sustainability

MDPI

Article

Bacterial Spore-Based Hygromorphs: A Novel Active Material with Potential for Architectural Applications

Emily Birch [1], Ben Bridgens [1], Meng Zhang [2] and Martyn Dade-Robertson [1,*]

[1] Hub for Biotechnology in the Built Environment, School of Architecture, Planning and Landscape, Newcastle University, Newcastle NE1 7RU, UK; e.birch@newcastle.ac.uk (E.B.); ben.bridgens@ncl.ac.uk (B.B.)
[2] Hub for Biotechnology in the Built Environment, School of Applied Sciences, Faculty of Health and Life Sciences, Northumbria University, Newcastle NE1 8ST, UK; meng.zhang@northumbria.ac.uk
* Correspondence: martyn.dade-robertson@ncl.ac.uk

Abstract: This paper introduces a new active material which responds to changes in environmental humidity. There has been growing interest in active materials which are able to respond to their environment, creating dynamic architectural systems without the need for energy input or complex systems of sensors and actuators. A subset of these materials are hygromorphs, which respond to changes in relative humidity (RH) and wetting through shape change. Here, we introduce a novel hygromorphic material in the context of architectural design, composed of multiple monolayers of microbial spores of *Bacillus subtilis* and latex sheets. Methods of fabrication and testing for this new material are described, showing that small actuators made from this material demonstrate rapid, reversible and repeatable deflection in response to changes in RH. It is demonstrated that the hygromorphic actuators are able to lift at least 150% of their own mass. Investigations are also extended to understanding this new biomaterial in terms of meaningful work.

Keywords: active material; bacterial spore; hygromorph; responsive material

Citation: Birch, E.; Bridgens, B.; Zhang, M.; Dade-Robertson, M. Bacterial Spore-Based Hygromorphs: A Novel Active Material with Potential for Architectural Applications. *Sustainability* **2021**, *13*, 4030. https://doi.org/10.3390/su13074030

Academic Editor: Antonio Caggiano

Received: 15 January 2021
Accepted: 17 March 2021
Published: 5 April 2021

1. Introduction

In this paper, we present a novel, responsive hygromorphic material for potential use in architectural applications. There is growing interest in active materials which exhibit reversable changes in response to dynamic environmental conditions. Active materials can be used in a range of different applications, including, for example, replacing complex and energy-consuming mechanical actuators in responsive building skins [1–3].

Hygromorphic materials are a subset of active materials which respond to moisture through shape change [4]. Architectural applications of wood-based hygromorphs have already been studied [2,5,6]. In this paper, we present a new class of hygromorphic materials for architecture based on bacterial spores. This new type of hygromorphic material offers a number of interesting functional characteristics, including rapid response and sensitivity to even small changes in ambient humidity. The spores themselves are also robust, and monolayer systems using this material have shown repeated and predictable deflections over thousands of cycles [6], and spores themselves are resilient to being degraded over many hundreds or even thousands of years [4]. Bacterial spore-based materials also represent a new field of research for architectural materials, combining a deep understanding of microbiology with material development at scales relevant for built environment applications. *B. subtilis* spores are dormant and so require no sustenance and are found commonly in the environment and are safe for humans. They have been studied for a range of applications, including as food probiotics [7]. While the fundamental principles for the hygromorphic material have been established [6] and a number of design experiments in other fields have been developed [8,9], there has been very little research on this material for architectural applications. Wang, for example, studied their application as biohybrid wearables [9]. There is additionally one published pilot study [10] which has

proposed their potential role in architecture, with limited scope, with student-based design proposals which are largely speculative. In this paper, we will develop this foundational work on bacterial hygromorphs by developing a number of novel fabrication and testing methods, establishing some baseline data for the use of spore-based hygromorphs.

In this paper, we establish some of the key principles for the development of bacterial spore-based hygromorphs. We explain some of the biological principles and protocols for developing and programming this new material. These experiments demonstrate material performance in terms of the actuation force, predictable deformation response and programmability. While this is a preliminary study in a new material system, these experiments add to our understanding of the effect of spore density on the deflection and force output of the material, and how this could contribute to future programmability. This research should provide a baseline for further experiments in the development of moisture responsive actuators, with the potential to scale up for architectural actuations.

2. Background

2.1. Hygromorphic Materials

Many materials, both natural and synthetic, shrink or swell by absorbing and desorbing water. This property of certain materials has been harnessed as a technology for centuries. For example, swelling wooden wedges were used to split stone for construction in pre-industrial quarries [11], and wooden barrels were self-sealed by adding wine to swell the curved planks [12]. Materials which change shape in response to environmental relative humidity (RH) or wetting are termed "hygromorphic" [4]. These materials will swell in response to water, when, for example, RH is high, and shrink when RH is low as water evaporates. Harnessing this shape change property offers the opportunity for passive actuators, where a mechanical system changes dynamically in response to changes in humidity, without the need for external power. Using the power of evaporation in this way is a potentially neglected opportunity in human-engineered systems [7,9] but is used widely in plants, where hygroscopic actuation creates movement without muscles. The tissue architecture of many plants is based on a cellular structure with resistive cellulose fibrils embedded in a swellable matrix vacuole. The orientation and density of these cellulose fibrils causes differences in the magnitude of expansion and contraction which, in turn, leads to the bending and twisting of the plant tissue [11,13]. Seed-producing (female) conifer cones use this principle of differential hygromorphic expansion in structured zonal arrangements to engineer precise bending in response to relative humidity [14]. In damp environments, the scales lie flat, trapping the seeds within the cone (Figure 1). In dry environments, the moisture evaporates from the spines and the outermost layers of the bilayered structure (Figure 2) shrink, causing them to bend away from the central cone, causing the seeds to be released [14]. This hygromorphic response occurs even if the tissues are dead and the cone has fallen from the tree, so it requires no cellular energy (ATP) input [4] but instead relies on natural evaporation as a reliable renewable energy resource [15]. Scott's "ResponsiveKnit" harnesses hygromorphic responses in textiles. Scott's system is programmed knitting with different yarn types and expansion and contraction inputs leading to shape change [16].

2.2. Application of Hygromorphic Mechanisms in Architecture

In architecture, there have been some attempts to harness the benefits of hygromorphic materials in building design [17] by utilising hygro-bending [12] and wood-based systems (Figure 3). Examples include wooden cladding panels which are secured at the upper edge. The outer, exposed surface of the cladding panels allows moisture to evaporate out of the wood, causing the panel to contract and open, much like pine-cone scales, allowing ventilation in low RH. In high RH, however, the expansion of the outer layers of the panels causes the panels to close, forming a weatherproofing layer [4]. By using different types and thicknesses of wood, and through the lamination of layers which expand at different rates, the response of these hygromorphic materials can be programmed in terms of both

the angle of deflection and the speed of response [5]. Wood-based hygromorphs have been shown to be promising but also present some limitations, which may be overcome with spore-based hygromorphs. For example, wood is not a homogenous material as growth patterns influence the mechanical and absorption properties of the wood [4]; by contrast, spore monolayers are homogenous, being derived from a monoculture [18]. The speed of deflection is governed by the rate of diffusion through the hygromorphic material [12]. When the diffusion distance is small, the response is relatively rapid. For wood-based bilayer materials, the thickness of the active layer is typically between 0.6 mm and 5 mm, with response times from around 15 min for the thinnest veneers to many hours or days for the thickest materials. These have potential for certain architectural applications where daily or even seasonal applications may be desirable, but they are unable to provide materials which rapidly respond to changing weather or temperature conditions [5]. The microscopic size of the spores allows the creation of hygromorphic layers which offer very small diffusion distances with resultantly rapid response times (3 s) [8]. Wood- or plant-based hygromorphs are prone to environmental degradation and may not be sufficiently resilient for architectural application, whereas spores have evolved for the purpose of environmental resilience. Composite wood-based laminate materials are also prone to signs of degradation at the interface after repeated deflections [17], and whilst this has not been studied explicitly, degradation has not been noted in any studies of spore:latex composite hygromorphic actuators [7,19].

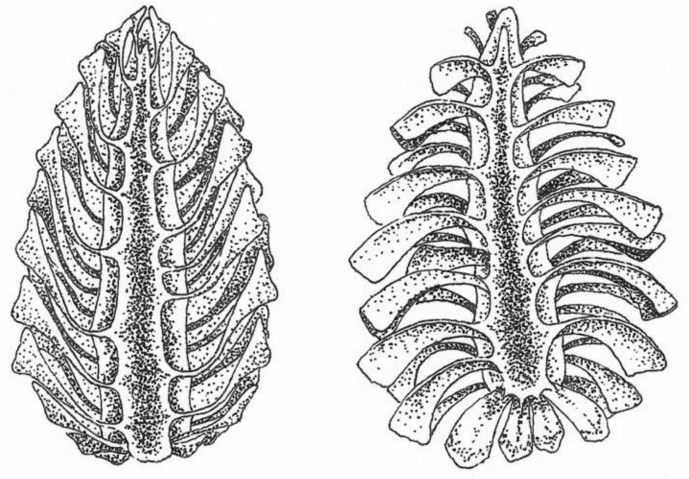

Figure 1. Pine cone in humid (left) and dry (right) environments.

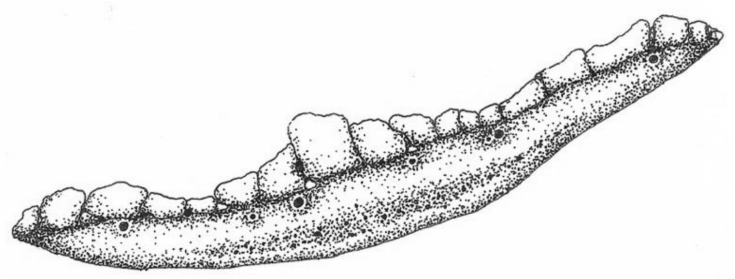

Figure 2. Trans-lateral cross-section of a pine cone scale highlighting bilayer structure of sclerenchyma fibre and sclereids.

2.3. Hygromorphic Nature of Bacillus subtilis Spores

Active *B. subtilis* cells will transform into dormant spores as a survival strategy when their environment is not conducive to their survival and reproduction—for example, when the cell is exposed to extreme temperature, pH, toxin build-up or, most importantly,

inadequate nutrient supply [20,21]. Spores are very resilient and can survive hostile environments for thousands of years [20]. *B. subtilis* spore germination is normally triggered by chemical and physical conditions through activation of nutrient receptors, which have been intensively studied for various applications (Vliet et al., 2015). Although scientists observed very low frequencies for spontaneous germination in *B. subtilis*, in which spores "wake up" at random, without a growth-permissive environment, i.e., rich nutrients, the cells will eventually die (Sturm & Dworkin 2015). A spore consists of tightly bound layers of proteins protecting the bacterial DNA (Figure 4) This unique structure, consisting of at least 70 proteins, is designed to control osmotic movements and provide a barrier to harmful substances entering the spore [22,23]. The spores are approximately one micrometre across, which allows water to diffuse through the protein layers quickly, and the cortex layer of the spores, which contains peptidoglycan, is highly hygromorphic, allowing the spores to swell and shrink by as much as 12% of their overall diameter [6].

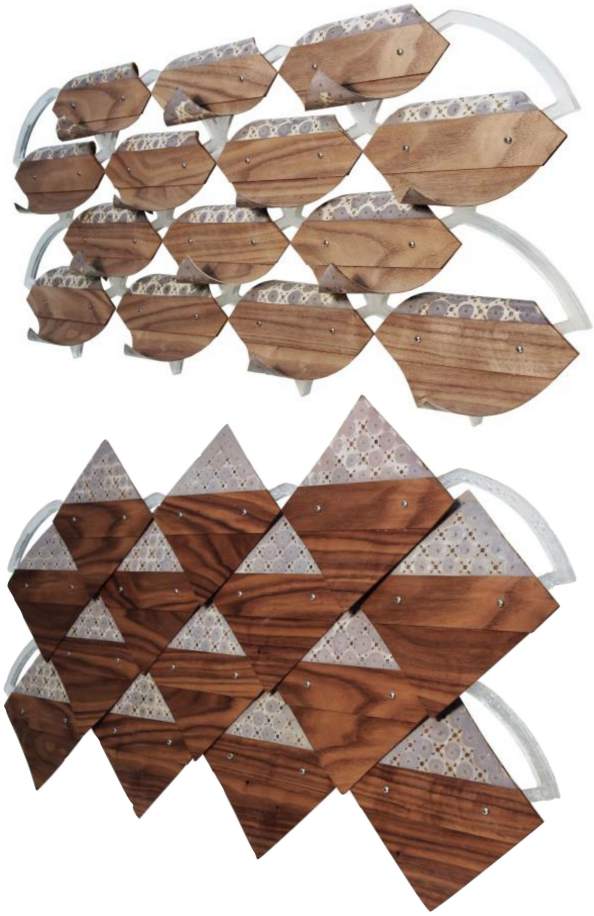

Figure 3. Prototype hygromorphic cladding panel comprising walnut veneer bonded to laser perforated fibre glass, with apertures which are open when dry (top) and closed when wet (bottom). Image by Artem Holstov.

The hygromorphic response of *B. subtilis* spores is augmented by the structural relationship between the cortex and spore coat, where a specialised structure facilitates the outer spore coat to fold into distinct and organised ridges over the cortex as it contracts during dehydration. This means that changes in diameter are highly conserved and therefore reproducible with characteristic spore surface morphology [22,24,25] (Figure 5). This mechanism also facilitates a rapid diffusion pathway, such that the spores begin to swell almost immediately (0.4 s, [6]) in response to elevated RH [26,27]. This hygromorphic expansion generates a force which, if kinetically harnessed, can be applied to do mechanical work [9,20,28]. It has been shown that the energy density produced by this expansion is

more than 10 MJ/m^3 [6], which, if a mechanism could be designed to harness it on a large scale, would be sufficient to lift a car [9,20].

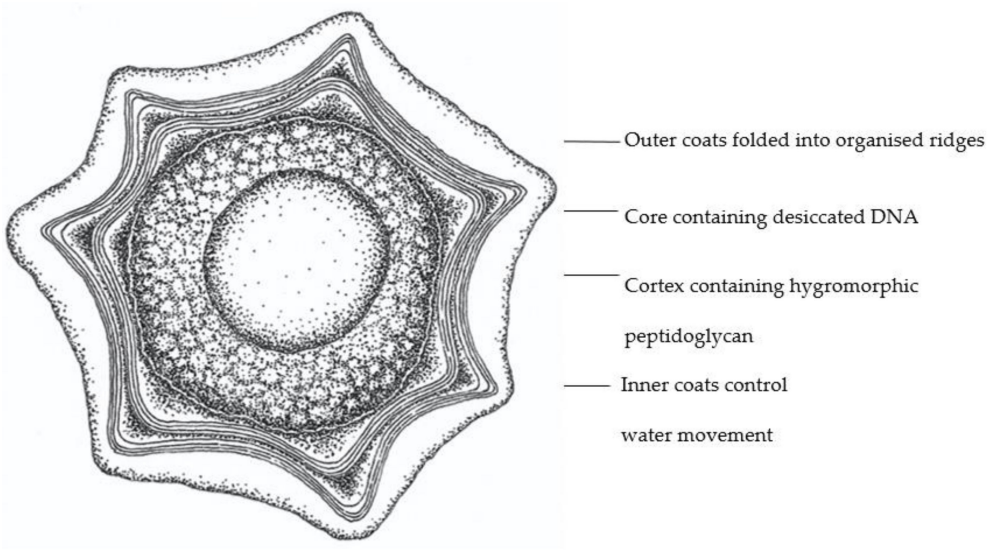

Figure 4. Artist's impression of cross-section structure of *B. subtilis* spore.

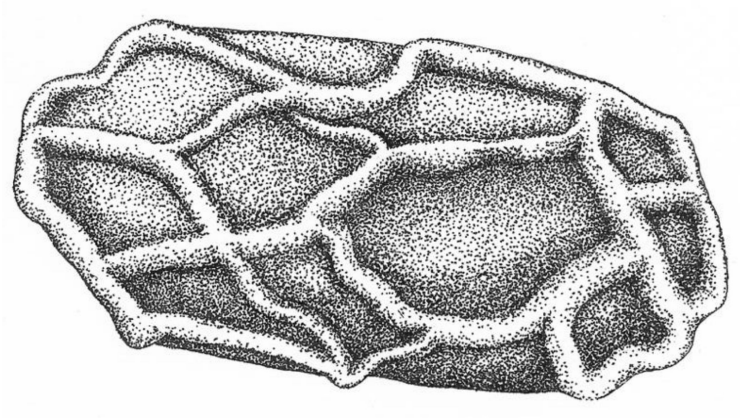

Figure 5. Three-dimensional morphology of a dehydrated *B. subtilis* spore, highlighting distinctive folding pattern.

2.4. Spores Implemented as Part of a Bio-Hybrid Material

Using the principle developed for bimetallic strips and implemented in wood-based hygromorphs, the small hygromorphic effects of *B. subtilis* spores can be amplified by applying them as a thin "active" layer on an inert (non-hygromorphic) material, where the resultant differential expansion will create a deflection in the composite bilayer material (Figure 6). Furthermore, it is predicted that the spores can be layered to increase the magnitude of the response. To this end, we have developed a series of experiments to optimise the production and fabrication of a spore-based hybrid material on an inert substrate layer of latex elastomer sheeting and to test the magnitude of the deflection response and the potential programmability of the material. This work was extended to investigate the force generated by these *B. subtilis* spore monolayers assembled as bio-hybrid hygromorphs.

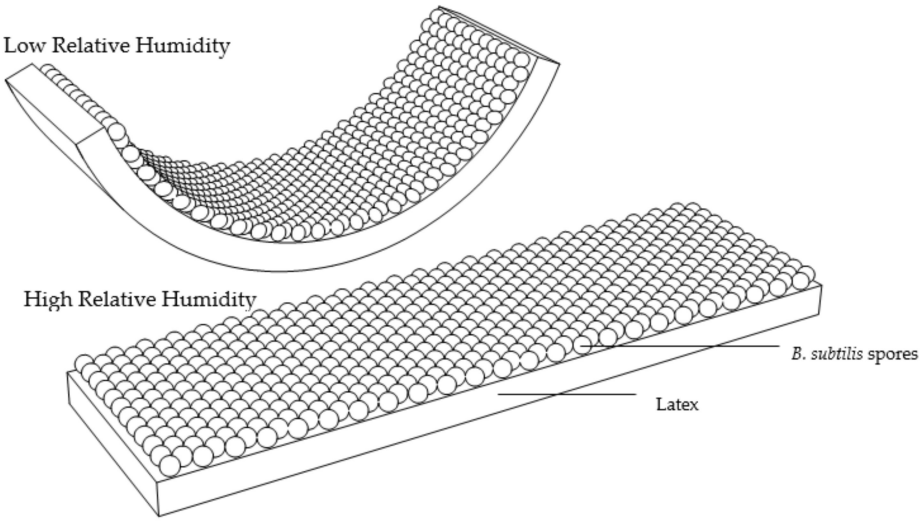

Figure 6. Diagram of bilayer at high and low RH (not to scale).

3. Materials and Methods

3.1. Selection of Spore Type

Three *Bacillus* subtypes which sporulate to form hygromorphic spores could act as potential actuators, including the *B. subtilis* wildtype, a mutated strain called *Bacillus subtilis cotE gerE* and a related species called *Bacillus thuringiensis*. Each of these spore types has strengths and weaknesses as potential hygromorphic actuators. *B. thuringiensis* has the greatest hygromorphic expansion and force output resulting from the larger spore cell size because it has an additional exosporium layer on the outside of the spore's outer coat [6]. However, whilst this may improve adherence to the substrate [28], it means that the spores cannot pack as densely in a monolayer and there is lower repeatability of the impact of hydration and dehydration on spore volume [6]. *B. subtilis cotE gerE* double mutant has spore coat changes, which increases the degree of expansion of the spores compared to the wildtype *B. subtilis* [6]. This means that, in theory, these mutated spores are preferable to the wildtype *B. subtilis.* However, because the *cotE gerE* mutation affects the protein elements of the spore coat, the mutated spores lack the ability to expand and contract repeatedly and, therefore, lack resilience [20] as a reusable hygromorph. We have, therefore, selected the wildtype *B. subtilis* spores, which are able to form a tight and regular monolayer with high packing density [6].

3.2. Culturing of Bacillus subtilis Spores

The first stage in the fabrication process is to develop a viable stock of spores. This means growing live (vegetative) cells at an appropriate density in a liquid culture and then reducing the available nutrients to trigger the sporulation process and create a pure culture of spores.

The spores were cultured from mother cells (laboratory stock in 40% glycerol) and inoculated with 10 mL DifcoO sporulation medium (DSM) containing 10% (*w*/*v*) KCl, 1.2% (*w*/*v*) $MgSO_4.7H_2O$, 1 M NaOH, 10 mM $Ca(NO_3)_2$, 10 mM $MnCl_2$ and 1 mM $FeSO_4$ with an adjusted pH of 7.6 and transferred to a culture tube. This tube was incubated in a shaker at 30 °C with a shaking speed of 250 rpm for 3–6 h. Once the optical density of the culture reached 0.4–0.6 at 600 nm, 1 mL culture medium was transferred to a 500-mL or 1-L shake flask containing 100 or 200 mL medium at 30 °C with a shaking speed of 250 rpm for another 5 days before autoclaving and harvesting. The culture samples were monitored by light microscope to examine the sporulation stages. When the percentage of spores in the total population reached >99% with trace amounts of vegetative cells (assessed microscopically), the spore suspension was centrifuged at 9000× *g* for 15 min at 4 °C. The spores sedimented, forming a dark brown pellet, with a light brown culture medium rising

to the top. The medium was removed by pipetting and the spores washed twice with deionised water using the same centrifugation conditions to remove the trace amount of culture medium. Then, the spores were resuspended in sufficient water to yield a *Bacillus* spore stock solution with a final optical density (OD_{600nm}) of 35–40 [29], corresponding to a cell density of 5–6 $\times$ 10^9 colony forming units/mL.

B. subtilis spores are classed as biosafety level 139 and GRAS status by the FDA [19] for risk assessment purposes, reflecting their use in food production, industrial applications [30] and presence in most mammalian gastrointestinal systems.

3.3. Selection of Inert Substrate Layer from Existing Studies

When evaluating potential substrate layer materials for use with the *B. subtilis* spores, the following (often conflicting) requirements must be considered:

- Negligible expansion and contraction with changing moisture content;
- Suitable surface for spore dispersion and adhesion;
- Sufficient axial stiffness to ensure that spore expansion induces curvature (as opposed to linear stretching of the substrate);
- Suitable bending stiffness (which is a function of axial stiffness and strip thickness) to give the required balance between deflection and actuation force.

A number of bilayer studies from other researchers have investigated two materials.

The first is polyimide tape, which, at only 0.02 mm thick, has very low bending stiffness, meaning that the spores produce a bilayer, demonstrating rapid deformation, high deflections but low force. These fast-acting bilayers rapidly "flick" forward and backward and have been integrated into, for example, biohybrid nano-engines, where multiple actuators turn a wheel similar in concept to a water wheel [9]. However, this material provides a rapid response at the expense of actuation force. The bending stiffness of the 0.02-mm polyimide tape is insufficient to move a significant mass beyond the material's self-weight. For architectural applications, responsive materials are usually required to actuate secondary systems such as shading fins or other material systems. Depending on the configuration of the system, the hygromorphic actuator will be required to push and/or pull a significant mass in addition to its own weight.

Thicker substrate materials have been used to provide greater actuation force by increasing the bending stiffness of the actuator. A key study [6] utilised 0.5-mm natural latex sheet constructed as a biohybrid actuator with *B. subtilis* spores, which were allowed to deform in a horizontal plane to minimise the effect of gravity, but, to date, no study has investigated actuation force to lift an external mass.

3.4. Application of Spores to Inert Layer (Latex) to Form a Hygromorphic Bilayer

Previous studies [6] calculated the ratio of elastic moduli of the passive sheet and the spore layer required to maximise energy transfer, and, on this basis, the inert layer used in this study was 0.5-mm-thick natural, translucent latex with a Young's modulus of elasticity of 3.0 MPa [9]. One side of the latex was roughened with wire wool (000 grade) to provide an increase in the adhesion of the spores [9]. The latex sheet was then washed with diluted laboratory detergent and rinsed with deionised water to remove surface residues and standardise the surface pH before drying thoroughly in a drying cupboard.

A scalpel and metal ruler were used to cut identical latex strips measuring 1 cm $\times$ 2 cm as the inert substrate layer for the replicate hygromorphic bilayers. Each latex strip was placed onto a microscope slide to provide support as a carrier whilst the spores were applied. A standard microscope slide adhesive (16.6 µL, poly-l-lysine solution) was smeared evenly across the surface of the latex strip using a spreader to increase spore adhesion [6]. This was air-dried (RH 40%) in a covered area to ensure that no airborne microorganisms became lodged in the adhesive layer. *B. subtilis* spores naturally produce a uniform, single-cell thick monolayer. The spore coat structure means that they form a very densely packed arrangement which is approx. 3 µm thick [8]. The spore stock solution was mixed with a vortex mixer to ensure uniform concentration before 16.6 µL was transferred

by micropipette and applied evenly across the surface of each 1 cm × 2 cm latex strip. To ensure that the same stock solution was used to produce the replicate strips for each trial, 200 µL was transferred to an Eppendorf tube for the preparation of the 9 hygromorphic bilayers for each trial. The latex strips were gently rocked by hand to spread the solution evenly once it had been applied (Figure 7).

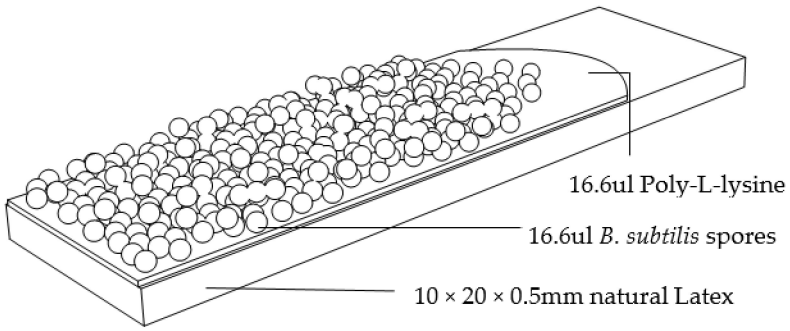

Figure 7. Diagram of bilayer construction (not to scale).

The freshly prepared hygromorphic bilayers were transferred to the grid platform in the humidity chamber set at above 95% RH and were equilibrated at room temperature for 24 h. The bilayers were re-examined under the light microscope to check for uniform monolayer coverage and were then stored in an airtight container for later testing. For the study investigating the effect of multiple *B. subtilis* monolayers on hygromorphic behaviour, additional monolayers were added sequentially. A micropipette was used to add a further 16.6 µL of the spore solution across the surface of the strip, which was then rocked gently by hand to ensure even distribution and was then allowed to air-dry in a covered area at room temperature and RH (~42% RH). This process was repeated for each additional layer, ensuring adequate drying time between the layers. The freshly prepared, multi-layered, hygromorphic bilayers were then transferred to the grid platform in the humidity chamber and incubated at 95% RH for 24 h.

3.5. Hygromorphic Bilayer Performance Testing

The hygromorphic bilayers were transferred from room humidity (~42% RH) into the humidity chamber using forceps to provide a high RH environment at >95% for 3 min. The response of the bilayers to the change in environmental humidity was filmed (Canon, EOS 4000D) for analysis later. After 3 min, the strips were removed and returned to room humidity (~42% RH) and the response again filmed for analysis later. The bend angle of each hygromorphic bilayer was also recorded at 42% RH by tracing the curvature in triplicate onto graph paper using a flexi-curve.

To analyse the raw data, graph paper and a protractor were used to trace the bend angles set up on a grid marking 0°, 90° and 180°, with the centre of the biohybrid strips as the origin of each angle (Figure 8). From this point, a straight line was drawn from the origin through the end of the curve on each side, forming a "v" shape. This line allowed accurate measurement of the overall deflection angle achieved by the biohybrid strips. As each side of the 90° marker was independent, this process was repeated on the other side and each angle was measured independently. The measurement was conducted in triplicate and all data summarised to give a mean ± the standard deviation (SD) before the relationship between deflection magnitude and number of monolayers was analysed using regression analysis, in which the number of monolayers was treated as a continuous predictor.

A simplified mathematical modelling approach adopted by Wang et al. for calculating the force required to bend the latex by the hygromorphic material was used to estimate the forces generated (Figure 9) by the *B. subtilis* hygromorphic bilayers with different monolayer numbers. To analyse the raw data, graph paper was used to trace the bend angles of each biohybrid strip. A straight line (c) was then drawn and measured between

the two ends of the strip and a further measurement taken of the height (h) of the curve. These data were used to calculate the radius of curvature and subsequently the force generated using the Stoney formula (Figure 9). Similar methodologies have been adopted by Chen et al. [6]. The relationship between deflection angle, force generated and number of monolayers was analysed using a correlation coefficient.

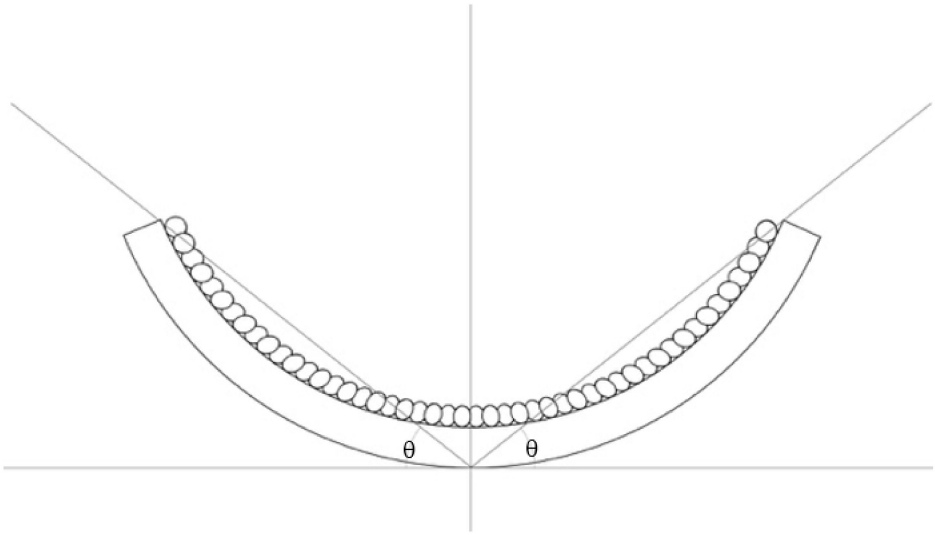

Figure 8. Deflection angle measurement method for a *B. subtilis* actuator.

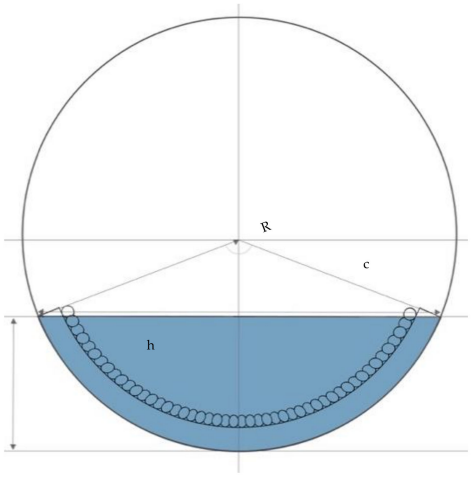

Figure 9. Calculation of force generated by a *B. subtilis* actuator.

3.6. Preparation of Hygromorphic Actuators for Loading Experiments

The spores were cultured using the previous methodology to produce a spore concentration of 5–6 $\times$ 10^9 colony forming units/mL and these were then used to prepare 2 cm $\times$ 1 cm latex strips with four layers of spore solution as before, since four layers of spore monolayers demonstrated the greatest deflection angle, suggesting the greatest force output out of all the number of monolayers tested.

The freshly prepared, hygromorphic bilayers were transferred to the grid platform in the humidity chamber set at above 95% RH and were incubated for 24 h to allow the spores to orientate themselves to produce a uniform monolayer. Four 1 cm $\times$ 2 cm strips of paper were cut using a ruler and scalpel and the mass recorded using a balance. When using 150-g/m^2 paper, the mass recorded was 0.06 g per 1 cm $\times$ 2 cm strip. Eight further paper strips were cut: four 1 cm $\times$ 2.5 cm, four 1 cm $\times$ 3 cm. The mass of each size was measured

using a balance. It was found that 1 cm × 2.5 cm strips were 0.07 g and 1 cm × 3 cm were 0.09 g.

The prepared actuators were removed from the humidity chamber and allowed to return to room RH (42%). The strips were observed during this process to ensure that a regular and even deflection was seen. Any actuators which did not achieve a minimum deflection angle of 42.4° (average deflection angle for 4 monolayers observed in our earlier experiment) or showed inconsistent deflection were rejected.

Glue (UHU—product no: 63625) was used to affix a pair of 1 × 2 cm paper weights to each side of one actuator (Figure 10) overlapping the paper behind the actuator by 0.2 cm and gluing directly onto the untreated side of the latex. This was repeated to create two actuators with a combined mass of 0.12 g (two 0.06-g paper weights), two actuators with a combined added mass of 0.14 g (two 0.07-g paper weights) and two actuators with a combined added mass of 0.18 g (two 0.09-g paper weights).

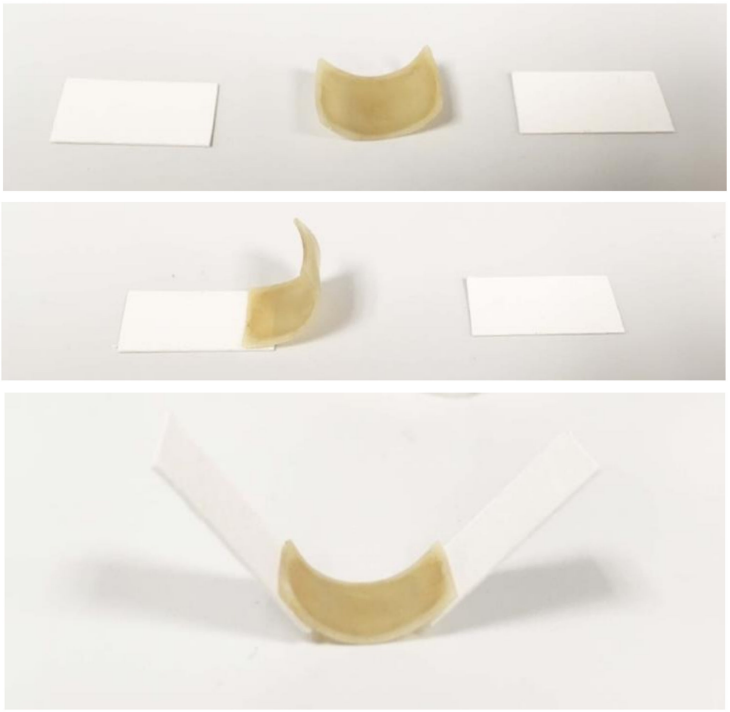

Figure 10. Process of attaching paper masses to a *B. subtilis* actuator.

Forceps were used to transfer the 6 prepared hygromorphic bilayers with added mass into the humidity chamber at >95% for 3 min. The process was filmed for subsequent analysis. After 3 min, the 6 strips were removed and placed at room humidity (~42% RH). This process was also filmed for subsequent analysis. The bend angle was recorded for each hygromorphic bilayer by tracing the curvature in triplicate onto graph paper.

4. Results

4.1. Actuator Deflection and Programmability Using Multiple Spore Layers

It was observed that the biohybrid actuators had a greater angle of deflection after 24-h equilibration at 95% RH than was seen pre-equilibration (Figure 11). The hygromorphic actuators showed a rapid deformation response upon transfer from an environment of high RH (>95% RH) to low relative humidity (~42% RH), which was reversible and repeatable. Maximum deformation response occurred in less than 3 min and there was no difference in deflection for a given RH, irrespective of whether the biohybrid strip was in a shrinking (dehydrating) phase (from high to low RH) or swelling (hydrating) phase (from low to high RH). For each dataset, eight actuators were tested three times, with the mean deflection

angle recorded each side of the centre line (shown in Figure 8) to provide two mean deflection angles per actuator. This method provided 16 data points per set (n = 16). This process was then repeated 10 days later, with results which were consistent with the original results, with no change in performance over this time period.

Figure 11. Hygromorphic actuators before (left) and after (right) 24-h equilibration period.

It was found that increasing the number of layers of *B. subtilis* spore solution applied (from 1 to 4 layers) to produce the hygromorphic bilayers resulted in an increase in the magnitude of deflection of the actuator ($p < 0.01$, n = 16 for each layer, at 42% RH). These deflections maintained the same basic performance characteristics as the monolayer hygromorphic bilayer actuators, which was still rapid (maximum deflections occurring within 3 min), reversible and repeatable. This would suggest that the deflection angle at a given RH could be programmed by designing the hygromorphic actuator with a specific number of Bacillus spore monolayers. This offers the potential to develop "programmable" actuators, deflecting by a known degree for a given RH, and offers enormous possibilities in designing environmentally responsive actuators.

The data also demonstrate that a single monolayer created insufficient force to deflect the inert latex substrate. In addition, the analysis of the deflection angle data shows that whilst the trend was linear across the range from 1 to 3 monolayers, it appears that there was a diminishing response at four monolayers. When a trendline was introduced as a linear function, it had a very good fit ($R^2 = 0.985$), but this was improved when a sigmoidal curve was used as a trendline—the fit was excellent ($R^2 = 0.997$) (Figure 12). This would suggest that there would be a diminishing increase in actuator deflection with the addition of further layers beyond the four used in the current investigation.

4.2. Forces Generated by the Hygromorphic Actuators

Force calculations based on the Stoney formula demonstrated that the biohybrid actuators were capable of generating a force of up to 26.7 N/m. This supports findings by Chen et al. [6], who also used *B. subtilis* spores as the hygromorph on an biohybrid actuator, using a 0.5-mm latex strip as a substrate, and reported that, at a similar RH (43%), the force generated was 16 N/m. The force generated was directly correlated with the number of spore monolayers applied to the latex across the range from 2 to 4 layers (Figure 13). This regression was linear and very highly significant ($p < 0.001$, n = 6, $R^2 = 0.999$, for individualised data) and showed no evidence of "declining returns" in additional force generation for the biohybrid strip with four monolayers.

A second study extended this investigation experimentally by loading masses to each arm of the hygromorphic bilayer actuators to test their performance.

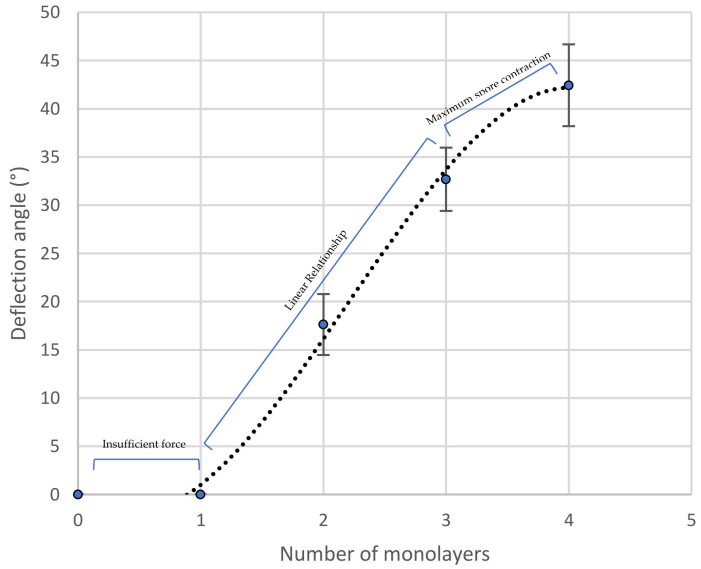

Figure 12. Effect of spore monolayer number (1, 2, 3 and 4) on maximum deflection angle (°, mean ± SD n = 16) achieved at 42% RH (19.4 °C in *B. subtilis* spore:latex hygromorphic actuators.

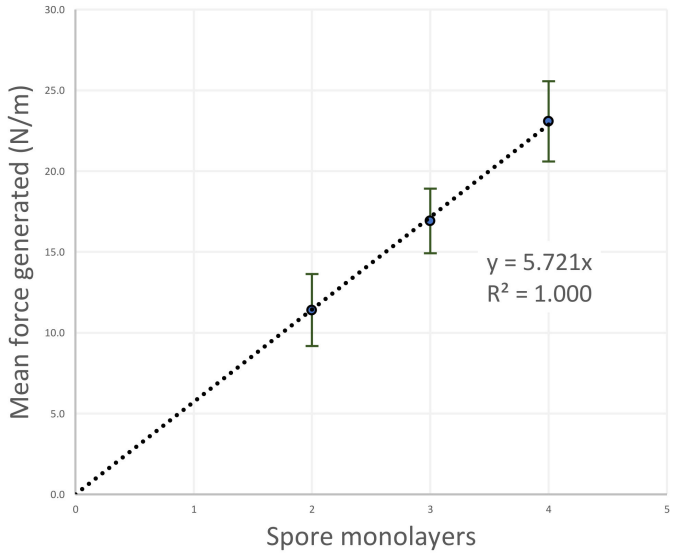

Figure 13. Effect of spore monolayer number (1, 2, 3 or 4) on maximum force generated (N/m, mean ± SD n = 6) achieved at 42% RH (19.4 °C) in *B. subtilis* spore:latex hygromorphic actuators.

4.3. Capability of Hygromorphic Actuators to Do Work

This study has demonstrated for the first time that *B. subtilis* biohybrid actuators are capable of doing useful work by lifting mass in addition to their own weight. This work was quantified by measuring the deflection angle when a known load was added. When no load was added, the deflection angle was 42.4 ± 4.2°. This was reduced with each additional load added, but this decrease was linear (R^2 = 0.9617, n = 4, $p < 0.05$, for individualised data R^2 = 0.816, n = 20, $p < 0.001$) across the range measured (Figure 14). Evidence of this relationship would again support the idea of programmability in the biohybrid actuator.

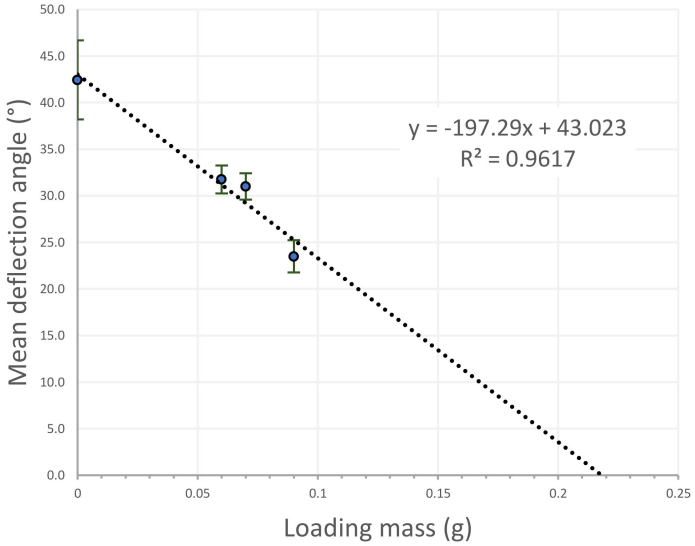

Figure 14. Effect of loading mass on maximum deflection angle (°, mean ± SD n = 4) achieved at 42% RH (19.4 °C) in *B. subtilis* spore:latex hygromorphic actuators (4 monolayers).

5. Discussion

5.1. Speed and Angle of Deformation

The actuators deformed rapidly in response to a change in environmental humidity, supporting previous findings [7,9,30] where responses in less than 5 min were reported. This rapid hydration and dehydration cycle reflects earlier studies [31,32], where it was found that water entered spores in two phases: the first, through the coat, was very fast, and the second was somewhat slower into the cortex. The Sunde et al. [31] study used dehydrated Bacillus spores and found that it took 50 s to transverse the coat and 500 s to rehydrate the cortex fully (i.e., 8.3 min). This is greater than the 3 min observed in the current study but two key differences in methodologies might account for this. Firstly, Sunde et al. [31] investigated the response from fully dehydrated to fully hydrated spores, whereas the current study investigated the time taken for response for spores in an environment from 42% to 95% RH, i.e., approximately half of the range—if hydration was linear across these RHs, it would be expected to take half the time, i.e., 4.1 min. Secondly, Sunde et al. [31] used *B. thuringiensis*, which has an exosporium, meaning that the diffusion distance across the coat layer is greater, predicting an increase in rehydration time compared to *B. subtilis*. This would suggest a response in less than 4.1 min, supporting the finding in the current study of a response time of 3 min.

As was observed in this study, the deflection angle increased post-24-h-incubation at 95% RH, which was also seen by Chen et al. [6]. This phenomenon could be due to the high-humidity environment, allowing the spores to realign themselves once in their swollen state (Figure 15), forming much greater regularity within the monolayer structure and therefore more coherent net expansion and contraction of the monolayer, leading to a greater deflection angle of the hygromorphic actuator.

This study demonstrated that deflections of the hygromorphic actuators were reversible and repeatable over 10 hydration/dehydration cycles. This supports and extends previous work in the field. Chen et al. [6] reported the same phenomenon for *B. subtilis* spores in a microcantilever study with silicon wafers as the substrate. This was confirmed by Yao et al. [33] in a study investigating the hygromorphic performance of *B. subtilis* vegetative natto cells, and by Chen et al. [8] with *B. subtilis* endospores incorporated into polyimide tape hygromorphic bilayers for potential use in developing artificial muscles. In the current study, the hygromorphic actuators showed no indication of degradation or reduction in deflection angle when tested multiple times. The maximum number of cycles of low to high RH tested on one strip was 10 cycles, with the starting and end maximum

deflections being identical. This suggests that there was no deterioration in actuator performance; however, 10 cycles would be insufficient to conclude that it could be maintained over an infinite number of cycles. However, in the study by Chen et al. [6], deflection was recorded over 1 million cycles without performance change, which bodes well for the predicted performance of our actuators over extended use and would be critical for application in an architectural setting, where the actuators would be designed to last for many years. This durability factor would exclude the possibility of using *B. subtilis* natto cells as potential hygromorphic actuators because these vegetative cells would not be sufficiently resilient in an architectural application and have poor adherence characteristics [34].

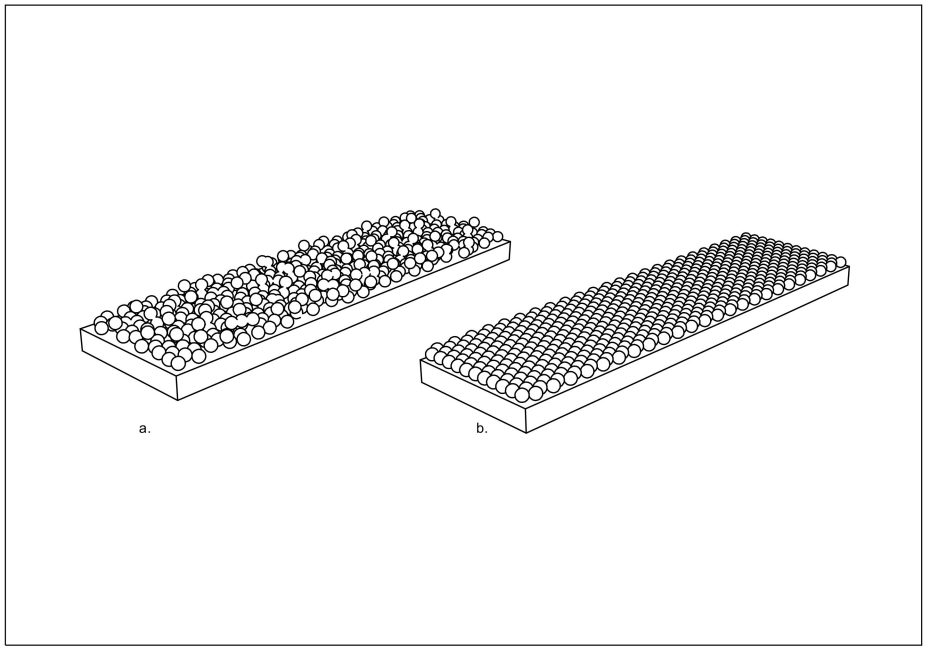

Figure 15. (**a**) Diagrammatic visualisation of irregularly aligned monolayer pre-24-h-incubation at 95% RH; (**b**) regularly aligned monolayer post-24-h-incubation at 95% RH.

As highlighted in the results, there was no deflection response in actuators with only one monolayer, suggesting that this created insufficient force to deflect the inert latex substrate. Similar studies by Chen et al. [6] showed some deflection but their methodology orientated the actuators such that they were deforming horizontally, therefore not requiring potential energy to lift the latex. The study by Wang et. al. [9] investigated genetically engineered *E. coli* vegetative cells rather than spores and so is not directly comparable. Differences in experimental design also mean that the study by Yao et al. [29] cannot be directly compared—here, the authors used a bio-printing system which dispensed the equivalent of multiple layers of *B. subtilis* spores to the actuators.

This performance investigation demonstrated that by increasing the number of *B. subtilis* spore monolayers, there is an increase in the maximum deflection angle of the actuator. This suggests that not only is there tight adhesion between the spores in each monolayer, but also that there is an adhesion between the monolayers. This highlights that individual monolayers must be contributing towards a combined force instead of each "pulling" separately, suggesting that the adhesion between the monolayers is sufficient to transfer force from one layer to another. Yao et al. [33] reported a similar finding for vegetative *B. subtilis* natto cells, where, for the same RH, the thicker the cell layers deposited on the actuator substrate, the greater the bending curvature. In this study, the thickness of the natto cell layer was determined by the flow rate of the bio-printer, so it was not formed from separate monolayers but demonstrated a similar response to the current study.

It was observed that, for actuators with four monolayers, the maximum deflection angle observed appeared to plateau. This maximum deflection angle could be a result of the spores reaching their maximum contraction. The spores can only contract up to an estimated 12% of hydrated volume due to internal cellular architecture [23] and then they will stop. Beyond this point, adding extra layers of spores will increase the actuation force, but not the deflection.

5.2. Force Output

Previous studies by Chen et al. [6] have investigated the force output of individual Bacillus spores using microcantilever studies. The force output of a biohybrid strip with a single *B. subtilis* monolayer on latex has also been investigated, through both calculation and direct experiments, but this is the first investigation of the performance of multiple monolayers. It was observed by Chen et al. [6] that the force output at 42% RH was recorded at 16 N/m, which is of similar magnitude to the findings of the current study, where actuators with two monolayers generated a force of 11.4 + 2.2 N/m. Whilst the force observed in our study is marginally lower, this can be explained through differences in the methodology of the two studies—our study placed the hygromorphic actuators horizontally, resulting in the actuators deforming vertically (i.e., the direction of movement was vertical; hence, they need to overcome gravity) in addition to overcoming the elastic resistance of the latex substrate. By contrast, Chen et al. [6] placed the hygromorphic actuators vertically (deformation was horizontal) so that they were only performing work to overcome the elastic forces.

This study has shown for the first time that the force output of these hygromorphic actuators has a strong linear relationship with the number of monolayers. With each increase in the number of monolayers, there is a predictable and therefore programmable force response given by these hygromorphic actuators. The results of this first study would suggest that, since the maximum force output of these actuators is 150% of their own weight, they would be sufficient to successfully actuate multiple folds in an origami-like structure.

Although it was suggested by Chen et al. [6] that, if an incomplete spore layer is seen when assessing the coverage on the latex, further spores could be added to ensure complete coverage, there was no reference made to these additional layers increasing the maximum displacement seen (bend angle) or force output as demonstrated in this experiment.

When viewing the biohybrid actuators under the microscope, it became apparent that some of the monolayer surfaces had cracked, supporting earlier observations by Chen et al. [6] (Figure 16). This was related to load, with increased added mass causing "cracks" to form across the spore layer, especially at low relative humidity. This observation could be explained by the spores physically pulling apart, with a tensile force which was greater than the adhesion force between each spore and to the latex substrate. This would mean that the spore monolayers crack and separate into segments and break away from the latex layer (when at low relative humidity).

Whilst poly-l-lysine improves adhesion to the latex, this is achieved by changing the electrostatic charge on the surface of the latex and it does not have any impact on adhesion between the spores themselves [9], but recent work has shown that culturing and sporulation conditions modify the spore coat, which affects adhesion [34] and merits further investigation. In addition, the germination of the spores was not observed during our experimental tests. As mentioned before, in Chen et al. [7], deflection was recorded over 1 million cycles without performance change. However, the stability of the dormant spores after long-term exposure to various humidified environments should be further investigated as it would be critical for application in an architectural setting, where the actuators would be designed to last for many years.

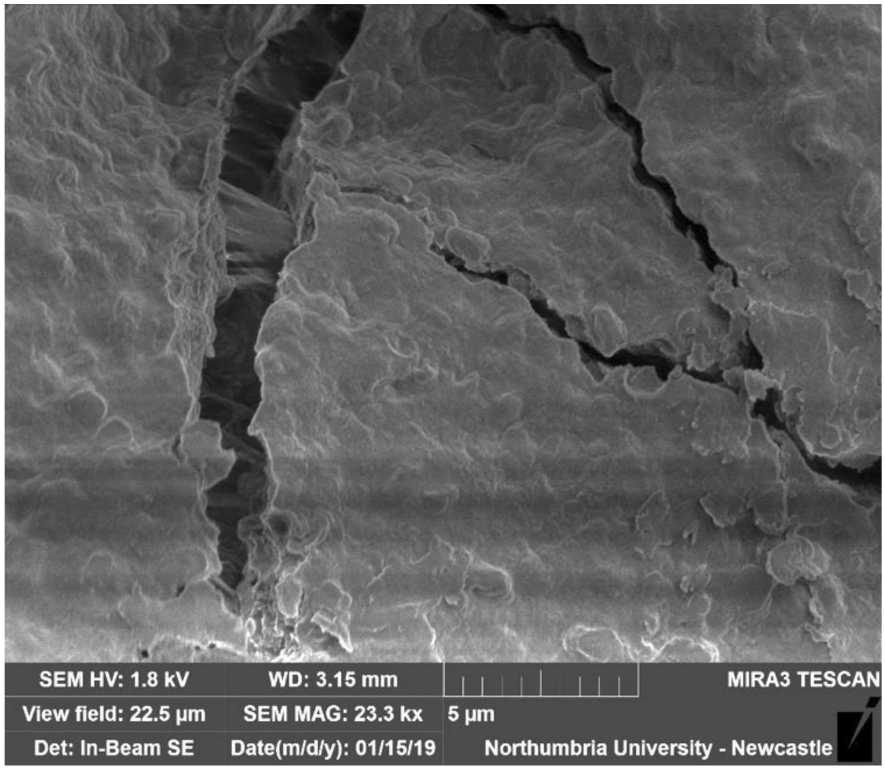

Figure 16. Scanning electron microscopy (SEM) of cracking observed at extremely low relative humidity.

6. Conclusions

We have developed a methodology to produce functional *B. subtilis* latex hygromorphic bilayers as actuators. These working *B. subtilis* spore actuators' properties were investigated and showed a rapid (less than 3 min), reversible and repeatable deflection of up to 43°, which is in agreement with the previous literature. We demonstrated that the magnitude of this deflection was increased by increasing the number of *B. subtilis* monolayers applied to the latex substrate up to a maximum value limited by the expansion of the spores. Mathematical modelling (using calculated radius of curvature in the Stoney formula for plane strain) was used to estimate the maximum force generated in bending the latex to the maximum measured curvature as 26.7 N/m, and it was shown that this force was achieved in the actuator with the greatest number of monolayers. The relationships between mass, spore layers and deflection angle are linear, demonstrating that the behaviour of spore-based actuators is predictable and potentially programmable (i.e., actuators could be designed to move a particular system a specified distance with a specified change in relative humidity). Further, we have demonstrated that a *B. subtilis* actuator is capable of generating sufficient force to lift a mass equivalent to 150% of the mass of the actuator.

With the UK aiming for "net zero carbon" buildings by 2050, and with many similar targets established globally, the future of architectural design must focus on designing buildings which can be constructed and operated with minimal energy input [3]. To minimise (or eliminate) energy requirements for heating and cooling, it is essential that buildings become better able to respond to the changing external environment. Bacterial spore-based hygromorphs, alongside other shape-changing materials, may provide the opportunity for a new generation of building materials which are able to sense and respond to their environment. The materials studied here have the benefit of being fast-acting, powerful (compared to their mass), sensitive and programmable (by altering the substrate and application of spore layers). These novel smart materials may be the key to unlocking some of the technological challenges involved in achieving passive systems in architecture.

The most commonly proposed application of morphing materials is as shading devices, to reduce solar gain through glazed facades in hot climates [4,5]. Arrays of small (centimetre-scale) spore-based hygromorphs could be installed within a double-skin façade to provide a highly responsive shading system, with the spacing and patterning of the many small elements providing opportunities to tune the level of shading depending on the orientation of the façade, with interesting aesthetic possibilities as the dynamic elements constantly change shape in response to the immediate microclimate. However, a significant challenge is that the spores respond to moisture, not temperature, and therefore the relationship between humidity and temperature within the double-skin façade must be understood in order to enable the responsive shading system to be tuned to operate effectively.

Spore-based hygromorphs also have potential to control ventilation within buildings. Whilst they could be used simply to open and close ducts (for example, to allow warm, moist air to be extracted through a heat recovery system), they could revolutionise breathable building envelopes. Most attempts to minimise building energy use focus on improving insulation and airtightness, but an alternative approach is dynamic insulation, where a porous wall construction, or skin, uses the thermal capacity of the wall materials to heat or cool incoming air, with air movement driven by natural ventilation at roof level [35]. However, dynamic insulation operates constantly, despite varying internal or external conditions, unless mechanical ventilation and control systems are added. Incorporating spore-based hygromorphs into dynamically insulated breathing wall systems has potential to deliver truly passive buildings which would autonomously combine high levels of ventilation with passive heating and cooling through the building fabric.

Our study establishes the baseline for this new material and its application in architecture. In future work, we intend to scale-up these materials to conduct longer-term studies of their behaviour in real environments. We also believe that there is potential to explore a wider range of material substrates—extending beyond latex and introducing a range of actuator types—extending beyond the fold. We will also need to investigate the biology of the spores. While bacterial spores are dormant (i.e., they have negligible metabolism), so they are, in theory, able to survive for hundreds of years; if the environmental conditions are favourable, they can germinate to become bacterial cells. Preventing this process will be important to the long-term resilience of the material and so finding biological methods for "switching off" this capacity for germination will also be a feature of future research in this area.

Author Contributions: Conceptualization, E.B. and M.D.-R.; methodology, E.B., B.B and M.Z; investigation, E.B.; writing—original draft preparation, E.B.; writing—review and editing, E.B., B.B., M.Z., M.D.-R.; supervision, B.B., M.Z., M.D.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded as part of Newcastle Universities Research Investment Fund (RIF).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Acknowledgments: The authors would like to thank staff and students at the Hub for Biotechnology in the Built Environment for their support. We would like to highlight the contributions of Beate Christgen and Jennifer Wright.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Koyaz, M. Adaptability Level of Facade Systems Regarding Facade Adaptability Level of Facade Systems. In Proceedings of the International Conference on Building Envelope Systems and Technologies (ICBEST 2017), Istanbul, Turkey, 15–18 May 2017.
2. Augustin, N. *Motion with Moisture: Creating Passive Dynamic Envelope Systems Using the Hygroscopic Properties of Wood Veneer*; University of Waterloo: Waterloo, ON, Canada, 2018.
3. Banfill, P.F.G.; Peacock, A.D.; Banȼll, P.F.G. Energy-efficient new housing–the UK reaches for sustainability. *Build. Res. Inf.* **2007**, *35*, 426–436. [CrossRef]

4. Holstov, A.; Bridgens, B.; Farmer, G. Hygromorphic materials for sustainable responsive architecture. *Constr. Build. Mater.* **2015**, *98*, 570–582. [CrossRef]
5. Reichert, S.; Menges, A.; Correa, D. Meteorosensitive architecture: Biomimetic building skins based on materially embedded and hygroscopically enabled responsiveness. *Comput. Des.* **2015**, *60*, 50–69. [CrossRef]
6. Chen, X.; Mahadevan, L.; Driks, A.; Sahin, O. Bacillus spores as building blocks for stimuli-responsive materials and nanogenerators. *Nat. Nanotechnol.* **2014**, *9*, 137–141. [CrossRef]
7. Hong, H.A.; Duc, L.H.; Cutting, S.M. The use of bacterial spore formers as probiotics: Table 1. *FEMS Microbiol. Rev.* **2005**, *29*, 813–835. [CrossRef]
8. Chen, X.; Goodnight, D.; Gao, Z.; Cavusoglu, A.H.; Sabharwal, N.; DeLay, M.; Driks, A.; Sahin, O. Scaling up nanoscale water-driven energy conversion into evaporation-driven engines and generators. *Nat. Commun.* **2015**, *6*, 7346. [CrossRef]
9. Wang, W.; Yao, L.; Cheng, C.-Y.; Zhang, T.; Atsumi, H.; Wang, L.; Wang, G.; Anilionyte, O.; Steiner, H.; Ou, J.; et al. Harnessing the hygroscopic and biofluorescent behaviors of genetically tractable microbial cells to design biohybrid wearables. *Sci. Adv.* **2017**, *3*, e1601984. [CrossRef]
10. Ramirez-Figueroa, C.; Hernan, L.; Guyet, A.; Dade-Robertson, M. Bacterial Hygromorphs. In Proceedings of the 36th Annual Conference of the Association for Computer Aided Design in Architecture, Cambridge, MA, USA, 2–4 November 2017.
11. Le Duigou, A.; Castro, M. Evaluation of force generation mechanisms in natural, passive hydraulic actuators. *Sci. Rep.* **2016**, *6*, srep18105. [CrossRef]
12. Arends, T.T.; Pel, L.L.; Huinink, H.H. Hygromorphic response dynamics of oak: Towards accelerated material characterization. *Mater. Struct.* **2017**, *50*, 181. [CrossRef]
13. Erb, R.M.; Sander, J.S.; Grisch, R.; Studart, A.R. Self-shaping composites with programmable bioinspired microstructures. *Nat. Commun.* **2013**, *4*, 1712. [CrossRef]
14. Reyssat, E.; Mahadevan, L. Hygromorphs: From pine cones to biomimetic bilayers. *J. R. Soc. Interface* **2009**, *6*, 951–957. [CrossRef]
15. Cavusoglu, A.-H.; Chen, X.; Gentine, P.; Sahin, O. Potential for natural evaporation as a reliable renewable energy resource. *Nat. Commun.* **2017**, *8*, 1–9. [CrossRef]
16. Scott, J. The University Of Leeds Responsive Knit: The evolution of a programmable material system. *DRS2018: Catalyst* **2018**, *4*. [CrossRef]
17. Holstov, A.; Farmer, G.; Bridgens, B. Sustainable Materialisation of Responsive Architecture. *Sustain. J. Rec.* **2017**, *9*, 435. [CrossRef]
18. Van Der Hofstadt, M.; Fabregas, R.; Millan-Solsona, R.; Juarez, A.; Fumagalli, L.; Gomila, G. Internal Hydration Properties of Single Bacterial Endospores Probed by Electrostatic Force Microscopy. *ACS Nano* **2016**, *10*, 11327–11336. [CrossRef]
19. Arora, S.; Kumar, N.; Yadav, A.; Raghu, H.V. Spore: Potential of Invaluable Bacterial Wrap. *Int. J. Life-Sci. Sci. Res.* **2016**, *2*, 513–518. [CrossRef]
20. Henriques, A.O.; Moran, C.P., Jr. Structure, Assembly, and Function of the Spore Surface Layers. *Annu. Rev. Microbiol.* **2007**, *61*, 555–588. [CrossRef]
21. Plomp, M.; Carroll, A.M.; Setlow, P.; Malkin, A.J. Architecture and Assembly of the Bacillus subtilis Spore Coat. *PLoS ONE* **2014**, *9*, e108560. [CrossRef]
22. Nicholson, W.L.; Munakata, N.; Horneck, G.; Melosh, H.J.; Setlow, P. Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments. *Microbiol. Mol. Biol. Rev.* **2000**, *64*, 548–572. [CrossRef]
23. Driks, A. The dynamic spore. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3007–3009. [CrossRef]
24. Zolock, R.A.; Li, G.; Bleckmann, C.; Burggraf, L.; Fuller, D.C. Atomic force microscopy of Bacillus spore surface morphology. *Micron* **2006**, *37*, 363–369. [CrossRef]
25. Ghosal, S.; Leighton, T.J.; Wheeler, K.E.; Hutcheon, I.D.; Weber, P.K. Spatially Resolved Characterization of Water and Ion Incorporation in Bacillus Spores. *Appl. Environ. Microbiol.* **2010**, *76*, 3275–3282. [CrossRef]
26. Sahin, O.; Yong, E.H.; Driks, A.; Mahadevan, L. Physical basis for the adaptive flexibility of Bacillus spore coats. *J. R. Soc. Interface* **2012**, *9*, 3156–3160. [CrossRef]
27. Garg, N. New Technologies in Renewable Power. Международный Научно-Исследовательский Журнал **2014**, *2*, 988–995.
28. Joshi, L.T.; Phillips, D.S.; Williams, C.F.; Alyousef, A.; Baillie, L. Contribution of Spores to the Ability of *Clostridium difficile* to Adhere to Surfaces. *Appl. Environ. Microbiol.* **2012**, *78*, 7671–7679. [CrossRef]
29. Yao, L.; Ou, J.; Wang, G.; Cheng, C.-Y.; Wang, W.; Steiner, H.; Ishii, H. bioPrint: A Liquid Deposition Printing System for Natural Actuators. *3D Print. Addit. Manuf.* **2015**, *2*, 168–179. [CrossRef]
30. Tehri, N.; Kumar, N.; Raghu, H.V.; Shukla, R.; Vashishth, A. *Microbial Bioprospecting for Sustainable Development*; Springer: Singapore, 2018; pp. 279–289.
31. Sunde, E.P.; Setlow, P.; Hederstedt, L.; Halle, B. The physical state of water in bacterial spores. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 19334–19339. [CrossRef]
32. Westphal, A.J.; Price, P.B.; Leighton, T.J.; Wheeler, K.E. Kinetics of size changes of individual Bacillus thuringiensis spores in response to changes in relative humidity. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 3461–3466. [CrossRef]
33. Yao, L.; Ou, J.; Cheng, C.-Y.; Steiner, H.; Wang, W.; Wang, G.; Ishii, H. bioLogic. In *Proceedings of the 33rd Annual ACM Conference on Human Factors in Computing Systems*; ACM: New York, NY, USA, 2015; pp. 1–10.

34. Zhou, K.X.; Li, N.; Christie, G.; Wilson, D.I. Assessing the Impact of Germination and Sporulation Conditions on the Adhesion ofBacillusSpores to Glass and Stainless Steel by Fluid Dynamic Gauging. *J. Food Sci.* **2017**, *82*, 2614–2625. [CrossRef]
35. Craig, S.; Grinham, J. Breathing walls: The design of porous materials for heat exchange and decentralized ventilation. *Energy Build.* **2017**, *149*, 246–259. [CrossRef]