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Ing. Hana Schreiberová

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Školitel: prof. Ing. Alena Kohoutková, CSc., FEng.

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Prohlášení

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Datum

Abstrakt

Tato disertační práce se věnuje problematice samohojitelného betonu na biologické bázi – cementovému kompozitu, který je za pomoci bakterií a živin přidaných do směsi schopen samovolně zacelovat vzniklé trhliny. Práce poskytuje rozsáhlý přehled existující literatury popisující studie, které se tomuto perspektivnímu tématu, především v posledním desetiletí, věnovaly. Na základě rešerše jsou v práci stanoveny cíle, kterých chce autor dosáhnout v rámci vlastní experimentální práce. Prvním výstupem experimentů je výběr vhodné bakterie a popsání jejího chování za optimálních i neoptimálních podmínek. Dále byl proveden výběr vhodných živin na základě jejich kompatibility s bakterií a vlivu na mechanické a reologické vlastnosti betonu. Byly navrženy a aplikovány různé způsoby ochrany bakterie v betonu a vytvořena vlastní směs samohojitelného biologického betonu. Pro tuto směs byla stanovena její schopnost zacelovat trhliny v optimálních i reálných podmínkách.

Abstract

This thesis focuses on the problem of self-healing bio-based concrete - a cementitious composite, which is able to spontaneously heal cracks due to bacteria and nutrients added to the mixture. The thesis provides an extensive review of the existing literature describing the studies that have addressed this promising topic, especially in the last decade. Based on the search, the thesis sets out the objectives that the author wants to achieve in his own experimental work. The first outcome of the experiments is the selection of a suitable bacterium and description of its behaviour under optimal and sub-optimal conditions. Further, suitable nutrients are selected based on their compatibility with the bacterium and their effect on the mechanical and rheological properties of concrete. Different methods of protecting the bacterium in concrete are proposed and analysed and a proprietary mixture of self-healing biological concrete is created. For this mixture, the ability to heal cracks under optimal and close-to-real-outside conditions is determined.

Poděkování

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Table of contents

PROHLÁŠENÍ 2

ABSTRAKT 3

ABSTRACT 4

PODĚKOVÁNÍ 5

TABLE OF CONTENTS 6

INTRODUCTION 11

MOTIVATION AND GOALS 12

PART A: LITERATURE REVIEW

CHAPTER 1: MICP AND CONCRETE 13

1.1 BIO-BASED SELF-HEALING CONCRETE: BASIC PRINCIPLES 14

CHAPTER 2: MECHANISMS OF BICP 17

2.1 AEROBIC RESPIRATION 17

2.2 UREA HYDROLYSIS 17

2.3 NITRATE REDUCTION 18

CHAPTER 3: THE SELF-HEALING AGENT 20

3.1 BACTERIAL STRAINS 20

3.1.1 CULTURE AND SPORULATION MEDIA 20

3.2 FACTORS INFLUENCING BACTERIAL GROWTH AND MICP 21

3.2.1 ALKALINITY 21

3.2.2 THE CONCENTRATION OF CALCIUM IONS 21

3.2.3 DEPENDENCE ON OXYGEN 22

3.2.4 REAL-LIFE CONDITIONS (LOW TEMPERATURES, SALINITY, ETC.) 22

3.3 BACTERIA PRODUCTION AND ADEQUATE CONCENTRATIONS 23

3.3.1 PRODUCTION OF AXENIC/ANOXIC CULTURES 23

3.3.2 APPROPRIATE CONCENTRATION OF BACTERIA 24

3.4 SOURCES OF CARBON, NITROGEN, AND CALCIUM 25

3.4.1 SELECTION AND OPTIMAL DOSAGE 25

3.4.2 IMPACT ON THE MECHANICAL PROPERTIES AND RHEOLOGY 27

3.4.3 IMPACT ON BACTERIAL ACTIVITY AND MICP 29

3.5 THE SELF-HEALING AGENT - CONCLUSIONS 31

CHAPTER 4: PROTECTION OF BACTERIAL SPORES 32

4.1 THE ACDC 33

4.2 MICROENCAPSULATION 34

4.3 LIGHTWEIGHT AGGREGATES 35

4.4 HYDROGELS AND SUPERABSORBENT POLYMERS (SAPS) 37

4.5 SILICA GEL AND POLYURETHANE 38

4.6 SILICA FUME 39

4.7 DIATOMACEOUS EARTH 39

4.8 FIBRES 40

4.9 CSA CEMENT CARRIES 41

4.10 PROTECTION OF BACTERIAL SPORES - CONCLUSIONS 41

CHAPTER 5: MATERIALS AND METHODS APPLIED TO DETERMINE THE SELF-HEALING POTENTIAL 42

5.1 SPECIMEN'S SHAPES 43

5.2 SPECIMEN'S MATERIALS 44

5.3 INTRODUCTION OF CRACKS AND CURING PERIOD 44

5.4 DETERMINATION OF THE HEALING POTENTIAL 46

5.4.1 DURABILITY EVALUATION 46

5.4.2 RECOVERY OF MECHANICAL PROPERTIES 49

5.5 MATERIALS AND METHODS APPLIED TO DETERMINE THE SELF-HEALING POTENTIAL – CONCLUSIONS 50

CHAPTER 6: OUTDOOR EXPERIMENTS 51

6.1 IRRIGATION CANAL IN ECUADOR 51
6.2 PANELS IN SOUTH WALES 51
6.3 A ROOF SLAB IN ANTWERP 53
6.4 GREEN BASILISK B.V. 53

PART B: EXPERIMENTAL WORK

CHAPTER 7: EXPERIMENTAL WORK BACKGROUND 55

CHAPTER 8: ANALYSIS OF BACTERIA BEHAVIOUR AND BICP MECHANISM 55

8.1 BACTERIA SURVIVAL AND GROWTH CURVES 55
8.1.1 RESISTANCE TO FREEZE-THAW CYCLES 55
8.1.2 THE EFFECT OF TEMPERATURE ON GROWTH 57
8.1.3 MATHEMATICAL MODELLING OF GROWTH CURVES OF *BACILLUS PSEUDOFIRMUS* 59
8.2 EVALUATION OF THE CALCITE PRODUCTION 63
8.2.1 MATERIALS AND METHODS 63
8.2.2 RESULTS 64
8.2.3 CONCLUSIONS 66
8.3 ANALYSIS OF BACTERIA BEHAVIOUR AND BICP MECHANISM – CONCLUSIONS 67

CHAPTER 9: SELECTION OF NUTRIENTS 67

9.1 IMPACT OF NUTRIENTS ON MATERIAL CHARACTERISTICS 67
9.1.1 MATERIALS AND METHODS 68
9.1.2 IMPACT OF NUTRIENTS ON MATERIAL CHARACTERISTICS – RESULTS 69
9.1.3 DISCUSSION AND CONCLUSIONS 71

CHAPTER 10: SELECTION OF PROTECTIVE CARRIERS 71

10.1 NANOFIBRE TEXTILE 72
10.1.1 MATERIALS AND METHODS 72
10.1.2 RESULTS 73
10.1.3 CONCLUSIONS 73
10.2 LIGHT WEIGHT AGGREGATES 74
10.2.1 MATERIALS AND METHODS 74

10.2.2 RESULTS 75
10.2.3 CONCLUSIONS 76
10.3 SUPERABSORBENT POLYMERS 76
10.3.1 MIX DESIGN AND ITS TESTING 77
10.3.2 RESULTS 78
10.3.3 CONCLUSIONS 78

CHAPTER 11: DETERMINATION OF THE CRACK-SEALING POTENTIAL 79

11.1 EXTERNAL CURING – MICROSCOPY ANALYSIS OF CRACK-SEALING 79
11.1.1 MATERIALS AND METHODS 79
11.1.2 RESULTS 80
11.1.3 CONCLUSIONS 82
11.2 CRACK-SEALING UNDER OPTIMAL IN-VITRO CONDITIONS 83
11.2.1 MATERIALS AND METHODS 83
11.2.2 RESULTS 84
11.2.3 DISCUSSION 86
11.2.4 CONCLUSIONS 87
11.3 CRACK-SEALING UNDER SIMULATED OUTSIDE CONDITIONS 87
11.3.1 MATERIALS 88
11.3.2 METHODOLOGY 88
11.3.3 TEST METHODS 90
11.3.4 MECHANICAL STRENGTH AND CONSISTENCY RESULTS 91
11.3.5 VISUAL INSPECTIONS OF THE CRACK HEALING EFFICIENCY 92
11.3.6 DYNAMIC YOUNG'S MODULUS RECOVERY RESULTS 95
11.3.7 DISCUSSION 96
11.3.8 CONCLUSIONS 97

CONCLUSIONS 99

ANNEX: MATERIALS AND METHODS

CHAPTER 1: MATERIALS 101

1.1 MEDIA USED FOR CULTURING/SPORULATION OF BACTERIA 101

CHAPTER 2: METHODS – MICROBIOLOGICAL RESEARCH 101

2.1 CULTIVATION AND SPORULATION OF BACTERIA 101

2.2 BACTERIAL GROWTH CURVE USING ELISA MEASUREMENT 101

2.3 BACTERIAL GROWTH CURVE USING BOD 102

CHAPTER 3: METHODS – CONCRETE RESEARCH 102

3.1 CEMENT FLOW TABLE TEST 102

3.2 THE FLEXURAL AND COMPRESSIVE STRENGTH 102

REFERENCES 104

Introduction

In the field of cementitious building materials, microbiology has traditionally been included in the research for the negative impacts of some microorganisms on concrete structures [1].

Biodeterioration, a type of concrete degradation caused by microbially produced acids and by microorganisms themselves in a form of biofilms, causes concrete corrosion which may lead to structural and functional defects [2–4]. Microbial stains, mainly caused by algae, on building facades and walls is another example of a negative impact. Although it is an aesthetic rather than a structural issue, researchers have been seeking an effective method of protection to avoid financially demanding cleaning actions [5,6]. Not surprisingly, a proliferation of microorganisms on building materials indoors has been also in the centre of interest as neglecting the problem can result in severe issues, not only esthetical but also health-related (irritations, infections, allergies, and other respiratory and skin diseases) [7,8].

It is crucial to further investigate the described negative impacts of microorganisms on cementitious building materials; however, it is also important to acknowledge that in some cases the interaction between building materials and microorganisms can result in positive actions [1].

Numerous applications of bacteria as a protective or repair strategy for concrete structures have been proposed and further investigated in the last decades. As mentioned earlier, microbial biofilms are known to damage concrete structures; however, in some cases, biofilms can also serve as a protective layer against biodeterioration by certain organic polymers or proliferation of the undesirable harmful species [1,9–11].

Another example of positive interaction between microbes and concrete is the application of certain biological agents for the material's strength and durability improvement. The MICP (microbially induced calcite precipitation, or, in other words, the biocalcification) process, production of calcium carbonate ($CaCO_3$) by specific microorganisms, has been widely studied in recent decades for its possible applications.

MICP has been proposed and investigated as a surface and internal treatment which could contribute to the durability of structures through autonomous sealing of formed damages by the mentioned calcium carbonate.

This thesis focuses primarily on the second method of the MICP application – the internal treatment in the form of the so-called bio-based self-healing concrete. This novelty material is a cementitious composite containing a biological agent (microorganisms capable of the MICP supplemented with suitable nutrients) which can autonomously seal formed cracks in the material by calcium carbonate. Thus, the transport of aggressive substances through the porous system of concrete is prevented and the possible risk of the materials degradation is significantly reduced. For this reason, the development of material with such extended durability is promising not only from an environmental point of view but also economically as the generally high maintenance costs could be reduced.

Motivation and goals

Nowadays, the idea of sustainable development is attracting widespread interest throughout society. In the field of building materials, research has been focused on materials with prolonged durability to reach more ecological and economic construction possibilities. This thesis deals with one of them – the self-healing biological concrete as introduced above.

Self-healing bio-based concrete will not be, in all probability, a material for common use. However, it could be a beneficial alternative for specific constructions where maintaining tightness and durability is crucial. One of the obvious examples is the current issue with bridges. Due to their financially demanding and complicated maintenance, especially the hard-to-reach parts of structures, they are often in an unsatisfactory condition which can lead, and have led in the past, to tragic events. Other possible places of application could be hard-to-reach structures that are in contact with moisture, e.g., underground waterproofing structures or tunnel linings. This newly developed material could find its place exactly in such exposed and crucial structures where a higher initial investment would be offset by future benefits.

The topic has been, especially in the recent decade, extensively studied; however, numerous questions and factors affecting the material's producibility and applicability remain unsolved.

The main aims of this thesis are to:

- **Provide an extensive literature overview as a base for further experimental research.**
- **Compare different bacterial species, describe their behaviour in various conditions, and further clarify the MICP mechanisms.**
- **Propose and investigate new methods of protection of bacterial spores and select appropriate nutrients considering the material properties of the resulting cementitious composite.**
- **Produce samples of the bio-based concrete and examine its self-healing potential in optimal and simulated realistic conditions.**

PART A: Literature review

Chapter 1: MICP and concrete

Despite the developments in the field of cementitious materials in recent decades, the formation of cracks remains the material's weak point. The formed damages largely contribute to the transport of various harmful substances through the material's porous system, thus increasing the risk of its damage and endangering the structure durability (the reinforcement corrosion or the concrete matrix deterioration) [12]. As some level of cracking is generally inevitable in concrete structures, frequent manual repairs or over-dimension of the protective concrete cover layer is necessary. However, both measures tend to be financially demanding. The repair actions can additionally pose a threat to the environment as the majority of the repair techniques are polymer-based, and they are difficult to implement in inaccessible parts of the structure.

Concrete is known for its autogenous healing capacity. In the literature, several chemical, physical, and mechanical processes that may contribute to this phenomenon are mentioned: swelling and hydration of cement paste, precipitation of calcite crystals, blocking of flow path by water impurities or by concrete particles broken from the crack surface [13–15]. Click or tap here to enter text.Investigations of Edvardsen [15] have shown that the most significant action in mature concrete exposed to water is the spontaneous precipitation of calcite crystals (SICP). The chemical reaction taking place in an alkaline environment (pH > 8) is the following:

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+ \qquad \text{Eq. 1}$$

Hydrogen carbonate HCO_3^-, one of the constituents of carbonate hardness of water, reacts with the calcium ions exuded from concrete. Calcium carbonate is then produced, and the cracks can be gradually sealed. Even though SICP can contribute to crack sealing to a considerable extent, it is not possible to rely on this natural phenomenon completely.

Based on the knowledge of SICP, research, especially in the last decade, has been focused on improving the phenomena in order to create the so-called self-healing cementitious material. This material should be able to autonomously fill formed cracks in the structure without the need of any human intervention. Thus, the risk of durability loss would be minimized while the economic and environmental costs reduced. Several approaches to the topic of self-healing have been proposed: the addition of crystalline admixtures [16,17], fibres [16,18], or microbial healing agents – the subject of this thesis.

The microbial induced calcite precipitation (MICP), a term introduced by Stock-Fisher *et al.* [19], was first reported in the late 19th century by Murray and Irvine and Steinmamm [20] with ureolytic bacteria in marine conditions. Since then, numerous researchers have been trying to deepen the knowledge of the mechanism in marine bacteria [21,22] and soil bacteria [23]. Following studies have been proposing various beneficial applications of MICP in the field of building materials, such as protection and remediation of stone surfaces [24,25]. Based on these findings, in 2008, Jonkers *et al.* proposed utilization of MICP as a way to achieve prolonged durability of concrete structures and introduced self-healing concrete with an incorporated biological agent [26].

Several studies successfully demonstrated that external applications of biological healing systems can be beneficial in the terms of crack-sealing [27]; however, this method still requires

regular inspection of the structure and manual execution. The self-healing bio-based concrete, as proposed by Jonkers, benefits from the ability of specific species of bacteria to survive in environments which seem to be inhospitable for any living forms (deserts, rocks etc.) [28], [29] for up to hundred years [30] in a form of spores – a dormant state characterized by no metabolism, and high chemical and mechanical resistance.

In the self-healing bio-based concrete, calcite-producing microorganisms (mostly bacteria) are embedded together with necessary nutritive organic compounds into the cementitious matrix. Then, MICP is triggered by the crack formation itself. Thus, the healing action does not require any human intervention at all.

1.1 Bio-based self-healing concrete: basic principles

To understand all the aspects of the development of a truly self-healing material, we must first look at the principles of material design in general. In the book "Self-Healing Materials: An Alternative Approach to 20 Centuries of Material Science" [31], the current material development was divided into two concepts: damage prevention and damage management.

As the name suggests, the damage prevention concept focuses on the changes of specific material properties to achieve stronger and more durable material in order to avoid any potential damages. Even though the concept has been successful, and it will certainly be applied in future development, it has its limits. The cohesion between atoms of any material ultimately limits its properties. For example, the strength of a material can be artificially enhanced by its composition or a production process; however, it is not possible to significantly tune its stiffness, as this property depends on the strength of the interatomic bonds and their packing density. In practice, it means that although we can delay or reduce the damage formation, we cannot exclude it completely. Consequently, the structures need regular inspections and repairs of the formed defects.

The self-healing bio-based concrete addressed in this thesis represents the second concept – damage management. In contrast with the first concept, the damage management concept accepts the damage formation but considers it non-threatening as long as the damage process is parallel to a "healing" process. It means that material with an absolute self-healing capacity would be infinitely durable. However, one can quickly realize that almost nothing is absolute in the real world, and we must reckon with certain limits. In this part of the chapter, we will briefly introduce the principles of the self-healing bio-based concrete and factors which may influence its efficiency.

The main principle of the bio-based self-healing concrete seems rather simple. During the mixing process, a specific self-healing agent (usually bacterial spores and certain nutrients) is added directly to a standard concrete mixture. Further processing does not differ from the traditional procedure. The healing action is initiated by water penetrating the material through the formed microcracks. The bacterial spores in the cracks germinate and their metabolism starts. The process leads to production of $CaCO_3$, thus to the targeted crack sealing (Figure 1).

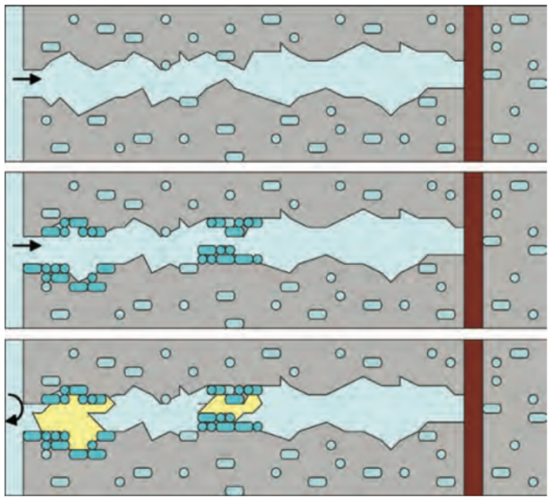

Figure 1. A scenario of the crack-healing by concrete-immobilized bacteria. Bacteria on fresh crack surfaces become active due to the water ingression and start to grow and precipitate calcite ($CaCO_3$). The crack is eventually sealed by the precipitates and the material is protected against any further external attacks [18].

Now, let us discuss some of the factors that may deviate the bio-based self-healing concrete from the ideal material of the damage management concept.

The first question is to what extent the damages are removed. The ideal self-healing material would have an infinite number of healing cycles without any accumulations of the damages. However, in a real self-healing material, not-complete healing actions and limited cycling potential are probably inevitable.

As studies showed, the efficiency of the currently investigated bio-based self-healing concrete largely depends on the number of the incorporated bacterial spores, the availability of nutrients, environmental conditions, crack widths, and many other factors. Due to these limitations, not-complete healing actions and a decrease in the crack sealing potential overtime need to be expected.

Further, we must ask how the damage will be detected. In order to create a truly self-healing material, the self-healing agent should detect the defect itself and start the healing process spontaneously. The bio-based self-healing concrete meets these conditions almost completely as the process of calcification is automatically triggered by the damage creation itself, or more precisely by the resulting water ingression. However, in the case of decreased humidity, the self-healing efficiency could be significantly limited or non-existent at all.

Another question is how the self-healing agent can be transported through the rigid material. There is a great discrepancy between the need to locate the self-healing agent in the damage destination, therefore the need for flexibility, and its purpose to fix the damage by becoming immobile. This issue is partially solved by an efficient spreading of the self-healing agent through the material. Thus, there should be a high probability that the formed crack reaches the incorporated agent, so the healing action can be initiated. However, a balance between the applied number of bacterial spores and the economic factor must be kept in mind as their production is generally a financially demanding process. A sufficient concentration and placement near the structure surface certainly increase both the healing potential and economic feasibility.

Finally, the healing of concrete microcracks does not largely differ from the healing of broken human bones. As it is important to put a broken hand in a cast so the bone stays in a fixed

position, the cracks cannot be sealed unless their surfaces are brought together and stable. Therefore, the cyclicity of load applied on the structure might influence the healing efficiency greatly as the crack surfaces would need to stay in a stable position for a sufficient time period.

Chapter 2: Mechanisms of BICP

Different pathways which lead to the $CaCO_3$ production by specific microorganisms have been described over the last decades. In all pathways, calcium carbonate ($CaCO_3$ in the mineral form of calcite, aragonite, or vaterite) [32] is formed due to the shift of carbonate ions (CO_3^{-2}) under the conditions of high pH and high calcium ion (Ca^{+2}) concentration [33]. The formation of carbonate ions is a result of the presence of carbon dioxide (CO_2) released by the metabolic activity of bacteria

In this chapter, different modes of MICP used in the self-healing concrete development are briefly introduced and their specific advantages and disadvantages are presented.

2.1 Aerobic respiration

In the aerobic respiration metabolic pathway, bacteria are the catalyst of degradation of organic compounds which leads to precipitation of calcium carbonate and carbon dioxide production according to the following reaction [34]:

$$Organic\ compounds + O_2 \rightarrow CaCO_3 + CO_2 + H_2O \qquad Eq.\ 2$$

Various nutrients (metabolic activators and calcium sources) have been proposed and successfully investigated as organic compounds. More information about the topic is given in the following chapters.

The produced molecules of CO_2 by the bacteria metabolism can also further react with portlandite ($Ca(OH)_2$) minerals. That results in even more production of calcium carbonate-based minerals according to the following reaction [34]:

$$CO_2 + Ca(OH)_2 \rightarrow CaCO_3 + H_2O \qquad Eq.\ 3$$

To further increase the production of $CaCO_3$, Chen *et al.* have proposed and investigated improvement of the phenomenon by providing a part of the carbon dioxide extra by yeast fermenting glucose [35].

The main drawback of the aerobic respiration metabolic pathway is its dependence on dissolved oxygen. It should not be an issue in the upper layers of the crack but in deeper parts, the metabolic activity is expected to be considerably lower [36].

2.2 Urea hydrolysis

Urea hydrolysis, another metabolic pathway which leads to MICP, has been widely investigated in previous studies. Similar to the previously introduced pathway, MICP based on the urea hydrolysis requires ureolytic bacteria accompanied by nutrients (organic compounds and/or urea) and calcium source.

The process will be further described with the summarization which Al-Salloum *et al.* [37] provided in their review of microbial mineral precipitation.

First, urea is enzymatically broken down into ammonia and carbamic acid, which is instantly hydrolysed to form ammonia and carbonic acid:

$$CO(NH_2)_2 + H_2O \leftrightarrow NH_3 + NH_2COOH \quad \text{Eq. 4}$$

$$NH_2COOH + H_2O \leftrightarrow NH_3 + H_2CO_3 \quad \text{Eq. 5}$$

Next, ammonia forms ammonium and hydroxide ions and carbonic acid forms bicarbonate ions:

$$2NH_3 + 2H_2O \leftrightarrow 2NH_4^+ + 2OH^- \quad \text{Eq. 6}$$

$$H_2CO_3 \leftrightarrow HCO_3^- + H^+ \quad \text{Eq. 7}$$

Hydroxide ions raise the pH, causing a shift in bicarbonate equilibrium, which results in the formation of carbonate ions:

$$HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \leftrightarrow CO_3^{-2} + 2NH_4^+ + 2H_2O \quad \text{Eq. 8}$$

Finally, in the presence of calcium ions, carbonate ions precipitate as calcium carbonate crystals:

$$Ca^{+2} + CO_3^{-2} \leftrightarrow CaCO_3 \quad \text{Eq. 9}$$

Ureolytic bacteria have been successfully applied in numerous studies, but it should be noted that not even this pathway is without its disadvantages. Urea hydrolysis leads to a production of ammonia which may increase the risk of reinforcement corrosion [38] and degradation of the concrete matrix, particularly when further oxidized by bacteria to yield nitric acid [39]. However, it is questionable whether the production of ammonia is large enough to cause the mentioned issues and further research would be needed.

Unlike the previous MICP mechanism, it was demonstrated by Wang *et al.* [40] that ureolytic activity is in fact not dependent on oxygen; that this, urea can be decomposed by vegetative cells disregarding the oxygen presence. However, for spores to germinate and active bacteria to grow, the oxygen presence is needed. Thus, MICP could be still limited in the deeper parts of the crack as germination of spores might be limited due to the oxygen shortage.

2.3 Nitrate reduction

Anoxic oxidation of organic carbon, a process called denitrification, is based on the reduction of nitrate (NO_3^-) or nitrite (NO_2^-). In the presence of organic carbon source under oxygen limited conditions, the denitrification process results in CO_3^{2-} production and HCO_3^- ions, which are necessary for the $CaCO_3$ precipitation [41], according to the following reaction:

$$5HCOO^- + 2NO_3^- \rightarrow N_2 + 3HCO_3^- + 2CO_3^{2-} + H_2O \quad \text{Eq. 10}$$

The nitrate reduction pathway, same as the previously described modes of calcite precipitation, requires a supply of certain compounds apart from the bacteria: an organic carbon compound and a source of NO_3^-.

The ability of nitrate-reducing bacteria to metabolize under oxygen-limited conditions is the most striking benefit of this pathway. As described earlier, in the case of aerobic pathways, reduced metabolic conversion may occur in the deeper parts of the crack where the oxygen concentration is not sufficient. Clearly, this should not be a problem in the case of anaerobic bacteria.

Recently, the potential of a steel reinforcement passivation as a by-product of denitrification has been investigated. In the process of NO_3^- reduction, production of NO_2^- takes place [41] according to the following reaction:

$$HCOO^- + NO_3^- + H^+ \rightarrow CO_2 + NO_2^- + H_2O \qquad \text{Eq. 11}$$

NO_2^- is commercially used as a steel reinforcement corrosion inhibitor in the form of $Ca(NO_2)_2$ [42]. Unfortunately, in the metabolic pathway, the NO_2^- reduction is mostly suppressed by a high rate of NO_3^- reduction. However, if the accumulation of NO_2^- would achieve $[NO_2^-]/[Cl^-]$ ratio in the range of 0.34-1.0 (the optimal range for corrosion inhibition [42,43]), the nitrate reduction pathway could potentially lead to not only crack sealing but also to the inhibition of corrosion of concrete structures [41].

Ersan *et al.* reported promising results [41], but direct addition of nitrite was still an easier and more reliable option because the amount of produced NO_2^- was not sufficient for effective passivation. It is, however, important to note that nitrite (in contrast to nitrate) has a negative effect on the concrete material characteristics, it is more toxic to the environment, and it tends to be more expensive. Thus, improvement of the NO_3^- reduction with NO_2^- production is favourable and further investigations of the potential should be carried out.

Chapter 3: The self-healing agent

The bio-based self-healing concrete is based on the addition of certain self-healing agent to a standard concrete mix. In the past decades, researchers have come across many requirements and limitations which affect the selection of the agent. However, not only its composition is crucial. The production methodology also needs to be taken into consideration as it is closely linked to the economic feasibility of the novelty material.

In this chapter, individual components of the self-healing agent, which have been most proposed in the previous studies, are presented. Further, the aspects which specify the final selection are introduced for each part of the agent.

3.1 Bacterial strains

Previous studies have dealt with many different spore-forming, alkaliphilic bacterial strains capable of MICP which may be able to survive the harsh and highly alkaline environment of concrete and restore its metabolic activity. Besides the chemical and mechanical resistances, the bacteria must also have no negative effect on health and the environment as the material would be produced and further function not in controlled laboratory conditions but *in-situ* as regular concrete.

In Table 1, some of the most frequently proposed bacteria species for MICP are listed. They are characterized by different metabolic pathways, resistance to pH, humidity and temperature requirements, and the need for nutrients supply.

Table 1. A list of the most frequently proposed bacteria species for the MICP.

Reference	Bacterial genotype	Metabolic pathway
[44–50]	*Bacillus sphaericus*	enzymatic hydrolysis of urea, nitrate reduction
[51–53]	*Bacillus pasteurii*	enzymatic hydrolysis of urea
[54], [55]	*Bacillus subtilis*	oxidation of organic carbon
[26], [34], [56]–[58]	*Bacillus pseudofirmus*	oxidation of organic carbon
[26,34,56]	*Bacillus cohnii*	oxidation of organic carbon
[35]	*Bacillus mucilaginous*	oxidation of organic carbon
[26,59]	*Bacillus halodurans*	oxidation of organic carbon
[60]	*Bacillus alkalinitrilicus*	oxidation of organic carbon
[61–63]	*Diaphorobacter nitroreducens*	nitrate reduction
[61–63]	*Pseudomonas aeruginosa*	nitrate reduction

3.1.1 Culture and sporulation media

A sufficient supply of nutrients is necessary for the growth of bacteria *in vitro*. Previous studies have mainly used culture media consisting of nutrient broth, yeast extract and peptone. After a growth period in the culture medium, the bacteria were usually inoculated into spore-forming medium, which is alkaline and poorer in nutrients, thus promotes sporulation.

Ting *et al.* [64] in their study compared sporulation rates of six strains of bacteria in different sporulation alkaline media. The study showed that it is crucial to identify the most suitable

medium for each bacterium separately, as the sporulation rates varied significantly based on its composition. For *Bacillus cohnii* and *Bacillus pseudofirmus* alkaline R2A agar (0.5 g casein acid hydrolysate, 0.5 g yeast extract, 0.5 g dextrose, 0.5 g soluble starch, 0.3 g K_2PO_4, 0.3 g Na pyruvate, 0.25 g casein peptone, 0.25 g peptic digest of animal tissue, 0.05 g $MgSO_4$, 15 g agar) sporulation medium seemed to be most efficient, whereas for *Sporosarcina pasteurii* it was trypticase soy agar TSA (15 g pancreatic digest of casein, 5 g peptic digest of soybean meal, 5 g NaCl, 15 g agar) supplemented with 2% urea and sporulation agar SAU (5 g peptone, 3 g meat extract, 0.01g $MnSO_4 \cdot H_2O$, 15 g agar) supplemented with 2% urea, and for *L. sphaericus* it was R2A agar supplemented with 2% urea.

These findings suggest that the composition of both media (culture and sporulation) should be investigated in more detail in future studies as it directly affects the behaviour of the selected bacteria. Moreover, as Al-Salloum *et al.* [37] mentioned in their review, it would be interesting to study the impact of the switchover between the culture/sporulation media and the nutrients present in the final healing agent, as it could greatly influence the viability of the bacteria.

3.2 Factors influencing bacterial growth and MICP

3.2.1 Alkalinity

Alkalinity of the surrounding environment is one of the key factors which contribute to the efficiency of the MICP. Although, as previously stated, the applied bacterial strains are known for their alkalinity resistance, research has shown that alkaline pH exceeding a certain level could hinder bacterial growth and metabolic activity.

Wang *et al.* [40] found that the optimal pH range for ureolytic strain *Bacillus sphaericus* is between 7 and 9. In a higher alkaline environment (pH 10 ~ 12), the growth and germination slowed down dramatically but did not stop completely. Similarly, Algaifi *et al.* showed that the pH value of 12 ~ 13 not only inhibited the growth of the selected bacteria (ureolytic strain *Sporosarcina pasteurii*) but even reduced the efficiency of urea hydrolysis greatly.

These findings raise a question of whether the bacteria will be able to metabolize in highly alkaline media such as concrete pore solution with its optimal pH between 12,5 ~ 13 [65]. It is difficult to determine the pH inside a crack, as the water/moisture ingress may lower it noticeably. The inner environment of a crack, although still highly alkaline, may be more suitable for the bacteria than the standard concrete pore solution. Further, the carbonation process, an undesirable but generally unavoidable phenomenon which causes a decrease in alkalinity, could contribute to bacterial activity.

3.2.2 The concentration of calcium ions

As described in the previous chapter, free calcium ions (Ca^{2+}) are necessary for MICP regardless of the metabolic pathway type. Calcium ions are available in the cement matrix itself and present in the bacterial spore's protective membrane [66]. The majority of conducted studies also applied an additional calcium source, mostly in the form of organic calcium salts (e.g., calcium lactate, calcium formate, or calcium chloride).

Some studies focused on the possible negative impact of high Ca^{2+} concentration. As the bacterial cell wall has a negative charge, it is believed that the positively charged Ca^{2+} ions could cling onto its surface easily. Consequently, this could lead to the precipitation of calcium carbonate on the bacterial cell wall, thus limiting further metabolic activity and growth [53,67].

Research concerning this issue provided rather inconsistent results. Algaifi *et al.* [53] in a study using ureolytic bacteria reported that the calcium ion concentration exceeding 250 mM has a negative impact on urea hydrolysis. As a favoured condition, the value of 150 mM was suggested. Zhang *et al.* showed that the calcium carbonate precipitation by non-ureolytic bacteria (sediment isolate close to *Bacillus Pseudofirmus*) dropped significantly when the Ca^{2+} concentration increased from 30 to 60 mM. In contradiction, Wang *et al.* [40] reported no significant influence on the ureolytic activity of *Bacillus sphaericus* even in the presence of 0.9 M Ca^{2+}.

3.2.3 Dependence on oxygen

The dependence of the MICP on oxygen has already been discussed in the previous chapters. Apart from anaerobic bacteria capable of nitrate reduction, bacteria capable of both aerobic respiration and urea hydrolysis need oxygen to produce calcium carbonate. Although the ureolytic bacteria need oxygen purely to germinate and grow [40], the shortage of oxygen could significantly decrease the healing efficiency in both cases. To address this issue, Zhang *et al.* [68] in their pilot study proposed an application of CaO_2 accompanied by lactic acid to produce oxygen-releasing tablets which could serve as an oxygen source for the bacterial self-healing system.

3.2.4 Real-life conditions (low temperatures, salinity, etc.)

Other factors affecting the MICP efficiency are related to real-life outside conditions. Bacteria investigated *in vitro* have a sufficient supply of nutrients, oxygen, optimal humidity, and temperature conditions, and they are protected from direct sunlight. The actual outside conditions, however, tend to be far from the optimum.

Only a few studies focused on the performance of the bacterial self-healing concrete under non-optimal conditions. Palin *et al.* [69] in their study found a bacterial psychrophile isolate that showed excellent germination/growth at high salinity (30 g/l sodium chloride) and low temperatures (8°C). Using this isolate as a base for the self-healing agent, cementitious composites reduced their permeability by 95% in the case of 0.4 mm wide cracks and by 93% in the case of 0.6 mm wide cracks when immersed in artificial seawater at 8 °C for 56 days.

A field study carried out by Paine *et al.* [56] did not report such optimistic results. In this study, a series of reinforced concrete wall panels were prepared partially with addition of coated expanded perlite with immobilized bacteria. The panels were cracked and situated at their planned location – as a conventional retaining wall structure at a highway. After 7 months, no complete crack healing could be observed, and bacterial calcite precipitation could not be precisely distinguished from autogenous healing. As relative humidity remained rather high throughout the experiment, researchers identified low temperature as one of the possible causes. The cultivation temperature of bacteria used in this experiment (*Bacillus pseudofirmus)* is as high as 30 °C. During preliminary in vitro experiments, the temperature has never dropped below 20°C; however, during the 8 months of the outside investigation, the temperature remained around 15 °C and lower. Thus, the explanation of the unsuccessful crack-sealing could be that the selected bacteria simply did not become active.

Gonzalez *et al.* [58] also did not observe any crack-sealing in mortar containing bacterial agent (*Bacillus pseudofirmus* and nutrients) at 4 °C after 68 days. In contrast to Paine and Gonzalez, Wang *et al.* [40] detected activity of ureolytic bacteria *Bacillus Sphaericus* at 10 °C.

Interestingly, in this experiment, the bacteria germinated and revived ureolytic activity even at this low-temperature value, although significantly later than under optimal conditions.

3.3 Bacteria production and adequate concentrations

Ersan in his study [70] claimed that 95% of the additional cost of the bio-based concrete is the production of bacteria itself. It implies that the selection of appropriate bacteria, optimized production process and sufficient dosages of bacteria and nutrients are the key factors that influence not only the self-healing efficiency, but also the cost-effectiveness.

The traditional approach to obtaining bacterial cultures/spores involves inoculating the culture medium with the bacteria in question and subsequent cultivation. To obtain spores, the culture is then inoculated into an alkaline medium to promote sporulation. The bacterial spores are then spun down from the alkaline medium and, usually, washed with saline solution. This whole process is carried out under controlled axenic conditions and is generally time-consuming (in a matter of weeks) and costly, especially if the aim is to obtain large quantities of a given culture/spore.

In this chapter, different ways of bacteria production and their possible influence on the efficiency of the self-healing process are presented. The appropriate concentration of bacteria to ensure crack healing is also addressed.

3.3.1 Production of axenic/anoxic cultures

To achieve effective and more affordable bio-based concrete, Ersan *et al.* [71] have introduced a self-protected nitrate-reducing culture – the so-called "Activated Compact Denitrifying Core" (ACDC). The idea was to develop granules with nitrate-reducing bacteria embedded in the core and protected against the outer environment by a shell from aerobic bacteria and $CaCO_3$. The ACDC proved to be effective in terms of crack sealing [71], water tightness [71], and permeability [72] regain. Furthermore, as the ACDC does not require any protective carries, the cost of bio-based self-healing concrete could be decreased by tenfold when compared to encapsulated axenic bacteria [71,73].

The application of mixed-culture-based self-healing agents was also proposed in several studies. As the production of pure cultures requires strict axenic conditions, preparation of mixed cultures under anoxic conditions may decrease the cost-effectiveness greatly (Zhang *et al.* reported 61% decrease in production cost [32]).

To further address this issue, a selection process to obtain a powderous material containing an efficient ureolytic microbial community (CERUP) has been developed by Da Sileva *et al.* [74]. In this study, a comparison of the operational expenses between CERUP and axenic culture of *Bacillus sphaericus* indicated, that the anoxic approach results in 40 times lower costs.

Some also believe that open cultures may also be characterized by better resistance against the microorganisms present in the real construction environment. This approach was successfully applied by Zhang *et al*. In [75], the majority of selected cracks (0.3 – 0.6 mm) were sealed using three microbial consortia capable of MICP under aerobic, anaerobic, and facultative anaerobic conditions.

Ersan in his work also proposed another cost-reducing solution and used a side-stream from a potato factory as inoculum [72]. However, the granulation process of the ACDC (as described above) was originally rather slow and although the use of waste products reduced the costs, the production speed was not affected. To accelerate the production process, an application of waste bulk biomass was suggested and investigated [72]. This process is traditionally used for

an anaerobic treatment of industrial wastewater where the present COD load (Chemical Oxygen Demand) is turned into biogas by anaerobic granular biomass [76]. Conveniently, some of the waste granular biomass from specific plants have the appropriate properties for ACDC production and may be successfully used. It has been shown that the cell production rate is increased significantly by the granular biomass application [72].

3.3.2 Appropriate concentration of bacteria

An evaluation of the appropriate dosage of bacteria for the bio-based self-healing concrete is a complex problem. The survival and growth rate of bacteria is difficult to estimate as it is influenced by many factors (pH range, Ca^{2+} concentration, temperature, water/humidity ingress, pore size, etc.).

The rate of $CaCO_3$ precipitation is mainly determined by the amount of CO_3^{2-} produced by the bacteria metabolism, in case of a sufficient Ca^{2+} supply [67]. Further, the formation of $CaCO_3$ crystals relies on the presence of nucleation sites. Results presented by Li and Qu [77] suggest that certain macromolecules on the bacterial cell walls may act as nucleation sites. Thus, an increased number of bacteria cells could promote the MICP.

Some studies focused on the estimation of a sufficient concentration of spores/bacteria cells in nutrient media to provide a basis for determining the amount needed in the bio-based self-healing concrete. Zhang *et al.* [67] identified 4×10^7 spores/ml as a suitable concentration of the selected bacteria as higher values did not result in an order of magnitude higher $CaCO_3$ formation. Wang *et al.* [40] showed that the concentration of 1×10^8 cells/ml ensures good Ca tolerance (up to 0.9M Ca^{2+}) while sufficient urea decomposition can be obtained (more about Ca tolerance in 3.4.1.3). In this study, at least 1×10^7 vegetative cells per millilitre in the crack zone was recommended in order to obtain a significant rate of urea decomposition and hence $CaCO_3$ precipitation. In agreement with Wang, Algaifi *et al.* also reported 1×10^8 cells/ml as a sufficient concentration based on the amount of decomposed urea in a liquid media. However, Algaifi pointed out that such quantity is almost impossible to achieve in the harsh concrete environment.

Jonkers *et al.* [34] showed that the viability of bacteria incorporated into concrete matrix without any protection is limited to young concrete. Not only the bacteria are exposed to the highly alkaline environment, but also in later stages, the bacteria may be crushed by crystallization pressures of hardening cement paste. The bacterial spores/cells are mechanically destroyed as the pore sizes cease to be sufficient for them. To resolve this issue, application of certain protective carries has been proposed and investigated by many researchers. A comprehensive overview of the protective methods will be provided in more detail in the following chapters.

Setting aside all the different encapsulation methods, the composition of the self-healing agent and survival rates of incorporated bacterial spores, not many studies have investigated the influence of the ratio of bacteria and nutrients to the self-healing efficiency. Most of the previous studies used concentration of bacteria between $2 – 4 \times 10^6$ to 10^7 spores per gram of calcium source; however, Alazhari *et al.* [56] in their study estimated that 8×10^9 is a minimal number of bacterial spores per gram of calcium source to ensure that enough cells will take part in the healing process. The bacteria concentration should be further investigated in future research in order to balance the minimal dosage required for efficient crack sealing and the economic feasibility.

3.4 Sources of carbon, nitrogen, and calcium

Autonomous bio-based crack sealing of concrete structures is generally based on the conversion of a mineral precursor compound to calcium carbonate. Although the exact composition of the nutritional part of the self-healing agent varies according to the given bacteria strain, the main components remain the same. Bacterial spores need to be accompanied by a metabolic activator (a source of carbon and nitrogen), and a calcium source, which serves as the mineral precursor compound.

The selection of nutrients is important from many points of view. Firstly, the self-healing agent is added into the concrete matrix during the mixing process, and it stays there incorporated for its whole lifespan. It means that it can, positively or negatively, influence the material's characteristics and behaviour. Although the nutrients can be encapsulated in protective carriers just like bacteria, possible leakage must be considered. For this reason, the nutrients not only have to be suitable for the MICP but also must not have any unacceptable negative effect on the material properties.

Secondly, in order to facilitate the implementation of the bio-based self-healing concrete into the commercial sphere, these compounds should ideally be allowed for use in cementitious materials according to the relevant standards and their dosage should not exceed 5% by mass of cement as prescribed for admixtures [78], [79].

3.4.1 Selection and optimal dosage

3.4.1.1 Yeast extract

In most previous studies, yeast extract, possibly accompanied by peptone, was used as a source of nutritional carbon and nitrogen to stimulate bacterial growth. Although Wang *et al.*[40] showed that ureolytic bacteria can still revive the ureolytic activity in medium without yeast extract, with an increasing concentration of the yeast extract, the germination and growth took place significantly faster.

Most of the studies pointed out that the addition of yeast extract into concrete has a negative effect on its mechanical properties and its dosage needs to be limited. Yeast extract can be encapsulated in a protective carrier same as bacteria; however, even in this case, a potential leakage during mixing must be taken into consideration. Wang *et al.* [44] in their study proposed 0.85% yeast extract by cement mass as a limit value in the case of a direct addition into the mortar. Higher additions resulted in a significantly delayed hydration of cement and a decrease in the hydration degree.

3.4.1.2 Urea

As the name suggests, ureolytic bacteria need urea as a substrate for MICP. Although not many studies investigated the impact of urea alone on the mechanical properties of concrete, it seems that certain dosages of urea do not have a negative effect on the compressive strength [50]. Urea is also commercially used as a hydration accelerator, thus compatibility with concrete can be expected. However, in the biological self-healing concrete, much larger doses are needed than in the case of the traditional application as a hydration accelerator.

Several studies determined an appropriate concentration of urea based on bacteria behaviour (the germination, growth, and urease activity). Generally, the more urea was supplied, the more $CaCO_3$ was formed under a sufficient concentration of free calcium ions. However, as Wang *et al.* [40] showed in their study, a certain number of bacteria can metabolize

only certain amount urea. When the upper limit is exceeded, the excess urea appears to hinder bacteria metabolism. Wang attributed this phenomenon to the inhibition of cellular processes by the extensive urea molecule transportation over the cell membrane. Wang estimated the upper limit of urea as 2 M for 2 x 10^7 cells/ml. An optimal value was recommended by Algaifi *et al.* [53] as 333 mM.

It is important to acknowledge that the addition of urea as a part of the self-healing agent is generally problematic for several reasons. Enzymatic hydrolysis of urea leads to the production of ammonia [80] which is toxic to the environment [81]. Further, it also might negatively affect reinforcement corrosion and degradation of the concrete matrix when further oxidized by bacteria. Also, as a consequence of hydrolysis, the pH of the environment is increased. This pH shift could have a negative effect on bacterial growth [19], thus limit the crack-sealing functionality (see 3.2.2).

3.4.1.3 Calcium source

A variety of mineral precursors (i.e., additional Ca^{2+} sources) have been applied and investigated in the MICP research. As previously stated, many factors must be considered when searching for the optimal calcium source for a self-regenerating agent, such as the effect on self-regeneration efficiency, compatibility with concrete, and commercial availability.

As we mentioned in chapter *3.2.2*, the presence of free calcium ions is indispensable for MICP; however, its dosage seems to be crucial as excessive amounts of Ca^{2+} were shown to have an inhibitory impact on the bacteria metabolism and MICP.

In the literature, no mention of studies in which the bio-based self-healing concrete would be prepared with no additional source of calcium was found. Some researchers disagree with this approach and argue that no additional calcium source is needed as its concentration should not exceed 30 mM and the free calcium present in the cement matrix should be sufficient [67]. Regardless of this point of view, an additional calcium source, mostly in the form of organic calcium salts, has been applied in the self-healing concrete in relatively high concentration (around 3% by cement mass).

In the beginnings of the MICP investigations, calcium chloride [19], [49], [82] was mostly applied as the calcium source. However, a greater presence of chloride in concrete can lead to its degradation and reinforcement corrosion. Other calcium sources were preferred in further studies such as calcium lactate, calcium formate, calcium nitrate, calcium acetate, or calcium glutamate. A closer look into the impact of each calcium salt on the MICP and material properties of concrete is provided in the following chapters.

As already mentioned earlier, the economic factor of the bio-based self-healing concrete must be also considered. In Table 2, there is a list of the most frequently employed calcium sources and their informative prices obtained from the global trade website Alibaba.com.

Table 2. Informative prices of Ca sources from Alibaba.com

Type of Ca source	Quantity [kg]	Price [$]
calcium lactate	1000	1400
calcium formate	1000	900
calcium nitrate	1000	200
calcium acetate	1000	1200
calcium glutamate	1000	7200

Conventional commercial use of the proposed calcium sources has a great impact on both price and applicability in self-healing concrete. Calcium glutamate and calcium lactate are commercially used as food additives [83], [84]. Calcium acetate is used primarily as a food additive, laboratory chemical, lubricant, and stabilization agent [85]. Advantageously, calcium formate and calcium nitrate are, apart from other industrial applications, used as concrete admixtures. Calcium formate is used as a setting accelerator [86] and calcium nitrate as a multifunctional concrete admixture (setting accelerator, strength enhancer, anti-freeze admixture and inhibitor against steel corrosion) [87].

From the economic perspective, calcium nitrate and calcium formate seem to be the most suitable candidates for the calcium source in the bio-based self-healing concrete as their price is favourable when compared with the other compounds. They are also both known for their compatibility with concrete and their incorporation into concrete should be unproblematic.

3.4.2 Impact on the mechanical properties and rheology

As already mentioned, the addition of the self-healing agent must not compromise the functionality of the final structure and its effect on the mechanical properties should be carefully examined. In most studies, compressive strength has been measured as an indicator of the effect, but the rheology of the bio-concrete has not usually been investigated further.

3.4.2.1 Impact on the compressive strength

In

Table 3, a list of metabolic activators/calcium sources and their impact on the compressive strength as measured in relevant studies is presented. The results are generally rather contradictory or incomplete, and it will be necessary to re-investigate the issue in further detail in future research. However, we can conclude that not only the composition of the nutrients, but also their dosage is a key factor which influences the mechanical properties of the bio-based concrete.

Table 3. Impacts of the selected carbon/nitrogen and calcium sources on the compressive strength measured at 28 days (compared to cement paste without any additives).

Type of nutrient	Dosage by cement weight	Compressive strength relative to ref. sample at 28 days	Ref.
calcium lactate	8.00%	58%	[50]
	4.00%	118%	[50]
	3.00%	122%	[88]
	2.00%	130%	[50]
	2.00%	114%	[88]
	1.00%	105%	[88]
	1.00%	118%	[34]
	1.00%	108%	[89]
calcium formate	3.00%	103%	[88]
	2.00%	105%	[88]
	1.00%	119%	[88]

calcium nitrate	8.00%	118%	[50]
	4.00%	119%	[50]
	3.00%	79%	[88]
	2.00%	109%	[50]
	2.00%	87%	[88]
	1.00%	108%	[88]
calcium glutamate	1.00%	103%	[89]
calcium acetate	1.00%	73%	[34]
urea	1.00%	101%	[50]
	0.50%	85%	[50]
	0.25%	67%	[50]
yeast extract	1.00%	65%	[34]
	1.00%	78%	[50]
	0.50%	76%	[50]
	0.20%	81%	[50]

Based on the overview in

Table 3 it seems that generally all of the calcium sources have no or even a positive impact on the observed property (especially in concentrations under 4.0%). The overview is still incomplete, and some results are rather contradicting (e.g., calcium nitrate). For that reason, an additional investigation of the impact of calcium salts on the compressive strengths is needed.

Based on the results available so far, calcium lactate, calcium formate, and calcium glutamate seem to be the most suitable choice as a calcium source based on their impact on the compressive strength. If we also compare the impacts of selected compounds on the compressive strength with the economic evaluation, it indicates that an addition of calcium glutamate could increase the price of the bio-based self-healing concrete significantly, thus other sources of calcium should be probably considered.

As it has been said in previous chapters, the addition of urea and/or yeast extract is necessary for an efficient metabolic activity of bacteria. Therefore, as their addition is practically inevitable, the investigations only determine their maximum and minimum dosages.

According to the results reported in [50], the addition of urea at a lower concentration had a rather surprisingly negative effect on the compressive strength, while at 1% by weight of cement the strength was comparable to the reference. However, based on the recommendation of [53], the urea concentration should be approximately 333 mM to achieve sufficient MICP. If a standard cement dosage of 500 kg/m3 is considered, then the required value corresponds to approximately 4 % by weight of cement. Therefore, the investigation of the effect of urea on material properties should focus on higher concentration values.

In line with the expectations, the addition of yeast extract resulted in a drop of the compressive strength in all the studies. Based on these findings, the recommended value of 0.85% by cement weight [44] seems like a reasonable compromise between ensuring the MICP efficiency and the mechanical properties preservation. Further, the negative impact might be to some extent balanced by the addition of a suitable calcium source.

3.4.2.2 Impact on fresh-state properties

As it was previously stated, not many studies have investigated the impact of nutrients on concrete rheology and other fresh-state concrete properties. In a study carried out by Luo and Qian [88], rheology of concrete with a biological self-healing agent (a bacterial spore powder with calcium sources) was investigated. Results indicated that addition of bacterial spore powder and calcium lactate, calcium formate, and calcium nitrate respectively in doses 1%, 2% and 3% by cement mass improved fluidity of the mortar as it can be seen in

Table 4.

The effect on the setting time was also investigated in the study. Results indicated that calcium lactate has a retarding effect on the setting time and calcium nitrate and calcium formate have an accelerating effect. These results are in agreement with preliminary estimations as the mentioned compounds are commercially used as setting time accelerators.

Table 4. Influences of the selected calcium sources on the fresh-state properties [88].

Type of nutrient	Dosage by cement weight	Shift in fluidity	Effect on setting time
calcium lactate	3%	112%	retarding
	2%	111%	
	1%	106%	
calcium formate	3%	109%	accelerating
	2%	102%	
	1%	101%	
calcium nitrate	3%	113%	accelerating
	2%	115%	
	1%	103%	

3.4.3 Impact on bacterial activity and MICP

Only a few studies investigated the impact of different calcium sources on $CaCO_3$ precipitation. In [90], a culture of *Sporosarcina pasteurii* was inoculated in a urea-calcium salt solution (20g/l urea, calcium salt 50 mM) and the impact on urea hydrolysis was observed. A wide range of calcium salts was tested: calcium acetate, calcium chloride, calcium lactate, calcium sulfate, calcium silicate, calcium metaborate, calcium gluconate. The results revealed that all the calcium salts decreased the urease activity when compared to the control. Further, all the salts caused a similar decrease apart from calcium silicate which did not affect the activity significantly. However, according to Goropse *et al*., this might be attributed to the low solubility of calcium silicate in water.

Further in this study, sand columns were bio-consolidated using the bacteria and a urea-calcium salt solution (30g/l urea, 20g/l as calcium ion) – calcium chloride, calcium acetate, and calcium lactate were applied. In Table 5, the applied calcium salts are listed and ordered by the consolidation efficiency.

In a study by Achal *et al.* [91], the influence of calcium sources (calcium chloride, calcium acetate, calcium oxide, and calcium nitrate) was investigated. Bacterial growth, urease activity, and $CaCO_3$ production was measured using ureolytic bacteria culture inoculated in nutrient broth containing urea (2%) and a calcium source (25/50/24 mM). In contradiction to [90], the addition of calcium salts resulted in higher urease activity compared to the control in all cases. Identically as in [90], calcium chloride was the most efficient source.

Based on these experiments, calcium chloride could be considered as the most suitable calcium source, at least in the case of ureolytic bacteria. However, as it was already stated, the addition of calcium chloride to concrete is problematic owing to the potential steel reinforcement corrosion. Therefore, other calcium sources should be preferred.

Table 5. Influence of calcium source on $CaCO_3$ precipitation efficiency.

	Type of nutrient (ordered by efficiency)	**Concentration**	**Reference**
1.	calcium chloride	30g/l urea + 20g/l as calcium ion	[90]
2.	calcium acetate		
3.	calcium lactate		
1.	calcium chloride	2% urea + 25/50/40 mM Ca source	[91]
2.	calcium nitrate		
3.	calcium acetate		
4.	calcium oxide		

Only a few studies have compared the effect of different types and doses on targeted crack sealing. Unfortunately, comparisons of the results of individual experiments are generally not sufficiently accurate because the variability in material composition and conditions under which healing was observed tends to be high.

Xu and Yao [89] in their study investigated a crack sealing potential of non-ureolytic bacteria with two different calcium sources (calcium lactate and calcium glutamate) by multiscale mechanical quantifications. Mechanical tests in macroscale (bending tests and ultrasonic pulse velocity) were carried out before and after the healing period. Results showed that the recovery rate of the flexural strength and the flexural modulus was higher in the case of calcium glutamate. Furthermore, nanoscale mechanical characteristics were evaluated by nanoindentation. The results agreed with the macroscale measurements.

In Figure 2, contour maps of modulus of the newly deposited layer from calcium lactate and calcium glutamate on mortar are shown. In both cases, the blue areas with low modulus values were mainly observed in the original mortar matrix region. On the contrary, the red/orange/yellow areas with the highest values were detected primarily in the deposited layer region. The average modulus value of the deposited layer was estimated to be in a range from 40 to 50 GPa regardless of the calcium source. However, the zone in the deposited layer on the border with the original mortar matrix was significantly thicker and stiffer (average modulus values about 20% higher compared to the inner precipitates) in the case of calcium glutamate. This layer may have acted as a strong bond between the original matrix and new deposit layer, thus ensuring better recovery rate of the observed mechanical properties. Based on this finding it could be assumed that a self-healing agent containing calcium glutamate will result in more reliable crack-sealing.

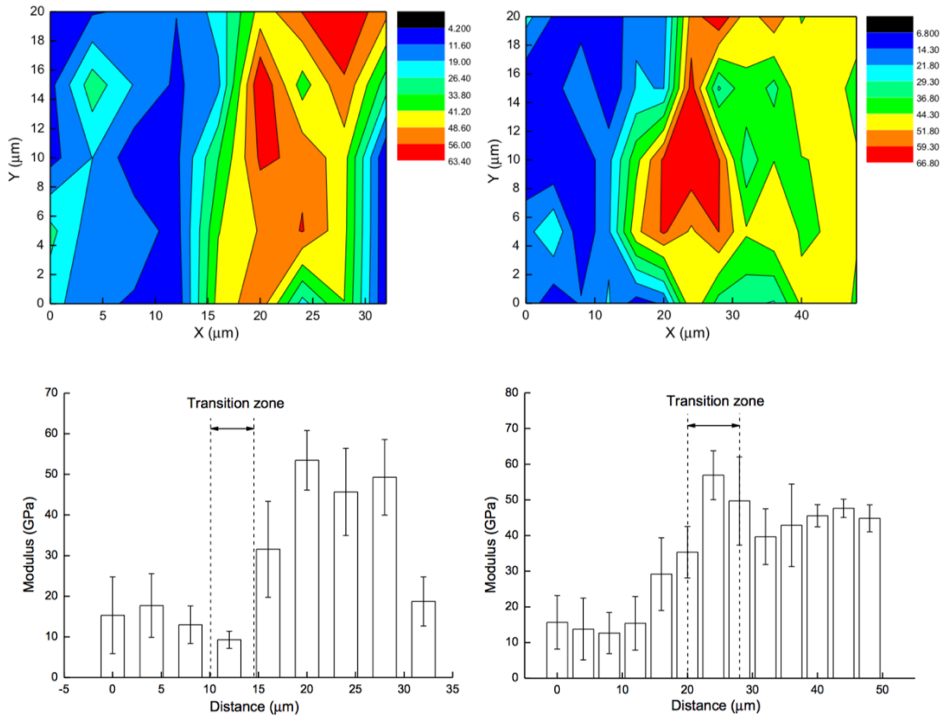

Figure 2. Contour maps of the modulus in GPa of the sealed cracks on mortar specimens with the self-healing agent with calcium lactate (left) and calcium glutamate (right) [89].

3.5 The self-healing agent - conclusions

- Anoxic bacteria should be preferred over aerobic bacteria due to its more efficient calcite precipitation in deeper parts of the crack. Although other factors must also be taken into account (ability to grow, behaviour at sub-optimal temperatures, etc.)
- The composition of culture and sporulation media should be further investigated as it influences the economic feasibility of the production process and the final crack-sealing efficiency.
- The application of open-cultures (prepared in axenic environment) should be favoured due its possible better resistance and lower production costs.
- Germination, growth, and calcite production achievable by the selected bacteria should be investigated under various conditions (e.g., high pH environment, high/low free Ca^{2+} concentrations, low temperature, or carbon/nitrogen concentrations).
- Utilization of waste products in the production process of bacterial spores should be considered. It reduces costs of the final product and potentially accelerates the production process significantly.
- A minimal number of bacterial spores per gram of calcium source should be considered when designing the self-healing agent.
- Calcium lactate, calcium formate, and calcium nitrate should be used as calcium sources owing to their impact on the mechanical properties, economic feasibility and compatibility with concrete.
- The positive effect of anoxic bacteria and calcium nitrate on steel reinforcement passivation should be further investigated.

Chapter 4: Protection of bacterial spores

Although spores are an extremely resistant form of bacteria, mixing of concrete and hydration threaten their survival greatly. Jonkers *et al.* [34] pointed out that their viable number significantly decreases after approximately 7 days, as it can be seen in Figure 3. This decrease could be attributed to both mechanical destruction by crystallization pressures during hydration and high alkalinity of the environment [44,60].

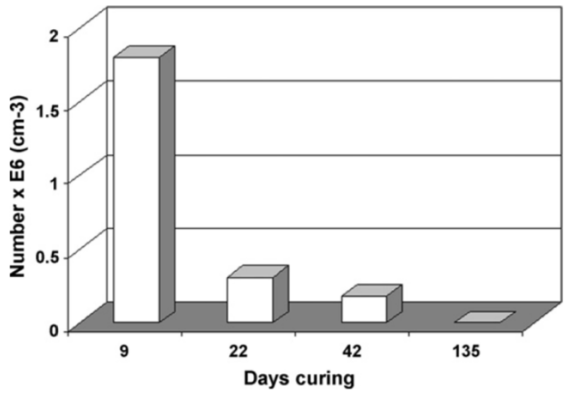

Figure 3. The most-probable number estimate of viable bacterial spores incorporated in aged cement stone specimens [34].

To overcome this limitation, researchers have been suggesting and investigating different methods of protection of bacteria (and possibly nutrients), which would extend bacteria viability, thus ensuring sufficient calcium carbonate deposition in cracks. In this chapter, various protective carriers will be presented and their influence on the mechanical properties and the crack-sealing efficiency will be evaluated.

When searching for a suitable protective method, numerous factors need to be considered. Firstly, the protection must not negatively influence the viability of the bacteria. Therefore, only non-harmful and compatible materials can be used. Secondly, the protection must be durable enough to withstand both the mixing process, the mechanical forces in aging concrete, and high alkalinity. On the other hand, it must release the bacteria (with nutrients) when cracking occurs so the penetrating water could initiate the MICP. Thirdly, the influence of the protection on the material characteristics must be considered. Especially the encapsulation of bacteria in porous materials can lead to a decrease in the concrete density, thus decrease of the compressive strength.

The economic factor is, besides the production costs, largely influenced by sufficient dosing of the protected self-healing agent. Still, one of the downsides which will need improvement in future research is the inequality of placement of the self-healing agent. Although microcracks appear only in the thin outer layer, the self-healing agent is generally spread throughout the structure volume. That implies that determination of an adequate dosage is crucial. The healing agent must be distributed densely enough to ensure that crack will interfere with it but on the other hand, with the increasing amount of the healing agent, the economic feasibility and possibly the mechanical properties decrease. Hence, a balance must be struck between these factors.

4.1 The ACDC

The "Activated Compact Denitrifying Core" (ACDC) was already briefly introduced in the previous chapters. Although it is not a typical example of encapsulation of bacterial spores, the ACDC is one of the most perspective methods of bacteria application.

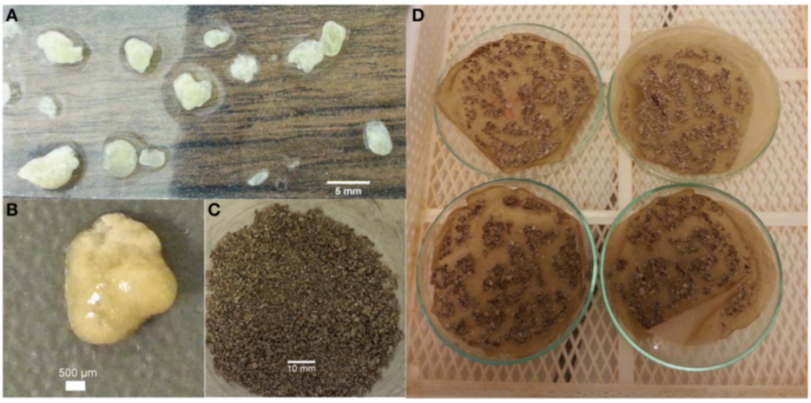

Figure 4. Harvested ACDC granules (A, B) wet and (C, D) after drying [71].

ACDC granules [41] are compact layered particles which consist of a wide range of axenic bacteria in outer layers and non-axenic denitrifying bacteria in the core. They are composed of approximately 70% of bacteria and 30% of inorganic salts.

These granules (Figure 4) have numerous functional benefits. Firstly, the contained species can be enriched by the cultivation process to obtain specific types to our advantage. Secondly, denitrifying bacteria in the core is naturally protected so that the ACDC does not need any further treatment prior to addition to the concrete matrix. Also, the production process proved to be significantly faster than traditional bacterial sporulation of pure cultures.

To investigate the ACDCs healing potential, a series of laboratory experiments were carried out [41]. Several cement specimens with different ACDC dosages were prepared. The mortar was also enriched with calcium sources: calcium formate 2% and calcium nitrate 3% by cement mass respectively.

The results clearly recorded a significant healing potential. An addition of 0.5 and 1% of the ACDC to cement weight led to a crack-closure up to 500 µm for more than 90%, whereas non-biological specimens reached crack-closure only up to 200 µm as it can be seen in Figure 5.

To prove the long-term efficiency of the ACDC, cracks were also created after 6 months from casting. Some decrease in the crack sealing potential was recorded, the cracks only up to 400 µm were closed up to 90%. However, this shift could be a result of the decrease in the natural autogenous capacity of the material as in the non-biological specimen cracks were closed only up to 135 µm.

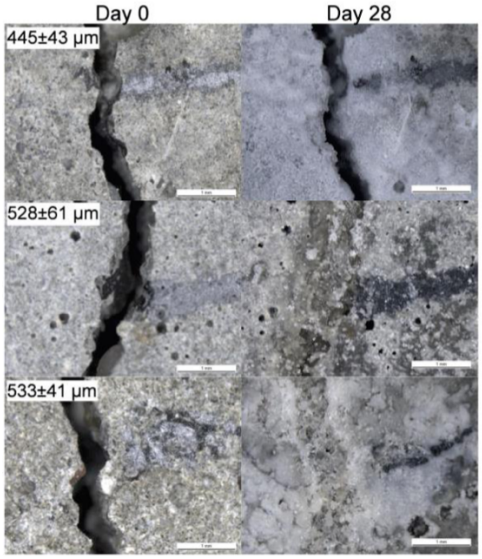

Figure 5. The initial (left) and final (after 28 days healing, right) appearance of cracks created in 28 days old specimens: reference specimen (top), specimen with 0.5 % ACDC and 5 % nutrients (middle), specimen with 1 % ACDC and 5 % nutrients (bottom) (scale bar is 1 mm) [41].

4.2 Microencapsulation

The process of encapsulation of bacterial spore powder (dried and ground paste of spores) in melamine-based microcapsules has been patented in 2014 [92]. The microcapsules contain an inert substance which protects the spores, and they are directly added into the matrix during the mixing process. The carrier is humid sensitive which means it is flexible in high humidity and becomes brittle in low humidity. It means that during the mixing process and hardening, the spores are protected against mixing water and mechanical forces and only when humidity later decreases, the microcapsules can be broken by the formed crack. Hence, the spores can be released, and the MICP takes place.

In a study carried out by Wang *et al.* [44], spores of *Bacillus sphaericus* were encapsulated into microcapsules (10^9 cells/g of microcapsules). To determine the self-healing potential and their impact on the mechanical characteristics, a series of cement specimens was prepared. The cement mortar further contained 0.85% of yeast extract, 8 % of calcium nitrate and 1,2,3,4, and 5 % of the microcapsules by cement mass respectively.

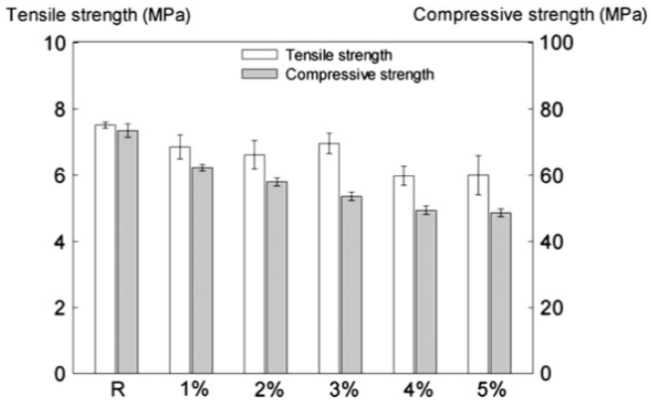

Figure 6. Mechanical properties of the specimens at 28 days (R - control without microcapsules; X % - dosage of microcapsules by cement mass) [44].

Not surprisingly, the microcapsules negatively influenced the compressive strength (Figure 6). The rate of decline varied depending on the quantity. With increasing addition of 1 % to 5 % microcapsules, the compressive strength decreased by 15 % to 34 %. However, the water absorption rate of the bacterial samples decreased significantly (up to 40 %) and the water permeability also reached lower values compared to the control samples.

To investigate the healing capacity, specimens were cracked 28 days after casting, and the crack-sealing was subsequently monitored. A cracked healed area of the non-bacterial specimen was around 18 % to 50 %, whereas in the case of bacterial specimen, it was from 48 % to 80 %. The maximum crack width healed in this experiment was 970 µm.

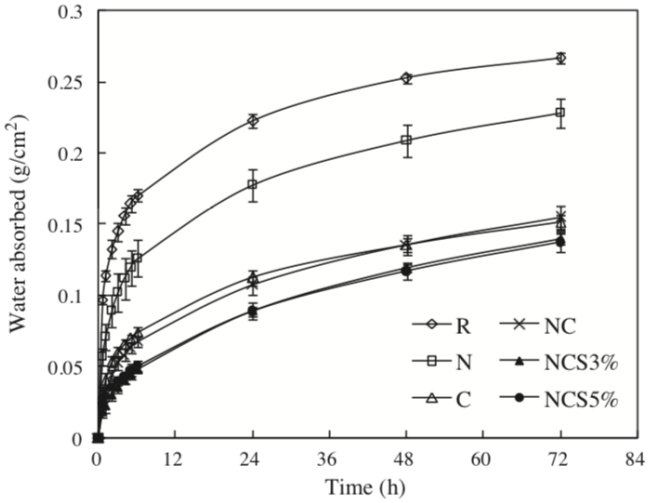

Figure 7. The water absorption of the specimens with or without microcapsules (R – reference, N – only nutrients, C – only blank capsules, NC – nutrients and blank capsules, NCS – nutrients and capsules with bacteria in 3 % and 5 % dosage by cement mass) [44].

4.3 Lightweight aggregates

Lightweight aggregates (expanded clay/perlite particles or expanded shale particles) have been used in many previous studies as protective carriers of bacterial spores (in some cases with nutrients as well). This method of protecting bacteria has also been patented by Jonkers in 2010 [93].

Usually, the lightweight aggregates (LWA) were dried and impregnated with the healing agent under pressure and added directly into the concrete matrix. Theoretically, the LWA should protect the spores from the mechanical forces in the matrix and release them only when the particles are broken by cracking.

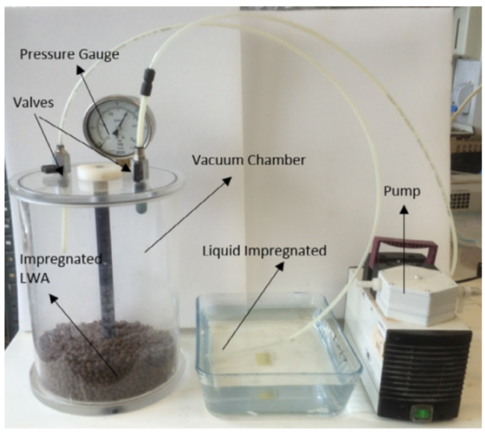

Figure 8. Vacuum impregnation set-up proposed by Algharmi *et al.* [94].

The process of efficient impregnation under pressure was described by Alghamri *et al.* [94] (see Figure 8); however, the subsequent treatments usually differ. Some researchers only dried the impregnated LWA before adding them into the concrete matrix [35,60,95], whereas others tried to enhance the protective ability of the carrier and underwent some additional procedures.

Chiu et al. did not dry the impregnated particles but added them soaked with the healing agent into the mixture to eliminate the leakage [57]. In other studies, an additional protective coating around the LWA particles was introduced, for example: a polyvinyl alcohol-based coating [94], a cement paste layer and/or sodium silicate layer [56]. Su *et al.* [96] crushed ceramsite particles prior to the impregnation with a healing agent and impregnated them using organic cover layer (curable epoxy resin and wrapped in sand).

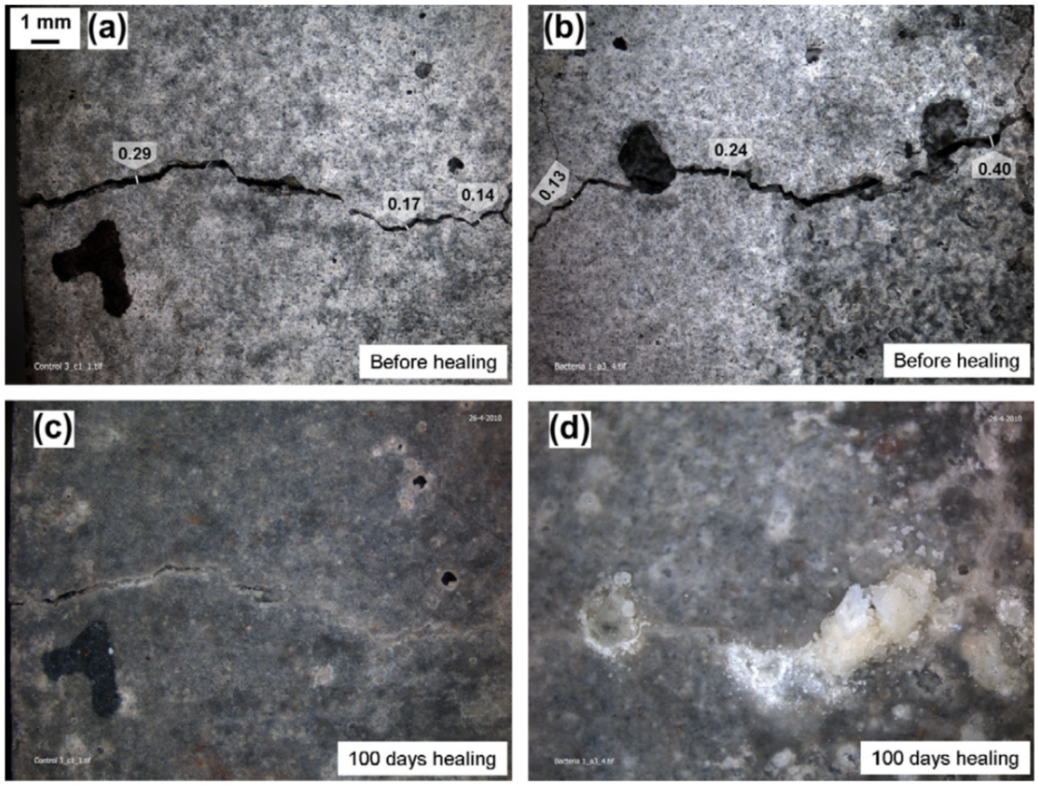

Figure 9. Stereomicroscopic images of crack-healing process in control mortar specimen with bacteria encapsulated in LWA before (a) and after 100 days healing (c), in bio-chemical agent-based specimen before (b) and after 100 days healing (d) [60].

Some experiments showed that encapsulation of bacterial spores in LWA significantly increases the long-term self-healing efficiency when compared with non-protected bacterial spores. However, other studies detected that the mean viable counts of bacteria encapsulated in the LWA dropped significantly when incorporated in the concrete matrix which could indicate insufficient protection [57].

Nevertheless, as it can be expected, the addition of LWA leads to a decrease in the bulk density, thus a decrease in the compressive strength. This drawback probably cannot be compensated by any additives, on the other hand, this bio-based self-healing concrete with the impregnated LWA could be applied as a cover layer or in parts of structures which are highly exposed to aggressive outside conditions.

4.4 Hydrogels and superabsorbent polymers (SAPs)

One of the factors which could potentially limit the efficiency of the MICP is the need for a sufficient water supply which triggers the bacterial spore's germination and growth. In the case of outside horizontal structures, the problem might not be so significant, as water is present naturally on their surface. However, in the case of vertical structures, insufficient humidity could limit the healing ability of the material.

For this reason, researchers proposed hydrogel as a protective carrier. Hydrogel, a network of polymer chains, is hydrophilic and highly absorbent material, thus it could not only protect the spores but also provide the needed humidity.

Wang *et al.* [47] in their experiment encapsulated *Bacillus sphaericus* spores in hydrogel and prepared gel-like sheets. Results showed that the selected bacteria were able to withstand the encapsulation process and preserve their viability. Furthermore, the crack-healing potential of specimens with the bio-hydrogel was significantly greater (crack up to 500 µm was closed) than in the case of the non-bio hydrogels specimens (crack only up to 300 µm was closed). However, the addition of hydrogel led to an unacceptable decrease (up to 50 %) in the compressive strength, probably owning to the voids left by the swollen hydrogel.

In the following experiment, new types of hydrogel were proposed and investigated to overcome this issue. The alginate-based hydrogel was proposed, and it reached significantly better compressive strength results (decrease of 16 – 23 %) [48] compared to previous experiments with standard hydrogel.

Wang *et al.* [97] further investigated pH-responsive hydrogel for the encapsulation of bacteria. The pH-responsive hydrogel should have very limited water absorption at extremely high pH (12-13) like in fresh concrete but have a high swelling capacity at lower pH (8-11). This ability hinders the negative impact on the compressive strength as the hydrogel would not become swollen in fresh concrete, but when the cracking occurs, the lowered pH would enable the hydrogel to swell, thus protecting the bacteria and creating a water reservoir.

From the investigated hydrogels, chitosan-based hydrogel proved to be a probably better candidate for self-healing concrete over alginate-based hydrogel (alg-H). Chitosan-based hydrogel (chi-H) showed clear biocompatibility with the selected bacteria, although alginate-based hydrogel showed better sealing efficiency. Furthermore, the addition of 1 % of chitosan-based hydrogel (by cement mass) led to a decrease of compressive strength by only 5 % (Figure 10).

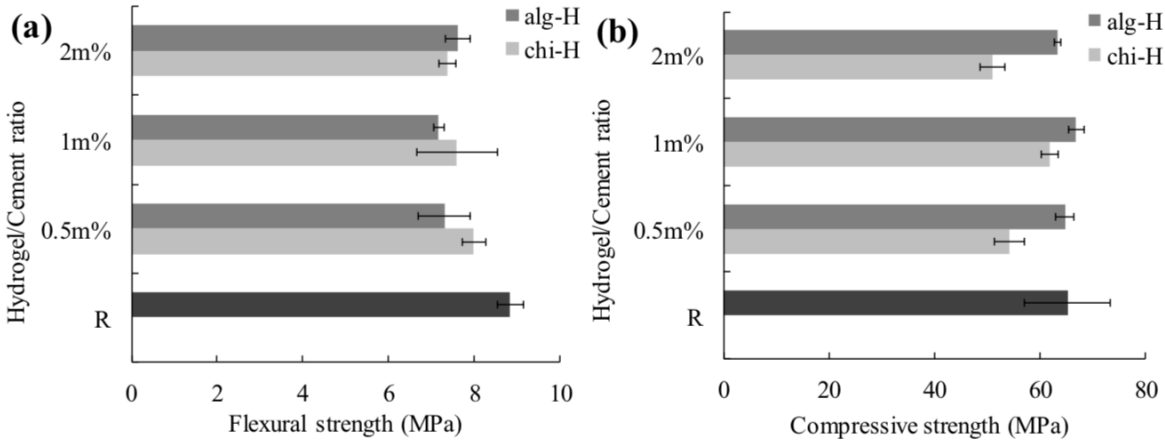

Figure 10. Flexural (a) and compressive strength (b) of the mortar with alg-H and chi-H incorporated [97].

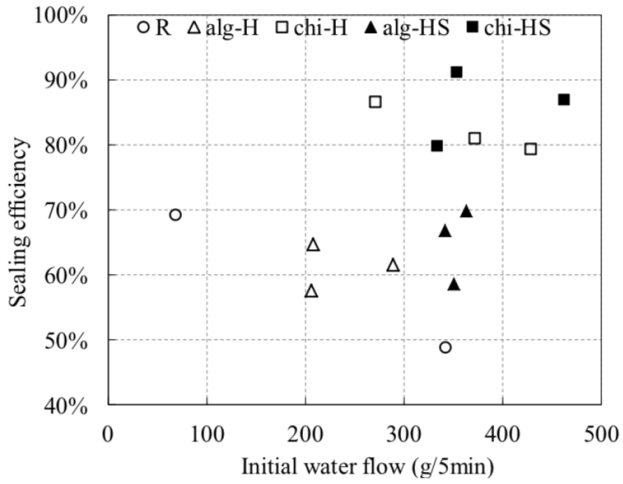

Figure 11. Sealing efficiency of the specimens with the additions of alg-H(S) and chi-H(S): R – control, H – without bacteria, HS – with bacteria [97].

Overall, the application of pH-sensitive hydrogels as protective carriers seems to be a promising concept and should be further investigated in the future research.

Superabsorbent polymers (SAPs) have also been proposed as protective carriers. Compared to hydrogels, SAPs have a significantly higher absorption capacity which reaches up to hundred times their own weight. SAPs have already been applied as a concrete additive for various purposes: mitigation of autogenous shrinkage [98], [99], improvement of frost resistance [100], rheological modification of shotcrete [101], or water-proofing [102]. Importantly, researchers have also been investigating its potential to enhance the natural self-healing mechanisms [103], [104]. In bio-based concrete, the swollen SAP particles (or voids after their drainage) could ensure enough space for bacteria to survive, and, at the same time, serve as a reservoir for the moisture needed for the bacteria metabolic activity.

This concept was used in a study by Kua *et al*. [105], in which the combination of SAP, biochar and bacteria led to closure of cracks up to 800 µm and higher recovery of mechanical properties compared to non-bacterial samples.

4.5 Silica gel and polyurethane

Silica gel and polyurethane was suggested as suitable carriers for biological compounds, especially owing to their biological inertness. This hypothesis was further verified in several

studies, where both materials employed to immobilize bacteria for external crack repair systems [51], [106].

In a study carried out by Wang et al. [45], ureolytic bacteria *Bacillus sphaericus* was immobilized in glass tubes filled with either silica gel or polyurethane. In the experiment, several groups of glass tubes (Figure 12) with bacterial or non-bacterial carriers and nutrients were added into cement paste in order to produce specimens. The specimens were later submitted to a three-point bending test to create realistic cracking. Subsequently, the strength-regain and water permeability of the cracked and cured specimens were monitored and analysed.

Results showed that bacteria can still revive its ureolytic activity after the immobilization in the selected carries. However, polyurethane foam seemed to result in a better self-healing potential as it reached a higher strength regain and lower water permeability.

The experiment proved the compatibility of the selected bacteria with silica gel and polyurethane foam; however, it is necessary to mention that no compressive tests were performed during the study. As the glass tubes were rather large (40 mm in length and 3 mm in diameter), it is possible to assume that their addition would affect the mechanical properties significantly and probably lead to a dramatic decrease in the material properties.

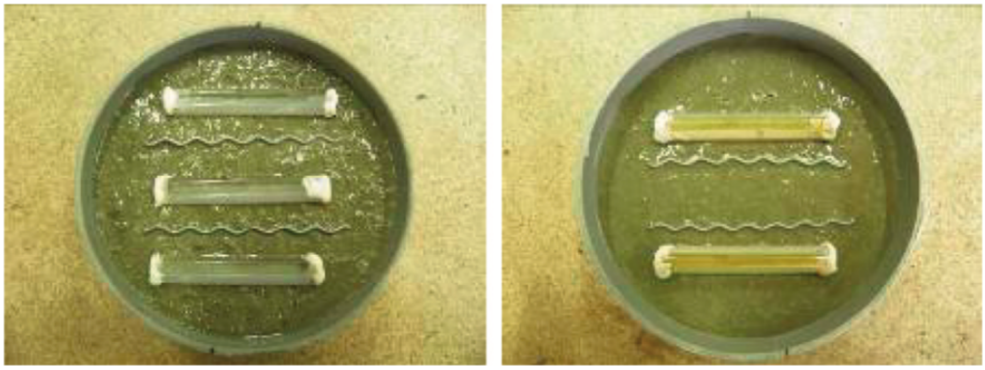

Figure 12. Tubes with silica sol (left) and polyurethane (right) [45].

4.6 Silica fume

Xu and Yao [89] in their study investigated the impact of different calcium sources and repair methods (direct addition or external application of the self-healing agent) on the crack sealing efficiency. All the prepared mortar samples contained basalt fibres (3.3 % by cement mass) to enable creation of larger cracks without losing their integrity and silica fume (10 % by cement mass) to alleviate high alkalinity and create isolated micropores in which the bacterial spores could survive.

After the introduction of cracks after 28 days from casting by four-point bending test, the specimens were submitted to different healing conditions for 30 days, and the crack-sealing capacity was recorded. Although the experiment did not involve any specimens without the silica fume as reference samples, crack-sealing was detected throughout the whole healing period. Thus, it could be assumed that the addition of silica fume truly increased the survivability of the applied bacterial spores.

4.7 Diatomaceous earth

Another potential protective carrier was introduced by Wang *et al.* [46]. The aim of their study was to use diatomaceous earth as a protective carrier for *Bacillus sphaericus*. Diatomaceous

earth (DE) is a naturally occurring, chalk-like, earthy, very-fine-grained siliceous sedimentary rock which consists of fossilized remains of diatoms – a type of hard-shelled algae [107]. The DE is commercially used in filters, as an abrasive, insecticides or in fire resistant safes. Conveniently, the DE is also proved to be compatible with concrete as a partial replacement for Portland cement in cement mortars [108]. Furthermore, the DE was already employed as a carrier for bacteria and non-pathogenic microbes [109]–[111].

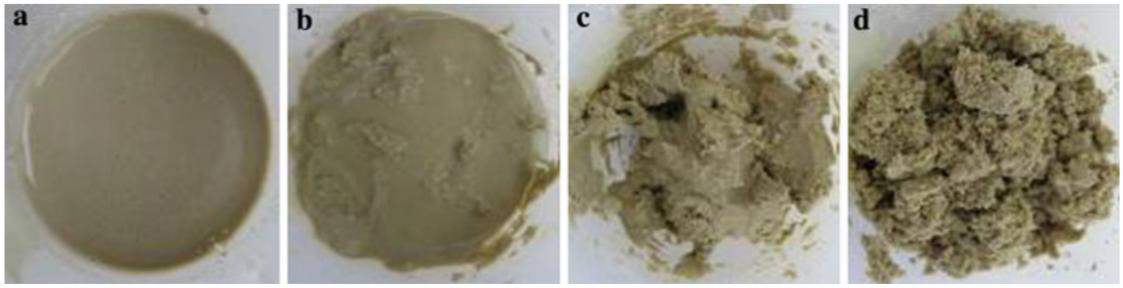

Figure 13. Mixtures of DE and bacteria suspension at different concentrations of DE (DE concentration in a, b, c and d were 40, 50, 60, and 70%, respectively) [46].

Wang *et al.* [46] in their experiment mixed the DE in different concentrations (40, 50, 60 and 70% respectively, Figure 13) with a bacterial suspension and added it into mortar specimens during mixing. The results showed that metabolic activity of the bacteria was not affected by the encapsulation into the DE and, furthermore, the bacteria immobilized in the DE in cement slurry showed much higher activity than free cells.

The mortar specimens were cracked after 14 days from casting and left to heal immersed in water or deposition media. Visual examinations of the cracks and capillary water absorption measurements were carried out after 40 days of healing. The results showed a considerable healing potential of encapsulated bacteria as the water absorption in the specimens with bacteria was about 50% (cracks were partly filled) or 70% (cracks were completely filled) lower than in the non-bacterial specimens.

4.8 Fibres

In the majority of conducted in-vitro studies, fibres were generally added into the cementitious mix in order to create a crack of the required width without completely breaking the specimen [89]. In real structures, the beneficial potential of the fibre addition to bio-based is quite clear. Higher rigidity would ensure smaller crack widths, making MICP more efficient in sealing them (with a decreasing crack width, the probability of its complete healing increases [112]).

Some studies have further addressed the possible protective functionality of fibres and their impact on MICP. Feng et al. [113] introduced cement composite containing bacterial agent and polypropylene (PP) and polyvinyl alcohol (PVA) fibres. Preliminary experiments with bacteria in culture media indicated, that the presence of both fibre types results in a decrease in bacteria cell concentration. Additionally, an investigation of MICP in liquid media the presence of fibres showed that the resulting calcium carbonate polymorphs (all calcite) had larger particle size in the case of PVA presence.

The crack healing monitoring revealed that the repair area was slightly lower for specimens with fibres and bacteria than that with bacteria only. However, water tightness and flexural strength regain were improved noticeably by the fibre addition (Figure 14). The calcium carbonate distribution in the crack, and calcium ions concentration in repair media indicated

that both PP and PVA fibres may capture calcium ions dissolved from crack, thus increasing the healing depth.

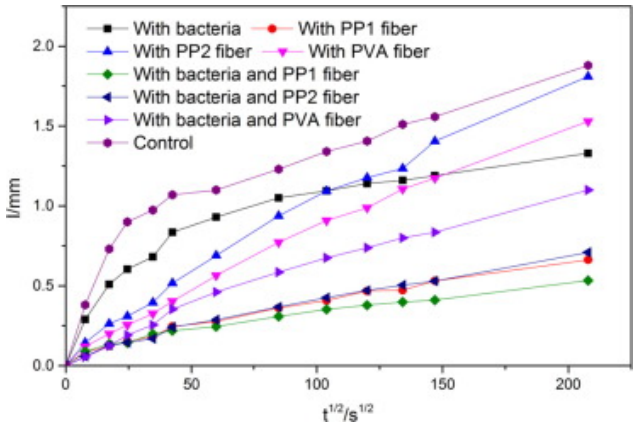

Figure 14. Water absorption/square root of time in cracked specimen with/without PP or PVA fibres [113].

Rauf et al. [55] proposed application of natural fibres (coir, flax, and jute) as protective carries. The fibres were soaked in a bacterial solution and added directly to the mix. In this study, the application of flex fibres resulted in better crack-healing (up to 800 µm), whereas mix containing coin fibres reached higher compressive strength. Similar results were reported by Singh [114] using micro cellulose fibres. The crack-healing ratio was improved by the fibre incorporation as they seemed to increase the availability of bacteria in the crack region. On the other hand, the compressive strength was decreased by the fibre-bacteria addition in this experiment as well.

4.9 CSA Cement Carries

Su *et al.* [115] addressed the issue of too high pH values of the concrete pore solution, which limits the bacteria metabolism by application of low alkaline suplhoaluminate (CSA) cement to produce bacterial pellets. The CSA proved to be a suitable carrier from the perspective of self-healing but unfortunately, the study did not include determination of the mechanical properties of the developed cement composite. This could be valuable information given that one of the most common negative side-effects of the protective carries is the reduction of strengths. In this case, the compatibility of the applied CSA cement carrier and Ordinary Portland cement in the concrete matrix could ensure that the mechanical properties are maintained.

4.10 Protection of bacterial spores - conclusions

- Protection of bacterial spores against the harsh environment and mechanical forces in the concrete matrix is a crucial requirement for achieving functional self-healing bio-based concrete.
- The chosen protective method must not have a negative effect on the bacteria viability.
- The impact of the chosen protective method on the mechanical properties of concrete must be investigated in detail to prevent their unacceptable variance.
- The long-term protective effect should be investigated as functionality seems to decrease in time.

- The dosage of the bacterial carrier, thus its availability in the crack zone, is a crucial factor which affects both functionality and economic feasibility of the bio-concrete.
- The resistance of the chosen protective method against non-optimal conditions should be investigated (lower and higher temperatures, high pH, mechanical forces etc.).
- Costs of the material and the production process need to be taken into consideration in order to develop a commercially applicable material.

Chapter 5: Materials and methods applied to determine the self-healing potential

In this chapter, some of the previous investigations of the self-healing bio-based concrete which attempted to determine the self-healing potential of the material will be introduced. In Table 6, a list of some of the in-vitro experiments investigating the efficiency of the crack sealing potential is presented. Individual procedures will be described in detail in the following chapters.

Table 6. A list of some of the experiments investigating the self-healing potential of the bio-based self-healing concrete.

Ref.	Specimens	Damage introduction	Curing	Investigations of the self-healing capacity
[116]	Prismatic with reinforcement	After 28 days from casting by 3-point bending test.	Wet-dry cycles for 28 days.	ESEM with EDS analysis, crack permeability via water flow, oxygen consumption
[112]	Cylindrical and prismatic	After 7, 14, 28, 60 and 90 days from casting. Specimens wrapped in adhesive tape, embedded nails to achieve different crack widths.	Immersion in water/ 90% RH/ wet-dry cycles.	SEM with EDS, XRD
[34]	Prismatic specimens	Broken into pieces after 7 days or 28 days from casting.	Immersion in water for 8 days.	ESEM
[89]	Prismatic specimens from fibre reinforced mortar	After 28 days from casting by four-point bending test.	External and internal applications of the self-healing agent, immersion in water/nutritional media.	UPV measurements, bending test, nanoindentation measurements
[95]	Prismatic with reinforcement	After 28 days from casting by three-point bending test.	Immersion in water/wet and dry cycles for 28 or 56 days.	Crack water permeability test, ESEM with EDS, FT-IR, oxygen consumption
[35]	Prismatic and cylindrical	After 28 days from casting by bending test. Specimens wrapped in adhesive tape, embedded nails to achieve different crack widths.	Immersion in water.	Water permeability test, flexural strength test, SEM with EDS, XRD
[44]	Prismatic with/without reinforcement, cylindrical	After 28 days from casting by tensile and splitting test.	RH > 95%/ immersion in water/ immersion in deposition medium/ wet-dry cycles with water/	Light microscopy, SEM, water permeability

			wet-dry cycles with medium.	
[45]	Prismatic and cylindrical with/without reinforcement	Crack width controlled three-pointed bending test, splitting test.	-	Bending test, water permeability test
[46]	Prismatic with reinforcement	After 14 days from casting, crack width controlled three-pointed bending test.	Immersion in water/deposition medium for 40 days.	Light microscopy, capillary water absorption test, SEM, SEI
[60]	Prismatic with reinforcement	After 56 days from casting by tensile test.	Immersion in water.	Stereomicroscopy and photographic imagining, ESEM with EDX, FT-IR, oxygen consumption
[74]	Prismatic with reinforcement	After 28 days from casting by tensile forces applied to the steel bar reinforcement.	Immersion in demineralized water/ immersion in urea solution for 4 weeks.	Microscopy
[117]	Mortar disks	After 28 days from casting, specimens wrapped with carbon fibre reinforcement polymer strips and submitted to splitting test.	In humidity close to 100%RH.	Microscopy, FT-IR, surface absorption test
[52]	Prismatic with reinforcement	After 28 days from casting by three-point bending test.	Wet-dry cycles for 14/28/120 days.	UPV measurements, electrochemical measurements on reinforcement.

* Explanatory notes of the measurement methods:

SEM (scanning electron microscopy), ESEM (environmental scanning electron microscopy), EDS (energy dispersive X-ray spectroscopy), XRD (X-ray diffraction), UPV (ultrasonic pulse velocity test), FI-IR (Fourier transform infrared spectroscopy), SEI (secondary electron imaging).

5.1 Specimen's shapes

As it can be seen in Table 6, most studies investigated the self-healing efficiency on small mortar prismatic or cylindrical specimens. In some of them, mortar specimens were reinforced with steel wires [71,95,118] or fibres [89], [119] in order to keep the integrity of the specimens while creating microcracks through bending or splitting tests. In others, the reinforcement was replaced by wrapping of the specimens in an adhesive tape [112].

When planning the experiment, the need for a relatively large number of specimens must be considered. Some specimens are required for the mechanical property investigations and others for the determinations of the self-healing efficiency by various methods which will be further introduced in this thesis.

As previously stated, studies were usually conducted on small mortar specimens (usually prismatic 40x40x160 mm^3 or 30x30x360 mm^3). Interestingly, when research was performed on a larger-scale specimen [27] (beam 150x250x3000 mm^3) cracked by four-point bending and submitted to wet-dry cycles, the results showed significantly lower crack-sealing efficiency than when a similar experiment was conducted on small mortar specimens. The difference in the crack-sealing potential could be caused either by an insufficient dose of the self-healing agent or, as researchers have suggested, by low water supply. Both findings indicate potential issues that could occur under outside conditions in real structures.

5.2 Specimen's materials

In the majority of conducted studies, ordinary cement stone specimens containing an encapsulated self-healing agent were used to determine the crack-healing potential. However, it can be assumed that the novelty bio-based self-healing concrete will most likely find its place especially in highly stressed key parts of structures. Nowadays, there is a tendency for such key structures to use special high-performance concretes with modified properties using a wide variety of additives and admixtures. Therefore, the compatibility between MICP and high-performance concrete compositions should be carefully investigated in detail.

Amiri *et al*. [120] addressed this issue by determining the effect of different chemical admixtures (a superplasticizer and an air entraining agent) on the crack-sealing capacity. Although it has been suggested that superplasticizers may affect the morphology of biogenic $CaCO_3$ [121], it was also believed that lower dosages of the additive could enhance the $CaCO_3$ precipitation as its formation rises with the increasing number of CO_3^- ions. Amiri *et al.* [120] confirmed this theory and demonstrated that not only the superplasticizer had no negative effect on the self-healing ability, moreover, its addition improved both water absorption and durability of the $CaCO_3$ precipitates.

The air entraining agent (AEA) was primarily suggested as a protective method as it could potentially increase the viability of spores by providing extra porosity. However, the experiment of Bundur *et al.* [122] demonstrated that an addition of AEA resulted in a decrease in the estimated number of viable cells in mortar samples when compared to mortar with unprotected bacterial cells. Researchers have attributed this negative effect to the AEA characteristics as they are composed of a hydrophilic head and a hydrophobic tail. Thus, the negative effect could be a result of the mechanism in which the bacteria cells are trapped in the voids, but the supply of nutrients is limited due to the hydrophobicity of the tails.

In contrast with this study, Amiri *et al.* [120] did not record any negative impacts of the AEA on the crack-healing efficiency. It could be contributed to different viscosity of nutrient media which were applied in the mentioned studies. However, the impact of the AEA to the viability of bacterial spores must be further investigated in the future research.

In order to provide better understanding of the interaction of MICP with real-life concrete compositions, Gonzalez et al. [58] evaluated the impact of Pozzolanic cement on the self-healing. Results of this study indicated that Portland cement better promotes the self-healing of cracks compared to Pozzolanic cement.

5.3 Introduction of cracks and curing period

In most *in-vitro* studies, the specimens described in the previous chapter were submitted to a bending or splitting test (the so-called natural cracking) in order to introduce microcracks. To create cracks within a certain width range, crack width was usually controlled by a linear variable differential transformer during the loading. To maintain integrity, the specimens were either reinforced (as described in previous chapter) or were additionally secured e.g., by plastic foil (Figure 15) or casted in a plastic tube [112], [123].

From studies investigating capillary absorption and self-healing in concrete, the term "artificial cracking" is known [124]. In artificial cracking, the cracks are represented by notches which can be characterized by certain width, depth, and length. Typically, a thin metal plate is inserted into fresh paste and taken out after some hardening of the concrete specimen, leaving an artificial crack behind. Conveniently, this approach leads to more or less uniform results, thus reproducibility and possible comparison across individual experiments are ensured. However,

this approach is not considered appropriate for the self-healing investigations by all as in some studies the autogenous crack-sealing was not observed in the case of the notch method, while in the case of natural cracks the crack-closure was unmissable [124].

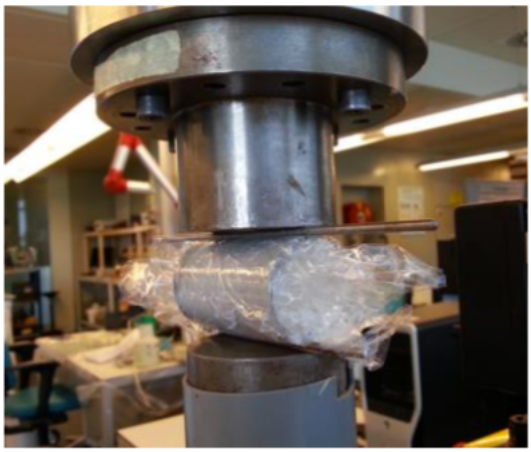

Figure 15. Splitting of the cylindrical specimens in plastic wrap under compressive loading [123].

The majority of studies introduced cracks after 28 days from casting; however, some researchers pointed out that the crack-sealing efficiency could differ for cracks in early and aged concrete. Also, usually there was an effort to create cracks with variable width as it was assumed to affect the crack-sealing potential greatly.

Luo *et al.* [112] in their research investigated factors which affect the crack repairing capacity, including cracking after 7, 14, 28, 60, and 90 days from casting with cracks with widths from 0.1 mm to 1.0 mm. Results indicated that the crack healing capacity decreases significantly with increasing cracking age. However, it is necessary to take into consideration that in this study, no method of protection of bacteria was applied. The massive decrease of the crack repair capacity might be caused by the decrease in the viability of the unprotected bacteria over time. The crack width turned out to be crucial factor in terms of achievable sealing. The area repair rate of cracks 0.4 mm wide was 70%, whereas in cracks 0.9 mm wide it was only around 10% [112].

Qian *et al.* [125] came to the same conclusion. In their study, small cracks with width bellow 0.3 mm were complete healed after 5 days. However, this rapid sealing on the surface could also have a secondary negative impact. As MICP is accelerated by CO_2, the early closure could prevent its ingression and cause insufficient sealing in deeper parts of the crack.

After the crack introduction, specimens were submitted to different curing periods. As previously stated, water is a crucial element which activates the bacterial metabolic activity, thus the MICP process. In laboratory conditions, the water/nutrient medium supply was usually ensured by either total immersion of the specimens in water/media, placement in higher humidity 90-95% RH, or submitting to wet-dry cycles (spraying of water in various time intervals). In a study by Van Mullem *et al.* [126], various curing conditions were compared. From the applied conditions, wet-dry cycling led to the highest crack-closing efficiency. This was attributed to the diffusion of CO_2 during the dry periods and so additional $CaCO_3$ could be formed. It can be considered a positive finding as the wet-dry cycles simulate the real outside conditions more precisely than the full immersion in water.

Other factors which might affect the crack sealing efficiency in real outside conditions were usually neglected. In most of the studies, the curing period was held in room conditions

(temperature, no direct sunlight, and constant water supply). The possible negative impact of low temperatures on MICP and the need for further investigations was already mentioned in chapter 3.2. It is important to acknowledge that not only the metabolic activity of bacteria, but also the properties of the new $CaCO_3$ precipitates should be investigated in further detail as its stability might be affected.

For example, Amiri *et al.* [120] in their study determined the impact of freeze-thaw cycles, rainwater and direct sunlight on the $CaCO_3$ precipitates. Results from this experiment indicated that biogenic $CaCO_3$ is durable against freeze-thaw cycles; however, the precipitates showed instability when exposed to rainwater and sunlight. Thus, in further research, more realistic environmental conditions should be simulated to prove the self-healing potential in real structures.

5.4 Determination of the healing potential

In the available literature, two main approaches to the determination of the healing potential of bio-based concrete in laboratory conditions are presented. The efficiency of the self-healing process is generally linked to the increase in durability or regain of mechanical properties.

As appropriate performance indicators of increased durability, several tests were performed in the previous studies: evaluation of the crack-closure and tests related to the transport processes (e.g., water-permeability, sorptivity, chloride penetration, air permeability, etc.) [127].

Although the regain of mechanical properties is not the primary aim of the bio-based self-healing concrete, their change over time is a good indicator of durable and functional crack-closure. For this reason, several studies involved estimation of strength or stiffness before and after the healing period.

In the review by Jakubovskis *et al.* [127], an overview of the applied test methods is provided. Further, a methodology suitable for laboratory/outdoor conditions is presented and crucial questions which should be answered are highlighted.

5.4.1 Durability evaluation

The most straight forward test method is a visual measurement of the crack-closure. In the majority of studies, high-resolution photography or light microscopy were commonly used to record the achieved crack closure. In order to quantify the healing efficiency, crack closure (or area closure) percentage was widely used [60]:

$$h = \frac{w_i - w_t}{w_i} \times 100\%, \qquad \textit{Eq. 12}$$

where w_i is the initial crack width (or area); w_t is the crack width (or area) after the healing.

Although visual investigation of the crack sealing is a good indicator of the healing potential, more information about the precipitate and its characteristic is generally needed (see Table 6).

Alongside with the light microscopy, investigations of the crack filling by scanning electron microscope (SEM) or environmental scanning electron microscope (ESEM) were applied in many studies. A typical ESEM image of biological precipitates on the crack surface can be seen in Figure 16. The electron microscopes were frequently equipped with an attached X-ray energy dispersive system (EDS) or, in other words, the energy diffraction analysis of X-rays (EDX) for a major element analysis. Further, the previous studies also used X-ray diffraction analysis (XRD) and/or Fourier-transform infrared spectroscopy (FT-IR), as high peaks of Ca, C, and O suggest the presence of $CaCO_3$.

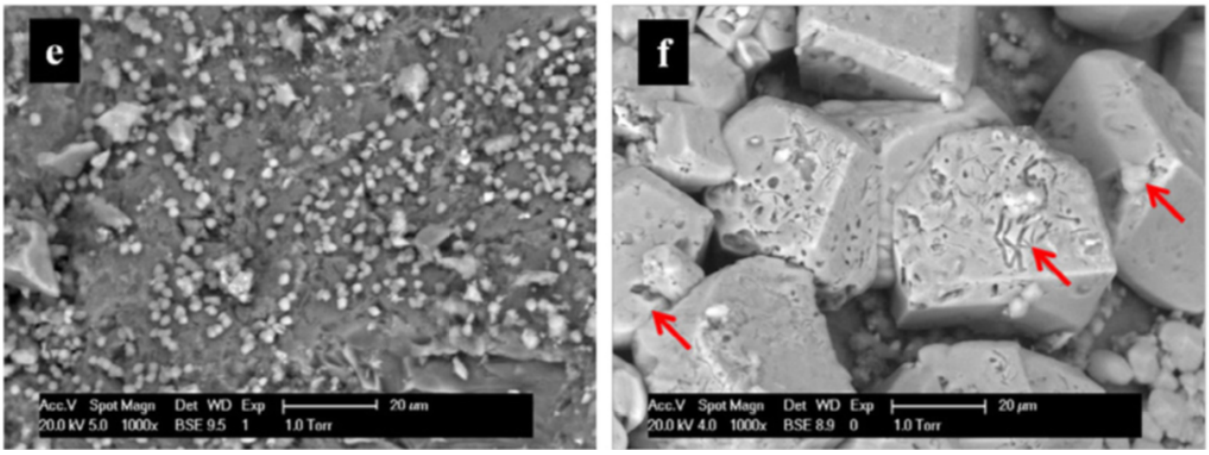

Figure 16. ESEM images of precipitates that were found on the surface of the crack after healing treatments; e: control specimen with non-bacterial LWA, f: specimen with LWA impregnated with bacteria after subjection to wet-dry cycles for 56 days [95].

In [89], visual observations were further complemented by nanoindentation in order to determine the nanoscale mechanical characteristics of the new precipitates and the transition zone which separates them from the original mortar. Although the method is rather laborious, the obtained information provides a valuable insight into the mechanism of the healing process, durability of the biologically produced material, and its interaction with the surrounding mortar (for results see chapter 3.4.3).

As described in chapter 1, cementitious materials are characterized by the ability of natural autogenous crack-sealing. Thus, the observed cracks can be closed due to the mentioned phenomenon and not as a result of MICP. To correctly distinguish the effect of bacteria from the autogenous concrete crack-sealing, control non-bacterial samples were used as reference samples in most studies.

The problem, however, is that the crack introduction by bending or splitting tests leads to rather non-homogeneous results and it is difficult to compare the samples to each other precisely. To overcome this issue, researchers often used the oxygen consumption/concentration measurement method [60,95] by which the oxygen decrease, therefore metabolic activity of axenic cultures, near to the surface of the investigated specimen can be detected. Typical results obtained from the oxygen consumption/concentration measurements can be seen in Figure 17. The decrease in the oxygen concentration on the surface of the bacterial sample B clearly indicates metabolic activity, whereas the stable concentrations in the case of REF and CTRL serve as non-bacterial reference curves.

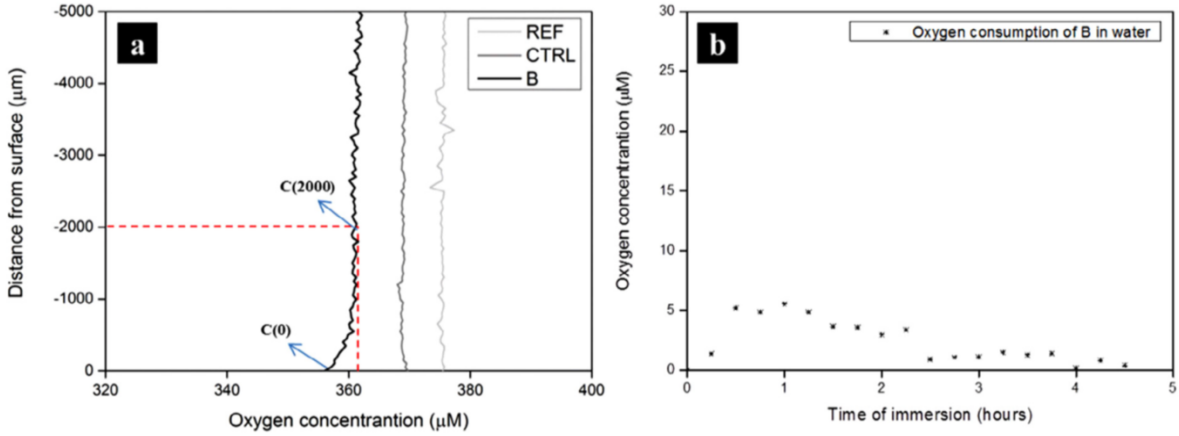

Figure 17. a. Typical profile obtained by specimens in tap water, b. Oxygen consumption in time from B specimen immersed in tap water [95]; CTRL and REF – non-bacterial specimens, B – bacterial specimen.

A large percentage of studies also used some kind of a water permeability measurement [95], [123], [128], [129] to estimate the healing potential of the proposed bio-based concrete composition. As durability is closely linked to the liquid ingression, these sorptivity-related characteristics are suitable indicators by which self-healing can be evaluated. An example of a water permeability test set up can be seen in Figure 18.

In general, the water ingress through the crack is expressed as the flow rate *q* [127]:

$$q = A \times w^3, \qquad \textit{Eq. 13}$$

where *A* is the fitting constant depending on the test set up; *w* is the crack width.

A problematic aspect of the water permeability assessment lays in the significant variability of the obtained results. Mullem *et al*. [130] highlighted this problem and suggested solutions such as a minimal number of specimens and a novel active crack width control method that should reduce the variation.

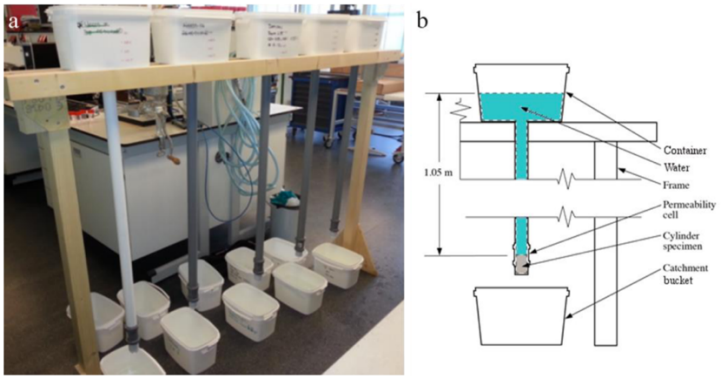

Figure 18. An illustration of a water permeability test, (a) the five-column set-up used during experiments; (b) the schematic representation of the set-up [123].

In Figure 18, an experimental set up for water permeability test is shown. In this study carried out by Ersan *et al.* [123], a mortar containing nitrite reducing bacteria was examined. Specimens prepared from the given mortar were cracked (with controlled crack width of 0.4 mm) and treated with tap water for 28 days. The water permeability test under 0.1 bar pressure was performed before and after the healing period. The results revealed that in non-bacterial

samples, the autogenous healing caused around 25% drop in water permeability, whereas in bacterial samples the decrease reached as high as 80 %.

Tziviloglou *et al.* [95] focused on the comparison of liquid tightness of cracked mortar samples containing bacterial self-healing agent encapsulated in LWA under different curing regimes (full water immersion and wet-dry cycles). In this study, water tightness recovery did not differ significantly for bacterial and non-bacterial samples when immersed in water (both around 70% recovery). However, when wet-dry cycles were applied during the healing period, the bacterial samples showed noticeably higher water tightness recovery (76% recovery at 28 days and 98% recovery at 56 days) than control samples in which no recovery was observed.

Although the regain of water tightness will provide information about the level of crack-sealing, as De Belie *et al*. [131] pointed out, more studies should address the actual threats to the durability such as chloride penetration [132]–[134], carbonation [52], and reinforcement corrosion [52], [132].

5.4.2 Recovery of mechanical properties

The primary goal of the bio-based self-healing concrete concept is to prolong durability of concrete structures; however, as the formed calcium carbonate bonds the crack surfaces, a recovery of the mechanical properties can be observed as well.

Ferrara *et al*. [17], in a study which dealt with crystalline admixtures introduced two indicators which can evaluate the mechanical properties regain: the index of load recovery (ILR) and index of damage recovery (IDR):

$$ILR = \frac{P_{max,reloading} - P_{unloading}}{P_{max,uncracked} - P_{unloading}}, \qquad \textit{Eq. 14}$$

$$IDR = \frac{K_{reloading} - K_{unloading}}{K_{loading,uncracked} - K_{unloading}}, \qquad \textit{Eq. 15}$$

where *P* is the external load; *K* is the stiffness.

When estimating the recovery of mechanical properties, the regain of residual strength (strength after cracking) is the primary concern. Most studies used compressive specimens due to the easy execution of the test. However, as stated by Jakubovskis *et al.* [127], the compressive samples are unfortunately unsuitable for the recovery estimations as the drastic decrease in their mechanical resistance is incomparably higher than the achievable recovery by MICP. Therefore, bending/splitting/tensile test should be preferred. To address the issue, Gribniak *et al.* [135] introduced a new testing procedure to estimate the residual flexural stiffness of reinforced concrete.

A study of Wan *et al.* [45] was one of the few that determined the residual strength using a three-point bending test. The repeated loading indicated that the addition of polyurethane-encapsulated bacteria results in a tensile strength up to 60 % (compared to 0 % in the case of reference samples). However, no information about the achievable strength values in the bacteria-polymer samples are provided. Thus, the question remains whether the bio-active agent did not result in an unacceptable decrease in the investigated mechanical properties.

Xu *et al*. [89] in their study showed that the recovery ratio of modulus was significantly higher in bacterial specimens (especially when the self-healing agent was applied externally) by using a ultrasonic pulse velocity (UPV) test. The modulus recovery in bacterial specimens reach up to 50 %, whereas only 25 % in the case of non-bacterial samples.

5.5 Materials and methods applied to determine the self-healing potential – conclusions

- Compatibility of the healing agent with mortars with various compositions should be further investigated (additives, pozzolanic materials etc.).
- Introduction of cracks should be performed at different ages to determine both short-time and long-term behaviour.
- Cracks of different widths should be investigated as it seems to affect the healing potential greatly.
- The healing period should simulate real outside conditions as much as possible. Different factors (e.g., temperature, humidity, or sunlight) appear to have a great impact on MICP.
- Investigations of the healing potential should involve measurements which simulate real durability threats (chloride penetration, carbonation, reinforcement corrosion, etc.), closer inspections of the biologically produced crack filling (element analysis, nanoindentation, crack sealed depth, etc.), and determination of the residual tensile strength using mechanical tests.
- It is important to correctly distinguish the crack closure as a result of the metabolic activity from the autogenous healing capacity of concrete.

Chapter 6: Outdoor experiments

Although the bio-based self-healing concrete has been investigated rather extensively in laboratory conditions over the past decades, not many outdoor experiments have been performed till this day. In this chapter, the carried out outdoor experiments are briefly introduced.

6.1 Irrigation canal in Ecuador

The first field application of the self-healing bio-based concrete was carried out in the highlands in Ecuador in 2014 in an irrigation canal which ensures water supply for local farmers [136]. New linings from natural-fibre reinforced concrete with alkaliphilic spore-forming bacteria encapsulated in LWA were built as a replacement of the existing cracked concrete linings to improve the efficiency of the water supply as most of the water was yield due to cracking.

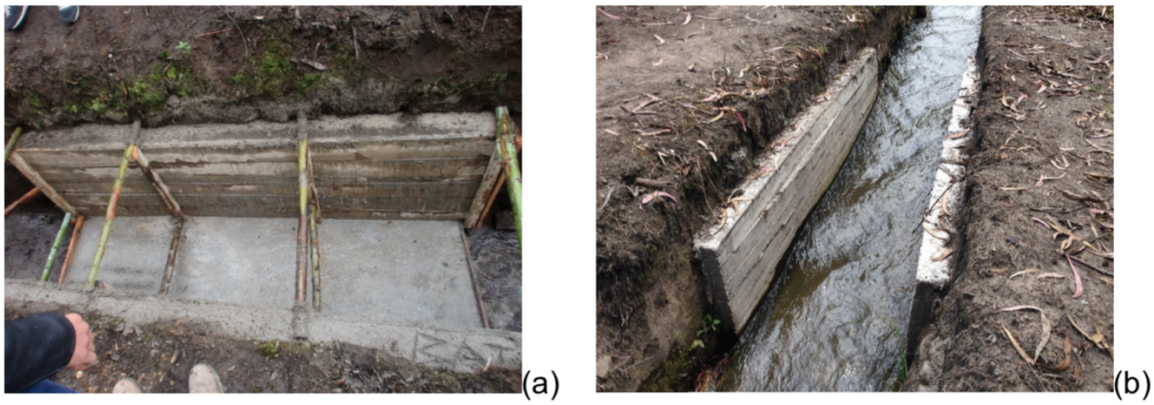

Figure 19. (a) Canal section 24 h after casting, (b) Five months after the casting [136].

The mixture was prepared on-site with local materials to prove the applicability of the novelty material on-field. After cleaning of the section of the canal (Figure 19), a wood formwork was placed, and bio-based concrete was casted. After 5 days from casting, the canal was reopened, and the structure was further inspected.

No cracking was observed up to 5 months from casting. Thus, it is impossible to draw any conclusions from the on-field experiment. Quite possibly, the original concrete linings were cracked due to poor construction, therefore the new well-designed experimental structure had no reason to crack.

6.2 Panels in South Wales

Paine et al. [56] in their study investigated the applicability of the bio-based concrete at a larger-scale. To study the self-healing capacity on-site, a wall panel was constructed using standard C40/50 concrete complemented with the self-healing concrete (an addition of coated expanded perlite with encapsulated bacteria or nutrients) which was situated in the part where the panel was designed to crack (Figure 20).

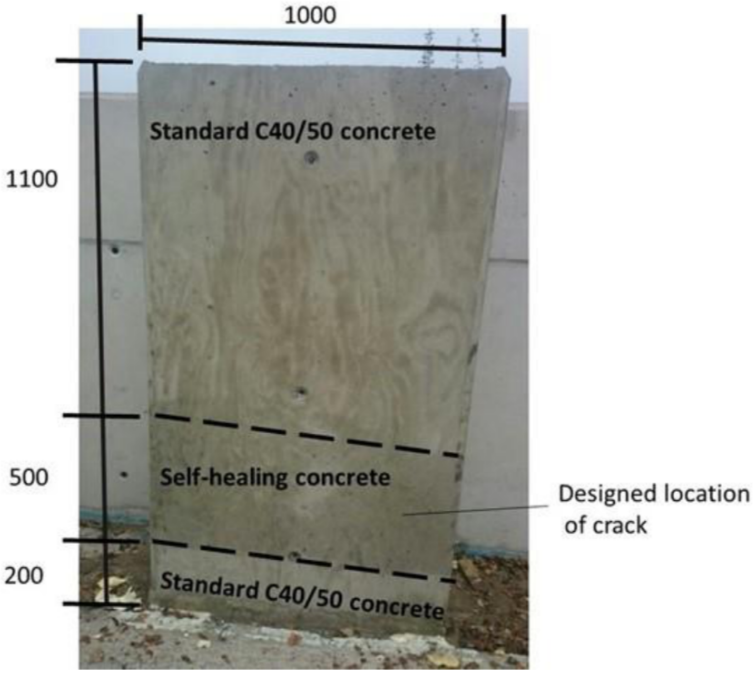

Figure 20. The panel containing self-healing concrete with impregnated expanded perlite [56].

The panels (bio-based and non-biological) were situated in real outside conditions at the A456 road as a replication of a conventional retaining wall structure. The panels were cracked at 36 days under specific loading conditions using a threaded bar and hydraulic jack system. Thereafter, the cracked panels were submitted to thorough monitoring.

Unfortunately, no complete crack healing was observed in this experiment. Although some crack closure was detected, it was not possible to definitely attribute it to bacterial activity rather than autogenous healing. Researchers have provided several possible explanations of the situation.

Firstly, as it was previously described, most of the proposed bacteria cultivation temperature is rather high (around 30°C). Although the in-vitro experiments were successfully carried out in room temperatures (around 20°C), the studies have never looked into temperatures as low as in this study (usually below 15°C,Figure 21). As humidity seemed to be sufficient for the calcite precipitation throughout the healing period (between 60% and 90%, Figure 21), the lack of self-healing capacity could be possibly contributed to the temperature factor.

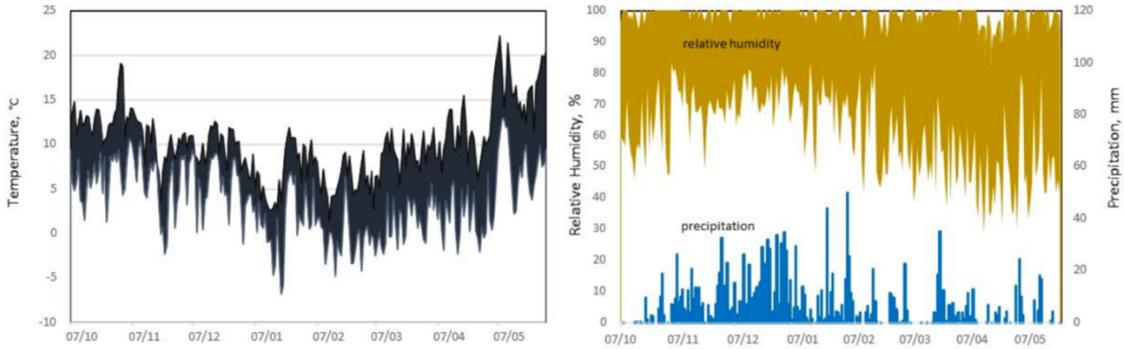

Figure 21. a) daily variations in temperature, (b) daily rainfall and variations in relative humidity from 7 October 2015 (casting date) to 31 May 2016 at the trial site [56].

Secondly, researchers have asserted that the ratio of spores to growth media was insufficient. Therefore, only a limited number of spores was available to generate healing. Furthermore, the uneven distribution of the spores encapsulated in expanded perlite could cause that the formed cracks did not penetrate the carrier, thus no metabolic activity was initiated.

6.3 A roof slab in Antwerp

Mullem *et al.* [126] in their paper described an outdoor experiment which involved casting of a reinforced concrete roof slab from bacterial self-healing concrete. The slab was located in an inspection chamber of a drainage pipe. This placement was chosen for the trail as it was expected that there would be the necessary liquid water, or at least high humidity, present most of the time. Furthermore, the bottom of the slab, where the cracks were expected to appear, would be accessible for inspections.

For the concrete production, a bacterial agent MUC$^+$ was employed. This agent consists of Mixed Ureolytic Culture (MUC) and anaerobic granular bacteria, and it was developed and produced by the company Avecom in the EU-FP7 project HEALCON. To promote the MUC$^+$ activity, urea and calcium nitrate tetrahydrate were added as nutrients and calcium source.

The bacterial concrete was transported to the construction site with a mixing truck and casted into standard formwork. Alongside with the outdoor application, laboratory specimens were prepared and investigated.

Unfortunately, no cracking was observed after 1 year from casting in the case of the roof slab. However, the laboratory specimens indicated several interesting findings. Consistently with the known results, even in this case, the wet-dry conditions led to the most efficient crack-closure. Furthermore, as also described elsewhere, the healing efficiency decreased with increased crack width (0.3 mm seemed to be the breaking point in this experiment). The lab experiment also revealed that the cracks at the bottom of the specimens showed a greater degree of healing than on the ones of the top or sides.

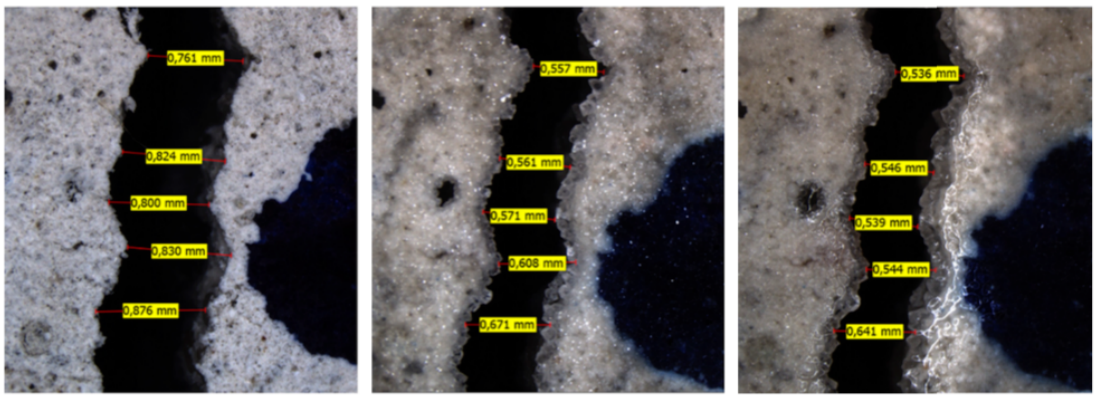

Figure 22. Crack on the bottom face of a specimen under wet/dry cycles (a) initial, (b) 3 weeks of healing, and (c) 6 weeks of healing [126].

6.4 Green Basilisk B.V.

In 2015, based on the research at TU Delft, a Green Basilisk B.V. company was founded. The company currently offers several solutions using MICP: concrete admixture containing biological

self-healing agent, self-healing biological mortar for existing concrete structures, and biological liquid repair solution for existing concrete structures.

The company offers tailored solutions and promises up to 60% savings on shrinkage reinforcement. However, no specific information about the materials, technologies or achievable results can be found on the company's website [137].

On the Basilisk's website, 10 examples of the application of Basilisk's products on real structures can be found and 6 of them is the application of the biological self-healing admixture: Het Loo Palace in Apeldoorn, water basin port in Rotterdam, waste-water treatment plant in Evides, proof-of-concept concrete slabs for Deutsche Bahn and retaining walls for a Dutch railway company. Unfortunately, the projects are described very briefly and there is no further information about the application and the results achieved.

PART B: Experimental work

Chapter 7: Experimental work background

This part of the thesis describes the author's own original contribution to the topic of self-healing biological concrete. The individual steps of the research and obtained conclusions are presented herein. It presents both published articles in which the author is the first author, but also articles that were created within the framework of a project founded by the Czech Science Foundation in which the author participated. The articles are partly reproduced in their original form, partly supplemented or shortened. For each paper, the author's contribution to the paper is indicated.

Now let us briefly present the objectives that were set based on the literature search:

- to identify a suitable bacterial species and describe its behaviour,
- to analyse the microbially induced calcite precipitation in greater detail,
- to select appropriate nutrients from the perspective of both bacteria metabolism and mechanical properties of concrete,
- to suggest and investigate possible protective carriers for bacteria,
- to prepare own experimental samples from biological self-healing concrete based on previous optimizations of individual components and determine its crack-sealing potential.

Chapter 8: Analysis of bacteria behaviour and BICP mechanism

8.1 Bacteria survival and growth curves

As discussed in Chapter 3.2, the bacteria used in self-healing concrete are highly tenacious and resistant. However, various factors may limit their growth and ability to metabolize (alkalinity, temperature, nutrient and oxygen deficiency, etc.). In order to properly determine the appropriate composition of the self-healing agent, it is first necessary to thoroughly investigate the behaviour of the selected bacterial species under different conditions. As the experiments are quite demanding, an attempt to simulate it using mathematical models was also made

8.1.1 Resistance to freeze-thaw cycles

This chapter is based on the following paper:

- **Applicability of Bio-Based Self-Healing Concrete in Central European Conditions: A Preliminary Study** [138]
  - type: conference paper
  - status: published in RILEM TC 253-MCI International Conference, 2018
  - authors: Schreiberova H, Bily P, Ryparova P
  - author's contribution: The author of this thesis is the first author. The author participated in planning and execution of the experiments, evaluated the experiments, and wrote the paper.

The first step in describing the behaviour of a bacterium suitable for self-healing concrete is to become familiar with its resistance to a range of temperatures. Firstly, it is necessary to

determine what different procedures can be applied to its handling (preparation of spores, encapsulation in protective carriers, etc.) and whether the bacterium is able to survive the freeze-thaw cycles that can occur in our climatic conditions. In [138], both temperature-related issues were investigated.

8.1.1.1 Materials and methods

In this preliminary study, bacteria *Bacillus pseudofirmus* was applied. At first, bacterial curves of both media (culture medium and mineral alkaline medium, see Annex 1.1) with *Bacillus pseudofirmus* was measured by photometry (see Annex 2.2) to determine the activity of the bacteria at optimum temperature (25 °C ± 2 °C).

To inspect the influence of conditions which may occur in the Central Europe region, we exhibited spores of *Bacillus pseudofirmus* to artificial freeze-thaw cycles (20 cycles from –10 °C to 10 °C). Two types of specimens were exposed to the cycles: BP-Spores and BP-O/N. The first group was prepared by centrifugation of inoculated alkaline mineral media which was preliminary exposed to 80 °C for 30 minutes to enhance sporulation of bacteria. The second group was prepared by centrifugation of over-night culture of *Bacillus pseudofirmus* in alkaline mineral media but without the heat treatment. It means that the BP-O/N samples contained spores, live bacteria and debris of bacteria. After the end of freeze-thaw cycles, the bacterial batch was inoculated into pure culture and alkaline mineral (see Annex 1.1) and bacterial growth was measured under optimum temperature (25 °C ± 2 °C) using photometer (see Annex 2.2).

The growth was evaluated based on the specific growth rate constants determined in the exponential phase according to:

$$\mu = \frac{\ln\left(OD_{630,2}\right) - \ln\left(OD_{630,1}\right)}{\left(t_2 - t_1\right)}, \qquad \textit{Eq. 16}$$

where OD_{630} is the optical density at 630 nm and $(t_2 - t_1)$ is the duration of the exponential phase of growth.

8.1.1.2 Results

A comparison (Table 7) of bacterial curves before and after the freeze-thaw cycles shows a slight decrease (Δμ) in the specific growth rates ($\Delta\mu_{BP\text{-}O/N}$ = -5,51 % and $\Delta\mu_{BP\text{-}Spores}$ = -0,32 %) of samples exposed to freeze-thaw cycles when cultured in culture medium. However, the specific growth rates of samples cultured in mineral alkaline medium before freeze-thaw cycles is substantially higher then after the cycles. The decrease after the freeze-thaw cycles reached $\Delta\mu_{BP\text{-}O/N}$ = -69,90 % and $\Delta\mu_{BP\text{-}Spores}$ = -84,19 % in mineral alkaline medium.

Table 7. Change in specific growth rate (Δμ) before and after the freeze-thaw cycles [138].

Sample	Type of medium	$\mu_{original}$ [h^{-1}]	$\mu_{after\ cycles}$ [h^{-1}]	Δμ [%]
BP-O/N	culture	0,0808	0,0763	-5,51
BP-Spores	culture	0,0808	0,0805	-0,32
BP-O/N	mineral alkaline	0,0681	0,0205	-69,90
BP-Spores	mineral alkaline	0,0681	0,0108	-84,19

8.1.1.3 Conclusions

Based on the results presented in [138] , it seems that *Bacillus pseudofirmus* could be a suitable candidate for the self-healing biological concrete in the conditions of the Central European

region, as it is able to restore metabolic activity after freeze-thaw cycles. However, regrowth occurred only in the presence of sufficient nutrients in the culture medium. When bacteria/spores were cultured in alkaline medium which is nutritionally poorer, only a fractional recovery of growth occurred. This could indicate a complication when using this bacteria type under real conditions, as the supply of nutrients for the bacteria can be expected to be limited in the self-healing concrete.

8.1.2 The effect of temperature on growth

This chapter is based on the following paper:

- **The effect of temperature on bacterial self-healing processes in building materials** [139]
  - type: conference paper
  - status: published in IOP Conference Series: Materials Science and Engineering, 2020
  - authors: Ryparova P, Tesarek P, Schreiberova H, Prosek Z
  - author's contribution: The author of this thesis is a co-author. The author participated in execution of the experiments, writing, and editing of the paper.

As noted in Chapters 3.2.4 and 6.2, insufficiently high temperature can be one of the critical factors that will limit the crack-sealing action. In [139], microbial properties of three different bacteria strains were investigated at quasi-optimal (30 °C) and sub-optimal (10 °C) temperature to determine the growth potential.

8.1.2.1 Materials and methods

The selected bacteria (*Sporosarcina pasteurii, Bacillus pseudofirmus, Bacillus cohnii*) were obtained from the Czech collection of microorganisms (CCM) and the Belgian Coordinated Collections of Microorganisms (BCCM). The access number of strains were: *Sporosarcina pasteurii* CCM 2056, *Bacillus cohnii* CCM 4369, *Bacillus pseudofirmus* (LMG 17944). For the respective culture media see Annex 1.1.

The bacterial inoculation for experiments was prepared by three-time repeated cultivation for starting the maximal growth rate in optimal condition for seven days. Further, this inoculum was applied in ratio 1 ml of the inoculum to 50 ml of the fresh media for the ELISA experiments (see Annex 2.2) and measured at 10 °C and 30 °C.

8.1.2.2 Results

The influence of different temperatures (10 °C and 30 °C) on the bacterial growth was determined as a change of optical density determined by ELISA measurements. The bacteria *Bacillus pseudofirmus* (Figure 23) had a longer lag phase at the lower temperature but the optical density was similar at hour 145 of the experiment. The ending of the log phase of the *Bacillus pseudofirmus* cultivation at 10 °C was between 45 – 76.5 hours. The log phase of the *Bacillus pseudofirmus* cultivation at 30 °C was shorthened to 10.5 hours.

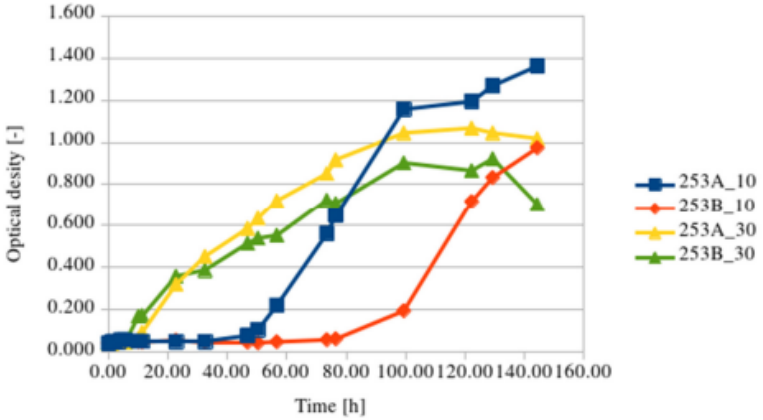

Figure 23. The growth curve for *Bacillus pseudofirmus* (253A/B), the number after label is the used temperature for the experiment, 10 °C or 30 °C [139].

In the case of *Sporosarcina pasteuri* (Figure 24), the log phase was shifted similarly to *Bacillus pseudofirmus*. The bacteria growth has started in range 32.5 to 50.6 hours in 10°C and in 22.5 hours in the 30 °C experiment. The limited supply of nutrients was detected in 140 hours.

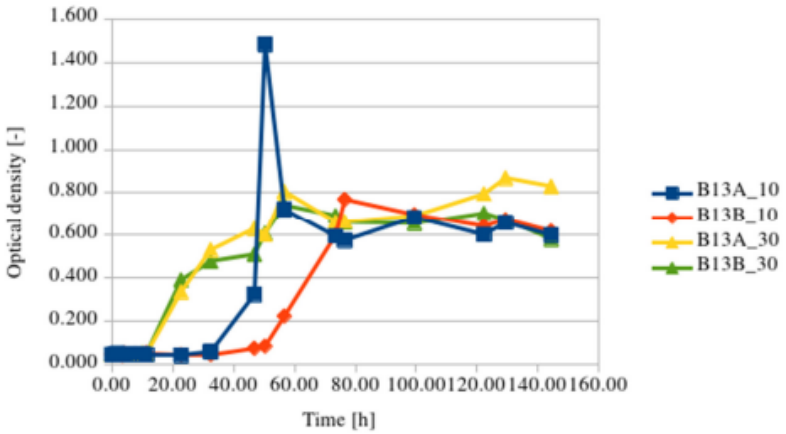

Figure 24. The growth curve for *Sporosarcina pasteuri* (B13A/B), the number after label is the used temperature for the experiment, 10 °C or 30 °C [139].

The multiplication of *Bacillus cohnii* can be seen in Figure 25. This cultivation has different results from the other investigated bacteria. The log phase was the longest of all used bacterial strains. The cultivation at 30 °C showed very different results across the prepared samples. The variance of the onset of growth was between 32.5 and 122.5 hours. It is clear from this result that *Bacillus cohnii* tend to be highly unstable and its behaviour can change under the same conditions. Unlike the other strains, *Bacillus cohnii* has not started to grow at 10 °C in the time of the experiment.

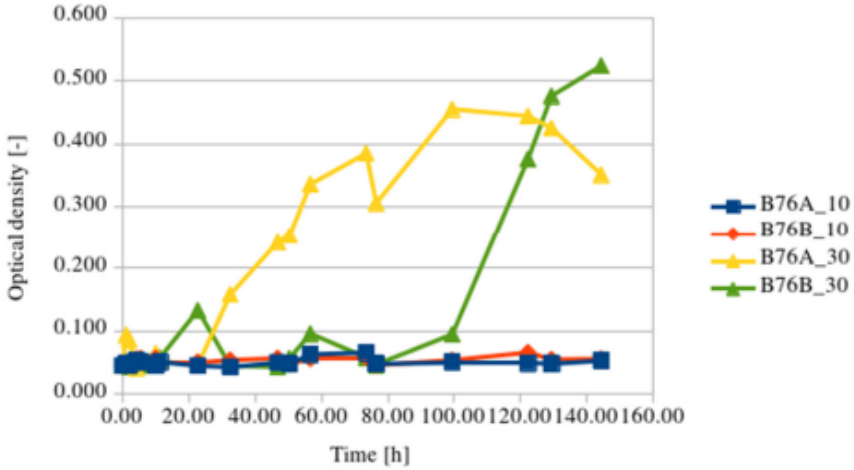

Figure 25. The growth curve for *Bacillus cohnii* (B76A/B), the number after label is the used temperature for the experiment, 10 °C or 30 °C [139].

8.1.2.3 Conclusions

In this study, three strains of bacteria were studied and the possibility of their application at lower temperatures was investigated. Generally, the time required for the metabolic activity recovery depends on the water content, nutrient source and temperature. In the case of *Bacillus pseudofirmus* and *Sporosarcina pasteurii*, the onset of growth was found to be delayed at the lower temperature compared to the optimum temperature, but the maximum values obtained were similar. On the other hand, *Bacillus cohnii* not only showed unstable behaviour even at optimal temperatures but was unable to recover even part of its metabolic activity at the lower temperature.

In this study, we were able to identify two species of bacteria that can grow at low temperature, but it must be kept in mind that the other conditions in the experiment (water, oxygen and nutrient levels) were optimal. The behaviour of the bacteria in real conditions, where these components may be deficient, could vary considerably and further research is needed.

8.1.3 Mathematical modelling of growth curves of *Bacillus pseudofirmus*

This chapter is based on the following paper:

- **Self-healing concrete: Application of Monod's approach for modelling *Bacillus pseudofirmus* growth curves** [140]
  - type: journal paper
  - status: published in European Journal of Environmental and Civil Engineering, 2022
  - authors: Nezerka V, Demo P, Schreiberova H, Ryparova P, Bily P
  - author's contribution: The author of this thesis is a co-author. The author participated in execution of the experiments, literature research and editing of the paper.

Experimental determination of bacterial growth curves is a cornerstone of self-healing concrete research. However, in order to standardize this new technology, proper mathematical models are needed to quantify the growing process. Based on the result from the conducted experiments (more in the Chapters 8.1.2, 8.2.3), we focused on the strain *Bacillus pseudofirmus* in the development of the mathematical models.

The Monod's model presented in the paper correctly predicts the onset of individual growth phases, quantifies the effects of temperature and concentration of nutrients on the concentration of bacteria, and reveals relationships between the concentration of nutrients and the maximum number of bacteria.

This thesis presents the main methods and conclusions from the paper, more details are available in its published version.

8.1.3.1 Background

The complexity of self-healing bio-based concrete lays, inter alia, in its interdisciplinarity. Despite a large body of research demonstrating the applicability of BICP for healing of cracks in concrete, proper mathematical models regarding growth of respective bacteria are missing. In order to address this issue, we must turn to the field of predictive microbiology.

Predictive microbiology estimates the response of microorganisms to specific circumstances through mathematical models [141]. Different equations and models describing the microorganism growth have been proposed, analysed, and compared; however, there is no clear consensus on which one is the most suitable. As stated by Pla et al. [142], Gompertz [143], [144], or Baranyi [144], logistic function, Monod's, and three-phase linear models are the most frequently used. Exponential models are valid only at the initial stage of bacterial growth, whereas logistic equations and Monod's model is more realistic as it assumes limited nutrient sources.

8.1.3.2 Monod's model

Monod's model has been proposed for description of microbial growth by Mankad and Bungay [145] and justified by Lobry et al. [146] few years later. One of the advantages of Monod's model is that its parameters have a clear biological meaning. The Monod's model relates bacterial growth rate to the size of population via the Monod's function $f(S(t))$:

$$\frac{dN(t)}{dt} = N(t)\frac{r_M S(t)}{\alpha + S(t)} = N(t)f\big(S(t)\big), \qquad \textit{Eq. 17}$$

Where $S(t)$ represents the concentration of nutrients in the system, r_M is the maximum growth rate, and α is the half-saturation, so that when $S \rightarrow \alpha, f(S) = r_M/2$.

By assuming that the nutrient consumption is proportional to the negative of the bacterial growth:

$$\gamma\frac{dS(t)}{dt} = -\frac{dN(t)}{dt} = -N(t)\frac{r_M S(t)}{\alpha + S(t)}, \qquad \textit{Eq. 18}$$

Integration of the equation leads to:

$$S(t) = S_0 + \frac{N_0 - N(t)}{\gamma}, \qquad \textit{Eq. 19}$$

And the equation for the growth rate becomes:

$$\frac{dN(t)}{dt} = r_M N(t)\left(\frac{\gamma S_0 + N_0 - N(t)}{\gamma\alpha + \gamma S_0 + N_0 - N(t)}\right), \qquad \textit{Eq. 20}$$

where N_0 is the initial concentration of the inoculate [g/l], S_0 is the initial concentration of nutrients [g/l] and γ can be interpreted as a growth efficiency of the nutrients ($\gamma = 1$ if dN of bacteria would consume dS of nutrients).

Therefore, when the concentration of nutrients decreases to $S = \alpha$, the growth rate is equal to one half of the maximum value; α is related to the interaction between bacteria and their substrate, affecting the transport of oxygen in the system. It is influenced by both the medium (composition, density, viscosity, temperature, and mixing rate if provided) and the bacteria (diffusion rate through its membrane, oxygen gradient, length of transport path, and enzymatic processes) [147], [148].

8.1.3.3 Experimental determination of growth

The growth of bacterial strain *Bacillus pseudofirmus* (cultured and sporulated in media according to Annex 1.1, 2.1) was measured using ELIZA data collecting device (see Annex 2.2). The initial concentration of inoculate was $N_0 = 0.033$ g/l. The growth rate was measured at three different temperatures (10 °C, 20 °C, and 30 °C) with concentration of nutrients $S_0 = 8$ g/l. Additionally, the growth was measured at 30 °C with three sub-optimal concentrations of nutrients $S_0 = 1.6, 0.8,$ and 0.4 g/l to simulate more real conditions when incorporated in concrete.

8.1.3.4 Sensitivity to changes in free parameters

The sensitivity of $N(t)$ to the change of α, γ, and r_M was identified by solving the Eq. 15 using SciPy library implemented in Python programming language. The known parameters were set according to growth $N(t)$ of *Bacillus pseudofirmus* measured at 30 °C with $S_0 = 8$ g/l and $N_0 = 0.033$.

The assessment of parameter sensitivity demonstrated high impact of γ (i.e., "efficiency" of nutrients) on the maximum value of $N(t)$. Conversely, the parameters α and r_M influenced the length (rate) of the exponential phase but did not change the maximum growth value $N(t)$. Individual results are available in the published version. Based on the sensitivity assessment, the best fit to *Bacillus pseudofirmus* growth at 30 °C with $S_0 = 8$ g/l was obtained with the following parameters: $\alpha = 60$, $\gamma = 0.2$, and $r_M = 1$ h^{-2}.

8.1.3.5 Modelling of growth at different concentrations of nutrients

The calibrated Monod's model with the parameters set to aforementioned values was applied on growth curves with different values of S_0 to validate the model (Figure 26).

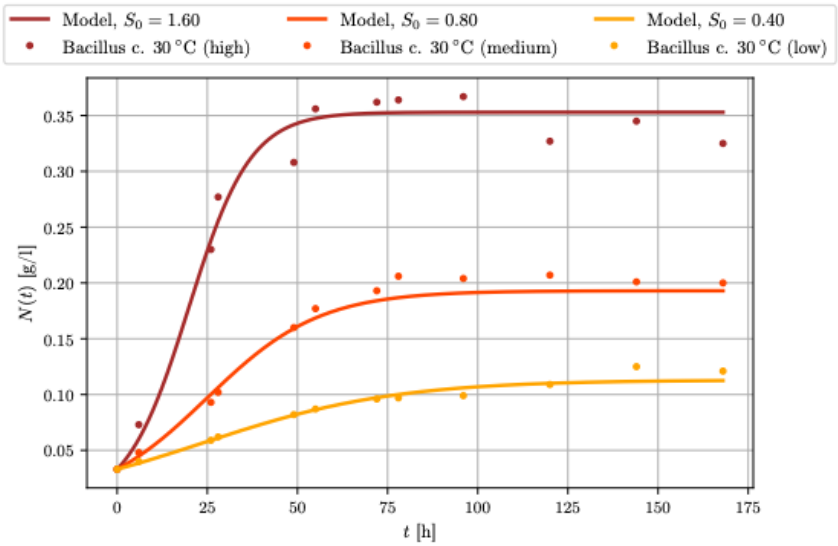

Figure 26. Modelling of Bacillus pseudofirmus growth at 30 °C for different initial concentrations of nutrients S_0.

8.1.3.6 Modelling of growth at different temperatures

The same calibrated model was used for the reproduction of *Bacillus pseudofirmus* growth at different temperatures and with $S_0 = 8$ g/l (Figure 27). The model predictions were controlled only by adjusting the γ parameter to obtain the best fits.

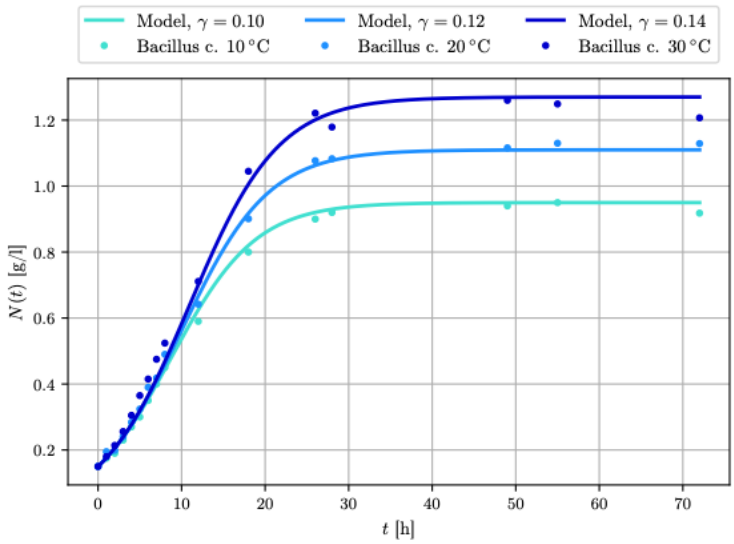

Figure 27. Modelling of *Bacillus pseudofirmus* growth at different temperatures with initial concentration of nutrients S_0 = 8 g/l.

8.1.3.7 Relationship between nutrient concentration and growth

Based on the Monod's model, the relationship between the initial concentration of nutrients S_0 and growth N(t) could be determined. It can be seen that these relationships are linear, increasing temperature results in higher maximum growth, and maximum growth increases linearly with the initial nutrient concentration.

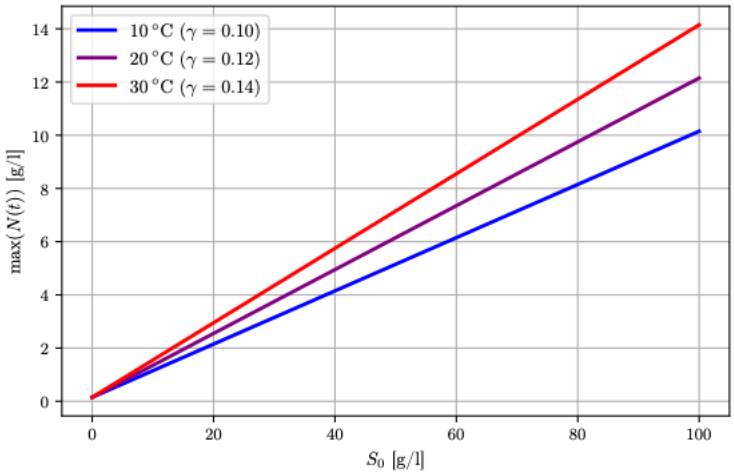

Figure 28. Relationships between max(N(t)) and S_0 for *Bacillus pseudofirmus* at different temperatures according to the Monod's model.

8.1.3.8 Conclusions

It was found that Monod´s model, with all its parameters having a biological meaning, allowed reproducing the behaviour of *Bacillus pseudofirmus* for a wide range of realistic parameters

affecting its growth. The model correctly predicted the onset of individual growth phases, quantified the effects of temperature and concentration of nutrients on the concentration of bacteria, and revealed relationships between the concentration of nutrients and the maximum number of bacteria. It must be noted, however, that cement solution is a hostile environment and many factors, such as high pH or the presence of Na/K/Ca vs. silicate ionic species would certainly affect the growth curves and biomineralization kinetics. Therefore, solid research must be carried out to address this issue in future.

8.2 Evaluation of the calcite production

This chapter is based on the following paper:

- **The role of bacterially induced calcite precipitation in self-healing of cement paste** [149]
  - type: journal paper
  - status: published in Journal of Building Engineering, 2021
  - authors: Ryparova P, Prosek Z, Schreiberova H, Bily P, Tesarek P
  - author's contribution: The author of this thesis is a co-author. The author participated in execution of the experiments, data collecting and writing the paper.

In existing studies, the effectiveness of the BICP (bacterially induced calcite precipitation) process in self-healing concrete has been primarily determined by indirect indicators such as changes in mechanical properties of the materials, water permeability, or visual assessment of crack closure. However, a closer understanding of BICP and determination of the calcite production potential of individual bacterial species has been neglected. In [149], we focused on the characterization of BICP using wide range of advanced research methods such as X-ray fluorescence (XRF), thermogravimetric analysis (TGA), thermal conductivity detection (TCDF), or Fourier transform infrared spectroscopy (FTIR). [149] thus provides a comprehensive view of BICP and compares different bacterial species based on direct measurements of calcite production.

8.2.1 Materials and methods

8.2.1.1 Bacteria strains and culture/precipitation media

The study is focused on three bacteria strains: *Sporosarcina pasteurii* (SP)*, Bacillus pseudofirmus* (BP)*,* and *Bacillus cohnii* (BC), obtained from the Czech collection of microorganisms (CCM) and the Belgian Coordinated Collections of Microorganisms (BCCM). For the respective culture media see Annex 1.1. To prepare precipitation media (for the calcite production investigation), 8 % wt/V of calcium lactate was added to culture media. The exact procedure of bacterial cultivation and preparation of bacterial pellets is available in [149].

8.2.1.2 Experimental methods

As stated in the introduction, several advanced experimental techniques were used for determination of the potential of calcite production by each bacteria strain.

Fourier transform infrared spectroscopy (FTIR) analysis was conducted by attenuated total reflection (ATR) sampling technique in order to determine the contents of functional groups indicating the characteristic compounds of the pellets. Bacterial pellets dried at 105 °C were placed on diamond ATR crystal and the spectrum was measured. The number of accumulated scans was 128 with resolution 4 cm^{-1}. The spectral curve was edited by software Omnic 9

(Nicolet, Thermo Fisher Ltd., USA). FTIR was used for qualitative determination and confirmation that the calcium ions are in the form of calcite. However, the determination of the amount of calcite is more complicated and requires implementation of another analysis methods as well.

X-ray fluorescence (XRF) on device XRF – Genius IF (Technopark Kralupy, Czech Republic) was used to determine the amount of all the elements (apart from light elements such as H, O or C) present in the prepared bacterial pellets.

Thermogravimetric analysis (TGA) was used to determine the amount of calcite and thus the amount of calcium and inorganic carbon in bacterial pellets. TGA is a method of thermal analysis in which the mass of a sample is measured over time as the temperature changes. This measurement provides information about various physical and chemical phenomena. The measurements were performed on Labsys EVO TG-DTA/DSC device with a differential thermal analysis (DTA) sensor in 80 µl corundum crucibles. The temperature range was set from 30 °C to 1000 °C, the temperature gradient was 10 °C/min. Synthetic air 5.0 (containing only N_2 and O_2, purity ≥ 99,999%) was used, the gas flow was 60 ml/min.

The amount of organic and inorganic carbon, sulphur, and nitrogen was determined through thermal conductivity detection (TCD) using Elementar Vario EL Cube device. In this device, the bacterial pellets were burned at high temperatures (up to 1000 °C) in pure oxygen. Gaseous combustion products (N_2, CO_2, H_2O and SO_2) were purified, separated and analysed in TCD detector. The 4-aminobenzenesulphonic acid was used as a standard in weight of 5 mg. The accuracy of the method is 0.1% for a particular element. To differentiate between organic and inorganic carbon, part of the samples was pre-treated by acidification by 2 M hydrochloric acid at room temperature. This way, all the inorganic carbon is transferred to carbon dioxide and released from the sample. As a result, the sample contains only organic carbon. The rest of the acid must be eliminated by evaporation before proceeding with the analysis. The analysis then yields the amount of organic carbon. The amount of inorganic carbon is calculated by subtracting the amount of organic carbon from the total amount of carbon determined on non-acidified samples.

8.2.2 Results

8.2.2.1 Elemental composition (XRF)

Table 8 provides an overview of the mass distribution of the individual detectable elements in the investigated bacterial pellets. The dominant element in all the of the cases was calcium (Ca), with the highest concentration of 30.56 % wt. in SP. However, as [149] points out, the lower content of potassium (K) in SP could indicate its lower viability, as the content of potassium is related to the maintenance of osmotic pressure.

Table 8. Content of elements in bacterial pellets [% wt.] [149].

Element	SP	BC	BP
Ca	30.56	26.51	25.21
Al	0.12	0.09	0.10
Si	0.11	0.08	0.10
P	0.91	1.40	1.40
S	0.14	0.17	0.21

Element	SP	BC	BP
K	0.16	0.24	0.29
Fe	0.05	0.08	0.06

8.2.2.2 Thermogravimetric analysis (TGA)

The paper [149] presents the results of TGA and describes the procedure that led to the conclusions determining the weight distribution of calcium and inorganic carbon in the bacterial pellets. In this thesis, we will note only the final mass fraction of calcium determined by TGA. In the presented analysis, the SP was characterized by the highest percentage of both calcium and inorganic carbon compared to other strains.

Table 9. Contents of calcium (Ca) and inorganic carbon (Ci) in pellets [% wt.] [149] .

Strain	Ca	C_i
SP	33	9.9
BC	31	9.3
BP	27	8.1

8.2.2.3 Elemental organic analysis using TCD

The results of elemental organic analysis are shown in Table 10. The highest organic carbon content (14.10%) was found in the BP sample. This indicates that BP has the highest potential for the cell division and highest capacity for BICP. The pellets of the other two strains contained significantly less organic carbon (30% less in the case of BC and 50% less in the case of SP).

Table 10. Concentration of elements in pellets determined by elemental organic analysis [% wt.]. Ct = total carbon content, Co = organic carbon content, Ci = inorganic carbon content [149].

Strain	Ct	Co	Ci
SP	17.53	7.38	10.10
BC	18.63	9.90	8.70
BP	21.80	14.10	7.70

8.2.2.4 FTIR analysis

Infrared spectroscopy was used for further characterization of bacteria pellets. In all three strains, absorbance peaks at 140.11 cm^{-1} wavelength were detected, which has an 80% similarity score to $CaCO_3$. The highest amount of calcite was recorded in the case of BP with the highest absorbance peak.

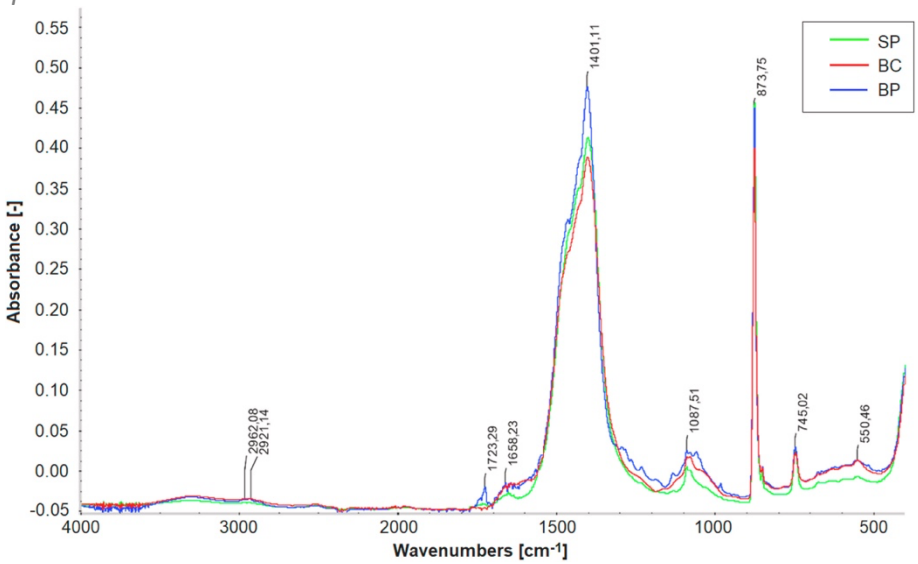

Figure 29. Results from FTIR analysis [149].

8.2.3 Conclusions

As can be seen from the above results, each method resulted in slightly different values of the measured variables such as the contents of calcium or inorganic carbon. This difference was expected as the methodologies of the different techniques differ significantly from each other. By combining these technologies, it is possible to refine the estimate of the amount of calcite that a given bacteria is able to produce, thus select the most suitable strain.

In order to obtain a result combined from the aforementioned methods, the contents of $CaCO_3$ in bacterial pellets was determined from the contents of particular elements by stoichiometric calculations based on the atomic masses of elements and the molar weight of $CaCO_3$. Calcite contents obtained from the results of individual methods and the resulting average content are presented in Table 11.

Table 11. $CaCO_3$ content in bacterial pellets calculated from amounts of Ca and Ci determined by different analytical methods [% wt.] [149].

Strain	Base element: Ca		Base element: Ci		Average
	from TGA	from XRF	from TGA	from EOA	
SP	82.41	76.32	82.50	84.16	81.35 ± 3.45
BC	77.42	66.20	77.50	72.50	73.40 ± 5.34
BP	67.43	62.96	67.50	64.17	65.51 ± 2.31

According to Table 11, the highest amount of precipitated $CaCO_3$ was in the case of SP, followed by BC and BP. However, the comparison of organic carbon content (Table 10), i.e. the potential of the bacterium to precipitate calcite by BICP, favours BP over the two remaining strains.

The amount of organic carbon correlated with the content of sulphur (S) and phosphorus (P) (Table 8). Both P and S amount indicates the level of the bacterial metabolic activity, as they are important elements found in DNA and proteins. This statement is further supported by the results of FTIR where BP exhibited the strongest $CaCO_3$ band (Figure 29).

8.3 Analysis of bacteria behaviour and BICP mechanism – conclusions

In this chapter, we have presented experiments that approximate the behaviour of several bacterial strains (*Sporosarcina pasteurii, Bacillus pseudofirmus*, and *Bacillus cohnii*). As mentioned, most studies dealing with self-healing concrete have focused on the resulting crack healing in concrete, which they evaluated based on indirect measurements, but a closer look at the behaviour and capabilities of the bacteria was almost absent. In our research, we focused on determining growth curves under different conditions: optimal and suboptimal temperatures and nutrient concentrations. Using several measurement techniques, we determined the possibilities of calcite production by the bacteria and by that compared different bacterial strains applicability. As a result of these experiments, a comprehensive view of bacterial characteristic and the BICP process itself has been obtained and a bacterium *Bacillus pseudofirmus* has been selected for further experiments involving direct incorporation of the bacteria into the cementitious material.

Chapter 9: Selection of nutrients

The selection of the nutritional compounds is primarily based on the mode of metabolic activity of the applied bacteria as described in Chapter 2. All the bio-calcification pathways need a suitable metabolic activator (e.g., nitrogen and carbon, urea, nitrite) and a calcium source. A wide range of these compounds was proposed and analysed from the perspective of the crack sealing efficiency [89], the impact on mechanical properties of concrete [50], [88], and economic factors [69]. However, as mentioned in the Literature review part, only a very limited number of studies compared the impacts of the nutrients to a larger extent and the reported results across studies differ quite significantly.

In this chapter, we determine how the compounds used as nutrients affect the material properties of the cementitious composite. The result provides a uniquely comprehensive comparison of the most applied substances.

9.1 Impact of nutrients on material characteristics

This chapter is based on the following papers:

- **Impact of the self-healing agent composition on material characteristics of bio-based self-healing concrete** [150]
  - type: journal paper
  - status: published in Case Studies in Construction Materials, 2019
  - authors: Schreiberova H, Bily P, Fladr J, Seps K, Trtik T, Chylik R
  - author's contribution: The author of this thesis is the first author. The author participated in planning and execution of the experiments, evaluated the experiments, and wrote the paper.

In the study, a variety of nutrients (yeast extract, urea, calcium nitrate, calcium formate, and calcium lactate) was proposed based on the reported impacts on the compressive strength, economic comparison, and compatibility with concrete. The pre-selected nutrients were directly added into a cement composite mixture and a large number of small prism specimens was prepared. This study then evaluated the impacts of the applied nutrients and discussed the most suitable self-healing agent composition.

9.1.1 Materials and methods

9.1.1.1 The nutrients selection

With the intention of optimizing the self-healing agent, applied nutrients and their dosages were pre-selected based on the already performed experiments presented in available literature, economic factors, and widely known compatibility with concrete.

The economic factor, hence the commercial applicability of the bio-based self-healing concrete, was approximately determined on the basis of the product prices on the web Alibaba.com to date 09/2018. This mode of evaluation was inspired by Palin et al. [69]. The price comparison was carried out only between the calcium sources, as the addition of yeast extract and possibly urea is generally unavoidable depending on the mode of the metabolic pathway of the selected bacteria. A detailed overview of the compounds from which the selection was made can be found in chapter 3.4.1.3 – Table 2 (financial demands) and

Table 3 (impacts on the compressive strength).

Based on the research, following nutrients were selected: calcium lactate ($C_6H_{10}CaO_6$), calcium nitrate ($Ca(NO_3)_2$), and calcium formate ($Ca(HCOO)_2$) at a concentration of 3.0 % to cement weight as calcium sources, yeast extract at 0.85 % to cement weight as nutritional carbon and nitrogen source, and urea at a concentration of 2.5 % to cement weight as addition in case of a self-healing agent with ureolytic bacteria.

9.1.1.2 Preparation of specimens

In order to determine the impacts of the pre-selected nutrients mentioned above, series of mortar specimens were prepared. The base for all the series was ordinary Portland cement CEM I 42.5 N (obtained from Závod Mokrá, Czech Republic) and sand with grains 0.1-1 mm and 1-2 mm (obtained from Provodínské písky a.s., Czech Republic). The water-to-cement ratio was 0.5 and the cement-to-sand ratio was 3.0 (Table 12).

Table 12. Mixing proportion used for all the series.

Material	**Amount** [kg/m³]
CEM I 42.5 N	586.00
Water	293.00
Sand (1-2 mm)	439.50
Sand (0.1-1 mm)	1318.50

The nutrient series were prepared by an addition of:

- calcium lactate ($C_6H_{10}CaO_6.5H_2O$, purity ≥ 98 %, obtained from Carl Roth GmbH + Co. KG, Germany) at a 3% concentration to cement weight (LAC)
- calcium nitrate ($Ca(NO_3)_2.4H_2O$, purity ≥ 99 %, obtained from Lach-Ner, s.r.o., Czech Republic) at a 3% concentration to cement weight (NIT)
- calcium formate ($Ca(HCOO)_2$, purity ≥ 99 %, obtained from Sigma-Aldrich spol. s.r.o., Czech Republic) at a 3% concentration to cement weight (FORM)
- yeast extract (obtained from Carl Roth GmbH + Co. KG, Germany) at a 0.85% concentration to cement weight (YE)
- and urea (NHCONH, purity ≥ 99.5 %, obtained from Ing. Petr Švec - PENTA s.r.o., Czech Republic) at a concentration 2.5 % to cement weight (UR).

The group without any further addition served as a control sample (CTRL). Mortar prisms 40x40x160 mm3 were prepared in triplicate sets for each group for the flexural and compressive test in 3, 7 and 28 days. Specimens were demolded after one day and placed in a room with temperature (20 ± 2 °C) into plastic water-filled containers where they were cured until 12 hours before testing.

9.1.1.3 The rheology of fresh cement paste

Cement flow test was performed to determine the impact of the selected nutrients on the rheology of fresh cement paste. Immediately after the mixing process, a part of the cement paste of all the series was removed and submitted to the cement flow test using a cement flow table. For detailed description of the procedure see Annex 3.1.

9.1.1.4 The flexural and compressive strength

After the end of the respective curing period (i.e., after 3, 7 and 28 days from casting), all the series were submitted to mechanical testing to determine the flexural and compressive strength. For detailed description of the procedure see Annex 3.2.

9.1.2 Impact of nutrients on material characteristics – results

9.1.2.1 The rheology of the fresh cement paste

In general, the obtained results have an overall strong concordance with our expectations. In all the cases, the addition of nutritional admixtures resulted in an increased viscosity of the fresh cement paste as it can be seen in Table 13.

The addition of calcium nitrate and calcium formate resulted in the increase of 13 % in the workability. As both chemical compounds are commercially used as hydration accelerators, it is known that they influence the consistency of fresh cement mortar quite dramatically, especially in higher dosages (139–141).

Calcium lactate and urea addition caused a noticeable increase of approximately 30 % in the fluidity; this tendency was also reported in previous research (88,142). The highest fluidity value was obtained in the cement mortar with yeast extract; the applied additive resulted in a substantial increase of 40 %.

Table 13. Results of cement flow table test.

Series	Diameter before drops [mm]	Diameter after drops [mm]
CTRL	100	115
LAC	100	150
NIT	100	130
FORM	100	130
YE	100	160
UR	100	145

9.1.2.2 The flexural strength

The performed three-point bending tests provide information about the impact of the applied nutrients on the flexural strength of prepared cement mortar. Overall, the shift due to the nutritional addition usually did not exceed 5 % when compared to the control series at 28 days.

The lowest values of the tensile strength in all ages were obtained in the cement mortar with yeast extract as a nutritional source, which is in line with our expectations. The data given in Figure 30 display slightly better results of the flexural strength (an increase of around 10-15 %) of mortar with urea (UR), calcium lactate (LAC), and calcium formate (FORM) in early ages (3 days) when compared to control mortar (CTRL). However, the final flexural strength in 28 days did not seem to be affected extensively by those admixtures. Furthermore, although calcium nitrate (NIT) is commercially used as a hydration accelerator, the flexural strength was not improved by the chemical admixture in any hardening stage.

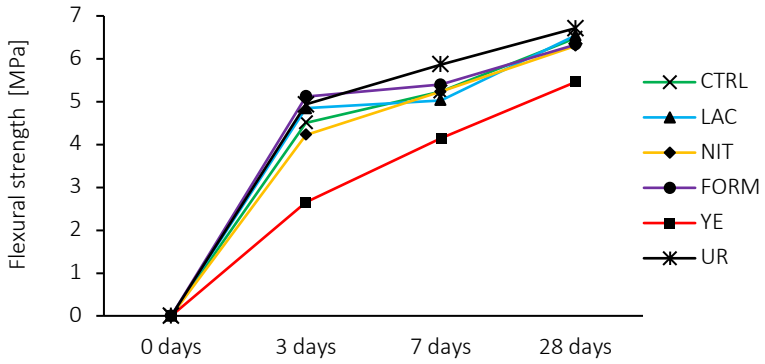

Figure 30. Mean values of the flexural strength at given time.

9.1.2.3 The compressive strength

In Figure 31, the mean values of the compressive strength results are presented. The results show that the pre-selected calcium sources (calcium formate, calcium nitrate, and calcium lactate) in the concentration of 3% to cement weight do not noticeably endanger the compressive values at any age. In this study, the addition of calcium lactate (LAC) and calcium formate (FORM) had a distinct tendency to even increase the values throughout the whole curing period.

As calcium nitrate (NIT) and calcium formate are used as hydration accelerators, a steeper growth of the compressive strength was expected, and the results are in agreement with our assumption. Additionally, unlike calcium nitrate, the mortar with calcium formate reached an even higher final value of the compressive strength when compared to the control series. The strength measured at 28 days was improved by 30% in the case of calcium lactate, whereas the addition of calcium nitrate resulted in a slight drop of 9 %.

In this study, a higher dose of urea (UR, 2.5 % to cement weight) had predominantly a positive impact on the compressive strength in the investigated ages. The final value of mortar with urea did differ only minimally from the control series.

The addition of yeast extract (YE) in the dose of 0.85 % to cement weight caused a drastic drop of the compressive strength. Especially at the early age (3 days), the strength of the YE series reached as little as 47 % of the strength of the control series. Furthermore, two of the samples prepared for the testing at 3 days and 28 days have been spontaneously damaged and excluded from mechanical tests; a large crack appeared on the samples when they were immersed in tap water in the storage containers, probably due to excessive shrinkage of the paste.

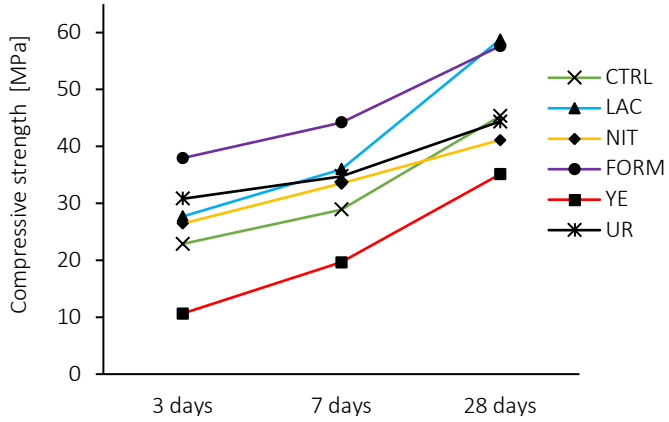

Figure 31. Mean values of the compressive strength at given time.

9.1.3 Discussion and conclusions

No negative impact of nutrients on rheology of cement paste was identified. Flexural strength was noticeably affected only by yeast addition. Therefore, the decisive parameter is the compressive strength.

In this study, the 3% addition of calcium lactate to the cement matrix caused a considerable improvement of compressive strength of about 30 %, which is in agreement with the result reported by Luo *et al.* [88]. The dose 3 % of calcium nitrate resulted in a slight decrease of about 10%, which is about 2 times less than in the experiment carried by Luo *et al.* [88]. The impact of 3% addition of calcium formate was also investigated by Luo *et al.* [88]. However, the positive effect of calcium formate in our study reached up to 30 %, whereas Luo et al. detected only a negligible difference from the control series. The impact of the urea and yeast extract addition in our concentrations were not previously directly determined by mechanical tests, thus our obtained data provide a useful extension of the known impacts.

Based on the conducted research and our own examinations, this paper concludes that all of the pre-selected calcium sources (calcium formate, calcium lactate, and calcium nitrate) are, at the proposed concentrations, applicable without any considerable negative impacts on mechanical properties of cement mortar. Furthermore, the addition of calcium formate and calcium lactate led to a cementitious material with significantly higher compressive strength values at all ages. In addition, the presence of urea, the nutritional compound for ureolytic bacteria, at a concentration of 2.5% to cement weight did not negatively affect the materials properties as well.

Based on the results, the addition of yeast extract to cement mortar seems to be the most problematic admixture. Our data suggest that the concentration of 0.85% to cement weight causes a dramatic fall of the material's strength at all ages. Potentially, this negative effect could be balanced by the presence of the other nutritional compounds.

Chapter 10: Selection of protective carriers

We have already described the issue of bacterial protection in the research part of this thesis. In short summary, experiments showed that the metabolic activity of bacteria incorporated in cementitious mortar decreases significantly after approx. 7 days [34] from casting. This decrease was contributed to mechanical destruction of the bacteria by crystallization pressures in the aging concrete.

In the studies presented in this chapter, we have not only tried the methods already applied (lightweight aggregates), but also proposed and examined our own ways of ensuring the survival of the bacteria in the material (nanofibre liquid polyvinyl alcohol textile, superabsorbent polymers, and liquid polyvinyl alcohol).

10.1 Nanofibre textile

This chapter is based on the following journal/conference papers:

- **Applicability of Bio-based Self-healing Concrete in Central European Conditions: A Preliminary Study** [138]
  - type: conference paper
  - status: published RILEM TC 253-MCI International Conference Proceedings, 2018
  - author's contribution: The author of this thesis is the first author. The author participated in planning and execution of the experiments, evaluated the experiments, and wrote the paper. The paper is based on the author's diploma thesis [155].

The study focused, among other topics, on the possible encapsulation of bacteria in polyvinyl alcohol (PVA) nanofibre textile. Encapsulation of biological material (bacteria and viruses) in PVA nanofibre textile has been already researched by Salalha *et al*. in 2006 [156]. The experiment showed that a range of organisms can be efficiently encapsulated and stay viable, thus it could be a promising method suitable for our purpose.

As mentioned in the literature overview, several types of fibres have been tested for the purpose of encapsulation; however, PVA nanotextile have not been previously applied. Thus, the research presented herein is of primary interest.

10.1.1 Materials and methods

The main issue which needed to be addressed is the solubility of PVA nanofibre textile in water. There are several methods of increasing stability and insolubility of the material; however, all the possible approaches pose a risk to the bacteria.

10.1.1.1 Encapsulation of bacteria spores in PVA nanofibre textile

To determine the potential of anchoring spores of *Bacillus pseudofirmus* into PVA nanofibre textile, samples (double layered circles with a diameter of about 4.4 cm) with different composition of the PVA solution were prepared by roller electrospinning (Nanospider, Elmarco CZ). The composition of samples is indicated in Table 14. The base for all the series was 10% aqueous PVA solution. Series 10PVA and 10PVAB also contain H_3PO_4 and glyoxal which improve the efficiency of the stabilization process. The samples 10PB and 10PVAB further contain bacterial spores which were added into the basic solution.

Table 14. Composition of PVA nanofabric samples.

Series	10% PVA	H_3PO_4	Glyoxal	Spores
10P	•			
10PB	•			•
10PVAB	•	•	•	•

Nanofibre samples were either stabilized by exposure to high temperature (140 °C for 20 minutes) or placed in desiccators with glutaraldehyde for two hours or left unstabilized. After the stabilization process, the bacterial samples (10PB and 10PVAB) were placed into 100 ml of culture media to examine the survival and viability potential via visual inspections and bacterial curves based on the values acquired from BOD sensors (Annex Chapter 2.3).

10.1.2 Results

The experiment showed that nanofibres without the addition of H_3PO_4 and glyoxal are almost impossible to be stabilized by both glutaraldehyde (GA) vapor or temperature. Stabilization (i.e., water insolubility) appeared only in series 10PVA and 10PVAB.

Measurements of oxygen demand of nanofabric samples with encapsulated bacterial spores in culture medium showed metabolic activity in all series (Figure 32). However, in our conditions and due to the method of production of PVA nanofibres, it was impossible to ensure complete sterility of the environment and the samples. Therefore, it is difficult to distinguish the activity of *Bacillus pseudofirmus* from unintentional contamination.

Generally, samples stabilized by high temperature (140 °C for 20 minutes) showed lower values of metabolic activity. This finding agrees with the investigation of the influence of the stabilization process to bacterial viability. Results also show that the series 10PVAB stabilized by GA vapor had the latest start of metabolic activity but reached the highest values first. This result could indicate that in our experiment, the stabilization by GA vapor was the most effective method. It seems that the bacterial spores stayed captured and protected within the fibres the longest when immersed in culture media.

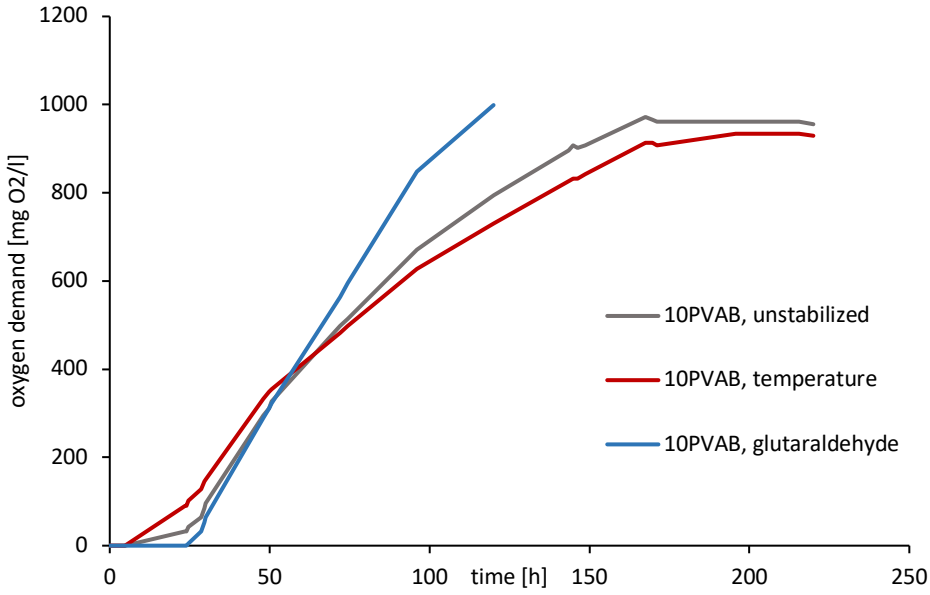

Figure 32. Oxygen demand measured in nanofabric samples containing bacteria immersed in culture media.

10.1.3 Conclusions

The method of bacteria encapsulation in PVA nanofibre textile has not been found in the previous studies focused on the self-healing concrete. Results showed that the most suitable stabilization method may be the exposure to glutaraldehyde vapour. However, it was problematic to draw any firm conclusions from the experiments with bacterial nanofibre textile

as it was impossible to ensure complete sterility. If PVA nanofibres were to be used for bacterial protection, further experimental verification of their applicability would be required. In our studies, we decided to abandon PVA fibres due to the complexity of material preparation and handling.

10.2 Light weight aggregates

This chapter is based on the following paper:

- **Applicability of Bio-based Self-healing Concrete in Central European Conditions: A Preliminary Study** [138]
  - type: conference paper
  - status: published RILEM TC 253-MCI International Conference Proceedings, 2018
  - authors:
  - author's contribution: The author of this thesis is the first author. The author participated in planning and execution of the experiments, evaluated the experiments, and wrote the paper. The paper is based on the author's diploma thesis [155].

The possibility of encapsulating bacterial spores in light weight aggregates (LWA) was already introduced in Chapter 4.3. This method of encapsulation has been used in many studies, but only a limited number have looked more closely at the effect of encapsulation process on bacterial viability. In this study [138], we have focused on the encapsulation of bacteria and nutrients in LWA and a detailed description of the final product.

10.2.1 Materials and methods

10.2.1.1 LWA impregnation and drying

Sterilized LWA (Liapor 4/8 mm) was impregnated with two different solutions – bacteria with 1g/l yeast extract (BY) and bacteria with 80 g/l calcium lactate and 1 g/l yeast extract (BCY and BCYH). The concentration of bacterial spores of *Bacillus pseudofirmus* was around $20x10^6$ spores/l in both cases. The exact procedure of cultivation/sporulation and applied materials are described in a greater detail in Annex A, chapters 1.1 and 2.1.

The process of impregnation was identical in all cases. Dried and sterilized LWA was placed into a container with the respective solution connected to a vacuum pump. The vacuum pump was turned on for 5 minutes to create a negative pressure and then the treated LWA was left to soak up for 20 minutes in atmospheric pressure. After this procedure, surplus solution was poured out and impregnated LWA was placed onto filter papers and let to dry out.

The drying process was carried out under different conditions to examine the influence of temperature and humidity on the efficiency of the drying process. Two of the series (BY and BCY) were placed into desiccators with silica gel and left at room temperature until further use. The BCYH series was at first placed into desiccator with 75 % humidity at 5 °C for 24 hours and then placed into desiccator with 55 % humidity and left at room temperature. Finally, after two days, BCYH was also placed into a desiccator with silica gel at room temperature and left there for further use.

10.2.1.2 Viability of bacteria after impregnation

For determination of the impact of impregnation and drying on bacterial viability, impregnated LWA was subjected to measurement of bacterial activity via BOD (biochemical oxygen demand)

sensors. To simulate future conditions of the bacterial LWA in cementitious material, all the series were cultured in media which would demonstrate real supply of nutrition. BY was cultured in 100 ml of calcium lactate solution (80 g/l) and BCY, BCYH in sterilized tap water.

10.2.2 Results

10.2.2.1 LWA impregnation and drying

At first, impregnated LWA particles were subjected to visual investigation after 7 days of drying. Grains from each series were selected, cut in half and inspected under a microscope (Olympus BX41 Fluorescence Microscope, magnification 10x). Aggregates from all of the series contained some yellow particles (Figure 33). These particles were not present in untreated LWA; therefore, it could indicate a successful impregnation. As all of the series were impregnated with yeast and bacteria, the yellow particles could be the result of their presence. In aggregates from series with the addition of calcium lactate (BCY and BCYH), copious amounts of white to transparent crystals (Figure 33) were present along with the yellow particles. These crystals were present neither in untreated LWA nor in LWA impregnated only with bacteria and yeast (series BY), thus it could indicate a successful impregnation with calcium lactate.

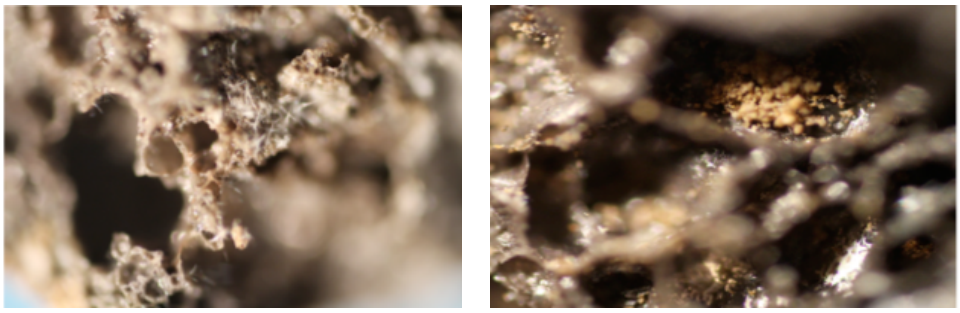

Figure 33. Crystalline particles (left) and yellow particles (right) in impregnated LWA.

10.2.2.2 Viability of bacteria after impregnation

Bacterial curves of impregnated LWA were derived from the oxygen demand measured with BOD sensors. Results show (Figure 34) that in all the series, spores of *Bacillus pseudofirmus* became active and their metabolic activity was restored. However, the beginning of oxygen demand, thus metabolic activity, occurred relatively late after the immersion of LWA into media. The metabolic activity of the series BY became noticeable after approx. 65 hours from the immersion, in BCYH after 160 hours, and in BCY after 190 hours. The metabolic activity of the series BY reached the highest values while the series BCY and BCYH reached similar values, despite the earlier beginning of the BCYH series.

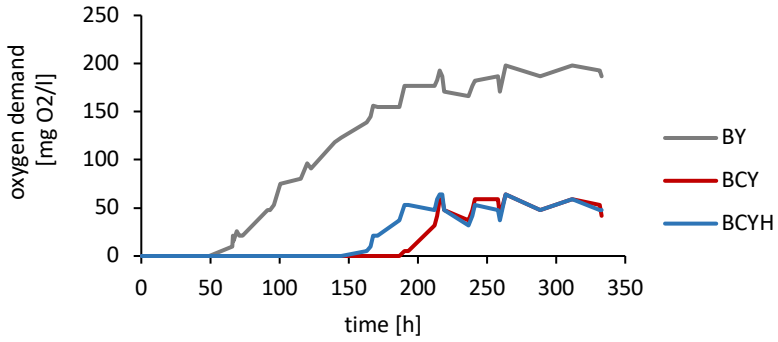

Figure 34. Measurements of oxygen demand of impregnated bacterial LWA in tap water/calcium lactate solution.

A possible explanation of the differences between the metabolic activity of the LWA series could be a different nutrition supply. The series BY was immersed in 100 ml calcium lactate solution (80 g/l), therefore the quantity of the Ca source was ensured. However, the series BCY and BCYH were immersed in sterilized tap water, thus the only Ca source available was incorporated inside the LWA particles in the crystallized form, as it could be seen under the microscope. The results show that the gradual drying, which was applied on the series BCYH, does not have a great influence on the final bacterial activity. The series BCYH became active approximately 30 hours earlier than the BCY series, but they both reached identical values of oxygen demand at their peaks.

10.2.3 Conclusions

The experiment carried out showed that the bacteria *Bacillus pseudofirmus* can survive the impregnation process, but the nutrient uptake in LWA is not sufficient with the given procedure. Therefore, this means that nutrients will probably need to be added directly to the cement matrix when applying LWA as a protective carrier. Furthermore, it must be remembered that LWA is traditionally applied to lightweight concretes with low strengths. It is therefore questionable whether the resulting material would have a chance of achieving the required strengths.

10.3 Superabsorbent polymers

This chapter is based on the following paper:

- **Design of Nutrient Enriched Cement Paste with a Superabsorbent Polymer for the Bio-based Self-healing Concrete Development** [157]
  - type: conference paper
  - status: published Material Science Forum (vol. 995)
  - authors: Schreiberova H, Fladr J, Šeps K, Kohoutkova A
  - author's contribution: The author of this thesis is the first author. The author participated in planning and execution of the experiments, evaluated the experiments, and wrote the paper.

The application of superabsorbent polymers (SAPs) has already been proposed as one of the possible ways to increase the self-healing ability of cementitious composites. This approach relies on the striking absorption capacity (up to hundred times its own weight) of SAPs. The watertightness of the material is believed to be enhanced due to SAP by various mechanisms –

it provides extra moisture for further hydration of cement grains (the so-called internal curing) as well as physically blocks the penetration of substances into formed cracks.

The SAP approach to self-healing described above could advantageously complement the biological approach in several ways. The swollen SAP particles (or voids left after their drainage) could provide enough space for the bacteria to survive and retain water penetrating through the crack, thus provide the needed space and moisture for bacterial activity and keep the healing products inside the crack. In the development of biological self-healing concrete, standard pH-responsive hydrogels, which are far less absorbent than the SAP, has been previously successfully applied; however, only little work regarding SAP application has been done.

10.3.1 Mix design and its testing

Generally, the absorption and retention behaviour of SAP relies on numerous conditions – size of the particles, pH, temperature, light, pressure, and presence of ions [104], [158]. Especially the lastly mentioned feature is highly significant in the concrete application. Researchers have been determining various "extra" water-cement ratios (w/c_e) which could partially make up for the water entrained by the SAP while providing the needed moisture for the targeted purpose (self-healing, internal curing, etc.) [159], [160]. As the ionic composition influence the SAP absorption capacity greatly, the needed extra water-cement ratios might differ significantly based on the concrete/cement paste composition. In this study, cement composite containing SAP and nutrient compounds needed for bacterial activity was proposed and investigated.

In this experiment, lower strength cement supplemented with slag was used to reduce the effect of autogenous crack healing. In future experiments with this material, it should then be easier to recognize the crack-sealing effect of the biological agent.

The SAP was added into the cement paste in two states – a dry state (SAPD) and a saturated state (SAPS), see Table 15. The concentration was identical for both series – 0.5 % by cement weight (2.93 g/l). In the case of the dry SAP, extra mixing water (73.3 g/l) was added into the mixture. The applied w/c_e ratio (w/c_e = 0.125) was determined based on our preliminary investigations which dealt with cement paste without the nutritional compounds.

For the wet application, a known quantity of SAP (2.93 g) was immersed in demineralised water (293 g) prior to the addition in cement paste. The amount of water was identical as the amount of mixing water in the reference paste thus the swollen SAP should serve as the mixing water substitute. As our preliminary investigations showed, the SAP was saturated only partially as the absorption capacity of the applied amount (2.93 g) should be several times higher (around 715 g of demineralised water).

Table 15. The cement pastes compositions for flowability tests and preparation of cement mortar specimens.

	REF [g/l]	**SAPD** [g/l]	**SAPS** [g/l]
CEM II 32.5 R	293.0	293.0	293.0
blast-furnace slag	293.0	293.0	293.0
distilled water	293.0	293.0	-
w/c	0.5	0.5	-
extra distilled water	-	73.3	-
w/c_e	-	0.125	-
sand 1-2 mm	439.5	439.5	439.5
sand 0.1-1 mm	1318.5	1318.5	1318.5
calcium lactate	17.58	17.58	17.58

yeast extract	4.98	4.98	4.98
dry SAP	-	2.93	-
saturated SAP (SAP + absorbed water)	-	-	2.93 + 293.0

To determine the suitability of the mixture design, cement flow table test (Annex 3.1) and three-point bending and compressive strength test (Annex 3.2) were carried out.

10.3.2 Results

The cement flow table test indicated that the application of 0.5 % dry SAP by cement weight and extra mixing water (w/c_e = 0.125, i.e., 25 g/g SAP) causes only a slight increase in the flowability when compared to the reference paste. Thus, it seems that a large part of the extra water was absorbed by the SAP. This finding rather contradicts with the most frequently considered value of the SAP absorbency in cement paste between 12-13 g/g SAP [159], [161] but this only illustrates the magnitude of the effect of the ionic composition of the aspirated fluid.

The flowability measurement of the cement paste with partially saturated SAP (SAPS) provides interesting insight into its retention behaviour. The partially saturated SAP carried the exact amount of mixing water applied in the reference paste. The swollen SAP was added alongside with cement and further mixing process was identical as in the reference case. The flow table test showed that the SAPS cement paste was significantly less flowable compared with the reference paste. It implies that majority of the liquid may have remained captured inside the SAP particles despite the thorough mixing process.

The strengths measurements were generally in line with the flowability tests. The strength values of SAPD were lower compared with the reference paste values at all times. On the other hand, in the case of the partially saturated SAP (SAPS), the mechanical tests indicated higher strength values than the reference samples (Figure 35).

Although the absorption and retention behaviour of the SAP in the cement matrix enriched with nutrients need further clarification, our results suggest that the concentration 0.5 % SAP by cement weight is applicable in the bio-based concrete from the perspective of mechanical properties. The strengths of the hardened paste seem to be predominantly affected by the water amount.

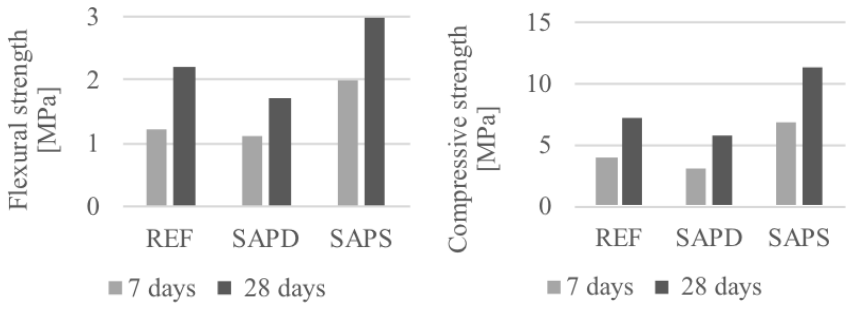

Figure 35. The mean flexural and compressive strengths of cement paste containing SAP.

10.3.3 Conclusions

In this section, the main observations obtained from the conducted experiments and future proposals will be briefly concluded:

- The concentration 0.5 % SAP by cement weight seems to be applicable in the nutrient enhanced cement paste.

- Our results indicate that partially saturated SAP particles are able to capture majority of the absorbed liquid throughout the mixing process. In our study, the replacement of mixing water with the saturated SAP led to a drier but significantly stronger cement paste.
- Future research should focus on investigations of the protective potential of SAP and determine the most appropriate method of its application (dry or saturated) from the bacterial protection perspective.

Chapter 11: Determination of the crack-sealing potential

This chapter describes the experiments that can be considered as the final part of this thesis. On the basis of the partial experiments described in the previous chapters, a suitable composition of the self-healing agent and the protective carriers was designed, and the crack-sealing efficiency was investigated under various conditions.

11.1 External curing – microscopy analysis of crack-sealing

This chapter is based on the following paper:

- **The role of bacterially induced calcite precipitation in self-healing of cement paste** [149]
  - type: journal paper
  - status: published in Journal of Building Engineering, 2021
  - authors: Ryparova P, Prosek Z, Schreiberova H, Bily P, Tesarek P
  - author's contribution: The author of this thesis is a co-author. The author participated in execution of the experiments, data collecting and writing the paper.

Paper [149] presents, among other things, an experiment in which small cement specimens were made in which a crack was created and a self-healing agent was externally applied to the created "crack". Subsequently, the samples were observed by electron microscopy to determine the nature of the newly formed material within the crack and to separate the two types of self-healing processes that take place simultaneously – spontaneous and bacterial (SICP and BICP).

11.1.1 Materials and methods

Microscopic sections were cut out of macroscopic cement paste samples to observe the calcite precipitation. The sections were embedded in epoxy resin and polished in an alcohol-based solution using Struers Tegramin grinding plate with 1200, 2000, and 4000 grain/cm^2 grit under 5 N compression. Subsequently, a groove of 50 µm average width was created in the surface of each microscopic section to simulate a crack. The grooves were created mechanically using stainless steel scalpel. Microorganisms were applied on the surface of the microscopic samples by dripping 0.05 ml of medium in the area of the groove. Samples were placed in a desiccator with 100% humidity.

Three different solutions were used to treat the artificially cracked specimens: set A (reference – pure water), set B (*Bacillus pseudofirmus* precipitation medium, see [149] for composition), and set C (*Bacillus pseudofirmus* precipitation medium with bacteria).

The samples were scanned continuously by optical microscopy. In the moment when the crack in one of the samples was visually closed, the experiment was terminated. The complete crack closure took 56 days. Further, Scanning electron microscope (SEM) FEG-SEM Merlin ZEISS

was exploited to study the precipitation of calcite and to investigate the morphology and quantity of calcite. The chemical analysis of each phase was carried out using an energy dispersive spectrometer (EDS).

11.1.2 Results

Figure 36 shows the degree of the healing in the individual simulated cracks from the optical microscope after 56 days of treatment. In the case of sample C (medium + bacteria) the crack was completely healed, while in other cases just isolated clusters of calcite crystals were identified. Figure 37 presents the results from the electron microscope.

In the case of the sample A (only water), the crack remained almost unhealed from the macroscopic point of view. SEM analysis showed isolated grains with cubic crystal lattice that did not form a more compact structure. These crystals were formed because of the spontaneous crystallization of free calcium in the cement paste. This assumption was confirmed by the microscopic elemental analysis as the crystals contained the following elements: calcite (34.6 % wt.), carbon (14.2 % wt.), oxygen (51.1 % wt.) and other elements (0.1 % wt.). When converted to atomic percent and using stoichiometry, it can be stated that it was $CaCO_3$ formed by spontaneous precipitation (SICP). The calcite crystal size was between 1 and 30 μm.

The analysis of the sample B was performed in order to determine what crystal formations were created in the crack because of the application of the medium without bacteria. The crack was partially filled with amorphous formations. The SEM images showed leaf-like structures that were probably the result of crystallization of proteins from the medium. The hypothesis was confirmed by determining the chemical composition from these areas. The crystallization structures had a high content of sodium (34.1 % wt.), oxygen (51.9 % wt.) and carbon (11.1 % wt.). Many other elements such as potassium and calcium (total 2.9 % wt.) were found it this area. The simulated crack and its immediate surroundings were filled with a larger number of crystals compared to the sample A; the size of crystals was identical to the sample A, i.e. between 1 and 30 μm. Chemical analysis confirmed that they were calcite crystals from the spontaneous healing. Larger amounts of these crystals were found due to the presence of calcium lactate in the medium. Calcium lactate has a high calcium content and thus supports the formation of calcite from SICP.

The best result was obtained in the sample C, i.e. the sample with added bacteria. In this case, the simulated crack was completely healed. The SEM image showed the resulting neoplasms that were more arranged compared to the previous samples. Distinct clusters of crystals smaller than 1 μm in size could be seen in the structure. The chemical composition was similar to that found in case of SICP, i.e. calcium (41.4 % wt.), carbon (12.2 % wt.), oxygen (44.2 % wt.) and other elements (2.2 % wt.). The chemical composition was slightly distorted by the proteins present from cultivation media, but still the results suggest that they were mainly crystals of calcite formed by BICP. Furthermore, it can be seen (Figure 37) that the structure did not contain larger amounts of crystallized proteins, and thus the bacteria consumed all nutrient medium to heal the cracks. Crystalline structures with the shape of bacteria were clearly visible, indicating the bacterial origin of calcite. These formations were probably bacteria covered with various forms of calcite, and the bodies of the bacteria themselves formed a nucleation site for crystal growth.

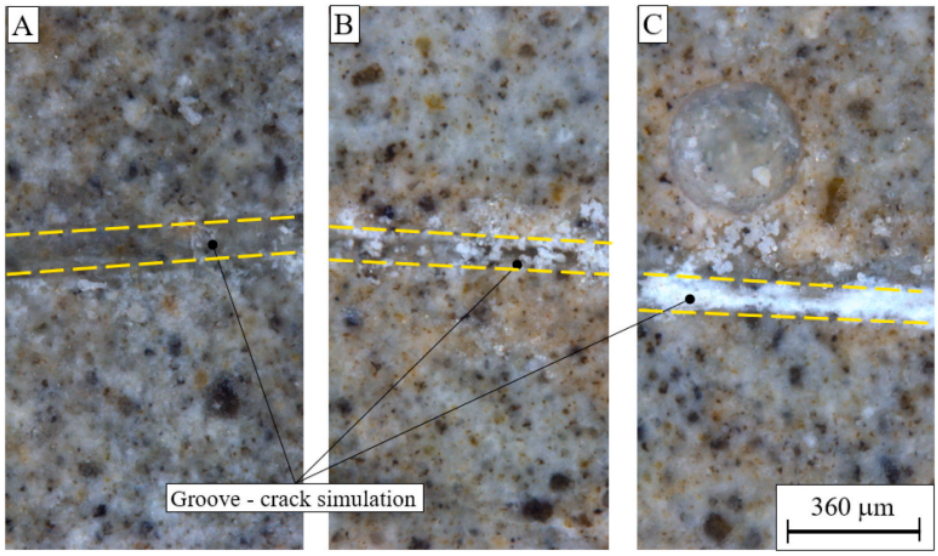

Figure 36. Grooved surfaces of cement paste samples after 56 days of treatment – images from the optical microscope (magnification 50 ×) [149].

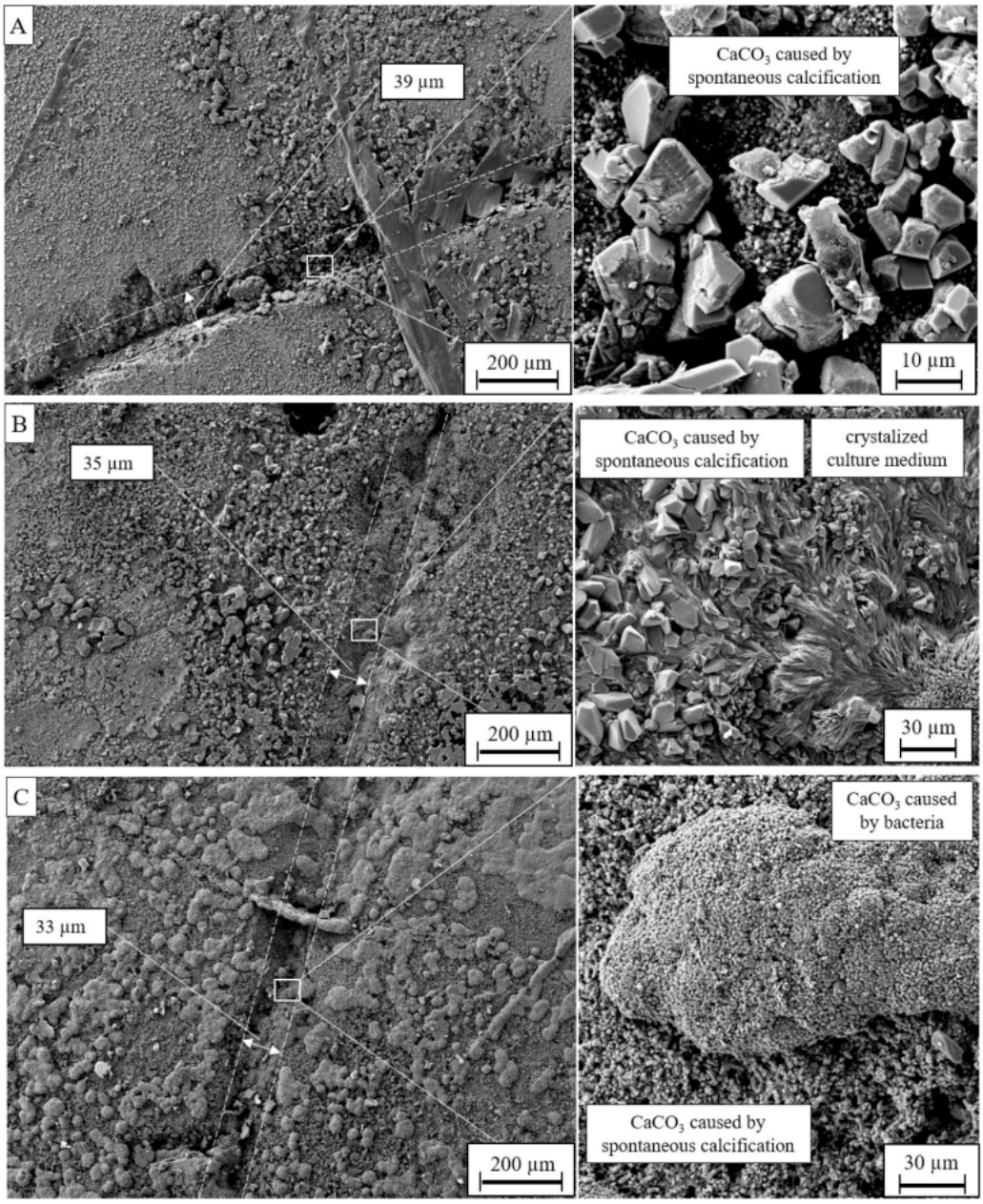

Figure 37. Grooved surfaces of cement paste samples after 56 days of treatment – images from the electron microscope.

11.1.3 Conclusions

The comparison of the precipitates formed in the artificially created cracks on the surface of cement paste samples allowed to determine the contribution of the selected bacteria (BP) to the self-healing process. Sporadic isolated calcite neoplasms were found on the surface of the samples that were treated just by water. The number of crystals was not sufficient to seal the cracks. Larger crystals (1–30 µm) were formed. When the samples were treated with the precipitation medium without bacteria, the cracks were mostly sealed, containing a mixture of calcite and crystallized proteins. However, such material probably won't have sufficient binding properties. Also, in this case, larger crystals (1–30 µm) creating porous infill of the crack were formed. By adding the bacteria to the medium, the crystallization of proteins was significantly reduced as they were consumed by the bacteria. The crack was completely sealed by a dense structure of small crystals that were a result of presence of bacteria. In conclusion, it can be said

that the direct beneficial influence of bacteria on calcite precipitation and self-healing of cement paste was proved.

11.2 Crack-sealing under optimal in-vitro conditions

This chapter is based on the following paper:

- **Applicability of Superabsorbent Polymer as a Protective Carrier in the Bio-based Self-healing Concrete** [162]
  - type: conference paper
  - status: published in Proceedings of the 2020 Session of the 13th fib International PhD Symposium in Civil Engineering, 2020
  - authors:
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This paper deals with an application of bacterial spores of *Bacillus pseudofirmus*, superabsorbent polymer (SAP), and two nutritional compounds (calcium lactate and yeast extract) in bio-based self-healing concrete. The crack-sealing potential is determined on several series of cement composite beams which were cracked by three-point bending test and further cured in water to trigger the healing.

11.2.1 Materials and methods

In Table 16, design proportions of the prepared cement mortars are given. Four series were prepared in total. The CTRL mix served as the control, as it contained neither bacterial spores nor SAP. Mix containing directly added spores without SAP (BAC) provides an evaluation of the contribution of SAP on protection of the spores, thus on the crack filling. Further, by comparing the CTRL_SAP mix (only SAP added) with control mix, the contribution of the polymer itself to the self-healing can be determined. Finally, the mix SAP_BAC was prepared in order to evaluate the self-healing potential of the SAP-bacteria combination.

Table 16. Mix composition of the investigated cement mortars with nutrient compounds.

Material		**CTRL**	**BAC**	**CTRL_SAP**	**SAP_BAC**
CEM I R 42.5	[kg/m^3]	586	586	586	586
Distilled water	[kg/m^3]	293	293	355	355
Sand (1-2 mm)	[kg/m^3]	440	440	440	440
Sand (0.1-1 mm)	[kg/m^3]	1319	1319	1319	1319
SAP	[wt.% of cement]	0	0	0.50	0.50
Calcium lactate	[wt.% of cement]	3.00	3.00	3.00	3.00
Yeast extract	[wt.% of cement]	0.85	0.85	0.85	0.85
Bacillus pseudofirmus	[CFU/ml]	-	1x10^6	0	1x10^6

To prepare specimens for the crack-introduction, the nutritional compounds and SAP, if applied, were added alongside with cement and premixed for 1 min in order to achieve distribution as uniform as possible. Mixing water with/without dispersed bacterial spores was added as prescribed by the standard.

All mixes were casted into 40x40x160 mm^3 moulds. Additionally, steel fibres were placed approx. 10 mm below the mould edge (Figure 38). This reinforcement should further facilitate

the crack creation by a three-point bending in later ages. Without the steel reinforcement, the specimens could be easily fractured completely and split in half. The moulds with the embedded steel fibres were then left covered at room temperature and humidity. After demoulding, the specimens were cured at 25 °C and approx. 90% RH for 28 days.

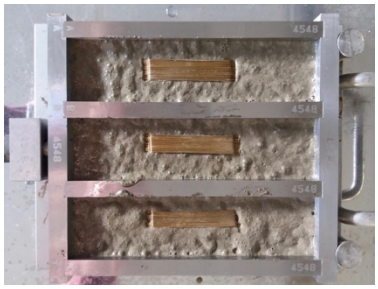

Figure 38. A mould for three 40x40x160 mm^3 specimens – a demonstration of the steel fibres placement.

In order to determine the self-healing potential of the SAP-bacteria concrete, cracks were introduced by a three-point bending test after the end of the curing period in every specimen. The placement and widths of the cracks were carefully recorded by photographic imagining. Additionally, the areas around the formed cracks were scanned by optical scanning microscope in order to obtain high resolution images. After the investigation of the pre-healed state, the samples were subjected to the healing process.

Continuous full immersion in water was applied in the healing period. The cracked samples were placed into open plastic containers filled with standard tap water and left open at room temperature for 28 days. After the healing period, the samples were removed from the containers and the crack sealing was inspected and recorded by high resolution photographic imagining and scanning optical microscopy.

11.2.2 Results

Self-healing efficiency, thus crack sealing, was in this paper evaluated by visual inspections through scanning optical microscopy and photographic imagining. The example of photographic images which compare the state before the 28-day long immersion in water at room temperature and after the healing period can be seen in Figure 39.

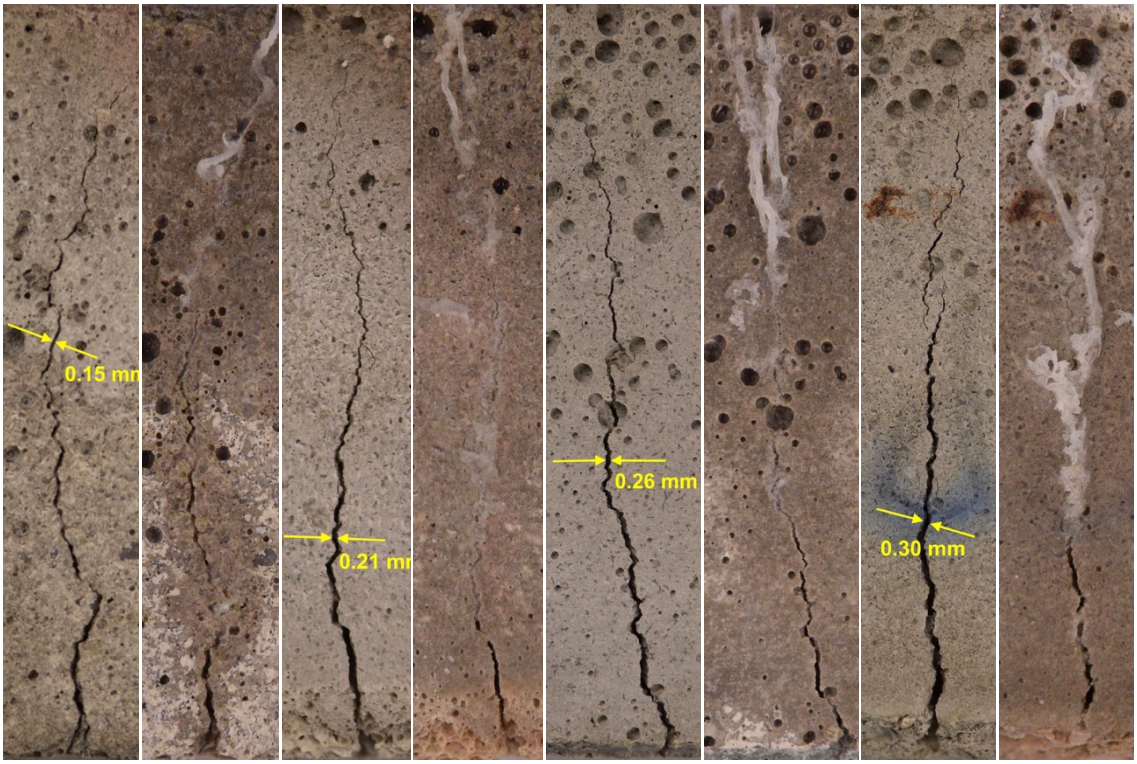

Figure 39. Comparison of the cracked cementitious specimens before and after the healing process – selected samples, the maximum initial width of the crack that was healed is marked.

It is evident from the results that certain crack-sealing has taken place in all of the investigated series. As it can be observed, newly formed white precipitates were detected in all of the samples. Following investigations revealed that maximum initial widths of the crack where the precipitates formed differed depending on the mix composition. The samples where produce in duplicate, thus four different cracked surfaces were available for each mix composition. Based on the photographic imagining, the maximum initial width of the cracks that were healed were measured and their average value was determined for each mix.

In Figure 40, the average maximum healed crack widths are showed. In control samples (CTRL), the average maximum initial crack width was 0.18 mm. In the BAC samples where bacteria were incorporated, the width increased to 0.23 mm. In samples where only SAP was added, the average initial crack width reached 0.26 mm. Finally, in samples SAP_BAC containing both bacteria and SAP, the precipitates could be observed in crack with the average initial width up to 0.28 mm.

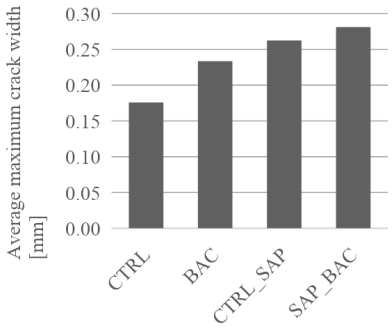

Figure 40. Average maximum initial widths of healed crack.

The scanning microscopy enabled closer visual inspection of the crack sealing. In Figure 41, a crack closed after the healing period can be seen. As visible from this example, the precipitates formed not only in the crack itself, but also in the area adjacent to it. The white depositions were primarily located in pores, although some of them remained empty. Figure 41 captures crack in an SAP_BAC specimen, thus specimen containing bacteria and SAP. However, the microscopic investigation showed that the precipitates did not visually differ in the other series.

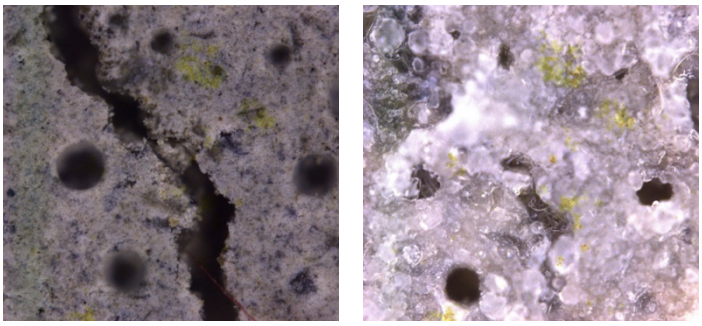

Figure 41. A microscopic image of the crack before (left) and after the healing process (right) in a sample from the SAP_BAC series.

11.2.3 Discussion

In accordance with the assumptions, due to the autogenous self-healing capacity of cement composites, the crack closure was observed in all of the series including the control (CTRL). The positive impact of the SAP addition, especially in the case of crack widths below 0.5 mm, on the autogenous healing was reported elsewhere [163]. Comparable to these findings, in this paper, the maximum healed crack width of the CTRL_SAP series was 49.3% greater compared to the control series. However, it must be acknowledged that the extra mixing water added alongside with SAP in order to preserve the flowability may have improved the autogenous crack sealing itself.

The addition of the bacteria (BAC) increased the average healed crack width by 32.7% compared to the control. The highest value of the average maximum healed cracked after 28-day immersion in water could be observed in the samples containing both bacteria and SAP (SAP_BAC). The value was 59.84% higher compared to the control.

Although the results presented in this paper indicate positive impact of the SAP and bacteria addition on the cement composite self-healing ability, the maximum crack width healed in this experiment was only 0.32 mm. This value is not in line with earlier studies where cracks up to 0.70 mm wide were completely sealed [163]. It may be a result of an insufficient distribution of the healing agent (bacteria and nutritional compounds) in the mortar. Further, the SAP may

successfully function as a moisture reservoir and increases the autogenous crack-sealing, but it may not provide enough protection of the bacteria spores against crystallization pressures.

11.2.4 Conclusions

In the current study, the combination of bacteria *Bacillus pseudofirmus*, nutritional compounds, and SAP was applied in cement composite in order to evaluate the biologically enhanced material's self-healing potential. The artificially cracked specimens were submitted to a 28-day long healing period and subsequently the crack closure was visually inspected. The following conclusions can be drawn based on the current experimental investigation:

- The SAP and extra mixing water addition increases the autogenous crack-sealing in cement composite.
- The bacterial self-healing agent (*Bacillus pseudofirmus*, calcium lactate, yeast extract) significantly improves the crack closure compared to plain cement composite samples.
- Protection of bacterial spores by SAP may not be sufficient and further additional methods of protection should be considered.

11.3 Crack-sealing under simulated outside conditions

This chapter is based on the following paper:

- **Self-healing in cementitious composite containing bacteria and protective polymers at various temperatures** [164]
  - type: journal paper
  - status: published in Magazine of Civil Engineering, 2021
  - authors:
  - author's contribution: The author of this thesis is the first author. The author participated in planning and execution of the experiments, evaluated the experiments, and wrote the paper.

This paper is focused on two main issues of the so-called bio-based self-healing concrete i.e., protection of the bacterial spores embedded in the cementitious matrix and behaviour of the material at low temperatures (as described in 3.2.4). The second aspect is particularly important as the impact of the conditions corresponding to real outside environment was rarely investigated before. An investigation of the influence of temperatures below the freezing point is a unique extension of the current state of the art.

The paper has dealt with the application and comparison of two polymer-based protective agents (SAP powder and PVA water solution) in cementitious composite containing bacteria and nutrient organic compounds. The influence of the applied protective agents on the material's properties (specifically on consistency and strengths) was determined via cement-flow table test, three-point bending, and compression test. Further, the self-healing was observed on cracked specimens, which were exposed for 28 days to various conditions (room temperature, low temperature, and freeze cycles). The healing efficiency was determined based on visual investigations using high-resolution photography, 3D scanning microscopy, and dynamic modulus recovery.

11.3.1 Materials

The composition of the mixture (Table 17) in this experiment was not too different from that presented in the previous chapter. A significant difference in this study was the application of polyvinyl alcohol (PVA) solution as an alternative solution to protect the bacteria.

There is no mention in the existing literature of the application of PVA water solution in self-healing bio-based concrete, even though several studies have already evaluated its effect on mechanical properties, reduced water absorption or increased acid resistance of cementitious composites [165]. In this study, it is expected that the polymer could form a protective layer around the spores, while the increased porosity (according to previous research) would provide additional protective space for the bacterial spores.

Table 17. Composition of the mixtures.

Compound	**CTRL** [kg/m³]	**CTRL_SAP** [kg/m³]	**CTRL_PVA** [kg/m³]	**BAC** [kg/m³]	**BAC_SAP** [kg/m³]	**BAC_PVA** [kg/m³]
Portland cem.	586	586	586	586	586	586
Distilled water	293	337	262	293	337	262
Medium agg.	440	440	440	440	440	440
Fine agg.	1319	1319	1319	1319	1319	1319
SAP	no	2.93	no	no	2.93	no
16 % PVA	no	no	36,63	no	no	36.63
Calcium lactate	17.58	17.58	17.58	17.58	17.58	17.58
Yeast extract	2.64	2.64	2.64	2.64	2.64	2.64
Bacillus Pseudofirmus [CFU/ml]	no	no	no	$8x10^8$	$8x10^8$	$8x10^8$

The mixing procedure was kept identical in all cases. Yeast extract was homogenized with cement prior to mixing and calcium lactate was dissolved in mixing water (containing dispersed bacterial spores if applied). In the case of SAP and PVA series, both polymers were applied alongside cement and mixed prior to the aggregate and water addition.

From the prepared mixes, two types of specimens were prepared - specimens for mechanical testing and specimens for crack-sealing investigations. All the specimens were prepared in triplicates for each mix design and testing method. Both types were casted in 40x40x160 mm^3 steel molds, thoroughly vibrated using a vibrating table. In the case of the specimens intended for the crack-sealing, around 20 profiled steel wires were placed in the middle of the span, approx. 1 cm from the mold top.

The molds were then left at room temperature covered with plastic foil for 24 hours to harden. Thereafter, all the specimens were unmolded and placed in a climate chamber with temperature 24 °C and relative humidity up to 95% for 28 days. After the end of the curing period, dimensions of the specimens were thoroughly measured, and the samples were weighed.

To estimate the healing capacity of the proposed combinations of bacteria and protective methods, the prepared reinforced samples were cracked after the end of the curing period. The cracks were introduced through three-point bending using a calibrated electric loading machine. The loading rate was controlled manually and operatively altered to avoid complete destruction of the sample.

11.3.2 Methodology

The cracked samples were subjected to three different healing conditions: optimal, low temperature and temperatures below the freezing point. The optimal environment (25 ± 2 °C) served as the reference. This value also more or less corresponds to the highest reachable

average month temperatures in the place of our research - the Central Europe region. To inspect the healing potential in the ideal conditions, the cracked samples were placed into separate plastic containers filled with tap water and left at temperature for 28 days. All the series were exhibited to the ideal conditions in order to obtain a complete overview of each material's healing capacity. Thus, the contribution of bacteria and each protective method to the healing process could be determined.

The temperature of 10 °C was chosen for the investigation at low temperatures. In the place of our research, Czech Republic, the long-term air temperature normal (1981-2010) reaches and exceeds this value from May to August, i.e., in 5 months of the year, according to the data from the Czech Hydrometeorological Institute. In the case of sufficient crack sealing at this temperature, the self-healing could potentially take place for a large part of the year, thus the material could be declared applicable in the Central European region. In order to inspect this hypothesis, identically as in the ideal conditions, the cracked samples were submerged in water in plastic containers. The containers were then placed in a climate chamber with a controlled temperature of 10 °C for 28 days. In this case, only specimens with protective polymers (i.e., BAC_SAP and BAC_PVA) and control (CTRL) were used.

An investigation of the impact of temperatures below the freezing point on the self-healing ability is a unique extension of the current state of the art. Although the crack sealing due to the metabolic activity of bacteria at freezing temperatures is not expected, it is crucial to answer the question, whether the bacteria immobilized in the cementitious material/protective polymer can withstand these conditions and restore its activity once the temperature raises. To simulate the freezing conditions, the cracked samples, prior to any water submersion, were placed into a freeze-thaw chamber. Through air flow, the temperature was precisely and gradually varied from 0 °C to -5 °C. The time of one cycle was 24 hours (Figure 42). The samples were left at the chamber for 14 days (i.e., 14 cycles).

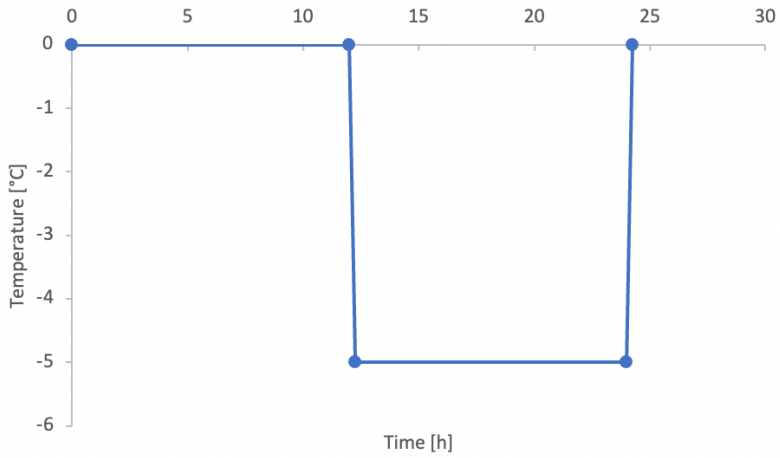

Figure 42. The temperature course of one freezing cycle.

Although such conditions do not necessarily correspond completely to reality, they are sufficiently testing a range of frequently occurring values in a relatively short test time. After the below-zero temperature cycles in the chamber, the samples were taken out and placed into water-filled containers in ideal conditions (as described above) for 28 days. As in the previous case, only specimens with protective polymers (i.e., BAC_SAP and BAC_PVA) and control (CTRL) were used.

11.3.3 Test methods

11.3.3.1 Consistency and mechanical tests

To determine the suitability of the mixture design, cement flow table test (Annex 3.1) and three-point bending and compressive strength test (Annex 3.2) were carried out. Both measurements were performed only on non-bacterial samples as no noticeable influence of bacteria on the consistency/compressive strength was expected.

11.3.3.2 Visual inspections of the crack-healing

The maximum sealed crack width was selected as the basic indicator of the self-healing potential. Through this value, the extent of the crack sealing can be easily compared through the individual series without the need for uniformed damages. The average maximum healed crack width (Δw_{max}) was determined as:

$$\Delta w_{max} = \frac{\sum w_{max}}{n}, \qquad \textit{Eq. 21}$$

where w_{max} is the maximum crack width that was sealed in each specimen and n is the number of specimens in each series.

In order to document the development of the crack sealing, all cracked reinforced specimens were subjected to high-resolution photography at the beginning of the healing period, and after 28 days in the respective environment. To obtain further information about the crack-closure, selected specimens were also additionally scanned with a 3D scanning optical microscope.

11.3.3.3 Dynamic Young's modulus recovery

The crack-sealing in the bio-based concrete primarily aims to the extension of the structure's durability, thus improvement of the material's watertightness through reduction of the crack area. However, recovery of mechanical properties would surely be a welcome side effect. Furthermore, the information about the changed properties may appropriately supplement the information obtained from the visual assessments.

In this paper, the dynamic modulus was measured on all the reinforced specimens before cracking, after the cracking and after the healing period. For the quantity evaluation, the Resonance Frequency dynamic methodology was applied.

The Resonance Frequency dynamic method is a non-destructive test for determination of dynamic modulus (E_d) based on the responses obtained from a vibrating signal induced in the specimen. The resonance frequency of the specimen, which produces the maximum amplitude of vibration, is then used to calculate the corresponding E_d value [31]. For the evaluation, the Brüel & Kjaer assembly (measurement station type 3560-B-120, type 4519-003 acceleration transducers, an 8206 impact hammer type, and a computer) was used.

The dynamic Young's modulus was evaluated based on the longitudinal natural frequency of the samples as

$$E_{d,l} = \frac{4lmf_l^2}{bt}, \qquad \textit{Eq. 22}$$

where $E_{d,l}$ is the dynamic Young's modulus [Pa], l is the sample length [m], m is the sample mass [kg], f_l is the basic longitudinal natural frequency of the sample [Hz], b is the sample thickness [m], and t is the sample height [m].

11.3.4 Mechanical strength and consistency results

11.3.4.1 Consistency

The comparison (Table 18) between the spreading of the control mix (CTRL) and mix containing SAP (CTRL_SAP) shows that the applied amount of extra mixing water (15 g distilled water per 1 g SAP) leads to a paste with consistency almost identical to the control one. Thus, the SAP liquid uptake in this specific mix design is close to the "extra mixing water" value which was applied. However, it must not be forgotten that due to the extreme sensitivity of the SAP absorption to the ionic composition of the soaking solution, the results might vary greatly with different w/c ratio or nutritive additions (calcium lactate and yeast extract).

The addition of PVA water solution led to appreciably more flowable paste compared to the control (Table 18). This result contradicts with most studies where the PVA addition generally caused increased viscosity but reduced consistency [166]. However, the studies dealing with the water-soluble cementitious composites frequently use much lower w/c ratios (close to 0.3) [165]. Thus, the results cannot be compared directly as the overall water content might influence the PVA behaviour in the material greatly.

Table 18. The results of the flowability test - the initial spreading diameter and final spreading diameter after the dropping.

Mix type	Initial spreading [mm]	Final spreading [mm]
CTRL	80	160
CTRL_SAP	80	159
CTRL_PVA	125	183

11.3.4.2 Tensile and compressive strength

The mechanical tests revealed several important aspects of the cementitious composite with polymer additions applied in this study. The mean values of the measured quantities are shown in Figure 43. Firstly, the proposed dosage of the nutritive compounds (3 % wt. of cement of calcium lactate and 0.45 % wt. of cement of yeast extract) proved to be suitable as the compressive and tensile strength reached sufficiently high values (mean values 39.4 MPa and 6.4 MPa, respectively).

The series CTRL_SAP evinced satisfactory behaviour in tension. Its tensile strength reached slightly higher values compared to the control mix (the mean value about 7% greater). On the other hand, the applied alterations of the mix caused rather significant drop in the compressive strength. The mean value of the compressive strength was about 30% lower compared to the control.

The addition of 1 % wt. of cement of PVA (in the form of 16% water solution) in the series CTRL_PVA resulted in a drastically weaker material in both cases. The tensile strength reached only 44% of the control mix strength, the compressive strength as low as 21%. This finding contradicts with the results presented elsewhere as generally, the compressive strength decreased similarly as in our case, but the tensile strengths tended to be improved [167].

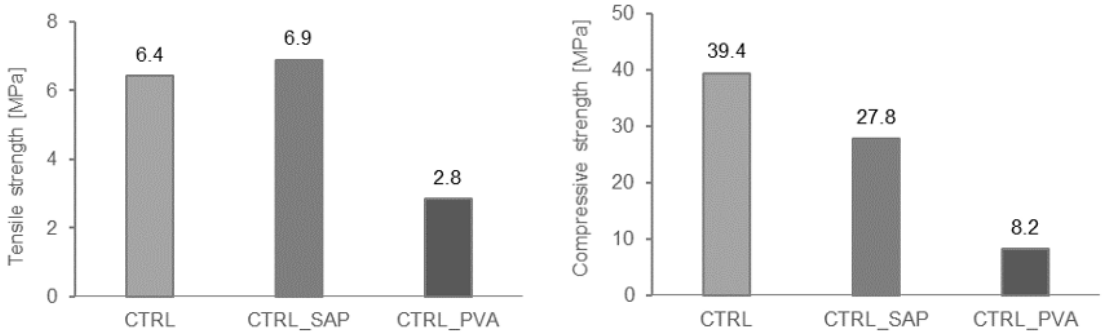

Figure 43. The mean values of tensile (left) and compressive (right) strengths of the non-bacterial mixes obtained through the mechanical tests

11.3.5 Visual inspections of the crack healing efficiency

In this work, we sought to establish the applicability of the proposed bio-based self-healing concrete in other than ideal in-vitro conditions, thus extending the scope of the majority of earlier studies. In Figure 44, for the sake of completeness, all values of the average maximum sealed crack width (Δw_{max}) that could be identified in each series are summarized.

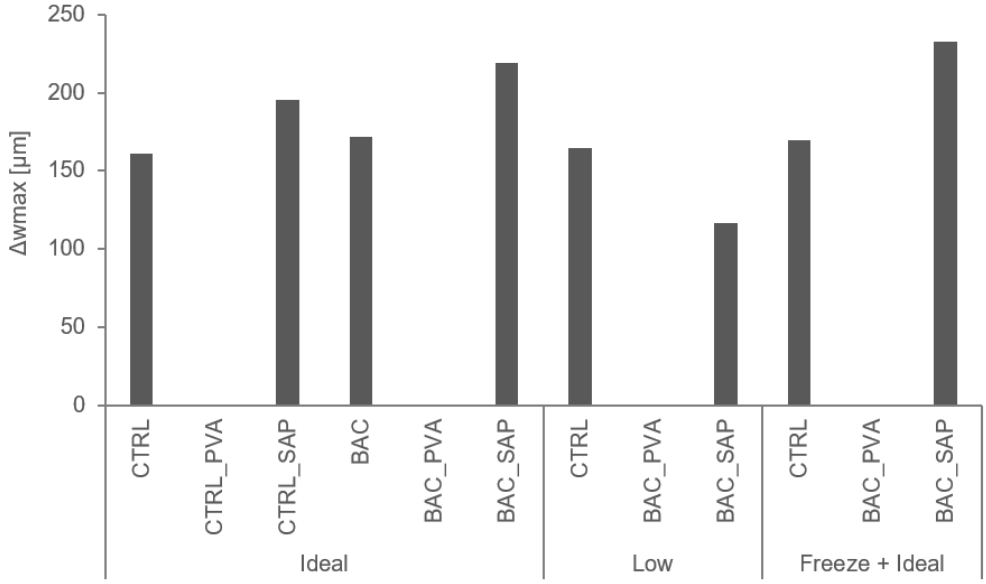

Figure 44. An overview of the average maximum healed crack widths (Δw_{max}) in each series.

11.3.5.1 Healing at ideal temperature

The widest range of the cement composite mix designs was subjected to the healing in ideal conditions (i.e. room temperatures as described in the Methodology section). In general, the data suggest that detectable crack-sealing took place in all of the prepared series except the ones containing liquid PVA (Figure 44). Further, in Figure 45 and Figure 46, a selection of the high-resolution photography results is provided.

In the reference series (CTRL), the value of Δw_{max} reached 161 µm. As in the CTRL series no enhancement of the self-healing capacity was applied, this value can be considered achievable through the natural autogenous crack-sealing ability of the cementitious material in this study.

A slightly higher value (172 µm) was recorded when bacterial spores without any protection (BAC) were incorporated into the cementitious composite. This would indicate that in this study, the natural autogenous crack-sealing potential could be increased by the bacteria-driven $CaCO_3$ precipitation by around 7%.

In the ideal conditions, the widest crack parts were sealed in the case of the SAP addition. In the composite with SAP alone (CTRL_SAP), the Δw_{max} increased to 195 µm. When a combination of SAP and bacterial spores was applied (BAC_SAP), the Δw_{max} reached as high as 219 µm. These results would indicate the overall positive impact of the SAP addition to the self-healing mechanisms as mentioned in the Introduction.

Furthermore, the difference between the series with only SAP and SAP-bacteria combination was higher (around 12%) compared to the difference between the reference series (CTRL) and series containing the unprotected bacteria (BAC). Thus, these results may suggest the possible SAP protective potential as it seems to improve the biocalcification process itself.

In this study, as mentioned previously, the self-healing potential of PVA-based cement composite series (CTRL_PVA and BAC_PVA) showed to be completely disappointing as no crack-sealing was detectable in the case of the liquid PVA addition. This result is somewhat surprising, as the PVA presence seems to not only inhibit the biocalcification, but also the natural autogenous self-healing.

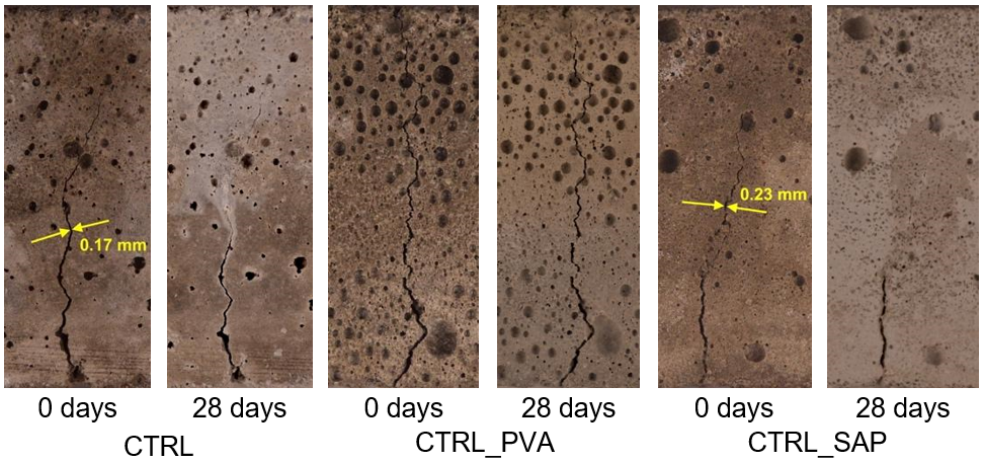

Figure 45. High-resolution photography before (0 days) and after the healing period (28 days) in ideal conditions of the non-bacterial samples. The maximum healed crack width on the individual samples is marked.

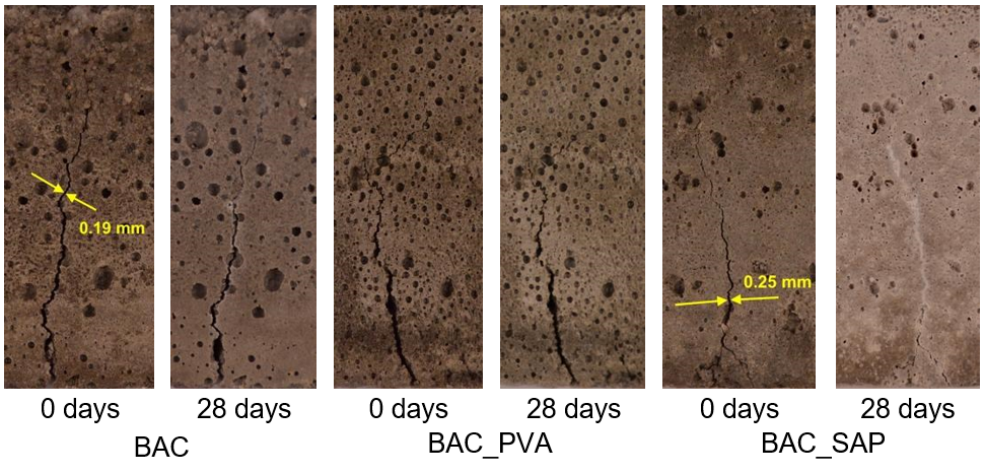

Figure 46. High-resolution photography before (0 days) and after the healing period (28 days) in ideal conditions of the bacterial samples. The maximum healed crack width on the individual samples is marked.

In Figure 47, details of selected cracked specimens after the healing period are presented. The images are in line with the previous findings. In the case of BAC and BAC_SAP, the cracks are almost completely sealed with white crystalline precipitates. In the cracked BAC_PVA specimen, some formation of the precipitates can be also seen on the crack surfaces; however, closing of the crack was not achieved.

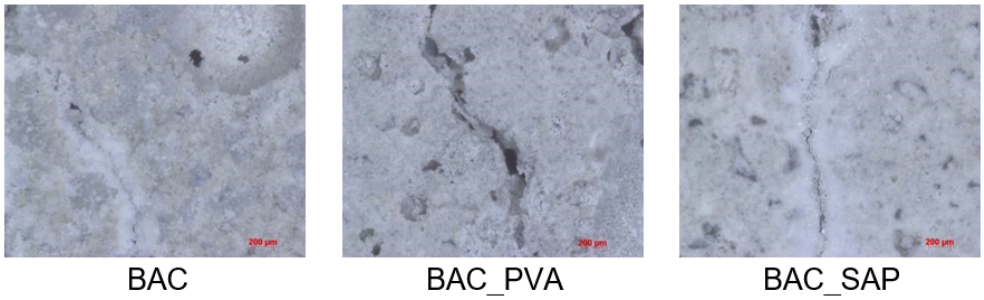

Figure 47. Images obtained using a 3D scanning microscope.

11.3.5.2 Healing at low temperature

As previously outlined, the problematic functionality of the bio-based self-healing concrete at lower temperatures was frequently mentioned in earlier studies. In our case, the findings are in line with the pessimistic presumptions (see Figure 44 for complete overview and Figure 48 for selected cracks).

In the 10°C environment, the autogenous crack-sealing detected in the case of CTRL did not noticeably differ from the values achieved in the ideal conditions (Δw_{max} = 165 µm). Interestingly, in the BAC_SAP series, the Δw_{max} dropped to 117 µm. Thus, it seems that not only the bacteria-driven biocalcification was limited at low temperatures as expected, but also the results indicate that the positive impact of SAP to the self-healing may be inhibited by the temperature as well. Further, it seems that the SAP at low temperatures possibly even limits the natural autogenous crack-sealing capacity as the Δw_{max} was even lower by 30 % compared to the control series.

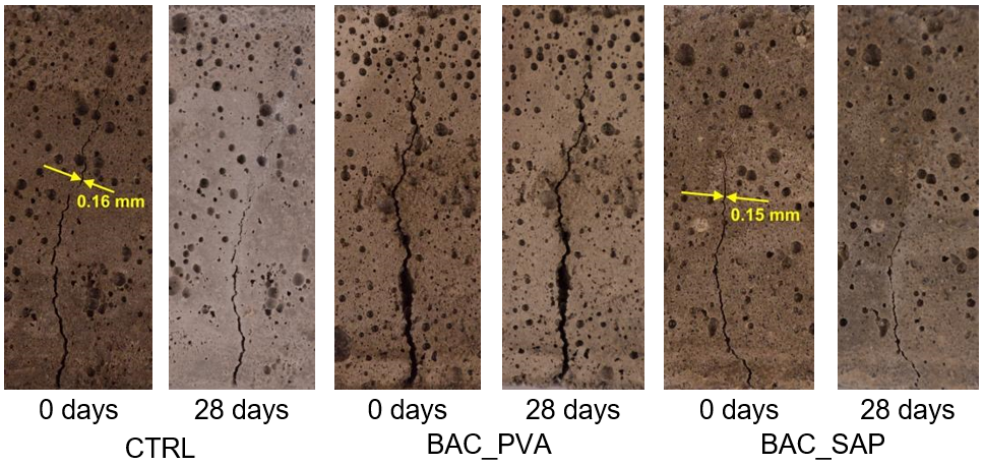

Figure 48. High-resolution photography before (0 days) and after the healing period (28 days) at low temperature of the bacterial (BAC_PVA and BAC_SAP) and non-bacterial (CTRL) samples. The maximum healed crack width on the individual samples is marked.

11.3.5.3 Healing at ideal temperature after freeze-thaw cycles

From Figure 44 it can be seen that, interestingly, the Δw_{max} reached in both CTRL and BAC_SAP even slightly higher values compared to the series without the freeze treatment (170 and 233 µm, respectively). However, the difference between the two mentioned series remained almost identical in both environments i.e., around 35% increase in the case of BAC_SAP. Thus, the bacteria viability was not negatively affected by the freezing cycles, possibly thanks to the SAP that served as a sufficient protective method. Consistently with the previous results, even after the freeze treatment, no crack-sealing could be observed in the series containing liquid PVA as illustrated in Figure 44 and Figure 49.

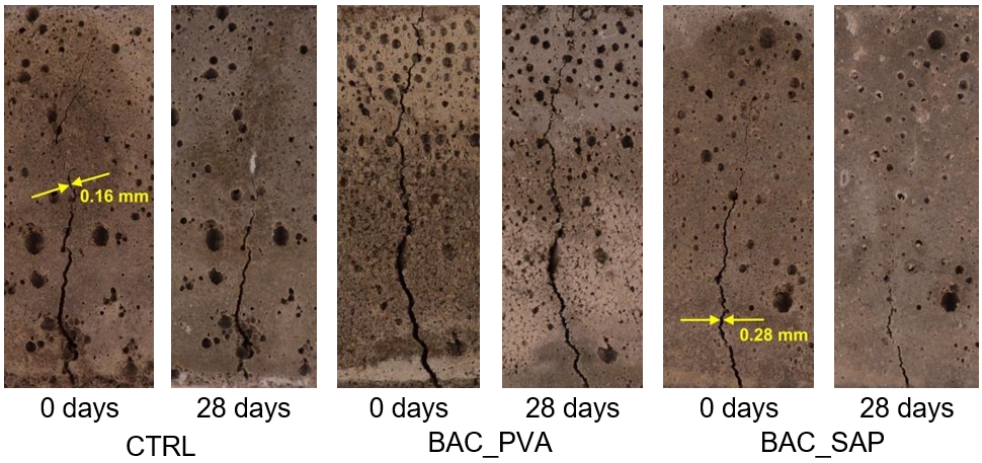

Figure 49. High-resolution photography before (0 days) and after the freeze cycles and healing period (28 days) in ideal conditions of the bacterial (BAC_PVA and BAC_SAP) and non-bacterial (CTRL) samples. The maximum healed crack width on the individual samples is marked.

11.3.6 Dynamic Young's modulus recovery results

In Figure 50, the mean values of $E_{d,I}$ evaluated from longitudinal vibration measured on the specimens before cracking can be seen. These values more or less correspond to the tendencies noticeable from the mechanical tests – the addition of PVA generally caused drop of the monitored quantity, whereas the SAP series values were around the control values. After the controlled cracking, the value of $E_{d,I}$ in all of the series was zero as expected.

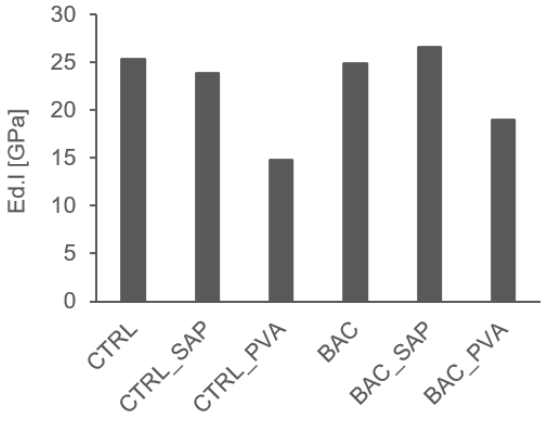

Figure 50. The mean values of the dynamic modulus of elasticity E_d measured on the uncracked specimens.

Measurements after the end of the healing period were far from complete as it was possible to detect the longitudinal frequency only for a fraction of the samples; for the rest $E_{d,l}$ remained zero. Overview of all the successfully measured samples is given in Tab. 2. Provided that the measurement of the longitudinal frequency could be accomplished only if the filling of the crack was sufficiently rigid and solid, the data would suggest that the combination of bacteria and SAP leads to the most reliable crack-sealing as the majority of measurable samples was from the BAC_SAP series in all of the temperature conditions. Further, the recovery rate (healed/uncracked specimen) seemed to be consistently the highest in the case of BAC_SAP series (as much as 51%).

Table 19. Values of Ed measured on uncracked specimens, after cracking, and after the healing period. Recovery represents the ratio Healed to Uncracked values.

Environment	Specimen n.	E_d [GPa]			Recovery
		Uncracked	Cracked	Healed	[%]
Ideal	CTRL 1.3	26	0	9	35
	CTRL_SAP 1.1	23	0	5	23
	BAC 1.1	25	0	8	32
	BAC 1.2	25	0	10	38
	BAC 1.3	24	0	6	25
	BAC_PVA 1.1	19	0	6	33
	BAC_SAP 1.1	27	0	10	39
	BAC_SAP 1.2	27	0	10	38
	BAC_SAP 1.3	26	0	13	51
Freeze	CTRL 3.1	27	0	8	31
	BAC_SAP 3.1	27	0	9	34
	BAC_SAP 3.2	27	0	6	22
Low	BAC_SAP 2.2	26	0	7	27

11.3.7 Discussion

In this paper, two different compositions of the bio-based self-healing concrete were proposed and investigated. Firstly, the materials characteristics of the proposed cementitious composites were determined. The flowability table test showed that when enriching the bio-based cement composite with SAP, a dose of extra mixing water (15 g distilled water per 1 g SAP) ensures preservation of the paste workability. The test further revealed that addition of PVA (1% wt. of cement) leads to a more flowable paste compared to the control.

The performed mechanical tests provided information about the strengths of the proposed cement composites. According to the 3-point bending test, the addition of 0.5% wt. of cement of SAP and extra mixing water lead to improvement of the material's tensile strength. On the other hand, the addition of liquid PVA caused a significant drop of the tensile strength. The compressive test showed that the cement composite containing SAP in the applied dosage is a slightly weaker material in compression compared to the control. In the case of PVA application, similarly to the tensile strength, the compressive strength was lowered dramatically compared to the reference in our study.

The main aim of this paper was to provide comparison of the crack-sealing potential of the bio-based self-healing concrete containing SAP/PVA in other than ideal conditions. First of all, however, it should be noted that the overall efficiency of the crack closure in this experiment is generally lower compared to the values reported elsewhere. In our case, the maximum healed cracked width was around 300 µm (BAC_SAP), whereas other studies described sealing of cracks with widths up to 400-700 µm [94], [114]. However, we must take into consideration that even

scattering of the autogenous crack-sealing itself throughout the studies is considerable. This phenomenon shows how complex it is to quantify the material's efficiency, as it is influenced by a large number of, in most cases, volatile factors (such as mix design, w/c ratio, state of the applied bacteria, possibly the cement chemistry etc.).

At the ideal temperature, the visual investigations indicated that SAP may increase the natural autogenous crack-sealing capacity as the observed value Δw_{max} was around 20% higher than control. This finding is in line with the results presented elsewhere [104]. Further the SAP presence seemed to improve the biocalcification efficiency based on the visual inspections – the Δw_{max} of bacteria-SAP composite reached around 35% higher value compared to control. This conclusion was also presented in an earlier study [163]. The investigation of dynamic modulus recovery indicated similar tendencies at the ideal temperature. The majority of the cracked specimens in which the value $E_{d,I}$ could be measured belonged to the BAC_SAP series.

Further research should be focused on the bacteria-SAP combination in conditions with a lower/inconsistent water supply. In this environment, the positive impact of SAP on autogenous crack-sealing and biocalcification might be even more pronounced as the SAP absorption capacity could ensure the needed moisture for both of the self-healing mechanisms.

Interestingly, in our study, the SAP had a slight negative impact on the self-healing at 10 °C based on the visual investigation. Although it is possible to assume that the biocalcification process was completely inhibited at this temperature, the SAP sample showed even worse results than the control. However, this finding completely contradicts with the dynamic modulus measurements as $E_{d,I}$ could be recorded only on the specimen containing bacteria and SAP.

As to the knowledge of the authors, this experiment is first of its own kind, and the mechanisms of the polymer's functionality in the cement composite at low temperatures are unknown. However, the findings provide an interesting indicator of what the future research should be focused on in terms of the SAP impact on the autogenous self-healing.

Unique results were obtained by research of the self-healing in the proposed materials after freeze cycles. In the case of SAP-bacteria composite, the visual inspections showed that the exposure to temperatures below zero did not result in any decrease in the crack-sealing capacity. After 28 days of healing in ideal conditions, the detectable crack-sealing was similar (or even higher) to the series non-treated by the freeze cycling. Similar results were indicated by the dynamic modulus recovery measurements as, again, the majority of successfully measured specimens were from the BAC_SAP series.

In our study, the impact of liquid PVA to the self-healing was disastrous in all of the healing environments. Not only it seems to inhibit the biocalcification process, but also not even any noticeable autogenous crack-sealing occurred in any of the monitored samples. The mechanism behind the negative impact is yet to be discovered as no records of its application in self-healing concrete was found in the existing literature. A possible explanation could lay in a decreased level of cement hydration caused by the polymer. This would also correspond with the results of mechanical characteristics as both the tensile and compressive strength were significantly lower compared to the control. However, further research dealing with this issue would be needed. The main focus should be on the procedure for preparing the mixture with PVA.

11.3.8 Conclusions

In the current study, the combination of bacteria *Bacillus pseudofirmus*, nutritional compounds, and SAP or PVA was applied in cement composite in order to evaluate the biologically enhanced

material's self-healing potential in various healing conditions. The following conclusions can be drawn based on the current experimental investigation:

- SAP in all probability has a positive impact on the natural autogenous crack-sealing.
- In this paper, the SAP addition seemed to improve the biocalcification process, thus the bacteria driven crack-sealing.
- The SAP functionality might be limited at lower temperatures; however, more research on the exact mechanism is needed.
- The efficiency of the proposed self-healing cement composite containing the combination of SAP and bacterial healing agent did not seem to be affected by the freeze cycles.
- The application of liquid PVA, given the mixing procedures, turned out to be unsuitable from the point of view of both material characteristics and self-healing efficiency. Other mixing procedure such as addition after mixing water should be examined in future research.

Conclusions

This thesis was devoted to a new and promising material – self-healing bio-based concrete that is sure to make a statement in the age of sustainable construction. The thesis was divided into two parts. In the first, the author provided a thorough literature review and presented a vast number of studies that have been carried out, mainly in the last decade. On this basis, the author then set out the objectives that she would like to achieve in the experimental part of the thesis. The experimental part of the thesis presented a considerable number of experiments that the author had conducted or participated in and that covered all aspects of the novel material. The preparation and organisation of the experiments was demanding mainly due to the interdisciplinary nature of the research that required close cooperation of specialists from different fields: concrete technology, microbiology, chemistry, microscopy and programming. It can be stated that the objectives in both parts of the thesis have been met and the thesis extends the current state-of-the-art.

In the experimental part of the work, all the predetermined objectives were achieved. To recapitulate, the objectives were:

- To identify a suitable bacterial species and describe its behaviour. Several bacterial strains (*Sporosarcina pasteurii, Bacillus pseudofirmus, Bacillus cohnii*) were compared. Their ability to grow under optimal and sub-optimal conditions was determined, and the growth of the selected bacterium was described by a mathematical model. Calcite production by the species itself was evaluated using advanced techniques. Based on these thorough investigations, the bacterial strain *Bacillus pseudofirmus* was selected.
- To analyse the bacterially induced calcite precipitation in greater detail. The mechanism of BICP was approached by means of optical and electron microscopy complemented by elemental analysis, and the autogenous and autonomous crack healing was clearly distinguished.
- To select appropriate nutrients from the perspective of both bacteria metabolism and mechanical properties of concrete. A comparison of the most frequently applied nutrients was carried out. Their impact on mechanical and rheological properties of cementitious composite was determined and the most suitable one (calcium lactate) was selected.
- To suggest and investigate possible protective carriers for bacteria. Several different methods of bacterial protection were tested, ranging from those already known (LWA, SAP) to completely new ones (liquid PVA and PVA nanofibres). SAP was recommended as the most promising carrier based on our current results.
- To prepare own experimental samples from biological self-healing concrete based on previous optimizations of individual components and determine its crack-sealing potential. Several mixes of self-healing concrete were designed and tested. Their material parameters were determined, and the ability of the material to autonomously seal cracks was evaluated using optical and electron microscopy and dynamic Young's modulus measurements. It was confirmed that mixes containing SAP had the best performance in both crack sealing and mechanical properties tests.

The experiments carried out yielded many insights. Now, let us summarize the main conclusions that have been made.

It is clear that bacterially induced calcite precipitation has huge potential in the field of research of cementitious composites. However, it must be realised, and this was evident in our experiments, that the closer we get to reality, the more problematic this new material becomes. The results of other authors published in the literature might be more encouraging than our results, but this should be attributed to two substantial facts. First, they were mostly obtained in sterile laboratory conditions, and second, the studies were narrowly focused on one aspect of the material.

In this thesis, we struggled to provide a broad and realistic view of the technology of bio-based self-healing concrete and to create our own opinion of its perspectives. In our experiments, it has been shown that selected bacteria accompanied by appropriate nutrients lead to the formation of calcite. The selected bacteria were able to metabolise even at low temperatures or after exposure to freeze-thaw cycles. When the self-healing agent prepared in the laboratory was applied externally on concrete samples, the benefit of the bacteria was quite evident. However, the moment we leave the sterile and controlled conditions of the microbiology laboratory and start to produce a material that more closely resembles its final form in concrete laboratories, the functionality of the material becomes somewhat questionable.

Annex: Materials and methods

Chapter 1: Materials

1.1 Media used for culturing/sporulation of bacteria

Culture medium for *Bacillus pseudofirmus* Culture medium consisted of 1 g lamb extract, 2 g yeast extract, 5 g pepton, 5 g NaCl, and the pH 9.7 was achieved by Na-sesquicarbonate solution after sterilization in an autoclave. The Na-sesquicarbonate solution consisted of $NaHCO_3$ 4.2 g, Na_2CO_3 anhydrous 5.3 g in distilled water 100 ml, the sterilization was performed by filtration.

Alkaline medium for *Bacillus pseudofirmus* Alkaline medium enhancing sporulation contained 0.2 g NH_4Cl, 0.02 g KH_2PO_4, 0.225 g $CaCl_2$, 0.2 g KCl, 0.2 g $MgCl_2.6H_2O$, 0.01 g $MnSO_4.2H_2O$, 0.1 g yeast extract, 0.516 g citric acid tridosium salt, 0.42 g $NaHCO_3$ and 0.53 g Na_2CO_3 per liter of distilled water.

Culture medium for *Bacillus cohnii* Culture medium consisted of beef extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l, NaCl 10 g/l, and the pH 9.7 was achieved by Na-sesquicarbonate solution after sterilization in an autoclave. The Na-sesquicarbonate solution consisted of $NaHCO_3$ 4.2 g, Na_2CO_3 anhydrous 5.3 g in distilled water 100 ml, the sterilization was performed by filtration.

Culture medium for *Sporosarcina pasteurii* Culture medium consisted of beef extract 10 g/l, peptone 10 g, NaCl 5 g/l, urea 20 g/l, and the pH 9.4 was achieved by Na-sesquicarbonate solution after sterilization in an autoclave. The Na-sesquicarbonate solution consisted of $NaHCO_3$ 4.2 g, Na_2CO_3 anhydrous 5.3 g in distilled water 100 ml, the sterilization was performed by filtration.

Chapter 2: Methods – microbiological research

2.1 Cultivation and sporulation of bacteria

To culture respective bacteria, liquid cultures were inoculated with viable bacteria or spores and incubated at room temperature (25 °C ± 2 °C) in Erlenmeyer flasks on shaker tables at 160 rpm from 5 to 7 days.

To obtain spores of the given bacteria, grown culture was inoculated into alkaline medium and collected by centrifugation and washed in physiological solution. The pellets with spores were kept in a fridge at 5 °C for further use.

2.2 Bacterial growth curve using ELISA measurement

One of the methods of measurement of the bacterial growth was photometry - a widely used measurement technique in biochemistry that takes advantage of the fact that many substances absorb electromagnetic radiation. The amount of light of a particular wavelength that a substance absorbs depends on the concentration of the substance. Our measurements used the ELISA method (using Opsys MR, Dynex), where the measurement is performed by a vertical beam on plates with inoculated media samples (200 µl). A wavelength of 630 nm was used for the measurement. The obtained values of the so-called optical density (ω or OD) were then used to determine the bacterial growth rate over time - the higher the bacterial concentration, the higher the optical density.

2.3 Bacterial growth curve using BOD

The biological oxygen demand (BOD) method is based on measuring the amount of oxygen consumed by aerobic microorganisms. In our studies, this method was used for the determination of the bacteria metabolic activity. BOD values are most commonly expressed in milligrams of oxygen consumed per litre of the sample during a five-day incubation. In our experiments, BOD analysis bottles were filled with 100 ml of fresh medium tempered to the selected temperature. Subsequently, 1 or 2 ml of bacterial inoculum was added. The bottles were sealed and maintained at given cultivation temperature. Measurements were taken continuously (i.e., once every 0.5 h for 5 days).

Chapter 3: Methods – concrete research

3.1 Cement flow table test

The cement flow table test was used to determine the consistency of the fresh mix according to a relevant standard [168]. To perform the test, a cement flow table, metal cone, choke, and meter are needed.

Before each test, the plate and the inner surface, including the edge of the metal cone, was cleaned with a damp cloth and wiped dry. Their surface was painted with a thin layer of mineral oil. The metal cone was placed in the centre of the flow tabletop and filled with mortar in two layers. Each layer was compacted with 10 light strokes of the choke. Excess mortar was wiped off and the free surface of the slab wiped clean and dry. After about 15 s, the cone was slowly lifted vertically upwards, and the mortar was spilled on the tabletop in 15 strokes at a constant frequency of one stroke per second. The diameter of the spilled mortar was measured in two mutually perpendicular directions with a gauge. The result was given in mm to the nearest 1 mm.

3.2 The flexural and compressive strength

To determine the flexural strength of the material, a three-point bending test was performed on 40x40x160 mm^3 mortar specimens using a calibrated electric press machine. The bending tests were run in a deflection-controlled mode with a loading speed 0.3 mm/min, and the maximal load values were recorded and analysed using a controlling software (SMAPS).

The final flexural strength of the tested mix designs was determined according to the following equation:

$$f_{cf} = \frac{3F_{max}l}{2bh^2}, \qquad \textit{Eq. 23}$$

Where f_{cf} is the flexural strength; l is the distance between support rollers; *b* and *h* are the width and height of the tested specimen, respectively; and F_{max} is the maximum applied load.

To determine the compressive strength, the pressure test was performed on the specimen halves that were created after the three-point bending test according to relevant standards. The specimens were evenly loaded with pressure (2.5 kN/s) using a calibrated hydraulic press machine, and the mode of failure was observed to exclude incorrectly damaged specimens. The specimens were in all cases tested in the direction of casting. The maximum applied load was recorded and analysed using a controlling software (SMAPS).

The final compressive strength of the tested mix designs was determined according to the following equation:

$$f_{cc} = \frac{F_{max}}{b^2}, \qquad \textit{Eq. 24}$$

Where f_{cc} is the compressive strength; b is the dimension of the loading plates; and F_{max} is the maximum applied load.

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