Technical University of Munich



Water repellency and durability of mortar enriched with
bacterial additives

A thesis presented by

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## TUM School of Engineering and Design

## Water repellency and durability of mortar enriched with bacterial additives

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# Summary

Materials derived from bacterial sources represent a new class of additives in the field of cementitious building materials, which has become increasingly popular in recent years. In addition to being non-hazardous, thus eco-friendly, bacterial additives can strongly alter various properties of cementitious building materials; therefore, they can contribute to the development of more sustainable cementitious materials in different ways. To date, several studies have been published showing promising results at laboratory scale; however, application-driven studies, which could prove the functionality of those systems in large-scale applications, are still rare. Furthermore, the exact mechanism of these bacterial additives is often not known.

In this work, various bacterial additives were tested for their effect on mortar and evaluated regarding their availability, cost, and processability. By doing so, it could be shown that a variety of different bacterial additives (produced by different bacterial strains) could be used to hydrophobize mortar, however, with varying efficiencies. In addition, a hybrid mortar formulation containing the most promising bacterial additive, freeze-dried bacterial biofilm produced by the bacterial strain *Bacillus subtilis* 3610, was developed further to improve application-specific parameters (tensile and compressive strength) which had suffered from the addition of the bacterial additive. This was achieved by enriching the formulation with common supplementary cementitious materials (SCMs); thereby, both tensile and compressive strength could be greatly improved, while the hydrophobic properties brought about by the addition of the freeze-dried bacterial biofilm powder, were maintained nearly completely. Furthermore, the properties of the obtained material could be directly linked to the pore structure of the material, which was found to greatly influence both, its ability to suppress the capillary water uptake as well as its mechanical strength. Finally, the long-term stability of this improved formulation was tested. It could be successfully shown that the material is intrinsically stable against a wide range of harmful environmental influences, thus proving its functionality under environmental conditions.

The results discussed herein may be of great value for the development of future generations of more sustainable cementitious building materials containing bacterial additives. With novel solutions for more sustainable cementitious building materials urgently needed, further research on bacterial materials should help to fully exploit their potential.

# Contents

# 1. Introduction

Cement is an inorganic, nonmetallic powder that has little to no use as a building material on its own. However, when mixed with water, cement forms a paste that sets and hardens as a result of a chemical reaction which is referred to as hydration.[1] The hydraulic hardening, strength development, and durability of the material can be primarily attributed to the formation of the main hydration product calcium silicate hydrates (CSH), and – to a smaller extent – other hydration products such as calcium aluminate (CAH) or calcium aluminate sulfate hydrates (Afm, Aft).[2] After hardening, which can take place either underwater or in air, the resulting material can preserve its strength and durability under various conditions. These properties make cement the key ingredient for various cementitious materials, where cement is used as a binder to hold together sand or coarser aggregates. Depending on the choice of additive, these cementitious materials are sorted into different categories, *e.g.,* mortar (with a maximum particle size of 4 mm) or concrete (with particle sizes >4 mm), which differ in material properties such as workability or compressive strength.

**Cement production and its environmental impact**

Although cementitious materials were already used more than 2000 years ago by the Romans,[3] modern cement was developed in the 19th century, when the hydraulic properties of cement were rediscovered and the commercial production of cement started. Over time, the cement production process was further improved.[4] Nowadays, cement is produced in a so-called dry process using rotary kilns. Here, the raw materials (mainly limestone and clay) are extracted as sources of calcium carbonate and silicon dioxide, processed, and mixed at defined ratios with smaller amounts of aluminum oxide and iron oxide. This mixture is then crushed into grains of ~90 μm, and the resulting 'raw meal' is burned in a rotary kiln at temperatures of up to 1,450 °C to produce the so-called cement clinker (**Figure 1**).
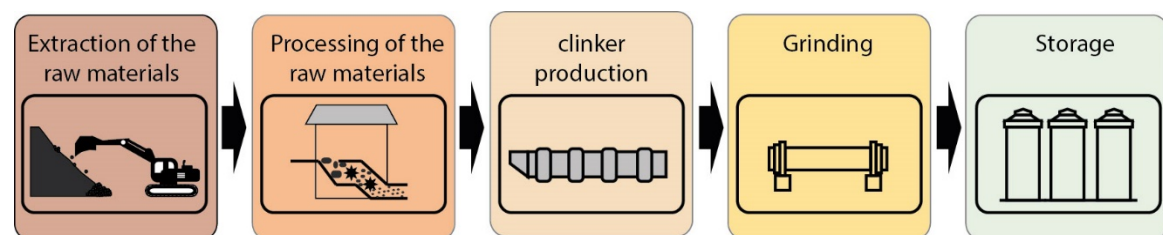


**Figure 1: Simplified process of cement production.** Simplified, the cement production process can be divided into five steps: extraction and processing of the raw materials, clinker production, grinding, and storage.

Cement clinker consists of calcium silicates and calcium aluminates that give cement its characteristic properties. To obtain reactive cement, the cement clinker, together with gypsum to control the setting time of the resulting cement, is then ground into a fine powder. This fine powder can easily be stored in silos or bags and represents the commonly known picture of cement. The production of cement is accompanied by the emission of large amounts of $CO_2$, especially during clinker production. Here, the emitted $CO_2$ not only originates from the burning of fuels but also from the chemical process of converting limestone into calcium oxide. In total, cement production accounts for ~8–10 % of the anthropogenic $CO_2$ emission – and the emitted amount of $CO_2$ has been increasing constantly over the past decades.[5, 6] The dominance of Portland cement as a binder in building materials is a direct consequence of the composition of the starting materials and their availability. Approximately 99% of the earth crust consist of eight elements only,[7] four of which – oxygen, calcium, silicon, and aluminum – together form the three most prevalent components of cement: calcium oxide, silicon dioxide, and aluminum oxide.[8]

**Approaches to reduce the environmental impact of cementitious materials**

For the past decades, extensive research has been conducted to reduce the environmental impact of cementitious materials. Although the manufacturing process of cement is highly energy-efficient,[9] there are still chances for improvements, *e.g.*, by using alternative fuels.[10-12] Moreover, alternative cementitious binders (such as calcium sulfoaluminate cements, alkali-activated binders, or calcinated clays),[9, 13, 14] which can be processed at lower temperatures or contain less lime compared to ordinary Portland cement (calcium aluminate cements),[13] moved into the focus of attention. Yet, traditional Portland cement will continue to be the first choice – at least until those alternative materials are fully developed and available at similar cost and insufficient amounts.[15] Of course, another way to reduce the level of $CO_2$ emission associated with cement production is to reduce the overall cement production, which requires a more targeted way of using cement. This approach has already been followed intensively by integrating so-called supplementary cementitious materials (SCMs) into the mixing design of cementitious materials; indeed, with this approach, the amount of Portland cement used can be reduced.[16] In this context, a variety of both, naturally occurring additives and industrial waste products, have been tested to replace a certain fraction of cement. Examples include fly ash[17-19], blast furnace slag[20], silica fume[21], matakaolin[22-24], and lime stone[25, 26]. Here, the elimination of the clinkering process entails a significant reduction in $CO_2$ emission per ton of

cementitious material.[13, 27, 28] In addition to industrial waste products, also other anthropogenic waste products such as those originating from agriculture were successfully used as SCMs.[29-33] A third approach to mitigate the ecological footprint of cementitious building materials is to increase their durability and resistance towards potentially harmful environmental influences thus extending their service life. In general, cementitious building materials are considered resistant to a variety of external attacks. However, under certain circumstances, even these sturdy materials can deteriorate.[34, 35] Water is a key factor for such destructive mechanisms: damaging substances, which often initiate such attacks can be transported by penetrating moisture. Accordingly, several approaches have been developed to protect cementitious structures from moisture ingress. One common method is the application of additional surface treatments.[36, 37] And indeed, hydrophobic coatings can protect the material well. However, there is also one major disadvantage: once such coatings are damaged, water can be dammed behind these hydrophobic layers. This, in turn, can even accelerate water-driven deterioration processes. Thus, integrating hydrophobic substances into the bulk of the building material with the intention to obtain a water repellent bulk material with intrinsic hydrophobic properties represents a second protection approach.[38-40] Especially in the long term, such a bulk modification could be advantageous; however, the materials used are often expensive and the resulting material may thus no longer be economical.

**Bacterial substances as additives to cementitious materials**

Bacterial biofilms or other bacterial substances may sound like a bad choice as additives to cementitious building materials: the chemical conditions during the cement hydration process (*i.e.*, high pH values and a lack of nutrients) do not represent ideal living conditions for bacteria. Nevertheless, bacteria and bacterial substances have triggered the interest of scientists as these additives may improve the properties of cementitious materials. For instance, biopolymers produced *via* bacterial fermentation[41] (such as welan gum[42-44] or xanthan gum[45]) are well-known viscosity-modifying agents for concrete. A similar shear-thinning behavior was reported for mortar samples that were supplemented with bacterial cells[46] or bacterial cell walls[47], which can both be easily obtained *via* centrifugation of bacterial cultures. Bacterial cell walls were also reported for their ability to improve the mechanical performance of concrete.[48] Here, however, the underlying mechanism is different: whereas viscosity-modifying agents establish their effect due to their composition (bacterial cell walls are composed of peptidoglycans, which are built similarly to biologically derived polysaccharides), bacterial cell walls are known to mediate

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microbially induced calcite formation (MICP). Here, bacterial spores have to be mentioned for their role in microbially induced calcite formation – the basic principle of self-healing concrete, a promising concept aiming at more sustainable concrete.[49-52] Bacterial substances, however, can also be used to achieve bulk modifications in mortar such that it becomes hydrophobic.[53, 54] These examples highlight the large potential that bacterial additives offer for the development of more sustainable cementitious building materials.

In this thesis, different bacterial additives and their effects on mortar are examined. In the first part of this thesis, the impact of both, solid (*i.e.* bacterial biofilms or bacterial spores) and liquid (*i.e.* bacterial cultures) bacterial additives on mortar are investigated. The influence of one particular bacterial biofilm formed by *Bacillus subtilis* 3610, had already been studied previously[53], and an increased wetting resistance as well as a partially suppressed water uptake were observed for those biofilm-enriched hybrid mortar samples. Yet, it remained unclear how the addition of bacterial biofilm affects the mechanical properties of the hybrid mortar. Here, it is shown that a similar hydrophobizing effect – however, with varying efficiencies – can be obtained by supplementing mortar samples with other forms of bacterial additives, *i.e.*, bacterial biofilm (fresh and freeze-dried) and liquid bacterial cultures of three different bacterial strains (*Bacillus subtilis* 3610, *natto*, and B-1). In addition, it is investigated how the addition of these different additives affects the mechanical strength, the hydration reaction, and the workability of the resulting material. It is shown, that freeze-dried bacterial biofilm powder (formed by the bacterial strain *Bacillus subtilis* 3610) outperforms the other tested additives. At the same time, its state of aggregation enables an easy integration into the mixing process of the hybrid mortar material.

The second part of this thesis aims at further improving the mortar formulation containing the best performing bacterial additive, *i.e.*, freeze-dried and ground bacterial biofilm (biofilm powder). An analysis of biofilm enriched hybrid mortar samples and unmodified mortar samples demonstrated that the reduced mechanical strength obtained for hybrid mortar does not result from different hydration products, but is rather a consequence of an increased porosity of hybrid mortar. To remedy the ensuing loss in mechanical strength, SCMs (which partially replace cement) are integrated into the hybrid mortar formulation. This has two positive effects: the mechanical strength is increased, and the environmental impact of the material is decreased. In fact, until a certain threshold is reached, SCMs can be added without compromising the hydrophobic properties the material exhibits as a result of the addition of

biofilm powder. By varying the SCM concentration, the pore structure of the resulting materials can be modulated, which has an influence on both, the mechanical strength and the hydrophobic properties.

Finally, in the last step, the durability of hybrid mortar and SCM-enriched hybrid mortar is tested. It is shown that an improved hybrid mortar formulation containing silica fume exhibits great durability towards the exposure to chemical and thermal stress. Here, the mechanical strength does not suffer from long-term storage under challenging conditions and more importantly, the hydrophobic properties are maintained at nearly all tested conditions.

# 2. Materials and Methods[1]

## 2.1 Microbial additives

### 2.1.1 Bacterial strains

Three different bacterial strains of the species *Bacillus subtilis* were used throughout this thesis. All three strains – *Bacillus subtilis* 3610 (*B. subtilis* 3610), *Bacillus subtilis natto* (*B. subtilis natto*), and *Bacillus subtilis* B-1 (*B. subtilis* B-1) – are non-pathogenic and used without further genetic modification. **Table 1** gives an overview about the used bacterial strains.

Table 1: Overview of the *Bacillus subtilis* strains used in this thesis.

| Bacterial strain | Specification | Source |
|---|---|---|
| *B. subtilis* 3610 | Wild type | Lab of Roberto Kolter (Harvard Medical School, USA) |
| *B. subtilis natto* | 27E3 | Bacillus Genetic Stock Center (BGSC) |
| *B. subtilis* B-1 | Wild type | Lab of Masaaki Morikawa (Hokkaido University, Japan)[55] |

### 2.1.2 Bacteria cultivation

Bacteria were kept in frozen glycerol stocks at –80 °C until they were used. Liquid cultures of all strains were prepared by inoculating a small piece of frozen glycerol stock of the respective bacterial strain in 10 mL of liquid Luria/Miller (LB)-Medium (Carl-Roth, Karlsruhe, Germany; specifications: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, pH-value 7.0 ± 0.2). After incubation at 37 °C and 90 rpm (200 rpm for *B. subtilis* B-1) in a shaking incubator (Sartorius, Göttingen, Germany) overnight (*i.e.,* for 16h), a so-called "overnight culture" (a solution containing a high density of planktonic bacteria) was obtained (**Figure 2a**). Overnight cultures were used within this thesis for biofilm cultivation or as hydrophobizing additive in mortar.

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[1] The following part is adopted from the publications Ertelt *et al.*, ACS sustainable Chemistry and Engineering (2020 and 2021), and Ertelt *et al.* Cement and Concrete Composites (2021).

### 2.1.3 Biofilm cultivation and harvesting

For biofilm cultivation, 100 µL of a freshly prepared overnight culture was plated on (1.5% v/w) agar plates (standard Petri dishes) enriched with Luria/Miller LB-Medium (2.5% v/w) and evenly spread using a *Drigalski* spatula. The liquid bacterial culture was then allowed to dry before the Petri dishes were placed into an incubator, where they were incubated at 37 °C for 24 h to grow bacterial biofilms (**Figure 2b**). Fresh biofilm was harvested using a PDMS-spatula, which allowed for collecting biofilm only while keeping the agar layer underneath intact. The collected biofilm was subsequently collected in commercial PET tubes and stored at –80 °C (if not used immediately).
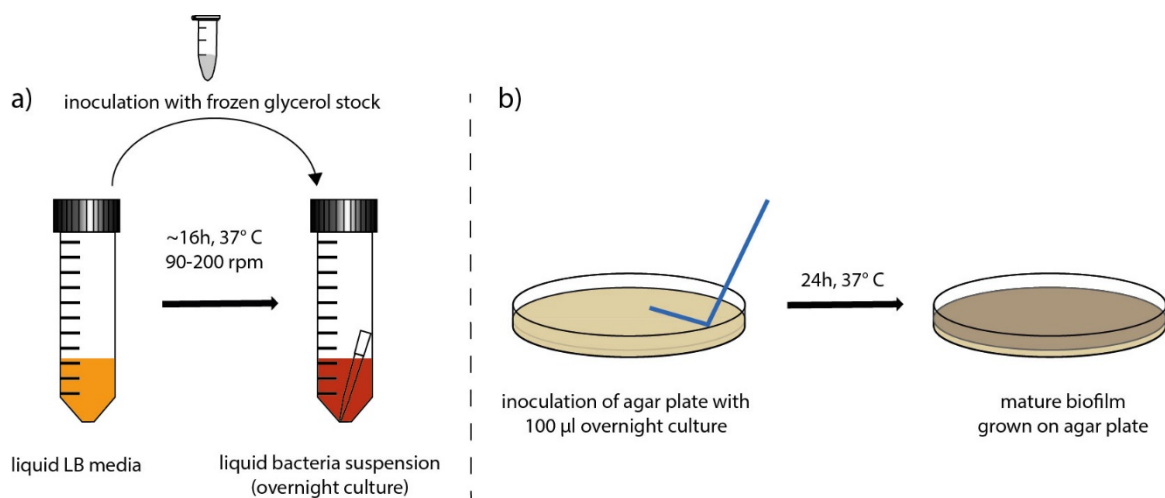


**Figure 2: Schematic overview of the production process of overnight cultures (a) and bacterial biofilm (b).**

### 2.1.4 Biofilm powder

Fresh biofilm is difficult to store and dose properly: thus, different forms of bacterial additives were tested regarding their hydrophobizing effects on mortar. One of them is freeze-dried and finely ground bacterial biofilm (which within this thesis is referred to as "biofilm powder"). To produce biofilm powder, fresh biofilm was stored at −80 °C for at least two hours. Subsequently, the frozen biofilm was freeze-dried for at least 72 h using a lyophilizer (Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). After freeze-drying, the dry biofilm was manually ground into a fine powder with an average particle size of 500 µm. Biofilm powder was subsequently stored in a closed container at room temperature (RT).

## 2.1.5 Bacterial spores

Bacterial spores were tested as hydrophobizing agents in mortar, as they represent a bacterial additive which can be produced at industrial scale. In this thesis, bacterial spores of six different non-pathogenic bacterial strains were used (**Table 2**).

Table 2: Overview of the used bacterial spore variants.

| Bacterial strain | Acronym |
|---|---|
| *Bacillus atrophaeus* ABi05 | Abi05 |
| *Bacillus subtilis* ABi26 | Abi26 |
| *Bacillus licheniformis* ABi53 | Abi53 |
| *Bacillus velezensis* FZB24 | FZB24 |
| *Bacillus velezensis* FZB42 | FZB42 |
| *Bacillus velezensis* FZB45 | FZB45 |

All spore variants were obtained from ABiTEP GmbH (Berlin, Germany) and used as is, *i.e.*, without any further purification or functionalization steps.

## 2.2 Cement and supplementary cementitious materials (SCMs)

Cement is the binder in all cementitious materials. Depending on *e.g.* the desired exposure class or the desired compressive strength, there is a large variety of different types of cement available for different applications. For all experiments in this thesis, ordinary Portland cement (CEM I, 42.5 N, Schwenk Zement KG, Ulm, Germany) was used. All mortar samples were based on the same basic formulation, which contains a 3:1 mixture of CEN standard sand (NORMENSAND GmbH, Beckum, Germany) and cement, distilled water, and (for hybrid mortar samples) the bacterial additive.

In addition to this standard mixture, common SCMs were used in further developed formulations. SCMs are naturally occurring or industrial waste products, that can be used to substitute part of the cement and contribute to the properties of the cementitious material through hydraulic and/or pozzolanic activity.[16]

All used SCMs as well as their chemical composition and specific surface area are listed in **Table 3**.

Table 3: Chemical composition, loss of ignition, and specific surface area of the used SCMs. The specific surface area was determined either according to Blaine (a) or Brunauer-Emmett-Teller (b).

| | | Fly Ash (FA) | Blast Furnace Slag (BFS) | Lime Stone (LS) | Silica Fume (SF) |
|---|---|---|---|---|---|
| Chemical composition [% (w/w)] | $SiO_2$ | 52.04 | 36.20 | 0.51 | 95.55 |
| | $Al_2O_3$ | 23.18 | 12.20 | 0.19 | 0.27 |
| | $Fe_2O_3$ | 7.35 | 1.60 | 0.12 | 0.67 |
| | CaO | 3.40 | 39.30 | 55.43 | 0.52 |
| | MgO | 1.88 | 6.80 | 0.19 | 0.20 |
| | $SO_2$ | 0.36 | 0.09 | 0.01 | 0.13 |
| | $K_2O$ | 3.41 | 0.45 | 0.02 | 0.51 |
| | $Na_2O$ | 1.09 | 0.39 | 0.01 | 0.10 |
| Loss of ignition | [%] | 3.48 | 0.10 | 43.29 | 1.85 |
| Physical properties | Surface area [m²/g] | 2101[a] | 4800[a] | 1588[a] | 21686[b] |

## 2.2.1 (Hybrid) Mortar sample preparation

Two ways of preparing mortar samples were used throughout this thesis. For initial contact angle measurements of new formulations, samples were produced manually in small scale. For all other tests, mortar samples were produced according to DIN EN 196-1[56]. For all hybrid mortar samples containing solid bacterial additives (bacterial biofilm powder or bacterial spore

powder), the additive was mixed with the dry cement. Fresh biofilm was added as an aqueous suspension and was added just like the other used liquid bacterial additives, *i.e.*, bacterial overnight cultures as a partial replacement of the mixing water.

For manually mixed samples, the bacterial additive was added to a 3:1 mixture of CEN standard sand and cement. In the next step, water was added to obtain a water to cement (w/c) ratio of 0.5. Finally, the mixture was stirred mechanically using a paddle mixer for at least 2 min.

To prepare mortar samples according to DIN EN 196-1, an automatic laboratory mortar mixer (ToniMix, Zwick Roell, Ulm, Germany) was used, and the mixing procedure was conducted like described in the norm. For the production of one batch of a mortar formulation according to DIN EN 196-1, quite a lot of the respective bacterial additive is needed (*i.e.*, 1.8 g of biofilm powder). Thus, only promising formulations (identified by their wetting behavior) were further investigated. A key difference compared to the procedure described in DIN EN 196-1 is that, if bacterial additives were used, the casting mold was removed after 48 hours. This modification was applied to take the reduced early strength, caused by the delayed hydration reaction, into account. Throughout this thesis, different casting molds were used, as appropriate for the desired testing: *e.g.* commercial PET tubes (outer diameter 40 mm) for water uptake experiments, flat prisms (1x4x16 cm) to monitor the sulfate resistance and standardized prisms (4x4x16 cm) to determine three-point-bending and compressive strengths.

## 2.3 Techniques to determine hydrophobic properties

To be able to assess the hydrophobic properties of different mortar formulations, the wetting resistance and the capillary water uptake was determined on cured samples.

### 2.3.1 Wetting resistance

The wetting resistance of cured mortar samples was examined *via* contact angle measurements. The contact angle (CA) is a measure for the ability of a liquid (*e.g.* water) to wet a solid surface. It is defined as the angle formed between the solid surface and the line tangent to the liquid/air interface and quantifies the wettability of the surface. CAs can be further divided into static and dynamic contact angles and can be used to distinguish between different wetting states. Static CAs are commonly used to categorize solid surfaces into hydrophilic (CA < 90°), hydrophobic (CA ≥ 90°), and superhydrophobic (CA ≥ 120°) samples.

In this thesis, only static contact angle measurements were conducted. To do so, five 10 µL droplets of double distilled water (ddH$_2$O) were placed onto each sample at different spots. Images were acquired from a lateral view using a digital camera (Flea3, Point Grey, Richmond, Canada). The contact angle was then evaluated from the digital pictures using the image analysis software ImageJ (public domain, ImageJ 1.52n) in combination with a drop-analysis plugin tool (**Figure 3**).



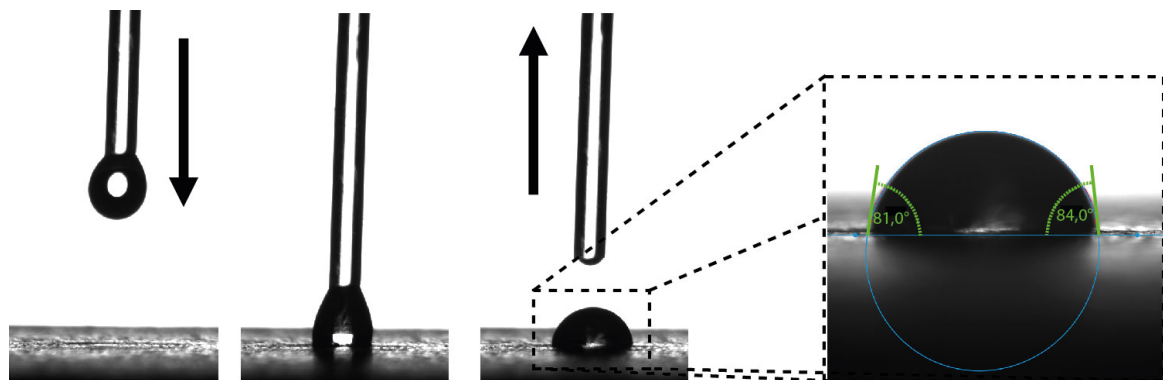**Figure 3: Schematic overview of a contact angle measurement.** For each measurement, a water droplet is placed on the sample surface and the formed contact angle is analyzed.

Automated contact angle measurements were conducted using a drop shape analyzer (DAS25S, Krüss GmbH, Hamburg, Germany). Here, five 4 µL droplets of ddH$_2$O were placed onto the surface of each mortar sample at different spots, and images were acquired from a

lateral view using the built-in high-speed camera (CF04, Krüss GmbH, Hamburg, Germany). The contact angle was then evaluated using the software ADVANCE (Krüss GmbH, Hamburg, Germany).

## 2.3.2 Capillary water uptake

There are different standards to determine the water uptake by capillary forces into cementitious materials. To save material of the different bacterial additives, here, the capillary water uptake was determined in a modified way of the procedures described in (DIN EN 1015-18:2003-03 [57] and DIN EN ISO 15148[58]): The mortar formulation to be tested was prepared according to DIN EN 196-1 (see 2.2.1) and poured into commercial polyethylene tubes (diameter: 40 mm, height: 120 mm), which served as casting molds. After three days of curing, the formwork was stripped, and after 11 additional days of storage at RT (r.h $\leq$ 50%) the mortar samples were coated with a resin (MC-Inject 1264 compact, MC Bauchemie, Bottrop, Germany). 24 h later, the resin was completely dried and one end of the sealed cylinders was cut open to create an open surface which enables water ingress (**Figure 4**). The mortar samples were then dried overnight at 80 °C and weighed before they were immersed into a water bath (water level set to 2 cm, samples placed with the open surface facing down). This weighing step was repeated at defined time intervals, and the amount of water taken up by capillary forces was monitored by determining the mass change of the mortar samples using a micro-scale (TLE 303, Mettler Toledo AG, Greifensee, Switzerland).
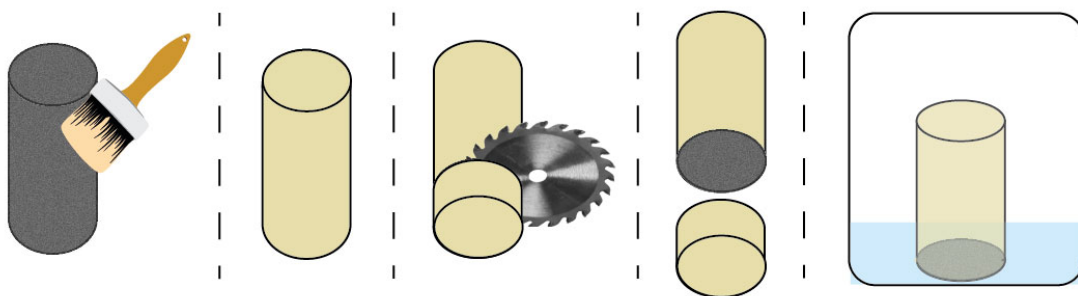


**Figure 4: Schematic illustration of the manufacturing process used to generate cylindrical mortar samples and the measurement setup for capillary water uptake tests.** In consecutive steps, cylindrical mortar samples are coated, cut open and placed into a water bath to determine the capillary water uptake.

## 2.4 (Mechanical) Testing methods for fresh and cured mortar

### 2.4.1 Macrorheology

Rheology can be used to describe and assess the deformation and flow behavior of materials. Rheology measurements determine the viscoelastic properties of a material based on its response to applied forces. A common way to measure these properties is using a shear rheometer equipped with a plate-plate measuring setup. Here, the material is placed between a stationary and a movable plate, and the movable plate is sheared at a defined frequency to apply the desired shear stress to the sample. This method works very well for homogeneous samples with small or without particles. However, even mortar with a maximum grain size of 4 mm, is not an ideal sample to be measured using a standard plate-plate measuring system, and special measuring instruments are needed to receive reliable results. Here, the Building Material Cell (BMC) is recommended (**Figure 5**). This measuring system consists of a cylinder made of stainless steel with an exchangeable inset cage and a paddle mixer. The inset cage prevents not only the segregation of the material but prevents also the material from sliding alongside the wall during the measurement.
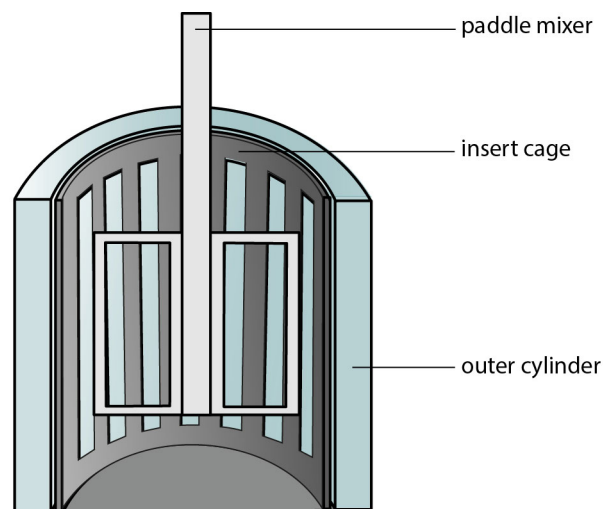
**Figure 5: Schematic illustration of the cross-section of the Building Material Cell (BMC).** Cross-section of the BMC showing the cylindrical housing, the exchangeable insert cage, and the paddle mixer in the actual measuring position.

To determine the workability of the mortar samples, measurements were performed using a commercial shear rheometer (MCR 302; Anton Paar GmbH) equipped with the BMC 90 measuring cell for building materials and a paddle mixer. For all measurements, the measuring cell was filled with 450 g of (hybrid) mortar. Those mortar samples were mixed manually outside the rheometer (as described for initial contact angle measurements, see 2.2.1, not according to DIN EN 196-1) and transferred into the measuring cell after 3 min of mixing.

With this procedure, the first measurement point was taken 4.5 min after the hydration reaction was initiated. The measurements were conducted without further cooling at RT. Every 30 s, the torque required to maintain a constant shear rate of 0.0001 s$^{-1}$ was measured for a time span of up to 30 min. If a critical torque greater than 200 N m was reached, the measurement was stopped manually.

## 2.4.2 Development of Elastic Parameters (FreshCon)

For a safe application of cementitious materials, knowledge of the setting and hardening process of the material used is of crucial importance. The FreshCon system (**Figure 6a**) uses ultrasonic technology to determine the setting and hardening behavior of mortar.[59, 60]

For the actual measurement, the mortar formulation to be mixed is filled into the FreshCon container (**Figure 6b**) and the transit time of ultrasonic signals is recorded at defined time intervals. From the transit times, the ultrasonic velocity in the material can be determined continuously. When two containers are used simultaneously, the shear wave velocity can be determined in addition to the compression wave velocity. This allows for calculating further material parameters, such as the modulus of elasticity and the *Poisson's* ratio.
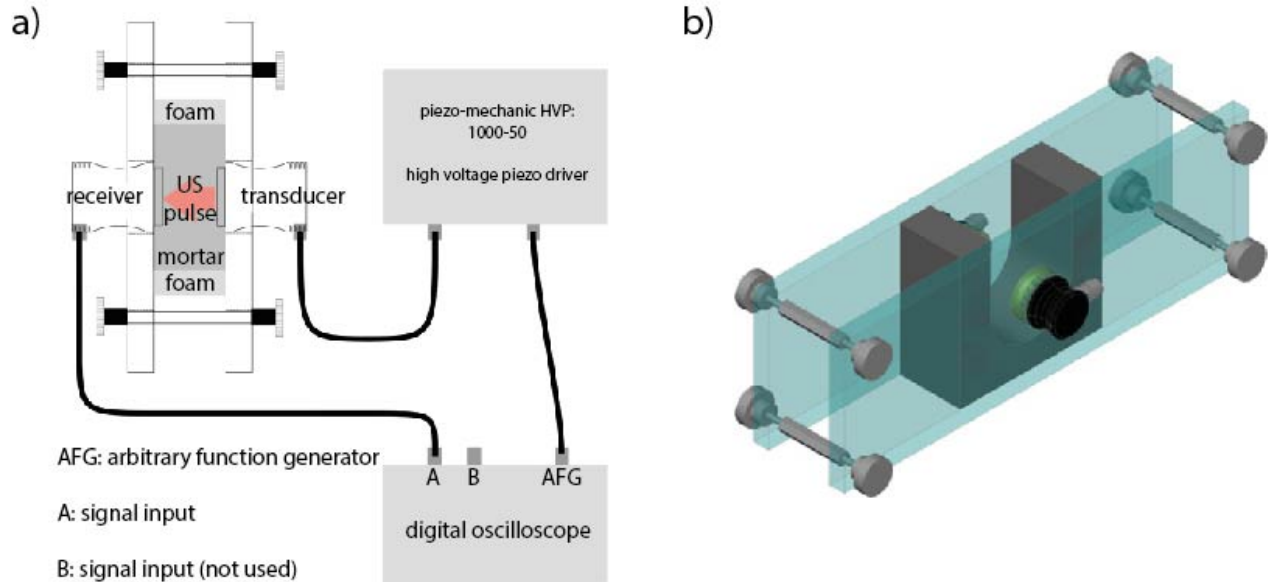


**Figure 6: Measurement setup for FreshCon measurements.** Schematic overview of the measurement setup for FreshCon measurements (a) and a 3D illustration of a used FreshCon container [61] (b).

In this thesis, the setting and hardening process of different mortar formulations was tracked continuously by using a modified version of the FreshCon system. The recording and trigger generation was carried out with a digital oscilloscope. Data acquisition was controlled using a

MATLAB script. Both compression and shear waves were excited and measured using a shear wave transducer with a 500 kHz center frequency. Compressional wave onsets were determined using Akaike's Information Criterion (AIC) as the onset determination method described in the literature.[62, 63] Shear wave onsets were determined using the method described by Krüger *et al.*.[64] Data points resulting from picking errors were manually determined and deleted. Missing data was filled using a smoothing spline interpolation using the MATLAB fit function and a smoothing parameter of 0.5.

### 2.4.3 Three-point bending and compression tests

By determining three-point bending and compressive strengths, the performance of building materials can be assessed with regard to mechanical loading.

Here, all three-point bending strength values of standard and hybrid mortar samples were determined according to DIN EN 196-1 using standardized test specimens (4cm × 4 cm × 16 cm). To determine the three-point bending strength, the test specimens were placed into a loading frame as shown in **Figure 7**, and then mechanically broken.

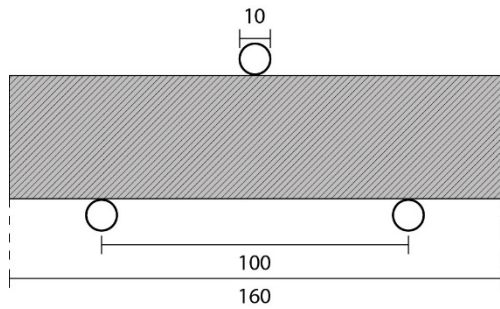The three-point bending strength $R_f$ [MPa] was then calculated according to equation 1, with $F_f$ [N] being the force applied to the center of the specimen, $l$ [mm] the distance between the lower load rollers, and $b$ [mm] the side length of the cuboid specimen.



$$R_f = \frac{1.5 \cdot F_f \cdot l}{b^3} \qquad (1)$$

**Figure 7: Standardized test prism in a loading frame.** Front view; graphical illustration adopted from DIN EN 196-1.[56]

After determining the bending strength, the compressive strength could be measured using both resulting fragments of the specimen. Therefore, each fragment is placed in between two plates, as shown in **Figure 8**, and the load on the specimen is increased constantly until failure. The compressive strength $R_c$ [MPa] was then calculated following equation 2, where $F_c$ [N] denotes the maximum load at the point of failure, which is divided by the area of the plates (1600 mm$^2$).
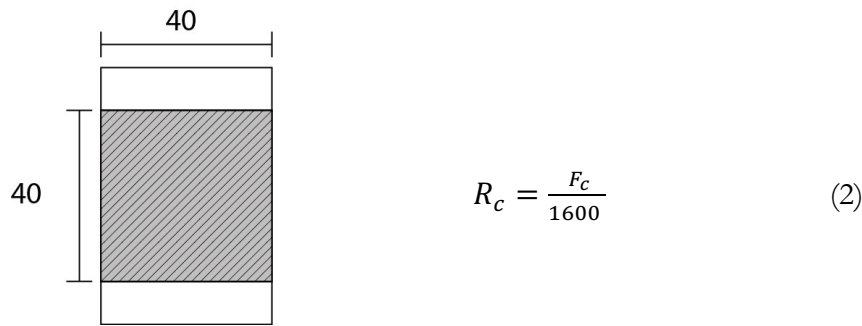
$$R_c = \frac{F_c}{1600} \qquad (2)$$

**Figure 8: Broken test specimen in loading frame to determine the compressive strength.** Front view; graphical illustration adopted from DIN EN 196-1.[56]

Here, all three-point bending and compressive strengths were determined according to DIN EN 196-1. The only difference was that for hybrid mortar samples the framework was stripped after 48 instead of 24 hours. Subsequent storage was conducted as stated in the norm. On each testing date (*i.e.*, after 7 and 28 days of curing), first the three-point-bending strength was determined; afterward, the compressive strength was measured using a loading frame (Series DB Super, Walter&Bai, Löhningen, Switzerland) as described above.

## 2.3.4 Durability tests

For newly developed bacterial additives that aim at improving the durability of mortar, the hydrophobizing effect has to persist over time and needs to withstand harmful environmental influences at the application side, such as extreme temperatures, humidity, or acid rain. Here, to assess the durability of the achieved hydrophobic properties and of the hybrid mortar itself, critical environmental influences were simulated and mortar samples were exposed to these conditions.

For these durability tests, standardized prims, produced according to DIN EN 196-1, were used. They were first cured at 20 °C (r.h. ≤ 50%) for 28 days and afterward exposed to the respective challenge. This procedure was conducted for all samples in the same way, with one exception: For analyzing the effect of freeze-thaw cycles, the mortar samples were first cured according to the CD testing procedure (DIN CEN/TS 12390-9[65]) and then subjected to freeze-thaw cycles (20 °C to –20 °C; 14, 28, or 56 cycles). To simulate high and low temperatures, test samples (standardized prims according to DIN EN 196-1) were stored in a closed container at –20 °C and 50 °C, respectively. To simulate moisture, acid rain, and sulfate attack, test samples were stored at 20°C in a closed container containing either distilled water, a HCl solution (pH 4), or a $Na_2SO_4$ solution (30 g $SO_4^{2-}$/L), respectively.

After seven, 14, and 28 days of exposure to each condition, contact angle measurements, capillary uptake tests as well as 3-point bending and compressive strength tests were conducted as described in sections 2.3.2 and 2.4.3.

### 2.4.5 Density determination

Over 2000 years ago, *Archimedes* formulated a principle that is today known as *Archimedes'* principle. When an object is immersed in a fluid, the buoyant force applied by the fluid is equal to the weight of the displaced fluid. This principle can be used to determine the density of solid materials by immersion weighing.

To determine the density of cured mortar samples, immersion weighing was conducted according to DIN EN 12390-7[66]. Therefore, cured mortar samples (cured for at least 28 days at 20 °C and r.h. ≥ 50%) were stored underwater for 7 days to achieve maximal saturation. The mass of the saturated test specimen was then measured underwater ($m_2$, **Figure 9**) and after the surface of the sample was dabbed above water ($m_3$).
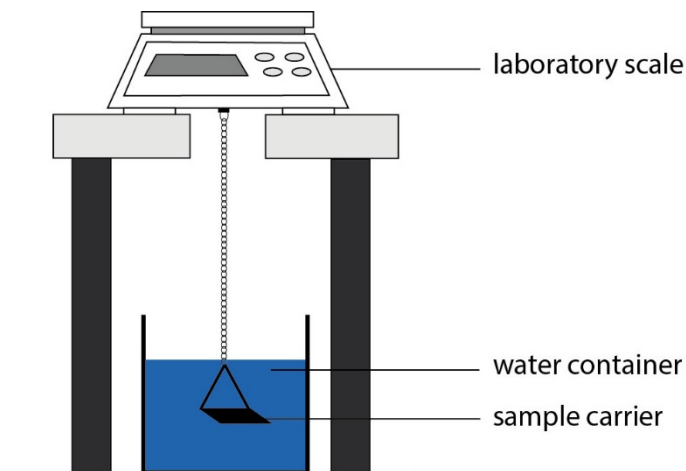


**Figure 9: Schematic measurement setup to determine the sample weight underwater.** Using a special measuring setup, the determination of the sample weight underwater is possible, which further allows for determining the density of the sample.

After these measurements, the test specimens were dried at 105 °C until a constant weight ($m_1$) was obtained. With these three measurements, the sample density was calculated according to equation 3, where $\delta_w$ denotes the density of water at 20 °C, *i.e.*, 998 kg/m$^3$.

$$\text{density} = \frac{m_1}{m_3 - m_2} \cdot \delta_w \tag{3}$$

## 2.5 Microscopy, light scattering, and spectroscopy

### 2.5.1 Phase contrast microscopy

Phase contrast microscopy is a contrast-enhancing optical technique invented in the 1930s. This technique became particularly important in biology, as it enables the examination of living cells without the need for previous staining or fixation, thus earning its inventor Frits Zernike the Nobel Prize in Physics in 1953.[67]

The technique uses the following principle: When light passes through a medium, in addition to the amplitude, also the phase of light waves changes, and the latter depends on the refractive index of the material. This phase change, however, cannot be perceived by the human eye, which is only sensitive to changes in amplitude. Thus, the phase contrast technique converts phase shifts into changes in amplitude, which are detected by the eye as differences in intensity, making these changes visible as differences in image contrast.[68]

To enable phase contrast microscopy, two special components are added to the light path of the microscope. Light emitted by the light source is first converted into a light cone by a phase diaphragm and then focused on the sample. Due to the interaction with the sample, one part of the light is scattered, while the remaining light (which did not interact with the sample) passes unscattered. After passing the specimen, scattered and unscattered light (background light) reach the phase plate, which is positioned in a way to allow light scattered from the sample to pass almost unchanged while manipulating both phase and amplitude of the unscattered background light. The resulting image is thus formed by the interference of light diffracted by the sample and undiffracted background light. The contribution of the diffracted light is therefore enhanced compared to the undiffracted light, which gives the resulting image a higher contrast.

In this thesis, phase contrast microscopy was used to optically characterize the microbial composition of different bacterial additives. Pictures were recorded using a DMi8 microscope (Leica, Wetzlar, Germany) equipped with a 63x objective (Leica) and a digital camera (OrcaFlash 4.0 C11440-22C, Hamamatsu, Japan).

## 2.5.2 Profilometry

Profilometry is an optical surface analysis technique, which allows to obtain topographic information of the sample surface. The method uses the principle of confocal microscopy, however, in an advanced approach: While confocal microscopy allows the recording of contrast and resolution enhanced pictures, profilometry enables to display a three-dimensional contour of the measured sample. Compared to a conventional light microscope, instead of illuminating the whole specimen at once the specimen surface is scanned spot-wise. Furthermore, a pinhole in the conjugate image plane to the light source is used. This way, the intensity of light, reflected from above or below the focus plane, is decreased (**Figure 10**).
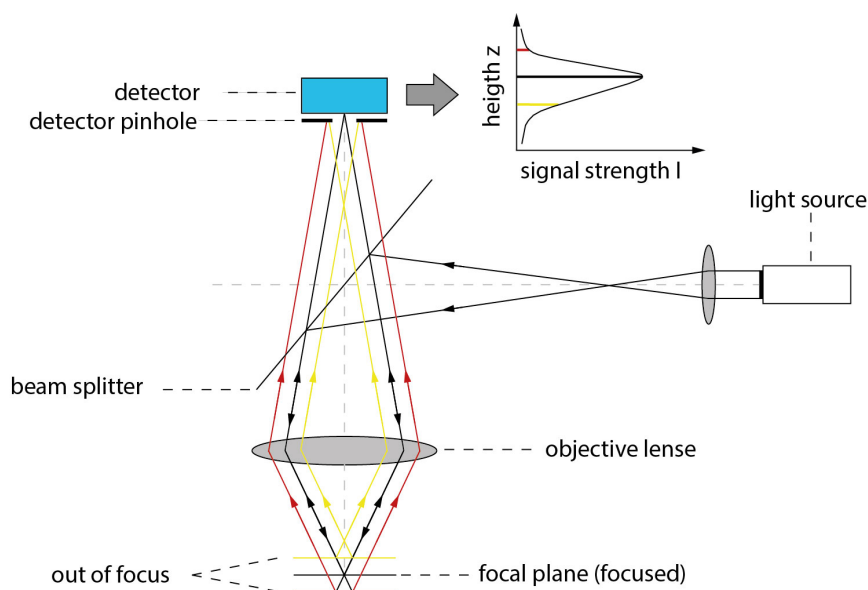


**Figure 10: Schematic illustration of the optical path for confocal imaging.** The intensity of light reflected from above (yellow rays) or below (red rays) the focal plane, is decreased due to the use of a detector pinhole. By scanning the Z axis, the focal plane (black rays) as the point of the maximum light intensity can be determined.

For each spot the point of maximum signal intensity of light, reflected from the sample surface, is determined by driving the objective lens in the Z axis. This Z axis position resulting in the highest intensity is determined to be the focal plane and this procedure is repeated spot-wise for the whole sample surface. The resulting images are obtained with increased resolution and contrast. In addition, three-dimensional contours can be generated by merging the height information of each point.

In this thesis, microscopic surface profiles of mortar samples were obtained using a laser scanning microscope (VK-X1000, Keyence, Oberhausen, Germany) equipped with a 50x lens (NA = 0.95; Nikon, Chiyoda, Tokyo, Japan). Images were acquired without any further sample treatment, *i.e.*, directly after curing of the mortar samples. On each sample, five spots with an

approximate area of 220 μm × 300 μm were scanned. The scanned area was then evaluated with the software MultiFileAnalyzer (Version 2.1.3.89, Keyence, Oberhausen, Germany) to obtain the developed interfacial area ratio (*Sdr*, equation 4).

$$Sdr = \frac{1}{A}\left[\iint_A \left(\sqrt{\left[1+ \left(\frac{dz(x,y)}{dx}\right)^2 + \left(\frac{dz(x,y)}{dy}\right)^2\right]} -1 \right) dxdy\right] \qquad (4)$$

Here, *A* denotes the scanned sample area, *x* and *y* the lateral dimensions, and *z* the height of the surface profile. Then, the developed interfacial area ratio *Sdr* quantifies the additional surface area contributed by a texture compared to a fully planar surface.

### 2.5.3 Scanning Electron Microscopy (SEM)

Instead of visible light, electron microscopy uses electrons for imaging. As a result, due to the much shorter wavelength of electrons compared to visible light (380 to 760 nm), higher resolution and therefore magnification can be achieved.[69] A scanning electron microscope operates by scanning the sample surface with a focused electron beam and subsequent processing of the interactions of the electrons with the sample surface. The most commonly used source of information for the analysis of a sample surface are the so-called secondary electrons, *i.e.*, electrons of low energy, which originate from near-surface regions of the sample. These electrons are generated by the interaction of the electron beam (primary electrons) with the sample surface and are used to generate an image of the scanned surface. Other effects caused by the primary electrons, such as backscattered electrons, or characteristic X-rays can be used as well to identify elements and to map their distribution in the sample.

Here, SEM was used to image single bacterial spores and the surface of (hybrid) mortar samples. To image single bacterial spores, spore powder was dispersed onto a piece of adhesive tape (approx. 3 cm x 3 cm), which was placed onto an aluminum sample holder and sputtered with gold (MED 020, BAL-TEC, Balzers, Liechtenstein). Pictures were acquired on a JEOL-JSM-6060LV scanning electron microscope (Jeol, Eching, Germany) at an acceleration voltage of 15 kV. SEM images of mortar surfaces were acquired without sputtering and obtained on a FlexSEM 1000 scanning electron microscope (Hitachi, Chiyoda, Japan) at an acceleration voltage of 5 kV.

## 2.5.4 Dynamic light scattering (DLS)

Dynamic light scattering is a powerful tool for the characterization of the diffusion behavior of small particles and macromolecules in solution. In principle, the Brownian motion of those objects is measured in solution, and this information is then used to determine their hydrodynamic size, which can be defined as the hypothetical hard sphere that diffuses in the same manner as the particle being measured.[70]

Brownian motion can be described as the random movement of particles, resulting from their collision with the surrounding molecules. The diffusive "speed" of particles undergoing Brownian motion is dependent on the temperature, the sample viscosity, and the particle size. The larger a particle, the slower it will diffuse through solution. For dynamic light scattering measurements, particles in solution are exposed to monochromatic light, and the scattered light (whose intensity is dictated by the particle movements) is quantified. The intensity of the scattered light results from the interference of light beams scattered from different particles. Owing to the constant movement of particles, the intensity of the scattered light changes continuously (due to constructive and destructive interference). Accordingly, the ratio of the measured intensity fluctuations can be related to the diffusion coefficient $D$ described in the Stokes-Einstein equation (5):

$$D = \frac{k_B T}{6\pi\eta r} \qquad (5)$$

Then by determining this diffusion coefficient D from the scattering signal, the hydrodynamic size of a diffusing object can be calculated (by assuming a spherical geometry, and a particle movement at a low Reynolds number).

In addition to the hydrodynamic size, a modern particle analyzer also offers the option to determine the zeta potential ($\zeta$-potential) of a particle. The zeta potential is a measure for the surface charge of dispersed particles and defines the degree of electrostatic repulsion or attraction between neighboring particles. The higher the zeta potential, the more stable the dispersion (and fewer agglomerates will form).

In this thesis, the hydrodynamic size and the zeta-potential ($\zeta$) of the different spore variants were measured using a Litesizer 500 (Anton Paar, Graz, Austria) equipped with a 35-mW laser diode light ($\lambda = 658$ nm). All measurements were conducted using aqueous spore suspensions at a concentrations of 1.5 mg/mL at pH 13. Measurements were performed in technical triplicates

## 2.5.5 Infrared (IR) spectroscopy

Infrared (IR) spectroscopy is a non-destructive analytical method that is used to identify chemical substances or functional groups. By irradiating a substance with infrared light (wavelength 800 nm – 1000 nm), molecular vibrations are excited, which are in the same energy range as the vibrational levels of molecular bonds. This causes a certain portion of the radiation to be absorbed. In the resulting IR spectrum, the transmission of the exciting light is plotted against the wavenumber (typically ranging from 4000 $cm^{-1}$ – 400 $cm^{-1}$), leading to characteristic absorption bands. The location of the individual absorption bands can then be used to infer the presence of certain functional groups.

Infrared spectra obtained for this thesis were recorded on a Spectrum 100 FT-IR spectrometer (PerkinElmer, Waltham, USA) in a wavenumber range from 4000 $cm^{-1}$ to 450 $cm^{-1}$. Measurements were performed at RT using the total reflection (ATR) sampling technique.

## 2.6 Analytical methods

### 2.6.1 X-ray diffraction (XRD)

XRD is a standard technique for the structural analysis of crystalline substances. It analyzes the diffraction of monochromatic radiation at the crystal lattice.[71] Materials can be either analyzed as single crystals or (which is the preferred option for poorly crystalline samples) a ground sample is analyzed, which is then called X-ray powder diffraction. With the wavelength of x-rays (0.1–10 Å) being similar to the interatomic distance between atoms in the crystal or crystalline material resolving atomic structures is possible.[72] For the actual measurement, the sample is placed in the x-ray diffractometer and is irradiated with x-rays from different angles while a detector measures the intensity of the reflected radiation. Thereby, most of the diffracted x-rays interfere destructively and cancel each other out. Only at certain conditions (when the differences in the travel path are equal to integer multiples of the wavelength, **Figure 11**) positive interference is possible. This condition is also known as Bragg's law (equation 6). Here, $\lambda$ denotes the wavelength, $d$ describes the distance between two parallel lattice planes, and $\theta$ gives the angle between the x ray and the lattice plane.
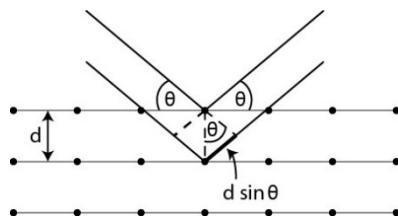


$$n\lambda = 2d \cdot \sin\theta \qquad (6)$$

**Figure 11: Schematic illustration of Bragg's law for constructive interference.** Graphical illustration adopted from literature.[71]

If Bragg's law is fulfilled, at these positions (or for these angles) increased intensities are measured. The result is an angle-dependent intensity distribution, whose maxima are called Bragg reflexes. By using a XRD structure database, the reflexes of the resulting diffractogram can be assigned to known structures or phases.

All X-ray diffraction (XRD) patterns analyzed in this thesis were recorded on a D8 ADVANCE diffractometer (Bruker, Billerica, Massachusetts, USA) using Cu Kα radiation and a high-resolution energy-dispersive detector (LYNXEYE-XE, Bruker, Billerica, Massachusetts, USA). The scanning angle $2\theta$ was varied from 5 to 70° using a step size of 0.02°

and a dwell time of 0.2 s. For the quantitative mineralogical analysis, the samples were ground into pieces ≤30 µm. As an internal standard, 20 % (w/w) ZnO was mixed with the specimens.

## 2.6.2 Mercury intrusion porosimetry

Mercury intrusion porosimetry was used in this thesis to determine the porosity and pore size distribution of cured mortar samples. Despite being toxic, mercury has the great advantage of acting as a non-wetting liquid towards most solid materials. Still, at ambient pressure, mercury does not penetrate porous materials such as concrete or mortar. If external pressure is applied, mercury is forced to fill those pores. By assuming that all pores are spherically shaped and knowing the surface tension $\gamma$ and contact angle of mercury $\theta$, the pore diameter $d$ can be calculated depending on the applied pressure $p$ according to the *Washburn* equation (equation 7).

$$d = \frac{4\gamma \cdot \cos\theta}{p} \tag{7}$$

For analyzing the porosity, average pore size, and pore size distribution, mortar samples were produced according to DIN EN 196-1 and cured for 28 days (RT, r.h. ≥50 %). Subsequently, all mortar samples were dried for three additional days at 105 °C and at ambient pressure. Measurements were acquired using an automated mercury porosimeter (AutoPore IV, Micromeritivs GmbH, Norcross, USA) in a pressure range of 0.01 - 413.7 MPa (2 – 60,000 psi).

## 2.6.3 $^{29}$Si-Nuclear Magnetic Resonance ($^{29}$Si-NMR)

Nuclear Magnetic Resonance spectroscopy is a useful technique to study molecular structures at atomic levels. In brief, NMR-active nuclei (nuclear spin unequal to zero) as charged particles generate their own magnetic field due to their nuclear spin. As soon as an external magnetic field $B_0$ is applied, the spins of the nuclei will align in specific spin states. The energy difference between these states ΔE is given as

$$\Delta E = \frac{h \cdot \gamma \cdot B_0}{2\pi} \tag{8}$$

with Planck's constant $h$ [J s], the gyromagnetic ratio $\gamma$ [T$^{-1}$ s$^{-1}$], and the applied magnetic field $B_0$ [T]. NMR signals can be obtained by measuring the absorption of energy from radiofrequency radiation matching $\Delta E$. This is the case at the so-called resonance frequency $\vartheta$ (equation 9) and leads to measurable NMR signals.

$$\vartheta = \frac{\gamma \cdot B_0}{2\pi} \tag{9}$$

Depending on their chemical environment, different NMR signals of the same isotope are obtained. Even though NMR spectroscopy is used mainly for liquid samples, solid state NMR is a useful tool to investigate solid samples. In comparison to liquid-state NMR, the anisotropy of the solid sample leads to line broadening. However, this can be overcome by a process called magic angle spinning (MAS). Here, the solid sample is tilted at a specific angle (54.74°), to minimize dipolar splitting and chemical anisotropy interactions,[73] resulting in similar behavior of solid samples compared to liquid-state NMR.

The $^{29}$Si NMR experiments within this thesis were performed on a Bruker Avance 300 spectrometer (magnetic field strength 7.0455 T, resonance frequency for $^{29}$Si: 59.63 MHz) in MAS mode using the single pulse technique (90° pulse). The samples were packed in 7 mm zirconia rotors and spun with 5 kHz. About 10000 scans were recorded for each spectrum using a repetition time of 5 s. The chemical shifts were referenced to an external sample of tetramethylsilane (TMS) at 0 ppm. Then, the obtained spectra were deconvoluted with the Bruker WINNMR software and interpreted using the Q$^n$ nomenclature.[74]

# 3. Summary of publications

## 3.1 Bacterial Materials: Applications of Natural and Modified Biofilms

*Elif N. Hayta, **Marvin J. Ertelt**, Martin Kretschmer, Oliver Lieleg* [75]

Involuntarily or not, almost everyone has come in contact with bacterial biofilms in one or more forms: a clogged pipe under the sink or plaque on teeth represent only two prominent examples of the various manifestations in which bacterial biofilms can occur. These common examples illustrate how – at least in popular perception – there is little to no expectation that these slimy substances might be useful for anything. Yet, bacterial biofilms, which can be described as bacteria embedded into a matrix of self-produced biopolymers, exhibit unique properties, making them an interesting research topic. When embedded into a biofilm matrix, bacterial cells develop a higher resistance against a variety of environmental and mechanical influences, *e.g.*, shear stress, fluctuations in the pH value, or towards exposure to potentially harmful chemicals (including antibiotics). In addition, some biofilms are able to efficiently repel water, whereas others are able to liberate electrons as a byproduct of their metabolism. This review article summarizes selected applications of natural and modified bacterial biofilms from various areas of life, thus demonstrating the benefits of these biological model systems for humankind.
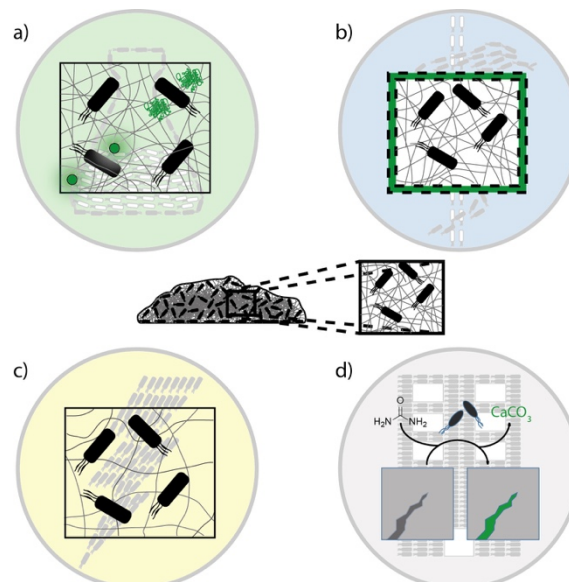


**Figure 12: Schematic overview of areas of application of biofilm inspired bacterial materials in biotechnology (a), medical applications (b), electricity generation (c), and civil engineering (d).**

The diverse and impressive properties of bacterial biofilms serve scientists as an inspiration for a variety of research approaches. For example, the ability to better protect bacteria embedded in the biofilm polymer matrix against external environmental influences can be used in medical applications to improve probiotic delivery systems. These systems can be further adjusted since biofilms can be tailored by integrating different additives into the biofilm matrix. Moreover, similar modifications can be achieved by genetically modifying natural biofilms or by mixing different bacterial strains. The resulting great variety of different properties enables the application of biofilms to be used in a wide range of different fields. For instance, a higher porosity of the biofilm matrix leads to an increased permeability, which, in turn, can improve the properties of bio-electrochemical systems. Moreover, by integrating enzymes or nanoparticles into a biofilm matrix, the functionality of the respective biofilm can be broadened: The immobilization of enzymes in a biofilm matrix could improve its specific activity towards specific targets, and adding nanoparticles into biofilms could improve their antimicrobial performance, thus, improving commonly used fertilizers.

In the construction sector, bacterial additives can improve the performance of building materials such as mortar. Considering the ever growing importance of sustainability, bacterial additives gained increasing attention over the last decades as they can improve the sustainability of cementitious building materials by introducing self-healing or water-repellent properties or by replacing previously used noxious additives. Microbially induced calcite precipitation (also known as biocementation) even represents one of the very few approaches to develop cement free building materials. Recent studies showed that the concept of using bacterial materials is also compatible with advanced manufacturing methods such as 3D printing, which might open new applications in the future.

In summary, this review article points out the high potential of bacterial biofilms by summarizing applications of bacterial biofilms from various fields of life such as agricultural and industrial biotechnology, medicine, power generation, and civil engineering. Given the large variety of bacterial species, there might be an even larger hidden potential, which still needs to be explored.

<u>Individual contributions of the candidate:</u> I contributed to the conception of the article, the design and creation of the figures, and to the writing of this review article.

## 3.2 Bacterial Additives Improve the Water Resistance of Mortar

**_Marvin Johannes Ertelt_**, _Manuel Raith, Josef Eisinger, Christian U. Grosse, and Oliver Lieleg_ [76]

Low costs and high durability made concrete – among other cementitious materials – the most used construction material worldwide. However, under certain conditions, even these robust materials can deteriorate. The most known mechanisms responsible for such deterioration processes occur in aqueous environments. Thus, the most common approach to prevent the ingress of water is the application of hydrophobic coatings. This additional working step, however, is time-consuming and cost-intensive. Moreover, if the protective layer gets damaged, water can be trapped inside the material, which will eventually even accelerate the deterioration process. A possible solution for this problem is a modification of the bulk material. Yet, commonly used additives for such a bulk modification drastically increase the price of the resulting material and/or are noxious.

To overcome those drawbacks, in this study, different bacterial additives were mixed with commercial mortar, and their hydrophobization potential on the bulk material was investigated. The obtained results demonstrated that three different bacterial additives generated from different bacterial strains are suitable to form hybrid mortar materials with increased hydrophobic properties.
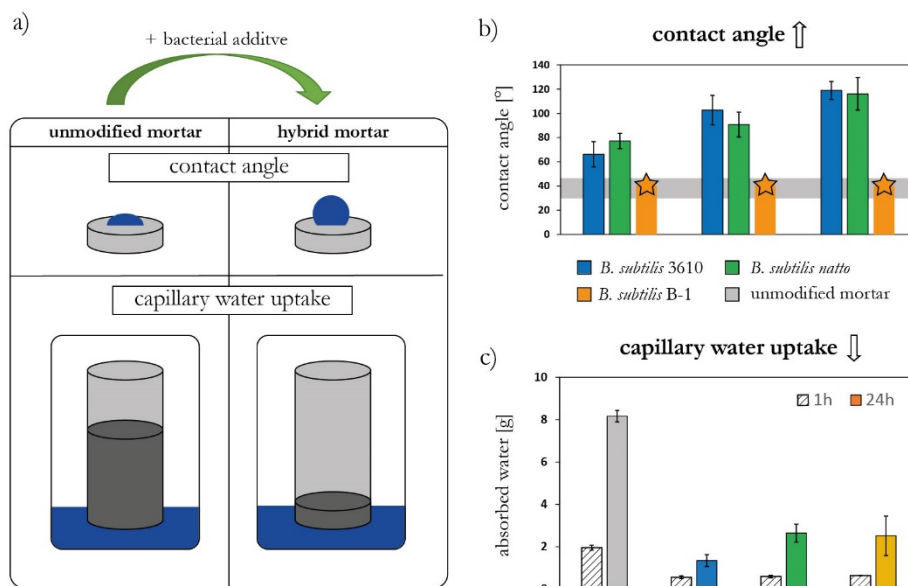


**Figure 13: Schematic overview of the effect of bacterial additives in mortar**. a) By incorporating bacterial biofilm into the mix design of mortar, so-called hybrid mortar is obtained. The addition of biofilm powder leads to b) increased wetting resistance and c) a partial suppression of the capillary water uptake.

The tested bacterial additives can easily be integrated into the hybrid mortar during the mixing process. Solid additives, *i.e.*, freshly cultivated or freeze-dried bacterial biofilms, can be mixed with cement, whereas bacterial overnight cultures (as a liquid additive) replace a fraction of the mixing water. In its cured state, the resulting hybrid mortar materials resist wetting by water, *i.e.*, they were successfully rendered hydrophobic. However, as a consequence to the addition of bacterial additives, the hydration reaction of cement is delayed and overall lower moduli of elasticity are obtained. In accordance with the latter, all hybrid mortar variants show lower three-point bending and compressive strengths, as well as reduced densities.

In summary, this study demonstrates that bulk modification of mortar is possible by adding various bacterial additives. The resulting cementitious hybrid materials are characterized by hydrophobic properties, which can significantly increase their durability. These new findings could prove useful for the development of new, sustainable cement additives and cementitious building materials.

Individual contributions of the candidate: I contributed to the conception of this study, was mainly responsible for the design and execution of the experiments, and the data analysis, and contributed to the writing of the article.

## 3.3 Bacterial spores as hydrophobizing agents in mortar

**_M. J. Ertelt_**, _Lea Bubendorfer, C. U. Grosse, O. Lieleg_ [77]

The use of bacterial additives for cementitious materials is an emerging trend in the field of developing new building materials. Biopolymers produced by bacterial fermentation, _e.g.,_ Welan gum or xanthan gum, which are widely used as viscosity modifying additives in concrete, are among the most prominent examples. However, to be used in cementitious materials, microbial substances have to fulfill certain requirements: on the one hand, they have to withstand the harsh conditions occurring during cement hydration, and, on the other hand, they have to be rather inexpensive for the resulting material to remain economical.

This study demonstrates that bacterial spores obtained from commercial sources can be used as hydrophobizing agents in mortar. The automated production of these bacterial additives at industrial scale can strongly reduce the price of the resulting hybrid mortar material compared to other bacterial additives, which, so far, can only be produced manually at laboratory scale.
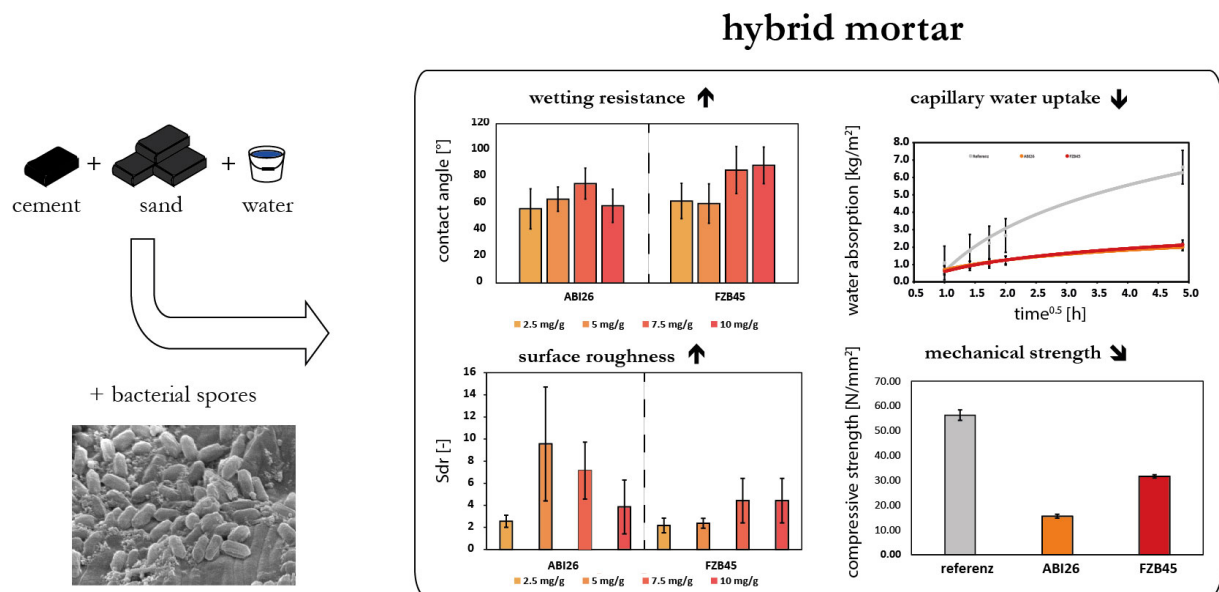


**Figure 14: Schematic overview of the effect of bacterial spores used as hydrophobizing agents in mortar.** Incorporating bacterial spores into the mix design of mortar affects the properties of the resulting hybrid mortar mortar: wetting resistance and surface roughness are increased, whereas capillary water uptake and mechanical strength are decreased.

Here, commercially available and industrially produced bacterial spores are used as hydrophobizing agents in mortar without any further purification and functionalization steps. To explain the different degrees of hydrophobicity observed for the different resulting hybrid mortars, all tested spore variants are analyzed with regard to functional groups on their outer shell, as well as their surface charge at a pH that occurs during cement hydration. Virtually

identical IR spectra as well as comparable negative surface charges suggest that the spores, although formed by different bacterial strains, are relatively similar in terms of their outer shell structure. Since bacterial spores (the dormant live form of bacteria) are likely to affect cement hydration through surface effects, a similar effect for all spore variants on the hydrophobic properties of the resulting hybrid mortar variants was expected. However, the different spore variants affect both, the wetting resistance and the capillary water uptake of mortar, differently. Moreover, for each spore variant, the most desirable mortar property modifications are achieved at different spore concentrations. Two favorized conditions are identified, at which the capillary water uptake is reduced and the wetting resistance is enhanced, at the same time. Similar to other bacterial and organic additives, these two spore variants also slow down the hydration reaction of cement and affect the strength of the cured material. Here, two different effects are observed: whereas one spore variant leads to increased values for both, tensile and compressive strength, the other spore variant leads to reduced values.

In conclusion, this study demonstrates, that bacterial spores produced at industrial scale can be used as hydrophobizing additives to mortar without requiring further purification or functionalization. However, compared to other bacterial additives, the overall performance of the tested bacterial spores is somewhat inferior. Still, the potential use of bacterial spores as a cheap and easily available alternative for other hydrophobizing additives should be further considered until other bacterial additives are available in larger quantities.

Individual contributions of the candidate: I contributed to the conception of this study, was mainly responsible for the design and execution of the experiments, and the data analysis, and contributed to the writing of the article.

## 3.4 Small pores, big impact - controlling the porosity allows for developing more sustainable construction materials

*__Marvin Johannes Ertelt__, Harald Hilbig, Christian Ulrich Grosse, and Oliver Lieleg* [78]

Although there is already intensive research on new, cement-free and thus more sustainable building materials, such materials can often not be produced at industrial scale. As a consequence, cementitious building materials can be expected to remain the dominating building material in the construction sector. However, more and more attempts are made to improve the sustainability of such cementitious materials: Often, supplementary cementitious materials (SCMs) are used to replace certain fractions of cement. Such SCMs emerge as waste products of industrial processes and, thus, do not release any additional $CO_2$ into the environment during their production. In addition to the $CO_2$ emission, the environmental impact and thus the sustainability of a building material is also strongly dictated by its durability. As a consequence, it is desirable to protect cementitious structures against the ingress of water and the associated deterioration processes. However, hydrophobic coatings on these materials, which are commonly used to prevent water uptake often make us of noxious chemicals. Accordingly, these additives do not represent ideal ingredients for the development of more sustainable materials.
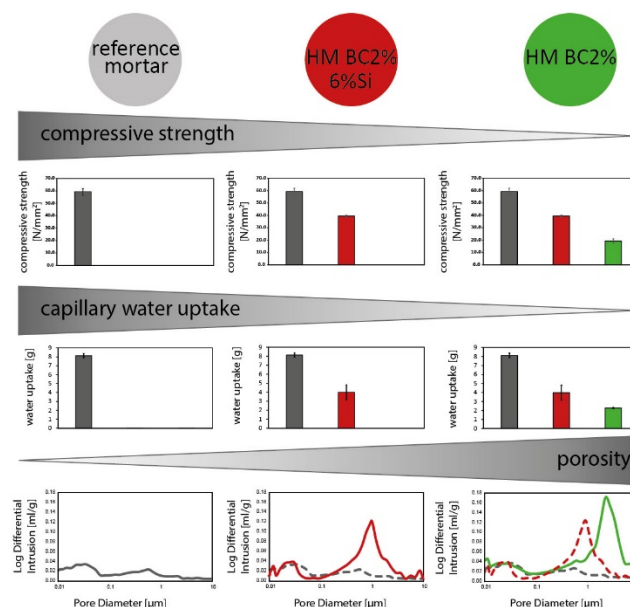


**Figure 15: Table of content figure:** Schematic overview of the effect of bacterial biofilm powder and SCM addition to mortar.

One intrinsic key property that dictates the applicability of a building material is its mechanical strength. Thus, in this study, the origin of the loss in strength of biofilm enriched mortar was investigated. $^{29}$Si NMR measurements in combination with XRD measurements could prove that identical hydration products were formed in the presence and absence of the bacterial additives. Accordingly, the observed loss in strength is most likely linked to the increased porosity of the hybrid mortar materials: here, an increased air entraining effect obtained by the addition of biofilm powder creates pores in the range of 2 μm.

Importantly, commonly used SCMs can be used to compensate the apparent loss of strength in biofilm-enriched hybrid mortar while, at the same time, maintaining its hydrophobic properties. Porosimetry experiments performed with these more complex mortar samples pinpointed a correlation of the hydrophobic properties of the different mortars (in particular, their capillary water uptake behavior) with their overall porosity, average pore size, and pore size distribution.

In conclusion, this study demonstrated that by combining two different additives both, the sustainability and the durability, of a cementitious building material can be increased simultaneously. The concept of modulating the wetting behavior and mechanical properties of cementitious materials by controlling their porosity might pave the way for designing more sustainable building materials – especially considering that such an approach might be applicable for concrete, the world's most used cementitious building material.

Individual contributions of the candidate: I contributed to the conception of this study, was mainly responsible for the design and execution of the experiments, and the data analysis, and contributed to the writing of the article.

## 3.5 Durability of biofilm-enriched hybrid mortar towards chemical and physical challenges

Unpublished manuscript – currently under revision

*Abstract*

To increase the service life of cementitious structures, it is important to protect them against penetrating moisture and molecules/ions transported by water ingress. For mortar, such a protection can be achieved by adding bacterial biofilm powder. However, the hydrophobizing effect needs to persist over time – even when the material is exposed to harsh conditions. Here, we show that the hydrophobic properties brought about by the addition of bacterial biofilm powder to mortar are maintained even when the material is challenged with difficult thermal or chemical conditions.

*Introduction*

Cementitious building materials can be both, a curse and a blessing: on the one hand, they can enable structures to last for centuries; on the other hand, depending on the formulation used, damage can also occur relatively early. Especially historical buildings are often in need of costly and time-intensive restorations; two prominent examples from Germany are the Frauenkirche in Munich and the Cologne Cathedral. In the latter, the scaffolding was recently removed from the North tower – after ten years of restoration work.

Damage to mortar is often triggered by moisture penetration accompanied by invading ions, and this problem affects all cementitious building materials alike. For concrete, where a moisture-driven ingress of chloride ions can lead to corrosion of the reinforcement steel, a protection towards water can be provided by hydrophobic coatings. However, such coatings can be damaged over time by weathering and abrasion, which reduces their effectiveness and requires regular maintenance. Thus, for a restoration of landmark buildings, a bulk modification of mortar would be preferable, as it promises a higher longevity. Previously, a biological bulk additive to mortar, bacterial biofilm, was introduced that provides the construction material with water-resistant properties.53 However, given the biological origin of this hydrophobizing agent, the durability of water-repellent properties established by this additive, are unclear.

*Materials and Methods*

*Mortar sample preparation*

Biofilm powder was produced as described in [76]. Mortar samples were then prepared according to DIN EN 196-1 as described in more detail previously [78]. A key difference compared to the procedure described in DIN EN 196-1 is, th  biofilm powder was added to cement before mixing.

*Durability tests*

To simulate high and low temperatures, test samples (standardized prims according to DIN EN 196-1) were stored in a closed container at –20 °C and 50 °C, respectively. To simulate moisture, acid rain, and sulfate attack, test samples were stored at 20°C in a closed container containing either distilled water, a HCl solution (pH 4), or a Na2SO4 solution (30 g SO42-/L), respectively. In each case, the mortar samples were first cured at 20 °C (r.h. ≤ 50%) for 28 days and then exposed to the respective challenge. For analyzing the effect of freeze-thaw cycles, the samples were first cured according to the CD testing procedure (DIN CEN/TS 12390-9) and then subjected to freeze-thaw cycles (20 °C to –20 °C; 14, 28, or 56 cycles). All samples were compared to reference samples of the same formulation, which were stored at 20 °C (r.h. ≥ 50%). Contact angle measurements, capillary uptake tests as well 3-point bending and compressive strength tests of mortar samples were conducted as reported previously.[78]

*Results and Discussion*

We here compared a biofilm-enriched hybrid mortar formulation with an improved version containing silica fume (a supplementary cementitious material, SCM) in addition to biofilm. The latter formulation was selected as it shows good hydrophobic properties but improved mechanical performance compared to SCM-free biofilm mortar [78]. First, we asked whether the materials will still possess an increased wetting resistance and reduced capillary water uptake after storage at high and low temperatures, after exposure to moisture, after freeze-thaw cycles, or after incubating them in sulfate or acidic solutions. Then, contact angle measurements (**Figure 16a**) and capillary water uptake tests (**Figure 16b**) were conducted.
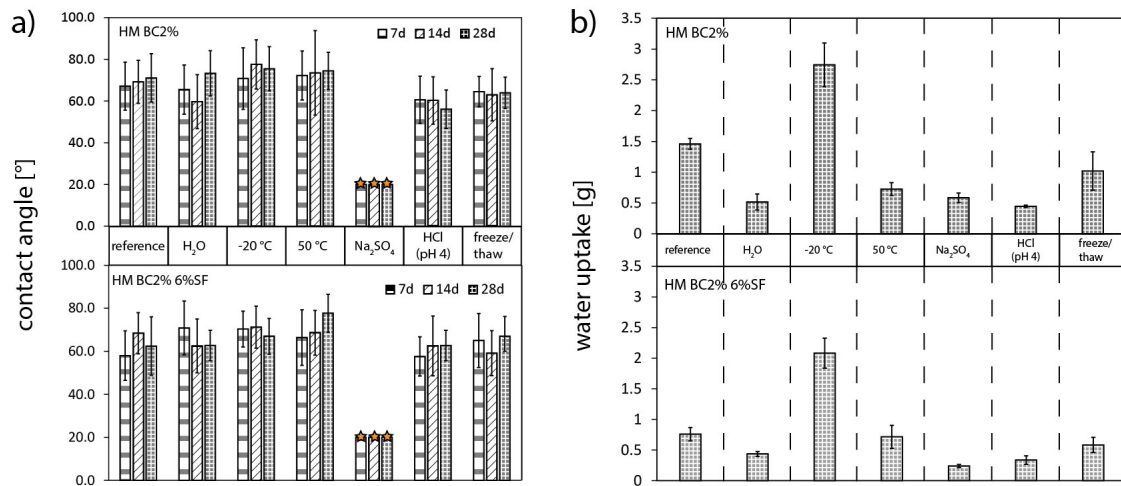
**Figure 16: Wetting resistance of and capillary water uptake into hybrid mortar and SCM enriched hybrid mortar after storage under various conditions.** (a) Contact angle measurements on the surface of hybrid mortar (bc = 2%, upper panel) and SCM-enriched hybrid mortar (bc = 2%, 6% SF, lower panel) after 7, 14, or 28 days of storage under conditions simulating environmental challenges. The values shown represent averages of 15 measurements conducted on three independent samples. Error bars denote the standard deviation. (b) Amount of water absorbed by hybrid mortar samples without (upper panel) and with silica fume (SF) addition (lower panel). All samples were cured for 28 days at 20 °C (r.h. ≥ 50%), before being stored for additional 28 days under the respective condition. The values shown represent averages of three measurements conducted on independent samples. Error bars denote the standard deviation.

Importantly, the good wetting resistance of the hybrid mortar samples was maintained for all tested conditions, but one (**Figure 16a**): Only storage in $Na_2SO_4$ reduced the surface wetting resistance. On those particular samples, determining contact angles was not possible as the water droplets placed onto the surface of the samples disappeared into the bulk of the samples. Interestingly, for all tested storage conditions, the amount of water that invaded the samples by capillary forces was strikingly low (**Figure 16b**). Compared to the unchallenged reference sample, only extended storage at –20 °C led to larger water uptake; but also here, the amount of water in the sample was considerably lower than what we found previously for standard mortar (*e.g.*, 8.2 g water after 24 h only[76]).

Importantly, as the results depicted in **Figure 17** show, most storage conditions did not compromise the mechanical properties of the samples either. Only for extended storage at –20 °C, we detected a noticeable reduction in the compressive strength. For samples subjected to 28 freeze-thaw cycles, we determined even higher compressive strength values than for all other samples. This was somewhat surprising; however, it is important to realize that these particular samples cannot be directly compared to the other ones: here, the storage conditions were chosen according to DIN CEN/TS 12390-9, which requires extended sample storage in water in addition to curing.
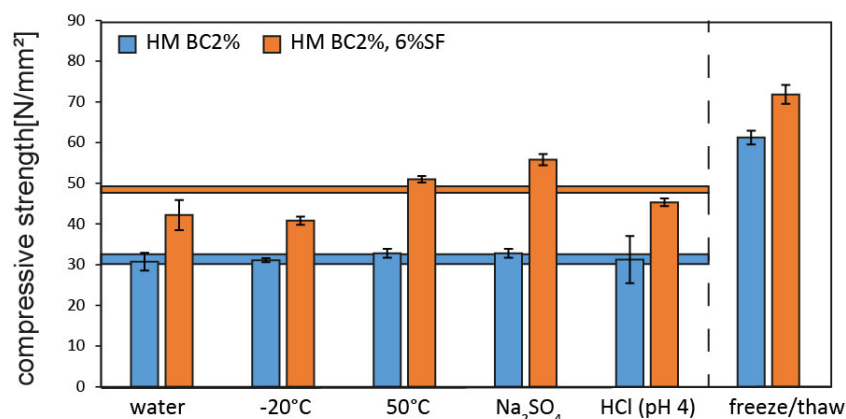
**Figure 17: Compressive strength of mortar samples.** Influence of different storage conditions on the compressive strength of hybrid mortar samples. Compressive strength tests were performed after 28 days of curing at 20 °C (r.h. ≥ 50%) and additional 28 days storage under the respective condition. Hybrid mortar samples containing biofilm powder only (blue) are compared to hybrid mortar samples containing biofilm powder and SCM (orange). The values shown represent averages of three independent measurements. Error bars denote the standard deviation.

Prolonged exposure to sulfate ions eliminates the wetting resistance provided by the biofilm additive; however, extensive exposure of mortar to sulfate ions is also known to trigger strong structural damage. When stored under dry conditions, all samples remained dimensionally stable (**Figure 18**). After storage in sulfate solutions, we observed cracks and strong macroscopic deformations for two of the three tested mortar formulations. For biofilm-enriched hybrid mortar, this effect occurred earlier than for standard mortar (**Figure 18b,c**). However, for mortar samples containing both, biofilm powder and silica fume, this issue was completely remedied (**Figure 18b,c**). This was somewhat surprising – although previous results already pinpointed, that SCM addition can increase the sulfate resistance of cementitious materials [79-83], mostly by inducing a refinement of the pore size distribution.
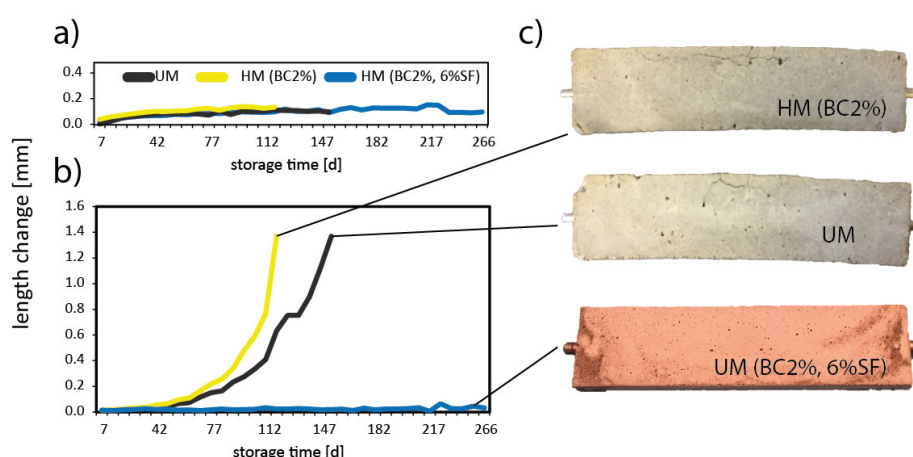


**Figure 18: Sulfate resistance of mortar samples.** (a,b) Change in sample length when stored at different conditions. Unmodified standard mortar (UM) is compared to biofilm-enriched hybrid mortar (HM, bc = 2%) and a hybrid mortar formulation containing both, bacterial biofilm and silica fume (HM, bc = 2%, 6% SF). Sample storage in the dry (a) is compared to storage in a $Na_2SO_4$ solution (b). (c) Pictures of the tested fragments on the indicated testing date.

In a last, more application-oriented test, we asked if commercially available paint can be stably applied onto the hybrid mortar material. As mortar is mostly used as an outer finish applied to walls or as binder material to hold bricks together, it would be highly desirable if the material could be painted. Indeed, not only the unmodified reference sample but also both hybrid mortar formulations could be very well colored with the blue logo of TUM (**Figure 19a**). Importantly, the applied color was also stable towards mechanical scrubbing, both when using only water (**Figure 19b**) or soap water (**Figure 19c**) when trying to remove the paint again.
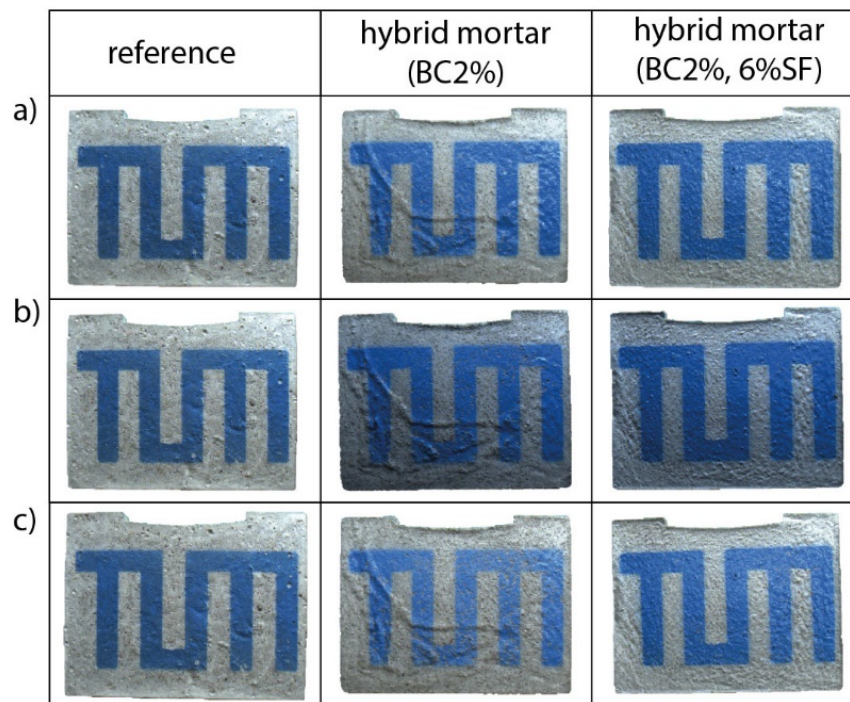


**Figure 19: Assessing the applicability of spray paint.** (a) Unmodified and hybrid mortar samples (with and without silica fume (SF) addition) were spraypainted with a commercially available spray can. After the applied paint was allowed to dry for several days, the surface of the materials was mechanically treated by scrubbing them with a root brush using first tap water (b), and then soap water (c).

*Conclusions*

The obtained results underline the high potential of biofilm-enriched hybrid mortar as a sustainable cementitious building material. The durability of this water-repellent hybrid mortar towards thermal and chemical exposure conditions is surprisingly good – and even better when the formulation contains silica fume in addition to biofilm. Since applying paint to the surface of the modified material is easily possible, the color of the hybrid mortar can be adjusted to match that of 'aged' spots from landmark buildings during their restoration. Of course, for an industrial application of bacterial biofilm as a hydrophobizing additive to mortar (and the same holds true for many other promising approaches making use of bacterial materials [75]), the

production of the bacterial additive has to be possible at a larger scale – even if only 'small volume' applications such as the restoration of historical buildings are targeted. Indeed, with the recent development of an automated biofilm-producing bioreactor [84], also this prerequisite has been achieved already. This opens the door for real-life applications of biological hybrid materials such as biofilm-enriched mortar.

<u>Individual contributions of the candidate:</u> I contributed to the conception of this study, was mainly responsible for the design and execution of the experiments, and the data analysis, and contributed to the writing of the article.

# 4. Discussion

Cement-based building materials will continue to dominate as construction materials to satisfy mankind's need for housing and infrastructure, at least in the near future; at this point, there is simply no suitable replacement. However, elongating the service life of cementitious structures could help to decrease the demand for cement. Here, bacterial additives, *e.g.* bacterial biofilm, come into play. They have the potential to drastically modify the properties of fresh and cured cementitious materials in a positive manner.

This may sound surprising since when thinking about bacterial biofilm, mostly negative associations *e.g.*, plaque[85], colonized pipes[86] or catheters[87-89] might come to mind. The ability of bacterial biofilms to adapt to various environments can be described as a double-edged sword: on the one hand, unwanted biofilm formation is hard to prevent; on the other hand, biofilms with their astonishing properties can serve as a model system for bioinspired materials and industrial applications (as described in chapter 3.1). Interestingly, especially in the field of construction materials, an area typically not associated with bacteria, intensive research is conducted concerning bacterial additives (**Figure 20**).
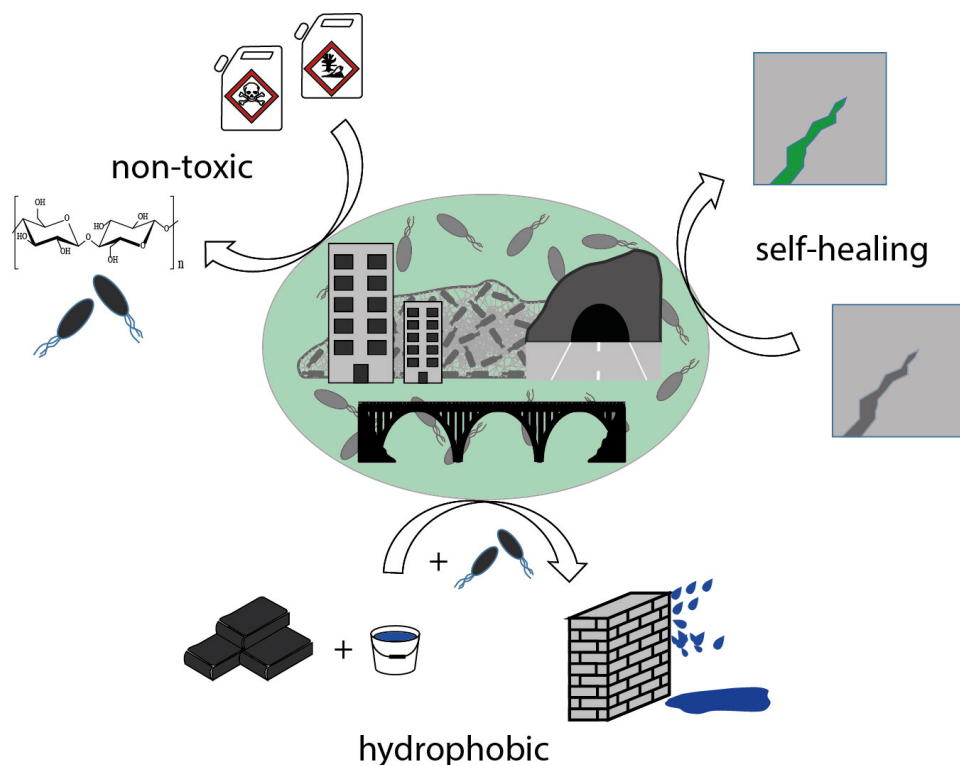


**Figure 20: Schematic overview of possible applications of bacterial additives in the construction sector.** Bacterial additives can lead to more sustainable building materials by replacing toxic or noxious chemicals and elongating the service life of cementitious structures by providing hydrophobic or self-healing properties.

The high potential of bacterial additives can be illustrated by the example of Welan gum, a viscosity-modifying agent produced by bacterial fermentation of *D*-glucose.[42, 90] This biological additive is among the most expensive bio-admixtures for cementitious materials currently in use.[41] Still, Welan gum and other biopolymers, produced via bacterial fermentation (*e.g.* Xanthan gum), are actually used despite their high cost, which demonstrates their high impact on the resulting material properties. In fact, high costs represent maybe the only real disadvantage of these biopolymers.

The arguably most popular application of bacterial additives in the field of construction engineering is their use in self-healing cementitious materials. This concept can be explained quite easily: bacterial spores are integrated into the bulk of the cementitious material. Then, cracks and subsequent penetrating moisture reactivate the calcium carbonate producing bacteria, which seal the cracks with metabolic products. The popularity of this concept can be explained by the great demand for more durable cementitious materials and the great variety of different bacterial strains able to induce calcium carbonate precipitation.[91-95] The first patent for a healing agent in cementitious materials was granted in 2014[96]; however, until today, a commercial application of this method is still not available. This demonstrates that some disadvantages of this approach still have to be solved.[97] One of them is the production of unwanted ammonia as a side product in most biological pathways. Additionally, studies evaluating large-scale applications,[98] which could successfully prove the long term stability and effectiveness of these systems, are still rare.

Another approach is to use bacterial cultures for bulk modifications: Qu *et al.*[54] presented a simple method to produce hydrophobic mortar by incorporating a bacterial suspension of *Bacillus subtilis* 3610. Bacterial solutions are also exploited as a source of bacteria cells[99, 100] or of bacterial cell fragments[47, 48], which can be used as additives to cementitious materials. Although detailed studies investigating the effect of such bacterial cultures on cementitious materials are rare, especially the low costs required for their preparation (compared to other bacterial additives) make them promising. A disadvantage of liquid bacterial cultures, however, could be that the effect of these additives might strongly be dependent on the exact composition and the bacteria density within – and both might be difficult to keep constant over time. Also, construction sites do not provide ideal, sterile conditions to produce these cultures, and they might require special storage conditions. Together, these disadvantages could make the use of this kind of additives laborious, and thus unprofitable. However, to investigate whether all these stated points apply, further research in this field has to be conducted.

As described in sections 3.2 and 3.3, different bacterial additives (liquid and solid), can be used to modify the bulk of mortar. Especially for the solid bacterial additives, most of the above stated points of concern do not apply. In addition, not only the storage conditions for these additives differ, but also do their efficiencies, if they are used to hydrophobize mortar *via* bulk modification. Therefore, the question arises, what component exactly triggers the hydrophobization, or in other words, which part of the bacterial additive has to be added to obtain a highly hydrophobic material? To answer this question precisely, the effect of the added bacterial additive has to be understood in detail. Grumbein investigated the major matrix components of *Bacillus subtilis* 3610 biofilms for their effect on the hydrophobization process of mortar. In an exclusion process making use of selected mutants, it was concluded, that neither the hydrophobic surface layer protein BslA nor other macromolecular key elements of the biofilm matrix are necessary for the hydrophobization.[101] By testing a variety of different bacterial additives from three bacterial strains and bacterial spores from six different bacterial strains (as described in sections 3.2 and 3.3) it could be shown in this thesis, that the hydrophobization mechanism is not limited to one bacterial strain or a particular bacterial additive. The different efficiencies in terms of wetting resistance and suppression of the capillary water uptake observed for bacterial spores in comparison to biofilm powder (Appendix A3) give reason to assume that two different mechanisms are triggered by different components of each bacterial additive. One mechanism leads to an increased wetting resistance of the mortar surface, with even superhydrophobic contact angles being obtained for the highest content of biofilm and biofilm powder (freeze-dried and ground biofilm) tested (Appendix 3.2). In nature, there is a famous way to obtain superhydrophobic surfaces as known from, *e.g.* the surface of lotus leaves[102-104] or the feathers of penguins[105, 106]. This is achieved by the combination of two features: a rough surface and a chemical wax layer. The increased surface roughness found for hybrid mortar samples enriched with bacterial biofilm[53] and bacterial spores (see Appendix A3) indicates a similar mechanism. A chemical contribution to the increased wetting resistance (water droplets retain their high contact angle on the hybrid mortar samples enriched with bacterial biofilm[53] for over 30 minutes) could not be identified yet. A similar wetting resistance of mortar was also reported by Qi *et al.*[54] using bacterial cultures as an additive; however, the mechanism leading to this hydrophobization remained unclear. Other studies using bacteria-free, functionalized waste products[39] or an emulsion of stearic acid[38] for a bulk modification of mortar and concrete, resulted in a similar or even higher wetting resistance; however, also here, an exact mechanism for the increased wetting resistance was not described either.

The second effect observed for hybrid mortar samples in this thesis is the reduction of capillary water uptake in hybrid mortar samples enriched with bacterial additives. As penetrating moisture and deteriorative ions transported by it can drastically affect the stability of cementitious materials, suppressing the water uptake could help to elongate the durability of the material. In hybrid mortar containing biofilm powder, the water uptake could be decreased by ~50–75% for the addition of bacterial biofilm, by ~70–80% for the addition of bacterial overnight cultures, and by ~40–70% for the addition of bacterial spores. The ability of a material to take up water by capillary forces is strongly related to its pore structure; this, in turn, can be affected by a variety of different factors: air-entraining agents can greatly increase the overall porosity,[107-109] whereas SCMs are known to reduce the overall porosity of cementitious materials.[110] However, also mixing parameters, *e.g.*, the water to cement ratio[111] or the curing conditions[112] affect the porosity. In this thesis, for all tested bacterial additives, decreased densities of the cured hybrid materials were found. As all samples compared here are based on the same basic formulation using the same type of cement and sand as well as the same w/c ratio, thus the bacterial additive was suspected to be the cause for this reduced density. Here, an increased porosity caused by the addition of the bacterial additive is consistent with the obtained results.

And indeed, when comparing the structure of the used bacterial additives with commonly used air-entraining agents (AEAs), similarities in the structural composition could be found (**Figure 21**). If an AEA is present during the mixing process of a cementitious material, air bubbles that are trapped in the cementitious paste are stabilized.[113] **Figure 21a** shows, that AEAs are usually organic molecules, which are basically composed of hydrophobic chains or cores and hydrophilic termini,[114] however, they can differ quite strongly in terms of size and structure. Regarding their working mechanisms, two general types of AEAs can be described.[113] The first type of AEA reacts with the cement paste to form insoluble calcium salts which accumulate at the solid–liquid–air interfaces and thus stabilizes the air bubbles.[115] The second type of AEA adsorbs at the air–water interface, thus strongly decreases the air–water surface tension which, in turn, stabilizes the formation of small air bubbles in the cement paste or mortar.

Selected macromolecules which are known as structural components of bacterial additives, are shown in **Figure 21b**. However, the exact composition of each bacterial additive used in this thesis is not known. For most bacterial biofilms, the main matrix components and sometimes even other matrix components have been identified; however, a complete catalogue of matrix constituents, is often not available yet – not even for intensively studied biofilms.[116] A similar

issue applies to bacterial spores, for which the basic structure is known;[117] their detailed architecture, however, can differ strongly for each bacterial strain. As both, biofilms and spores, are mostly composed of polysaccharides and polypeptides, almost every building block of their structure bears chemical functionalities that should allow them to act as AEA.

The conducted density measurements (Appendix A.2) as well as the MIP measurements (for samples enriched with biofilm powder, AppendixA.4) confirm an air-entraining effect for biofilm powder and liquid bacterial cultures. For all the other bacterial additives tested in this thesis, an air-entraining effect is likely, but was not proven.
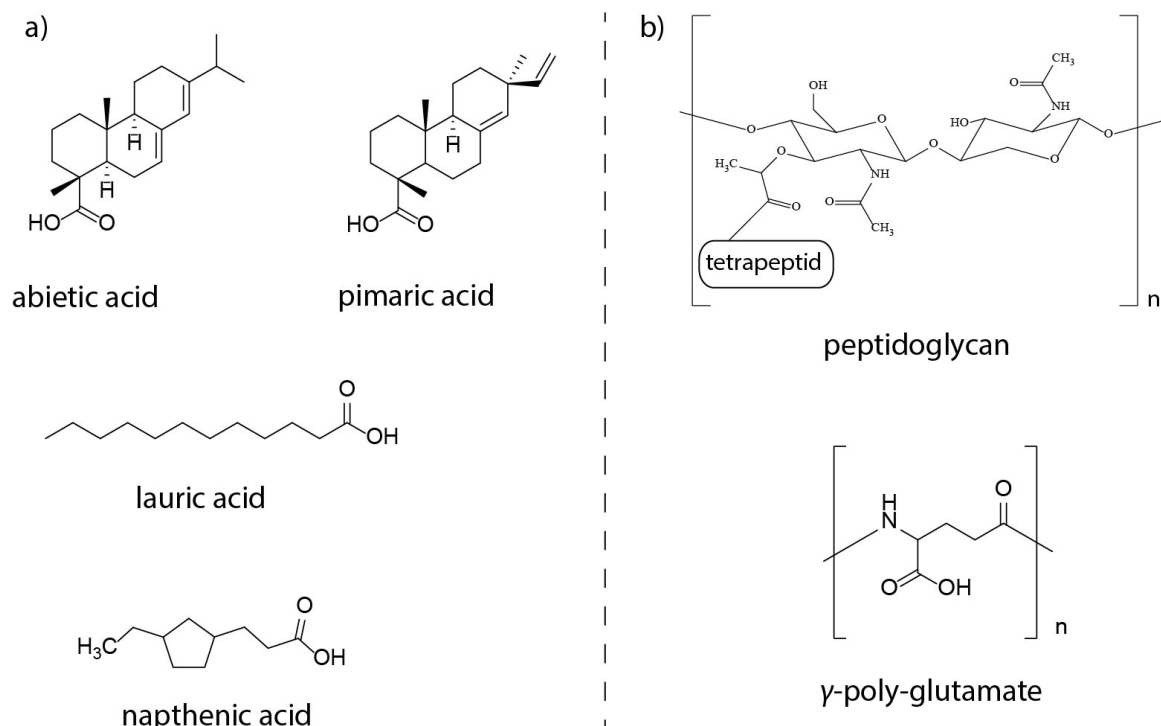


**Figure 21: Overview of the structure of selected air-entraining agents (a) and selected structures found in bacterial additives (b).** (a) Abietic and pimaric acids are the main compounds in wood resins. Other examples of commonly used air-entraining agents are lauric acid and naphtenic acid. (b) Peptidoglycan, a macromolecule found in bacterial cell walls and γ-polyglutamate as the main matrix component of some protein-based biofilms, represent selected structures found in bacterial additives.

To exclude the possibility of different hydration products being formed in unmodified mortar samples and hybrid mortar samples, XRD and $^{29}$Si-NMR measurements were conducted. These measurements confirmed, that in hybrid mortar samples enriched with biofilm powder of the bacterial strain *Bacillus subtilis* 3610 the same hydration products were formed as in unmodified reference samples (section 3.4). Therefore, the reduced density of these samples is attributable to the air-entraining effect brought about by the biofilm powder.

A more detailed investigation of the pore structure in biofilm enriched hybrid mortar samples, revealed an increased overall porosity, an increased average pore size, and a different pore size distribution for this mortar variant. The average pore size shifted compared to unmodified reference samples to higher values. Whereas ~60 % of the pores have a diameter <0.1 µm in unmodified reference samples, hybrid mortar enriched with biofilm powder contains ~80 % pores with a diameter >0.1 µm (**Figure 5**, Appendix A.4). Consistent with the finding of a reduced capillary water uptake in mortar exhibiting a certain pore structure, Feng *et al*[38] reported a reduction of the capillary water uptake of ~86 % for the use of a stearic acid emulsion as hydrophobizing additive in mortar. Here, the addition of the stearic acid emulsion lead to an increase in the total pore volume as well as a shift of the average pore size from ~60 nm to ~0.3 µm.

The pore system does, however, not only affect the capillary water uptake, but also the mechanical strength of the resulting material. [118-121] In this thesis, for all tested hybrid mortar variants but one, decreased mechanical strengths were observed. Only at an unusually high w/c ratio of 0.6, one spore variant resulted in a slightly higher compressive strength than the unmodified reference. At more common w/c ratios of 0.5 or lower, the addition of bacterial additives leads to a (sometimes drastic) decrease of the compressive strength, e.g. by 60 – 70 %, upon the addition of biofilm powder. In accordance, Ersan *et al.* reported a decrease in compressive strength of ~40 % for the addition of *Bacillus sphaericus* spores.[122] Here, also an increased porosity was suggested to be the cause for the observed loss in strength. A similar decrease in compressive strength was also reported for the integration of bacteria-free, organic admixtures such as stearic acid emulsion[38] or paper sludge ash powder functionalized with stearic acid[39] – and those additives also increased the overall porosity of cementitious materials. Having verified *via* XRD and ^{29}Si-NMR measurements that similar hydration products were formed in unmodified and hybrid mortar enriched with biofilm powder, it should be possible to increase the compressive strength by decreasing the porosity of the material. Therefore, in this thesis, SCMs were used. Especially silica rich SCMs are known to influence the amount and type of hydrates formed in cementitious systems, thus affecting the volume, the porosity and finally the durability of such systems.[16] And indeed, the compressive strength of hybrid mortar formulation enriched with both, one of four tested SCMs and biofilm powder, could be increased by 10 – 32 % compared to hybrid mortar samples containing biofilm powder only. Importantly, the increased wetting resistance of the hybrid mortar and its ability to partially suppress capillary water uptake was maintained at least until a certain SCM concentration was reached; higher concentrations led to a complete loss of the wetting

resistance. An analysis of the microscopic surface profiles of hybrid mortar samples showed that, at such high SCM concentrations, the increased surface roughness (a key feature for superhydrophobic surfaces) is strongly decreased. Interestingly, already small concentrations of each SCM affected the capillary water uptake of mortar; however, the amount of water taken up by these samples was still half of what was determined for unmodified reference samples. With increasing SCM concentrations, the capillary water uptake did not increase further, but remained at an almost constant level. MIP measurements of selected hybrid mortar samples enriched with SCMs and biofilm powder showed a medium porosity for these samples when comparing to both, unmodified reference samples (which exhibited a low porosity) and hybrid mortar samples containing biofilm powder only (which exhibited a very high porosity). Compared to hybrid mortar samples containing biofilm powder only, in hybrid mortar samples additionally enriched with SCMs, the porosity and the average pore size was decreased. The obtained results indicate that, at a constant biofilm content, the pore structure and the overall porosity of the resulting materials can be modulated by varying the SCM content. The resulting pore structure, in turn, influences both, the mechanical strength and the capillary water uptake of the sample. A similar concept was reported by Oltulu *et al.*[123], who described a relationship between the pore size distribution and the capillary water absorption behavior of a mortar containing silica fume and different nanopowders. Here, the porosity was regulated by tuning the composition of the different binary or ternary mixtures.

An additional, well-known effect, which also applies for bacterial additives, is the delay of the hydration reaction due to the addition of organic additives to cementitious systems. As described in section 3.2, the addition of bacterial biofilm powder, as well as bacterial cultures, led to a delayed hydration reaction. A similar retardation was also observed in previously published studies investigating bacterial cultures.[54, 124, 125] However, although reported for a variety of different molecules[54, 124-128], the exact mechanism could not always be identified, yet.[129] Possible hypotheses assume a complexation of calcium ions or the inhibition of growth of hydrates.[128]

Finally, in the last step of this thesis, the durability of the best performing hybrid mortar formulation (hybrid mortar enriched with biofilm powder and silica fume) was examined. To do so, possible challenging environmental influences, *e.g.*, high and low temperatures, moisture, and freeze-thaw cycles were simulated at laboratory scale. These exposures were designed to provide insights into the durability of the tested hybrid mortar materials and, more importantly,

the longevity of the hydrophobic properties. The obtained results underlined the high potential of the tested SCM enriched hybrid mortar formulation: The hydrophobic properties and the mechanical strength of the samples were maintained under almost all tested conditions. SCMs[130] and especially silica fume,[131, 132] are also known for their ability to improve the resistance of cementitious materials against sulfate attack. However, when silica fume and biofilm powder are added simultaneously, both additives affect the pore structure antagonistically. Thus, a question was if the resulting intermediate porosity could still successfully protect the test samples from sulfate attack. And indeed, the damage formation due to sulfate attack could completely be prevented in the tested hybrid mortar formulation containing biofilm powder and silica fume.

In conclusion, this thesis provides many novel insights into the effects of bacterial additives on the hydrophobic and mechanical properties of mortar. However, some aspects concerning the role of bacterial additives during cement hydration remain unclear. Most notably, the exact mechanism leading to the increased wetting resistance still needs to be deciphered. Furthermore, this work presents a cementitious hybrid material whose mechanical and hydrophobic properties can be tuned by the addition of two different additives. The obtained properties can be directly correlated with the pore structure of the hardened material. Finally, an investigation of the long-term stability of hybrid mortar confirmed the suitability of the previously developed hybrid material for real-life applications: This material is intrinsically stable against a wide range of harmful environmental influences. Especially with the rising demand for more sustainable additives for cementitious materials, the insights gained in this thesis may be of great value for the future development of new, sustainable additives for cementitious building materials.

# 5. Outlook

The bacterial additives presented in this thesis have the potential to greatly improve the durability of mortar; however, some challenges still have to be overcome for those additives to be used in large scale applications. One issue might be the addition of the bacterial additive to the building material. Here, particularly powdery additives, *e.g.* lyophilized biofilm powder, have the benefit of easily and effectively being integrated into the mixing design of mortar and potentially other cementitious building materials. However, the low costs of producing bacterial cultures, compared to other bacterial additives, in combination with the promising results obtained with this kind of additive so far make them interesting objectives for future studies.

Another challenge is that, in addition to the aggregate state of different bacterial additives, their effectiveness regarding the hydrophobization of mortar can vary strongly. Especially the exact mechanism which leads to an increased wetting resistance is worth of future investigations. Understanding this mechanism could enable the future development of novel additives, which only increase the wetting resistance without increasing the porosity of mortar and other cementitious materials. This might be particularly important, as the effect of the current used bacterial additives – a simultaneous increase of the wetting resistance and the porosity – might not be suitable for all cementitious materials, especially not for reinforced concrete. Here, an increased porosity could lead to faster corrosion of the reinforced steel, thus even shortening the service life of this material. Adapting the mode of action of bacterial additives to enable their use as an additive in concrete, the world's most used material, might not only be economically profitable, but could significantly improve the sustainability of the whole construction sector.

A third challenge for the use of the current state of bacterial additives are their currently high production costs. The current manufacturing processes for bacterial additives often require time and cost intensive manual work. This makes the resulting material expensive, and not well-suited for a large-scale application. The recently developed bioreactor for the continuous cultivation of *B. subtilis natto* biofilms[84] could, however, help to solve this problem by enabling a semi-automatic large-scale production of bacterial biofilm. Such a production method could drastically reduce the costs of this bacterial additive in the near future. Additionally, an automatic production could strongly reduce the amount of plastic trash resulting from the manual cultivation of bacterial biofilm, thus making the production process more eco-friendly. Genetic modifications of the used microorganisms could further help to improve the yield.[133]

With the increasing importance of sustainability, research also focuses on cement-free building materials. Again, bacteria can be a key component in the development of such materials. The concept of "biocementation" is based on microbially induced calcite precipitation, similar to the strategy employed for self-healing concrete. However, instead of healing cracks, the produced calcium carbonate is used to solidify sand or coarser aggregates – without the need for a cementitious binder. [134, 135] One step further goes the relatively new field of engineered living materials, an approach combining biology and material science to develop more sustainable building materials. Heveran et al.[136] developed a material in which the microorganisms stay alive and continue to produce the material until a change in the environmental conditions shuts off the material growth. In addition to avoiding cement as a binder, after its life cycle is over, the material is reported to be well recyclable. Similarly, McBee et al.[137] described a high-performance, lightweight and biodegradable fungi–bacteria–based composite material, which represents another recyclable, cement-free material with astonishing properties. However, future studies have to prove, whether one of these, at laboratory scale, promising approaches has the potential to compete against the supremacy of cementitious building materials.

# Appendix

This appendix contains the following items:

    A.  Full-text publications presented in this thesis
    B.  Licenses for publication
    C.  Full list of publications

## A. Publications

## A.1    Bacterial Materials: Applications of Natural and Modified Biofilms

# Bacterial Materials: Applications of Natural and Modified Biofilms

*Elif N. Hayta, Marvin J. Ertelt, Martin Kretschmer, and Oliver Lieleg\**

Over millennia, bacteria have developed clever strategies to build biopolymer-based communities in which they can survive even extremely challenging conditions. Such bacterial biofilms come with a broad range of fascinating material properties that—in settings such as medicine, food production, or other areas of industry—make it difficult to remove or inactivate them: they can stick to many surfaces, repel water and oils, and can even transport electrons. Inspired by the outstanding versatility and sturdiness of such bacterial biofilms, material scientists have set out to harness those properties and to create bacterial materials for different applications. However, as the range of technological applications employing biofilms keeps expanding, improved material properties or broader functionalities are desired. Here, such attempts where materials with improved properties were created by making use of either natural or modified bacterial biofilms are reviewed. The areas in which those bacterial materials may be used range from agriculture and (environmental) biotechnology over biomedical and electrical engineering to construction engineering.

## 1. Introduction

Bacterial biofilms are sticky and slimy substances that come with a variety of unique and sometimes annoying properties. In those biofilms, bacteria embed themselves into self-secreted, extracellular polymeric substances (EPSs).[1–6] Depending on the particular bacterial strain and the growth conditions during biofilm generation, the composition of this EPS can vary quite a bit.[7] Yet, in any case, these EPS crucially determine many biofilm properties and allow the resident bacteria to survive in challenging environments.[4,8]

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Biofilms can colonize a broad range of different surfaces including those of natural materials such as stones, teeth, and plant roots[9–11] as well as man-made objects including pipes, hulls, and catheters.[12–14] Yet, bacterial biofilms not only adhere well to the surface of objects they colonize; the upper surface of many biofilms is sticky as well, and this property enables biofilms to adhere to each other and to neighboring materials.[15–19]

Another central property of bacterial biofilms is their slimy consistency. In most cases, bacterial biofilms can be described as viscoelastic solids, i.e., materials that combine liquid-like and solid-like characteristics but are dominated by the latter.[8,20–26] Depending on the bacterial species, the stiffness of biofilms grown in the lab ranges from a few hundred to several kPa.[15,20,27] However, when exposed to certain metal ions, which can be part of the natural environment the biofilms grow in, those stiffness values can be increased up to 1000-fold.[15,20,21] This finding already indicates the high adaptability of this biomaterial. Even more curious is the ability of biofilms to self-heal: even after exposure to large shear forces, they are able to quickly and fully recover their initial viscoelastic properties.[20,22] Together, those properties enable biofilms to permanently settle on solid surfaces—even in the presence of shear forces.[21,28,29]

Another key property some bacterial biofilms are able to develop is the ability to efficiently repel a broad range of fluids ranging from water to oils.[30,31] With such a high wetting resistance, biofilms can withstand erosion by flowing or dripping water, and they can protect themselves from toxic substances (such as antibiotics or metal ions) dissolved in liquids.[21,31,32] Moreover, even if bacterial biofilms can be successfully wetted, they still can restrict the diffusive entry of molecules into their core.[33–35] The macromolecular network established by the bacterial EPS is mainly responsible for this effect: molecules (or particles) that bind to the EPS are prevented from reaching the bacteria—and this can limit the efficiency of antibiotics or other antibacterial substances.[21,36]

Of course, the viability and proliferation of biofilm bacteria requires the metabolic conversion of nutrients, and certain biofilms have developed a specific internal architecture to allow for their perfusion.[25,37] As a side product of their metabolic activity, a subset of biofilm bacteria liberate electrons, which originate from the chemical decomposition of organic substances;[38] and there are even conductive biofilms that are able to generate an electric current.[39]

Altogether, these properties render bacterial biofilms sturdy and unique materials. In many cases, typically in industrial or medical
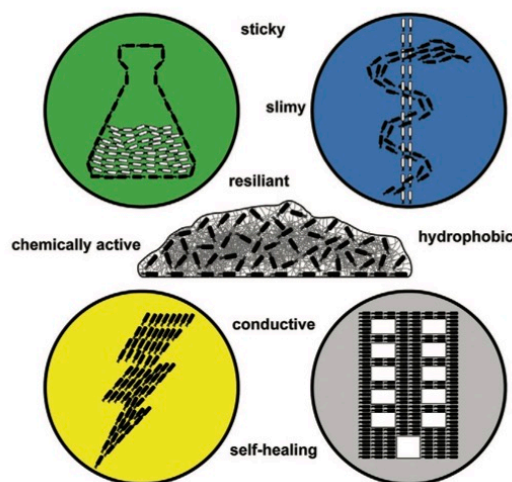
**Figure 1.** The multifaceted material properties of bacterial biofilms can be useful in different fields of application. Due to their unique material properties, bacterial biofilms and materials generated thereof have been tested in several areas including agriculture and (environmental) biotechnology (green), (bio)medicine (blue), electricity generation (yellow), and civil engineering (gray).

settings, biofilm growth has negative consequences for humans as the bacteria can contaminate food production processes or lead to infections.[4,40–43] With the range of properties discussed above, it is typically quite difficult to remove biofilms from the surfaces they colonize or to chemically inactivate bacteria residing within a protective biofilm matrix. However, from a material scientist's point of view, some of the unique properties of bacterial biofilms do not have to be a burden only—they also offer a variety of possibilities for applications in biotechnology, medicine, and even civil engineering (**Figure 1**). One option to make this happen is using genetic engineering tools, and there are many examples where this strategy was successfully implemented.[24,44,45] As an alternative approach, the properties of biofilms can also be modified by manipulating the composition of this biomaterial, e.g., by adding (bio)polymers, nanoparticles (NPs), small molecules, or other bacteria. In the following section, we highlight selected examples of the latter. There, either natural biofilms or artificial combinations of bacteria with microscopic objects have demonstrated high potential to serve mankind by doing exactly what they are good at: being resilient, sticky, and liquid-repellent as well as chemically converting molecules into other products.

## 2. Applications of Natural and Modified Biofilms in Different Fields

### 2.1. Biofilms for Agricultural, Environmental, and Industrial Biotechnology

Several biotechnological applications highly benefit from mankind's ability to make use of bacteria and their products. For instance, a large range of bacterial biocatalysts (enzymes or whole cells) have been developed to produce valuable molecules such as fine chemicals, pharmaceuticals, and ingredients of cosmetics.[46] In addition, bacteria themselves can be interesting products themselves, e.g., as food ingredients[47–49] or as additives to increase the sustainability of agriculture. The latter is achieved by the microorganisms acting as biocontrol agents,[50] plant-growth promoters,[51] or biofertilizers.[52] Of course, also bioremediation approaches heavily depend on microbial activity[53]—without them, wastewater treatment would be not efficient at all. In the following section, we highlight a few examples from those areas, where bacterial biofilms with dedicated properties were developed.

To obtain biofilms with tailored functionalities, synthetic biology tools can be employed, where genetic modifications on the bacterial genome are employed to change the biofilm properties.[24,45] Typically, such a strategy is based on the bacteria secreting additional (or altered) biofilm matrix components, enzymes, or other functional molecules. Alternatively, different bacterial strains can be combined with each other or with synthetic components (molecules, polymers, or nanoparticles) during biofilm cultivation (**Figure 2**). In nature, biofilms comprise multispecies microbial consortia, which follow a symbiotic life style to better adapt to the environment. Inspired by this natural collaboration, cocultivation of bacteria producing cellulose (e.g., acetic acid bacteria or acetobacteria) with other, catalytic microorganisms can result in functional, living materials with increased production efficiency. Here, the cellulose-environment generated by one bacterial strain can act as an encapsulation agent for the other strain and thus provides protection to the latter.[54,55] In another example where several additional functionalities were installed into a biofilm, the cellulose matrix secreted by the biofilm bacteria was modified by enzymes produced by yeast cells. There, engineered yeast cells were artificially integrated into the biofilm matrix by cocultivation, and this resulted in biofilms with altered mechanical properties: the biofilms were converted into viscoelastic fluids.[56]

A different strategy aims at immobilizing enzymes in biofilms to obtain an enhanced bioprocess performance such as increased activity, robustness toward alterations in pH and temperature, and reusability. For instance, Romero et al.[57] demonstrated how biofilm matrix components contribute to the immobilization of an extracellular bacterial enzyme. They showed that secreted lipase molecules are fully trapped in the biofilm matrix—there was no (undesired) loss of enzyme from the biofilm pellicle into the aqueous phase it was grown on. In the protected microenvironment of the biofilm matrix, the specific activity of this immobilized enzyme was increased, and the immobilized enzymes maintained 42% of their activity even after three catalytic cycles. Botyanszki et al.[58] achieved an immobilization of $\alpha$amylase onto the curli fibers of *Escherichia coli* biofilms; to make this possible, they used genetic tools to achieve site-specific binding to the curli fibers, and this entailed improved enzymatic activity: As a consequence of this immobilization, the pH range within which the enzyme has good activity, was increased and the biocatalyst maintained a high activity even in the presence of solvents. Such improvements and those building on them[59] may provide a big advantage in industrial applications, where organic solvents are necessary—
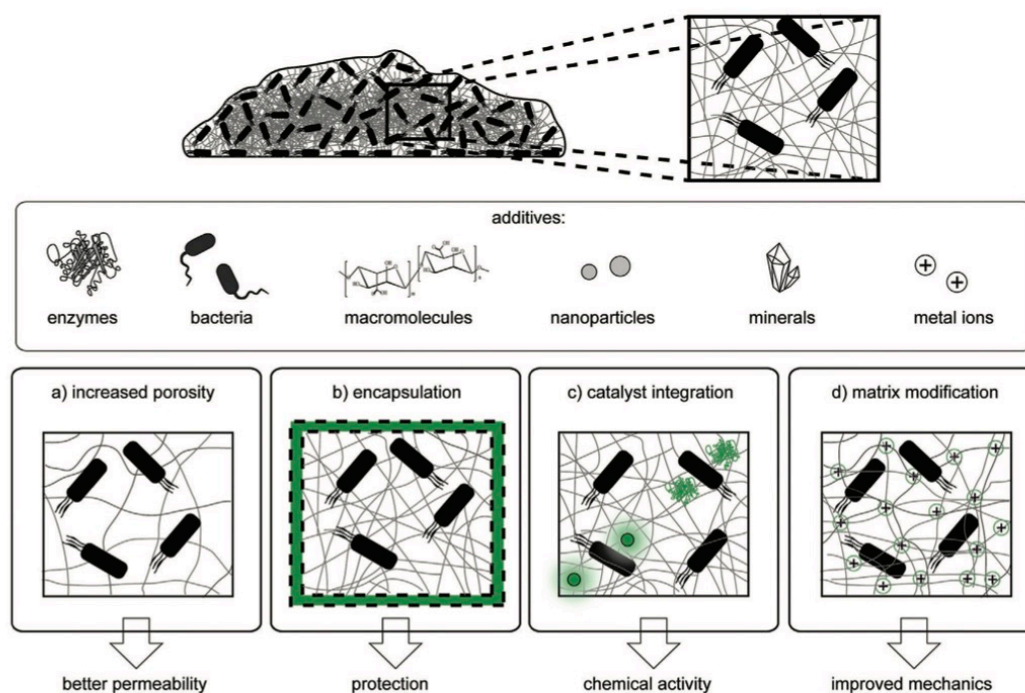
**Figure 2.** Strategies to obtain biofilms with improved properties. Integration of various entities such as (macro)molecules, nanoparticles, minerals, metal ions, enzymes, or other bacteria into the matrix can alter their properties such that they become better suitable for certain applications. a) For instance, increasing the porosity of the matrix improves the biofilm permeability toward nutrients and electrons, and this typically improves bacterial viability within the biofilm. b) Encapsulation of biofilms with polymers or minerals protects the bacteria from environmental stresses. c) By integrating enzymes or catalytic nanoparticles into the biofilm structure, the functionality of the biofilms can be broadened. d) Adding ionic compounds such as metal ions or extracellular DNA to the biofilm matrix induces crosslinking effects, which boosts the stiffness of the bacterial material.

thus broadening the range of possible applications. Similarly, Dong et al.[60] made use of a chemical immobilization strategy based on carbodiimide coupling to covalently link an enzyme to the EPS of a *Bacillus subtilis* biofilm matrix without reducing enzymatic activity. Moreover, in the same study, magnetic nanoparticles were added to the enzyme-enriched biofilm. The authors suggested that this second modification may help retrieving the biofilm by magnetic forces, e.g., after it has been added to a complex environment, in which it is supposed to perform its catalytic activity.

Of course, the benefit of including nanoparticles into biofilms can go beyond enabling material recovery. The antimicrobial properties of certain nanoparticles are well established, and there are many biotechnological applications where nanoparticles are combined with biofilms to harness this property.[61] In agriculture, nanoparticles have already been used quite often as biocontrol agents against plant pathogens.[62,63] Recently, Mahawar et al.[64] combined silver nanoparticles with cyanobacteria and observed improved plant growth as well as better resistance toward pathogens than when those two agents were applied individually. Timmusk et al.[65] formulated different bacterial inoculations with titanium dioxide ($TiO_2$) nanoparticles

to improve the resilience of plants toward drought, salt, and pathogen stresses. Here, the addition of nanoparticles to the bacterial inoculates considerably amplified biofilm formation on the rhizosphere of the plant; consistently, NP-containing formulations performed better than bacterial fertilizers alone. Similarly, in a study conducted by Vishwakarma et al.,[66] combining rhizobacteria with silicon gave rise to better protection of plants against toxic effects than a rhizobacterial biofilm could provide itself.

Similar to equipping biofilms with enzymes, some nanoparticles can also convey catalytic activity to bacterial materials. For instance, Wang et al.[67] immobilized several nanomaterials in engineered *E. coli* biofilms to enable the reduction of polynitrophenol, the photocatalytic degradation of organic dyes, or photoinduced hydrogen production. In addition to establishing catalytic processes, nanoparticles can also boost the existing catalytic activity of a biofilm by enhancing existing extracellular electron transfer mechanisms. For instance, bioremediation of hexavalent chromium by *Shewanella oneidensis* biofilms formed in carbon nanotube (CNT)-enriched alginate beads was improved compared to biofilms formed without CNTs.[68] Adding quinone-based electron mediators to the CNT-enriched

biofilm materials further enhanced this effect, and the resulting bacterial material turned out to be useful for the bioremediation of uranium.[69]

For many biotechnological applications, e.g., for protein or metabolite biosynthesis, planktonic bacteria are highly suitable;[70] however, when other bacterial properties are required as part of a functional material, biofilms are typically preferred over planktonic cells.[71] To a large extent, this is due to the superior material properties of biofilms. Nevertheless, there are efforts to further improve these material properties, e.g., by incorporating functional entities (molecules, ions, minerals, or polymers) into the biofilm matrix. As one of the main components of natural biofilm matrices, extracellular DNA (eDNA) has been shown to play a crucial role in biofilm formation,[72] bacterial aggregation,[73] and adhesion;[74,75] moreover, eDNA can affect the mechanical strength and integrity of biofilms,[76–78] modulate extracellular electron transfer throughout the biofilm matrix,[79] and provide enhanced resistance against antibiotics.[80] In fact, Chaves et al.[81] suggested that tuning the viscoelastic properties and even surface topography of biofilms by controlling the amount of eDNA within the biofilm may offer opportunities in biotechnological applications—yet this still needs to be explored.

From a physicochemical point of view, interactions between eDNA and biofilm matrix components (or antibiotics) can be rationalized by electrostatic forces acting between the strongly anionic eDNA molecules and cationic groups from biofilm constituents.[82,83] Another strategy to alter the interactions between certain biofilm matrix components makes use of ionic crosslinks, which can be generated by incorporating cationic metal ions into the biofilm material.[20,21,84] Kretschmer and Lieleg[22] showed that the size and valency of the ions in combination with the molecular configuration of anionic residues on the biofilm matrix polymers dictates if and how strongly the stiffness of the biofilm is increased by this approach. The ability to boost the biofilm stiffness may open the door for novel applications: for instance, by adding $Fe^{3+}$ ions to the biofilm matrix, Zhang et al.[85] could increase the internal mechanical strength of biofilms, which were genetically engineered to become highly sticky. With such a "living glue," surface damage could be successfully repaired. A similar self-healing activity accompanied by a strong anticorrosion protection was observed by Liu et al.[86] when they enriched a culture of cellulose-overproducing bacteria with $Ca^{2+}$. Here, calcite ($CaCO_3$) formation within the cellulose-rich biofilm provided improved stability of biofilm coatings. The authors suggested that such anticorrosive biocoatings could be useful tools to increase the life time of metallic objects in marine environments. A (reversibly) increased biofilm stiffness as achieved by the addition of metal ions can also result in enhanced erosion resistance[21,29]—and such a property can be a desirable feature for biotechnological applications. A similar result was obtained by Hayta and Lieleg;[28] yet, there, bacteria were allowed to establish a biofilm matrix in the presence of purified biopolymers. As a consequence, not the shear stiffness of the biofilm but its surface topography and thus its mode of interaction with water was modulated such that the stability of the biofilm toward erosion was enhanced.

Embedding bacteria with polymers is a strategy not only used by naturally occurring biofilms—the same can be achieved arti-

ficially. In fact, the effects of embedding bacteria into purified biopolymers present in the matrix of certain biofilms (such as alginate and cellulose) has already been extensively studied—both, with the goal to investigate the bacterial behavior in different polymeric matrixes[87,88] and to create more robust bacterial catalysts for biotechnological applications.[89–91] Recently, novel encapsulation techniques have been introduced to keep up with the latest developments in biotechnology. For instance, in a study conducted by Jaroch et al.,[92] in situ encapsulation of mature biofilms was achieved, and the biofilms were grown on a hollow fiber membrane. As a result, the encapsulated biofilm could better resist the shear forces it was exposed to in a bioreactor. Importantly, this covering layer was permeable to air and nutrients, which guaranteed good cell viability. A different approach was followed by Panchal et al.,[93] who filled liquid marbles generated from halloysite nanotubes with a bacterial culture. Here, the bacterial EPS produced inside the liquid marbles enhanced the mechanical strength of the spheres and stabilized their shape and volume by preventing evaporation. With these improvements, the encapsulated bacteria could be stored at room temperature for more than a week. Interestingly, a similar approach could be applied to nonbiofilm forming bacteria when the spheres were artificially enriched with polymers. A new, bioinspired method based on the self-assembly process of chitosan macromolecules was introduced by Park et al.[94] Here, tyrosinase-producing bacteria modified the chitosan biopolymers such that they bound to the bacteria and formed a network around them. Artificial biofilms produced this way showed better cell loading capacity and cellular viability than those obtained via conventional encapsulation strategies. Also here, an application has already been identified: The authors showed that these synthetic biofilms can be employed in the bioremediation of crude oil: within 28 days, they could remove ≈90% of oil from contaminated water.

Overall, those examples clearly highlight that, with further improvements in terms of production time, stability and recoverability, artificial biofilms have the potential to contribute to many other areas of biotechnology in the future.

## 2.2. Biofilms for Medical Applications

One of the natural habitats of bacterial biofilms is the gastrointestinal tract (GIT).[95] In fact, in humans, there are approximately ten times more procaryotic cells than eucaryotic ones. Commensal bacteria are not only crucial for regulating our metabolism and immune system, they can also protect us against pathogens. Hence, avoiding (and, if necessary, curing) GIT dysbiosis is increasingly considered as a therapeutic approach to deal with GIT disorders. To maintain or regain a balanced microflora in the GIT, diet regulation, antibiotic treatment, and consumption of prebiotics or probiotics may be needed.[96] The latter are living microorganisms which, when administered in adequate amounts, confer a health benefit to the host. More specifically, consumption of probiotics aims at regulating the gut microbiota by manipulating interspecies interactions.[97]

During the industrial production process that is required to turn bacteria into food products suitable for oral consumption,

the probiotics are exposed to harsh conditions such as heat or cold; after production is completed, the prolonged storage, e.g., in fridges or cooling cabinets (4 °C), is not ideal for the bacteria either. In addition, until they reach the desired area (i.e., the intestines), probiotics pass through the extreme environment of the stomach—yet they need to be viable in large numbers when arriving in the gut where they are supposed to take effect. Thus, those beneficial bacteria require protection. Microencapsulation of probiotic bacteria is a well-established method to produce functional probiotic food products,[98] and several biopolymers or smaller molecules (such as milk proteins) have been employed to achieve this.[97,99] However, also the natural shield produced by the bacteria, i.e., the EPS, can provide the required protection: biofilm-embedded bacteria exhibit better resistance against extreme conditions and trigger a better immune response in the host.[100,101]

Even though probiotics in biofilm form come with a range of advantages compared to their planktonic counterparts, they can still be further improved. For instance, Cheow and Hadinoto[102] encapsulated *Lactobacillus rhamnosus* bacteria into double-layered, chitosan-coated alginate or carrageenan polymeric beads and then further incubated these microcapsules to enable the formation of biofilms in their core. As expected, those shielded biofilms process showed superior freeze-drying resistance and thermotolerance. Moreover, bacterial release into the intestinal mucosa was higher for such encapsulated biofilms. In 2014, the same group of researchers improved their probiotic delivery system by adding locust bean gum to their chitosan-coated alginate formulation, which boosted the resilience of the probiotic.[103] Similarly, biofilm loaded calcium pectinate beads produced by Heumann et al.[104] lead to sturdier probiotics; from those biofilm-spheres, the bacteria were released to the colon as clusters which provided a better anti-inflammatory effect and protection against GIT disorders than other probiotic forms of this bacterial strain. A better release of biofilm bacteria was also achieved by Vega-Sagardía et al.,[105] who enriched their formulation with vegetal oil to increase the residence time of the probiotic biofilm in the stomach so that a *Helicobacter pylori* infection could be efficiently dealt with. A different approach proposed by Praveschotinunt et al.[106] aimed at enriching a probiotic biofilm with a therapeutic peptide to promote epithelial restitution. By introducing these modified biofilms, the authors were able to achieve mucosal healing and immunomodulation in vivo.

In addition to enriching the biofilm matrix, also fine-tuning the growth conditions of biofilms can render probiotics more resilient. For instance, Kiew et al.[107] examined the effect of biofilm age and growth medium affect the stress-resistance of biofilms. Moreover, also adjusting the detailed production process of encapsulated biofilms[108] and cocultivation with a second bacterial strain, e.g., combining lactic acid bacteria with *B. subtilis*[109] can improve the resilience of the probiotic. In the latter example, the EPS produced by *B. subtilis* is mainly responsible for the obtained protection effect. Importantly, the presence of this second bacterial strain comes with another advantage: in addition to their ability to secrete exopolymeric substances, *B. subtilis* bacteria have recently been reported to be able to help maintaining the balance of the GIT microbiota.[110,111] Another promising usage of biofilms in the GIT

was described by Duraj-Thatte et al.[112] Here, a robust, self-regenerative hydrogel containing living bacteria was developed that showed an increased retention time in the GIT in vivo. Expression of mucin binding proteins by genetic modifications resulted in specific and strong adhesion of the bacteria-loaded hydrogels to the GIT tissue. Furthermore, the viscoelastic properties of this bacterial hydrogel could be adjusted by varying the type of mucoadhesive protein and the DNA content of the gel, and the authors suggested that such a system has the potential to serve as a drug delivery system.

However, the benefits of bacterial biofilms for our health are not limited to regulating the gut flora. Bacterial pellicles generated by certain bacteria belonging to the genera *Agrobacterium*, *Acetobacter*, *Pseudomonas*, *Rhizobium*, *Azotobacter*, *Alcaligenes*, *Achromobacter*, and *Sarcina* comprise almost exclusively bacterial cellulose (BC), and those have applications in biomedicine—with the bacteria being inactivated and washed out.[113,114] Owing to their high biocompatibility, water uptake capacity, permeability to gases and liquids, and desirable mechanical properties such as high tensile strength and flexibility, those BC materials have turned out to be good candidates for drug delivery systems[115] and wound treatment.[116,117] In addition, the structural similarity of BC-biofilms and human collagenous extracellular matrix enables the use of the former as tissue scaffolds.[118] Also here, the chemical and physical properties of BC-based materials can be further improved by incorporating polymers, nanoparticles, minerals, or functional molecules.[113,119,120]

More recently, engineered living materials have been introduced. Here, genetic engineering tools are combined with material science approaches to create novel materials for medical applications. For instance, Wang et al.[121] employed light-inducible biomineralization of hydroxyapatite to repair site-specific damages: *E. coli* biofilms expressing adhesins act as a glue that connects polystyrene microspheres thus creating a biohybrid filler material that autonomously solidifies via mineralization processes. Possible future applications of this technique could be in the field of bone regeneration. A similar *E. coli* biofilm producing adhesive molecules (adhesin and DOPA) was used by An et al.[122] to fight blood-leakage. In the lab, this already works: using a microfluidic setup mimicking a (slightly) bleeding blood vessel, it was shown that this living glue can autonomously repair small damages, and this is triggered by exposing the bacteria to the molecule heme. Although those examples still need to be developed further to be applicable in vivo, they present innovative new concepts of how bacterial biofilms could serve as promising tools for medical problems.

## 2.3. Biofilms with Enhanced Electrochemical Activity

Already in 1911, Potter could demonstrate that the decomposition of organic substances by bacteria or fungi can generate an electrical current.[38] However, this finding did not receive much attention until it was realized how the microbes make use of electron mediators in such a biological, electrogenic system:[123,124] Exoelectrogenic bacteria can transfer electrons directly to each other or to the surface of electrodes, and they

achieve this by employing outer membrane cytochromes, excreted mediators (i.e., electron shuttles) or biological "nanowires" (i.e., conductive pili).[39,123] Recent improvements in the electron transfer capability of microbial communities promoted the development of applications making use of them. Examples of such bioelectrochemical systems (BESs) include microbial fuel cells (MFCs),[124,125] microbial electrolysis cells (MECs),[126,127] biological photovoltaics (BPVs),[128,129] microbial desalination cells (MDCs),[130,131] microbial electrosynthesis (ME),[126,132,133] and microbial electrochemical biosensors (MEBs).[134]

In BESs, a diverse range of microorganisms (typically, those are bacteria; however, there are also examples where algae or fungi are used) can be employed—both as isolated strains and mixed cultures,[135] and either in form of planktonic cells[136] or as biofilms.[137] One of the major factors hindering the practical application of BESs is the low electron transfer efficiency at the electrode. To overcome this issue, biofilms can be a convenient solution as they come with the advantage that they can grow directly on the electrode surface; moreover, in protective biofilm matrix, the bacteria are well connected to each other, which facilitates the electron transfer process from one bacterium to another. However, when this biofilm matrix becomes too thick, it may become an obstacle that limits the diffusive transport of nutrients and electrons.[138] Hence, there is still a need to maximize the transport properties within biofilms as well as the electron transfer efficiency at the biofilm–electrode interface.

To improve the electron transfer process to electrodes, researchers have pursued several approaches, and they can be divided into two groups: the first strategy is based on a manipulation of biofilm growth to increase both, biofilm formation and the electroactivity of the biofilm bacteria. The latter is typically achieved by creating more options to transfer electrons from donors to acceptors. One option to achieve this is to increase the number of extracellular electron carriers (cytochromes, flavin- or quinone-based mediators, or conductive pili) by means genetic engineering.[44,139] Of course, maximizing the number of microbial cells producing these carriers, e.g., by nutrient optimization, has a similar effect.[139,140] An alternative approach aims at manufacturing electrodes with enhanced conductivity of with larger surface areas. Here, lots of effort has been made to investigate various electrode materials and surface treatments.[123,141]

The second strategy aims at improving extracellular electron transfer through the BESs by integrating artificial components into either the liquid part of the BES (containing planktonic bacteria) or into the biofilm matrix—and the latter is typically achieved by growing the biofilms in the presence of those artificial objects. The first steps taken in this area were based on the addition of soluble electron mediators to the bacterial culture during microbial growth. Flavin and quinone containing compounds such as riboflavin, Neutral Red, Brilliant Blue, Methyl Violegene, and humic acid have been approved as electron mediators for indirect electron transfer purposes.[124,142–145] Wu et al.[146] have studied the performance of a BES making use of a *S. oneidensis* strain in combination with five different mediators. They observed that, after 4 days of incubation, the current generated by the mediator-enriched samples was 20–60 times higher than the one generated in the control sample. Further-

more, they showed that it was indeed the biofilm formed on the anode in combination with the artificially supplied electron mediators that was responsible for the obtained effect—and that the contribution of planktonic cells was weak. Moreover, in this particular setting, supplying the mediators did not only promote the electron shuttling process, but also enhanced biofilm formation by a factor of >15. Arinda et al.[147] added riboflavin-functionalized magnetic beads to biofilms to enable recovery and reusage of the mediators. However, the effect of riboflavin on biofilm formation and current generation was weaker than when it was added in its free, unbound form.

In addition to electron mediators, several polymers have been employed to improve the performance of a BES, and examples include both, biological and synthetic polymers.[79,148–152] In a recent study, Zhang et al.[153] mixed a bacterial culture of *S. oneidensis* with the conductive polymer PMNT (poly(3-(3′-*N,N,N*-triethyloamino-1′-propyloxy)-4methyl2,5-thiophene hydrochloride)). There, a combination of electrostatic and hydrophobic interactions between PMNT and *S. oneidensis* cells was suggested to help the bacteria transfer electrons between each other and to the electrodes. This enhanced bidirectional electron transfer throughout the biofilm was also suggested to help obtaining thicker biofilm layers with improved bacterial viability (even within the inner layers of biofilm); probably, this was made possible by boosting the metabolic activity of bacteria, which—otherwise—would be limited by diffusion. An additional advantage brought about by this PMNT enrichment was that the lifetime of the biofilm electrodes was prolonged from 100 to 250 h. Another example of how polymer incorporation improves the functionality of a BES was described by Du et al.[154] Here, the researchers could produce a robust BES by encapsulating a mature biofilm with polydopamine (PDA): even at strongly acidic conditions, this PDA-coated bioelectrode contained a very high density of viable cells—and in such extreme environments, unprotected bacteria would die.

Similar to conductive polymers, also carbon-based materials have been used—either as electrode materials or as components for artificial biofilms. Due to their chemical stability, high conductivity, high specific surface area, and good biocompatibility, graphene and CNTs have been widely used in BESs.[155] By growing bacteria on graphene oxide (GO) nanosheets in situ, Yong et al.[156] produced an *S. oneidensis* biofilm with an increased pore size of 10–200 μm. Yuan et al.[157] showed that that such a GO-enriched biofilm with increased porosity not only exhibits improved transport of nutrients but also enhanced kinetics of electrochemical activity. An application of such GO-enriched biofilms was demonstrated by Song et al.:[158] here, within 48 h, these semiartificial biofilms could remove all Cr(IV) from wastewater; the control sample was only half as efficient. Carbon-based additives to electrode biofilms resulted in similar results.[155] Also here, wastewater treatment benefitted from those engineered biofilms as they allowed for rapidly determining the biological oxygen demand (BOD).[159] Of course, there also combined approaches where both, graphene-type and carbon-type objects were added to enhance the properties of the enriched biofilm.[160–162] Finally, using similar strategies as described above, it is even possible to create a conductive biofilm from nonexoelectrogenic bacteria.[149,163–165] This demonstrates the great potential that combining microbes with artificial objects holds.

### 2.4. Bacterial Construction Materials

Creating more sustainable building materials is one of the major goals in the field of civil engineering;[166] especially alternatives for cementitious construction materials are needed to reduce the $CO_2$ emission originating from the production of cement.[167] Interestingly, bacteria and bacterial products can also help here.[168,169] Even though the chemical conditions inside cementitious materials (such as the high pH levels occurring during the hydration reaction and the lack of nutrients) are not ideal for promoting bacterial growth, innovative concepts have been introduced that improve the functionality of construction materials by using bacterial additives (**Figure 3**).

One reason why biobased admixtures derived from bacterial sources have attracted lots of interest is their ability to replace commonly used, partially noxious or even toxic additives; at the same time, those biological additives can often be produced such that their environmental impact is comparably low. Prominent examples for such bacterial products are biopolymers generated by bacterial fermentation: examples include welan gum[170–172] or xanthan gum,[173–175] both of which are used as viscosity modifying agents in concrete.[176,177] Similar effects were obtained with other bacterial additives, such as extracellular polysaccharides,[178] bacterial cell walls,[179] whole prokary-

otic cells,[180,181] or bacterial biofilms.[182] This is important as the viscosity determines the workability of the uncured construction material—and this parameter often needs to be adjusted to meet the requirements of different applications.

In the literature, the viscosity-increasing effect of bacterial additives was attributed to a combination of different mechanisms:[183] First, water molecules can be bound by the additives via hydrogen bonds, and this can increase the viscosity of the hybrid material. Second, long polysaccharide chains present in the bacterial additives can, in combination with water, create a gel-like structure, and this boosts the viscosity. Third, bacterial additives are often charged; different anionic motifs from a polymer chain can interact with several positively charged cement particles, leading to bridging flocculation, and also this effect can tune the viscosity of the material.

In addition to modifying the viscosity of cementitious materials, a second important effect brought about by bacterial additives aims at improving the properties of the cured material. For a variety of different bacterial additives, e.g., bacterial cell walls,[184] and bacterial solutions,[185,186] such an improvement of the mechanical competence of the final, cured material has been reported.[187–189] Moreover, bacterial additives can enhance the corrosion resistance of load-bearing steel elements in reinforced concrete[190–192]—and this increases the durability and
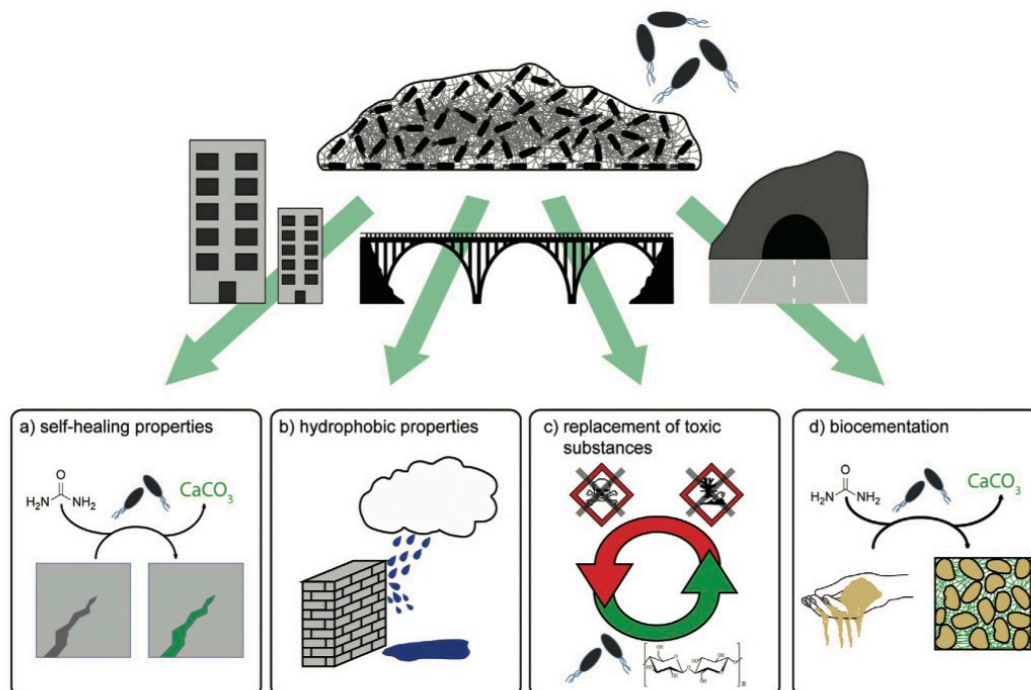


**Figure 3.** Benefits obtained by using bacterial additives in construction materials. Bacterial additives such as bacterial suspensions, spores, secreted macromolecules or cell fragments, as well as whole biofilms have been shown to improve the functionality of cementitious materials. For instance, a) self-healing of microcracks and b) water repellency was achieved. Moreover, c) by replacing toxic chemicals with bacterial ingredients and d) by enabling biocementation processes, more sustainable construction materials were developed.

thus lifetime of objects making use of this class of building materials, e.g., bridge pillars, walls in high rise buildings, and tunnel constructions.

The microscopic mechanisms responsible for these properties can be as follows: as the porosity and the strength of cementitious materials are related,[193] strength improvement is often achieved by calcite precipitation, which reduces the porosity. In addition, precipitated calcite can act as a diffusion barrier and therefore protect steel elements in concrete from corrosion, and reduced rates of oxygen ingress can contribute to a higher corrosion resistance as well.[194]

Corrosion of steel elements in concrete is driven by the ingress of chloride and sulfate ions into the bulk of the material—and this is made possible by invading water transporting the ions. The latter can occur via rain or water splashes, or it can originate from capillary water uprise when cementitious structures are erected in moist environments. Importantly, also in this context, bacterial additives have turned out to be extremely helpful: both, the external wetting resistance of mortar and the suppression of the capillary water uptake into the material can be enhanced using fresh[195,196] or freeze-dried bacterial biofilm,[197] bacterial solutions,[197] or bacterial spores.[198]

In those cases, it was suggested that a modification of the microstructure of the mortar material is responsible for the increased water resistance: increased roughness features on the inner and outer surface of the mortar as well as alterations in the density of the material were observed when bacterial additives were used. Yet, it remains to be shown which particular microarchitecture of bacterial hybrid mortar provides the overall optimal set of material properties—and how this ideal microstructure can be achieved.

Increasing the service life of cementitious structures is certainly a great step toward more sustainable building concepts. Yet, emerging trends from this field aim at developing cement-free building materials to completely erase the greenhouse gas emission caused by the cement production. Here, alternative binders, e.g., alkali activated slag, may offer a possible option.[199] One limitation of this approach is the considerable material shrinkage triggered by alkali activation as well as insufficient containment of moisture in the material volume. Again, by using bacterial biofilm as an additive, those two issues could be successfully addressed.[200]

Whereas, in the examples discussed above, a modification of the material properties was directly achieved by the addition of bacteria or bacterial products, a second strategy employed in the area of civil engineering aims at exploiting the unique ability of bacteria to take part in, control, or initiate biomineralization processes.[201] Indeed, also this approach has led to many new developments toward the creation of more sustainable building materials,[202] and most of them make use of microbial-induced calcite precipitation. One prominent example from this area is the concept of self-healing cementitious materials.[203–205] In this approach, bacterial spores are added to the bulk of concrete. Due to their unique structure, bacterial spores can withstand harsh conditions without losing their viability.[206,207] Instead, they remain dormant without any perceivable metabolic activity until they are reactivated by contact with moisture and oxygen. The latter is made possible when cracks have formed in the material through which water

and air can enter. In other words, damage to the material serves as a "wake-up call" which then triggers autonomous repair: The metabolic activity of the reactivated bacteria induces calcite precipitation,[201] and this, in turn, can seal microcracks (in the range of 0.46 mm).[208] For such self-healing concrete, different types of bacteria have been identified,[209] and they utilize different precipitation mechanisms[210] to achieve this effect.

Bacterial precipitation is also the basis for the patented concept of "biocementation."[211,212] Here, instead of sealing cracks in the cured construction material, bacteria are employed to produce calcium carbonate, and this mineral can solidify sand or other gravel particles without the need of a binding agent.[213] The properties of such "bacterial soil" depend on several factors including the concentration of added bacteria and urea, and the grain size distribution.[214,215] However, real-life applications of this idea have not been tested yet. Along the same lines—yet taking this idea one step further—the concept of "living building materials" is discussed by Heveran et al.[216] As the name already suggests, microorganisms inside such a material remain viable and thus can react to alterations in environmental conditions such as temperature and humidity by switching on (or off) material growth.

For such a novel class of living buildings, it was even suggested that—once the end of the service life of the building is reached—the material can be largely recycled. Whether or not this is really possible, future research will have to show. Overall, the results we highlight above clearly demonstrate the great potential innovative bacterial materials hold for developing a novel, more sustainable class of construction materials with improved properties.

## 3. Outlook

Bacterial biofilms have the potential to be so much more than just a nuisance. The examples we highlight here stem from selected areas of bioprocess, biomedical, agricultural, environmental, electrical, and civil engineering and demonstrate how different material properties of biofilms can be used to generate objects with tailored functionalities. Together with fundamental insights into how those material properties can be further boosted or modified, a broad range of applications have already been identified that make use of bacterial materials. With the current improvements in additive manufacturing techniques,[217–219] our ability to control the composition, architecture and shape of objects is improving day by day.

Indeed, there are already a few recent examples where such advanced manufacturing methods have been applied to create bacterial materials.[220–222] For the purpose of wastewater treatment, an artificial biofilm was printed into a grid-like structure to obtain an object with a very high surface area. As a bacterial strain for this particular application, *Pseudomonas putida* was selected, which is capable of degrading phenol and converting it into biomass.[223] As an example of a medical application, we would like to highlight 3D-printed *Acetobacter xylinum* bacteria, which—once embedded into a hydrogel matrix—produced cellulose.[223] Once enough cellulose was secreted, the bacteria and the hydrogel were removed by washing leaving a cellulose

scaffold in a predefined shape as realized by the printing process. Such cellulose scaffolds were suggested to support the wound healing process—especially in areas having complex shapes such as the face. Also in electrical engineering, 3D-printing of bacterial structures has been attempted and living anodes for microbial fuel cells were produced. With this technique, bioelectrodes could be fabricated with a high level of control in terms of geometry and porosity.[224] A different approach was realized by Moser et al.,[225] who used light signals to pattern *E. coli* on solid surfaces to induce the biofilm formation in a desired shape.

To arrange any printable material into a dedicated 3D shape, the viscoelastic properties of the "ink" need to be just right—and the same holds true when attempting to print biofilms. During printing, the biofilm needs to have the properties of a liquid; yet afterward, it has to stay in place and maintain its shape which requires elastic properties. Owing to their viscoelasticity and stickiness in combination with their self-healing abilities, "naturally grown" bacterial biofilms meet these requirements. However, when specific functions are desired, artificial biofilms are preferred and a viscoelastic matrix (typically a hydrogel comprising either alginate, hyaluronic acid, carrageenan, or fumed silica) is loaded with the bacteria of choice[223,226] or the expression of bacterial EPS is manipulated.[227] Here, the artificial biofilm matrix not only needs to provide the required mechanical stability but has also to ensure bacterial survival and metabolic activity. Duraj-Thatte et al.[228] used a nanofiber gel produced by bacteria as a matrix for 3D-printing. Then, the original bacteria were removed from the gel by washing and replaced with different bacterial cells and selected additives.

In addition to 3D-printing, several other methods have been reported to control the structure of cellulose-based biofilms and to create complex shapes. For instance, biofilm spheres could be produced by adding PTFE nanoparticles,[229] by employing microfluidics methods using alginate–agarose as a shell structure,[230] or by making use of water-in-oil emulsions.[231–233] With this range of methods, spherical biofilms with tunable sizes can be produced which might be useful for encapsulation purposes in food engineering and biomedical applications.

At this point, material science, microbiology, and manufacturing science meet and open up a plethora of new avenues that still need to be explored. Considering the huge variety of bacterial species and our growing ability to control the properties of bacterial biofilms, many new and exciting developments are possible in this area.

## Acknowledgements

## Conflict of Interest

The authors declare no conflict of interest.

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## A.2   Bacterial Additives Improve the Water Resistance of Mortar

# Bacterial Additives Improve the Water Resistance of Mortar

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**ABSTRACT:** The ingress of water into mortar and concrete is an ongoing problem which can reduce the lifetime of cementitious structures. Commonly used approaches that aim at preventing water ingress mainly employ an additional surface treatment after the casting process. Thus, they are time-consuming and make use of synthetic, nonsustainable additives. In contrast, it was shown recently that a biological material, i.e., a bacterial biofilm generated by *B. subtilis* 3610 bacteria, can be used as a bulk additive which leads to hybrid mortar with increased wetting resistance. Here, we demonstrate that a similar enhancement of the water resistance of mortar can be achieved by using different bacterial additives, i.e., wet biofilm, freeze-dried biofilm powder, and bacterial suspensions, each of which can be produced by one of three selected variants of *B. subtilis* bacteria. We characterize the mechanical properties of the different hybrid mortar variants regarding their setting behavior, tensile and compressive strength, and density. Our results imply that bacterial additives could be an eco-friendly and sustainable alternative to existing synthetic mortar additives.

**KEYWORDS:** *Hydrophobic mortar, Biofilm, Water uptake*
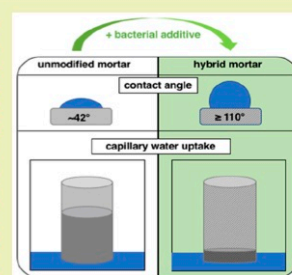
### ■ INTRODUCTION

Cementitious materials, such as concrete, mortar, and stucco, are the most commonly used materials worldwide. In 2018 alone, 33.7 million tons of cement were produced in Germany.[1] With an increasing world population exceeding 8.5 billion in 2030,[2] the demand for cementitious building materials is unlikely to decrease in the near future. Cured cementitious materials represent durable and reliable building materials; however, it is increasingly appreciated that the production of cementitious materials significantly contributes to the global carbon dioxide emission,[3] which calls for cement or mortar formulations with increased durability and lower environmental impact. Cementitious materials are exposed to a range of factors that can lead to their degradation. Among those are environmental challenges such as the ingress of water and the resulting transport of aggressive chemicals or ions into the material. Carbonation, freeze−thawing cycles, chemical attack, and corrosion of reinforcement are among the most common deleterious processes for cementitious materials caused by penetrating moisture.[4] In practice, very often a combination of these processes occur.

Preventing moisture-based deterioration mechanisms and thus increasing durability and extending the residual lifetime of cementitious structures is a key approach to develop cementitious materials with lower environmental impact.[5−8] Commonly used strategies to prevent the ingress of moisture into cement or concrete involve surface coatings, hydrophobic impregnation, pore-blocking surface treatments, and multi-functional surface treatments.[9] To generate (super)-hydrophobic coatings, various materials are used. Examples include traditional organic polymers, which are typically based

on epoxy,[10−12] acrylic[13] or polyurethane resins,[14−17] or—more recently—polymer nanocomposites.[18,19] The water-repelling effect of such coatings is, however, not only dependent on the material used. Also the air permeability, bonding strength, penetration depth, and thickness of the coating are important factors that affect the performance of the surface treatment.[9] Another issue is the limited durability of the coatings itself. Moreover, they require a cost-intense renewing and—when tunnels or bridges require maintainance—very often entail an obstruction of traffic.

Despite some beneficial results obtained with newly developed superhydrophobic coating materials, the application of such coatings to cementitious materials is controversial, as the corrosion rate and other water-based degradation mechanisms can be increased rapidly in defective coating areas.[9] Thus, another approach is to incorporate (hydro-phobic) materials into the bulk of the cementitious material to alter its volume and surface properties and thus to provide additional protection against degradation. Interestingly, this strategy has existed for centuries; already the Romans used pozzolanic additives to increase the durability of cementitious building materials.[20] Today, numerous natural materials (some of which are created as byproducts of other processes) are tested as additives to prolong the lifetime of cement, mortar,

and concrete. Examples include blast-furnace slag,[21] fly ash,[22,23] and limestone[24] with pozzolanic or hydraulic properties. These additives can also be used to substitute some of the cement in mortar or concrete formulations, and such an approach led to the development of so-called inorganic polymer concrete, a cementitious material with a very small greenhouse footprint compared to traditional concrete variants.[25,26]

In the last decades, also synthetic materials were taken increasingly into consideration as additives for cementitious materials, and indeed, the properties of such a synthetic additive can strongly influence the resulting material. For instance, the addition of expanded polystyrene beads results in a super lightweight concrete with improved thermal and sound insulating properties.[27,28] More recently, a novel biobased (and thus maybe more sustainable) approach was introduced, which aims at increasing the lifetime of cementitious structures by incorporating bacteria into the material.[29−32] Certain bacteria can produce the enzyme urease that catalyzes the hydrolysis of urea into ammonia and $CO_2$. Caused by ammonia production, the pH-value is increased, which results in an increased formation of carbonate precipitating on the bacterial cell wall. Such bacteria-induced calcite formation has the potential of crack remediation and increases the resistance of cementitious materials to aggressive substances. Yet, this strategy does not prevent the ingress of water into the construction material.

The latter, however, was achieved by incorporating a more complex bacteria-based additive into mortar during the mortar mixing process, i.e., bacterial biofilm generated by the species *Bacillus subtilis* 3610.[33] Different from individual bacteria, biofilms are ubiquitous communities of microorganisms where the bacteria encase themselves in a matrix of self-produced biopolymers.[34] Surprisingly, with this approach, a hydrophobic hybrid mortar material was obtained, which not only resists wetting but also suppresses the capillary water uptake— although the bacterial biofilm itself has hydrophilic properties. Our current understanding of this effect suggests that the addition of the bacterial biofilm stimulates a biomineralization process that confers hydrophobic properties to the whole material—not only to the outermost layer. Such a biobased approach would be a sustainable and eco-friendly alternative to existing hydrophobizing strategies. However, if using other biofilm variants different from the one tested in Grumbein et al.[33] leads to similar results has not been tested yet. Also, it remains to be shown if and to what extent such a bacterial additive affects the mechanical properties of the generated hybrid material.

Here, we investigate the suitability of three different bacterial additives as hydrophobizing agents for mortar. In detail, we compare freshly harvested and lyophilized biofilm as well as liquid overnight cultures. Each additive variant is generated from three different *B. subtilis* strains, and we aim at identifying the most suitable mixture with respect to the wetting resistance, capillary water uptake, hydration kinetics, and mechanical competence of the created hybrid mortar. Our results suggest that—depending on the particular application of the mortar material—different bacterial additives might perform better than others.

■ **MATERIALS AND METHODS**

**Biofilm Cultivation and Production of Bacterial Additives.** *Bacillus subtilis* NCIB 3610 (*B. subtilis* 3610) was obtained from the lab of Roberto Kolter (Havard Medical School, USA). *Bacillus subtilis*

*natto* (27E3) was obtained from the Bacillus Genetic Stock Center (BGSC). *Bacillus subtilis* B-1[35] was obtained from Masaaki Morikawa (Hokkaido University, Japan). Biofilms were cultivated by first inoculating 10 mL of liquid Luria/Miller LB-Medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, pH-value 7.0 ± 0.2, Carl-Roth, Karlsruhe, Germany) with a frozen bacterial/glycerol stock. After incubation at 37 °C and 90 rpm (200 rpm for *B. subtilis* B-1) in a shaking incubator (Sartorius, Göttingen, Germany) overnight (i.e., for 16h), an "overnight culture" (a solution with a high density of planktonic bacteria) was obtained (Figure S1). Then, 100 μL of this overnight culture was plated on (1.5% v/w) agar plates (standard Petri dishes) enriched with Luria/Miller LB-Medium (2.5% v/w) and incubated at 37 °C for 24 h to grow biofilms (Figure S2). Fresh biofilm was harvested using a PDMS-spatula (Figure S3), collected in a tube and frozen at −80 °C. After storage at −80 °C for at least 2 h, the frozen biofilm was freeze-dried (Figure S4) for at least 72 h (Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Please note that the bacterial additives used here may also contain remnants of the culture media (LB) used in the bacterial cultivation process. The relative contents of this culture medium are expected to be highest for the overnight cultures and lowest for the biofilm powder, which have undergone the lyophilization process.

The three variants of bacterial additives contain either viable bacteria, bacterial spores, or a mixture of both. Microscopy images of the nine different bacterial additives are shown in the supplement.

**Mortar Sample Preparation.** Fresh biofilm was harvested from agar plates by manual scraping with a PDMS spatula. The scraped biofilm material was pooled, weighed, and split into small amounts as needed. For biofilm lyophilization, the scraped biofilm was pooled and freeze-dried for 72 h. The lyophilized biofilm was finely ground into a powder using a ceramic mortar and pestle and then stored in a closed container at room temperature until further use.

For initial contact angle experiments, test specimens were produced in smaller scales (i.e., not according to DIN EN 196-1). For the addition of fresh biofilm into mortar, an aqueous suspension needs to be generated. Therefore, fresh biofilm was mixed with water and homogenized with a pestle for 2 min. This suspension was then mixed into a 3:1 mixture of CEN standard sand (NORMENSAND GmbH, Beckum, Germany) and 10 g of cement (Portland cement CEM 42.5 N, Schwenk Zement KG, Ulm, Germany) and stirred mechanically using a paddle mixer for 2 min. Lyophilized biofilm powder was added directly to the dry mixture of cement and sand, and double distilled water (ddH$_2$O) was added to this mixture as needed to obtain a w/c ratio of 0.5. If a bacterial overnight culture was used as an additive, the overnight culture was added to the sand/cement mixture (ratio 3:1) instead of water. All mortar samples were prepared with a w/c ratio of 0.5 and cured for 3 days at room temperature before they were used for any experiment.

For sample preparation according to DIN EN 196-1,[36] cement and biofilm powder were added to water (or bacterial overnight culture) within 10 s, and the mixing process was immediately started in a bowl at a stirring speed of 140 rpm. After 30 s, sand was added within a time window of 30 s, and the mixture was stirred at a stirring speed of 285 rpm for an additional 30 s. Then, the mixing process was stopped for 90 s. The mortar adherent to the stirring head and/or the upper part of the bowl was transferred back to the bottom of the bowl using a rubber scraper, and the stirring process was continued for additional 60 s at a stirring speed of 285 rpm. The prepared mortar was then poured into the desired mold within 120 s, while being compacted using a vibrating table.

**Contact Angle Measurements.** For determining the wetting properties of the mortar samples, five 10 μL droplets of ddH$_2$O were placed onto each sample at different spots, and images were acquired from a lateral view using a digital camera (Flea3, Point Grey, Richmond, Canada). The contact angle was then evaluated from the digital pictures using the image analysis software ImageJ in combination with a drop-analysis plugin tool.

**Water Uptake Experiments.** To assess capillary water uptake into mortar, cylindrical samples were prepared according to DIN EN
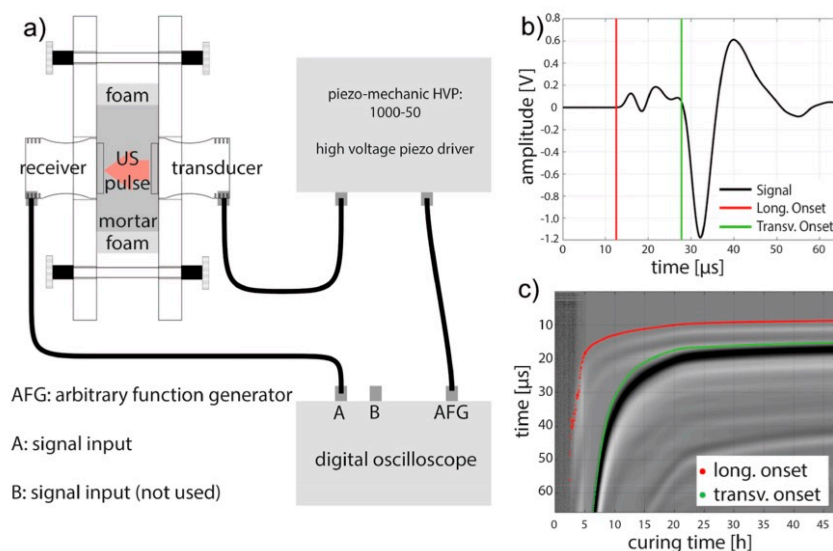
**Figure 1.** Schematic representation and example data of FreshCon measurements. (a) Measurement setup. (b) Example of an ultrasound signal measured on a standard mortar reference 11 h after water addition. Vertical lines mark the compressional and shear waves onset. (c) Color-coded representation of all recorded ultrasound signals from the reference measurement with gray scale representing amplitudes.

196-1 and poured into commercial polyethylene tubes (diameter: 40 mm). After 3 days of curing, the samples were removed from the tubes, and after 11 additional days of storage at room temperature, they were coated with the injection resin MC-Inject 1264 compact (MC Bauchemie, Bottrop, Germany). After 24 h, one of the ends of the coated cylinders was cut off to create one open surface vulnerable to water ingress. Although this cutting process could be conducted at any height of the cylinders (which have received a bulk hydro-phobization as a consequence of the bacterial additive), we—for practical reasons—selected a cutting plane such that the remaining cylinders had a size of approximately 10 cm. The open surface generated by this cutting process was then immersed into a 2 cm high water bath. To quantify water uptake, the moisture content of these specimens was measured at two conditions, i.e., in a "dry" state (before exposure to water) and after exposure to water for 24 h. Water uptake was quantified by determining the mass of the mortar sample by weighing using a microscale (TLE 303, Mettler Toledo AG, Greifensee, Switzerland).

**Workability Characterization.** To determine the workability of the mortar samples, measurements were performed using a commercial shear rheometer (MCR 302; Anton Paar GmbH) equipped with a BMC 90 measuring cell for building materials and a paddle mixer. For all measurements, the measuring cell is filled with 450 g of (hybrid) mortar. Those mortar samples were mixed outside the rheometer as described above and transferred into the measuring cell after 3 min of mixing. With this procedure, the first measurement point was taken 4.5 min after the hydration reaction was initiated. The measurements were conducted without further air or water cooling at RT. Every 30 s, the torque required to maintain a constant shear rate of 0.001 s$^{-1}$ was measured for a time span of up to 30 min. If a critical torque greater than 200 N m was reached, the measurement was stopped.

**Curing and Development of Elastic Parameters.** In order to monitor the setting and hardening of the biomodified mortar, ultrasonic measurements were performed. The used system, known by the name "FreshCon", was developed by the University of Stuttgart and TTi GmbH (TGU Smartmote).[37] When knowing the distance

between transmitter and receiver, the propagation velocities of the compressional and shear waves can be determined measuring the travel times of the waves through the specimen. With the aide of several assumptions, the evolution of the elastic parameters can be computed from these velocities.[38] This technique is well introduced and commonly used to monitor the curing of cementitious materials.[39,40] A detailed description can be found in refs 41 and 42. Figure 1a shows the technical implementation such a FreshCon measurement. The design of the mortar container was taken from ref 43. In addition to the measurement setup, Figure 1b and c shows an exemplary signal and a phase picking result at this signal. The recording and trigger generation was carried out with a digital oscilloscope. Data acquisition is controlled using a MATLAB script. To ensure precise onset determination, a high sampling rate of 40 MHz was used at 16 bit, and 1000 individual signals were averaged to improve the signal-to-noise ratio. Signal-averaging takes about 20 s and is repeated every second minute. Along with the recorded ultrasound signal, a time stamp is generated and stored to compensate execution time changes. Both, the compression and shear waves, were excited and measured using a shear wave transducer with a 500 kHz center frequency. Compressional wave onsets were determined using Akaike's Information Criterion (AIC) as the onset determination method described in the literature.[44,45] Shear wave onsets were determined using the method described by Krüger et al.[46] Depending on the mortar type, signal features become visible for 1 h after water was added.

The distance between transmitter and receiver (d) as well as the specimen density ($\rho$) were measured using hardened specimens. Then, the propagation velocity $c_p$ was calculated according to eq 1, where t denotes the travel time

$$c_p = \frac{d}{t} \tag{1}$$

On the basis of the velocities determined, the dynamic modulus of elasticity E and Poisson's ratio $\nu$ are calculated using eqs 2 and 3. There, $C_l$ denotes the longitudinal wave velocity, and $C_t$ denotes the transversal wave velocity

**Table 1. Overview of Production Yields of Fresh Biofilm and Biofilm Powder as Well as Weight Loss during Freeze-Drying of Biofilm Created by Three Variants of *B. subtilis* Bacteria[a]**

| bacterial strain | wet biofilm mass (per Petri dish) (g) | weight loss after freeze-drying (%) | biofilm powder (per Petri dish) (mg) | mass loss factor | required amount of biofilm powder for bc of 2% (mg) |
|---|---|---|---|---|---|
| *B. subtilis* 3610 | 0.22 ± 0.04 | 80 ± 1 | 45 ± 1 mg | 5.0 ± 0.4 | 40 |
| *B. subtilis natto* | 0.25 ± 0.04 | 79 ± 1 | 52 ± 1 | 4.9 ± 0.2 | 41 |
| *B. subtilis* B-1 | 1.35 ± 0.2 | 94 ± 1 | 83 ± 1 | 16.3 ± 1.6 | 13 |

[a]With the mass loss factor (which is defined as the ratio of fresh biofilm mass with respect to the mass of lyophilized biofilm), the required amount of biofilm powder can be calculated for the desired biofilm content (bc, which describes the ratio of fresh biofilm with respect to the mass of dry cement). The yield values shown represent the average amount of biofilm harvested from one Petri dish. Error bars denote standard deviation as determined from biofilm samples grown on 300 Petri dishes each.

$$C_l = \sqrt{\frac{E(1-\nu)}{\rho(1+\nu)(1-2\nu)}} \qquad (2)$$

$$C_t = \sqrt{\frac{E}{2\rho(1+\nu)}} \qquad (3)$$

Data points resulting from picking errors were manually determined and deleted. Missing data was filled by means of smoothing spline interpolation using the MATLAB fit function and a smoothing parameter of 0.5.

**Three-Point Bending and Compression Tests.** The three-point bending and compressive strengths of standard and hybrid mortar samples were tested using cuboid specimens of standardized geometry (4cm × 4 cm × 16 cm). These specimens were produced according to DIN EN 196-1 as described above. After 24 h, the test specimens were removed from the casting framework and stored at 20 °C until they were tested. On each testing date (i.e., after 7 and 28 days of curing at 20 °C and a relative humidity greater than 50%), first the three-point-bending strength was determined, and afterward, the compressive strength was measured using a loading frame (Series DB Super, Walter&Bai, Löhningen, Switzerland) and then the six fragments obtained from the bending tests.

**Density Determination.** The density of cured mortar samples was determined by immersion weighing according to DIN EN 12390-7.[47] In brief, cured mortar samples were stored under water for 7 days to achieve maximal saturation. The mass of the saturated test specimen was then measured underwater ($m_2$) and dabbed above water ($m_3$). After these measurements, the test specimens were dried at 105 °C until a constant weight ($m_1$) was obtained. With these three measurements, the sample density was calculated as

$$\text{density} = \frac{m_1}{m_3 \cdot m_2} \cdot \delta_w$$

where $\delta_w$ denotes the density of water at 20 °C, i.e., 998 kg/m³. We note that, for materials with hydrophobic bulk properties as we study them here, this method might not return fully accurate results. It does, however, provide a good first indication on whether or not the density of the hybrid mortar is affected by the bacterial additive.

## ■ RESULTS AND DISCUSSION

The three variants of bacterial additives tested here are freshly harvested biofilm (which is further processed into a suspension), lyophilized biofilm powder, and a liquid culture of planktonic bacteria ("overnight culture"). In the first step, we ask if the three *B. subtilis* variants, *B. subtilis* 3610, *B. subtilis natto*, and *B. subtilis* B-1, generate biofilms with different efficiencies, i.e., if the amount of produced biomass per incubation period differs. To test this, we cultivated continuous biofilm layers on agar plates overnight, harvested the grown biofilm by manual scraping, and determined the wet biofilm mass per agar plate by weighing. As summarized in Table 1, *B. subtilis* 3610 produces biofilm in similar amounts as *B. subtilis*

*natto*; in contrast, *B. subtilis* B-1 produces approximately 4 times more biofilm mass than the other two strains.

To add freshly harvested biofilm to mortar, an aqueous suspension needs to be generated, which is then added to the mixture of sand and cement. Due to the sticky consistency of fresh biofilm, creating this suspension is not trivial. Also, storage and transport of such a biofilm-based suspension are not ideal. For practical applications, it would be much easier to add the biofilm in the form of a freeze-dried powder. Such a biofilm powder is easy to store, can be portioned precisely, and can be added directly to the mixture of sand and cement before those are mixed with water. A biofilm powder can easily be prepared by freeze-drying fresh biofilm and subsequently grinding the lyophilizate by using a pestle. For the *B. subtilis* 3610 biofilm, this approach was tested before and returned biofilm-enriched samples with very good hydrophobic properties. Thus, in the next step, biofilm samples of each strain were freeze-dried, and the mass loss due to lyophilization was determined. This step was conducted so that we are able to better compare the biomass yields obtained for the fresh, water-containing biofilms to that of the freeze-dried biofilm powder. As shown in Table 1, the *B. subtilis* B-1 biofilm loses more weight during the freeze-drying process than the other two biofilm variants; in other words, this biofilm has the highest water content among the biofilm samples compared here. However, when we calculate the amount of biofilm powder obtained per Petri dish, we find that *B. subtilis* B-1 bacteria still generate about twice the biomass as the other two *B. subtilis* strains.

As a third dosage form of bacterial material, we tested liquid overnight cultures. These are nutrient solutions, which are inoculated with a frozen glycerol stock of the respective bacterial strain and incubated overnight. During this incubation, the bacteria proliferate and start secreting biomolecules. In some cases, some small amounts of thin biofilm layers (so-called pellicles) form on the surface of this liquid; however, if this happened, these pellicles were removed before the overnight culture was used as an additive for the generation of mortar samples.

For samples containing biofilm and biofilm powder, we refer to the amount of added bacterial material as the biofilm content (bc). In a previous study, we had defined this biofilm content as the ratio of added wet biofilm mass with respect to the dry mass of inorganic cement. For consistency, when lyophilized biofilm powder is added, the weight loss during freeze-drying is corrected for. Due to the significant differences in biofilm water content, the amount of biofilm powder required to obtain a mortar sample with a bc of 2% varies between 13 and 41 mg (Table 1). When employing bacterial liquid cultures as additives, those are used instead of water
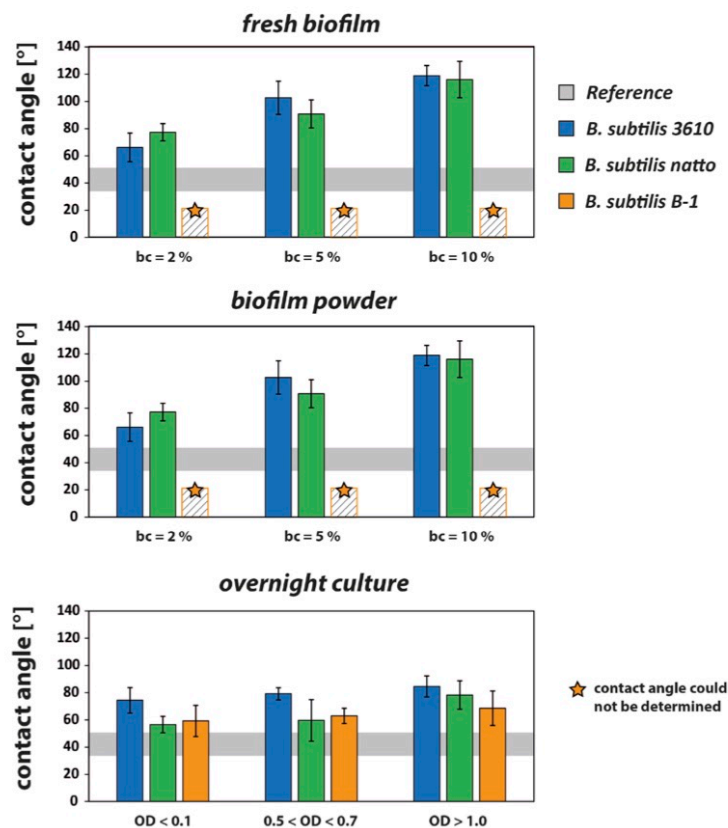
**Figure 2.** Wetting resistance of mortar enriched with different bacterial additives. The wetting properties of different mortar samples are characterized by the contact angle with water, and samples with different biofilm contents (bc) and different optical densities of the added overnight cultures, respectively, are compared. Contact angles determined on unmodified reference samples are indicated by the gray horizontal bars. The values shown depict averages obtained from approximately 15 measurements conducted on at least three independent samples. Error bars denote the standard deviation calculated from those 15 data points. Contact angles for samples containing biofilm and biofilm powder of *B. subtilis* B-1 could not be determined since the high porosity of these mortar samples entailed rapid penetration of wetting water into the bulk volume of those samples.

during the sample production process. Consequently, the amount of additive—in this particular case—is limited by the water to cement ratio (w/c) the sample is supposed to have. To limit the complexity of our study, we here maintained a w/c ratio of 0.5 for all tested conditions. Nevertheless, also in liquid bacterial cultures, the content of biological material can be varied. This is achieved by stopping bacterial growth at different time points of the incubation period. To quantify bacterial growth, the optical densities of the overnight cultures were measured before they were added to the dry mortar mix. Higher optical densities indicate a combination of both, higher concentrations of bacteria and higher concentrations of metabolic products/macromolecules secreted by the bacteria.

The next step was to test the ability of these bacterial additives to increase the wetting resistance of mortar. In a previous study, we had already shown that the addition of either fresh *B. subtilis* 3610 biofilm or freeze-dried biofilm powder leads to mortar with contact angles up to 100−120°

and that this is achieved in a dose-dependent manner. Here, we not only determined the wetting resistance of mortar enriched with different variants (*B. subtilis* natto and *B. subtilis* B-1) of fresh biofilm and biofilm powder, respectively, but also tested the influence of the liquid bacterial additive (overnight culture). As depicted in Figure 2, the dose-dependent hydrophobizing abilities of *B. subtilis* 3610 biofilms reported earlier were successfully reproduced. Similarly, the addition of *B. subtilis* natto biofilms yielded good results as well. Also for this biofilm variant, we measured contact angles as high as 110° when a biofilm content (bc) of 10% was chosen. Surprisingly, when *B. subtilis* B-1 biofilms were used as an additive, the created hybrid mortar samples did not show hydrophobic properties at all; instead, for all three bc values tested (and both for fresh biofilm and biofilm powder used as additive), water droplets placed onto the surface of those hybrid mortar samples quickly entered the volume of the sample, which made the determination of a contact angle

impossible. Interestingly, when the optical appearance of those *B. subtilis* B-1-enriched samples were inspected (Figure 3), we
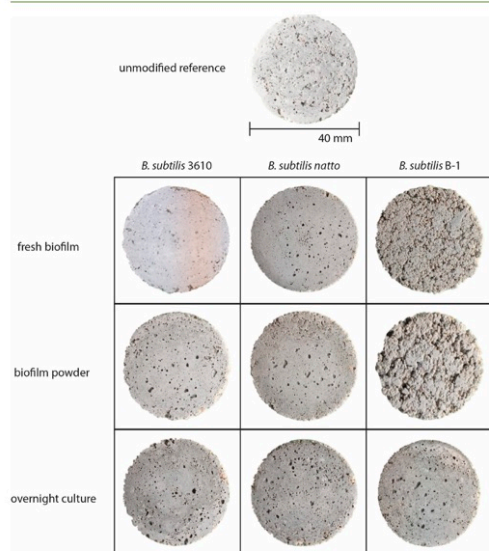


**Figure 3.** Addition of certain bacterial additives affects the macroscopic surface structure of the hybrid mortar samples. Samples containing *B. subtilis* B-1 biofilm and biofilm powder, respectively, develop macroporous surfaces. All other samples containing fresh biofilm or biofilm powder generated from the other two tested bacterial strains as well as samples containing bacterial overnight culture as an additive exhibit very similar surface morphologies as the unmodified reference samples. The scale bar in the top picture applies to all pictures of this figure.

found that their surface was much more porous than those of the other biofilm-enriched samples (which, in terms of surface porosity, all looked similar to unmodified standard mortar). We speculate that the high content of the hygroscopic biopolymer γ-polyglutamate might be responsible for the increased porosity of the *B. subtilis* B-1-enriched samples.

This polyanionic macromolecules is the main component of *B. subtilis* B-1 biofilms but is also present in *B. subtilis* natto biofilms—albeit at lower concentrations.[35] When adding a hygroscopic component to mortar while keeping the water/cement ratio constant, a certain amount of added water might be absorbed by the hygroscopic biomacromolecules, and less water will be available for the hydration reaction of cement. For mortar samples enriched with *B. subtilis* B-1 biofilm powder, the increase in surface porosity seems to be even stronger than when fresh *B. subtilis* B-1 is used as an additive. We speculate that, since almost all biofilm water is removed during the freeze-drying process used for creating the biofilm powder, this dosage form further increases the water demand of this particular hybrid mortar variant. Thus, problems associated with insufficient mortar hydration should be dominant for this particular hybrid mortar variant, which could explain why those samples exhibit the strongest heterogeneity in their macroscopic surface texture (Figure 3).

Interestingly, also the addition of bacterial liquid cultures resulted in hybrid mortar samples with increased wetting resistance. However, for all three bacterial variants tested here, the obtained effect was only moderate, and the obtained contact angles only reached values up to approximately 85°.

A key difference between the two "biofilm" additives (fresh biofilm, biofilm powder) compared to the bacterial overnight culture is that they both contain larger amounts of biological macromolecules, which were secreted by the bacteria to construct the biofilm matrix. This suggests that a certain amount of those matrix macromolecules is required for obtaining a strong hydrophobization effect. A second parameter in which the composition of the different additives differs is the amount of bacterial spores (i.e., metabolically inactive variants of bacteria, which have switched into a dormant, sturdy state). Indeed, we find the largest density of such bacterial spores in the biofilm powder samples and the lowest density in the bacterial liquid culture (Figure S5). Control experiments, where only culture media (components) used for bacterial cultivation were used as an additive, show that the hydrophobizing effect achieved with this bacteria-free supplement is only mild (Figure S6), which demonstrates that the presence of bacteria or spores is important for obtaining a strong improvement of the water repellency. On the basis of previous findings obtained with freshly harvested biofilms,[33] we expect that a combination of two effects is responsible for conveying hydrophobicity to the hybrid mortar samples presented here: first, an alteration of the mortar microstructure (driven by biomineralization events) and, second, the addition of hydrophobic biocomponents to the mortar mix. In principle, both mechanisms may be triggered by bacteria, bacterial spores, and biofilm matrix components alike, and more detailed research will be required to identify a detailed link between the different components of the bacterial additives and the mechanism by which they influence the water repellency of mortar.

However, from the experiments conducted so far, we can already conclude that freshly harvested biofilm and biofilm powder perform equally well (or, in the case of *B. subtilis* B-1, equally bad). However, from an application-driven perspective, using fresh biofilm as an additive is less practical than the other two dosage forms. Thus, for the remainder of this Article, we conduct all further experiments with biofilm powder and liquid bacteria cultures only.

Our next objective was to test if the different hybrid mortar formulations would also suppress capillary water uptake into the bulk volume of the samples. Indeed, previous experiments conducted with fresh *B. subtilis* 3610 biofilm had shown such behavior.[33] Other biofilm variants and other types of bacterial additives, however, have not been tested yet regarding this material property. In our previous study, we had used X-ray imaging for visualizing differences in the capillary water uptake behavior of standard and biofilm-enriched hybrid mortar. However, this method is rather complex as it requires an X-ray scanner and thus less useful for screening different material compositions. Therefore, we chose here a different, methodically simpler approach that allowed us to quantitatively determine the amount of water that has invaded a (hybrid) mortar sample (Figure 4a). In brief, cylindrical mortar specimens were prepared and (after 14 days of curing at RT) sealed with resin (see Materials and Methods for details). Then, the bottom part of those sealed cylindrical test specimens was removed by a saw to create a well-defined
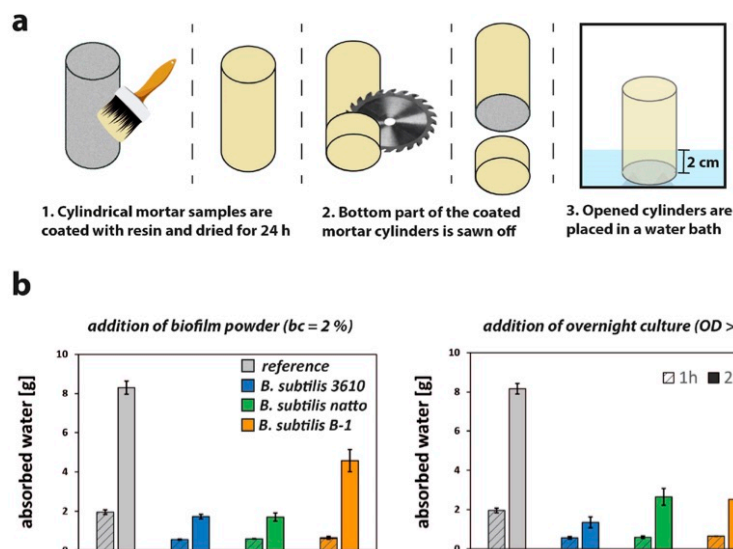
**Figure 4.** Capillary water uptake into hybrid mortar samples. (a) Schematic illustration of the manufacturing process used to generate cylindrical mortar samples for capillary water uptake tests. (b) Amount of water absorbed by the different samples after partial immersion into a water bath for 1 and 24 h, respectively. Samples enriched with biofilm powder (bc = 2%, left) and samples enriched with a bacterial overnight culture (OD > 1.0, right) are compared to unmodified control samples (gray bars). Control samples containing only the culture media used for bacterial growth show slightly decreased capillary water uptake as well; however, here, the effect is much weaker than for the bacteria-containing additives (Figure S7). The values shown represent averages of three measurements conducted on independent samples. Error bars denote the standard deviation.

interface that was (putatively) vulnerable for the uptake of water by capillary forces. To quantify the capillary water uptake, the moisture content of the cylindrical test specimens was determined gravimetrically before and after exposure to water.

Indeed, we observed strong suppression of capillary water uptake for both classes of hybrid mortar samples, i.e., for those containing biofilm powder and those containing a bacterial overnight culture (Figure 4b). Compared to unmodified standard mortar, the amount of water taken up within 1 h of exposure was reduced by approximately 50% for all hybrid mortar samples tested. Interestingly, when inspected visually, all samples appeared to be completely saturated with water within the first centimeter above the water-exposed surface. Nevertheless, the hybrid mortar samples maintained their water resistance at longer exposure times, and the difference regarding the water uptake became even more pronounced. After 24 h, the best-performing sample (i.e., mortar enriched with *B. subtilis* 3610 overnight culture) contained only 1/8 of the water amount invaded into standard mortar, and the worst-performing sample (i.e., mortar enriched with *B. subtilis* B-1 biofilm powder) still suppressed approximately 50% of the water uptake compared to the unmodified control.

Having established that several bacterial additives convey water resistance to hybrid mortar, we have investigated if other material properties relevant for an industrial application were affected by the addition of biofilm or bacterial overnight culture. As a first parameter, which is highly important for hybrid mortar to be used as an industrial product, we have evaluated its workability by investigating its flow properties. These were assessed by shear rheology, where the torque

required to maintain a constant shear rate of $0.001 \ s^{-1}$ was measured (Figure 5).

Here, based on the manual mixing of hybrid mortar samples conducted so far and due to the increased water demand of the bacterial additives discussed above, we expected this workability to be somewhat lower compared to unmodified mortar. However, we observed the opposite behavior. With the measuring parameters chosen, the reference sample, i.e., unmodified mortar, could be sheared to approximately 25 min. Then, the torque required to maintain the preset shear rate became too large, and the rheometer was stopped. In contrast, for all tested hybrid mortar formulations, the time-dependent shear resistance was lower, and measurements were possible for 30 min. All samples containing biofilm powder exhibited a very similar behavior. After 25 min of testing, they all required approximately 50% of the torque measured for the control at this time point. For mortar samples enriched with bacterial overnight culture, the behavior depended on the bacterial species. For liquid cultures generated by *B. subtilis* 3610 or *B. subtilis* natto, the torque measured after 25 min was approximately 60% of that required for the control, whereas for liquid cultures generated by *B. subtilis* B-1, the corresponding torque level was approximately only 40%. Together, these rheology measurements show that the workability of the bioenriched mortar samples is rather improved than reduced, which suggests that cement hydration is delayed in the hybrid mortar.

To test this hypothesis, measurements using the FreshCon system (see Materials and Methods) were conducted with the same set of samples. With this technique, the progression of the hydration reaction of a cementitious material can be
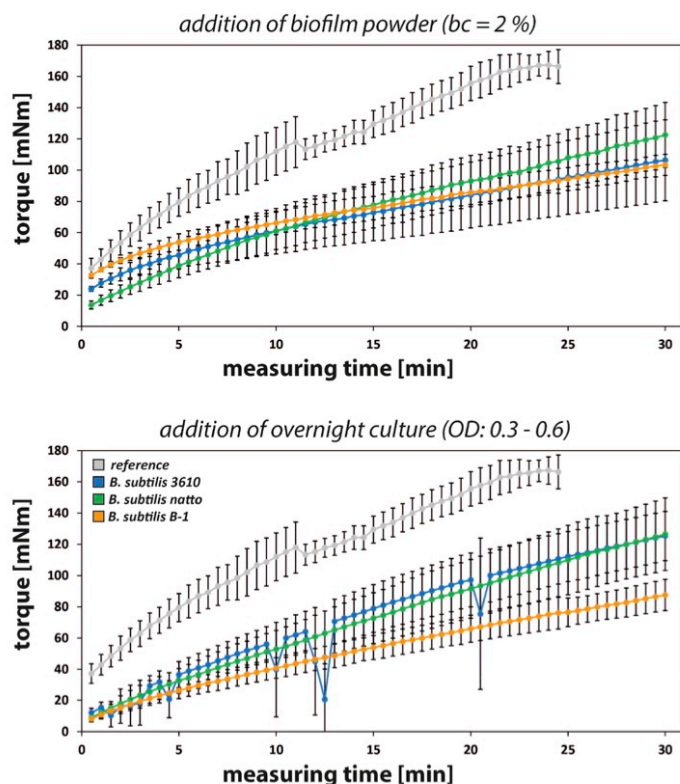
**Figure 5.** Influence of bacterial additives on the hardening process of mortar. The graphs depict the time-dependent increase of the torque required to maintain a constant shear rate of 0.001 s$^{-1}$ on different mortar variants. Hybrid mortar samples containing biofilm powder (upper panel) and overnight culture (lower panel) are compared to unmodified reference samples (gray curves). The values shown represent averages of three independent measurements. Error bars denote the standard deviation.
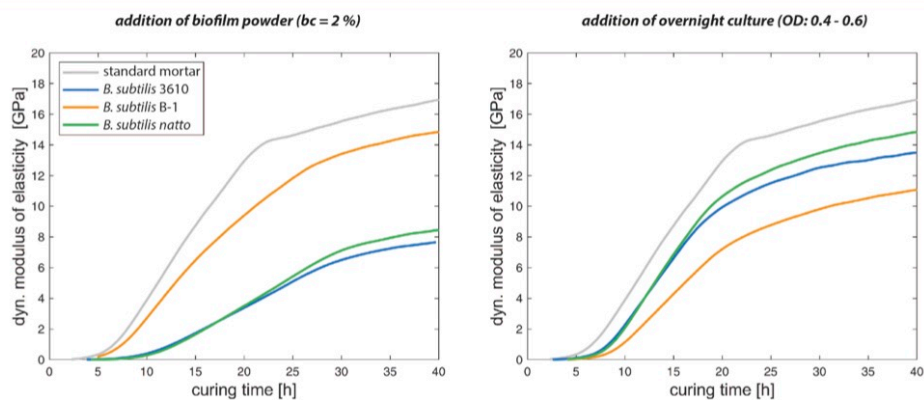


**Figure 6.** Change of the modulus of elasticity during the setting process. On the basis of the measured propagation speed of the longitudinal wave as well as the transversal wave, the development of the dynamic elastic modulus is computed. This development is shown for hybrid mortar formulations containing biofilm powder (left graph) and overnight cultures (right graph).
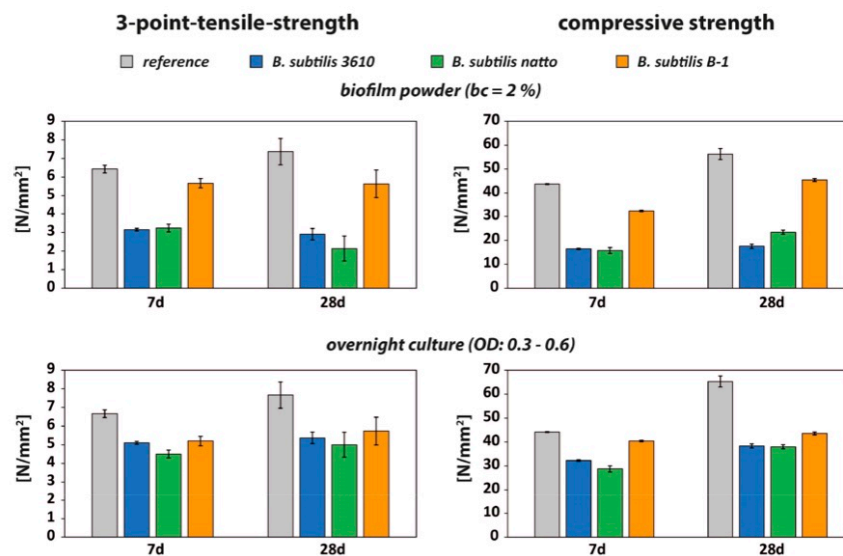
**Figure 7.** Influence of bacterial additives on the tensile and compressive strengths of mortar samples. Tensile strength (left) and compressive strength (right) tests were performed after 7 and 28 days, according to DIN EN 196-1.[36] Hybrid mortar samples containing biofilm powder (upper panels) and overnight culture (lower panels) are compared to unmodified reference samples (gray columns). The values shown represent averages of three independent measurements. Error bars denote the standard deviation.

monitored using ultrasound by following the change of sound propagation velocities and elastic parameters. In brief, ultrasonic signals are recorded and analyzed after they have traveled across the hardening material. Here, the duration (velocity), amplitude (energy), and waveform (frequency) of the signal are influenced by material properties of the mortar—mainly by its transition from a suspension to a solid. From such FreshCon measurements, the development of wave velocities in standard and biomodified mortar is obtained. As anticipated based on the results from shear rheology, standard mortar showed the steepest increase in compressional wave and shear wave velocities. In other words, here, the dynamic modulus of elasticity increased faster than for the biomodifed mortar variants (Figure 6). Additionally, the reference mortar achieved the highest absolute dynamic elasticity modulus value during the observed time window.

The development of the dynamic modulus of elasticity is strongly influenced by the biological additive used. Using bacterial overnight culture as an additive, the kinetics of the dynamic modulus development are quite similar to that of unmodified mortar, yet the final values reached after 40 h are approximately 20−45% lower. With the addition of biofilm powder, the temporal development of the dynamic modulus of elasticity is affected more strongly, and the result depended on the strain of bacteria used. Only the hybrid mortar containing biofilm powder generated from the bacterial strain *B. subtilis* B-1 showed, with a slight delay, a similar increase in the dynamic modulus of elasticity as unmodified mortar; here, also similar final values were reached. In contrast, when biofilm powder of *B. subtilis* 3610 or *B. subtilis* natto, respectively, were used as an additive, a noticeble delay in the modulus development was observed. Moreover, here, the final value of the dynamic modulus of elasticity was approximately half of that obtained

for the reference sample. It has been shown earlier that the cement hydration reaction can be influenced by a number of organic[48,49] or inorganic molecules.[50,51] In our case, the hydration retardation can be caused by the bacterial additives or the presence of carbohydrates in the culture media, and indeed, we detected a similar retardation if only culturing media (without any bacteria) were added to the mortar instead of water (Figure S8).

In the last step, the mechanical properties of different hybrid mortar samples were compared in their hardened state (see Materials and Methods). As depicted in Figure 7, when compared to unmodified reference samples, all hybrid mortar samples showed lower tensile and compressive strength—both after 7 and 28 days of storage. The hybrid mortar samples seem to reach their final mechanical properties earlier than the unmodified reference. In almost all cases, very similar values were obtained for the biomortar samples at the two tested time points; only for the *B. subtilis* B-1 biofilm powder additive, we detected a noticeable increase in compressive strength over time. With this particular additive, we also found bending and compressive strength values closest to the reference, with a reduction of 20−25% only.

In contrast, with the other biofilm powder variants, we determined a reduction in mechanical strength of approximately 60%−70%. Interestingly, hybrid mortar samples containing bacterial overnight cultures showed higher flexural and compressive strength than samples containing biofilm powder. Also here, the additive generated from *B. subtilis* B-1 bacteria performed slightly better than the other two variants. Overall, the relative differences between the samples we determined with those two sets of mechanical tests agreed very well with the results obtained with the FreshCon setup discussed above (Figure 6).

One parameter that could help rationalize those differences in mechanical competence is the porosity of the hybrid mortar samples, which we determined by immersion weighing. As summarized in Table 2, this material property was indeed

**Table 2. Influence of Bacterial Additives on Density of Mortar Samples**[a]

| bacterial additive | bacterial strain | density (kg/m³) |
|---|---|---|
| none (standard mortar) | – | 2141.4 |
| biofilm powder (bc = 2%) | *B. subtilis* 3610 | 1693.2 |
|  | *B. subtilis natto* | 1702.2 |
|  | *B. subtilis* B-1 | 2060.9 |
| overnight culture (OD = 0.4–0.6) | *B. subtilis* 3610 | 1911.3 |
|  | *B. subtilis natto* | 1976.6 |
|  | *B. subtilis* B-1 | 1750.0 |

[a]Mortars enriched with biofilm powder and bacterial overnight culture, respectively, are compared to unmodified control samples. Densities were determined through immersion weighing (see Materials and Methods).

affected by the different bacterial additives. We determined a reduction in density ranging from approximately 20% (*B. subtilis* 3610 biofilm powder) to about 5% (*B. subtilis* B-1 biofilm powder) with the other samples showing intermediate behavior.

With all the measurements conducted so far, are hybrid mortar samples enriched with bacterial additives suitable for applications in construction engineering? Due to the addition of bacterial additives, we observed that hybrid mortar samples developed lower densities compared to unmodified reference samples. A lower density or a higher porosity of cured cementitious materials can lead to a drastic decrease in the compressive strength.[52] Here, we indeed observed such a reduction in compressive strength, but this effect was too weak to moderate. In turn, the porosity and the pore distribution in mortar (including the presence of microcracks) also play crucial roles regarding water ingress.[53] We found that all hybrid mortar samples suppress such water ingress very well, which is a clear benefit of the bacterial additives studied. Depending on the desired application, the benefits related to suppressing capillary water uptake might outweigh possible disadvantages associated with a decreased compressive strength.

Synthetic additives can also create hybrid mortar with hydrophobic properties; for instance, when silica particles are used as an additive,[54] the resulting hybrid mortar shows contact angles toward water as high as approximately 120° and, at the same time, can reduce water absorption by up to 45%. However, even though the strength of those hydrophobizing effects is comparable to what we describe here, these silica particles need to be synthesized and functionalized to display their hydrophobic behavior, and they are not biodegradable. However, incorporating bacterial additives into the bulk of cementitious materials to modify their volume and surface properties might be a strategy that is compatible with other approaches aiming at the development of more environmental friendly construction materials. For instance, an existing biobased strategy pursues the goal to partially replace cement with other hydraulic additives such as fly ash thus reducing the $CO_2$ emission associated with the fabrication of cement.[55,56] In combination, these two biobased approaches could lead to

cementitious materials with lower environmental impact and, at the same time, improved water resistance and therefore durability.

## ■ CONCLUSION

We here evaluated different hybrid mortar materials containing bacterial additives. Compared to unmodified standard mortar, all biomortar variants possess increased surface hydrophobicity and suppress capillary water uptake—albeit with different efficiencies. However, this water repellency comes at a cost. As a consequence of the bacterial additive, the density, as well as the flexural and compressive strength of the obtained hybrid mortar samples, is decreased. The strength of this loss in mechanical competence depends on the type of bacterial additive, which may limit the applicability of selected hybrid mortar variants in certain fields of construction engineering. Yet, in such applications, where water ingress damages the building structure, having a cementitious material with increased water repellency might outweigh the decrease in mechanical strength we report here. Moreover, further modifications of the mix including other additives could lead to a compensation of the strength reduction effect we observed here. Of course, the small-scale production process of bacterial additives we currently conduct in the lab strongly relies on manual labor and uses a large amount of consumables—and this is neither practical nor economical. Thus, for the presented biobased hybrid material to become an economical, eco-friendly alternative to existing hydrophobizing strategies, a mass production of bacterial biofilm and/or bacterial overnight culture needs to be established, and the latter appears to be more easily feasible as existing methods from the field of biotechnology already allow for the large-scale bioproduction of bacterial cultures.[57] In addition, the long-term stability of the different bacterial-based hybrid materials needs to be assessed before the most suitable additive can be identified. Nevertheless, incorporating bacterial additives into the bulk of cementitious materials seems to be a promising route toward creating sustainable, more environmental friendly construction materials.

## ■ ASSOCIATED CONTENT

**ⓈI Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.0c00547.

Detailed manufacturing process of different bacterial additives, optical characterization of microbial composition of different bacterial additives, and additional data showing influence of bacterial growth media (components) on the material properties of mortar (PDF)

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**Author Contributions**
M.J.E. and O.L. designed the experiments. M.J.E., J.E., and M.R. performed the experiments and analyzed data. The manuscript was written by M.J.E., M.R., O.L., and C.U.G.

**Notes**
Approval of ethics is not required for the experiments conducted in this manuscript.
The authors declare no competing financial interest.

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Supplemental Information for

# Bacterial additives improve the water resistance of mortar

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**1. Detailed manufacturing process of the different bacterial additives**

*1.1 Overnight culture:*

10 mL of liquid Luria/Miller LB-Medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L sodium chloride, pH 7.0 ± 0.2, Carl-Roth, Karlsruhe, Germany) were inoculated with a frozen bacterial/glycerol stock. After incubation at 37 °C and 90 rpm (or 200 rpm for *B. subtilis* B-1) in a shaking incubator (Sartorius, Göttingen, Germany) overnight, a solution of planktonic bacteria (= an 'overnight culture') was obtained (**Figure S1**).
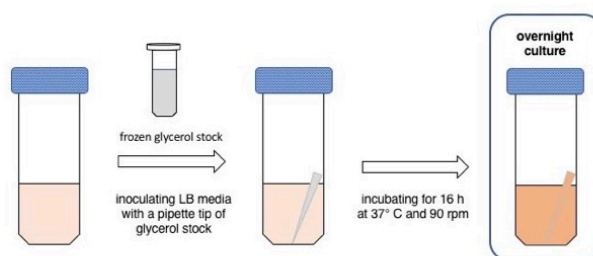


*Figure S1*: **Preparation of bacterial overnight cultures.** Each overnight culture was incubated for 16 h to reach the stationary phase of the bacteria growth rate and thus ensure the highest possible density of live planktonic bacteria in the liquid media.

*1.2 Bacterial biofilm:*

To generate bacterial biofilm, agar plates were produced first. Therefore, 20 g LB media and 12 g Agar were dissolved in 800 mL distilled water, and subsequently the solution was autoclaved. The mixture (hereafter referred to as LB-agar) was allowed to partially cool down to 60°C. 25 mL of (still liquid) LB-agar was then poured into a sterile petri dish and allowed to fully cool down. After the LB-agar had solidified, 100 µL overnight culture was plated on every agar-filled Petri dish and incubated at 37° C. After 24 h, a continuous layer of biofilm has formed on the agar plates (**Figure S2**).
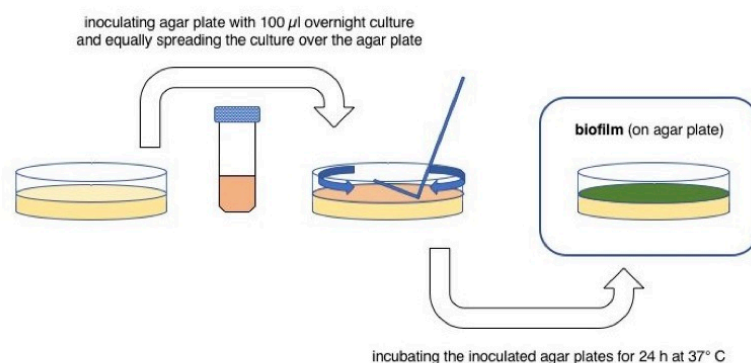


*Figure S2*: **Formation of bacterial biofilm on agar plates.** 100 µL of Overnight culture (highly concentrated solution of planktonic bacteria) was applied on and evenly distributed over each agar plate. On the nutrient rich agar, the bacteria start to produce bacterial biofilm.

Finally, using a customized PDMS-spatula, the generated biofilm could be harvested without collecting any agar pieces (**Figure S3**).
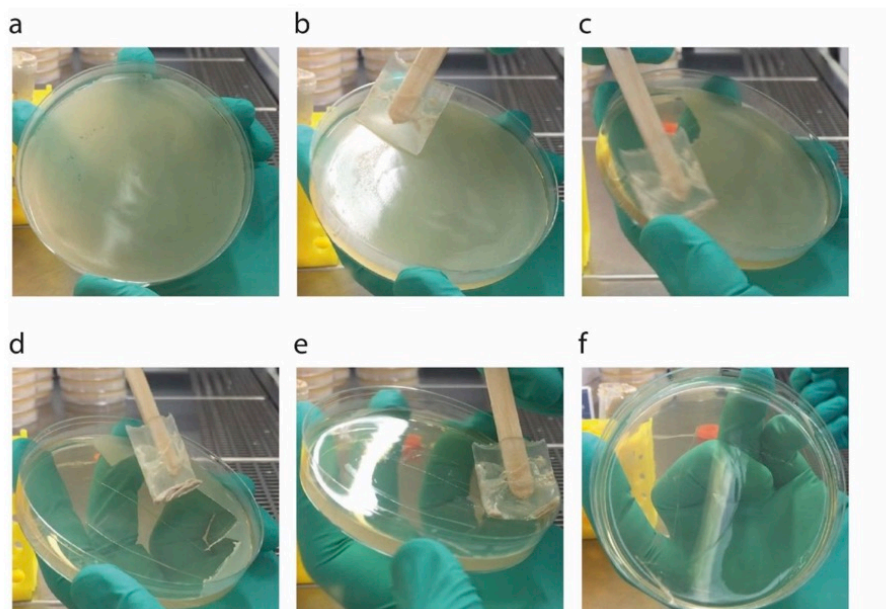


***Figure S3***: **Harvesting process to collect bacterial biofilm from agar plates using a PDMS spatula.** a) After incubating the inoculated agar plates for 24 hours, a continuous biofilm layer is formed on the whole agar surface. b) Using a custom-made PDMS-spatula, the biofilm can be collected from the agar (c - e) without damaging the agar substrate (f).

### 1.3 Biofilm powder:

Bacterial biofilm was generated and collected as described above, and then stored at -80° C for at least 2 h. Subsequently, the frozen biofilm was freeze-dried for 72 h. To obtain biofilm powder, the freeze-dried biofilm was finely ground using a mortar and pestle.
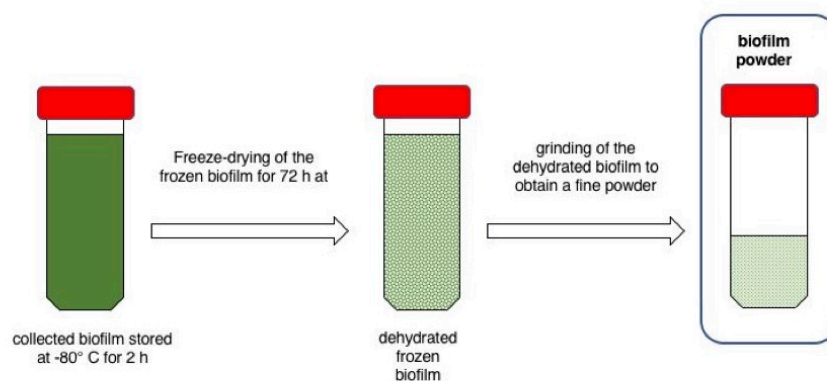


***Figure S4***: **Production procedure to obtain a powder from a freshly collect, wet bacterial biofilm.** After collection, the biofilm was dehydrated in a freeze-drying process, and finally the dehydrated biofilm was finely ground into a powder.

## 2. Optical characterization of the microbial composition of the different bacterial additives
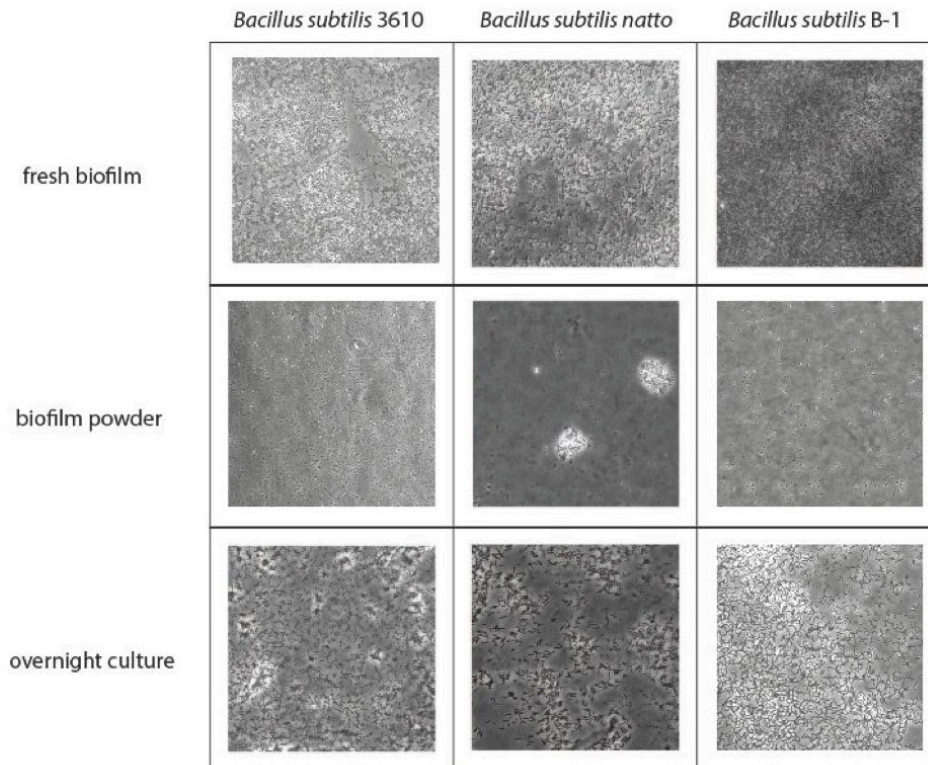


**Figure S5: Phase contrast images of the nine different bacterial additives used in this study.** Fresh biofilm (top row) contains a mixture of active cells (rod shaped objects) and spores (circular objects), *i.e.*, bacteria in a metabolic inactive state. In contrast, biofilm powder (middle row) contains exclusively spores, and bacterial suspensions ('overnight culture´) contain mostly active bacteria cells and almost no spores.

## 3. Influence of bacterial growth media (components) on the material properties of mortar
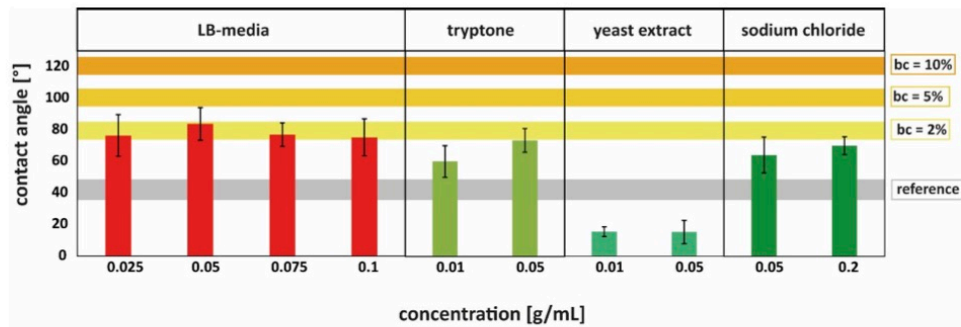


**Figure S6: Contact angles obtained for mortar samples containing different concentrations of LB-media and single media components, respectively.** Standard LB-media (0.025 g/mL) used for the cultivation of bacteria as described in the main paper contains tryptone (0.010 g/mL), yeast extract (0.005 g/mL) and sodium chloride (0.010 g/mL). If those components are added to mortar alone (i.e., without biofilm components or bacteria), the measured contact angles never reach the values above 85° and thus are much lower than what we obtain with the different bacterial additives. The contact angle values obtained for biofilm additives (as described in the main text) are indicated by the three yellow/orange stripes and refer to samples with different biofilm content.
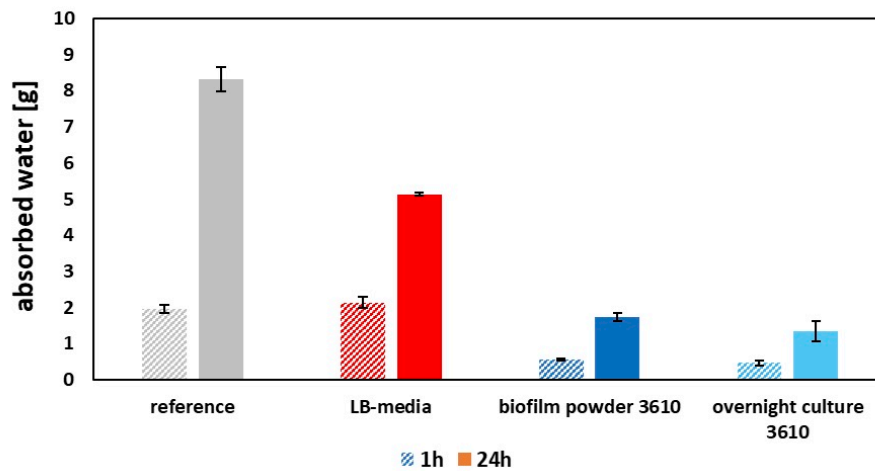


**Figure S7: Capillary water uptake into hybrid mortar samples.** The amount of water absorbed by the different samples after partial immersion into a water bath is depicted for immersion times of 1 h and 24 h, respectively. Samples enriched with biofilm powder (bc = 2 %, left) and samples enriched with a bacterial overnight culture (OD > 1.0, right) are compared to both, unmodified control samples (grey bars) and samples containing culture media. The values shown represent averages of three measurements conducted on independent samples, error bars denote the standard deviation.

S5
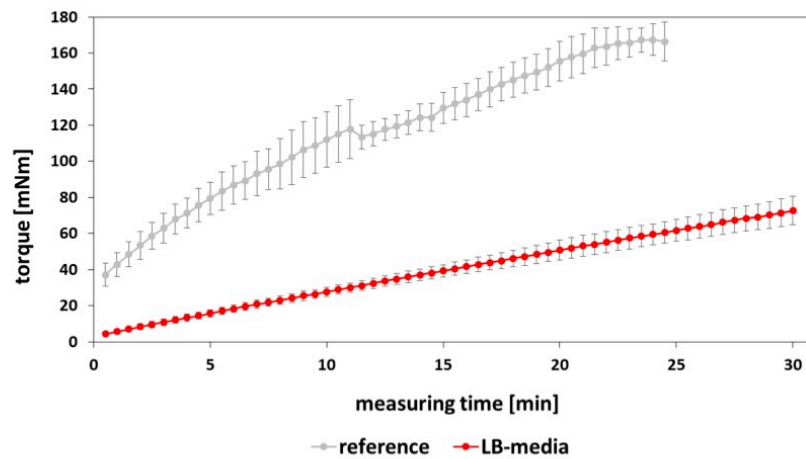
***Figure S8:*** **Influence of LB media on the hardening process of mortar.** The graphs depict the time-dependent increase of the torque require to maintain a constant shear rate of 0.001 s$^{-1}$ on different mortar variants. Mortar samples containing LB media instead of mixing water (red curve) are compared to unmodified reference samples (grey curve). The values shown represent averages of three independent measurements, error bars denote the standard deviation.

## A.3 Bacterial spores as hydrophobizing agents in mortar

# Bacterial spores as hydrophobizing agents in mortar

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ABSTRACT

In the last decade, biological additives have gained increased attention as admixtures to cement based materials. One example are bacterial additives, which can improve the wetting resistance and/or the mechanical properties of cementitious materials. However, the production process of most bacterial additives investigated so far is typically time consuming and comparably expensive. Here, we investigate six different commercially available bacterial spores as an alternative bacterial additive to mortar and characterize the wetting resistance, capillary water uptake, and mechanical properties of the resulting hybrid mortar formulations. Our results imply that selected bacterial spores are indeed able to enhance the water-resistance of mortar; however, compared to other bacterial additives such as biofilm, the overall performance of the resulting hybrid material is decent but still inferior.

## 1. Introduction

Microbes and concrete might not sound like a match made in heaven – in the end, mold development in basements and moist living rooms are a major nuisance in the area of civil engineering. Yet, biological additives [1,2] – including such containing bacteria [3–5] – are increasingly gaining attention as alternative supplements that could help making the cement industry more sustainable. One key challenge such bio-based attempts have to face is the high pH value in the cement paste (which can reach levels above 12.5 during the hydration reaction), the lack of nutrient sources in classical cement mixtures, as well as the limited access to moisture or oxygen; in other words, cementitious materials do not provide an ideal environment for most bacteria. However, certain microbial variants, so called spores, do not require similarly gently conditions as normal bacteria [6]. Spores represent a highly resilient, yet metabolically inactive life form of bacterial cells, and they are generated when the microbes encounter adverse environmental conditions [7]. Due to their complex, multi-layered organization, spores exhibit high resistance to heat, dehydration, chemicals and radiation [8]. Typical spores possess the following structural features (listed from the inside to the outside): a central core, an inner membrane, a germ cell wall, a cortex, an outer membrane, as well as an exosporium [9].

Owing to their high sturdiness, spores have already been successfully tested as additives to cementitious building materials: for instance, bacterial spores can convey self-healing abilities to concrete, i.e., via microbially induced calcite precipitation (MICP) [5,10,11]. To achieve this, the spores are typically embedded (e.g., into microcapsules enriched with nutrients and urea) and then integrated into the bulk of a cementitious material. Then, structural damages and the ensuing ingress of moisture can reactivate the spores into vegetative bacteria, which in turn, enable the formation of calcium carbonate crystals that grow from the anionic bacterial cell walls.

Such stimulation of biomineralization events can, however, also be triggered by bacterial additives in which the bacterial cells are not alive – and in those cases, an unwanted reproduction of the bacteria is prevented. For instance, recent studies have shown that bacterial cell walls (in other words, fragments of dead cells) can significantly increase the compressive strength of concrete [12], or they can be used as a viscosity-modifying additive for concrete [13]. Also, other bacterial additives, e.g., wet or freeze-dried bacterial biofilm [3], as well as bacterial solutions [4], have been shown to convey beneficial properties to cementitious materials: they can increase the wetting resistance of mortar and reduce capillary water uptake – and both effects should be able to extend the service life of cementitious structures thus reducing their environmental impact.

However, those various bacterial additives differ not only in terms of

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composition but also in terms of efficiency and practicability. To date, a critical limitation of most bacterial additives is their production process which can be both, time and cost intensive. For bacterial spores, however, this is not a critical issue: some of them (*e.g.*, those used for agricultural applications) are already produced at industrial scale [14] and commercially available as ready-to-use powders. Such a spore-based powder can easily be integrated into the mixing process of cementitious building materials, which renders bacterial spores a potentially very interesting candidate for a biological mortar additive.

Here, we investigate the suitability of selected bacterial spores as a mortar admixture that conveys hydrophobic properties to the hybrid mortar. We compare six different, commercially available *Bacillus* spores which are used as additives as is, *i.e.*, without any further purification or functionalization. In detail, we focus on spores of well-studied, harmless (*i.e.*, apathogenic) bacterial strains that are used in agricultural applications, which renders them all suitable candidates for the development of marketable products. We aim to find the most suitable *Bacillus* spore variant that – at a given, moderate dosage – maximizes the wetting resistance of mortar while minimizing capillary water uptake. Moreover, the hydration kinetics and the mechanical competence of the resulting hybrid mortar variants are examined to assess if improving the water resistance of the material comes with an impairment of other important material properties. Our results indicate that, depending on the type of bacterial spore used, an improvement of the water-repellent properties of mortar is possible – albeit at reduced efficiency compared to bacterial biofilm.

## 2. Materials and Methods

### 2.1. Biofilm powder production

Biofilm powder was produced as described in Ref. [4]. In brief, *Bacillus subtilis* NCIB 3610 (*B. subtilis* 3610) was obtained from the lab of Roberto Kolter (Harvard Medical School, USA), and planktonic over-night cultures were cultivated in luria broth (LB) medium (LB broth, Luria/Miller, Carl Roth). Then, biofilm was cultivated on LB-agar, harvested, freeze-dried for three days, and afterward ground into a fine powder. Using the mass loss factor from Ref. [4] (which is defined as the ratio of fresh biofilm mass with respect to the mass of lyophilized biofilm), the required amount of biofilm powder could be calculated to obtain the desired biofilm content of 2% (bc, which describes the ratio of fresh biofilm with respect to the mass of dry cement).

### 2.2. Bacterial spores

Bacterial spore powders were obtained from ABiTEP GmbH (Berlin, Germany). Spores from six different *Bacillus* variants are investigated here: *Bacillus atrophaeus* ABi05 (Abi05), *Bacillus subtilis* ABi26 (Abi26), *Bacillus licheniformis* ABi53 (Abi53), *Bacillus velezensis* FZB24 (FZB24), *Bacillus velezensis* FZB42 (FZB42), and *Bacillus velezensis* FZB45 (FZB45). The spore powders were all used as is, *i.e.*, without any further purification or functionalization step.

### 2.3. IR-spectroscopy

Infrared spectra (IR) were recorded on a PERKIN ELMER Spectrum 100 FT-IR instrument (Waltham, USA) in a wavenumber range from $4000 \text{ cm}^{-1}$ to $450 \text{ cm}^{-1}$. Measurements were performed at room temperature (RT) using the solid spore powder without any further purification. To analyze the outer spore surface (which is the most likely part of the spore to influence the hydration reaction of mortar), the total reflection (ATR) sampling technique was selected. With this method and the settings used here, the typical penetration depth of the IR signal is $\sim$100 nm [15]. Considering that the thickness of the outer spore coat ranges from 70 to 200 nm [6], it is unlikely that the recorded spectra return information from the spore volume. Instead, the measured signal

will – to a large extent – originate from the spore surface and the outer layers of the spore coat/exosporium.

### 2.4. Zetasizer measurements

The Zeta-potential ($\zeta$) and hydrodynamic size of the different spore variants were measured using a Litesizer 500 (Anton Paar, Graz, Austria) equipped with a 35-mW laser diode light ($\lambda = 658$ nm). All measurements of spore suspensions (concentration of 1.5 mg/mL, pH = 13) were performed in technical triplicates.

### 2.5. Light profilometry

The microscopic surface profiles of mortar samples were obtained using a laser scanning microscope (VK-X1000, Keyence, Oberhausen, Germany) equipped with a 50x lens (NA = 0.95; Nikon, Chiyoda, Tokyo, Japan). Images were acquired without any further sample treatment, *i.e.*, directly after curing of the mortar samples. On each sample, five spots with an area of $213 \times 284$ μm were scanned. The scanned area was then evaluated with the software MultiFileAnalyzer (Version 2.1.3.89, Keyence, Oberhausen, Germany) to obtain the developed interfacial area ratio,

$$Sdr = \frac{1}{A} \left[ \iint_A \left( \sqrt{1 + \left( \frac{dz(x,y)}{dx} \right)^2 + \left( \frac{dz(x,y)}{dy} \right)^2} - 1 \right) dxdy \right].$$

Here, $A$ denotes the scanned sample area, $x$ and $y$ the lateral dimensions, and $z$ the height of the surface profile. Thus, the developed interfacial area ratio $Sdr$ quantifies the additional surface area contributed by a texture compared to a plane surface.

### 2.6. Mortar sample preparation

For initial contact angle experiments, test specimens were produced at lab-scale (*i.e.*, using $\sim$45 g of material and thus not according to DIN EN 196-1). Such smaller specimens were prepared to be able to test different formulations without wasting too much material. For further testing (bending and compressive strength, water uptake measurements), the mortar samples of promising formulations were prepared according to DIN EN 196-1.

When preparing mortar samples for contact angle measurements, bacterial spores were added to a 3:1 mixture (w/w) of CEN standard sand (NORMENSAND GmbH, Beckum, Germany) and cement (Portland cement CEM I 42.5 N, Schwenk Zement KG, Ulm, Germany). Distilled water (ddH$_2$O) was added as needed to obtain a w/c ratio of 0.5, and the mixture was stirred mechanically using a paddle mixer for 2 min. All mortar samples were cured for 3 days at RT before they were used in any experiments.

For water uptake tests as well as bending and compressive strength measurements, samples were prepared according to DIN EN 196-1 [16] using an automatic mortar mixer (ToniMix, Zwick Roell, Ulm, Germany). Within 10 s, cement and spore powder were added to a bowl together with the desired amount of water, and the mixing process was immediately started: The first step of this mixing procedure comprises 30 s of stirring at speed of 140 rpm. Then, within 30 s, the sand was added at a mixing speed of 140 rpm and the mixture was stirred for an additional 30 s at an increased stirring speed of 285 rpm. Then, the mixing process was stopped for 90 s; this was necessary to transfer mortar pieces, which adhered to the stirring head and/or the upper part of the bowl, back to the bottom of the bowl using a rubber scraper. After an additional 60 s of mixing at a stirring speed of 285 rpm, the prepared mortar was poured into the desired mold within 120 s, where it was compacted using a vibrating table.

### 2.7. Contact angle measurements

To investigate the wetting behavior of the mortar samples, 10 μL droplets of ddH$_2$O were placed onto the surface of each sample at five

different spots, and images were acquired from a lateral view using a digital camera (Flea3, Point Grey, Richmond, Canada). The locations for conducting wetting tests were chosen following a fixed scheme: first, one droplet was placed in the middle of the sample; then, four additional droplets were deposited in the corners of a rectangle having the first testing spot as its center. The resulting contact angles were then determined by evaluating the digital pictures using the image analysis software ImageJ (public domain, ImageJ 1.52n) in combination with a drop-analysis plugin tool, which was also downloaded from the same source.

### 2.8. Water uptake experiments

To determine the capillary water uptake of mortar, cylindrical samples were prepared according to DIN EN 196-1 using commercial polyethylene tubes (diameter: 40 mm) as casting molds. Those tubes were removed after 3 days of curing, and the mortar samples were coated with the injection resin MC-Inject 1264 compact (MC Bauchemie, Bottrop, Germany); after 11 additional days of storage at RT, this resulted in completely sealed mortar samples. After 24 h, one of the ends of the sealed cylinders was cut off to create an open surface which enables water ingress. The mortar samples were then immersed into a 2 cm high water bath, with the open surface facing down. To quantify the amount of water taken up by capillary forces, the mortar samples were weighed using a micro-scale (TLE 303, Mettler Toledo AG, Greifensee, Switzerland). This weighing step was conducted before and after the samples were exposed to water for defined time intervals.

### 2.9. Workability characterization

To determine the workability of the different mortar samples, rheological measurements were performed using a commercial shear rheometer (MCR 302; Anton Paar GmbH, Graz, Austria) equipped with a BMC 90 measuring cell for building materials and a paddle mixer. For each measurement, 450 g of each mortar formulation was prepared and mechanically mixed for 3 min. After transferring the mortar mass into the measuring cell, the measurement was started 4 min after the hydration reaction was initiated. After 1 min of stirring at a constant shear rate of $0.0001\ s^{-1}$, the torque required to maintain this constant shear rate was measured every 30 s for a total duration of up to 30 min. If a critical torque >200 Nm was reached, the measurement was stopped. These measurements were conducted at RT, *i.e.*, without any further air or water cooling.

### 2.10. 3-Point bending and compression tests

The 3-point bending and compressive strength of standard and hybrid mortar samples were determined using test specimens of standardized geometry (4 × 4x16 cm). For every formulation and testing date, three test specimens were produced according to DIN EN 196-1 as described above. After 24 h, the test specimens were removed from the formwork and stored in a closed container inside an air-conditioned room; here, the storage conditions were selected according to DIN EN 196-1, i.e., (20 ± 2) °C and ≥50% relative humidity (r.h.) until they were tested. On each testing date (*i.e.*, after 7 and 28 days of curing, respectively) first, the 3-point-bending-strength was determined; afterward, the compressive strength was measured with a loading frame (Series DB Super, Walter&Bai, Löhningen, Switzerland) using the six fragments obtained from the bending tests.

### 2.11. Scanning electron microscopy

For SEM images of bacterial spores, the spore powder was dispersed onto a piece of adhesive tape, which was placed onto an aluminum sample holder and sputtered for 40 s with gold (MED 020, BAL-TEC, Blazers, Lichtenstein). Pictures were acquired on a JEOL-JSM-6060LV

(Jeol, Eching, Germany) scanning electron microscope at an acceleration voltage of 15 kV.

For SEM images of mortar surfaces, the mortar samples were dried at 80 °C for at least 24 h and then placed onto an aluminum sample holder. Pictures were acquired without sputtering on a FlesSEM 1000 (Hitachi, Chiyoda, Japan) scanning electron microscope at an acceleration voltage of 5 kV.

### 3. Results and discussion

When particular matter is used as a mortar additive, the surface properties of the particles are important as they can influence the hydration reaction and mineralization processes occurring during setting. Thus, in a first step, we asked if the biological particles used here, *i.e.*, the spores generated by different *Bacillus* bacteria, exhibit differences regarding their physicochemical surface properties. Accordingly, for all tested spore variants, we measured the Zeta-potential and the hydrodynamic size of the spore particles and recorded an IR spectrum characterizing the surface chemistry of the exosporium, *i.e.*, the outer surface of the spores (Fig. 1).

The Zeta potential is a measure for the overall surface charge of a microscopic object, and we determined strongly negative values for all tested spores. This result was encouraging as negatively charged objects are reported to be well suited to interact with calcium ions during the cement hydration reaction [17,18]. Size measurements conducted on the same set of samples, however, returned surprisingly large differences and values as high as 5–8 μm (especially for the Abi53, FZB24 and FZB42 spore variants, where sample-to-sample variations were high as well).

This was unexpected as, for example, the size of *B. subtilis* bacteria (and, thus, also the spores generated from them) are typically all in the range of 1–2 μm (corresponding to a typical cell volume of 0.4–3 fL [19]). Indeed, those expected dimensions could be confirmed for the *B. subtilis* (Abi26) spores using SEM (Fig. 2).

However, if the spores were to possess hydrophobic surface properties, then they would tend to form aggregates in aqueous suspensions. Indeed, the poor solubility of all the spore variants tested here (resulting in sedimentation of the spores from aqueous suspension that occurs, *e.g.*, overnight) could explain the large values we obtained for some of the tested spore variants.

An analysis of the recorded IR spectra indicated similar bands and therefore the presence of the same set of functional groups for each spore variant. The broad peak at 3300 $cm^{-1}$ indicates that the outer spore surface carries hydroxyl groups. Considering the other peaks occurring in the IR spectra, a possible explanation for this result would be the presence of (oligo)saccharide motifs on the outer spore surface, which is typical feature for bacterial cell walls [13]. We would like to emphasize that, even though the structure of bacterial spores differs considerably from those of vegetative bacterial cells, most bacterial spores are structured in a similar manner: In many bacterial spores, the exosporium constitutes the outermost layer; the spore coat, which is a component of this exosporium, comprises mostly proteins, lipids and carbohydrates – and all of those components are possible candidates that could give rise to the IR band corresponding to hydroxyl groups. However, from such IR spectra alone, the number and exact location of the hydroxyl groups we detect as well as the overall hydrophobicity of the spore surface cannot be assessed.

The different behavior of the spores in aqueous suspension as observed by dynamic light scattering, however, suggests that the different spore variants might form aggregates of different sizes; this, in turn, could result in differences regarding their impact on the hydration reaction of mortar. However, we found that the size distribution of only one spore variant tested here was sensitive to ultrasonication treatment (Fig. S1), and this result did not correlate well with differences in the hydrophobizing potential of the different bacterial additives (*vide infra*).

In a second step, the suitability of the different bacterial spores as a hydrophobicity conveying mortar additive was examined. Therefore,
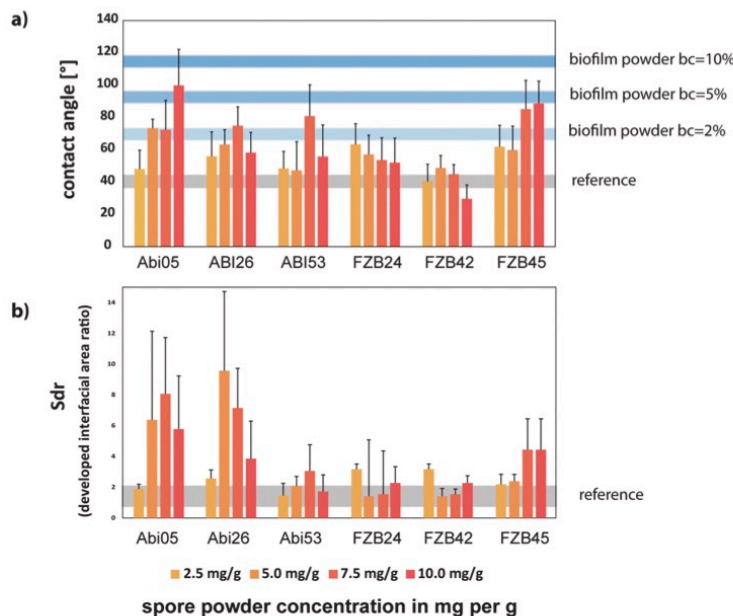
3

**Fig. 3. Wetting resistance and surface roughness of mortar enriched with different bacterial spores.** a) The wetting properties of different mortar samples were characterized by the contact angle water droplets form on their surface. Samples with different spore powder contents are compared. The contact angle values shown represent averages obtained from 15 measurements conducted on a set of three samples; the error bars denote the standard deviation. The horizontal stripes in the background of the figure represent the contact angles of unmodified reference samples (grey) and hybrid mortar samples containing an increasing amount of *B. subtilis* 3610 biofilm powder (blue, data taken from Ref. [4]), respectively. b) The surface roughness of each sample is investigated by measuring the Sdr value. Each value shown represents the average of five measurements obtained on different spots on the surface of one sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

surface using light profilometry (Fig. 3b). From those images, we calculated the developed interfacial area ratio, *Sdr* (see Methods). This parameter describes the percentage of an area's additional surface as generated by its texture compared to a planar sample of the same size. Indeed, previous studies already showed that an increased surface roughness can be related to an increased wetting resistance of mortar.

In the next step, we tested if the addition of bacterial spores would reduce the capillary water uptake in the resulting hybrid mortar. For this purpose, we used the same experimental procedure as described in Ref. [4] (see Materials and Methods for details). Interestingly, we observed that all tested hybrid mortar samples but one suppress capillary water uptake – at least to a certain degree (Fig. 4 and S2): the sample that could not be characterized with regard to this property was the one containing Abi05 spores; this particular hybrid mortar sample was
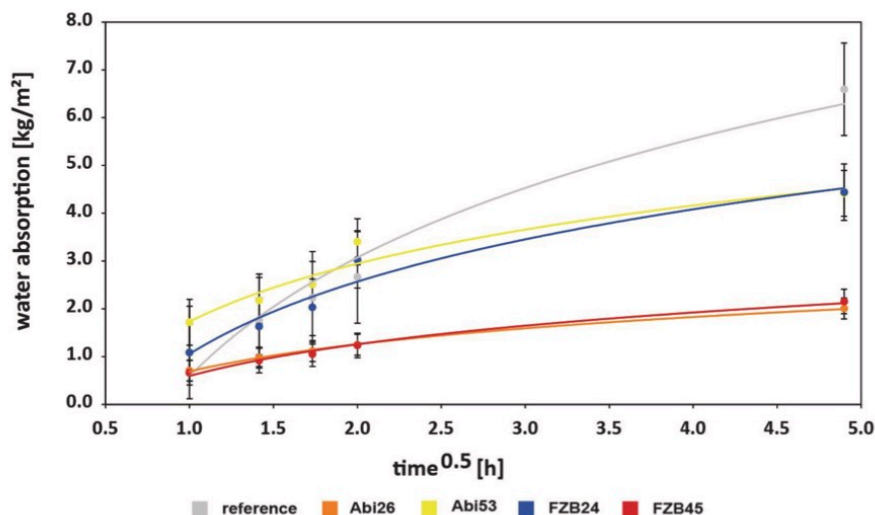


**Fig. 4. Capillary water uptake into hybrid mortar.** The amount of water absorbed by hybrid mortar samples after partial immersion into a water bath for 1, 2, 3, 4, and 24 h, respectively, is shown for samples containing bacterial spores (at a concentration of 10 mg spores/g cement) compared to unmodified reference samples. The values shown represent averages of three measurements conducted on independent samples, the error bars denote the standard deviation.
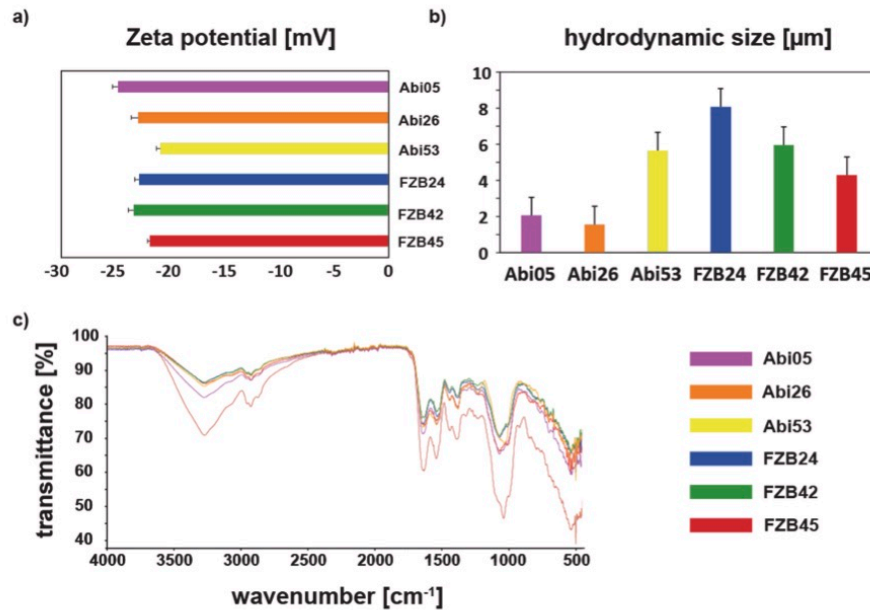
**Fig. 1. Physico-chemical characterization of the tested spores.** Each spore powder variant was analyzed by measuring the zeta potential **a)**, the hydrodynamic size **b)**, as well as infrared spectra **c)**. Error bars denote the standard deviation as calculated from 3 independent replicates.
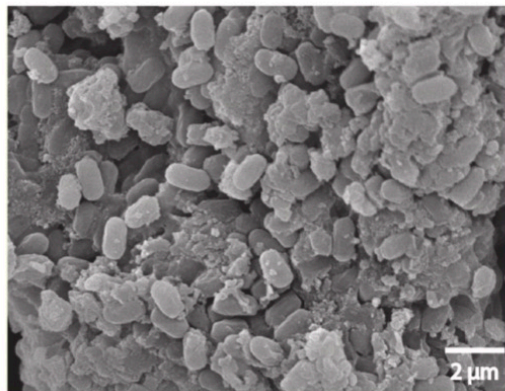


**Fig. 2. SEM image of the B. subtilis Abi26 spore powder.** Individual spores with sizes in the range of 1.5–2 μm can be identified. The scale bar represents 2 μm.

mortar samples containing different amounts of bacterial spores were prepared and – after curing – their wetting resistance was evaluated by measuring the contact angles of water droplets placed onto the mortar surface (Fig. 3a). Results obtained with other (hydrophobic or hydrophilic) bacterial additives, *i.e.*, bacterial biofilm or bacterial cell walls, suggested that charged residues present in those biological materials may influence the hydration process of mortar, *e.g.*, via complexation of calcium ions and/or via precipitation of hydration products onto the surface of the mortar material. Similarly, taking into account the negative Zeta potential of all the spore powder variants we study here, we assume that those particular biological objects do not lead to

different hydration products in the hybrid mortar either but rather alter the microstructure of the material, and thereby its wetting resistance.

As in our previous study [4], where the effect of different bacterial additives was observed to be dose-dependent, an increase of the contact angle with an increasing amount of added spore powder was also observed here for most of the spore variants tested – although a linear dependency was only obtained for the Abi05 spores (Fig. 3a). The lowest concentration we tested (*i.e.*, 1 mg spores per g cement) was inspired by the concentration those spores naturally occur in soil. However, with this low dosage, there was almost no effect on the contact angle (data not shown). Thus, we here only show data obtained with higher dosages, where the majority of the tested samples returned contact angles higher than the unmodified reference samples. For most of the tested spore variants, a dosage between 7.5 mg/g and 10.0 mg/g returned the best results. The relatively large error bars obtained in this set of experiments reflect the inherent difficulties associated with measuring contact angles on inhomogeneous surfaces such as concrete or mortar [3,20,21]. Similar complications brought about by the addition of bacterial additives, *e.g.,* an increased macroporosity of the hybrid mortar were observed for other bacterial additives in a previous study [4], and also this effect leads to larger error bars in contact angle measurements.

Nevertheless, the addition of bacterial spores to mortar resulted - in most cases – in a hybrid material that had an increased wetting resistance compared to unmodified reference samples (Fig. 3a, grey horizontal stripe). However, the addition of FZB42 did not improve the wetting resistance of the mortar sample; instead, we here even detected even a slight decrease in the wetting resistance of the samples at the highest tested dosage form (which is why this particular spore variant is not evaluated further). Overall, when compared to samples, which had received bacterial biofilm powder as an additive (blue horizontal stripes in Fig. 3a), the spore additives tested here appear to be less potent in establishing hydrophobic surface properties in the mortar samples.

To better understand the differences in the wetting behavior we observed between the different samples, we further analyzed their

unstable; *i.e.*, it fell apart when removing the tubes the samples were cured in. Therefore, from this point on, this particular spore variant was not evaluated further.

For all tested samples, we obtained a time-dependent uptake of water (Fig. 4 and S2). Compared to the unmodified reference samples, however, the capillary water uptake into hybrid mortar samples containing bacterial spores was reduced by at least ~35% for all tested spore variants when added at a concentration of 10 mg/g (for lower dosages, weaker effects occurred as shown in the supplemental information (SI)). Moreover, in hybrid mortar formulations containing the spore types Abi26 and FZB45, respectively, the suppression of capillary water uptake was even stronger: here, we found a reduction by ~75% compared to the unmodified reference samples. This efficient suppression of capillary water uptake is similar in strength to what we obtained previously using *B. subtilis* 3610 biofilm powder as an additive (there, a suppression of the capillary water uptake > 85% was observed [4]). Because of this good result, the two corresponding spore variants (Abi26 and FZB45) were selected as favorites and further characterized. Considering that spore dosages below 10 mg/g returned less favorable results than higher doses, we selected this high additive concentration for all further tests.

The next question was, how the added spore powder would affect the flow properties and therefore the workability of the resulting hybrid mortar. This is an important aspect since, depending on the desired application of the cementitious material, sufficient processability is required. For instance, similar to concrete [22], masonry mortar is - according to its consistency - classified into different groups [23,24]. Moreover, it is well established that many additives affect the viscosity of cementitious materials [25,26], and they can either retard [27,28] or accelerate [29] the hydration reaction. Therefore, to evaluate the workability of the different mortar variants, rheological measurements were conducted. Here, the torque needed to shear the freshly mixed mortar formulations at a constant shear rate of $0.0001 \text{ s}^{-1}$ was measured in a time-dependent manner, which allows for following the change in mortar workability as the hydration reaction takes place (Fig. 5). Previous studies had already shown, that the addition of bacterial additives, such as bacterial cell walls [13], bacterial liquid culture, or freeze-dried bacterial biofilm [4] influences the hydration kinetics and thus the viscosity of cementitious systems. Thus, we expected that using bacterial spores as an additive could have a similar effect. Indeed, as depicted in

Fig. 5, the mechanical strength of the unmodified reference sample developed faster compared to hybrid mortar containing Abi26 and FZB45 spore powder, respectively.

Interestingly, this effect was more pronounced for the Abi26 spore variant: After 30 min, only ~70% of the torque needed to shear the unmodified reference was required to maintain the same shear rate for hybrid mortar variant containing FZB45 spores. For the hybrid mortar enriched with Abi26 spores, only ~45% of the reference torque was required. This increase in workability is comparable to what we obtained previously for other bacterial additives, where a reduction in the shear torque by ~50–75% was observed for mortar samples containing bacterial liquid cultures or bacterial biofilm powder. Such a delay in the early strength development of mortar is typically associated with a retardation of the hydration reaction [30]. And indeed, the hydration reaction can be affected by a number of for organic molecules such as carbohydrates [31–33] or superplasticizers [34–37]; yet, the exact mechanism underlying this effect (which we also reported previously for other bacterial additives [4]) is not fully understood. It was, however, shown that stimulated biomineralization processes can significantly affect the strength development in cementitious materials [38–40], and bacterial additives have been suggested to lead to such biomineralization [3,4,41].

Previous work had already indicated that bacterial additives not only affect the strength of hybrid mortar at early time points but also at later stages of the hardening process. Thus, in the last step, we here also evaluated the two remaining spore powder variants with regards to this parameter. For this particular set of experiments, standard test specimens were prepared according to DIN EN 196-1 (see Materials and Methods for details) and both, tensile and compressive tests were conducted at two time points, *i.e.*, 7 and 28 days after the samples were produced (Fig. 6). Moreover, samples with different water/cement ratios (ranging from 0.4 to 0.6) were prepared to cover the range of most commonly used water/cement ratios in civil engineering applications.

In full analogy to other bacterial additives tested previously, at a w/c ratio of 0.5, hybrid mortar samples containing spore powder showed lower tensile and compressive strength than the unmodified reference. The empirical Abram's law [42] predicts that the compressive strength of standard mortar samples should decrease as the w/c ratio increases. Surprisingly, even though we study spore-enriched hybrid mortar here, most of our results fully agree with this prediction. Only when Abi26
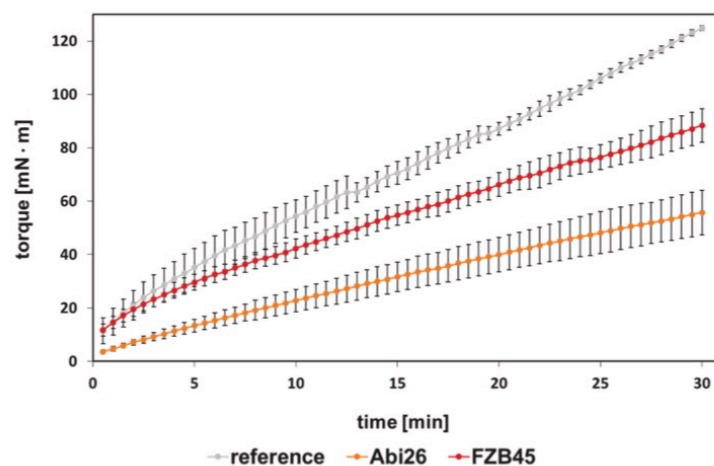


**Fig. 5. Influence of bacterial spores on the hardening process of mortar.** The graph depicts the time-dependent increase of the torque required to maintain a constant shear rate of $0.0001 \text{ s}^{-1}$ on different mortar variants. Hybrid mortar samples containing bacterial spores are compared to unmodified reference samples. The values shown represent averages of three independent measurements, the error bars denote the standard deviation.
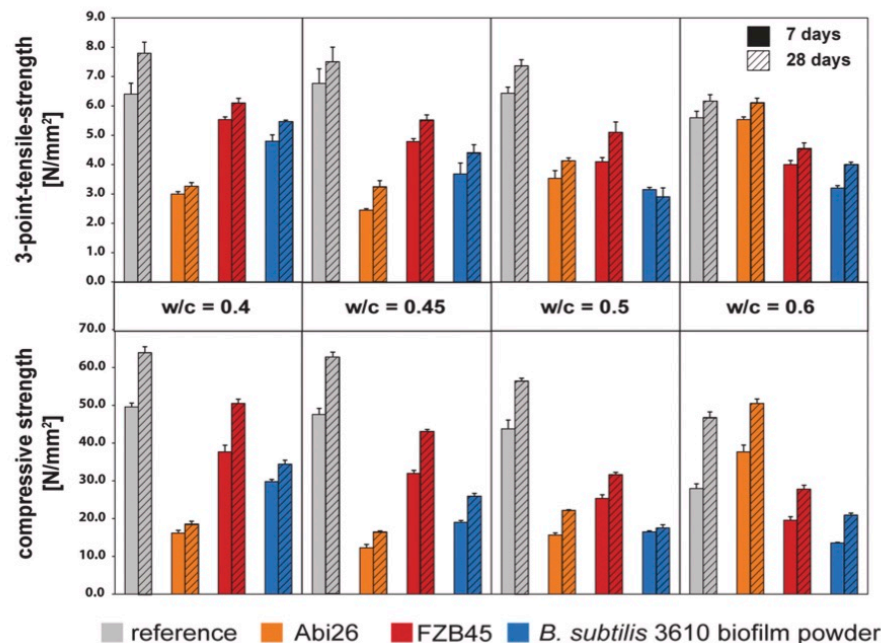
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**Fig. 6. Influence of bacterial spores on the tensile and compressive strength of mortar.** Tensile (upper panel) and compressive strength (lower panel) tests were performed according to DIN EN 196-1; samples were studied 7 and 28 days after their production. Hybrid mortar samples containing bacterial spores (orange and red) are compared to unmodified reference samples (grey) and samples containing biofilm powder (blue) at different w/c ratios. The values shown represent averages of three independent measurements, the error bars denote the standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

spores were used as an additive, the opposite behavior was observed: for those particular samples, both the tensile and compressive strength increased with increasing w/c ratio. In particular, at the highest w/c ratio tested here (w/z = 0.6), the generated hybrid mortar samples showed a mechanical stability as high as that of the unmodified reference.

The measurements and analyses conducted here demonstrate that a hydrophobization of mortar is, in principle, possible by using bacterial spores as an additive. In terms of hydrophobization efficiency, however, we found considerable differences between the tested spore variants; this is interesting and somewhat surprising considering that microscopic characteristics of the different spores are rather similar: they all exhibit an overall negative surface charge and return nearly identical IR absorption spectra. The strongest differences we obtained from size measurements conducted on aqueous spore suspensions: Here, Abi26 and FZB45 spores seem to be least prone to aggregation – and it is those two spore variants that, overall, performed best as hydrophobizing agents. We speculate that those two spore variants might be distributed most homogeneously across the volume of the hybrid mortar thus conveying better (and more homogeneous) hydrophobic properties (e.g., by stimulating biomineralization events) than the other spores tests here. However, SEM images obtained from unmodified mortar and Abi26/FZB45-enriched hybrid mortar do not return a clear picture of how the respective nanomorphology of the samples might differ (Fig. S3). Nevertheless, we would like to mention again that, for those two hybrid mortar variants, light profilometry measurements did return increased surface roughness values compared to the control – and this seems to be the main structural alteration we detected here.

## 4. Conclusion

Here, we find that, when bacterial spores are used as an additive to mortar, interesting and positive hydrophobizing effects occur; nevertheless, the overall performance of this particular bio-additive is somewhat inferior to what can be obtained with bacterial biofilms - especially with lyophilized biofilm. Yet, this may not be totally surprising: As shown in the SI [4], bacterial spores are a component of bacterial biofilm, and their number is particularly high in freeze-dried biofilm powder. In addition to bacterial spores, however, a bacterial biofilm also contains dead bacterial cells (and thus cellular debris) and a broad range of secreted biopolymers. Which of those other biofilm components is responsible for the superior hydrophobizing performance of bacterial biofilm compared to simple spore powder remains a question, which future research will have to address. Yet, for certain applications of cementitious building materials, especially where an increased wetting resistance is considered more relevant than a loss of mechanical strength, e.g. the use as masonry or render mortar, the increased water resistance obtained with bacterial spores might be a good first step. In the end, one big advantage those spores have compared to bacterial biofilm is their commercial availability [14], which makes it easy to generate hydrophobic mortar samples without the need for biofilm growth equipment.

**Statement of ethics approval**

Approval of ethics is not required for the experiments conducted in this manuscript.

## Declaration of competing interest

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cemconcomp.2021.104002.

## Author contributions

MJE and OL designed the experiments; MJE and LB performed the experiments and analyzed data. The manuscript was written by MJE, CUG, and OL.

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**Supplemental Information for**

# Bacterial Spores as Hydrophobizing Agents in Mortar

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## 1. Hydrodynamic size measurements with and without ultrasonic treatment
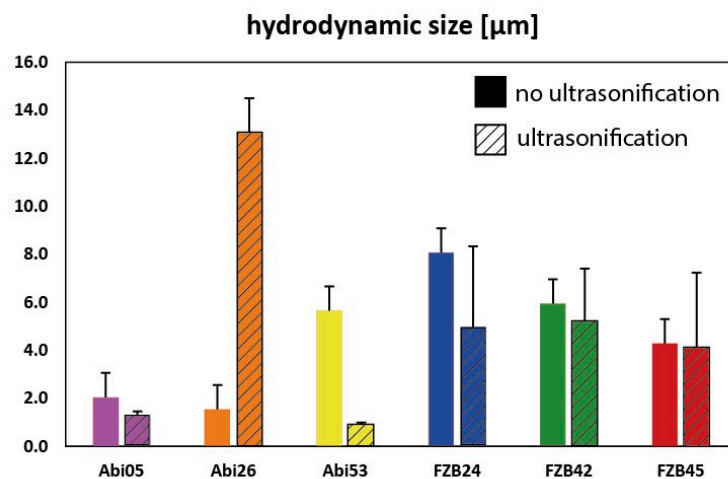


**Figure S1: Hydrodynamic size measurements of the different spore variants tested in this study.** Measurements were conducted as described in the main text; the data obtained without ultrasonification is the same as depicted in Fig. 1b of the main text. To possibly reduce the formation of spore agglomerates, a second series of measurements was conducted where the aqueous suspension was treated with a SONOPLUS ultrasonic homogenizer (BANDELIN, Berlin, Germany) for 30 s. The detailed settings of this treatment were: frequency: 20 kHz; amplitude: 20 %; 1 pulse/second. Error bars denote the standard deviation as calculated from 3 independent samples.

1

## 2. Water uptake measurements
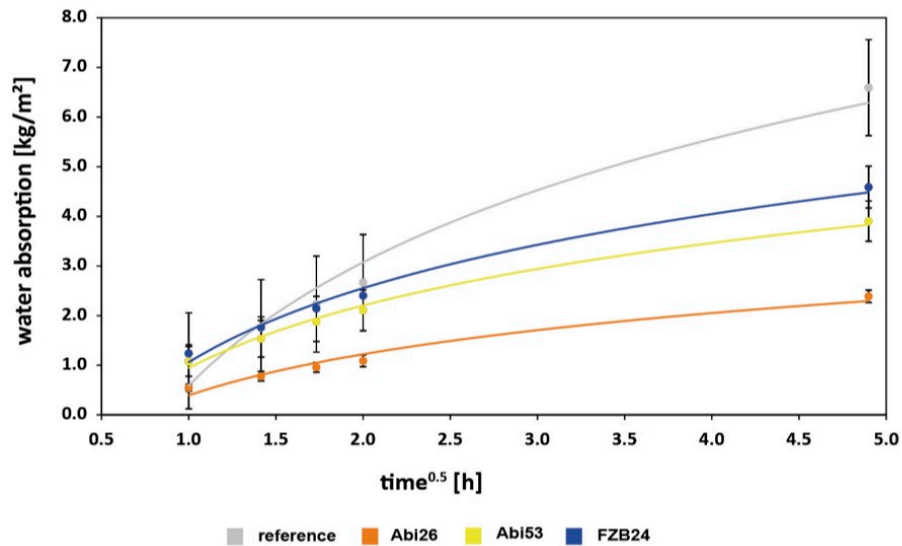


**Figure S2: Capillary water uptake into hybrid mortar samples enriched with lower amounts of spores.** The amount of water absorbed by hybrid mortar samples after partial immersion into a water bath for 1, 2, 3, 4, and 24 h, respectively, is shown for samples containing bacterial spores at lower concentrations than those samples described in Fig. 4 of the main text. In detail, here, a spore concentration of 2.5 mg (FZB24) and 7.5 mg (Abi26, Abi53) spores/g cement), respectively, is compared to unmodified reference samples. These concentrations were chosen as they represent conditions at which the respective spore variant has returned the highest contact angle (see Fig. 3a of the main text). The values shown represent averages of three measurements conducted on independent samples, and the error bars denote the standard deviation.

2

3. SEM pictures of reference and hybrid mortar samples (enriched with Abi26 and FZB45 spores, respectively)
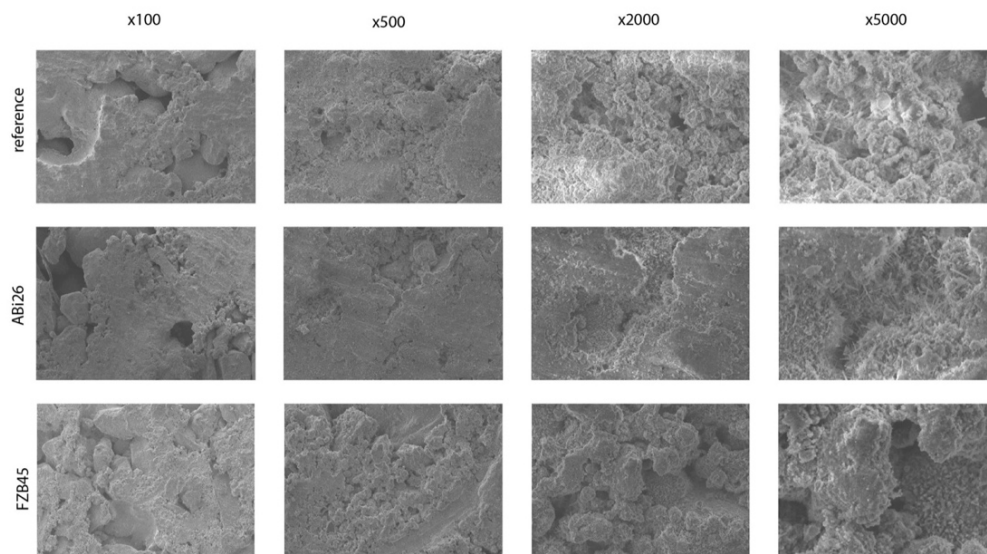


**Figure S3: SEM images of the surface of unmodified and hybrid mortar samples.** Images were acquired at different magnifications (x100, x500, x2000, x5000) without prior sputtering using an acceleration voltage of 5kV.

3

## A.4    Small Pores, Big Impact - Controlling the Porosity Allows for Developing More Sustainable Construction Materials

# Small Pores, Big Impact—Controlling the Porosity Allows for Developing More Sustainable Construction Materials

Marvin Johannes Ertelt, Harald Hilbig, Christian Ulrich Grosse, and Oliver Lieleg*

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**ABSTRACT:** Owing to the ongoing increase in world population, two challenges in the field of construction materials need to be solved: first, the sustainability and, second, the durability of the materials used. Whereas there are first concepts to address either issue independently, combined approaches are still scarce. We here present a hybrid mortar system, in which two different additives achieve this dual goal: a biological additive minimizes the ingress of water into mortar, thus improving the durability of the material, and a second group of additives reduces the ecological impact of the material by lowering the amount of carbon dioxide emission associated with cement production. Our results indicate how either additive affects the pore structure of the hybrid material and how this affects its mechanical competence and resistance to water ingress. If a similar concept can also be applied to other cementitious materials, it may present an urgently needed short-term solution to improve the sustainability of construction materials.

**KEYWORDS:** *hydrophobic mortar, $^{29}Si$ MAS NMR, pore structure, water uptake*
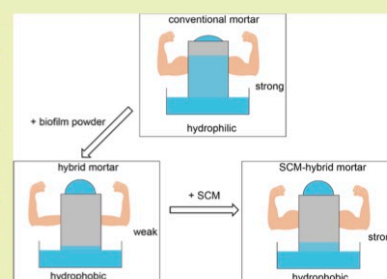
## ■ INTRODUCTION

Already more than 2000 years ago, cementitious materials were employed by the Romans;[1] today, owing to its simple mode of application and low cost, concrete is the most used building material—and the demand for cementitious materials is expected to increase even more. As of now, the annual cement production is already responsible for 8% of the anthropogenic greenhouse gas (GHG) emissions.[2] Thus, developing more sustainable building materials is a major challenge for our society.

One emerging trend in the area of material design draws inspiration from synthetic biology; here, design principles borrowed from biology are used to develop new functional materials.[3–5] Indeed, combining conventional cementitious materials with living cells or organisms has been attempted.[6,7] However, this approach is rather difficult as the conditions present in mortar or concrete, for example, the lack of nutrients and the high pH values occurring during the hydration reaction, are not well suitable for supporting life. Cement-free building materials, in contrast, do not suffer from this limitation. Accordingly, promising first results were obtained there, and living building materials could be successfully engineered.[8,9]

Still, until satisfactory solutions are identified to fully replace cementitious materials with ecofriendly alternatives, short-term solutions are needed to improve the sustainability of existing construction strategies. One way to decrease the GHG emission accompanied by cement production is to reduce the amount of cement used. In modern cementitious materials, part of the cement is substituted with so-called supplemental cementitious materials (SCMs); with this approach, the $CO_2$ emission originating from the cement production process can be reduced.[10–12] Examples of commonly used SCMs include granulated slag (a byproduct of the steel production process[13]), fly ash (FA) (a waste product generated in coal-fired power plants[14]), and silica fume (SF) (a byproduct of the silicon production process[15]). In addition, agricultural waste materials, such as rice husk, palm oil fuel, or bagasse ash, have been successfully used as cement clinker substitutes.[16]

The second main strategy to lower the environmental impact of cementitious structures aims at increasing their lifetime. However, harsh environmental conditions at the operation sites, such as humidity and temperature changes, as well as air pollution, limit the durability of cementitious structures as they give rise to various deterioration mechanisms. Several techniques and approaches have been developed to minimize the ingress of water. Examples include hydrophobic coatings,[17,18] the integration of hydrophobic substances,[19–21] or the use of additives, which alter the

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microstructure or the surface roughness of the material.[22−24] Biobased examples for the latter approach employ bacterial solutions,[25] bacterial spores,[26] or freeze-dried bacterial biofilm[22] (referred to as biofilm powder) as a hydrophobizing agent in mortar. In addition to affecting the surface chemistry (the exact mechanism is yet to be identified) similar to what the wax layer achieves on lotus leaves,[27] biofilm additives were suggested to establish water repellent properties in the resulting hybrid materials by increasing the inner and outer surface roughness—both on the micro- and nanoscale. Such bacterial additives, however, can also impact the mechanical strength of hybrid mortar (HM).[25,26]

Here, we show, how the reduced mechanical competence of biofilm-enriched mortar can be remedied while maintaining the excellent hydrophobic properties of the hybrid material. This is achieved by using SCMs in the material formulation. As we demonstrate, this strategy affects the pore structure of the mortar samples such that intermediate pore distributions are obtained. These midlevel porosities are sufficiently high to suppress capillary water uptake and, at the same time, low enough to allow for good mechanical stability of the mortar samples. Thus, our results demonstrate the dual influence the pore structure of cementitious materials has on the hydrophobic and mechanical properties of the material. Moreover, our study introduces an easy, cost-efficient, and sustainable strategy to improve cementitious materials in two ways at the same time.

## ■ MATERIALS AND METHODS

**Supplementary Cementitious Materials.** The chemical composition, as well as the specific surface area [determined according to Blaine[28] (a) or Brunauer−Emmett−Teller[29] (b)] of the SCMs used in this study, are listed in Table 1.

**Table 1. Chemical Composition and Specific Surface Area of the Used SCMs**

| | | fly ash (FA) | blast furnace slag (BFS) | lime stone (LS) | silica fume (SF) |
|---|---|---|---|---|---|
| chemical composition [% (w/w)] | $SiO_2$ | 52.04 | 36.20 | 0.51 | 95.55 |
| | $Al_2O_3$ | 23.18 | 12.20 | 0.19 | 0.27 |
| | $Fe_2O_3$ | 7.35 | 1.60 | 0.12 | 0.67 |
| | CaO | 3.40 | 39.30 | 55.43 | 0.52 |
| | MgO | 1.88 | 6.80 | 0.19 | 0.20 |
| | $SO_2$ | 0.36 | 0.09 | 0.01 | 0.13 |
| | $K_2O$ | 3.41 | 0.45 | 0.02 | 0.51 |
| | $Na_2O$ | 1.09 | 0.39 | 0.01 | 0.10 |
| loss of ignition | [%] | 3.48 | 0.10 | 43.29 | 1.85 |
| physical properties | surface area [$m^2$/g] | 2101[a] | 4800[a] | 1588[a] | 21686[b] |

**Biofilm Powder Production.** Biofilm powder was produced as described in ref 25. In brief, *Bacillus subtilis* NCIB 3610 was obtained from the lab of Roberto Kolter (Harvard Medical School, USA), and planktonic overnight cultures were cultivated in Luria Bertani (LB) medium. Then, biofilm was cultivated on LB agar, harvested, freeze-dried for 3 days, and afterward ground into a fine powder with an average particle size of ∼500 μm.

**Mortar Sample Preparation.** The mortar sample preparation (component selection and mixing procedure) was performed according to DIN EN 196-1. Using the mass loss factor published in ref 25 (this factor describes the ratio of fresh biofilm mass with

respect to the mass of lyophilized biofilm), the required amount of biofilm powder can be calculated to obtain a biofilm content (bc, which describes the ratio of fresh biofilm with respect to the mass of dry cement) of 2% in the HM samples. Cement and biofilm powder were added to water within 10 s, and the mixing process was immediately started in a bowl at a stirring speed of 140 rpm. After 30 s, sand was added within a time window of 30 s, and the sample was mixed at a stirring speed of 285 rpm for an additional 30 s. Then, the mixing process was stopped for 90 s, mortar adherent to the stirring head and/or the upper part of the bowl was transferred back to the bottom of the bowl using a rubber scraper, and the stirring process was continued for an additional 60 s at a stirring speed of 285 rpm. The prepared mortar was then poured into the desired mold while being compacted for 120 s using a vibrating table. Table S1 lists all used mortar formulations used in this study.

**NMR Spectroscopy.** The $^{29}Si$ NMR experiments were performed on a Bruker AVANCE 300 spectrometer (magnetic field strength 7.0455 T, resonance frequency for $^{29}Si$: 59.63 MHz) in the magic angle spinning (MAS) mode using the single pulse technique (90° pulse). The samples were packed in 7 mm zirconia rotors and spun with 5 kHz. About 10,000 scans were recorded for each spectrum using a repetition time of 5 s. The chemical shifts were referenced to an external sample of tetramethylsilane at 0 ppm. Then, the obtained spectra were deconvoluted with the Bruker WINNMR software and interpreted using the $Q^n$ nomenclature.[30]

**Mercury Intrusion Porosimetry.** The pore structure of the cured mortar samples was investigated using mercury intrusion porosimetry (MIP) (AutoPore IV, Micromeritics GmbH, Norcross, USA) in a pressure range of 0.01–413.7 MPa (2–60,000 psi). Before conducting the measurements, the mortar samples were cured at room temperature (RT) for 28 days and then additionally dried for 3 days at 105 °C and ambient pressure in a desiccator.

**X-ray Diffraction.** The X-ray diffraction (XRD) patterns were recorded on a D8 ADVANCE diffractometer (Bruker, Billerica, Massachusetts, USA) using Cu $K_α$ radiation and a high-resolution energy-dispersive detector (LYNXEYE-XE, Bruker, Billerica, Massachusetts, USA). The scanning angle 2θ was varied from 5 to 70° using a step size of 0.02° and a dwell time of 0.2 s. For the quantitative mineralogical analysis, the samples were ground into pieces ≤30 μm. As an internal standard, 20% (w/w) ZnO was mixed with the specimens.

**Water Uptake Experiments.** The capillary water uptake was determined in a modified way to refs 31 and 32 as described in ref 25. Cylindrical samples were prepared using commercial polyethylene tubes (diameter: 40 mm) as casting molds. After 3 days of curing, the formwork was stripped, and after 11 additional days of storage at RT, the mortar samples were coated with a resin (MC-Inject 1264 compact, MC Bauchemie, Bottrop, Germany). Cutting one end of the sealed cylinders created an open surface, which enabled water ingress. The mortar samples were then immersed into a 2 cm high water bath (with the open surface facing down), and the amount of water taken up by capillary forces was monitored by determining the mass change of the mortar samples using a microscale (TLE 303, Mettler Toledo AG, Greifensee, Switzerland). This weighing step was conducted before and after the samples were exposed to water for defined time intervals.

**Three-Point Bending and Compression Tests.** The three-point bending and compressive strength of mortar samples were tested using cuboid specimens of standardized geometry (4 × 4 × 16 cm), which were produced according to DIN EN 196-1,[33] as described in ref 25. After 24 h, the test specimens were removed from the casting framework and stored at 20 °C until they were tested. On each testing date (i.e., after 7 and 28 days of curing at 20 °C and a relative humidity > 50%), first, the three-point bending strength was determined; afterward, the compressive strength was measured using a loading frame (Series DB Super, Walter&Bai, Löhningen, Switzerland), and the six fragments obtained from the bending tests.

**Light Profilometry.** The microscopic surface profiles of the mortar samples were obtained using a laser scanning microscope (VK-X1000, Keyence, Oberhausen, Germany) equipped with a 50× lens
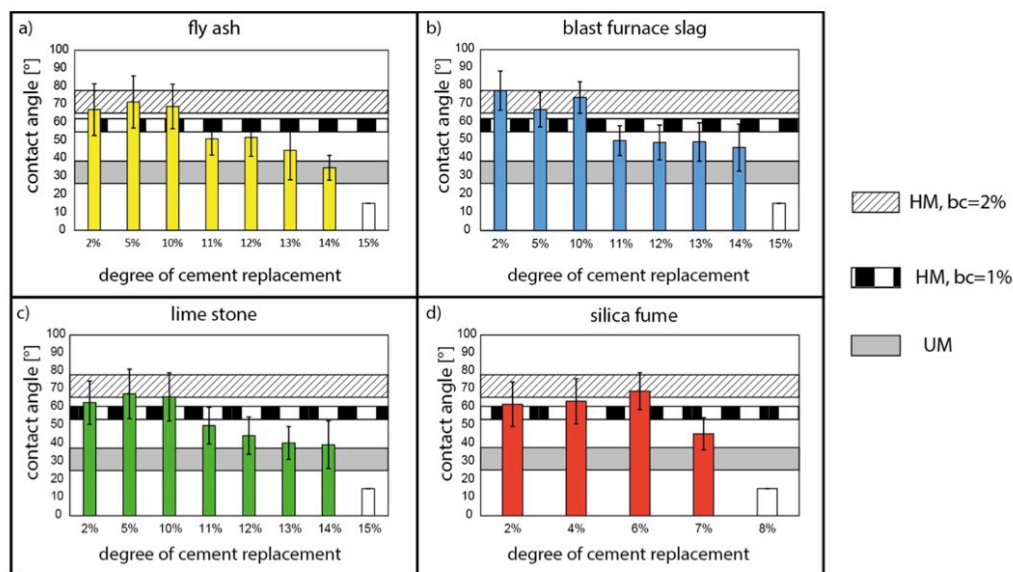
**Figure 1.** Wetting resistance of HM samples containing different SCMs. The wetting resistance of the different mortar samples is characterized by the contact angle which water droplets form on their surface. HM samples containing different concentrations of the four tested SCMs are compared to SCM-free HM samples (bc = 2%: striped horizontal bar; bc = 1%: patterned horizontal bar) and UM (gray horizontal bar). The values shown depict averages obtained from five measurements conducted on three independent samples each. Error bars denote the standard deviation calculated from these 15 data points.
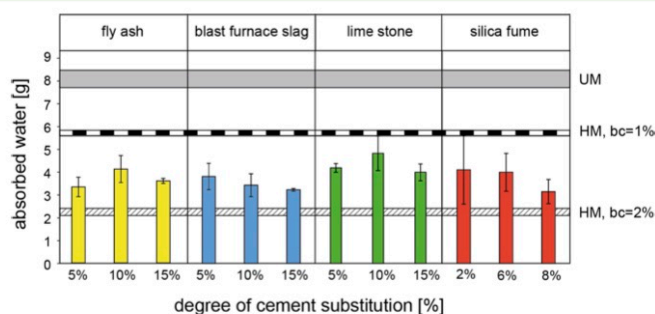


**Figure 2.** Capillary water uptake into HM samples containing different SCMs. The amount of water absorbed by different mortar samples after partial immersion into a water bath for 24 h is shown. More detailed data quantifying water uptake after 1, 2, 3, 4, and 24 h can be found in Supporting Information (Figure S1). HM samples containing different concentrations of the four tested SCMs are compared to SCM-free HM samples (bc = 2%: striped horizontal bar; bc = 1%: patterned horizontal bar) and UM (gray horizontal bar). The values shown represent averages of three measurements conducted with independent samples; the error bars denote the standard deviation.

(NA = 0.95; Nikon, Chiyoda, Tokyo, Japan). The images were acquired without any further sample treatment, that is, directly after curing of the mortar samples for at least 3 days. On each sample, five spots with an area of 213 × 284 $\mu$m were scanned. The scanned area was then evaluated with the software MultiFileAnalyzer (Version 2.1.3.89, Keyence, Oberhausen, Germany) to obtain the developed interfacial area ratio according to the following equation

$$Sdr = \frac{1}{A}\left[ \iint_A \left( \sqrt{\left[ 1 + \left( \frac{dz(x, y)}{dx} \right)^2 + \left( \frac{dz(x, y)}{dy} \right)^2 \right]} - 1 \right) dx dy \right] \tag{1}$$

here, $A$ denotes the scanned sample area, $x$ and $y$, the lateral dimensions, and $z$, the height of the surface profile. Thus, the developed interfacial area ratio $Sdr$ quantifies the additional surface area contributed by a texture compared to a fully planar surface.
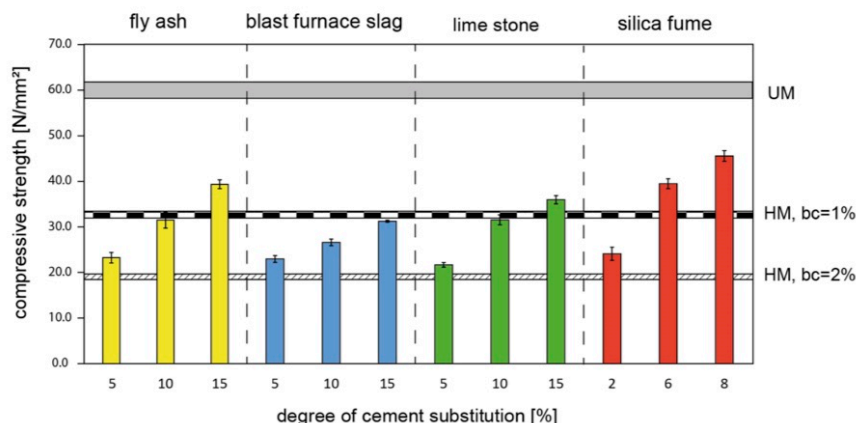
**Figure 3.** Compressive strength of HM samples containing different SCMs. Compressive strengths values were determined according to DIN EN 196-1, that is, 28 days after sample preparation. HM samples containing different concentrations of the four tested SCMs are compared to SCM-free HM samples (bc = 2%: striped horizontal bar; bc = 1%: patterned horizontal bar) and UM (gray horizontal bar). The values shown represent averages of three independent measurements; error bars denote the standard deviation.

## ■ RESULTS AND DISCUSSION

**Influence of SCM Addition on the Hydrophobic Properties of the HM.** Despite the great water repelling properties HM samples containing different bacterial additives exhibit, they all suffer from a loss in mechanical strength. In contrast, SCMs are known to enhance the strength of cementitious materials[15,34−36] but cannot provide hydrophobic properties. Thus, in a first step, we ask if a hybrid material containing both, a bacterial hydrophobizing agent and an SCM can combine both beneficial properties the additives can contribute. Of course, it is possible that, when the mechanical properties of the biofilm-enriched HM are improved by using an SCM, this may compromise the hydrophobic properties of the HM. We assess this question by determining the wetting resistance of the HM enriched with different concentrations of four classical SCM variants: FA, blast furnace slag (BSF), SF, and limestone (LS) (Figure 1). For all wetting tests, mortar samples with a fixed biofilm content of bc = 2% (bc is defined as in ref 22) are prepared. The content of the respective SCM is then varied from 2% (lowest dosage) up to a maximum dosage, which is selected to match the composition of different CEM II cements according to DIN EN 197-1.[37] The wetting resistance of each SCM-enhanced HM sample is then evaluated by measuring contact angles of water droplets on the HM surface and comparing these values to those measured on the unmodified mortar (UM, gray vertical bars in Figure 1), as well as biofilm-enriched HM, where no SCM was used (patterned vertical bars in Figure 1). These measurements show that the SCM-containing samples exhibit a similarly good (or even slightly better) wetting resistance than the standard HM—at least for small and moderate SCM contents. At higher SCM contents, however, the wetting resistance of the HM samples is reduced; the particular dosage, at which this undesired effect sets in, depends on the respective SCM (7% for LS and 11% for the three other SCMs).

As shown previously, bacterial additives can not only increase the external wetting resistance of mortar but may also reduce the capillary water uptake of the material. Thus, in

the next step, we test if the SCMs affect this second important property brought about by the bacterial additive (Figure 2). As SCMs are known to reduce both the overall porosity and the average pore size of cementitious materials, we actually expect that the SCMs negatively impact this material property of the HM. To test the influence of the four different SCMs on capillary water uptake, we use the same experimental procedure as described in ref 25 (see Materials and Methods for details). For this set of experiments, we select three different concentrations of each tested SCM: the first tested dosage represents the highest SCM concentration for which we still obtained a similarly high wetting resistance as for the HM devoid of SCM (i.e., 10% for FA, BSF, and LS; 6% for SF). The second SCM concentration is chosen to be smaller than this first selected value, and the third concentration is chosen to be high enough that the wetting resistance of the HM is completely lost (i.e., 15% for LS, FA, and BSF and 8% for SF). Interestingly, different from the results of the wetting tests discussed above, we do not observe a clear dependence of capillary water uptake on the SCM concentration. Even in formulations containing the highest SCM content tested here, the capillary water uptake is reduced by ∼35 to 50% compared to the UM. However, none of the SCM-containing samples performs as well as the pure HM, which is in full agreement with our expectations.

Overall, these results suggest that mortar samples containing both biofilm and SCM differ in terms of their pore structure from both UM and HM devoid of SCM. To confirm this notion, we next investigate the mechanical strength of the different mortar formulations. This is motivated by the well-established realization that the porosity of a cementitious material also strongly affects its strength.[38] In detail, we determine the three-point-tensile and compressive strength of the samples after 28 days of storage. Figure 3 shows the compressive strength values obtained for different formulations (tensile strength values can be found in Supporting Information, Figure S2); here, the same mixtures were tested as discussed above with regard to capillary water uptake, and indeed, for all mortar variants containing both biofilm and
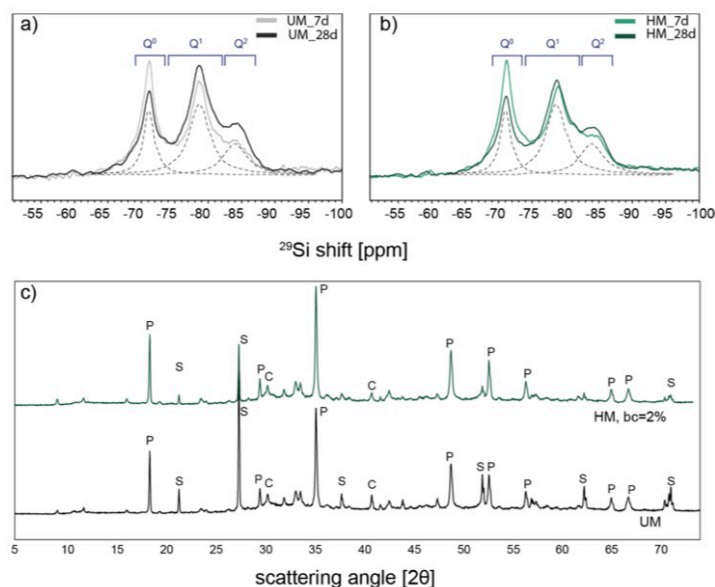
**Figure 4.** $^{29}$Si-NMR spectra and XRD pattern of UM and HM samples. The stack plot of the $^{29}$Si NMR signals, recorded after 7 and 28 days, respectively, are shown for (a) UM and (b) HM. A decrease of the NMR signal for isolated $[SiO_4]$ tetrahedrons (Qo, −71 ppm) with a simultaneous increase of the NMR signal for linked $[SiO_4]$ units (Q1, −79 ppm; Q2, −85 ppm) can be observed with the ongoing process of the hydration reaction. (C) XRD patterns of the UM and HM (bc = 2%) after 28 days of sample hydration (P = portlandite, C = calcite, S = silicon dioxide). With the recorded spectra, the same mineralogical composition for the UM and HM could be verified.

SCM, we obtain intermediate results compared to UM and HM samples (Figure 3).

Overall, two different trends can be observed: first, for samples devoid of the SCM, the compressive strength of the material decreases with increasing bc. Second, for samples containing a fixed amount of biofilm (*i.e.*, bc = 2%), the compressive strength increases with increasing SCM concentration. The former trend coincides with the observation of air bubbles during the mixing process: this effect is more strongly pronounced when a higher biofilm content is used in the HM mixture. Conceptually, such an air entraining effect brought about by the biofilm powder additive can explain the decreased strength of the HM material: it has been established before that the density of a cementitious material crucially impacts its strength.[39−43] The second, SCM-induced change in the material properties, was expected as the SCMs studied here have all been selected due to their well-documented ability to increase the strength of cementitious materials.[35,44,45] Overall, the best results are obtained with SF: here, using 8% SF largely compensates for the loss of compressive strength brought about by the biofilm addition (Figure 3): now, the material reaches ~77% of the compressive strength (and 76% of the tensile strength; see Figure S2) of the standard mortar. Also, a somewhat lower concentration of this particular SCM (*i.e.*, 6% SF; this dosage fully maintains the hydrophobic properties of the HM; see Figure 1d) boosts the mechanical competence of the HM quite a bit: here, the compressive strength of the material is doubled compared to the standard HM, and the tensile strength of the material is improved by 73%.

The results we discussed so far demonstrate that by modifying the composition of the standard mortar in two ways—*via* biofilm addition and replacement of cement with SCM—a HM material with hydrophobic properties can be obtained that has better mechanical strength than biofilm-enriched mortar alone. Yet, the microscopic reason for the loss in strength resulting from biofilm addition and how SCMs compensate for this effect is not fully clear yet. As mentioned above, alterations in the porosity of mortar can strongly influence both the resistance of a cementitious material toward capillary water uptake and its mechanical strength. However, different hydration products created during the hydration reaction of the material or different levels of cement hydration could cause similar effects as well.[46,47] To examine the latter, we conduct Si$^{29}$-NMR measurements with UM and HM (bc = 2%) samples (Figure 4a,b). Such Si$^{29}$-NMR measurements are not only a useful tool for monitoring the development of the different hydrate phases but they can also indicate the degree of mortar hydration and the chain length of the C−S−H phase (Formulas 2 and 3, see Supporting Information).[48−50] When we compare UM samples at ages of 7 and 28 days, respectively, the proportion of hydrated silica phases in the cement slightly increases from 61 to 72%. This result was expected as it reflects the ongoing hydration reaction of the material. For the HM (bc = 2%), this increase is very similar as we measure 60% hydrated silica phases at day 7 and 68% at day 28. Moreover, differences in the average chain length calculated from the NMR spectra were similar for both UM and HM samples (see Supporting Information, Table S2). In addition, XRD measurements (Figure 4c) demonstrate that the mineralogical
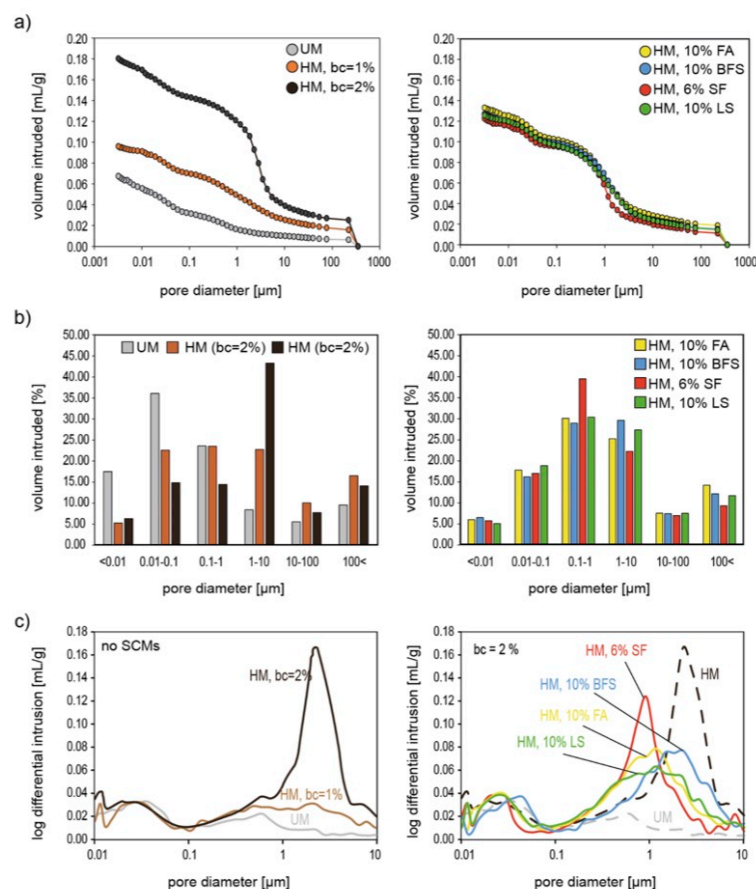
**Figure 5.** Pore size distributions in the UM, HM, and SCM-enriched HM. Cumulative intrusion volume (a), cumulative intrusion volume percentage (b), and the pore size distribution of UM as well as (SCM-enriched) HM.

composition of UM and HM samples is virtually identical. Thus, we conclude that the addition of bacterial biofilm powder does not interfere with the hydration reaction of the mortar, and it does not create new hydration products either.

As mentioned above, we observed an air-entraining effect for HM samples containing biofilm powder. Moreover, capillary uprise experiments and mechanical stability tests returned results consistent with the idea that the different samples we study here might differ in terms of porosity. Thus, in the last step of this study, we apply MIP to determine the porosity and the pore size distribution of UM, HM, and SCM-enriched HM samples (Figure 5). When comparing SCM-free samples, we find a strong increase in porosity upon addition of biofilm powder (Figure 5a): with 0.18 mL/g, the total pore volume in the HM (bc = 2%) is 3 times higher than in UM, where we calculate 0.06 mL/g. For the HM containing 1% biofilm only, we find an intermediate level of porosity, that is, 0.09 mL/g. This result agrees very well with the dose-dependent

alterations of HM properties—both with regard to capillary water uptake and mechanical competence.

Importantly, the measured pore size distributions show that this increase in overall porosity is due to the formation of larger cavities with diameters in the range of 1 $\mu$m and above. Such large pores are not present in the UM samples. When we analyze the SCM-containing HM formulations, we find overall porosity levels that are larger than in UM but lower than in the HM (bc = 2%; see Supporting Information, Table S3). In addition, these SCM-containing samples show pore size distributions with the main peaks shifted toward smaller pore diameters (compared to the HM, bc = 2%, see Figure 5b). The latter effect is strongest for the sample containing SF—and it was this particular SCM that rendered the biofilm-enriched HM with the best mechanical stability (see above).

The results we obtained for the capillary water uptake tests showed that UM samples, where large pores (>1 $\mu$m) are not present, absorb the largest amount of water. In contrast, HM samples (no SCM, bc = 2%) showed the lowest water uptake,

and for this mortar variant, we detected the largest proportion of such big pores. Interestingly, a very similar pore structure as obtained for those HM samples was described in a recent study,[19] where hydrophobic concrete was obtained by adding a stearic acid emulsion. Compellingly, SCM-enriched HM samples showed a slightly higher water uptake than the SCM-free HM—yet, this agrees with the observation that their pore size distribution is in between those of the UM and SCM-free HM, respectively. With this realization in mind, we conclude that a certain pore structure is required to reduce water uptake by capillary forces (and this effect strongly contributes to the overall hydrophobic properties of the material).

In other words, the addition of bacterial biofilm powder results in a HM material with greatly improved hydrophobic properties. At the same time, additional pores are introduced, which affect the density and thus the mechanical strength of the resulting hybrid material. As we demonstrate here, SCMs keep the porosity of the HM at bay, and this improves the stability of the hybrid material. At too high SCM contents, the increase in surface roughness brought about by the biofilm additive is eliminated, which explains the loss of the wetting resistance we observe once a certain SCM threshold is passed (see Supporting Information, Figure S3). When comparing the dose-dependent effects brought about by the different SCMs, it is interesting to note that we obtained similarly strong effects for SF as for the other SCMs even though this additive was used at much lower concentrations. We speculate that differences in the specific surface area of the SCMs might play an important role here: this parameter is $\sim 5-10$ times higher for SF than for the other SCMs tested here (Table 1). Together, our results indicate that the SCM-enriched HM constitutes a highly promising hybrid material, where a broad range of important material properties (such as the wetting resistance, suppression of capillary water uptake, and mechanical strength) can be controlled by the amount of SCM used—offering the possibility to tailor the material according to the desired application.

## CONCLUSION

Our results indicate that the loss of strength observed for the biofilm-enriched HM does not result from the formation of unwanted hydrate phases as it can, for example, occur for calcium aluminate cement.[51,52] Instead, it seems that hydrophobizing biofilm additives act (in part) as air-entraining agents that increase the porosity of the mortar material—and this compromises the mechanical stability of the hybrid material. By combining bacterial biofilm powder and SCMs, this issue is remedied as we obtain a material that exhibits both good water repellent properties and better mechanical behavior than the biofilm-enhanced mortar alone. With this improvement, the biological hybrid material is brought one step closer to become an interesting candidate for several areas of construction engineering—either as mortar or, potentially, also as concrete or as render. At the same time, our results also indicate that the hydrophobic properties of a cementitious material, especially the capillary water uptake, can be strongly affected by the overall porosity, as well as the average pore size and pore size distribution. Moreover, the concept of controlling the porosity of a cementitious material to regulate its hydrophobic properties can open new doors in the search for more sustainable building materials with increased service life.

## ASSOCIATED CONTENT

**Ⓢ Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c03625.

HM formulations, detailed capillary water uptake curves, calculation of the average chain length of the C—S—H gel, tensile strength measurements, pore structure analysis, and surface roughness analysis of different mortar formulations (PDF)

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**Author Contributions**

M.J.E., H.H., and O.L. designed the experiments; M.J.E. and H.H. performed the experiments and analyzed data. The manuscript was written by M.J.E. and O.L. and was critically revised by all authors.

**Notes**

The authors declare no competing financial interest.
Approval of ethics is not required for the experiments conducted in this manuscript.

## ACKNOWLEDGMENTS

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**Supplemental Information for**

# Small pores, big impact – controlling the porosity allows for developing more sustainable construction materials

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Total number of tables: 3

1. **(Hybrid) mortar formulations**

**Table S1: Detailed composition of the different hybrid mortar formulations investigated in this study.**

| Sample | Biofilm content [%]/ biofilm powder [g] | Degree of cement substitution [%] | Cement [g] | SCM [g] | Sand [g] | Water [g] |
|--------|------|------|--------|-------|---------|--------|
| UM | - | - | 450.00 | - | 1350.00 | 225.00 |
| HM | 1 / 0.9 | - | 450.00 | - | 1350.00 | 225.00 |
| HM | 2 / 1.8 | - | 450.00 | - | 1350.00 | 225.00 |
| HM | 2 / 1.8 | 2 | 441.00 | 9.00 | 1323.00 | 220.50 |
| HM | 2 / 1.8 | 4 | 432.00 | 18.00 | 1396.00 | 211.50 |
| HM | 2 / 1.8 | 5 | 427.50 | 22.50 | 1282.50 | 213.75 |
| HM | 2 / 1.8 | 6 | 423.00 | 27.00 | 1269.00 | 211.50 |
| HM | 2 / 1.8 | 7 | 418.50 | 31.50 | 1255.50 | 209.25 |
| HM | 2 / 1.8 | 8 | 414.00 | 36.00 | 1242.00 | 207.00 |
| HM | 2 / 1.8 | 10 | 409.50 | 40.50 | 1228.50 | 204.75 |
| HM | 2 / 1.8 | 11 | 405.00 | 45.00 | 1215.00 | 202.50 |
| HM | 2 / 1.8 | 12 | 400.50 | 49.50 | 1201.50 | 200.25 |
| HM | 2 / 1.8 | 13 | 396.00 | 54.00 | 1188.00 | 198.00 |
| HM | 2 / 1.8 | 14 | 391.50 | 58.50 | 1174.50 | 195.75 |
| HM | 2 / 1.8 | 15 | 387.00 | 63.00 | 1161.00 | 193.50 |

S2

2. **Capillary water uptake into hybrid mortar samples**
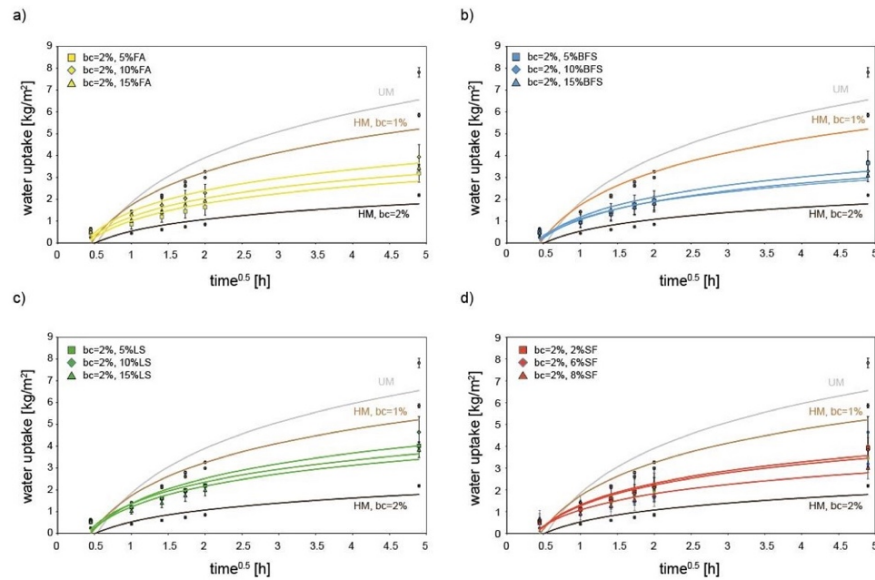


**Figure S1: Capillary water uptake into SCM-enriched HM samples**. The amount of water absorbed by hybrid mortar samples after partial immersion into a water bath for 1, 2, 3, 4, and 24 h, respectively, is shown for samples containing different concentrations of SCMS. As references, data obtained for unmodified mortar (UM) and SCM-free hybrid mortar (HM) with a biofilm content of 1% and 2%, respectively, is depicted as well. The values shown represent averages of three measurements conducted on independent samples, and the error bars denote the standard deviation.

S3

### 3. Tensile strength values of different HM formulations as determined by 3-point bending
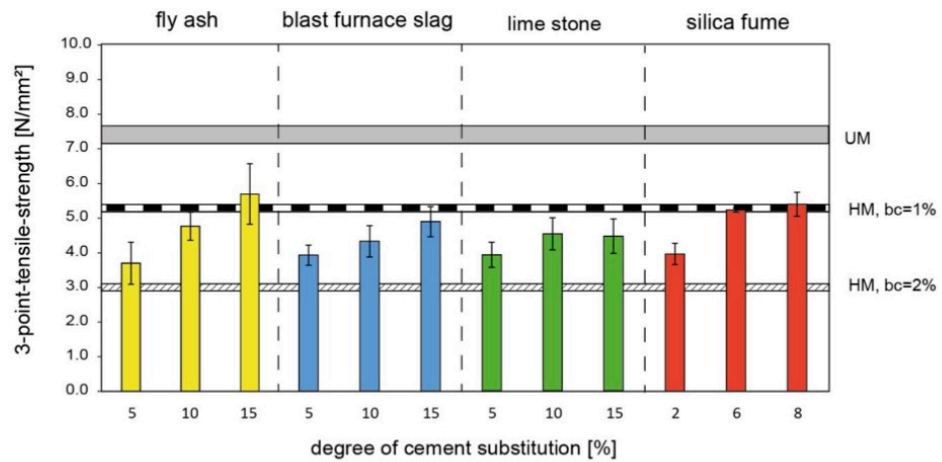


**Figure S2: Tensile strength of hybrid mortar samples containing different SCMs.** Tensile strengths values were determined according to DIN EN 196-1, *i.e.*, 28 days after sample preparation. HM samples containing different concentrations of the four tested SCMs are compared to SCM-free HM samples (bc = 2%: striped horizontal bar; bc = 1%: patterned horizontal bar) and UM (grey horizontal bar). The values shown represent averages of three measurements conducted with independent samples; error bars denote the standard deviation.

S4

**4. Calculation of the average chain length of the C-S-H gel**

By combining the integrated intensity of the different NMR signals of each, it is possible to calculate the degree of hydration of cement $H_{Si,z}$ (equation 2) as well as the average chain length $\bar{C}$ of the silica phases (equation 3):

$$H_{Si,z} = 1 - Q^0 \qquad (2)$$

$$\bar{C} = \frac{2(I(Q^1) + I(Q^2_p) + I(Q^2))}{I(Q^1)} \qquad (3)$$

Here, $Q^i$ indicate the number of bonds generated by the silicate tetrahedron. Each silicate tetrahedron can form a bond over each of its 4 oxygens. The $Q^0$ phase is observed for unreacted tricalcium silicate (C3S) and dicalcium silicate (C2S), $Q^1$ represents end-chain groups and $Q^2$ and $Q^3$ middle-chain groups in the C-S-H gel.[1]

**Table S2: Average chain length in HM and UM samples.** Average chain length of the silica phases as calculated using formula (2).

| Sample | Sample age [d] | Average chain length |
|---|---|---|
| UM | 7 | 2.85 |
| UM | 28 | 3.12 |
| HM, bc = 2% | 7 | 3.27 |
| HM, bc = 2% | 28 | 3.25 |

### 5. Pore structure analysis of UM, HM and SCM-enriched HM samples

**Table S3: Pore structure analysis of UM, HM and SCM-enriched HM.** Values for porosity, median pore diameter and total intrusion volume are depicted for UM, HM and SCM-enriched HM. Measurements were conducted using mercury intrusion porosimetry (see Methods section of the main paper).

| Sample | BC | SCM | Porosity [%] | Median pore diameter [μm] | Total intrusion volume [mL/g] |
|--------|-----|---------|--------------|---------------------------|-------------------------------|
| UM | - | - | 14.5 | 0.06 | 0.06 |
| HM | 1% | - | 19.4 | 0.96 | 0.09 |
| HM | 2% | - | 31.2 | 2.13 | 0.18 |
| HM | 2% | 10% FA | 24.7 | 0.92 | 0.13 |
| HM | 2% | 10% BFS | 24.0 | 1.00 | 0.12 |
| HM | 2% | 10% LS | 23.8 | 0.87 | 0.12 |
| HM | 2% | 6% SF | 23.3 | 0.78 | 0.12 |

### 6. Surface roughness analysis of UM, HM and SCM-enriched HM samples
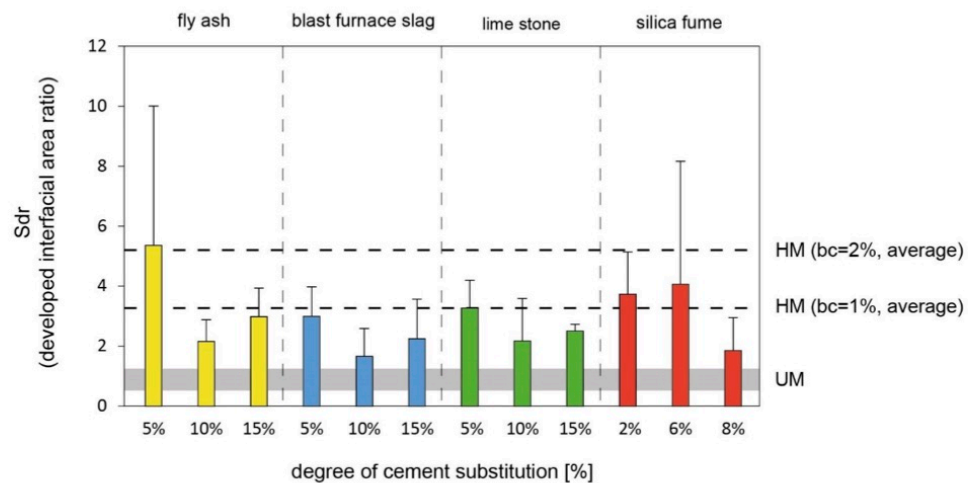


**Figure S3: Surface roughness of hybrid mortar samples containing different SCMs.** The surface roughness of each mortar sample is characterized by the *Sdr* value calculated from topographical images (see Methods). HM samples containing different concentrations of the four tested SCMs are compared to SCM-free HM samples (bc = 1% and 2%; for the sake of clarity, only the mean values of the corresponding data sets are shown as dashed lines) and UM (grey horizontal bar). Each value shown represents the average of five measurements obtained on different spots on the surface of one sample.

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## B.2   Bacterial Additives Improve the Water Resistance of Mortar

## B.3    Bacterial spores as hydrophobizing agents in mortar

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## B.4 Small Pores, Big Impact - Controlling the Porosity Allows for Developing More Sustainable Construction Materials

## C. Full list of publications

Journal articles (peer-reviewed)

1. **Ertelt, M. J**.; Raith, M.; Eisinger, J.; Grosse, U; Lieleg, O., Bacterial Additives Improve the Water Resistance of Mortar. *ACS Sustainable Chemistry & Engineering*, **2020**, *8(14)*, p. 5704–5715.

2. **Ertelt, M**; L. Bubendorfer; Grosse, C. U.; Lieleg, O., Bacterial spores as hydrophobizing agents in mortar. *Cement and Concrete Composites*, **2021**, 120, 104002.

3. **Ertelt, M**; Hilbig, H; Grosse, C. U.; Lieleg, O., Small Pores, Big Impact – Controlling the Porosity Allows for Developing More Sustainable Construction Materials. *ACS Sustainable Chemistry & Engineering*, **2021**, *9(39)*, p. 13188–13195.

4. Hayta, E. N.; **Ertelt, M. J.**; Kretschmer, M.; Lieleg, O., Bacterial Materials: Applications of Natural and Modified Biofilms. *Advanced Materials Interfaces*, **2021**, 2101024.

5. M. Kretschmer, E. N. Hayta, **M. J. Ertelt**, M. A. Würbser, J. Boekhoven, O. Lieleg, A rotating bioreactor for the production of biofilms at the solid-air interface. *Biotechnol. Bioeng*, **2022**, 119, 895–906.

Journal articles (not peer-reviewed yet)

1. Ertelt, M.J.; Eicheler, C.; Machner, A.; Lieleg, O.; Durability of biofilm-enriched hybrid mortar towards chemical and physical challenges. *Material Letters* (In review process).

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