Artificial biocrust establishment on materials of potash tailings piles along a salinity gradient

V. Sommer^{1,2} · A. Palm² · A. Schink¹ · P. Leinweber³ · N. Gose² · U. Karsten¹ · K. Glaser¹

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Abstract

Biocrust communities provide a pallet of ecosystem services, such as soil stabilization, altering of hydrological cycles and primary production, and often are the first colonizers of unvegetated surfaces during succession. Therefore, artificially establishing biocrusts can improve soil properties, for example, by stabilizing bare soil surfaces against erosion or by accumulating nutrients. In this study, the establishment of artificial biocrusts was tested for the restoration of potash tailings piles that result from potash fertilizer production and mostly consist of NaCl. A biocrust cover as primary vegetation could decrease the saline seepage waters by trapping rainwaters, thereby reducing the environmental pollution. In a laboratory experiment, we created a salt gradient by mixing the tailings materials with non-saline dune sand. Surface material of the abandoned potash tailings pile Neuhoof-Ellers (NE) and material of the Infiltration Hampering Stratum (IHS) were tested, along with a treatment with bone char^{plus} (BC^{plus}) and sodium alginate. A mixture of 50% (w/w) IHS and dune sand was most successful for the establishment of green biocrust microalgae, based on increased biomass and photosynthetic performance. The chlorophyll *a* content was negatively correlated with the electrical conductivity (EC), and was significantly increased in the BC^{plus} and sodium alginate treatment, while biocrusts failed to establish on pure tailings piles substrates. The limit of the substrates EC for biocrust establishment was 35 mS cm⁻¹. This limit provides a baseline for future studies that should use BC^{plus} and sodium alginate to increase the success of biocrust establishment on potash tailings piles.

Keywords Biocrust · Restoration · Potash industry · Inoculation · Green algae

Introduction

Biological soil crusts (biocrusts) are often overlooked, but ecologically significant communities found at the soil-atmosphere interface. A biocrust is defined as an association of various micro- and macro-organisms that inhabit the uppermost millimeters of the soil. Filamentous cyanobacteria and green algae interweave the soil surface and excrete sticky extracellular polymeric substances (EPS) that bind soil particles to each other and to the organisms (Rossi et al. 2017). Together with unicellular microalgae, for example,

green algae and diatoms, as well as other bacteria, archaea, and sometimes also mosses and lichens, a robust biocrust is formed.

Biocrusts dominate drylands worldwide, creating biodiversity hotspots (Weber et al. 2016a, b), but are also found in other biomes. In newly emerging habitats, such as glacier forefields, biocrusts are one of the first colonizers (Breen and Lévesque 2006; Baumann et al. 2017).

Biocrust communities provide a range of ecological services. As soil stabilizing surfaces, biocrusts reduce erosion by wind and water (Belnap and Büdel 2016), and their important role in hydrological processes has been discussed in various studies (Belnap 2006; Bowker et al. 2013; Chamizo et al. 2016; Gypser et al. 2016; Kidron 2016, 2019; Whitney et al. 2017; Rodríguez-Caballero et al. 2018). In their mostly harsh habitats, biocrust plays an important role in nitrogen (Barger et al. 2016) as well as carbon cycles (Sancho et al. 2017), thereby accounting for 7% of the global terrestrial net primary production and about 50% of terrestrial biological N fixation (Elbert et al. 2012). Despite their adaption to harsh

✉ U. Karsten
ulf.karsten@uni-rostock.de

¹ Applied Ecology and Phycology, University of Rostock, Albert-Einstein-Str. 3, 18059 Rostock, Germany

² Upi UmweltProjekt Ingenieurgesellschaft, Breite Straße 30, 39576 Stendal, Germany

³ Soil Sciences, University of Rostock, Justus-von-Liebig-Weg-6, 18059 Rostock, Germany

environmental conditions, biocrusts should be regarded as vulnerable communities worthy of protection. They are strongly affected by disturbances such as trampling and grazing by cattle (Belnap and Eldridge 2003; Yang et al. 2020), vehicle traffic (Zaady et al. 2013), and mining activities (Williams et al. 2019). A recent study reported the negative effect of biocrust disturbance on soil CO₂ emission (Yang et al. 2020). To prevent the loss of that valuable vegetation cover, several studies aimed at the rehabilitation of biocrusts. As an example, fencing is a passive method to exclude herbivory and trampling (Condon et al. 2020). However, the natural recovery of disturbed biocrusts in water-limited drylands is rather slow (Belnap and Eldridge 2003), and the time for recovery strongly depends on several parameters, such as environmental conditions (e.g., water availability) or timing of the disturbance (Weber et al. 2016a, b).

Therefore, active intervention may be required for faster re-establishment of a viable and dense biocrust cover. This was realized in studies that directly applied representative biocrust organisms to the soil surface, using cyanobacteria (Chen et al. 2006; Rossi et al. 2017; Fattahi et al. 2020; Zhao et al. 2021), bryophytes (Xiao et al. 2015; Bu et al. 2018; Cruz de Carvalho et al. 2018), or lichens (Ballesteros et al. 2017). Furthermore, the application of stabilizers (Park et al. 2017; Peng et al. 2017; Li et al. 2021) showed accelerated development of cyanobacterial biocrusts.

Besides the restoration of disturbed biocrusts, artificial biocrust development can be actively initiated in areas without previous natural biocrust cover, in order to prevent or reverse soil degradation (Chen et al. 2006; Rossi et al. 2017; Chi et al. 2020; Li et al. 2021) or to stabilize sand and other surfaces, such as artificial water-body margins (Cruz de Carvalho et al. 2018). So far, research on artificial biocrusts is restricted to dryland areas, while such studies are absent from Central Europe.

Degraded landscapes in Europe emerged, for example, following potash mining. During potash fertilizer production, vast amounts of waste salt material (mostly NaCl) need to be deposited in so-called potash tailings piles. In central Germany, potash tailings piles shape the landscape due to their large scales with heights of up to 200 m, and due to the impact of salt on the surrounding environment. Rainfall washes out saturated salt brines and thus creates extremely saline conditions in habitats far from the coast. Several studies evaluated covering potash tailings piles with higher plants, in order to trap rainwater and reduce the salt output (Schmeisky and Podlacha 2000; Niessing 2005). However, these methods require large amounts of non-saline substrates, which is difficult to apply to the steep slopes of larger potash tailings piles. A recent approach used insoluble additives mixed to a small percentage with the salt waste (Kockx 2017). This created an Infiltration Hampering Stratum (IHS) that increased the surface evaporation by

50%, thereby decreasing the leakage of saturated salt brine (Kockx 2017). Moreover, in a pilot field study, a spontaneous settlement of young biocrusts was recorded from the IHS (Sommer et al. 2019). Therefore, the application of biocrusts moved into focus as a potential biotool for the surface restoration of potash tailings piles. A biocrust cover could potentially decrease the amount of saline seepage water by trapping rainwater, and increasing water retention as well as evaporation, and in this way lower the salt impact of potash tailings piles on the environment. Biocrusts have recently been studied in the surrounding of several tailings piles in Germany, and numerous green algal strains have been isolated, established as clonal cultures, and identified for further experiments (Sommer et al. 2020a, b). These isolates are promising candidates for artificial biocrust formation since they are expected to tolerate saline conditions.

Salt-tolerant microalgae are able to survive saline conditions to a certain level. EPS contribute to salinity tolerance since these compounds also act as an extracellular barrier to reduce the salt impact (Chen et al. 2006). Furthermore, the intracellular production and accumulation of salt-protective organic osmolytes has been characterized in several terrestrial green algae (Gustavs et al. 2010, 2011). These osmotically active substances increase intracellular osmotic pressure to withstand extracellular osmotic pressure caused by salt ions. However, the production of organic osmolytes requires energy and thus is metabolically costly (Erdmann and Hagemann 2001). At high extracellular salinity level, a tipping point of the osmotic balance may be reached, with water flowing out of the cell due to the rising osmotic gradient, leading to shrinkage and, eventually, to physical damage.

Therefore, knowledge of the upper limits of substrate salinity for biocrust algae is important for the establishment of biocrusts on potash tailings piles. The inoculated algal cells have to (1) survive the inoculation on the saline substrate, and (2) be able to accumulate biomass over a longer period of cultivation. Consequently, the substrate salinity should not induce instant cell death, but should allow the cells to survive over time and, importantly, grow. The aim of the present study was to assess the establishment of artificial biocrusts by inoculating selected green algae on potash tailings piles materials along a salinity gradient. The results should provide information on the limit for both, short-term inoculum survival and long-term biomass accumulation.

In addition, the usage of a stabilizer (sodium alginate) as well as a fertilizer (bone char^{plus}, BC^{plus}) to enhance biocrust establishment was evaluated. Sodium alginate had been proven to increase biocrust establishment (Peng et al. 2017). Bone char as a recycling product is made by pyrolysis of animal bones from slaughterhouse waste. Because bone char is rich in P, it is discussed as a promising alternative P-fertilizer for sustainable agriculture (Siebers and

Leinweber 2009). Furthermore, bone char has been enriched with sulfur, the result being BC^{plus} (Zimmer et al. 2019). BC^{plus} application in a pot experiment with rye grass showed a yield comparable to a common P-fertilizer application, whereas conventional bone char did not reach that level (Zimmer et al. 2019). Therefore, providing BC^{plus} as a P source for artificial green algal biocrusts could increase their biomass and thus support the establishment of biocrusts.

Material and methods

Green algal strains

Three green algal strains (Supplementary Fig. 1) that were isolated from biocrusts in the surrounding of potash tailings piles were used for inoculation: *Pseudostichococcus monallantoides* WT-3-1-H (MT991551; Sommer et al. 2020b), *Chloroidium* sp. SY-1-2-R, and *Nannochloris* sp. TTF-3-1-J, which were identified based on 18S rRNA sequencing. These strains were isolated using saline medium (agarized, 3% (w/v) NaCl); the details on the isolation as well as habitat descriptions have been published earlier (Sommer et al. 2020a, b). Pre-cultures were maintained separately in aerated Erlenmeyer flasks filled with 3 N-BBM + V medium (Bischoff and Bold 1963; modified by Starr and Zeiskus 1993) that was enriched with 6% (w/v) NaCl. Culturing was approximately for 8 weeks, until sufficient amounts of biomass for inoculation were obtained. All cultures were kept at 20 °C in a 16:8 h light:dark cycle (Lumilux daylight L36W/954, Osram, 80,807 Munich, Germany) with 30 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$.

Description of the saline and non-saline materials

Four types of saline materials were used for the experiment: two potash tailings piles materials and two salt materials. Surface material (uppermost 5 cm) of the Infiltration Hampering Stratum (IHS) was collected from residuals of a pilot study on a small-scale potash tailings pile model (Kockx 2017; Sommer et al. 2019). In addition, surface material derived from the plateau area of the abandoned potash tailings pile in Neuhof-Ellers (NE), Germany, was collected in 2018. The pure waste salt (WS), i.e., the overburden representing the solid, fresh tailings from the potash industry, was collected in April 2016. In addition, sodium chloride (NaCl) was used. Moreover, non-saline dune sand was collected in March 2018 from the Baltic Sea coast, Warnemünde beach, Rostock, Germany. All the mentioned substrates were air-dried and sieved (< 2 mm) prior to utilization, whereas WS was additionally dried in the oven (70 °C, overnight) before sieving.

Preparation and characterization of the salt gradient and the treatments

The saline materials IHS, IES, WS, and NaCl were partly mixed with non-saline dune sand (Table 1) in order to create a salinity gradient. The 25% (w/w) IHS substrate, for example, was prepared by mixing 25 g of the IHS material with 75 g dune sand. The substrates 2.5% (w/w) NaCl and NoSalt (100% (w/w) dune sand) were used in addition. The additive treatment (A⁺) was prepared for each substrate by adding 1% (w/w) sulfur-enriched bone char (1–2 mm, BC^{plus}, Burgstaller et al 2012, Zimmer et al. 2019). The treatment without additive (A⁻) received no BC^{plus}. Figure 1 provides an overview over all substrates and the two treatments.

The electrical conductivity (EC) and the pH of the substrates were measured before the experiment. Ten grams of substrate was mixed with 50 mL distilled water ($\text{EC} < 5 \mu\text{S cm}^{-1}$) to obtain a soil solution for the EC measurement. The samples were shaken for 1 h. After 30 min of sedimentation, the EC was determined in the supernatant using a conductivity probe (Seven MultiTM, Mettler Toledo, in Lab 731 probe, Switzerland). The pH was measured as previously described (Sommer et al. 2020a).

The premixed substrates were added into 6-well plates (9.6 cm² surface per well) that were perforated three times

Table 1 Overview over the substrates used for artificial biocrust formation. Saline materials were the potash tailings piles IHS (Infiltration Hampering Stratum), material from the potash tailings pile NE (Neuhof-Ellers), fresh potash tailings WS (waste salt), and NaCl; NoSalt (non-saline dune sand)

Substrate	Concentration of saline material [% (w/w)]	Concentration of non-saline dune sand [% (w/w)]
25% (w/w) IHS	25	75
50% (w/w) IHS	50	50
75% (w/w) IHS	75	25
100% (w/w) IHS	100	0
25% (w/w) NE	25	75
50% (w/w) NE	50	50
75% (w/w) NE	75	25
100% (w/w) NE	100	0
1% (w/w) WS	1	99
5% (w/w) WS	5	95
10% (w/w) WS	10	90
15% (w/w) WS	15	85
1% (w/w) NaCl	1	99
2.5% (w/w) NaCl	2.5	97.5
5% (w/w) NaCl	5	95
10% (w/w) NaCl	10	90
15% (w/w) NaCl	15	85
NoSalt	0	100

Fig. 1 Overview on the 6-well plates filled with the substrates used for artificial biocrust formation, including concentrations of added saline materials (w/w). **a** IHS (Infiltration Hampering Stratum); **b**

NE (Neuhof-Ellers); **c** WS (waste salt); **d** NaCl; **e** NoSalt (non-saline dune sand). Additives: BC^{plus} and sodium alginate

at the bottom; each well was filled 1 cm high. Three replicates without (A⁻) and three replicates with additive treatment (A⁺) were used for each substrate. In addition, the wells for the additive treatments were sprayed with a Na-Alginate solution using a sterile pump spray, resulting in 2 g m⁻² Na-alginate (Peng et al. 2017). All plates were closed with transparent plastic lids and watered from the bottom using artificial rainwater (Williams et al. 2005) until the substrates reached saturation.

Preparation and characterization of the inoculum

The unialgal strains were harvested from culture medium using sterile glass pipettes, and afterwards carefully suspended using sterile syringes. The collected cells of the three individual strains were mixed afterwards in a small vessel. Microscopic examination of the mixed inoculation suspension was performed to ensure vitality of the cells and to roughly estimate the proportional percentage of each

green algal strain in the mixture. Three microscope slides with one drop inoculation suspension each were evaluated to roughly estimate the proportion of the three strains, which was approximately 9:3:1 (*Pseudostichococcus:Chloroidium:Nannochloris*).

The average chlorophyll *a* content (Chl *a*) of 4 mL inoculation suspension was measured in four replicates. The biomass was filtered with Whatman GF/6 glass fiber filters and was stored at -20°C until Chl *a* extraction. A pinch of MgCO_3 was added to the samples, and Chl *a* was extracted with 3 mL 96% ethanol in a water bath (78°C , 5 min). After centrifugation ($4.640 \times g$, 5 min), the absorbance was measured in 1 cm cuvettes at 750 and 665 nm. The Chl *a* content of the inoculum was calculated (HELCOM 2015; LUNG SOP-Nr: 640-Chlorophyll-KG):

$$\mu\text{g Chl } a \text{ L}^{-1} = \frac{(E_{665} - E_{750}) * v * 10^6}{83 * V * d} \quad (1)$$

where v = extraction volume [mL], V = filtered volume [mL], and d = length of cuvette.

The average dry mass (DM) of 4 mL inoculation suspension was determined by filtration of four replicates. Pre-weighed Whatman GF/6 glass fiber filters with the attached biomass were dried in the oven overnight (40°C) and weighted subsequently. The DM was calculated by subtracting the empty weight of the respective filter from the filter with attached dried biomass.

Inoculation and experimental set-up

The inoculation suspension was transferred to sterile pump spray bottles, and 450 μL ($\text{DM } 405 \pm 19 \text{ mg m}^{-2}$; Chl *a* $1.46 \pm 0.11 \text{ mg m}^{-2}$) was equally sprayed on the substrate surface of each well from approximately 5 cm distance. The lids were closed but the plates remained unsealed in order to provide constant gas exchange. Each 6-well plate was placed in a plastic coaster and transferred to mini indoor greenhouses ($36 \times 22 \times 13 \text{ cm}$; Pflanzen-Kölle, Germany). The cultivation was set at 20°C and $25\text{--}30 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (16:8 h light:dark cycles; Lumilux daylight L36W/954, Osram, Germany) reaching the sample surface. The samples were kept constantly moistened by adding artificial rainwater to the empty spaces between the wells, providing saturated air humidity in addition to the watering from the bottom before the experiment. The artificial rainwater was refilled once a week. The experiment was run for 4 weeks.

Biocrust monitoring during the experiment

During the experimental phase, the biocrusts were inspected every week. Photographs were taken from each 6-well plate, and imaging PAM (pulse amplitude modulation, I-PAM)

measurements were performed for each well using an imaging PAM IMAG-CG/S equipped with an IMAG-MIN/B measuring head (blue light) and an IMAG-K-6 camera (G-145B, Heinz Walz GmbH, Germany). The effective quantum yield of the photosystem II (PSII), in short Y(II), was measured in the center of each well. The measuring area ($24 \text{ mm} \times 32 \text{ mm}$) corresponded to 81.7% of the well surface.

Measurement of Chl *a* and water content

After the experimental phase, the surface (i.e., the uppermost 3 mm) of each well was collected for the determination of Chl *a*. The Chl *a* extraction was performed as described above. The absorbance of the extract was measured in 1 cm cuvettes at 750, 696, 665, 649, and 632 nm. The Chl *a* content [mg m^{-2}] was calculated as described by Ritchie (2008) and normalized to area.

The water content of the remaining sub-crust substrate was determined as follows: The mass of the substrate was recorded immediately after removing the surface for the Chl *a* measurements. Samples were then dried overnight at 70°C and subsequently cooled in a desiccator, followed by DM measurement. The water content (w/w) was calculated by subtracting the DM from the fresh mass.

Microscopic examination

In order to monitor any changes in microalgal composition, the biocrusts were studied under an Olympus BX51 light microscope after 4 weeks using differential interference contrast and $400\times$ magnification (DIC; Olympus, Germany). For this, a small amount of the biocrust (e.g., biocrusts attached to a few sand grains) from each well was carefully collected, aiming at minimal disturbance of the biocrust. The microalgal composition was assessed by estimating the proportions of the three green algal strains.

Statistical analyses

The correlation between the determined properties (pH, EC, Chl *a*, and Y(II)) at week four was calculated using the Spearman's rank correlation test and displayed as a correlation matrix. The Wilcoxon rank sum test was performed to evaluate significant differences between the measured variables in the two treatments (A^- , A^+). Statistical analyses were computed with R (V 3.6.3) in the R studio interface (V. 1.2.5033, RStudio Inc.). In order to identify the maximum EC suitable for biocrust establishment, the substrates EC that allowed successfully established biocrusts ($\text{EC}_{\text{BSC_growth}}$) was subset from the mean EC of the respective substrate. The $\text{EC}_{\text{BSC_growth}}$ was defined as the EC only of samples with $\text{Chl } a_{\text{End}} > \text{Chl } a_{\text{inoculum}}$ (Chl a_{End} : Chl *a*

content after the experiment; Chl a_{inoculum} : Chl a content of the inoculum).

Results

Characteristics of the substrates

The EC of the substrate-water solution was between 0.04 and 62 mS cm⁻¹ (Table 2). Comparing the EC of the non-mixed materials (100%), IHS had a higher EC than NE, whereas the NoSalt (dune sand) had the lowest EC. WS and NaCl had similar ECs. The substrates treated with BC^{plus} had slightly higher ECs than the samples without the additive.

The pH of the substrates ranged between 6.7 and 8.8. The majority of the samples was neutral (8 > pH > 6.5), whereas a few samples without added BC^{plus} (100% (w/w) IHS, 5–15% (w/w) NaCl) were basic (pH > 8). The lowest pH was measured in WS as well as in the NoSalt samples. Overall, the pH was significantly lower with added BC^{plus} (Table 3).

Development of the induced biocrusts observed by visual inspection

Directly after inoculation at the beginning of the experiment, biocrusts were undetectable. However, over the course of 4 weeks, visible biocrusts developed on several substrates

Table 3 Wilcoxon rank sum test results to test for significant differences in the respective variables between the two treatments (without additives, with additives). The pH and EC of the premixed substrates were measured before the experiment, Y(II) was measured during the experiment, and Chl a and the water content were measured after 4 weeks. Significant p -values are marked with asterisks (*)

	w	p
pH	1821	0.026*
EC	1341	0.474
Y(II) week 0	1491.5	0.837
Y(II) week 1	1362	0.519
Y(II) week 2	1371.5	0.564
Y(II) week 3	1398	0.679
Y(II) week 4	1362	0.512
Chl a content	1883	0.009*
Water content	1084	0.152

(Fig. 2), recognizable by the appearance and subsequent deepening of green color.

On the IHS substrate, a green surface was recorded in one replicate of 25% (w/w) IHS A⁺ after 4 weeks, and in three replicates of 50% (w/w) IHS A⁻. Due to the dark coloring of the NE materials, only a slightly green cover was detectable, namely on three replicates of 25% (w/w) NE A⁺. The biocrusts on WS and NaCl were most prominent on all 1% (w/w) A⁺ replicates, whereas the three 1% (w/w) A⁻ replicates showed a slightly less greenish surface. One replicate of 2.5% (w/w) NaCl A⁺ showed a visibly green cover.

Several biocrusts completely covered the substrates surfaces, whereas some replicates of the IES and NE substrates had less evenly distributed algal covers. These replicates showed either an accumulation on the edges of the wells

Table 2 Characteristics of the substrates used for artificial biocrust establishment. The percentages refer to (w/w) of the saline materials mixed with non-saline dune sand: IHS (Infiltration Hampering Stratum), NE (Neuhof-Ellers), WS (waste salt), and NaCl. The treatment A⁻ was without additives and A⁺ with additive (BC^{plus})

Substrate	EC [mS cm ⁻¹]				pH			
	A ⁻		A ⁺		A ⁻		A ⁺	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
25% (w/w) IHS	18.443	0.597	17.520	1.394	7.556	0.007	7.521	0.080
50% (w/w) IHS	33.167	2.346	35.067	0.899	7.533	0.159	7.476	0.243
75% (w/w) IHS	43.133	0.450	48.967	1.053	7.852	0.120	7.606	0.233
100% (w/w) IHS	54.033	5.792	62.067	0.125	8.051	0.069	7.517	0.048
25% (w/w) NE	8.510	0.757	9.433	0.249	7.657	0.030	7.798	0.033
50% (w/w) NE	16.177	0.313	16.294	1.019	7.768	0.022	7.855	0.038
75% (w/w) NE	22.767	0.330	24.733	0.450	7.884	0.102	7.855	0.058
100% (w/w) NE	28.167	0.047	29.433	0.478	7.758	0.036	7.832	0.503
1% (w/w) WS	2.890	0.659	4.300	1.126	6.744	0.023	6.797	0.035
5% (w/w) WS	11.860	0.991	14.917	2.208	6.915	0.035	6.746	0.070
10% (w/w) WS	34.300	2.707	35.600	2.142	6.970	0.035	7.088	0.033
15% (w/w) WS	42.200	4.825	48.267	4.969	7.004	0.021	7.154	0.166
1% (w/w) NaCl	3.817	0.126	4.200	0.259	7.549	0.023	7.331	0.330
2.5% (w/w) NaCl	8.840	0.313	9.720	0.306	7.009	0.173	6.947	0.337
5% (w/w) NaCl	12.433	0.192	22.467	1.470	8.072	0.022	7.609	0.300
10% (w/w) NaCl	32.967	0.806	34.400	1.023	8.315	0.081	7.592	0.435
15% (w/w) NaCl	48.300	0.216	47.300	1.417	8.445	0.014	7.665	0.395
NoSalt	0.041	0.000	0.141	0.012	7.869	0.066	7.444	0.070

Fig. 2 Photographs of the 6-well plates filled with saline substrates 4 weeks after inoculation with a green algal mixture, including the percentages (w/w) of saline materials mixed with non-saline dune

sand: **a** IHS (Infiltration Hampering Stratum); **b** NE (Neuhof-Ellers); **c** WS (waste salt); **d** NaCl; **e** NoSalt (100% dune sand); additives: BC^{plus} and sodium alginate

(25% (w/w) NE A⁺, Fig. 2b) or a slightly uneven coverage at the surface (25% and 50% (w/w) IHS, Fig. 2a).

Microscopic observation of the induced biocrusts

The biocrusts were inspected under the microscope after 4 weeks. The dominant microalga was *Pseudostichococcus monallantoides* that formed filaments interweaving the substrate particles, which easily disintegrated during handling under the microscope. The other two green algae used for inoculation, *Chloroidium* sp. and *Nannochloris* sp., were much less present. The proportion was estimated to be

50:5:1 (*Pseudostichococcus*:*Chloroidium*:*Nannochloris*). There was no specific pattern of the biocrust community found among the substrates regarding both material type and salinity. The cells of *P. monallantoides* appeared to be the most vital, showing intact cell components with evenly green chloroplasts. In contrast, some cells of *Chloroidium* sp. exhibited irregular shapes, instead of the elliptical cells typical for the genus (Supplementary Fig. 1). Furthermore, chloroplasts in cells of *Chloroidium* sp. and *Nannochloris* sp. were also often irregularly shaped and pale green, while cells appeared to accumulate intracellular grains/droplets (probably starch/lipids).

Fig. 3 Effective quantum yield Y(II) of the surface of saline substrates (a–d) as well as of a non-saline substrate (e) 0, 2, and 4 weeks after biocrust inoculation using a green algae mixture. Percentages refer to (w/w) of the saline materials mixed with non-saline dune

sand; **a** IHS (Infiltration Hampering Stratum); **b** NE (Neuhof-Ellers); **c** WS (waste salt); **d** NaCl; **e** NoSalt (100% dune sand). Additives: BC^{plus} and sodium alginate

Fig. 4 Spearman's rank correlation test of properties determined in an experiment on artificial biocrust formation in a salinity gradient on materials from potash tailings piles. Y(II), Chl *a*, and water content were measured after 4 weeks, EC and pH indicate the initial substrate properties. Numbers refer to Spearman's correlation coefficient (ρ)

Photosynthetic performance of the induced biocrusts

The photosynthetic performance of the biocrusts changed over the course of the experiment (Fig. 3, Suppl. Figure 2). The I-PAM measurements directly after inoculation (Suppl. Figure 2a) showed the highest Y(II) in the NoSalt samples, which was on average 0.54 for NoSalt A⁻ and 0.59 for NoSalt A⁺. Other samples exhibited no signal in either treatments (75% (w/w) IHS, 12.5% (w/w) WS, 15% (w/w) NaCl). After 1 week (Suppl. Figure 2b), both treatments of 1% (w/w) NaCl and 1% (w/w) WS showed similar high Y(II) values as NoSalt, whereas those on all IHS substrates decreased compared to the previous week. The samples 100% (w/w) IHS, 5% and 12.5% (w/w) NaCl did not display a signal in addition to the inactive samples of the previous week. In the IHS samples, the measured Y(II) increased in the next 2 weeks, whereas in NoSalt, it decreased. After 4 weeks, one replicate of the 25% (w/w) IHS A⁺ samples showed the highest photosynthetic performance (Fig. 3), whereas the mean Y(II) was particularly pronounced in both treatments of the 50% (w/w) IHS samples (Suppl. Figure 2e). There was a positive correlation between the Chl *a* content and the Y(II) measured at week 4 (Fig. 4).

Furthermore, the 50% (w/w) IHS samples with and without additives reached the highest increase from start to end of the experiment, calculated as the relative change of the effective quantum yield $\Delta Y(II)$ after inoculation vs. end of the experiment (Fig. 5), i.e., photosynthetic performance in these IHS samples increased on average by 79% for 50% (w/w) IHS A⁻ and by 59% for 50% (w/w) IHS A⁺,

respectively. Both treatments of the 25% (w/w) IHS samples also exhibited an increase of the Y(II). Some replicates of these two substrates further reached a Y(II) that exceeded the value of week 0 ($\Delta Y(II) > 100\%$). In contrast, biocrusts on every other substrate did not reach a Y(II) that was higher than that after inoculation ($\Delta Y(II) < 0\%$).

Chl *a* and water contents

The Chl *a* content was measured at the end of the experiment as a proxy for the biomass of the biocrusts (Fig. 6a). The Chl *a* values were highest on 50% (w/w) IHS A⁻ (mean 40.33 mg m⁻²) and 25% (w/w) IHS A⁺ (22 mg m⁻²), while 25% (w/w) NE A⁺, 1% (w/w) WS A⁺, 1% (w/w) NaCl A⁺, and NoSalt A⁺ had values around 15 mg m⁻². Among the treatments, the range of the Chl *a* content of the three replicates was larger by up to factor three, compared to the mean. The maximum range was observed in the 25% (w/w) IHS A⁺ sample (span 60.9 mg m⁻²); the three replicates showed a Chl *a* content of 0 mg m⁻², 7.2 mg m⁻², and 60.9 mg m⁻², respectively. On other substrates, the range of the Chl *a* content was around 10 to 20 mg m⁻² (15% (w/w) NE A⁺, 1% (w/w) WS A⁺, 1% (w/w) NaCl A⁺, 1% (w/w) NaCl A⁻, 2.5% (w/w) NaCl A⁺).

The overall highest water content was in the IHS substrates (Fig. 6b). It increased gradually following the substrate concentrations, from a mean of 16% in the 25% (w/w) IHS A⁻ substrate to 29% in the 100% (w/w) IHS A⁺ substrate. The same pattern was recorded in the NE substrates, but with about 30% lower values. Low water contents of about 5% (w/w) were measured in 15% (w/w) WS A⁻, 15% (w/w) NaCl A⁻, and 15% (w/w) WS A⁺. The NoSalt substrates had a mean water content of 8% (w/w) in A⁻ and 10% (w/w) in A⁺, respectively. Overall, Chl *a* content values were negatively correlated with both EC and the water content (Fig. 4).

Linking Chl *a* content of the induced biocrust to the substrates EC

The EC (EC_{BSC_growth}) of samples with established biocrusts that showed higher Chl *a* values at the end of the experiment compared to the inoculum ($Chl a_{End} > Chl a_{Inoculum}$) was plotted in order to evaluate the EC limit for biocrust establishment (Fig. 7). The highest EC_{BSC_growth} was measured in IHS A⁺ and was 35.1 mS cm⁻¹. In IHS A⁻, the EC_{BSC_growth} was slightly lower but the respective biocrust samples showed higher Chl *a* contents compared to IHS A⁻. One replicate of WS A⁻ had an increased Chl *a* content in conjunction with an EC of 34.3 mS cm⁻¹. However, the respective Chl *a* content was the lowest compared to the other established biocrust. All other biocrusts that established on WS showed a much lower EC_{BSC_growth} of 2.9–4.3 mS cm⁻¹. The second

Fig. 5 Relative change of the effective quantum yield $Y(II)$ from week 0 to week 4 after biocrust inoculation on the surface of saline substrates as well as of a non-saline substrate. Percentages refer to (w/w) of the saline materials mixed with non-saline dune sand; IHS, Infiltration Hampering Stratum; NE, Neuhof-Ellers; WS, waste salt; NoSalt, non-saline dune sand. Each bar represents a mean value, with dots and asterisk representing single measurements of the three replicates. Positive values: increased $Y(II)$; negative values: decreased $Y(II)$; $\Delta Y(II)=0$: no change (e.g., no signals in both measurements); $\Delta Y(II)=-100$: signal in week 0 but no signal in week 4. Additives: BC^{plus} and sodium alginate

highest EC_{BSC_growth} was also found in IHS substrates. The maximal EC_{BSC_growth} of NE was 16.2 mS cm^{-1} . All other biocrusts established on substrates with $EC_{BSC_growth} < 10 \text{ mS cm}^{-1}$. On substrates with an $EC > 35.1 \text{ mS cm}^{-1}$, the Chl *a* content after the experiment was always lower compared to the inoculum.

Discussion

Biocrust establishment: assessing overall success

The success of artificial biocrust establishment strongly depends on the inoculum (Román et al. 2018; Malešević et al. 2021) and can be determined by evaluating biocrust

algal viability macroscopically, and under the microscope, as well as measuring Chl *a* contents and photosynthetic performance. The visible green surfaces that partly or fully covered several substrates (Fig. 2) indicated successful biocrust establishment. The microscopic observation further detected vital microalgal communities strongly dominated by *Pseudostichococcus monallantoides*. The genus *Pseudostichococcus* occurs worldwide in a variety of habitats (Pröschold and Darienko 2020) and was frequently found in biocrusts in the surroundings of potash tailings piles (Sommer et al. 2020a, b). Therefore, it was used as the main green algal taxon of the inoculum mixture. In addition, *Pseudostichococcus* easily breaks into fragments, making fragmentation, which is, besides cell division, a common form of vegetative reproduction in this genus (Pröschold and Darienko 2020).

Indeed, the homogenization of the microalgal mixture before inoculation may have led to increased fragmentation of the *P. monallantoides* filaments, thereby initiating fast growth when applied to the substrate. In contrast, the unicellular taxa *Chloroidium* sp. and *Nannochloris* sp. do not reproduce by fragmentation, and the homogenization process probably did not initiate autospore development. This may be the reason for the overall dominance of *P. monallantoides* in the established biocrusts after 4 weeks. On the other hand, the unicellular *Chloroidium* sp. and *Nannochloris* sp. may become important at a later stage of biocrust development, as they were commonly encountered in natural biocrusts growing near potash tailings piles (Sommer et al. 2020b).

The Chl *a* content of several samples indicated increase of microalgal biomass, and thus successful biocrust establishment and growth (Fig. 6a). Overall, the highest mean Chl *a* content measured after the experiment (50% (w/w) IHS A[−]) was 2.5-fold lower than that of induced non-saline cyanobacteria biocrusts in a laboratory experiment under similar culturing conditions (Malešević et al. 2021). Salinity as an abiotic stressor is known to inhibit microalgal growth in liquid medium (Gustavs et al. 2010), suggesting slowed-down development of the inoculated microalgae on the saline substrates. However, the NoSalt samples did not exhibit significantly higher Chl *a* contents compared to the biocrusts that established on saline substrates. The study of Malešević et al. (2021) is based on cyanobacterial inoculation on a fertile loess surface, whereas the presented experiment used green algae on a less fertile substrate. Differences in mineral composition, nutrients, and physical structure may explain different biomass developments on these substrates. Studies on induced biocrusts using green algae are still lacking, although these eukaryotic microalgae often dominate biocrusts, particularly in mesic habitats (Glaser et al. 2018; Mikhailyuk et al. 2019). Natural green algal biocrusts on nutrient-depleted sandy soils showed Chl *a* contents of 200–600 mg m^{−2} (Caesar et al. 2017), which is much higher compared to those in the experiment, but this may be explained by different age of the biocrusts, i.e., the biocrusts that established in the present study are very young and an early developmental stage. Nevertheless, the data of the present study document for the first time the successful application of green algae as an inoculum for the induction of biocrusts.

The cell numbers used for inoculation could be crucial for biomass development and biomass accumulation over time. For the inoculation using cyanobacteria, a dry weight of 5–7.5 g m^{−2} led to successful biocrust establishment (Chamizo et al. 2018; Román et al. 2018; Roncero-Ramos et al. 2019; Mugnai et al. 2020). This is more than tenfold higher than the dry weight of the inoculum mixture used in this study. Furthermore, a thicker layer of inoculated organisms may have a positive influence on their survival

on the saline substrates. The cells closest to the substrate could die off earlier, but still provide physical shelter from the direct influence of the salt for the organisms in upper layers of the crust. This could prolong their time for acclimation to the condition in the substrates. Therefore, a larger inoculated biomass may better support biocrust establishment on the substrates of potash tailings piles, which would lead to higher Chl *a* contents after 4 weeks.

The highest measured Y(II) after the experiment reached comparable levels to that of natural cyanobacterial biocrusts under wet conditions (Williams et al. 2014) and was also comparable to those of green algae under standard culturing conditions (Schreiber et al. 2007). This indicates that the photosynthetic activity was on a high level reflecting healthy and unstressed green algae in the young biocrusts on the respective substrates, as well as a feasible methodological approach to follow artificial biocrusts development.

It is noteworthy that the Chl *a* content as well as the Y(II) showed a wide range of values for some substrates and treatments. While some replicates showed no biocrust development at all, biocrusts managed to establish on others. This suggests that the process of biocrust establishment is not homogenous, which should be considered in future experiments, for example, by optimizing the inoculation procedure and increasing the number of replicates.

Biocrust establishment: evaluating the substrates and their EC

The establishment of biocrusts differed between the substrates used in the experiment, revealing the crucial role of the substrate for artificial biocrust induction (Rozenstein et al. 2014; Román et al. 2018; Mugnai et al. 2020). Physico-chemical soil parameters shape the micro-habitat characteristics by providing both a physical matrix and a pool of beneficial, detrimental, and neutral chemical substances for the microorganisms. Water-holding capacities are further determined by the substrate. The highest Chl *a* content in combination with the highest increase of the photosynthetic performance was observed on the 50% (w/w) IHS, followed by the 25% (w/w) IHS substrates. Consequently, biocrust establishment was most successful on the IHS. The fact that biocrusts naturally appeared on IHS surfaces in the field (Sommer et al. 2019) underlines the potential of biocrust establishment on this particular material.

The Chl *a* content of the NoSalt samples was not the highest observed in this experiment, even though the collected salt-free dune sand is a habitat for natural biocrusts along the Baltic Sea coast (Schulz et al. 2016; Schaub et al. 2018; Khanipour Roshan et al. 2020). Likewise, the Y(II) of the NoSalt samples in both treatments decreased during the experiment, indicating a likely preference of the green

Fig. 6 Chlorophyll *a* content of the surface (**a**) and water content of the sub-surface (**b**) 4 weeks after biocrust inoculation on saline substrates as well as of a non-saline substrate. Numbers refer to percentages (w/w) of the saline material mixed with non-saline dune sand;

IHS, Infiltration Hampering Stratum; NE, Neuhoef-Ellers; WS, waste salt; NoSalt, 100% non-saline dune sand. The bars represent mean values; values of each replicate are shown in addition. Additives: BC^{plus} and sodium alginate

algae used for inoculation of saline over non-saline substrate. This is not surprising, since these algae were isolated from saline habitats. However, on the saline 1% (w/w) NaCl and 1% (w/w) WS substrates, the Y(II) similarly decreased

during the experiment. These substrates mostly consisted of dune sand, and this material is not as hygroscopic as the more saline substrates. Gypsum, a component of the IHS (Kockx 2017), attracts water more easily than sand. Thus,

Fig. 7 Electrical conductivities (ECs) of the substrates in all tested salinities that were suitable for biocrust induction. Dot positions represent the mean EC (of one substrate and treatment) referring to samples with successfully induced biocrusts showing increased biomass after the experiment ($\text{Chl } a_{\text{End}} > \text{Chl } a_{\text{Inoculum}}$). EC values of the substrates without biomass increase ($\text{Chl } a_{\text{End}} \leq \text{Chl } a_{\text{Inoculum}}$) not shown. The dot size indicates the $\text{Chl } a_{\text{End}}$ of each sample. IHS, Infiltration Hampering Stratum; NE, Neuhoof-Ellers; WS, waste salt; NoSalt, non-saline dune sand. Additives: BC^{plus} and sodium alginate

the humidity on dune sand alone could have been insufficient to maintain an optimal water level for the biocrust algae. Indeed, the water content of the sub-crust of the NoSalt, as well as the 1% (w/w) NaCl and 1% (w/w) WS substrates, was about one-third lower compared to the samples with the highest Chl *a* content (25% (w/w) IHS A⁺, 50% (w/w) IHS A⁻). This suggests that the photosynthetic performance of the biocrusts on dune sand declined due to drier conditions, which finally led to growth inhibition. Overall, these data are in congruence with a recent study that showed much lower cyanobacteria biocrust development in a dry chamber compared to in a wet chamber (Malešević et al. 2021).

Biocrusts did not establish on the 75% and 100% (w/w) NE and IHS, nor on the NaCl/WS substrates of > 5% (w/w). This correlated with the higher EC (as a proxy for soil salinity). Most of the samples showed photosynthetic activity after inoculation, whereas, for example, on 12.5% and 15% (w/w) WS, no chlorophyll fluorescence signal was detected. Thus, on most tested substrates, the algae survived the inoculation, except on strongly saline materials. The decreasing Y(II) values of some samples during the following weeks reflect the harmful effect of salt over a longer time period. The substrate salinity in these samples most probably exceeded the upper salt tolerance of the green algae, and consequently no biocrust could establish. An increased Y(II) at the end of the experiment in turn suggests that the salinities of the respective substrates were in a range compatible for survival. The critical salinity limit for biocrust establishment on potash tailings piles materials appears to be an $\text{EC}_{\text{BSC_growth}}$ of 35.1 mS cm^{-1} (Fig. 7). This value resembles the maximum EC that was recorded in natural saline soil algal habitats (Tsujimura et al. 1998), whereas other studies on natural biocrust mostly reported much lower

values in a range of $0\text{--}5 \text{ mS cm}^{-1}$ (Kakeh et al. 2018, 2020; Nyenda et al. 2019). However, the substrates 10% (w/w) WS, 10% (w/w) NaCl, and 100% (w/w) NE also showed an EC of $30\text{--}45 \text{ mS cm}^{-1}$, but no biocrusts developed. Therefore, factors other than the EC alone influence the development of biocrusts. The EC is a sum parameter and refers to the electrical conductivity in a medium provided by the dissolved ions. Typical salt ions are Na^+ , K^+ , and Cl^- . The solubility of gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) is more than a hundred times lower than that of NaCl, but 2.1 g L^{-1} dissolves in water (ions: Ca^{2+} , SO_4^{2-}), thereby increasing the EC. Therefore, gypsum, as a main component of the IHS (Kockx 2017), could lead to an elevated EC in the IHS substrates of the present study. Interestingly, a study on *Chlorella vulgaris* showed an increase of the Chl *a* content by adding gypsum to saline media, whereas the Chl *a* content in saline media alone declined (Mathad and Hiremath 2010). This might be explained by the beneficial role of Ca^{2+} for algae, for example, as a secondary messenger in calcium-signaling pathways. The gypsum as a component of the IHS may further protect the algal cells from more toxic salt ions, such as Na^+ and Cl^- , and thereby allowing the biocrust establishment on substrates with a higher EC.

Soil texture is another factor influencing artificial biocrust development. Cyanobacterial biocrust establishment on fine sand was more successful than on coarse sand (Rozenstein et al. 2014; Mugnai et al. 2020). In contrast, the Chl *a* content of cyanobacterial biocrusts after 30 days was lower on silt loam to loamy sand than on sandy sand; however, biocrusts on sandy sand were more fragile (Chamizo et al. 2018). The additives as a component of the IHS belong to the soil texture “silt” (0.002 to 0.05 mm particle diameter; Kockx 2017), whereas dune sand from the Baltic Sea coast

has been classified as “sandy sand” (0.05 to 2.0 mm particle diameter; Schulz et al. 2016). As the silt fraction of the IHS substrates provided a larger reactive surface than the coarse-textured dune sand, this addition of finer particles may have provided reactive surface and nutrient reservoirs supporting the biocrust establishment.

Moreover, mixing the potash tailings piles materials IHS and NE with dune sand aimed to simulate natural weathering processes. Under outdoor conditions, rain dissolves the soluble components of the tailings (mostly NaCl) that will then be washed out, whereas the insoluble components remain on the surface, leading to a desalinization of the surface layer. The insoluble and hardly soluble components in the IHS derive from the IHS-additives, as well as from the 1–2% insoluble and hardly soluble components of the overburden (gypsum/anhydrite, clay; Kockx 2017). After 4 years in an outdoor pilot study, the IHS showed a layer thickness of 3–9.4 cm and a mean NaCl content of 25% (Kockx 2017), suggesting that the salinity declines with time. Consequently, more time may be required to further reduce the salinity of the IHS, in order to allow biocrust establishment on the IHS in the field.

Biocrust establishment: evaluating the treatment

The application of sodium alginate as a surface stabilizer in combination with BC^{plus} as a nutrient source significantly increased the biocrusts Chl *a* content on the NE, WS, NaCl, and NoSalt substrates (Table 3). Therefore, we confirm statements on beneficial effects of surface stabilizers on artificial biocrust formation (Peng et al. 2017; Chi et al. 2020). Sodium alginate increases the compressive strength of the soil by forming a film on the surface, which supported the induction of cyanobacterial biocrusts on sand (Peng et al. 2017). Our results showed that green algae likewise benefit from this stabilizer. The stabilizing effect could be even more important for single-celled green algae (*Chloroidium* sp., *Nannochloris* sp.) as well as the easily fragmenting filaments of *P. monallantoides* compared to the robust, EPS-excreting filamentous cyanobacterium *Microcoleus vaginatus* used by Peng et al. (2017). Furthermore, the water content of biocrusts with sodium alginate application was higher compared to the control (Peng et al. 2017), which is in accordance with our results. It is thus reasonable to assume that a higher water availability could have supported biocrust establishment on the respective substrates.

The increased Chl *a* content on several substrates with the additive treatment furthermore suggests a promotion of the biocrust growth by the second additive, BC^{plus}. This can be explained by the release of additional nutrients like phosphate, sulfate, Ca²⁺, and Mg²⁺ from BC^{plus}, corresponding to positive crop yield effects of BC^{plus} application

in agronomic experiments (Zimmer et al. 2019). Also, BC^{plus} (pH 5) application can decrease the soil pH (Zimmer et al. 2019). Indeed, the BC^{plus} treatments showed a lower pH than the samples without treatment (Table 3). This is an advantage in sodic substrates, since nutrient availability strongly depends on soil pH. The availability of phosphorous and trace metals strongly decreases at pH > 7.5, as it was the case for most of the substrates used in this study (Table 3). Future studies could further evaluate the nutrient availability in potash tailings piles substrates enriched with BC^{plus}.

Since the additive treatment generally enhanced biocrust establishment on substrates of moderately high salinity, sodium alginate and BC^{plus} are beneficial for the development of biocrusts. However, both additives did not enhance the salinity tolerance of the inoculated microalgae. Determining the optimal additive concentration under controlled laboratory conditions is another crucial step for the application in future outdoor experiments. Sodium alginate was applied in a concentration that has been shown to be effective before (Peng et al. 2017), although some modifications of the protocol may be useful because of the specific EC, pH, and soil texture conditions of the materials from the potash tailings piles.

Conclusion

In this pioneer study, green algae were for the first time successfully applied to the artificial induction of biocrusts on saline substrates containing potash tailings pile materials in the laboratory. *Pseudostichococcus monallantoides* was the dominant alga in the established biocrusts and thus represents a suitable halotolerant green algal inoculum for restoration purposes. However, many other green algal strains, several of them belonging to undescribed taxa, have been reported from these biocrusts (Sommer et al. 2020a, b), and the potential to identify strains that could be even more salt-tolerant has so far not been systematically investigated. Moreover, several cyanobacterial isolates were obtained from biocrusts growing in the vicinity of potash tailings piles surroundings (Sommer et al. 2020b). These strains should be also tested for the restoration of potash tailings piles, since inoculation of filamentous cyanobacteria led to successfully established biocrusts in several studies (Rossi et al. 2017; Román et al. 2018; Malešević et al. 2021; Zhao et al. 2021). The focus of the present study, however, was set to green microalgae, since this group was more prominent in young biocrusts collected from the closest potash tailings piles environments (Sommer et al. 2020a) and on the IHS in the field (Sommer et al. 2019). In addition, green algae are more abundant in temperate biocrusts compared to drylands,

where cyanobacteria are more important (Glaser et al. 2018).

The establishment of biocrusts was most successful on the potash tailings piles material IHS that was mixed with dune sand at proportions of 25% (w/w) IHS and 50% (w/w) IHS, resulting in an $EC < 35 \text{ mS cm}^{-1}$, whereas substrates with a higher EC were not suitable for biocrust establishment. These findings provide a first baseline on the physico-chemical conditions required for biocrust establishment on potash tailings piles. The biocrust establishment on the IHS on potash tailings piles materials possibly bears the potential to improve water retention that may in consequence lower the harmful salt output after precipitation events. Eventually, a living biocrust cover, which provided beneficial effects like surface stabilization, nutrient accumulation, and water retention, may be the first step towards a renaturalization of potash tailings piles.

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Data availability All data is provided in the article.

Declarations

Conflict of interest The authors declare no competing interests.

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