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Use of carrier materials to immobilise and supply cementation medium for microbially mediated self-healing of biocement

C A Spencer¹ and H Sass²

¹School of Engineering, Cardiff University, Cardiff, UK

²School of Earth and Ocean Sciences, Cardiff University, Cardiff, UK

E-mail: SpencerCA1@Cardiff.ac.uk

Abstract. Microbially induced calcium carbonate precipitation (MICP) has been attracting growing interest in respect of its use for biocementation, as a means of improving the engineering properties of granular soil. Recent studies have demonstrated the potential of MICP to enable self-healing of biocement, through the injection of nutrients and precursor chemicals required for MICP into degraded biocement. This paper documents the early stages of research into the development of an autonomous self-healing system for biocement, whereby the nutrients and precursor chemicals are provided from within the biocement matrix. This system has the potential to improve the durability and sustainability of geotechnical structures. The effectiveness of a variety of carrier materials for the immobilisation and release of the nutrients and precursor chemicals, also referred to as the cementation medium, has been explored. Materials tested include expanded perlite, diatomaceous earth and natural fibres such as jute and coir. Studies have subsequently been undertaken to investigate the effect of these carrier materials on the MICP process, in aqueous solutions and within the biocement matrix, and thus the potential to enable self-healing. Ureolytic, spore forming *Sporosarcina ureae* has been utilised to induce the precipitation of calcium carbonate.

1. Introduction

Microbially mediated self-healing, as an autonomic process, is of interest for use in construction, to enable the development of low cost, sustainable, and environmentally friendly biocements and biogroups. Self-healing, in the context of construction materials, can be defined as, ‘the partial or total recovery of at least one property of a material’ [1]. Soil properties often need to be enhanced to support infrastructure, to help meet the needs of a growing population [2]. Microbially induced calcium carbonate precipitation (MICP) can improve the mechanical properties of loose, saturated sand, by increasing its strength and stiffness and by reducing its tendency to dilate [2]. Successful application of self-healing microbially induced calcium carbonate precipitation can potentially reduce infrastructure maintenance and repair costs and improve the durability and sustainability of geotechnical structures.

Self-healing of biocement has previously been achieved by injecting the nutrients and precursor chemicals (cementation medium) required for MICP into degraded biocement [2,3]. To enable a truly autonomous healing process, the nutrients and precursor chemicals will need to be readily available within the biocement matrix. This research explores the use of carrier materials to facilitate the storage and release of cementation medium within biocement, to enable self-healing.

The use of carrier materials to store and supply cementation medium is a new concept in respect of studies on self-healing in biocement. Studies on self-healing in cementitious materials have utilised a



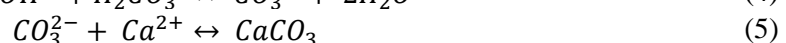
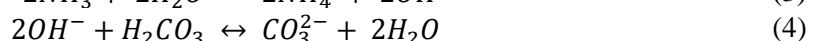
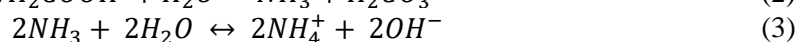
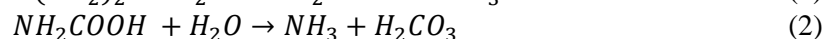
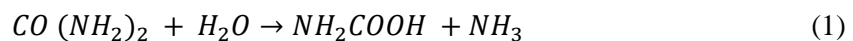
variety of porous materials to immobilise bacteria and/or cementation medium. Within cementitious materials, immobilisation has been used to protect bacterial cells/ spores from the mechanical forces exerted during the mixing stage of concrete or mortar production and from the highly alkaline environment within cementitious materials. These carrier materials may also be used as internal nutrient reservoirs [4]. Reference to nutrients within this context refers to the nutrients and precursor chemicals, otherwise known as the cementation medium, required for MICP. Immobilisation has been reported to be an efficient approach for bacteria-based self-healing in cementitious materials [5].

The various carrier materials utilised in cementitious materials incorporating MICP based healing systems, along with crack widths healed through MICP where reported, are summarised in table 1. For many of these studies the bacteria, in the form of vegetative cells or spores, has been immobilised along with the required nutrients and precursor chemicals. However, it has been demonstrated that it is not necessary to immobilise bacteria along with the cementation medium to enable self-healing. Khaliq and Ehsan [6] demonstrated that comparable amounts of self-healing can be achieved by immobilising the bacteria only. This particular study utilised graphite nanoplatelets to immobilise bacteria, with nutrients and precursor chemicals added directly to the concrete mix. Bundur et al. [4] used expanded shale aggregates to immobilise nutrient medium only.

Table 1. Materials utilised for immobilisation in cementitious materials incorporating MICP.

Carrier material	Immobilised constituents	Crack width healed (mm) (Curing duration)	Ref.
Graphite nanoplatelets	<i>Bacillus subtilis</i> bacteria	0.81 (28 days)	[6]
Expanded perlite	<i>Bacillus cohnii</i> bacterial spores and nutrients	0.79 (28 days)	[5]
Ceramsite	<i>Bacillus mucilaginous</i> bacteria and nutrients	0.50 (28 days)	[7]
Expanded clay particles	Calcium lactate and bacterial spores from alkaline lake soil, with 98.7% homology to <i>Bacillus alkalinitrilicus</i>	0.46 (100 days)	[4]
Diatomaceous earth	<i>Bacillus sphaericus</i> bacteria	0.17 (28 days)	[8]
Zeolite	<i>Sporosarcina ureae</i> and <i>Sporosarcina pasteurii</i> bacteria	0.10 (6 months)	[9]
Lightweight aggregate	<i>Bacillus cohnii</i> bacteria and nutrients	–	[10]
Expanded shale aggregates	Nutrient medium	–	[4]

This research utilises MICP to produce biocement through ureolysis. Ureolysis increases the alkalinity of fluid in soil pore spaces as a result of the degradation of urea to carbonate and ammonium, and induces calcium carbonate precipitation. The chemical process, as reported by De Belie [11], is detailed in equations (1) to (5).



Active ureolytic bacteria produce urease, which catalyses the hydrolysis of urea, resulting in the production of carbamate (NH_2COOH) and ammonia (NH_3), as per equation (1). Carbamate hydrolyses spontaneously to produce ammonia and carbonic acid (2). Simultaneously, in the presence of water, the ammonia and carbonic acid products equilibrate to produce ammonium (NH_4^+), hydroxide (OH^-) (3) and

carbonate (CO_3^{2-}) ions (4). This results in a pH increase. Since carbonic is a weak acid it will only partially dissociate into ions, until equilibrium is reached. In the presence of calcium, in an alkaline environment, calcium carbonate will precipitate (5). Calcium chloride dihydrate has been used as the calcium source in this research. In the presence of calcium chloride (CaCl_2), crystals of calcium carbonate, often in the form of calcite, form inside the soil matrix [12]. Calcite binds soil particles together, creating a biocement.

Due to the alkaline conditions produced during the MICP process, the type of bacteria used needs to be alkaliphilic, to enable the survival of vegetative cells. To enable long term self-healing, the bacteria will need to be able to sporulate to ensure its survival within the biocement. Ureolytic *Sporosarcina ureae* was selected for use in this research due to its spore forming ability, as tested using MacDonald and MacDonald [13] sporulation medium and the Schaeffer and Fulton [14] spore staining procedure. Ureolytic bacteria are commonly found in soils of varying type and mineralogy and in a variety of environments [15].

2. Materials and Methods

Since encapsulation and immobilisation technologies have only been used in cementitious materials to date their behaviour within soil systems is unknown. Laboratory studies have been undertaken to test the effectiveness of selected carrier materials for the immobilisation and release of a cementation medium. Subsequently a preliminary study has been undertaken whereby carriers loaded with cementation medium have been incorporated into biocement.

2.1 Microorganism, growth conditions and cell harvesting

Sporosarcina ureae was obtained from the National Collection of Industrial and Marine Bacteria, UK (NCIMB 9251, ACDP Group 1) as a freeze-dried culture. Cultures of *S. ureae* were grown on Luria-Bertani (LB) medium amended with urea. This medium comprised per litre of deionised water; 10 g tryptone, 5 g yeast extract, 10 g sodium chloride, 15 g agar powder and 20 g urea. The LB medium without urea was sterilised by autoclaving at 121°C for 15 min. A urea solution was then added to this medium using a 0.2 µm syringe filter. The medium was then allowed to cool to 55°C before pouring onto 9 cm diameter petri dishes, to minimise condensation on plates. After incubation at 30°C for 48 hrs pure single colonies from the plates were used to inoculate a liquid broth medium consisting of 5 g peptone, 3 g meat extract and 20 g urea per litre of tap water. This liquid broth medium without urea was autoclaved at 121°C for 15 min, to which a urea solution was added using a 0.2 µm syringe filter. Aseptic technique was used throughout. Multiples of 50 ml liquid broth cultures were produced as required for experiments, in 250 ml Erlenmeyer flasks incubated at 30°C, 150 rpm for approximately 19 hrs to produce cultures with an optical density at a wavelength of 600 nm (OD_{600}) of 0.9-1.2 (10^7 - 10^8 cells/ml), as measured using a spectrophotometer (Hitachi U-1900 UV-Vis).

2.2 Production of cementation medium

The standard cementation medium produced for biocementation, as per table 2, to provide the necessary nutrients and precursor chemicals for the MICP process, was adopted from Stocks-Fischer et al. [16]. To increase the efficiency of the immobilisation process, a concentrated cementation medium was produced using the chemical components and quantities listed in table 2, in deionised water. This medium was produced in 300 ml quantities. Oxoid CM001 nutrient broth was dissolved in 25 ml of deionised water, adjusted to pH 6.0 using concentrated HCl, and sterilised by autoclaving at 121°C for 15 min. The remaining chemicals, excluding calcium chloride dihydrate, were thoroughly mixed in 275 ml deionised water, using a magnetic stirrer. This solution was then adjusted to pH 6.0 using concentrated HCl, to prevent the calcium precipitating out of the calcium chloride dihydrate. Calcium chloride dihydrate was then added as a powder. This solution was then syringe filtered into the Oxoid CM001 nutrient broth solution, using a 0.2 µm syringe filter.

Table 2. Cementation medium components and sterilisation methods.

Medium component	Standard medium (g/l)	Concentrated medium (g/l)	Sterilisation method
Urea (NH ₂ (CO)NH ₂)	20	133.33	Syringe filtered
Ammonium chloride (NH ₄ Cl)	10	66.67	Syringe filtered
Calcium chloride dihydrate (CaCl ₂ .2H ₂ O)	7.35	49	Syringe filtered
Sodium bicarbonate (NaHCO ₃)	2.12	14.13	Syringe filtered
Oxoid CM001 nutrient broth	3	20	Autoclaved

2.3 Selection and preparation of carrier materials

Diatomaceous earth (Celite S) and expanded perlite (Harborlite 800), abbreviated to DE and EP respectively, were selected from carriers reported to have been used in cementitious materials incorporating MICP based self-healing systems. Availability, cost and particle size of materials were taken into consideration. Properties of these carriers are listed in table 3. Specific gravity was determined using the small pycnometer method, in accordance with BS 1377-2. Loss on ignition values are as per specifications provided by Sigma Aldrich for Celite S and Imerys Filtration Minerals for Harborlite 800.

Table 3. Powdered carrier material properties.

Carrier	Specific Gravity	Loss on ignition (%)
Diatomaceous Earth (DE)	2.01	3.2-10.0
Expanded Perlite (EP)	1.99	<1.0

In addition, natural absorbent fibres were tested for their suitability as carriers. Constituents of coir and jute fibres, as reported by Rowell and Stout [17] and Banerjee [18] respectively, are summarised in table 4.

Table 4. Constituents of Coir and Jute.

Fibre	α -cellulose (%)	Hemicellulose (%)	Lignin (%)
Jute (J)	58-63	21-24	12-14
Coir (C)	32-43	0.15-0.25	40-45

Prior to use, the fibres were washed thoroughly using a sieve and deionised water. All carrier materials were sterilised by autoclaving at 121°C for 15 min, then oven dried at 90°C. Drying at 105°C for 24 hrs was observed to result in a significant darkening of the coir fibres. Lignocellulosic fibres, which include coir and jute, thermally degrade through dehydration, depolymerization, and oxidation when heated [19]. The extent of delignification has also been reported to be dependent on the duration of heating [19]. Lignin has been reported to undergo thermal degradation at temperatures as low as 100°C [20]. For this reason, the oven temperature for drying was reduced to 90°C, for all materials for consistency, and to prevent the decomposition of sodium bicarbonate which is reported to occur at temperatures above 100°C [21].

2.4 Immobilisation capacity of carrier materials

50 ml polypropylene centrifuge tubes were filled with 1 g of carrier material, in sets of six for each carrier material type. Centrifuge tubes along with the lids were weighed before adding carriers. Concentrated cementation medium was added to each tube, to fully immerse the carrier material. The tubes were left at room temperature (approximately 21°C) for 24 hrs, then centrifuged (3200 rcf, 30 min, Varifuge 3.0 Heraeus) to separate the liquids and solids, after which the supernatant was carefully poured from the tubes, leaving the solids remaining. For tubes containing fibres, these were then inverted for 2 hrs and the liquid drained after, to remove any excess adsorbed medium. Since there was still a

small amount of EP at the surface of the supernatant following centrifugation, this was gravity filtered to establish mass, this having found to be between 4 mg and 9 mg and hence negligible. The tubes containing the carriers were then oven dried at 90°C until the mass was constant. After weighing, to determine the mass of solid cementation medium immobilised, the process was repeated. For those materials for which the overall mass had increased for all six samples, a third loading with cementation medium was undertaken.

2.5 Cementation medium release from carrier materials

A preliminary test was first undertaken using samples from the immobilisation test, to determine the amount of water to be added to samples. 0.5 g carrier material was added to 50 ml polypropylene tubes in sets of four for each material and loaded once with cementation medium, as per the process outlined above. Deionised water was then added to each tube. The quantity of water used was equal to 25 times the mass of dried cementation medium in each tube. These tubes were then left at room temperature (approximately 21°C), with one tube being drained after 1 hr, the second after 3 hrs and the third and fourth after 6 and 24 hrs respectively. Tubes were then dried and weighed to quantify loss of cementation medium. These results gave an indication of potential release of cementation medium for self-healing following water ingress into the biocement. This test was repeated three times. An additional test for a period of 50 hrs was later undertaken under the same conditions.

2.6 Microbiologically induced CaCO₃ precipitation in aqueous media

This test was undertaken to establish whether sufficient quantities of each component of the cementation medium had been immobilised by the carrier materials to enable MICP. For each carrier material, six 50 ml centrifuge tubes were filled with 1 g of carrier material and loaded once with cementation medium. 20 ml autoclaved sterilised deionised water was added to each tube. The liquid was drained from the tubes after 24 hrs and syringe filtered after measuring the pH. Standard cementation medium was used in the control tubes. Before these solutions were inoculated with *S. ureae*, the drained tubes were dried and weighed, to enable the concentrations of cementation medium in the solutions to be determined. A small amount of additional deionised water was then added to the tubes containing cementation medium released from jute and the standard cementation medium, to ensure that each set of six tubes contained solutions with an average cementation medium concentration of 3.6 %.

Phosphate buffered saline (PBS) was prepared using 8 g NaCl, 1.42 g Na₂HPO₄ and 0.24 g KH₂PO₄ per litre of deionised water and autoclaved at 121°C for 15 min. Twenty-four 50 ml centrifuge tubes were each filled with 10 ml of freshly cultured liquid broth of *S. ureae*. After centrifugation at 3200 rcf for 20 min, and washing twice with PBS, the tubes containing the vegetative *S. ureae* bacterial cells were filled with the prepared solutions containing cementation medium and incubated at 30°C, 150 rpm. After one day of incubation, three of each set of six tubes were removed and the contents filtered using a vacuum driven Millipore filter system and 0.2µm filter papers. The pH was measured. 10 µl samples were then taken and added to 990 µl autoclaved deionised water to make 1:100 dilutions, to determine calcium ion concentrations using ICP-OES. This process was repeated after three days of incubation for the remaining samples.

2.7 Cementation of sand through microbially induced CaCO₃ precipitation

To harvest the vegetative *S. ureae* cells required for the biocement production, six 50 ml polypropylene centrifuge tubes were each filled with 19 ml of liquid broth culture, this quantity equating to the estimated pore space to be filled within the sand in each 50 ml tube. The approximate pore volume had been established via the wet pluviation technique using deionised water and a vortex mixer to vibrate and densify the saturated sand in a 50 ml centrifuge tube, which also gave an estimate of the mass of sand required. Tubes containing the liquid broth were centrifuged at 3200 rcf for 20 min. The supernatant was then drained and replaced with 19 ml PBS, followed by centrifugation at 3200 rcf for a further 20 min to wash any remaining metabolic waste and metabolism by-products from the cells. This washing was repeated twice. After draining the PBS, this was replaced with 19 ml of cementation medium.

Using a funnel secured 5 cm above the tube, tubes containing the bacteria and cementation medium were filled in triplicates, with sand only and then a premixed sand and loaded EP mixture. The EP had been mixed with the sand using a pestle and mortar. Once the tubes were half full, and again after the tubes were almost filled, these were vibrated to densify the sand. The lids were placed on the tubes which were then inverted in an incubator at 30°C. After 24 hrs a 1 mm hole was pierced in the base of the tubes, through which the effluent was extracted using a vacuum, as per figure 1, utilising a flask in which the centrifuge tube was held firmly within the neck.

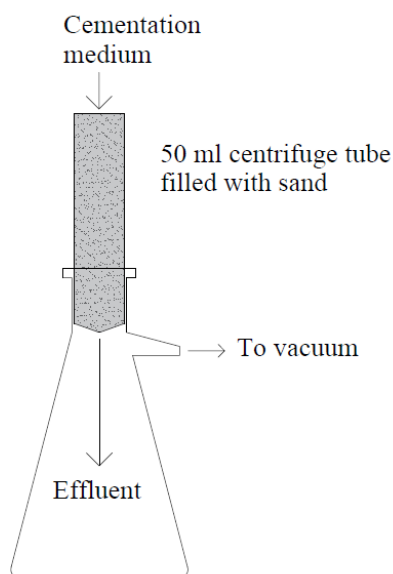


Figure 1. Vacuum assisted extraction of effluent and percolation of cementation medium.

The effluent was syringe filtered for ICP-OES analysis of calcium ion content. This was replaced with cementation medium via surface percolation. Excess cementation medium was drawn through the sand using a vacuum, while continuing to pour cementation medium into the top of the tube, so that approximately 1.5 times the pore volume was used to flush through any remaining effluent. This process was repeated for all tubes, with the holes then being covered after and tubes returned to the incubator inverted. The extraction of the effluent and addition of cementation medium was repeated on weekdays over a 14-day period. After three weeks of drying at 30°C, samples were taken from the biocement to test for the presence of spores using the plate counting method.

3. Results

3.1 Cementation medium storage

The immobilisation capacity of the carrier materials, following up to three loadings with concentrated cementation medium, is shown in Figure 2. Coir was loaded twice only since following the second loading there had been some mass reduction in four of the six tubes, this can be attributed to the dissolution of immobilised cementation medium into the surrounding liquid cementation medium. This effect, albeit to less of an extent, was also noticed after the second loading of expanded perlite. It was evident that the immobilisation capacity had been reached after the first loading of coir fibres, and after the second loading of EP. The capacity of coir to immobilise cementation medium was relatively low and the variable fibre thicknesses likely contributed to inconsistencies in results, this carrier is therefore not used in later tests.

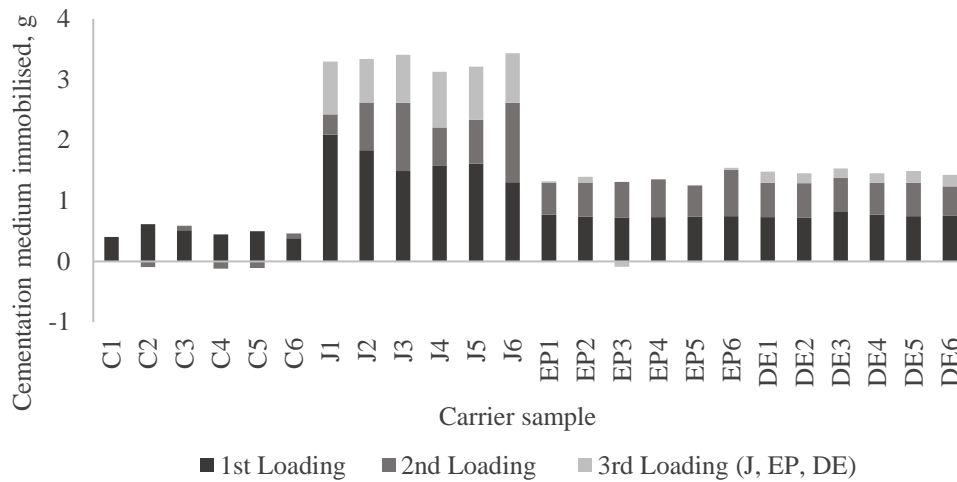


Figure 2. Quantities of cementation medium immobilised by one-gram samples of coir (C), jute (J), expanded perlite (EP) and diatomaceous earth (DE), following repeated loadings.

3.2 Release of cementation medium

Percentages of immobilised cementation medium released from the carrier materials over a period of up to 50 hrs are shown in figure 3. The rate of release of cementation medium from jute within the first 4 hrs is the fastest of the carriers tested. Following this initial fast release from jute equilibrium appears to have been reached with the surrounding solution. This quick release may be beneficial to the self-healing process, in addition to the potential to retain cementation medium for later release. For the first 9.5 hrs cementation medium release is the slowest from EP. Figure 3 shows the R^2 values of the fitted regression curves, these values being close to 1 for cementation medium release from EP and DE. Results for EP and DE show similar trends, with regression lines converging at approximately 30 hours. The rate of cementation medium release from DE then slows slightly compared to EP after 34 hours. The lower R^2 value of 0.65 for cementation medium release from jute may be attributed to the slight variations in length and thickness of the individual fibres within samples, and therefore variations in surface area of fibres. More testing is required to better understand the behaviour of the jute fibres in respect of the release of immobilised chemicals.

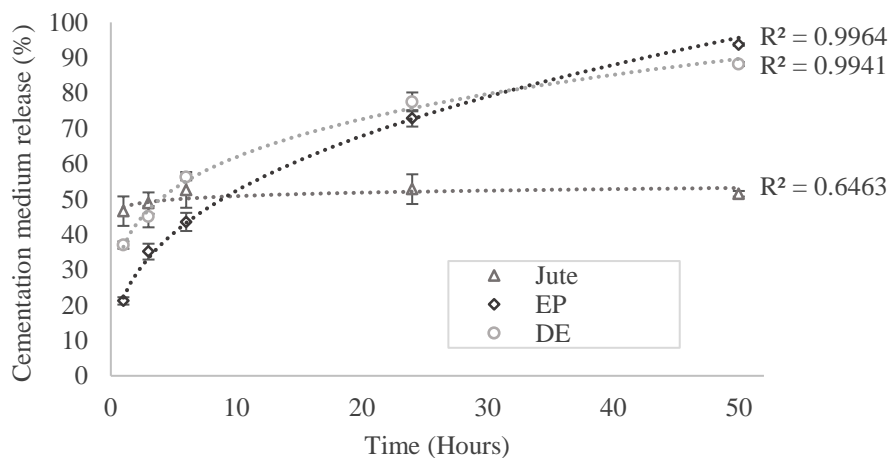


Figure 3. Percentage of immobilised cementation medium released from carrier materials over a fifty-hour period, at 21°C.

3.3 MICP in aqueous solutions

On the basis of calcium ion depletion in solutions, as shown in figure 4, it can be deduced that all carriers tested immobilise and release the cementation medium constituents to the extent that MICP can be enabled. This is further supported by results shown in figure 5, which show a pH increase following inoculation of solutions with *S. ureae*. This pH rise is indicative of MICP and is consistent across all samples. The solutions containing cementation medium constituents which had been immobilised by and released from jute have a noticeably lower pH prior to inoculation with *S. ureae*. The lower reduction in calcium ion concentration for these samples suggests that the jute fibres may not be immobilising or releasing the cementation medium constituents as effectively as EP or DE. However, a slower release over time could help ensure that more nutrients and precursor chemicals are retained by the carrier material during biocementation. These results again suggest that more testing is required on jute prior to use within biocement. The calcium ion reduction in the solutions containing cementation medium which had been immobilised by DE is greater than that of the control solution. This result indicates that the use of DE as a carrier for cementation medium may have a beneficial effect on the MICP process.

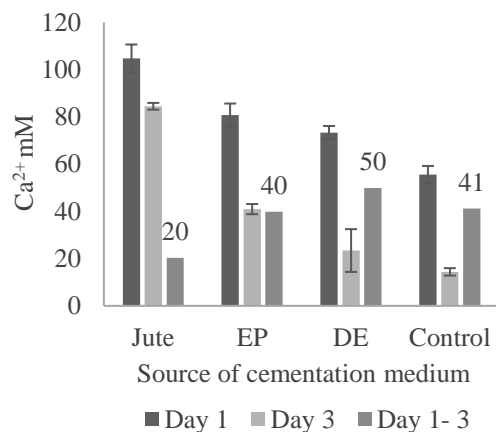


Figure 4. Calcium ion concentration of solutions containing cementation medium released from carriers and standard medium as the control, measured one day and three days after inoculation with *S. ureae*.

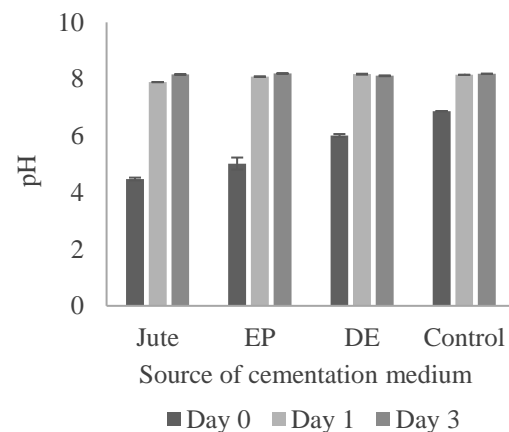


Figure 5. pH of solutions containing cementation medium released from carriers, compared to standard cementation medium, before (Day 0) and after inoculation with *S. ureae*.

3.4 Biocementation

Results from the biocementation tests are shown in figures 6 and 7. Elevated calcium ion levels in the effluent extracted from the tubes containing cementation medium loaded EP, in comparison to the controls, up to day five, can be attributed to leaching of cementation medium constituents from carriers. This appears to have accelerated the biocementation process. At day fourteen, after ten cementation medium treatments, the calcium ion content of the effluent drained from tubes containing loaded EP drops below that of the controls, at this point the test had been ended since the flow in tubes containing the loaded EP had stopped as a result of pores in the sand being plugged with calcium carbonate. Similarly, Stocks-Fischer et al. [19] reported that the flow in biocement columns stopped after ten days of medium treatment, after supplying the medium continuously via gravity flow. For those tubes containing the sand only, further treatments would have been required before flow ceased. Plate counts from samples taken from the dried biocement showed variable results and were deemed unreliable, the growth of cultures on plates did however indicate the presence of viable spores within the biocement.

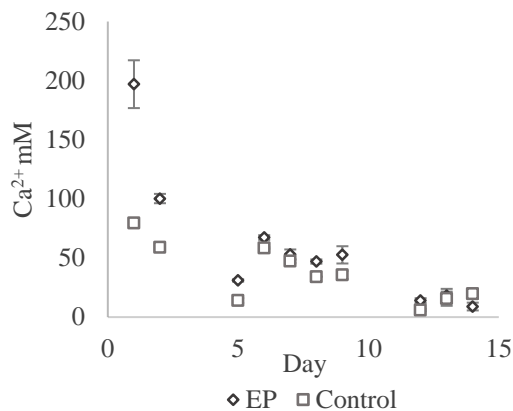


Figure 6. Calcium ion concentrations of effluent extracted during biocementation.

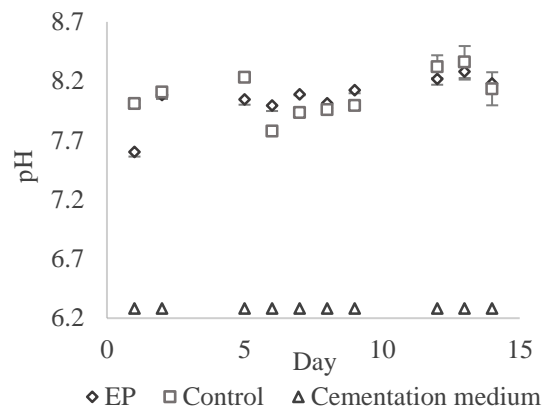


Figure 7. pH of effluent extracted during biocementation, compared to the initial pH of the cementation medium.

Figure 6 shows a significant reduction in calcium ion concentration in the effluent extracted on days 5 and 12, when compared the calcium ion concentration in effluent extracted on days 2 and 9 respectively. This decrease follows weekend periods during which there are no cementation medium treatments and hence calcium ions are depleted. The pH increase shown in figure 7, when comparing the pH of the effluent to that of the cementation medium treatments, is indicative of MICP.

4. Conclusions

Results obtained demonstrate that diatomaceous earth, expanded perlite and jute fibres have the potential to be utilised for the immobilisation and supply of the required nutrients and precursor chemicals to enable self-healing via MICP. Furthermore, diatomaceous earth has been shown to have a beneficial effect on the MICP process by increasing calcium carbonate precipitation. Coir was deemed unsuitable due to the low capacity for immobilisation of cementation medium when compared to other carriers tested.

To ensure longevity of cementation medium supply for self-healing, the retention of cementation medium components during the biocementation process requires improvement. The next stage of this research will involve testing the effectiveness of geopolymer coatings consisting of calcined kaolin and sodium silicate applied to the powdered carriers, to reduce loss of immobilised cementation medium during biocementation. The behaviour of fibres such as jute in respect of immobilisation and release of cementation medium will also be explored further.

The production of the biocement was achieved through the use of vegetative bacteria. Self-healing will require production and germination of spores. *S. ureae* has been selected for use in this study due to its spore forming ability, it presents the potential to be used for self-healing purposes, the presence of spores in the biocement will need to be quantified in future tests using a reliable method.

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