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Coupling Sewage Sludge Amendment with Cyanobacterial Inoculation to Enhance Stability and Carbon Gain in Dryland Degraded Soils

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Abstract: Sewage sludge (SS) is widely used as a soil conditioner in agricultural soil due to its high content of organic matter and nutrients. In addition, inoculants based on soil microorganisms, such as cyanobacteria, are being applied successfully in soil restoration to improve soil stability and fertility in agriculture. However, the combination of SS and cyanobacteria inoculation is an unexplored application that may be highly beneficial to soil. In this outdoor experiment, we studied the ability of cyanobacteria inoculum to grow on degraded soil amended with different concentrations of composted SS, and examined the effects of both SS concentration and cyanobacteria application on carbon gain and soil stability. We also explored the feasibility of using cyanobacteria for immobilizing salts in SS-amended soil. Our results showed that cyanobacteria growth increased in the soil amended with the lowest SS concentration tested (5 t ha^{-1}, on soil 2 cm deep), as shown by its higher chlorophyll a content and associated deeper spectral absorption peak at 680 nm. At higher SS concentrations, inoculum growth decreased, which was attributed to competition of the inoculated cyanobacteria with the native SS bacterial community. However, SS significantly enhanced soil organic carbon gain and tightly-bound exopolysaccharide content. Cyanobacteria inoculation significantly improved soil stability and reduced soil's wind erodibility. Moreover, it led to a decrease in the lixiviate electrical conductivity of salt-contaminated soils, indicating its potential for salt immobilization and soil bioremediation. Therefore, cyanobacteria inoculation, along with adequately dosed SS surface application, is an efficient strategy for improving carbon gain and surface stability in dryland agricultural soil.

Keywords: organic waste; biocrust cyanobacteria inoculation; organic carbon content; aggregate stability; wind erosion susceptibility; agricultural soil

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1. Introduction

Expansion and intensification of usage of agricultural lands to meet the increasing global food demand poses a serious risk to sustainability and food security [1]. Inadequate agricultural practices such as intensive tilling, overgrazing, removal of vegetation, and excessive use of chemical fertilizers and pesticides have resulted in increased soil erosion rates, rapid mineralization of organic matter, higher CO_2 emissions, less soil fertility, and loss of biodiversity [2–5]. Agricultural soil erosion is one of the main threats to soil sustainability [6]. Soil erosion rates in agricultural lands are estimated at 400 m My^{-1}, which greatly exceeds natural soil formation rates, estimated at 50–200 m My^{-1} [2]. Problems are aggravated in drylands, where climate conditions (low rainfall and high temperatures) and edaphic characteristics (poor soil structure and low organic matter content) reduce the availability of a substrate suitable for crop development [7]. In addition, drylands are extremely vulnerable to the increased aridity predicted for the end of this century [8,9]. Growing human pressure on drylands will exacerbate soil degradation [10,11] and compromise their suitability for cropping [12]. New approaches are necessary to maintain and/or increase crop yield while ensuring environmental protection in drylands.

Various solutions for improving or restoring agricultural soil quality are being explored, with a special focus on increasing soil stability and organic carbon content, as these are considered very efficient strategies for overcoming soil degradation and the most reliable indicators for monitoring it. There have been attempts at reforestation using shrub-like plants typical of dryland regions [13] or the return of agricultural wastes to soil, such as local crop straw [14]. The two techniques achieved good results, with a significant amelioration in both soil aggregate stability and total organic carbon content. Another practice employed is the use of organic waste, which has become an important waste recycling measure in the European circular economy strategy [15]. One of the most important organic wastes used as a soil amendment is sewage sludge (SS), a by-product of municipal and industrial wastewater treatment. Due to its high content in organic matter and macro- and micronutrients, SS is applied as a soil conditioner to improve and maintain soil quality and stimulate plant growth [16]. Sewage sludge applications have also gained in importance due to the growing population and urban and industrial development, which make efficient recycling and management necessary to reduce environmental impacts [17,18]. Around 50% of SS generated is used in agriculture in Europe [19], and up to 65% in Spain [20]. Spreading SS over the ground benefits crops by improving soil chemical, physical, and biological properties and providing nutrients to plants [21–23], reducing the need for inorganic fertilizers [24]. Nitrogen contained in SS is rapidly mineralized and made available for plant uptake, stimulating plant growth [16,25]. Sewage sludge also increases total organic and microbial biomass carbon [26], improving soil aggregation [27] and water retention capacity [28].

However, in spite of the indisputable benefits resulting from the use of SS in agriculture, its long-term or mismanaged soil application leads to a variety of undesirable qualities that can cause adverse effects on the environment [29,30]. For example, even when SS has been subjected to secondary treatments and dehydration, a wide range of pollutants, such as heavy metals, organic contaminants, or human bacteria and potentially pathogenic organisms [31] that could be transferred to the food chain may still persist. Another common detrimental effect of SS in drylands is the risk of soil salinization [32,33] which may limit its fertility. Furthermore, irrigation in dryland agriculture may also cause salts to accumulate in the soil profile, and this in turn can promote the displacement of undesirable substances, including heavy metals [34,35]. Both salts and heavy metals can affect groundwater quality. Moreover, water and wind erosion could potentially transport pollutants from surface-applied SS offsite [36], as well as part of C, N, and other nutrients added with the SS amendment. The loss of these nutrients from runoff and water erosion is especially important when crops are located on relatively steep slopes or when the hydrophobic effects commonly associated with dry sludge persist after application [37]. To reduce such nutrient losses and the environmental and health risks of the agricultural use of SS, new strategies able to counteract these potential risks and at the same time support the circular economy and sustainable agriculture have been implemented. Many of them focus on stabilizing the contaminants through adsorption, surface complexation, and precipitation processes using natural or synthetic materials (for example fly ash, carbonates, zeolites, biochar, clay minerals, marble waste, etc.), alone or with organisms, particularly in combination with microorganism inoculation [38,39].

It has been suggested that native soil microbial communities can degrade or reduce the bioavailability of contaminants, as well as offset the losses of carbon and nutrients from erosion or gaseous emission and even increase bioavailability of P compounds. Thus, the combined application of SS with these microorganisms represents an important opportunity for recycling nutrients for agriculture while limiting the environmental risks. Of the potential native microorganisms that could be combined with SS in soil, cyanobacteria are ideal candidates for use in drylands.

The capacity of soil cyanobacteria to survive and grow at high temperatures [40] and under long exposure to UV radiation [41] and drought [42] makes them suitable for use in drylands. In addition, several other characteristics make them excellent candidates for reducing the negative effects of SS. For example, soil cyanobacteria, through their capacity for photosynthesis, are able to reduce GHGs by fixing atmospheric CO_2 and N_2 [43]. Soil cyanobacteria also secrete exopolysaccharides (EPS), which, in addition to increasing soil fertility [44] and soil water retention [45], have several features crucial to ameliorating the impact of sludge. EPS bind soil particles, increasing soil stability [46] and reducing soil water and wind erosion [47], and, as a result, reduce the loss of SS nutrients and dissemination of pollutants by wind and water. Cyanobacteria have also been demonstrated to be effective for the remediation of salt-affected soils, as the secreted EPS can bind sodium ions and form biofilms, protecting plants from salt stress [48,49], and their trichomes can remove soluble sodium from the soil by biosorption [50].

Finally, cyanobacterial strains of interest can be isolated from soil, and cultured ex-situ in liquid media for large-scale inoculation. The production cost of this biomass has been reduced by optimizing growing temperatures, making use of natural sunlight [51], employing wastewater instead of a freshwater enrichment medium, or utilizing media made with agricultural fertilizers [52,53].

Thus, the application of SS combined with soil cyanobacteria inoculation is hypothesized to be appropriate for agricultural management, as it increases soil stability and reduces erosion, increases potential carbon sequestration in soils, and contributes to the circular economy and GHG mitigation. In this study, we examined the effect of combining SS application and soil cyanobacteria inoculation on soil properties in degraded agricultural soil under outdoor conditions. The specific objectives were: (i) to analyze the ability of cyanobacterial inoculum to grow on soils amended with SS at different concentrations; (ii) to examine the effect of SS application and cyanobacteria inoculation on physicochemical soil properties; and (iii) to explore the capability of cyanobacteria for salt immobilization in SS-amended soils.

2. Materials and Methods

2.1. Sewage Sludge Collection

The composted SS was collected from the "Montes Orientales" composting plant in the province of Granada, Spain. The sludge, coming from a municipal wastewater treatment plant in Almeria, was centrifuged, air-dried and composted by mesophilic aerobic digestion. To do this, the material is compiled in small piles and turned every ten days, maintaining the temperature range between 55 and 65 °C, with the process lasting for three months. The main characteristics of the compost are shown in Table 1.

Table 1. Heavy metal content and physicochemical characteristics of the composted sewage sludge. The number in brackets indicates limit values (ppm) of heavy metals for application in agricultural soils with pH > 7, according to the RD 1390/1990 Spanish regulation.

Heavy Metals		Physicochemical Characteristics	
Chromium (ppm)	25.1 (1500)	Total organic carbon (%)	19.19
Nickel (ppm)	16.8 (400)	Labile organic carbon (%)	0.23
Copper (ppm)	106.36 (1750)	Total carbon (%)	19.28
Zinc (ppm)	264.84 (4000)	Total nitrogen (%)	2.88
Lead (ppm)	31.28 (1200)	pH	6.17
Cadmium (ppb)	754.74 (40,000)	Electrical conductivity (ms cm^{-1})	7.52
Mercury (ppb)	32.5 (25,000)		

2.2. Soil Collection

Soil samples were collected from an abandoned agricultural site (36°52′21″ N, 02°12′07″ W) located in the province of Almeria (Spain) adjacent to the municipal wastewater treatment plant from which the SS was collected. Soil texture was sandy loam (68.6% sand, 16.8% silt, and 14.6% clay), pH was 9.06 and electrical conductivity was 0.53 mS cm^{-1}. The soil was scarcely developed and had a poor structure, with a low field capacity (<13%) and hydraulic conductivity of 1.27 cm h^{-1}. Total organic carbon and nitrogen contents were low, with average values of 1.15% and 0.22%, respectively.

2.3. Culture of Cyanobacterial Inoculants

Four native cyanobacterial strains common in semiarid soils [54] and previously isolated and identified from different soils in the province of Almeria [55] were selected as inoculants: two non-heterocystous cyanobacteria, *Trichocoleus desertorum* (CAU7 UAM 832) and *Leptolyngbya frigida* (CAU10 UAM 837), and two filamentous heterocystous, *Nostoc commune* (CANT2 UAM 817) and *Tolypothrix distorta* (CANT7 UAM 825). N-fixing and non-fixing cyanobacteria were used together as a consortium to combine the benefits of the two groups. The non-heterocystous species are those that dominate in the early stages of biocrust succession, since their morphologic and physiologic features let them colonize unfavorable environments while facilitating later-successional organisms' colonization by improving soil properties. On the other hand, heterocystous cyanobacteria are commonly found in the later stages of biocrust development; they increase soil fertility by fixing atmospheric nitrogen [54]. Single trichomes were transferred separately to sterilized Erlenmeyer flasks (0.25 L) containing BG11 media (for the non-heterocystous) and BG110 (for the heterocystous). The flasks were continuously aerated with sterilized air by filtering (0.22 µm, Millex EMD Millipore™), and the cultures were incubated in a room at 25 ± 1 °C under a constant irradiance of 200 µmol photons m^{-2} s^{-1}. Cyanobacterial biomass was harvested at the exponential phase (2.5 g L^{-1}) and scaled up to larger containers (5 L) to maximize biomass growth. Once enough biomass for soil inoculation experiments was grown, the inoculum was harvested by filtration.

2.4. Experimental Design

The experiment was conducted in June 2020. Small aluminum trays (19.1 cm × 13.6 cm) were filled with 350 g of soil amended with different SS concentrations. Five SS doses, applied on soil 2 cm deep, were tested: (1) no SS application to the soil (hereafter "D0"); (2) SS application at a dose of 5 t ha^{-1} (D5); (3) SS at a dose of 10 t ha^{-1} (D10); (4) SS at a dose of 30 t ha^{-1} (D30); and (5) SS at a dose of 40 t ha^{-1} (D40). Although the standard application of SS in agriculture often involves the first 30 cm of the soil profile, this experiment was designed to analyze the combined effect of SS amendment and cyanobacteria inoculation on soil properties. Thus, SS was applied on a thin soil layer (2 cm) where the induced cyanobacterial biocrust is expected to have a major impact. To prepare the amended substrate at the different SS doses, a large tray was filled with the collected soil and the SS was added at the desired dose and mixed to ensure proper mixing. Samples were irrigated with 200 mL of distilled water to allow the substrate to stabilize prior to soil inoculation.

For each SS dose, two treatments were applied: control or no cyanobacteria inoculation, and cyanobacteria inoculation. Four replicates of each treatment (control/inoculated for each SS dose) were prepared, with a total of 40 trays. Cyanobacterial biomass was inoculated on an equal mixture of the four selected species (1:1:1:1 weight) on the amended substrates, at a concentration of 6 g m^{-2}. This concentration has been successfully employed in previous studies involving the same species [56–58]. To achieve that concentration, 150 mg of the cyanobacterial mixture was resuspended in 200 mL of distilled water (0.75 g L^{-1}) and applied uniformly onto the substrate with a sprayer three consecutive times (~65 mL each time). An equivalent amount of distilled water was applied to the uninoculated samples. Samples were placed outdoors for four months. The study period included the whole summer and the daily mean air temperature during the study period was 23.4 °C. Samples were irrigated with a quantity of water equivalent to the mean annual rainfall in a wet year (380 mm) according to local rainfall records and calculated for the duration of the experiment. Thus, the resulting irrigation was 60 mL of water twice a week.

Additionally, an experiment was set up to evaluate cyanobacterial growth on soil with the lowest and the highest SS doses (5, 30 and 40 t ha^{-1}), sterilized to remove the native biological community by autoclaving it. SS was mixed with soil to obtain sterilized and unsterilized SS treatments; then, four control and four inoculated samples of both were prepared. Experiment preparation, duration, and physical conditions were maintained as in the previous experiment.

2.5. Assessing Inoculum Growth

2.5.1. Chlorophyll a Content

Chlorophyll a content, which is considered one of the best indicators to evaluate cyanobacteria growth [59], was determined at the end of the experiment by following the procedure developed by Castle et al. [60]. A composite sample per tray, obtained from three individual soil samples (0–3 mm depth), was used for the chlorophyll a determination. Thus, one gram of fine powder was mixed with 5 mL ethanol, heat ed at 80 °C in a water bath for 5 min, vortexed at maximum speed, and then cooled at 4 °C for 30 min. The sample was centrifuged at 4000× g for 20 min and the supernatant recovered. This procedure was conducted twice for each soil sample. Afterwards, chlorophyll a content was determined immediately after extraction by measuring the absorbance at 665 and 750 nm with a spectrophotometer (Helios Zeta UVVIS, Thermo, UK) and applying the equation developed by Ritchie [61]:

$$Chlorophyll\ a\left(\mu g\ g^{-1}\ soil\right) = \frac{11.9035 \times A(665-750) \times V}{soil\ weight(g) \times L} \quad (1)$$

where A is the absorbance value at the specific wavelength, V is the volume of the extract (L), and L is the optical path length of the spectrophotometer cuvette (cm).

2.5.2. Soil Spectral Response Measurements

To assess cyanobacterial development over the whole soil surface, the spectral response of the surface was also analyzed with an Analytical Spectral Device (ASD) hand-held portable spectroradiometer (ASD Inc., Boulder, CO, USA). The instrument was equipped with an optic fiber capable of sample intervals of 3.5 nm from 325 nm to 1075 nm. The fiber was placed 16 cm above the soil sample to cover half of the tray on each acquisition, so two measurements per tray were required to cover its entire surface. All measurements were conducted under constant light conditions by using two ASD lamps that uniformly illuminated samples during spectral acquisitions. The signal was pre-calibrated using a white reference Spectralon® panel. Three measurements were taken per sample, each one being the average of three individual spectra, and then averaged to produce a single surface spectrum per tray. Data were pre-processed by removing noisy bands in the range between 325 and 400 nm and between 950 and 1075 nm, later applying a cubic polynomial smoothing filter with a 17 bands-window size [62]. The smoothed spectra were used to extract the continuum removal (CR), a technique that normalizes soil reflectance by dividing the original spectrum by a continuum curve resulting from applying a convex hull fit over the top of the spectrum that connects local maxima with straight line segments, which have a value of 1.0 [63]. Then, values equal to 1.0 indicate no absorption, while lower values indicate the presence of absorption features. This was employed to estimate the maximum absorption at ~680 nm (CR680), close to the natural absorption peak produced by the presence of chlorophyll a, which has been found to be strongly correlated with chlorophyll a content in cyanobacterial biocrusts [64].

2.6. Influence on Soil Properties

2.6.1. Soil Stability Measurements

To test the effectiveness of the treatments on soil stability, we measured soil susceptibility to wind erosion using a wind tunnel experiment and conducted a test to evaluate aggregate stability at the end of the experiment. Trays were placed at the center of the wind tunnel (0.35 m long with a cross-section of 0.15 × 0.15 m), and a turbine attached to one side of the tunnel was employed to produce a laminar and non-turbulent flow with a speed of 18 m s^{-1} for one minute. Wind speed was measured with a thermic anemometer PCE-423 with 0.01 m s^{-1} resolution. The wind speed was selected according to the range of threshold friction velocities found by Liu et al. [65]. A bag was attached to the other side of the tunnel to recover all the particles detached from soil surfaces. At the end of the experiment, these particles were air-dried and weighted to produce a single value of wind erosion (g) per sample.

To measure aggregate stability, the soil stability kit described by Herrick et al. [66] was used. Briefly, six surface aggregates (~6–8 mm diameter × 2–3 mm depth) per tray were collected and placed in a 1.5 mm mesh wire basket, immersed in distilled water for 5 min, then followed by five dipping cycles at the rate of one cycle every 2 s. Each aggregate was assigned to a class on a scale of 1 to 6, with greater values indicating higher aggregate stability. Finally, the average percentage of each stability class for each treatment was determined.

2.6.2. Exopolysaccharide and Soil Organic Carbon Content

After conducting the wind tunnel and soil stability measurements, three surface samples (3 mm depth) were collected from each tray and mixed together to obtain a composite sample. The soil was air-dried at room temperature, and ground with a mortar and pestle for EPS and soil organic carbon content determination.

Both loosely- (LB) and tightly-bound (TB) EPS fractions were determined. For this, 0.1 g of soil was weighed and three consecutive extractions with 3 mL of distilled water were conducted to recover the LB-EPS. The three extractions were combined into one single solution. After this, three consecutive extractions with 3 mL of 0.1 M Na_2EDTA were used and mixed to recover the TB-EPS [67]. The exopolysaccharide content of each solution was determined by using the phenol–sulfuric acid assay, measuring the absorbance at 488 nm with a UV–VIS spectrophotometer [68] and using glucose as the standard.

Soil organic carbon (SOC) was determined via wet oxidation using the Walkley and Black method modified by Mingorance et al. [69].

2.7. Inoculation of Saline Soils with Native Cyanobacteria

Additionally, a second experiment was conducted to test the ability of cyanobacteria to immobilize salts in the soil. The soil amended with the optimum SS dose for the cyanobacterial colonization (i.e., 5 t ha^{-1}) was selected. Three doses of NaCl salt were simulated: (1) no salt added to the soil; (2) 0.3% NaCl w w^{-1}; and (3) 0.6% NaCl w w^{-1}. The soil and the SS were sterilized prior to soil inoculation to account solely for the response of cyanobacteria for salt immobilization without the interference of the native soil community. To facilitate a more homogeneous incorporation of the salt into the substrate, the SS-amended soil and the salt were previously ground using a mortar and pestle and then mixed together following the same procedure as in Chen et al. [70]. This experiment was conducted in Petri dishes filled with 80 g of the amended substrate. Finally, a mixture of the four cyanobacterial strains was inoculated on the substrate following the same methodology described above (Section 2.4), and the same irrigation pattern was applied. Samples were placed into a growth chamber under constant light and a light–dark cycle of 16:8 h for three months.

The ability of cyanobacteria to survive under increasing salt concentration was evaluated by analyzing the spectral absorption by chlorophyll a at the end of the experiment. The maximum absorption at ~680 nm (CR680), obtained with the spectroradiometer as explained in Section 2.5.1, was used to estimate the development of the inoculum over the soil surface.

To assess if cyanobacteria were able to immobilize NaCl from the soil, electrical conductivity was measured in the lixiviates of the salt-contaminated samples at the end of the experiment. For that, soil samples were irrigated with distilled water until saturation and the lixiviate was recovered by vacuum filtration. The electrical conductivity (mS cm^{-1}) was directly measured in the lixiviate using a Crison Conductivity meter 522 (Crison Instruments SA, Barcelona, Spain).

2.8. Statistical Analyses

To test the effect of the treatments (SS dose and inoculation) on both cyanobacteria growth and soil properties, a two-way analysis of the variance (ANOVA) test was conducted. The same test was used to test the effect of salt concentration and inoculation on the electrical conductivity of the lixiviates. Variables were tested for normality and homogeneity of variance using the Shapiro–Wilk and Levene's test. Data were log-transformed before performing parametric analysis if assumptions of normality were not met. The Tukey post-hoc test was applied to test differences between means. Significance was established at $p < 0.05$. All the analyses were performed using RStudio version 4.1.3.

3. Results

3.1. Cyanobacterial Inoculum Viability Assessment

Four months after cyanobacteria application, the soil amended with the lowest SS dose (5 t ha^{-1}, on soil 2 cm deep) showed the deepest absorption peak at 680 nm (CR680 = 0.986). This absorption peak decreased (lower CR680 value) with increasing SS in the inoculated samples (Figure 1). On the contrary, all the uninoculated samples, regardless of SS dose, had CR680 values close to 1, indicating the absence of photosynthetic pigments (Figure 1).

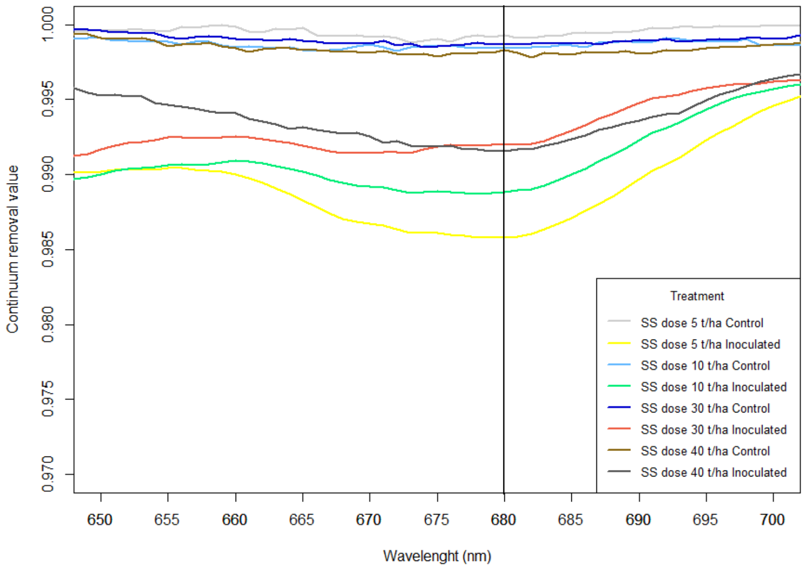

Figure 1. Continuum removal curves between 650 and 700 nm obtained for the control and inoculated samples for the different sewage sludge doses tested in the experiment, after 4 months.

The same pattern was observed in the chlorophyll a concentration, with even stronger differences than those observed in the continuum removal values (Figure 2). Chlorophyll a content was significantly higher in the inoculated soil amended with the lowest SS dose (2.74 ± 0.24 μg g^{-1}) than in the uninoculated soil amended with the same SS dose (0.58 ± 0.1 μg g^{-1}). On the contrary, there were no statistically significant differences between the control and inoculated samples without SS or with increasing SS concentrations, which showed chlorophyll a content from 0.5 to 1 μg g^{-1} (Figure 2).

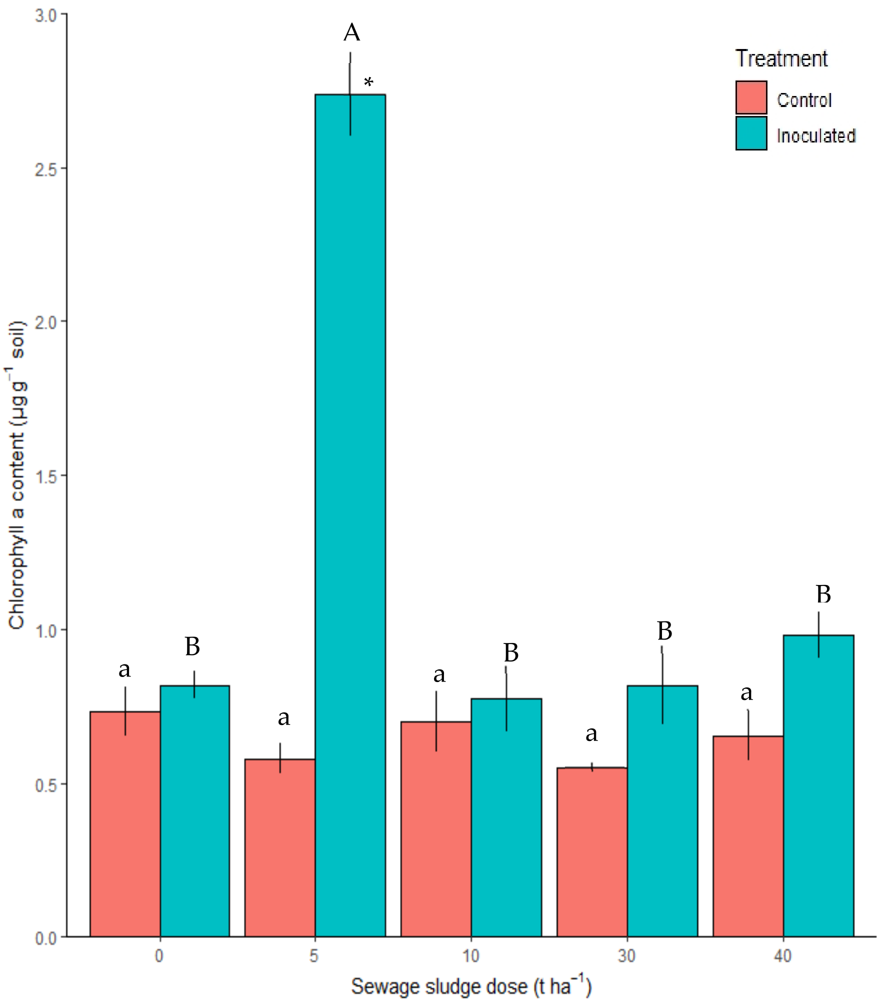

Figure 2. Chlorophyll a concentration (µg g^{-1} soil) in the control and inoculated samples for the different sewage sludge doses, after 4 months. Lowercase letters indicate significant differences among control treatments at the different SS doses, while capital letters indicate significant differences among inoculated treatments at the different SS doses. * indicates statistically significant differences between the control and the inoculated treatment for each sewage sludge dose.

3.2. Effects on Soil Physico-Chemical Properties

3.2.1. Soil Stability

At the end of the experiment, aggregates in all the control treatments were in the lowest stability class, regardless of the sewage sludge concentration in the amendment (Figure 3). On the contrary, four months after the application of the cyanobacterial inoculum, the aggregate stability of the inoculated samples had increased significantly, with a large percentage in aggregate stability classes 4 and 5. The best results were with the lowest doses of the organic amendment, particularly with 5 t ha^{-1}, with more than 10% of the aggregates in the highest stability class (Figure 3).

Regarding wind erodibility, the amount of sediment displaced by the wind force applied increased with SS dose because of the higher coarse particle content in the amended samples (Figure 4). Cyanobacteria inoculation strongly decreased particle displacement, and significant differences were found between inoculated and control soils for almost all the SS concentrations tested (Figure 4).

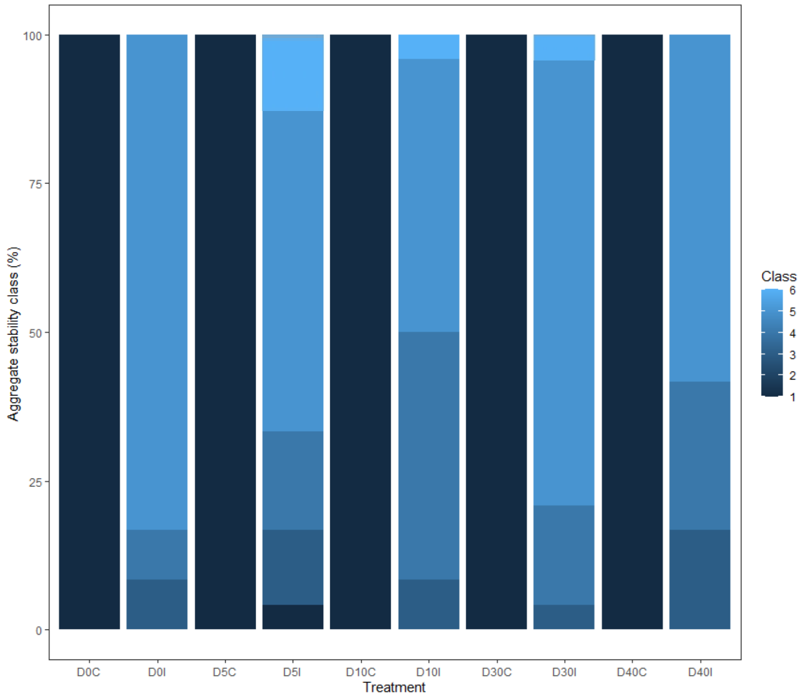

Figure 3. Percentage of each aggregate stability class in the samples of all doses of sewage sludge (t ha^{-1}) tested for both control (C) and inoculated (I) samples 4 months after cyanobacteria inoculation.

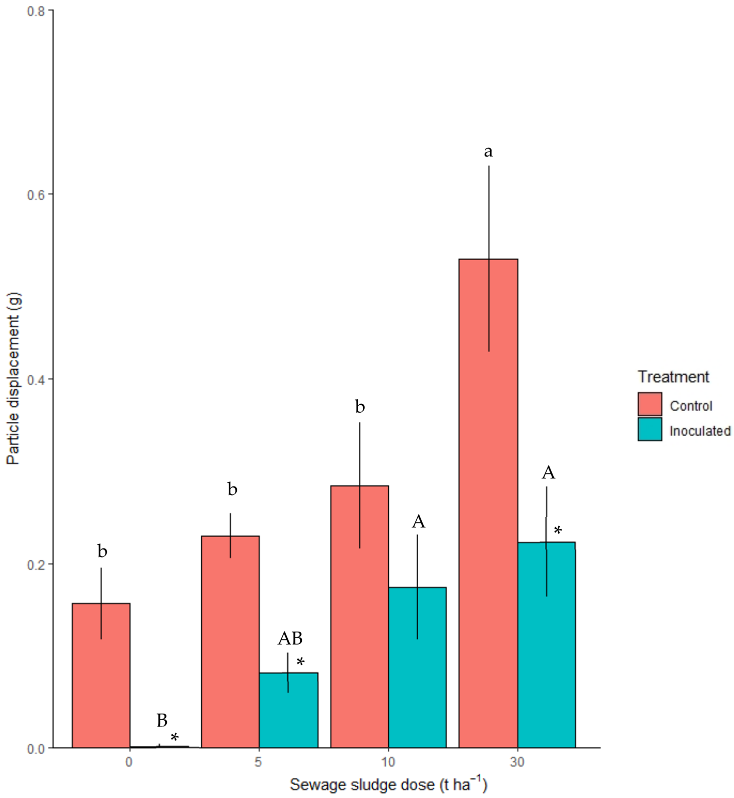

Figure 4. Sediment displaced (g) following wind simulation at a constant speed of 18 m s^{-1} for one minute. Lowercase letters indicate significant differences among control treatments at the different SS doses, while capital letters indicate significant differences among inoculated treatments at the different SS doses. * indicates statistically significant differences between the control and the inoculated treatment inside the sewage dose considered.

3.2.2. Total Organic Carbon and EPS Content

Total organic carbon concentration rose with SS dose, ranging from about 1% in soil without any SS amendment to over 3.5% with the highest dose (40 t ha^{-1}) (Figure 5). However, there were no significant differences between the control and the inoculated treatments at any dose tested.

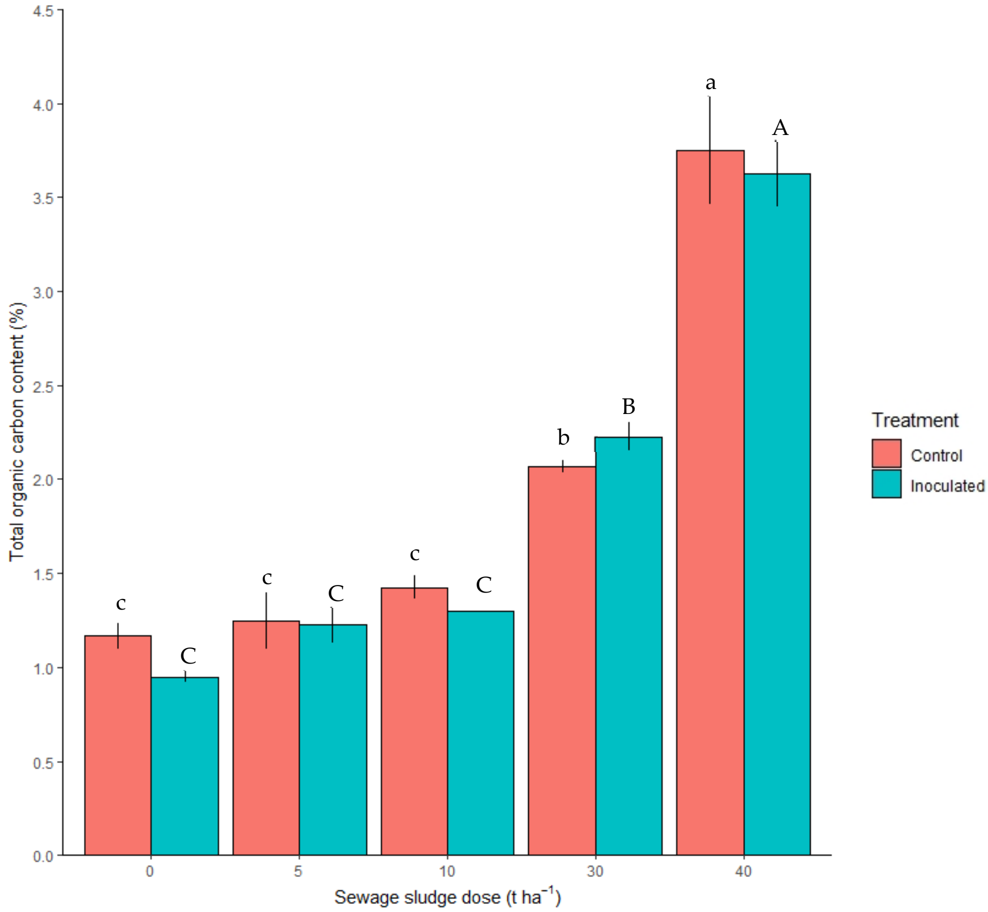

Figure 5. Organic carbon content (%) in the control and inoculated soils amended with the different SS doses, after 4 months. Lowercase letters indicate significant differences among control treatments at different SS doses, while capital letters indicate significant differences among inoculated treatments at different SS doses.

No significant differences in total EPS content were found between the control and the inoculated samples. Like the trend observed in organic carbon content, total EPS concentration rose with SS dose, from around 1 mg g^{-1} with no or very low SS amendment to 2.5 mg g^{-1} in the soil with the highest SS (Figure 6). EPS was from 6% to 10% of the total organic carbon. The LB-EPS fraction of the total EPS was smaller (around 30%) than the TB-EPS. It should also be mentioned that LB-EPS concentrations remained similar at all SS doses except 40 t ha^{-1}, where they were significantly higher. On the other hand, TB-EPS increased with SS dose, and was significantly higher in the soil with the highest SS doses (30 and 40 t ha^{-1}).

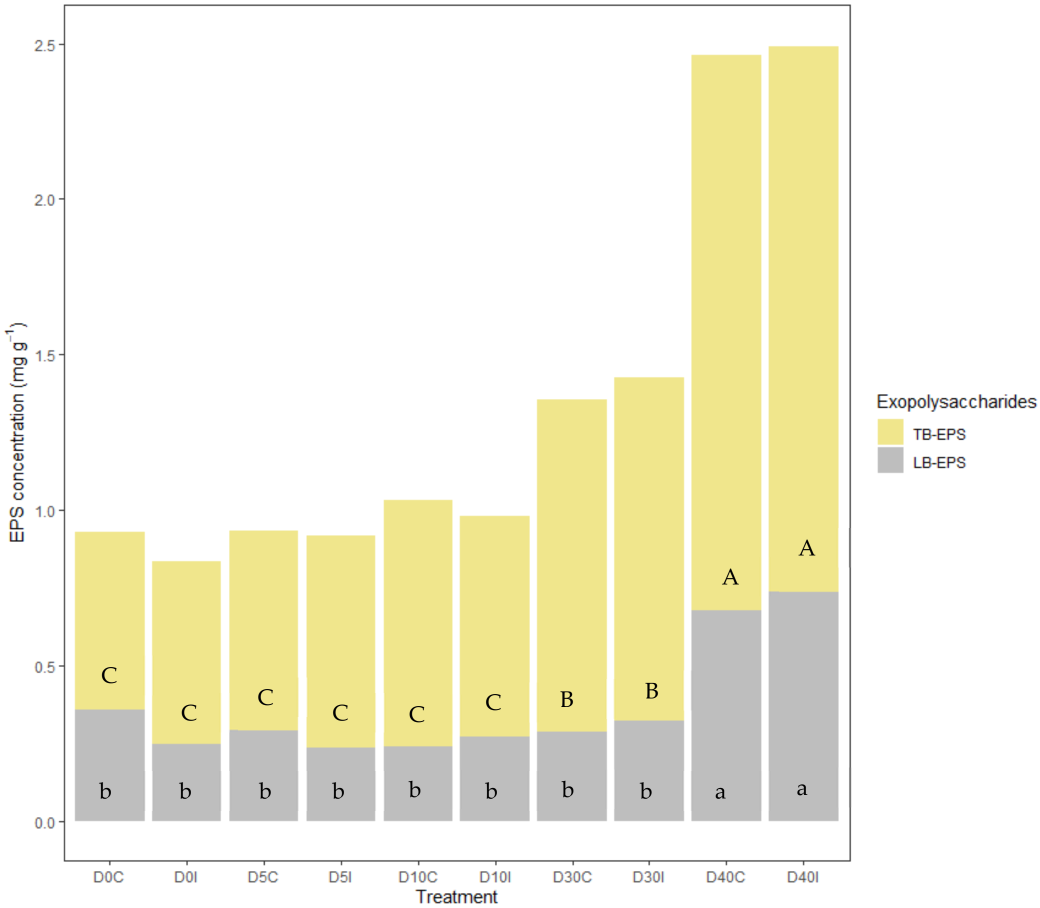

Figure 6. Exopolysaccharide content (mg EPS g^{-1} soil) in the control (C) and inoculated (I) soils for the different SS doses (t ha^{-1}), after 4 months. Lowercase letters indicate significant differences in LB-EPS content at the different SS doses, while capital letters indicate significant differences in TB-EPS content at the different SS doses.

3.3. Cyanobacterial Effects in Saline Soils

The soil used to evaluate the response of cyanobacteria in the presence of high salt concentrations was amended with the lowest SS dose (5 t ha^{-1}), as it showed the best cyanobacterial growth in the previous experiment (see Section 3.1). It was observed that cyanobacteria considerably reduced lixiviate electrical conductivity in soils contaminated with 0.3 and 0.6% NaCl, the two salt concentrations tested, reaching values of about 4.5 mS cm^{-1}, while in the control soils, the values were 12.5 and 12.2 mS cm^{-1}, respectively (Figure 7). EC value in the two saline treatments inoculated with cyanobacteria were also similar to the non-saline samples, indicating that the inoculum was able to remove almost all the NaCl ions added (Figure 7).

The cyanobacteria's ability to grow in two salt-contaminated soils was assessed by the chlorophyll a spectral absorption peak. As expected, cyanobacteria survival three months after soil inoculation was better in the soil without added salt (Figure 8). However, the soils at the two NaCl concentrations inoculated with cyanobacteria also showed absorption peaks at 680 nm, revealing the ability of the inoculum to survive in these saline soils. The CR680 value was lower (higher absorption by chlorophyll a) in the soil with lower NaCl concentration, indicating more growth than in soil with a higher NaCl content (Figure 8).

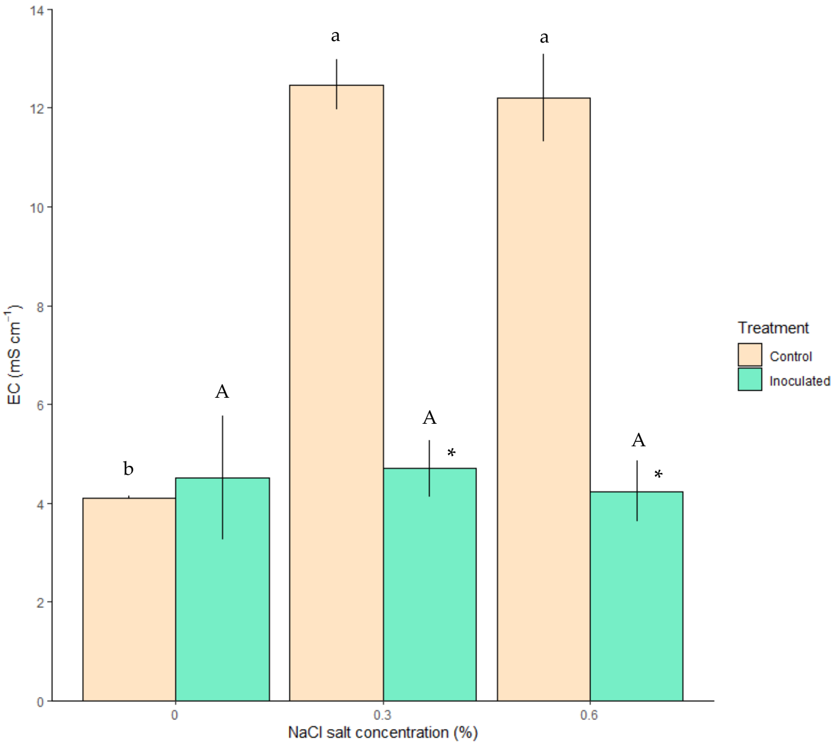

Figure 7. Electric conductivity, EC, (mS cm^{-1}) measured three months after the beginning of the experiment in the lixiviate of the soil spiked with NaCl salt at two different concentrations. Lowercase and capital letters indicate significant differences among control and inoculated trea tments, respectively, at each NaCl soil concentration. * indicates statistically significant differences between the control and the inoculated treatment for each NaCl concentration.

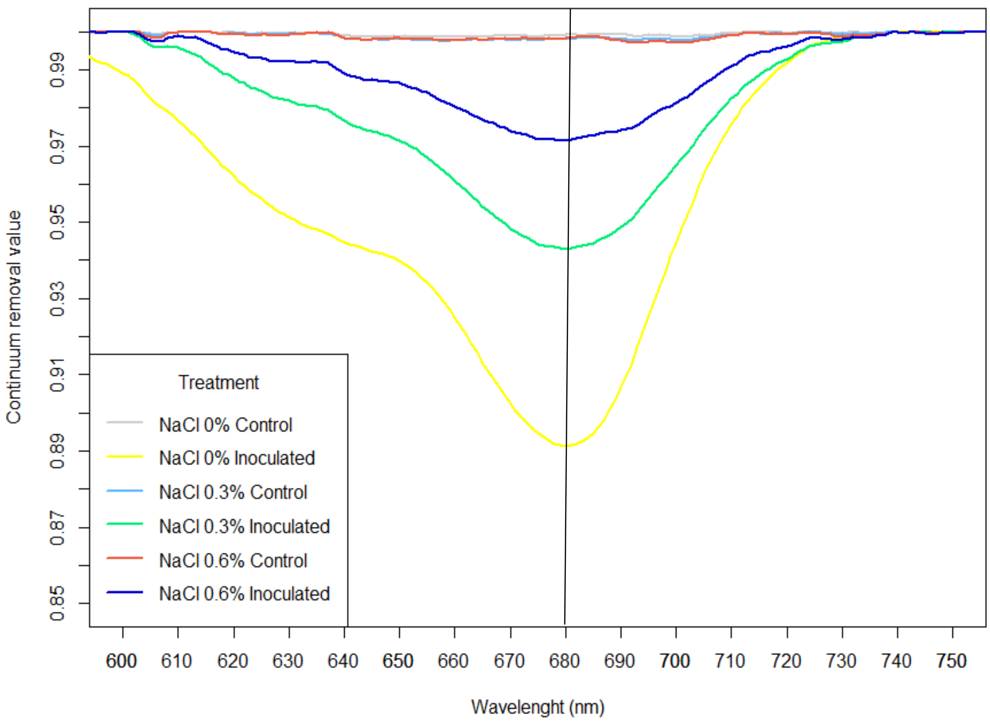

Figure 8. Continuum removal curves between 600 and 750 nm obtained for the control and inoculated samples three months after the application of the different NaCl concentrations tested in the experiment.

4. Discussion

4.1. Inoculum Viability in Soils Amended with different SS Doses

The application of organic wastes such as SS has been shown to increase organic matter, nutrient concentration, and microbial growth in soils, and is therefore a suitable strategy for mitigating soil degradation and improving the quality of agricultural soils [71,72]. Technologies based on the use of microbial cultures and inoculants in agricultural soils have also increased in use enormously, because they improve soil fertility, decompose organic waste, prevent plant diseases, and enhance crop productivity [73,74]. Use of SS amendments and microbial inoculants together is an underexplored technique that can potentially maximize the beneficial effects of both techniques in croplands.

In this study, the application of SS as a soil amendment at different doses increased the organic carbon content (Figure 5), and its combination with cyanobacteria inoculation resulted in significant improvements in soil stability (Figures 3 and 4). However, cyanobacterial inoculum growth on the amended soil depended heavily on the SS dose applied (Figures 1 and 2).

Our results showed that the lowest SS concentration (5 t ha^{-1}, on soil 2 cm deep) was the best to promote inoculum growth, reporting better results than the unamended soil (Figures 1 and 2). Maestre et al. [75] also found that cyanobacteria from biocrust were able to colonize the surface of soils amended with SS and that the addition of the composted SS significantly increased chlorophyll a content. However, the addition of SS favored the survival and growth of the cyanobacterial inoculum only when SS concentrations applied were low, contrary to what usually occurs with plants, which would increase their productivity at the highest dose tested [76,77]. This could be due to the excessive presence of nutrients or harmful compounds [78], or the presence of other microorganisms in the SS that compete with cyanobacteria [79], substantially lowering their survival rates [80]. Considering that the concentration of the hazardous heavy metals analyzed remained well below legal limits (Table 1), a more plausible explanation seems to be the presence of competing microorganisms. To corroborate this, an additional experiment with sterilized SS was set up, as explained in Section 2.4. The findings of this experiment demonstrated that inoculum growth increased in the soils with the sterilized SS at higher doses (assessed by the CR680 value), confirming our hypothesis. (Figure 9). Previous studies have also found that high SS concentrations could be detrimental to crop yield, enzyme activity, and carbon microbial biomass [26]. Furthermore, the increase in coarse particle content added with increasing SS dose could also have hindered establishment of the inoculum on the surface.

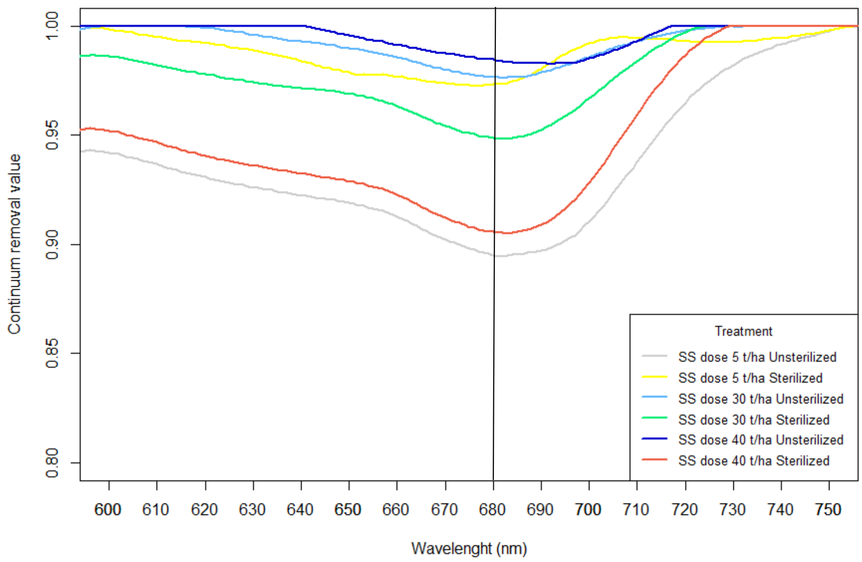

Figure 9. Continuum removal curve of the cyanobacteria-inoculated soils with the sterilized and unsterilized SS at the different SS doses, after 4 months.

4.2. Effects of the Inoculum and the SS Dose on Soil Properties

Due to its high content of organic matter and valuable nutrients, application of SS can be highly profitable, as it reduces the cost of fertilizers [81]. As in this study, previous studies have highlighted the essential nutrients for plant growth such as nitrogen, phosphorous, and carbon that SS can provide [72,82–85], and shown linear enhancement of soil organic carbon content with increasing SS application rate (Figure 5). From 6 to 10% of the soil organic carbon in our treatments was made up of extracellular polymeric substances (EPS), which are produced by the microorganisms naturally present in SS (Figure 6) [86]. The EPS matrix provides an improved environment for bacterial survival and growth and constitutes a protective layer against any hazardous compounds [87]. In this study, cyanobacterial EPS seemed to be negligible compared to the EPS already present in the SS, so EPS content in the inoculated soils was similar to the uninoculated soil, and increases in EPS were mainly associated with higher SS dose (Figure 6). Interestingly, the largest fraction of these EPS compounds was made up of TB-EPS, which are more recalcitrant and in the mid to long term could have a major role in soil particles adherence and soil structure improvement [67,88]. Thus, the use of SS may contribute to enhancing soil stability and protecting soil from wind and water erosion by improving its EPS content. Nonetheless, our results showed that the presence of cyanobacteria was crucial in increasing aggregate stability (Figure 3), which is essential to reducing soil water and wind erodibility. The soil inoculated with the lowest SS concentration, which was also the one in which cyanobacterial growth was higher as shown by its high chlorophyll a content, had the highest soil stability (Figure 2). The cyanobacterial inoculum also reduced wind erosion susceptibility from 40 to 99% by stabilizing the soil surface (Figure 4). These results are supported by previous studies, which reported a soil loss reduction of 77–89% and 82%, respectively, in soils with cyanobacterial inoculation compared to uninoculated soil [89,90]. Due to their significant effect on soil stabilization, cyanobacterial inoculum is being used extensively to restore degraded soil in arid ecosystems with outstanding mitigation of soil loss [47,89,91,92].

4.3. Effect of Cyanobacteria Inoculum on Salt Immobilization in SS-Amended Soils

Some studies have suggested that adding SS to soil could also lead to a significant increase in salt concentration [93,94]. Since high soil salinity is a major abiotic stress in agriculture, the ionic imbalance in plants due to excessive accumulation of Na^+ and Cl^-, which limits absorption of essential mineral nutrients, must be mitigated [95,96]. In this study, cyanobacteria were able to reduce the electrical conductivity of the leaching solution, showing their ability to immobilize salts and reduce their solubility and leaching in the soil solution. This effect was noticeable at both NaCl concentrations tested (Figure 7). Cyanobacteria EPS have a complex chemical composition including high molecular weight organic molecules [97] rich in negative-charged aminic and carboxylic groups [98]. As reported by Nishanth et al. [99], these functional groups are able to sequester both heavy metal and salt ions due to the simple electrostatic attraction between the protonated ions and the negatively charged active sites in the EPS extracellular matrix.

The EPS absorption capacity of heavy metal and other ions by EPS rises with increasing metal ion concentration until a certain limit, after which it starts to go down again due to active site saturation [100]. In our case, cyanobacteria were able to immobilize soil salt at both NaCl concentrations, as shown by the electrical conductivity in the lixiviates from the saline samples with cyanobacterial inoculation, which was similar to the non-saline samples (Figure 7). Thus, with the species used, the biomass concentration applied, and the pH of the soil measured, the salt concentration of 0.6% was not enough to saturate the active groups on the cyanobacterial wall. The continuum removal values at 680 nm demonstrated that cyanobacteria were able to survive in these saline soils, even if the biomass was lower than in the control soil. Indeed, many cyanobacteria have developed diverse response mechanisms to high soil salt concentration [101,102]. However, this may depend on the cyanobacterial species and salt concentration. Singh et al. [103] reported that *Nostoc calcicola* showed more inhibited growth and metabolism in hypersaline soils than at low NaCl concentrations, and the same was found by Kumar et al. [104] with *Nostoc muscorum*. Lan et al. [105] found that the growth of *Microcoleus vaginatus* was inhibited by salt treatments, and this effect was more noticeable at higher NaCl concentrations and longer times.

Despite the good results obtained, it is necessary to address some limitations in order to transfer the small-scale experiments to field conditions. For example, the studied treatment needs two successive steps to be completed, given that SS must be applied and mixed with the soil prior to the inoculation with cyanobacteria, which makes its application more time-consuming. The feasibility of applying the inoculum with irrigation or spreading dry cyanobacterial biomass with a fertilizer spreader might be assessed to optimize the strategy. It would also be interesting to carry out subsequent studies to test strategies focused on reducing the competition of microorganisms with the cyanobacteria inoculum to increase its growth and colonization on soils amended with higher doses of SS, which usually work in agriculture.

5. Conclusions

The application of soil cyanobacteria to SS-amended soils has been proven a successful strategy for improving soil qualities. In this study, application of the nutrient-rich SS amendment led to a considerable increase in soil fertility by significantly enhancing organic carbon and exopolysaccharides content. In addition, the cyanobacterial inoculum had a major role in stabilizing the soil against both wind and water erosion, although its growth on the amended soil depended on SS dose and the greatest growth was observed at low SS. Cyanobacteria can also grow on salt-contaminated soils and immobilize salts in the soil, reducing their solubility and providing a promising solution as a soil bioremediation technique. Therefore, application of SS amendments and cyanobacterial inoculants together can combine the beneficial effects of the two strategies alone, leading to synergistic effects benefiting the recovery of soil fertility and improving soil stability, while minimizing contamination such as soil salinization. Although not tested in this study, the efficiency of cyanobacteria for immobilization of heavy metals should also be evaluated. To summarize, in view of dryland vulnerability to global change and constraints on crop productivity in these regions, the strategy based on SS application as a soil conditioner coupled with cyanobacteria inoculation appears to be a powerful technique for the recovery and/or improvement of degraded soils, and would potentially enhance crop productivity.

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