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Short Communication

# Cyanobacteria inoculation enhances carbon sequestration in soil substrates used in dryland restoration

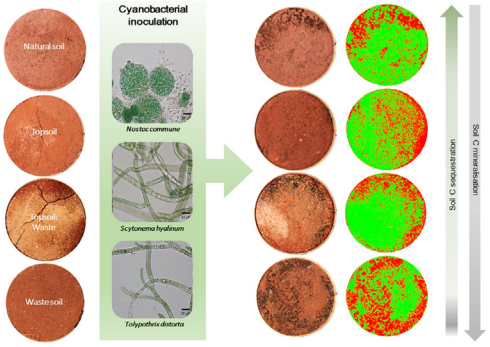
M. Muñoz-Rojas [a,b,c,*], J.R. Román [d], B. Roncero-Ramos [d], T.E. Erickson [a,b], D.J. Merritt [a,b], P. Aguila-Carricondo [d], Y. Cantón [d]

[a] The University of Western Australia, School of Biological Sciences, Crawley 6009, WA, Australia
[b] Kings Park Science, Department of Biodiversity, Conservation and Attractions, Kings Park, 6005, WA, Australia
[c] University of New South Wales, School of Biological, Earth & Environmental Sciences, Sydney 2052, NSW, Australia
[d] University of Almería, Agronomy Department, 04120 Almería, Spain

## HIGHLIGHTS

- Cyanobacteria rapidly colonised mine substrates used in restoration.
- Cyanobacteria biocrust cover increased 31.1–40.6% after three months.
- Cyanobacteria inoculation resulted in increased levels of soil organic C.
- In mine waste, soil organic C increased 3-fold following cyanobacteria inoculation.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Despite significant efforts to restore dryland ecosystems worldwide, the rate of success of restoration is extremely low in these areas. The role of cyanobacteria from soil biocrusts in reestablishing soil functions of degraded land has been highlighted in recent years. These organisms are capable of improving soil structure and promoting soil N and C fixation. Nevertheless, their application to restore functions of reconstructed soils in dryland restoration programs is yet to be harnessed. In this study, we used microcosms under laboratory conditions to analyse the effects of inoculating soil substrates used in post-mine restoration with a mixture of N-fixing cyanobacteria isolated from soil biocrust (*Nostoc commune*, *Tolypothrix distorta* and *Scytonema hyalinum*) on i) the recovery of the biocrust, and ii) the carbon sequestration and mineralisation rates of these substrates. Soils were collected from an active mine site in the mining-intensive biodiverse Pilbara region (north-west Western Australia) and consisted of previously stockpiled topsoil, overburden waste material, a mixture of both substrates, and a natural soil from an undisturbed area. Our results showed that cyanobacteria rapidly colonised the mine substrates, with biocrust coverage ranging from 23.8 to 52.2% and chlorophyll *a* concentrations of up to 12.2 μg $g^{-1}$ three months after inoculation. Notably, soil organic C contents increased 3-fold ($P < 0.001$) in the mine waste substrate (from 0.6 g $kg^{-1}$ to 1.9 g $kg^{-1}$) during this period of time. Overall, our results showed that cyanobacteria inoculation can rapidly modify properties of reconstructed soil substrates, underpinning the potential key role of these organisms as bio-tools to initiate recovery of soil functions in infertile, reconstructed soil substrates.



* Corresponding author at: The University of Western Australia, School of Biological Sciences, Crawley 6009, WA, Australia
*E-mail addresses:* miriam.munozrojas@dbca.wa.gov.au, miriammunozrojas@gmail.com (M. Muñoz-Rojas).

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## 1. Introduction

During extractive operations such as open-cut and strip mining, it is common practice to remove and stockpile the topsoil layer for future re-spreading in areas targeted for restoration. This topsoil is a crucial source of seeds, nutrients, and microorganisms, but is a scarce resource which challenges the success of many restoration programs (Bateman et al., 2018; Golos and Dixon, 2014). Alternative soil substrates, such as mine wastes, are increasingly being used as growth media to reconstruct soil profiles in land restoration programs. However, these growth media are often depleted in organic materials and nutrients, and have poor soil structure and low water retention (Kneller et al., 2018; Merino-Martín et al., 2017). The establishment of native plant species on these altered substrates can be extremely difficult, limiting the capacity to restore ecosystem biodiversity and original plant community composition (Muñoz-Rojas et al., 2016a;Shackelford et al., 2018).

Biological soil crusts (biocrusts) are communities of microscopic and macroscopic organisms that include cyanobacteria, algae, fungi, lichen and moss that live in the uppermost soil layers (Bowker et al., 2010; Chilton et al., 2017; Eldridge and Greene, 1994). Soil biocrusts are present in most environments, but they are particularly important in drylands where they comprise a large portion of the living cover of soils (Belnap et al., 2003; Concostrina-Zubiri et al., 2014).

Cyanobacteria form the most ancient group of biocrusts and contribute to multiple ecosystem functions, including enhancement of soil structure, stability, fertility, surface hydrology, and soil C and N cycling (Chamizo et al., 2012; Zhao et al., 2014). These photosynthetically active microorganisms can promote biocrust re-establishment and may enhance seed germination and plant colonisation of some native plant species (Muñoz-Rojas et al., 2018; Singh et al., 2016; Zaady et al., 1997). Moreover, cyanobacteria can excrete exopolysaccharides (EPSs) – specialised compounds that facilitate soil aggregation and stability and improve soil water infiltration (Adessi et al., 2018; Rossi et al., 2017). But despite the increasing interest of these organisms as biotools in restoration of degraded lands (Maestre et al., 2017), their ability to kick-start soil function by promoting soil C sequestration in post-mining reconstructed soil substrates is yet to be harnessed.

Here, we present a case study in the resource-rich, biodiverse, semi-arid Pilbara region (north-west Western Australia), where hundreds of thousands of hectares are disturbed due to established and emerging iron-ore mine operations (Erickson et al., 2017). At this scale, cost-effective solutions are needed to restore these landscapes in order to preserve biodiversity and achieve functional ecosystems (Muñoz-Rojas et al., 2016b). In this study, we investigated the effects of cyanobacterial inoculation of soil substrates used in dryland restoration on soil biocrust re-colonisation, and enhancement of soil function through soil C sequestration. The specific objectives were to: (i) determine the coverage (%) and photosynthetic biomass (chlorophyll *a*) of cyanobacteria biocrust on soil substrates inoculated with a consortium of three N-fixing cyanobacteria strains (*Nostoc commune*, *Tolypothrix distorta* and *Scytonema hyalinum*); (ii) assess soil organic C contents and C mineralisation rates of these inoculated soil substrates, and; (iii) discuss the implications of this study in dryland restoration.

## 2. Methods

### 2.1. Soil substrates collection

Soil substrates were collected from an active mine in the southern parts of the Pilbara in October 2016 (dry season). The Pilbara biogeographical region (north-west Western Australia, 22°03S, 118°07E to 23°19S 119°43E), covers 178,060 km$^2$. The climate is semi-arid climate with extreme temperatures that range between 19.4 and 33.2 °C on average (25–40 °C during the summer and 12–29 °C during the winter). Mean annual precipitation varies from 250 to 400 mm (Bureau of Meteorology, 2017). Soils consisted of previously stockpiled topsoil (upper 10 cm of the soil profile; TS), overburden mine waste previously extracted from within the mine pit (WS), and a 50:50 blend of both substrates (TWS) (Bateman et al., 2018; Kneller et al., 2018). Additionally, natural soil (NS) from an undisturbed area adjacent to the mine site was collected from the upper 10 cm. Composite samples from each soil substrate (five random samples per soil type) were taken to the soil laboratory at Kings Park and Botanic Garden (Perth, Australia), air-dried and sieved to 2 mm, and divided in two subsamples. One subsample was shipped to the soil laboratory at the University of Almeria (Soil UAL Lab, Spain) and the other was analysed for soil physicochemical properties at Kings Park's soil lab (Table 1).

### 2.2. Cyanobacteria culturing and inoculation

The cyanobacteria strains used in this study were *Nostoc commune* CANT2 UAM817, *Tolypothrix distorta* CANT7 UAM825 and *Scytonema hyalinum* CAU6 UAM820, selected based on their broad presence in soil in arid lands in Australia and worldwide (Aboal et al., 2016; Dojani et al., 2014; Flechtner et al., 2008). Individual cyanobacterial cultures were obtained from available strains at the Soil UAL lab that belonged to the culture collection of the Universidad Autónoma de Madrid (UAM). Cyanobacterial isolates were cultured with $BG11_0$ at 28 °C under light:dark cycles (16:8 h), with a constant input of sterile air, and an irradiance of 70 μmol m$^{-2}$ s$^{-1}$, to obtain enough biomass for inoculation. Cultures were then filtered, resuspended in distilled water ($H_2O_d$), and mixed in equal proportions until a biomass concentration of 2 g L$^{-1}$ was achieved. Biomass concentration was determined by dry weight, through filtering the biomass and keeping it in an oven at 80 °C for 24 h.

### 2.3. Experimental design and methods

The experiment was conducted over three months (December 2016 to February 2017) in a phytotron-plant growth chamber (Air-Frio) maintained at 25 ± 1 °C to simulate average air temperature conditions in the Pilbara. The multi-factorial experiment included the four different soil substrates: NS, TS, WS and TWS, and two treatments (control soils, versus soils inoculated with the cyanobacteria consortium). These

**Table 1**
Soil physicochemical characteristics of collected soil substrates (mean ± SE, n = 4). EC: electrical conductivity, TOC: total organic carbon, TN: total nitrogen, C/N: carbon to nitrogen ratio.

| Soil properties | Natural soil | Topsoil | Topsoil:waste | Waste |
|---|---|---|---|---|
| EC (ms m$^{-1}$)[a] | 32.1 ± 4.6 | 41.7 ± 5.8 | 49.8 ± 4.1 | 55.7 ± 10.7 |
| pH[a] | 6.4 ± 0.1 | 8.0 ± 0.2 | 7.2 ± 0.20 | 7.3 ± 0.0 |
| Clay (%)[b] | 3.8 ± 0.2 | 4.6 ± 0.1 | 3.5 ± 1.0 | 2.1 ± 0.3 |
| Silt (%)[b] | 12.5 ± 0.8 | 24.9 ± 0.7 | 18.6 ± 0.9 | 11.8 ± 1.0 |
| Sand (%)[b] | 83.4 ± 1.3 | 70.5 ± 0.9 | 83.1 ± 1.5 | 86.1 ± 1.0 |
| TOC (g kg$^{-1}$)[c] | 8.0 ± 0.8 | 2.5 ± 0.3 | 2.3 ± 0.7 | 0.6 ± 0.3 |
| TN (g kg$^{-1}$)[d] | 0.2 ± 0.1 | 0.1 ± 0.1 | 0.05 ± 0.0 | 0.0 ± 0.0 |
| C/N | 10.1 ± 0.3 | 9.2 ± 3.8 | 9.5 ± 2.3 | 10.4 ± 1.3 |
| P (mg kg$^{-1}$)[e] | 4.0 ± 0.8 | 4.3 ± 0.3 | 3.0 ± 0.2 | 1.2 ± 0.2 |
| K (mg kg$^{-1}$)[e] | 190.0 ± 10.0 | 170 ± 1.2 | 96.7 ± 2.8 | 38.7 ± 1.9 |
| Mn (mg kg$^{-1}$)[e] | 110.0 ± 8.0 | 86.5 ± 1.7 | 54.7 ± 1.5 | 12.0 ± 2.2 |
| Microbial respiration ($CO_2$)[f] | 32.0 ± 4.4 | 2.8 ± 0.2 | 2.3 ± 0.0 | 2.7 ± 0.4 |
| C mineralisation (qM, %)[g] | 4.0 ± 0.5 | 1.1 ± 0.1 | 1.1 ± 0.2 | 3.9 ± 0.9 |

[a] Analysed using an AD8000 microprocessor-based pH.
[b] Analysed by laser diffraction using a Mastersizer 176 2000 (Malvern Instruments, Malvern, England) after removing the organic matter with $H_2O_2$.
[c] Measured using the *Walkley and Black* method modified by Mingorance et al. (2007).
[d] Measured using the *Kjedhal* method.
[e] Measured with Mehlich Extraction followed by inductively coupled plasma atomic emission spectroscopic (ICP-AES) analysis.
[f] Determined with the 1-day $CO_2$ Solvita test (Muñoz-Rojas et al., 2016b,c).
[g] Determined as described by Francaviglia et al. (2017): qM = $CO_2$/TOC.

treatment combinations were replicated four times and arranged in a completely randomised order of individual microcosms (total n = 32). Microcosms consisted of Petri dishes (66 $cm^2$) each filled with approximately 80 g of each substrate. Soil substrates were inoculated at a concentration of 6 g of cultured biomass dry weight $m^{-2}$ by spreading small volumes of inoculum uniformly, and the equivalent amount of $H_2O_d$ was used in the control soils. Each microcosm was irrigated with a simulated precipitation regime of 12 mm pulses consisting of five events of 2.4 mm (16 ml) every 12 h, followed by dry down events of 4.5 d. The quantity and frequency of water added to the soil samples were calculated based on the mean annual rainfall of the study site (Muñoz-Rojas et al., 2016b) and considering the duration of the experiment (90 d).

### 2.4. Cyanobacteria biocrust and soil measurements

Biocrust cyanobacterial growth was evaluated by digital image analysis and recorded as a percentage of the total soil surface coverage. The concentration of chlorophyll *a* (μg $g^{-1}$) was also used as a surrogate of cyanobacterial biomass in the biocrust. At the end of the experiment, a photo was taken 25 cm above each microcosm after irrigation with a digital camera (CANON EOS 600D; 18 Megapixels resolution). Using these photos, we performed an image analysis that identified training and validation points, selecting 500 training points and 300 validation points for each class (colonised and bare soil). Then, cyanobacteria coverage (%) on the soil substrates was determined by using a supervised maximum likelihood classification, and accuracy of the classification was assessed based on the validation points acquired by image analysis using a confusion matrix for calculating the commission and omission errors for each class, the overall accuracy, and Cohen's Kappa coefficient (K) (Congalton and Green, 2008). Image analysis was carried out with ENVI 4.3 (ITT VIS, Boulder, CO, USA). Chlorophyll *a* (μg $g^{-1}$) was determined as in Castle et al. (2011) by the double ethanol extraction technique measuring absorbance at 665 nm using a Helios Zeta UV–vis spectrophotometer (Thermo, England). Soil organic C contents (TOC, g $kg^{-1}$) and the C mineralisation quotation (qM), which determine the fraction of organic C mineralised over a period of time (%) (Francaviglia et al., 2017; Kneller et al., 2018; Mocali et al., 2008), were determined as detailed in Table 1.

### 2.5. Statistical analyses

All variables analysed, i.e. biocrust coverage, chlorophyll *a*, soil organic C, and C mineralisation were tested for normality and homogeneity of variance using the Kolmogorov-Smirnov and Levene's test. Data were log transformed as necessary for analysis but all presented data are non-transformed for ease of interpretation. Differences across treatments were then tested using a two-way ANOVA and comparisons between means (where significance was found) were performed with the Tukey's HSD test. Pearson's correlations were used to test relationship between analysed parameters. Analyses were performed with R statistical software version 3.1.2 (R Core Team, 2017).

## 3. Results and discussion

### 3.1. Cyanobacteria colonisation of soil substrates

After 90 d of incubation at 25 ± 1 °C, the upper surface of all the control soil substrates remained uncovered by cyanobacteria biocrust, while the inoculated soils were all partially colonised (Fig. 1A). Surface coverage across inoculated soil types ranged between 31.1 ± 5.5% and 40.6 ± 5.2%. We observed highest cover percentage in the inoculated NS followed by the inoculated TWS and WS substrates, and lowest in the TS. However, differences across these four soil types were not significant (Table 2). The recovery of the cyanobacteria coverage in the inoculated substrates was remarkably fast considering the short period of incubation. Previous studies have shown similar cyanobacteria cover rates in inoculated soils of desert areas (Li et al., 2013; Wang et al., 2009). However, some of these studies were conducted in field conditions, and measurements were taken after longer periods following inoculation. For example, Wang et al. (2009) estimated 41.5, 45.5 and

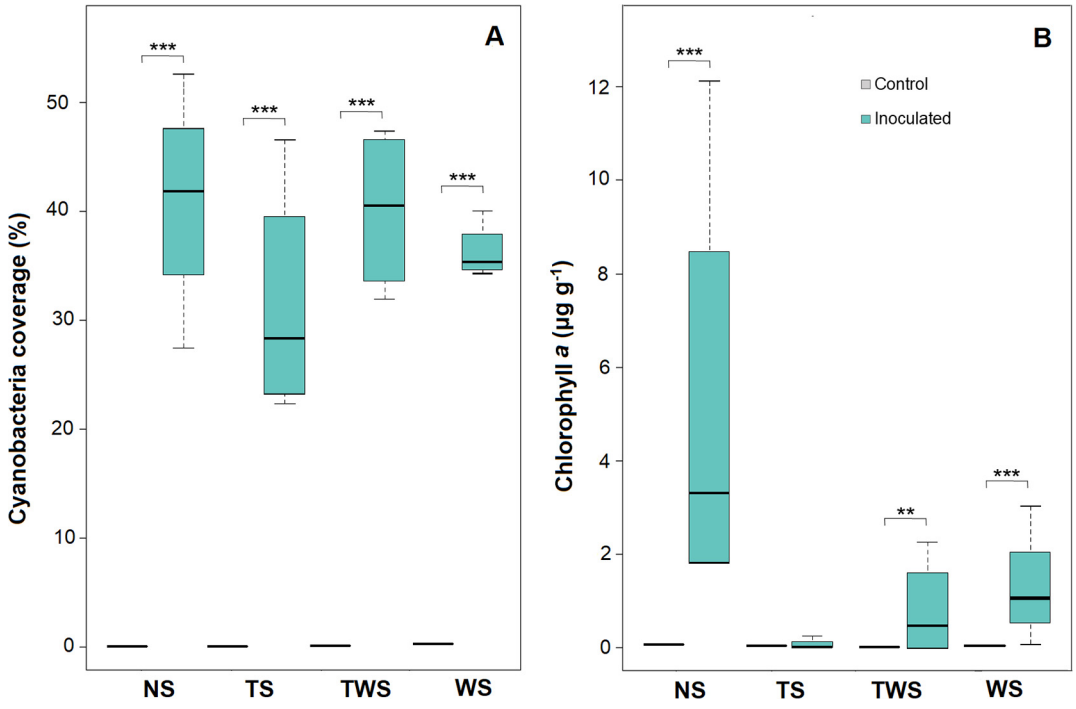


**Fig. 1.** (A) Coverage (%) of cyanobacteria biocrust and (B) chlorophyll *a* (μg $g^{-1}$) of soil substrates (control vs inoculated with cyanobacteria). NS (natural soil), TS (topsoil), TWS (topsoil: waste soil), WS (waste soil). Statistical significance levels: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Note: In (B), chlorophyll *a* values in the control samples are zero.

**Table 2**
Effects of soil substrate (S) and treatment (T, control versus inoculated), and interactive effects of these factors on cyanobacteria coverage (%), chlorophyll *a* (μg $g^{-1}$), total soil organic C (TOC, g $kg^{-1}$) and C mineralisation (qM, %). Statistical significance levels: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, ns - not significant.

| Response | | Coverage | | Chlorophyll *a* | | TOC | | qM | |
|---|---|---|---|---|---|---|---|---|---|
| | df | F value | *P* value | F value | *P* value | F value | *P* value | F value | *P* value |
| S | 3 | 1.06 | ns | 3.10 | * | 89.63 | *** | 11.98 | *** |
| T | 1 | 299.75$e^{-15}$ | *** | 1134.92 | *** | 12.12 | ** | 15.2 | *** |
| S × T | 3 | 1.06 | ns | 3.33 | * | 5.27 | ** | 3.8 | * |

48.5% cover of cyanobacterial and algal forming biocrusts 1, 2 and 3 years, respectively, after inoculation with cyanobacteria.

Chlorophyll *a* contents were zero in all the control soils, and varied between 0 and 12.2 μg $g^{-1}$ in the inoculated samples. These contents were significantly ($P < 0.05$) higher in the inoculated NS (5.2 ± 2.4 μg $g^{-1}$) compared to the other soil substrates (Fig. 1B). This result may be explained by the favourable niche that these undisturbed soils, with higher initial levels soil nutrients, provide to cyanobacteria, as well as possible positive synergies with present soil microorganisms. Park et al. (2017) obtained similar values of chlorophyll *a* (3.01 μg $g^{-1}$) after 4 months following inoculation of a cyanobacteria consortium in a natural soil. Differences in chlorophyll *a* concentrations among the substrates used for rehabilitation (TS, WS and TWS) were not significant. These concentrations ranged between 0.1 ± 0.1 and 1.3 ± 0.6 μg $g^{-1}$ and were similar to those obtained by Wang et al. (2009) who reported values of 1.59 μg $g^{-1}$ one year after inoculating a degraded soil with cyanobacteria from the *Microcoleus* and *Scytonema* genera.

### 3.2. Soil carbon sequestration of inoculated soil substrates

Soil organic C contents were significantly ($P < 0.001$) higher in the inoculated NS, TS and WS, compared to their control counterparts (Table 2, Fig. 2A), and correlated positively ($P < 0.01$) with the chlorophyll *a* values ($r^2 = 0.66$). It is plausible then, that the increased C contents are a result of C fixation by the cyanobacteria. The ability of the photosynthetically active cyanobacteria to sequester C in inoculated soils under optimal light, moisture and pH conditions, has been evidenced in a number of studies (Büdel et al., 2018; Rossi et al., 2017; Zhao et al., 2014). Arid soils inoculated with *Nostoc* spp. have shown increased levels of soil organic C that range from 0.4 g C $kg^{-1}$ soil to 9.0 g C $kg^{-1}$ soil (Pardo et al., 2010). Nisha et al. (2007) also reported an increase of soil organic C (50% of initial values) 90–180 d following cyanobacterial inoculation. Remarkably, our results showed that TOC in the inoculated mine waste substrate (WS) increased 3-fold during the 90-day incubation. These contents raised from 0.6 g $kg^{-1}$ (Table 1) to 1.9 g $kg^{-1}$ (Fig. 2A), highlighting the ability of cyanobacteria to colonise infertile substrates. Differences in TOC were small in the TS, and not significant in the TWS, which may indicate a potential negative interaction of cyanobacteria with present root and seed exudates, or soil microorganisms affected by changes in nutrient dynamics during topsoil stockpiling (Birnbaum et al., 2017).

Levels of microbial activity did not change after cyanobacterial inoculation in any of the studied substrates (results not shown). However, the carbon mineralisation quotient (qM) decreased with cyanobacterial inoculation in all substrates (Fig. 2B). In particular, we found significantly ($P < 0.001$) lower values of qM in the inoculated NS, TS and WS compared to their non-inoculated counterparts, with higher differences observed in the NS and WS substrates. The soil C mineralisation and $CO_2$ production is inversely proportional to the stability of soil organic C. High rates of C mineralisation are therefore associated with labile C that is rapidly accessible to soil microbes (Mganga et al., 2016; Tian et al., 2016). Decreased soil C mineralisation rates following cyanobacterial inoculation of the studied soil substrates indicate a potential production of more stable forms of soil C, as suggested by previous studies. For example, Pardo et al. (2010) reported an increase in stable forms of soil organics, mostly in the form of humic and fulvic substances in soils inoculated with cyanobacteria. Similarly, Miralles et al. (2012) analysed different chemical fractions of organic matter produced in soil biocrusts and underlying soils, and found high contents of humic acid-like forms of soil organic C.

### 3.3. Implications for recovery of soil function of reconstructed soils in restoration programs

Several methods have been investigated in drylands to promote soil health and increase soil carbon contents, including the addition of

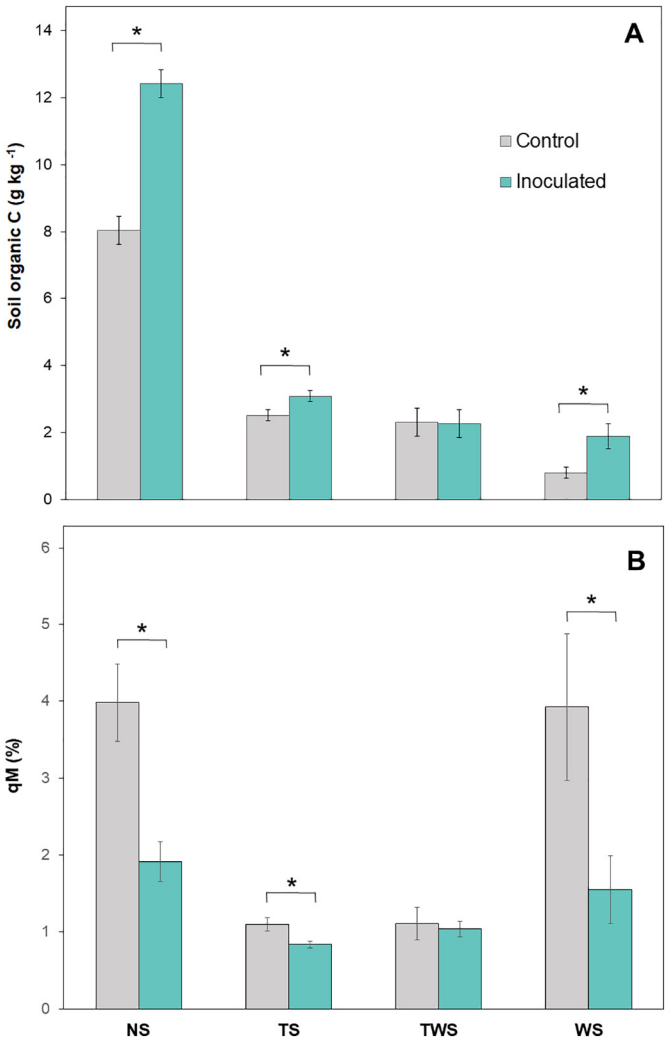


**Fig. 2.** (A) Soil organic C contents (%) and (B) soil C mineralisation quotient (qM, %) of soil substrates (control vs inoculated with cyanobacteria). NS (natural soil), TS (topsoil), TWS (topsoil:waste soil), WS (waste soil). Statistical significance levels: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

exogenous organic C in the form of biochar or organic amendments (Hueso-González et al., 2018; Luna et al., 2016). These methods can potentially increase soil organic C levels in reconstructed soils; however, they may be only effective in the long-term, as several months or years are often required before a significant effect on treated soils is evident (Kneller et al., 2018).

Our results showed that cyanobacteria inoculation with N-fixing species that are present worldwide, can rapidly modify properties of reconstructed soil substrates, resulting in a considerable increase in biocrust coverage, cyanobacteria biomass, and soil organic C levels. The benefits of applying cyanobacteria as bio-tools to enhance soil fertility and initiate recovery of soil functions in infertile substrates may be substantial, e.g. by reducing associated costs of external fertiliser and amendment inputs (Maestre et al., 2017; Singh et al., 2016). Furthermore, cyanobacteria from soil biocrust occur naturally in arid landscapes, and, despite being in a dry, dormant stage for several years, have the ability to reactivate after rainfall (Belnap et al., 2013; Büdel et al., 2018; Williams et al., 2014). Nevertheless, using these organisms under field conditions for large-scale restoration may bring several challenges, including abiotic and biotic factors such as water limitations at early stages of biocrust and plant development, and interactions with native soil microbial communities. Future research should consider how inoculating indigenous, locally-adapted cyanobacteria species can be achieved in large-scale restoration settings. This will likely involve the development of suitable tools and technologies to deliver targeted cyanobacteria cultures, and the design of effective strategies for large-volume production of cyanobacteria biomass (Antoninka et al., 2016; Bowker, 2007; Muñoz-Rojas et al., 2018; Rossi et al., 2017).

## 4. Conclusions

Overall, our results showed a positive and rapid effect of inoculated N-fixing cyanobacteria on the biocrust cover and the levels of soil organic C of soil substrates used in post-mine restoration. After 90 d following cyanobacterial inoculation, 30–40% of the soil surface of the treated soil substrates was covered by biocrust cyanobacteria, and chlorophyll *a* contents increased from 0 to 12.2 μg $g^{-1}$. During this period, soil organic C contents were significantly ($P < 0.001$) higher in the inoculated NS, TS and WS, compared to the control (non-inoculated) soils. In particular, soil C contents increased 3-fold in the mine waste substrate, which originally contained the lowest levels of organic C. Moreover, soil C mineralisation rates were generally lower in the inoculated soils, suggesting a stabilisation of the accumulated soil C, and therefore highlighting the potential of cyanobacteria to increase soil function of reconstructed soils by promoting soil C sequestration.

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