrochloride extraction as described previously (34). Co(III)-EDTA reduction was determined spectrophotometrically by the decrease in absorbance at 535 nm. Chromate was analyzed by the *sym*-diphenylcarbazide method (55). Fe(III) reductase activity was determined in 10 ml of anaerobic PBS buffer with H_2 as the electron donor (headspace of 100% H_2) and ferric pyrophosphate (0.5 mM) (Pfaltz and Bauer, Inc., Waterbury, Conn.) as the electron acceptor by end-point analysis after 5 min of incubation at room temperature.

Metabolic responses to starvation. Non-spore-forming bacteria typically respond to starvation by exhibiting a reduction in cell size and decreasing endogenous respiration rate while still maintaining an intact genome (29). Starved bacterial forms have been called ultramicrobacteria (53), dwarf cells (28), or filterable bacteria (2). A proportion of the starved population survives and can be resuscitated to prestarvation cell physiology when nutrients are supplied.

Culture absorbance, cell viability, and Fe(III) reductase activity were measured weekly during the 9-week starvation regimen of *S. alga* BrY cell suspensions. The absorbance at 600 nm gradually decreased from 1.95 to 0.213, and the viable cell

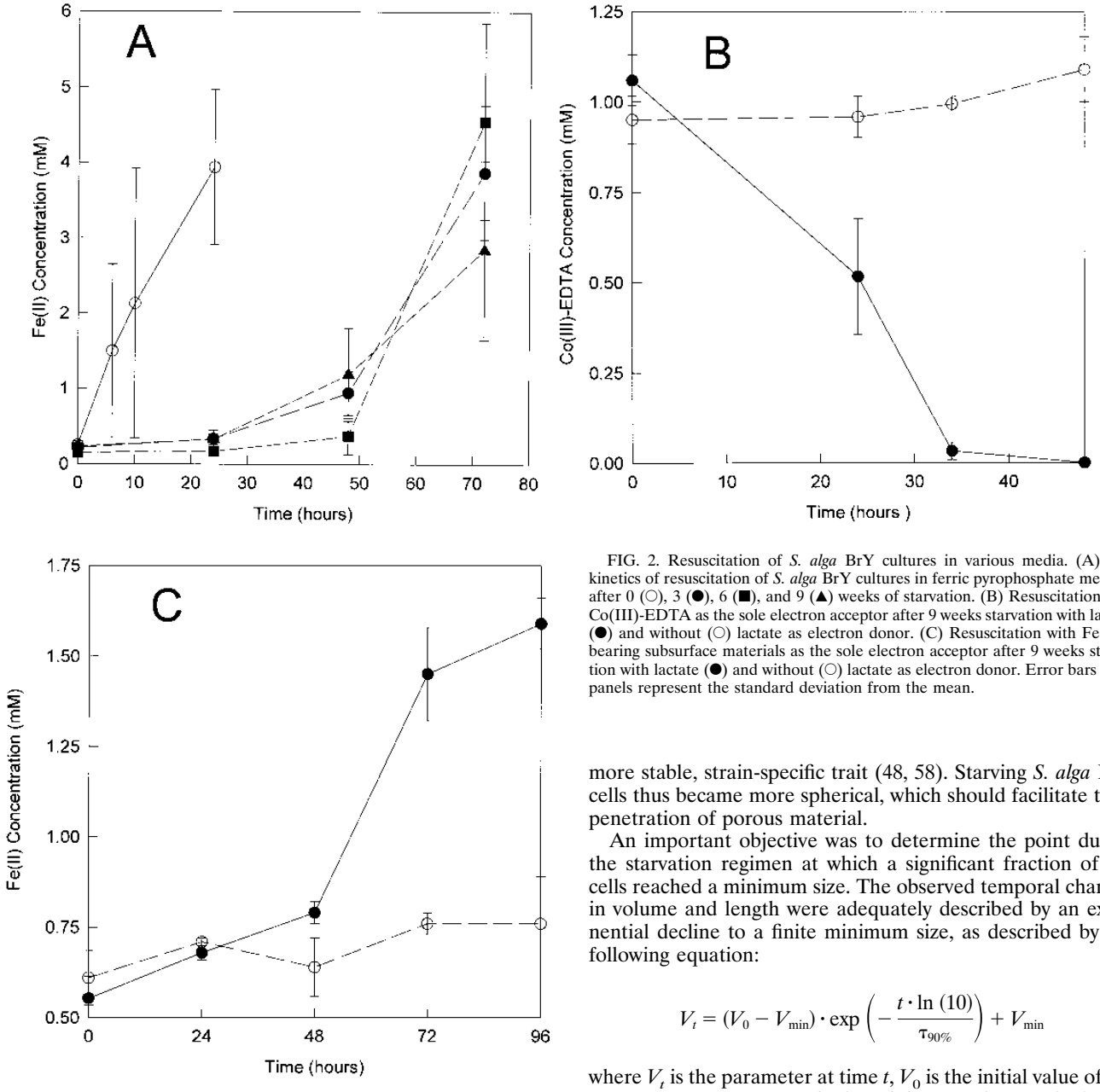

FIG. 2. Resuscitation of *S. alga* BrY cultures in various media. (A) The kinetics of resuscitation of *S. alga* BrY cultures in ferric pyrophosphate medium after 0 (○), 3 (●), 6 (■), and 9 (▲) weeks of starvation. (B) Resuscitation with Co(III)-EDTA as the sole electron acceptor after 9 weeks starvation with lactate (●) and without (○) lactate as electron donor. (C) Resuscitation with Fe(III)-bearing subsurface materials as the sole electron acceptor after 9 weeks starvation with lactate (●) and without (○) lactate as electron donor. Error bars in all panels represent the standard deviation from the mean.

count decreased from 7.6×10^9 to 1.1×10^7 CFU ml^{-1}. The Fe(III) reductase activity decreased during the 9-week starvation regimen from $8.53 \pm 0.28 \times 10^{-7}$ nmol Fe(II) min^{-1} CFU^{-1} to below detection limits after 5 weeks of starvation and remained so for the remainder of the starvation regimen.

Morphological responses to starvation. Starvation of *S. alga* BrY also resulted in a gradual decrease in cell size during the 9-week starvation regimen. The mean cell volume decreased over time from 0.48 ± 0.03 to 0.20 ± 0.03 μm^3. This decrease in volume is attributed to a decrease in the mean length of the starving *S. alga* BrY cells from 2.20 ± 0.07 to 1.04 ± 0.12 μm, whereas the mean width of these cells remained constant (0.63 ± 0.02 μm) over time (Fig. 1). This is in agreement with previous observations that cell length is highly variable depending on growth conditions, whereas the cell diameter is a more stable, strain-specific trait (48, 58). Starving *S. alga* BrY cells thus became more spherical, which should facilitate their penetration of porous material.

An important objective was to determine the point during the starvation regimen at which a significant fraction of the cells reached a minimum size. The observed temporal changes in volume and length were adequately described by an exponential decline to a finite minimum size, as described by the following equation:

$$V_t = (V_0 - V_{\min}) \cdot \exp\left(-\frac{t \cdot \ln(10)}{\tau_{90\%}}\right) + V_{\min}$$

where V_t is the parameter at time t, V_0 is the initial value of the parameter at $t = 0$, $V_{\min}$ is the minimum value reached after prolonged starvation, and $\tau_{90\%}$ is the time after which 90% of the parameter change has happened. For the average cell volume we found that $V_0 = 0.47$ μm^3, $V_{\min} = 0.21$ μm^3, and $\tau_{90\%} = 7.3$ weeks, as indicated by the best fitting curve in Fig. 2A. An identical model for the change in average cell length, L_t, gave the following parameters: $L_0 = 2.15$ μm, $L_{\min} = 1.01$ μm, and $\tau_{90\%} = 7.4$ weeks. The similar 90% response times, $\tau_{90\%}$, for both parameters indicated that the cells had reached a stable starvation state when the resuscitation experiments were performed (i.e., after 9 weeks of starvation).

Induction of growth in starved cultures. Cultures of *S. alga* BrY that were starved for 9 weeks could be resuscitated and grown with O_2 as an electron acceptor when inoculated into tryptic soy broth medium. Cell growth commenced after an initial 3-h lag and exhibited typical log and stationary growth phases. The effects of starvation were reversed under these growth conditions, as cells reached a mean volume of $0.60 \pm$

0.09 μm^3 after 30 h of resuscitation, which was even greater than the mean cell volume prior to starvation (0.48 ± 0.03 μm^3). Resuscitated cells reached a mean length of 2.77 ± 0.12 μm, while the mean cell width remained 0.64 ± 0.04 μm (Fig. 1). Resuscitated cells exhibited a Fe(III) reductase activity of $13.83 \pm 4.43 \times 10^{-7}$ nmol Fe(II) min^{-1} CFU^{-1}, which exceeded the Fe(III) reductase activity of the original vegetative inoculum. These results demonstrate that a DMRB can be starved to produce small cells which fully maintain their capacity to reduce Fe(III).

Starved cells of *S. alga* BrY could also be resuscitated after 9 weeks in a minimal medium with lactate as the carbon and energy source and amorphous ferric oxyhydroxide as the sole electron acceptor (data not shown). No iron reduction was observed in controls which did not contain lactate. The kinetics of resuscitation with ferric pyrophosphate as the sole electron acceptor are shown in Fig. 2A. No significant difference in the rate of resuscitation was observed in cultures resuscitated after 3, 6, or 9 weeks of starvation.

The ability of starved cells of *S. alga* BrY to grow by directly reducing a potential subsurface contaminant was demonstrated by resuscitation with Co(III)-EDTA as a sole terminal electron acceptor (Fig. 2B). The concentration of Co(III)-EDTA decreased over time when lactate was added as an electron donor. There was no Co(III)-EDTA reduction in controls which did not contain lactate. $^{60}Co(III)$ is a nuclear byproduct that is often codisposed with the synthetic chelator EDTA. The Co(III)-EDTA complex is extremely stable to chemical reduction and exchange reactions and is thus highly mobile in saturated subsurface environments. Microbial reduction of Co(III)-EDTA may limit the far-field migration of ^{60}Co in contaminated subsurface environments (10), as Co(II)-EDTA strongly adsorbs to aluminum oxides in soils and subsoils while Co(III)-EDTA does not (10).

Starved cells of *S. alga* BrY could also be resuscitated in minimal medium with Fe(III)-bearing subsurface material as the sole electron acceptor (Fig. 2C). Resuscitating cells used Fe(III) as an electron acceptor, as shown by the increase in Fe(II) concentration over time when lactate was added as an electron donor. There was no Fe(III) reduction in controls which did not contain lactate. Resuscitating cells reduced 90% of the Fe(III) present in the subsurface material within 4 days. These results suggest that a potential subsurface bioremediation scenario using starved *S. alga* BrY cells is, at least metabolically, feasible.

Chromium contamination is a common environmental problem associated with its widespread industrial use. The reduction of chromium represents a potential mechanism for reducing its toxicity and limiting its migration in contaminated subsurface environments (42, 43, 49, 52). The iron-bearing subsurface minerals that were reduced by starved *S. alga* BrY cells rapidly reduced chromate. Reduced minerals that had been killed by autoclaving prior to the addition of chromate reduced chromate at an identical rate to that of reduced minerals containing live cells. No chromate reduction was observed in sterile oxidized minerals. Although *S. alga* BrY is able to reduce chromate enzymatically (11), the kinetics of abiotic chromate reduction by Fe(II) are much faster than those of direct enzymatic reduction. Thus, we assumed that most of the chromate was reduced chemically by Fe(II) and not enzymatically. Previous studies have shown that ferrous iron is capable of rapidly reducing chromate and precipitating the reduced chromium (18). This study is the first to demonstrate that microbially produced Fe(II) can reduce chromate. These results also suggest that a living biobarrier would not have to be maintained once the groundwater and solid materials in a contaminated subsurface environment were microbially reduced.

In conclusion, this study has demonstrated that the DMRB *S. alga* BrY responds to starvation by decreasing in cell size and endogenous metabolic activity and that starved *S. alga* cells can be resuscitated with a variety of electron acceptors, including oxygen, Fe(III), Co(III)-EDTA, and natural subsurface materials. These results suggest that starvation may provide a means of delivering DMRB to contaminated subsurface environments and that the in situ resuscitation of starved DMRB cells may provide a mechanism for the bioremediation of groundwater contaminants.

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