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„Substrate and irrigation media preferences of terrestrial
cultivated *Nostoc commune*“

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Anna Broschek, BSc

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ao. Univ.-Prof. Mag. Dr. Michael Schagerl

Kurzfassung

Terrestrische Arten des Cyanobakteriums *Nostoc* sind austrocknungstolerante Pionierorganismen in nährstoffarmen Böden. Aufgrund menschlicher Einflüsse sind sie in einigen Regionen vom Aussterben bedroht. Restaurationsmaßnahmen wurden durch das langsame Wachstum von *Nostoc* im Freiland erschwert. Versuche, den Biomasseertrag durch Flüssigkultivierung und anschließende Ausbringung auf Böden zu erhöhen, erzielten bislang keinen Durchbruch. Zudem kann sich in Flüssigkultur gezüchteter *Nostoc* von terrestrischem *Nostoc* unterscheiden. Die Studie zielte darauf ab, *Nostoc* auf festen Substraten zu kultivieren, um seine koloniebildende Lebensweise zu berücksichtigen und seine Substrat- und Bewässerungsmedienpräferenzen sowie die Auswirkung periodischer Austrocknung zu untersuchen. Zu diesem Zweck wurde *Nostoc commune* auf fünf Substraten (Filterpapier, Glasperlen, Marmorgranulat, Quarzsand, Schotter) kultiviert und mit drei Medien (BG11, BG11₀, Regenwasser) bewässert. In einem Versuchsaufbau blieben die Proben feucht, während sie im anderen regelmäßig austrockneten. Das Wachstum wurde sowohl durch Messung der Anfangs- und Endtrockenmasse als auch alle zwei bis 14 Tage mit einem Imaging Pulse-Amplitude-Modulation (PAM)-Fluorometer überwacht, um die Chlorophyll-Fluoreszenz sichtbar zu machen und die Koloniegroße zu bestimmen. Größe und Trockenmasse von *N. commune* nahmen im feuchten Versuchsaufbau auf den Substraten Glasperlen, Marmor und Quarzsand bei Bewässerung mit BG11₀ und Regenwasser signifikant zu. Austrocknung zwischen den zwei- bis dreitägigen Bewässerungsintervallen reduzierte das Wachstum drastisch. *N. commune*, das mit BG11 besprüht oder auf Filterpapier kultiviert wurde, wuchs in beiden Versuchsaufbauten nicht. Dieses Ergebnis erfordert weitere Untersuchungen, um die Auswirkungen von Austrocknung und Nährmedien auf das Wachstum von *N. commune* besser zu verstehen.

Schlagwörter: Kulturmedien, Cyanobakterien, Austrocknung, Wachstum, Imaging-PAM-Fluorometrie, Mikroskopie, terrestrische Kultivierung, Substrattypen

Abstract

Terrestrial species of the cyanobacterium *Nostoc* are desiccation-tolerant pioneer organisms in nutrient-poor soils. Due to human impact, they have become endangered in some regions. Restoration efforts have been hampered by the slow growth of *Nostoc* in the field. Attempts have been undertaken to increase the biomass yield through liquid cultivation and subsequent application to soils, but a breakthrough has yet to occur. Moreover, liquid-cultured *Nostoc* can differ from terrestrial *Nostoc*. The study aimed to cultivate *Nostoc* on solid substrates to consider its colony-forming lifestyle and to investigate its substrate and irrigation media preferences and the effect of periodical desiccation. For this purpose, *Nostoc commune* was grown on five substrates (filter paper, glass beads, granulated marble, quartz sand, crushed stone) irrigated with three media (BG11, BG11₀, rainwater). In one treatment, the samples remained moist, while in the other they dried out regularly. Growth was monitored every two to 14 days using an Imaging Pulse-Amplitude-Modulation (PAM) fluorometer to visualise chlorophyll fluorescence and derive colony size, and by measuring initial and final dry mass. The size and dry mass of *N. commune* significantly increased in the moist treatment on the substrates glass beads, marble, and quartz sand when irrigated with BG11₀ and rainwater. Desiccation between the two- to three-day irrigation intervals drastically reduced its growth. *N. commune* sprayed with BG11 or cultured on filter paper did not grow in both treatments. This result calls for further investigations to better understand the role of desiccation and nutrient media in the growth of *N. commune*.

Key words: culture media, cyanobacteria, desiccation, growth, Imaging-PAM fluorometry, microscopy, solid cultivation, substrate types

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List of abbreviations

BG11	blue-green medium number 11
BG11 ₀	blue-green medium number 11 without nitrogen
EPS	extracellular polysaccharides
F	filter paper
F ₀	dark fluorescence yield
Fig.	Figure
F _m	maximal fluorescence yield
G	glass beads
GPS	Global Positioning System
<i>H</i> test	Kruskal-Wallis <i>H</i> test
IQR	interquartile range
M	marble
N.	<i>Nostoc</i>
PAM	Pulse-Amplitude-Modulation
Q	quartz sand
Q1	first quartile
Q3	third quartile
RW	rainwater
S	crushed stone
SAG	Sammlung von Algenkulturen der Universität Göttingen (Culture Collection of Algae at Göttingen University)
SAT-Pulse	Saturation Pulse
SodF	superoxide dismutase
<i>U</i> test	Mann-Whitney <i>U</i> test
WSP	water stress proteins
WSR test	Wilcoxon's signed-rank test

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1. Introduction

Overexploitation of wild species is a major threat to biodiversity (Maxwell et al. 2016). It leads to species decline and can cause habitat degradation (Roney et al. 2009). An alternative to harvesting wild species is the cultivation of these organisms (Schippmann et al. 2002). Although this practice is controversial for conservation purposes, some international organisations and governments recommend it (WHO et al. 1993, Schippmann et al. 2002, Gao et al. 2005). Yet, bringing wild species into cultivation can be challenging – such as the cultivation of the cyanobacterium *Nostoc* (Gao and Ye 2003, Katoh et al. 2012).

Nostoc is an aerobic, photoautotrophic microorganism that produces oxygen via photosynthesis (Adams and Duggan 1999). The ability to photosynthesise is a reason why cyanobacteria are studied traditionally by phycologists (Dodds et al. 1995) and have been called blue-green algae in the past (Haselkorn 2006, Garcia-Pichel et al. 2020). However, this term is misleading and no longer used today, as cyanobacteria belong to the domain of bacteria (Garcia-Pichel et al. 2020). Unlike eukaryotic algae, they do not have a nucleus or chloroplasts, which makes them prokaryotic organisms (Adams and Duggan 1999, Haselkorn 2006). Cyanobacteria perform photosynthesis in their thylakoid membranes using chlorophyll *a* and phycobilisomes (Grossman et al. 1995). In addition to being able to fix carbon dioxide, cyanobacteria can also fix nitrogen (Haselkorn 2006). Cyanobacteria form a large and heterogeneous phylum with a wide variety of morphologies (Adams and Duggan 1999, Garcia-Pichel et al. 2020). One order that belongs to this phylum is the Nostocales, which include the genus *Nostoc* (Rajaniemi et al. 2005).



Fig. 1: *Nostoc* sp. in a desiccated state (left) and in a wet state (right) (picture from Michael Schagerl).

Nostoc forms macroscopic colonies, which means it is visible to the naked eye as a green or blackish-brown structure (Cameron 1962, Mollenhauer et al. 1999; Fig. 1). The shape of these colonies varies between species as well as depending on the state of moisture and ranges from hair-like to discoid to spherical (Cameron 1962, Gao 1998, Briones-Nagata et al. 2007). If *Nostoc* gets wet, it swells rapidly and tends to form elastic, gelatinous structures that smell earthy and feel jelly-like (Potts 1997). In a dried state, *Nostoc* changes its appearance to a flat, crust-like, brittle layer (Potts 1997). Dry *Nostoc* can be easily overlooked in the environment, whereas the slimy swollen *Nostoc* masses which appear after heavy rains are conspicuous (Dodds et al. 1995, Potts 1997). This led to the former belief

that *Nostoc* could be an ejection from the stars, which is why it was called a “shot star” (Dodds et al. 1995, Potts 1997). Paracelsus (Theophrastus Bombastus von Hohenheim) also had theories about these legends and created the name “Nostoch” in the 16th century (Potts 1997).

The *Nostoc* colonies contain numerous trichomes embedded in an extracellular polysaccharide matrix (Gao 1998, Billi and Potts 2000). Trichomes are pearl necklace-like cords of cells consisting of two different cell types: the dominating vegetative cells and specialised cells, called heterocytes (Fig. 2). The normal vegetative cells are spherical cells that perform photosynthesis (Adams and Duggan 1999, Billi and Potts 2000). When *Nostoc* lacks combined nitrogen, some vegetative cells differentiate into heterocytes to fix atmospheric nitrogen (Adams and Duggan 1999). Heterocytes can no longer perform normal photosynthesis because they lose their photosystem II activity to protect their nitrogen-fixing enzyme nitrogenase from oxygen (Adams and Duggan 1999). Visually, heterocytes can be distinguished from vegetative cells as they are slightly larger, have thicker cell walls, and have fewer cytoplasmic granules (Adams and Duggan 1999). The ability to obtain energy through different metabolic pathways enables *Nostoc* to colonise various habitats, especially nutrient-poor environments (Sand-Jensen 2014). *Nostoc* has a global distribution in aquatic and terrestrial habitats (Cameron 1962, Dodds et al. 1995, Mollenhauer et al. 1999).



Fig. 2: *Nostoc* trichomes with 10x magnification (left) and 40x magnification (right). The red h indicates the heterocytes.

The (semi-)terrestrial species *Nostoc commune* and its close relative *Nostoc flagelliforme* are of ecological importance as pioneer organisms in nutrient-poor and bare soils (Sand-Jensen and Jespersen 2012, Gao et al. 2016). *Nostoc* can stabilise degraded soils and enhance soil fertility by increasing the content of nutrients such as nitrogen (Falchini et al. 1996, Obana et al. 2007, Gao et al. 2016, Román et al. 2018, Roncero-Ramos et al. 2019b). The two species *N. commune* and *N. flagelliforme* can be distinguished morphologically, but Aboal et al. (2016) described morphological transitional forms and found no distinct genetic differences. *N. flagelliforme* forms hair-like filaments with straight, parallel-oriented trichomes, whereas *N. commune* forms extended, layered structures with coiled trichomes oriented in various directions (Aboal et al. 2016, Cui et al. 2017). Differences were also described in their tolerance to wetness and their water absorption speed (Scherer et al. 1984, Scherer and Zhong 1991). *N. flagelliforme* absorbs water more rapidly than *N. commune* and degrades

under constant moisture (Scherer and Zhong 1991, Gao 1998). Therefore, this species occurs predominantly in arid and semi-arid areas of the world, such as deserts and steppes (Gao 1998, Aboal et al. 2016). *N. commune* is a cosmopolitan taxon, distributed from the Poles to the Tropics, and occurs in arid, semi-arid, as well as moist habitats (Mollenhauer et al. 1999, Johnson et al. 2008, Kvíderová et al. 2011).

Both species tolerate drought and can thus survive for a long time in a desiccated state (Scherer et al. 1984, Dodds et al. 1995). Lipman (1941) successfully revived an 87-year-old herbarium specimen of *N. commune*. Cameron (1962) continued and surpassed this experiment in longevity by reactivating parts of this *N. commune* specimen, which had then been in dried form for 107 years. Desiccated *N. commune* is metabolically inactive and can withstand extreme temperatures ranging from -269 °C up to 70 °C and a pH between 3 and 10 (Sand-Jensen and Jespersen 2012). After rehydration, *N. commune* resumes its metabolism by starting respiration, followed by photosynthesis, and subsequently nitrogen fixation (Scherer et al. 1984). The reactivation speed of these processes depends on the duration of the previous drying period; respiration can resume instantly after rewetting (Scherer et al. 1984). The extracellular matrix and the production of extracellular polysaccharides (EPS) are mechanisms that contribute to the high stress tolerance of *N. commune*, for example, by protecting its photosynthetic apparatus from damage caused by desiccation or freezing (Tamaru et al. 2005, Inoue-Sakamoto et al. 2018). The extracellular matrix consists mainly of water stress proteins (WSP) and further contains a superoxide dismutase (SodF) and photo-protective pigments (Scherer and Potts 1989, Wright et al. 2005). The pigments act as sunscreen for *N. commune* by absorbing UV radiation (Böhm et al. 1995).

These adaptations allow *N. commune* to colonise habitats where dry and wet periods alternate and where high UV radiation is present (Becerra-Absalón et al. 2019). Ye et al. (2021) showed that *Nostoc* sp. even survives in a stratospheric environment for at least a few hours and would thus be a potential candidate for Mars colonisation. Previous to the study of Ye et al. (2021), other authors have already explored the potential of *N. commune* in extra-terrestrial environments, for example, as a water-storing biofilm under Mars-like conditions or as a fertilising soil additive for space agriculture (Kato et al. 2012, Janchen et al. 2016). The use of *Nostoc* sp. as a fertiliser has also been studied for soils on Earth, showing that *Nostoc* increases the nitrogen and carbon content of nutrient-poor soils and thus promotes plant growth (Obana et al. 2007, Maqubela et al. 2009). Other applications of *N. commune* include the remediation of contaminated soils and wastewater treatment, as it can absorb certain heavy metals and radionuclides (Kato et al. 2012, Sasaki et al. 2013, Lavado-Meza et al. 2023). Furthermore, compounds with antimicrobial, anti-inflammatory, and anti-carcinogenic properties have been isolated from *N. commune* (Jaki et al. 1999, Ninomiya et al. 2011, Itoh et al. 2014, Guo et al. 2015), making *Nostoc* interesting for pharmaceutical and medicinal research (Nowruzi et al. 2018). Some indigenous people have long been aware of the therapeutic value of *N. commune* and use it, for example, to treat inflammation (Johnson et al. 2008). However, it is mainly collected for nutritional purposes (Briones-Nagata et al. 2007, Johnson et al. 2008). Both *N. commune* and *N. flagelliforme* are used as food in some Asian and South American countries (Gao 1998, Briones-Nagata et al. 2007, Johnson et al. 2008, Liang et al. 2022).

Due to steadily increasing market demand, *N. commune* and *N. flagelliforme* have been decimated in China (Gao 1998, Diao and Yang 2014). Habitat loss, use of fertilisers and

pesticides, as well as pollution have exacerbated the threat to the edible *Nostoc* species (Gao 1998, Qiu et al. 2002, Diao and Yang 2014). The lack of *N. flagelliforme* led to soil erosion and subsequently to sandstorms and accelerated desertification, so the Chinese government banned the harvest and trade of this species in 2000 and placed it under protection (But et al. 2002, Roney et al. 2009). In Europe, *N. flagelliforme* is considered rare and would be worth protecting there as well (Aboal et al. 2016). *N. commune* is abundant in some regions of Europe but vulnerable or extinct in others due to threatened habitats (Mollenhauer et al. 1999). In Peru, fertiliser and pesticide use are thought to be a reason why *N. commune* has no longer been found in former habitats (Johnson et al. 2008). To conserve *N. commune* and *N. flagelliforme*, the protection of their habitats is crucial (Mollenhauer et al. 1999, Aboal et al. 2016). As *Nostoc* has already disappeared from some existing habitats, which can lead to massive environmental problems, restoration is another goal (Gao et al. 2016). This requires the successful propagation of terrestrial *Nostoc*, whereby cultivation in the laboratory plays an essential role (Gao et al. 2015, Román et al. 2021).

Research on cultivating *Nostoc* has been ongoing for decades, and after initial difficulties, scientists were able to achieve success (Gao and Yu 2000, Feng et al. 2012, Diao and Yang 2014, Roncero-Ramos et al. 2019a). One challenge is the slow growth of *N. commune* and *N. flagelliforme* (Gao 1998, Katoh et al. 2012). To accelerate their growth rate, both *Nostoc* species are cultivated in liquid media, leading to higher biomass (Su et al. 2008, Roncero-Ramos et al. 2019a). However, *N. flagelliforme* is sensitive to wetness and can decompose under constant wet conditions (Scherer and Zhong 1991). Regular desiccation periods or prior disinfection of the sample surface reduce the risk of its decomposition by limiting the growth of epiphytic microorganisms that can disintegrate the sheath of *Nostoc* (Gao and Ye 2003, Su et al. 2005). Nevertheless, the liquid-cultured *N. flagelliforme* differed from its wild-growing relatives, for example, by forming a more transparent sheath (Gao and Ye 2003). The characteristic filamentous structure of *N. flagelliforme* is also lost in liquid cultivation unless countermeasures are taken (Liu and Chen 2003). If the agitation speed in liquid suspension culture is too low, *N. flagelliforme* forms globular colonies, but increasing the agitation speed can prevent this (Su et al. 2008). Even when *N. flagelliforme* is grown on solid agar plates in the laboratory, its morphology may differ from naturally growing colonies (Liu and Chen 2003). Without desiccation, *N. flagelliforme* formed spherical colonies on solid agar, but under periodic desiccation, it formed filamentous colonies, which could be elongated by additional treatment with UV irradiation (Feng et al. 2012).

Colonies of *N. commune* were grown in liquid culture in discoid morphology by controlling the shaking speed (Diao and Yang 2014). This was achieved by disinfecting trichomes after collecting, followed by cultivation under continuous shaking speed of 40 rpm. After about one and a half months, macro-colonies formed until then developed into discoid structures (Diao and Yang 2014). Some researchers applied *N. commune* grown in liquid culture to solid substrates (Román et al. 2018, Roncero-Ramos et al. 2019b, 2019a). Nevertheless, differences were found between the EPS production of *N. commune* in liquid culture and that of *N. commune* on solid substrate (Roncero-Ramos et al. 2019a). Laboratory strains of *N. commune* appear to have lower EPS concentrations than wild strains, resulting in lower desiccation tolerance in the former (Inoue-Sakamoto et al. 2018). After a cyanobacterial consortium containing *N. commune* cultivated in liquid medium failed to establish permanently on different outdoor soils, it was treated with a hardening protocol that included periodical desiccation and light exposure for increasing the robustness (Román et al. 2021).

However, also this treatment did not increase the survival under natural conditions without the support of a plant fibre net (Román et al. 2021).

Although some cultivation challenges have already been overcome, restoring degraded soils with pre-cultured *Nostoc* still does not work properly (Gao et al. 2016, Román et al. 2021). Therefore, alternative ways of cultivating terrestrial *Nostoc* are desirable, especially since liquid-cultured *Nostoc* properties obviously differ from terrestrial ones. In the current study, terrestrial *Nostoc* was collected from natural habitats and then cultured in the laboratory on solid substrates without previous liquid cultivation. *Nostoc* was grown on five substrate types irrigated with three different media, once with and once without periodical desiccation. We aimed to gain insight into growth preferences by examining the influence of substrate types, irrigation media, and desiccation status. Specifically, we studied to what extent the substrate type and the irrigation medium influence the development of *Nostoc* and how periodical desiccation affects the performance of this taxon.

2. Material and methods

2.1. Sampling

We collected fresh samples of wild-growing *Nostoc* sp. from a concrete wall in the “Wassercluster Lunz” in Lunz, Austria (Fig. 3).

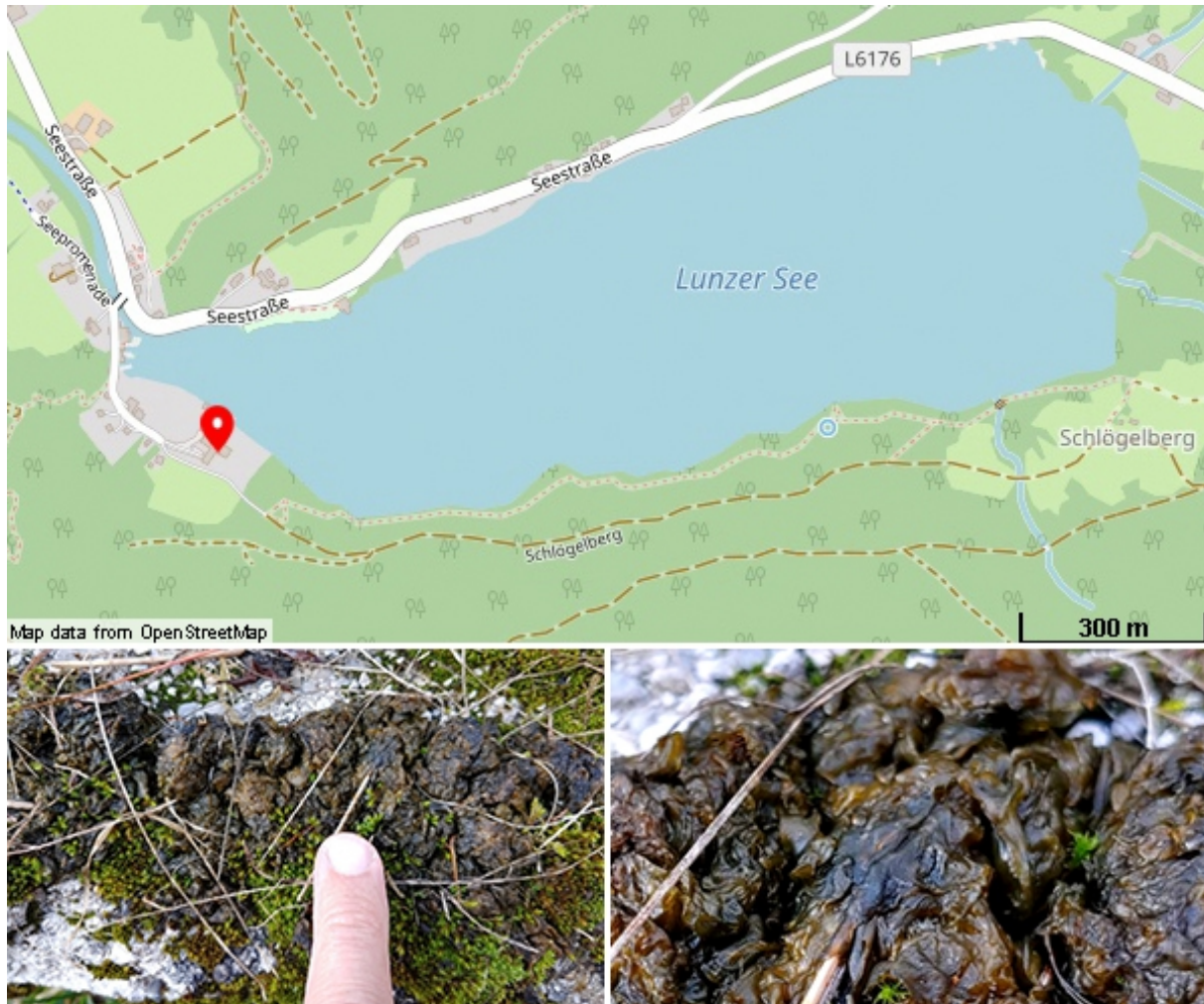


Fig. 3: The map illustrates the sampling area (GPS coordinates: 47.85148, 15.04208) of *Nostoc* sp. (OpenStreetMap 2023). The pictures below show the wild-growing *Nostoc* collected for the study (pictures from Michael Schagerl).

Nostoc was kept cooled in a closed plastic bottle until it was cleaned a few days later to remove attached soil and foreign organisms, such as mosses and small animals like worms. Visible impurities were removed using tweezers and then *Nostoc* was rinsed with tap water. Large *Nostoc* colonies were crushed, either by disintegrating on their own through the washing process or by dividing them with the help of tweezers. Cleaned *Nostoc* was placed on paper towels for air-drying and covered with an air-permeable lid to protect it from dust. After about one month, the dried *Nostoc* was removed from the paper towels and stored in jars in the dark until use.

2.1.1. Species identification

A sample of *Nostoc* sp. was sent to an external laboratory to identify *Nostoc* at the species level using 16S rDNA sequencing. A comparison with the SILVA database revealed that the amplicon is identical to three *Nostoc* species and could originate from *N. commune*, *N.*

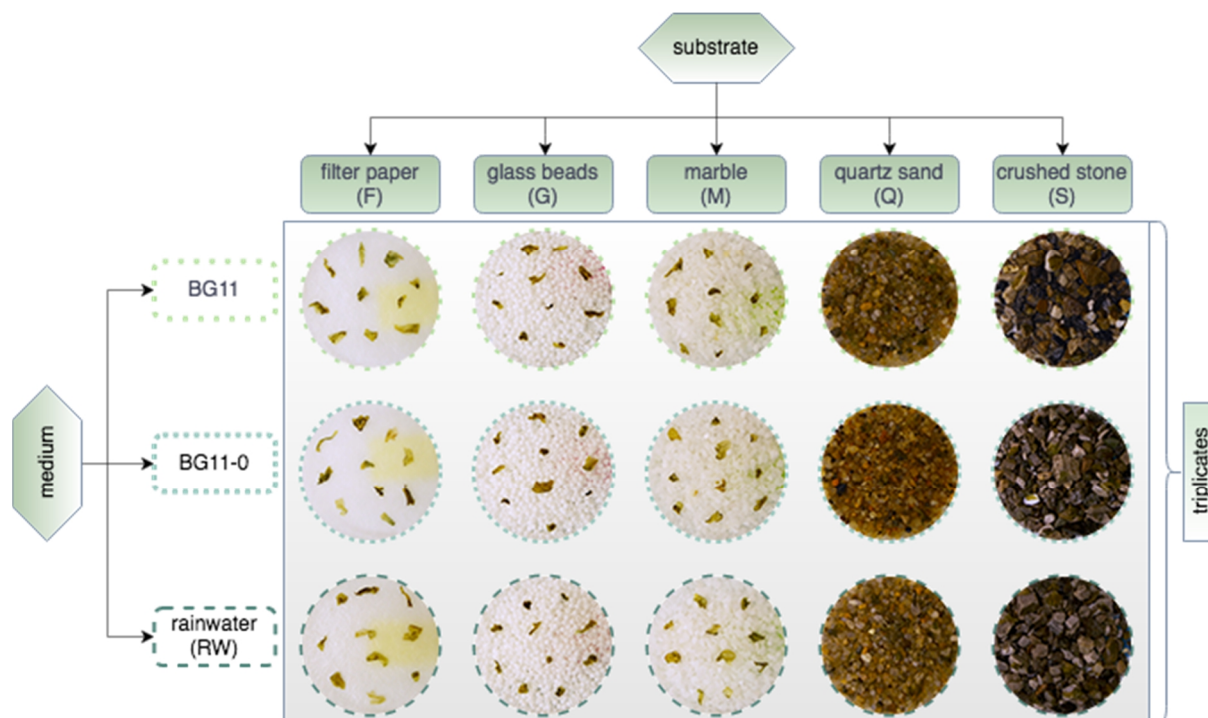
flagelliforme, or *N. piscinale*. Based on its distribution area and morphology, *N. flagelliforme* can be excluded (see chapter 1). The collected *Nostoc* species neither forms hair-like filaments nor is the sampling site Lunz an arid region. Using Komárek (2013), the species was identified as *N. commune*. (Komárek 2013)

2.2. Experimental setup

We performed two experimental runs to investigate the effect of regular desiccation on *N. commune*, each lasting about 50 days. It was tested if continuously humid conditions or desiccation/rehydration cycles are more favourable for the growth of *Nostoc* and to what extent desiccation protects against contamination with foreign microorganisms. Five substrate types and three irrigation media were tested in triplicate for each treatment (Fig. 4). Glass Petri dishes with a diameter of 5 cm were filled with approximately 10 g of substrate. They were autoclaved at 121 °C for 30 min for sterilisation and dried overnight at 60 °C. The following substrate types were applied:

- Filter paper (F): white filter paper
- Glass beads (G): white 2 x 1.5 mm “glass seed beads” from “PandaHall Elíte”
- Granulated marble (M): white marble litter granules from “ideen mit herz” measuring 2 – 4 mm
- Quartz sand (Q): Min2C from "Natural Minerals" with a grain size of 0.5 – 2 mm
- Crushed stone (S): collected from a sandpit near Herzogenburg, Austria

These solid substrates were used to test whether they affect growth of *N. commune* or not. The filter paper served to verify if a granular substrate is required. Glass beads represented an artificial substrate with a granular structure. The substrate types marble, quartz sand, and crushed stone are substrates on which *Nostoc* has already been found in nature (Mollenhauer et al. 1999).



N. commune was irrigated with the media BG11, BG11₀, and rainwater. Rainwater was chosen because it is the natural water supply for *Nostoc*. Moreover, it has the advantage of being readily available and free of charge. Both the culture media BG11 and BG11₀ (without sodium nitrate) have already been used successfully for the cultivation of *Nostoc* (Feng et al. 2012, Roncero-Ramos et al. 2019a). The intention was to investigate whether the presence of this nutrient influences the terrestrial development of *N. commune*. This is of interest because of the ability of *Nostoc* to fix atmospheric nitrogen (Haselkorn 2006). BG11 and BG11₀ medium for cyanobacteria were made according to the medium recipe (version 05.2013) of the Culture Collection of Algae (SAG 2013). It is a modified protocol from Rippka and Herdman (1992). These two culture media were also sterilised at 121 °C for 30 min in an autoclave. To keep the rainwater untreated, it was not autoclaved in the first trial with the open Petri dishes. However, since contaminants were more likely to occur in the closed Petri dishes, the rainwater was sterilised for the second trial. The three media were filled into spray bottles to sprinkle *N. commune* regularly during the experiment.

2.2.1. Procedure of the experiment

In every Petri dish, ten pieces of dried *N. commune*, each with a mass between 0.1 mg to 0.5 mg were placed, resulting in a total weight of around 2.7 mg *Nostoc* in each Petri dish. To ensure that the *Nostoc* pieces adhered well to the substrate, they were soaked previously for a few seconds in their respective medium. The Petri dishes were placed on a table and illuminated from above with 50 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ photosynthetically active radiation from a white fluorescent light in a 16:8 light-darkness rhythm. The Petri dishes were rearranged regularly on the table to compensate for possible irradiation differences. The samples were cultivated at room temperature, as it did not deviate excessively from the optimal growth temperature for *N. commune* of about 25 °C (Tripathi et al. 2012, Møller et al. 2014).

The first experiment run was conducted for 49 days with open Petri dishes in which *N. commune* followed a periodic cycle of desiccation and rewetting. *Nostoc* was irrigated every two to three days with ten spray strokes from a spray bottle (one stroke equals 0.03 mL to 0.09 mL (median: 0.06 mL)).

For the second run, closed Petri dishes were used in which *Nostoc* remained permanently hydrated. *Nostoc* was sprayed less frequently because it did not lose moisture as quickly and should not be covered with medium. Due to the humid microclimate in the closed Petri dish, *Nostoc* did not dry out even though it was sprinkled with only one to two spray bottle strokes. The second trial lasted 50 days.

In both trials, triplicates were used for each treatment, leading to 45 Petri dishes with ten *Nostoc* pieces each to be investigated. This resulted in a total sample size of 450 per trial and a sample size of 30 samples per treatment.

2.3. Analytical methods

We used different methods to observe the development of *N. commune* and to detect the potential growth of individual colonies. Non-invasive digital imaging was carried out during the experiments to monitor any changes in size and to obtain general information about the condition of *Nostoc*. Individual samples were examined microscopically at the end of the experiments to gain insights into the appearance of their trichomes and sheath. Furthermore,

the dry mass of *Nostoc* was measured before and after the experiments to determine possible weight changes.

2.3.1. Growth monitoring via images

Every two to fourteen days, the Petri dishes were photographed to track changes in the size, orientation, and colour of the colonies. The Petri dishes were marked to keep them oriented in the same direction, and top shots were always taken from the same distance with the compact camera Lumix DMC-TZ71 (Panasonic, Hamburg, Germany).

In addition to the photos, pictures were taken with an Imaging Pulse-Amplitude-Modulation (PAM) fluorescence device to visualise the photosynthetically active areas of the *Nostoc* colonies by imaging their chlorophyll fluorescence. PAM fluorescence offers a non-destructive way to measure chlorophyll *a* fluorescence (Consalvey et al. 2005). Photosynthetic parameters can be derived from this, which can provide information about the physiological state of the species. Furthermore, Imaging-PAM fluorometry is suitable for monitoring the growth of phototrophic microorganisms (Stock et al. 2019). In this study, the IMAGING-PAM M-Series in MINI version (Heinz Walz GmbH, Effeltrich, Germany) was used to monitor the development and potential growth of *N. commune*. The following configurations and settings were applied: measuring head: IMAG-MIN/R (red), measuring light intensity: 9, measuring light frequency: 1, gain: 5, and damping: 4. The *F_o*, *F_m*, and the SAT-Pulse measurement were performed, and the image files were saved for later analysis. Before the measurements, the samples were mostly sprayed with medium (see chapter 2.2.1, where exceptions in the second experiment are described in order not to flood the closed Petri dishes) and pre-darkened for at least ten minutes.

2.3.2. Microscopy

At the end of the experiments, one out of the ten colonies of each Petri dish was examined under the microscope BA400 (Motic®, Barcelona, Spain). Pictures of each specimen were taken at exemplary positions using the microscope camera AxioCam ERc 5s (Carl Zeiss AG, Oberkochen, Germany). Attention was paid to whether the sample was contaminated with foreign organisms, and the general condition of the trichomes and cells was analysed.

2.3.3. Dry mass

Before the experiment, the dry mass of each *N. commune* piece was measured with an ultrafine scale. After the trials, mostly only nine of ten samples from a Petri dish could be weighed, as one was microscoped (see chapter 2.3.2). *Nostoc* air-dried for at least 21 days before measurement to achieve a constant dry mass.

2.4. Data analysis

2.4.1. Image analysis

Nostoc was hardly distinguishable from the background in the photos on the darker substrates (Q and S), so the PAM fluorescence images were taken to monitor growth. The fluorescent pictures were examined with the software ImageJ 1.53k (Schneider et al. 2012) to calculate the number of pixels of each piece. The pixel analysis was carried out with the following settings: Size (pixel²): 0-Infinity, Circularity: 0.00-1.00, Show: Nothing, ticked “Summarize” box. As each *Nostoc* piece was selected individually, the value in the “Total

Area” field could be used. The microscope images were interpreted descriptively and scale-wise.

2.4.2. Statistics

Statistical analysis was performed with the software R (version 3.6.3) using the package R Commander (version: 2.6-2) and the plugins KMggplot2 and EZR (Fox 2005, R Core Team 2020). As data were not normally distributed according to the histograms and the Shapiro-Wilk test, non-parametric tests were applied. A Mann-Whitney U test (U test) was used to test for differences in the central tendencies between two independent groups. Potential differences in the distributions of more than two unpaired groups were tested using the Kruskal-Wallis H test (H test). Wilcoxon’s signed-rank test (WSR test) was applied to compare two paired samples, and the Friedman test was used for multiple paired samples. Box-Whiskers-Plots in the style of Tukey (McGill et al. 1978) were chosen for the graphical representation, as they make the distribution of the values easily recognisable. The margins of the box represent the first (Q1) and the third quartile (Q3), and the whiskers represent values that lie within the interquartile range (IQR) * 1.25. All values outside this range are considered extreme values and are displayed as dots.

3. Results

Both the growth and the decline of *N. commune* biomass were observed during the study. The development of the colonies differed depending on the treatment (see chapter 3.2). Fig. 5 shows an example of proliferating colonies, and Fig. 6 shows an example where *N. commune* was not performing well. A comparison of the initial and final mass of these colonies confirmed this observation. As the development of *N. commune* was monitored with different methods, the comparability of their results is presented in chapter 3.1.

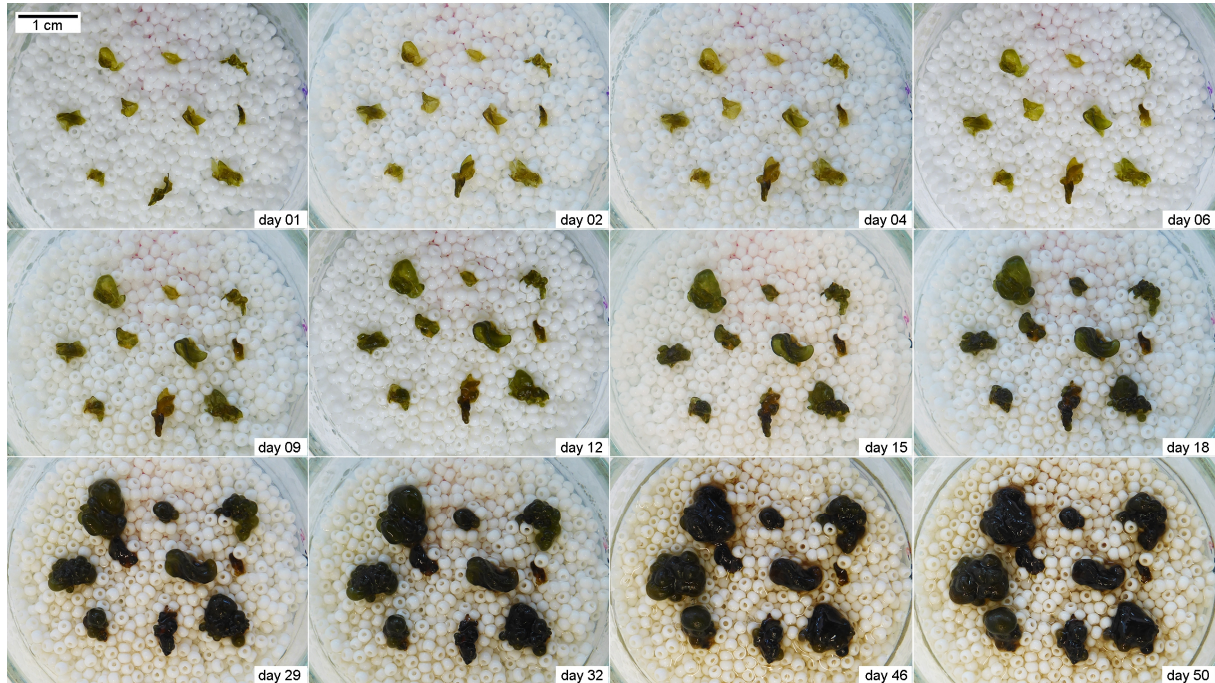


Fig. 5: Photos of a Petri dish filled with the substrate glass beads and irrigated with BG11₀ in the second trial using closed Petri dishes show a size increase of *N. commune* from day 1 to day 50.

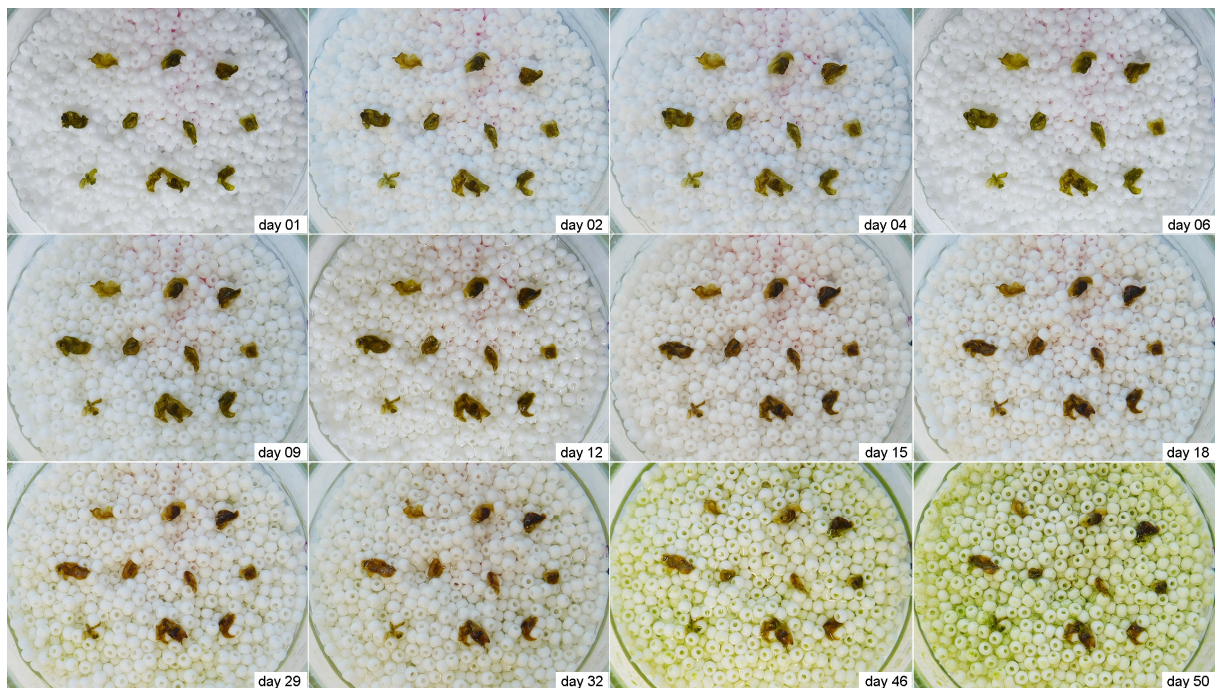


Fig. 6: Photos of a Petri dish filled with the substrate glass beads and irrigated with BG11 in the second trial using closed Petri dishes show almost no change in size of *N. commune* between day 1 and day 50.

3.1. Method evaluation

The methods used to determine the potential growth of the samples complemented each other. The camera photos documented changes in the size and colour of the individual *Nostoc* pieces. However, the photo monitoring was unsuitable for the dark substrates (see substrate types Q and S in Fig. 4), as *Nostoc* was hardly distinguishable from the substrate. Therefore, chlorophyll fluorescence of the colonies was visualised with Imaging-PAM fluorometry. This method additionally served to obtain information on the overall photosynthetic performance of *Nostoc* and provided alternative images to size active *Nostoc*. In these images, *Nostoc* was clearly visible even on the dark substrates. At the beginning of the experiment, the visualisation of the photosynthetic activity of *Nostoc* corresponded to the diameter and shape of the *Nostoc* pieces in the photos (Fig. 7).

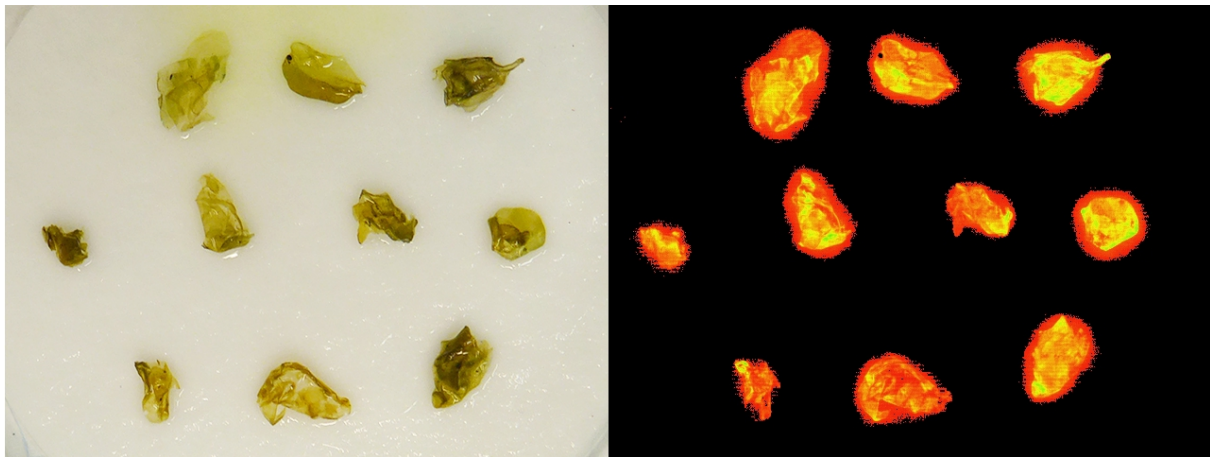


Fig. 7: Ten *N. commune* pieces on filter paper in a Petri dish on the first day of the experiment, taken with an ordinary compact camera (left) and with the Imaging-PAM fluorometer (right).

For these reasons, the PAM fluorescence images were used for determining the size of the colonies. The number of pixels of each *Nostoc* piece was calculated using a pixel-counting programme to quantify the data. The fluorescent pictures, just like the photos, were taken at two to fourteen-day intervals and were used to observe the development of the individual *N. commune* pieces during the experiment. A comparison of the quantitative pixel values derived from the fluorescence images with the dry mass shows that the results of these two methods correlate with each other (Fig. 8). Due to this relationship, the results of these methods appear to be comparable.

Repeating the first experiment with open Petri dishes in a second experiment with closed Petri dishes also demonstrated the reliability of the size determination using the calculated pixel size from the PAM fluorescence measurement. *N. commune* was, again, weighed in a range of 100 μg to 450 μg at the beginning of the second experiment. This, in turn, led to a comparable starting point of the calculated pixel size (Fig. 9). A Mann-Whitney U test confirmed that there were no significant differences between the initial values of the two experiments (initial dry mass $p = 0.966$, initial pixel number $p = 0.939$). This indicates that this method is reliable.

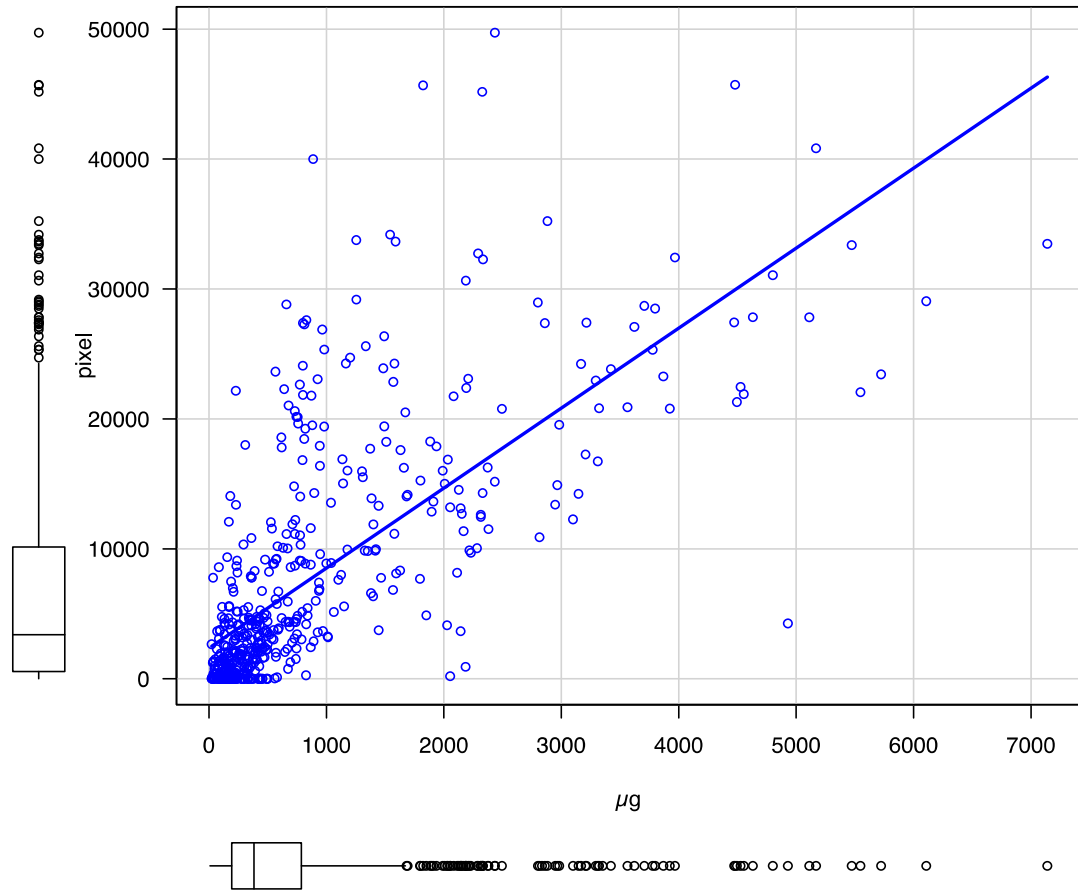


Fig. 8: The Scatterplot shows the correlation between the dry mass (μg) of *N. commune* after the experiments on the x-axis and the pixel amount calculated from the fluorescence measurement on the last day of the experiments on the y-axis (Kendall's tau = 0.551, p -value < 0.001). The blue line represents the line of the least squares.

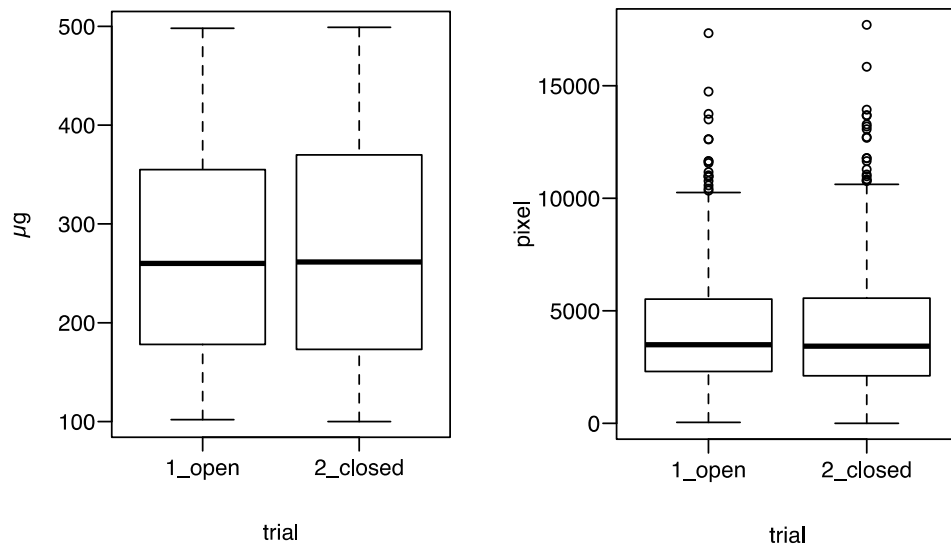


Fig. 9: On the left side, the similar initial dry mass (μg) of the *Nostoc* colonies at the beginning of the trials is shown. This is reflected in the boxplots on the right side in a similar number of initial pixels.

In addition to the previously described methods, individual colonies were observed under the microscope at the end of the experiments. The condition of the cells within the colonies was assessed and the presence of potential contaminants was verified. The microscopic

examination corresponded to the data obtained from the PAM fluorescence measurement. For example, Fig. 10 shows two samples where the number of pixels has increased (indicated by the numbers 1 and 2) and two samples where the number of pixels has decreased due to declining or absent chlorophyll activity (indicated by 3 and 4). The sample labelled with the number 2 was taken from the Petri dish shown in Fig. 5, which demonstrates growing colonies. The sample labelled 4 is from a piece from the Petri dish in Fig. 6, which contains pieces that do not grow. On the 46th and 50th day, a greenish discolouration of the glass beads can be seen in Fig. 6, indicating emerging contaminants. This assumption can be verified with the help of the microscopic image 4b shown in Fig. 10. The microscopic examination and a physical observation support the data generated by the PAM fluorescence and the weight measurements.

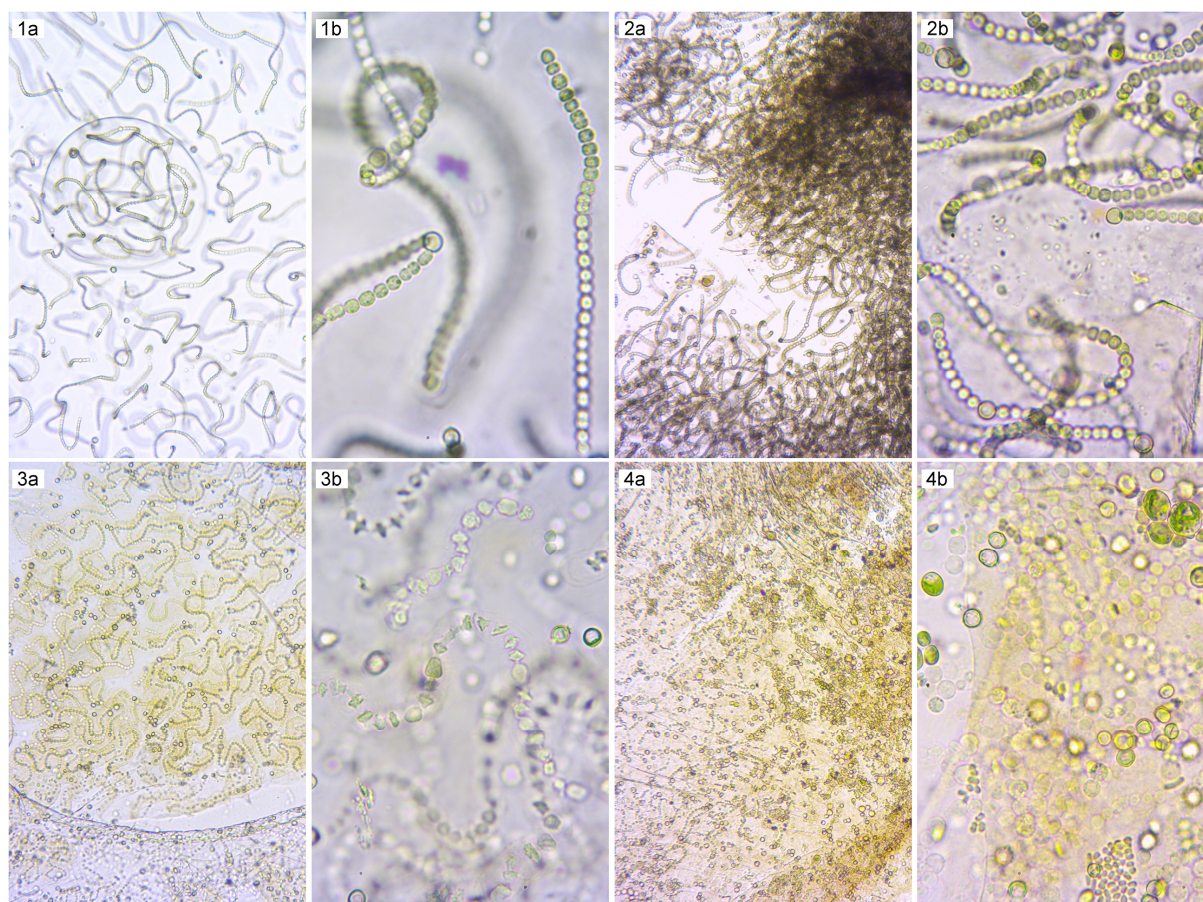


Fig. 10: Microscope images of *N. commune* (a = 10x magnification, b = 40x magnification). The upper row shows samples with intact trichomes and healthy-looking cells (1, 2). In contrast, the lower row shows samples that did not perform well in the PAM fluorescence measurement and whose cells look damaged (3, 4). Picture 4b, moreover, reveals foreign organisms.

Overall, the microscopic and macroscopic observations captured on photographs support the data generated by the fluorescence measurement and the weight determination.

3.2. Influencing factors

The development of the *N. commune* colonies depended on the treatment. To illustrate this, the 900 colonies are presented from different perspectives in the following sub-chapters. In the next three sub-chapters, the main influencing factors of this study (desiccation, substrate type, and irrigation medium) are analysed separately, followed by a breakdown of the combinations of influencing factors in the final sub-chapter.

3.2.1. Desiccation

To investigate the influence of regular desiccation, the run with open Petri dishes ($n = 450$) was compared to the other with closed Petri dishes ($n = 450$). In the open Petri dishes, *N. commune* dried out regularly, while it remained moist in the closed ones. This treatment showed a significant impact on the development of *N. commune*. Colonies developed faster in the closed Petri dishes and had a higher area increase at the end of the trials (Fig. 11). The figure represents an example of growing *Nostoc* colonies. However, depending on other influencing factors (see chapter 3.2.2 and chapter 3.2.3), also non-growing colonies were observed in both setups.

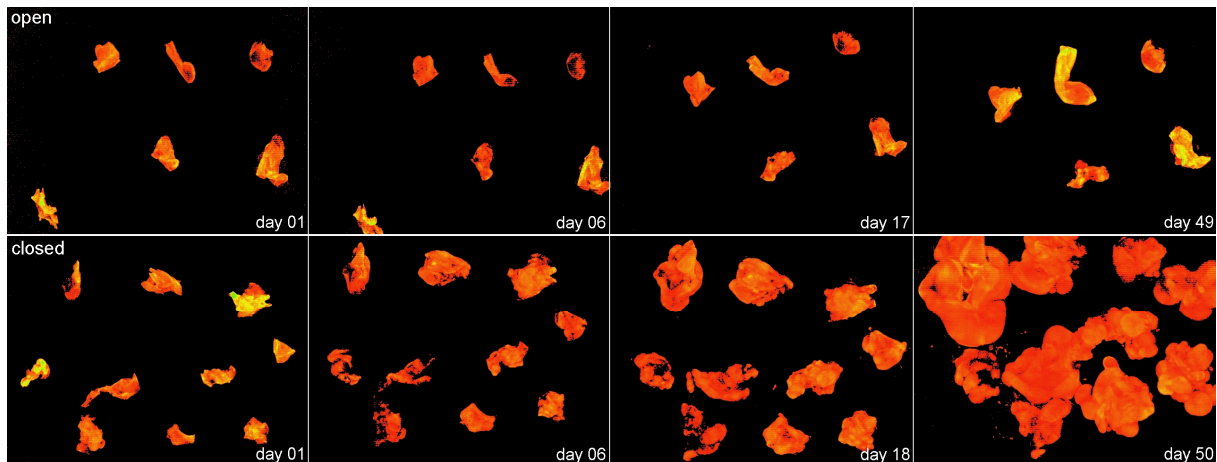


Fig. 11: The PAM fluorescence images in the top row show the development of single *N. commune* colonies in an open Petri dish filled with quartz sand and irrigated with rainwater every two to three days. From day 1 (left) to day 49 (right), the chlorophyll fluorescence and, thus, the area of some colonies increased. The bottom row shows *N. commune* in a closed Petri dish, which was also cultivated on quartz sand and irrigated with rainwater. The increase in the area of all colonies from day 1 (left) to day 50 (right) is considerably higher than in the row above.

The example shown in Fig. 11 is supported by the pixel count results (Fig. 12). On day one (top left; $n = 841$), the boxplots of the two trials look similar, indicating the same initial conditions (U test, day 1 $p = 0.939$). On day six (top right; $n = 878$), the median of the second trial is already higher than the median of the first trial and the range of the data starts to expand in the closed Petri dishes. The Mann-Whitney U test confirms the presence of significant differences between the two trials on day 6 ($p < 0.001$). These observations are even more pronounced when comparing day 17 of the first trial with day 18 of the second trial (bottom left; $n = 867$). The median is higher in the second trial with the closed Petri dishes than in the first trial with the open ones, and the second trial has a broader range (U test, day 17/day 18 $p < 0.001$). Comparing the open Petri dishes on day 49 with the closed Petri dishes on day 50 (bottom right; $n = 576$) affirms a higher pixel count in the second trial. Again, the median and the maximum value are higher in the second trial than in the first trial (U test, day 49/day 50 $p < 0.001$). Day 49 from trial one and day 50 from trial two should only be used to compare the two trials with each other, but should not be compared with the other days, as their sample size (n) is lower due to the absence of an irrigation treatment (see chapter 3.2.3 for an explanation).

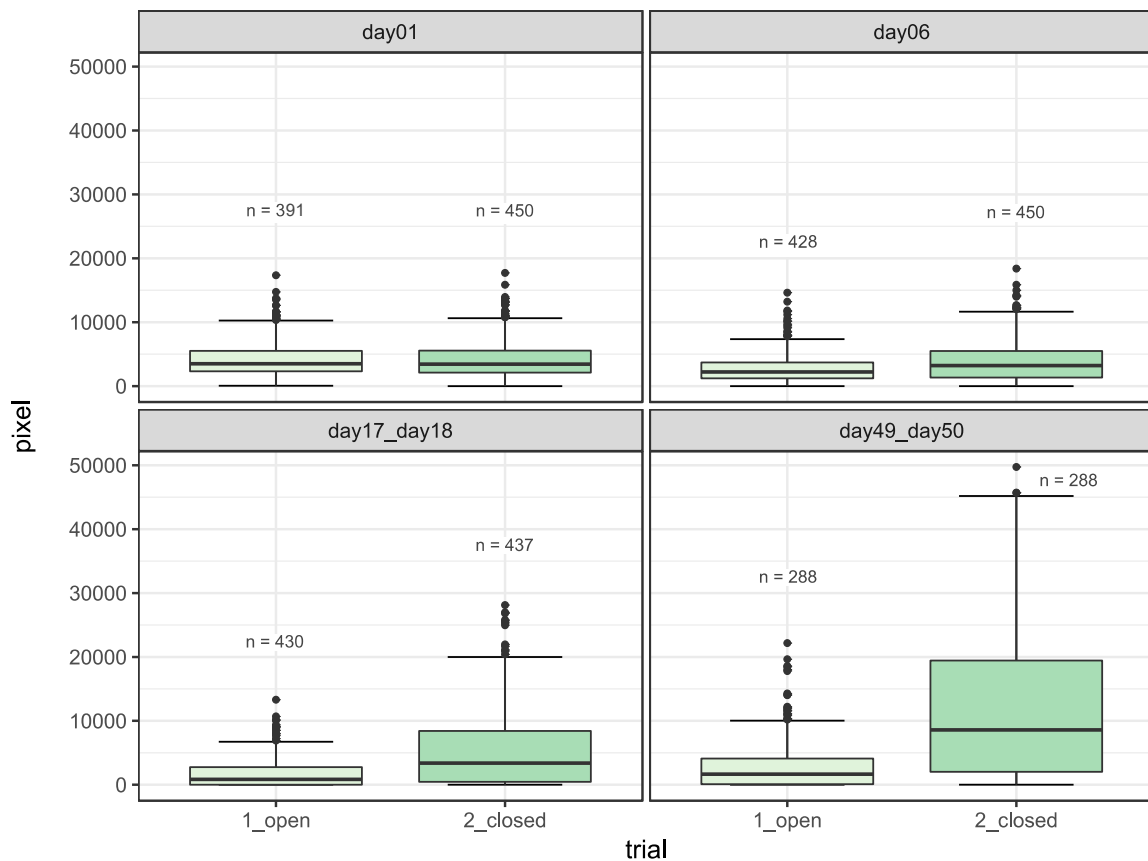


Fig. 12: The boxplots show a comparison of the two trials (1 = open Petri dishes with regular desiccation, 2 = closed Petri dishes without desiccation) on different days of the experiment. The y-axis displays the pixel size of *N. commune*, which was calculated from the PAM fluorescence measurement.

A comparison of day one with day six shows a decreasing median in the open Petri dishes (WSR test, $p < 0.001$) and an almost unchanged median in the closed Petri dishes (WSR test, $p = 0.157$). From the sixth to the seventeenth day, the median and the maximum value in the open Petri dishes decrease even more, and the range becomes smaller. Wilcoxon's signed-rank test demonstrates a significant difference in the distributions of the open Petri dishes between day six and day seventeen ($p < 0.001$). In contrast, the median, the maximum value, and the range in the closed Petri dishes increase from day six to day eighteen (WSR test $p < 0.001$). In summary, there is a decrease in the number of pixels from day one to day 17 in the open Petri dishes (WSR test, $p < 0.001$) and an increase from day one to day 18 in the closed Petri dishes (WSR test, $p < 0.001$).

Comparing *Nostoc*'s final dry mass of the two trials with each other, the medians look similar (Fig. 13). However, with the Mann-Whitney U test, a difference in the distribution between the trials is detected ($p = 0.027$). In agreement with the PAM fluorescence data, the range in the second trial is much broader. In these permanently humid Petri dishes, the third quartile and the maximum value were noticeably higher than in the regularly desiccated open Petri dishes. This was not the case at the beginning of the trials (see the similar boxplots in Fig. 9 on the left side with a p -value of 0.939). So there is a difference in the final dry mass between the first trial with open Petri dishes and the second trial with closed Petri dishes. A Wilcoxon's signed-rank test verifies a significantly different distribution between the initial and the final dry mass ($p < 0.001$).

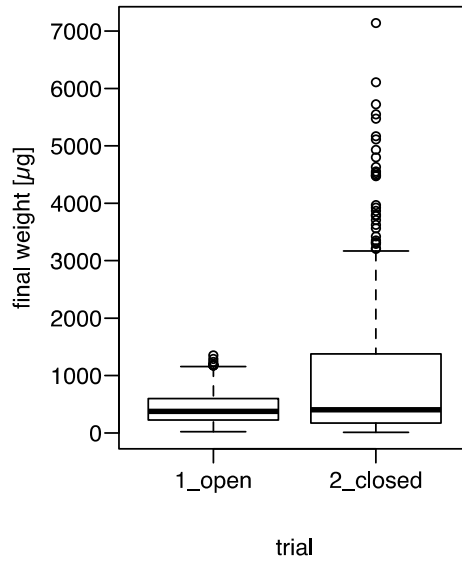


Fig. 13: The boxplots illustrate the distribution of the weight data after the two trials (1 = open Petri dishes with regular desiccation, n = 400; 2 = closed Petri dishes without desiccation, n = 424). The final weight [μg] is shown on the y-axis.

Contamination with foreign organisms was lower in the regularly desiccated, open Petri dishes than in the humid, closed ones. Indications were already found in the macroscopic observation of the Petri dishes and in some fluorescence images. The microscope images support this observation. No foreign organism was found in the majority of the microscopically examined *N. commune* pieces from the open Petri dishes, whereas foreign organisms were detected more frequently and in higher numbers in the microscope slides from the closed Petri dishes (Table 1). In the second trial with the closed Petri dishes, nine samples were overgrown with foreign organisms, whereas this was the case in only one sample in the first trial.

Table 1: Presence of foreign organisms in the microscopically examined samples of the first (1_open) and the second trial (2_closed).

Contamination with foreign organisms	1_open	2_closed
No	25	14
Yes	20	31
One or two species, ≤ 3 specimen	15	8
One or two species, > 3 specimen	3	7
> 2 species, not dominant	1	7
> 2 species, predominant	1	9

The microscopic examination further revealed that the trichomes of *N. commune* from the open Petri dishes often appeared longer and thinner, and more cells looked desiccated. The length of *Nostoc*'s trichomes from closed Petri dishes seemed to be more diverse, and the cells appeared fuller. However, the condition of the cells also depended on the other factors.

3.2.2. Substrate type

N. commune did not grow on filter paper in both experimental setups. Colonies cultivated on the substrate types glass beads (n = 180), marble (n = 180), quartz sand (n = 180), and

crushed stone (n = 180) differed from specimens cultivated on filter paper (n = 180) in that they grew at least partially, depending on the other influencing factors.

The *Nostoc* pieces were difficult to distinguish from the darker substrates, which is why the Imaging-PAM fluorometer was used for visualisation and area determination. However, *Nostoc* growing on the brighter substrate types filter paper, glass beads, and marble emitted more strongly (Fig. 14, Table 2).

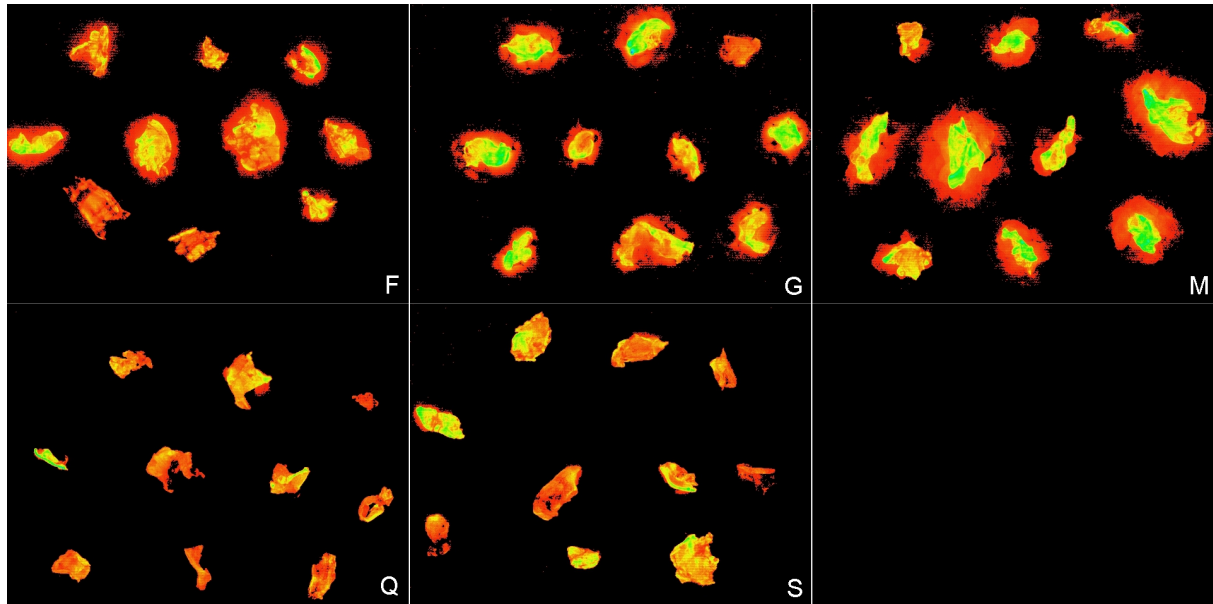


Fig. 14: Imaging-PAM fluorometry pictures from the first day. In the upper row, the brighter substrate types filter paper (F), glass beads (G), and marble (M) are shown, where *N. commune* has a higher light scattering. The lower row shows *N. commune* on the darker substrates quartz sand (Q) and crushed stone (S).

Table 2: Pixel count from PAM fluorescence measurement on the first day. *N. commune* growing on the bright substrate types filter paper (F), glass beads (G), and marble (M) have a noticeably higher median and maximum value than *N. commune* growing on the dark substrate types quartz sand (Q) and crushed stone (S).

Substrate	Mean $\pm$ SD	Minimum	Q1	Median	Q3	Maximum	n
F	5016 $\pm$ 2757	814	3059	4420	6438	12688	166
G	6022 $\pm$ 3202	44	3646	5259	8072	17705	174
M	5313 $\pm$ 3103	0	3029	4571	6870	17336	167
Q	2286 $\pm$ 1046	208	1487	2122	2922	5564	174
S	2581 $\pm$ 1201	482	1661	2414	3296	7237	160

To obtain comparable values, all pixel counts were divided by their respective initial pixel counts. The resulting normalised values were used for further calculations and graphical representation (Fig. 15).

Therefore, in Fig. 15 on the first day, the initial value for all substrate types is 1. On day six, the median of the normalised PAM fluorescence data is between 0.82 and 0.88 for all substrate types except filter paper (F), while for filter paper it is lower at about 0.47. A Kruskal-Wallis *H* test confirms significant differences in the data distribution between the substrate types ($p < 0.001$). The subsequent pairwise comparison according to Bonferroni

reveals that the substrate types glass beads (G), marble (M), quartz sand (Q), and crushed stone (S) all differ from filter paper ($p < 0.001$), but not from each other ($p = 1$).

On day 17 and day 18, respectively, the median of *N. commune* growing on filter paper drops to 0.04. The median of the substrate types glass beads and marble remains quite stable at 0.85 and 0.83. For quartz sand it decreases to 0.74, and for crushed stone to 0.62. The *H* test again finds significant differences between the substrate types ($p < 0.001$). The substrate types G, M, and Q differ highly significantly from the substrate F (pairwise comparison according to Bonferroni, $p < 0.001$), but not from each other ($p = 1$). The data distribution of substrate type S differs from that of F with a *p*-value of 0.002.

On the last day of the experiments (day 49/day 50), it becomes apparent that there was no increase in the area of *N. commune* with the substrate type F. The median value is 0, and the distribution of the data is clearly below that of the other substrate types (*H* test and subsequent pairwise comparison according to Bonferroni, $p < 0.001$). No significant differences were found between the other substrate types. In Fig. 15, as before in Fig. 12, it should be noted that the sample size (*n*) on day 49/50 is lower than on the days six and 17/18 due to the absence of an irrigation medium and, therefore, should not be compared with them.

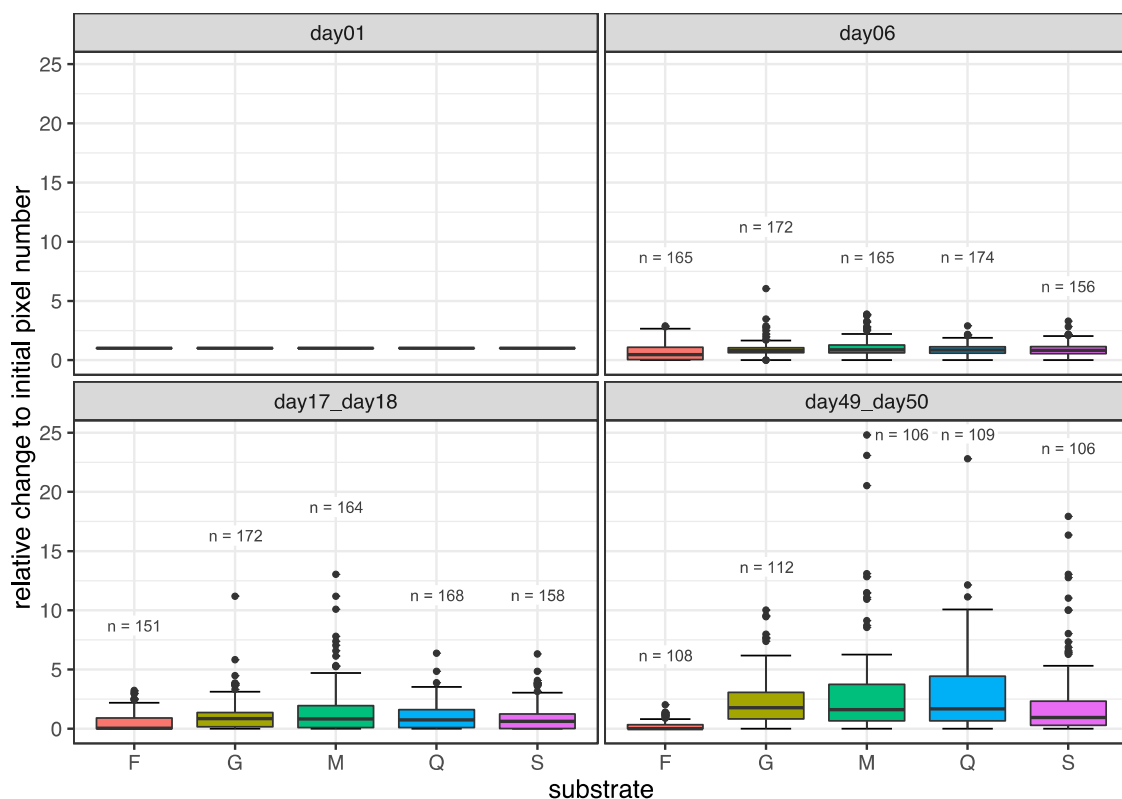


Fig. 15: Normalised pixel count of *N. commune* separated by substrate types on different measuring days.

In general, the expansion of *N. commune* cultivated on filter paper steadily decreased. The chlorophyll fluorescence became continuously weaker and mostly disappeared completely. This was not so clear for the other substrate types. Depending on the other influencing factors, there was an increase in area for some samples and a decrease for others (see chapter 3.2.4).

In accordance with the PAM fluorescence data, there is also a difference between the substrate filter paper and the other substrates regarding the final weight (Fig. 16). The median of F (198 μg) is lower than that of G (526 μg), M (548 μg), Q (493 μg), and S (322 μg). With the H test, the presence of differences between the substrate types can again be confirmed ($p < 0.001$). A pairwise comparison according to Bonferroni shows significant differences in the data distribution of the substrate F compared to the other four substrates ($p < 0.001$). No differences were found between substrate types G, M, and Q ($p = 1$). The distribution of weight data differs between substrate type S and G ($p = 0.004$), S and M ($p < 0.001$), but not between S and Q ($p = 0.06$). All substrate types show a significant change in their final mass compared to their initial mass (WSR test, F: $p = 0.017$; G, M, Q, and S: $p < 0.001$).

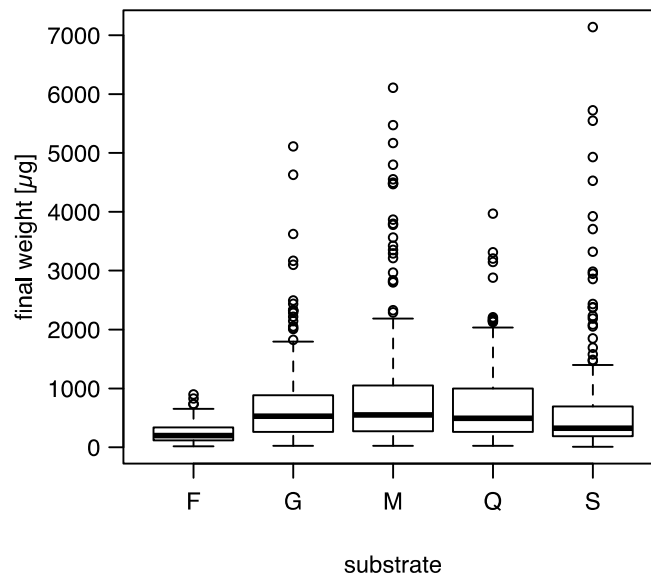


Fig. 16: Weight [μg] of *N. commune* at the end of the experiment depending on the substrate type.

Macroscopically, *N. commune* growing on filter paper differed from *N. commune* on the other substrates by remaining relatively flat. In most cases, *Nostoc* turned brownish on filter paper.

Table 3: Presence of dissimilar organisms in the microscopic samples separated by substrate type.

Foreign organisms	F	G	M	Q	S
No	4	9	8	11	7
Yes	14	9	10	7	11

In the microscopic examination, short cell chains and sheaths surrounding the cells were most evident in *Nostoc* cultivated on filter paper. In all samples, damaged cells were occasionally found, but the filter paper samples had the highest proportion of damaged cells. Foreign organisms were most frequently found in samples grown on filter paper, and the fewest foreign organisms were present in samples grown on quartz sand (Table 3).

3.2.3. Irrigation medium

The type of irrigation medium played a significant role in the colony development. After irrigation with BG11₀ ($n = 300$) and rainwater ($n = 300$), respectively, *N. commune* grew well

in certain Petri dishes, depending on the other influencing factors. In contrast, *N. commune* irrigated with full BG11 medium (n = 300) did not grow (see Fig. 6 and the top row of Fig. 19). Similar to the substrate filter paper, chlorophyll fluorescence was dropping to zero during irrigation with BG11: In the first approach with open Petri dishes, the chlorophyll fluorescence decreased continuously until no chlorophyll fluorescence was detected after day 17 indicating death of colonies (therefore this approach was stopped here). In the second trial with the closed Petri dishes, the chlorophyll fluorescence in samples irrigated with BG11 also decreased continuously. However, a few samples with low residual fluorescence were detected on day 18, therefore this survey was continued. Nevertheless, the data from the subsequent measurement days could not be used mostly, as contaminants took over in the Petri dishes and overgrew *Nostoc*. In these cases, it was not possible to distinguish *N. commune* from the foreign organisms with the PAM fluorescence images. For these reasons, fluorescence data from *Nostoc* irrigated with BG11 are only available up to day 17 and day 18, respectively (Fig. 17).

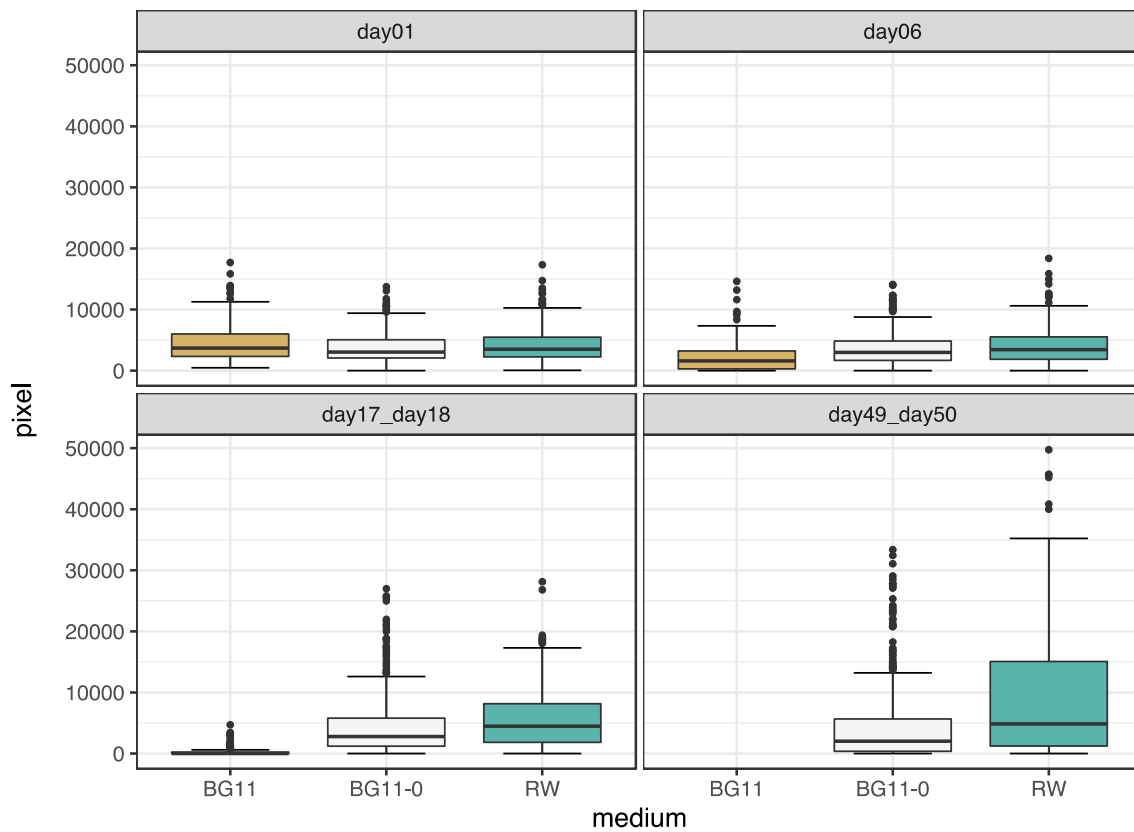


Fig. 17: Comparison of the pixel size of *Nostoc* calculated from the PAM fluorescence measurement depending on the irrigation medium on four measurement days.

The boxplots on day one in Fig. 17 look similar, but an *H* test reveals differences in the distribution of the pixel values between the three groups at the beginning of the experiment ($p = 0.02$). For this reason, the normalised values were used for the statistical calculations, as previously in the differentiation of the substrate types. All values were divided by their initial pixel count (see Table 4 and Fig. 25 in the appendix for the normalised plot). On the sixth day, the pixel number of *Nostoc* irrigated with BG11 already decreases and is below that of the other two irrigation media. The normalised distribution of the values differs significantly between the irrigation media on day six (*H* test, $p < 0.001$). The difference is in *Nostoc* cultivated with BG11 (subsequent pairwise comparison according to Bonferroni with

U test, $p < 0.001$). There are no significant differences in the data distribution between the irrigation media BG11₀ and rainwater (subsequent U test, $p = 0.44$). On day 17, resp. 18, the differences between the irrigation media are more obvious (H test, $p < 0.001$). The pixel area of *Nostoc* irrigated with BG11₀ and rainwater differs significantly from *Nostoc* irrigated with BG11 (subsequent U test, $p < 0.001$). The BG11₀ samples now as well differ from the rainwater samples (subsequent U test, $p = 0.011$). On day 49, resp. 50, the median of the normalised pixel area of *N. commune* in BG11₀-irrigated samples is lower than in rainwater-irrigated samples, and there is a significant difference between the samples (U test, $p < 0.001$).

Table 4: Normalised pixel area of the *N. commune* colonies calculated from the PAM fluorescence images.

Day	Medium	Mean $\pm$ SD	Minimum	Q1	Median	Q3	Maximum	n
1	BG11	1 $\pm$ 0	1	1	1	1	1	278
	BG11 ₀	1 $\pm$ 0	1	1	1	1	1	283
	RW	1 $\pm$ 0	1	1	1	1	1	279
6	BG11	0.5 $\pm$ 0.5	0	0.1	0.5	0.8	3.5	274
	BG11 ₀	1 $\pm$ 0.7	0	0.7	0.9	1.2	6	281
	RW	1.1 $\pm$ 0.6	0	0.7	1	1.4	3.9	277
17/18	BG11	0.1 $\pm$ 0.1	0	0	0	0.1	1.1	269
	BG11 ₀	1.4 $\pm$ 1.7	0	0.6	0.9	1.7	13	274
	RW	1.5 $\pm$ 1.3	0	0.8	1.2	2.2	7.8	270
49/50	BG11 ₀	1.7 $\pm$ 3.1	0	0.2	0.6	1.9	24.8	268
	RW	2.8 $\pm$ 3.5	0	0.7	1.6	3.5	22.8	273

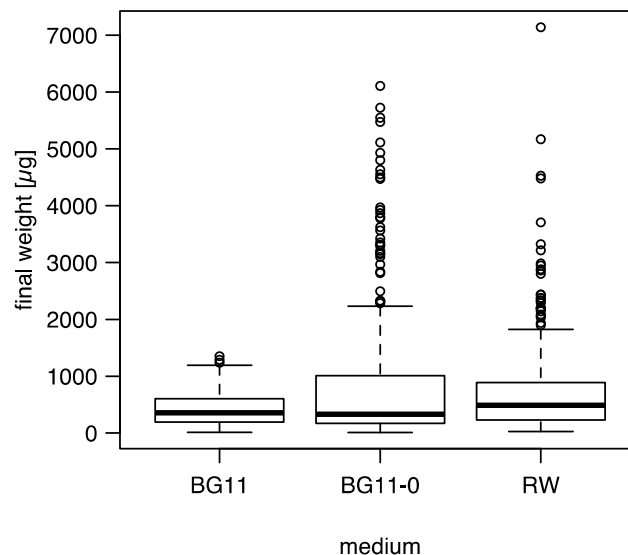


Fig. 18: Comparison of the final dry mass [µg] of *N. commune* according to the irrigation medium.

A comparison of the final dry mass (Fig. 18) again shows significant differences between the irrigation media (H test, $p < 0.001$). Looking closer, the following pairwise comparison with a U test identified different central tendencies between BG11 and rainwater irrigated samples ($p < 0.001$), but not for the groups BG11 and BG11₀ ($p = 0.91$) as well as BG11₀ and

rainwater ($p = 0.1$). It is worth to mention that the weight data of BG11 irrigated samples presented here refer to different final times of the experiments. As previously described, in the first run with open Petri dishes, *Nostoc* was no longer irrigated after day 17, but in the second run with closed Petri dishes, it was irrigated until the end of the experiment on day 50. The premature stopping of BG11 irrigation in the first trial was not optimal and led to biased weight data (compare chapter 3.2.4).

The weight measurement at the end of the experiments was also influenced by the fact that colonies adhered differently to the substrate depending on the irrigation medium. *Nostoc* irrigated with BG11₀ stuck firmly to the substrate, and the dried pieces were often porous. This meant that the pieces often disintegrated and small remnants sometimes could not be detached from the substrate leading to an underestimation of the biomass yield. Samples sprinkled with BG11 and rainwater, on the other hand, were usually easier to remove from the substrate. Especially the rainwater samples were elastic and mostly remained in one piece. *Nostoc* pieces that were irrigated with BG11 crumbled more often.

The differently irrigated samples showed differences in colour, especially in the second trial (Fig. 19). Samples sprinkled with BG11 changed their colour from green to pale brownish. *Nostoc* supplied with BG11₀ became steadily darker green and formed distinct three-dimensional structures. *Nostoc* cultivated with rainwater, on the other hand, became lighter green and more translucent.

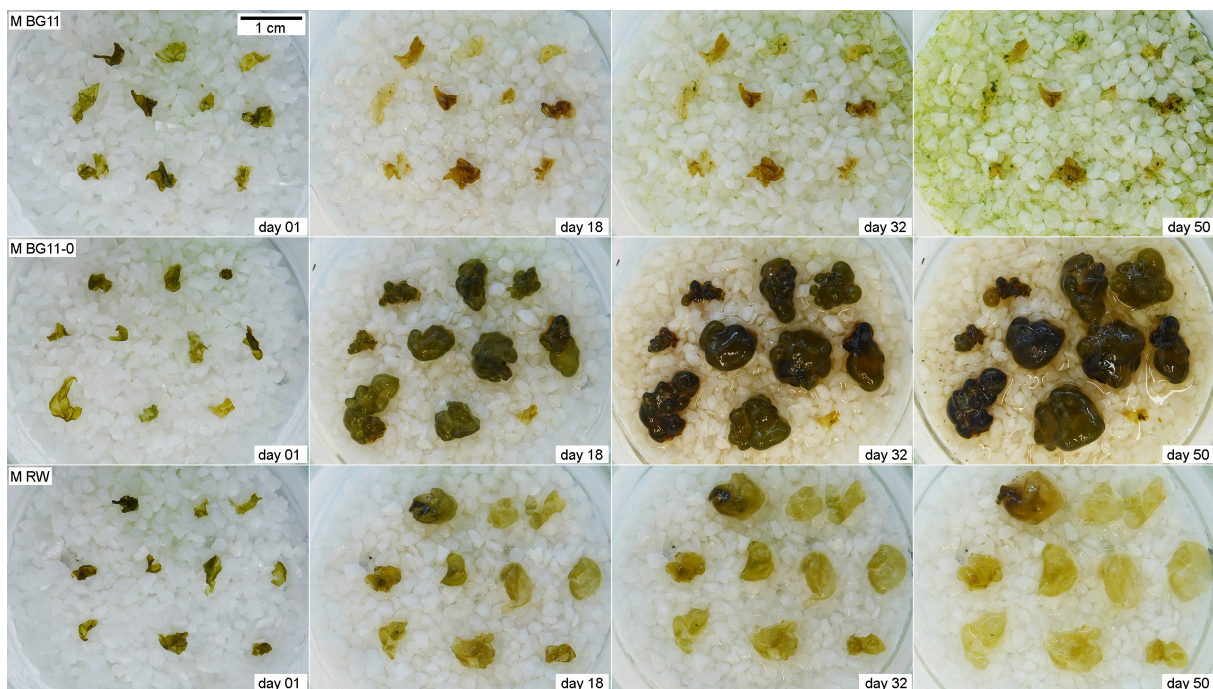


Fig. 19: Change in appearance of *N. commune* cultivated on marble in closed Petri dishes depending on irrigation medium.

In the top row of Fig. 19, a greenish discolouration of the marble is clearly visible on day 32 indicating the presence of alien organisms in the BG11-irrigated Petri dish. Additionally, dark green coloured dots appear on the *Nostoc* pieces. On day 50, this discolouration is even more prominent. This example shows that in closed Petri dishes sprinkled with BG11, contamination with foreign organisms increased dramatically in the progress of the experiment and demonstrates why the fluorescence images could no longer be used for pixel counts.

The microscopic analysis confirmed the presence of a large number of foreign organisms in samples that were irrigated with BG11 (Table 5). These were, for example, other cyanobacteria, green algae, or unspecified bacteria. In particular, overgrown samples in which more than two foreign taxa were present, were predominantly found in samples cultured with BG11. Foreign organisms were also frequently found in samples irrigated with BG11₀, but not in such large numbers, and they were generally not dominant. The fewest alien species were found in *Nostoc* samples sprinkled with rainwater.

Table 5: Presence of alien species in the microscopic examination of selected *Nostoc* specimens.

Contamination with foreign organisms	BG11	BG11 ₀	RW
No	9	11	19
Yes	21	19	11
One or two species, ≤ 3 specimen	8	8	7
One or two species, > 3 specimen	1	6	3
> 2 species, not dominant	3	4	1
> 2 species, predominant	9	1	0

During microscopic observation, most of the damaged cells were found in BG11-irrigated samples and their trichomes often had orange sheaths around their cells. In some cases, the trichomes were no longer recognisable, and an orange-to-brownish opaque mass dominated. Occasionally this was also seen in BG11₀ sprinkled *Nostoc* samples, while it was rare in the rainwater sprinkled samples.

3.2.4. Breakdown of the combinations

The previous sub-chapters proved that the colony development of *N. commune* is influenced by desiccation (see chapter 3.2.1), substrate type (see chapter 3.2.2), and type of medium provided (see chapter 3.2.3). These growth factors, however, had different effects on *N. commune* depending on their combination. We, therefore, present the sample types (Fig. 4) separately in the current chapter, which enable to identify the factor combinations with significant positive or negative effects on the colony development. Since the two setups (open and closed Petri dishes) represent two independent experiments, the results are visualised in separate graphs (Fig. 20, Fig. 21, Fig. 23, and Fig. 24). The two trials show similar trends, such as the negative influence of the substrate type filter paper and the irrigation medium BG11, but differ in their growth rates and the influence of the irrigation medium BG11₀.

The first two figures show the results of the first trial, in which *N. commune* followed a desiccation-rehydration cycle. Fig. 20 illustrates the results of the PAM fluorescence measurement from the first trial on different measurement days.

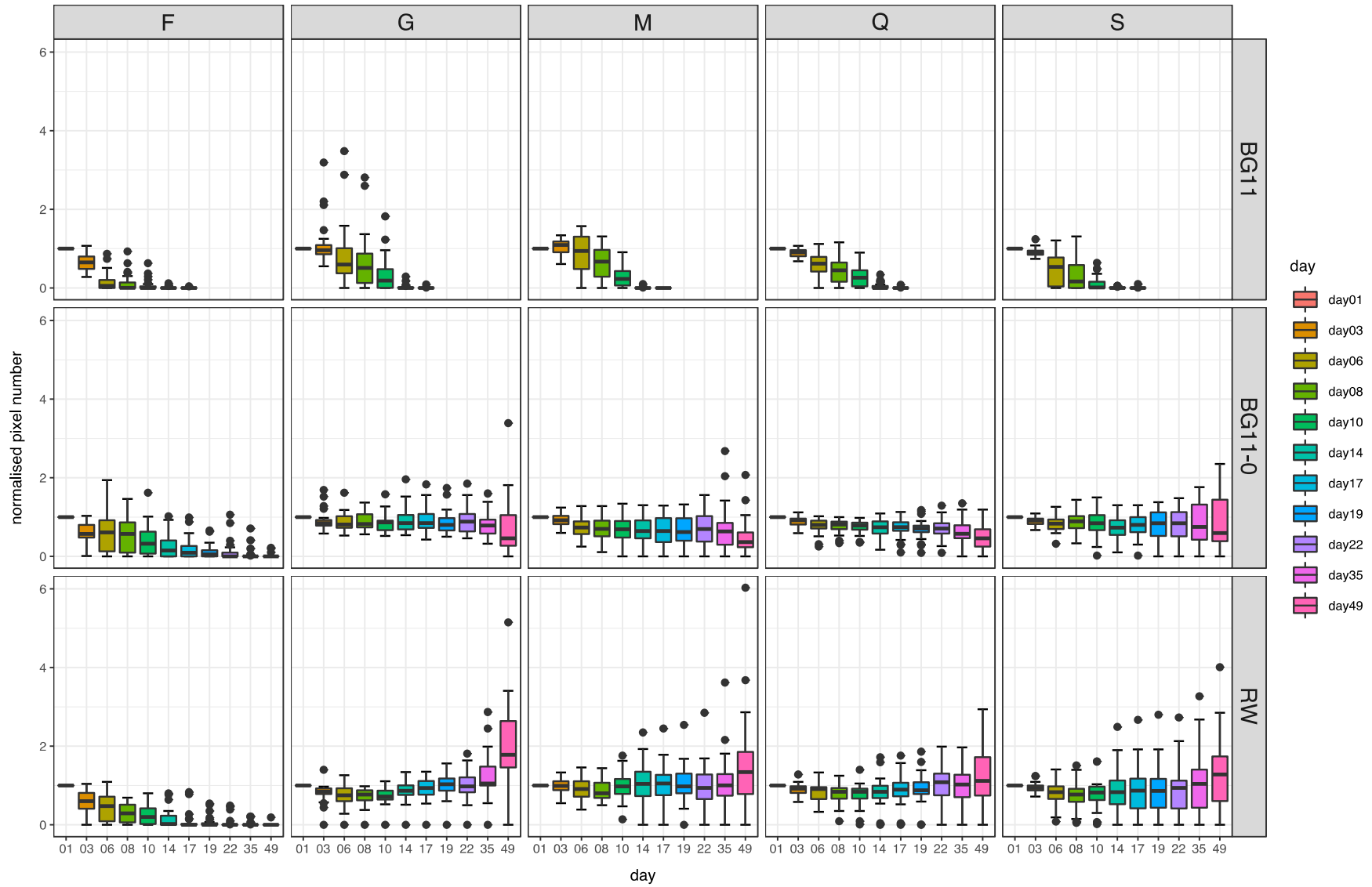


Fig. 20: The normalised pixel numbers (y-axis) from different measurement days (x-axis) were calculated from the PAM fluorescence images of the first trial with open Petri dishes. The graph represents each sample type in a single box, and the information in the grey boxes shows the combination of influencing factors forming the sample type.

In line with the results presented before, the breakdown shows that *Nostoc* irrigated with BG11 stopped its chlorophyll fluorescence until day 17 (WSR test, $p < 0.001$), regardless of the substrate type provided (pairwise comparison with U test, $p = 1$). *Nostoc* cultivated on filter paper also lost its chlorophyll fluorescence in the course of the experiment (WSR test, $p < 0.001$). The fluorescence decreased faster when *Nostoc* grown on filter paper was irrigated with BG11, and the decrease lasted the longest with BG11₀ irrigation. BG11₀ irrigation was nevertheless inferior to rainwater irrigation in the first experiment run. Looking at the median of *Nostoc* grown on the other substrates, the chlorophyll fluorescence increases towards the end of the experiment with rainwater irrigation, while it decreases with BG11₀ irrigation (Fig. 20, Table 6). The cultivation of *N. commune* in open Petri dishes achieved the highest median with the substrate type glass beads combined with rainwater irrigation. This sample differs significantly from the samples irrigated with BG11₀ and from the samples on the substrate filter paper, but not from the samples on the other substrate types that were irrigated with rainwater (see Table 8 in the appendix for pairwise comparisons). The highest maximum value, with a six-fold increase in area, was found with the combination of the substrate type marble and the irrigation medium rainwater. In the samples irrigated with rainwater, the final pixel area of *Nostoc* cultivated on the substrate types filter paper, glass beads, and marble differed significantly from their initial pixel area (see p -value in Table 6). In BG11₀ irrigated samples, there was a significant difference in all samples except those grown on crushed stone.

Table 6: Final fluorescence values (normalised by their respective initial pixel counts) and final dry mass from the samples cultivated in open Petri dishes.

Sample (1_open)	Final fluorescence (normalised pixels)					Final dry mass (µg)				
	Min	Median	Max	n	p (WSR)*	Min	Median	Max	n	p (WSR)**
F_BG11	0	0	0	24	< 0.001	193	436	897	27	< 0.001
G_BG11	0	0	0.1	30	< 0.001	261	583	1238	27	< 0.001
M_BG11	0	0	0	23	< 0.001	217	751	1352	27	< 0.001
Q_BG11	0	0	0.1	28	< 0.001	263	679	1175	27	< 0.001
S_BG11	0	0	0.1	23	< 0.001	123	369	1187	26	< 0.001
F_BG11 ₀	0	0	0.2	27	< 0.001	119	243	488	27	0.02
G_BG11 ₀	0	0.5	3.4	27	0.023	26	200	711	27	0.22
M_BG11 ₀	0	0.4	2.1	26	< 0.001	67	238	820	27	0.683
Q_BG11 ₀	0	0.5	1.2	27	< 0.001	38	270.5	788	26	0.839
S_BG11 ₀	0	0.6	2.4	24	0.338	22	272	744	25	0.914
F_RW	0	0	0.2	25	< 0.001	112	256.5	560	28	0.616
G_RW	0	1.8	5.2	25	< 0.001	171	419	811	27	< 0.001
M_RW	0	1.3	6	27	0.032	138	545	941	27	< 0.001
Q_RW	0	1.1	2.9	23	0.186	78	434	749	26	0.002
S_RW	0	1.3	4	23	0.438	27	354.5	891	26	0.014

*WSR test compared paired values from day 01 with day 17 (BG11 samples) or day 49 (BG11₀ and RW samples); **WSR test compared the initial with the final weight

PAM fluorescence and the dry mass result do not match well in the open Petri dishes setup. *N. commune* from open Petri dishes sprinkled with BG11 shows the highest dry mass increase (Fig. 21, Table 6), although chlorophyll fluorescence continuously decreased and eventually stopped. It is important to consider that the BG11 irrigation was terminated after

day 17 when all chlorophyll fluorescence had disappeared. The weight measurement, therefore, refers to an earlier day than with the other two irrigation media with which *Nostoc* was irrigated until day 49. After removing all *Nostoc* pieces from the filter paper for weighing, the empty filters were weighed, and it was found that the filters that had been sprinkled with BG11 were heavier than the others (see Fig. 26 in the appendix). This media-dependent difference in the final weight of the filter papers existed in both trials, and we assume that salt precipitation was responsible for the dry mass increase. More consistent with the pixel analysis, the average dry mass of *Nostoc* irrigated with BG11₀ decreased or remained approximately the same in the first trial (Fig. 21, Table 6). The dry mass of *Nostoc* irrigated with rainwater tended to increase in all substrate types except for filter paper, but a significant difference was confirmed only between the substrate types marble and filter paper (see pairwise comparisons in Table 9 in the appendix). The final dry mass of *Nostoc* irrigated with BG11 did not differ significantly from each other, indicating that the substrate type did not have a considerable effect on the weight of these samples. This was also the case for the BG11₀ irrigated samples. There were some significant differences in final dry mass between the differently irrigated samples (Table 9).

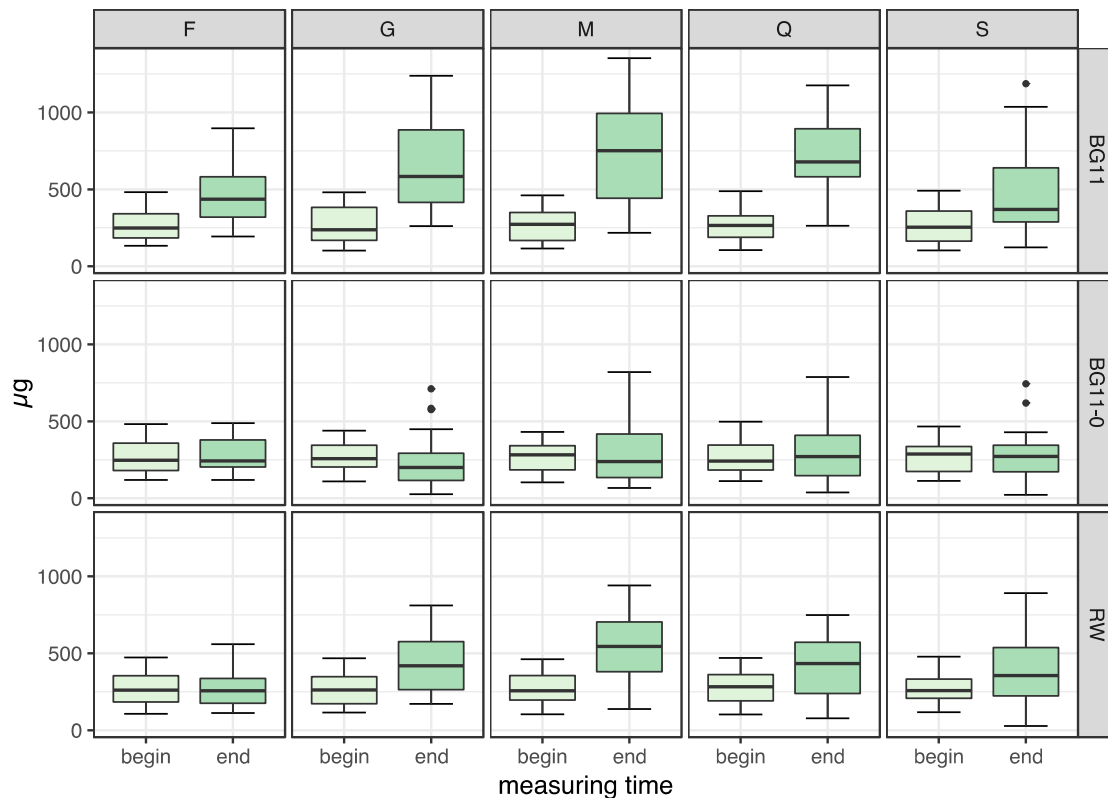


Fig. 21: Dry mass [µg] of *Nostoc* at the beginning and the end of the first trial with open Petri dishes.

Microscopic observations also showed more pronounced differences between the differently irrigated samples than between the samples cultivated on different substrates. *Nostoc* samples from the first trial irrigated with BG11₀ were mostly difficult to microscope, as hardly any individual cells or trichomes were recognisable. Instead, they consisted of thick gel layers, damaged cells (see sample M_ BG11₀ in Fig. 22) and were heavily contaminated with other organisms. The rainwater-irrigated samples, on the other hand, were the least contaminated with alien species. In these samples, the individual cells were easily recognisable and formed chains of various lengths. BG11 irrigated *Nostoc* most often had thin, long trichomes.

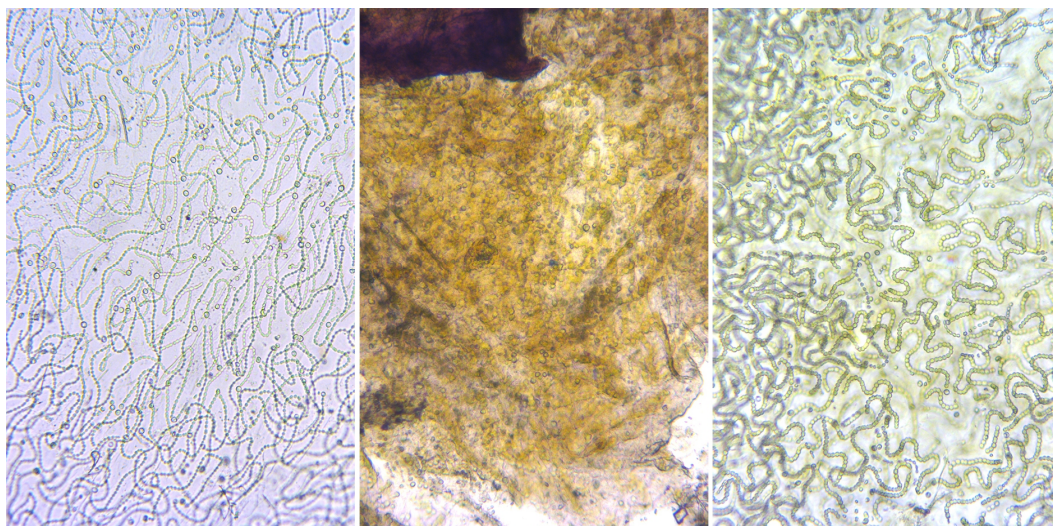


Fig. 22: Microscopic examples of *N. commune* cultivated in open Petri dishes (10x magnification). The following samples are shown from left to right: F_BG11, M_BG11₀, and G_RW.

In the course of the second run with constantly humid conditions, colonies died with BG11 irrigation, regardless of the substrate type (Fig. 23). Chlorophyll fluorescence continuously decreased or stopped, and the data analysis was terminated after day 18 and day 29, respectively, due to contamination with other organisms. The measured pixel number was significantly lower at day 18 than at the beginning of the experiment (Table 7). Termination of chlorophyll fluorescence of BG11-irrigated *Nostoc* occurred at a similar time in both trials. As in the first trial, *Nostoc* did not grow on the substrate filter paper (Fig. 23), but residual fluorescence was still detectable in BG11₀- and rainwater-irrigated *Nostoc* on day 18 (Table 7). Unlike in the first trial, we found a significant increase in pixel area for samples G_BG11₀, M_BG11₀, and Q_BG11₀. *Nostoc* cultured on marble sprinkled with BG11₀ even showed a maximum chlorophyll fluorescence value with a nearly 25-fold increase. The final fluorescence of S_BG11₀ is not significantly different from its initial fluorescence (WSR test, $p = 0.665$), like in the first trial. A few *Nostoc* pieces showed an increase in the pixel area, but most of them decreased (Fig. 23). The chlorophyll fluorescence area of *N. commune* irrigated with rainwater increased significantly on all substrate types except filter paper (Fig. 23, Table 7). This increase is consistent with the results of the first trial, but is considerably higher in the second trial. *Nostoc*, cultivated on quartz sand and irrigated with rainwater (Q_RW), shows the largest increase of all samples, with the median increasing five-fold. Q_RW is thus significantly different from samples F_BG11₀, G-BG11₀, S-BG11₀, and F_RW, but not from the other rainwater-irrigated samples and not from Q-BG11₀ and M-BG11₀ (see Table 10 in the appendix for pairwise comparisons).

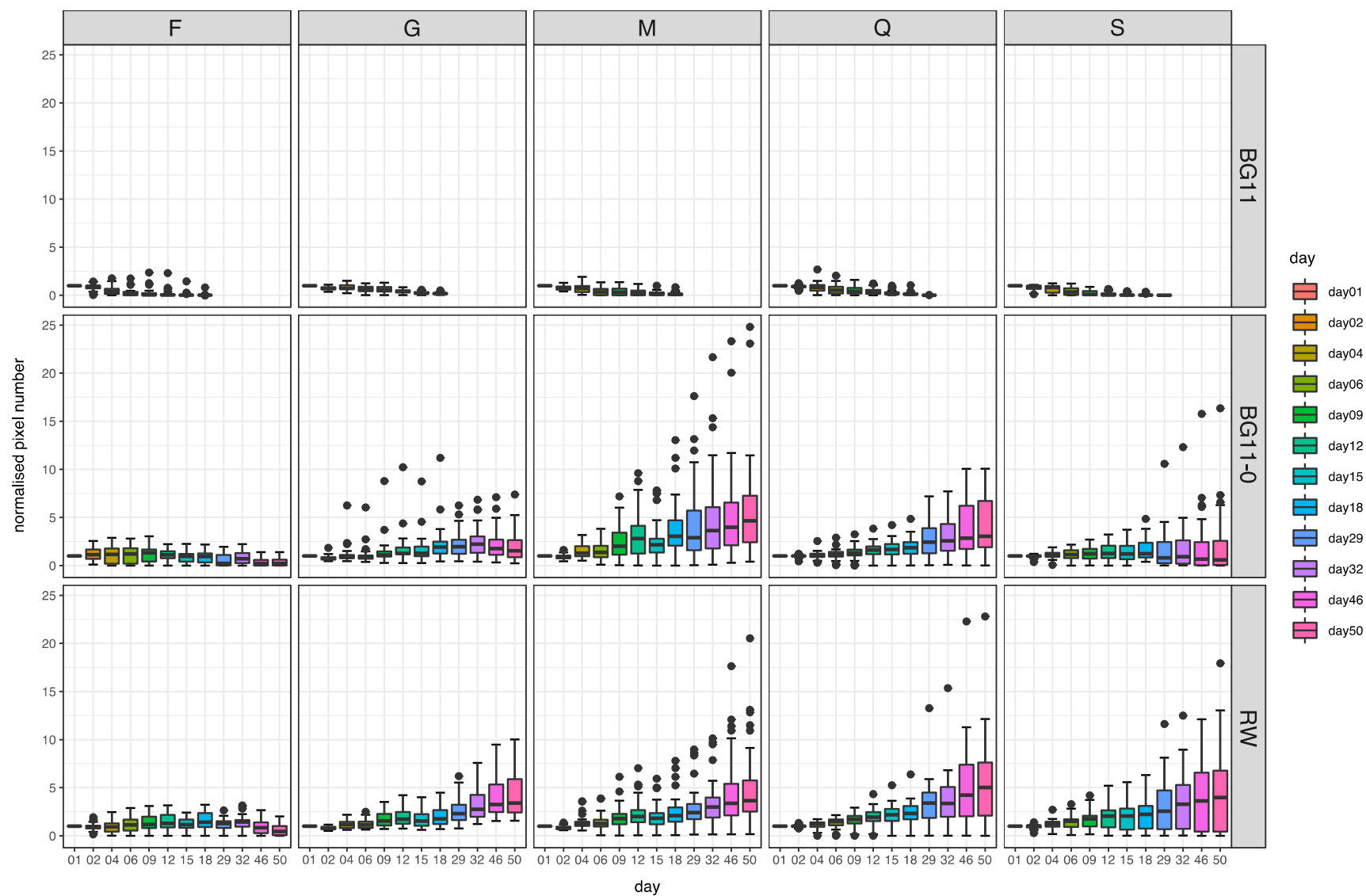


Fig. 23: The normalised pixel numbers (y-axis) from different measurement days (x-axis) were calculated from the PAM fluorescence images of the second trial with closed Petri dishes. The graph represents each sample type in a single box and the information in the grey boxes shows the combination of influencing factors forming the sample type.

Table 7: Final PAM fluorescence values (normalised by their respective initial pixel counts) and final dry mass from the samples cultivated in closed Petri dishes.

Sample (2_closed)	Final fluorescence (normalised pixels)					Final dry mass (µg)				
	Min	Median	Max	n	p (WSR)*	Min	Median	Max	n	p (WSR)**
F_BG11	0	0	0.8	21	< 0.001	35	141	499	26	0.001
G_BG11	0	0.2	0.5	30	< 0.001	103	325	731	27	0.073
M_BG11	0	0.1	0.9	30	< 0.001	25	212	657	25	0.833
Q_BG11	0	0.1	1.1	30	< 0.001	39	196	810	26	0.006
S_BG11	0	0	0.4	30	< 0.001	11	191.5	379	28	< 0.001
F_BG11 ₀	0	0.2	1.4	27	< 0.001	14	91	334	27	< 0.001
G_BG11 ₀	0.2	1.6	7.4	30	0.001	211	1728.5	5112	30	< 0.001
M_BG11 ₀	0.4	4.6	24.8	23	< 0.001	30	2825	6108	30	< 0.001
Q_BG11 ₀	0	3	10.1	29	< 0.001	106	1125	3968	29	< 0.001
S_BG11 ₀	0	0.6	16.4	29	0.665	8	500.5	5723	30	< 0.001
F_RW	0	0.4	2	30	< 0.001	43	135	555	28	< 0.001
G_RW	1.6	3.4	10	30	< 0.001	188	816	2435	30	< 0.001
M_RW	0.2	3.7	20.5	30	< 0.001	32	1172.5	5169	30	< 0.001
Q_RW	0	5	22.8	30	< 0.001	24	1363	2882	28	< 0.001
S_RW	0	4	17.9	30	< 0.001	35	1393.5	7139	30	< 0.001

*WSR test compared paired values from day 01 with day 18 (BG11 samples) or day 50 (BG11₀ and RW samples); **WSR test compared the initial with the final weight

Comparison of dry mass with PAM fluorescence measurements showed a much higher accordance in the second trial. The dry mass of all samples sprayed with BG11 decreased significantly or remained stable (Table 7, Fig. 24). There was a significant weight reduction in all samples cultured on filter paper (WSR test, $p < 0.001$). The dry mass of *N. commune* cultivated on all other substrates and irrigated with BG11₀ or rainwater increased significantly (WSR test, $p < 0.001$). The highest final dry mass was measured in BG11₀-irrigated *Nostoc* grown on marble. The final weight of the M_BG11₀ samples was significantly different from the final weight of all samples irrigated with BG11 as well as that of *Nostoc* grown on filter paper, but not from the other substrate types that were irrigated with BG11₀ or rainwater (see Table 11 in the appendix for pairwise comparisons). Unlike the normalized pixel area, where rainwater-irrigated samples tended to have the greatest increase in area, the final weight of BG11₀-irrigated samples tended to be higher (Table 7).

The poor condition of *N. commune* cultivated on filter paper or irrigated with BG11 was also evident under the microscope. *Nostoc* sprinkled with BG11 had many single and broken cells. Cell sheaths surrounding the trichomes and yellow layers were predominant. In contrast to the first experiment, *Nostoc* irrigated with BG11₀ looked mostly healthy this time. Individual cells and trichomes of varying lengths were visible. Again, the fewest foreign organisms were found in the rainwater-irrigated samples, whereas most were found in the BG11-irrigated samples in the second trial.

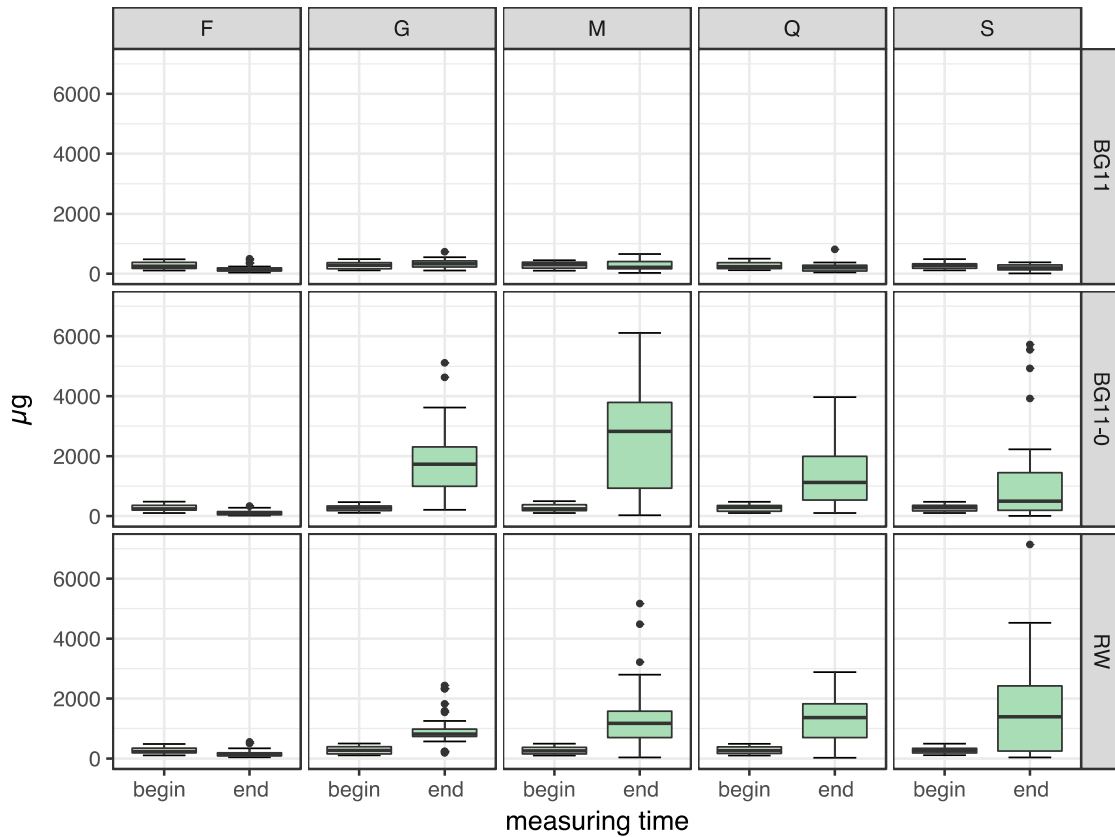


Fig. 24: Dry mass [μg] of *Nostoc* at the beginning and the end of the second trial with closed Petri dishes.

In summary, *Nostoc* irrigated with BG11 did not grow at all and generally, it did not perform well on the substrate filter paper. *Nostoc* increased in size and dry mass on all other substrate types when it was irrigated with rainwater. This increase was considerably higher in the second trial, in which closed Petri dishes were used. The performance of *Nostoc* that was irrigated with BG11₀ was dependent on the state of moisture. In the first trial, where *Nostoc* dried out regularly, it did not grow with the irrigation medium BG11₀, but in the second trial, where *Nostoc* stayed humid, it had the highest dry mass increase with BG11₀.

4. Discussion

Although various methods are described in the literature to measure growth of *Nostoc*, no general approach is available. The choice of a technique depends, for example, on the type of cultivation, the research question, and the available analytical instruments. In some studies, an increase of liquid-cultured *Nostoc* was detected spectrophotometrically via optical density or chlorophyll *a* content (Su et al. 2008, Feng et al. 2012, Diao and Yang 2014), but these methods rely on homogeneously distributed biomass, which is not very likely in a colony-forming culture. Growth can also be assessed via biomass gain by measuring the dry weight (Gao and Yu 2000, Gao and Ye 2003, Yu et al. 2009). Differences in filament length are suitable for showing *N. flagelliforme* growth (Su et al. 2005, Feng et al. 2012). Some researchers used photographs to analyse an increase in cyanobacterial cover on soils (Roncero-Ramos et al. 2019b, 2019a, Del Mondo et al. 2021). All these methods have advantages and disadvantages, which is why several approaches were combined in the current study to monitor the development of *N. commune*.

We used the digital camera photographs to document and compare the macroscopic colony development. Changes in colour, shape, and size were clearly visible on the bright substrates marble, glass beads, and filter paper, but not on the dark gravel, and quartz sand. Therefore, the photos could not be used for pixel analysis but were helpful to complement information of the PAM fluorescence images. The photos made it easier to track *N. commune* pieces that had been moved or twisted by spraying with an irrigation medium.

Digital photographs and the PAM fluorescence images matched well at the beginning of the study, indicating the successful revival of the desiccated *N. commune* pieces and confirming the suitability of the PAM fluorescence method for measuring their projected area. The PAM fluorescence measurement was applicable to all samples regardless of the brightness of the substrate type. Normalising the data eliminated minor differences that existed in radiation intensity between the substrate types. Other researchers have also reported differences in the chlorophyll *a* content depending on the soil type (Román et al. 2018, 2021). The fluorescence images were ideal for analysis with a pixel-counting programme due to the dark background. It allowed continuous monitoring of the cultures as it is a non-invasive method that does not affect or harm the organisms. In addition, the Imaging-PAM fluorometry provides valuable information on the physiological state of *Nostoc*. The photosynthetically active zones of the colonies were recorded and an increase, decrease, or stopping of chlorophyll fluorescence could be observed. A drawback was the undetectable three-dimensional structure of *Nostoc*, and it could not be ruled out that fluorescent components were covered by the substrate.

Microscopy serves as a complementary method but it is not suited to quantify growth of large three-dimensional macro colonies. However, it provides detailed insight into the health state of cells and is an ideal supplement to verify contamination. The microscopy pictures agreed well with the macroscopic observations, results of the PAM fluorescence, and weight measurements. Growing colonies had healthy-looking, intact cells and non-growing colonies had destroyed cells with thick, yellowish sheaths and were contaminated with other organisms. One limitation of microscopy is the requirement to remove parts or whole samples. This resulted in only a small number of samples being examined microscopically at the end of the study, as most of them were needed for weight determination.

Measuring dry mass offers the advantage of taking into account the entire biomass of *Nostoc*. In contrast to the fresh mass, which depends on the hydration state, the dry mass remains constant (Kvíděrová et al. 2011). As with microscopy, it was not possible to determine the dry mass during the study without manipulating samples, so it was just measured at the beginning and end of the experiments. Another disadvantage is that biomass does not provide information about the actual physiological state of the sample. Dead *Nostoc* may have gained weight before dying, and foreign microorganisms proliferating on *Nostoc* also contribute to the overall dry mass. In addition, we found media-dependent differences in the stickiness to the substrate (see chapter 4.1.3) that influenced the measurements. Some samples adhered firmly to the substrate and were hard to detach so that they partially crumbled, and in rare cases, even crumbs were lost.

Overall, dry mass and pixel analysis correlated with each other and showed a linear relationship. A correlation between the fresh weight and photochemically active colony area of *N. commune* has been discovered previously (Kvíděrová et al. 2011). Nevertheless, certain differences existed, which could be caused, for example, by the fact that photosynthetically inactive *Nostoc* dropped to zero in the PAM fluorescence measurement but still had weight. Furthermore, the three-dimensional structure was lost in the pixel evaluation, which could also lead to slight discrepancies in the outcomes.

In summary, the methods complement each other. However, using various analyses is time-consuming and does not provide conclusive results, as each method has its limitations. We recommend the PAM fluorescence measurement because it is non-invasive and offers additional information on the health state of the colony. In this study, the parameters for determining the state of health were measured but not further analysed. Another advantage of Imaging-PAM fluorometry is that it can be performed during the experiment. Statements about the physiological state of *Nostoc* were possible, changes in size could be analysed, and the emergence of foreign organisms was also detected. Only the three-dimensional structure of *N. commune* was not optimally captured and was completely lost during monochromatic pixel counting. However, this could be optimised in the future with an evaluation method that takes into account the different colours in the fluorescence images.

4.1.1. Desiccation

The moisture content of the samples had a strong influence on the colony development. Periodical desiccation resulted in lower growth rates of *N. commune* in the open Petri dishes compared to closed Petri dishes with constantly high humidity. Permanently moist *N. commune* grew faster and more samples showed an increase in size. At the end of the experiment, this resulted in a higher projected area and dry mass compared to regularly desiccated *N. commune*. Although *Nostoc* is considered a slow-growing organism (Kato et al. 2012, Sand-Jensen 2014, Guljamow et al. 2017), a comparison of the trials on the sixth day of the experiment already showed the first differences in the growth rate, which further increased with the duration of the trials.

The enhanced growth of *N. commune* kept under constant humid conditions compared to regularly desiccated samples confirmed former studies. Thus, the volume increase of *Nostoc* sp. on rocks tended to be more intense under high than under low humidity (Del Mondo et al. 2021). Sufficient moisture in the solid culture could also promote rapid growth of *N. flagelliforme* (Yuan et al. 2021). Wild *N. commune* also grows particularly well in moist

environments (Mollenhauer et al. 1999, Sand-Jensen 2014). In addition, *Nostoc* is often cultivated in liquid culture, as it is considered the most efficient way to generate high biomass quickly (Guljamow et al. 2017, Roncero-Ramos et al. 2019a). In contrast to liquid culture, however, the permanently moist *N. commune* on solid substrate retained its natural morphology, even when not exposed to regular desiccation cycles. Whether and how the lack of desiccation periods affects its desiccation tolerance was not investigated in the present study. The results of another study suggest that a prolonged hydration state causes *Nostoc* to be more sensitive to desiccation, whereas regular desiccation cycles promote its desiccation tolerance (Scherer and Zhong 1991).

In this study, however, the difference in growth between the regularly desiccated *N. commune* and the constantly moist colonies was so significant that the desiccation approach appears to be unsuitable for maximising biomass yield. Maybe the desiccation periods provided were too long, as *N. commune* dried out quickly in the open Petri dishes. The size of the sample pieces might have favoured the rapid desiccation. Kvíderová et al. (2011) described that small *Nostoc* pieces desiccate faster than larger ones due to the higher surface-area-to-volume ratio. Moreover, rapid drying seems to impair the regenerative capacity of *Nostoc*, which is why slow dehydration proved to be beneficial (Kvíderová et al. 2011, Yuan et al. 2021). *Nostoc*'s metabolic activity decreases progressively with declining water content: first the nitrogenase activity decreases, then the photochemical activity, and finally the metabolic processes stop completely (Kvíderová et al. 2011). This causes the desiccated *Nostoc* to enter a dormant state. After rewetting, the metabolic processes resume one after the other (see chapter 1), but it was shown that nitrogen fixation in a two-day desiccated sample needed about 50 hours to reach its maximum value again (Scherer et al. 1984). Since *N. commune* was always dried out after two days during the first trial, it could be that the nitrogen fixation could never reach its initial capacity again. Furthermore, microscopic examination indicated that some cells were completely shrivelled.

Surprisingly, the irrigation medium seemed to influence the desiccation tolerance of *N. commune*. Only *Nostoc* irrigated with rainwater thrived under regular desiccation, while *Nostoc* irrigated with the other media degenerated during the first trial. *Nostoc* sprayed with BG11₀, on the other hand, proliferated under constant humidity in the second trial (see chapter 4.1.3). Despite all this, the regular desiccation periods also had certain advantages. There was considerably less contamination with other organisms in the desiccated open Petri dishes than in the moist closed Petri dishes. This is in line with the findings of other research groups who promote desiccation as a measure to prevent the spread of foreign bacteria (Gao and Ye 2003, Su et al. 2005). A sufficient water supply is of importance for the propagation of certain bacteria (Su et al. 2005). The growth of these bacteria is one reason why cultivated *N. flagelliforme* often disintegrates (Gao and Ye 2003, Su et al. 2005). *N. flagelliforme* usually decomposes after a few days if kept permanently wet (Scherer and Zhong 1991, Gao and Ye 2003, Su et al. 2005). In contrast, permanent moisture in this study resulted in increased growth of *N. commune* and most samples did not decompose even after 50 days of moist cultivation. However, in the present study, it is not *N. flagelliforme* but another *Nostoc* species, which could explain the difference.

4.1.2. Substrate type

The substrate type influenced the development of *N. commune*. The provided substrate is, therefore, relevant for its growth performance.

N. commune did not grow on filter paper but did grow on the other substrate types. Unlike the other substrates, the filter paper was smooth and flat. The irrigation medium could not seep away and got absorbed by the filter paper, or larger quantities of the medium flooded the Petri dish. Scherer and Zhong (1991) showed that *N. flagelliforme* disintegrated under wet conditions on filter paper after about five days, but *N. commune* formed daughter colonies. The microscopic examination of the filter paper samples in this study also indicated daughter colonies of *N. commune* in isolated cases. However, these samples still had the highest proportion of destroyed cells and foreign microorganisms compared to the other substrates offered. In the PAM fluorescence images, a decrease in the chlorophyll fluorescence of *N. commune* cultivated on filter paper was already visible on the third day of the experiment. This decline proceeded at different rates depending on the other influencing factors and resulted in some samples no longer fluorescing on the sixth day, while others showed a low residual fluorescence until the end of the experiment. However, for every *N. commune* grown on filter paper, the fluorescent area decreased and the weight hardly changed or decreased as well. Macroscopically, *N. commune* cultivated on filter paper did not look healthy. It was flat and turned brownish. Taken together, the examinations indicated that *N. commune* was not performing well on filter paper. Further investigations are needed to determine, if the filter paper itself or the low structure it provides is the key factor for this poor performance.

The other substrate types were granular, leading to drier and wetter areas in the Petri dish. The *N. commune* colonies on these substrates propagated well in some cases, depending on the other influencing factors. Possibly, the granular structure of the substrates was advantageous, as *Nostoc* adhered better to these substrates and medium-filled depressions developed. Shallow, wet depressions have been described as a suitable habitat for *N. commune*, because it receives water and nutrients from below and light and atmospheric gases from above (Sand-Jensen 2014). Regarding the fluorescence measurement, the substrate types glass beads, marble, quartz sand, and crushed stone did not differ significantly from each other. Nevertheless, *N. commune* seemed to grow slightly worse on crushed stone than on the other three substrates. The weight measurement confirmed this assumption since *N. commune* grown on crushed stone weighed significantly less than that grown on glass beads and marble. Falchini et al. (1996) already reported different growth rates of *Nostoc* sp. depending on soil type, and other scientists also demonstrated the influence of the soil type on the colonisation rate of *N. commune* and other cyanobacteria (Román et al. 2018, Roncero-Ramos et al. 2019b, Del Mondo et al. 2021). Falchini et al. (1996) suggested in their study that the influence of the soil type on the growth of *Nostoc* sp. could depend on the content of the nutrient phosphorus in the soil, and Sand-Jensen (2014) also considered phosphorus to be a limiting growth factor for *N. commune*. However, the phosphorus content of the substrates was not determined in this study and is therefore not comparable. In another study, the differing colonisation rates of *Nostoc* sp. on several mineral substrates were attributed to the different water absorption coefficients of the substrates (Del Mondo et al. 2021). Another possibility for differences in the growth performance of *N. commune* could be the brightness of the substrates. Crushed stone was the darkest substrate and thus reflected less light. The lower light availability could have negatively influenced *Nostoc*'s growth. Concerning irradiance supply, some contradicting results were published: higher light intensities of up to $414 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ promoted the growth of *N. flagelliforme* (Gao and Yu 2000), although light intensities exceeding $150 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ also negatively impacted the growth of *Nostoc* (Nowruzi et al. 2013).

The artificial substrate, glass beads, did not appear to have disadvantageous effects on the development of *N. commune* since it was not inferior to the natural substrate types. Moreover, as a bright substrate, it is well suited for macroscopic observations. The latter was also the case with marble and filter paper. Quartz sand stood out due to the lowest number of contaminations with foreign organisms. Sand does not seem to be an optimal habitat for the alien organisms, whereas *Nostoc* grows well on it.

In our study, no provided substrate was clearly the best for the terrestrial cultivation of *N. commune*. However, filter paper proved to be unsuitable. Depending on the measurement method and the combination of influencing factors, the largest volume increase of *N. commune* was found on different substrates. In the moist trial, *Nostoc* cultivated on quartz sand sprayed with rainwater reached the highest median in the PAM fluorescence measurement. In contrast, *Nostoc* cultivated on marble sprayed with BG11₀ had the highest median in the biomass yield.

4.1.3. Irrigation medium

The irrigation medium influenced the growth, colour, and elasticity of *N. commune*, as well as the emergence of alien organisms. *N. commune* irrigated with BG11 did not grow, rather the chlorophyll fluorescence decreased steadily until it was finally absent or foreign organisms prevailed and the *Nostoc* pieces degraded. The colonies turned yellowish to brownish, lost their three-dimensional structure, and flattened. Likewise, Liu and Chen (2003) observed a yellowish colour change when their *N. flagelliforme* liquid culture collapsed, which was attributed to the release of pigments due to the disintegration of the colonies. This could also be the reason for the colour change in the present experiment. Another reason for a yellow colouration may be an increase in light intensity (Gao and Ye 2003, Cui et al. 2017), but the irradiance supply remained unchanged in the current study. Microscopic examination of the BG11 samples revealed thick yellow-brownish sheaths around the trichomes and partly loose, dense yellow-to-brown layers in the preparation. The thick sheaths around the *Nostoc* trichomes contradict reports that *N. flagelliforme* cultures grown with nitrogen had no sheath and that sheath formation was associated with nitrogen-free BG11₀ medium (Nowruzi et al. 2013). However, the formation of thick sheaths could also be an indication that *Nostoc* switches to dormancy due to unfavourable conditions. In the second trial with BG11 irrigation, numerous contaminations with foreign organisms as well as partly faded and loose *N. commune* cells were also visible under the microscope.

The degenerative changes of BG11 irrigated *N. commune* occurred independently of the other influencing factors and are surprising because this medium has been successfully used to cultivate *Nostoc* in several other studies (Gao and Yu 2000, Su et al. 2005, Feng et al. 2012, Roncero-Ramos et al. 2019a, Yuan et al. 2021). Some study authors even described BG11 as the medium used to achieve the highest growth rate of *Nostoc*, but these were liquid cultures (Cui et al. 2017, Roncero-Ramos et al. 2019a). Due to the ability of *Nostoc* to fix atmospheric nitrogen (Haselkorn 2006), the type of cultivation makes a difference: in aquatic culture, dinitrogen is not that easily available compared to terrestrial culture, because diffusion in liquids is much lower compared to gas phases.

Nevertheless, BG11 was not assumed to have deleterious effects on the terrestrial development of *N. commune*. The observed detrimental effect of the medium does not fit with the results of other studies either, in which another species, *N. flagelliforme*, was

successfully cultured in the air on solid substrates with BG11 liquid medium or on BG11 agar (Gao and Yu 2000, Su et al. 2005, Feng et al. 2012, Yuan et al. 2021). Cultivation on solid BG11 agar did not require additional irrigation with medium (Su et al. 2005, Feng et al. 2012, Yuan et al. 2021), possibly resulting in different conditions. However, Gao and Yu (2000) soaked the *N. flagelliforme* mats in BG11 once or twice daily for 30 min and Su et al. (2005) submerged *N. flagelliforme* grown on a sandy substrate each day for 90 min in BG11. These samples were, therefore, even more frequently exposed to BG11 than *N. commune* in this study. The reason why *N. commune* did not grow with full BG11 medium remains unclear. At present, we can only speculate that the *N. commune* used in our study is sensitive to sodium nitrate (NaNO_3), as *N. commune* irrigated with BG11₀ grew at least in the second experimental run.

In contrast to the previously mentioned studies (Cui et al. 2017, Roncero-Ramos et al. 2019a), *Nostoc* performed better with the medium BG11₀ than with BG11, although the development of *N. commune* with BG11₀ irrigation differed considerably between the two trials. In the first trial with regular desiccation, the fluorescent area of some samples decreased. However, unlike with BG11 irrigation, it extinguished more slowly and not completely. Nevertheless, the combination of BG11₀ and desiccation had a negative effect on the growth of *N. commune* and the condition of its cells. In the microscopic examination of the BG11₀ irrigated samples from the first trial, hardly any trichomes or single cells were visible. Instead, thick, impenetrable, dark layers and cell debris dominated. Contrasting results were obtained in the BG11₀ irrigated samples in the second trial: trichomes of diverse lengths predominated and the individual cells were easily recognisable. Trichomes of diverse lengths may indicate *Nostoc* at different stages of development, for example, long cell filaments were associated with early *N. flagelliforme* colonies and shorter filaments with more mature colonies (Liu and Chen 2003). Consistent with the microscopic image, the fluorescent area and dry mass increased significantly under continuous moisture with BG11₀ irrigation. We assume that a component in the BG11₀ medium lowers the desiccation tolerance of *N. commune*, as this moisture-related effect did not occur with rainwater irrigation.

The rainwater-irrigated samples grew in both trials, although less under regular desiccation than under permanent moisture. Microscopically, they did not differ much from each other. Overall, rainwater was the most suitable irrigation medium as it stimulated growth with and without drying periods. Since *Nostoc* can grow well in nature under nutrient-poor conditions (Sand-Jensen 2014), the additional nutrients in the BG11 and BG11₀ nutrient media may be unnecessary or even counterproductive.

Media rich in nutrients favour the growth of other microorganisms than *Nostoc* and thus result in unwanted competition. We found considerably more contamination with foreign algae and bacteria in the Petri dishes sprinkled with BG11 and BG11₀ than in those sprinkled with rainwater. Moreover, *N. commune* pieces irrigated with rainwater remained elastic and were easily detachable from the substrate at the end of the experiments. The elasticity could be related to the low number of foreign microorganisms, for example, *N. flagelliforme*, which was locally decomposed by the increasing presence of foreign bacteria, showed reduced elasticity (Su et al. 2005). The more contaminated BG11₀ irrigated *N. commune* lost its elasticity as the experiment advanced and developed a crumbly structure. Apart from that, it also adhered firmly to the substrate.

The stronger adhesion to the substrate could be due to the release of EPS. The EPS matrix contains substances that make it sticky and promote its adhesion to surfaces (Rossi and De Philippis 2015). EPS synthesis, in turn, is influenced by environmental conditions, including the availability of nutrients such as nitrogen, phosphorus, carbon, and sulphate (Rossi and De Philippis 2015) and, therefore, probably varies depending on the irrigation medium. The variability in EPS composition has been previously noted, with the culture medium being one of the influential factors (Han et al. 2017).

Not only did the elasticity and adhesion of the samples differ due to the media, but their colour also changed in the progress of the experiment. Rainwater-irrigated *N. commune* became lighter green, while colonies irrigated with BG11₀, especially those in the second trial, became darker. *N. commune* that did not grow, such as the samples sprayed with BG11 or cultivated on filter paper, often turned pale yellowish or brownish. In another study, a colour change of *N. flagelliforme* was attributed to nitrogen availability in the culture medium, as samples treated with BG11 turned darker green and those treated with BG11₀ turned lighter green (Feng et al. 2012). However, this does not agree with the present results, where BG11₀ sprayed *N. commune* turned darker and not lighter green, while the BG11 sprayed one turned yellow.

It is possible that not a specific substance in the irrigation media was responsible for the colour change but that the colour was an expression of the physiological state of *Nostoc*. A developmental colour change of *N. flagelliforme* has already been described by Liu and Chen (2003), in whose study the cultures became darker in the course of their growth and a collapsing colony turned yellowish. Although yellowing has been explained with the release of pigments or increased carotenoid synthesis, it often seems to be associated with poorer *Nostoc* development (Liu and Chen 2003, Cui et al. 2017). In the present study, the yellow colouration indicated stagnant growth and the decline of chlorophyll fluorescence. However, why the BG11₀ samples became darker and the rainwater samples lighter remains unclear, as both developed well.

The area increase of the rainwater irrigated samples cultivated on quartz sand reached the highest median with a five-fold increase and the rainwater samples showed the most intense chlorophyll fluorescence in the PAM fluorescence images towards the end of the experiment. The highest maximum area increase, with a nearly 25-fold increase, was found in the BG11₀ irrigated samples cultivated on marble, which also had the highest dry mass median and macroscopically formed a distinct three-dimensional structure. In the moist trial, it is therefore not possible to clearly determine which of the two irrigation media was better suited for the cultivation of *N. commune*.

For future experiments, it would be interesting to find out what triggers the colour change of *Nostoc* and to explore why it performed poorly with the combination of BG11₀ and desiccation. Due to the existing advantages of desiccation, it would be of interest to optimise the cultivation of *Nostoc* on a solid substrate under regular desiccation periods. For future surveys, we recommend keeping *Nostoc* moist for longer periods or letting it dry only slightly. A minor loss of water could allow nutrients to enter the cells more easily, while metabolic processes would still remain active (Kvíděrová et al. 2011). Since an increased amount of media would have flooded the Petri dishes, more frequent irrigation intervals are advisable and larger sample pieces. A simpler alternative could be to cultivate *Nostoc* in closed Petri dishes but occasionally open the lid slightly to let *Nostoc* dry out slowly in between.

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Appendix

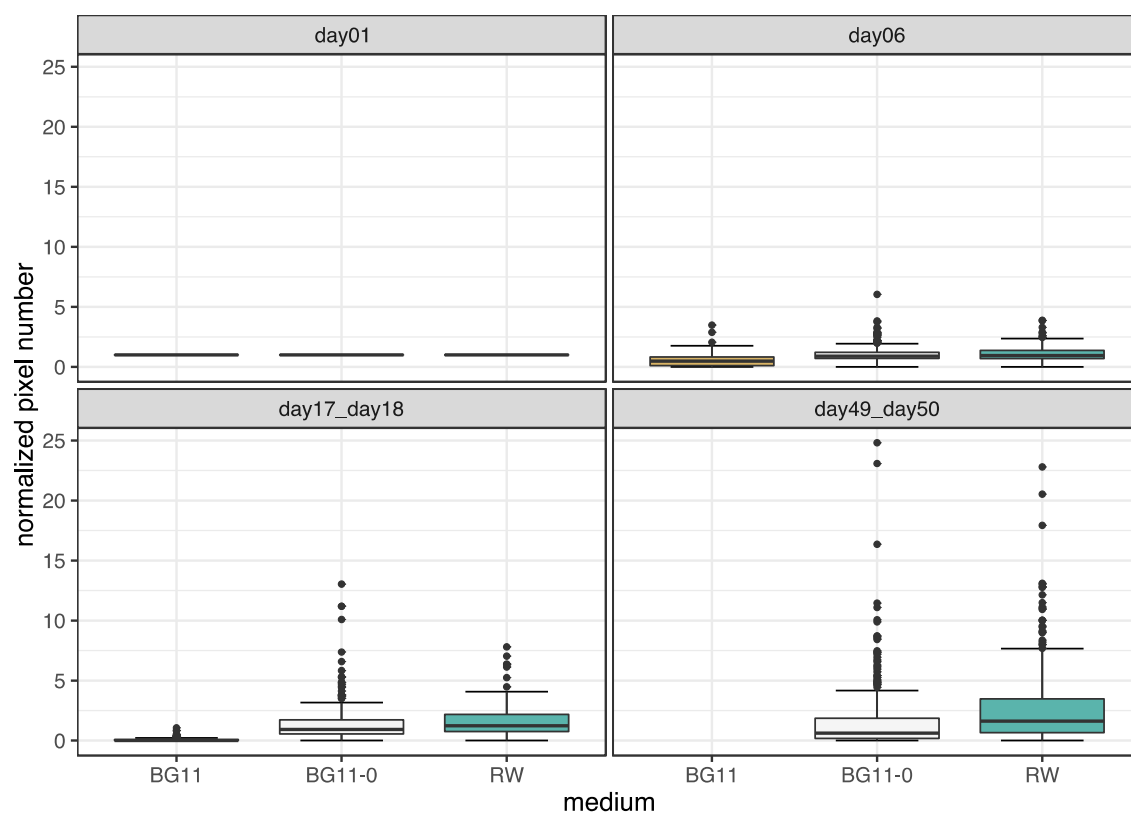


Fig. 25: The boxplots show the normalised pixel count of *Nostoc* depending on the irrigation medium on four measurement days.

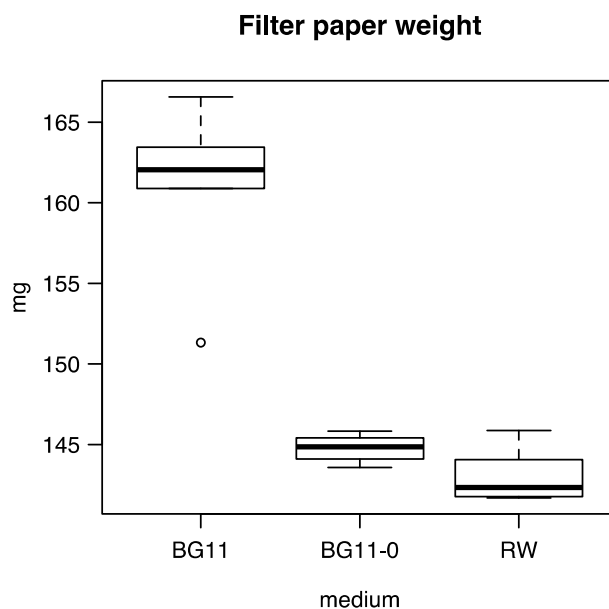


Fig. 26: Weight of empty filter papers after removal of *Nostoc* at the end of the experiment, itemised according to the applied irrigation medium.

Table 8: *p*-values (according to Bonferonni) of the pairwise comparisons (using *U*-test) of the PAM fluorescence values of day 49 from the samples of the first experiment with open Petri dishes.

	F_BG11-0	G_BG11-0	M_BG11-0	Q_BG11-0	S_BG11-0	F_RW	G_RW	M_RW	Q_RW	S_RW
F_BG11-0	-	< 0.001	< 0.001	< 0.001	< 0.001	1	< 0.001	< 0.001	< 0.001	< 0.001
G_BG11-0	< 0.001	-	1	1	1	< 0.001	< 0.001	0.051	0.513	1
M_BG11-0	< 0.001	1	-	1	1	< 0.001	< 0.001	< 0.001	0.008	0.127
Q_BG11-0	< 0.001	1	1	-	1	< 0.001	< 0.001	< 0.001	0.003	0.043
S_BG11-0	< 0.001	1	1	1	-	< 0.001	0.007	0.449	1	1
F_RW	1	< 0.001	< 0.001	< 0.001	< 0.001	-	< 0.001	< 0.001	< 0.001	< 0.001
G_RW	< 0.001	< 0.001	< 0.001	< 0.001	0.007	< 0.001	-	1	0.445	0.431
M_RW	< 0.001	0.051	< 0.001	< 0.001	0.449	< 0.001	1	-	1	1
Q_RW	< 0.001	0.513	0.008	0.003	1	< 0.001	0.445	1	-	1
S_RW	< 0.001	1	0.127	0.043	1	< 0.001	0.431	1	1	-

Table 9: *p*-values (according to Bonferonni) of the pairwise comparisons (using *U*-test from the final dry mass of the samples of the first experiment with open Petri dishes.

Sample	F_BG11	G_BG11	M_BG11	Q_BG11	S_BG11	F_BG11-0	G_BG11-0	M_BG11-0	Q_BG11-0	S_BG11-0	F_RW	G_RW	M_RW	Q_RW	S_RW
F_BG11	-	0.968	0.104	0.052	1	0.015	0.002	0.307	0.208	0.062	0.007	1	1	1	1
G_BG11	0.968	-	1	1	0.626	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.245	1	0.68	0.089
M_BG11	0.104	1	-	1	0.113	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.064	1	0.06	0.016
Q_BG11	0.052	1	1	-	0.122	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.035	1	0.026	0.009
S_BG11	1	0.626	0.113	0.122	-	0.888	0.032	1	1	1	0.288	1	1	1	1
F_BG11-0	0.015	< 0.001	< 0.001	< 0.001	0.888	-	1	1	1	1	1	0.362	0.001	1	1
G_BG11-0	0.002	< 0.001	< 0.001	< 0.001	0.032	1	-	1	1	1	1	0.018	< 0.001	0.208	1
M_BG11-0	0.307	< 0.001	< 0.001	< 0.001	1	1	1	-	1	1	1	1	0.023	1	1
Q_BG11-0	0.208	< 0.001	< 0.001	< 0.001	1	1	1	1	-	1	1	1	0.033	1	1
S_BG11-0	0.062	< 0.001	< 0.001	< 0.001	1	1	1	1	1	-	1	0.476	0.006	0.866	1
F_RW	0.007	< 0.001	< 0.001	< 0.001	0.288	1	1	1	1	1	-	0.105	< 0.001	0.805	1
G_RW	1	0.245	0.064	0.035	1	0.362	0.018	1	1	0.476	0.105	-	1	1	1
M_RW	1	1	1	1	1	0.001	< 0.001	0.023	0.033	0.006	< 0.001	1	-	1	1
Q_RW	1	0.68	0.06	0.026	1	1	0.208	1	1	0.866	0.805	1	1	-	1
S_RW	1	0.089	0.016	0.009	1	1	1	1	1	1	1	1	1	1	-

Table 10: *p*-values (according to Bonferonni) of the pairwise comparisons (using *U*-test) of the PAM fluorescence values of day 50 from the samples of the second experiment with closed Petri dishes.

Sample	F_BG11-0	G_BG11-0	M_BG11-0	Q_BG11-0	S_BG11-0	F_RW	G_RW	M_RW	Q_RW	S_RW
F_BG11-0	-	< 0.001	< 0.001	< 0.001	1	1	< 0.001	< 0.001	< 0.001	0.002
G_BG11-0	< 0.001	-	0.109	0.232	1	< 0.001	0.004	0.077	0.038	1
M_BG11-0	< 0.001	0.109	-	1	0.016	< 0.001	1	1	1	1
Q_BG11-0	< 0.001	0.232	1	-	0.046	< 0.001	1	1	1	1
S_BG11-0	1	1	0.016	0.046	-	1	0.003	0.014	0.018	1
F_RW	1	< 0.001	< 0.001	< 0.001	1	-	< 0.001	< 0.001	< 0.001	0.017
G_RW	< 0.001	0.004	1	1	0.003	< 0.001	-	1	1	1
M_RW	< 0.001	0.077	1	1	0.014	< 0.001	1	-	1	1
Q_RW	< 0.001	0.038	1	1	0.018	< 0.001	1	1	-	1
S_RW	0.002	1	1	1	1	0.017	1	1	1	-

Table 11: *p*-values (according to Bonferonni) of the pairwise comparisons (using *U*-test from the final dry mass of the samples of the second experiment with closed Petri dishes.

Sample	F_BG11	G_BG11	M_BG11	Q_BG11	S_BG11	F_BG11-0	G_BG11-0	M_BG11-0	Q_BG11-0	S_BG11-0	F_RW	G_RW	M_RW	Q_RW	S_RW
F_BG11	-	0.011	0.951	1	1	1	< 0.001	< 0.001	< 0.001	0.006	1	< 0.001	< 0.001	< 0.001	0.001
G_BG11	0.011	-	1	0.439	0.419	< 0.001	< 0.001	< 0.001	< 0.001	1	0.004	< 0.001	< 0.001	< 0.001	0.337
M_BG11	0.951	1	-	1	1	0.002	< 0.001	< 0.001	< 0.001	1	0.421	< 0.001	< 0.001	< 0.001	0.052
Q_BG11	1	0.439	1	-	1	0.218	< 0.001	< 0.001	< 0.001	0.223	1	< 0.001	< 0.001	< 0.001	0.008
S_BG11	1	0.419	1	1	-	0.047	< 0.001	< 0.001	< 0.001	0.259	1	< 0.001	< 0.001	< 0.001	0.009
F_BG11-0	1	< 0.001	0.002	0.218	0.047	-	< 0.001	< 0.001	< 0.001	< 0.001	1	< 0.001	< 0.001	< 0.001	< 0.001
G_BG11-0	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	-	1	1	0.227	< 0.001	0.404	1	1	1
M_BG11-0	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	1	-	1	0.273	< 0.001	0.09	1	1	1
Q_BG11-0	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	1	1	-	1	< 0.001	1	1	1	1
S_BG11-0	0.006	1	1	0.223	0.259	< 0.001	0.227	0.273	1	-	0.002	1	1	1	1
F_RW	1	0.004	0.421	1	1	1	< 0.001	< 0.001	< 0.001	0.002	-	< 0.001	< 0.001	< 0.001	< 0.001
G_RW	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.404	0.09	1	1	< 0.001	-	1	1	1
M_RW	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	1	1	1	1	< 0.001	1	-	1	1
Q_RW	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	1	1	1	1	< 0.001	1	1	-	1
S_RW	0.001	0.337	0.052	0.008	0.009	< 0.001	1	1	1	1	< 0.001	1	1	1	-