Accelerated Biodegradation of Cement by Sulfur-Oxidizing Bacteria as a Bioassay for Evaluating Immobilization of Low-Level Radioactive Waste

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Disposal of low-level radioactive waste by immobilization in cement is being evaluated worldwide. The stability of cement in the environment may be impaired by sulfur-oxidizing bacteria that corrode the cement by producing sulfuric acid. Since this process is so slow that it is not possible to perform studies of the degradation kinetics and to test cement mixtures with increased durability, procedures that accelerate the biodegradation are required. Semicontinuous cultures of *Halothiobacillus neapolitanus* and *Thiomonas intermedia* containing thiosulfate as the sole energy source were employed to accelerate the biodegradation of cement samples. This resulted in a weight loss of up to 16% after 39 days, compared with a weight loss of 0.8% in noninoculated controls. Scanning electron microscopy of the degraded cement samples revealed deep cracks, which could be associated with the formation of low-density corrosion products in the interior of the cement. Accelerated biodegradation was also evident from the leaching rates of Ca^{2+} and Si^{2+} , the major constituents of the cement matrix, and Ca exhibited the highest rate (up to 20 times greater than the control rate) due to the reaction between free lime and the biogenic sulfuric acid. Leaching of Sr^{2+} and Cs^+ , which were added to the cement to simulate immobilization of the corresponding radioisotopes, was also monitored. In contrast to the linear leaching kinetics of calcium, silicon, and strontium, the leaching pattern of cesium produced a saturation curve similar to the control curve. Presumably, the leaching of cesium is governed by the diffusion process, whereas the leaching kinetics of the other three ions seems to be governed by dissolution of the cement.

Sulfur-oxidizing bacteria are known to be the main causal agents of the corrosion and degradation of concrete in various facilities, including sewage systems (7, 15, 19), wastewater treatment facilities (16), and cooling towers (23). These chemolithotrophs oxidize various sulfur compounds to produce sulfuric acid, which is responsible for the corrosion and degradation of the concrete. The sulfuric acid reacts with free lime [$\text{Ca}(\text{OH})_2$] in the concrete to form gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$), which produces a corroding layer on the concrete surface that penetrates into the concrete, increasing the degradation due to the large density difference between the reaction products and the concrete (1, 14). A far more destructive reaction occurs between the newly formed gypsum crystals and calcium aluminate in the concrete. This reaction leads to the production of ettringite ($3\text{CaO} \cdot \text{Al}_2\text{O}_3 \cdot 3\text{CaSO}_4 \cdot 32\text{H}_2\text{O}$), which further contributes to the degradation of the concrete by increasing the internal pressure, leading to the formation of cracks. The cracks, in turn, provide a larger surface area for corrosion processes and provide additional sites for acid penetration (6).

In contrast to the large number of reports on the role of sulfur-oxidizing bacteria in the corrosion of cement paste and concrete, very little information has been published on the possible effects of these bacteria on the concrete or cement paste used to immobilize radioactive and heavy metal wastes. Immobilization of low-level radioactive waste in cementitious

mixtures, which are buried in soil, is becoming a common practice for the disposal of short-lived isotopes, such as strontium and cesium (8). It is required that the immobilized radioactive elements not be leached out of the concrete for a period equivalent to 10 half-lives (i.e., about 300 years for the isotopes of strontium and cesium). Isolation of *Thiobacillus thiooxidans* and other sulfur-oxidizing bacteria from soils at disposal sites for low-level radioactive wastes (18) has increased awareness of possible environmental pollution by leakage of radioactive isotopes from the buried cement. Biodegradation of cement in natural environments due to exposure to microbially generated sulfuric acid is a very slow process, which may take many years, and it may therefore be difficult to evaluate the resistance of various cementitious materials to microbial corrosion. To facilitate such an evaluation, a number of experimental procedures have been developed to accelerate the natural microbial corrosion of cement induced by sulfur-oxidizing bacteria (11, 19) or by the fungus *Fusarium* (9) cultured under optimal nutritional and environmental conditions. The main drawback of these procedures is that they may require time on the order of months to determine the degradation kinetics.

In the present study, we developed a simple procedure to accelerate biodegradation of cement pastes by incubating samples of the neutrophilic sulfur-oxidizing bacteria (NSOB) *Halothiobacillus neapolitanus* and *Thiomonas intermedia* in semicontinuous culture. The biodegradation kinetics of the cement was evaluated by monitoring the concentrations of elements leached from the cementitious mixture and by measuring the gravimetric weight loss of the cement samples. Non-radioactive strontium and cesium ions were used to simulate

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the immobilized ions in cement and leakage of the corresponding radionuclides.

MATERIALS AND METHODS

Sulfur-oxidizing bacteria and growth conditions. *T. intermedia* strain ATCC 15466 and *H. neapolitanus* strain ATCC 23638 were purchased from the American Type Culture Collection. The bacteria were cultured in 250-ml flasks containing 50 ml of a mineral salts solution supplemented with thiosulfate as the sole energy source (9). The flasks were incubated on a rotary shaker at 30°C.

Preparation of cement samples. Cement specimens were prepared by mixing 1,500 g of Portland cement (PC 250; Nesher Israel Cement Enterprises, Ramla, Israel) with 450 ml of 1 M NaOH with an N-50 mixer (Hobart, Troy, Ohio). To simulate radioactive waste immobilized in the cement, the NaOH solution was supplemented with strontium and cesium ions [10 g of $\text{Sr}(\text{NO}_3)_2$ and 5 g of CsNO_3 , respectively]. The mixture was cast into a cylindrical mold and allowed to stand for 24 h. The demolded paste was then sealed in a polypropylene bag and cured at room temperature for 28 days. After curing, the specimen was cut into cubes (approximately 1 by 1 by 1 cm) with an electric saw equipped with a 35-cm diamond blade (Buehler, Lake Bluff, Ill.).

Adjustment of the pH of cement samples. The high pH of cement, which ranges from 11 to 13 (12), prevents the growth of sulfur-oxidizing bacteria in media containing cement specimens. However, exposure of cement to atmospheric CO_2 results in a carbonation reaction, which reduces the surface pH (10). In the present study, the cement paste samples were first exposed to 100% CO_2 in a sealed jar for 14 days. The pH of the carbonated cement was then evaluated by immersing three cement cubes in 100 ml of distilled water for 1 h and then determining the pH of the water. The pH obtained, pH 9.0, was further reduced by washing the samples under running tap water for 10 h and then under running distilled water for 4 h. This reduced the pH to 8.0. The pH of cultures containing the cement samples could be reduced still further to values below 8.0 by increasing the concentration of KH_2PO_4 in the culture medium.

Assay of biodegradation of cement in semicontinuous cultures of sulfur-oxidizing bacteria. An assay was performed in 500-ml flasks, each containing 100 ml of thiosulfate-mineral salts medium at pH 5.5 (adjusted by increasing the concentration of KH_2PO_4 from 0.6 to 4.0 g/liter) amended with 13 disinfected cement cubes. Disinfection of the cubes was accomplished by immersing them in 70% ethanol for 12 h and then drying them at 80°C for 72 h to allow evaporation of the residual ethanol from the cubes. Each flask was inoculated with 5 ml of a logarithmic-phase culture of *T. intermedia* or *H. neapolitanus* containing 10^8 cells/ml. The cultures were incubated at 30°C on a rotary shaker at 100 rpm. Once every 4 days, the cement samples were transferred to flasks containing fresh medium, which were subsequently inoculated as described above. After each transfer, samples of the spent medium were analyzed to determine the concentrations of ions leached out of the cement, pH, and bacterial count. After specified periods of time, a cement cube was aseptically removed from each flask for determination of changes in the dry weight of the cement. Flasks containing sterile medium and the same initial number of cement samples served as controls.

Analyses of ions in cultures of *T. intermedia* or *H. neapolitanus* amended with cement cubes. Aliquots withdrawn from the cultures were centrifuged at $11,950 \times g$ for 10 min at 4.0°C to pellet the bacterial biomass. The concentration of thiosulfate was determined by titration with a KI solution (0.005 N) with starch as the indicator. The sulfate concentration was determined with a Dionex (Sunnyvale, Calif.) ion chromatograph. Ca^{2+} , Sr^{2+} , and Si that had been leached or dissolved from cement samples were analyzed with an optical inductively coupled plasma (ICP) spectrometer (Perkin-Elmer, Wellesley, Mass.), while Cs^+ was analyzed by atomic absorption spectrometry (Spectra A+250; Varian Palo Alto, Calif.). Since the leaching of immobilized nonradioactive ions of Sr^{2+} and Cs^+ was expected to be similar to that of the radioactive isotopes, we tested only leaching of the nonradioactive ions.

Gravimetric determination of cement. Weight reduction of cement samples was used as an additional measure of the degree of biodegradation. Since cement is highly porous and has a high labile water content, the following standardized weighing procedure was used. The cement cubes were first immersed in distilled water for 24 h so that the drying procedure would start with water-saturated samples. The samples were dried in an oven at 80°C for 3 days (the time required for maximal evaporation), transferred to a chamber kept at a humidity of 100% for 24 h, and then weighed. At various times during incubation with the bacterial cultures, cement cubes were removed from the cultures for determination of weight changes. Before weighing, each cube was washed for 1.5 h in 50 ml of distilled water, then for 15 h in 2% sodium dodecyl sulfate to remove any residual bacterial biomass, and finally for 1 h in running distilled water. Prior to weighing,

FIG. 1. Culture pH of *H. neapolitanus* and *T. intermedia* amended with 10 cement cubes in a medium buffered to an initial pH of 5.5 or 6.5. The data are cumulative concentrations and are means $\pm$ standard deviations for three replicates.

the washed samples were subjected to the same procedure as the preincubated cubes.

SEM of cement samples. For scanning electron microscopy (SEM) cement samples were removed from the medium, washed with tap water to remove medium residue, and dried in a desiccator for 24 h under a vacuum. The samples were vapor fixed at room temperature for 3 days in a sealable glass container containing two beakers, one with 10 ml of 25% (vol/vol) glutaraldehyde in H_2O and the other with 5 ml of 5% OsO_4 in 0.1 M phosphate buffer (pH 7.0). After fixation, the container was aerated for 20 h. The samples were gold coated in a deep vacuum and visualized with a JSM-35CF SEM (JOEL, Tokyo, Japan).

RESULTS

Since the high alkalinity of cement paste may inhibit the growth of sulfur-oxidizing bacteria and hence reduce the rate of biodegradation, we first had to monitor the changes in pH and in the growth of *H. neapolitanus* and *T. intermedia* in the liquid cultures containing cement specimens. For 100 ml of culture containing eight carbonated and washed cement cubes, the initial pH was 6.5, but the pH increased to 11.0 after 24 h, which inhibited bacterial growth (data not shown). These findings indicated that excessive washing of the cement samples reduced only their surface alkalinity, while the internal pH of the samples remained high. To stabilize the pH of the cement-amended cultures, the concentration of KH_2PO_4 was therefore increased from 0.6 to 4.0 g/liter. In this way, the initial culture pH was reduced from the original value, pH 6.5, to 5.5, and the pH was maintained within a range from 6.2 to 6.5 during the whole experiment, which supported the growth of *H. neapolitanus* and *T. intermedia* (Fig. 1). The corrosion of the cement by the biogenic sulfuric acid was evaluated in terms of the weight lost by the cement and the concentrations of leached ions. Our working hypothesis was that optimal acceleration of the biodegradation required maximal production of sulfuric acid by the bacteria. Thus, it is important that the amount of thiosulfate was not limiting. In cultures containing the cement samples, both thiobacilli completely oxidized thiosulfate to sulfate within 3 to 4 days after inoculation (data not shown). We therefore used a semicontinuous culture procedure, which comprised intermittent transfers (every 4 days) of the cement

FIG. 2. Leaching of calcium and silicon from cement cubes exposed to semicontinuous cultures of *H. neapolitanus* and *T. intermedia*. Ion concentrations in cell-free aliquots withdrawn from the cultures every 4 to 5 days were analyzed with an ICP spectrometer. The data are cumulative concentrations and are means $\pm$ standard deviations for three replicates.

samples to fresh thiosulfate medium inoculated with *H. neapolitanus* or *T. intermedia*.

Figures 2 and 3 show concentrations of ions leached from the cement samples over 39 days of incubation in semicontinuous culture. The calcium and silicon concentrations were determined since these elements are the major constituents of the cementitious matrix. The high leaching rate of calcium, which was up to 20-fold higher than that in the control, indicated that dissolution and structural failure of the cementitious matrix occurred (Fig. 2). In a cementitious matrix, the dissolution of $\text{Ca}(\text{OH})_2$ is followed by the dissolution of calcium-silicate hydrate, which is the main backbone of the cement paste. This could explain the high Ca^{2+} leaching rate compared with that of Si. The leaching kinetics for strontium and cesium (simulating the behavior of the radioactive isotopes) showed markedly different patterns (Fig. 3). The leaching of strontium from cement incubated with the bacterial cultures

exhibited a linear pattern, similar to the pattern for calcium and silicon, and the amount of leached strontium was about 7.5-fold higher than that of the control (Fig. 3). In contrast, the leaching of cesium ions from the cement samples resembled a saturation pattern, and the cumulative amount of leached cesium was only slightly higher than that of the control.

In all leaching experiments, no significant differences were found in the leaching patterns for the elements tested between the two bacterial species. Measurements of the weight loss of cement samples during incubation with the bacterial cultures showed that there was rapid degradation of the cement samples, as expressed by weight losses of 16 and 11% after 39 days of incubation with *H. neapolitanus* and *T. intermedia*, respectively (Fig. 4).

The exposure of cement samples to semicontinuous cultures of the two bacteria resulted in major morphological modifications of the cement surface. SEM analysis showed that there

FIG. 3. Leaching of strontium and cesium from cement cubes exposed to semicontinuous cultures of *H. neapolitanus* and *T. intermedia*. Ion concentrations in cell-free aliquots withdrawn from the cultures every 4 to 5 days were analyzed with an ICP spectrometer. The data are cumulative concentrations expressed as the ratio of the amount of the leaching ion to the initial amount in the cement and are means $\pm$ standard deviations for three replicates.

FIG. 4. Weight loss for the cement cubes during exposure to semicontinuous cultures of *H. neapolitanus* and *T. intermedia*. The data are means $\pm$ standard deviations for three replicates.

were deep cracks in the cement exposed to *H. neapolitanus*, apparently resulting from the activity of sulfuric acid, and that the density of cracks was much lower on the surface of the control samples (Fig. 5). No bacterial cells were detected on the surface of the cement samples.

DISCUSSION

Consideration of cementitious mixtures as potential matrices for immobilization of low-level radioactive waste requires the concomitant development of procedures for testing the resistance of the cement to microbially induced corrosion. This corrosion is caused predominantly by the sulfuric acid produced by sulfur-oxidizing bacteria. Since the biodegradation of cement in natural environments is a slow process, procedures for accelerating the biodegradation of concrete and cement

pastes are required if a reliable bioassay is to be developed. The present study reports a procedure for accelerating the biodegradation of cement by incubating samples in semicontinuous cultures of sulfur-oxidizing bacteria grown on thiosulfate as the sole energy source. This experimental setup enabled continuous exposure of the cement samples to sulfuric acid and resulted in rapid biodegradation of the cement (up to 16% weight loss in 4 weeks). To the best of our knowledge, this accelerated biodegradation is faster than the biodegradation previously reported for acceleration systems based on sulfur-oxidizing bacteria. Sand (19), for example, reported a 1.8% weight loss for concrete incubated for 9 months with *T. thiooxidans* in a device designed to accelerate concrete corrosion. In other simulation experiments, using the same apparatus, Sand and his coworkers (20, 21) showed that exposure of concrete to biogenic sulfuric acid over a period of 270 days resulted in a maximal weight loss of 5.8%. A higher rate of weight loss (up to 31% in 5 months) was demonstrated by Schmidt et al. (22) in their tests of the effect of sulfuric acid on various types of concrete.

The rapid degradation of the cement obtained in our system may be attributed to the semicontinuous bacterial culture, in which cement samples were exposed to a sequence of several corrosion cycles and thus to a larger amount of biogenic sulfuric acid compared with batch culture systems. Acceleration of the process in our system might also have been enhanced by the high surface/volume ratio of the cement samples. Indeed, Monteny et al. (13) found that processes of concrete biodegradation start at the concrete surface and then penetrate (layer by layer) into the internal part of a sample. Hence, our use of cubic 1-cm³ cement samples gave a relatively high surface/volume ratio (6:1), which also enhanced the biodegradation. On the other hand, the concrete cubes used by Sand et al. (21) in their accelerated corrosion system had a lower surface to

FIG. 5. SEM photomicrographs of cement surfaces after 30 days of exposure to a sterile medium (left image) and to a semicontinuous culture of *H. neapolitanus* (right image). Bars = 100 μ m.

volume ratio (3.3:1), which might explain the lower weight loss (5.7% over a period of 270 days).

Further evidence demonstrating the corrosion and biodeterioration of the cement samples was provided by SEM photomicrographs. These images clearly showed the formation of cracks in the cement incubated with the sulfur-oxidizing bacteria. Such cracks are typical of cement pastes corroded by sulfuric acid, which reacts with calcium hydroxide and calcium aluminate in the cement to form gypsum and ettringite crystals, respectively. Both these substances are formed in the cement, where they expand and increase the internal pressure (due to their lower density), which leads to the formation and widening of cracks (3, 6, 14).

Considering the relatively high concentration of phosphate in the medium, the possibility of some precipitation of calcium phosphate on the surface and in the pores and channels of the cubes cannot be ruled out. However, the fact the cracks and pores were clearly visible by SEM indicates that such precipitation was not major. Moreover, if precipitation had filled the pores and channels, we assumed that this would have increased the stability of the cement and interfered with the degradation process. However, our results demonstrating that there was rapid biodegradation of the cement compared with the biodegradation in other systems does not support this assumption. Furthermore, it is unlikely that such precipitation, by itself, could affect the structural stability of the cement because no degradation was observed in the controls (which contained the same concentration of the phosphate buffer).

In natural environments, the microbial corrosion of cements and concrete usually does not start before the pH, which is 10 to 13 (12), is reduced to about 9.0 by a carbonation step mediated by atmospheric CO₂ (17). This process is followed by the action of a succession of sulfur-oxidizing bacterial populations, starting with NSOB, which reduce the pH to about 4.0, followed by acidophilic sulfur-oxidizing bacteria (ASOB), which continue to produce sulfuric acid, resulting in a further reduction of the pH to about 1.0 (11, 17). Interestingly, our accelerated biodegradation was obtained with cultures of the NSOB and *T. intermedia* growing at pH 6.2 to 6.5, whereas in most other accelerated systems ASOB such as *T. thiooxidans* are used, resulting in a pH of 0.5 to 2.0. Nevertheless, the acceleration of biodegradation in our system was greater than that obtained in systems in which ASOB were used.

The kinetics of leaching of ions from cementitious mixtures can serve as an additional evaluation tool (in addition to weight loss) for assessing concrete biodegradation. We chose to monitor the leaching of Ca and Si, since these elements are the main constituents in the cementitious structure. Leaching of Ca and Si would indicate structural changes and failure of the cement matrix due to the action of biogenic sulfuric acid. The amount of Si leached from the cementitious matrix was much lower than the amount of Ca leached. The reason for this difference is not completely clear, since both ions are integral parts of the cement and therefore both should have been damaged by the sulfuric acid. Nevertheless, the high levels of calcium that leached out of the cement indicate that the Ca(OH)₂ phase played a major role in the corrosion of the cementitious paste. The high concentration of Ca in the leachate may thus be associated with the higher dissolution rate of Ca(OH)₂ in sulfuric acid compared with that of calcium

silicate hydrate (C-S-H), which is the backbone of the cementitious matrix.

Leaching becomes an important factor when concrete is used as a matrix for the immobilization of low-level radioactive waste. In our experiments, strontium and cesium were added to the cement mixture with the aim of simulating immobilization of their corresponding radionuclides in buried concrete. The finding that the pattern of leaching of strontium was similar to that of calcium was not surprising, since the strontium ion is bivalent, like calcium, and could therefore replace the calcium in the cement structure. There is evidence that strontium is absorbed by or precipitated in cement, which therefore becomes a suitable matrix for its immobilization (1).

The leaching pattern obtained for cesium was different from that obtained for all other ions tested. It appears that cesium was not strongly bound to the cement, and hence, leaching of cesium was limited by its diffusion and not the dissolution of the cementitious matrix (as observed for the other ions tested). Furthermore, this leaching behavior was not affected by the degradation of the cement; for both the samples exposed to the bacteria and the control samples, the leaching was excessively high, reaching 77 and 90% of the initially added amount of cesium, respectively. For comparison, the overall leaching of strontium from cement exposed to the bacteria was approximately 12% of the amount initially added. The difficulty in immobilizing cesium in cement paste is due to the fact that this element belongs to the alkali monovalent group of metals, which are highly soluble. In addition, since the cesium ion is relatively big, substitution in the cement hydrates is not possible. These findings are in agreement with previous reports that also indicated that there were low levels of binding of cesium in cement (2, 4, 5). Thus, immobilization of cesium is challenging and requires careful selection of additives to be introduced into the cement mixture to aid in binding the cesium ions.

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