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PII: S2352-7102(21)00692-6

DOI: https://doi.org/10.1016/j.jobe.2021.102834

Reference: JOBE 102834

To appear in: *Journal of Building Engineering*

Received Date: 10 March 2021

Revised Date: 13 May 2021

Accepted Date: 2 June 2021

Please cite this article as: I. Justo-Reinoso, A. Heath, S. Gebhard, K. Paine, Aerobic non-ureolytic bacteria-based self-healing cementitious composites: A comprehensive review, *Journal of Building Engineering*, https://doi.org/10.1016/j.jobe.2021.102834.

Aerobic non-ureolytic bacteria-based self-healing cementitious composites: A comprehensive review

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The use of bacteria for self-healing cement-based materials has shown great potential in recent years. Microbially induced calcium carbonate precipitation (MICP) is a novel technology that relies on the metabolic activity of bacteria, principally from the genus *Bacillus* and close relatives, to precipitate calcium carbonate ($CaCO_3$) inside the cracks to heal them. Among the different bacteria that can be used for this purpose, aerobic non-ureolytic bacteria have shown promising results to improve healing efficiencies and engineering properties of self-healing cementitious composites in a more sustainable way. Unfortunately, research results involving these specific bacteria species are scattered throughout the literature. Therefore, this review aims to present in one place the state-of-the-art knowledge relevant to the development of self-healing cementitious composites that rely on aerobic non-ureolytic bacteria. In this review, the most recent advances regarding the various aerobic non-ureolytic bacteria species normally used (e.g., *B. cohnii, B. pseudofirmus*, etc.), the methods for embedding these bacteria, the effects of these bacteria on the healing performance of cementitious composites, the results from various outdoors trials and the economic feasibility of these systems are reported, and the principal findings discussed and summarised at the end of each section. Finally, research gaps and future research work are identified and presented in the last section.

Keywords: Bacteria; Concrete; MICP; Non-ureolytic; Self-healing.

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1. Introduction

Globally, with an estimated yearly consumption approaching 30 Gt, concrete is considered the most widely consumed human-made product [1,2]. This global consumption rate is equivalent to 1.3 tonnes of concrete for every living human being, making concrete the second most consumed resource on the planet, only after water. Concrete is predominantly a combination of cement, aggregates, chemical admixtures and water, where the cement paste fills the spaces between aggregates. Cement, with an estimated global production in 2019 of 4.1 Gt [3] and expectations to increase this number up to 5 Gt in 2050 [4,5], is an essential component of concrete. In 2018, China, the world's largest concrete producer, transformed alone 2.2 Gt of cement into 13-16 Gt of concrete [1,3,6]. Theis huge consumption of concrete could result in our period being remembered by future generations as the "Concrete Age". Concrete plays an essential role in the infrastructure that supports health services, human refuge and socioeconomic activities. One reason of the extensive use of concrete is that its principal components are considered relatively inexpensive and commonly accessible in most parts of the world, resulting in an average price in Europe between €60 and €75 per m^3 of concrete [7]. Nevertheless, despite many advantages, concrete has a significant drawback as a consequence of its limited tensile strength: a tendency to form cracks [8,9]. Concrete cracks are a natural phenomenon that weakens the mechanical properties of concrete [7,10] and can affect durability. For this reason, concrete is usually combined with steel reinforcement to bear the tensile stresses and limit the crack width. However, even though this reinforcement steel has a positive effect on crack width restriction, it is not able to completely prevent crack formation. Moreover, it is well known that cracks are one of the principal causes that accelerate concrete deterioration by allowing the migration of aggressive gases and liquids through the cement matrix. As a result, reinforcement steel may be exposed to different environmental conditions that could trigger its corrosion. A recent study by Van der Bergh *et al.* states that even hairline cracks have a direct influence on the corrosion of reinforcement steel [11]. To extend the service life of concrete structures when cracks appear, it is essential to repair them. These repairs often involve chemical materials such as epoxy resins, polyurethane-based polymers and latex emulsions, among others. However, significant problems remain owing to cracks that are not visible or accessible, incompatible interfaces, toxic gases from chemicals, moisture sensitivity or that

the repair effect is not permanently maintained [12-14]. Therefore, crack repair becomes a complicated process with high direct costs estimated at €130 per m^3 of concrete for a one-event crack injection [7], more costly than the initial material per unit volume. Moreover, in Europe, costs related to repair works of concrete structures can amount to up to half of the annual construction budget of government agencies [10,15]. In addition to the direct costs of these repairs, also indirect costs due to the loss in productivity that results from disruptions in commerce, industry and traffic carry a significant economic impact. To counteract the negative effects that cracks have on the durability of concrete structures, self-healing technologies have emerged in the last decade as a viable alternative to produce concretes with the potential to self-heal their cracks [10,12,16-24].

In general, self-healing materials are broadly classified in two families depending on the self-healing mechanism: extrinsic and intrinsic [25-27]. Extrinsic self-healing materials are those in which the process depends on an external healing agent, commonly present in the form of capsules or vascular networks. These healing agents are released to heal the damage and do not specifically interact with the matrix. In contrast, intrinsic self-healing materials are those in which the material can restore by itself after a damage event [25]. Recent studies are adopting a similar categorisation for self-healing in cementitious materials [28]. The criterion proposed is based on whether there are any chemical interactions occurring between the cementitious matrix and the components within or externally embedded in the cementitious matrix. When these chemical interactions exist, the mechanism is termed intrinsic healing, while extrinsic healing is often performed by the physical filling into cracks [28]. Consequently, intrinsic self-healing in cementitious materials can be achieved through autogenous or autonomous processes. Intrinsic autogenous self-healing, a natural process occurring in concrete structures in the presence of water, is an old and well-known phenomenon [10,29-31]. This mechanism can fill microcracks through the hydration of unhydrated cement particles or from the reaction of calcium hydroxide ($Ca(OH)_2$) with atmospheric carbon dioxide (CO_2) to produce calcium carbonates ($CaCO_3$) [31]. However, autogenous healing is limited to microcracks smaller than 0.3 mm [10,11,14,17], and its success depends strongly on factors such as the presence of water and the amount of unhydrated cement [32]. For intrinsic autonomous self-healing, two methods are generally

employed: encapsulation and vascular networks. Encapsulation relies on capsules that can safeguard the healing agent (*i.e.,* chemical-healing agents or bacterial spores) during the initial mixing but also for an extended period [32]. A variety of chemical healing agents have been used such as epoxy [33,34], sodium silicate [35-37], methyl methacrylate (MMA) [38], cyanoacrylate [39] and dicyclopentadiene (DCPD) [40]. Nevertheless, when a capsule system is used, the repeatability of the healing process is restricted [41]. On the other hand, vascular systems consisting of hollow channels or interconnected networks can be incorporated within the cement matrix to provide a continuous healing agent delivery. Contrary to capsule systems, vascular networks are able to ensure the supply of larger volumes of healing agents or repeated damage repair [42]. However, there have been some criticisms that the inclusion of these networks in concrete could actually provide a route for more rapid deterioration processes. The production of $CaCO_3$ as a side effect of microbiological activity [23] is an intrinsic autonomous method to "engineer" the self-healing capacity of concrete. It is well known that bacteria can induce different minerals, such as carbonates, sulphates, silicates and phosphates [43]. However, among all the different minerals that these microorganisms can induce, precipitation of $CaCO_3$ has attracted interest due to the efficient bonding capacity and compatibility with cement matrix [32]. The effectiveness of this strategy highly relies on the availability of dissolved inorganic carbon, moisture, pH, nucleation sites, calcium sources and temperature [32]. In a similar way, a plant-derived urease enzyme (*i.e.,* jack bean urease) was recently shown to precipitate calcium carbonate in the absence of these microorganisms [44].

Several comprehensive reviews have been published in recent years about bacteria-based self-healing materials, where the different pathways to reach this objective (*i.e.*, ureolytic, non-ureolytic, denitrifying, etc.) have been included and discussed all together. Consequently, the information and results related to aerobic non-ureolytic bacteria in cementitious materials are scattered through different sources and mixed with other self-healing technologies that make their access less than straightforward. In this regard, this review paper critically compiles the information available on the use of aerobic non-ureolytic bacteria for self-healing purposes on cementitious materials. To the best of the authors' knowledge, there is no comprehensive review that gathers in one place all the scientific

advances related exclusively to the use of aerobic non-ureolytic bacteria when these are utilized to produce self-healing cementitious materials.

2. Microbially induced calcium carbonate precipitation (MICP)

Cell surfaces of bacteria are negatively charged; as a result, bacteria draw Ca^{2+} ions from the environment to accumulate on their cell surfaces [45]. Afterwards, these ions will react with the CO_3^{2-}, which can be increased by bacterial metabolism, leading to the precipitation of $CaCO_3$ at the cell surface, provided that the medium is oversaturated with respect to $CaCO_3$ [46,47]. Under laboratory conditions, it has been proven that MICP can self-heal cracks widths up to 1.0 mm with pure cultures of bacteria (*Bacillus sphaericus*) [48], and up to 1.2 mm when using mixed cultures [49], much larger crack widths than can be healed through autogenous healing. Different MICP strategies have been used for self-healing purposes on cementitious materials, including urea degradation, nitrate reduction and oxidation of organic carbon sources as the main options [16] (**Fig. 1**).

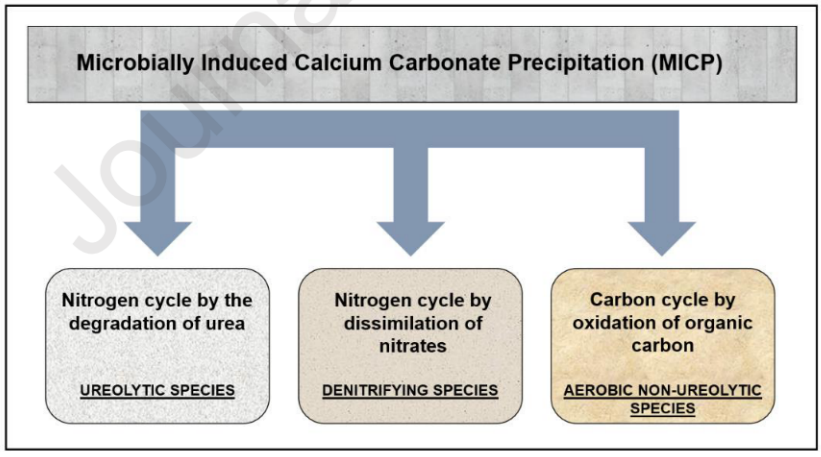

Fig. 1. Principal microbially induced calcium carbonate precipitation (MICP) strategies used for self-healing cement-based materials.

2.1. MICP involving nitrogen cycle by the degradation of urea (Ureolytic species)

The most commonly used bacteria for self-healing cementitious materials are alkali-tolerant ureolytic species. Ureolytic bacteria, through urease activity, can decompose urea ($CO(NH_2)_2$) into ammonia

(NH_3)/ammonium (NH_4^+) and carbonate ions (CO_3^{2-}) [50]. The general reaction can be written as follows (**Equation (1)**):

$$CO(NH_2)_2 + H_2O \rightarrow \underline{\text{Bacterial urease}} \rightarrow NH_4^+ + CO_3^{2-} \quad (1)$$

Ureolytic bacteria present the fastest $CaCO_3$ precipitation rates compared with other types of bacteria [16,51]. Reeksting *et al.* [52] studied a bacterial isolate (*i.e.,* CG7_3) capable of switching between ureolytic and non-ureolytic states depending on the availability of urea to observe the differences in $CaCO_3$ precipitation in the presence or absence of urea (20 g/L). Under identical environment conditions (*i.e.,* LB medium supplemented with $Ca(OAc)_2$ (10 g/L)), it was found that in the presence of urea, the bacterial isolate reached the maximum theoretical level of $CaCO_3$ precipitation after one day. In contrast, in the absence of urea, the same amount of $CaCO_3$ precipitation took 13 days.

Although the use of ureolytic species has proven to be successful, some significant drawbacks exist. Production of ammonium ions (NH_4^+) results in nitrogen oxide (NO_x) emissions into the atmosphere, causing health and environmental concerns [16,22,53]. Moreover, the presence of excessive ammonium in the concrete matrix increases the risk of salt damage by conversion to nitric acid [32], and this could exacerbate corrosion of reinforcement steel [50,54,55]. Additionally, ammonia accumulation inhibits the MICP process resulting in less than optimal precipitation [56,57]. In this context, Reeksting *et al.* [52] observed a rapid loss of the viability of ureolytic bacteria, likely due to high ammonia production, potentially indicating a limited time over which the mineralisation process can take place. Furthermore, Gat *et al.* [57] observed that the volatilisation of ammonia produced in ureolytic systems resulted in a local decrease in pH, which likely caused the dissolution of previously precipitated $CaCO_3$ [57]. It was observed that ammonia volatilisation amounted to 50% of produced ammonia that resulted in a maximum dissolution of 30% of precipitated $CaCO_3$. Standard species in the group of ureolytic bacteria for self-healing are *Sporosarcina pasteurii (*formerly named *Bacillus pasteurii*) [12], *Sporosarcina ureae, Bacillus sphaericus* and *Bacillus megaterium.*

2.2. MICP involving nitrogen cycle by dissimilation of nitrates (Denitrifying species)

Precipitation of $CaCO_3$ through nitrate-reducing bacteria has been used for concrete self-healing due to the capability of these bacteria for functioning under oxygen-limited conditions [58]. Denitrification (NO_3^- reduction) occurs through the microbial oxidation of organic carbon by using NO_3^- (nitrate) as the electron acceptor in the absence of oxygen (**Equation (2)**) [16]. This process generates CO_3^{2-} and HCO_3^- ions, which are then precipitated as $CaCO_3$ in the presence of dissolved calcium (**Equations (3)** and **(4)**).

$$5HCOO^- + 2\,NO_3^- \rightarrow N_2 + 3HCO_3^- + 2CO_3^{2-} + H_2O \quad (2)$$

$$Ca^{2+} + HCO_3^- + OH^- \leftrightarrow CaCO_3\,(precip) + H_2O \quad (3)$$

$$Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3\,(precip) \quad (4)$$

Different nitrate-reducing bacteria species have been used, including *Pseudomonas denitrificans, Castellaniella denitrificans* and *Diaphorobacter nitroreducens.*

2.3. MICP involving carbon cycle by oxidation of organic carbon (Aerobic non-ureolytic species)

Aerobic non-ureolytic bacteria, commonly referred to simply as non-ureolytic bacteria, have also been widely used in self-healing cementitious composites. Precipitation of $CaCO_3$ in the presence of non-ureolytic bacteria is likely driven principally by two factors: the bacteria serving as a nucleation site for $CaCO_3$ precipitation and an increase of the surrounding pH due to the production of bicarbonate ions (HCO_3^-) [59-61]. The presence of negatively charged groups on the surface of bacterial cells attracts Ca^{2+} ions and favours calcium precipitation as $CaCO_3$ [61,62]. These calcium precipitates gradually accumulate and cover the bacterial bodies resulting in the bacterial cells embedded within the growing $CaCO_3$ crystals [60,63]. On the other hand, the rise in pH results mainly from the use of organic acids by non-ureolytic bacteria as their only source of carbon and energy. The consumption of these organic acids produces bicarbonate ions (HCO_3^-). Since these bicarbonate ions are alkaline, an increase in their concentration results in a rise of pH in the surrounding environment [59,61-63]. Furthermore, as a result of concrete being an alkaline and calcium-rich environment, the produced bicarbonate ions further react to form $CaCO_3$ (**Equation (5)**):

$$Ca^{2+} + HCO_3^- + OH^- \rightarrow CaCO_3 + H_2O \quad (5)$$

This induced $CaCO_3$ precipitation process occurs at a slower pace with aerobic non-ureolytic when compared to other types of bacteria used for self-healing concretes [52]. Therefore, the majority of bacteria-based self-healing concrete (BBSHC) publications have been focused on studying other types of bacteria, especially ureolytic species despite the environmental drawbacks of this approach described in section 2.1. (**Fig. 2**). Photosynthetic cyanobacteria have also been investigated in recent years, principally for soil restoration [64,65]. Moreover, in a recent study, cyanobacteria were inoculated to an inert structural sand-hydrogel scaffold to induce $CaCO_3$ biomineralisation [66,67]. Nevertheless, the use of cyanobacteria has so far been limited to surface areas, and no successful results have been reported for healing cracks in cementitious materials.

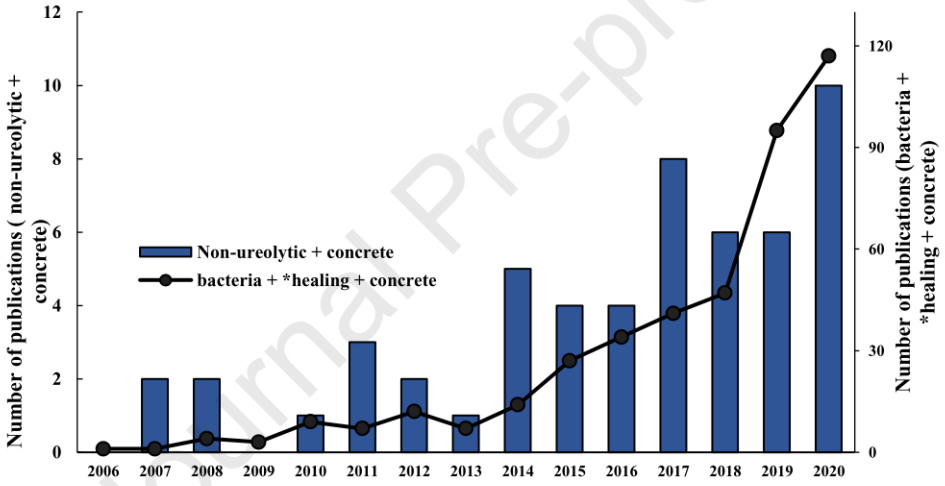

Fig. 2. Number of publications on non-ureolytic + concrete and bacteria +*healing + concrete over the last years (Source: Web of Science, Clarivate Analytics, 11 Feb 2021 [68])

To summarise, microbially induced calcium carbonate precipitation (MICP) has shown great potential for healing cracks and improve the overall behaviour and durability of cementitious materials. MICP comprises a series of complex biochemical reactions that vary according to the different metabolic pathways. Ureolytic bacteria have been historically more investigated due to their fastest $CaCO_3$ precipitation rates when compared to other types of bacteria. However, in recent years, aerobic non-ureolytic bacteria have emerged as a very promising alternative due to their ability to induce similar $CaCO_3$ precipitations to the ones produced by ureolytic bacteria but with significant environmental advantages.

3. Non-ureolytic bacteria

The most commonly used non-ureolytic bacteria in BBSHCs are species belonging to the genus *Bacillus*. Gram-positive bacteria of this genus are rod-shaped aerobic or facultative anaerobic cells that are 1-10 μm long [69]. *Bacillus* species are the preferred option for BBSHC as they are able to increase the pH of the neighbouring environment at the same time that they produce CO_2 by cellular respiration, creating efficient conditions for $CaCO_3$ precipitation [70]. Another important reason for their use is their ability to form spores, which are an extremely durable survival state [71]. As spores, the bacteria can withstand the initial harsh concrete environment conditions that include high pH values (pH ~13), dense matrix, and unsuitable humidity conditions [17,56]. However, it should be noted that *Bacillus* bacteria are not the only genus of bacteria that are capable of precipitating $CaCO_3$ through non-ureolytic pathways, and indeed there is no certainty that they are necessarily the best option for developing self-healing of concrete.

Non-ureolytic bacteria capable of living and precipitating $CaCO_3$ in high pH environments have been isolated from different locations around the world. Three non-ureolytic alkali-resistant bacterial isolates were obtained by Wiktor *et al.* from alkaline natural lakes located in Chiprana-Playa (Spain), Kulunda (Siberia, Russia) and Wadi Natrum (Egypt) [72]. Phylogenic analysis of these isolates revealed that they were most closely related to the alkaliphilic species *Bacillus cohnii* and *Bacillus alkalinitrilicus*. Similarly, Reeksting *et al.* were able to isolate non-ureolytic bacteria from calcium-rich sites (*i.e.,* exposed limestone, caves, and soils in areas with a carboniferous limestone bedrock) within the southwest of the United Kingdom [52]. The principal isolate obtained was identified as MM1_1 (closely related to *Bacillus licheniformis*). Moreover, a very promising alkaliphilic and halotolerant non-ureolytic bacteria (*Bacillus miscanthi* AK13) was recently isolated by Jung *et al.* from the rhizosphere of a wild grass (*Miscanthus sacchariflorus*) from South Korea. This specific strain can grow at pH 13 and withstand conditions of high salinity (11% (w/v) NaCl) [56]. Consequently, nature provides a valuable and versatile toolbox of different species of bacteria that could have the potential to be used for MICP in cementitious materials. However, it is unlikely that a

singular species of bacteria will be found that is able to meet all requirements for self-healing concrete under different exposure conditions. In this regard, further research using genetically modified microorganisms could deepen our understanding of the role different determinants may have in bacterial activity to aid in the selection of the most appropriate species for the required application.

Non-ureolytic bacteria used in BBSHC systems have an optimum range of pH to thrive and produce $CaCO_3$ precipitates. However, on aged concrete structures, the degree of carbonation may change the surface pH, resulting in a direct effect on the kinetics and efficiency of the healing process. Therefore, it has been indicated by some researchers that there is a need to embed the bacteria along with a buffering agent able to maintain the pH at an optimum range of alkalinity [73]. An alternative option is the use of specific bacteria that are known to change external pH value to a pH suitable for growth and create their own required environment [74,75]. For instance, Horikoshi found that the alkaliphilic *B. clausii* 221, which is a strong alkaline protease producer, can grow slowly at neutral pH, changing the pH of the surrounding environment [74]. Once this bacteria (*B. clausii* 221) was able to change the surrounding pH to 9, it began to grow rapidly and produced a large amount of alkaline protease [74].

The non-ureolytic bacterial species most commonly used for BBSHC studies include *Bacillus cohnii* [76-82], *Bacillus pseudofirmus* [54,77,78,83], *Bacillus subtilis* [47,84,85] and *Bacillus alkalinitrilicus* [21,86]. Other non-ureolytic bacteria species that have also been used include: *Bacillus thuringiensis* [12], *Lysinibacillus boronitolerans* YS11 [75,87-89], *Bacillus licheniformis* [52], *Bacillus halmapalus* [90,91], *Bacillus alkaliphilus* [88], *Lysinibacillus sp* [92], *Bacillus halodurans* [78], *Bacillus miscanthi* AK13 [56], *Bacillus mucilaginous* [93] and phylogenetically related facultative aerobic strains [17,52,84,94].

3.1. Nutrients and calcium precursors

It is of great importance to select appropriate nutrients and calcium precursors to ensure optimal bacterial performance in terms of metabolic activity and $CaCO_3$ precipitation, but also to avoid adverse effects on the fresh and hardened properties of BBSHCs. Calcium acetate [72,83,95] and calcium lactate [21,72,80,96] have been widely used in these bacteria-based systems, both as a source

of nutrients and calcium precursors, while calcium nitrate [73,92,97] and yeast extract [83,88,98] have been used separately as a calcium precursor and nutrient, respectively. These nutrients and calcium precursors have been combined with non-ureolytic bacteria as direct additions, either during initial mixing or encapsulated within the same carrier [21,81] or added separately (as a two-component system) [82,83] (**Table 1**).

Table 1: Overview of non-ureolytic bacteria and nutrients which have been used to precipitate $CaCO_3$ in cementitious composites.

Non-ureolytic bacteria	*Growth Medium*	*Cementitious composite*	*Embedment method*	*References*
Bacillus halmapalus PSY 4 and PSY 5	Calcium alginate, yeast extract and magnesium acetate	Mortar	Immobilized (beads)	[91]
Bacillus cohnii	Nutrient broth	Mortar	Direct (w/ mixing water)	[45]
Lysinibacillus sp	Calcium nitrate, calcium lactate	Mortar	Direct (w/ mixing water)	[92]
Lysinibacillus boronitolerans YS11 and *Bacillus alkaliphilus* AK13	Calcium lactate, yeast extract	Mortar	Direct (w/cement)	[88]
Bacillus subtilis AP91	None	Mortar	Direct (w/ mixing water)	[84]
Bacillus subtilis	NaCl, tryptone and yeast extract	Mortar	Direct (w/ mixing water)	[99]
Bacillus cohnii	Calcium lactate and yeast extract	Concrete	Immobilized (EP)	[80]
Bacillus cohnii	Calcium lactate, calcium glutamate, calcium chloride, yeast extract.	Mortar	Liquid treatment	[100]
Bacillus subtilis	Calcium chloride, calcium lactate, sodium alginate, yeast extract	Mortar	Immobilized (beads)	[101]
Bacillus subtilis and *Sporosarcina pasteurii**	NBu medium and urea	No/ medium	Direct (Inoculated)	[102]
Bacillus pseudofirmus	Calcium acetate, dextrose, yeast extract	Mortar	Immobilized (EP)	[83]
Bacillus alkalinitriculus	Calcium lactate, yeast extract	Mortar	Immobilized (EC)	[21]
Bacillus cohnii	Calcium lactate, yeast extract	Mortar with PVA fibres	Immobilized (EC)	[81]
Bacillus cohnii	Calcium lactate, yeast extract	Cement paste and concrete	Immobilized (PCP)	[96]
Bacillus cohnii	Calcium lactate, yeast extract	Mortar	Immobilized (EC)	[103]
Bacillus cohnii	Calcium lactate, yeast extract	Mortar samples (commercial dry mixture)	Immobilized (PCF)	[104]
Bacillus cohnii	Calcium nitrate, yeast extract	Mortar	Immobilized (ACG)	[82]
MM1_1 (close related to *B. licheniformis*)	Calcium nitrate, yeast extract	Mortar	Immobilized (ACG)	[52]
Bacillus pseudofirmus, Bacillus cohnii and Bacillus halodurans	Calcium acetate, glucose, yeast extract	Mortar	Liquid treatment	[78]
Bacillus subtilis	Calcium lactate	Concrete	Direct (w/ mixing water) and Immobilized (LWA and GNP)	[105]
Bacillus cohnii	Nutrient broth	Concrete	Direct addition	[106]
Bacillus pseudofirmus	Calcium lactate, yeast extract	Mortar	Immobilized (EC)	[107]
Bacillus pseudofirmus, Bacillus cohnii and Bacillus halodurans	Calcium nitrate, sodium gluconate and yeast extract	Concrete	Liquid treatment	[108]
Bacillus mucilaginous	Calcium nitrate, yeast extract and sucrose	Concrete	Direct (w/cement)	[93]

* Ureolytic bacteria

EP = Expanded Perlite
EC= Expanded Clay
PCP= Powder-Compressed Particles
PCF= Powder-Compressed Flakes
ACG= Aerated Concrete Granules
LWA= Light Weight Aggregate
GNP= Graphite Nano Platelets

Three different organic compounds (*i.e.,* calcium lactate, calcium acetate and sodium gluconate) were investigated by Tziviloglou *et al.* to determine the most suitable organic carbon source to be used by non-ureolytic bacteria. Based on continuous and non-continuous oxygen consumption measurements of washed bacterial cultures (*Bacillus* isolates closely related to *B. cohnii* and *B. alkalinitrilicus*), a

preference for calcium lactate and calcium acetate, but an indifferent behaviour for sodium gluconate, were observed [72]. Moreover, Xu and Yao found that the type of calcium source has a profound impact on healing effectiveness. The recovery ratio of flexural strength in the case of calcium glutamate was always higher than when calcium lactate was used [79].

Another crucial component that forms part of the growth medium of non-ureolytic BBSHCs systems is yeast extract. This is usually included to aid germination of the spores and growth of the cells when combined with organic carbon sources such as calcium lactate or calcium acetate [21,72,80,83,108]. However, recent work by Tan *et al.* has demonstrated the viability of using yeast extract as the only nutrient source for non-ureolytic bacteria (*B. cohnii*) [82]. Calcium nitrate was the calcium precursor used, and the results showed that yeast extract alone was able to provide sufficient carbon for the bacteria to promote the precipitation of $CaCO_3$. The quantity of the latter was directly related to the amount of yeast extract provided [82]. In contrast, Zhang *et al.* observed a decline of viable bacterial spores when high concentrations of yeast extract have been used (*i.e.,* 5 g/L), although it was not clear which components were affecting the viability of the spores [109].

The amount of Ca^{2+} ions available for the bacteria to induce $CaCO_3$ precipitation is also a key parameter. If insufficient calcium precursor is added during the production of BBSHC, the efficiency of the crack healing will be compromised as not enough $CaCO_3$ will precipitate to close the cracks. In a recent study, Tan *et al.* investigated the availability of Ca^{2+} ions for non-ureolytic bacterial spores (*B. cohnii*) embedded in carbonated and non-carbonated mortar samples [82]. Calcium nitrate was added as the calcium source directly with the mixing water during the casting process. Tan *et al.* concluded that in non-carbonated (28-days) mortar samples, the calcium hydroxide created as a hydration product was enough to produce sufficient $CaCO_3$ precipitates to heal the cracks. Moreover, the additional Ca^{2+} ions from the calcium nitrate helped to improve healing crack efficiency [82]. In contrast, when evaluating the carbonated (aged) mortar specimens, neither the calcium hydroxide produced during the hydration process nor the calcium nitrate directly added to the mixing water were a sufficient source of Ca^{2+} ions to achieve satisfactory crack healing. Consequently, the direct addition of calcium precursors is not recommended for BBSHCs that are likely to carbonate before the crack formation. In this

scenario, Ca^{2+} ions become locked in a form that is not suitable for the bacteria to use. For aged concrete (*i.e.,* carbonated), there is a need to supply an additional source of Ca^{2+} ions via encapsulation, vascular systems or direct application to be available at the moment of cracking [82]. Similarly, Tziviloglou *et al.* compared two batches of non-ureolytic bacteria-based mortar (*B. cohnii* and *B. alkalinitrilicus*) containing expanded clay (EC) particles impregnated with two different concentrations of calcium lactate to elucidate the influence of the amount of calcium precursor. However, the comparison showed that the crack healing trend was similar for both batches regardless of the two different calcium lactate concentrations. These results were attributed to a possible oxygen limitation during the healing process as the specimens were kept permanently submerged in water [95].

On the other hand, an excess in the amount of calcium precursor added to BBSHC could result in negative effects. Zhang *et al.* [94] concluded when using a bacterial strain named H4 (closely related to *B. pseudofirmus*) that the presence of excessive Ca^{2+} ions not only inhibited the calcium precipitation process but also resulted in waste of the Ca^{2+} resource. They suggested that maintaining a Ca^{2+} concentration lower than 30 mM was a good strategy since the free Ca^{2+} concentration of the pore solution inside the concrete usually is less than 30 mM [110]. A further negative consequence of high calcium concentrations was found by De Muynck *et al*. They observed that the crystallinity and the size of the $CaCO_3$ crystals could be influenced by the concentration of calcium in the culture medium, whereby the crystal size and crystallinity became lower as the concentration of calcium increased [111].

3.2. Bacteria concentration and viability

Spore concentration has a direct influence on the effective precipitation of $CaCO_3$. Zhang *et al.* observed that a minimum spore concentration of 4×10^7 spores per mL of culture medium was required to obtain adequate $CaCO_3$ precipitation when using *B. pseudofirmus* H4 [109]. In a similar context, but linking the number of bacterial spores to the amount of calcium precursor, Alazhari *et al.* found that the spore concentration necessary for optimal self-healing was 8×10^9 spores per g of

calcium acetate when using *B. pseudofirmus* [83]. However, lower ratios were reported by Wiktor and Jonkers, between 5 x 10^5 and 2.8 x 10^6 spores per g of calcium lactate, when using *B. alkalinitriculus* [21,103]. It is important to mention that between these two studies, different calcium sources were used, where theoretically, one mol of calcium lactate yields six moles of $CaCO_3$ while calcium acetate may yield only four moles of $CaCO_3$ [83]. Furthermore, other variables likely affecting these results could be attributable to the type of cement used (Alazhari *et al.* used a Portland-fly ash cement), the efficiency of bacteria to precipitate $CaCO_3$ and the effect of combining the spores and calcium source within the same carrier [83].

When bacterial spores are directly added to cementitious composites, their viability appears to be low compared to theoretical estimations [78,112]. Sharma *et al.* found that the detection of viable *B. pseudofirmus* cells after 93 days was only between ~1-4% of the originally incorporated spores (~3 x 10^6 spores/cm^3), while for *B. cohnii,* it was even lower, with spore viability values in a range between 0.5-2.4 %. Moreover, between 7 and 28 days, the spore viability significantly declined to 0.15-0.09% [78]. In this study, the dried spores were directly added to cement before the addition of water. Considering the well-documented resilience of *Bacillus* spores to adverse conditions and the consistent survival found beyond 42 days and up to 93 days in cement, they suggested that the aggressive extraction procedure that was employed for recovering the spores may have been the limiting factor to recover viable spores between 7 and 28 days [78]. The recovery procedure involved crushing and pulverizing the cement paste using high mechanical forces and then suspension by ultrasonic treatment. In contrast, Jonkers *et al.* found that viability of directly added *B. cohnii* spores (2.4 x 10^8 spores/cm^3) in cement paste specimens after nine days was 1%, with this value decreasing to insignificant levels after 135 days. Jonkers *et al.* associated these observations with the continuing reduction in matrix pore diameter sizes when pore sizes in the cement matrix decreased to ~1 μm at later ages, causing the majority of the incorporated spores to apparently become crushed [76]. In this regard, Sharma *et al.* were able to detect similar or even higher spore viability values at 93 days (*i.e.,* 0.5-2.4%) to the values observed by Jonkers *et al.* at nine days (*i.e.,* 1%). The extraction procedure, cement type (32.5R), curing conditions (20°C) and water-to-cement ratio (*w/c*= 0.5) were similar for

both studies. All these factors likely produced comparable cement pastes. However, likely differences in the process of casting the samples (*i.e.,* consolidation of the paste or mixing time) could have resulted in internal differences (*i.e.,* entrapped air) that may likely have protected some of the spores added by Sharma *et al.* and therefore resulted in a higher recovery of viable spores. Moreover, even though both studies used a standard microbiological dilution-to-extinction method (*i.e.,* most-probable-number (MPN)), the process to obtain the total counts was different between these two studies. Sharma *et al*. used LB alkaline agar plates maintained at 30°C overnight, and total viable counts were recorded manually after 18 h. In contrast, Jonkers *et al.* relied on the sample partitioning and analysis automation by using multiwell plates filled with mineral medium and incubated for two weeks at room temperature. The 96-wells were read using a computer program to quantify the turbidity in each of them. It is known that this automation of the counting process significantly reduces the variability in the interpretation of the results and the possibility of human error [1]. Differences in the process of casting the mortar samples and during the total counting process, could potentially have had an influence on the total counts obtained in each study.

The viability of the bacterial spores is significantly increased when different carriers (e.g., lightweight aggregates or microcapsules) are used to protect the spores until the crack formation. Sierra-Beltran *et al.* [81] found that bacterial spores (*B. cohnii*) impregnated in lightweight aggregate (*i.e.,* Catsan®) were viable three months after casting, and they could, in the presence of water, metabolize the provided organic nutrient source and consume oxygen. Similar behaviour was observed by Wiktor and Jonkers on freshly fractured samples of 9-month-old mortar specimens cured in water, where the bacterial spores (*B. alkalinitriculus*) had been immobilized in expanded clays, and their viability was evaluated through oxygen consumption measurements [21].

Benjamin *et al.* [113] observed an increase on the compressive strength response when a live bacterial culture, containing vegetative (*i.e.,* non-spore) cells of non-ureolytic bacteria (*B. subtilis*), was used to replace the mixing water of cement mortar specimens. Three different bacteria concentrations (*i.e.,* 10^4, 10^6 and 10^8 cells/mL) were used. The optimum concentration observed was 10^6 cells/mL, where an increase of 30% in the compressive strength after 28 days was observed when compared to a

reference sample with no bacteria added. Nevertheless, the mechanism responsible for the compressive strength increase observed was not clear. There was a lack of microstructural evolution tests (e.g., TGA or SEM-EDS) to support that the strength improvements observed could be associated with the formation of additional calcium carbonates resulting from bacterial activity. Furthermore, in a recent study by Skevi *et al.* (2021), it was observed that similar compressive strength responses were observed when dead or live cells were directly added to mortar formulations without nutrient addition. They concluded that strength improvements were not likely attributable to MICP, but more probably due to bacteria promoting deposition of hydration products (*i.e.,* nucleation sites) or related to the composition of their cell walls [114]. The viability differences between the use of vegetative bacterial cells or spores have been recently studied by Jang *et al*. [88]. They investigated the bacterial viability at different curing ages of cement mortar specimens containing *L. boronitolerans* YS11, *B. alkaliphilus* AK13, or a mixture of both, added directly as vegetative cells or spore forms (**Fig. 3**).

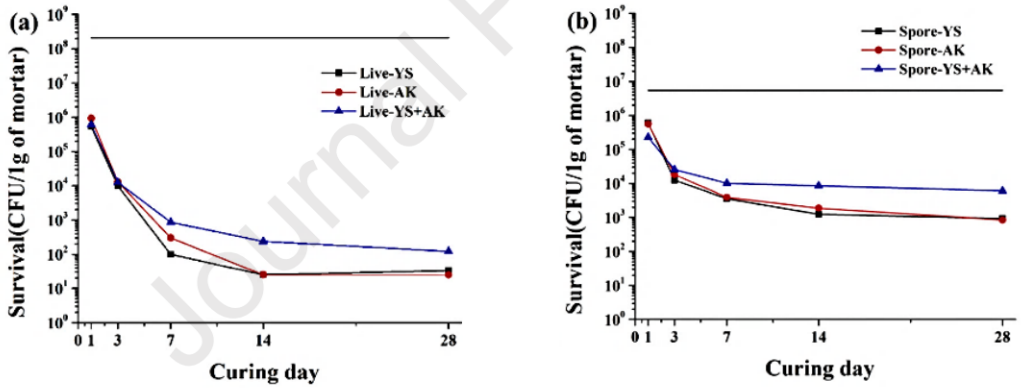

Fig. 3. Survival rate (number of viable cells (colony forming units, CFU) in 1 g of mortar specimen) of *L. boronitolerans* YS11, *B. alkaliphilus* AK13, and mixed bacteria, added as the (a) vegetative and (b) spore forms, in cement mortar after different curing ages submerged in tap water. The black lines on the top of both graphs represent the theoretical number of bacteria incorporated initially in the mortar (during mixing) [88].

When adding vegetative cells, barely between 0.002 and 0.006% of the original bacteria survived after 28 days. In contrast, the corresponding extinction rate was much reduced when incorporating spores, where approximately 0.2% of the initial spores were viable after a similar time [88]. Moreover, both the vegetative and spore mortar specimens revealed higher survival rates in the case of mixed bacteria compared with the single species (*i.e.,* survival rate of 2%). They considered this survival rate as high

despite the few studies on the bacterial survival in cement mortars via the colony-forming unit (CFU) measurement technique [88].

3.3. Temperature

Despite the large differences in temperatures between the different populated places around the world, BBSH systems employing non-ureolytic species have principally only been investigated under typical laboratory temperature conditions (20-30°C). These laboratory temperatures are within the optimal range for the activity of these bacteria [81,83,88]. However, if BBSHCs are to be considered for building around the world, then the bacteria-based agents used to generate self-healing concrete will need to function under a wide range of temperature conditions.

In general, microbial processes slow down when temperatures are close to the freezing point [17], and this is a possible limitation for the use of BBSHCs in low-temperature environments such as underground, arctic zones or deep-sea locations. Palin *et al.* [91] studied the self-healing performance of BBSH mortar specimens in low-temperature (8°C) marine environments using two non-ureolytic bacterial isolates, PSY 4 and PSY 5, which presented a 99% sequence similarity with *B. halmapalu*s. These bacterial isolates, PSY4 and PSY5, were obtained from a microbial soil sample from La Salada de Chiprana, a hyper-saline inland lake in the north of Spain [90]. *B. halmapalus* is known to grow in a temperature range of 10-40°C [90]. However, even though the bacterial isolates used in this study were closely related to *B. halmapalus*, they were able to successfully grow at even lower temperatures (4°C) [90]. Moreover, *B. halmapalus* does not reduce nitrate, whereas PSY4 and PSY5 were both able to reduce nitrate to nitrite under anoxic conditions. These differences made PSY4 and PSY 5 better bacterial candidates than *B. halmapalus* for the development of BBSHC for low temperature marine concrete applications. Another recent study involving the use of non-ureolytic bacteria at low temperatures was published by Su *et al.* [92] where the ability of *Lysinibacillus sp.* to survive, germinate and induce $CaCO_3$ precipitation at 7°C when vegetative cells were directly incorporated into BBSH cement mortar specimens (10^{13} cells/ m^3 of mortar) with different calcium sources (calcium nitrate or calcium lactate). After healing for 14 days, satisfactory closure results for cracks

with widths below 0.5 mm were achieved, while cracks between 0.3-0.4 mm width were completely repaired. Su *et al.* also observed that the $CaCO_3$ precipitates were densely packed at a depth of 0-0.8 mm from the cracking surface, while between 0.8 and 1.6 mm, these $CaCO_3$ presented a smaller particle size (less packed). Beyond 1.6 mm depth, the results suggested no presence of $CaCO_3$ precipitates. They concluded that non-ureolytic *Lysinibacillus sp.* strains have the potential to be used in BBSHC exposed to low temperatures (7°C) [92].

For temperatures higher than 30°C, even less research has been carried out. Abdulkareem *et al.* [99] investigated the effect of high temperature (*i.e.,* 40°C) on the compressive strength of bacteria-based mortar specimens when these were exposed to different relative humidities (*i.e.,* 50, 72 and 95% RH). Non-ureolytic bacteria (*B. subtilis*) were added as vegetative cells during the initial mixing process. When the bacteria-based mortars were maintained at 40°C with the highest relative humidity (95%) condition, a higher compressive strength response was observed (3.2%) when compared to similar water-cured bacteria-based control formulations maintained at laboratory conditions. Moreover, higher temperatures also have a significant influence on the precipitation of $CaCO_3$. Rodriguez-Navarro *et al.* [115] and Zamarreno *et al.* [116] observed that at higher temperatures, $CaCO_3$ crystals are rapidly formed as a result of high saturation environments. The biogenic crystals formed in these supersaturated conditions presented a lower consolidative effect and lower adherence compared to crystals that were slowly formed at low supersaturation values (*i.e.,* under lower temperatures) [115].

3.4. Oxygen

The biochemical reactions during $CaCO_3$ precipitation rely on oxygen. Consequently, the limitation of its availability will have a negative effect on the rate of carbonate precipitation. However, when oxygen is not available in the environment surrounding steel reinforcement in concrete, the rate and risk of corrosion of this steel are greatly minimized. Therefore, the presence of active oxygen-respiring bacteria close to these rebars could potentially prolong the service life of steel-reinforced concrete structures even without $CaCO_3$ formation. [17]. Oxygen releasing compounds have been studied as components of healing agent formulations for their potential to increase $CaCO_3$ precipitation yield

when non-ureolytic bacteria are used under oxygen-limited conditions [17]. Zhang *et al.* found that calcium peroxide (CaO_2) tablets improved $CaCO_3$ precipitation by *B. pseudofirmus* H4 at an optimal dosage of 7.5 g/L [94,109]. It was observed that when oxygen was available, spores germinated more effectively and were able to maintain high metabolic activity. Moreover, the reaction of CaO_2 with water led to the production of additional Ca^{2+} ions. Overall, the inclusion of this oxygen-releasing agent resulted in three times more induced $CaCO_3$ precipitation than that obtained without an oxygen supply [109]. Nevertheless, the inclusion of these oxygen-releasing agents implies additional costs and challenges. The size of the bacterial carrier will increase to include these agents with likely effects on the mechanical response of these BBSH materials. Moreover, further research is needed to elucidate the effects of this additional oxygen when liberated in the proximity of embedded reinforcement steel. On the other hand, when oxygen cannot be made available, an alternative solution could be the use of facultative anaerobe non-ureolytic bacteria. Palin *et al.* [90] observed that bacterial isolates closely related to non-ureolytic *B. halmapalus* (PSY 4 and 5) were capable of respiring using "free" oxygen when available and then switch to nitrate (NO_3^-) when oxygen became limited. This ability may offer an attractive option for enhancing healing in complex situations such as marine environments or deep inside the cracks.

3.5. Mixed culture systems

To date, most of the research on BBSHC has been confined to the use of pure cultures of either non-ureolytic or ureolytic bacteria in an isolated way. However, some recent studies have begun to consider mixing both types of microorganisms to understand the interactions and efficiencies that these mixed communities may have in BBSHC. In this context, the effects of mixing a well-known ureolytic bacterium (*S. pasteurii*) with non-ureolytic bacteria were studied by Gat *et al*. and Son *et al.* using *B. subtilis* and *B. thuringiensis*, respectively. Gat *et al.* investigated the effect of these interactions on the $CaCO_3$ precipitation in natural soils [47] and artificial coastal groundwater medium [102], while Son *et al.* investigated the effects on the self-healing of cement mortars with cracks between 0.04 and 0.19 mm wide [12]. The results obtained when non-ureolytic and ureolytic bacteria were mixed give a valuable understanding of the complexity of interactions between these two

different bacteria. It has been shown that the non-ureolytic bacterial species used displayed higher growth rates, which resulted in a higher bacterial concentration [12,47,102]. This relatively higher growth led to a decrease in pH of the surrounding environment, which resulted in lower $CaCO_3$ accumulations even with higher total dissolved inorganic carbon (DIC) concentrations. Nevertheless, the presence of non-ureolytic bacteria resulted in a higher rate of $CaCO_3$ precipitation. These studies [12,47,102] have suggested that the non-ureolytic bacteria, when mixed with ureolytic bacteria, promoted $CaCO_3$ precipitation by supplying additional nucleation sites. However, for scaling up this approach, more research is needed not only to fully understand the complex interactions existing between both types of bacteria when they are grown together but also to know the effects that these interactions have on the $CaCO_3$ precipitation process [47].

Another strategy that recently has been explored involves the culture exclusively of different non-ureolytic species. In this context, Jang *et al.*[88] employed spray-dried mixed bacterial spores from non-ureolytic alkali-tolerant (*L. boronitolerans* YS11) and alkaliphilic (*B. alkaliphilus* AK13) strains [88]. This combination of non-ureolytic species produced a synergistic effect, where *L. boronitolerans* YS11 increased the pH of the surrounding environment during its metabolic activities [89]. This increase in alkalinity stimulated the growth of alkaliphilic *B. pasteurii* AK13 and improved the biofilm formation helping the bacterium to withstand conditions that it cannot tolerate by itself [75]. Jang *et al.* concluded that the use of mixed non-ureolytic bacterial spores has favourable properties for producing self-healing concrete, such as high internal survival (2% after 28 curing days) and well-crystalline $CaCO_3$ precipitation [88]. However, two major disadvantages were observed: an increase in macropores (0.1–0.2 mm) from the metabolic reactions of bacteria that resulted in a high water-porosity (twice that of the control sample) and a significant reduction of the 28-day compressive and flexural strengths, 39.3% and 55.7%, respectively [88].

To summarise, among aerobic non-ureolytic bacteria, the *Bacillus* genus has been the preferred option for developing self-healing cementitious materials, where *B. cohnii, B. pseudofirmus, B. subtilis* and *B. alkalinitrilicus* have been the most investigated isolates. The type and amount of nutrients and calcium precursors added profoundly impact the healing effectiveness and final cost of these self-healing

systems. While yeast extract has historically been included to aid germination of the spores and growth of the cells when combined with other organic carbon sources such as calcium lactate or calcium acetate, it has been shown in a recent study by Tan *et al*. [82] that it could be used as the only nutrient source. Bacterial spore concentration is another crucial parameter with a direct influence on the effective precipitation of $CaCO_3$, where the viability of these spores is significantly increased when different carriers (e.g., microcapsules or lightweight aggregates) are used to protect them until the crack formation. Up to now, most laboratory experiments using non-ureolytic bacteria have considered germination of their spores and precipitation of $CaCO_3$ at conditions close to optimum for these bacterial species (20-45°C) [81,83,88]. Some research has gone further to investigate BBSHC exposed to cold temperatures (4-8°C) [90-92]. Nevertheless, more research is needed on BBSH systems capable of efficiently performing under a broader range of temperatures and when these systems are exposed to cycles of low and high temperatures. Non-ureolytic bacteria able to survive and thrive in a wider temperature range and the use of mixed bacteria communities will be the clue to achieve this.

4. Bacteria delivery strategies

Non-ureolytic bacteria, generally in the form of spores, frequently have been added to self-healing cementitious composites formulations during the initial mixing process. However, to be viable when cracks occur, these spores must be able to withstand several limiting factors, such as high pH values (pH ~13), dehydrating conditions within cement matrix, and high mechanical compressive pressures during the initial curing period [76]. Different studies have corroborated that bacterial spores can survive up to 200 years under dry conditions [112], but also that they can withstand aggressive chemicals and ultraviolet radiation [10,21,32,112]. Nevertheless, to maximize the viability of the bacterial spores in cementitious materials, different protection strategies have been developed to overcome the initial conditions, with encapsulation and immobilization of bacteria within porous granules being the principal two methods [17]. Other methods also used for delivering viable bacterial spores directly to the cracks include external liquid agents [100,117] and vascular networks [118].

4.1. Direct addition

Non-ureolytic vegetative bacterial cells or spores can be added directly to the mixing water [19,45,79,84,85,92,105,119] or, in the case of spores, dry mixed with the cement before adding the mixing water. Chaurasia *et al.* investigated the densification of the interfacial transition zone (ITZ) of concrete aggregates when non-ureolytic vegetative bacteria (*B. cohnii*) were added directly with mixing water [106]. This densification was obtained by additional production of C-S-H and portlandite at the ITZ. For obtaining bacterial spores as a dry powder for direct addition, two principal methods have been used: spray-drying [88,120] and freeze-drying (lyophilization) [78]. Spray-drying produces bacterial spores as a dry powder from a slurry by rapidly drying it with hot air (**Fig. 4**), while freeze-drying involves freezing the spores, lowering the pressure, and then removing the ice by sublimation.

Fig. 4. Spray-dried bacterial spore powder: (*left*) powder and (*right*) 45x magnified image [88].

When bacterial spores are directly added to cementitious materials, these spores may have the capacity of filling pores during the initial curing period [84]. In a recent study, Schwantes-Cezario *et al.* analysed three different methods of porosity testing (*i.e.,* water absorption, X-ray computed microtomography (μ-CT), and mercury intrusion porosimetry (MIP)) to validate if the direct addition of bacterial spores was able to decrease the porosity of cement mortars [84]. Bacterial spores (3.3 x 10^4 spores/cm^3 of mortar) from a non-ureolytic bacterium isolated in Brazil (*B. subtilis* AP91) were added directly to the mixing water. It was shown that the direct addition of bacterial spores caused small changes in porosity, which were detected only by MIP. Blockage of pores smaller than 20 μm

due to $CaCO_3$ precipitation evidenced by SEM-EDS and an increase in the compressive strength (*i.e.,* 31%) were observed and related to the direct inclusion of the bacterial spores [85].

4.2. Immobilization and encapsulation

Due to concerns about the survival of bacterial spores through the mixing phase and hydration period of cementitious composites, most BBSH systems require spores to be immobilized or encapsulated prior to their addition. Common immobilization/encapsulation methods include the use of: (i) porous particles; (ii) polymers; and (iii) powder-compressed particles.

4.2.1. Immobilization in porous particles

Many porous materials have been considered for non-ureolytic BBSHCs; however, the nature of the pores within these materials is crucial to incorporate the required bacterial spores efficiently. As a result, not all porous particles are automatically suitable for the immobilization of these spores [121]. The most commonly used lightweight aggregates (LWAs) for this purpose are: (i) expanded clay, (ii) expanded perlite and (iii) aerated natural quartz sand. Porous natural fibres have also been used recently to immobilize non-ureolytic bacterial spores (**Fig. 5**).

Fig. 5. (*left*) Expanded Clay (EC) particle with styrene-acrylic protective layer [87]; (*middle*) Expanded Perlite (EP) particles [80]; and (*right*) flax porous fibre [122].

Expanded clay (EC) is a LWA that has been commonly used as a bacteria-carrier. EC is produced by heating clay to around 1,200°C in a rotary kiln resulting in highly porous particles with macropores

(*i.e.,* 10 to 100 μm) and rounded shapes (**Fig. 5 (left)**) [87]. Wiktor and Jonkers used EC particles (1-4 mm) to encapsulate calcium lactate (6% by mass of aggregate) and yeast extract (less than 0.1% by mass of aggregate) along with *B. alkalinitrilicus* spores (1.7 x 10^5 bacterial spores/g of EC). They observed that upon cracking, these encapsulated particles were able to heal cracks in mortar specimens after 100 days of being immersed in water [21]. Furthermore, Tziviloglou *et al.* incorporated EC particles (0-4 mm) with a healing solution containing non-ureolytic alkaliphilic bacterial spores (1x10^8 spores/L), calcium lactate (200 g/L) and yeast extract (4 g/L) [103]. The oxygen depletion measurements and SEM observations validated that the origin of the crack healing was bacterial activity. Stuckrath *et al.* [107] also used EC particles impregnated with calcium lactate (2% wt. of EC) and yeast extract (0.04% wt. of EC) in combination with vegetative non-ureolytic bacteria (1.3 x 10^8 *B. pseudofirmus* cells/g of EC). In a recent study, Han *et al.* [87] compared the protection efficiency between coated (*i.e.,* styrene-acrylic coating) and uncoated EC particles (2-5 mm) impregnated with a suspension of vegetative bacterial cells. After these two types of EC particles were exposed for 48 h to a harsh environment (60°C and pH 12), bacterial viability was found higher for coated EC (2.4 x 10^4 CFU/g of EC) than for the uncoated EC (5.0 x 10^3 CFU/ g of EC). Even though viability tests clearly demonstrated a better survival rate when coated EC particles were used, water permeability tests showed only a slight difference (*i.e.,* 5%) between these two types of EC particles. According to Han *et al.*, the observed differences in water flows were probably because of $CaCO_3$ precipitation, which is more affected by the amount of nutrients near the bacteria rather than the amount of bacteria at the time of crack formation. So, even though these bacteria-impregnated EC particles have been proven to protect bacteria and to improve crack-healing of cementitious composites, the inherent porous nature of these particles presents a significant drawback. The volume of EC particles needed for obtaining these self-healing effects compromises the strength of cementitious composites significantly. This loss in strength is because only 20% of the EC particle volume can be utilized as bacteria and nutrients storage space as most (80%) of the internal pores are not interconnected [123].

Expanded perlite (EP) is another LWA commonly used for immobilizing bacteria spores. Raw perlite rock, which is an amorphous volcanic glass with relatively high water content (2-5%), is rapidly

heated to above 870°C to vaporize its water content and create a greyish-white expanded and highly porous material known as EP (**Fig. 5 (middle))** [124]. EP is commonly used as an insulating material within the construction industry, and it is classified as chemically inert with a pH of ~7. Zhang *et al.* [80] compared the use of EP as a bacteria-carrier with EC particles. Both types of particles (2-4 mm) were impregnated under vacuum with a solution containing *B. cohnii* spores and then oven-dried for two days. Following this, a solution of calcium lactate and yeast extract was sprayed onto their surfaces. The particles were then covered with a geopolymer coating (metakaolin and sodium silicate solution). Significant amounts of voids (up to 100 µm in size) were observed in the EP particles. The highly porous structure in these EP particles could provide sufficient cover and "oxygen reservoirs" to the immobilized bacterial spores, while its high-water absorption capacity could allow embedded bacterial spores to contact with sufficient water [80]. Even more important is that these attributes allow a small increase in the number of *B. cohnii* spores that can be immobilized in EP particles when compared to EC particles. The contents of bacteria in EP and EC particles observed were 1.0×10^9 and 9.1×10^8 per cm^3 carrier, respectively (12% increase). As a result of its higher porosity and bacteria immobilization potential, lower amounts of EP particles (11% less) were needed to immobilize a similar number of bacterial spores. Likewise, Alazhari *et al.* utilized coated EP particles (1-4 mm) for a two-component encapsulated system containing, separately, EP particles with non-ureolytic bacteria spores (*B. pseudofirmus*) and EP particles loaded with nutrients (calcium acetate and yeast extract). The spore-containing EP particles (4.1×10^9 spores/g of EP) and nutrients-containing EP particles (with 0.3 g and 0.03 g of calcium acetate and yeast extract, respectively) were coated with a dual-layer of sodium silicate solution and a final application of dry cement powder to prevent leaching of spores and nutrients [83].

Lightweight fine-pored natural granules composed of natural quartz sand and lime have also been investigated as a potential bacteria carrier. Sierra-Beltran *et al.* used a commercial LWA (*i.e.,* Catsan®) with particles size between 0.25 and 2 mm as the bacteria and nutrient carrier [81,108]. The first step was to impregnate the porous carrier with a calcium lactate (150 g/L) and yeast extract (7.5 g/L) solution, followed by a second impregnation with a bacterial spore solution (*B. cohnii*). The

impregnated LWA held 15% (by mass) calcium lactate and 1.2 x 10^7 bacterial spores per gram of particle [81]. It was observed that the cracks break the LWA particle exposing the bacteria impregnated in it to the water present in the crack. Similarly, Tan *et al.* [82] utilized aerated concrete granules (ACG) commercially available from Cellumat SA (Belgium). These ACG were coated with polyvinyl acetate (PVA) and used to independently encapsulate non-ureolytic bacterial spores (*B. cohnii*) and growth medium (calcium nitrate and yeast extract). They demonstrated that these PVA-coated ACG particles were able to survive the mixing and hardening process intact, caused no retardation from growth medium leaking and fractured when cracks were formed.

Natural fibres are another type of porous material that has recently emerged as a potential carrier for bacterial spores. Rauf *et al.* investigated the potential use of coir, flax and jute fibres to immobilize *B. subtilis* and *B. cohnii* [122]. Flax fibres provided the best protection to bacteria with improved crack-healing and regain in compressive strength (**Fig. 5 (right)**). This better performance of the flax fibres reported by Rauf *et al.* was inferred to be the result of the high sorption property of flax fibres (*i.e.,* 985%) that allowed the spores in the solution to penetrate deep inside these fibres, consequently protecting them during the initial mixing. In contrast, coir fibres rendered a less suitable bacterial carrier than flax and jute fibres, this as a result of its low sorption capacity when compared to the other fibres (74% and 67% less sorption than flax and jute fibres, respectively).

4.2.2. Encapsulation in polymers

Palin *et al.* [91] utilized a polymer-based bead technology to encapsulate non-ureolytic bacterial spores (*B. halmapalus* PSY and PSY5). These 1 mm diameter bacteria-based beads were composed of bacterial spores and nutrients (yeast extract and magnesium acetate) encapsulated in calcium alginate. In contact with water, the beads along the crack swelled by 300% (up to a 3 mm diameter), clogging the cracks and releasing the bacterial spores and nutrients. This liberation of nutrients and bacterial spores resulted in the production of magnesium-based minerals and induced $CaCO_3$ precipitations (in and on the surface of the bacteria-based beads) that resulted in the healing of the cracks [91]. According to Palin *et al.*, 0.112 g of beads (~30 beads) can potentially produce ~1 mm^3 of calcite over

14-days [125]. In a recent study, Hamza *et al.* [101] encapsulated *B. subtilis* in calcium alginate to protect the bacterial spores in cement mortar formulations to be healed buried in the soil. To achieve this, a bacterial sodium alginate solution containing 6.1 x 10^6 CFU/mL was manually dropped via syringe into a coagulate solution consisting of calcium chloride and calcium lactate to produce calcium alginate beads with a particle size of 150 μm. Similarly, microcrystalline cellulose (MC) was used by Liu *et al.* to produce bacteria-based microcapsules via the extrusion-spheronisation and spray-drying method [126]. MC was first mixed with *B. pseudofirmus* spores to form the core material and then were encapsulated in a shell of ethyl cellulose (EC). It was revealed that the spores survived the initial mixing process and that some of the microcapsules were able to break upon the formation of a crack. An interesting finding was that liberation of the bacterial spores from these EC microcapsules could be controlled by pH values, where an increase of the pH will result in a considerably decrease in the delivery rate [126].

While the encapsulation of non-ureolytic bacterial spores has been successfully demonstrated, there has been less advance on the encapsulation of the required nutrients, mainly because these water-soluble materials can readily escape during the encapsulation process [17]. For encapsulation of calcium-based precursors by complex coacervation, this problem is exacerbated as these precursors can influence the pH of the environment, which is a crucial parameter for wall deposition. Moreover, the Ca^{2+} ions can interrupt the complexation of the two polymers forming the wall [17]. Nevertheless, some success has been recently obtained in this aspect [127]. Paine *et al.* [121], in partnership with the company Lambson (UK), produced gelatin/acacia gum microcapsules to encapsulate non-ureolytic bacterial spores (*i.e., B. subtilis* and *B. pseudofirmus*) and growth media using a complex coacervation process. They reported that an emulsion stabiliser was required to develop acceptable microcapsules due to the components of the growth media (*i.e.,* calcium nitrate and yeast extract) being water-soluble [127]. This encapsulation process was reported as simple and resulted in good yields with microcapsule sizes around 180 μm. Additionally, isothermal calorimetry results demonstrated that the microcapsules had few impacts on the hydration kinetics of cement and that they were able to survive the mixing process. A recent study by Zamani *et al.* [54] presented the novel use of synthesized

polyurea as a medium to encapsulate non-ureolytic bacteria (*B. pseudofirmus*). An in-situ polymerization method was employed for the synthesis and encapsulation of the bacterial spores and calcium lactate powder (growth medium) in polyurea capsules. It was observed that upon capsule rupturing, the bacterial spores were able to switch from dormant to active state and consume the available nutrients to precipitate the $CaCO_3$ needed for crack healing. Moreover, the results showed that this encapsulation process can provide significant benefits over current self-healing carriers, such as reasonably short curing time, adjustable brittleness, water insensitivity and longevity [54].

4.2.3. Powder-compressed particles

Mors and Jonkers incorporated non-ureolytic bacterial spores in powder-compressed particles to enhance water tightness on the surface of concrete specimens to improve the protective cover of the steel reinforcement [96]. Bacteria-based powder-compressed (BBPC) particles (2 mm diameter x 2-3 mm long cylinders) composed of lactic acid derivatives, calcium lactate, bacterial spores (*B. cohnii*-related strains) and activation nutrients (yeast extract) were developed in conjunction with Corbion Purac (Netherlands). Concrete specimens were cast using two different types of cement: CEM I (Portland cement) and CEM III (blast furnace slag cement). Surface densification took place upon BBPC particles addition (15 kg/m^3) to concrete specimens made with CEM I. In contrast, when CEM III containing a high ground granulated blast furnace slag (ggbs) content was used, the surface had a higher water absorption. Moreover, BBPC addition to concrete specimens containing CEM III exhibited an important decline of the ggbs reaction and concrete strength evolution, likely caused by potential competition over calcium hydroxide with BBPC particles addition. According to Mors and Jonkers [96], BBPC, in the form used, was suitable for addition only to CEM I (and potentially CEM II) concretes, while for concretes using cement with clinker content below 50%, a reduction in the concentration or modification of the BBPC was recommended. Advancing further in this research line, Mors and Jonkers [104] developed powder-compressed flakes (PCFs) by roller compaction of powders to sheets, with subsequent milling to flakes in the size range of the sand fraction (1-4 mm). These PCFs were also developed in collaboration with Corbion Purac, and they contained the same healing agents used by Mors and Jonkers to produce previous BBPC cylindrical particles [96]. These PCFs

were mixed directly with a commercially available dry mix mortar to evaluate the mechanical responses. The results revealed a negligible influence on mortar strength evolution in time (after 28-days). De Koster *et al.* studied the use of a geopolymer coating to protect powder-compressed particles. A tablet-type particle composed of nutrients (calcium lactate and yeast extract) and non-ureolytic *Bacillus* spores was produced using powder compression techniques. The compressed particles were coated with metakaolin (aluminosilicate source) and an activator liquid (sodium silicate or sodium aluminate) using a rotating disk granulator. They observed that the interaction between the cement paste and the coated particles appeared to be sufficient to ensure that the crack interface will go straight through the particle [128].

4.3. Sprayable liquids

In recent years, liquid bacteria-based systems have been developed as a surface treatment strategy to improve durability or repair existing concrete structures. Nevertheless, few studies have investigated the use of non-ureolytic bacteria in these bacteria-based agents. In a series of studies, Wiktor *et al.* developed a bacteria-based healing liquid (BBHL) system containing non-ureolytic bacteria for long-lasting and sustainable repair of cracked and porous concrete structures [117]. They produced a two-component system that requires sequential application of both components to avoid leaching of minerals (*i.e.,* portlandite) from the cement matrix. The first solution (component A) contained non-ureolytic bacterial spores ((1.6 x 10^8 spores/L) that included *B. cohnii* [129]), sodium silicate, sodium gluconate and yeast extract, while the second solution (component B) contained calcium nitrate, bacterial spores (1.6 x 10^8 spores/L) and yeast extract [117]. When “component A” is applied prior to “component B”, the two solutions start to produce a soft gel due to the chemical reaction between silicate and calcium ions contained in components A and B, respectively. With time, this gel is transformed into $CaCO_3$ due to bacterial metabolic conversion resulting in a permanent sealing of the cracks [117]. The effectiveness of this BBHL system was demonstrated through different field trials on concrete structures located in the Netherlands [73,76,123]. In a similar manner, Sharma *et al.* [78] investigated the performance of a non-ureolytic BBHL agent to repair microcracks in cement mortar specimens with a maximum width of 0.25 mm. This BBHL contained non-ureolytic bacterial spores (3

x 10^6 cells of *B. pseudofirmus*/L), calcium acetate (100g/L), yeast extract (4 g/L) and glucose (2 g/L). Application of the BBLH to microcracks resulted in obstruction by the deposition of $CaCO_3$. Cracks were effectively sealed based on water absorption tests. They concluded that the $CaCO_3$ precipitated remained intact within the crack, due to its low solubility at high pH, therefore providing a lasting and effective seal.

In a slightly different approach, Xu *et al.* explored a bio-deposition technique to be used as a surface treatment strategy [100]. Vegetative non-ureolytic bacterial cells (*B. cohnii*) were used along with an organic calcium-containing liquid medium. This liquid medium was composed of NH_4Cl, KCl, $MgCL_2$, KH_2PO_4, yeast extract and a calcium source, either calcium lactate or calcium glutamate. Surface treatment was applied by ponding. First, a layer of high-concentrated bacteria solution ($1x10^9$ cells/mL) was brushed on the upper surface of the specimens and left to induce bacteria attachment. After 24 h, the calcium-containing liquid medium was poured into the surface forming a pond, and the samples maintained at 30°C. After two weeks, the solution was removed, and the samples were left to dry at room temperature. $CaCO_3$ mineral formed a continuous layer covering the surface of the mortar specimens, where the thickness of the layer precipitated from the calcium glutamate medium (~280 μm) was considerably larger than that from the calcium lactate medium (~120 μm) [100]. The surface treatment resulted in decreased capillary water absorption (more than 50%) and increased resistance to carbonation (~50%) for all the bio-deposition surface treatments when compared with untreated specimens [100]. It was observed that the primary protective effect was due to pore blocking rather than resulting from a precipitated layer thickness.

4.4. Vascular networks

A vascular healing concept in concrete takes a biomimetic approach to deliver liquid healing agents to damaged sites. This is similar to how the human cardiovascular system uses its network of arteries and veins to provide oxygen-rich blood cells to build new tissue in wounds. A significant advantage of these systems is that when the bacteria and the nutrients are provided from an external source, there is theoretically no limitation to the volume of damaged material that can be restored. On the other hand,

a disadvantage of having a vascular network in a concrete structure, if left open to the atmosphere, is that it could provide a preferential route for detrimental elements (e.g., chloride ions or oxygen) to bypass the concrete cover protection layer and be detrimental to the durability of concrete structures. An approach to provide a vascular network utilizing non-ureolytic bacteria has been developed by Sangadji and Schlangen [118]. They designed a porous network concrete (PNC) containing a pervious concrete embedded in the interior of a concrete main body, imitating the hard-spongy structures of human bones [130]. This porous core functioned as a medium to transport the healing agents directly to the cracks (**Fig. 6**).

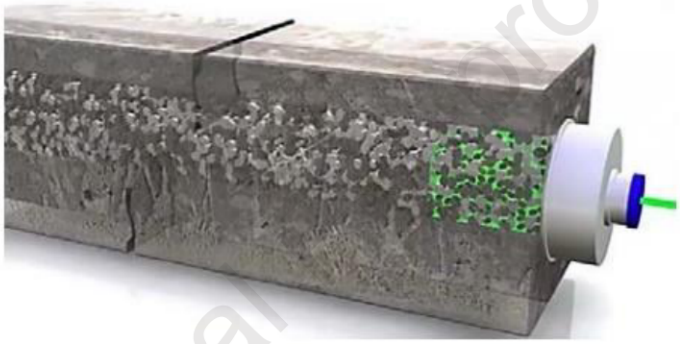

Fig. 6. Conceptual principle of healing agent transport in a Porous Network Concrete (PNC) [118].

The healing solution used was prepared based on the BBHL system containing non-ureolytic bacterial spores previously developed by Wiktor and Jonkers [21]. PNC prisms were elaborated and cracks intentionally produced in their mid-spans. They observed that complete permeability reduction through the cracks up to 0.25 mm, was achieved in the specimens after 28-days of wet curing (95% RH and ± 20°C). In contrast, samples that were dry-cured (30% RH ± 20°C) for 28-days showed only a limited reduction of permeability, and consequently self-healing. Multiple injections of the BBHL appear to be the most efficient way to obtain a sufficient volume of $CaCO_3$ precipitates to heal the cracks completely [118]. It was concluded that the results obtained showed promising potential for PNC structures as self-healing vascular systems.

To summarise, delivery strategies for non-ureolytic bacteria systems include different strategies such as direct addition, encapsulation, sprayable liquids, and vascular networks. The most economical and straightforward delivery strategy remains to be the direct addition of the bacterial spores during the initial mixing process. However, the amount of bacterial spores viable once the crack is formed is severely compromised as the spores must withstand very aggressive conditions and high mechanical compressive pressures during the initial curing period. To maximise the viability of these bacterial spores is that they are immobilized or encapsulated using different carriers prior to their addition. Different porous materials have been considered for this purpose; however, not all porous materials are automatically suitable for immobilizing the spores, as the nature and connectivity of the pore system within these materials play a crucial role [121]. Furthermore, coating these porous carriers can improve bacteria viability and efficient nutrients and precursors delivery [80,83,87]. Expanded clays and perlites, along with aerated concrete granules and more recently porous fibres, have been investigated for this purpose. Among the advantages of using porous carriers are a higher spore viability obtained and the ease of application by just adding them along with the fine aggregate during the initial mixing. In contrast, the main disadvantage of these porous materials lies in the fact that due to their porous nature, their inclusion results in a significant decrease of the mechanical properties of these bacterial-healing systems and a higher cost associated with the encapsulation process. Moreover, a potential problem with bacterial-healing systems is that whilst the bacteria can actively germinate, grow and theoretically sporulate once the conditions become unfavourable, the nutrients and calcium precursors required for growth and $CaCO_3$ precipitation are only available in a finite quantity. To address this problem, vascular networks have emerged as a mechanism to deliver nutrients and even additional bacterial spores with theoretically no limitations to the volume of damaged material that can be restored. However, the use of these networks is not without problems, and research conducted up to now has focused primarily on the delivery of chemical agents and not of nutrients for non-ureolytic bacterial-healing systems. Significant disadvantages are not limited to the additional work needed to prearrange the vascular network inside the concrete structure and the application of a slight additional pressure to improve the healing agent delivery, but also to the possibility of providing a preferential route for harmful elements (e.g., chloride ions or oxygen) to bypass the concrete cover protection

layer. Moreover, there is the potential risk that when placing the concrete, the vascular network could become crushed or strangled, which would cause the system not to work properly or not work at all. Further intensive research should focus on solving the disadvantages mentioned above as this technology shows promising potential.

5. Effects of bacteria on the performance of BBSHCs

Comparing data from the literature on the different impacts that the incorporation of non-ureolytic bacteria may have in cementitious composites is challenging. This is as a result of multiple differences in materials, experimental methodology and environmental conditions, as well as in bacterial species, concentrations, spore preparation, immobilization methods, curing and incubation conditions, nutrients, calcium precursors and cementitious materials used. All of these could potentially have a direct influence on the observations generated. The following section aims to give not only an overview of the main environmental conditions when using non-ureolytic bacteria, but also some of the efficiencies obtained regarding crack healing, improvement of mechanical properties and $CaCO_3$ precipitation.

5.1. Environmental conditions

Different ambient conditions can be found around the world, and these variations will inevitably affect the germination, growth and calcite precipitating capability of non-ureolytic bacteria. The impact of different environmental conditions on the compressive strength of cementitious composites when using vegetative non-ureolytic bacteria (*B. subtilis*) was investigated by Abdulkareem *et al.* [99]. Two curing scenarios were used: (i) a combination of varying temperatures (10°C, 26°C and 40°C) and relative humidities (50%, 72% and 95%), and (ii) a combination of different sunlight exposures (2 h, 5 h and 8 h) and wind speeds (0 m/s, 3 m/s and 6 m/s). It was observed that environmental conditions significantly affected the compressive strength of bacteria-based mortars (BB-mortars) after 28-days of curing. The compressive strength attained by all the BB-mortars exposed to these environmental conditions was higher than the water-cured no-bacteria control sample. This difference was more pronounced in the following two scenarios: (i) with a temperature of 40°C and 95% RH (increased by

110%) and (ii) with a wind speed of 6 m/s and sunlight exposure time of 2 h (increased by 133%) [99]. Moreover, the water-cured control BB-mortar always presented a higher compressive strength when compared to the BB-mortars exposed to all the different environmental conditions, except for the following two combinations: (i) with a temperature of 40°C and 95% RH (increase by 3.2%) and (ii) with a wind speed of 6 m/s and 2 h sunlight exposure (increase by 13.5%) [99]. They concluded that increased relative humidity, temperature and wind speed were beneficial for these BB-mortars as they resulted in higher compressive strength responses. In contrast, increased sunlight exposure time was detrimental, as it resulted in lower compressive strength responses. Similarly, Wood *et al.* [131] investigated how the viability of *B. subtilis* spores on the surface of the cement mortar degrades over time when exposed to direct sunlight. Cement mortar coupons were inoculated with *B. subtilis* spores (1×10^8 CFU per coupon) and exposed to simulated sunlight using ultraviolet radiation (UV-A/B). Wood *et al.* [131] observed that with exposure to UV radiation, the decay of *B. subtilis* spores viability occurs as an initial rapid decline followed by a slower inactivation period. It was shown that exposing the bacterial spores to this UV radiation for 56 days resulted in approximately a 1 to 2 log reduction when compared to initial values obtained after two days. In contrast, without UV radiation, they observed only an approximately 1 log reduction in the recovery of these spores from the mortar surfaces. It was concluded that the recovery of viable spores is greatly diminished when cement mortar surfaces are exposed to direct sunlight.

In laboratory conditions, the time from the moment cracks are generated until bacteria can heal them is known as the incubation phase. It is well known that the activities of non-ureolytic bacteria to produce $CaCO_3$ can be affected by different factors such as the presence of nucleation sites [132], pH [133], Ca^{2+} ions concentrations [134] and dissolved inorganic carbon (DIC) [29]. Additionally, different environmental parameters can come into play and influence the mentioned factors (e.g., the partial pressure of CO_2 and temperature) [135,136]. In general, BBSH specimens have been mainly incubated in three different scenarios: (i) submerged in water, (ii) in moist conditions (~95 ± 5% RH) and (iii) wet/dry environments [83]. Crack healing in wet/dry environments has been shown to significantly enhance bacterial growth and $CaCO_3$ precipitation when compared with wet conditions [103]. On the

other hand, incubation in moist environments has been demonstrated to require longer times for crack healing, needing in some cases up to 165 days for crack widths of 0.35 mm [83]. Up to now, most tests involving non-ureolytic bacteria have been done under typical laboratory conditions (*i.e.,* 20-30°C and using tap water). In order to better understand the effects that different environmental factors may have on the crack-healing efficiency of BBSH systems, more studies with less conventional scenarios are required. Among these non-conventional scenarios, Palin *et al.* investigated the crack healing capacity of BBSH cement mortar specimens when they were incubated in cold artificial seawater [91]. The mortar samples were incubated submerged in artificial seawater for 56 days (permeability tests) and 168 days (compressive strength) in a refrigerator at 8°C, and the bacterial spores (*B. halmapalus* PSY4 and PSY5) and growth media (magnesium acetate and yeast extract) were encapsulated using calcium alginate beads. The mortar samples displayed excellent crack-healing capacity under these specific ambient conditions; however, compressive strength was decreased by the presence of the beads.

Even though research to date has mainly focused on the incubation in moist conditions and water-submerged environments (either freshwater or seawater), a significant number of concrete structural elements are exposed to a broad range of soil conditions, where *Bacillus* species are naturally present. In this context, Hamza *et al.* [101] investigated the effects that the encapsulation of *B. subtilis* spores in calcium alginate beads have on the self-healing of cement mortar samples buried under a fine-grained soil. The soil used for the experiments comprised a dark brown silty sandy clay with a small portion of organic matter and an average pH value of 7.05. They found that the healing ratio after 28-days for cracks up to 0.42 mm that were present in the mortar specimens buried within saturated soil was similar to the healing ratio found for similar water-submerged samples. However, this was found to be applicable only when the soil pore-water pressure was positive or near-equilibrium (*i.e.,* fully-saturated soil) [101]. In contrast, it was observed that partially-saturated fine-grained soil likely developed suction that overcame the capillary pressure of the cracks and therefore interrupted the water ingress to them [101]. This probably resulted in the delay of bacterial $CaCO_3$ precipitation. Hamza *et al.* [101] expected that impregnation with *B. subtilis* spores was not necessary in order to

accomplish self-healing of the cement mortars cured within natural soil, as a result of the naturally occurring *Bacillus* bacteria in the soil. Nevertheless, the results indicated that the specimens with no bacteria, either in part or fully-saturated soil conditions, did not exhibit significant self-healing within the 28-days testing duration. It was hypothesized that the potential healing effect of the naturally existing bacteria in soil might require a longer healing time and an added nutrient source [101]. In a similar study by Esaker *et al.* [137], the healing ratio for mortar specimens embedded within a non-cohesive soil (sand) has been reported under different pH (*i.e.,* 4,6 and 7) and humidity soil conditions. It was observed that for the mortar specimens containing encapsulated bacteria (*i.e.,* perlite + *B. subtilis* + nutrients), healing ratios in the range of 47-83% were achieved. In contrast, significant lower healing ratios (between 33-38%) were obtained with mortars not containing encapsulated bacteria under similar conditions. SEM-EDX analyses revealed $CaCO_3$ precipitation inside the cracks of the mortars containing encapsulated bacteria, while ettringite and C-S-H were observed on non-bacteria mortars. The latter indicates that no $CaCO_3$ precipitation from bacterial activity occurred on these non-bacteria mortars despite naturally present soil microorganisms. In the two studies mentioned above, the only calcium source for the naturally occurring bacteria corresponded to the calcium present within the cement matrix as no additional calcium was added to any of these no-bacteria mortars. Consequently, the effects of the naturally existing bacteria within the soil on cementitious materials' self-healing process are still unknown and require further research [101,137].

5.2. Crack closure efficiency

Crack width is a critical element in order to obtain faster and efficient healing when using bacteria-based systems [138]. Different crack closure efficiencies have been observed depending on different factors such as the non-ureolytic bacteria species, growth media, incorporation method and incubation conditions used (**Table 2**).

Table 2: Overview of crack healing in cementitious materials by non-ureolytic bacteria.

Non-ureolytic microorganism	*Bacterial carrier*	*Type of specimen / Incubation condition*	*Growth Medium / via*	*Incubation period (days)*	*Max. crack width healed (mm)*	*References*
Bacillus alkalinitrilicus	Expanded Clay (EC)	Mortar / Submerged in tap water	Calcium lactate and yeast extract / impregnated in EC	100	0.46	[21]
Bacillus cohnii	Expanded Clay (EC)	Concrete / Submerged in tap water	Calcium lactate and yeast extract / impregnated in EC	28	0.45	[80]
Bacillus cohnii	Expanded Perlite (EP)	Concrete / Submerged in tap water	Calcium lactate and yeast extract / impregnated in EP	28	0.79	[80]
Bacillus pseudofirmus DSM8715	Expanded Perlite (EP)	Mortar / Moist conditions (~100% RH and 30°C)	Calcium acetate and yeast extract / impregnated in EP	165	0.35	[83]
Bacillus cohnii	Lightweight Aggregate (LWA)	Mortar/ Submerged in water @ 25 ±2°C	Calcium lactate and yeast extract / impregnated in LWA	56	0.20	[81]
Bacillus cohnii	Direct addition	Fibre reinforced mortar / Moist conditions (90 ±10% @ 20 ±2°C)	Calcium lactate or calcium glutamate and yeast extract / Direct addition	30	0.40	[79]
MM1_1 (close related to *B. licheniformis*) and RC1_1 (closely related to *B. anthracis*)	Lightweight aerated concrete granules (ACG)	Mortar / Partially submerged in tap water at room temperature.	Calcium nitrate and yeast extract / impregnated in ACG	60	0.50	[52]
PSY 4 and PSY 5 (99% coverage with *Bacillus halmapalus*)	Calcium alginate beads	Mortar / Submerged in artificial seawater @ 8 °C	Calcium acetate, magnesium acetate and yeast extract / encapsulated in calcium alginate beads	56	0.60	[91]
Lysinibacillus sp	Direct addition	Mortar / Submerged in DI water @ 7 ±3°C	Calcium lactate or calcium nitrate and yeast extract / Direct addition	14	0.50	[92]
Lysinibacillus boronitolerans YS11 and *Bacillus alkaliphilus* AK13	Direct addition	Mortar / Submerged in tap water @ 23 °C	Calcium lactate and yeast extract / Direct addition	28	0.43	[88]
Bacillus subtilis H50620/9	Calcium alginate beads	Mortar / Fully and partially saturated alluvial soil @ 20 °C	Calcium lactate and yeast extract / encapsulated in calcium alginate beads	28	0.42	[101]
Bacillus cohnii	Powder-compressed flakes	Mortar / Submerged in tap water or 95% RH room	Calcium lactate and yeast extract / encapsulated in flakes	100	0.60	[104]
Bacillus cohnii	Lightweight aerated concrete granules (ACG)	Mortar / Wet-dry cycle (16h wet- 8h dry)	Calcium nitrate and yeast extract / impregnated in ACG	84	0.50	[82]
Bacillus pseudofirmus,	Bacteria-based liquid agent	Mortar / Moist conditions (100% RH at 30 °C)	Calcium acetate, glucose and yeast extract / Liquid bacteria-based agent	8	0.25	[78]
Alkaliphilic bacteria of the genus *Bacillus*	Expanded Clay (EC)	Mortar / Submerged in water @ 20 ±2°C and wet-dry cycle (12h wet- 12h dry)	Calcium lactate and yeast extract / encapsulated in EC	56	0.35	[103]
Bacillus pseudofirmus	Expanded Clay (EC)	Mortar / submerged in tap water	Calcium lactate and yeast extract / impregnated in EC	100	0.22	[107]
Bacillus subtilis	Graphite nano platelets (GNP)	Concrete / Moist conditions	Calcium lactate / Direct addition	7	0.81	[105]
Bacillus mucilaginous	Direct addition	Concrete/submerged in water	Calcium nitrate, yeast extract and sucrose	28	0.49	[93]

It is well known that different external factors influence the crack healing and induce a different $CaCO_3$ yield by the same non-ureolytic bacteria species [99], where more efficient crack healing has been observed under wet-dry conditions [83]. Wiktor and Jonkers [21] stated that *B. alkalinitrilicus* was able to heal cracks up to 0.46 mm width when a two-component bio-chemical self-healing agent consisting of bacterial spores and calcium lactate was impregnated in expanded clay (EC) particles. Likewise, Zhang *et al.* [80] compared two different carriers, expanded perlite (EP) and expanded clay (EC), to immobilized *B. cohnii*. After 28 days, they reported the healing of 0.79 mm and 0.45 mm

width cracks when using EP and EC, respectively [49,80]. Alazhari *et al.* [83] found that self-healing of cracks with widths of 0.35 mm could be achieved when coated EP particles were used as a 20% replacement of fine aggregate, provided that a suitable ratio of *B. pseudofirmus* spores to calcium acetate was used (8 x 10^9 spores per g of calcium acetate). Sierra-Beltran *et al.* [81] found that crack widths below 0.20 mm were healed on specimens of an engineered cementitious composite (ECC) when these were incubated underwater for 28-days. An EEC containing PVA-fibres (2% by total volume), LWA impregnated with nutrients (calcium lactate and yeast extract) and non-ureolytic bacterial spores (*B. cohnii*) was used. The use of PVA-fibres guaranteed a maximum crack width of 0.20 mm. As the quantity of $CaCO_3$ precipitates was not substantially different between the no-bacteria specimens and the bacteria-based specimens, it was suggested that this could probably be attributed to limited amounts of nutrients impregnated into the LWAs (calcium lactate at ~3.5% cement weight). However, when using the same bacterial strain, Xu and Yao found that the direct addition of bacteria cells (*B. cohnii*), yeast extract and an organic calcium salt (*i.e.,* calcium lactate or calcium glutamate) completely sealed cracks within a width range between 0.10 and 0.40 mm width [79]. It should also be noted that the concentration of calcium lactate used in this latter study (1% by mass of cement) was significantly lower than the one used by Sierra-Beltran *et al.*

Other researchers have investigated the crack closure healing efficiency of BBSH systems using non-ureolytic bacteria under radical conditions such as in cold seawater or when buried in the soil. Crack closure efficiency in cold temperature artificial seawater (*i.e.,* 8°C) was investigated by Palin *et al.* using non-ureolytic bacteria encapsulated in calcium alginate beads [91]. They observed that the mortar samples displayed an excellent crack-healing capacity by reducing the permeability of cracks widths of 0.4 mm and 0.6 mm by 95% and 93%, respectively. This healing was attributed to autogenous precipitation, bead swelling, magnesium-based mineral precipitation, and bacteria-induced $CaCO_3$ precipitation inside and outside the surface of the beads [91]. In a recent study by Hamza *et al.* [101], crack closure efficiency of bacteria-based mortar (BBM) specimens buried within saturated natural alluvial soil was compared to water submerged samples. They found that the healing ratio (HR) of the cracks, with widths between 0.18 mm and 0.42 mm, for the BBM specimens buried within

soil was similar to the HR found for the water submerged samples after 28-days of incubation (*i.e.,* ~60% HR) [101]. However, they observed that the BBM specimens in both conditions (*i.e.,* buried and water submerged) did not reach 100% HR, especially along the larger cracks and they suggested that this may have been due to the encapsulation method (*i.e.,* calcium alginate beads) used and the short incubation times.

Recently, Reeksting *et al*. compared the performance of non-ureolytic and ureolytic bacteria species for self-healing of cracked cement mortars [52]. They found that non-ureolytic bacteria caused more consistent recovery of water tightness and more complete healing recovery of cracks (widths up to 0.50 mm) when compared to ureolytic species. They suggested that a possible explanation for these differences may be that the rapid precipitation and small crystal size observed in ureolytic isolates do not reliably lead to retention of the $CaCO_3$ precipitates within the crack. Thus they may not perform as reliably as the larger precipitates with organic components observed with non-ureolytic species [52].

5.3. Mechanical properties

The performance of non-ureolytic bacteria to fill up minuscule voids in cementitious composites to decrease porosity and increase the mechanical properties have been investigated by Schwantes-Cezario *et al.* [85]. They evaluated the influence of *B. subtilis* AP91 on the mechanical response of cement mortars. Bacterial spores were added directly with the mixing water or by immersion of the specimens in a solution containing the spores. They found that the 28-days compressive strength of cement mortars was increased, especially when bacterial spores (3.3×10^4 spores/cm^3) were added directly (31% increase). Tensile strength was also increased. Additionally, a small gradual increase in the modulus of elasticity of the mortars was observed when the spores were added, either by immersion or in the mixing water. It was concluded that the bacteria activity produced $CaCO_3$ crystals within pores of the cement mortar, especially when the bacterial spores were added directly to the mixing water. In a more recent study, Schwantes-Cezario *et al.* investigated the capacity of these bacteria (*i.e., B. subtilis* AP91) to fill pores and decrease porosity during the curing period of mortars when these were cured by immersion in a phosphate buffer solution (pH 7.2) [84]. As expected, the

addition of *B. subtilis* AP91 spores by immersion caused a reduction in porosity in pore sizes between 1.84 and 0.006 μm. However, no significant increase in compressive strength was observed when compared to control formulations [85]. Chaurasia *et al.* observed that due to the negative zeta potential found on bacterial cell surfaces, Ca^{2+} ions were able to adhere to these surfaces resulting in the additional formation of calcium hydroxide and calcium silicate hydrate (C-S-H) along with calcite in BBSHC specimens [106]. In this study, *B. cohnii* was cultured in a nutrient broth and used to replace the mixing water. It was found that *B. cohnii* provided extra nucleation sites that helped increase the amount of hydrated products. The results showed ~16% higher C-S-H and ~37% more calcium hydroxide formation in BBSHC specimens. The formed mineral phases (*i.e.,* C-S-H, calcium hydroxide and calcite) acted as fillers that clogged pores and increased the packing density of the concrete matrix, which subsequently, resulted in overall performance enhancement.

Khaliq and Ehsan investigated the effects of the addition of non-ureolytic bacteria (*B. subtilis*) on the compressive strength response when bacterial spores were directly added with mixing water or impregnated in graphite nanoplatelets (GNP) [105]. Compressive strength trends of the two formulations showed a slight increase in the compressive strength (*i.e*., 12% increase). Similar increases in compressive strength were observed by Sierra-Beltran *et al.* when *B. cohnii* was immobilized using LWAs in PVA-fibre reinforced mortars [81]. Mors and Jonkers investigated the effect on compressive strength when flakes containing a bacteria-based (*B. cohnii*-related strains) healing agent were mixed with a commercially available dry mixture mortar (15 kg flakes/m^3) [104]. Influence on compressive strength development of the mortar specimens was apparent before, but negligible after seven days. The effect was more significant at 1-day age when the average compressive strength ratio to control reported for the bacteria-based specimens was ~40%. The ratios observed at 3 and 7 days gradually improved to ~65% and ~90%, respectively. In a recent study by Rauf *et al.* [122], non-ureolytic bacteria (*B. subtilis* and *B. cohnii)* were immobilized into natural fibres (*i.e.,* flax, jute and coir fibres) when added to concrete specimens. The maximum increase in strength among these two bacterial species was 36% and 33% when using coir fibres to immobilize *B.*

subtilis and *B. cohnii*, respectively. Higher differences in compressive strength between both bacteria were observed with the other fibres (*i.e.,* 6 and 13% for flax and jute fibres, respectively)

In contrast, a decrease in compressive strength was observed by Jonkers *et al.* when *B. pseudofirmus* spores were added directly without organic compounds additions to cement mortars. They concluded that the direct incorporation of a high number of bacterial spores (6 x 10^8 spores/cm^3) with the mixing water resulted in a decrease in compressive strength of less than 10% for the 3, 7 and 28-days cured specimens [76]. Additionally, mercury intrusion porosimetry (MIP) analysis revealed that while pores inside the cement matrix of young samples (up to 7 days) were able to allow bacterial spores with typical diameters of 0.8-1 μm, the majority of incorporated spores were apparently crushed in aged specimens (28 days), resulting in decreased $CaCO_3$ precipitation capacity [76].

When porous aggregates are used to immobilize bacteria, the mechanical properties of these particles have a considerable influence on the overall mechanical properties of BBSHCs. Significant reductions in compressive and flexural strengths were reported by Tziviloglou *et al.* when 33% of the fine aggregate (by mass) was replaced with expanded clay (EC) particles (0-4 mm) in cement mortars. Along with the expected reduction on the compressive strength, they found that the presence of the healing agent (bacterial spores and nutrients) incorporated into these EC particles, delayed the hardening of the mortars by approximately 1-day. The latter resulted in significantly lower values for flexural and compressive strength at 3-days, 54% and 63%, respectively, when compared to control mortars. However, at a later age (>7 days), the healing agent presence did not seem to have affected these mechanical properties [103]. Similarly, Palin *et al.* found that compressive strength was reduced by 45% when using calcium alginate beads, representing 5% of the materials' volume, compared to otherwise similar plain mortar cubes [91]. In contrast, Khaliq and Ehsan observed an increase in the compressive strength when *B. subtilis* were impregnated into EC particles (12% improvement compared to reference formulation). They attributed this increase to the presence of calcite producing bacteria and a smaller size of EC. This smaller size permitted better packing and compaction of concrete matrix around the particles, which gave the concrete specimens much higher compressive strength than reference specimens [105].

5.4. Calcium carbonate precipitates

$CaCO_3$ can be formed through abiotic or biotic processes in cement-based materials. The abiotic process involves the production of $CaCO_3$ precipitates due to the carbonation of calcium hydroxide present in the cement matrix. In contrast, the biotic process relies on the bacterial activity to convert calcium ions, from calcium precursors or calcium hydroxide, into $CaCO_3$ precipitates. Abiotic carbonation is a very slow process compared to bacterial precipitation processes, as it relies on the availability of CO_2 dissolved in the pore water. Furthermore, as calcium hydroxide is soluble in water, it is dissolved whenever it comes in contact with permeated water, leaving less calcium hydroxide on the cementitious matrix to convert in $CaCO_3$. On the other hand, it has been observed that the presence of non-ureolytic bacteria along with the required nutrients catalyses the production of $CaCO_3$ crystals [105]. In this context, similar findings have been reported when ureolytic bacteria are used for MICP purposes. Okyay *et al.* [139] observed that biotic $CaCO_3$ precipitation depended on the bacterial isolate and the microbial growth conditions present. The cell surface properties of bacteria, including cell walls, proteins and extracellular polymeric substances (EPS), have key effects on the morphology and mineralogy of the produced $CaCO_3$ [32,115]. Okyay *et al.* also observed that abiotic $CaCO_3$ precipitation was significantly affected by environmental conditions, as higher $CaCO_3$ precipitation resulted when more nutrients were available in the environment. The observed higher abiotic precipitation was explained as a result of the CO_2 diffusivity, which is known to be dependent on the viscosity and temperature [139].

The type of calcium source used is also an important factor that affects $CaCO_3$ morphology [56]. Accordingly, different morphologies of $CaCO_3$, such as calcite (rhombohedral crystal), vaterite (hexagonal crystal) or aragonite (needle-like crystal) (**Fig. 7**), can be precipitated based on chemical properties of the bacteria cell surface, calcium sources used and the environment [32,140].

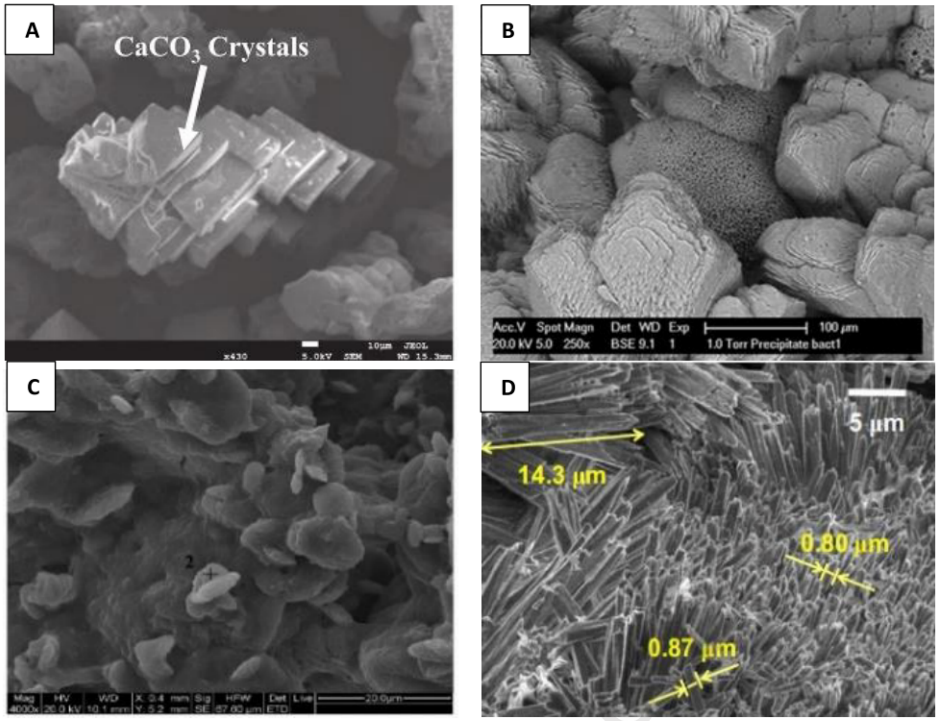

Fig. 7. SEM images of: A) calcite by *B. cohnii* [80], B) calcite deformed lamellar rhombohedra precipitated by *B. alkalinitrilicus* [21], C) granular grains of vaterite by *B. cohnii* [100] and D) aragonite by mixed culture (MC-Aa) under anoxic conditions [49]. Mineralogy classification was confirmed using XRD (A, C and D) and FTIR (B) analyses.

Calcite is the most common $CaCO_3$ polymorph found in MICP studies [49]. Bacterial species have a significant effect on $CaCO_3$ precipitation. Wiktor and Jonkers [21] characterized the $CaCO_3$ precipitations lining the crack surfaces after the healing process when *B. alkalinitrilicus* spores were used in combination with calcium lactate and yeast extract. The $CaCO_3$ precipitate morphologies observed differ from the typical rhombohedral form of calcite [100]. The precipitates in this study appeared in two distinctive shapes, as "deformed" lamellar rhombohedra (calcite) (**Fig. 7 (B)**) and as needle-like clusters assembled in dumbbell shapes (aragonite), with 51% and 49% formations, respectively [49]. Similar morphology was observed by Zhang *et al.* when a different non-ureolytic bacteria strain was used (*B. cohnii*). $CaCO_3$ precipitates were confirmed to be in the form of calcite crystal [49]. The crystal morphology observed was proven to be the same deformed lamellar rhombohedra observed by Wiktor and Jonkers [21]. Calcite and aragonite were also observed by other researchers [78,107,122]. Sharma *et al.* compared the $CaCO_3$ precipitates of three different non-ureolytic bacteria (*B. pseudofirmus, B. cohnii* and *B. halodurans)* [78]. FTIR and Raman spectroscopy

techniques were employed to characterize the precipitates. FTIR spectrum revealed four major absorption peaks at 1530, 1426, 875 and 712 cm-1 peculiar to calcite, while in the Raman spectra the most intense bands corresponded to calcite ((~282 and 713 cm-1) and aragonite (~207 and 704 cm-1) [78]. In contrast, vaterite-like $CaCO_3$ minerals were observed in a recent study by Yoonhee *et al.* when using alkaliphilic and halotolerant *B. subtilis* AK13 in combination with yeast extract and either calcium acetate or calcium lactate [56]. Crystals were found tightly attached to the bacterial cells, and isotope ratio mass spectroscopy analysis was used to confirm that the majority of the CO_3^{2-} ions in these precipitates were produced by cellular metabolism rather than being derived from environmental CO_2 [56]. Stuckrath *et al*. [107] found that the presence of bacteria (*B. pseudofirmus*) promoted the formation of larger crystals when compared to formulations containing only the chemical agent (*i.e.,* calcium lactate) with no bacteria. Jonkers *et al.* [76] observed similar behaviour. They reported that smaller sized calcite crystals (between 1 and 5 μm) were formed by abiotic factors, while larger sized calcite crystals were formed due to bacterial activity. In a recent study, Rauf *et al*. [122] found that the morphology and size of $CaCO_3$ precipitate varied between *B. subtilis* and *B. cohnii*, with deformed lamellar crystals (50 μm) and rhombohedral crystals (20 μm), respectively.

Yoonhee *et al.* [56] noted that the $CaCO_3$ crystals precipitated by non-ureolytic bacteria varied in size depending on the type of calcium source utilized. The $CaCO_3$ crystals formed with calcium acetate were smaller than those formed when calcium lactate was used. Likewise, Xu *et al.* noted that the type of calcium source had a profound impact on the crystallization of bacterially mediated $CaCO_3$ [100]. Poorly crystallized calcite (clustered lamella (< 10 μm)) was formed when either calcium chloride or calcium lactate were used as the calcium source. For crystals formed from calcium glutamate, grains indicated the presence of vaterite with a size of 3-10 μm. Vaterite is very unstable, which is caused by its higher solubility and lower density as compared with more stable crystal forms, such as calcite and aragonite [141]. Vaterite is expected to transition to the more stable forms of $CaCO_3$ over time under the conditions likely to be encountered in BBSH concrete. However, it has been observed that the presence of organic molecules associated with living organisms, particularly those including amino acids, helps to improve vaterite formation and stabilization [100,141]. In this study, calcium glutamate

acted as a template for vaterite nucleation or inhibiting the transition to more stable forms (*i.e.,* calcite). Moreover, the highest amount of total precipitated $CaCO_3$ was obtained when using calcium glutamate, while the lowest was obtained when using calcium chloride. Furthermore, Zhang *et al.* [142] observed that the crystal type of the $CaCO_3$ precipitates when using calcium acetate was mainly aragonite presenting an acicular morphology, while for the others calcium sources (*i.e.,* calcium chloride and calcium nitrate), it was mainly calcite presenting a hexahedral morphology.

The differences in $CaCO_3$ precipitates between non-ureolytic and ureolytic bacteria have been investigated recently by Reeksting *et al*. [52]. The non-ureolytic isolate MM1_1 (closely related to *B. licheniformis*) and the ureolytic isolate CG7-3 (closely related to *Lysinbacillus fusiformis*) were used in this study. Initial precipitates consisted of spherical $CaCO_3$, typical of the polymorph vaterite, which eventually converted into the rhombohedral more stable morphology associated with calcite [52]. They found that when $CaCO_3$ precipitation was rapid, such as with the ureolytic strain, inorganic, homogeneous crystals were produced. In contrast, the non-ureolytic strain precipitated $CaCO_3$ more slowly, producing crystals containing significant proportions of bacterial cells that appeared as mixed organic/inorganic crystals (**Fig. 8**).

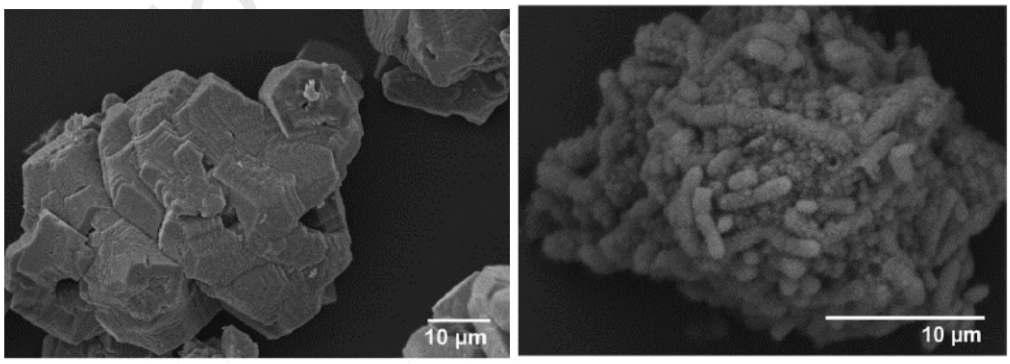

Fig. 8. Differences between ureolytic and non-ureolytic $CaCO_3$ precipitates: (*left*) ureolytic calcite precipitated by CG7_3 strain (closely related to *L. fusiformis*) and (*right*) non-ureolytic calcite precipitated by MM1_1 strain (closely related to *B. licheniformis*) [52].

Reeksting *et al*. observed that non-ureolytic bacteria showed a strikingly consistent recovery in water tightness. On the other hand, with ureolytic bacteria, there was more variability on the degree of

healing obtained which was most likely due to the mixed nature of the non-ureolytic $CaCO_3$ precipitates, where for the same amount of calcium being used, a larger volume of $CaCO_3$ precipitate can be formed [52]. Although it is not possible to generalise that the type of $CaCO_3$ precipitate obtained through MICP is completely related to the pathway mechanism, as many key factors such as pH, calcium concentration, dissolved inorganic carbon or availability of nucleation sites also have a significant effect on these precipitates, the study by Reeksting *et al.* revealed fundamental differences in the way in which different bacteria precipitate $CaCO_3$. In a similar context, some researchers have suggested that when $CaCO_3$ precipitates are found in a less dense form than calcite (*i.e.,* vaterite), they are likely able to fill more space for a given mass of precipitate and improve healing efficiencies [32].

5.5. Effects of healing on different mechanical properties

Most of the research work done on bacteria-based self-healing cementitious materials have been focused on demonstrating the capability of these systems to self-heal the cracks formed under very different experimental conditions and by employing different bacteria isolates and nutrients. Therefore, the regain or improvement of the original mechanical properties of the bacteria-based materials after successful healing has been constantly relegated to a second plane. In this context, the available literature on self-healing and restoration of the original mechanical properties when specifically non-ureolytic bacteria are used is currently scarce and dispersed.

A few studies can be found where the regain of the mechanical properties has been measured following self-healing of cementitious materials by non-ureolytic bacteria. In these studies, ultrasonic pulse velocity (UPV), compressive strength and three-point bending tests have been used to investigate the recovery of the initial mechanical properties when self-healing has been successfully achieved. Rauf *et al.* [122] studied the use of different natural fibres (*i.e.,* coir, flax and jute) as a protective carrier for *B. cohnii* spores in bacteria-based self-healing (BBSH) concretes. The recovery of the initial cement matrix conditions was assessed through ultrasonic pulse velocity (UPV) and recovery of compressive strength. Ultrasonic pulse velocity (UPV) was used to observe the sealing of internal cracks after 28 and 56 days of healing. The percentage difference between the initial and final

transmission velocities showed an improvement of 14.1% at 56-days when *B. cohnii* spores were embedded into flax fibres. Slightly lower UPV percentage values were observed at a similar healing time for the other two fibres (*i.e.,* ~11%). These differences were likely attributable to the different hydrophilic nature of these fibres and their bonding with the cementitious matrix. Moreover, two ureolytic bacterial isolates (*i.e., B. subtilis* and *B. sphaericus*) were also investigated by Rauf *et al*. to have a direct comparison from the perspective of the efficiency of different bacterial species and different pathways (i.e., non-ureolytic vs ureolytic). In this context, higher UPV percentage improvements (*i.e.,* ~21.5%) were observed for the two ureolytic bacteria isolates, which was likely due to their higher calcite forming ability [122]. Xu and Yao [79] also employed UPV to demonstrate the recovery of the original mechanical properties when a non-ureolytic live culture solution was either directly added to the mixing water or externally applied on fibre-reinforced self-healing mortars. The results suggested a relatively low self-healing effectiveness when compared to the external application of the bacterial solution.

Flexural stress regains from three- and four-point bending tests have also been investigated in non-ureolytic bacteria-based self-healing systems. Sangadji *et al.* [118] obtained the flexural stress regain of a reinforced porous network concrete (PNC) beam from a three-point bending test. The PNC specimens, injected with a bacteria-based repair solution containing non-ureolytic bacterial spores (*i.e., B. alkalinitrilicus*) and prepared based on Wiktor and Jonkers [21], were tested in a pre- and post-healing state (after 28 days) and compared to a control specimen, which only received a tap water injection after the crack was formed. Sangadji *et al.* observed that the post-healing stiffness values were lower for both the control and the bacteria-injected specimens when compared to the values of the pristine samples, and the crack reopened when the second loading was applied. As the specimens contained embedded rebar, the post-healing strength observed resulted from the steel reinforcement and not due to complete and effective crack closure [118]. In contrast, Sierra-Beltran *et al.* [81] observed a slight recovery of the deflection capacity and flexural strength when four-point bending tests were used to evaluate engineered cementitious composites (ECC) containing PVA fibres and lightweight aggregates impregnated with calcium lactate and non-ureolytic bacterial spores (*i.e., B.*

cohnii). The ECC specimens were preloaded at seven days and then cured in water until age 56 days. At this age, the ECC specimens were tested to failure to evaluate the mechanical properties of the "healed" specimens. After cracking and healing, the specimens containing the bacteria healing agent showed a slightly better recovery of the original mechanical properties when compared to the control formulations. Furthermore, Sierra-Beltran *et al.* observed that in the control specimens, most of the cracks under reloading passed through the pre-existing cracks, while in the specimens containing the bacterial spores, some cracks deviated from the healed pre-existing crack and formed new cracks. These observations suggest that regain of the original mechanical properties could be possible on these bacteria-based systems. Xu and Yao [79] also employed a four-point bending test to demonstrate the recovery of flexural strength when a *B. cohnii* live culture solution was either directly mixed with the initial water or externally applied to cement mortars. The highest recovery of the flexural strength ratio was observed when the bacterial culture solution was externally applied. The fact that the recovery of mechanical properties after self-healing was lower than that after external applied healing, but higher than the control, suggests a relatively low healing effectiveness by self-healing. These differences between the external repair and the self-healing method could likely be the result of the number of active bacteria present and the amount of nutrients available for them. For the external repair method, a sufficient amount of liquid medium could be applied to fill the crack while for the self-healing method an enormous amount of nutrients would need to be incorporated to obtain similar results. Moreover, the nutrients may become part of the cement matrix with time, and consequently, less available for the bacteria. Xu and Yao also observed that the calcium source has a significant impact on the recovery ratio of flexural strength, where the use of calcium glutamate presented constantly higher values compared to the use of calcium lactate. This higher efficiency was related to the strong bond formed in the interfacial transition zone (ITZ) between the matrix and the deposited layer from calcium glutamate [79].

Compressive strength has also been used to assess the regain of the original mechanical properties on non-ureolytic bacteria-based self-healing cementitious materials. In this aspect, Rauf *et al.* studied the recovery of compressive strength on BBSH concretes when natural fibres were used as protective

carriers for the bacterial spores. After 28 days of healing in 28-days cracked specimens, the recovery in compressive strength of the fibre-reinforced specimens with embedded *B. cohnii* spores was 60%, 78% and 92% for coir, flax and jute fibres, respectively. The relatively low efficiency observed with the coir fibres was likely due to the low sorption capacity observed for this type of fibre compared to the other two, resulting in most of the bacterial solution adsorbed onto the surface and not inside the fibres. Consequently, bacterial spores were likely not adequately protected. Moreover, when comparing the recovery of compressive strength of the non-ureolytic bacteria with the other two ureolytic bacteria, it was observed a similar recovery value between *B. subtilis* and *B. cohnii* when either coir or flax fibres were used, but a slightly higher regain percentage for *B. subtilis* when jute fibres were the protective carrier. On the other hand, when *B. Sphaericus* was used, it presented a significantly higher recovery of compressive strength when coir, flax and jute fibres were used (80%, 100% and 100%, respectively). As the testing conditions and the number of spores were identical, this higher regain in compressive strength was likely the result of a more efficient calcite precipitation activity by this ureolytic bacteria when compared to the other two bacterial strains.

To summarise, environmental conditions influence the germination, growth and calcite precipitation of non-ureolytic bacteria. Exposure to sunlight radiation has been found to be detrimental for vegetative bacterial cells [99,131]. Crack healing in wet/dry environments has been shown to significantly enhance bacterial growth and $CaCO_3$ precipitation when compared with wet conditions [103], while in moist environments, cracks require longer times to heal [83]. Different crack closure efficiencies and mechanical properties results have been obtained in various studies, and comparing the data is challenging as these discrepancies could be due to multiple factors. In general, compressive and flexural strength responses have been observed to improve when vegetative cells or bacterial spores are embedded within the cement matrix, likely by providing extra nucleation sites that helped increase the amount of hydrated products [81,85,105,106,122]. In contrast, when porous materials are used to immobilise bacterial spores, significant reductions in compressive and flexural strengths have been reported due to the replacement of solid fine aggregate grains with these porous particles [91,103]. Furthermore, few studies have assessed the regain of the mechanical properties following bacterial

healing, either for ureolytic or non-ureolytic systems [79,81,118,122]. Therefore, we need to expand our research scope to further evaluate to what extent the original mechanical properties are recovered. Healing of cracks with widths up to 0.81 mm have been reported by Khaliq and Ehsan when immobilising *B. subtilis* into graphite nano-platelets (GNP) [105]. Under more extreme healing conditions, Palin *et al.* were able to heal cracks with widths up to 0.6 mm when bacteria were encapsulated in calcium alginate beads and the mortar samples submerged in cold temperature artificial seawater (8°C) [91]. Calcium carbonate precipitation has been found to be dependent on different factors such as the bacterial isolate, type of calcium source and the microbial growth conditions present [32,56,122,139,140]. Different morphologies of $CaCO_3$ can be precipitated in the presence of non-ureolytic bacteria, being calcite and aragonite, the most common polymorphs found in these systems. Furthermore, the size of $CaCO_3$ crystals is influenced by the type of calcium source [56] but also by the presence of bacteria [76,107], where larger $CaCO_3$ precipitates could be observed when compared to precipitates formed by abiotic factors. Moreover, $CaCO_3$ precipitates morphology varies between non-ureolytic and ureolytic bacteria, where non-ureolytic bacteria produce mixed organic/inorganic crystals while the more rapid precipitation achieved by ureolytic bacteria results in homogeneous inorganic calcite crystals [52].

6. Full-scale outdoors applications

Until now, most research has been focused on mortar specimens at a lab-scale due principally to the high costs of self-healing additives (*i.e.,* nutrients and bacteria). In the end, the aim of any self-healing material should be to prove its self-healing potential in an in-situ real-life concrete structure. However, this brings significant challenges with regards to the upscaling of the production of these materials. Some challenges include the structural element, its long-term accessibility for monitoring performance, inherent complications resulting from upscaling mortar formulations to concrete, effects of commercial chemical admixtures and the expected environmental conditions [143]. Furthermore, BBSH systems require contact with liquid water or very humid environments for their activation and maintenance [16,20,21]. Due to all these challenges, few MICP field-scale applications have been successfully performed. The majority of these field-scale applications have been conducted using the

urea hydrolysis pathway (ureolytic bacteria) not only for BBSHCs [143] but also to improve the geotechnical quality and erosion control of soils [144,145], fracture sealing below the ground surface [146,147], and more recently for enhancing wellbore cement integrity [148]. Field trials where only non-ureolytic bacteria have been used are scarce and are presented in this section [20,93,108,149,150].

6.1. Bacteria-based self-healing concretes (BBSHCs)

In July 2014, Jonkers *et al.* at Delft University of Technology (TU Delft), in collaboration with the Catholic University of Santiago de Guayaquil, conducted the first field application of a non-ureolytic BBSHC [108,129]. A linear 3 m concrete section of an irrigation canal was cast on-site in the Andean highlands of Tungurahua (Ecuador). Expanded clays (EC) particles were impregnated in situ with calcium lactate and non-ureolytic bacterial spores (*B. cohnii*). Local natural fibres were used to assure a maximum crack width. No cracks were observed after five months; thus, the self-healing capacity of this bacteria-based concrete structure could not be evaluated. After this first field application, Mors and Jonkers conducted two more ambitious field trials involving large-scale concrete water tanks (Netherlands) [20]. The first project, built in March 2016, involved the construction of a wastewater treatment tank where three of the applied precast concrete sections were fabricated using BBSHC. An innovative pelleted bacterial healing agent developed by TU Delft was used for these precast BBSHC sections. The pelleted agent, containing *B. cohnii* spores, yeast extract and calcium lactate, was used in a dose of 10 kg/m^3 of concrete [96]. Almost four years later, the Mors and Jonkers inspected the structure, and no signs of damage or degradation were observed in both the BBSHC or reference elements. The second full-scale BBSHC by the TU Delft research group involved a rectangular concrete water tank that was cast on-site in October 2017 [20]. In this project, a BBSHC incorporating a lower dosage (5 kg/m^3 of concrete) of the pelleted bacterial healing agent used during the first full-scale field trial was employed to supply half of the tank's concrete. BBSHC for this application was not designed to reduce the amount of steel reinforcement but as an additional safety measure to guarantee water tightness. Consequently, the quantity of applied steel reinforcement necessary to allow only the appearance of cracks with a maximum width of 0.1 mm was the same in BBSHC and reference concrete. In 2018, the tank was entirely filled with water and officially placed in service.

After more than one year, no active leaking cracks have been observed on any of the concrete walls [20]. The mentioned projects will be continuously monitored during the following years to estimate service-life performance and directly related maintenance costs.

The first full-scale field trial employing non-ureolytic bacteria in the UK (2015) was reported by Davies *et al.* [150,151]. This field trial was part of the Materials for Life (M4L) research project focused on developing self-healing cementitious materials. For this on-site trial, a vertical wall was constructed with a combination of BBSHC and a vascular flow network. It was located at a highway construction site to purposely expose it to the same weather conditions as this type of infrastructures. (**Fig. 9**) [150,152-154].

Fig. 9. Bacteria-based self-healing concrete (BBSHC) field trial in the UK: (*left*) vascular flow network installed prior to casting of the BBSHC concrete; and (*right*) vertical wall containing BBSHC (middle zone) and standard C40/50 concrete (dimensions in mm) [153,155].

The experimental wall was constructed using both BBSHC and standard C40/50 concrete. Growth media (yeast extract and calcium acetate) and non-ureolytic bacteria spores (*B. pseudofirmus*) were embedded independently into expanded perlite (EP) particles. After 36 days, the wall was slowly loaded to induce cracking in the proposed location (*i.e.,* BBSHC section). After almost eight months, no relevant self-healing that could be attributed to bacteria was achieved. The reasons for this were likely related to: (i) suboptimal ratio of bacterial spores (an order of magnitude lower than the optimal ratio of 8 x 10^9 spores/g of calcium acetate) [83]; (ii) scale-up issues from mortar tests to concrete due

to aggregate effects; (iii) low-temperature conditions; and (iv) the spatial distribution of spores throughout the concrete [153]. After six months, BBSHC samples were obtained from the wall to test the viability of the bacterial spores. It was observed that the spores survived the mixing process and remained viable. This site trial showed the feasibility to cast BBSHC on-site using standard concrete practices and that there were no negative repercussions on setting properties [153].

Zhang and Qian [93] conducted a full-scale site trial of BBSHC during the reconstruction and expansion of the Mangdao River ship lock chamber in China. The BBSHC was used for the channel walls on both sides of the lock chamber (**Fig. 10 (left)**).

Fig. 10. (*left*) Pouring of the BBSHC during the reconstruction of the Mangdao River ship lock chamber in China; (*right*) crack healing (after four months) in the channel walls of the ship lock [93].

A spray-dried bacterial powder containing *B. mucilaginous* spores, sucrose, yeast extract and calcium nitrate was added directly to a 25 MPa commercial concrete (6.8 kg of healing agent per cubic metre of concrete) [93]. The main aim of this full-scale site trial was to validate the adaptability of the healing agent to existing commercial concrete designs. It was observed that the inclusion of the microbial healing agent did not have any negative effects on the workability and 28-days compressive strength response when added to a commercial concrete formulation. As expected, early cracks on the sidewalls of the lock were formed on both types of concrete due to temperature stress and the shrinkage of bottom concrete. After two months, cracks on the surface of standard concrete were not healed, while a significant presence of $CaCO_3$ appeared on the surface of the cracks present on the BBSHC that completely blocked the free-water flow (**Fig. 10 (right)**). An important observation was

that not all the calcium carbonate precipitates induced by the bacteria remained within the crack zone, as some of these precipitates were washed out of the crack with the flowing water. Therefore, they considered that water flow must be considered when designing self-healing elements. Four months after the initial casting, the BBSHC had completed the effective closure of the early cracks in the concrete surface, allowing the water channel to be placed in service again.

6.2. Bacteria-based repairing materials

Full-scale outdoors applications of bacteria-based repairing materials involving non-ureolytic bacteria have been led over the last eight years by TU Delft [156]. The group has demonstrated the functionality and market potential of two different products: a sprayable bacteria-based liquid repair (BBLR) system and a PVA-fibre bacteria-based repair mortar (BBRM) [157]. Based on the successful results obtained from the different field applications, in 2014, a spin-off company of TU Delft (*i.e.,* Basilisk Self-Healing Concrete) was created to commercialize these bacteria-based systems [108,158].

6.2.1. Bacteria-based liquid repair (BBLR) systems

In 2012, Wiktor and Jonkers conducted the first field application of a BBLR system [97]. They evaluated its performance on an outdoor cracked concrete structure located in Breda (Netherlands). Despite being recently built, numerous cracks with widths up to 1 mm were observed on the vertical walls and roof of the structure. The BBLR used in this field trial comprised a two-component technique involving the sequential application of two different solutions [117]. By successively applying these two solutions, a gel is formed and transformed into $CaCO_3$ due to bacterial activity, resulting in the sealing of the cracks. The results obtained were promising as they showed bacterial activity less than 24 h after the application of the repair liquid. Additionally, all the BBLR impregnated cracks showed the presence of mineral formation inside them four months after the initial application. After this initial successful field application of the BBLR system, in October 2014, Wiktor and Jonkers tested this system on a large scale. They treated a critically damaged concrete surface area (6,000 m^2) from a two-story underground parking garage located in the Netherlands [73,117,149]. The concrete structure presented two different problems: (i) the concrete deck was

suffering from cracking which resulted in considerable leakage of the structure, and (ii) the concrete pavement on each side of the access ramp was deteriorated due to freeze/thaw cycles. For the cracked concrete deck, the BBLR agent was sprayed directly on three visible cracks with cracks widths between 1 and 3 mm. The results showed that the permeability of two of the three cracks, eight weeks after the application of the BBLR, was reduced by more than 90%, whereas the third crack was completely waterproof. For the porous concrete pavement, the affected area was sprayed with the BBLR agent and left for two months to allow bacteria to precipitate $CaCO_3$. After this time, concrete cores were drilled and subjected to freeze/thaw cycles in the laboratory. The obtained concrete cores showed significantly less scaling (50% reduction) when compared to the reference cores. It was suggested that the BBLR treatment tended to improve the resistance of concrete to freeze/thaw cycles but was not able to inhibit scaling completely. Nevertheless, the results were interpreted with caution by Wiktor and Jonkers, as no information was available about the original concrete mix composition or the history of this concrete structure [73]. After testing this BBLR system in more than ten different locations [129], they concluded that the results were very promising as the BBLR treatment showed to be effective to heal cracks up to 2 mm width after six weeks [108].

6.2.2. Bacteria-based repair mortar (BBRM) systems

Between 2013 and 2014, a PVA-fibre bacteria-based repair mortar (BBRM) developed by Sierra-Beltran and Jonkers was applied as a patch repair system in several outdoors trials in the Netherlands. As a result of the PVA-fibres, the crack pattern in these BBRMs consisted of multiple cracks not exceeding a width of 0.02 mm each. Non-ureolytic bacteria (*B. cohnii*), along with calcium lactate and yeast extract, were impregnated into LWAs (*i.e.,* Catsan®) and then added during the production of the BBRM formulation [108,123,129,149]. In May 2013, the first field application was led by Sierra-Beltran and Jonkers on a garage exposed to the weather elements where a severe steel corrosion problem was detected [20,108,129,149]. Corroded steel bars were cleaned and completely covered with BBRM (**Fig. 11**).

Fig. 11. Use of bacteria-based self-healing mortar (BBRM) for: (*left* and *middle*) on-site structural repair of damaged steel-reinforced concrete; and (*right*) manual repair of actively leaking cracked concrete basement walls [20].

After one and a half years, the repair was inspected and reported in good condition not exhibiting cracks or deterioration [129]. A second field application was implemented on a cracked and leaking underground retaining wall from a parking garage [20,108,149]. The BBRM was applied in situ without any prior application of primer. Six months after the repair, the patch was inspected, and no visible signs of spalling or debonding were observed [129]. In October 2014, a third application involving a higher volume of applied BBRM was conducted on an old tunnel that was built circa 1930 [117,129]. An extensive area, containing very corroded steels bars that were causing spalling of the concrete cover, was treated. After two months, only negligible cracks were observed, and there were no delamination issues. The most recent field trial using BBRM was in 2019 during the basement expansion of the Palace Het Loo (Netherlands) [158]. Even though the behaviour of BBRMs have been promising, continued monitoring of the applied patch repairs during these field trials must be done to guarantee that the performance of these BBRMs is, in fact, superior to conventional repair systems [117].

To summarise, applications of BBSH systems in the field for any of the different pathways associated with MICP are rare. This is principally due to different challenges associated with the upscaling of the production of these BBSH materials. Among these challenges are the high cost of the self-healing additives (bacteria, nutrients and precursors), the long-term accessibility for monitoring the performance of the concrete structure, and the need for very humid environments for their activation

and maintenance. In this regard, full-scale applications are very recent as the first field trial using non-ureolytic BBSHC was conducted less than ten years ago (2014) by Jonkers *et al.* in Ecuador [108,129]. Following this initial application, only a few others have been conducted in other countries such as in the United Kingdom (2015) [150], The Netherlands (2017) [20] and China (2019) [93]. In any case, these initial full-scale applications have been an important step in gaining the confidence of contractors, designers and quality assurance engineers at concrete mixing plants to consider BBSHC as an option for future applications. Nevertheless, it is essential to have more large-scale application projects where the entire process, including the design and mass production of the bacterial healing agents, and the production, pouring and curing of the BBSH concrete is reported along with the field performance of the structure through time. These full-scale outdoors applications should also cover different environmental conditions and concrete mix designs to further extend the applicability range of this technology.

7. Economic feasibility

The use of non-ureolytic bacteria avoids the production of environmentally harmful products such as ammonium (NH_4^+) [16,22,53]. Therefore, for commercial applications, they appear more suitable for use than ureolytic bacteria because no addition of nitrogen precursors or removal of excess ammonium are required [117]. Moreover, the principal drawback for ureolytic systems comes from the claim in many published studies that this MICP pathway is environmentally friendly and sustainable, which to date appears to be unsubstantiated [117]. Life-cycle assessment (LCA) studies are needed to quantitatively compare the sustainability performance of ureolytic versus non-ureolytic systems. In LCA studies, the monetarisation of the environmental impacts can be quantified. In this context, environmental costs associated with ureolytic systems surpass equivalent costs for non-ureolytic systems due to the ammonia released. For example, environmental costs for a kg of emitted CO_2 is set at €0.05, while for a kg of emitted ammonia is set to €10.7 [159]. The management of these environmental costs may likely give an economic advantage to non-ureolytic over ureolytic systems. Regarding sporulation, germination, in vitro $CaCO_3$ precipitation, and the ability of spores to survive within the concrete, the non-ureolytic bacteria *B. pseudofirmus* and *B. cohnii* are currently considered

the most appropriate species to be used in cementitious materials for temperate environments [78]. Under optimum conditions, 100% sporulation can be achieved by these bacteria within 24 hours [78]. Nevertheless, the main challenge for the commercial implementation of bacteria-based strategies is still the production cost of the specific bacteria needed. So far, most of the studies have been performed by employing pure cultures (axenic) under controlled lab conditions. Economic concerns arise with the demanded sterile conditions and specific substrates when considering an industrial production. To overcome the high cost of pure-culture processes, mixed cultures (non-axenic) have been considered recently. In an initial attempt, Zhang *et al.* demonstrated the feasibility of mixed cultures to completely heal crack widths up to 0.6 mm [98]. The mixed cultures used were obtained from activated sludge and aged garden soil that were enriched in anoxic, anaerobic, and facultative-anaerobic conditions. However, it was not until recently that Zhang *et al.* compared the crack healing efficiency of these mixed cultures to a non-ureolytic pure culture (*B. cohnii*) [49]. The anoxic-enriched mixed culture was able to heal cracks up to 1.22 mm, while the non-ureolytic pure culture (*B. cohnii)* was only capable of healing cracks with a maximum width of 0.79 mm. Additionally, the economic evaluation by Zhang *et al.* verified that the anoxic microbial consortia resulted in a 61% decrease in production costs when compared to the *B. cohnii* pure culture [49]. Silva *et al.* and Ersan *et al.* also found similar economic advantages, whereby using non-axenic cultures reduced the cost of BBSHC by up to ten times without negatively affecting the crack healing performance [58,160]. The results from these mixed culture studies are promising, and they have the potential to make these bacteria-based self-healing systems more economically feasible. Bacterial carriers, such as microcapsules and LWAs, also represent a significant variable for the cost equation for BBSHC. In relation to this, Zhang *et al.* investigated the costs associated when different bacteria carriers were used for BBSH systems [80]. The direct cost for immobilizing *B. cohnii* in extended perlite (EP) and extended clay (EC) particles was compared. The results showed that the cost-effectiveness of EP particles was superior to that of EC particles. The cost of EP particles was reported as 18 US$/m^3, while the cost reached up to 100 US$/m^3 for EC particles considering costs in China (Shanxi province). Nevertheless, future research regarding bacteria carriers should be directed towards powder compression techniques.

Powder compression allows the manufacture of particles with healing agents composed almost entirely of ingredients that can be used for healing.

Another important economic benefit from using BBSHCs is that they can potentially permit significant reductions in the steel reinforcement needed for crack width restriction, especially in water-holding concrete structures. In this case, the use of BBSHC represents a paradigm change where a "crack management" strategy will be preferred to a "crack prevention" design concept. BBSHC application would therefore not only likely result in a reduction of costs but also in the improvement of environmental efficiency (lower CO_2 impact) and ease of in-place casting due to the reduction of steel reinforcement. The latter will permit faster construction times and will also improve final quality by reducing the risk of forming honeycombs in the structure [20]. To evaluate the feasibility of this "crack management" strategy, Mors and Jonkers [104] estimated the savings in reinforcement steel that could be obtained by using bacteria-based powder-compressed flakes (BBPCF) in reinforced concrete structures. The aim of including these BBPCFs is to increase the width of the crack allowed to occur and consequently reduce the amount of steel required by design. A considerable amount of reinforcement steel is included for the sole reason of crack width control, only to satisfy recommendations from practical guidelines or codes. The minimum reinforcement percentage is considered to be 0.20% of the element area, but in order to reach the recommended crack widths, usually, an area of 0.60% is required [161]. The principal norm for strict crack width limitations is the capability for autogenous healing to recover initial water tightness [104]. However, if BBSHC is to be used, then an increased crack width can be considered during the design phase, and the steel requirements can be relaxed. As a result, crack width compensating reinforcement can be significantly reduced. In an example given by the Mors and Jonkers [104], assuming 1 m^3 of concrete (2400 kg/m^3) and a 0.40% $V/V_{element}$ saving on reinforcement steel (7850 kg/m^3), approximately 31.4 kg less steel can be used (**Fig. 12**). With this reinforcement steel reduction and considering average market steel unit prices at the moment the study was carried out, it was estimated that approximately €73.5 could be saved per cubic metre of concrete element. Consequently, if BBPCF is dosed at 15 kg per m^3 of concrete, then the BBPCF can have a maximum cost of €4.9 per kg.

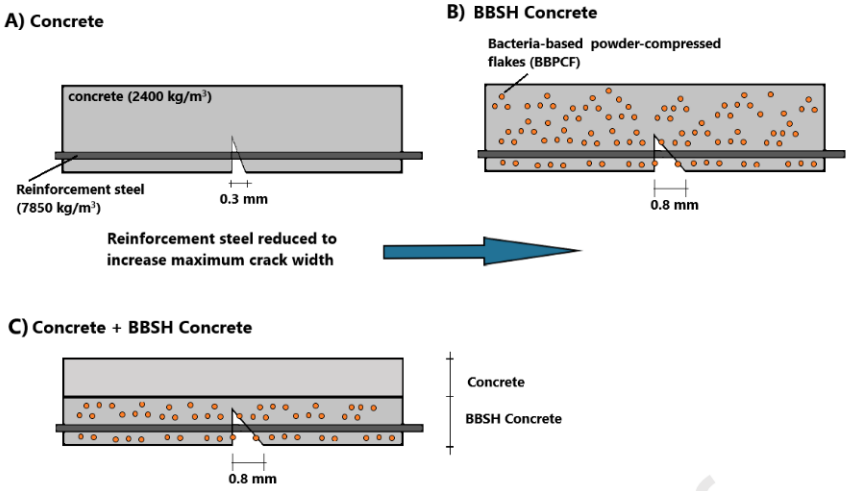

Fig.12. Use of bacteria-based powder-compressed flakes (BBPCF) in reinforced concrete structures to allow the formation of bigger cracks by reducing the amount of reinforcement steel. **A)** Reinforced concrete beam with the amount of reinforcement steel (*i.e.,* 0.60% of the element area) required to allow cracks with a maximum width of 0.3 mm; **B)** Reinforced concrete beam where the amount of reinforcement steel has been reduced to allow cracks with a maximum width of 0.8 mm and where BBPCF have been included in the cementitious matrix; and **C)** Reinforced concrete beam where only the bottom layer (where the cracks will be formed) contains BBPCF.

Mors and Jonkers proposed an additional optimization by including the BBPCF solely in the concrete cover layer or other specific volumes/elements, instead of the bulk volume of the structure (**Fig. 12(C)**) [104]. By placing the BBSHC in specifically targeted locations, an increased price per kg of this bacteria-based concrete could be justified, as the total mass of BBSHC per m^3 of concrete structure would be significantly reduced. Another example considered a 1 m^2 concrete element of 350 mm thickness [104]. If bacteria-based flakes are only added to the cover layer, a competitive price for the BBPCF can be up to €20 per kg for twice a cover of 50 mm thick and a concentration of 15 kg BBPCF/m^3 concrete. Further economisation could be achieved by reducing the BBPCF concentration from 15 to 10 kg/m^3, as this reduction is considered viable by Mors and Jonkers.

To summarise, bacteria-healing agents are considered the most expensive component of BBSH cementitious materials. The cost of these bacterial-healing agents is mainly due to the use of pure cultures (axenic), the need for sterile conditions and expensive specific substrates to produce the bacterial spores and the specialised labour required to produce them [160]. Moreover, the protection process (e.g., encapsulation) of these spores is also expensive and contributes significantly to the total

cost of the healing agent. In this context, the use of non-ureolytic bacteria appears to be more economically feasible than the use of more commonly used ureolytic bacteria, as no addition of nitrogen precursors or removal of excess ammonium are required [117]. Consequently, recent attempts have been done to demonstrate the economic feasibility of using mixed cultures (non-axenic) trying to overcome the high costs associated with pure-culture The production costs of these mixed cultures have been reported to be between 60% and 90% lower when compared to the production costs associated with non-ureolytic bacteria pure cultures [58,98,160]. These results are promising and have the potential to make these BBSH systems more economically feasible. Nevertheless, the production process must furthermore be optimised by also finding an inexpensive protection strategy for the bacterial spores, able at the same time to provide the necessary protection and maintaining or slightly modifying the concrete properties. In this aspect, future research regarding bacteria carriers should be directed towards powder compression techniques. Additionally, the use of BBSHCs could help reduce the amount of steel required by design, allowing faster construction times and improvements in the final quality of the concrete element [104]. This steel reduction will also reduce costs and improve environmental efficiency by lowering the overall CO_2 impact. However, the reduction of reinforcement steel due to the use of BBSHCs represents a paradigm change to move from a "crack prevention" design concept to a "crack management" strategy, which could be a complicated step to achieve. Another alternative involves optimising the use of these BBSHCs solely in the concrete cover layer or other specific elements and not on the bulk volume of the structure. In this case, an increased price per kg could be justified for these targeted locations, facilitating the initial commercial penetration of this technology.

8. Conclusions

This review has presented the state-of-the-art knowledge relevant to the development of self-healing cementitious composites that rely on aerobic non-ureolytic bacteria. Non-ureolytic bacteria-based self-healing (BBSH) systems present several important advantages when compared to BBSH strategies that have been historically more investigated (*i.e.,* ureolytic pathway). For example, no harmful emissions of ammonium are released during the self-healing process, avoiding environmental concerns,

exacerbation of steel corrosion and high accumulations that could inhibit the necessary bacterial activity. Moreover, aerobic non-ureolytic bacteria are able to produce larger, mixed organic/inorganic crystals, meaning that for the same amount of calcium consumed, a larger volume of $CaCO_3$ precipitate can be formed. The latter could present a significant advantage for industrial applications, where soluble calcium is only available in limited amounts within the cement matrix. Despite the recent studies involving different bacterial-carriers, novel non-ureolytic bacterial strains and the understanding of the effects of bacteria and nutrients on the precipitation of $CaCO_3$, more studies are needed to upscale these non-ureolytic BBSH systems from lab scale (where tests are usually performed on mortar specimens) toward real-life concrete applications. Likewise, more field trials involving different bacterial carriers, temperatures, humidity conditions, bacterial strains and cementitious composites formulations are required to build up the confidence of policymakers and contractors to promote future use of these BBSH materials. Apart from the mechanical and durability performance of non-ureolytic BBSH concrete elements, the production cost is another significant challenge. There is a need for more research into the reduction of the different costs associated with this strategy, like industrial production of bacteria, mixed cultures, nutrients and calcium precursors. Achieving better efficiencies of these BBSH systems at the same time that the costs associated with them are reduced will encourage construction managers in charge of design specifications to propose these materials in the early future.

8.1. Future research

Current research has been focused on demonstrating that non-ureolytic bacteria-based self-healing (BBSH) cementitious composites are able to 'self-heal' themselves by successfully closing the cracks formed within a specific width range. Unfortunately, the aspects related to the recovery of the original mechanical properties of these non-ureolytic BBSH materials have not been systematically investigated. Moreover, current research primarily focuses on a laboratory scale with very few outdoor scale trials. This has resulted in most experimental work being conducted on mortars and not on concrete, where the behaviour is likely to be different due to the influence of the aggregates and differing crack patterns. Additionally, further research is needed to evaluate the efficiency of these

BBSH systems under real environmental conditions, meaning with this at nonideal healing temperatures and humidity conditions, at later ages of the concrete, repeated cracking (cyclic healing) or under different sustained stresses. In this regard, it can be considered that future research needs to be carried out on the following themes to further improve the application level of non-ureolytic BBSH materials:

- Future work should focus on the protection of bacteria in situ and the maintaining of a continuous supply of nutrients.
- Expand research scope to extensively cover the effects of healing in the recovery of the original mechanical properties of these BBSH materials.
- There is still a lack of large relevant experience in commercial applications. Application of BBSH materials in actual engineering projects is required.
- Long-term durability tests are required to fully evaluate if healed BBSH concrete elements will achieve a similar or equivalent lifetime performance compared to uncracked conventional concrete elements.
- More research is needed to economically up-scale the different processes involved in the production of BBSH materials.
- Use of standardised methodology (e.g., life-cycle assessment (LCA)) to evaluate the cradle-to-gate sustainability of different BBSH alternatives
- To further expand the available bacterial isolates for case-specific bespoke solutions.
- Use of genetically modified microorganisms to deepen our understanding of the precise underpinning determinants of an optimal MICP bacterium to aid in the targeted selection of the most appropriate species.

Acknowledgements

This work was supported by the EPSRC through the Resilient Materials for Life (RM4L) (EP/PO2081X/1) and the Engineering Microbial-Induced Carbonate Precipitation *via* Meso-Scale Simulations (eMICP) (EP/SO13997/1) projects.

Disclosure statement: No potential conflict of interest was reported by the author(s).

References

1. Cen X, Wang Q, Shi X, et al. Optimization of Concrete Mixture Design Using Adaptive Surrogate Model. Sustainability. 2019;11(7):1991.
2. Monteiro PJ, Miller SA, Horvath A. Towards sustainable concrete. Nature materials. 2017;16(7):698-699.
3. (USGS) TUSGS. Cement Statistics and Information. January 2020 ed. United States of America: National Minerals Information Center- USGS; 2020. p. 2.
4. (CEMBUREAU) TECA. Activity report 2018. In: Association TEC, editor. Brussels, Belgium: The European Cement Association; 2019. p. 56.
5. Machado-Lopez I, Bacallao F, Moya I, et al., editors. Considerations for the Energy Balance and Preliminary Design of an Experimental LC 3 Cement Pilot Plant. Proceedings of the International Conference of Sustainable Production and Use of Cement and Concrete; 2020: Springer.
6. Statista. Statista: Statista; 2020 [cited 2020 10/05/2020]. Available from: https://www.statista.com/statistics/219343/cement-production-worldwide/
7. Filipe Bravo Silva NB, Nele de Belie and Willy Verstraete. Industrial Application of Biological Self-healing Concrete: Challenges and Economical Feasibility. Journal of Commercial Biotechnology. 2015 January 2015;21(1):31-38.
8. Mehta P, Paulo, Monteiro, Paulo JM. Concrete: microstructure, properties, and materials. 2006.
9. Zongjin L. Advanced concrete technology. Hoboken, New Jersey: John Wiley & Sons, Inc. 2011.
10. Van Tittelboom K, De Belie N. Self-healing in cementitious materials—A review. Materials. 2013;6(6):2182-2217.
11. van der Bergh JM, Miljević B, Šovljanski O, et al. Preliminary approach to bio-based surface healing of structural repair cement mortars. Construction and Building Materials. 2020;248:118557.
12. Son HM, Kim HY, Park SM, et al. Ureolytic/Non-ureolytic bacteria co-cultured self-healing agent for cementitious materials crack repair. Materials. 2018;11(5):782.
13. Ibrahim M, Al-Gahtani A, Maslehuddin M, et al. Effectiveness of concrete surface treatmentmaterials in reducing chloride-induced reinforcement corrosion. Construction and Building Materials. 1997;11(7-8):443-451.
14. Das BB, Neithalath N. Sustainable Construction and Building Materials: Select Proceedings of ICSCBM 2018. Vol. 25. Springer; 2018.
15. Cailleux E, Pollet V, editors. Investigations on the development of self-healing properties in protective coatings for concrete and repair mortars. Proceedings of the 2nd International Conference on Self-Healing Materials, Chicago, IL, USA; 2009.
16. De Belie N, Wang J, Bundur ZB, et al. Bacteria-based concrete. Eco-efficient repair and rehabilitation of concrete infrastructures: Elsevier; 2018. p. 531-567.
17. De Belie N, Gruyaert E, Al-Tabbaa A, et al. A review of self-healing concrete for damage management of structures. Advanced materials interfaces. 2018;5(17):pp. 1-28.
18. Van Tittelboom K, De Belie N, De Muynck W, et al. Use of bacteria to repair cracks in concrete. Cement and Concrete Research. 2010;40(1):157-166.

19. Jonkers HM, Schlangen E, editors. Crack repair by concrete-immobilized bacteria. Proceedings of the first international conference on self healing materials; 2007.
20. Mors R, Jonkers HM. Bacteria-based self-healing concrete: evaluation of full scale demonstrator projects. RILEM Technical Letters. 2020;4:138-144.
21. Wiktor V, Jonkers HM. Quantification of crack-healing in novel bacteria-based self-healing concrete. Cement and Concrete Composites. 2011;33(7):763-770.
22. Zhang W, Zheng Q, Ashour A, et al. Self-healing concrete composites for sustainable infrastructures: A review. Composites Part B: Engineering. 2020:107892.
23. Wu M, Johannesson B, Geiker M. A review: Self-healing in cementitious materials and engineered cementitious composite as a self-healing material. Construction and Building Materials. 2012;28(1):571-583.
24. Paine K, Al-Tabbaa A, Gardner D, et al., editors. Resilient materials for life: Biomimetic self-healing and self-diagnosing concretes. UKIERI Concrete Congress, Concrete: The Global Builder: Working together for durable and sustainable infrastructure; 2019.
25. Utrera-Barrios S, Verdejo R, López-Manchado MA, et al. Evolution of self-healing elastomers, from extrinsic to combined intrinsic mechanisms: a review. Materials Horizons. 2020;7(11):2882-2902.
26. Boehm AV, Meininger S, Gbureck U, et al. Self-healing capacity of fiber-reinforced calcium phosphate cements. Scientific Reports. 2020 2020/06/10;10(1):9430.
27. Ghosh SK. Self-healing materials: fundamentals, design strategies, and applications. Wiley Online Library; 2009.
28. Li W, Dong B, Yang Z, et al. Recent advances in intrinsic self-healing cementitious materials. Advanced Materials. 2018;30(17):1705679.
29. Talaiekhozan A, Keyvanfar A, Shafaghat A, et al. A review of self-healing concrete research development. Journal of Environmental Treatment Techniques. 2014;2(1):1-11.
30. Li VC, Herbert E. Robust self-healing concrete for sustainable infrastructure. Journal of Advanced Concrete Technology. 2012;10(6):207-218.
31. Hearn N. Self-sealing, autogenous healing and continued hydration: what is the difference? Materials and structures. 1998;31(8):563.
32. Seifan M, Samani AK, Berenjian A. Bioconcrete: next generation of self-healing concrete. Applied microbiology and biotechnology. 2016;100(6):2591-2602.
33. Perez G, Erkizia E, Gaitero J, et al. Synthesis and characterization of epoxy encapsulating silica microcapsules and amine functionalized silica nanoparticles for development of an innovative self-healing concrete. Materials Chemistry and Physics. 2015;165:39-48.
34. Chowdhury RA, Hosur MV, Nuruddin M, et al. Self-healing epoxy composites: preparation, characterization and healing performance. Journal of Materials Research and Technology. 2015;4(1):33-43.
35. Huang H, Ye G, Leung C, et al., editors. Application of sodium silicate solution as self-healing agent in cementitious materials. International RILEM conference on advances in construction materials through science and engineering; 2011: RILEM Publications SARL: Hong Kong, China.
36. Mao W, Litina C, Al-Tabbaa A. Development and Application of Novel Sodium Silicate Microcapsule-Based Self-Healing Oil Well Cement. Materials. 2020;13(2):456.
37. Litina C, Al-Tabbaa A. First generation microcapsule-based self-healing cementitious construction repair materials. Construction and Building Materials. 2020;255:119389.
38. Dry C. Matrix cracking repair and filling using active and passive modes for smart timed release of chemicals from fibers into cement matrices. Smart Materials and Structures. 1994;3(2):118.
39. Joseph C, Jefferson AD, Isaacs B, et al. Experimental investigation of adhesive-based self-healing of cementitious materials. Magazine of Concrete Research. 2010;62(11):831-843.

40. Gilford III J, Hassan MM, Rupnow T, et al. Dicyclopentadiene and sodium silicate microencapsulation for self-healing of concrete. Journal of Materials in Civil Engineering. 2014;26(5):886-896.
41. Zwaag S. Self healing materials: an alternative approach to 20 centuries of materials science. Vol. 30. Springer Science+ Business Media BV Dordrecht, The Netherlands; 2008.
42. Li Z, de Souza LR, Litina C, et al. A novel biomimetic design of a 3D vascular structure for self-healing in cementitious materials using Murray's law. Materials & Design. 2020;190:108572.
43. Simkiss K, Wilbur KM. Biomineralization. Elsevier; 2012.
44. Dakhane A, Das S, Hansen H, et al. Crack healing in cementitious mortars using enzyme-induced carbonate precipitation: Quantification based on fracture response. Journal of Materials in Civil Engineering. 2018;30(4):04018035.
45. Bisht V, Chaurasia L, Singh L, et al. Bacterially Stabilized Desert-Sand Bricks: Sustainable Building Material. Journal of Materials in Civil Engineering. 2020;32(6):04020131.
46. De Muynck W, De Belie N, Verstraete W. Microbial carbonate precipitation in construction materials: a review. Ecological Engineering. 2010;36(2):118-136.
47. Gat D, Tsesarsky M, Shamir D, et al. Accelerated microbial-induced CaCO3 precipitation in a defined coculture of ureolytic and non-ureolytic bacteria. Biogeosciences. 2014;11(10):2561.
48. Wang J, Soens H, Verstraete W, et al. Self-healing concrete by use of microencapsulated bacterial spores. Cement and concrete research. 2014;56:139-152.
49. Zhang J, Zhao C, Zhou A, et al. Aragonite formation induced by open cultures of microbial consortia to heal cracks in concrete: Insights into healing mechanisms and crystal polymorphs. Construction and Building Materials. 2019;224:815-822.
50. Stocks-Fischer S, Galinat JK, Bang SS. Microbiological precipitation of CaCO3. Soil Biology and Biochemistry. 1999;31(11):1563-1571.
51. De Muynck W, Verbeken K, De Belie N, et al. Influence of temperature on the effectiveness of a biogenic carbonate surface treatment for limestone conservation. Applied microbiology and biotechnology. 2013;97(3):1335-1347.
52. Reeksting BJ, Hoffmann TD, Tan L, et al. In-depth profiling of calcite precipitation by environmental bacteria reveals fundamental mechanistic differences with relevance to application. Applied and environmental microbiology. 2020;86(7).
53. Zhang W, Wang D, Han B. Self-healing concrete-based composites. Self-Healing Composite Materials: Elsevier; 2020. p. 259-284.
54. Zamani M, Nikafshar S, Mousa A, et al. Bacteria encapsulation using synthesized polyurea for self-healing of cement paste. Construction and Building Materials. 2020;249:118556.
55. Neville A, Brooks J. Concrete Technology, copyright© 1987. Pearson Education (Singapore) Pte. Ltd, Indian Branch, Delhi; 2002.
56. Yoonhee J, Wonjae K, Wook K, et al. Complete Genome and Calcium Carbonate Precipitation of Alkaliphilic Bacillus sp. AK13 for Self-Healing Concrete [Complete Genome and Calcium Carbonate Precipitation of Alkaliphilic Bacillus sp. AK13 for Self-Healing Concrete]. Journal of Microbiology and Biotechnology. 2020;30(3):404-416.
57. Gat D, Ronen Z, Tsesarsky M. Long-term sustainability of microbial-induced CaCO3 precipitation in aqueous media. Chemosphere. 2017;184:524-531.
58. Erşan YÇ, De Belie N, Boon N. Microbially induced CaCO3 precipitation through denitrification: an optimization study in minimal nutrient environment. Biochemical Engineering Journal. 2015;101:108-118.
59. Braissant O, Verrecchia EP, Aragno M. Is the contribution of bacteria to terrestrial carbon budget greatly underestimated? Naturwissenschaften. 2002;89(8):366-370.
60. Rivadeneyra M, Delgado G, Ramos-Cormenzana A, et al. Biomineralization of carbonates by Halomonas eurihalina in solid and liquid media with different salinities: crystal formation sequence. Research in Microbiology. 1998;149(4):277-287.
61. Hoffmann TD, Reeksting BJ, Gebhard S. Bacteria-induced mineral precipitation: a mechanistic review. Microbiology. 2021;167(4):001049.

62. Dhami NK, Reddy MS, Mukherjee A. Application of calcifying bacteria for remediation of stones and cultural heritages. Frontiers in microbiology. 2014;5:304.
63. Castanier S, Le Métayer-Levrel G, Perthuisot J-P. Ca-carbonates precipitation and limestone genesis—the microbiogeologist point of view. Sedimentary geology. 1999;126(1-4):9-23.
64. Fattahi SM, Soroush A, Huang N, et al. Durability of biotechnologically induced crusts on sand against wind erosion. Journal of Arid Environments. 2021;189:104508.
65. Chamizo S, Mugnai G, Rossi F, et al. Cyanobacteria inoculation improves soil stability and fertility on different textured soils: gaining insights for applicability in soil restoration. Frontiers in Environmental Science. 2018;6:49.
66. Heveran CM, Williams SL, Qiu J, et al. Biomineralization and successive regeneration of engineered living building materials. Matter. 2020;2(2):481-494.
67. Rahbar N. Extending the Life of Self-Healing Structural Materials. Matter. 2020;2(2):289-291.
68. Web of Science [Internet]. Clarivate Analytics. 2020 [cited 30 May 2020].
69. Wong LS. Microbial cementation of ureolytic bacteria from the genus Bacillus: a review of the bacterial application on cement-based materials for cleaner production. Journal of Cleaner Production. 2015;93:5-17.
70. Zhu T, Dittrich M. Carbonate precipitation through microbial activities in natural environment, and their potential in biotechnology: a review. Frontiers in bioengineering and biotechnology. 2016;4:4.
71. Shashank B, Dhannur B, Ravishankar H, et al. Study on Development of Strength Properties of Bio-concrete. Sustainable Construction and Building Materials: Springer; 2019. p. 423-437.
72. Tziviloglou E, Wiktor V, Jonkers HM, et al. Selection of nutrient used in biogenic healing agent for cementitious materials. Frontiers in Materials. 2017;4:15.
73. Wiktor V, Jonkers H. Field performance of bacteria-based repair system: Pilot study in a parking garage. Case Studies in Construction Materials. 2015;2:11-17.
74. Horikoshi K. 2.8 enzymes isolated from alkaliphiles. Extremophiles Handbook. 2011:162-177.
75. Lee YS, Park W. Enhanced calcium carbonate-biofilm complex formation by alkali-generating Lysinibacillus boronitolerans YS11 and alkaliphilic Bacillus sp. AK13. AMB Express. 2019;9(1):49.
76. Jonkers HM, Thijssen A, Muyzer G, et al. Application of bacteria as self-healing agent for the development of sustainable concrete. Ecological engineering. 2010;36(2):230-235.
77. Jonkers HM, Schlangen E, editors. Development of a bacteria-based self healing concrete. Proc. int. FIB symposium; 2008: Citeseer.
78. Sharma T, Alazhari M, Heath A, et al. Alkaliphilic Bacillus species show potential application in concrete crack repair by virtue of rapid spore production and germination then extracellular calcite formation. Journal of applied microbiology. 2017;122(5):1233-1244.
79. Xu J, Yao W. Multiscale mechanical quantification of self-healing concrete incorporating non-ureolytic bacteria-based healing agent. Cement and concrete research. 2014;64:1-10.
80. Zhang J, Liu Y, Feng T, et al. Immobilizing bacteria in expanded perlite for the crack self-healing in concrete. Construction and Building Materials. 2017;148:610-617.
81. Sierra-Beltran et al. MG, Jonkers, HM, Schlangen, E. Characterization of sustainable bio-based mortar for concrete repair. Construction and Building Materials. 2014;67:344-352.
82. Tan L, Reeksting B, Ferrandiz-Mas V, et al. Effect of carbonation on bacteria-based self-healing of cementitious composites. Construction and Building Materials. 2020;257:119501.
83. Alazhari M, Sharma T, Heath A, et al. Application of expanded perlite encapsulated bacteria and growth media for self-healing concrete. Construction and Building Materials. 2018;160:610-619.
84. Schwantes-Cezario N, Camargo GSFN, do Couto ÁF, et al. Mortars with the addition of bacterial spores: Evaluation of porosity using different test methods. Journal of Building Engineering. 2020;30:101235.

85. Schwantes-Cezario N, Porto MF, SANDOVAL G, et al. Effects of Bacillus subtilis biocementation on the mechanical properties of mortars. Revista IBRACON de Estruturas e Materiais. 2019;12(1):31-38.
86. Wiktor V, Jonkers H. Un nouveau béton auto-cicatrisant grâce à l'incorporation de bactéries. Matériaux & Techniques. 2011;99(5):565-571.
87. Han S, Jang I, Choi EK, et al. Bacterial Self-Healing Performance of Coated Expanded Clay in Concrete. Journal of Environmental Engineering. 2020;146(7):04020072.
88. Jang I, Son D, Kim W, et al. Effects of spray-dried co-cultured bacteria on cement mortar. Construction and Building Materials. 2020;243:118206.
89. Lee Y, Suk K, Hyun Jung, et al. Non-ureolytic calcium carbonate precipitation by Lysinibacillus sp. YS11 isolated from the rhizosphere of Miscanthus sacchariflorus. Journal of Microbiology. 2017;55(6):440-447.
90. Palin D, Geleijnse MA, Abbas BA, et al. A bacteria-based agent for realizing cost-effective self-healing concrete in low-temperature marine environments. AMB Exp Submitted. 2018.
91. Palin D, Wiktor V, Jonkers H. A bacteria-based self-healing cementitious composite for application in low-temperature marine environments. Biomimetics. 2017;2(3):13.
92. Su Y, Feng J, Zhan Q, et al. Non-ureolytic microbial self-repairing concrete for low temperature environment. Smart Materials and Structures. 2019;28(7):075041.
93. Zhang X, Qian C. Engineering Application of Microbial Self-healing Concrete in Lock Channel Wall. Case Studies in Construction Materials. 2020:e00398.
94. Zhang J, Wu R, Li Y, et al. Screening of bacteria for self-healing of concrete cracks and optimization of the microbial calcium precipitation process. Applied microbiology and biotechnology. 2016;100(15):6661-6670.
95. Tziviloglou EW, V.; Jonkers, H. M.; et al. Bacteria-based self-healing mortar: A comparison of two studies. In: Solutions C, editor. Conference: 6th International Conference on Concrete Repair JUN 20-23, 2016; Thessaloniki, GREECE: Concrete Solutions; 2016. p. 565-569.
96. Mors R, Jonkers H. Effect on concrete surface water absorption upon addition of lactate derived agent. Coatings. 2017;7(4):51.
97. Wiktor V, Jonkers H. Bio-réparation des matériaux cimentaires: les bactéries au service des mortiers fissurés. Matériaux & Techniques. 2013;101(4):401.
98. Zhang J, Zhou A, Liu Y, et al. Microbial network of the carbonate precipitation process induced by microbial consortia and the potential application to crack healing in concrete. Scientific reports. 2017;7(1):1-10.
99. Abdulkareem M, Ayeronfe F, Majid MZA, et al. Evaluation of effects of multi-varied atmospheric curing conditions on compressive strength of bacterial (bacillus subtilis) cement mortar. Construction and Building Materials. 2019;218:1-7.
100. Xu J, Yao W, Jiang Z. Non-ureolytic bacterial carbonate precipitation as a surface treatment strategy on cementitious materials. Journal of materials in civil engineering. 2014;26(5):983-991.
101. Hamza O, Esaker M, Elliott D, et al. The effect of soil incubation on bio self-healing of cementitious mortar. Materials Today Communications. 2020;24:100988.
102. Gat D, Tsesarsky M, Shamir D. Ureolytic calcium carbonate precipitation in the presence of non-ureolytic competing bacteria. Geo-Frontiers 2011: Advances in Geotechnical Engineering2011. p. 3966-3974.
103. Tziviloglou E, Wiktor V, Jonkers H, et al. Bacteria-based self-healing concrete to increase liquid tightness of cracks. Construction and Building Materials. 2016;122:118-125.
104. Mors R, Jonkers H. Feasibility of lactate derivative based agent as additive for concrete for regain of crack water tightness by bacterial metabolism. Industrial crops and products. 2017;106:97-104.
105. Khaliq W, Ehsan MB. Crack healing in concrete using various bio influenced self-healing techniques. Construction and Building Materials. 2016;102:349-357.

106. Chaurasia L, Bisht V, Singh L, et al. A novel approach of biomineralization for improving micro and macro-properties of concrete. Construction and Building Materials. 2019;195:340-351.
107. Stuckrath C, Serpell R, Valenzuela LM, et al. Quantification of chemical and biological calcium carbonate precipitation: performance of self-healing in reinforced mortar containing chemical admixtures. Cement and Concrete Composites. 2014;50:10-15.
108. Wiktor V, Jonkers H. Bacteria-based concrete: from concept to market. Smart Materials and Structures. 2016;25(8):084006.
109. Zhang J, Mai B, Cai T, et al. Optimization of a binary concrete crack self-healing system containing bacteria and oxygen. Materials. 2017;10(2):116.
110. Dong B, Wang Y, Fang G, et al. Smart releasing behavior of a chemical self-healing microcapsule in the stimulated concrete pore solution. Cement and Concrete Composites. 2015;56:46-50.
111. De Muynck W, Verbeken K, De Belie N, et al. Influence of urea and calcium dosage on the effectiveness of bacterially induced carbonate precipitation on limestone. Ecological Engineering. 2010;36(2):99-111.
112. Schlegel HG, Zaborosch C. General microbiology. Cambridge university press; 1993.
113. Benjamin B, Sabbavarapu A, Sudhakumar J, et al., editors. Experimental Study on Microbial Concentration Optimization in Cement Mortar using M-Sand as Fine Aggregate. IOP Conference Series: Materials Science and Engineering; 2020: IOP Publishing.
114. Skevi L, Reeksting BJ, Hoffmann TD, et al. Incorporation of bacteria in concrete: The case against MICP as a means for strength improvement. Cement and Concrete Composites. 2021:104056.
115. Rodriguez-Navarro C, Rodriguez-Gallego M, Chekroun KB, et al. Conservation of ornamental stone by Myxococcus xanthus-induced carbonate biomineralization. Appl Environ Microbiol. 2003;69(4):2182-2193.
116. Zamarreño DV, May E, Inkpen R. Influence of environmental temperature on biocalcification by non-sporing freshwater bacteria. Geomicrobiology Journal. 2009;26(4):298-309.
117. Pacheco-Torgal F, Ivanov V, Karak N, et al. Biopolymers and biotech admixtures for eco-efficient construction materials. Woodhead Publishing; 2016.
118. Sangadji S, Wiktor V, Jonkers H, et al. The use of alkaliphilic bacteria-based repair solution for porous network concrete healing mechanism. Procedia engineering. 2017;171:606-613.
119. Jonkers HM, Schlangen E. Self-healing of cracked concrete: a bacterial approach. IA-FraMCoS [internet][Acesso em: 2017 out 04]. 2007.
120. Ryu Y, Lee K-E, Cha I-T, et al. Optimization of bacterial sporulation using economic nutrient for self-healing concrete. Journal of Microbiology. 2020:1-9.
121. Pacheco-Torgal F, Melchers R, de Belie N, et al. Eco-efficient repair and rehabilitation of concrete infrastructures. Woodhead Publishing; 2017.
122. Rauf M, Khaliq W, Khushnood RA, et al. Comparative performance of different bacteria immobilized in natural fibers for self-healing in concrete. Construction and Building Materials. 2020;258:119578.
123. Jonkers HM, Mors R, editors. Full scale application of bacteria-based self-healing concrete for repair purposes. Concrete Repair, Rehabilitation and Retrofitting III: 3rd International Conference on Concrete Repair, Rehabilitation and Retrofitting, ICCRRR-3, 3-5 September 2012, Cape Town, South Africa; 2012: CRC Press.
124. Jedidi M, Benjeddou O, Soussi C. Effect of expanded perlite aggregate dosage on properties of lightweight concrete. Jordan Journal of Civil Engineering. 2015;9(3).
125. Palin D, Wiktor V, Jonkers H. A bacteria-based bead for possible self-healing marine concrete applications. Smart Materials and Structures. 2016;25(8):084008.
126. Liu B, Zhang J, Cheng W, et al., editors. The feasibility of encapsulation of self-healing bacterium with ethyl cellulose. Proceedings of the Fifth International Conference on Self-Healing Materials, Durham, NC, USA; 2015.

127. Paine K, Horne I, Tan L, et al., editors. Microencapsulated spores and growth media for self-healing mortars. Sixth International Symposium on Life-Cycle Civil Engineering; 2018: CRC Press.
128. De Koster S, Mors R, Nugteren H, et al. Geopolymer coating of bacteria-containing granules for use in self-healing concrete. Procedia Engineering. 2015;102:475-484.
129. Beltran M, Jonkers H. Crack self-healing technology based on bacteria. J Ceram Process Res. 2015;16(1):33-39.
130. Sangadji S, Schlangen E. Self healing of concrete structures-novel approach using porous network concrete. Journal of Advanced Concrete Technology. 2012;10(5):185-194.
131. Wood JP, Meyer KM, Kelly TJ, et al. Environmental Persistence of Bacillus anthracis and Bacillus subtilis Spores. PLOS ONE. 2015;10(9):e0138083.
132. Zhong W, Yao W. Influence of damage degree on self-healing of concrete. Construction and building materials. 2008;22(6):1137-1142.
133. Achal V, Mukherjee A, Reddy MS. Microbial concrete: way to enhance the durability of building structures. Journal of materials in civil engineering. 2011;23(6):730-734.
134. Li VC, Yang E-H. Self healing in concrete materials. Self healing materials: Springer; 2007. p. 161-193.
135. Joshi S, Goyal S, Mukherjee A, et al. Microbial healing of cracks in concrete: a review. Journal of industrial microbiology & biotechnology. 2017;44(11):1511-1525.
136. Hammes F, Verstraete W. Key roles of pH and calcium metabolism in microbial carbonate precipitation. Reviews in environmental science and biotechnology. 2002;1(1):3-7.
137. Esaker M, Hamza O, Souid A, et al. Self-healing of bio-cementitious mortar incubated within neutral and acidic soil. Materials and Structures. 2021;54(2):1-16.
138. Gupta S, Dai Pang S, Kua HW. Autonomous healing in concrete by bio-based healing agents–A review. Construction and Building Materials. 2017;146:419-428.
139. Okyay TO, Rodrigues DF. Biotic and abiotic effects on CO2 sequestration during microbially-induced calcium carbonate precipitation. FEMS microbiology ecology. 2015;91(3):fiv017.
140. Wei S, Cui H, Jiang Z, et al. Biomineralization processes of calcite induced by bacteria isolated from marine sediments. Brazilian Journal of Microbiology. 2015;46(2):455-464.
141. Rodriguez-Navarro C, Jimenez-Lopez C, Rodriguez-Navarro A, et al. Bacterially mediated mineralization of vaterite. Geochimica et Cosmochimica Acta. 2007;71(5):1197-1213.
142. Zhang Y, Guo H, Cheng X. Influences of calcium sources on microbially induced carbonate precipitation in porous media. Materials Research Innovations. 2014;18(sup2):S2-79-S2-84.
143. Van Mullem T, Gruyaert E, Caspeele R, et al. First Large Scale Application with Self-Healing Concrete in Belgium: Analysis of the Laboratory Control Tests. Materials. 2020;13(4):997.
144. Gomez MG, Martinez BC, DeJong JT, et al. Field-scale bio-cementation tests to improve sands. Proceedings of the Institution of Civil Engineers-Ground Improvement. 2015;168(3):206-216.
145. Meng H, Gao Y, He J, et al. Microbially induced carbonate precipitation for wind erosion control of desert soil: Field-scale tests. Geoderma. 2021;383:114723.
146. Cuthbert MO, McMillan LA, Handley-Sidhu S, et al. A field and modeling study of fractured rock permeability reduction using microbially induced calcite precipitation. Environmental science & technology. 2013;47(23):13637-13643.
147. Phillips AJ, Cunningham AB, Gerlach R, et al. Fracture sealing with microbially-induced calcium carbonate precipitation: A field study. Environmental science & technology. 2016;50(7):4111-4117.
148. Phillips AJ, Troyer E, Hiebert R, et al. Enhancing wellbore cement integrity with microbially induced calcite precipitation (MICP): a field scale demonstration. Journal of petroleum science and engineering. 2018;171:1141-1148.
149. Tziviloglou E, Van Tittelboom K, Palin D, et al. Bio-Based Self-Healing Concrete: From Research to Field Application. In: Hager MD, van der Zwaag S, Schubert US, editors. Self-healing Materials. Cham: Springer International Publishing; 2016. p. 345-385.

150. Davies R, Teall O, Pilegis M, et al. Large scale application of self-healing concrete: design, construction, and testing. Frontiers in Materials. 2018;5:51.
151. Davies R, Pilegis M, Kanellopoulos A, et al. Multi-scale cementitious self-healing systems and their application in concrete structures. 2016.
152. Teall O, Davies R, Pilegis M, et al., editors. Self-healing concrete full-scale site trials. Proceedings of the 11th fib International PhD Symposium in Civil Engineering; 2016.
153. Kevin Paine TS, Mohamed Alazhari, Andrew Heath, and Richard Cooper. Application and Performance of Bacteria-Based Self-Healing Concrete. In: 253-MCI RT, editor. Microorganisms-Cementitious Materials Interactions; Toulouse, France. France: RILEM; 2018. p. 594.
154. Lark R, Al-Tabbaa A, Paine K. Biomimetic multi-scale damage immunity for construction materials: M4L project overview. 2013.
155. Paine K, Alazhari M, Sharma T, et al., editors. Design and performance of bacteria-based self-healing concrete. The 9th International Concrete Conference 2016: Environment, Efficiency and Economic Challenges for Concrete; 2016.
156. JonNersa HM. Toward bioYbased geoY & civil engineering for a sustainable society. Procedia engineering. 2017;171:168-175.
157. Jonkers H, Wiktor V, Sierra Beltran M, et al. Limestone-producing bacteria make concrete self healing. Self-Healing Materials. 2015:137-148.
158. Basilisk. Admixture Basilisk healing agent now also CE certified Netherlands: Basilisk Self Healing Concrete; 2020 [cited 2020 23 July]. Available from: https://www.basiliskconcrete.com/en/admixture-basilisk-healing-agent-now-also-ce-certified/
159. De Bruyn S, Korteland M, Markowska A, et al. Shadow Prices Handbook. Valuation and weighting of emissions and environmental impacts. 2010.
160. Da Silva FB, De Belie N, Boon N, et al. Production of non-axenic ureolytic spores for self-healing concrete applications. Construction and Building Materials. 2015;93:1034-1041.
161. ACI-224R-01: Control of cracking in concrete structures. In: Barth F, editor. ACI Committee 2242001.

Highlights

- Overview of non-ureolytic bacterial species, nutrients and calcium precursors.
- Review of non-ureolytic bacteria delivery strategies.
- Crack healing performance of non-ureolytic bacteria in cementitious materials.
- Full-scale outdoors applications are presented.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: