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Application of expanded perlite encapsulated bacteria and growth media for self-healing concrete

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HIGHLIGHTS

- The crack healing ability of spores and nutrients encapsulated separately was proven.
- Crack healing requires an appropriate ratio of spores to calcium salt.
- A minimum number of bacterial spores are required for the healing process.
- Addition of growth components to the media results in greater calcite precipitation.

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ABSTRACT

Self-healing concrete based on calcium carbonate precipitation induced through bacterial activity has been investigated in recent years by teams around the world. For the first time, optimisation of the self-healing performance was considered in terms of the number of bacterial spores required, the concentration and composition of nutrients and precursors, and whether a two-component system was likely to efficiently produce self-healing in concrete. This information is required if efficient and cost-effective self-healing systems based on bacterial activity are to be implemented. For this research, coated expanded perlite was used to immobilise bacterial spores and encapsulate nutrients as two separate components for self-healing concrete. Self-healing capacity was evaluated by imaging and by initial surface absorption of water. The results indicated that healing could be achieved when coated expanded perlite containing self-healing agents was used as a 20% replacement of fine aggregate and if a suitable ratio of spores to calcium acetate was provided. This research is the first to show that self-healing is not simply a requirement of having sufficient healing compounds (e.g. calcium acetate) but that a minimal number of bacterial spores are also required to ensure that sufficient cells take part in the healing process.

1. Introduction

The effect of some water-borne ions (e.g. chlorides) on the durability of reinforced concrete is well documented, and cracked concrete has been shown to be more susceptible to permeation of these deleterious ions. Consequently, research is being undertaken in an attempt to develop concrete that can self-heal cracks; potentially reducing repair and maintenance costs on key infrastructure [1–3]. One approach to autonomic self-healing is the utilization of microbiologically induced calcite precipitation (MICP). This approach utilises the metabolic activity of bacteria and biomineral precursors embedded within the concrete to form an inorganic compound as a healing material. This is usually calcium carbonate, typically in the form of calcite but sometimes as vaterite [4,5]. This healing material can precipitate in small cracks soon after they form and it has the potential to limit the permeation of water and dissolved ions. Thereby the life of concrete structures can be extended without the need for manual intervention; which can be both costly and dangerous, particularly for structures with poor access.

While there have been a number of studies into the feasibility of using MICP for self-healing in concrete, there have not been studies on optimising the self-healing performance through consideration of the number of bacterial spores required, the concentration and composition of nutrients and precursors or whether a two-component system is likely to efficiently produce self-healing in concrete.

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There are three key pathways for delivering a MICP healing process: (i) enzymatic hydrolysis of urea [6], (ii) dissimilation of nitrates [7] and (iii) aerobic metabolic conversion of calcium salts [8]. In the aerobic metabolic conversion pathway healing occurs because the bacteria induce the oxidation of an organic calcium salt (precursor), for example calcium acetate or calcium lactate, to calcium carbonate under favourable conditions. These include environmental conditions for the bacteria to thrive (appropriate temperatures, pH and other environmental factors), and the presence of water, oxygen and nutrients for the bacteria to grow. The by-products of the conversion of calcium acetate to calcium carbonate are carbon dioxide and water, both of which are compatible with concrete (Eq. (1)). Furthermore, a weak carbonic acid may form in the presence of carbon dioxide and water that can lead to carbonation of calcium hydroxide within the concrete (Eq. (2)). This leads to a form of enhanced autogenous healing as the carbonated molecule is larger than the uncarbonated version [9].

$$Ca(C_4H_6O_4) + 4O_2 \rightarrow CaCO_3 + 3CO_2 + 3H_2O \quad (1)$$

$$3CO_2 + 3Ca(OH)_2 \rightarrow 3CaCO_3 + 3H_2O \quad (2)$$

Most bacteria-based self-healing concrete systems require spores to be immobilised and separated from any germination triggers, normally via encapsulation, prior to their addition to concrete. This also overcomes concerns with their viability in the aggressive conditions that occur in hydrating concrete [5,8]. Three standard approaches to immobilization have been studied: (i) encapsulation in porous solids, (ii) microencapsulation in gels [10], and (iii) use of pellets and flakes [11].

In addition to the encapsulation of the spores, the extra components required for self-healing (the calcium precursor) and to aid germination of the spores and growth of the cells (usually just yeast extract) need to be included into the concrete. In many self-healing systems, these extra components are added directly to the concrete during the mixing process: this is partly due to difficulties with encapsulating water-soluble compounds [10]. When added directly to concrete these compounds may affect the setting and hardening of concrete, but there has been little consensus on this to date. Reviews of these factors are provided in Paine [12] and De Belie et al. [13] but these do not include an in-depth investigation into how the compounds influence fresh or hardened concrete properties or how they affect the self-healing efficiency.

Because of concerns over the effect of the additional components on cement hydration, Wiktor and Jonkers [14] encapsulated calcium lactate (6% by mass of aggregate) and yeast extract (less than 0.1% by mass of aggregate) along with the spores in 1–4 mm expanded clay aggregates in order to eliminate as much as possible any effect on early-age properties. Impregnation of *Bacillus alkalinitriculus* spores was carried out twice under vacuum. It was shown that upon cracking these encapsulated particles were capable of providing healing in mortars. There was no significant effect on setting, which demonstrates that there was no leaching of detrimental compounds from the expanded clay aggregates. More recent research has used expanded perlite to immobilize spores of *Bacillus cohnii*. The volumes used were microscopically measured to be 3.6×10^9 cell/ml [15]. Calcium lactate (8 g/l) and yeast extract (1 g/l) were sprayed onto the surface of the particles but were not encapsulated or prevented from interfering with hydration reactions.

Diatomaceous earth, a fine porous powder, has also been considered as a carrier for bacterial spores for self-healing concrete applications [16]. However, the spores were found to sorb on the surface of the particles and not within the powder itself, while the bacteria were shown to maintain their ureolytic activity. In other work, Erşan et al. [7] investigated expanded clay particles of 0.5–2 mm in size as carriers of bacterial cells to precipitate calcium carbonate from conversion of calcium nitrate and calcium formate. In this case, impregnation of the particles was carried out under vacuum saturation. The resulting particles contained approximately 10% by mass of cells of *Diaphorobacter nitroreducens* or *Pseudomonas aeruginosa* and 0.15 M sodium chloride. No additional protection was considered necessary to prevent leaching of the cells from the particles. It is worth noting that using sodium chloride as a precursor may raise concerns relating to corrosion of reinforcement in concrete because of an increase in chloride ions.

Essential to self-healing concrete is a requirement for a sufficient quantity of Ca^{2+} to be available in the concrete to enable sufficient calcium carbonate to form and fill cracks. Conversion of the soluble calcium precursor to relatively insoluble calcium carbonate relies on the presence of bacterial cells. Because cells grow and multiply it has been considered that healing can be generated initially in the presence of relatively few cells. However, it has been shown that the spore concentration necessary to deliver calcium carbonate precipitation needs to be greater than 4×10^7 spores/ml [17]. Interestingly, it was suggested that the required spore concentration may be independent of calcium precursor content.

Any method that includes encapsulation of spores, precursors and nutrients in the same capsule can create a problem with ensuring that germination of the spores does not occur within the aggregate. Therefore a dual approach in which the spores and other ingredients are encapsulated separately has potential benefit.

In this study, expanded perlite (EP) was used as a carrier of spores, precursor and essential nutrients. Differently from earlier work, the (i) spores and (ii) the precursor and nutrients were encapsulated separately. Upon cracking of concrete, both the EP containing spores and the EP containing the precursor and nutrients in the crack zone were expected to break and allow the two to come together, provided that there was sufficient water to carry the water-soluble precursor and nutrients to the spores, or vice versa. Spores would then germinate and precipitate calcium carbonate crystals to heal the cracks. The aim of this research was to demonstrate the suitability of a two-component encapsulated system and ascertain the necessary ratio of spores to precursor needed to ensure healing. In order to fully understand the processes involved, microbiological experiments were undertaken to determine spore germination and carbonate productivity.

2. Materials and methods

2.1. Bacterial strain

Bacillus pseudofirmus DSM 8715 (German collection of microorganisms and cell cultures (DSMZ)) was used in this study. Living cells were routinely cultured on buffered lysogeny broth (LB) which contained 100 ml/l Na-sesquicarbonate to achieve pH 9.5. Spores were prepared in a sporulation media [8] and incubated at 30 °C on an orbital shaker for 72 h. Spores were harvested by centrifugation at 10,000 rpm for 15 min. Spore formation was confirmed by phase contrast microscopy. The spore pellet was washed three times with a chilled 10 mM Tris HCl buffer, pH 9.5. The spore pellet was then freeze dried to obtain a spore powder and stored in a desiccator prior to use.

2.2. Growth media

Three growth media were investigated (Table 1). GM1 consisted of a multi-component media based on initial microbiological studies and was selected to maximise as much as possible the germination of bacterial spores, growth of bacterial cells, precipitation of calcite and sporulation of bacteria [5]. GM2 consisted of just three

Table 1
Composition of the three growth mediums investigated (g/l).

Ingredients	GM1	GM2	GM3
Growth components			
Calcium acetate	7.1	5.0	100.0
Sodium citrate	2.0	0.0	0.0
Yeast extract	2.2	1.0	4.0
Dextrose	0.0	1.0	4.0
Na-glutamate	0.4	0.0	0.0
Alanine	0.2	0.0	0.0
Inosine	0.2	0.0	0.0
$MgCl_2$	0.2	0.0	0.0
NaCl	0.2	0.0	0.0
$MnSO_4$	0.1	0.0	0.0
KH_2PO_4	0.1	0.0	0.0
Buffering components			
100 mM $Na_3C_6H_5O_7$	5.2	5.2	5.2
50 mM $NaHCO_3$	4.2	4.2	4.2
25 mM Na_2CO_3	5.3	0.0	0.0

ingredients: calcium acetate (as the precursor), and yeast extract and dextrose as the nutrients. The ingredients were used in proportions similar to those in research elsewhere [14,15]. GM3 consisted of the same three constituents as GM2 but in much higher proportions, such that the solution was almost saturated with these components. When used in microbiological experiments, the growth media were buffered with tri-sodium citrate, sodium bicarbonate and sodium carbonate (in the case of GM1) to provide an alkaline environment suitable for growth of *B. pseudofirmus*.

2.3. Expanded perlite

EP is a lightweight, amorphous, mineral aggregate commonly used in horticultural applications in plant growth media. The particle size distribution of the EP as determined in accordance with BS EN 933-1 is shown in Fig. 1 and it conformed to a 0/4 mm aggregate according to BS EN 12620. The EP had a water absorption capacity of 146%, an apparent density of 292 kg/m^3 and a loose dry bulk density of 122 kg/m^3.

2.4. Encapsulation process

The EP particles were impregnated with the nutrients and bacteria by soaking in an appropriate volume of liquid until all liquid was absorbed. For immobilization of spores, the EP was soaked in a spore-containing suspension that resulted in a concentration of approximately 4.1×10^9 spores per g of EP.

For encapsulation of the nutrients, the EP was soaked in a solution containing calcium acetate and yeast extract of similar composition to growth medium GM2. The concentration of the nutrients per g of EP was approximately 0.3 g calcium acetate and 0.03 g yeast extract.

The EP particles were then coated with a dual layer of sodium silicate solution and cement powder to prevent leaching of the spores and nutrients into the mortar. Firstly, an initial layer of sodium silicate was applied by soaking the impregnated EP in sodium silicate solution until the surface of the EP was completely wet. The EP particles were then dried at 20 °C for 24 h. A second layer of sodium silicate was then applied to the EP, as above, followed by the application of dry cement to the wet sodium silicate surface. The cement powder reacted with the water in the EP and in the sodium silicate solution, as well as the soluble compounds in the sodium silicate, to produce a brittle shell. The coated EP was then cured in water for 48 h. The mass of the coating was approximately 70% of the overall mass of the coated EP and consequently the number of spores was approximately 8.2×10^8 spores per g of coated EP. The resulting coated EP particles are henceforth referred to as coated EP containing spores (CPS) and coated EP containing nutrients (CPN) throughout the paper. The effect of the coating on the particle size distribution is shown in Fig. 1 and resulted in aggregates with less than 1% of particles passing a 2 mm sieve; compared with approximately 50% passing this sieve prior to coating.

2.5. Germination of bacteria in growth media

Spore germination in the three growth media was determined by a qualitative procedure involving the percentage decrease in OD_{600} (optical density measurement at 600 nm) of spore suspen-

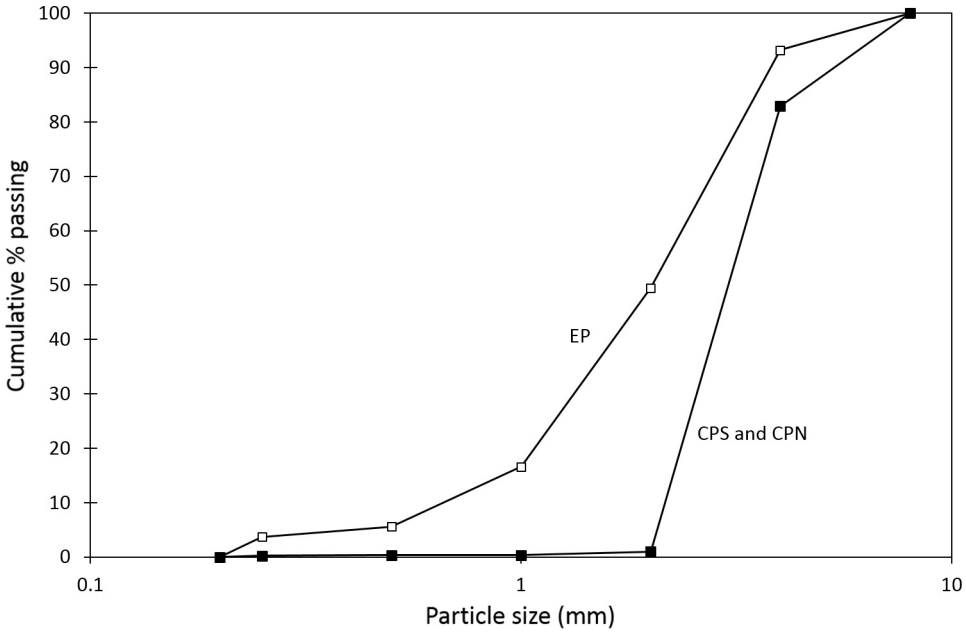

Fig. 1. Particle size distribution of EP and coated EP (CPN and CPS).

sions during germination [18–19]. Spores were suspended in the growth media and incubated at 30 °C at 150 rpm in an orbital shaker. Sample solutions were obtained every two hours up to eight hours and overnight.

2.6. Carbonate productivity in growth media

From the cultures of *B. pseudofirmus*, ten-fold serial dilutions were taken to obtain samples with cells (CFU) ranging from 7.4×10^9 to 7.4×10^{10} (7.4×10^9, 1.85×10^{10}, 3.7×10^{10}, 5.55×10^{10}, 7.4×10^{10}). Cells were added to 10 ml of each of the growth media (triplicates of each) as shown in Table 2. Growth media without the addition of cells were observed for comparison. Cultures were incubated on a shaker at 30 °C and at 150 rpm for eight days. Following this they were withdrawn and centrifuged at 10,000 rpm for 10 min. After decanting the supernatant, 2 ml of sterile water was added to the pellet and mixed until a homogenous suspension was obtained. This homogeneous suspension was subjected to filtration using three μ nitrocellulose membrane, and calcium carbonate crystals were collected from the membrane. The crystals were dried for 72 h at 50 ± 2 °C and then weighed.

2.7. Application of perlite encapsulated spores and nutrients in mortar

2.7.1. Preparation of mortar specimens

A series of mortar mixes was produced using Portland fly ash cement (CEM II/B-V), standard sand (BS EN 196-1) and tap water. The sand to cement ratio in the control mortar (MC) was 3.0 and the water to cement ratio was 0.5 by mass. Mortar mix proportions (in g) are given in Table 3. Combinations of CPN and CPS were added to the concrete as self-healing agents as a combined replacement of 20% by volume of sand. In mortar M100 the sand was replaced with CPN only. In M90 to M50, a combination of CPN and CPS were added in ratios of 9:1, 4:1, 7:3, 3:2 and 1:1, respectively. The mix number reflects the percentage of CPN to total coated EP (CPS + CPN) by volume.

Mixing was carried out in accordance with BS EN 196-1, with the coated perlite added at the same time as the sand. The specimens produced were disks with a diameter of 100 mm and thickness of 10 mm. After casting, the moulds were placed in a controlled environment room (20 °C, 40% RH) for 24 h. All specimens were demoulded at 24 h and stored in water at 20 °C until an age of 28 days.

Table 2
Approximate number of bacteria cells per g of calcium acetate for the carbonate productivity test.

Approx. cells per ml of growth media	GM1	GM2	GM3
7.4×10^9	1.0×10^{11}	1.5×10^{11}	7.4×10^9
5.6×10^9	7.9×10^{10}	1.1×10^{11}	5.6×10^9
3.7×10^9	5.2×10^{10}	7.4×10^{10}	3.7×10^9
1.9×10^9	2.7×10^{10}	3.8×10^{10}	1.9×10^9
7.4×10^8	1.0×10^{10}	1.5×10^{10}	7.4×10^8

2.7.2. Crack creation and healing

After 28 days the specimens were dried at room temperature for 24 h, and the perimeter of each of the specimens was wrapped with carbon fibre reinforced polymer strips to enable a controlled width crack to be generated without the specimen falling apart. Specimens were subjected to cracking using a splitting test. Load was applied to the specimens and crack opening was measured using a linear variable differential transformer (LVDT). Load was applied to maintain a crack growth of 25 μm per minute. Loading was stopped when a crack width of 350 μm was achieved. After cracking the specimens were placed in self-contained sealed containers and situated on spacers so that they were 2 mm above 100 ml of water, resulting in a relative humidity close to 100%. The containers were placed in an incubator at 30 °C for 165 days.

2.8. Investigation of self-healing efficiency

2.8.1. Visualisation of crack-filling

Visualisation of crack filling was performed under a Leica M205C light microscope. Detection of calcium carbonate was carried out using Fourier transform infrared spectroscopy (FTIR).

2.8.2. Initial surface absorption

There is no established test for determining the ability of self-healing mortar to resist the ingress of water or other constituents or for recovery of impermeability despite recent attempts to establish a suitable test method [20]. For this research a scaled-down and slightly modified version of the initial surface absorption method in BS 1881-208, as previously described by Sharma et al. [5], was used to determine the rate at which water is absorbed into the surface of the mortar specimens and to quantify the reduction in water ingress as a result of healing (Fig. 2). This was used as a proxy for quantifying the extent of self-healing. Although the test does not measure the bulk permeability of the concrete, it provides a measure of the quality of near surface properties by the rate at which water absorbs into the surface of concrete. A cap of 1600 mm^2 was sealed to the surface and filled with water. The rate at which the water was absorbed into the mortar under a pressure head of 200 mm was measured by movement along a capillary tube attached to the cap. The rate of surface absorption was measured at intervals of 10, 30, 60 and 120 min from the start of the test.

3. Results

3.1. Bacteria germination and growth

The growth curves of *B. pseudofirmus* in the three calcite precipitating media (GM1, GM2 and GM3) are presented in Fig. 3. Growth conditions in GM3 were more favourable than in GM1 and GM2.

Table 3
Mix designs for mortar samples.

Mix	CPN:CPS, by volume	Constituents, g					
		Water	Cement (CEM II/B-V 32.5 N)	Standard sand	CPN	CPS	Approximate number of spores per g of calcium acetate
MC	–	225	450	1350	–	–	0
M100	100:0	225	450	1080	108	0	0
M90	90:10	225	450	1080	97	10	1.4×10^9
M80	80:20	225	450	1080	87	21	3.2×10^9
M70	70:30	225	450	1080	76	31	5.5×10^9
M60	60:40	225	450	1080	65	41	8.6×10^9
M50	50:50	225	450	1080	54	51	13.0×10^9

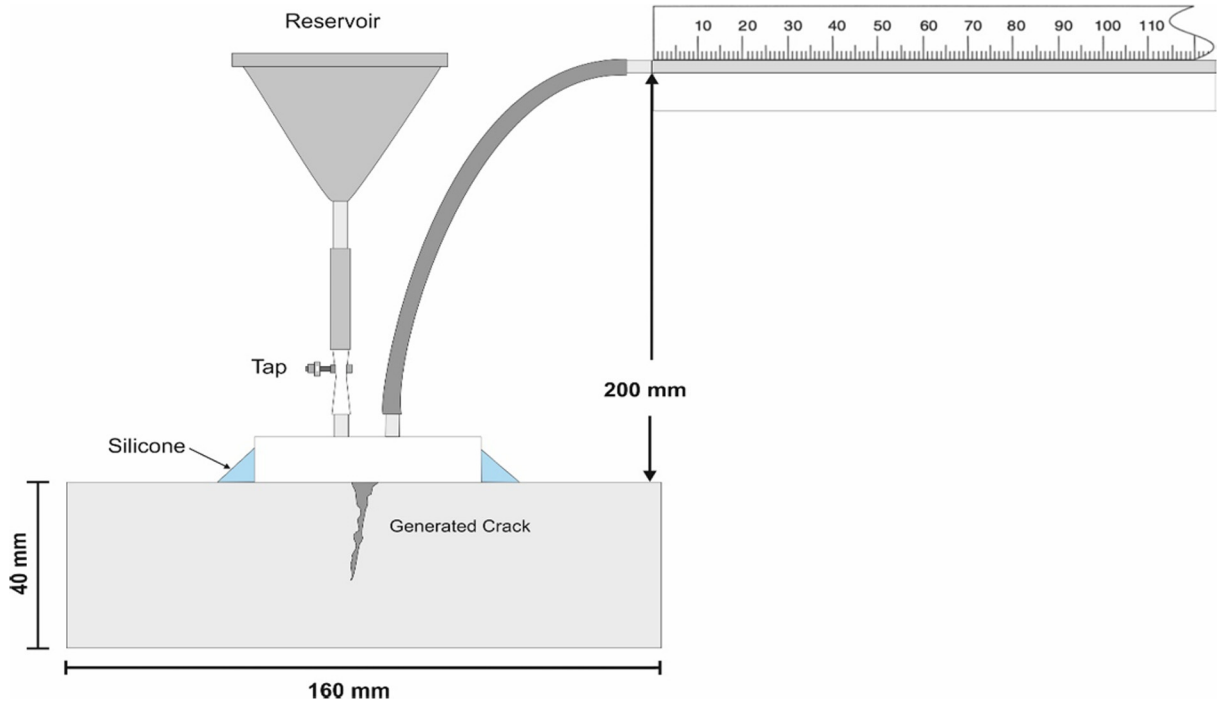

Fig. 2. Modified initial surface absorption test.

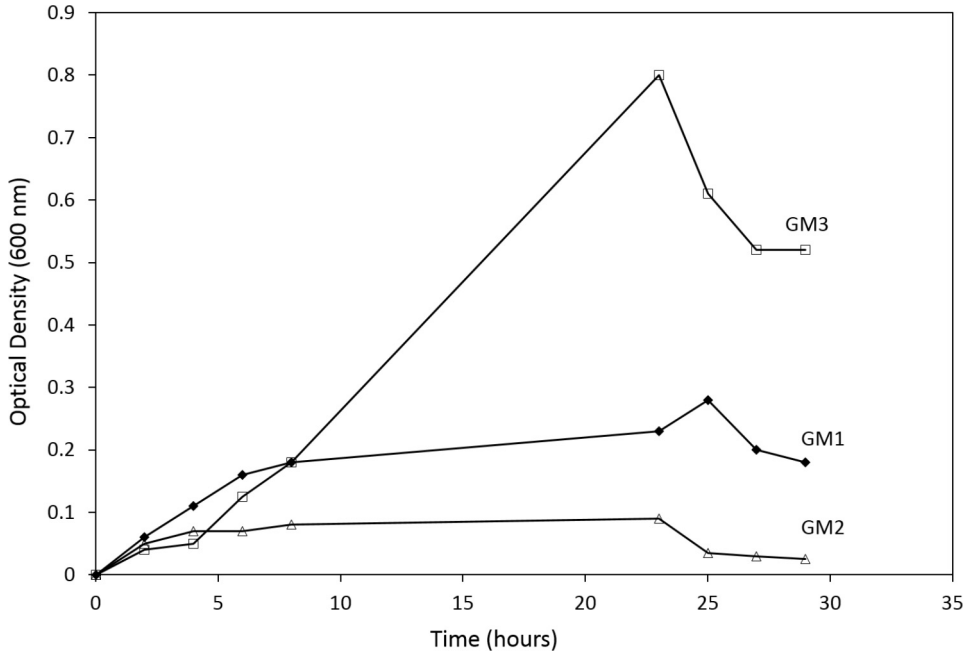

Fig. 3. Growth of bacteria in the three growth media.

However, growth in GM1 was faster during the first eight hours. This is not surprising as this growth medium was specifically designed to maximise germination and growth. However, overnight (8–23 h) growth in GM3 was greatest until the end of the test. This may be due to the greater amount of calcium acetate and quantity of energy sources (yeast extract and glucose) compared to GM2 and GM1. While the speed at which self-healing occurs is important in preventing the ingress of water, it is unlikely that a few hours delay in the initiation of self-healing will have a marked effect on the final outcomes.

3.2. Carbonate productivity by growth media

Fig. 4 shows the quantity of $CaCO_3$ produced in each of the growth media. It can be seen that as the number of bacterial cells added to the growth media increases, the mass of $CaCO_3$ precipitated after 8 days also increases in each case.

As expected, the mass of $CaCO_3$ precipitated from each of the growth media for a given concentration of cells differs given the differences in calcium acetate and nutrients used. For example, with the addition of 5.6×10^9 cells/ml it can be seen that GM1

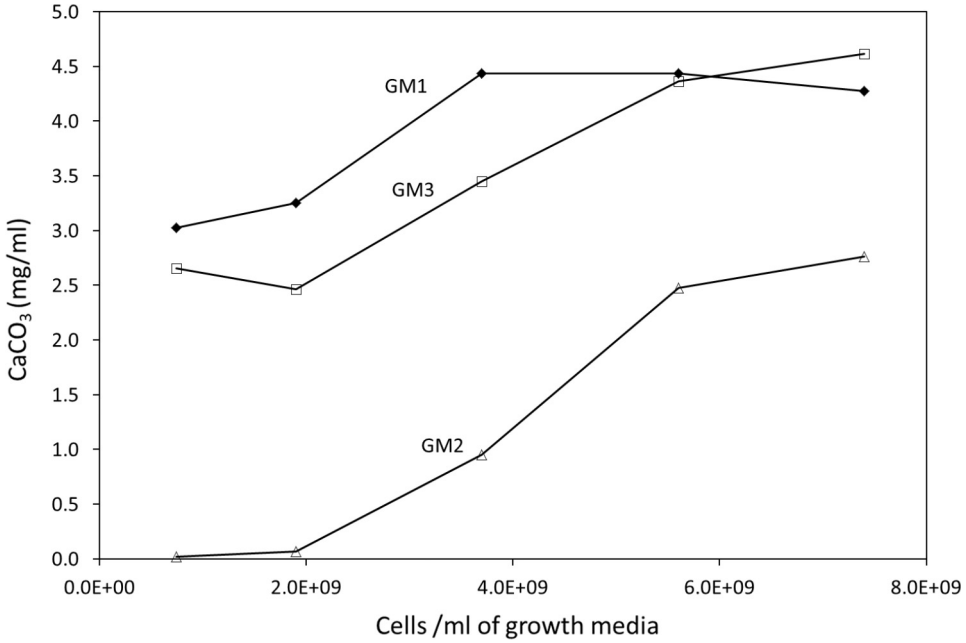

Fig. 4. Effect of cell concentration on calcium carbonate formation for all three growth media.

and GM3 produce approximately 4.5 mg/ml of $CaCO_3$, whilst GM2 resulted in around 2.5 mg/ml of $CaCO_3$.

It is therefore necessary to consider the amount of $CaCO_3$ precipitated in relation to the availability of calcium acetate in each of the growth media. Fig. 5 shows the same data normalised to the quantity of calcium acetate and includes the maximum stoichiometric $CaCO_3$ amount which would indicate that all of the precursor is converted to $CaCO_3$.

In the case of GM1 it can be seen that the use of 5×10^{10}–1×10^{11} cells per g of calcium acetate (equating to 3.7–7.4 $\times$ 10^9 cells/ml) produced approximately 0.62 g of $CaCO_3$ for every g of calcium acetate. This is close to the stoichiometric maximum of 0.63 (derived from Eq. (1)). This suggests that the conversion of calcium acetate to calcium carbonate was nearly 100% efficient with this number of cells. However, in the case of GM3 where a much larger proportion of calcium acetate was used in the growth media (100 g/l), this number of cells was unable to convert much of the calcium acetate to calcium carbonate. So whilst the actual mass produced was similar to that of GM1, the efficiency of conversion was much less at around 8%.

GM2 contained a similar amount of calcium acetate as GM1 and consequently showed a similar magnitude of conversion of calcium acetate to calcium carbonate as GM3. However, it can be seen that the conversion was much lower for all cell to calcium acetate ratios. This most probably represents the lack of additional growth components in this media and will be discussed later.

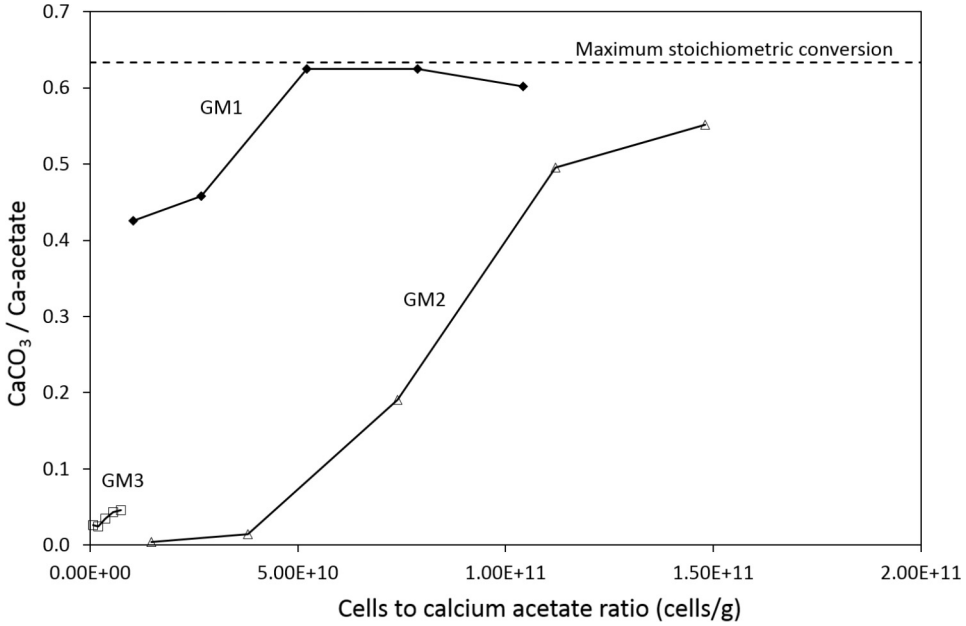

Fig. 5. Effect of cell concentration on calcium carbonate formation for all three growth media normalised per g of calcium acetate.

3.3. Self-healing of mortars

3.3.1. Visualisation of crack filling

Fig. 6 shows the cracks after 165 days, where it can be seen that the controls MC and M100 showed no healing. Furthermore, no visual healing was observed in M90, M80 or M50. However, images of M70 and M60 showed apparent complete crack filling, at least at the surface. It can be postulated that in M60 and M70 there is an optimum ratio between the number of spores and the volume of nutrients to germinate those spores to cells, and a suitable concentration of calcium acetate to precipitate calcium carbonate. The lack of healing in M80 and M90 may be due to the low number of spores, whilst in M50 there was perhaps insufficient calcium acetate to precipitate calcium carbonate. While these visual images provide some insight into whether crack healing has occurred, they are of little use in quantifying performance.

3.3.2. Initial surface absorption

Fig. 7 shows the change in initial rate of surface absorption with time. As expected, the rate of surface absorption reduced with time, as the moisture content of the mortar progressively increased during the test and parts of the sample approached full saturation. The mean initial surface absorption of an uncracked mortar at 10 min was 0.16 ml/m^2/s and at 120 min was 0.06 ml/m^2/s. Typically

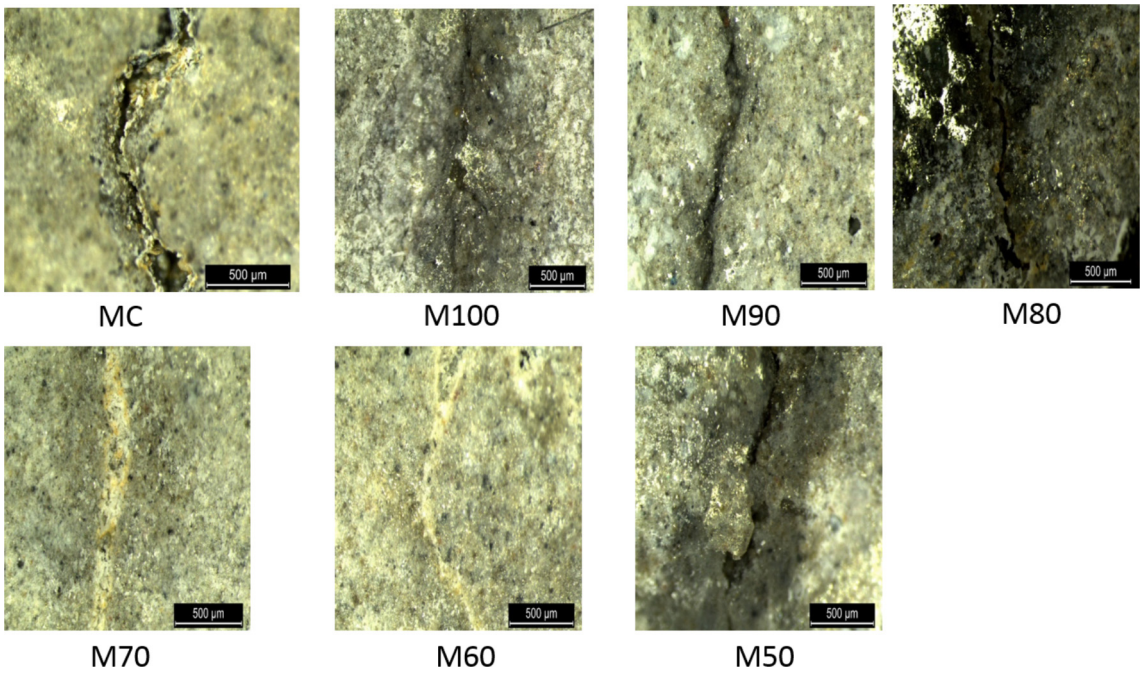

Fig. 6. Appearance of cracks after 165 days of healing.

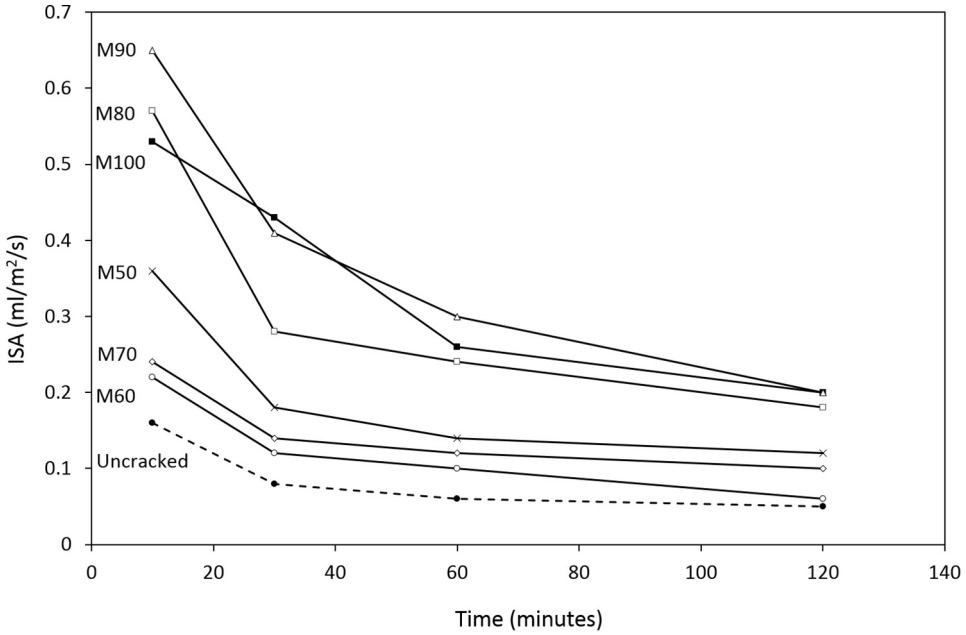

Fig. 7. Initial surface absorption of each mortar after 165 days of healing.

mortars are considered to have excellent resistance to ingress of water if they have values less than 0.25 and 0.07 ml/m^2/s at 10 min and 120 min, respectively [21]; and these properties were evident for the uncracked mortar.

Each of the cracked mortars had higher initial surface absorption values than the uncracked mortar, suggesting cracking and any healing did not result in a complete return to the original uncracked state. For M100, the mortar containing only CPN and no CPS, the mean initial surface absorption at 10 min was 0.53 ml/m^2/s and at 120 min was 0.2 ml/m^2/s. Since values greater than 0.5 and 0.15 ml/m^2/s at 10 and 120 min, respectively, are typical of mortars with high permeability [21] it can be suggested that little effective healing has taken place. Both M90 and M80 had similar surface absorption to M100. These values reflect the lack of visual healing observed.

However, the two mortars in which visual healing was observed, M60 and M70, had much lower surface absorption values than M100. At 10 and 120 min, values were less than 0.25 and 0.15 ml/m^2/s, respectively, suggesting that these mortars had excellent resistance to water absorption. Clearly, the precipitate observed in Fig. 5 has reduced the width of the crack, which has, in turn, reduced the water flow through it. Indeed for M60, surface absorption at 120 min was similar to that of the uncracked specimen.

Fig. 8 shows the relationship between initial surface absorption and spore to calcium acetate ratio present in each of the mortars. There appears to be a loose relationship between the two values. It can be suggested that at low spore to calcium acetate ratios there were an insufficient number of spores available to generate healing. This may be because the concentration of CPS was so low that none of these particles lay in the path of the crack and subsequently no spores were actually released, or that spores were released but the concentrations were too low to provide a sufficient number of cells to activate healing mechanisms.

In contrast, the low ISA values observed for M60 and M70, in which the spore to calcium acetate ratio lies between 5×10^9 and 9×10^9 spores/g, may reflect an efficient ratio between the number of spores and the volume of nutrients, as discussed above. At higher spore to calcium acetate ratios the efficiency of healing appears to reduce, which may again reflect the low quantity of calcium acetate and, consequently, the volume of calcite that can form in a crack.

4. Discussion

4.1. Quantity of cells and nutrients required for self-healing concrete

For self-healing to occur there needs to be sufficient calcium acetate available to convert into sufficient calcium carbonate within the crack to reduce the permeability. The theoretical quantities of calcium carbonate that can be formed are described in Eqs. (1) and (2), and the amount of calcium acetate required depends on the volume of the crack. Conversion of the calcium acetate into calcium carbonate is dependent upon the presence of bacteria cells. Due to continuous cell growth and multiplication it has been suggested that calcium carbonate precipitation can be generated initially in the presence of relatively few cells. However, for rapid and efficient precipitation to occur, the microbiological experiments described in this paper have shown that the number of cells required is greater than 3×10^9 cells/ml and that a greater concentration of cells is necessary as the quantity of calcium acetate increases. This is consistent with work elsewhere, where it has been shown that the initial biomass concentration affects $CaCO_3$ precipitation yield and rates. Wang has shown that for the urea hydrolysis pathway it is preferable to have at least 10^8 cells/ml [13].Elsewhere it has been shown that for the denitrification pathway, an increase in cells from 10^8 cells/ml to 10^9 cells/ml increases $CaCO_3$ precipitation significantly and that the same amount of $CaCO_3$ can be precipitated in half the time [22]. From Fig. 5 it can be suggested that 5×10^{11} cells are required for every g of calcium acetate depending on the presence of other nutrients (as discussed later).

In self-healing concrete these cells have to germinate from spores and then multiply to sufficient numbers. It appears from the results in this paper that the proportion of available spores to available calcium acetate and yeast extract is an important factor in generating efficient self-healing. The most effective ratio of spores to calcium acetate appears to be in the order of 8×10^9 spores per g of calcium acetate when coated EP is used as a 20% replacement of aggregate under the conditions used in this paper.

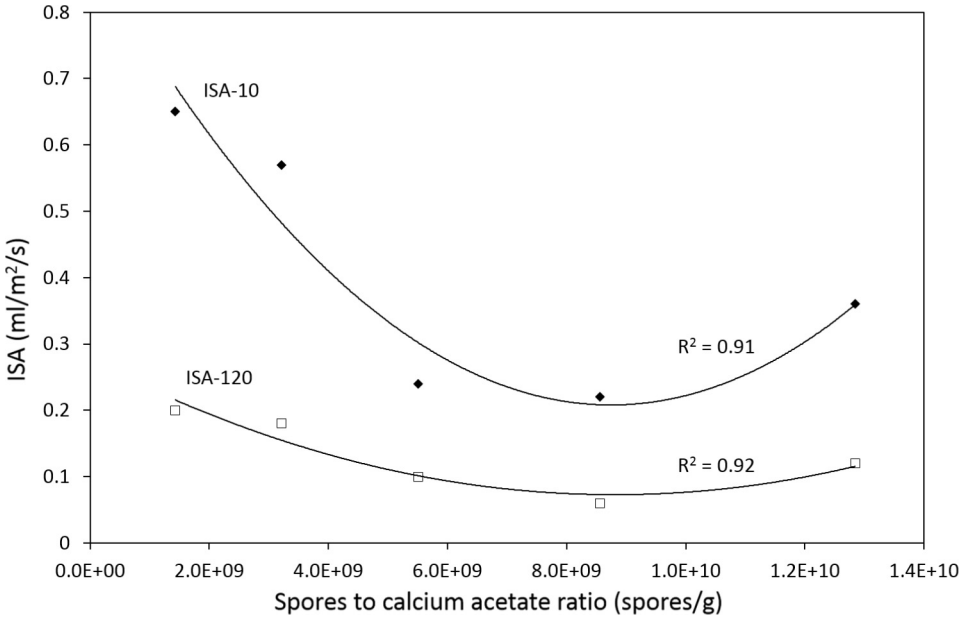

Fig. 8. Relationship between ISA-10 and ISA-120 and the potential spore to calcium acetate ratio.

It is noticeable that the most practically efficient number of spores per g of calcium acetate within concrete is two orders of magnitude lower than the proposed number of cells from microbiological experiments performed when the concrete was excluded. This suggests that there is a requirement for cells germinated from the spores to multiply before efficient self-healing: although it is probable that greater healing could have occurred if the number of spores had been increased and the quantity of calcium acetate maintained. For encapsulation in coated perlite, as described in this paper, this could only be achieved by adding more than 20% of coated perlite by mass of aggregate to the mortar. This would have detrimental effects on the mechanical properties of the concrete. It should also be noted that the calcium carbonate produced in the microbiological experiments and shown in Fig. 3 is due solely to conversion of the calcium acetate. However, in the concrete experiments additional calcium carbonate can be produced due to conversion of calcium hydroxide by the CO_2 produced as a product of primary conversion (Eq. (2)) or from atmospheric CO_2, assuming there is calcium hydroxide still present in the matrix at the time of self-healing.

It is observed that the required number of spores to calcium acetate found here is higher than that in work elsewhere. For example, the encapsulated clay particles for self-healing developed by Wiktor and Jonkers [14] included 1.7×10^5 spores and 0.06 g of calcium lactate per g of aggregate; this equates to approximately 2.8×10^6 spores per g of calcium lactate. Later work by the same team used 10^8 spores per litre in combination with 200 g/l of calcium lactate [23]. This equates to an even lower ratio of 5×10^5 spores per g of calcium lactate.

There are a number of possible reasons for this. Firstly, the materials used are different and it is possible that *B. alkalinitriculus,* as used by Jonkers' team, is a more efficient producer of calcium carbonate than the *B. peudofirmus* used in this work. Also it should not be ignored that, theoretically, conversion of one mole of calcium lactate yields six moles of calcium carbonate when the additional carbonation of calcium hydroxide is accounted for; whereas the equivalent conversion of a mole of calcium acetate yields only four moles of calcium carbonate (Eqs. (1) and (2)). Furthermore, the additional calcium carbonate by carbonation relies on the presence of sufficient calcium hydroxide which may not always be present in sufficient quantities when a pozzolanic cement is used, as was the case in this study. A further consideration is that Jonkers' team [14,23] combined calcium lactate and the spores within the same lightweight aggregate. Consequently, upon cracking, there was always the same ratio of spores to calcium lactate. In contrast, in our work we require both the CPS and CPN carriers to crack. Nevertheless, the research reported in this paper has shown that, for the first time, the required spore concentration is not independent of calcium precursor content as has been previously suggested [17].

4.2. Need for additional growth components

In developing self-healing concrete, the only nutrients typically added to aid growth and germination of the bacteria in the concrete are those nutrients present in yeast extract. This was also the case in the work reported in this paper. However, where greater precipitation is sought, for example in microbiological experiments, B4 or LB media tend to be used despite neither of these being the ideal media for any particular choice of bacteria used [24]. Research in this paper using three growth media showed the value of the additional growth components in GM1 over the use of yeast extract alone, as in GM2 (Fig. 3). Use of GM1 led to effectively complete conversion of calcium acetate at 8 days with 3.7×10^9 cells/ml, whereas the same number of cells with GM2 converted only around 30% of the calcium acetate, indicating it is possible to improve the efficiency of self-healing systems through a change in the growth medium.

Clearly, the precipitating environment present in concrete is different to that in microbiological experiments, but it is highly likely that the use of known germination and growth aids to the nutrients used in CPN will most probably enhance bacterial growth and maximise calcium carbonate precipitation in self-healing concrete. As bacteria-based self-healing of concrete continues to advance, it is the opinion of the authors that growth media more appropriate to the concrete environment and the specific bacteria used will need to be developed. The use of germination and growth aids as an integral part of the nutrients needs to progress and should be an element of any future work in bacteria-based self-healing concrete, although the effects of these on the long-term performance of concrete need to be considered.

4.3. Healing conditions

The aerobic respiration method for conversion of calcium acetate for self-healing requires the presence of oxygen, and consequently healing is likely to be slow under water. Indeed, healing in wet/dry environments has been shown to enhance bacteria growth and calcite precipitation when compared with wet conditions [23,25]. As additional calcium carbonate can be produced due to the reaction between atmospheric or generated CO_2 and calcium hydroxide, it is likely that wet/dry environments are beneficial, as carbonation has been shown to be more rapid in such environments [26]. In this work the cracked mortars were placed in a moist and humid environment rather than in a wet/dry environment. For this reason samples were maintained in the environment for 165 days to generate what we considered to be a suitable amount of healing – a similar healing period as was used by Wiktor and Jonkers (2011). Further unpublished work on cracked mortars containing CPS and CPN has shown that healing can occur within a matter of days in a wet/dry environment. In addition, the temperature at which healing takes place needs to be considered. In this work the cracked mortars were maintained at 30 °C, as this was known to be suitable for the growth of the bacteria [5]. However, this was not necessarily the optimum temperature for MICP. Consequently, these results have relevance for how self-healing concrete is used, in that it is most efficient in environments where it is exposed to wetting and drying, and appropriate temperatures. These mechanisms also need to be considered in the selection of healing scenarios in future research.

5. Conclusions

The self-healing capability of a dual system consisting of bacterial spores and nutrients encapsulated separately in EP was demonstrated based on microscopy and initial surface absorption of water. It was shown that crack healing could be achieved when coated EP was used as a 20% replacement of aggregate, provided a suitable ratio of spores to calcium acetate was achieved. For the particular method and conditions used in this paper this ratio appears to be in the order of 8×10^9 spores per g of calcium acetate. The results showed for the first time that self-healing is not simply a requirement of having sufficient healing compounds (e.g. calcium acetate) but that a minimal number of bacterial spores are also required to ensure that sufficient cells take part in the healing process.

Based on microbiological experiments it was shown that more rapid and efficient precipitation of calcium carbonate occurs in the presence of other nutrients rather than in a growth media comprising of just yeast extract. This has important consequences for further research and it is the opinion of the authors that growth

media more appropriate to the concrete environment and the specific bacteria used needs developing, whilst bearing in mind the consequences of adding extra components on the properties of the concrete.

In this research healing was achieved in a moist and humid environment which required up to 165 days for healing to occur. It is likely that faster healing can occur in a wet/dry environment, however the actual environments and exposure conditions that real structures are exposed to need to be considered in further research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.conbuildmat.2017.11.086.

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