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Evidence for a mutualistic relationship between the cyanobacteria *Nostoc* and fungi *Aspergilli* in different environments

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Abstract

Symbiotic partnerships are widespread in nature and in industrial applications yet there are limited examples of laboratory communities. Therefore, using common photobionts and mycobionts similar to those in natural lichens, we create an artificial lichen-like symbiosis. While *Aspergillus nidulans* and *Aspergillus niger* could not obtain nutrients from the green algae, *Chlorella*, and *Scenedesmus*, the cyanobacteria *Nostoc* sp. PCC 6720 was able to support fungal growth and also elevated the accumulation of total biomass. The *Nostoc–Aspergillus* co-cultures grew on light and CO_2 in an inorganic BG11 liquid medium without any external organic carbon and fungal mycelia were observed to peripherally contact with the *Nostoc* cells in liquid and on solid media at lower cell densities. Overall biomass levels were reduced after implementing physical barriers to indicate that physical contact between cyanobacteria and heterotrophic microbes may promote symbiotic growth. The synthetic *Nostoc–Aspergillus nidulans* co-cultures also exhibited robust growth and stability when cultivated in wastewater over days to weeks in a semi-continuous manner when compared with axenic cultivation of either species. These *Nostoc-Aspergillus* consortia reveal species-dependent and mutually beneficial design principles that can yield stable lichen-like co-cultures and provide insights into microbial communities that can facilitate sustainability studies and broader applications in the future.

Key Points

- *Artificial lichen-like symbiosis was built with wild-type cyanobacteria and fungi.*
- *Physical barriers decreased biomass production from artificial lichen co-cultures.*
- *Artificial lichen adapted to grow and survive in wastewater for 5 weeks.*

Keywords Artificial lichen co-cultures · Microbial consortia · Cyanobacteria · Filamentous fungi · Cell–cell interactions

Introduction

Co-culture systems are becoming widely used as a synthetic biological platform to mimic cell–cell interactions present in natural systems such as lichens, and as a potential innovative platform for the generation of products of industrial, medical, and environmental interest (Aanen and Bisseling 2014; Eymann et al. 2017; Goers et al. 2018; Hom and Murray 2014). For example, lichens, containing both photobionts and mycobionts, are well known for their ability to survive in extreme environmental conditions and to produce a variety of secondary metabolites that display diverse biological activities as potential biochemicals, biofuels, or pharmaceuticals (Oksanen 2006). Evaluating model co-cultures in the laboratory can serve to elucidate insights about metabolic and other

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interactions between natural populations, how symbionts survive in extreme environments, and the potential for added-value product generation from multispecies systems. However, with a radial growth expansion of only 0.5–5 mm/year (Ranković and Kosanić 2015), the extremely slow growth of natural lichens also imposes practical limitations for carrying out fundamental and applied research.

A co-culture set-up typically includes two or more different populations of microbes in order to explore how their interaction changes each partner's behavior. Schroeckh et al. (2009) used bacterial–fungal interaction to activate the silent gene clusters in *Aspergillus nidulans* and produced new secondary metabolites. The co-culture of fungal endophyte *Fusarium tricinctum* with the bacterium *Bacillus subtilis* increased the accumulation of constitutively present secondary metabolites up to 78-fold and observed seven novel compounds not detected in axenic fungal or bacterial controls (Ola et al. 2013). Kranner et al. (2005) showed that lichened alga and fungus upregulated the protective systems in each other during extreme desiccation and irradiation, while the axenic alga and fungus suffered oxidative damage. Co-cultures were also found to benefit biomass production, robust growth, and enhanced bioproducts yield (Do Nascimento et al. 2013; Hom and Murray 2014; Picard et al. 2013). The mycobionts described above typically depended on an external organic carbon source rather than photosynthetically produced sugars or proteins provided by the photobiont. This interaction differs from the functional symbiotic interplay in natural lichen and requires additional costly carbon inputs (Eymann et al. 2017).

To avoid external carbon feeding, genetic tools have been applied to improve the extracellular sugar production of the photobiont to support the growth of the mycobiont. As a result, the cyanobacteria *Synechococcus elongatus* has been engineered to express heterologous genes encoding an invertase and a glucose facilitator for the secretion of glucose and fructose (Niederholtmeyer et al. 2010) as well as a proton-sugar symporter (*cscB*) for secretion of sucrose (Ducat et al. 2012). The *cscB*-expressing cyanobacteria were paired with heterotrophs capable of uptaking sucrose effectively, including *Saccharomyces cerevisiae* (Ducat et al. 2012), *Rhodotorula glutinis* (Li et al. 2017), *Pseudomonas putida cscAB* (Lowe et al. 2017), and *B. subtilis* (Hays et al. 2017). These synthetic symbioses represented proof of concept studies to demonstrate the feasibility of creating mutualistic partnerships between photoautotrophs and heterotrophs facilitating cross-feeding between organisms, control of redox environment, and other interactions.

In natural lichens, green algae represent the partners in the vast majority of chlorobionts (Honegger and Nash 2008), and *Nostoc* is a commonly observed cyanobiont (Rikkinen 2013) for generating energy-rich glucans and sugar alcohols provided to the fungal partner (Eymann et al. 2017). The lichenized fungal partners often include some *Ascomycota*-related species and free-living filamentous fungi such as *Aspergillus* may have evolved from lichen-forming ancestors (Lutzoni et al. 2001; Stenroos and DePriest 1998). Furthermore, some *Aspergilli* species are commercially important to industrial production of food ingredients, pharmaceuticals, and enzymes (Meyer et al. 2011). As a result, we examined the potential for a mutualistic relationship of eukaryotic chlorobionts (*Chlorella* and *Scenedesmus*) or cyanobacteria from the genus *Nostoc* to grow in co-cultures with filamentous fungi, *A. nidulans* and *Aspergillus niger*. After confirming the partners composed of symbiosis, biomass accumulation and interaction behavior were observed for each microorganism in both chemically defined BG11 liquid and solid media. This co-culture stability is important but often missing in synthetic syntrophic communities (Kim et al. 2008). Hence, we also observed the adaptation and sustainable growth of the synthetic co-cultures in wastewater, in order to find co-cultures that can serve as useful synthetic lichen model for investigating growth promotion in mixed cultures and for evaluating sustainable co-cultures capable of adaptation to harsh environments such as wastewater.

Methods

Strains, inocula preparation, and culture conditions

Chlorella sorokiniana BRWWTP 002 (GenBank Accession number: KP645221) was previously isolated from the Back River Wastewater Treatment Plant and deposited at the Culture Collection of Algae at the University of Texas at Austin (UTEX) as UTEX B3011 (Bohutskyi et al. 2015). *Scenedesmus acutus f. alternans* UTEX B 72 was purchased from UTEX. The *Nostoc* PCC 6720 was obtained from the Pasteur Culture Collection (PCC). Heterotrophic *A. nidulans* (FGSC A991) and *A. niger* (FGSC A1121) were obtained from the Fungal Genetics Stock Center (FGSC) as freeze spore stock in 25% glycerol.

Algae and cyanobacteria strains were propagated in BG11 medium for inocula under the following conditions: standing cultivation, illumination of 100 μmol/m^2/s with 16:8 h light/dark cycle, temperature of 28 ± 1 °C, inorganic carbon supplement with filter-sterilized air enriched with 1% (v/v) CO_2. All the cultures in this study were grown under these conditions unless otherwise specified. BG11 medium contains 1.5 g/L $NaNO_3$, 40 mg/L K_2HPO_4, 75 mg/L $MgSO_4$ $7H_2O$, 36 mg/L $CaCl_2{\cdot}2H_2O$, 6 mg/L citric acid, 6 mg/L ferric ammonium citrate, 1 mg/L EDTA-Na_2, 20 mg/L Na_2CO_3, and 1 mL/L A5. A5 is a trace metal solution containing 2.86 g/L H_3BO_3, 1.86 g/L $MnCl_2{\cdot}4H_2O$, 0.22 g/L $ZnSO_4{\cdot}7H_2O$, 0.39 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.08 g/L $CuSO_4{\cdot}5H_2O$, and 0.05 g/L $Co(NO_3)_2{\cdot}6H_2O$.

For activation of fugal strains, the stored spores were grown at 28 ± 1 °C for 14 days on potato dextrose agar (PDA) plates. Spores were gently scraped from the plates with adding sterile water, filtered with sterile glass wool, and washed three times to remove any media components before incubating at 4 °C for up to 30 days. The spore solution was counted and used as the inoculum.

The co-culture was inoculated with a photobiont optical density at 750 nm of 0.1, and a heterotroph concentration of 5–6 × 10^5 spores/mL. Axenic controls were parallelly carried out with same initial cell density. For axenic phototroph and co-cultures, the cultivation conditions were same with that of inocula preparation: standing cultivation, illumination of 100 μmol/m^2/s with 16:8 h light/dark cycle, temperature of 28 ± 1 °C, inorganic carbon supplement with filter-sterilized air enriched with 1% (v/v) CO_2. Axenic fungi grew in flasks containing stirring bar on a magnetic stirring plate.

The primary effluent (PE) tested as a substitute medium was collected from Back River Wastewater Treatment Plant (Baltimore, MD, USA) and autoclaved at 121 °C for 30 min prior to growth experiments. Its characteristics include total carbon of 94 ± 1 mg/L, inorganic carbon of 24 ± 0 mg/L, total nitrogen of 23 ± 1 mg/L, and total phosphorus of 5 ± 0 mg/L.

Spent medium collection

To prepare algal and cyanobacterial supernatant, axenic phototrophs were cultured to stationary phase in BG11 medium or PE. The spent medium was collected by centrifugation at 4000 rpm for 5 min and sterilized by passing through a 0.22-μm filter.

BG11 medium or PE supplemented with glucose (2 g/L) and vitamins was used to grow fungi with initial concentration of 5–6 × 10^5 spores/mL for 4 days and prepare fungal supernatant. Post-growth preparation of the supernatant was the same process used for algae and cyanobacteria spent media.

For the supernatant trial, the sterilized spent media were mixed with fresh BG11 or PE medium at different ratios and fresh BG11 or PE medium was set as negative control.

Quantification of the cultures

For axenic algal culture, growth was monitored spectrophotometrically by measuring OD_{750}. For axenic cyanobacterial cultures, axenic fungal cultures, and co-cultures, dry weight of the biomass was measured by centrifuging at 4000 rpm for 5 min, washing three times with distilled water, and lyophilizing in a freeze dry vacuum (LGJ-25, Xiangyi, China).

Solid media experiments of *Nostoc* and *Aspergilli*

Spots of phototrophs were achieved by dropping 100 μL of *Nostoc* (0.5 of OD750) on solid BG11 or PE plates and incubating for 24 h until no observable liquid remained. Then, 100 μL fungal spore suspensions (5–6 × 10^5 spores/mL) were spotted on the plates slightly overlapping with the phototrophic green trace circle. For axenic *Nostoc* cultures, equal volume of sterilized deionized water replaced the fungal cultures as a negative control. Plates were then grown at 28 ± 1 °C, in a 16:8 h light/dark photoperiod with an illumination of 100 μmol/m^2/s.

Transwell experiments of *Nostoc* and *Aspergilli*

Transwell chambers (Millipore Sigma, Billerica, USA) were employed in the co-culture system with a physical barrier. The upper and lower cultures were separated by 0.4-μm pore size polyester membrane. Briefly, the lower compartment of the chamber was loaded with 3 mL of *Nostoc* at an initial OD_{750} of 0.1, and the upper compartment was seeded with a 1 mL fungal spore suspension at an initial concentration of 5–6 × 10^5 spores/mL. One milliliter of *Nostoc* (OD_{750} of 0.1) was added into the upper chamber as negative control. Separate positive controls consisted of a premixed culture containing a phototroph and a heterotroph and were added into both chambers. Environmental conditions were identical to those in plate cultivation in last section.

Microscopic analysis

Microscopic examination of cultures was performed using an Axiovert 200 fluorescence microscope (Carl Zeiss, Jena, Germany) and a Quanta 200 Environmental scanning electron microscope (SEM, FEI, Hillsboro, USA). For fluorescent images taken, transmitted and TxR channels were used for the cell information under white light and fluorescent light, respectively. The excitation wavelength of fluorescent light ranged from 460 to 550 nm that results in chlorophyll in cells emitting red light.

For SEM analysis, cell pellets were formed by centrifuging at 10,000 rpm for 5 min, fixed in 3% formaldehyde in 0.1 M Cacodylate buffer for 1 h at room temperature, and then rinsed in three 15-min washes with Cacodylate buffer. Samples were postfixed in Palade's osmium tetroxide for 30 min at room temperature and 1 h at 4 °C, rinsed three times in distilled water, and then dehydrated with graded series of cold ethanol (70, 95, 100%). Finally, samples were then critical point dried using CO_2, mounted on stubs using carbon pads, and silver-palladium sputter coated. Coated specimens were viewed at 2.7 kV.

Results

Appropriate partners composed of symbiosis

In order to achieve the goal of evaluating mutualistic symbioses for creating synthetic lichen-like microbial communities for research and applications, the first step is to pair an organic carbon generating autotroph with an obligately heterotrophic partner. In this study, we tested freshwater green algae (*Chlorella* and *Scenedesmus*) and cyanobacteria species (*Nostoc*), as candidate photobiotic partners paired with heterotrophic *Aspergilli* fungi.

The co-culture of microalgae and fungus

Aspergilli species have filamentous hypha and form flocs readily in liquid culture (Supplemental Fig. S1), which facilitates the recognition of the artificial lichen containing fungus and unicellular microalgae. Based on that, we concluded that few fungal spores were germinated in the co-culture of CS02-*A. nidulans*, due to the homogeneous green culture suspension absent of flocculant (Fig. 1a and b). Meanwhile, the spent medium of *C. sorokiniana* at stationary phase failed to support the growth of *A. nidulans* as no fungal hypha formed (Fig. 1c).

In contrast, green flocculants with macroscopic structures formed in the flask of *C. sorokiniana–A. niger* co-culture after 3 days (Fig. 1d). The flocs enlarged and turned brown after 8 days, suggesting that numerous algal cells had died (Fig. 1e). The appearance of flocs represents the germination and growth of *A. niger*, and the observation of mycelia (gray filaments) decorated with algal cells (red circle cells) indicates cohabitation (Fig. 1g and h).

For another algal species *Scenedesmus*, neither *A. nidulans* nor *A. niger* could access the carbon source in the co-culture system judging by the homogeneous cultures observed without floc formations (Supplemental Fig. S2).

The co-culture of cyanobacteria and fungus

Nostoc PCC 6720 are filamentous cyanobacteria and formulate flocs in axenic cultures (left flask in Fig. 2a and b). In contrast, axenic cultures of either *A. nidulans* or *A. niger* were unable to grow in the BG11 medium (data not shown). Alternatively, the flasks inoculated with both PCC 6720 and

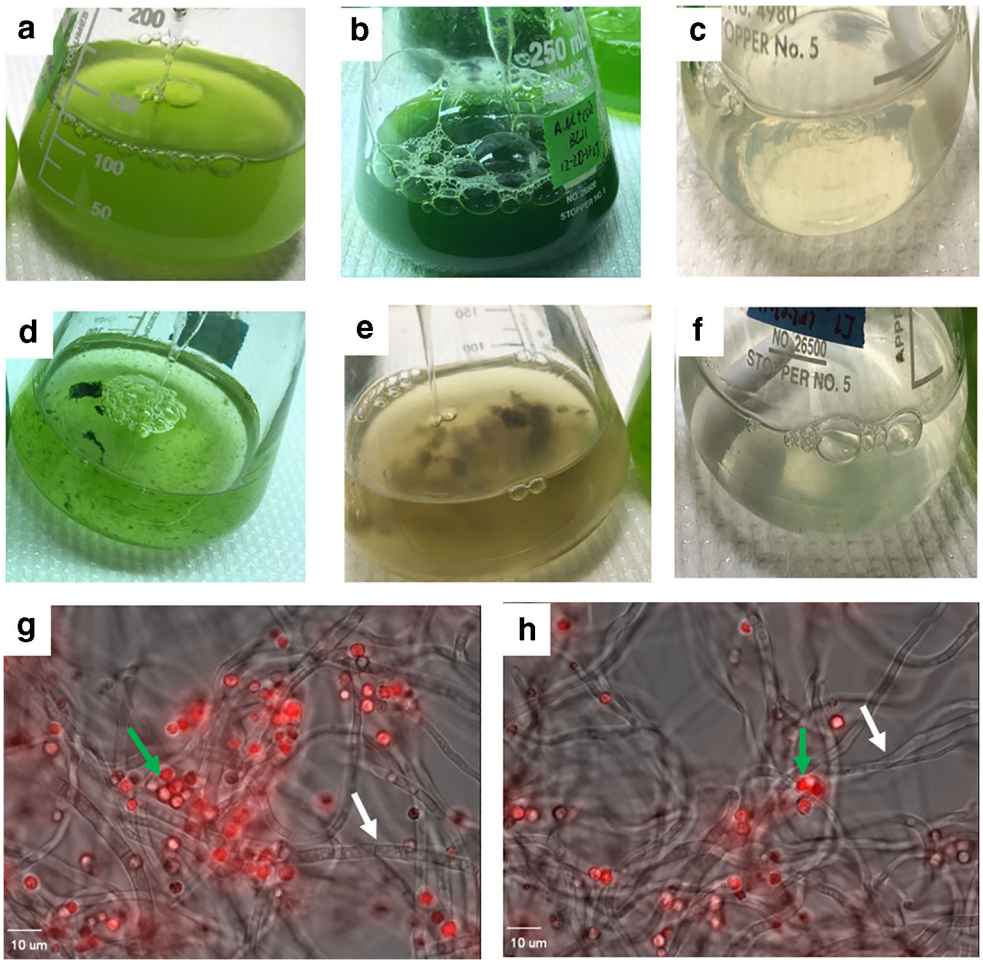

Fig. 1 The feasibility of co-culturing algae and fungus. **a**, **b** The co-culture of *C. sorokiniana* and *A. nidulans* after 3 days of postinoculation and 8 days of postinoculation, respectively. **c** Axenic culture of *A. nidulans* in supernatant of *C. sorokiniana*. **d**, **e** The co-culture of *C. sorokiniana* and *A. niger* after 3 days of postinoculation and 8 days of postinoculation, respectively. **f** Axenic culture of *A. niger* in supernatant of *C. sorokiniana*. **g**, **h** Representative fluorescent microscopy images of flocculants formed in *C. sorokiniana–A. niger* co-cultures from **d** and **e**, respectively, where red colored cells pointed by green arrow indicate *C. sorokiniana*, and white arrows indicate fungal hyphal fragments, scale bar, 10 μm

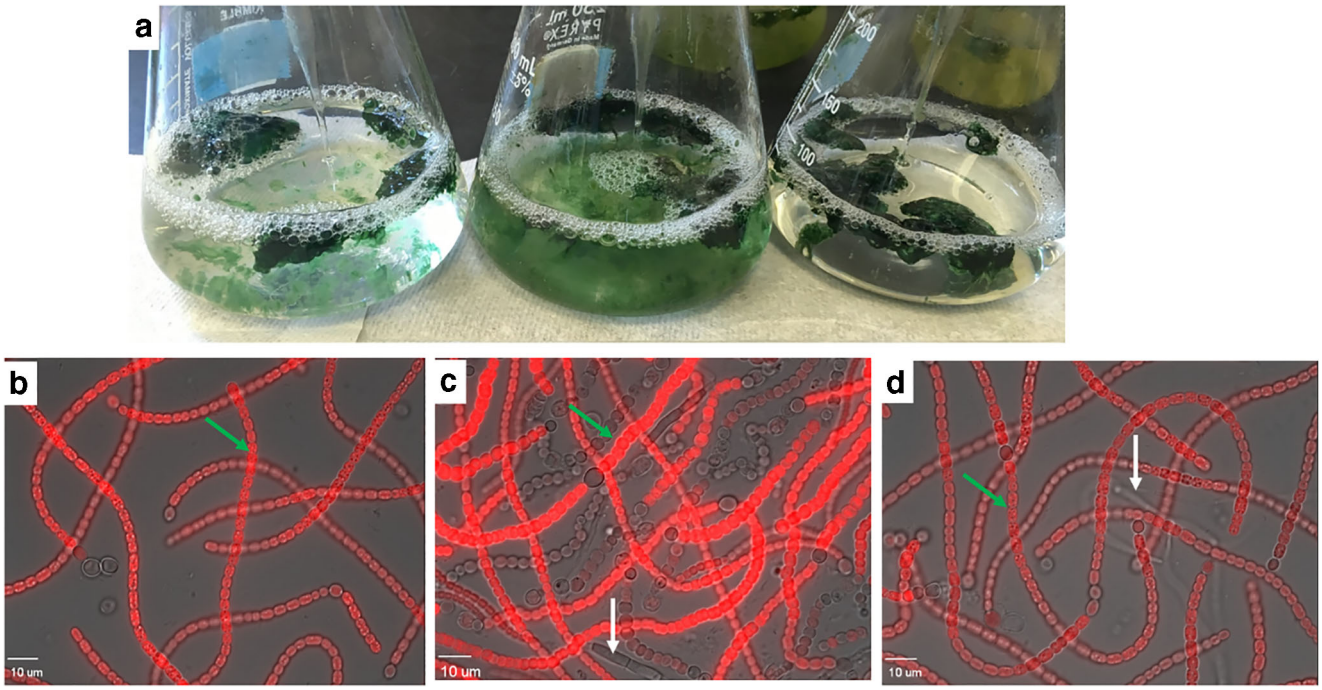

Fig. 2 The feasibility of co-culturing cyanobacteria and fungus. **a** The picture of flasks culturing axenic *Nostoc* PCC 6720, co-culture of *Nostoc* PCC 6720 and *A. nidulans*, and co-culture of *Nostoc* PCC 6720 and *A. niger*, from left to right, after 2 weeks of postinoculation. **b**–**d** Representative fluorescent microscope images of cultures in axenic culture of *Nostoc* PCC 6720, co-culture of *Nostoc* PCC 6720 and *A. nidulans*, and co-culture of *Nostoc* PCC 6720 and *A. niger*, respectively, where red colored cells pointed by green arrow indicate *Nostoc* PCC 6720, and white arrows indicate fungal hyphal fragments, scale bar 10 μm

Aspergilli species contained dark green flocs during the whole growth period (middle and right flasks in Fig. 2a). Nonetheless, it was not clear whether the co-culture system involved was established containing both cyanobacterial and fungal species. Therefore, fluorescent microscopic images were taken which indicate the hyphae of *A. nidulans* and *A. niger* interweaved together with the fluorescing *Nostoc* cells (Fig. 2c and d). Since axenic culture of both *Aspergillus* species was unable to grow in the BG11 medium alone, the *Nostoc* PCC 6720 must have supported the heterotroph growth of both *Aspergilli* and furthermore the *Aspergillus* did not substantially block the growth of *Nostoc* PCC 6720, suggesting that the pairing of *Nostoc* PCC 6720 and either *A. nidulans* or *A. niger* could represent a promising synthetic lichen.

The growth of fungi in spent medium of *Nostoc* PCC 6720

In order to the evaluate the source of the nutrients that were utilized by the fungi to grow, spent medium of *Nostoc* PCC 6720 at stationary phase was obtained through filtration followed by inoculation with the 2 *Aspergilli* species. Considering the possible exhaustion of needed elements for fungal growth in spent *Nostoc* medium, we also included flasks containing mixtures of fresh BG11 medium and the supernatant (SN) at different ratios. As noted above and shown in Fig. 3, biomass accumulation for both *A. nidulans* and *A. niger* was negligible in pure BG11 medium without any organic carbon supplementation. However, substantial fungal flocs were observed along with significant accumulated fungal biomass after incorporating spent medium from *Nostoc* PCC 6720 (Fig. 3).

When using the supernatant diluted with the same amount of fresh media (1BG11:1SN) or media consisted of 3 times as much fresh media (3BG11:1SN), there was no substantial difference in the levels of *A. nidulans* biomass accumulated and the amount was almost an order of magnitude higher than the negative control. The maximum amount of *A. nidulans* was achieved in the 100% supernatant media with no BG11 added, which indicated that the nutrients in spent medium of *Nostoc* were rich enough to satisfy the growth requirements of *A. nidulans*. For *A. niger*, the higher proportion of spent medium in 1BG11:1SN led to a slight more fungal biomass than with 3BG11:1SN ($p < 0.01$). Complete cyanobacterial spent medium also supported the growth of *A. niger*, although lower amounts of biomass were achieved in pure cyanobacterial spent medium, compared with the optimal medium containing 1 part BG11 and 1 part SN ($p < 0.05$). This difference suggests the depletion of nutrients or accumulation of certain toxins which have affected *A. niger* biomass accumulation in supernatants of *Nostoc*.

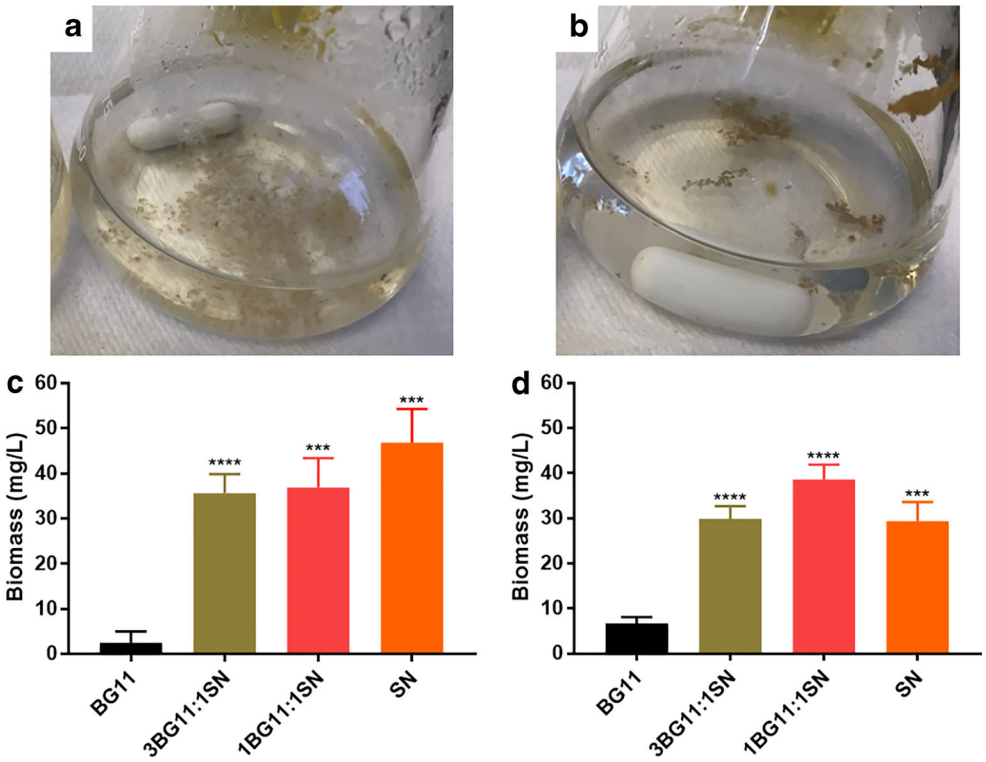

Fig. 3 The biomass production of **a**, **c** *A. nidulans* and **b**, **d** *A. niger* in the supernatant (SN) of *Nostoc* PCC 6720. All data are averages of biological four replicates ± standard deviation. Stars stand for the significant difference in comparison to BG11 control by *t* test

The growth of *Nostoc* PCC 6720 in fungal spent medium

We also grew fungi in BG11 medium supplemented with glucose and vitamins, collected the spent medium after 4 days, and then tested their effect on the growth performance of *Nostoc*. Compared with fresh BG11 medium, the growth of *Nostoc* was stimulated by supplementation with the spent medium of *Aspergilli*, with a significant increase in harvested cyanobacterial biomass (Fig. 4).

Dry weight of cyanobacterial biomass gradually increased as the proportion of *A. nidulans* supernatant increased from 1/10, 1/4 1/2, to 3/4. Even with only 1/10 of supernatant of *A. nidulans*, cyanobacterial biomass was 1.6-fold higher than that of fresh BG11 medium. The maximum level of biomass with medium containing the highest portion of *A. nidulans* supernatant (3/4) was 840 mg/L, which is 2.7-fold higher than the BG11 control.

For the supernatant of *A. niger*, the promotion of biomass occurred with supernatant addition in ratios higher than 1/10, and the highest amount of biomass was achieved with 1/2 supernatant in the culture medium. The quantity of biomass declined when using 3/4 supernatant compared with 1/2. The declined growth of *Nostoc* in 1BG11:3SN medium may be attributed to a shortage of available nutrients necessary for *Nostoc* or the accumulation of potentially toxic metabolites.

Collectively, these experiments show that the exudate of fungi can significantly improve the growth of cyanobacteria.

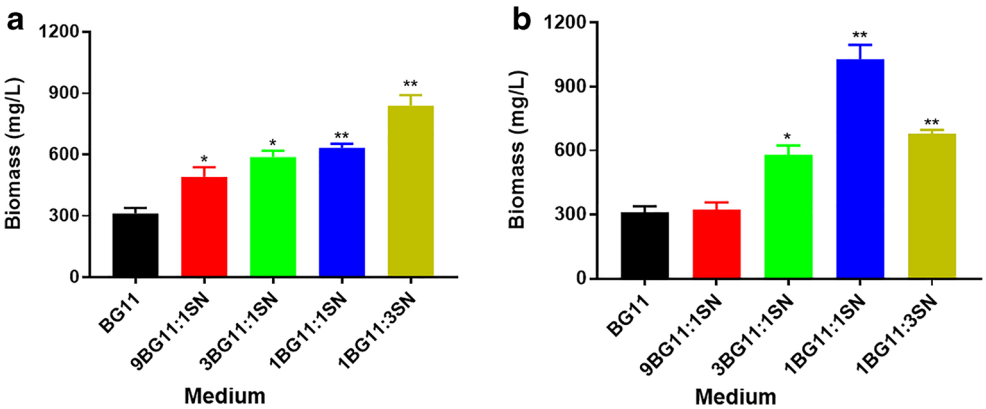

Fig. 4 The biomass production of *Nostoc* PCC 6720 in the supernatant (SN) of **a** *A. nidulans* and **b** *A. niger*. All data are averages of biological triplicates ± standard deviation. Stars stand for the significant difference in comparison to BG11 control by *t* test

The co-culture of *Nostoc* PCC 6720 and *Aspergilli* species

The above results indicated that *Nostoc* and *Aspergilli* growth could benefit from the presence of the other participant in a co-culture environment. Therefore, we designed pairwise consortia of *Nostoc* PCC 6720 and *Aspergilli* species and evaluated their performance in response to media conditions and physical interactions.

In liquid media, increased numbers and sizes of the flocs in co-culture flasks were observed relative to the axenic *Nostoc* PCC 6720 during the testing of partnerships of *Nostoc* PCC 6720 with *A. nidulans* and *A. niger* (Fig. 2a), suggesting mutual benefit in pairing the phototroph and heterotrophs. In order to evaluate the impact of co-culturing, the accumulation of total biomass was determined for *Nostoc–Aspergilli* co-culture (Fig. 5a). For both co-culture pairings, the dry weight of biomass significantly increased compared with axenic culture of *Nostoc* PCC 6720 for measurements starting at 5 days until the culture was terminated. By 12 days, the biomass in the two co-cultures was 80–100% higher than the levels obtained for mono-cultures of *Nostoc* PCC 6720. No growth was observed over the same period for the fungal mono-cultures due to the absence of available carbon source.

The effect of laying down adjacent cultures of *Nostoc* and *Aspergilli* on solid media is shown in Fig. 5d to g. We initially spotted 100 μL of liquid *Nostoc* cultures on BG11 agar plates and then incubated the plates for 24 h until no observable liquid remained. Next, 100 μL spore suspensions of *Aspergilli* were dropped on the BG11 plates so that they slightly overlapped with the cyanobacterial circular deposit, as indicated schematically in Fig. 5b. For the axenic cultures of *Nostoc*, equal volume of sterilized MilliQ water replaced the fungal culture as a negative control. After growing for 2 weeks, more intensely green colonies appeared for *Nostoc* PCC 6720 on the petri dishes in the presence of heterotrophic microbes than the axenic *Nostoc* culture alone (Fig. 5d and e versus c). Viewing these interactions under a microscope, fungal hyphae intertwined with the nummular cyanobacterial cells without any visible inhibition or zone lines, which represented a greater physical interaction than observed for the initial overlay conditions (Fig. 5g versus f). However, few of the fungal mycelia were able to penetrate the innermost cores of the thickest cyanobacterial colonies.

The greater thickness and green color intensity of the *Nostoc* cultures in the presence of overlaid fungal cells suggest that the fungi offer a growth enhancing effect on *Nostoc* cultures on solid media.

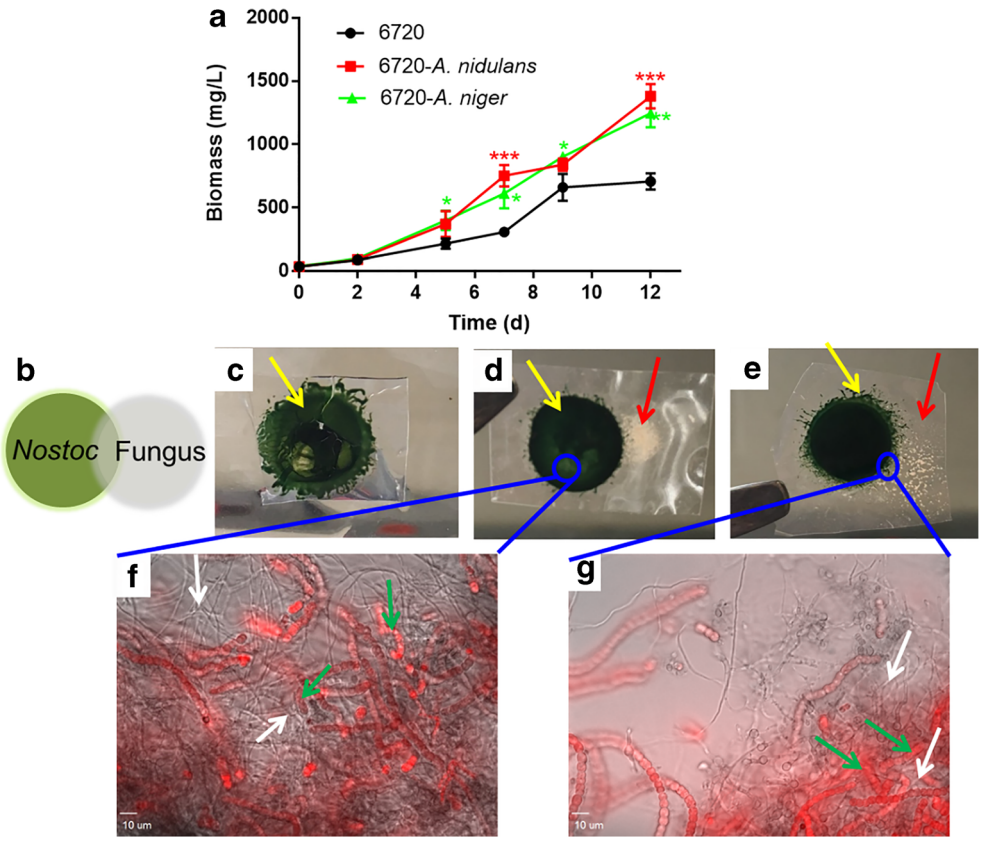

Fig. 5 The real co-culture of *Nostoc* PCC 6720 and *Aspergilli* species in liquid BG11 medium or on solid agar-containing petri dishes. **a** Biomass production in liquid medium. **b** The schematic diagram of culture spots on solid medium. **c-e** The spots of axenic *Nostoc* PCC 6720, co-culture of *Nostoc* PCC 6720 and *A. nidulans*, and co-culture of *Nostoc* PCC 6720 and *A. niger*, respectively, where yellow arrows point towards cyanobacterial spots and red arrows point towards fungal spots. **f** and **g** Representative fluorescent microscopy image of spots on **d** and **e**, respectively, where red color cells pointed by green arrow indicate *Nostoc* PCC 6720, and white arrows indicate fungal hyphal fragments, scale bar, 10 μm. All data are averages of biological triplicates ± standard deviation. Stars stand for the significant difference in comparison with axenic *Nostoc* control at the same day by *t* test

The effect of physical interaction between *Nostoc* and *Aspergilli* in co-culture

To compare the importance of physical interactions with nutrient exchange alone, *Nostoc* and *Aspergilli* were cultivated in transwell culture systems. In one case, species were physically separated by into separate transwell systems (Fig. 6a, separate) that have semi-permeable membranes prohibiting the direct contact of two partners but allowing metabolite diffusion and media exchange between compartments. Considering that more cyanobacterial filaments were observed in *Nostoc–Aspergilli* co-culture (Figs. 2c and d and 5f and g), the larger lower compartment (3 mL) was inoculated with *Nostoc*, while *Aspergilli* were cultured in the smaller upper compartment (1 mL). Alternatively, other cultivations were performed in which the two species were mixed and then added into the two different compartments as co-cultures. After culturing for 14 days, cyanobacterial biomass increased in the physically separated *Nostoc–Aspergilli* system with medium exchange, in comparison with the axenic *Nostoc* culture ($p < 0.05$) (Fig. 6b), consistent with the previously observed beneficial impact of providing fungal spent medium on cyanobacterial growth (Fig. 4).

In addition, the mixed cultures of *Nostoc–A. nidulans* produced total biomass level that exceeded the levels produced from physically separated co-cultures comprised by 40%. Similarly, biomass obtained in mixed *Nostoc–A. niger* co-cultures was 66% higher when compared with the physically separated co-cultures.

In order to more closely examine the interactions of cyanobacteria and fungi in co-culture, scanning electron microscope (SEM) was performed showing *Aspergilli* species interwoven with *Nostoc* cells in the mixed co-culture systems (red circled in Fig. 6c and d). *Nostoc* and *Aspergilli* species appeared to grow in tightly knit mats with filament and hyphae forming a connecting knob when there was no physical barrier between the phototroph and heterotroph.

Primary effluent as an alternative source of nutrients for co-culture

Since mutualistic symbiosis was established in co-cultures of *Nostoc* PCC 6720 and *Aspergilli* species in standard BG11 medium for cyanobacteria, we wondered if this symbiosis could also occur in harsher environments (e.g., municipal wastewater) such as primary effluent (PE) collected from Back River Wastewater Treatment Plant (Baltimore, MD, USA).

In 6-well culture plates, axenic *Nostoc* PCC 6720 produced notably less biomass when grown in PE than in BG11 control ($p < 0.0001$, Fig. 7b versus a and e). However, co-cultures of *Nostoc* and *A. nidulans* in PE achieved visibly higher amounts of biomass (Fig. 7c versus b), which was more than 2.7 times higher than the biomass accumulating with *Nostoc* alone and comparable with the biomass levels formed in axenic *Nostoc*

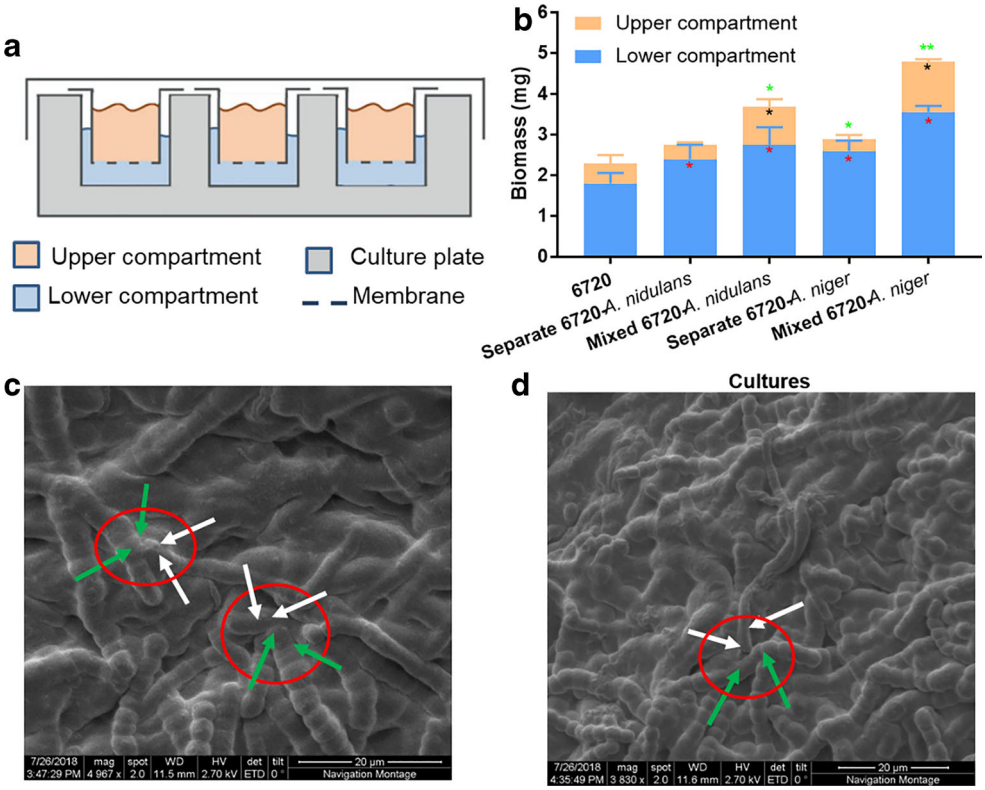

Fig. 6 The physical interactions between *Nostoc* PCC 6720 and *Aspergilli* species. **a** The schematic diagram of transwell system. **b** The biomass production from transwell compartment. **c**, **d** Scanning electron micrograph of *Nostoc–A. nidulans* co-culture and *Nostoc–A. niger* co-culture, respectively, where fungal mycelia are thread-like (white arrows) and cyanobacterial filaments are chained beads-like (green arrows). Stars stand for the significant difference in comparison with axenic *Nostoc* control by *t* test. Red stars represent lower compartment, black ones stand for upper compartment, and green stand for total biomass

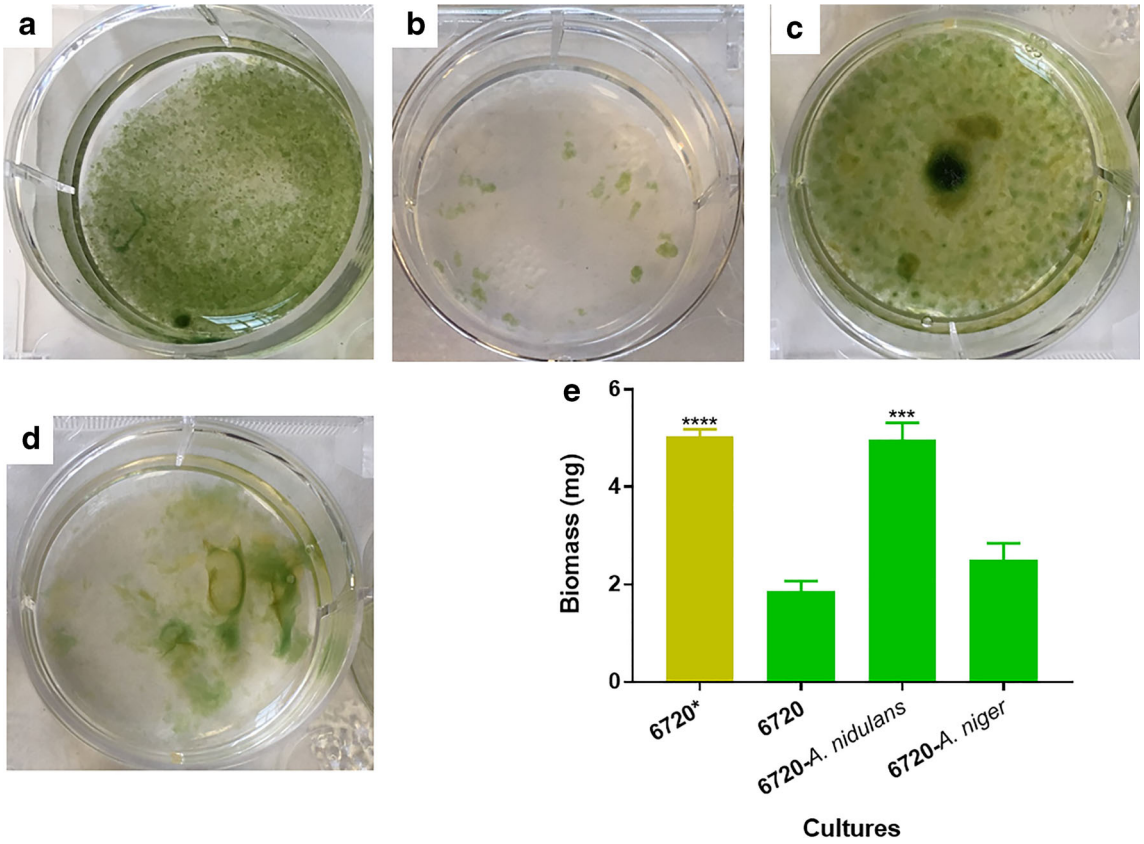

Fig. 7 The culture in 6-well plate with primary effluent. **a** Axenic culture of *Nostoc* PCC 6720 in BG11 medium. **b**–**d** Axenic *Nostoc* PCC 6720, co-culture of *Nostoc* PCC 6720 and *A. nidulans*, and co-culture of *Nostoc* PCC 6720 and *A. niger* in primary effluent, respectively. **e** Biomass collected from the 6-well plates. Stars stand for the significant difference in comparison with axenic *Nostoc* in PE by *t* test

culture in chemically defined BG11 medium (Fig. 7e). However, the co-culture of 6720-*A. niger* did not exhibit significant promotion of biomass production (Fig. 7d and e). The results show that PE is not ideal for axenic *Nostoc* PCC 6720 growth, but its co-culture with *A. nidulans* allows for successful growth of the *Nostoc*–*A. nidulans* system in wastewater, establishing a low-cost resource for co-culture cultivations.

The 6720–*A. niger* co-cultures showed no difference in growth compared with *Nostoc* mono-cultures in shake flasks, with biomass reaching approximately 600 mg/L after 6 days for both cultures (Fig. 8a and b). However, for the first 6 days, the 6720–*A. nidulans* co-cultures exhibited a higher growth rate (0.37 day^{-1}), compared with the *Nostoc* mono-culture (0.29 day^{-1}). Furthermore, the final biomass achieved in the 6720–*A. nidulans* co-cultures (990 mg/L) was far more than the sum of biomass obtained in the axenic culture of *Nostoc* PCC 6720 (560 mg/L) and the axenic culture of *A. nidulans* (70 mg/L) operated in parallel.

Similarly, when examined under a microscope, the cultures containing 6720–*A. nidulans* cultures contained overlapping beads of 6720 (green arrow in Fig. 8c) intertwined with *A. nidulans* mycelia (white arrow) as observed previously in Fig. 2c. The fungal mycelia were also observed as control in the axenic fungal cultures grown in PE added with glucose (Supplemental Fig. S3). In contrast for the 6720–*A. niger* cultures, few mycelia were observed under the microscope (Fig. 8d), which differed from the co-culture of 6720–*A. niger* in BG11 (Fig. 2d).

Trials evaluating the growth of cyanobacteria in spent fungal PE medium and vice versa were also carried out to explore the change of PE by *Nostoc* or fungi. As shown in Fig. 9a, the fungal supernatant (orange bars for *A. nidulans* and blue bars for *A. niger*) significantly promoted cyanobacterial biomass accumulation. Lower biomass levels were observed in pure supernatant of *A. nidulans* (SN1) than in PE amended with SN1, which might result from a lack of inorganic nutrients that were consumed by the fungus. The fungal growth in spent medium of *Nostoc* was also evaluated (Fig. 9b and c). Introducing the supernatant of *Nostoc* into PE increased the biomass production of *A. nidulans*, while adding *Nostoc* supernatant from growth in PE produced a negligible difference in *A. niger* biomass, suggesting metabolites from *Nostoc* offered no benefit to these fungi.

Finally, another experiment was performed in which the individual and mixed cultures in PE were added to transwells and the biomass was followed as performed previously in BG11 medium (Fig. 9d). For the 6720–*A. nidulans* cultures,

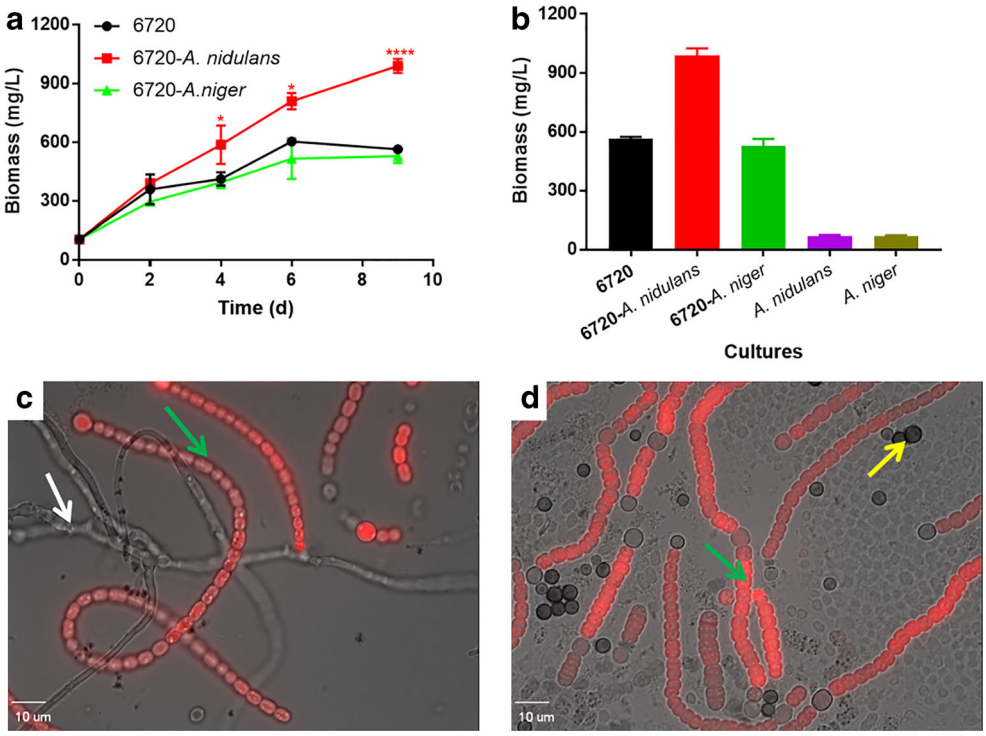

Fig. 8 The cultures of *Nostoc* PCC 6720 and *Aspergilli* species in primary effluent. **a** Biomass production. **b** The final biomass production. **c**, **d** Representative fluorescent microscopy image of co-culture of *Nostoc* PCC 6720 and *A. nidulans*, and co-culture of *Nostoc* PCC 6720 and *A. niger*, respectively, in primary effluent, where red color cells pointed by green arrow indicate *Nostoc* PCC 6720, white arrows indicate fungal hyphal fragments, and yellow arrow points to fungal spores. Scale bar, 10 μm. All data are averages of biological triplicates ± standard deviation. Stars stand for the significant difference in comparison with axenic *Nostoc* control at the same day by *t* test

the mixing of the two cultures resulted in significantly higher biomass levels compared with those achieved with separate cultivation of 6720 and *A. nidulans*. Conversely, co-culture of mixed 6720–*A. niger* cultures exhibited lower total biomass than the corresponding physically isolated co-culture (Fig. 9d).

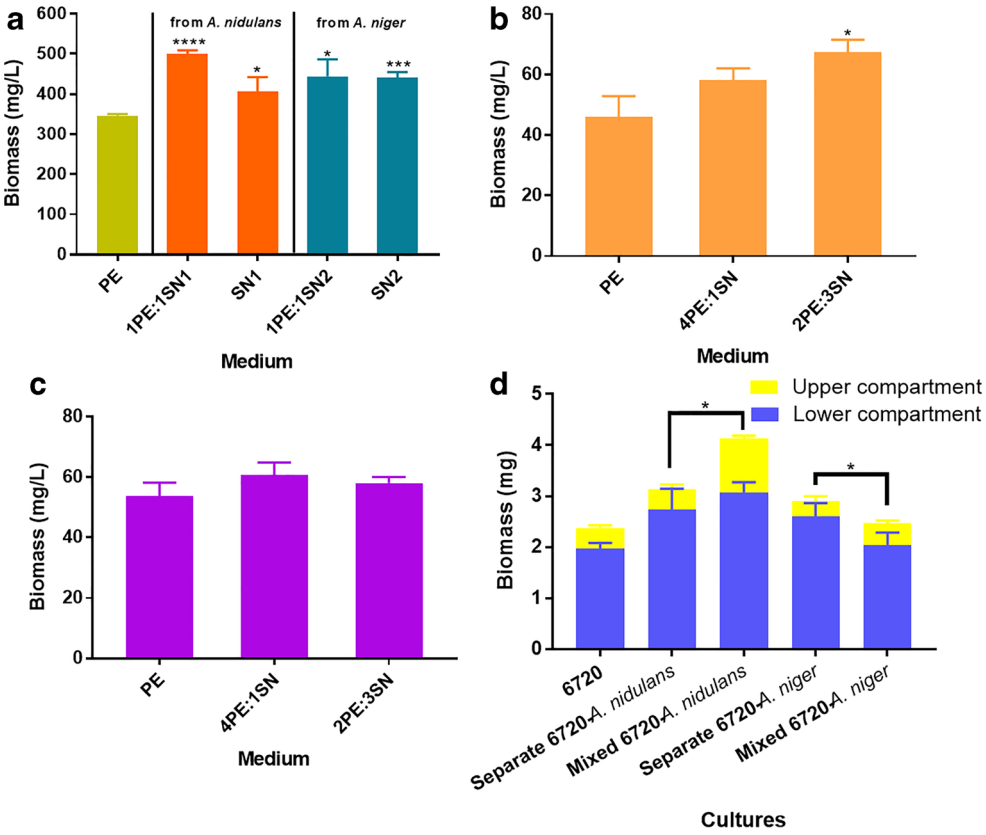

Fig. 9 The axenic cyanobacterial and fungal biomass production in the supernatant (SN) of each other grown and in transwell system with primary effluent as medium. **a** The biomass of *Nostoc* PCC 6720 in the supernatant of *A. nidulans* (SN1) and *A. niger* (SN2). **b**, **c** The biomass of *A. nidulans* and *A. niger* in the supernatant of *Nostoc* PCC 6720, respectively. **d** The biomass production from transwell compartment. All data are averages of biological four replicates ± standard deviation. Stars in **a**–**c** stand for the significant difference in comparison with PE control by *t* test. Stars in **d** represent the significant difference of total biomass in paired comparison by *t* test

The sustainability of *Nostoc* PCC 6720–*Aspergillus nidulans* co-culture in primary effluent

Primary effluent, as a renewable and inexpensive medium, could sustain the growth of *Nostoc* PCC 6720–*A. nidulans* co-culture and sustain their mutualistic relationship. In order to preliminarily analyze the application potential of this co-culture system, a long-term semi-continuous experiment was proposed and carried out for mixed cultures with physical interactions in order to evaluate its sustainability. On a weekly basis, half of the semi-continuous culture volume was collected for biomass analysis and was replaced by fresh primary effluent (Fig. 10). The co-culture recovered rapidly from the half reduction of cells and culture media and re-suspension in equal parts fresh PE and spent culture medium. A difference in biomass between the co-cultures and axenic 6720 cultures was evident after 2 weeks, demonstrating the superiority of the co-cultures, at least in terms of biomass accumulation, over the mono-culture. Moreover, the biomass production of co-culture continuously increased with time during the semi-continuous trial culture period, in which the co-culture biomass was nearly 98% higher at the 5-week time point, and the total biomass was more than 73% enhanced over all 5 weeks of cultivations. These findings indicate that the *Nostoc*–*A. nidulans* co-culture adapted to unfavorable PE environments more rapidly than the mono-culture, demonstrating the advantages and sustainability of co-cultivating two mutualistic organisms over a single microbe for growing maximizing biomass in unfavorable environments such as those from wastewater.

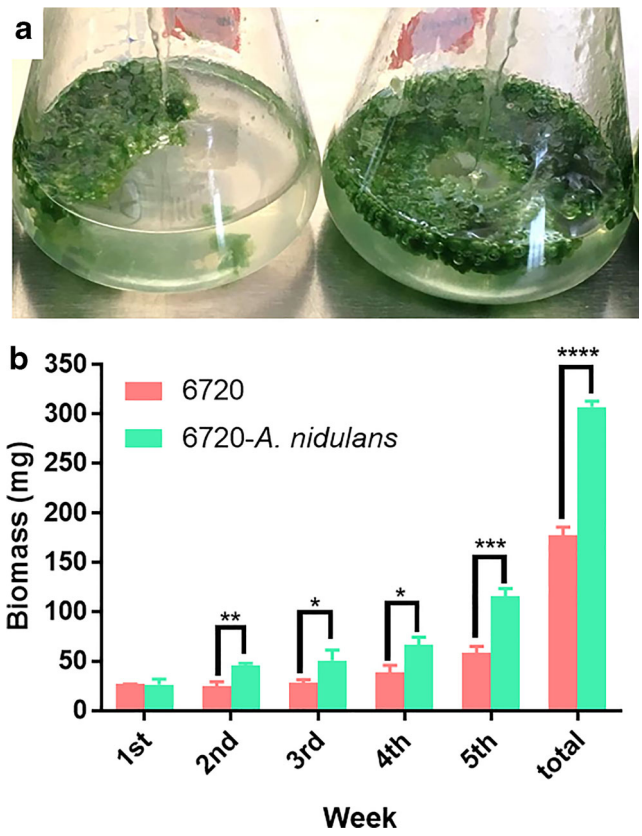

Fig. 10 Semi-continuous axenic culture of cyanobacteria and co-culture of *Nostoc* PCC 6720 and *A. nidulans* using primary effluent as medium. **a** The picture of flasks culturing axenic *Nostoc* PCC 6720 and co-culture of *Nostoc* PCC 6720 and *A. nidulans*, from left to right, after 5 weeks of postinoculation. **b** The biomass harvested every week and total biomass obtained from the semi-continuous cultures. All data are averages of biological four replicates ± standard deviation. Stars stand for the significant difference in paired comparison by *t* test

Discussion

In this study, we examined the potential mutualistic relationship of eukaryotic chlorobionts (*Chlorella* and *Scenedesmus*) or cyanobacteria (*Nostoc*) to grow in co-cultures with filamentous fungi (*A. nidulans* and *A. niger*).

Regarding chlorobionts, only the filaments of *A. niger* were observed when co-cultured with *C. sorokiniana*, while no filaments occurred in co-cultures of *A. nidulans*–*C. sorokiniana*, *A. nidulans*–*Scenedesmus*, and *A. niger*–*Scenedesmus*. These results suggested that *A. nidulans* could not obtain carbon from *C. sorokiniana*, and *Scenedesmus* was not a good provider of organic carbon for fungi. Therefore, these partnerships cannot be classified as artificial lichens. *A. niger* could grow when in co-culture with *C. sorokiniana*, perhaps utilizing components secreted or released from dead algal cells. Interestingly, no spores germinated within the hypha (Fig. 1f) when *A. niger* was cultured in the spent medium of *C. sorokiniana*. Considering dead algal cells were accompanied by enlarged flocs, it is reasonable to speculate that algal cells might be damaged by the fungus in order for the fungus to obtain an organic carbon source. Moreover, given the reality that *A. niger* can produce cellulase (Srivastava et al. 2018) and *Chlorella* was categorized as a cellulosic based organism (Laurens et al. 2017), we hypothesize that the growth of *A. niger* depends principally on the carbohydrates of algal cell wall as a carbon source. The relationship between *C. sorokiniana* and *A. niger* is similar to parasitism which is not a good combination for a lichen, either.

A stable synthetic partnership was constructed consisting of the cyanobacterial species *Nostoc* PCC 6720 and the *A. niger* growing on light and CO_2 in the absence of external carbon. Unlike our previous study using sucrose-secreting cyanobacterium *cscB*$^+$ *S. elongatus* as a carbon provider (Li et al. 2017), this partnership did not require any genetic manipulation of either species in order to achieve a successful co-culture.

The different behaviors observed when combining different phototrophs and heterotrophs in co-culture indicated that the symbiotic relationship depends on the specific species, which has been previously reposted. For example, Du et al.

(2018) co-cultured the marine alga *Nannochloropsis oceanica* with *Mortierella elongata* or *Morchella americana* in which algal growth was observed in the co-culture with *M. elongata*, while it turned deadly brown in the co-culture with *M. americana*. Further test of interactions between *N. oceanica* and other fungi also demonstrated that positive interactions only occurred from a few *Mortierella* species and the alga, while the other fungi neutrally or negatively impacted algal activities (Du et al. 2019). Hence, these previous findings and our studies suggest that multiple reasons may exist for encouraging or inhibiting mutualistic relationships that include but are not limited to: (1) accessible organic carbon provided by the phototroph to the heterotroph; (2) similar desirable cultivation environments (e.g., pH, temperature, aeration, etc.) for the partners; (3) absence of toxic or lethal factors produced by one organism against the partner.

The cyanobacterial supernatant supported the axenic fungal growth, indicating access of fungi to extracellular metabolites of *Nostoc* as a carbon and/or energy supply. *Nostoc* species are capable of producing both extracellular proteins and extracellular polysaccharides (EPS) (Xue et al. 2017) which have the potential to serve as energy and carbon source for the *Aspergilli* species. The carbon source available to fungal species from cyanobacteria in natural lichens may include released metabolites, EPS, cell-bonding substances bound to the cell surface, and even the cell itself (Crittenden and Porter 1991).

Adding *A. nidulans* and *A. niger* supernatant to the culture media boosted the axenic cyanobacterial biomass accumulation by a factor of up to three, although at different dilution levels of fresh media and fungal spent media. The benefits between the syntrophic microbes elevated the total biomass production in batch liquid co-culture systems by nearly 2-fold compared with the axenic *Nostoc* cultures in defined BG11 media. Previous research has demonstrated that the presence of heterotrophs can enhance the growth of phototrophs. For example, "helper heterotrophic bacteria" have been shown to reduce the oxidative stress observed for the cyanobacteria, *Prochlorococcus* (Morris et al. 2008), and *Rhizobium* strains have been shown to improve the growth of eukaryotic *Ankistrodesmus* sp. through secreting indol-3-acetic acid and/or vitamin B12 (Do Nascimento et al. 2013). In another study, the fungus *Rhizidium phycophilum* was shown to enhance the biovolume of the unicellular eukaryotic *Bracteacoccus* sp. (Picard et al. 2013). Our study represents the first case in which a filamentous fungal species has been shown to enhance the growth of filamentous cyanobacteria in co-cultures.

On solid medium, fungi grew on the periphery but not within the dense *Nostoc* biomass, perhaps because the *Nostoc* cells limit fungal growth possibly due to nutrient competition or oxidative stress from photosynthetic reactions. These results motivated us to consider further the relative importance of the physical interaction versus exclusively nutrient exchange on the growth of the *Nostoc* or *Aspergilli* species. Two possible reasons for the physical interaction are that (1) *Aspergilli* rely on contact with *Nostoc* for fast carbon and energy uptake, and (2) Signaling between the species may require physical contact. Interestingly, the *Aspergillus* did not flourish within very dense *Nostoc* colonies and instead accumulated on the periphery (Fig. 5D). Thus, if the *Nostoc* cultures become too dense, these may inhibit fungal growth due to nutrient competition or perhaps the activation of oxidative stress. Oxygenic photosynthetic organisms tend to produce reactive oxygen species, such as highly reactive singlet oxygen from light-harvesting chlorophyll, and superoxide, peroxide, and hydroxyl radicals from electron transfer reactions (Kihara et al. 2014), can impose detrimental effects on many biological macromolecules, including DNA, proteins, and membrane of fungi (Abrashev et al. 2008).

However, imposing physical barriers lead to a decrease in the total biomass obtained to suggest that some direct physical contact between cyanobacteria and heterotrophic microbes can promote the overall growth of the co-culture system. The reduction in biomass production for these separate co-culture systems may result from the decreased diffusion rates of nutrients and metabolites between compartments caused by the transwell membrane. High diffusion rates have been observed to be important to sustain adequate metabolite exchange among microbial populations whose expansion depends on nutrients, stimulants, or signals provided by other species (Weber et al. 2007). The physical interaction between cyanobacteria and fungi enables access to substances from each other and may also stimulate both parties to produce and secrete certain beneficial metabolites. Taken together, we can posit that physical interactions between *Nostoc* and *Aspergilli* are not a requisite but a promotive condition for augmenting biomass production in the cyanobacterial-fungal co-cultures.

Besides enhanced growth of cyanobacterium and fungi in co-cultivation, potentially adaptive benefits of this partnership were also tested under wastewater conditions. Although primary effluent is not an ideal medium for axenic *Nostoc* growth, co-culture with *A. nidulans* is shown to improve the growth of *Nostoc* in this harsh environment. That the biomass obtained in co-cultures was greater than the sum of biomass in axenic *Nostoc* and *A. nidulans* cultures demonstrated that a mutualistic association between *Nostoc* PCC 6720 and *A. nidulans* was established in primary effluent. Combining the two species may be advantageous when considering approaches to maximize utilization of these wastes for generation of biomass and bioproducts.

Interestingly, different metabolic events in *Nostoc* may change the PE such that the altered medium improves the growth of *A. nidulans*. However, unlike in the BG11 medium, *Nostoc* could not enhance the growth of *A. niger*, in the PE.

Instead of fungal mycelia observed in co-culture of 6720–*A. niger* in BG11, *A. niger* were observed primarily in the form of spores in the PE co-culture (yellow arrow in Fig. 8d) to suggest that *Nostoc* might synthesize certain metabolites or cause nutrient deficiencies that could contribute to the failure of *A. niger* spore germination in PE. Alternatively, certain toxins to *A. niger* that are not problematic to *A. nidulans* may appear in the PE which are not present in BG11 and prevent fungal germination and growth in co-cultures with *Nostoc* 6720.

To examine these alternatives, trials evaluating the growth of cyanobacteria in spent fungal PE medium and vice versa were carried out. The enhanced growth of *Nostoc* and *A. nidulans* by adding each other's spent medium into PE confirms the mutualistic symbiosis and metabolite exchange between *Nostoc* PCC 6720 and *A. nidulans*.

Furthermore, the impact of physical proximity of *Nostoc* on *A. nidulans* enhances the growth of at least one and perhaps both species in PE while the physical interactions are problematic for the 6720–*A. niger* interactions. The higher total biomass in mixed cultures of 6720–*A. nidulans* than the system with physical boundaries (Fig. 9d) shows that the mutualistic association, while involving some metabolite exchanges, is enhanced with the closer physical intimacy that comes with the mixed cultures, consistent with what was observed previously in BG11 medium (Fig. 6b). The lower total biomass of mixed 6720–*A. niger* co-culture than the corresponding separate system indicates that the *Nostoc* growing in PE may produce metabolites or signaling molecules that are toxic to *A. niger* but the lower diffusion rates and accessibility present in the transwell membrane cultures may reduce their toxicological impact. Thus, when setting up co-culture, the degree of partners' contact should be considered whether well-mixed or partially separated.

Furthermore, the synthetic *Nostoc*–*A. nidulans* co-culture was also able to grow stably in wastewater over a long-term 5-week semi-continuous condition with biomass levels that were 73% higher than the *Nostoc* mono-cultures. In this mutually beneficial symbiosis, besides the possible exchange of biological macromolecules as nutrients between fungi and cyanobacteria, *A. nidulans* might also afford protection to *Nostoc* in this harsh environment, which extends the application of this artificial lichen to wastewater treatment. The benefits of co-culture were also observed by Muradov et al. (2015) who screened fungal impact on microalgal flocculation and bioremediation efficiency in swine wastewater. Zoller and Lutzoni (2003) also found that the presence of fungi helped to retain water and serve as a larger capture area for algae to access minerals in natural lichens exposed to abiotic environmental stresses.

Based on our results, these synthetic lichen-like platforms including cyanobacteria and fungi can serve as a valuable model platform for exploring sustainable mutualism involving metabolite exchange and other interactions as well as a potential bioproduction platform for generating valuable fungal or cyanobacterial products such as secondary metabolites in the future.

Author contributions LJ participated in the study design, carried out the whole experiments, and drafted the manuscript. TL, JJ, and YH assisted the laboratory work and performed the manuscript revision. CB collected wastewater and revised manuscript. HP contributed to the manuscript revision. MJB conceived the experimental design, participated, and supervised manuscript preparation and editing. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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