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A refinement of the biofilm formation method for waste forms stability evaluation

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Abstract

A refinement of the biofilm formation method for waste form stability evaluation was carried out in this study. Refinement of the biofilm formation method became necessary because of the reduced contrast in degradation between control and experimental samples. The reduction in contrast was occasioned by the long duration of exposure (12 days) of the control samples to sterile medium of low pH in the first stage. Results of evaluation carried out reveal that the duration of the first stage of the biofilm formation method can be reduced to 24 h, with substantial increase in the contrast between degradations experienced by control and experimental samples. Reduction of the first stage can be done without compromising the efficiency of the inoculation process, which the longer duration of the first stage was originally intended to ensure. A doubt as to actual formation of biofilms on experimental samples, resulting from the use of non-sterile tubings and glass wares in the second stage, was also addressed in this study. Results reveal that substantial attachment of microbes occur on the surfaces of experimental samples in the first stage, thus any supply of microbes via the tubings and glass wares in the second stage is only additional and inconsequential.

Keywords: Biofilm; *Thiobacillus thiooxidans*; Waste forms; Stabilization/solidification; Leaching

1. Introduction

The isolation of microorganisms from low-level radioactive waste (LLW) environments, and other areas previously thought to be hostile to the existence of microbes, have raised serious concerns about long-term stability of disposed waste [1,2]. This apprehension partly

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explains why the United States Nuclear Regulatory Commission (NRC) now requires that microbial activity be addressed as one of the requirements for determining the stability of Classes B and C LLW [3].

Waste encapsulation and immobilization in stabilization and solidification (s/s) waste forms remains one of the promising technological approaches for waste disposal [4]. Cement is the most popular material used for waste immobilization, and has been the focus of most of the efforts at developing protocols for the testing of microbial stability. The first comprehensive package for evaluation of cement based waste forms stability to microbially induced degradation (MID) was put together for the NRC by researchers at Idaho National Engineering and Environmental Laboratory (INEEL) [2]. Under the NRC protocol, experimental samples are exposed to fermenter grown broth of *Thiobacillus thiooxidans* while control samples are exposed to sterile media of pH 4.0. This single stage arrangement allows for substrate limitation in the fermenter broths, making estimation of exact contribution of microbes to observed degradation impossible. Our laboratory developed a biofilm formation method, a two stage process, as part of the efforts to address the defects associated with the NRC methodology [5]. The first stage of the biofilm formation method was designed to run for 12 days, during which control samples are exposed to sterile medium of pH $\sim$ 2, and experimental samples to fermenter grown *T. thiooxidans* of similar pH. The 12 days duration is considered too long because of the magnitude of the degradation experienced by the control samples, which tend to reduce the overall contrast between them and the experimental samples. This is not withstanding the benefits of sufficient inoculation of the experimental samples with *T. thiooxidans* that may be derived from such a long duration of exposure. This paper reports efforts made to reduce the duration of the first stage of the biofilm formation method, in a bid to improve the method's reliability.

2. Materials and methods

2.1. Microorganism and growth

The microorganism used in this study was *T. thiooxidans*, which was supplied by INEEL. The medium used for the growth of the microorganism consisted of the following (g/l): $MgSO_4{\cdot}6H_20$ (0.4), $(NH_4)_2SO_4$ (0.5), $CaCl_2$ (0.1), $FeSO_4$ (0.01), $K_2S_4O_6$ (3.0), KH_2PO_4 (3.0). The bioreactor used for the growth of the microorganism was a New Brunswick Scientific Bioflow III batch/continuous fermenter equipped with accessories for temperature, pH and dissolved oxygen control.

2.2. Waste formulation

The waste formulations used in this study consist essentially of a locally purchased Portland type 1 cement (called Tuskegee cement). They were formulated by mixing two parts of Tuskegee cement with one part of water, and allowing the mix to set in a 5 ml plastic vial serving as the mould. The waste forms were cylindrically shaped after setting and had the following dimensions: 2.0 cm height $\times$ 1.5 cm diameter.

2.3. Waste form stability evaluation

Evaluations of waste form stability to MID were carried out using the biofilm formation method of Idachaba et al [5] with modifications. The biofilm formation approach involves exposure of control waste forms to sterile medium of pH 1.9 and experimental waste forms to fermenter broth of *T. thiooxidans* (pH 1.88), for the first 12 days. This stage of the process is meant for colonization of the experimental waste forms by *T. thiooxidans* and ends with the shutting off of supply of *T. thiooxidans* to the experimental waste forms and the supply of sterile medium of pH 1.9 to the control waste forms. The second stage of the biofilm formation process begins and continues to termination, with the supply of a fresh normal medium for *T. thiooxidans* (pH about 4.00) to both control and experimental waste forms. Formation of biofilm was confirmed from changes in pH and sulfate concentration. Substantial modifications to the biofilm formation method were carried out in this study. In the experiments dealing with minimum inoculation time, the 12 days duration of the first stage of the biofilm formation method is replaced with a varied time regime of 7 and 24 h, 3, 7 and 12 days. In the experiment establishing the formation of biofilm on experimental samples, a more significant modification was introduced. The first stage was followed with the washing of the experimental sample in three portions of 20 ml 0.85% saline and its transfer to a completely sterile soxhlet system, as opposed to simply shutting off the *T. thiooxidans* broth in the biofilm formation method of Idachaba et al [5]. Effluents were collected in vessels and removed for analysis at predetermined periods.

2.4. Analytical

Analyses of the metals in the media, fermenter broth, and the effluents were carried out using inductively coupled plasma (ICP) Optima 3300 DV (Perkin-Elmer) spectrophotometer. The sulfate concentration of the media and the effluents was estimated by UV method at 420 nm using the HacH 2010 DR spectrophotometer. This method is based on barium sulfate formation [6]. The pH of the media, fermenter broth and effluents was determined using a corning pH meter 345.

3. Results and discussion

3.1. Evaluation of minimum time required for inoculation of experimental samples

Evaluations were carried out involving different time regimes in a bid to reduce the duration of the first stage of the biofilm formation method. Results obtained using the original duration of the first stage (12 days) of the biofilm formation method are presented in Fig. 1. Results indicate that the total calcium leached from the experimental sample within 30 days of evaluation was only slightly (1.2 times) higher than the total calcium leached from the control within the same period. The total magnesium leached from the experimental sample however, was about two times higher than that leached from the control.

Results obtained when 7 h was used as the duration of the first stage are presented in Fig. 2. Results indicate that the total calcium and magnesium leached from the experimental sample

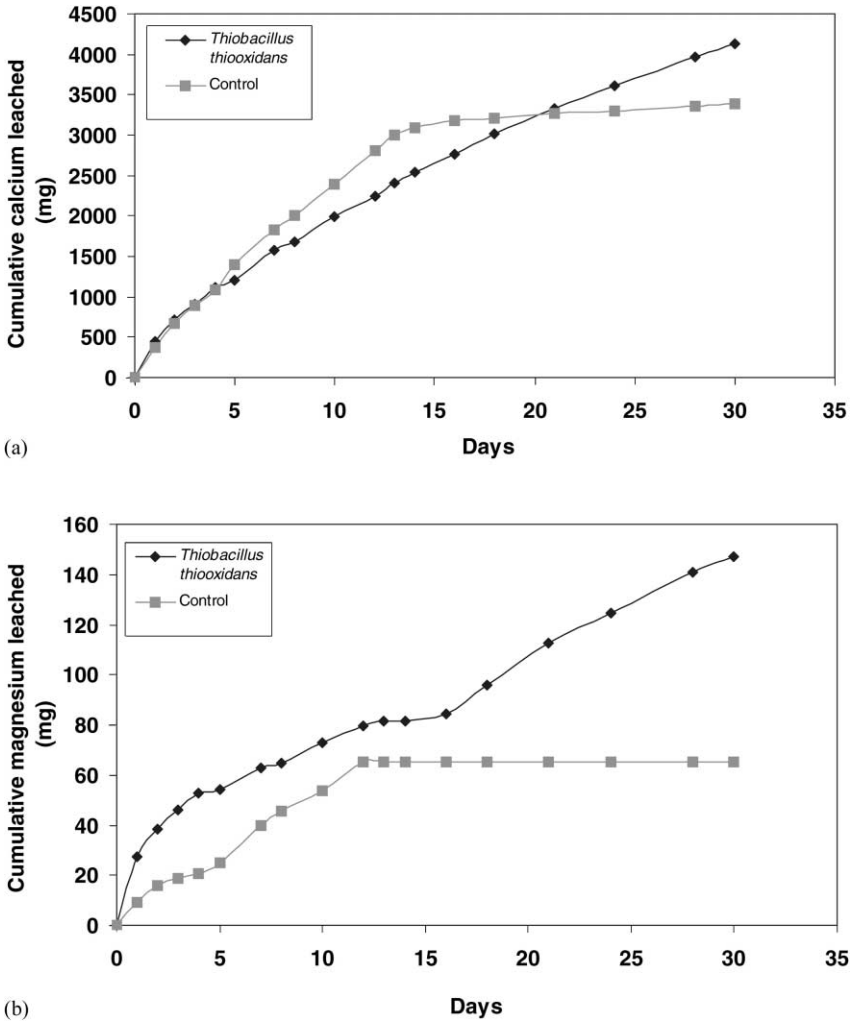


Fig. 1. Profiles of calcium (a) and magnesium (b) leached from 100% cement waste forms using biofilm formation method with duration of the first stage set at 12 days.

were substantially (about 10 times for calcium and 85 times for magnesium) higher than the total calcium and magnesium leached from the control sample within 30 days of evaluation.

Results obtained when the duration of the first stage was 24 h are presented in Fig. 3. It is clear from the figure that the total amount of calcium and magnesium leached from the experimental sample was substantially higher (5.2 times for calcium and 18.8 times for magnesium) than those leached from the control. However, their numerical magnitudes were substantially lower than those obtained when the duration of the first stage was 7 h.

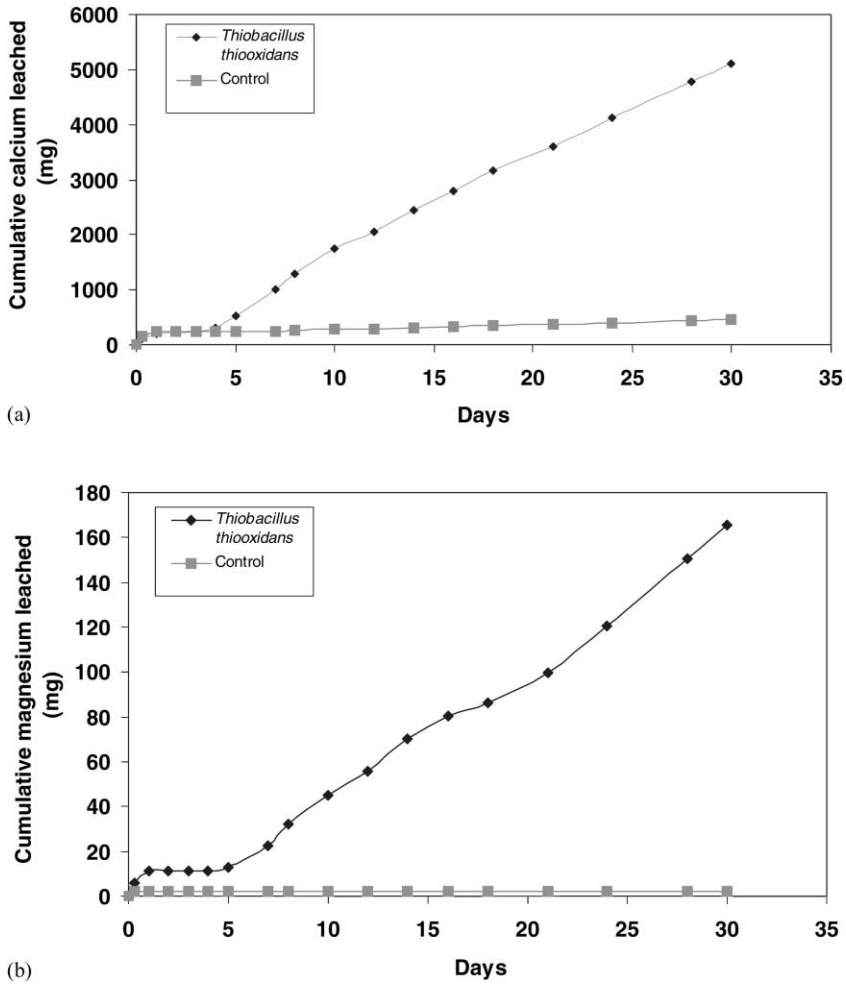


Fig. 2. The profiles of calcium (a) and magnesium (b) leached from 100% cement waste forms using biofilm formation method with duration of the first stage set at 7 h.

Results obtained when the duration of the first stage was 3 days are presented in Fig. 4. Results indicate that the total calcium and magnesium leached from the experimental sample were substantially higher (2.9 times for calcium and 15 times for magnesium) than those leached from the control within 30 days of evaluation. However, their magnitudes were much lower than those obtained for the 24 h duration of the first stage.

Results obtained from the evaluation of samples for which the duration of the first stage was 7 days are presented in Fig. 5. Results indicate that the total calcium and magnesium leached from the experimental sample were comparatively higher (1.6 times for calcium and 4.7 times for magnesium) than those from the control within 30 days of evaluation.

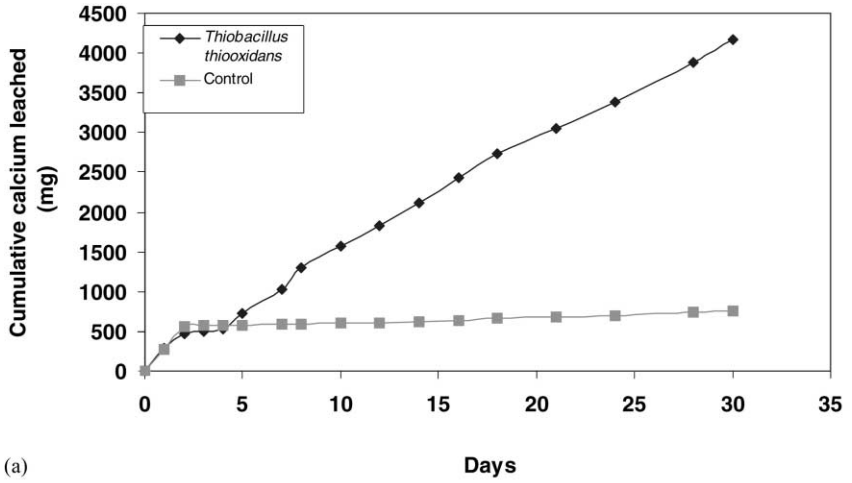
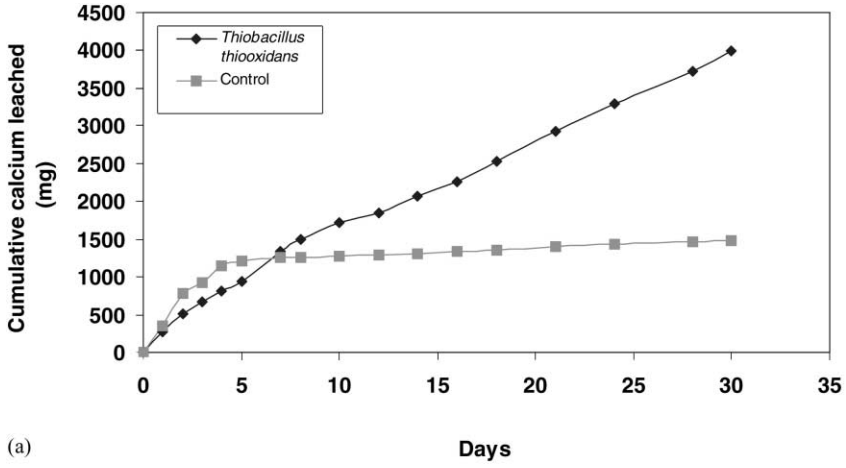


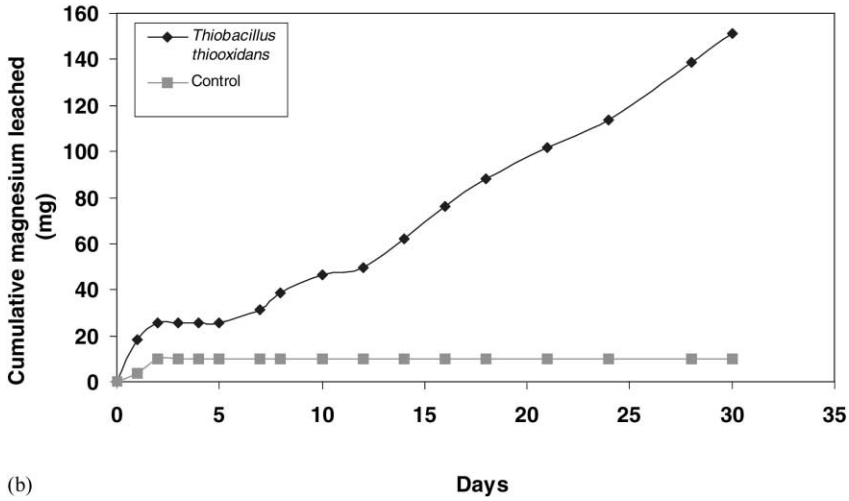
Fig. 3. Calcium (a) and magnesium (b) leached from 100% cement waste forms using biofilm formation method with duration of the first stage set at 24 h.

However, their magnitudes were much lower than those obtained when the duration of the first stage was 3 days.

The observed comparable degradation of the 100% cement experimental waste forms at all the inoculation times evaluated (see Table 1) suggest that the duration of the first stage could be substantially reduced without compromising the effectiveness of the method. In addition it is obvious that the shorter inoculation times make the contrast between the experimental and the control to be sharper. This is clearly exemplified with the leaching profile



(a)



(b)

Fig. 4. Calcium (a) and magnesium (b) leached from 100% cement waste forms, using biofilm formation method, with duration of the first stage set at 3 days.

of calcium. It is evident from Table 1 that the total calcium leached from the experimental samples with 7 h (minimum duration) and 12 days (maximum duration) inoculation times are essentially the same (4500 mg for 7 h and 4200 mg for 12 days). In contrast the total calcium leached from their controls are substantially different (500 mg for 7 h and 3400 mg for 12 days). The high value of total calcium leached from the 12 days control sample is undoubtedly due to the long exposure of the sample to sterile medium of low pH at stage 1 of the biofilm evaluation method.

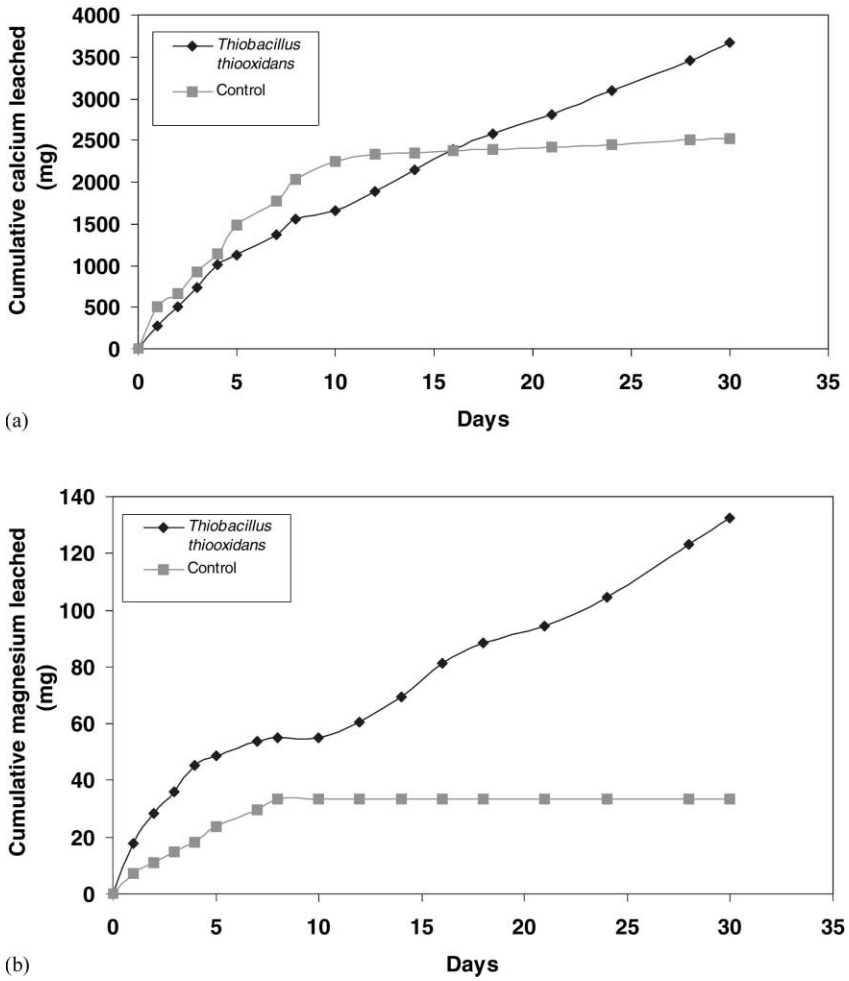


Fig. 5. Profiles of calcium (a) and magnesium leached (b) from 100% cement waste forms using biofilm formation method with duration of the first stage set at 7 days.

3.2. Confirmation of biofilm formation on experimental samples pre-exposed to microbes

Tubings and glass wares used in the first stage of the biofilm formation method were often used in the second stage as part of a routine procedure. This practice has raised some doubts as to whether the microbial activity being evaluated in the second stage of the biofilm formation method is actually due to the biofilm on the experiment samples. Experiments were therefore carried out to confirm the formation of biofilm on experimental samples pre-exposed to microbes in stage 1 of the biofilm formation method. Confirmation of biofilm formation was carried out using net sulfate balance measurements. Net sulfate

Table 1
Maximum sulfate concentration (mg/l), average pH (during the last 10 days of second-stage when system is fully stable), and total metals leached (mg) from experimental and control samples, during evaluation of biofilm formation at different duration of the first stage

Duration of first stage	Ca	Mg	SO_4^{2-}	pH
Experimental samples				
7 h	5000	170	4500	2.0
24 h	4200	150	4500	2.4
3 Days	4000	150	4500	2
7 Days	4000	140	4300	2
12 Days	4200	150	4300	2
Control samples				
7 h	500	2	0	6.0
24 h	800	8	0	5.5
3 Days	1400	10	0	6.0
7 Days	2500	30	0	5.5
12 Days	3400	60	0	5.5

balance is the difference in sulfate concentration between the sterile medium (before exposure to simulated experimental or control waste samples) and effluents (after exposure to simulated experimental or control waste samples). Theoretically, the net sulfate balances for the control samples should be zero. However, it is possible that some of the sulfate in the sterile medium could be retained by the samples resulting in negative values for net sulfate balances. Unusually high positive or negative net sulfate balances could also be obtained for both the control and experimental samples at the beginning of the second stage. This is because of change over from high sulfate solutions to low sulfate solution. The initial high positive and negative net sulfate balances occur during washing of the high sulfate solutions from the system. Results of experiments carried out on the confirmation of biofilm formation on experimental samples pre-exposed to microbes are presented in Figs. 6 and 7. Results in Fig. 6 indicate that both washed and unwashed experimental samples pre-exposed to *T. thiooxidans* exhibited varying degree of microbial activity during the second stage of the biofilm formation method, as evident in increased net sulfate balance. A maximum net sulfate balance of about 4500 mg/l was obtained for effluents from the unwashed pre-exposed sample within 30 days of evaluation, while a maximum net sulfate balance of about 1200 mg/l was obtained for the washed and transferred sample within the same period. Effluents from the control of both samples gave a net sulfate balance of approximately zero during the second stage of the evaluation. It is clear from Fig. 6 that a steady increase in net sulfate balance for effluents from the unwashed sample began at about the fifth day of evaluation (second day of stage 2). On the other hand, steady increase in net sulfate balance for effluents from the washed and transferred sample did not begin until day 8 of the evaluation (fifth day of stage 2). The profile of calcium leached from both washed and unwashed experimental samples presented in Fig. 7 indicate substantial difference between the calcium leached from the washed and unwashed samples pre-exposed to *T. thiooxidans*. A total of about 1400 mg of calcium was leached from the washed sample while a total of about 4000 mg of calcium was leached from the unwashed sample.

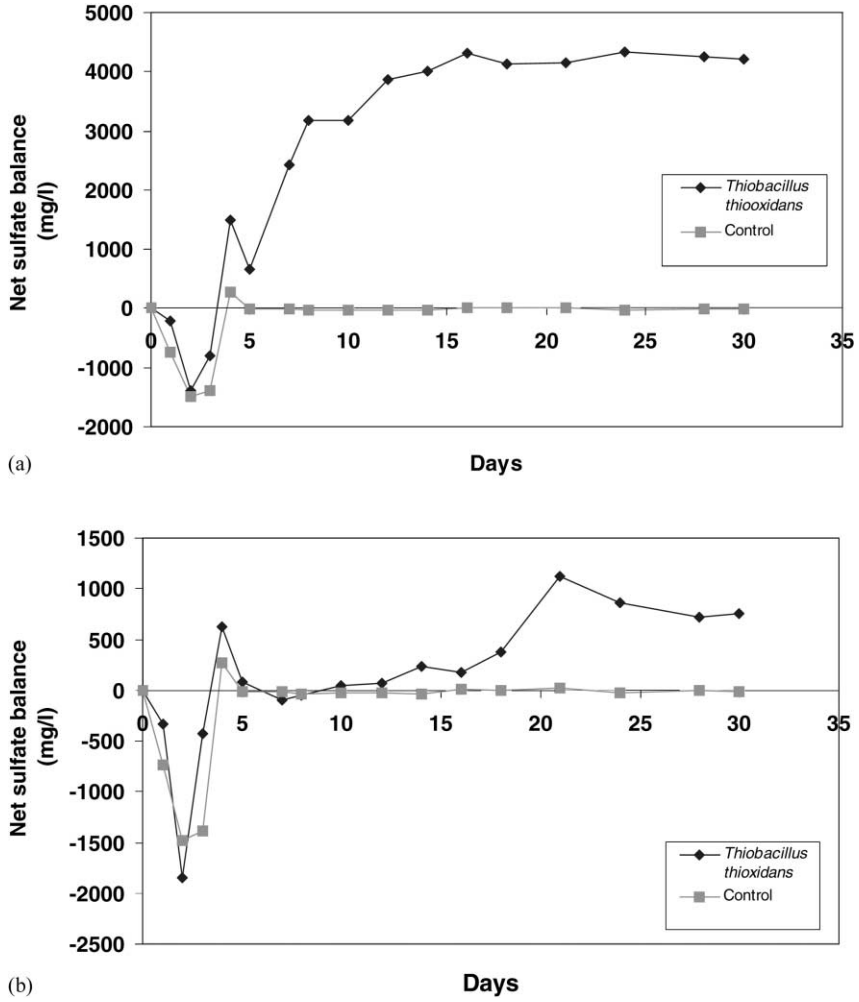


Fig. 6. Comparison of net sulfate balance of effluents from unwashed (a) and washed (b) 100% cement waste forms using biofilm formation method.

The results of evaluation on the confirmation of the formation of biofilm on experimental samples pre-exposed to microbes strongly suggest that a viable microbial population (stable biofilm) can be formed in and around experimental waste forms. This position is supported by the substantial depression of pH and the increase in net sulfate balance exhibited by the effluents from the *T. thiooxidans* pre-exposed sample thoroughly washed with saline, and transferred to a completely sterile soxhlet system. The washing procedure would by all account remove any loosely attached particles and materials, leaving only those strongly attached to the sample. Given that the washing solution was sterile and the procedure was carried out in a sterile environment, the depression of the pH and the increase in net sulfate

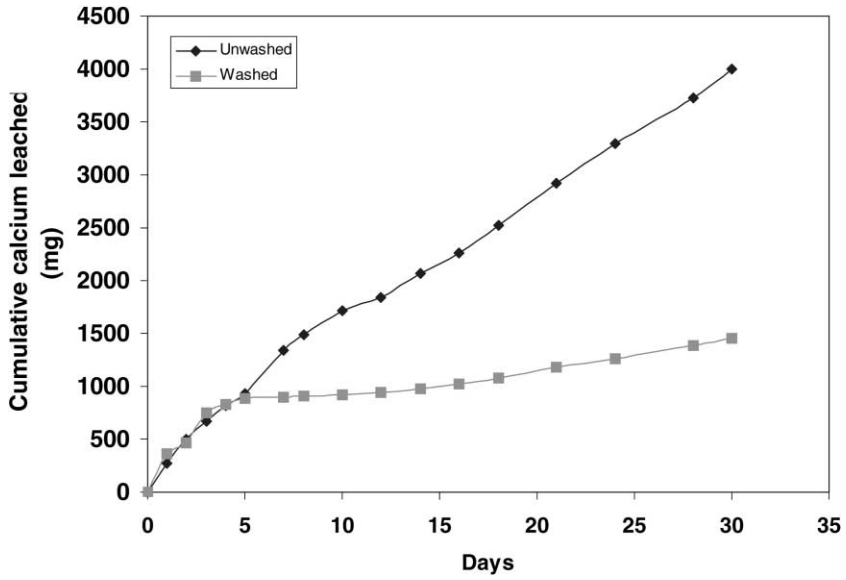


Fig. 7. Comparison of calcium leached from unwashed and washed 100% cement waste forms pre-exposed to *T. thiooxidans* during the first stage of the biofilm formation method.

balance could not have resulted from anything else apart from attached microbes to the sample. Microbes generally experience lags when they are transferred to a completely new environment. It is likely that the observed long delay for the presence of the microbe to become evident is connected to the lag phenomenon. The procedure of washing and transferring of a sample to another sterile system used in this study to confirm biofilm, appears to be the best approach to ensure the non-interference of other sources of the microbe in the experimental system, on the analysis. However, this approach for practical purpose is cumbersome, time consuming, and on the whole unnecessary. It is therefore not recommended for routine analysis.

4. Conclusions

No substantial difference in overall degradation was observed for experimental samples at different duration of the first stage of the biofilm formation method. Thus, exposure of experimental samples for only 7 h is sufficient for inoculation with microbes. Substantial differences in overall degradation were observed, however, for the control samples at different duration of the first stage of the biofilm formation method. The longer the duration of the first stage the greater the degradation of control samples. To ensure minimum degradation of the control samples, it is recommended that the duration of the first stage be as short as possible. Duration of 24 h is considered adequate and recommended for future evaluations. The formation of active biofilms on experimental samples is confirmed by this study.

However, the procedure for confirmation is not recommended for routine analysis as it is cumbersome and takes longer time than the conventional biofilm formation approach.

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