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Microbubble suspension as a carrier of oxygen and acclimated bacteria for phenanthrene biodegradation

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ABSTRACT

The applicability of microbubble suspension made of saponin as a biodegradation-enhancing carrier where oxygen and pollutant-degraders are limited was studied. The saponin-microbubble suspension was used to deliver phenanthrene-degrading bacteria, inorganic nutrients, and oxygen. Bench-scale study was carried out to determine the physical properties of the microbubble suspension and to verify whether the delivered bacteria and oxygen were effectively used to degrade phenanthrene. A concentration of 2 g saponin/L H_2O generated stable microbubble suspension with a long half-drainage time and a high gas hold-up, and the addition of phenanthrene-degraders and inorganic salts to the saponin solution did not affect such properties. The flow of the microbubble suspension through a heterogeneous sand/clay-packed column occurred in two phases, with the liquid front advancing faster and the retarded gas front. The retarded gas front provided oxygen with bacteria, which enables phenanthrene biodegradation. Approximately 30% of the spiked phenanthrene was degraded in 21 days when one pore volume of 2.0 g/L saponin-microbubble suspension was applied whereas no phenanthrene decrease was observed following the application of the same saponin solution without microbubble generation. The decrease mainly occurred at the lower part of the column where the supply of oxygen by the microbubble was concentrated.

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1. Introduction

Organic contaminants in the subsurface have a well known tendency for natural attenuation, which is mostly achieved by microbial degradation [1]. However, the efficiency and duration of the microbial degradation cannot be assured due to the wide variety of environmental factors involved in the process that do not work independently [2,3]. In particular, organic contaminants suitable for aerobic biodegradation are less degradable when located in the subsurface due to the shortage of electron acceptors and micronutrients [4]. Therefore, providing limiting factors such as oxygen and inorganic nutrients might enhance the natural biodegradation potential to change organic contaminants into harmless water and carbon dioxide.

Microbubbles, which can be generated by simply agitating a surfactant solution with a high-speed stirrer [5], have many advantages in material transport, subsurface remediation and soil flushing. These merits can be usefully adapted for more successful introduction of microbubbles to bioremediation. First, microbubbles have the potential to enhance bacterial transport [6]. Second, it shows plug-flow characteristics, which can overcome the matrix heterogeneity [7]. Therefore, it can provide the materials essential for aerobic biodegradation uniformly to the subsurface. Furthermore, it is easy to control microbubble flow because they can be stably pumped due to its enhanced stability and water-like viscosity [8]. Third, it can be used in a soil flushing process [9,10], because surfactant shells have layers of surfactant molecules in a tail-to-tail manner that can act as a partitioning medium for hydrophobic compounds [6,11].

Microbubble suspensions, same as other foam systems, are a collection of gas bubbles dispersed in an aqueous surfactant solution. When generated from a surfactant solution, a gas–liquid dispersion is formed with a high gas content of around 50% [12], which can be enhanced successfully up to 70% using combination of surfactants [13]. This enables the microbubble suspensions to act as a carrier for materials in gaseous form (e.g., gas phase oxygen) and dissolved form (e.g., micronutrients). Moreover, microbubble suspensions can carry microorganisms suspended in bulk liquid and/or attached to the bubbles. Therefore, oxygenation, bioaugmentation, and biostimulation effects can be achieved simultaneously through the application of the microbubble suspensions when generated from a solution containing target contaminants' degraders and micronutrients.

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A comprehensive bench-scale study was carried out to test the feasibility of a microbubble suspension as an *in situ* bioremediation technology. The physical parameters such as gas hold-up and half-drainage time of the microbubble suspension were measured and its flow properties were visually observed by visualization cell test. In addition, the biological degradation of a target contaminant (i.e., phenanthrene) by the introduced microbubble suspension containing phenanthrene-degraders and micronutrients was determined. The patterns of the materials distribution (i.e., bacteria and gas) after the introduction of the microbubble suspension into a test column were also investigated.

2. Experimental

2.1. Components for microbubble generation

The solution used to generate the microbubble suspension consisted of a surfactant, a phenanthrene-degrading bacterial species, and inorganic nutrients. A heterogeneous saponin mixture extracted from *Quillaja* bark (S7900, Sigma–Aldrich, St. Louis, MO, USA) was used as a base surfactant. Saponin is a plant-origin biosurfactant that is commonly found in soil [14]. The critical micelle concentration (CMC) of saponin was determined experimentally to be 1.0 g/L by measuring the changes in surface tension of a saponin solution as a function of concentration.

The phenanthrene degrader was isolated from an oil-contaminated soil and identified by 16S rDNA sequencing as *Burkholderia cepacia* and named *B. cepacia* RPH1. The bacterium was maintained in an inorganic salt medium containing 1.0 g/L $(NH_4)_2SO_4$, 0.8 g/L K_2HPO_4, 0.2 g/L KH_2PO_4, 0.2 g/L $MgSO_4{\cdot}7H_2O$, 0.1 g/L $CaCl_2{\cdot}7H_2O$, 0.005 g/L $FeCl_3$, and 0.001 g/L $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ with phenanthrene at the concentration of 100 mg/L as the sole energy and carbon source. After allowing the bacteria to grow for 9 days at 30 °C, the bacterial cells were harvested by centrifugation, washed twice with a phosphate buffer (pH 7.0), and resuspended in an appropriate solution prior to use. The same concentrations of inorganic salts were added to a 2.0 g/L saponin solution containing phenanthrene degraders for microbubble generation below. The supplement of inorganics maintained the pH of the saponin solution near neutral, otherwise the pH was about 4.5.

2.2. Microbubble generation

A generating device was developed as suggested by Sebba [15]. The generator consisted of a mixer (Silverson SL2T Model, Silverson Machines, Chesham Bucks, England) and a generating vessel (15 cm diameter × 23 cm height) with two baffles. One liter of each microbubble-generating solution (i.e., 0.5, 1.0, or 2.0 g/L saponin solution) was agitated thoroughly at 7000 rpm for 5 min [6,16]. The size of the microbubbles generated, which was determined using a particle counter (Coulter Multisizer II 9900266-F, Beckman Coulter, Miami, FL, USA), ranged from 20 to 250 μm with an average value of 49 μm.

2.3. Determination of microbubble suspension characteristics

Half-drainage time and gas hold-up were chosen as physical parameters to represent the stability and quality of the generated microbubble. A drainage curve of the changes in the drainage ratio as a function of time was constructed. Two hundred milliliters of the microbubble suspension were transferred to a graduated cylinder and the volume of the drained liquid was read with time. Half-drainage time was determined to be the time taken for half the total volume of liquid in microbubble suspension to drain away [8]. At the same time, gas hold-up (ε) was calculated using the initial microbubble suspension volume (V_{a0}) and the total liquid volume (V_{l0}) as follows [12]:

$$\varepsilon = \frac{V_g}{V_{a0}} = \frac{V_{a0} - V_{l0}}{V_{a0}} \quad (V_g, \text{gas volume})$$

The effect of the saponin concentration (0.5, 1.0, and 2.0 g/L) on the properties of the microbubble suspension was investigated by observing the above mentioned parameters.

2.4. Visualization cell test for microbubble suspension flow properties

A transparent visualization cell (15 cm × 1.5 cm × 25 cm) was designed to observe the flow properties of the microbubble suspension. The cell test was conducted in two ways; (1) a homogeneous medium packed with 920 g of Ottawa sand (20–30 mesh; Fisher Scientific, Fairlawn, NJ, USA) to give a final porosity of 0.334 and (2) a heterogeneous medium with a low permeability patch consisting of Ottawa sand mixed with 10% kaolinite at one side. The homogeneous medium was used to examine the effect of saponin concentration on microbubble suspension flow, and the heterogeneous medium was used to determine the plug-flow characteristic of microbubble suspension in comparison with a saponin solution (i.e., direct injection of saponin solution into the test cell without microbubble generation). After flushing the cell with carbon dioxide and saturating it with distilled water, a saponin-microbubble suspension was injected into the cell from the bottom using a peristaltic pump at a flow rate of 10 mL/min. A blue dye, bromophenol blue was added (0.25 g/L) to the saponin solution before microbubble generation to improve microbubble observation.

2.5. Biodegradation enhancement by microbubble suspension

The enhancement in biodegradation by saponin-microbubble suspension was determined using a semi-batch type column (4 cm diameter × 15 cm height) made of Pyrex® glass with Teflon seals. The influent and effluent ports were connected to Viton® tubing in order to prevent phenanthrene and bacterial adsorption. The inlet part of the column (i.e., bottom section) was packed with glass beads (4 mm diameter) to generate uniform influent flow.

Phenanthrene was dissolved in acetone (10^4 mg/L) and was spiked onto 120 g of Ottawa sand to yield a phenanthrene concentration of 100 mg/kg. The acetone to sand ratio was set to 1:100 (v:w) in order to prevent a cosolvent effect [17]. The spiked sand sample was then mixed vigorously by spatula and left to stand in a fume hood for 3 h to evaporate the acetone. A 20-g portion of the spiked sand was taken for phenanthrene analysis, the remaining 100 g was packed at the bottom of the column and another 200 g of clean Ottawa sand was then overlaid on top of the spiked sand to give a final porosity of 0.346.

After the column was saturated with distilled water, one pore volume (PV) of microbubble suspension was injected into the phenanthrene-spiked sand column at a flow rate of 10 mL/min in an upflow manner. The microbubble suspension was generated from a 2.0 g/L saponin solution containing *B. cepacia* RPH 1 (10^8 CFU/mL) and inorganic salts at the same concentrations as described earlier. The column was incubated at 25 °C for 21 days to allow biological degradation. One pore volume of 2.0 g/L saponin solution containing the same bacterial species and inorganic salts, but without microbubble generation, was also injected in the same manner for comparison. An additional column was also prepared to determine the initial distribution of phenanthrene and mass recovery. All experiments were duplicated to confirm the results of each column set.

2.6. Phenanthrene analysis

In order to analyze the phenanthrene mass in sand, 20 g of sand was taken from top, middle, and bottom of the column and mixed with 100 mL acetone for 24 h for extraction. In addition, 10 g of sand from each part of the column was sampled to measure the moisture content of each sample. Phenanthrene in the effluent was concentrated by using Sep-Pak® C18 cartridges (Millipore, Bedford, MA, USA) and dissolved in acetone. Phenanthrene concentration was analyzed using a high performance liquid chromatograph (Waters Alliance System, 2690 Separation Module, Waters, Milford, MA, USA) equipped with photodiode array detection (Waters 996) and Waters® PAH column packed with C18 (5 μm pore size, 4.6 mm in diameter, and 250 mm in length). The mobile phase was a 30:70 mixture of water and acetonitrile at a flow rate of 1.2 mL/min. The column temperature was maintained at 27 °C, and the absorbance was measured at a wavelength of 254 nm. All the samples were filtered through a 0.45-μm PTFE filter prior to analysis.

2.7. Materials distribution by microbubble suspension

The delivery of gas and bacteria by the microbubble suspension was investigated. For this purpose, the column was packed with clean 300 g Ottawa sand without the phenanthrene spiking. After saturating the column with distilled water, one PV of a saponin-microbubble suspension (2.0 g/L) containing *B. cepacia* RPH1 (3.8×10^7 CFU/mL) and the inorganic salts described above was injected at a flow rate of 10 mL/min. For bacterial distribution, the cells in effluent samples were determined by direct plate counting and the cells associated with the Ottawa sand were counted, as suggested by Zuberer [18]. Briefly, 10 g each of Ottawa sand was removed from the top, middle, and bottom of the column. Each sample was transferred to 95 mL of a phosphate-buffered saline solution (pH 7.0) in a 125-mL Wheaton bottle containing 20–40 of glass beads (4 mm diameter). The bottles were then shaken vigorously by hand for 30–60 s and subsequently mixed for 20 min at 200 rpm using a vortex mixer. After allowing the bottles to stand for 30 s, the aqueous phase was sampled and the number of cells was determined by plate counting. Another 10 g of sand from each part of the column was taken to analyze the moisture content. From the moisture content data, including those from the 21-day biodegradation tests, the gas saturation in each part of the column was calculated.

3. Results

3.1. Characteristics of microbubble suspension

Half-drainage time and gas hold-up were chosen as indicators representing stability and quality of the microbubble suspension, respectively. Drainage curves of the microbubble suspensions generated from the solutions with different saponin concentrations were developed. The drainage curve shifted rightward as the concentration of saponin increased (Fig. 1), indicating that more stable microbubble suspension was obtained from the higher saponin-containing solution. As shown in Table 1, the half-drainage time for the microbubble suspension from the 0.5 g/L saponin solution could not be obtained because more than half of the liquid was drained from the suspension during sampling. From linear extrapolation, the half-drainage times were calculated to be 1.76 and 4.78 min for 1.0 and 2.0 g/L saponin-microbubble suspension, respectively. The microbubble suspension generated from 2.0 g/L saponin also had the highest gas hold-up value of 0.419, indicating 41.9% of gas fraction. Moreover, the addition of inorganic salts at the same level

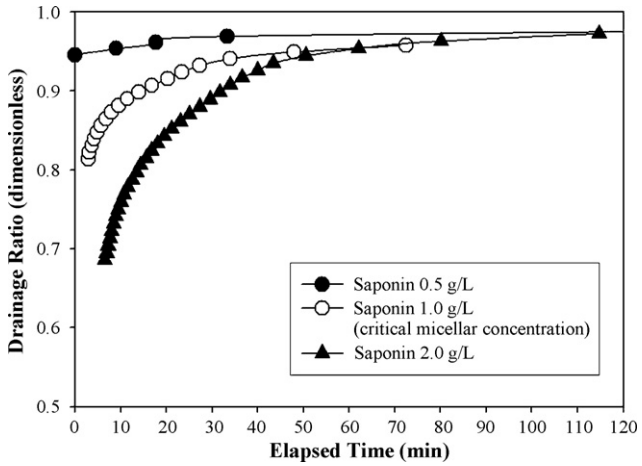

Fig. 1. Drainage curves of microbubble suspensions generated from different saponin concentrations.

Table 1
Characteristics of the microbubble suspensions generated from different saponin concentrations

	Saponin 0.5 g/L	Saponin 1.0 g/L[a]	Saponin 2.0 g/L
Gas hold-up	0.223	0.298	0.419
Half-drainage time (min)[b]	NA[c]	1.76	4.78

[a] Experimentally determined critical micellar concentration level.
[b] From the extrapolation data based on the assumption that there is a linear relationship between time and the drainage ratio.
[c] Not applicable.

as mentioned earlier and *B. cepacia* RPH1 (i.e., 9.6×10^4 CFU/mL) hardly affect the drainage characteristics of the microbubble suspension (Fig. 2), yielding gas hold-up and half-drainage time of 0.429 and 4.87 min, respectively.

3.2. Microbubble suspension flow properties

The flow properties of the microbubble suspension through a homogeneous medium were observed using a visualization test cell, and the results are shown in Fig. 3. In all cases, the liquid and gas fractions of the microbubble suspension were separated immediately after being introduced into the test cell. The liquid

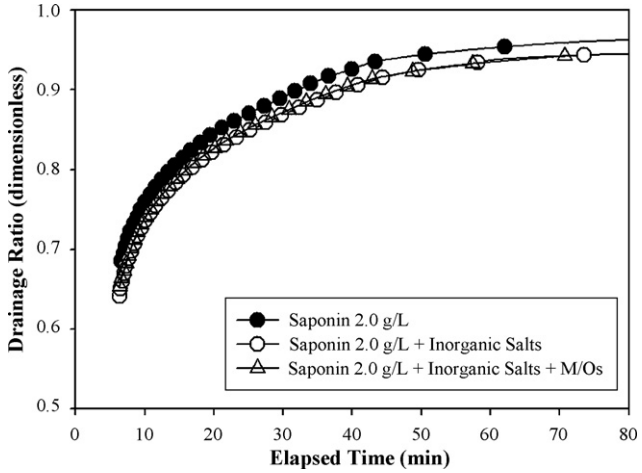

Fig. 2. Effect of microorganisms (M/Os) and inorganic salts addition on the stability of 2.0 g/L saponin-microbubble suspension.

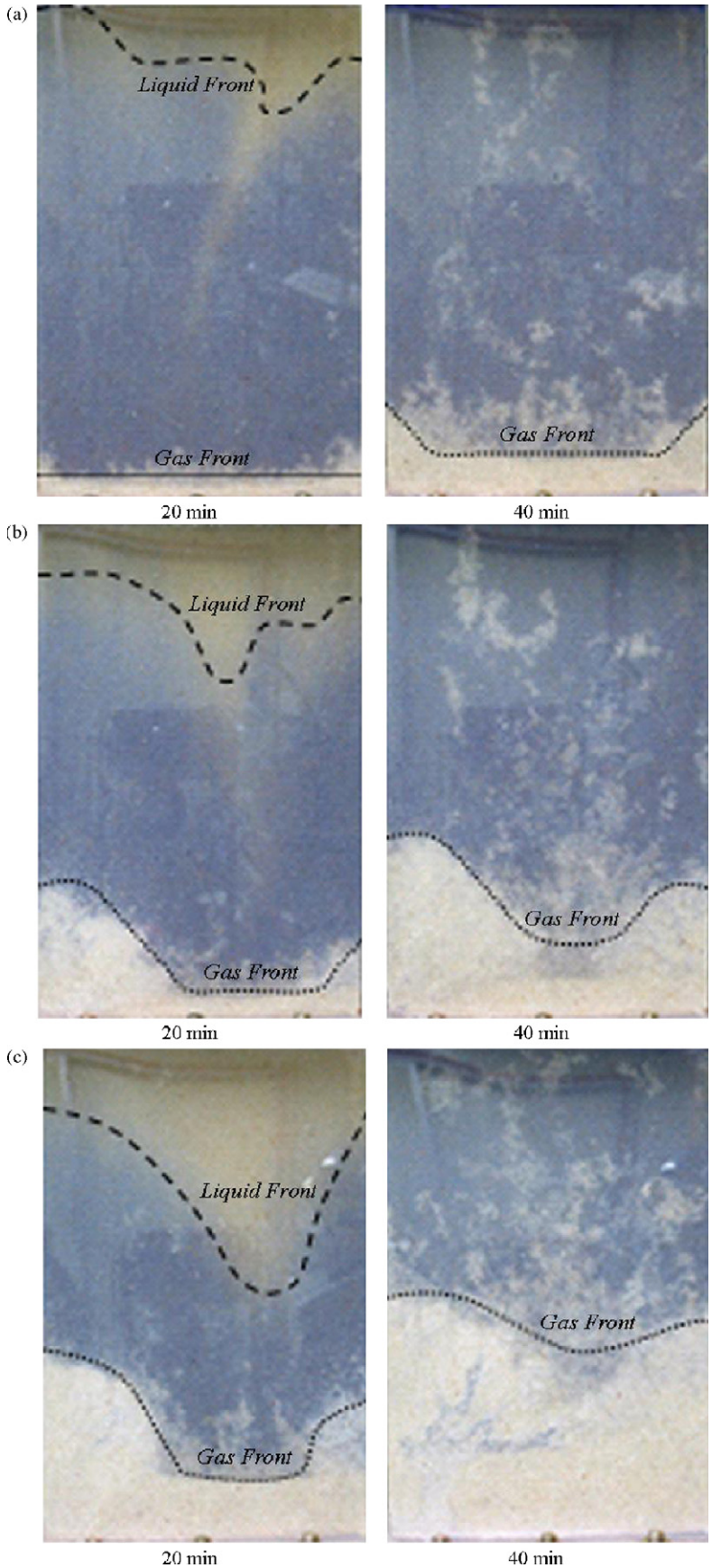

Fig. 3. Time-dependent flow properties of the microbubble suspensions generated from (a) 0.5 g/L, (b) 1.0 g/L and (c) 2.0 g/L saponin solution observed by the homogeneous visualization cell test.

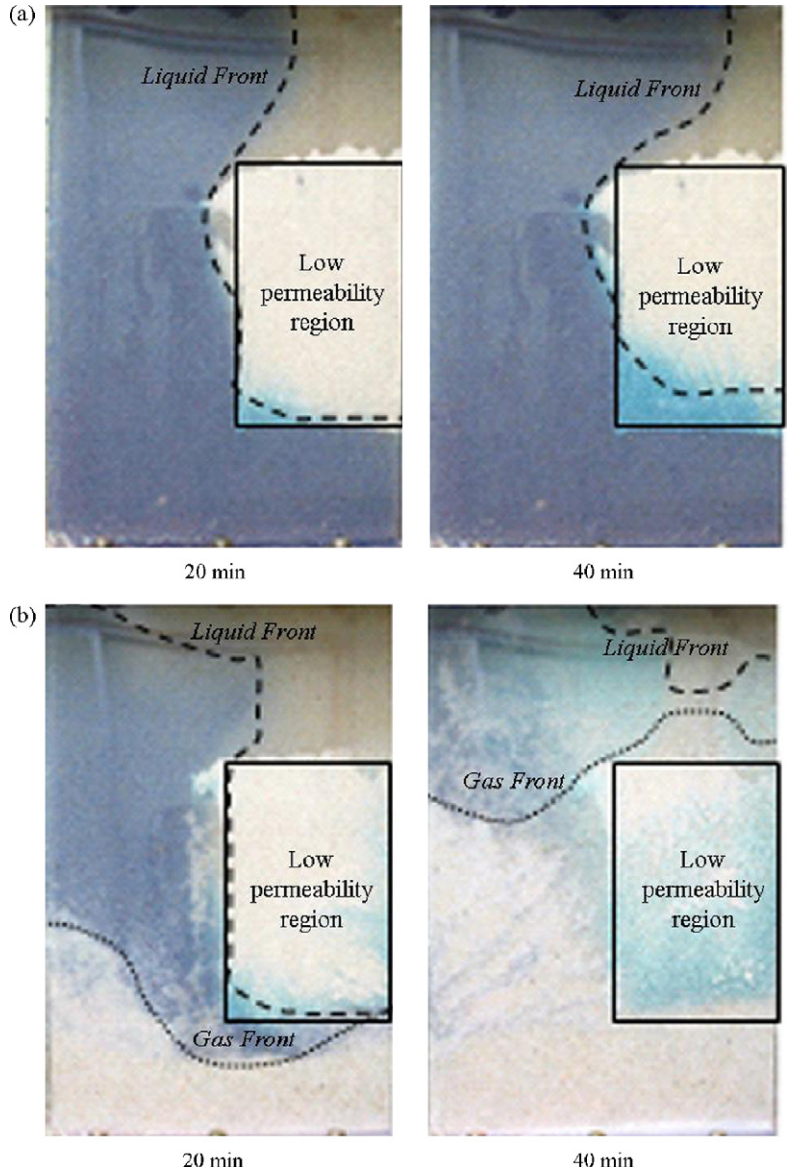

Fig. 4. Different flow properties of (a) 2.0 g/L saponin solution and (b) 2.0 g/L saponin-microbubble suspension observed in the heterogeneous medium visualization cells.

front (i.e., blue color due to dye) advanced faster than the gas front, and the movement of gas bubbles was retarded by the matrix. The gas front initially created an irregular interface, which tended to be flattened, probably by compensating the pores that had not been filled with the gas phase by continuous microbubble injection. In addition, some white spots were observed in the liquid-occupied region, indicating that some fraction of gas bubbles had been carried along with liquid flow. The area of the gas-occupied region increased significantly with increasing saponin concentration, indicating that the microbubble suspensions generated from higher saponin concentrations delivered larger amounts of air.

The same experiment was carried out in a heterogeneous medium. The heterogeneous medium was created by inserting a kaolinite patch (200 g) into the right side the homogenous sand medium used above. As shown in Fig. 4, the saponin solution (i.e., without microbubble generation) flowed preferentially through the high permeability region (i.e., Ottawa sand region). The similar flow was also observed with the liquid front of saponin-microbubble suspension flow. The gas front of the microbubble suspension flow also did not propagate into the kaolinite patch until 20 min after introduction. However, as the flow advanced, there was an abrupt penetration of bubbles (or the gas fraction) into the low permeability patch. The pressure accumulated from the gas trapped in the high permeability region and the associated blockage of subsequent flow appeared to result in such a sudden penetration of bubbles into the low permeable region. Indeed, the pressure drop in the heterogeneous medium after 50 min was 1.5 times greater than that observed in the homogeneous medium.

3.3. Enhancement of aerobic biodegradation

A soil isolate *B. cepacia* RPH1 was used for biodegradation. The bacterium was found to efficiently utilize phenanthrene in a liquid culture, degrading more than 80% in a week, and to survive and maintain its activity during the microbubble generation process. Our preliminary study verified that the presence of saponin did not affect phenanthrene-degrading capability though an increased lag phase up to 3–4 days was identified.

Immediately after introducing one PV of the 2.0 g/L saponin-microbubble suspension (i.e., at 0 day), phenanthrene recovery

Table 2
Phenanthrene remaining along the test columns (%) after introducing one pore volume of the microbubble suspension or saponin solution[a]

	Bottom	Middle	Top	Effluent	Total
Microbubble suspensions, after 0 day					
Test#1	76.73	17.66	0.37	0.15	94.91
Test#2	83.92	8.73	1.20	0.68	94.53
Mean value	80.33	13.20	0.79	0.42	94.72
Microbubble suspensions, after 21 days					
Test#1	38.40	21.90	6.91	1.41	68.62
Test#2	38.78	14.47	6.66	3.64	63.55
Mean value	38.59	18.19	6.79	2.53	66.09
2.0 g/L saponin solution, after 21 days					
Test#1	91.66	14.02	0.00	1.80	107.48
Test#2	80.03	10.07	1.42	6.43	97.95
Mean value	85.85	12.05	0.71	4.12	102.72

[a] Both saponin solution and microbubble suspensions consisted of the same composition including 2.0 g/L saponin, *Burkholderia cepacia* RPH1, and inorganic salts.

from the column showed more than 94%, however, only a mean value of 66.9% of the initial phenanthrene was recovered after 21 days, showing approximately 30% reduction (Table 2). In contrast, essentially no phenanthrene was biodegraded in the columns introduced with the same concentration of saponin solution without microbubble generation. When first prepared, all the spiked phenanthrene was present at bottom region of the column (i.e., one-third region from the bottom). However, some portion of the phenanthrene was flushed upward by introducing one PV of the saponin-microbubble suspension, and the amounts increased after 21 days (Fig. 5).

3.4. Materials distribution by microbubble suspension

The distribution of gas and microorganisms by the microbubble suspension was determined. The number of bacteria at different column depths (i.e., top = 2.5 cm, middle = 7.5 cm, bottom = 12.5 cm) after one PV of microbubble suspension injection are shown in Fig. 6. In the same figure, the gas saturation in the sand pores, which was calculated from the moisture content, was also presented as a function of depth. Bacterial cells were distributed almost uniformly throughout the column; 2.1×10^6 CFU/g at the bottom, 2.7×10^6 CFU/g at the middle, and 2.9×10^6 CFU/g at the top. The gas phase of the microbubble suspension was concentrated at the bottom of the column after introduction and such distribution pattern did not change too much after 21 days. At 0 day, about 84% of

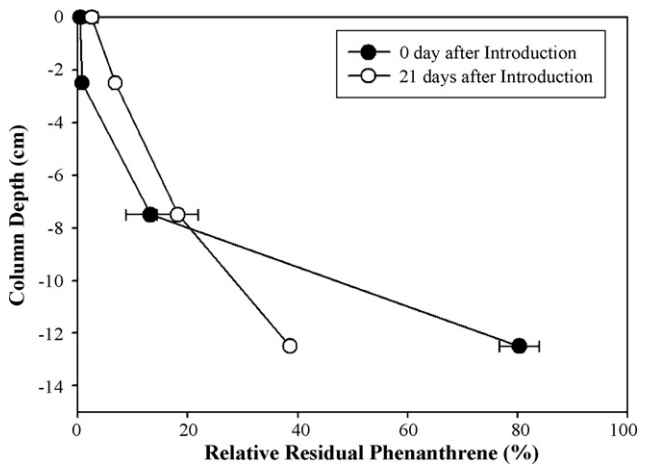

Fig. 5. Phenanthrene distribution after 0 and 21 days after introducing microbubble suspension.

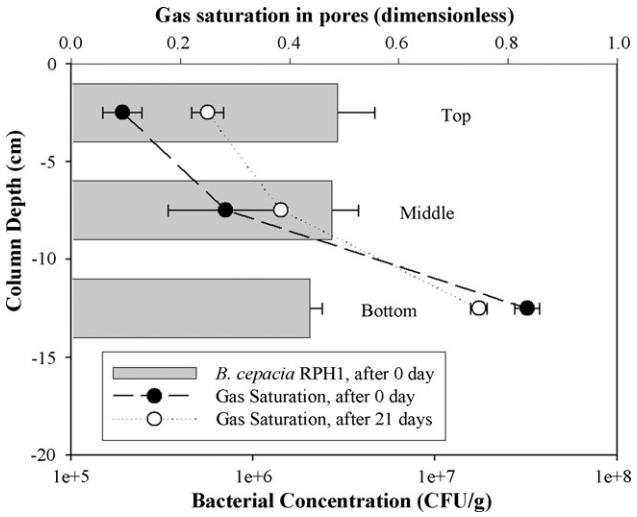

Fig. 6. Patterns of bacteria and gas distribution along the column after one pore volume of microbubble suspension application.

gas was present at the bottom region of the column and about 11% of them moved upward with time, leaving still about 73% at the bottom after 21 days.

4. Discussion

The ability of a microbubble suspension to deliver oxygen, microorganisms, and micronutrients to the target site is critical for successfully enhancing the aerobic biodegradation potential where such environmental factors are limited. When a microbubble suspension was generated at a saponin concentration of 2.0 g/L, the addition of microorganisms and inorganic salts did not alter the properties of the microbubble suspension (i.e., half-drainage time and gas hold-up) to a measurable extent, showing gas hold-up and half-drainage time of 0.429 and 4.87 min, respectively. Chaphalkar et al. [19] reported that the properties of microbubbles made of nonionic surfactants were not affected by the addition of electrolytes, whereas the study of Kommalapati et al. [10] showed that the half-drainage time of a microbubble suspension made of nonionic plant-based surfactant was altered by the addition of salts. In the case of the saponin mixture used in this study, the effect of electrolytes was negligible, and so was the effect of microorganisms.

It should be noted that the half-drainage time of a microbubble suspension does not mean that half of microbubbles in the suspension would collapse after that period. The microbubble suspensions generated go through phase separation, which the half-drainage time directly indicates, followed by a bubble collapse due to a structural failure [5]. In the case of a 2.0 g/L saponin-microbubble suspension, >90% of the microbubbles remained 30 min after generation. Furthermore, a gentle mixing would be effective for enhancing the longevity of the microbubbles, which should be considered in the field application.

The enhancement of phenanthrene biodegradation by the saponin-microbubble suspension was evident compared to the application of the 2.0 g/L saponin solution. Although the compositions of the two carriers were the same (i.e., saponin concentration, the number of phenanthrene-degrading bacteria, inorganic salts concentrations), the microbubble suspension made approximately 30% phenanthrene biodegradable in an oxygen-limited column while the same column received saponin solution showed no indication of biodegradation. The phenanthrene and gas distribution patterns before and after microbubble suspension introduction indicate that biodegradation occurred dominantly at the bottom

of the column where oxygen supply was enough, which explains why most phenanthrene degradation was observed at the bottom region even though the distribution of phenanthrene-degraders was similar throughout the column.

The overall amount of phenanthrene degradable by the microbubble supply can be estimated using a simple stoichiometric calculation. Considering the gas hold-up of 0.429, 60 mL pore volume (i.e., one PV), and 21% of oxygen in the air, 0.241 mmol of oxygen can be supplied by one PV of the microbubble suspension introduced. This is greatly larger than the estimated value of 0.011 mmol of dissolved oxygen which can be supplied by saponin solution. With 0.241 mmol of oxygen supply, 0.0146 mmol of phenanthrene (i.e., 2.60 mg) can be mineralized, which corresponds to 26% of the initially spiked phenanthrene (i.e., 10 mg). This value is consistent with the actual amount of phenanthrene biodegraded (i.e., approximately 30% in this study) by the microbubble suspension. The stoichiometric calculation also suggests that the phenanthrene biodegradation efficiency can be enhanced when more than one PV of microbubble suspension is applied.

The visualization cell test confirmed that the flow of the microbubble suspension yielded two separate phases; an advancing liquid front and a retarded gas front. This corresponded to the observation by Longe [7] with microbubbles generated from sodium dodecylbenzene sulfonate (NaDBS), alkyloxypolyethylenoxyethanol (T-15S12), and cetyl pyridinum chloride (CPC). Although the overall flow patterns of the gas and liquid phase were similar among the saponin concentrations tested (i.e., 0.5, 1.0 and 2.0 g/L), the area of the gas-occupied region was quite different. The 2.0 g/L saponin-microbubble suspension passing through the homogeneous medium showed the largest gas-occupied region compared to others. More importantly, microbubble suspension indicated a possibility to overcome preferential flow problem in the subsurface. In the heterogeneous medium tested, the liquid and gas fronts advanced to the high permeable region (i.e., sand), showing a typical preferential flow property. However, as the pressure from trapped gas accumulated, microbubbles in the gas front began to penetrate into the low permeable patch (i.e., kaolinite), which was believed to provide oxygen. Since the liquid front advanced faster than the gas front the gas content throughout the column was different: After applying one PV of the microbubble suspension, the gas saturation was 0.84 at the bottom of the column while it was 0.28 and 0.09 at the middle and top, respectively.

Our data suggest a possibility that the introduction of a microbubble suspension containing oxygen, pollutant-degraders, and micronutrients can be a useful tool for enhancing the rate of aerobic biodegradation in the subsurface. For better phenanthrene biodegradation, a means to deliver more oxygen using the microbubble suspension is being developed. An extended study to increase materials transport efficiency is also underway.

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