Waterborne Coatings Encapsulating Living Nitrifying Bacteria for Wastewater Treatment

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Biofilm bioreactors are attracting growing interest in the wastewater industry, as they allow higher cell densities and thus higher reaction rates compared to conventional bioreactors. However, some commonly used nitrifying bacteria, such as *Nitrosomonas europaea*, are slow-growing and need a prolonged period of time to develop a mature biofilm. Here, a biocoating or “living paint” is introduced, which is a synthetic biofilm made from a colloidal polymer (synthetic latex) binder encapsulating viable nitrifying bacteria at high density. Conventionally, the film formation of biocoatings is achieved by drying a bacteria/latex mixture. However, this fabrication is detrimental to the viability of the encapsulated bacteria because of the osmotic stress induced by desiccation. A nondesiccating film formation process is presented for biocoatings, which exploits two colloid science phenomena: coagulation and wet sintering. Desiccation-sensitive, nitrifying bacteria are employed in the biocoatings to convert NH₄⁺ to NO₂⁻ and then NO₃⁻. These biocoatings have a conversion rate (NO₂⁻ and NO₃⁻ production) of 3 mg N g⁻¹ d⁻¹ that is five times higher than in conventionally desiccated biocoatings. The reactivity continues over a period of 1 month. The processing method for these living paints is transformative for wastewater treatment and other applications using delicate, desiccation-sensitive microorganisms.

1. Introduction

The release of ammonium from municipal wastewater is known to cause significant problems in aquatic environments. Modern wastewater treatment facilities use biological nutrient removal processes in which nitrifying bacteria convert ammonium to nitrates.[1–2] In conventional bioreactors, such as an active sludge bioreactor, the nitrifying bacteria are in the form of a suspension.[2] However, more recent bioreactor designs, such as the moving bed biofilm reactor, encourage nitrifying bacteria to grow on the surface of plastic carriers and develop a natural biofilm.[2–4]

Biofilms are communities of bacteria that are attached to a surface and/or to each other and embedded in a self-produced polymeric matrix.[5–6] Biofilms facilitate high bacterial density and offer protection for bacteria against toxic compounds and environmental shocks.[6–7] The carriers have a high surface area-to-volume ratio, allowing more bacteria to attach efficiently. They also have a specially designed geometry that protect the growth of the biofilms.[1–8]

For these reasons, biofilm reactors are receiving growing interest in the wastewater industry.[6] However, there is a major technical difficulty in the start-up of biofilm reactors. Some commonly used nitrifying bacteria, such as *Nitrosomonas europaea*, are slow-growing and need a prolonged period of time to develop a mature biofilm.[7] As a result, nitrifying bacteria are always a minority in the broader biofilm community in an open system.[9] Meanwhile, the formation of their biofilms is most often the rate-limiting factor in the wastewater treatment start-up process. A potential solution to solve this problem is to encapsulate nitrifying bacteria at a high density inside a synthetic polymeric matrix that mimics a natural biofilm.[10–11] This synthetic biofilm, called a biocoating, is ready to perform functions in the same way as natural biofilms, but without the need for a prolonged growing period for biofilm formation. Moreover, synthetic biofilms can be engineered to offer increased mechanical robustness and adhesion.

In previous research, biocoatings were made using a synthetic colloidal polymer (i.e., latex) film to encapsulate metabolically active bacteria.[10–11] The idea of encapsulating viable bacteria in a latex coating first appeared in the 1980s.[12] In 1996, landmark publications that systematically studied and characterized biocoatings were published by Flickinger and colleagues,[13–15] who later coined the term “biocoating”[16] to refer to an adhesive biocatalytic coating. Biocoatings or “living paints” are attracting growing interest, but there are to-date only about 50 publications on the topic. Although great progress has been
made regarding the efficiency and the application of biocoatings, their fabrication process has remained mostly unchanged for these 30 years.\textsuperscript{[10,11]} In most of the research, the biocoatings are fabricated by applying a mixture of wet latex and bacterial suspensions onto a substrate followed by the complete drying of the mixture, sometimes at elevated temperatures. However, this fabrication process is not ideal for the viability of the encapsulated bacteria because of the osmotic stress induced during the desiccation.\textsuperscript{[10,11]} To tackle this problem, researchers have added osmotic protectants, such as glycerol and sucrose during the desiccation.\textsuperscript{[10,11]} There is no existing systematic study that proves the effectiveness of such additives. The reduced viability caused by desiccation is always considered as one of the major challenges in the exploitation of biocoatings.\textsuperscript{[10,11]} The need to encapsulate living bacteria in materials without loss of viability from desiccation has been raised as essential for future progress.\textsuperscript{[18,19]}

In this research, we propose a new approach to biocoating fabrication that is radically different from the previous methods, as it does not involve desiccation in the film formation process. This is realized by combining two colloid science phenomena: coagulation and wet sintering (Figure 1). We derived our inspiration from the coagulant dipping process that is used in the manufacture of latex gloves,\textsuperscript{[20,21]} wherein a latex dispersion is first coagulated into a gel film on a hand-shaped mold, after which the colloidal particle coalesces to provide cohesion.

In a typical latex dispersion, negatively charged colloidal particles repel each other and impart colloidal stability. To induce coagulation, a coagulant (typically ions in solution) is added to the dispersion to screen the charge of the colloidal particles.\textsuperscript{[22]} As is described by the well-known theory of Derjaguin-Landau-Verwey-Overbeek,\textsuperscript{[23]} when the repulsive electrostatic force is weakened, the energy barrier to coagulation is lowered. In our method, water-soluble salt is coated onto a substrate prior to the coating of the wet colloidal film. The dissolution of the salt induces coagulation and the formation of a gel (see Figure 1a,b).

Sintering of polymer colloids is the process wherein spherical particles deform to fill space to lower their total surface free energy by reducing their surface area. Thereafter, molecules diffuse across the boundaries of adjacent particles to achieve coalescence,\textsuperscript{[24–26]} and the colloidal particles fuse together at their contact points to form a cohesive phase.\textsuperscript{[24–27]} Sintering can happen either in air or in liquid. The former mechanism is called “dry sintering” and the latter is “wet sintering.” In a conventional film formation process, water evaporation proceeds from the onset and particle viscoelastic resistance is high, so that wet sintering is not active during the film formation process.\textsuperscript{[24–26]}

We propose to use wet sintering of a coagulated gel (Figure 1b,c) to prevent the desiccation and subsequent loss of viability of bacteria during film formation. It is worth noting that neither water evaporation nor desiccation occur in the processes of coagulation and wet sintering. Hence, no desiccation stress develops and enhanced viability is expected in the bacteria with this proposed fabrication method.

In this study, we investigated the feasibility and effectiveness of this new proposed method. A commercial nitrifying bacteria consortium was encapsulated using our biocoatings and their reactivity after the encapsulation was measured in controlled experiments. The nitrifying bacteria are in a mixed culture of ammonia oxidizing bacteria (AOB) and a nitrate oxidizing bacteria (NOB).\textsuperscript{[28]} The AOB first oxidizes ammonium into nitrite:

\[
\text{NH}_3 + \text{O}_2 \rightarrow 2\text{H}^+ + \text{H}_2\text{O} + \text{NO}_2^- \quad (1)
\]

Then, the NOB further oxidizes the nitrite into nitrate:

\[
\text{NO}_2^- + \text{O}_2 \rightarrow \text{NO}_3^- \quad (2)
\]

Some of the commonly used AOB and NOB microorganisms are from the genus \textit{Nitrosomonas} (e.g., \textit{Nitrosomonas eutropha} and \textit{Nitrosomonas europaea}) and from \textit{Nitrobacter} (e.g., \textit{Nitrobacter winogradskyi}), respectively.\textsuperscript{[28]} While these species are well characterized because of their importance for the wastewater industry, there is limited information on their desiccation tolerance.\textsuperscript{[29,30]} Nevertheless, it is widely acknowledged that nitrifying bacteria are generally sensitive to desiccation. For example, researchers found that riverbeds that naturally contain nitrifying bacteria lose their function of nitrification after periods of drought.\textsuperscript{[31,32]} Even though there is a huge need to contain nitrifying bacteria for wastewater treatment in bioreactors, there are no previous publications reporting the successful encapsulation of these bacteria within biocoatings, which are easier to transport, often provide larger surface areas and can be used to seed beneficial communities. In using these desiccation-sensitive bacteria, we showcase the advantages of our proposed nondesiccation film formation process to make “living paints” that are very promising for applications in wastewater treatment.

2. Results and Discussions

2.1. The Fabrication of Biocoatings Using Coagulation and the Wet Sintering Process

First, the coagulation and wet sintering process (abbreviated hereafter as C + WS) will be elucidated. The coagulation process is illustrated in Figure 2a–e and there are more details in the Experimental Section. Organic substrates, such as loofah, have been used in previous research, due to their low cost,
hydrophilicity, and large surface area.\textsuperscript{33,34} However, we had concerns that the irregular, 3D structure of the loofah would make the characterization of the film formation difficult. Instead, we chose flax as our substrate, which has a periodic 2D structure and is widely available, while still possessing the desired properties of loofah. The flax substrate was first dipped into a coagulant (MgCl\(_2\) solution) for 10 s (Figure 2b) to allow wetting. After drying, a layer of salt remained deposited on the surface of the flax (Figure 2c). A mixture of latex and nitrifying bacteria was then applied by spreading onto the salt-coated substrate to induce the coagulation and the formation of a gel film (Figure 2d). Clay nanoparticles (halloysite nanotubes) were included in the coatings formulation to increase the porosity and hence the permeability of the coating and viability of the bacteria, as we demonstrated previously.\textsuperscript{27}

For the coagulation to occur, the concentration of the counterions must exceed the critical coagulation concentration (C\(\text{\*}\)) of the latex,\textsuperscript{22} which was measured to be 0.05 m of Mg\(^{2+}\) for the latex used here. However, this value applies to the situation where the counterion is added directly to a latex dispersion. In a coagulant dipping process, and in the process presented here, the colloid is added onto a salt-coated substrate to create an ionic concentration gradient emanating from the substrate. Previously, researchers have optimized coagulant dipping processes by trial-and-error.\textsuperscript{20} According to an existing theoretical model for the process,\textsuperscript{20} coagulation occurs when the local concentration of ions in the gradient exceeds C\(\text{\*}\). In the fabrication of biocoatings, we propose that the concentration of the coagulant should be kept as low as possible to avoid a high osmotic stress on the bacteria. The commonly used coagulant in dipping processes, Mg(NO\(_3\))\(_2\), is not appropriate for this application, as it contains NO\(_3^-\) ions that inhibit the reactivity of nitrifying bacteria.\textsuperscript{28} We instead used a concentrated cell culture medium that contains Mg\(^{2+}\), Ca\(^{2+}\), etc. as the coagulant for the colloidal film gelation. We speculated that the salts in both the bacteria and the polymer. Normal chemical fixation was not applicable in this situation, as the organic molecules would inevitably alter the polymeric structure. The samples were freeze-dried to preserve the structure of the resulting biocoatings. The microscopic structure of the resulting biocoatings was studied by scanning electron microscopy (SEM) (Figure 3b–e). The samples were freeze-dried to preserve the structure of both the bacteria and the polymer. Normal chemical fixation was not applicable in this situation, as the organic molecules would inevitably alter the polymeric structure. The microscopic images show that the biocoating is a relatively uniform layer that covers the surface of the flax substrate (Figure 3b). Examination of the cross-section (Figure 3c) shows that the coating is about 200 µm in thickness. It appears that the coating material is primarily on the surface of the flax substrate instead of penetrating deeply into it. It is likely that the particles coagulated immediately after they contacted the salt layer on the surface. Higher magnification (Figure 3d,e) reveals that the biocoatings fabricated by the C + WS process had a highly porous structure comprised of particles in an open ring-like arrangement. Looking closely, some of the latex particles can still be identified by their spherical shapes, which suggests a relatively low degree of coalescence. Individual bacteria can also be found on the surface of the biocoatings (Figure 3d). In this SEM image, there are at least two different types of bacteria in the mixed culture. One has a relatively smooth surface and is rod shaped (yellow boxes). The other one is more rounded and has

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**Figure 2.** A schematic diagram of the experimental process of a nondesiccation film formation process consisting of coagulation (a–e) and wet sintering (e–f). The flax substrate (blue) is dipped into the coagulant salt (yellow). The latex binder (in coral pink) is spread on the salted substrate. For simplicity of the illustration, bacteria and halloysite are not included. The disc in (f) represents a sealed Petri dish that stops the water from evaporating.
a reticulated surface (brown box). Some halloysite nanotubes can also be seen on the surface of the biocoatings (Figure 3e, red boxes). From the known composition of the biocoatings, we estimate that the density of the bacteria within them to be on the order of $5 \times 10^{12}$ cells m$^{-2}$ after the film formation. (This density includes both live and dead bacteria.)
Porosity is a highly sought-after property for a biocoating.\textsuperscript{[16,17,27,35]} A higher porosity means a greater rate of transport of the nutrients and products into and out of the coatings and enhanced bacterial viability. Extensive research was conducted in the past to increase the porosity of biocoatings.\textsuperscript{[16,17,27,35]} To investigate the formation of this porous structure, we studied the effects of the individual components of the biocoating to identify the key elements that create this porosity. For comparison, Figure 3f shows the surface morphology of a biocoating film-formed by the conventional full-desiccation method. No visible porosity can be observed from this coating, suggesting that the porous structure in Figure 3e is a result of the C + WS processing. In particular, the gelation stage “freezes in” an open random packing of particles, whereas standard drying favors denser particle packing. In some places, indentations (red arrows) can be seen on the surface of the desiccated coating. Judging from their size, these features are most likely created by the bacteria that have escaped the coating and left a vacant space behind. For comparison, the surface morphology of a coating of the original latex film-formed by the same C + WS process is shown in Figure 3g. In this coating, pores are uniformly distributed on the surface, indicating that by coagulating the latex particles, a porous coating is created. However, the size of the pores in the latex film is still considerably smaller than that in the fully formulated biocoating (Figure 3e). The surface morphology of a latex/halloysite coating (without bacteria) film-formed by the same C + WS process is shown in Figure 3h. Here, the porous structure is very similar to the structure seen in Figure 3e when bacteria were present. Following this microstructural examination, we conclude that the C + WS process creates a porous structure, and the pore size of the structure is further increased by the addition of halloysite nanotubes, which could be acting as a type of scaffold. This method of fabricating porous coatings is simple yet effective. As there is great interest in creating porous polymer films,\textsuperscript{[36,37]} this method might offer another approach to achieving this objective and is worthy of further investigation.

2.2. The Viability and Reactivity of the Encapsulated Nitrifying Bacteria in Biocoatings

To determine how the method of fabricating the biocoatings affects the viability and reactivity of the encapsulated bacteria, we compared the nitrification rate of the biocoatings fabricated by the C + WS process to the conventional method of desiccation achieved with 3 h of water evaporation at 30 °C. The resulting coatings were touch dry, which is an indicator that evaporation was nearly complete. A gravimetric analysis (Table S1, Supporting Information) showed that the biocoatings lost 78% of their initial water after 3 h of evaporation, while it took 6 h for a biocoating to lose all of its water (Figure S1, Supporting Information). Bacteria in the original form of a suspension in the medium to NO\textsubscript{3}-N, and then to NO\textsubscript{2}-N. The results of the reactivity experiments, which are presented in Figure 4, show that the bacteria are active in the C + WS biocoatings with reactivities that approach that of the bacteria in suspension (PC). In all samples, the initial mass of the bacteria suspension was 1 g.

The concentration of the converted nitrogen (NO\textsubscript{x}-N), which is the sum of nitrogen in nitrite (NO\textsubscript{2}-N) and nitrogen in nitrate (NO\textsubscript{3}-N), over the entire duration of the experiment is shown in Figure 4a for one of the three biological replicates. It is clear that the C + WS sample (blue line) has a higher reactivity than the 3HD sample (orange line) throughout the entire experiment. The same trend in the reactivity was found in the other two replicates (Figure S3, Supporting Information). The C + WS processing of biocoatings preserves the reactivity of the nitrifying bacteria (both AOB and NOB), where the drying in the 3HD biocoatings reduces the reactivity sharply with a likely loss of viability.

The reaction media in the bioreactors were changed every 24 h over the first 72 h. By changing the reaction media daily, NH\textsubscript{4}+ was replenished while the NO\textsubscript{2}- and NO\textsubscript{3}- were removed along with any free bacteria in the suspension. Thus, any recorded reactivity is likely to be derived primarily from the encapsulated bacteria rather than from any suspended bacteria. From day 4, the reaction media were no longer changed.

There is a clear increase in the NO\textsubscript{x}-N production amounts over time for both samples (Figure 4a). Such a strong increase in reactivity over time has not been reported in previous research on biocoatings.\textsuperscript{[38]} We suggest that there are two possible reasons for this increase in reactivity. One reason is that the bacteria experienced increased stress during the film formation process, which triggered them to become less metabolically active and suspend the nitrifying process until the environment became more favorable.\textsuperscript{[39]} Another possible reason is that as the polymer matrix is highly porous, there is space for the encapsulated bacteria to grow during the course of the reaction. Hence, the reactivity increased as a result of a growing number of bacterial cells. This explanation was examined with electron microscopy (see Section 2.3) and found to be at least a contributing factor. The relative contributions of these two effects to the increase in the reactivity cannot be quantified within the limits of this study.

After day 4 (96 h), without a change of media, the increase in reactivity rate slowed and the reactivity leveled out (Figure 4a) as the AOB activity decreased. This leveling of reactivity was not caused by the depletion of ammonia, as suggested by measurements of the concentration of the remaining NH\textsubscript{4}+. At day 6, after 3 d of reaction without a media change, the remaining NH\textsubscript{4}+ in the media ranged from 92 to 128 mg L\textsuperscript{-1} for the three C + WS replicate samples. The initial NH\textsubscript{4}+ concentration for these three samples had an average value of 192 mg L\textsuperscript{-1}. After ruling out the possibility of NH\textsubscript{4}+ depletion, a more likely explanation for this leveling in reactivity is the inhibition effect of NO\textsubscript{3}- on nitrification as it accumulated.\textsuperscript{[22]}
The average N conversion rates of the three replicates over the first 4 d are presented in Figure 4b for the four types of sample. The reactivities are presented in units of mg g\(^{-1}\) d\(^{-1}\), which is the mass of total elemental N in mg produced by 1 g of bacteria in 1 d. These units are used to allow easier comparison with other data in the literature.\(^{[40]}\) Data from only the first 4 d of reaction are considered here, before the reactivity started to level (Figure 4a). From Figure 4b, throughout the time period evaluated, the nondesiccated (C + WS) sample showed higher reactivity than the desiccated samples (3HD). A paired Student’s t-test shows a statistically significant difference (*, \(p = 0.035\), \(N = 3\)) between the reactivity of the two methods on the fourth day of the reaction. The C + WS process, which keeps the bacteria in an aqueous medium, achieves reactivity similar to the control. The 3HD process, which desiccates the coating, results in significantly lower reactivity.

Figure 4c,d provides the dynamics of NO\(_2\)\(^-\) and NO\(_3\)\(^-\), respectively. The NO\(_2\)\(^-\) levels were negligible in the first few days of the reaction, apart from that of the positive control (bacteria in suspension). This is probably because the concentrations of the nitrite produced by AOB were not high enough to initiate the NOB reaction. When the NOB reaction started, the nitrite concentrations started to drop, as the nitrite is consumed by the NOB to produce nitrate. There are many factors that decide the reactivity of AOB and NOB, such as the ammonium concentration, oxygen level, pH, and C/N ratio.\(^{[28]}\) Although it is of interest to evaluate the effects of these parameters, the underlying biology is beyond the scope of this paper, which is focusing on the materials processing.

The reactivity of the positive control was higher than in the C + WS across all times. One explanation for the difference is that the transport of reactants and products was restricted in the biocoatings. Additionally, there is a possibility of cell growth in the positive control, whereas growth was hindered (at least initially) when the bacteria are confined in the biocoatings.

2.3. The Morphology of the Biocoatings after the Reaction

After 6 d of reaction in the bioreactor, the biocoatings were freeze-dried and the surface morphology of the biocoatings was studied using SEM (Figure 5). Figure 5a shows two different phases existing on the surface of the biocoating. The bottom left phase (blue arrow) is the surface of the biocoating, and the phase in the top right (red arrow) is the biofilm formed by the bacteria. In an image of the biocoating phase (Figure 5b), bacteria of different shapes and also halloysite nanotubes can be found scattered on the surface. Latex particles can no longer be seen, and the porous structure observed previously (Figure 3d,e) has also disappeared from the image. The structural change is because of the ongoing process of wet sintering. The bioreactor temperature was set at 30 °C, which is the same used for the wet sintering process during the film formation.
a result, the same mechanism that coalesced the colloidal particles during the film formation process also took place during the bioreaction. There are still a few pores left on the surface of the biocoating which are remnants of the porous coating.

Figure 5c shows the morphology of the biofilm phase that covers the biocoating surface. The morphology of biofilms varies depending upon many factors, which include the species composition and growth stage. The morphology of the natural biofilms formed from our nitrifying bacteria (Figure S4, Supporting Information) exhibit similar characteristics to the biofilms observed in Figure 5c,d. It is worth noting that the morphology of this natural biofilm, as well as those from the literature,[41–44] commonly exhibits an open porous structure, suggesting that is is favored naturally by the bacteria. The porous structure in our synthetic biocoatings offers the benefit of being structurally similar to the natural biofilm.

When considering Figure 5a, it is now reasonable to suggest that what we see there is a biofilm growing on the surface of the biocoatings. We conclude that as the bioreaction takes place, the encapsulated bacteria multiplied and migrated to the surface of the biocoatings through those large pores. Once there are enough bacteria on the surface, they begin to form biofilms on the surface of the synthetic biocoatings. This finding suggests significant bacterial growth, which explains the increase in the reactivity of the biocoatings.

### 2.4. Longevity Study of the Biocoatings

In order to be efficient in applications, a biocoating should maintain its high and stable reactivity over a prolonged period of time. To investigate the longevity of our biocoatings, we studied the reactivity of C + WS samples (with three biological replicates) over a period of 1 month. The NO$_2$-N and NO$_3$-N concentrations were measured every 3 d, immediately before the media were changed. Changing of the media was required to allow sufficient nutrition for the bacteria.

The results show that the biocoatings have a relatively high and stable reactivity over the period of 1 month (Figure 6). The blue columns here show the average value of N conversion rate from the three C + WS replicates. The values of the other replicates follow a similar trend and can be found in Figure S6 in the Supporting Information. In comparison to a positive control of bacteria in suspension (PC), the C + WS biocoating samples had a relatively low reactivity in the first 3 d, which corresponds to the findings shown previously. The reactivity peaked at day 6 and exceeded the positive control. After that, there was a clear drop in the reactivity. This is probably due to the bacteria growth shifting from an exponential phase to a stationary or death phase. From day 15 onward, the bacteria recovered and presented a gradual increase in reactivity over the next few days, with values very similar to the positive control. The N conversion rates of the C + WS samples in this experiment were comparable to those data shown in the previous section (Figure 4b). This result is suggestive of biofilm maturation, but we do not have other direct evidence. The overall longevity of the biocoating is highly promising for applications, as their reactivities continued over a duration of 1 month.

### 2.5. The Effect of Desiccation and Salt on the Reactivity of the Encapsulated Bacteria

In Section 2.2, we demonstrated the greater reactivity of the biocoatings fabricated by the nondesiccating method (C + WS)
compared with the conventional desiccation method (3HD). In making this comparison, there are two major factors that might affect the viability of the encapsulated bacteria: the desiccation time and the presence of added salt in the aqueous phase of the coating. To understand better the independent effects of desiccation and salt on the reactivity of the encapsulated bacteria, comparisons were made in additional experiments. For a valid comparison to investigate the effects of the parameters, all variables were kept the same in these pairs, apart from the one parameter being studied.

To study the effect of desiccation on the reactivity, two other types of sample are investigated. They were fabricated by coagulation (using salt-coated flax) followed by evaporation to remove water for either 1 h (denoted hereafter as C+1HD) or 3 h (C+3HD) at 30°C. These samples are good comparators to C+WS to observe the effects of desiccation, as they underwent gelation in the same way. All three types of sample have particles coagulated by salt, and all three formed a cohesive coating by the end of the film formation process (Table S1, Supporting Information). The only difference is in the amount of the desiccation they experienced. Figure 7a shows that the biocoatings with no desiccation (C+WS) show higher reactivity compared to those with 1 h of desiccation (C+1HD). Meanwhile, the reactivity of the C+1HD samples was higher than that of the fully desiccated coating (C+3HD), which shows barely any reactivity. A paired Student’s t-test finds that the reactivities of C+WS and C+1HD at Day 4 have a significant difference (**, p = 0.005). These results demonstrate the detrimental effect of desiccation on the reactivity of the encapsulated bacteria and point to the advantages of wet sintering.

To study the effect of salt on the reactivity, we next compared the samples made using coagulation with 3 h of desiccation (C+3HD) to the desiccated biocoatings (3HD). Both types of coating experienced the same amount of desiccation (3 h). However, the 3HD sample was not exposed to salt on the flax substrate. The results (Figure 7b) show that the salt has a pronounced negative effect on the reactivity of the encapsulated bacteria. This negative effect is expected, as ions in aqueous solution will create osmotic pressure on the bacteria and consequently reduce their viability. However, the degree of the negative effect is larger than first anticipated, as there was only a minor effect of salt on bacteria viability when the bacteria was cultured in media with high salt concentrations in a preliminary test.

Fortunately, this negative effect could be largely avoided in future development. The amount of salt needed for coagulating a latex depends on the C* value of the latex. Although the latex used here had a C* of 0.05 m of Mg^2+, in future research, a latex with lower C* could be used to allow the reduction of the salt concentration on the substrate. An increased reactivity of the encapsulated bacteria should result.

2.6. Partial Desiccation with Wet Sintering Method

To avoid any negative effect of the presence of salt on the viability of the encapsulated bacteria, we propose here an
alternative way to achieve wet sintering via the **partial desiccation** of the biocoating mixture (Figure 8a–c). In this method, enough water is removed to allow the latex particles to make physical contact with each other, so that the contacting particles can undergo wet sintering. In the random close-packing of spheres, 66% of space is filled.\(^{[45]}\) It is possible—in theory—to remove uniformly the precise amount of the water to create a close-packing of particles, and thereafter allow the particles to be wet sintered in the remaining water. In our experiments, we prepared biocoatings by drying by evaporation for over 1 h followed by 1 h at 30 °C with evaporation arrested to allow wet sintering (designated as 1HD + WS). Gravimetric analysis (Table S1, Supporting Information) revealed that 30% of the initial water was evaporated after 1 h of desiccation and resulted in a solids content in the final biocoating of \(\phi_{fb} = 32 \text{ wt} \%), which is \(\approx 29 \text{ vol} \% \) (see Supporting Information for the defining equations.) Although this value is far lower than the value for random close-packing, it is comparable to the values obtained in colloidal gels made by coagulant dipping.\(^{[20]}\) Furthermore, some of the remaining water was absorbed by and trapped in the flax substrate, which leads to an underestimate of the volume fraction of solids in the biocoating. The formation of a cohesive film suggests that wet sintering does indeed occur (Figure 8d). The microstructure of the coating is relatively porous (Figure 8e). However, the degree of the porosity of this sample is not as high as that of the coagulated sample shown previously.

Figure 8f shows the reactivity of the resulting 1HD + WS biocoatings, in comparison to that of the C + WS biocoatings (using salt). At a glance, the 1HD + WS biocoatings appear to have a higher reactivity throughout 4 d of reaction. However, no statistically significant difference can be observed here. Nevertheless, this result provides some indication for the negative effect of the salt on the reactivity (and presumably the viability of the bacteria). Any negative effect can be avoided by removing the presence of the salt. It is worth remembering here that the reactivity of the biocoatings processed with salt (C + WS) still achieved reactivity comparable to the positive control.

Although 1HD + WS showed a slightly higher reactivity in this experiment, we argue that the coagulation method still offers obvious advantages over the partial desiccation method. In the latter method, it is difficult to control precisely the desiccation to remove the target amount of water throughout, as the coatings tend to dry nonuniformly with the edges drying faster than the center. A strong advantage of the C + WS method is that with the use of coagulation, complex substrates can be covered in a thick coating (as will be presented in the next section). Adequate and uniform partial desiccation is challenging to achieve on complex shapes.

### 2.7. Coating 3D Substrates Using Nondesiccating Method with Dip Coating

Apart from increasing the viability of the encapsulated bacteria and creating porosity in the polymer matrix, the C + WS method offers the advantage over the conventional desiccation method of coating 3D substrates. In conventional coating methods, biocoatings are applied to a planar surface using applicators or Mayer rods.\(^{[38,66]}\) However, with the coagulation method, coatings can be applied to a complex 3D structure. This capability has already been demonstrated in the latex glove manufacturing industry, where a uniform layer of latex is coated onto hand-shaped formers.\(^{[20]}\) In this final section, we demonstrate the ability of coating 3D objects using the C + WS method.

Wooden objects with complex shapes are used as an example for this demonstration. In wastewater facilities, 3D objects...
called carriers (typically made of wood or plastic) are used as substrates on which natural biocoatings grow.[8] Carriers are used to increase the surface area while taking up little space and are suited for a variety of potential applications.

The substrates were dipped vertically into a coagulant (1 M CaCl$_2$ solution) and dried at room temperature. The salt-coated substrates were then dipped into a latex/halloysite mixture (without bacteria) for 10 s and held for 1 h at 30 °C in a closed container at a high relative humidity to allow wet sintering. In a photograph of the resulting coating (left side of Figure 9a), the upper part of the substrate is seen to be coated with a uniform layer of coating. This coating covers the inner surface of the cut-out holes uniformly without blocking them. For comparison, another piece of wooden substrate was directly dipped into the latex/halloysite mixture without first being coated with a layer of salt. In the right-hand side of the photograph (Figure 9a), no coating is apparent by eye.

Optical microscopy images suggest that by directly dipping into the latex, a thin and transparent coating is formed on the surface of the substrate (Figure 9c), while the coating formed by coagulation is thicker and opaque (Figure 9b). The effect of coagulation on the thickness of the coatings was further investigated by measuring the mass of the wet gel (Figure 9d) and the thickness of the resulting coatings (Figure 9e) as coagulant concentration was increased. The mass and the thickness both increase in proportion to the coagulant salt concentration. This preliminary study suggests that the C + WS method combined with dip coating is capable of coating 3D objects. The thickness of the coatings can be controlled by adjusting the concentration of the coagulant. This finding opens up new possibilities for the application of our biocoatings. In the future, we imagine that our nondesiccating method could be used to directly coat biofilm carriers. Those carriers would be ready to use in the reactors with the bacteria already encapsulated in the biocoatings, catalyzing biofilm formation and improving activity, which is often slow and difficult to propagate.

### 3. Summary and Conclusions

We have introduced a new film formation process for the fabrication of biocoatings for applications in wastewater treatment. This nondesiccating process was designed especially for the encapsulation of desiccation-sensitive bacteria. This process consists of two steps: coagulation and wet sintering. As neither of these two steps involves the removal of water, the encapsulated bacteria do not experience any desiccation stress during the film formation process. Desiccation stress is the primary reason for the loss of bacterial viability in biocoatings. Using this method, we successfully encapsulated desiccation-sensitive bacterial species that remained metabolically active and formed biofilm communities. We showed that this method can be used to dip-coat a porous substrate with a complex 3D form, for which a standard dipping method produced a thinner coating.
Our research points to the possibility of coating biofilm carriers, which use their complex form to increase their surface area and protect the biofilms that grow inside.

A benefit of our nondesiccating film formation process is the porous structure it creates. The porous structure allows the unhindered flow of nutrients and metabolites, which is considered to be one of the most sought-after properties for cell encapsulation materials.

We confirmed that desiccation has a negative effect on the reactivity of the biocoatings. When the desiccation time was longer, the reactivity was lower. This finding justifies the logic behind developing the nondesiccating film formation process. However, the coagulant salts had a small detrimental effect on the bacteria at the concentrations that were used. Fortunately, this negative effect can be avoided in future development by using latex having a lower critical coagulation concentration, C*. Another key finding is that there is a clear increase in the bacterial reactivity over time. This increase was caused by the growth of the bacteria and the formation of a complex biofilm on the surface of the biocoatings, as observed in SEM images. This multi-community biofilm formation on our biocoatings is ideal for the application in biofilm bioreactors for wastewater treatment as a means to seed the growth of natural biofilms.

We successfully encapsulated viable nitrifying bacteria in biocoatings using the newly developed nondesiccating film formation method. The bacteria in these “living paints” have a higher reactivity in comparison to coatings prepared by a conventional desiccation process. By encapsulating a high density of nitrifying bacteria (≈5 × 10^12 cells m⁻²), ready for reaction in a bioreactor, we have solved the wastewater treatment problem posed by slow-growing nitrifying bacteria. We anticipate that these synthetic biocoatings can be successfully deployed to transform the wastewater industry and to contribute to meeting the United Nations Global Development Goal of clean water and sanitation. Following the development of our process, bacteria encapsulation by biocoatings is no longer limited to desiccation-tolerant bacteria, with numerous applications in environmental remediation, carbon capture, and biofuels production.⁴⁶

### 4. Experimental Section

**Materials:** The latex is based on a copolymer of butyl acrylate, methyl methacrylate and acrylic acid in a 49.6:49.5:1 ratio. It was synthesized by emulsion polymerization at Clariant and used as received. It was made with an initiator of ammonium persulfate and a surfactant of alkylphenyl oxide disulfonate (Dowfax 2A1, Dow Chemicals). The polymer has a T_g of 17 °C according to differential scanning calorimetry (Q1000, TA Instruments). The latex particles have a size of 120 nm and a zeta potential of −22.73 mV according to analysis by dynamic light scattering (Zetasizer, Malvern Instruments).

The commercially available bacteria product was provided by Novozymes. It is a consortium of nitrifying bacteria that consists primarily of both AOB and NOB. All bacteria used in this study were from the same batch, obtained in a 1 L bottle. It was stored at 4 °C in a cold room for the entire span of this study (6 months). The halloysite nanoclay (diameters of 30–70 nm and nanotube lengths of 1–3 μm) was purchased from Merck. The halloysite was pretreated using a procedure described in the previous publication.²⁷ The flax substrate (FL303510, 420 g m⁻², 2 × 2 twill weave) was purchased from Goodfellow, UK. The cell culture media was made based using a recipe provided by Novozymes (Table S2, Supporting Information). All ingredients of the media were purchased from Merck. The nitrite (range from 0.5 to 25 mg L⁻¹) and nitrate (3–90 mg L⁻¹) test strips were purchased from Merck.

**Preparation of Biocoatings Using Coagulation and Wet Sintering Methods:** For the coagulation process, a piece of fabric with dimensions of 5 × 10 cm was used as the substrate of the biocoatings. 50× concentrated cell media (Table S2, Supporting Information) was used as the coagulant. The substrate was immersed in the 50× media for 10 s, then placed inside a 60 °C drying oven for 2 h to allow it to dry completely. Following drying, 5 g of a freshly prepared latex/halloysite/bacteria mixture (2:2:1 by mass) was dropped onto the substrate and was spread evenly over the surface of the substrate using a pipette tip. Next, the substrate with the wet mixture on it was immediately transferred into a Petri dish and sealed with laboratory film (Parafilm). The enclosed coatings were then placed in a temperature-controlled hot room at 30 °C (relative humidity of 20–25%) for 1 h to allow the coagulated particles to wet sinter. It was then rinsed three times with 50 mL of production media. For each round of rinsing, the substrate with biofilm on it was gently shaken inside the media for 30 s and then left to rest on a bench at room temperature for 10 min.

**Preparation of Biocoating Samples for the Reactivity Test:** All samples that appeared in the results section (prepared according to the parameters in Table 1) were prepared and studied at the same time using the same batch of bacteria to allow direct comparisons between different samples. 25 g of a mixture of latex, halloysite, and bacteria (2:2:1 by mass) was prepared. 5 g was aliquoted for each sample. For samples with the coagulation process (C + WS, C + 1HD, C + 3HD), the substrates were treated with coagulant using the procedure described above. After drying the substrates, 5 g of mixture was dropped onto each substrate and spread over the entire area of 50 cm². Then all samples were transferred into the 30 °C hot room. The desiccated and partially desiccated samples (C + 1HD, C + 3HD, 1HD + WS, 3HD) were placed on poly(tetrafluoroethylene) sheets to dry and form films. The wet sintering samples (C + WS, 1HD + WS) were placed in Petri dishes (BRAND, glass, 150 mm × 25 mm, Merck) and sealed with laboratory film (Parafilm). After film formation, the samples were rinsed three times as described previously. They were then stored overnight in production media at room temperature, before the reactivity study commenced on the second day. The negative controls were treated using exactly the same process as the C + WS samples; the only difference was that no bacteria were added into the mixture. The positive controls were 1 g of

### Table 1. Five different film formation methods for the fabrication of biocoatings.

| Film formation method         | Acronym | Salt on substrate? | Evaporation during film formation | Processing time [h] |
|-------------------------------|---------|---------------------|-----------------------------------|---------------------|
| Coagulation and wet sintering  | C + WS  | Yes                 | Hindered                         | 1                   |
| Coagulation and partial desiccation | C + 1HD | Yes                 | Free                            | 1                   |
| Coagulation and desiccation   | C + 3HD | Yes                 | Free                            | 3                   |
| Standard desiccation          | 3HD     | No                  | Free                            | 3                   |
| Partial desiccation and wet sintering | 1HD + WS | No                  | Free → hindered                 | 1 → 1               |

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bacteria in suspension. They were also left in the 30 °C hot room for 1 h and left at room temperature overnight before the reactivity study.

**Reactivity Test Using Bioreactors:** The reactivity tests were performed on all five samples (Table 1) and repeated three times for three biological replicates. Seven 500 mL Duran bottles were used as the bioreactors for each test (Figure S2, Supporting Information), with each containing 150 mL of reaction media (145.5 mL of deionized water, 3 mL of 50x media, 1.5 mL of 5% NH₄Cl aqueous solution (with a starting concentration of 168 mgNH₄⁺ L⁻¹) and 150 mg of Ca(HCO₃)₂). The accelerating voltage was set to 2.00 kV and the beam current was 0.10 nA. On the second day after the sample preparation, all five samples along with the two controls were added into bioreactors at the same time and the reactivities of the bacteria were monitored for the next 6 d. For biocoating samples and the negative control, the sample was added by suspending the flax substrate by a string and partially immersing it in the media. For the positive control, the bacteria were added by pipetting the bacteria suspension into the reactor and allowing it to mix with the media. The reaction temperature in all cases was set at 30 °C, which is the same temperature as the film formation. A stirring speed of 200 rpm was provided by a multi position stirrer (Cimarec I Poly 15, Thermo Fisher). Home fish tank air pumps (Uniclife Quiet 20, Amazon.co.uk) were used to provide the aeration (0.6 L min⁻¹). Two 1 mL samples (for two technical replicates) were taken from each reactor to measure the nitrite and nitrate concentration twice a day for the first 3 d and once a day from day 4 to day 6. The time points for the sampling were 0, 8, 24, 32, 48, 56, 72, 96, 120, and 144 h (10 sampling points in total). Deionized water was added in the reactors to replenish the evaporated liquid before each sampling. Reaction media were changed every day for the first 3 d (at 24, 48, and 72 h) after the sampling. For the positive control, the media were centrifuged at 3000 g for 5 min to recover the bacteria. The cell pellet was then resuspended and added into the fresh media to continue the reaction.

Small liquid samples were taken from the bioreactors for analysis. The values of nitrite and nitrate concentration were read using a reflectometer (RQflex 20, Merck). The method used separate test strips for nitrite (range of 0.5–25 mg L⁻¹) and nitrate (range of 3–90 mg L⁻¹). The reflectometer was used to record the change in color and to correlate it with concentration. The total elemental N concentration (in ppm) was found from the concentration of nitrite (Cₙitrite) and nitrate (Cₙitrate) by the formula Cₙitrite × 0.304 + Cₙitrate × 0.226. Samples were diluted as needed. The mean value of two measurements of each solution (technical replicates) was used.

**Scanning Electron Microscopy:** Samples were freeze-dried to preserve the structure of both the bacteria and the latex matrix. Biocoatings were first placed overnight in a freezer at −80 °C. Then they were transferred into a freeze drier (Modulyo, Edwards Vacuum, UK) and freeze-dried overnight. After the freeze drying, the samples were mounted onto SEM stubs and gold coated (Q 150 V ES Plus, Quorum, UK) with 3 nm of gold. Electron microscopy was performed using an Apreo 2 SEM (Thermo Fisher) to study the surface morphology of the biocoatings. The accelerating voltage was set to 2.00 kV and the beam current was set to 0.10 nA.

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Data Availability Statement**

The data that support the findings of this study are openly available in Figshare at https://doi.org/10.6084/m9.figshare.20805937.

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bacteria, biofilms, film formations, nitrification, wastewater treatments

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