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Massachusetts Institute of Technology

Degradation of Regenerated Silk Fibroin in Soil and Marine Environments

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KEYWORDS – silk, degradation, sea, water, soil, bacteria, environment

ABSTRACT

There is a compelling need to find new materials that meet stringent performance requirements for application in food, water and agriculture industries while addressing biodegradability, circular life cycle, and sustainable sourcing at scale. Regenerated silk fibroin (SF) is a structural biopolymer with applications in biomedicine, optoelectronics, food, water, and agriculture. Extracted from largely available *Bombyx mori* cocoons through a water-based process, SF is fabricated into advanced materials that have competitive performance and merits of natural origin and non-toxicity. As a protein, SF is considered slowly degradable in the human body but as a material it is known to be environmentally stable, and its biodegradation is mostly unknown. In this study, the degradation of SF in different soil and water environments is investigated. The effects of SF polymorphism, ionic strength, and presence of microorganisms on the proteinaceous material degradation are investigated. Modulation of beta sheet content allowed to control the degradation rate of SF films in soil of increasing NaCl concentration. Microbial activity was a key driver for silk degradation in different environmental conditions. Bacterial colonization accelerated silk film degradation, process that was further enhanced by encapsulation of bacteria in SF materials at the point of material assembly. Together, these data show that SF biodegradation can be controlled by material design and by regulating the interaction with microorganisms present in the environment.

INTRODUCTION

The development of new materials that combine performance with mitigation of environmental impact is an instrumental step to address the challenges that humanity will face in the next few decades.[1–3] Principles of sustainability, green chemistry, biodegradation and circular life cycle have been defined as key elements in reducing environmental pollution and decreasing greenhouse gas emissions while enhancing quality of life. Nonetheless, the ever increasing human population pressures the AgroFood, water and energy infrastructures to rapidly rise their outputs using already available, cost efficient, technological solutions, which are mostly based on linear materials and resource models that follow make-take-discard practices.[4,5] To challenge these systems, several new policies are forcing stakeholders to transition to new technological solutions that minimize environmental impact. In 2019, the European Chemicals Agency (ECHA) proposed a wide-ranging restriction on intentional uses of microplastics in products placed on the EU/EEA market to avoid or reduce their release to the environment, with non-biodegradable intentionally added microplastics (IAMPs) foreseen to be completely banned from the market in 2025.[6,7] These laws require to include principles of pre-determined biodegradation in polymer design and as a result new materials are under investigation to substitute the current standard materials used in IAMPs for applications in cosmetics, household chemicals, and agriculture, with the opportunity to expand the applications to other formats such as membranes, films, thermoformed etc. While new monomers and polymers that may favor a circular life cycle are investigated, an attractive option is to provide new scopes for structural biopolymers extracted from natural fibers or from the waste of the food and agriculture industry.

Natural polymers are generally abundant, non-toxic, and biodegradable and possess chemical and physical properties that allow for nano- and micro-fabrication.[7] Of particular interest is silk,

a 5000-year-old textile fiber that is increasingly studied as biomaterials for biomedicine, optics, photonics, water filtration, food and agriculture.[8–11] Silk fibroin (SF) is the structural protein present in silk fibers. SF is available from sericulture through the transformation of mulberry tree leaves in cocoons by the 5th instar larva of the *Bombyx mori*. In 2020, $1.1 \cdot 10^5$ tons of silk cocoons have been produced globally (0.1% of the whole fiber production market), with a percentage of 15-35% considered unreelable or pierced. SF fibers are renowned for their low diameter (brin size of circa 10 µm), high flexibility, non-toxicity, and mechanical strength, with applications from high-quality gowns to parachutes and suture threads. For applications beyond textile, silk cocoons (both textile grade and unreelable) can be regenerated in a water suspension similar to the dope present in the gland of the *Bombyx mori* larvae.[12–15] In this state, SF can be considered as a water-soluble polymer that can be nano- and micro-fabricated into several materials such as coatings, microparticles, nanofibers, prints, hydrogels, foams, films, membranes, photonic crystals, and hierarchical materials. The assembly process of SF molecules generally occurs in water at mild temperature and pH conditions, allowing for the encapsulation of dopants, e.g. enzymes, cells and microorganisms, nanoparticles, bioinorganics, and biomolecules that add orthogonal functionalities to the final material format.[16] Additionally, SF polymorphism, i.e. folding of the protein in different molecular structures that range from random coils to beta-sheets, regulates material solubility in water: disordered SF is readily water soluble, while an ordered folding of the protein makes it water insoluble and more stable in the environment.[17]

Due to the economic importance as textile, many previous studies evaluated the degradation of silk fibers under different environmental conditions.[18] UV irradiation leads to the cleavage of chemical bonds in the protein chains, which creates free radicals that further degrade the proteinaceous material, ultimately decomposing the protein into short peptides.[18,19] High

humidity and high temperature conditions are also known to accelerate silk fibers loss in molecular weight and tensile strength through oxidation and hydrolysis.[18] Mazibuko et al. have also reported silk fiber degradation in soil and suggested that the textile degradation was mostly the result of microbial activity.[20] Another interesting case is the maritime environment; in 1840 Major-General Charles Pasley, a Colonel of the Royal Engineers, recovered silk garments of satin weave from the HMS Royal George, which sunk in 1782.[21] The silk was "intact and perfect", while leather was found only in pieces and no woolen clothing was recovered in the wreckage.[21]

Despite the extensive body of knowledge on degradation of silk textile in the environment, little is known on biodegradation of regenerated SF (SF), except for the *in vivo* studies.[22–24] This is surprising as applications of SF are rapidly expanding towards nanofibrillar matrices for water filtration, coatings to extend food shelf life, films for transient electronics, and microneedles for foodborne pathogen sensing and for delivery of bioactive payloads in plants.[25–33] As SF is poised to become a technical material, understanding its biodegradation in different environmental conditions becomes paramount to embed the material life cycle as a design parameter and to comply with emerging policies that require polymers to be biodegradable with pre-determined rates of mass loss. In this study, we provided a first general attempt to understand mechanisms of SF degradation in the environment. Using the film format as a model, we investigated SF degradation in soil, freshwater, and sea water, as a function of salt (NaCl) concentration, microbial activity, and silk polymorphism. The findings provide critical knowledge to design silk materials with pre-defined performance (e.g., mechanics, degradation rates) in relationship to the environment in which they are deployed and to meet new environmental guidelines for polymeric materials.

EXPERIMENTAL SECTION

An overview of the silk film degradation studies conducted in several environmental conditions is depicted in **Figure 1**.

Preparation of silk solutions. *Bombyx mori* silk fibroin solutions were prepared according to previously published procedures by Rockwood et al.[16] Cocoons were boiled for 45 min in an aqueous solution of 0.02 M Na_2CO_3, and then rinsed thoroughly with water to remove sericin proteins. The extracted silk fibers were dissolved in a 9.3M LiBr solution at 60℃ for 4 hours. This solution was then dialyzed against Milli-Q water using Slide-a-Lyzer dialysis cassettes (3.5 kDa MWCO) for 72 h. The resulting SF suspension was then purified by centrifugation at 9000 rpm (~12,700g) over two 25-minute-long periods at 4℃.[16]

Preparation of silk and CIAT 899 solution. Suspensions were prepared by mixing gram negative plant growth promoting rhizobacteria's (*Rhizobium tropici* CIAT 899 Martinez-Romero et al. - ATCC 49672) with silk fibroin. 50% tryptic Soy Broth (Becton Dickinson, Franklin Lakes, NJ, USA) was prepared by adding 500 ml of H_2O to 2.5 g Bacto-peptone (Soybean-Casein Digest Medium) (Becton Dickinson, Franklin Lakes, NJ, USA), 1.5 g Yeast extract and 0.7 M $CaCl_2$ (autoclaved, working concentration dilute 100X) . The media was autoclaved for 60 min at 121ºC. CIAT 899 was sourced and cultured in a shaker incubator at 200 rpm and 30ºC up to an OD600 measure of 1. Once bacteria reached an OD600 of 1, 10 ml of bacteria broth solution was centrifuged at 4300 rpm for 20 min. The bacteria formed a pellet, and the supernatant was discarded. Lastly, 10 ml of 6 wt% silk fibroin was pipetted into the pelleted bacteria strain and uniformly mixed by thoroughly pipetting up and down.

Silk fibroin film fabrication by drop casting. 1 ml of ~7 wt% silk solution was cast on polydimethylsiloxane (PDMS) sheet to obtain regular silk fibroin films. The film physico-

chemical and mechanical properties have been extensively studied previously.[34–36] For silk fibroin films embedded with *rhizobium tropici* CIAT 899, CIAT 899 was grown and centrifuged as reported above. The CIAT 899 pellet was mixed with silk fibroin solution and was cast on PDMS. The films were air-dried in a biological hood to control the drying rate. Once dried, water insoluble silk fibroin films with a thickness of 80± 10 μm and diameter of 31 ± 2.9 mm. were also prepared by the water annealing treatment. Water annealing (enhancement of beta-sheet content) was achieved through exposure of silk fibroin films to water vapors under vacuum at 22℃ for 3 hours, 6 hours and 9 hours as required to obtain an average beta sheet of content of 44%, 50% and 54%, respectively. Water vapor drives the crystallization (beta sheet formation) of silk fibroin.[37] .

Degradation in soils. Dulytek premium nylon 100-micron mesh bags were loaded with 6 hour annealed silk fibroin films, unless stated, and sealed. The bags were then placed in soil with 25% water content at standard room conditions. Soil moisture was maintained by watering the soil every third day. The sealed bags were carefully taken out with tweezers at relevant time points. The samples were rinsed with distilled water and air dried for 48 hours. During the drying process, samples were repeatedly weighed and no further water loss was consistently measured after 48 hours of drying. The degree of degradation was determined gravimetrically at t=1, 2 and 4 weeks. Initial sample mass was determined before degradation and final sample mass was determined after degradation. n=5 samples were used for soil degradation studies and for each time point different samples were used. Two factors were tested in the soil degradation studies. 1. Effect of silk film beta sheets content; 2. effect of varying soil salinity by varying NaCl concentration in soil (0 mM, 50 mM, 100 mM and 200 mM).

Degradation in bacteriostasis was also investigated, by mixing 2.5% sodium azide solution into the test soil (ml per g of soil) to inhibit microbial activity

Degradation in water environments. The mass of silk fibroin specimen was noted and samples were added to constant volumes of NaCl solution. The silk specimen mass to NaCl solution volume were kept constant for all experiments and repeats. Measurements of dissolved silk fibroin (protein) in NaCl solution was used as a proxy for silk fibroin film degradation. The total loss of mass from silk fibroin specimens would give a total protein content (100%) in NaCl solution. Using a BCA Protein Assay kit (Sigma Aldrich), the protein content was measured for each sample considered. For every experiment, n=3 was used. Seawater was purchased from Worldwide Imports AWW84130 Live Nutri Seawater. The following NaCl solution concentrations were used: 0 mM, 50 mM, 100 mM and 200 mM as proxy for fresh and salty water.

Polysaccharide detection test. The anthrone test was performed to detect carbohydrates in degraded SF film samples.[38] SF films and SF films with encapsulated *R. tropici* were compared before and after 30 days of degradation in soil (n=5). . The films were tested before and after 30 days of degradation in soil (n=5). After 30 days of silk film degradation in soil, the films were carefully removed from the filter bags and cleaned with a blotting paper to get rid of any remaining soil residue. Each sample was weighed and added to a vial with 1 ml of distilled water. 5 ml of anthrone reagent (2 mg/ml) dissolved in conc. sulfuric acid was added to each vial and heated at 90°C while stirring with magnetic stir bars at 400 RPM for 17 minutes. The optical density of each sample was measured using a spectrophotometer at a wavelength of 620 nm (OD620) and compared to a standard curve prepared by testing standard samples with known glucose concentrations.

Protease activity. *R. tropici* was grown in a broth solution as previously described. Once the bacteria reached an OD600 of 1, 1 ml of the broth solution was centrifuged at 4300 rpm for 20 minutes and the pellet was resuspended in 30 ml of PBS solution in a 50 ml centrifuge tube and incubated at room temperature. In 3-day intervals, 500 µl samples were removed from the tube, centrifuged at 4300 rpm for 10 minutes, and the supernatant was used to measure protease activity using the EnzChek Protease Assay Kit (E6639 ThermoFisher Scientific). The fluorescence-based assay contains casein derivatives that release red fluorescent dye-labeled peptides through a protease-catalyzed hydrolysis, where the increase in fluorescence is proportional to protease activity. Protease activity in units/ml can be quantified by comparing against a standard curve of a protease with known activity. Fluorescence was measured on a plate reader with excitation and emission wavelengths of 590 nm and 645 nm, respectively.

pH changes. A drop of universal pH indicator (1.09175 Sigma Aldrich) was added to the silk solution before the drop casting step when preparing SF films and SF films loaded with *R. tropici*, as described earlier. The films were loaded in mesh bags and placed in soil as described earlier for degradation in soil. The films were taken out at relative time points for up to 14 days (t = 0,2, 4, 8 and 14 days) and the color changes due to the pH indicator were observed.

Fourier Transform Infrared Spectroscopy (FTIR). Drop cast films (thin films formed by dropping silk onto a flat surface followed by evaporation of the solution) were analyzed using Thermo Fisher FTIR6700 Fourier Transform Infrared Spectrometer through attenuated total reflection (ATR) with a germanium crystal. For each sample, 64 scans were coadded with a resolution of 4 cm^{-1}, at wave numbers between 4000 and 650 cm^{-1} . The background spectra were collected under the same conditions and subtracted from the scan for each sample.

Scanning Electron Microscopy (SEM). Drop cast films were freeze cracked after being dipped in liquid nitrogen and analyzed with a Zeiss Merlin High-resolution scanning electron microscope. Samples prepared did not charge, therefore no gold plating or any preparation of samples was required. An EHT of 1.00kv was used with a 100pA probe.

Nanoindentation. Nanoindentation measurements were performed on a Hysitron TriboIndenter with a nanoDMA transducer (Bruker). Samples were indented in load control mode with a peak force of 500 μN and a standard load-peak hold-unload function. Reduced modulus was calculated by fitting the unloading data (with upper and lower limits being 95% and 20%, respectively) using the Oliver-Pharr method and converted to Young's modulus assuming a Poisson's ratio of 0.33 for all samples. Each type of sample was prepared and indented in triplets to ensure good fabrication repeatability. For each sample, indentation was performed at a total of 49 points (7×7 grid with an increment of 20 μm in both directions) to ensure statistical reliability of the modulus measurements.

Statistical Analysis. One-way ANOVA to determine statistical significance differences in groups with one independent variable. Two-way ANOVA was used to determine how two factors affected the response variable. For experiments in solution, the independent variable was NaCl concentration. For experiments in soil, NaCl and beta-sheets content were the independent variables. Post-hoc Tukey test was used to investigate significant difference in the means ($p<0.05$) of the different groups considered.

RESULTS AND DISCUSSION

In nature, cocoonase is indispensable for Lepidoptera insects breaking the sealed cocoon and it is believed to be the most effective enzyme that can digest silk materials.[39] As human body lacks cocoonase, studies of SF biodegradation have mostly focused on in vivo resorption of the protein

post-implant to elucidate the host-biomaterial interactions for applications in regenerative medicine and drug delivery.[22–24] These studies were conducted also in response to the definition of silk fibers as non-biodegradable material by the United States Pharmacopeia, due to the negligible tensile strength loss *in vivo* of silk suture threads in the first six months post implant. Despite this definition, it is nowadays accepted – and supported by an extensive body of literature – that silk materials are biodegradable. Several studies have, in fact, shown that hydrolysis catalyzed by enzymatic activity is the major driving force for protein resorption in the body, with gelatinase, chymotrypsin, trypsin and collagenase I being the most effective proteases that our body produces to digest SF materials.[40,41] Additionally, Ghezzi et al have shown, in a multipocketed stroma rabbit model, how SF films polymorphism and the protein molecular weight are the main parameters that modulate degradation rate, *in vivo*.[22] Nonetheless, biotic and abiotic factors present in environments such as soil and water are very different from the human body, with major distinctions being water content, temperature, pH, microorganisms, salt concentrations and mechanical stresses. Studies that generally modeled SF materials' degradation were conducted with protease XIV, proteinase K, and papain and have shown SF biodegradation at different pH, temperatures and when the protein is fabricated in different material formats.[40,42]

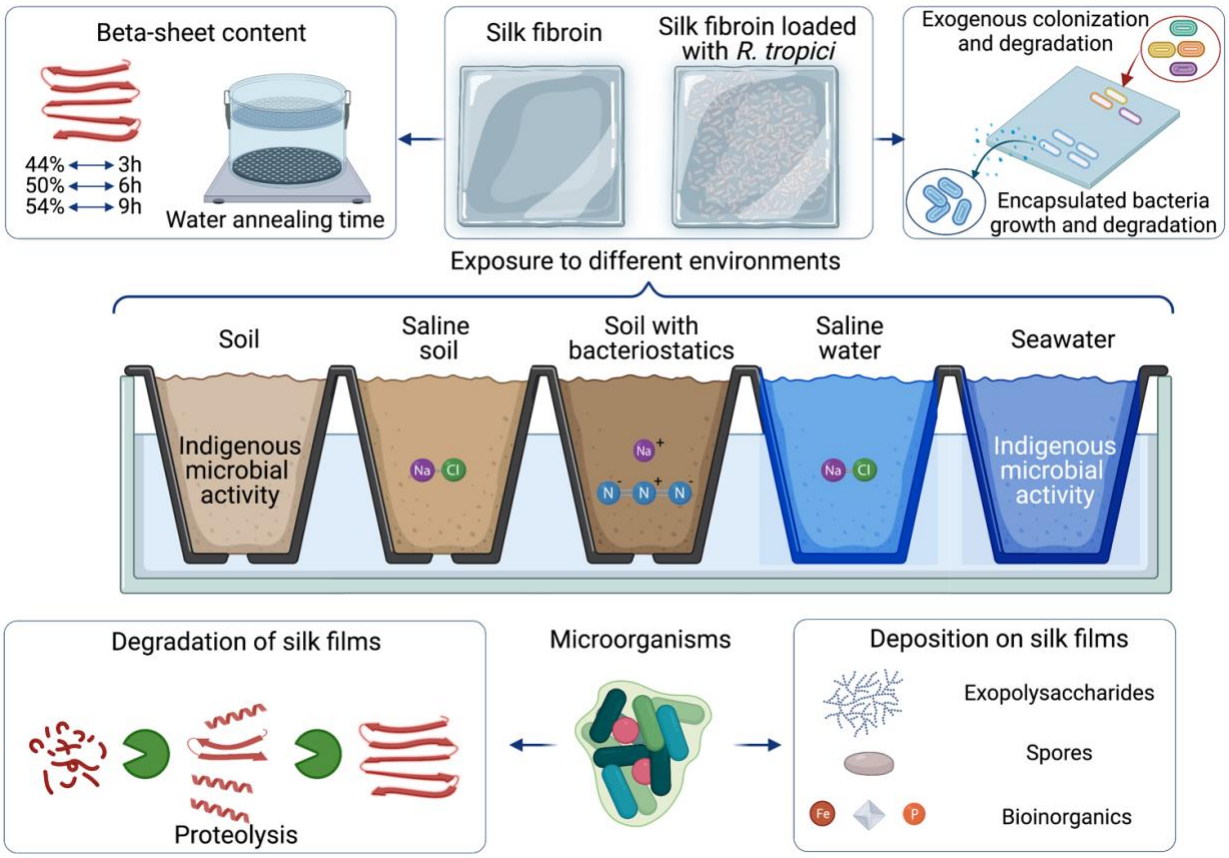

Figure 1. Schematic of silk film degradation in several environmental conditions. Parameters explored include protein's beta-sheet content, encapsulation of rhizobacteria, sodium chloride concentration, soil and water milieux. Resuscitation of encapsulated bacteria (i.e. indigenous) and exogenous colonization result in film degradation and deposition of exopolysaccharides, spores and bioinorganics.

Despite this extensive body of knowledge, there is a lack of understanding of degradation phenomena of silk materials in environments such as soil and water. To address this need, we investigated silk film degradation in soil and water as a function of NaCl concentration, microbial activity, and silk polymorphism, as summarized in **Figure 1**. We used the film format due to the ease of fabrication, reproducibility of material properties and wide interest for end material applications such as coating, membrane, packaging, and substrate for transient electronics.[28,30–32,43,44] SF polymorphism was modulated using water annealing post processing, which allows to slowly drive a disordered to ordered conformational change in silk fibroin molecules from random coils to beta-sheet structures thorough exposure to water vapors.[45] SF

films with a thickness of 80± 10 μm were exposed to water vapors for 3, 6 and 9 hours to obtain an average beta sheet of content of 44%, 50% and 54%, respectively. To investigate the effects of microbial activity on the degradation profile, *Rhizobium tropici* CIAT 899 – a strain of endosymbiotic, nitrogen fixing bacteria commonly used as biofertilizer for legumes – were encapsulated in SF film at the point of material assembly.[32] The effects of CIAT 899 encapsulation on SF degradation in soil was compared to negative controls composed of neat SF films and was studied as a function of soil NaCl concentration, presence of bacteriostats, and protein beta sheet content. Additionally, SF films degradation was studied in water at increasing sodium chloride concentration and in sea water.

Figure 2 summarizes the influence of beta sheet content on SF film degradation in soil. ANOVA test conducted on measurements of weight loss over time indicated that a statistically significant difference of material degradation occurred across time ($p<0.05$) and across different beta sheet content ($p<0.05$). At week 4, silk fibroin films lost between 50 and 60% of their original weight. At week 8, the films were heavily degraded (between 8 and 20% of the original weight remained) and lost their structural integrity (Figure 2a). SEM was used to investigate SF films morphology at week 4 of exposure to soil.

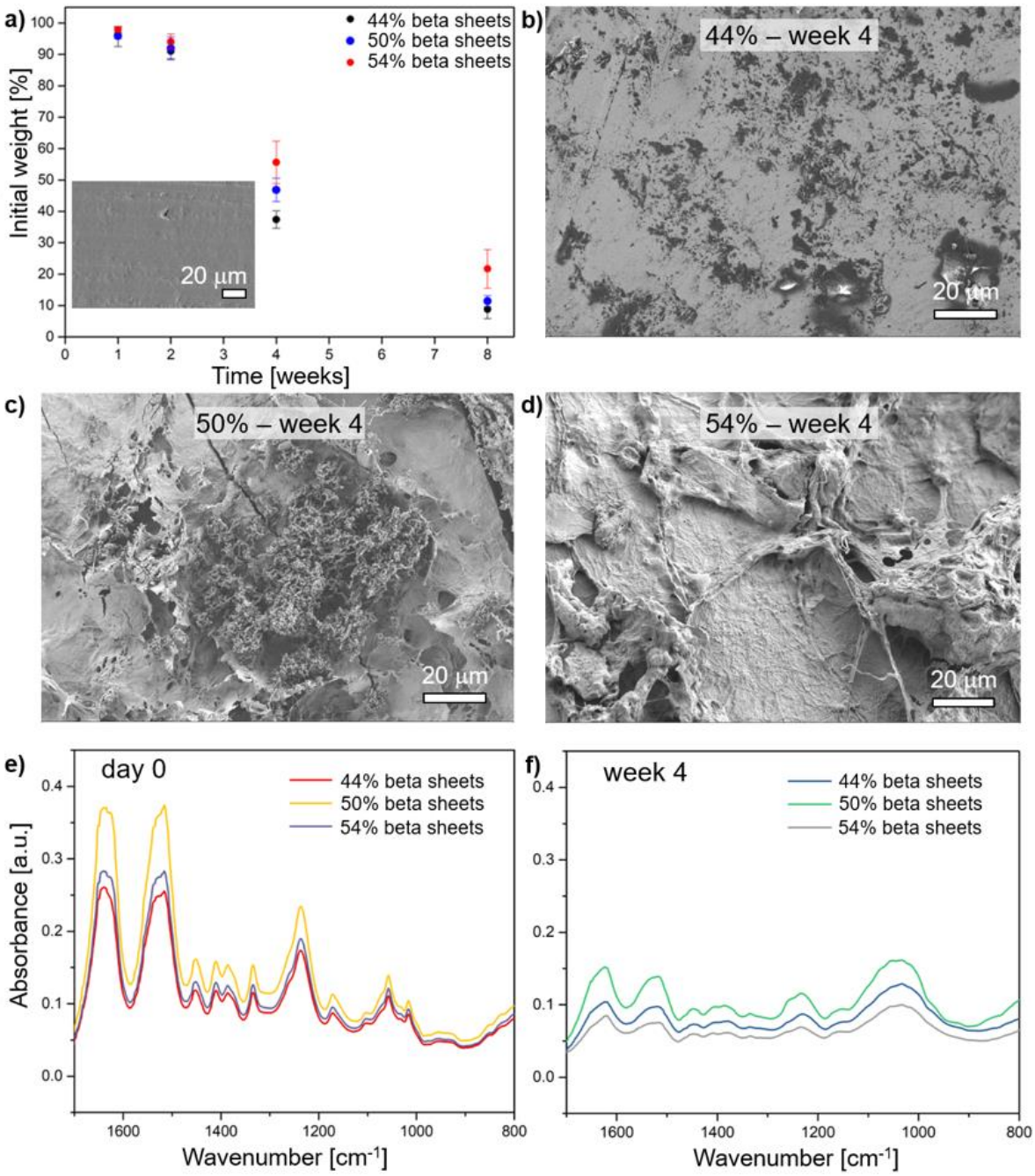

Figure 2. Influence of beta sheet content on silk fibroin film degradation in soil. Beta sheets content in silk fibroin films was modulated with post-processing water annealing. a) Silk fibroin film degradation profile as a function of different beta sheets content. Statistically significant difference of films degradation occurred across time ($p<0.05$) and across different beta sheets content ($p<0.05$). b-d) SEM images of silk fibroin film with increasing beta sheet content at week 4 in soil. When compared to a 50% beta sheet silk film at day 0, SEM image inset in panel a), silk films showed surface erosion and deposition of exogenous materials. Samples at week 8 were too degraded to be analyzed. e-f) ATR-FTIR analysis of silk fibroin films of increasing beta sheets content at (e) day 0 and (f) week 4 in soil. Amide I (1700-1600 cm-1) peak is dominated by random coil (1643 cm^{-1}) and beta sheet (1622 cm^{-1}) vibrations. The fingerprint region of silk is in the (1200-900 cm^{-1}) is visible at day 0 but altered at week 4. C–C and C–O stretching and CH_3 bending vibrations in the 1100-1000 cm^{-1} region present at week 4 are typical of polysaccharides.

For all the levels of beta sheet content considered, SF films showed surface degradation and deposition of exogenous material (Figure 2b-d). SF films at day 0 are in fact generally smooth with no features on the surface identifiable at the micrometer scale (Figure 2a, inset). We speculate that deposition of exogenous material and surface erosion can be attributed to microbial activity. To further investigate chemical deterioration of the films surface post treatment in soil, we conducted ATR-FTIR analysis on silk films of increasing beta sheet content at day 0 (Figure 2e) and compared the spectra with the one obtained at week 4 (Figure 2f).[46] Amide I (1700-1600 cm^{-1}) analysis of samples at week 4 depicted an increase in beta sheet content (1622 cm^{-1}) when compared to the day 0 controls.[46] Silk fibroin degradation occurs in molecules that have a non-ordered configuration (1643 cm^{-1}), first. Hence, the contribution in the Amide I of non-ordered fibroin molecules is reduced over time, leaving silk with mostly beta sheet structure. The fingerprint region (1200-900 cm^{-1}) of silk fibroin is also altered at week 4 due to the deposition of exogenous biopolymers, e.g. exopolysaccharides (EPS) symmetric CH_3 bending in the 1100-1000 cm^{-1} region.[47] Surprisingly, silk films developed a slight pinkish color at week 4 (Supporting Figure S1), which may be due to deposition of bioinorganics on the surface of silk. ICP-MS analysis of silk samples at week 4 of soil treatment showed in fact accumulation of iron in silk films (Supporting Table S1).

The influence of biofertilizers, sodium chloride concentration and bacteriostasis on silk fibroin film degradation was also studied in soil (**Figure 3**). *Rhizobium spp*. are soil-dwelling α-Proteobacteria that can fix nitrogen in a symbiotic relationship with leguminous plants and that can boost seed germination and mitigate biotic and abiotic stressors.[48] As biofertilizers for precision and restorative agriculture, *Rhizobium spp*. hold a high potential as their use would decrease the need to deploy nitrogen fertilizers, the production of which accounts for 1-2% of the

world energy consumption and ~3-5% of the natural gas produced globally.[49,50] However, preservation of *Rhizobium spp*. outside the soil is challenging.[51] Building on previous studies that have reported *Rhizobium* encapsulation in silk materials as an effective strategy to preserve and deliver biofertilizers, we encapsulated *Rhizobium tropici* CIAT 899 in silk fibroin films to study the effects of indigenous bacterial activity on material degradation when compared to silk fibroin film controls (Figure 3a and b, respectively).[32]

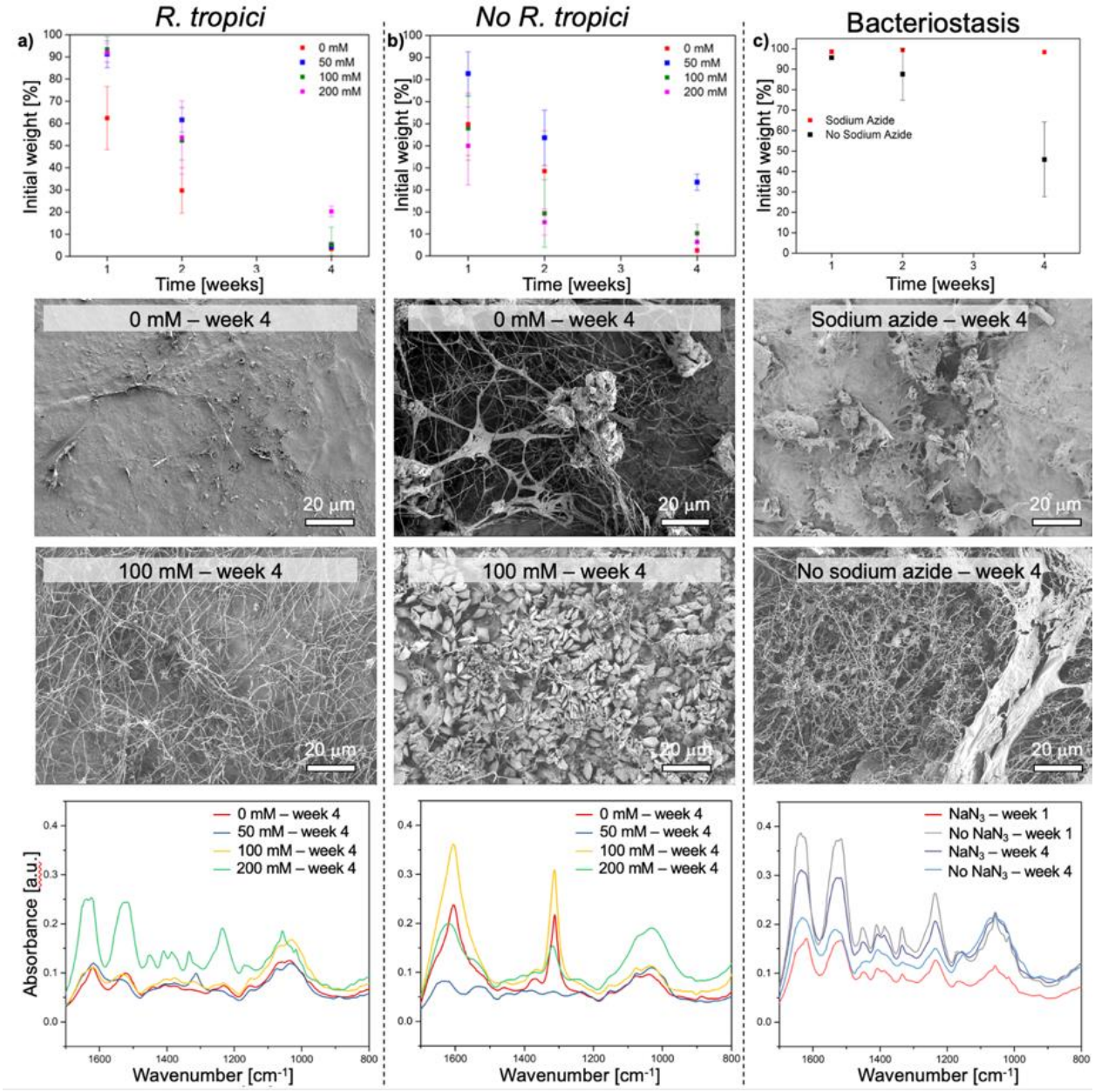

Figure 3. Influence of biofertilizers, soil sodium chloride concentration and bacteriostasis on silk fibroin film degradation in soil. a-b) Encapsulation in silk fibroin films of (a)biofertilizers such as *Rhizobium tropici* accelerates material degradation when compared to (b) silk fibroin film controls. Soil sodium chloride concentration also affects silk film degradation over time ($p<0.05$). ATR-FTIR spectra suggest the deposition of exopolysaccharides on the surface on silk films. c) Effect of bacteriostasis on silk film degradation. When sodium azide (NaN_3) is added to soil, silk fibroin degradation is hindered, indicating that silk degradation in soil is prevalently microbial ($p<0.05$).

R. tropici were encapsulated in silk films at the point of material assembly and preserved in dry state. As saprophytes, rhizobia survive in a complex microbial community by adopting an oligotrophic lifestyle.[52] Upon exposure to the moist soil, *R. tropici* activity increases and it can be speculated that proteases released by the bacteria and local changes in pH induced by bacterial activity accelerate silk fibroin degradation.[53,54] To test this hypothesis, we monitored over time the expression of proteases in *R. tropici* cultured in PBS and found a time-dependent increase in proteolytic enzymes content in the bacterial culture media (Supporting Figure S2a). A one-way ANOVA test was performed and showed a significant difference between the groups, followed by a Tukey test which showed a significant difference ($p<0.05$) between day 0 and day 9, as well as between day 3 and day 9. The linear increase in the protease activity of *R. Tropici* may indicate that *R. tropici* constantly express proteases. Additionally, we studied pH changes in silk films encapsulating *R. tropici* and degrading in soil by adding a universal pH indicator to the silk materials at the point of film assembly. The universal pH indicator dye is incorporated in the films and undergoes a color change when the pH of the environment shifts. Over time, the dye indicated that the presence of *R. tropici* in silk films generated a more acidic environment, when compared to the films left in soil with no bacteria encapsulated (Supporting Figure S2b). Also, the encapsulation of non-motile bacteria in SF films may have induced a bulk degradation when compared to surface erosion that occurs during the exogenous colonization of silk fibroin films exposed to soil microorganisms. *R. tropici* encapsulated films were in fact more degraded over time compared to control films, when there was no NaCl treatment. Anthrone test was used to quantify the amount of carbohydrates present in silk films degraded in soil for 30 days when

compared to the as made ones. The carbohydrates content for silk films with and without *R. tropici* was also measured. The amount of carbohydrates was statistically significant higher ($p<0.05$) after 30 days of exposure to soil and in silk films containing the biofertilizer ($p<0.05$) (Supporting Table S2). As the increased presence of carbohydrates in silk films degrading in soil can be due to the deposition of EPS, SEM analysis was performed on the surface of silk films and showed the deposition of nanofibrillar matrices on the surfaces of silk films loaded with *R. tropici*. These matrices were identified as biopolymers rich in saccharides from FTIR analysis (stretching vibration of the carboxylate group at 1700-1500 cm^{-1}, and C–C and C–O stretching and symmetric CH_3 bending in the 1100-1000 cm^{-1} region). Together, these analyses suggest that EPS were deposited on silk films. Nanofibrils were more evident on silk films at higher soil sodium chloride concentration (Figure 3a), in agreement with previous studies that showed an increased EPS synthesis in response to abiotic stressors.[47] Interestingly, the samples that were not loaded with *R. tropici* and exposed to soil at increasing sodium chloride concentration showed a more prominent deposition of nanofibrils on the film surface at week 4, when compared to the samples loaded with the biofertilizers, even though film degradation remained comparable to R. *tropici* films at 100 mM NaCl concentration. This result may suggest that an exogenous colonization on the surface of the silk films was more prominent when endogenous *R. tropici* were not encapsulated in the degrading materials or that the presence of biofertilizers in silk films partially inhibits nanofibrils deposition on the films' surface (Figure 3b). Nonetheless, more studies are necessary to elucidate microbial communities' interactions at the silk-soil interface. Sodium chloride concentration affected silk film degradation over time ($p<0.05$) and SEM and ATR-FTIR analyses indicated the deposition of nanofibrillar biopolymers on the surface on silk films. Additionally, at high sodium chloride concentrations (i.e. 100mM),

electron microscopy revealed the presence of spores on the surface of the films. The experiment of *R. tropici*-loaded SF films degradation in soil was repeated upon addition of a bacteriostatic agent such as sodium azide (NaN_3) to soil (Figure 3c). Sodium azide is a very common bacteriostat that inhibits most gram-negative bacteria, including *R. tropici*, but that is less effective with gram-positive bacteria (streptococci, pneumococci, lactobacilli, etc.).[55] Exposure of *R. tropici*-loaded SF films resulted in a decrease degradation rate and a reduced deposition of nanofibril lar biopolymers on the surface of the silk materials. Together, these experiments showed that SF film degradation in soil is driven by microbial activity and that the degradation profile is influenced by biotic (i.e. microbial communities) and abiotic (i.e. salt concentration) environmental parameters as well as silk properties (i.e. polymorphism). In particular, NaCl concentration – for the ranges studied - positively affected bacterial-driven degradation, also indicating an interplay between the material, biotic and abiotic parameters. A higher NaCl concentration may in fact promote the biological activity by maintaining a suitable osmotic pressure in the soil. However, future studies will be required to better understand the effects of soil salinity on the proteolytic activity of microbial communities in soil.

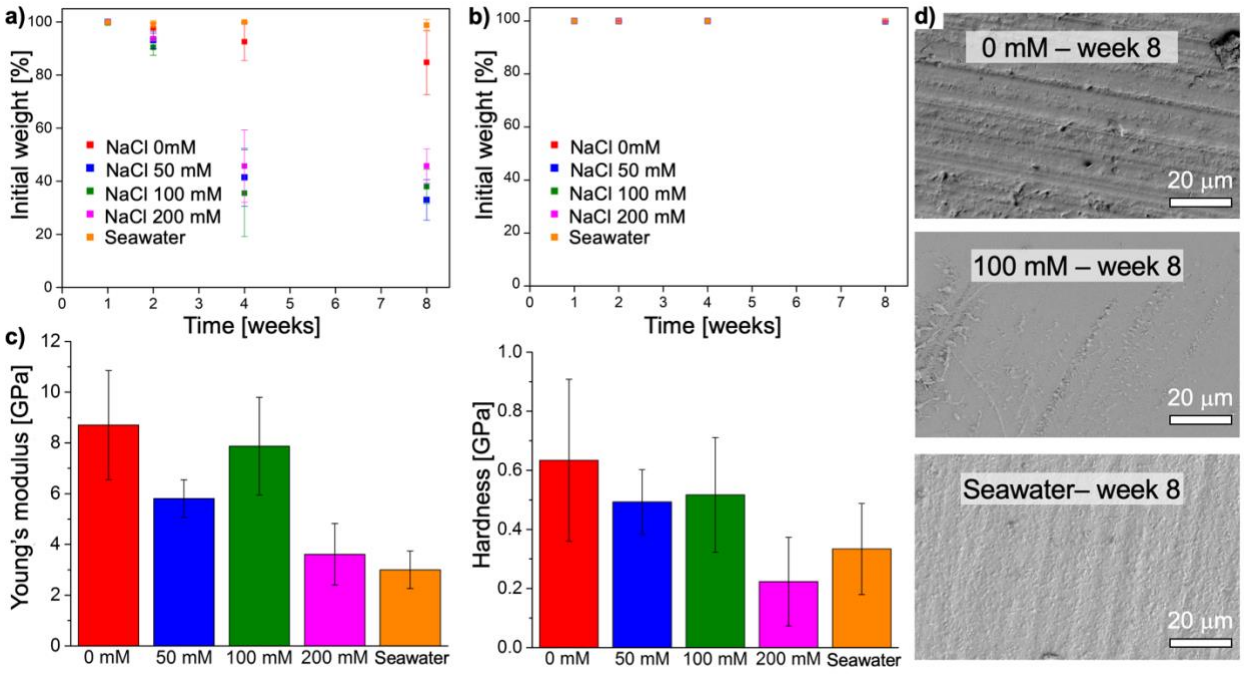

Figure 4 – Silk fibroin film biodegradation in water environments. Effects of increasing sodium chloride concentration on silk fibroin film biodegradation in water for films a) loaded with *R. tropici* and b) not loaded with the biofertilizers. Seawater and deionized water are used as controls. c) Negligible weight loss was measured in seawater for the 8 weeks of the study, although a statistically significant decrease ($p<0.05$) in the mechanical properties (i.e. Young modulus and hardness) of the films was measured through nanoindentation, when compared to films at week 8 of exposure to 0, 50 and 100 mM of NaCl solution. d) SEM microscopy of *R. tropici*-loaded silk films at week 8.

There is an increasing interest in investigating the degradation of polymers (synthetic and natural) in water. From an environmental impact perspective, accumulation of synthetic materials in freshwater and seawater produces 4.8-12.7 million tons of plastic waste every year, with 80% of marine debris originating from land and being transported to seas via water streams.[56,57] Additionally, biodegradation of biopolymers (typically polysaccharides such as alginate, chitin, and starch) in freshwater and marine environment by heterotrophic microbial communities has global implications on biogeochemical carbon cycles and its effects on climate change. For structural proteins, biodegradation of ubiquitous tissue components like collagen is well documented, while little is known on the hydrolysis rate of silks synthesized by arthropods to perform in freshwater and seawater (e.g. spider silk of aquatic spiders *Argyroneta aquatica* and *Desis marina*, respectively). **Figure 4** illustrates SF film biodegradation in water

environments. For freshwater, the effects of increasing sodium chloride concentration (0-200 mM) were investigated when *R. tropici* were encapsulated in the silk material at the point of material assembly and using non-loaded SF films as control (Figure 4a and b, respectively). Degradation of SF films loaded and non-loaded with *R. tropici* was also investigated in seawater. SF mass loss measurements over time showed that *R. tropici* induced silk fibroin biodegradation in water and the degradation rate was higher at sodium chloride concentrations that allow for the biofertilizers survival (NaCl 50-200mM). Negligible weight loss was in fact measured in deionized water (NaCl = 0 mM). For seawater, minor weight loss was also measured for the 8 weeks of the study; this may be caused by the limited number of microorganism present in seawater when compared to soil and by the low survival of *R. tropici* in a non-natural environment for the rhizobacteria. However, a statistically significant decrease ($p<0.05$) in the mechanical properties (i.e. Young's modulus and hardness) of the SF films exposed to seawater was measured with nanoindentation, when compared to SF films at week 8 of exposure to 0, 50, and 100 mM of NaCl solution (Figure 4c), which may suggest that hydrolysis causes a decrease in the protein MW in the time frame considered. SEM microscopy of *R. tropici-loaded* silk films at week 8 showed no sign of biopolymers deposition on the surface when exposed to deionized water (NaCl 0 mM) and in seawater, while nanofibrous biopolymers were visible in samples exposed to NaCl 100 mM solution. Together, these results suggest that degradation of SF films in seawater and freshwater requires the presence of active heterotrophic bacteria that possess the capability of digesting the silk material. The higher silk degradation rate in soil when compared to freshwater and seawater may be due the higher density of bacteria in soil (10^8-10^9 bacteria / g of soil) when compared to seawater (10^5 bacteria / g of surface sea water) and freshwater (10^3-10^5 bacteria / g of freshwater). Additionally, soil dwelling microorganisms may have evolved to

better digest structural proteins produced by terrestrial organisms when compared to water counterparts; e.g. Protease XIV produced by *Streptomyces griseus*, a gram-positive species of bacteria commonly found in soil, is a mixture of at least three caseinolytic activities and one aminopeptidase activity and is a well-known for its efficacy in degrading silk materials.

CONCLUSIONS

In this manuscript, we report for the first time the degradation of silk fibroin films in soil and water environment as a function of abiotic and biotic factors such as sodium chloride concentration, presence of biofertilizers, and bacteriostasis. The effects of silk fibroin polymorphism on degradation in soil was also investigated and showed that beta sheet content can be used to modulate silk fibroin film biodegradation rate. Microbial activity is a main driving force for silk fibroin film degradation in soil, mainly due to the production of proteases, and results in the complete degradation of the silk materials considered in soil in 8 weeks. When bacteriostats were added to the soil, biodegradation was hindered but not stopped. Further, incorporation of biofertilizers in silk fibroin films at the point of material assembly accelerated the material degradation, promoting a bulk degradation whereas biodegradation through microbial colonization from soil resulted in a surface degradation. Studies of biodegradation in freshwater and seawater environments indicated slower biodegradation profiles, when compared to the measurements obtained in soil, even when bacteriostats were added. Particularly interesting was the case of exposure to seawater, resulting in <10% of weight loss in 8 weeks. Given the rising need to predict materials' biodegradation in the environment and to embed circular life principles in the design of technical innovations, this study will provide the basis to engineer silk fibroin materials for applications in food, energy, water, and agriculture.

ASSOCIATED CONTENT

Supporting Information is available and show: microscopic pictures of degraded silk fibroin film at week 4 of treatment in soil, the result of ICP-MS analysis conducted on the same specimen, the results of the Antrhone test, expression of proteases in *R. tropici* and pH changes within silk films degrading in soil.

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Augustine T. Zvinavashe and Zeina Barghouti contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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CONFLICT OF INTEREST

BM is co-founder of Mori, Inc, a company that develops silk-based technologies to prolong food shelf life. BM and ATZ are co-inventors on IP positions that protect the use of silk-based materials to deliver biofertilizers in the soil.

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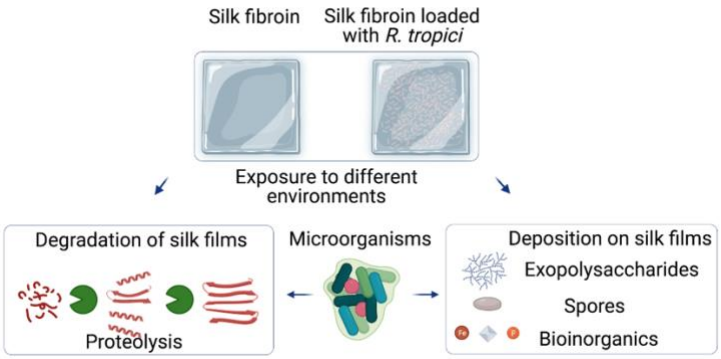

We investigate the biodegradation of silk films in different environmental conditions ranging from soil to seawater, elucidating the role of the environment and protein properties on material end-life.

Synopsis

Biodegradation of silk-based films in various environmental conditions with exogenous factors and encapsulated microbes are discussed.