



Ecology and Molecular Biology of Microbial Soil Crusts in the Deserts of Western USA

PhD. Thesis



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Annotation: The thesis includes studies of the microbial soil crusts in the desert of Western USA. Development of immobilized cyanobacterial amendments for reclamation of microbiotic soil crusts, and morphological and molecular characterization of four *Nostoc* species isolated from desert soils.

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Declaration: Hereby I declare that I wrote the thesis by myself using relevant references.

Klára Kubečková



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Preface

Soil algae have been around a long time. It is likely that during Precambrian times cyanobacteria colonized the soil, and that they were in turn followed by eukaryotic coccooid and filamentous algae (JOHANSEN & SHUBERT 2001).

The most detailed accounts of subaerial communities with abundant cyanobacteria come not from the wet tropics, but from the arid regions, such as western USA (SHIELDS 1957, ST. CLAIR & JOHANSEN 1993), Israel (LANGE ET AL. 1992, DOR & DANIN 1996) or Australia (ELDRIDGE 2001)

Cyanobacteria are the important component of many soils, including the surface crusts that cover extensive areas in cold and hot semiarid regions (BROADY 1996, EVANS & JOHANSEN 1999,) and mine spoil waste (STARK & SHUBERT 1982, LUKEŠOVÁ & KOMÁREK 1987). In the hot deserts of western USA they play a lot of important roles. Soil microbial crusts appear to aggregate soil surfaces and decrease the soil erosion due to water and wind too. BOOTH (1941) made suggestion about their importance for the soil surface stabilization. The empirical evidence for this hypothesis was valid at the first by KLEINER & HARPER (1972) and after it by many workers (BELNAP & GARDNER 1993, LEYS & ELDRIDGE 1998, BELNAP 2001A). The communities of heterocystous cyanobacteria, lichenised cyanobacteria and the nitrogen fixing eubacteria supply the desert soil with nitrogen and increase the soil fertility (BELNAP 2001B, MEEKS & ELHAI 2002). The increasing fertility of soil is reflected in the mineral nutrition of vascular plants. Microbiotic crusts have been influenced the water runoff, infiltration rates, evaporation (WARREN 2001) and seedling of vascular plants (BOUDELL et al 2002).

The microbiotic soil crusts are very fragile ecosystem and it is very easy to disturb them by many activities. Mechanical disturbance includes activities such as vehicle traffic (military and recreational), trampling livestock and people and land clearing (mining) (BEYMER & KLOPATEK 1992, BROTHERRSON ET AL. 1983). Range fire can also be very damaging. The main effect of the fire is loss of cover, biomass, and species diversity of soil crusts (JOHANSEN ET AL. 1993). The recovery of the species composition and functions of the crusts is slow. The recovery of cyanobacterial diversity can take from 10 to 30 years (ANDERSON ET AL. 1982, JOHANSEN ET AL. 1984). Recovery of ecosystem function, such as nitrogen fixation activity, may take longer than simple recovery of biomes.

Some of the most rapid and dramatic changes in ecosystem dynamics are occurring in arid and semiarid regions. These ecosystems cover 33 to 40% of the terrestrial land surface (KASSAS 1995) and are home to 35% of the world population (BROOKS & POKSHISHEVSKY 1986). MELILLO ET AL. (1993) predict a 50% increase in desert in response to increasing atmospheric CO₂ concentrations, and 20% increase for dry-lands in general. It is estimated that 35% of the world land area is at risk for desertification (CLOUDSLEY- THOMPSON 1988). Those numbers are alarming and are also the reason for the study of desert ecosystems around the world.

This thesis integrates experimental, ecological studies and taxonomical studies of microbiotic soil crust in the deserts of western USA. The first part was concerned on the development of immobilized cyanobacterial amendments for reclamation of microbiotic soil crusts. The amendment should help to speed recovery of the crusts in the military training areas and protected the soil surface again water and wind erosion. The second part is aimed at the taxonomical studies of *Nostoc* species from desert soil. The polyphasic approach was used for description of four *Nostoc* strains. At least one strain is unique according the morphological and molecular results. Other tree strains are different in the morphological features but the molecular results are not significant.

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**Development of immobilized cyanobacterial amendments for reclamation
of microbiotic soil crusts.**

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With 8 figures and 2 tables in the text

Abstract

Cyanobacteria were immobilized on hemp cloth, which was subsequently cut into fine pieces for use as a soil amendment. The amendment is intended for speeding recovery of microbiotic soil crusts in semi-arid and arid lands where such crusts have been destroyed by anthropogenic activities. *Microcoleus vaginatus*, *Schizothrix calcicola*, and *Nostoc* were used to create amendments, but most of the experiments in this study utilized the *Microcoleus* amendment, as it is the most cosmopolitan and ecologically important cyanobacterial taxon in desert soil crusts. The amendment was found to retain its viability in storage for at least 18 months. *M. vaginatus* grew best in CT and Z8+ (plus vitamins) media with aeration and addition of KHCO_3 . A field test at Fort Irwin National Training Center, Mojave Desert, California, showed significant increases in phototrophic biomass 6 and 12 months after inoculation, but the stimulus was not evident at 18 months. Four other field tests did not show significant gains. We suspect the failure of the field tests is due to a loss of cyanobacterial viability after inoculation but before favourable growth conditions. Cyanobacteria were possibly killed by UV irradiation or photo-oxidation in the hot desert environment. *M. vaginatus* is known to live below the surface in desert soils, and so likely is sensitive to full sunlight. Laboratory experiments in full sunlight verified this hypothesis.

Key words: Cyanobacteria, cyanobacterial amendments, desert soil, microbiotic crusts, *Microcoleus vaginatus*, *Schizothrix calcicola*.

Introduction

Microbiotic crust communities are common in many arid and semiarid regions of North America (ST. CLAIR & JOHANSEN 1993). Biological soil crusts have been well studied in semi-arid regions of the western United States (EVANS & JOHANSEN 1999, ROSENTRETTER & BELNAP 2001, WEST 1990), although there is a growing body of literature on crusts of other parts of the world (BÜDEL 2001a, 2001b, 2001c, ELDRIDGE 2001a, GALUN & GARTY 2001, ULLMANN & BÜDEL 2001). In semi-arid lands crusts can often make up the majority of plant cover (JOHANSEN 1993). Cover is reduced in hot deserts, often being replaced by desert pavement or coarse-textured sands and gravels.

Microbiotic crusts play several roles in the arid and semi-arid regions in which they occur. The most important of these roles are soil stabilization and nitrogen fixation (BELNAP 2001a, EVANS & LANGE 2001). Other potential roles include improved infiltration of rain water (ELDRIDGE 2001b, WARREN 2001), enhancement of shrub seedling establishment, reduced success of weedy annuals such as *Bromus tectorum*, improved mineral nutrition in plants, and increased carbon fixation in soils. Emerging from recent studies is the increased understanding that the ecosystem function of microbiotic crusts varies considerably by region and climate, and that assumptions true for one well-studied geographic region may not be true for other regions.

Increased soil erosion frequently occurs following disturbance. This has been documented repeatedly by many workers from around the world (BELNAP & ELDRIDGE 2001). Microbiotic crusts can be disrupted by grazing livestock, recreational and military vehicles, and human trampling (BELNAP 1996, BEYMER & KLOPATEK 1992, BROTHERTON et al. 1983). Rangefire can also be very damaging in those areas where sufficient vascular plant cover exists to carry a fire (CALLISON et al. 1985, JOHANSEN et al. 1982, 1984, 1993). Effects of disturbance include both a loss of soil stability and a loss of nitrogen fixation activity (BELNAP & ELDRIDGE 2001). Recovery estimates have varied, but long-term recovery studies

indicate that recovery of the cyanobacterial community often takes 5-30 years, and the moss and lichen components can take 40-100 years (ANDERSON et al. 1982, JOHANSEN et al. 1984). Recovery of ecosystem function, such as nitrogen fixation activity, may take longer than simple recovery of biomass (BELNAP 1996).

In an effort to speed the process of recovery, ST. CLAIR et al. (1986) inoculated a severely burned "blow out" site with the soil crust slurry made from material taken from a nearby undisturbed site. After six months significant recovery of the cyanobacteria and selected lichen species was demonstrated. BELNAP (1993) inoculated scalped plots with crushed cryptogamic soil and demonstrated significant recovery over uninoculated scalped plots. These studies strongly suggest a beneficial correlation between inoculation and recovery. However, if used on a larger scale, the method of applying crushed soil would necessitate the destruction of one area to restore another (CAMPBELL et al. 1989).

Recently, methods of preparing pelletized cyanobacteria as a soil amendment has been developed (BUTTARS et al. 1998). Briefly, cyanobacterial species were encapsulated in alginate, dried, ground and applied to soils. During field studies concerns developed as to the survivability and escapability of cyanobacteria from the alginate pellets. Although these studies provided some evidence that the pellets promoted recovery (JOHANSEN & ST. CLAIR 1994), a different carrier for the cyanobacterial amendment was subsequently sought. Cyanobacteria were grown on hemp fabric, and found to have much higher viability than pelletized cyanobacteria.

This paper reports the results of tests of this newly developed cyanobacterial amendment. Experiments leading to the choice of the carrier and its superiority over pelleted inoculants will be reported, followed by examination of long-term viability of the product, conditions favoring mass culture, and finally, field tests conducted thus far. Given the fragility of desert ecosystems, and the ecosystem degradation experienced when crusts are disturbed, it is important that means of speeding the recovery of microbiotic crusts be developed. Cyanobacterial amendments could restore both soil stability and nitrogen fixation activity, and so our efforts are focused on these elements of the crust community.

Materials and Methods

Isolation of Cyanobacterial Strains

Soil samples were collected from throughout the United States and used for the isolation of strains to be used in this study. All samples were dilution-plated on to agar-solidified Z-8 medium (CARMICHAEL 1986), and individual cyanobacterial isolates were made from these plates into liquid Z-8 medium. In some cases the dilution plates did not produce oscillatoriacean cyanobacteria. In these instances, soils were moistened with liquid Z-8 and filaments of cyanobacteria were isolated directly from the moistened soils. Although many strains were isolated, the work reported here is based on *Microcoleus vaginatus* (Vaucher) Gomont (JT-15), *Nostoc* species (JT-7), and *Schizothrix calcicola* (Agardh) Gomont (*sensu* Drouet) (JT-24) from Joshua Tree National Park. Two strains of *M. vaginatus* (JT-15, OTA) were used for the field tests at Orchard Training Area (Idaho), Yakima Training Center (Washington), and Fort Irwin (California). *Schizothrix calcicola* from undisturbed soils of Fort Bliss, New Mexico was used to inoculate plots at a nearby burned site. Cyanobacterial strains were maintained on agar-solidified Z8 media at 7 °C on a 12:12 hr light:dark cycle.

Preparation of Inoculum

Cyanobacterial strains were cultured in liquid media in small batch cultures of 1-3 liters. These cultures were harvested, homogenized in a kitchen blender, and sprayed onto autoclaved hemp fabric. The fabric was kept moist for 1-2 weeks to allow the cyanobacteria to establish a blue-green lawn on the fabric. The fabric was then air-dried, chopped by hand with a rotary cutter into small pieces (ca. 0.5x0.5 cm), and stored in the dark in Rubbermaid® plastic food-storage containers at room temperature until time of use.

Laboratory Experiments

Laboratory tests of viability and growth were conducted on wet sand. Four to six teflon coated metal trays that had been perforated with small holes in the bottom (27x15 cm x 5 cm deep) were filled with sand and placed in shallow aluminium trays. All trays and sand were autoclaved prior to set up. Liquid Z8 medium was added to the shallow trays and allowed to move by capillarity into the sand. Standing liquid Z8 medium was maintained in the shallow trays for the course of the experiment. Perforated plexiglass plates were placed over trays to minimize aerial contamination. At the start of each experiment, half of the trays were inoculated with 0.5 g hemp/tray (=10 g/m²), and half were left as uninoculated controls. The experiments were run in controlled conditions of 16:8 hrs light:dark cycle, with day temperature at 18 °C and night temperature at 10 °C. Trays were randomly sampled (3 replicates) usually every 3 days for 24 days, although in an early experiment trays were sampled every 2 days for 27 days. Chlorophyll *a* was extracted using the DMSO method recommended by BELL & SOMMERFELD (1987) as modified by JOHANSEN et al. (2001). Chlorophyll *a* was then measured fluorometrically (with triplicate readings for each replicate sample) and calculated as ng chlorophyll *a*/cm².

Growing the cyanobacteria in liquid media was often slow and unsuccessful. Several experiments were run to optimise growth of the cyanobacteria. These experiments took place in the same controlled environment as described above, and each was harvested after 21 days. In the first experiments, substrates were provided to see if added structure within the liquid media was beneficial for growth. In addition to growth in empty 125 ml Erlenmeyer flasks, growth was tested in the same flasks with glass beads, soft agar (0.4%), and sand. Growth in three different media was tested: Z8, BG-11 (STANIER et al. 1971), and CT. A vitamin stock was used in the preparation of CT medium, but not BG-11 and Z8. A vitamin stock was added to these latter two media to create what we refer to as BG-11+ and Z8+ media. The vitamin-enriched media contained 1 µg B₁₂, 1 mg Thiamine-HCl, and 2 µg Biotin per liter. We also added aeration with and without 2.0 mM KHCO₃ to see if additional inorganic carbon sources stimulated growth. Others have demonstrated that cyanobacteria can be stimulated by elevated CO₂ concentrations (LUKAVSKÝ 1975). In all of these experiments, chlorophyll *a* was measured fluorometrically using the DMSO extraction method mentioned above.

We were concerned about the effects of full sunlight on viability of hemp-immobilized cyanobacteria. An experiment was run on the roof of the science building at John Carroll University in which the experimental design for viability tests on sand was copied except 4 levels of light. The trays were shaded by cloth to obtain 100%, 60%, 40%, and 7% ambient light levels, and environmental conditions were not controlled. The maximum light on a sunny day observed by us during this experiment was just under 1600 µE/cm². This experiment was run for 14 days. Due to contamination by air-borne green algae, we measured cyanobacterial biovolume in this experiment using direct counts under epifluorescence microscopy (JOHANSEN & RUSHFORTH 1985).

Field Methods

Five study sites were chosen for field studies. The first site inoculated was at Fort Irwin National Training Center (Fort Irwin NTC), California, in an area that had been graded and leveled by machinery (35°14'50" N, 116°39'40" W). This site was studied November 1998 to April 2000. The other four sites were studied a year later, and included: an abandoned unpaved road at Fort Irwin NTC, California, a site in the Mojave Desert (35°11'44" N, 116°45'28" W); a burned site in the firing range of Fort Bliss, New Mexico, representing the Chihuahuan Desert (32°28'33.0" N, 105°55'03.6" W); a burned and grazed area in Orchard Training Area (OTA), Idaho, representative of the northern Great Basin (43°16'47.1" N, 116°13'50.2" W); and a burned and graded field at Yakima Training Center (YTC), Washington, in the Lower Columbia Basin (46°37'11.1" N, 120°07'29.3" W).

A grid of seventy-two 0.25 m² plots was set up at all sites. For the first Fort Irwin NTC site, OTA, and YTC the grid was a rectangle of 6 by 12 plots. The shape of the disturbed areas at the Fort Bliss and second Fort Irwin NTC sites was small and irregular, and so the arrangement of plots was not in a rectangular grid. In the first Fort Irwin site, the 72 plots were in blocks of six, while at the other four sites, the plots were arranged in blocks of four. Each plot was 0.5 by 0.5 m (0.25 m²) and positioned in the upper left-hand corner of a 2 by 2 m cell, to provide walking space between plots. Metal stakes 45 cm long were in the upper left-hand corner of each plot, and were color-coded with spray paint according to treatment.

Treatments at the first site were inoculation (inoculated, not inoculated) crossed with watering (no watering, watering monthly, watering bimonthly). Treatments at the other sites were inoculation (inoculated, not inoculated) crossed with application of tackifier (tackified, not tackified). The tackifier is a glue-like substance used specifically to consolidate bare soil surfaces against erosion, and was intended in these studies to hold the amendment in place in the plots. Treatments were assigned randomly to each block (of 6 or 4 plots) before arriving in the field. In the first site, small berms were constructed out the soil around each plot, so that the sample area was in a shallow basin. This was done to prevent overland run-off when watering. At the other four

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sites, no berm was established. Each plot was thoroughly trampled, kicked, and scraped by foot at OTA, YTC, and Fort Bliss, to disrupt any potential crust that had come in since disturbance was removed at these sites. The Fort Irwin NTC abandoned road area was not so disturbed because we did not want to cause erosion to take place on the site, which had a slight grade and was considered especially sensitive to trampling disturbance.

Soil samples were collected after disturbance but before treatments were applied from 12 of the 72 plots at each site. These were for the purpose of establishing a baseline measure of chlorophyll *a* in the disturbed plots. Three blocks of plots were chosen by random sample, with each of the four plots in the block being sampled with a composite of five teaspoons of soil. Three larger composite soil samples were taken from the interspaces between plots so that soil chemistry could be determined for each site.

Amendments were applied after plots were laid out, disturbed, and sampled. Each plot received 2.5 g of cyanobacteria-treated hemp fabric pieces. This is the equivalent of 10 g/m². In the first Fort Irwin study, hemp inoculum was dusted with soil by hand from the surrounding area, to help keep the inoculum in place.

At the other four sites, Soil Cement[®] tackifier was applied at a rate of 0.60 liters/m². The tackifier was diluted with water in a 1:2 tackifier:water mix. The biocide in the soil cement was deactivated by mixing with a sodium hypochlorite solution (3 ml Chlorox[®] bleach/gallon). The biocide is a weak antibacterial agent (chloromethyl/methyl isothiazolone), but may be inhibitory to cyanobacteria.

The first Fort Irwin NTC site was sampled at 6, 12, and 18 months after inoculation. The other four sites were sampled only once according to the following schedule: Fort Bliss, July 2000 (7 months after inoculation), Fort Irwin NTC, September 2000 (9 months), YTC, October 2000 (11 months), OTA, November 2000 (12 months). At the time of sampling, each plot was examined for the presence of hemp fabric pieces. The frequency of fabric pieces in twenty 10x10 cm squares in the centre of each plot was recorded. Each plot was then sampled in a grid consisting of 9 sample points. A teaspoon of soil was collected from each point, and composited in a single whirl-pak bag.

Soil Analyses

Soil chemical and physical analyses were conducted by the Soil Testing Laboratory at Brigham Young University using standard methods (SOIL CONSERVATION SERVICE 1972, SOIL SURVEY STAFF 1962). Analyses included percent gravel, soil texture (hydrometer method of BLACK 1965), pH (saturated paste), electrical conductivity, and percent organic matter (Walkley-Black method). Nitrate-N, calcium, magnesium, and sodium levels were determined from soluble extracts. Phosphorus and potassium were extracted using sodium carbonate via the Olsen method (standard for alkaline soils). Sodium absorption ratio (SAR) was calculated using levels of calcium, magnesium, and sodium.

Statistical Analyses

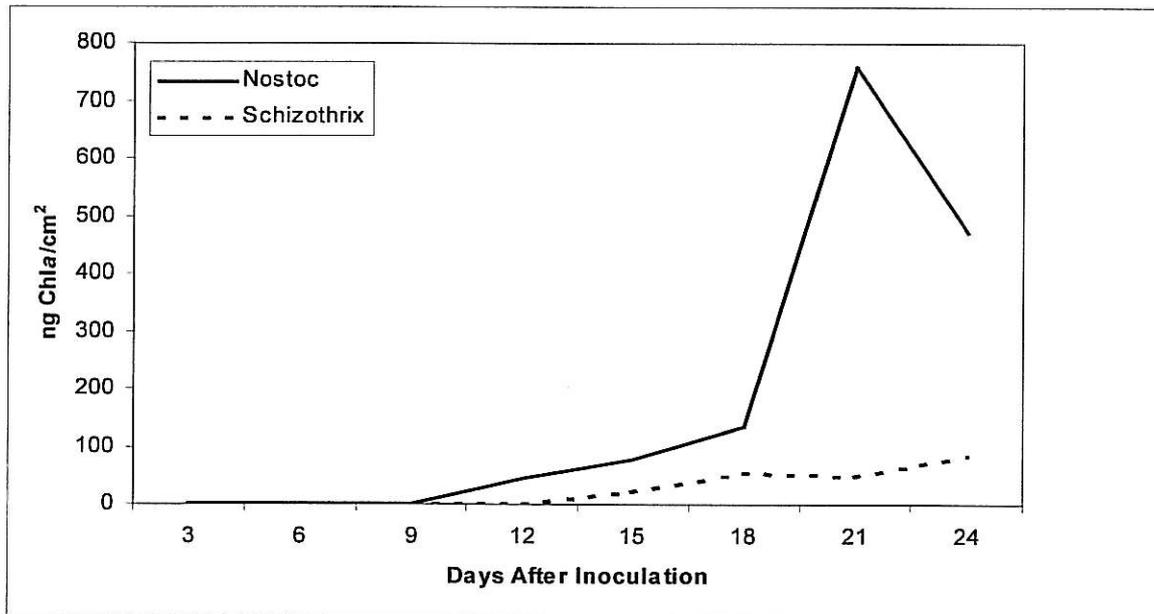
All statistical tests were run using SPSS statistical software. The Student's t-test and Kruskal-Wallis test were run on the data from the roof-top light experiment. Multifactor analysis of variance (ANOVA) was used to examine the effects of field treatments on chlorophyll *a* concentrations, with blocks used as a random factor nested in the interaction of treatments. Inoculation, watering, and tackifier treatments were considered to be fixed factors. A one-way alternative hypothesis was used in assessing the inoculation treatment (mean chlorophyll *a* is higher in inoculated plots). The watering and tackifier treatments were non-directional.

Results

Development of Amendment

Previous studies of alginate-pelletized cyanobacteria indicated that cyanobacteria may not be able to escape the pellets. In developing the current amendment, it was thought that cyanobacteria grown on the exterior of a substrate would have little trouble escaping the substrate, if the cyanobacteria survived desiccation. Several substrates were examined, including: kenaf, cheesecloth, burlap, cotton fabric (coarse and fine weave), hemp/cotton fabric blend, 100% natural hemp fabric, and a plastic stabilizing fabric called Bioblock[®]. Of these, only the hemp and hemp/cotton blend gave good results (mean growth over 50 times as high as other substrates).

Fig. 3. Viability of *Nostoc* species and *Schizothrix calcicola* from amendment stored 32 and 28 months, respectively.



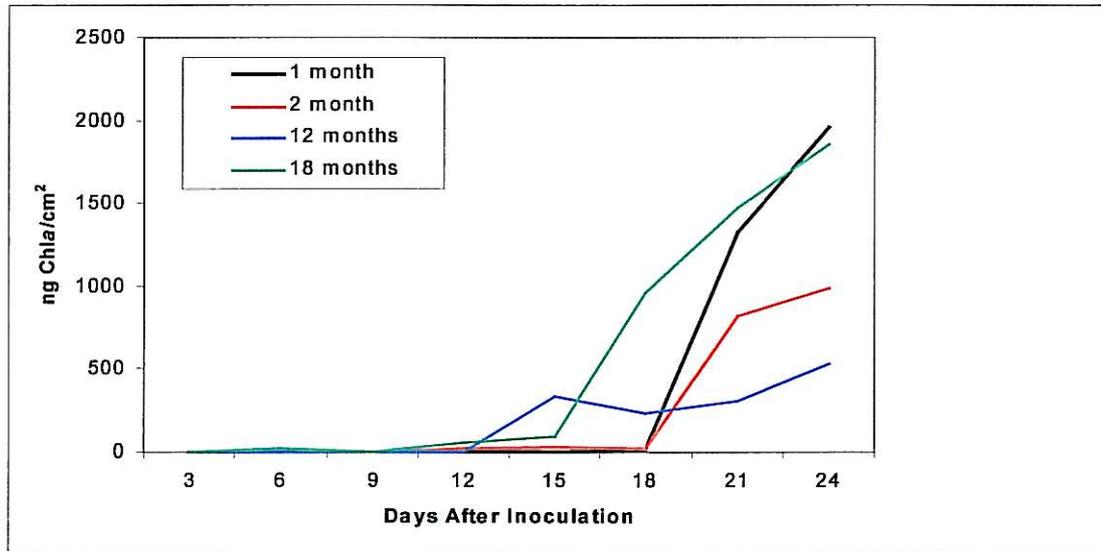
Optimising Growth in Liquid Media

All optimisation experiments utilized *Microcoleus vaginatus* (JT-15). Our initial experiment tested growth in liquid media (Z8, Z8+, BG-11, BG-11+) with various substrates (sand, glass beads, no substrate). Differences in this test were small, but indicated that vitamin enrichment improved growth and that sand was not a suitable substrate. Glass beads seemed to improve growth over no substrate in this experiment. The second experiment in this series tested the several liquid media (Z8+, BG-11, BG-11+, CT) against substrates (glass beads, soft agar, no substrate). The soft agar gave very poor growth regardless of medium type (Fig. 4). Culture flasks with glass beads gave similar levels of growth in all four media, with slightly higher growth in CT medium. The best growth was achieved in liquid media with no added substrate, with high levels of growth in both Z8+ and CT (Fig. 4). Unfortunately, the samples for chlorophyll extraction for the CT treatment were lost. We subsequently compared only Z8+ and CT media, with and without glass beads as a substrate. In this final test, growth was equal in Z8+ and CT, and best without glass beads.

In every one of the above experiments, we found that vitamin enrichment improves growth of *M. vaginatus*. Z8+ and CT appeared to give better growth than BG-11 or BG-11+. Substrates did not enhance growth.

Fig. 4. Growth of *M. vaginatus* (JT-15) in four different liquid media and with three substrates. Note that the samples for chlorophyll *a* extraction were lost for the CT/no

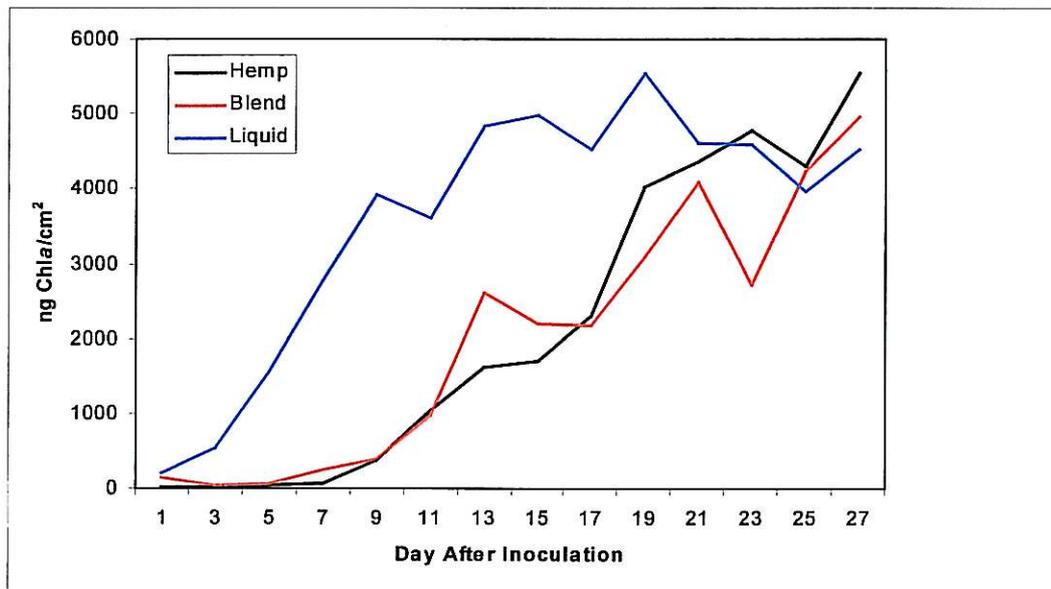
Fig. 2. Viability of *M. vaginatus* (JT-15) amendments 1, 2, 12, and 18 months after production.



We also tested two other batches of amendments that were prepared early in the project. A batch of *Schizothrix calcicola* (JT-24) was prepared in October 1998 and tested 28 months after preparation. The amendment was still viable, but did not give ideal growth (Fig. 3). Growth with this strain was highly localized due to its very limited motility. Dark green patches developed around the fabric pieces, but the random sampling often missed these patches. A batch of *Nostoc* (JT-7) was prepared in March 1999 and tested 32 months after preparation. This amendment was likewise viable (Fig. 3), and due to the mobility of the hormogonia, was more successful at moving away from the hemp pieces and onto the surrounding sand surface.

Both hemp and hemp/cotton blend were used to manufacture cyanobacterial amendments. *Microcoleus vaginatus* (JT-15) was used to make both amendments, which were tested simultaneously with fresh-growing cultures of *M. vaginatus* in liquid. *M. vaginatus* from actively growing cultures grew quickly on the sand, but soon the other two amendments gave comparable growth (Fig. 1). Indeed, by day 13 the growth from both fabric-based inocula was equivalent to that seen with alginate-pelletized cyanobacteria after 3 months of similar testing (Buttars et al. 1998). A patent for this product has since been obtained (US 6,228,136 B1). All subsequent studies used the 100% natural hemp, since this substrate gave slightly better cyanobacterial growth.

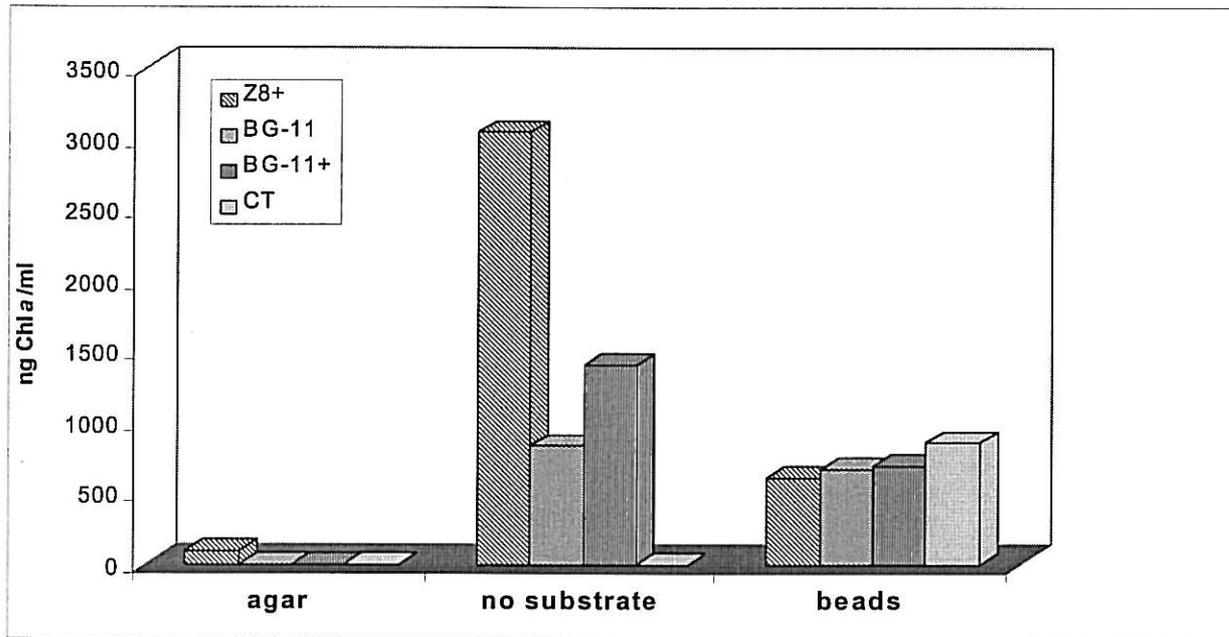
Fig. 1. Growth of *M. vaginatus* (strain JT-15) from dry amendments on hemp and hemp/cotton blend fabrics, with growth from actively growing cultures in liquid serving as a control.



Viability Studies

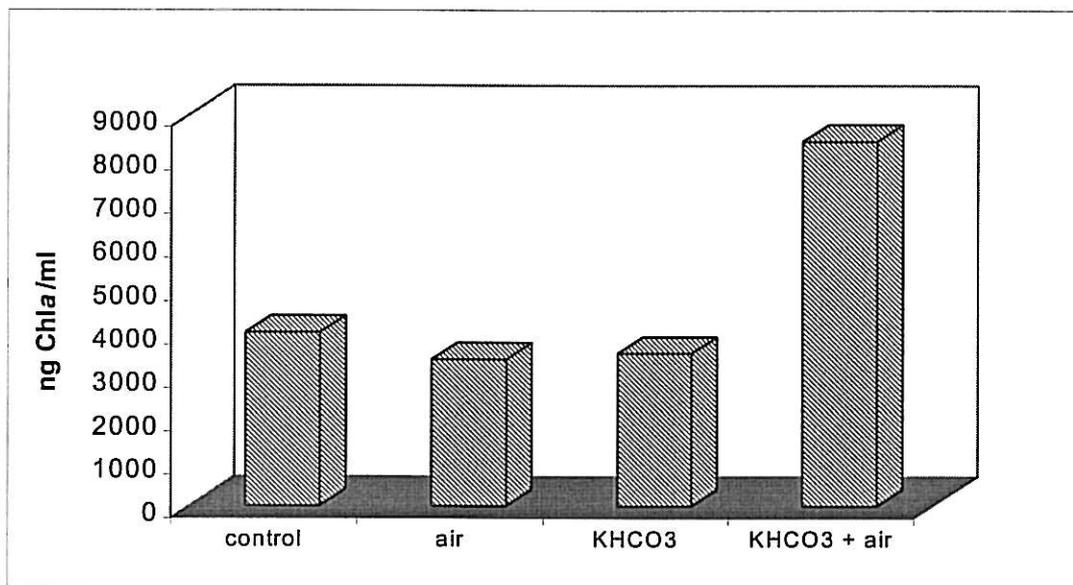
An important question for development of a commercially available product is the viability of the product in storage. A large batch of *M. vaginatus* (JT-15) amendment was produced in April 2000, and put into storage for the purpose of testing viability. Experiments were run on this batch at 1 month, 2 months, 6 months, 1 year, and 18 months after production. The run at 6 months became contaminated and results were discarded. In all cases, there was a lag period of 12-18 days before chlorophyll *a* showed significant increases. Data from the other periods indicated that the hemp-immobilized amendment retained its viability for at least 18 months, with no apparent loss in viability (Fig. 2). Growth of 2 month-old and 12 month-old inocula was somewhat lower than growth of 1 month-old inoculum. However, 18 month-old amendments gave a very similar final outcome after 24 days on the sand (Fig. 2).

Fig. 4. Growth of *M. vaginatus* (JT-15) in four different liquid media and with three substrates. Note that the samples for chlorophyll *a* extraction were lost for the CT/no substrate combination. Photo records of the experiment indicate growth in this treatment was similar to that in the Z8+/no substrate flask.



Aeration and added KHCO_3 had a synergistic effect on growth. Either alone did not enhance production, but together growth was nearly doubled (Fig. 5).

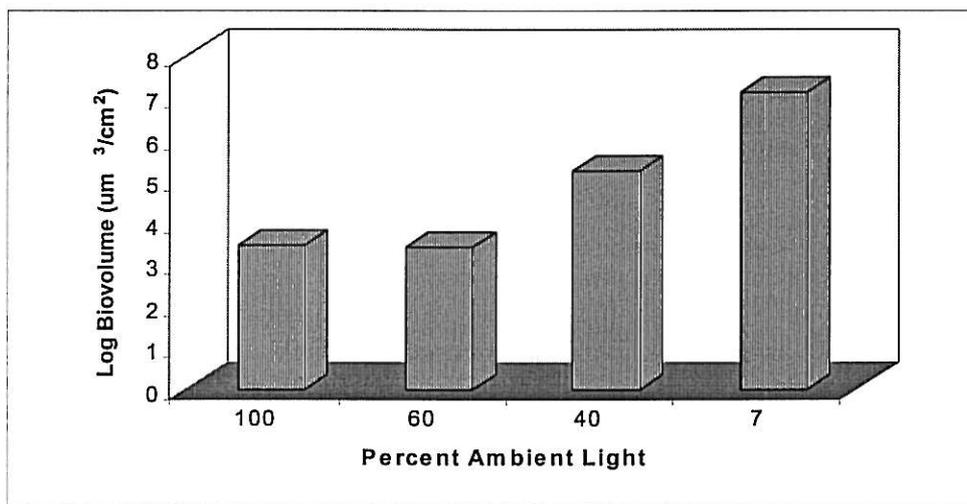
Fig. 5. Growth of *M. vaginatus* (JT-15) in the presence of aeration, added KHCO_3 , aeration plus added KHCO_3 , and a control with neither aeration nor added KHCO_3 .



Effects of High Intensity Irradiance

Cyanobacterial biovolumes measured on day 14 of the roof-top experiment demonstrated that high light has a deleterious effect on cyanobacterial survival and growth (Fig. 6). Biovolume was clearly highest at the lowest light intensity. We noted that in the two highest light intensities the hemp appeared bleached within two days of starting the experiment. We suspect that the cyanobacteria must move off of the hemp into the soil, where they are protected from high light, or it becomes bleached by UV light.

Fig. 6. Final biovolumes of *M. vaginatus* (JT-15) two weeks after inoculation in the roof-top experiment. Cyanobacteria were grown outdoors in the absence of shade with screens to achieve different light levels. Sand was kept damp for the duration of the experiment.



Field Studies

First Fort Irwin Study

The initial results from Fort Irwin were promising (Table 1). Mean chlorophyll *a* was actually higher in unwatered plots than in watered plots both at 6 months and 12 months following inoculation. This may mean that a small amount of watering not accompanied by cooler, humid weather actually causes the cyanobacteria to die off. If the cyanobacteria were to become physiologically active and then dryness occurred too quickly, this could lead to death and breakdown of chlorophyll *a*.

Chlorophyll *a* was significantly higher in inoculated plots at both 6 and 12 months, with the mean values in inoculated plots averaging about 20-30% more than values in uninoculated plots. It is especially interesting in light of the fact that the collectors at Fort Irwin picked noticeable pieces of fabric out of the samples they took, and the workers at John Carroll University screened the soil before analyzing chlorophyll *a*. This means that the increase in chlorophyll *a* noted in the amended plots is likely due the cyanobacteria that moved off the fabric into the soil. The alternate explanations are 1) the hemp alone stimulated growth in the plots, or 2) the significant result is artifactual and not really indicative of higher

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random chance). Although we have no way of assessing the first alternative with the design of the current experiment, we consider it less likely than that the cyanobacteria moved off the fabric and grew in the soil.

We sampled again at 18 months, but the affect of inoculum was no longer significant. The block effect was not significant at any time.

Tab.1. Mean chlorophyll *a* for the inoculation plots in ng/g soil. Means \pm SE are given for each watering-inoculation combination, and summarized for main effects. Watering was not a significant effect ($p = 0.054$ at 6 months, 0.353 at 12 months), but inoculated plots had significantly higher chlorophyll *a* values than uninoculated plots ($p=0.001$ at 6 months, 0.006 at 12 months). Interaction between watering and inoculation was not significant.

	No amendment	With amendment	Mean
<i>6 month trial</i>			
No watering	589 \pm 76	684 \pm 68	637 \pm 51
Low watering	496 \pm 69	711 \pm 101	604 \pm 62
High watering	523 \pm 74	558 \pm 70	541 \pm 51
Mean	536 \pm 42	651 \pm 47	
<i>12 month trial</i>			
No watering	314 \pm 38	363 \pm 35	338 \pm 26
Low watering	254 \pm 25	317 \pm 47	286 \pm 27
High watering	240 \pm 29	363 \pm 48	302 \pm 29
Mean	270 \pm 18	348 \pm 25	

Subsequent Studies

The soils of these four sites were very distinct (Table 2).

Tab.2. Soil texture and chemistry for four study sites. Means for Yakima Training Center, Orchard Training Area, and Fort Irwin National Training Center are based on 3 samples collected from within each grid. Data for Fort Bliss are based on 5 samples collected from within the area during a previous study. SAR is sodium absorption ratio.

Soil Parameter	Fort Irwin	Fort Bliss	Orchard	Yakima
Percent Sand	84.0%	44.1%	35.8%	51.9%
Percent Silt	8.7%	35.8%	41.6%	38.8%
Percent Clay	7.3%	20.2%	22.6%	9.3%
Percent Organic Matter	0.41%	1.48%	3.11%	2.80%
pH	7.37	7.67	6.59	6.24
Electrical conductivity (uS)	0.39	0.72	0.90	0.41
Phosphorus (ppm)	6.21	16.20	47.01	28.09
Nitrate-N (ppm)	3.21	5.58	14.17	14.94
Soluble Ca (ppm)	63.5	134.0	125.1	41.2
Soluble Mg (ppm)	12.3	17.6	45.7	15.9
Soluble K (ppm)	20.2	238.0	109.2	27.6
Soluble Na (ppm)	11.31	6.00	64.08	18.64
SAR	0.36	0.13	1.23	0.63
Chl <i>a</i> (ng/g soil)	1441	1258	9519	3587

The second, Fort Irwin NTC site had a loamy sand low in both phosphorus and nitrate and slightly above neutral in pH. The other three sites had finer soils, all classified as loam. Phosphorus and nitrate were also higher in all three, with OTA and YTC having markedly higher nutrients and below neutral pH.

At YTC we compared chlorophyll *a* in the burned and graded area with adjacent unburned islands. Chlorophyll *a* was significantly higher in the unburned area (mean of 10 samples = 13,774 ng chl *a*/g soil) than in the burned area (mean of 10 samples = 3,587 ng chl *a*/g soil) before treatment. The area was thoroughly disturbed by the combination of burning and mechanical disturbance.

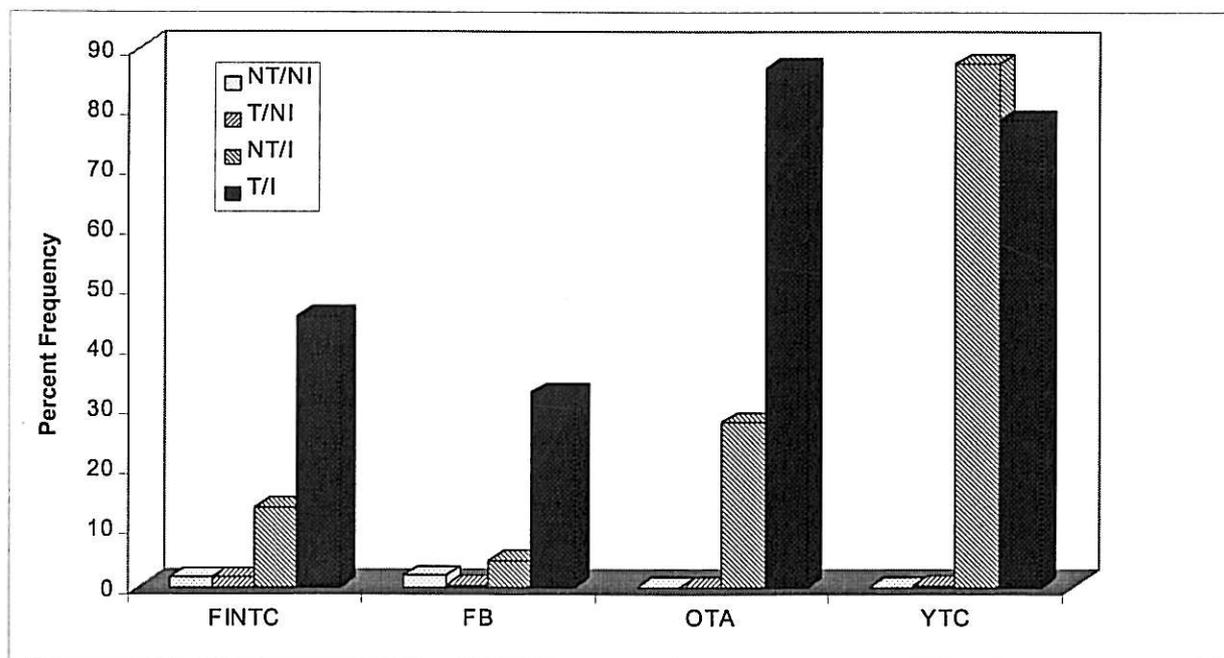
a/g soil) before treatment. The area was thoroughly disturbed by the combination of burning and mechanical disturbance.

At the other three sites, comparisons were made among inoculation plots before inoculation. At all three sites, the plots "to be inoculated" were not significantly different than those "not to be inoculated."

The OTA site had unexpectedly high chlorophyll *a* concentrations. Although this area was only recently fenced to keep out cattle, and was recently burned as well, we found evidence of fairly substantial microbiotic cover, particularly with regard to mosses. The foot trampling applied to all plots did have a significant effect (trampled 9,519 ng chl *a/g* soil, untrampled 14,754 ng chl *a/g* soil, $p=0.001$), but even with trampling the chlorophyll *a* was higher than at any other study site in this study. Indeed, the value compares favourably with chlorophyll *a* concentrations in undisturbed plots we have seen in the Mojave, Great Basin, and Chihuahuan Deserts.

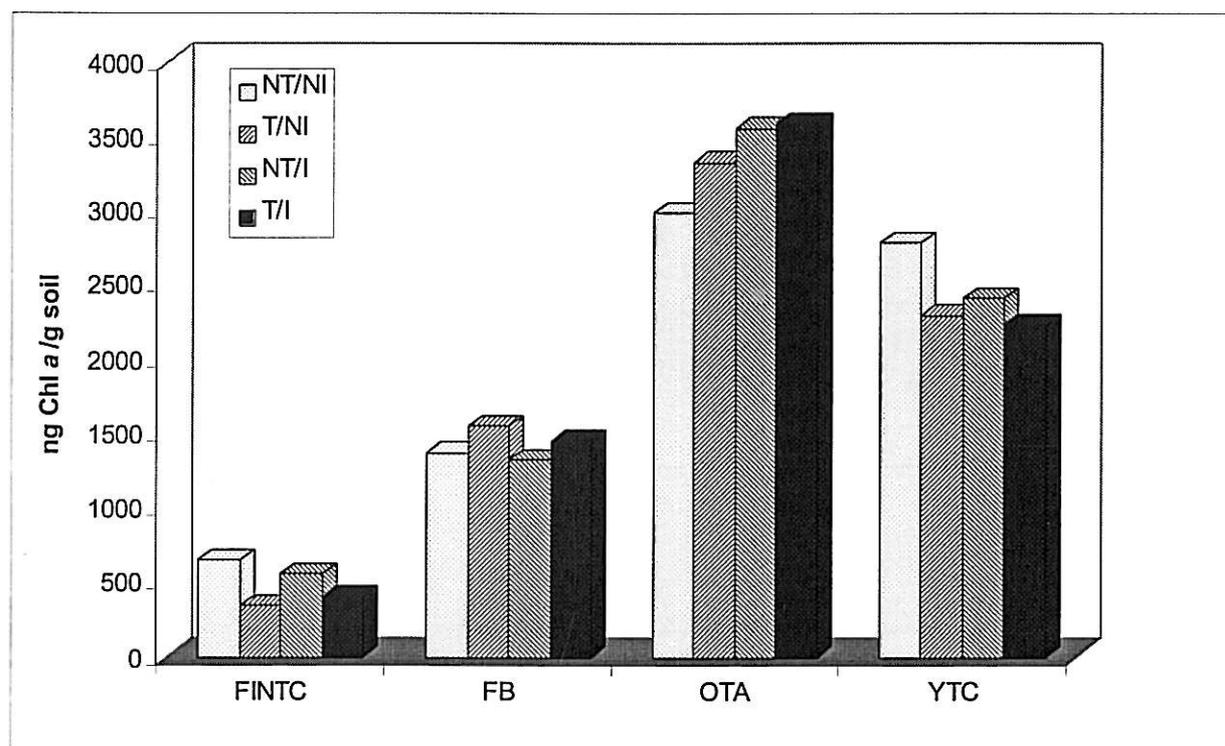
Retention of the inoculum was usually significantly higher in plots receiving both tackifier and inoculum. This was so for Fort Irwin NTC, Fort Bliss, and OTA, where most of the inoculum in non-tackified plots was lost (Fig. 7). In all sites except Fort Irwin NTC, the inoculated, non-tackified plots had significantly higher frequency of fabric pieces than the uninoculated plots, indicating that at least some of the amendment was not lost even as much as a year after inoculation. Fort Bliss had the greatest loss of inoculum (Fig. 7). The soil at Fort Bliss slakes quickly, causing extensive overland flow of water during heavy storms. Thus, it is not surprising that the inoculum was moved around in these plots. Fort Irwin NTC also had a good deal of loss, and it was noted that some erosion had occurred in the plots when they were sampled at the end of the experiment. YTC was surprising in that tackifier had no effect on retention. Where fabric pieces appear in uninoculated plots (FINTC, FB, and YTC), we assume they were washed or blown there from neighbouring plots.

Fig.7. Percent frequency of quadrats with hemp amendment still detectable at the conclusion of field trials. NT=no tackifier, NI=no inoculum added, T=tackifier used during inoculation, I=inoculum added, FINTC=Fort Irwin National Training Center, FB=Fort Bliss, OTA=Orchard Training Area, YTC=Yakima Training Center.



At every site, there were no significant differences in chlorophyll *a* due to inoculation or application of a tackifier. Although treatment means differed slightly at each site, they did not vary in a consistent way (Fig. 8). OTA and YTC had higher mean chlorophyll *a* than either Fort Irwin NTC or Fort Bliss, likely due to milder climate and greater abundance of mosses and green algae.

Fig.8. Phototrophic biomass as estimated by chlorophyll *a* at the conclusion of field trials. NT=no tackifier, NI=no inoculum added, T=tackifier used during inoculation, I=inoculum added, FINTC=Fort Irwin National Training Center, FB=Fort Bliss, OTA=Orchard Training Area, YTC=Yakima Training Center.



It is interesting to note that the mean chlorophyll *a* in OTA plots was considerably lower than it had been the year before. We suspect this is due at least in part to the rigorous trampling treatment we provided at the time of inoculation. The mosses and algae were still present in the soil after disturbance the year before, but likely died out with the disruption applied at that time. The trampling disturbance appeared to have lowered chlorophyll *a* content at YTC as well. Mean chlorophyll *a* was slightly higher at the Fort Bliss site at the conclusion of the field trial, and slightly lower at Fort Irwin. These changes are likely attributable to annual variations in climate. We suspect hot desert soils show a greater fluctuation from year to year than the soils in more mesic and temperate shrub-steppe. However, climate may have played a role in the interannual differences noted in those sites as well.

The hemp-immobilized cyanobacterial amendment developed in this project shows promise in a number of ways. First, growth from this amendment in laboratory studies greatly exceeds growth observed for alginate-pelletized cyanobacteria (BUTTARS et al. 1998). The cyanobacteria appear to survive the drying process better, and escape more easily to soil surfaces. Second, the product appears to have long-term viability in storage. If stored in the dark at room temperature, survival is not reduced even after 18 months. Even 32 month-old product (with *Nostoc*) retained its viability. This long-term viability is not surprising, given reports of even longer-term viability in herbarium preparations of cyanobacteria (DOR & DANIN 2001, VENKATARAMAN 1969) and eukaryotic algae (BUZER et al. 1985, LEESON et al. 1984). Third, this methodology is adaptable to many different strains. *Microcoleus vaginatus* has been our major focus due to its importance in initial crust formation and soil stabilization (BELNAP 2001b, BELNAP & GARDNER 1993, MAZOR et al. 1996). However, other strains of the same species and other species in different genera could be treated in the same way with similar outcomes. Our limited work with *S. calcicola*, *M. steenstrupii*, and *Nostoc* indicates that immobilization potential and viability are not limited to *M. vaginatus*. Fourth, the growth of the cyanobacteria directly on the fabric substrate seems to speed production of the cyanobacteria. The soil strains we have studied do not grow well in liquid media, and the time required to produce a batch of inoculum is shorter than the time required to obtain a dense liquid culture. Finally, the product is relatively inexpensive to produce.

Despite the promise in this technology, there are several obstacles to creating a commercial product at this time. We need to be able to increase the speed with which cultures are taken from isolate stage on agar to large liquid batch culture. Field studies were not successful in this study, and indicate that improvements in application of the inoculum are needed. It is vital to demonstrate that cyanobacterial amendments can indeed significantly stimulate crust recovery.

We demonstrated in this study that *M. vaginatus* growth rates in liquid culture could be increased with addition of vitamins, aeration, and potassium bicarbonate. Aeration likely increased growth due to increased carbon dioxide availability. Aeration was especially effective with the addition of the KHCO_3 , which buffered the media and provided a source of inorganic carbon for photosynthesis. The filaments do not attach well to the sides of the flasks, and tend to concentrate in small, slow-growing masses. Providing additional surface complexity in the liquid did not improve growth, and yet did make harvesting the cyanobacteria more difficult. Soft agar has been used successfully to grow chytridomycete fungi (AMON & ARTHUR 1979), but did not enhance growth of *Microcoleus*. Even though the agar is very diffuse, *Microcoleus* does not appear to be mobile in the agar. The agar may slow air exchange between the liquid and the atmosphere as well. Soft agar may be good for other cyanobacterial taxa, such as *Leptolyngbya*, which can penetrate 1.5% hard agar. In a recent paper, FISCHER et al. (1997) found that it was possible to grow cyanobacteria on white cotton fabric in vertical incubation chambers. This methodology might be useful for growing soil cyanobacteria as well, and could provide an additional means for getting even growth of cyanobacteria on the hemp fabric used in our application.

Four likely explanations exist for the failure of the amendment to stimulate crust recovery in the field studies. First, the inoculum is light-weight, and may have been blown away by wind or washed away by overland flow of storm water. It was evident that this is a problem when we examined the retention of the fabric on the soil with and without a tackifier. It not only stabilized the soil in our plots, but successfully glued the hemp fabric pieces to the soil. Although it kept the hemp in place, we are concerned that it may have reduced infiltration of rainwater, or even prevented the cyanobacteria from moving into the soil. This fear is supported by the fact that the plots with immobilized hemp pieces did not have higher chlorophyll *a* concentrations than soils that were not immobilized and showed loss of hemp

pieces over time. It appeared from the first Fort Irwin NTC study that dispersing a shallow layer of soil over the hemp pieces was effective at retarding loss of amendment. Since this is the only field experiment that showed positive effects, we will to try this method of retarding amendment loss in future trials.

Second, it is possible that high light, or the UV component in high light, was damaging to the cyanobacteria before the trichomes could move off of the hemp into the soil. This would be especially true for the *Schizothrix* amendment used at Fort Bliss, as this taxon is immotile even in ideal situations. From our work with cyanobacterial cultures, we know that *M. vaginatus* is very sensitive to light, especially when not in dense cultures. Indeed, light sensitivity in cyanobacteria is common, and has been observed in other taxa by other workers (FISCHER et al. 1997). If the cyanobacteria are quickly killed by UV radiation or photo-oxidized by the high light intensities and high temperatures experienced on the surface of desert soils, then the amendment would naturally be ineffective. Some cyanobacteria make UV-screening pigments (scytonemin) in their sheaths (GARCIA-PICHEL & CASTENHOLZ, 1991, 1993). These yellow sheath pigments are present in some desert cyanobacteria (*Nostoc*, *Scytonema*, *Tolypothrix*, *Calothrix*), but not in the nonheterocystous filamentous taxa. *Microcoleus*, *Phormidium*, *Schizothrix*, and *Leptolyngbya* are known to live below the surface of the soil in the desert, and migrate to the surface when soil moisture is sufficient and light intensity is diminished (DOR & DANIN 2001, GARCIA-PICHEL & CASTENHOLZ, 2001). They are able to use the soil itself to achieve proper light intensities, and so can be low-light adapted even in this most highly lighted environment. Our roof-top experiments confirm that death of the cyanobacteria can occur quickly in high light, even when moisture is provided immediately after exposure to the environment. Improved success may be obtained if the hemp amendment is worked into the surface 1cm layer of soil when it is applied.

Third, it is possible that the field studies were not successful due to improper climatic conditions. Although all plots were inoculated in late fall/early winter, when temperatures are milder and precipitation more likely, it is still possible in desert reclamation studies to have extended periods of unfavourable conditions. If in our studies a heavy rain did not fall for several months, it is possible that the amendment was either blown off the site or the cyanobacteria killed in place by heat and light before the rain came. Our laboratory studies indicate a long lag period is present before significant growth occurs. This may mean that an extended cool, moist period (15-20 days) is required for the amendment to be successful. A moist period of this duration is rare in the hot deserts (the south-west USA including Fort Bliss and Fort Irwin NTC). Indeed, precipitation at Fort Irwin NTC is very limited and sporadic every year, and combined with very high temperatures, this site may simply not be conducive to heavy crust development. JOHANSEN et al. (2001) found that crust development at Fort Irwin NTC was indeed limited when compared to other desert regions. OTA and YTC receive relatively higher amounts of precipitation, and one would expect good crust development in these sites. OTA, although burned and grazed, demonstrated considerable recovery of mosses and green algae, and this may have masked any effect of the amendment.

Fourth, two of the sites had very little cyanobacteria in undisturbed crusts nearby. OTA and YTC may not support abundant populations of cyanobacteria. Very limited cyanobacterial development was found in crusts in the Columbia Basin in an earlier study (JOHANSEN et al. 1993), and efforts to stimulate cyanobacterial growth in both sites may have been doomed from the start by their unsuitability for cyanobacterial growth. Soil pH may be partly responsible for limited cyanobacterial growth (OTA and YTC were slightly acidic), but differences in pH seem relatively minor to us (Table 2).

We now have over 100 strains of cyanobacteria from desert soil crusts in culture. These strains are from numerous areas in the western United States, and include multiple isolates of all of the genera we regard as common and important to ecosystem function. Our

laboratory experiments demonstrate that trichome mobility is an important feature for successful establishment of a cyanobacterial mat. Hence, *M. vaginatus*, which is highly motile, is still considered our best candidate species. It is possible that in the future we may wish to develop a mixed inoculum with a combination of heterocystous strains (for soil fertility) and motile oscillatoriacean strains, such as *M. vaginatus*. Despite their universal occurrence, the narrow trichomed taxa attributed to the genera *Leptolyngbya* and *Schizothrix* may not be ideal for reclamation technologies due to their very limited motility. *M. steenstrupii* and related species (*M. chthonoplastes*, *M. paludosus*, *M. sociatus*) reported from soil crusts (BELNAP & LANGE 2001, ROSENTRETTER & BELNAP 2001) probably function to stabilize the soil in the same manner as *M. vaginatus*. However, like *Leptolyngbya* and *Schizothrix*, they have much more limited motility, and so are not suitable either.

Summary

We developed a cyanobacterial amendment with *M. vaginatus* that was superior to alginate-pelletized cyanobacterial amendments (BUTTARS et al. 1998) in both viability and escapability. The amendment gave excellent results in laboratory experiments, being nearly equal to inoculation with liquid cultures of actively growing cyanobacteria. The *Microcoleus* amendment remains viable in storage for at least 18 months. Viability testing of *Schizothrix* and *Nostoc* amendments at 28 months and 32 months, respectively, showed that these taxa also retained their viability. *Microcoleus* grew best in Z8+ and CT media with added aeration and KHCO₃. The addition of substrates in liquid media (sand, glass beads, agar granules) did not appreciably increase growth, but did make harvesting of the cyanobacteria more difficult.

Field tests were conducted at five different sites, two at Fort Irwin NTC (Mojave Desert), one at Fort Bliss (Chihuahuan Desert), one at Orchard Training Area (Great Basin Desert), and one at Yakima Training Center (Columbia Basin). Only the first test at Fort Irwin gave significant results. This test differed from the other four in that the amendment was slightly covered with soil after inoculation, and we suspect this may have protected the cyanobacteria from damage by direct full sunlight. Roof-top experiments confirmed that direct sunlight was damaging. Growth was best at less than 10% full sunlight. In the first field test at Fort Irwin, three watering regimes were applied. Phototrophic biomass was highest in plots receiving no additional water.

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Morphological and molecular characterisation of four *Nostoc* species isolated from desert soils

Abstract

Soil samples from throughout the western United States (California, Utah, Nevada, New Mexico and Arizona) were collected by Dr. Jeff Johansen and used for the isolation of strains. The description of sites is on the web site (<http://www.jcu.edu.mcp>). Over 100 algal strains were isolated (*Nostoc*, *Microcoleus*, *Leptolyngbya*, *Phormidium*, *Scytonema*, *Tolypothrix* and others). Four strains of *Nostoc* species from the desert soil of western USA were chosen for the study. Those four *Nostoc* strains were described according the polyphasic approach (morphological and molecular characterisations).

The morphological characteristics of four-studied *Nostoc* were compared with the species of *Nostoc* described in the literature (GEITLER 1932, DESIKACHARY 1959, DROUET 1978, MOLLENHAUER 1994). The observed desert *Nostoc* species can be forced into the described species such as *N. punctiforme*, *N. paludosum*, *N. commune*, *N. microscopicum* and *N. muscorum*, but they did not fit those taxa well when all characters (including ecology) were used.

The sequences of the strains CM1-VF10, CM1-VF14, phycobiont of *Collema tenax* and JT2-VF2 were aligned with published *Nostoc* sequences from GenBank and *Nostoc* sequenced by other workers from J.R. Johansen laboratory, John Carroll University, Ohio, USA. Sequence identity was compared. *Cylindrospermum* sp. and *Anabaena "spiroides"* from GenBank were used as outgroups. All aligned sequences were analysed using PAUP* 4.0b (SWOFFORD 1998) to conduct heuristic searches (100 replicates each) for most parsimonious trees (SWOFFORD 1998).

One of the observed strains (JT2-VF2) can be described according to the botanical and bacteriological nomenclature like a new species for the science. The other three strains (CM1-VF10, CM1-VF14 and phycobiont of *Collema tenax*) could be describe like a new species only according to the botanical nomenclature because the sequence similarity was higher than 97%. The boundary between two different species is 97,5% of sequence similarity according to bacteriological approach (FLECHNER ET AL 2002).

According to morphological and molecular characteristics the phycobiont of *Collema tenax* is not *N.commune* like is mentioned in some lichenological literature.

Introduction

Cyanobacteria are one sub-group of Gram negative bacteria with oxyphototroph metabolisms and plant photosynthetic apparatus. Cyanobacteria often create the dominant in the terrestrial and aquatic environments (PAERL 2000, OLIVER & GANF 2000, BELNAP 2001) and are important primary producers in the number of food web. They have similar 16S and 5S rRNA to other members of eubacteria (CASTENHOLZ & WATERBURY 1989). Their nomenclature contains an extensive number of different genera and species, according to our current knowledge and the approaches. An entirely satisfactory classification of the cyanobacteria is difficult to achieve because of widely divergent views regarding what constitutes taxonomically valid criteria and how these may be recognized (BOLD & WYNNE 1985).

The oldest approach is traditional botanical nomenclature. This approach is based upon the morphological or cytological characteristics like a dimension and a shape of vegetative cells, heterocytes and akinetes. BORNET & FLAHAULT (1888) began the morphologicaly – based taxonomy for the heterocytous blue-green algae (Nostocales), and

were followed by GOMONT (1892), who proposed the initial classification for nonheterocystous, filamentous cyanobacteria (Oscillatoriales) (PAYNE 2001). 40 years later, Geitler provided a comprehensive taxonomic review that recognized 145 genera of cyanobacteria (GEITLER 1932). Many workers made a lot of revisions of the Geitlerian system. The most different taxonomical system was done by Drouet and Daily (DROUET 1968, 1973, 1978, 1981, DROUET & DAILY 1956). They reduced the number of genera from 145 to 24. This reduction was based on their theory that a numerous of species are actually different ecophenes that represent a small number of species. Drouet's classification system failed because it did not reflect the genetic diversity in nature.

The taxonomic system introduced by Stanier in the 1970s aimed to treat the cyanobacteria in the same way as other prokaryotes. An early step was to convince everyone of the need for a shift in name from blue-green algae to cyanobacteria (WHITTON, POTTS 2000). It was the beginning of the bacteriological approach. The system is based on use of the axenic, clonal cultures and the microbial techniques. This approach has had a lot of representatives who studied the morphological variability of the population in the culture (STAM & HOLLEMAN 1979, RIPPKA ET AL. 1979, 1981). When this approach is used, a lot is lost from the natural diversity because not all algae are able to grow in the controlled environmental conditions of algal collections, or the features used to identify the classical blue-green algal taxa may not be expressed (WHITTON 1992).

Komárek & Anagnostidis (ANAGNOSTIDIS & KOMÁREK 1985, 1990, KOMÁREK & ANAGNOSTIDIS 1986, 1989, KOMÁREK & ANAGNOSTIDS 1999) proposed the almost recent reclassification of the botanical nomenclature system. They arranged organisms according to the various possible combinations of pattern of cell division, arrangement of thylakoids, cell shape and other features. They also pointed to the fact that we are still using the taxonomic criteria established more than 100 years ago, often based on the unstable features (presence/absence of sheath or false branching). We have also used the key books from Europe for the determination of species in the tropic and antarctic regions.

Currently, there is an increasing interest in the molecular taxonomy (molecular approach). Some of the molecular techniques used for the taxonomic studies of bacteria and their results have been applied to resolve some problems of the present state of cyanobacterial taxonomy. "Recent approaches include: DNA-DNA hybridization (WILLMOTTE ET AL. 1997), fingerprinting based upon PCR with primers from short or long repeated elements (RASSMUSSEN ET AL. 1998), classification of clone cultures based upon 16S rRNA sequences (RUDI ET AL. 1997) and amplified ribosomal DNA restriction analysis of the internally transcribed spacer (SCHELDEMAN ET AL. 1999)." (WHITTON & POTTS 2000).

In my opinion it is important to use all of the knowledge and information that has been obtained in the recent past. In other words, put together all approaches: traditional botanical, molecular and bacteriological. In this way we will have the fourth approach – polyphasic. Today is increasing number of studies used the polyphasic approach (LEHTIMÄKI ET AL 2000, LAAMANEN ET AL 2001, FLECHNER ET AL. 2002). We have used the polyphasic approach to study four *Nostoc* species from the desert soil of the western USA.

Method

Isolation of strains

Soil samples from throughout the western United States (California, Utah, Nevada, New Mexico and Arizona) were collected by Dr. Jeff Johansen and used for the isolation of strains. The description of sites is on the web site (<http://www.jcu.edu.mcp>). Over 100 algal strains were isolated (*Nostoc*, *Microcoleus*, *Leptolyngbya*, *Phormidium*, *Scytonema*, *Tolypothrix* and others). All samples were dilution plated on to agar-solidified Z-8 medium (CARMICHAEL 1986), and individual cyanobacterial isolates were made from these plates onto agar

slants Z-8 or N- medium (Z-8 without nitrogen components). N- medium was used for the species from the family Nostocales. This medium supports the production of heterocysts. Cyanobacterial strains were maintained on agar-solidified Z8 or N-media at 7 °C on a 16:8 hr light:dark cycle under fluorescent light (200 $\mu\text{Es}^{-1}\text{cm}^{-2}$).

I chose 4 strains of *Nostoc* (CM1-VF10, CM1-VF14, JT2- VF2) and phycobiont of lichen *Collema tenax* for the morphological and molecular comparison.

Morphological observation

CM1-VF10 and CM1-VF14 from Clark Mountains, San Bernardino County, California, JT2-VF2 from Cadiz Valley, Joshua Tree National Park, California and "*Collema*" *Nostoc* from Canyonlands National Park, Utah were chosen for the morphological observations.

The strains were spread on the N- agar solidified Petri dishes in the 5 repetitions. The morphological characteristics were measured throughout the life cycle from young to senescent stadium. The morphology was measured during a 4 months period. At the beginning characteristics were measured every 3 days, after that every 5 days and later every 14 days. The cultures were kept and examined after 6 months also. The colour, shape of macrocolonies on agar, shape and colour of microcolonies, shape and dimension of vegetative cells, heretocytes, akinetes and hormogonia, the position of heterocysts, akinetes in the filaments, the arrangement of filaments into the mucilage, colour and character of sheath was observed and measured on the Olympus BH-2 microscope and Nikon dissecting scope.

ANOVA analysis

ANOVA tests in the program Statistic were used for the analysis of morphometrical characteristics. Tukey HSD test was used for pairwise comparisons of significant result post-hoc.

Molecular Methods

DNA Extraction

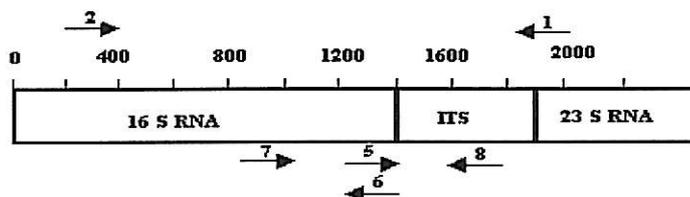
Total genomic DNA was extracted from cultures using the CTAB method as modified by CULLINGS (1992) for the isolation and purification of DNA from mucilaginous organisms (DOYLE & DOYLE, 1987). The extraction of DNA was checked using 1% agarose/ethidium bromide gels. Extracted DNA samples were stored at -20°C for at most 3 months.

PCR Amplification of 16S rRNA Genes

DNA samples were amplified using primer 1 and 2, which are specific cyanobacterial primers. The all amplified product ("log PCR") has 1600 base pairs. The results of amplifications were checked on 1% agarose/ethidium bromide gels. The long PCR was used like a template for reamplification. Three combinations of primer were used: primer 2 and 6, primer 1 and 5, primer 7 and 8. Primer 6 is the reverse complement of primer 5. Primers were ordered from the Midland Certified Reagent Company. The location and position of these primers are show in Fig.1.

Fig.1.

The location and position of the primers.



Primer 2: GGG GAA TTT TCC GCA ATG GG (Nübel et al. 1997)

Primer 6: GAC GGG CCG GTG TGT ACA (Wilmotte, 1994)

Primer 1: CTC TGT GTG CCT AGG TAT CC (Wilmotte, 1994)

Primer 5: TGT ACA CAC CGG CCC GTC (Wilmotte et al. 1993)

Primer 7: AAT GGG ATT AGA TAC CCC AGT AGT C (Nübel et al. 1997)

Primer 8: AAG GAG GTG ATC CAG CCA CA (Wilmotte et al. 1993)

After reamplification were obtained 3 "short" PCR. Primers 2 and 6 were used to amplify the middle region (base pairs 359-1350) of the 16S rRNA. Primers 1 and 5 amplified end region of the 16S rRNA, ITS and the first 30-50 bp of 23 rRNA gene (base pairs 1350- around 2000). Primers 7 and 8 were used to amplify the region overlapping portions of both the newly amplified 16S region and the terminal 16S associated with the ITS region. Primer 7 lies around bp800. This permitted the three sequences to be aligned continuously. Amplification and reamplification were done according to the established protocol for cyanobacteria (BOYER ET AL. 2001).

Cloning

Amplified "short" PCR products were cloned into pCR 4-TOPO plasmids using the TOPO™ TA cloning kit (Invitrogen®). Transformed *E. coli* cells (Invitrogen®) were plated onto Luria Broth plates containing 100 mg/L of ampicillin. Ampicillin restricted the growth of the bacterial contaminants. Additionally, 40 µ L of Xgal (20 mg/ml) and 4 µ L of IPTG (200 mg/ml) were spread onto the plates prior to the plating of transformed cells. Plates were incubated at 37° C overnight. On the following day, plates were refrigerated at 4°C for at least 2 to 4 hours to enhance the detection of blue colonies. Clones containing the cloned fragment appeared as white colonies, whereas clones lacking the gene insert appeared as blue colonies. Colonies containing the gene insert were selected and cultured in 4 ml of LB broth containing 8 µL of ampicillin (50 mg/ml) at 37°C overnight.

Plasmid DNA isolation

Cultured *E. coli* cells containing cloned inserts were harvested by centrifugation at 17000 rpm for 10 minutes. Plasmids were isolated according to the instructions provided in the QIAprep Mini-prep kit®. To verify the presence of a cloned insert in clones, plasmid DNA was digested using EcoR I. Two restriction sites for EcoR I flank both sides of the cloning site in the pCR-4-TOPO vector. Digests were resolved on 1 % agarose/ethidium bromide gels to detect plasmid inserts (PAYNE 2001).

Sequencing

Automated sequencing of at last two clones from each "short" PCR reaction was completed by Cleveland Genomics with the universal infrared primers M13 forward and reverse.

Data Analysis

Complementary sequence was done from the reverse sequence using Omega™. Forward and complementary sequences were aligned using the CLUSTAL W Multiple Sequence Alignment Program (THOMPSON ET AL., 1994). Ambiguities and PCR errors were checked by eye and the corrections according to the chromatograms were done where it was appropriate. Corrected sequences were aligned with published *Nostoc* sequences from GenBank and *Nostoc* sequenced by other workers from J.R. Johansen laboratory, John Carroll University, Ohio, USA (Tab.1). Sequence identity was compared. *Cylindrospermum* sp. and *Anabaena "spiroides"* from GenBank were used as outgroups. All aligned sequences were analyzed using PAUP* 4.0b (SWOFFORD 1998) to conduct heuristic searches (100 replicates each) for most parsimonious trees (SWOFFORD 1998). The following analyses were completed: 1) neighbor-joining using logdet distance metric and equal substitution rates, 2) neighbor-joining using HKY85 distance metric and equal substitution rates, 3) neighbor-joining using HKY85 distance metric and gamma substitution rates, 4) neighbor-joining using Jukes-Cantor distance metric and gamma substitution rates, 5) maximum parsimony using logdet distance metric and equal

substitution rates, 6) maximum likelihood using HKY85 distance metric and equal substitution rates. Support for each tree was subsequently obtained by running bootstrap analyses with 1000 replicates each. A similarity matrix for all strains was constructed using MEGA to compare sequence identity. The tree reported has the maximum likelihood topology, with branch lengths adjusted for distance using an unweighted group average algorithm (PIELOU 1984) for averaging distance among clusters. The graphic program Treeview 32 ver.1.6.1. (PAGE 2000) was used for the reconstruction of cladogrames.

Tab.1. 16S sequences used in this study

strain	accession	origin
<i>Anabaena "spiroides"</i>	AJ293118	Unknown
<i>Cylindrospermum</i> sp.	AJ133163	Soil in greenhouse, Stockholm, Sweden
<i>Nostoc</i> sp. JT1-VF3	Vincent 2001, Xuesong 2000	Desert soil, Joshua Tree NP, CA, USA
<i>Nostoc</i> sp. JT1-VF7	Vincent2001, Xuesong 2000	Desert soil, Joshua Tree NP, CA, USA
<i>Nostoc</i> sp. JT2-VF2	Vincent 2001, Xuesong 2000	Desert soil, Joshua Tree NP, CA, USA
Phycobiont <i>Collema tenax</i>	Vincent 2001, Xuesong 2000	Desert soil, Canyonlands NP, UT, USA
<i>Nostoc</i> cf. <i>microscopicum</i> SAG 2028	AJ344563	Cave in ctarens sandstone, Dragensberg Mts., South Africa
<i>Nostoc</i> sp. ATCC 53789	AF062638	Phycobiont of lichen, Arrow Island, Scotland
<i>Nostoc punctiforme</i> ATCC 29133	AF027655	Roots symbiont of <i>Macrozamia</i> sp. Australia
<i>Nostoc</i> sp. GSV 224	AF062637	Unknown
<i>Nostoc commune</i> EV1	theses	Soil, České Budějovice, Czech Republic
<i>Nostoc</i> sp. CM1-VF14	theses	Desert soil, Clark Mountains, CA, USA
<i>Nostoc</i> sp. CM1-VF10	theses	Desert soil, Clark Mountains, CA, USA
<i>Nostoc</i> sp. F15-VF12	Vincent 2001, Xuesong 2000	Desert soil, Fort Irwin, CA, USA
<i>Nostoc</i> sp. F15-VF4	Vincent 2001, Xuesong 2000	Desert soil, Fort Irwin, CA, USA
<i>Nostoc</i> sp. F15-VF1	Vincent 2001, Xuesong 2000	Desert soil, Fort Irwin, CA, USA
<i>Nostoc</i> sp. SV1-VF6	Vincent 2001, Xuesong 2000	Desert soil, Silurian Valley, Mojave Desert, CA, USA
<i>Nostoc</i> sp. Fin	AJ 133161	Lake Säaksjärvi, Finland

Results

Phenotypic features of the strains

Nostoc "Collema" (Fig.2)

Colonies microscopic in the thallus of *Collema tenax* in the desert soil. On agar colonies macroscopic. On the agar, they start as conglomerations of small, spherical to lobate

colonies which remain distinct for at least three months. Very old (6 months) macrocolonies fuse to produce a wrinkled, raised colony with a rough, broken margin along which small satellite colonies are evident (Fig.2A). Macrocolonies are dark green for most of their life cycle, becoming yellowish brown when very old (6 months). Microcolonies are spherical to oblong to irregular, with trichomes loosely arranged in the mucilage at all stages of the life cycle and growing distant (2-10 μm) from the outer margin of the sheath (Fig.2B). The sheath is colorless, even after exposure to sunlight. Trichomes are curved and bent, arranged loosely throughout the mucilage, 2-5 μm wide (Fig.2C). Hormogonia are very rare. Vegetative cells are spherical to longer than wide, distinctly constricted at the cross-walls, although the constriction is less evident in young trichomes (hormogonia) which have cells more compressed and quadrate, small (2-4 μm in diameter) in hormogonia, getting up to 5 μm in mature trichomes. Heterocytes are spherical to elongated oval, usually intercalary and solitary, occasionally apical following trichomes breakage, 3-6(7) μm long, 2-6(7) μm wide. Akinetes are rare, in series of up to 6 cells, oval to rounded, with smooth or granular cell surface, intercalary, apoheterocytic, 4-9(10) μm long, 3-7 μm wide (Fig.2D). Cell contents of all cells are non-granular, although some akinetes appeared granular.

Nostoc CM1-VF14 (Fig.3)

Colonies are microscopic in desert soils. On agar, macroscopic colonies start as conglomerations of small, spherical to lobate colonies, which remain distinct throughout the life cycle. Even old colonies consist of aggregations of microcolonies, and form fairly flat masses on the agar surface (Fig.3A). Macrocolonies start dark green, and become yellow-green to gray green. Microcolonies are rounded, irregular, not becoming macroscopic, averaging 40-45 μm long by 30-35 μm wide (Fig.3B). Sheaths are thin, firm, and colorless, with trichomes lying less than 4 μm from the outer surface. Trichomes are unrecognizable in the younger colonies, becoming visible but still very compact after one month of growth, curved and bent, 2-7 μm wide (Fig.3C, D). Hormogonia with oval to quadratic compressed cells are 2-6 μm long by 2-5 μm wide. Vegetative cells are rounded to irregularly compressed in compact colonies before trichomes are visible; in the trichome they are usually barrel-shaped to oval, or quadratic after division, constricted at the cross-walls, 2-7 μm long by 2-7 μm wide. Heterocytes are oval to rounded, intercalary or apical in position, 3-8 μm long by 2-8 μm wide (Fig.3D). Akinetes are round to oval, intercalary to apical in position, with smooth to granular surface, yellowish, 6-11 μm long by 5-11 μm wide (Fig.3E).

Nostoc CM1-VF10 (Fig.4)

Colonies are microscopic in desert soils. On agar, colonies are macroscopic gelatinous at all stages of life cycle, with smooth margins and no satellite colonies green in color. After three months, large air-filled bubbles form in the macrocolonies (Fig.4A). Microcolonies are spherical to irregular, only observed in first 60 days of life cycle, becoming completely confluent after that time, with trichomes compact and indiscernible in first three days, then becoming loosely arranged and growing distant (2-9 μm) from the outer margin of the sheath (Fig.4B, C). The sheath is colorless, even after exposure to sunlight. Trichomes are curved and bent, arranged throughout the mucilage, 2-5 μm wide. Hormogonia are common, fairly straight, with rounded or irregularly compressed cells. Vegetative cells are longer than wide, oval with distinct constrictions at the cross-walls to quadratic or cylindrical with less evident constrictions, granular in older cells, 2-5 μm wide by 2-5 μm long. Heterocytes are spherical to elongated oval, usually apical, very rarely intercalary, 3-7 μm long by 2-6 μm wide, with the larger heterocytes in older colonies (Fig.4D). Akinetes rounded or oval, intercalary, apoheterocytic, finely granular, yellowish, 4-9 μm long by 4-7 μm wide.

***Nostoc* JT2-VF2 (Fig.5)**

Colonies are microscopic in the desert soil. On agar, colonies do not grow very well. They grow better into liquid medium. Into the liquid they start as flat conglomerations of the small dots, in this stadium they remain for 2-3 months. Later macroscopic colonies create erected filaments (Fig.5A, B). Macroscopic colonies are dark green for most of life cycle, becoming yellowish brown when very old (6 months). Microcolonies spherical to irregular, they are arranged into the filaments (Fig.5C). The sheath around the microscopic colonies is firm, thin, and colorless at the beginning of life cycle and later yellowish brown. Vegetative cells grow in the 1-2 μm distance from the outer margin of sheath. In the microscopic colonies are unrecognizable trichomes throughout the entire life cycle (Fig.5D). Hormogonia are very rare, with oval or compressed cells. The dimensions of the cells of hormogonia are 10 μm long, 7 μm wide. Vegetative cells are rounded to irregularly compressed in the compact colonies, 4-10 (12) μm long, 4- 10 (11) μm wide. The color of vegetative cells is greenish brown. Heterocytes oval to rounded, intercalary or apical in position, 4-10 (12) μm long, (2) 4-8 (10) μm wide (Fig.5D). Akinetes were not observed during 6 months of my observation.

Comparison of the phenotypic features of strains (Tab.2)

All chosen strains are isolated from the desert soils in the western United States. In nature they are microscopic and for observation it is necessary to isolate them. In the laboratory, 3 of them grow very well on the solid "nitrogen- free" Z- medium (CM1-VF10, CM1-VF14, phycobiont of *Collema tenax*). The strain JT2-VF2 grows better in the liquid nitrogen free Z- medium. All observed strains create in the laboratory conditions the macroscopic colonies. They are different in this feature and the character of colonies on the agar or in the liquid is characteristic for each strain. CM1-VF10 creates bubbly, gelatinous colonies. CM1-VF14 creates colonies whose start as conglomerations of small spherical to lobate colonies. Phycobiont *Collema tenax* starts as conglomerations of small, spherical to lobate colonies. In this stadium it is very similar to CM1-VF14. Very old (6 months) colonies fuse to produce a wrinkled, raised colony with a rough, broken margin along which small satellite colonies are evident. In old age the character of macroscopic colonies is different from CM1-VF14. JT2-VF2 is very different from other strains. At the beginning of the life cycle, JT2-VF2 grows as a flat conglomeration of small dots, and remains in this stadium for 2-3 months. Later it creates erected filaments up to 2-3 cm long. The color of colonies by all strains is dark green at the beginning of life cycle, and later starts to be yellow brown.

The shape and dimension of microcolonies are the same by all observed strains, rounded to irregular.

Also the character and color of the sheath are the same by all strains. The sheath is thin, firm, and colorless to yellowish brown by the older strains. What seems to be an important characteristic is the distance of the sheath from the cells or trichomes. The distance from the outer margin of the sheath is 2-9 μm by CM1-VF10, 1-4 μm by CM1-VF14, 2-10 μm by phycobiont of *Collema tenax* and 1-2 μm by JT2-VF2.

Another feature in which the strains differ is arrangement of trichomes in the mucilage. CM1-VF10 has trichomes loosely arranged and they are recognisable throughout the entire life cycle. CM1-VF14 has trichomes, which are dense in the mucilage and are unrecognisable in the small microcolonies; later in the larger microcolonies they are recognisable. Phycobiont of *Collema tenax* has trichomes arranged very loosely and recognisable throughout the entire life cycle. JT2-VF2 has unrecognisable trichomes. Cells are very dense and do not create trichomes in the microcolonies.

Table 2. The morphological characteristics of studied strains. The highlight information is the important differences among strains. Abbreviation u – trichomes unrecognisable in the mucilage of microcolony, r- trichomes recognisable in the mucilage of microcolony.

strains	in nature	macrocolony		sheath			
		shape	color	shape	color	remove from veg. cells	arrangement of trichome
CM1-VF10	microscopic	gelatinous	dark green	thin	colorless	2-9 um	loosely
		bubbly		firm			r
CM1-VF14	microscopic	dotted	green	thin	colorless	1-4um	dense
		later flat	yellow-green	firm			u in small microc. r in large microc.
Collema Nostoc	microscopic	small	dark green	thin	colorless	2-10um	very loosely
		heaps		firm			r
JT2-VF2	microscopic	flat dots or trichomes	dark green	firm	colorless	1-2 um	u
				thin	yellow-brown in older		

strains	veg.cells			heterocytes				akinetes				
	shape	dimension um		shape	place	dimension um		shape	place	surface	dimension um	
CM1-VF10	oval	2-6	2-6	rounded	apical	3-7	2-6	rounded	intercalary	granular	4-9	4-7
	irregular			oval	intercalary			oval		yellowish		
	quadratic											
CM1-VF14	oval	2-7	2-7	rounded	apical	3-8	2-8	rounded	intercalary	smooth	6-11	5-11
	irregular			oval	intercalary			oval	apical	yellowish		
	quadratic											
	rounded											
Collema N.	rounded	2-6	2-7	rounded	intercalary	3-6	2-6	rounded	intercalary	smooth	4-9	3-7
	oval			oval	apical			oval		granular		
	quadratic											
JT2-VF2	oval	4-10	4-10	oval	apical	4-10	4-8	no observed				
	rounded			rounded	intercalary							
	irregular											

The dimensions of the vegetative cells, heterocytes and akinetes were analysed in ANOVA tests. Tukey HSD test was used for pairwise comparisons of significant results. The comparison of length and width of the vegetative cells and heterocytes were done for all four strains. The comparison of length and width of akinetes was done only for the three strains, because the akinetes were not observed in the strain JT2-VF2. The analyses were significant for five tests (length and width of vegetative cells and heterocytes, width of akinetes) (Tab.3). The length of akinetes did not differ significantly among the strains (Tab.3, Figure 6E). The strain JT2-VF2 is very different from the other three strains (Tab.3, Figures 6A, B, C, D). This strain had longer and wider vegetative cells and heterocytes than other strains. The differences in dimensions of the other strains were small. Only the width of akinetes differed significantly among the three strains (Figure 6F).

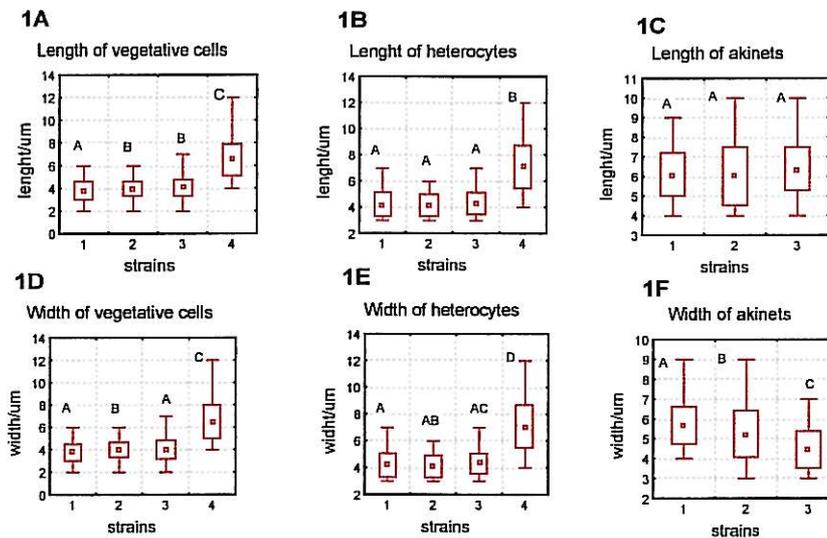
Table 3.

Results of measures ANOVA, HSD test for the dimensions of vegetative cells and heterocytes for the strains CM1-VF10, CM1-VF14, phycobiont of *Collema tenax* and JT2-VF2. The dimensions of akinetes were analysed only for CM1-VF10, CM1-VF14 and phycobiont of *Collema tenax*.

Characteristics	df Effect	MS Error	F	p-level
veg. cell length	3	0.949798	697.0141	0.00
veg. cell width	3	0.913276	321.9765	0.00
heterocyt length	3	1.181854	527.6475	0.00
heterocyt width	3	1.168153	184.1325	0.00
akinet length	2	1.485732	2.5568	0.079251
akinet width	2	1.051738	34.8312	0.00

Figure 6.

Results of measures ANOVA, HSD test for the dimension of vegetative cells and heterocytes for the strains CM1-VF10, CM1-VF14, phycobiont *Collema tenax* and JT2-VF2. The dimensions of akinetes were analysed only for CM1-VF10, CM1-VF14 and phycobiont of *Collema tenax*. The akinetes were not observed by the strain JT2-VF2. Legend to the figure: bar – Max-Min, dot – Mean and rectangle – Mean +/-Mean- SD.



Phylogenetic relationships between genus *Nostoc*

The sequence of the 16S rRNA gene was determined for 4 soil *Nostoc* strains (CM1-VF10, CM1-VF14, phycobiont *Collema tenax* and EV1). Those sequences were compared with other sequences of desert *Nostoc* done in Dr. J.A. Johansen's laboratory, John Carroll University, Ohio, USA and with the sequences of heterocystous cyanobacteria available in GenBank. A total of 1 100 nucleotide sites were used to generate the phylogenetic trees on the basis of the neighbour-joining method and the DNA parsimony method. The topology and

branch support observed in the maximum likelihood tree, distance and parsimony tree was nearly identical. Consensus tree is illustrating phylogenetic relationships among all strains in this study (Fig.7). Overall, bootstrap support for major clades was low the terminal branches were moderately supported. The clusters are not very well supported by bootstrap analysis because the tree reflects the molecular similarity of the strains of *Nostoc* genus from different regions and biotops.

Nostoc species studied here were divided into two distinct branches. The sequences of three strains from desert in western USA (JT1-VF3, JT1-VF7 and phycobiont of *Collema tenax*) and strain SAG 2028 from the cave in Dragonsberg Mts., South Africa created one main branch with poor support (29%). But in this main branch were terminal branches well supported. For example, the clade including JT1-VF3, JT1-VF7 and phycobiont of *Collema tenax* was well-supported (82%). The second main branch was created by the strains: ATCC 53789 isolated from lichen, Arrow Island, Scotland, GSV224 unknown origin and *Nostoc punctiforme* ATCC 29133 isolated from *Macrozamia* sp. root, Australia.

It could be said that our isolate of *Nostoc* from *Collema tenax* is not same species as the symbiont from *Macrozamia* sp. and lichen from Arrow Island, Scotland. Also *Nostoc* from *Collema tenax* from the desert soil of USA is not *Nostoc commune*. Other strains did not create clades, but despite the tree showed genetic differences between the strains CM1-VF14, CM1-VF10 and EV1 (*Nostoc commune* from middle Europe) on one side and their close relationship on other side. Strain JT2-VF2 was the most different strain of *Nostoc* studied in this project. The highest 16S sequence similarity among JT2-VF2 and other species was 95.9% (Tab.4).

The results from the molecular study were supported by the results from the morphological observation. Strain JT2-VF2 was the most different in the morphological and molecular characteristics from other observed *Nostoc* (CM1-VF10, CM1-VF14 and phycobiont of *Collema tenax*). CM1-VF10, CM1-VF14 and phycobiont of *Collema tenax* were similar each to other but not identical.

Figure 7.

Parsimony tree constructed from 16S rRNA gene sequences of the *Nostoc* strains. An alignment of 1116 nucleotides after excluding positions with gaps was used. Bootstrap values for parsimony was based on 1000 bootstrap replicates. *Anabaena spiroides* and *Cylindrospermum* sp. were used like outgroup. The sequences sequenced by myself have blue color and sequences from GenBank and co-workers from the Jeff Johansen's lab have red color.

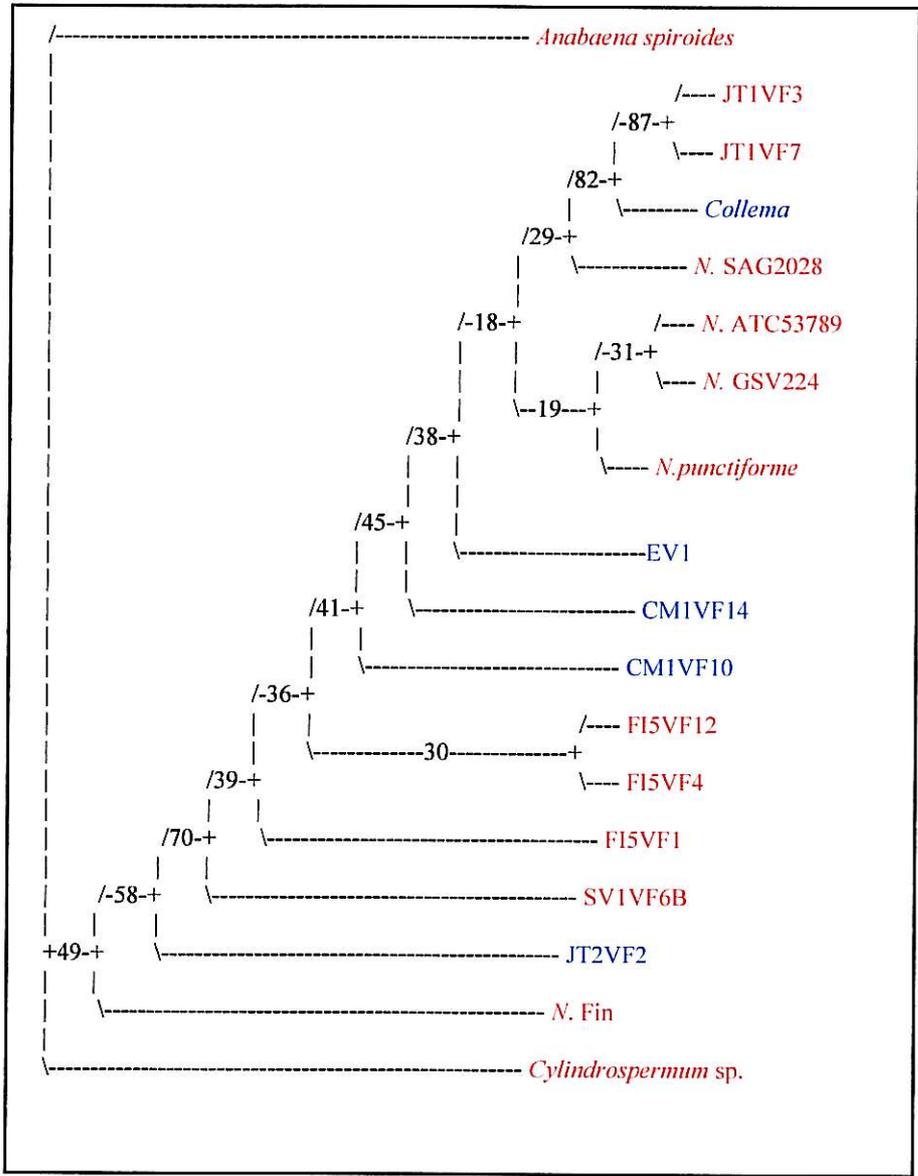
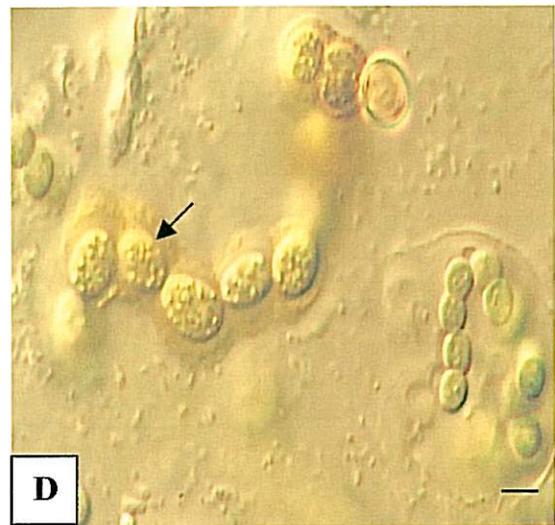
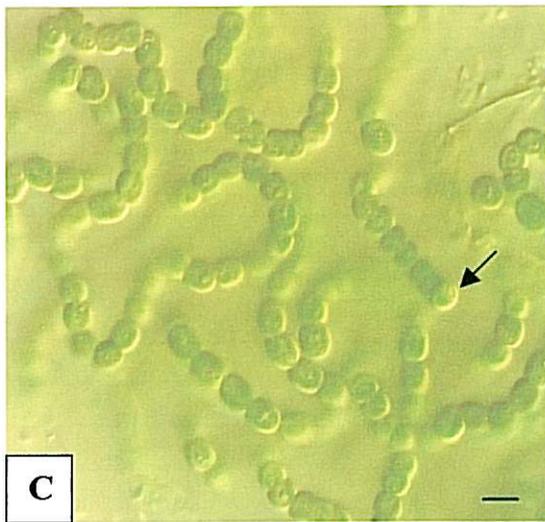
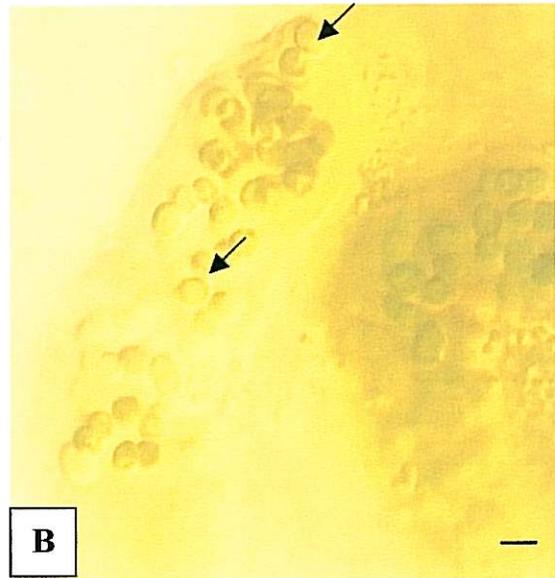
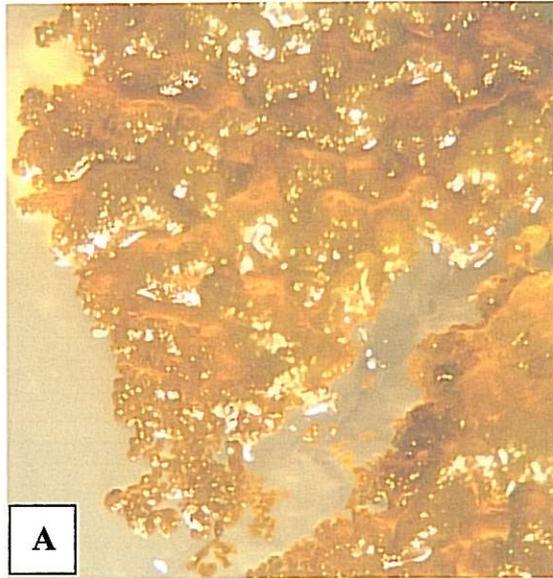


Table 4.
16S sequence similarities among taxa.

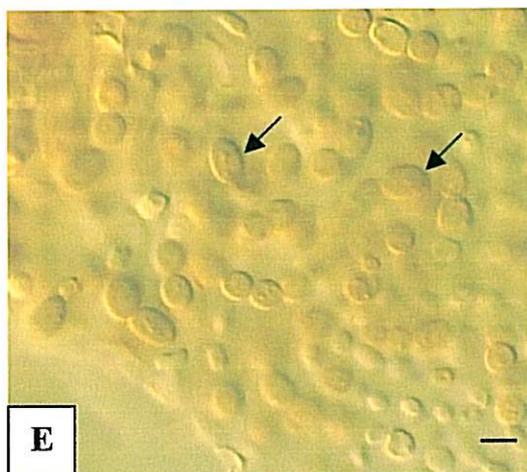
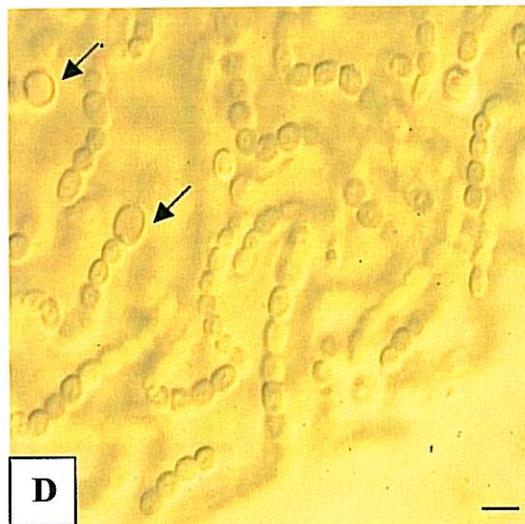
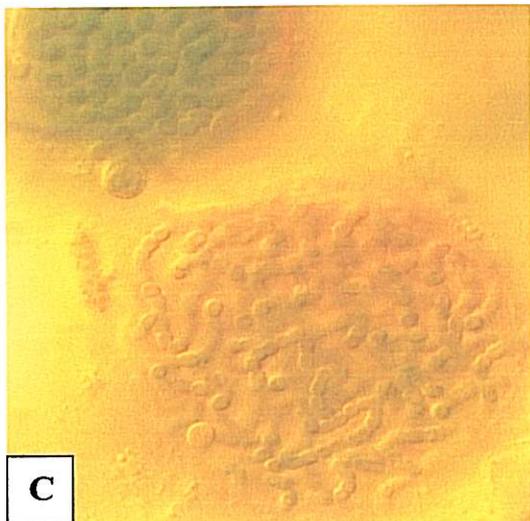
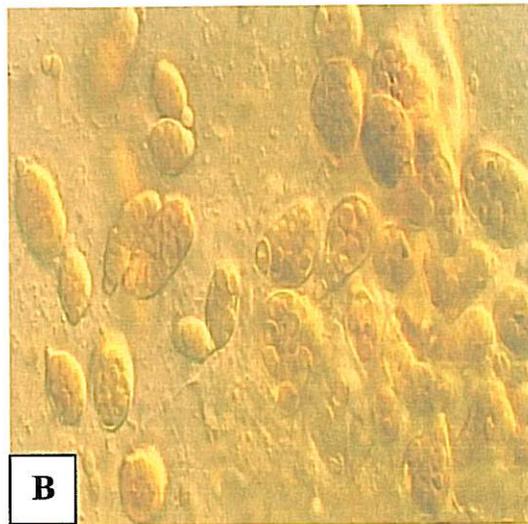
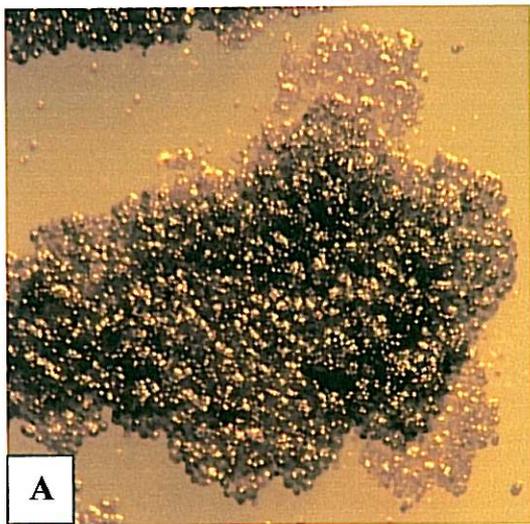
	N. SV1VF Fin. 6	SAG A 29133 96.5	GSV 224 95.8	EV1 A 53789 96.1	JT2VF 2 95.1	JT1VF 7 95.6	JT1VF 3 96.2	FISVF 4 95.8	FISVF1 2 95.7	FISVF 1 96.4	Cylindr 0 94.9	Collema 0 96.0	CM1VF1 0 94.7	CM1VF1 4 95.0	A. spir. 4 94.3
Nostoc Finland	95.5	96.5	96.3	95.8	96.1	95.9	95.1	95.6	96.2	95.8	94.9	96.0	94.7	95.0	94.3
SV1-VF6		97.0	97.2	96.7	97.4	96.6	97.4	98.4	98.4	98.4	95.4	96.9	95.6	96.8	94.0
SAG 20281			98.2	97.9	98.1	97.4	98.0	98.1	97.9	97.7	95.7	98.1	95.7	96.2	94.5
ATCC 29133				99.1	99.3	95.0	97.5	98.3	97.8	98.5	94.8	98.5	97.1	97.6	94.4
GSV 224				99.2	98.7	94.4	96.9	98.0	97.3	98.1	94.4	98.0	96.5	97.0	93.9
EV1						94.8	97.1	98.4	97.9	98.2	94.7	98.1	96.9	97.7	94.3
ATCC 53789						95.4	97.4	98.0	98.0	98.1	95.3	98.4	96.2	96.8	94.0
JT2-VF2						94.3	97.4	94.9	95.8	95.8	94.9	94.9	93.9	94.5	92.9
JT1-VF7							98.6	95.9	97.1	97.3	94.1	98.2	95.6	95.4	93.5
JT1-VF3								98.1	97.7	97.9	94.6	98.8	96.4	96.0	94.1
F15-VF4								99.2	97.1	97.3	95.3	98.3	96.5	97.9	94.3
F15-VF12									99.2	98.9	95.1	97.7	96.0	97.4	94.4
F15-VF1										98.6	95.4	97.5	96.4	97.7	94.8
Cylindrospermum												94.4	96.4	97.7	93.7
Collema													92.9	93.7	93.7
CM1-VF10													96.7	96.3	92.1
CM1-VF14														95.7	93.2
Anabaena spirales															

Fig.2. *Collema Nostoc*



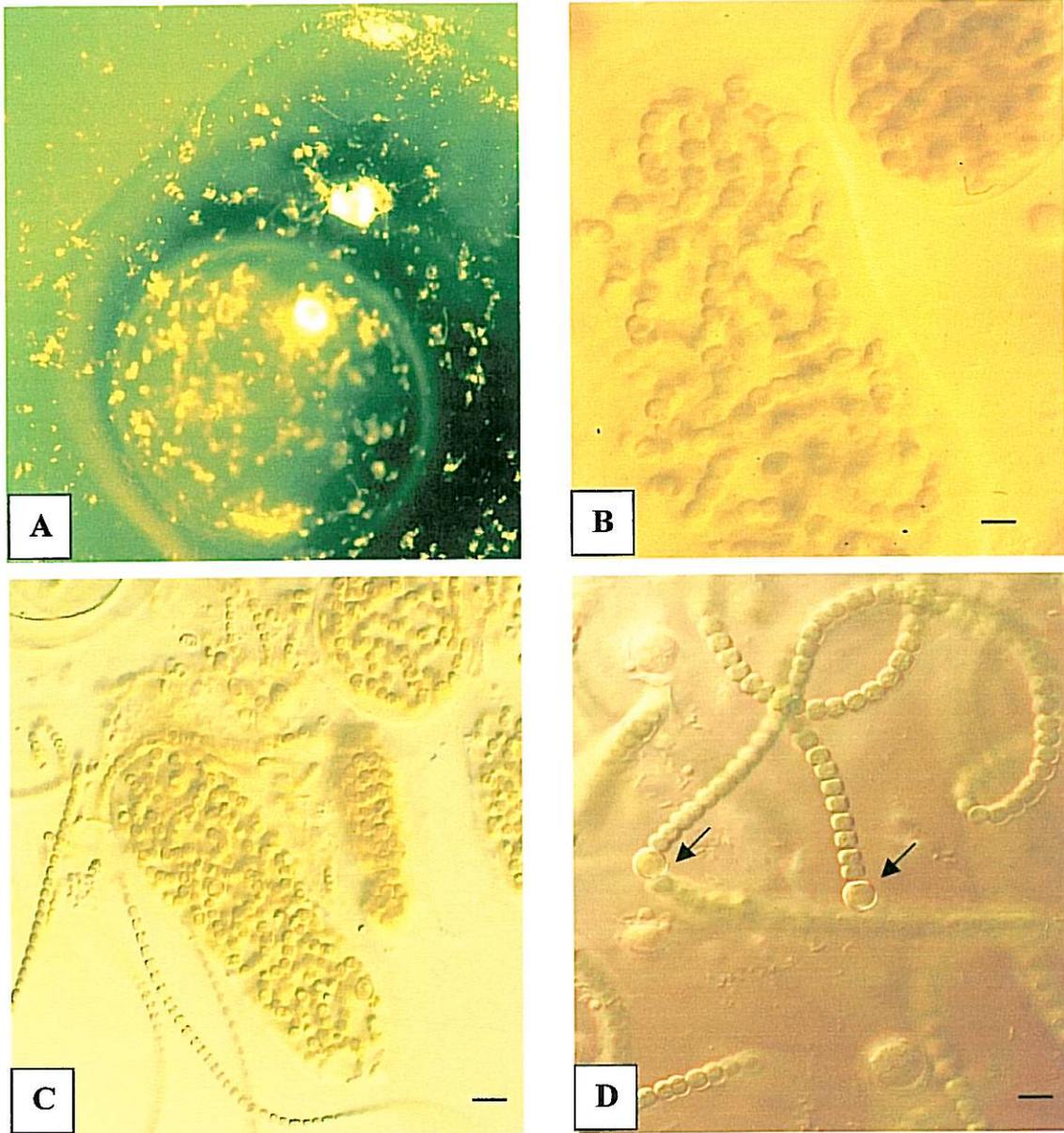
The bars are 5 μ m in the Fig.2B, C and D. Arrows in Fig.2B,C are showed heterocytes and in Fig.2D akinetes.

Fig.3. CM1-VF14



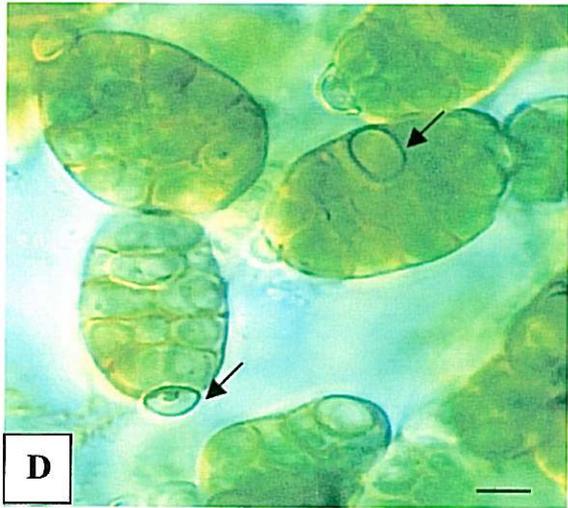
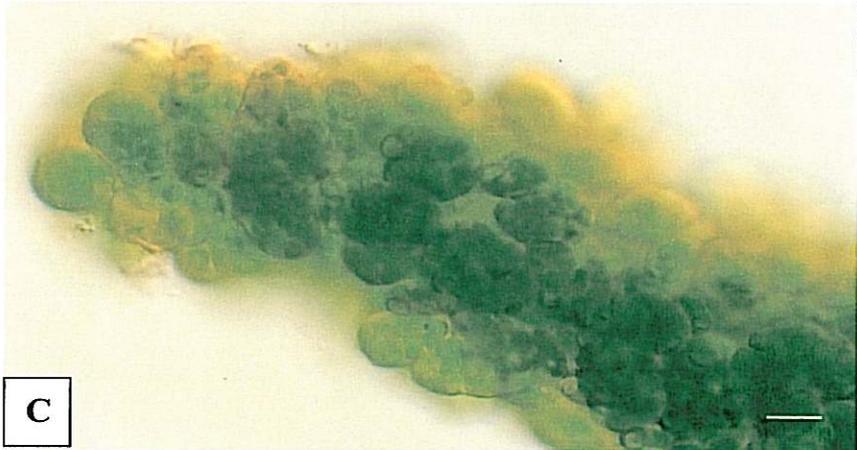
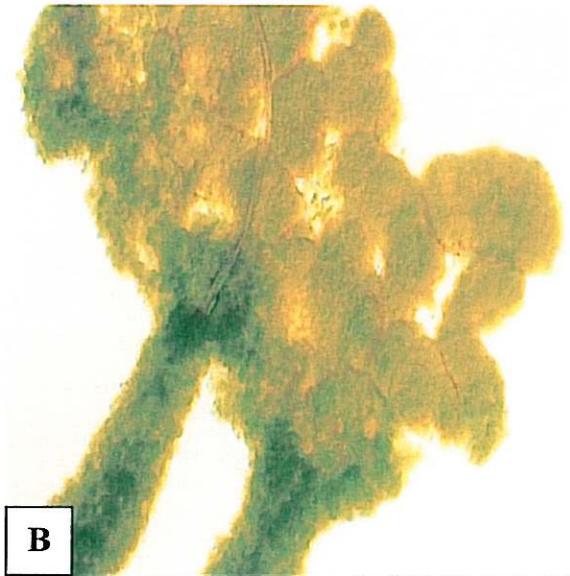
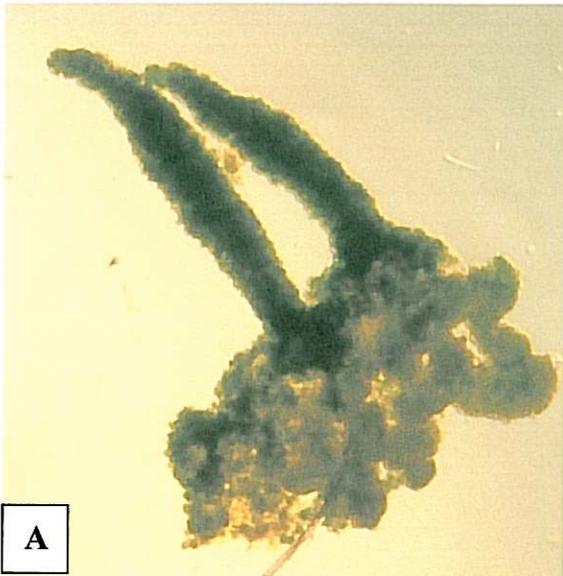
The bar in Fig.3D,E is 5 μ m. Arrows in Fig.3D are showed heterocytes and in Fig.3E akinetes.

Fig.4. CM1-VF10



The bars are 5 μ m in the Fig.4B,D and 10 μ m in Fig.4C. Arrows are showed heterocytes.

Fig.5. JT2-VF2



Original size of Fig.4A is 0.5cm. The bar in Fig.4C is 30 μ m and in Fig.4D 10 μ m. Arrows are showed heterocysts.

Discussion

Nostoc spp. is alga with a long history and has attracted the interest of people in different ways. For example, it plays important roles in the human diet and medicine. *Nostoc* is a common dietary supplement for the people from Thailand, Peru, China, Ecuador, Fiji, Java, Japan, Mexico, Mongolia and Siberia (JASSBY 1988).

The first note about *Nostoc* from Europe is 500 years old. It referred to the "earthy" smell created by the massive development of *Nostoc* colonies (NAES 1988). People in the Middle Age observed appearance of rapid growth of *N. commune* after thunderstorms. They called *Nostoc* "Sternschnuppen" or "Falling star" (MOLLENHAUER 1986A, FRENCH 1650). Vaucher described the genus *Nostoc* in the 19th century (BOURNET & FLAHAULT 1886).

Today, there is a considerable accumulation of literature and studies on *Nostoc* spp. Many of them are concerned with the eco-physiology, biochemistry and cell structure (MOLLENHAUER 1970, 1985A, 1985B, 1986A, 1986B, DODDS ET. ALL 1995, POTTS 1994).

In my work I was concerned with the polyphasic approach to the taxonomy of *Nostoc* from desert soil and the comparison of those species with the described *Nostoc* species from GEITLER (1932), DESIKACHARY (1959), MOLLENHAUER ET AL. (1994), MOLLENHAUER ET AL. (1999).

Morphological comparison

After the selection of the described *Nostoc* species from literature only five species have similar morphological characteristics like the observed *Nostoc* from desert soil. These five are *N. punctiforme*, *N. paludosum*, *N. commune*, *N. microscopicum* and *N. muscorum*. The results of the comparison of four studied *Nostoc* strains and species from literature is in the Table 5.

Strain **CM1-VF14** resembled *N. punctiforme* and *N. commune*. CM1-VF14 was also compared with *N. microscopicum* and *N. muscorum*. CM1-VF14 did not exactly match with these taxa. *N. muscorum*, *N. microscopicum* and *N. commune* create in nature macroscopic colonies observable without microscopy (GEITLER 1932), CM1-VF14 was not possible to observe without cultivation. CM1-VF14 had a colorless sheath throughout the life cycle and also after exposure to UV radiation. According to the description (GEITLER 1932) *N. muscorum*, *N. microscopicum* and *N. commune* have a yellow to brown sheath. Shape and dimension of vegetative cells, heterocytes and akinetes were similar among CM1-VF14 and *N. punctiforme*, *N. paludosum*, *N. commune*, *N. microscopicum* and *N. muscorum*. The best match from the selective taxa had *N. punctiforme*, which is microscopic like CM1-VF14. But if the ecophysiological characteristics were used, like ecology of species, *N. punctiforme* did not match. CM1-VF14 grew in the desert soil of western USA, but selected taxa were described from the stagnant water, intercellular space of *Gunnera*, wet soil and mosses (GEITLER 1932, MOLLENHAUER 1994).

Strain **CM1-VF10** was most similar to *N. paludosum* in cell size, arrangement of trichomes with similar character of colonies. The difference was in the color of sheath. *N. paludosum* has yellowish color of sheath (GEITLER 1932, DESIKACHARY 1959). CM1-VF10 did not change the color of sheath during its life cycle nor after the exposure to UV radiation. The surface of akinetes of CM1-VF10 was granular in contrast to *N. paludosum* with smooth surface of akinetes. The ecology of both taxa was different too. *N. paludosum* was described from stagnant water and CM1-VF10 was isolated from desert soil.

Other observed strains were **phycobiont *Collema tenax*** and **JT2-VF2**. They did not fit any described taxa. They were unique in shape and color of colony, and arrangement of trichomes into the mucilage. I tried to compare phycobiont of *Collema tenax* with

N. commune, because in the lichenology literature phycobionts of lichens are called *N. commune* (RAI 1990). Why is phycobiont of *Collema tenax* not *N. commune*? *N. commune* has much denser trichome arrangement into mucilage, smaller, smooth akinetes, and macroscopic colonies. None of those characteristics were observed by phycobiont of *Collema tenax*.

Table 5. The comparison of morphological characteristics of the strains studied in this study (blue) and similar species described in GEITLER (1932) and DESIKACHARY (1959) (red). Abbreviations: r= trichomes recognizable in the mucilage of microcolony, u= trichomes unrecognizable in the mucilage of microcolony.

	macrocolony on agar		size in the nature	sheath		veg. cells	
	shape	color		color	arrangement of trichom	dimension um	
CM1-VF10	gelatinous bubbly	dark green	microscopic	colorless	loosely r	2-6	2-6
CM1-VF14	dotted later flat	green yellow green	microscopic	colorless	dense u in small r in larger	2-7	2-7
<i>Collema Nostoc</i>	spheres in groups	dark green	microscopic	colorless	very loosely r	2-6	2-7
JT2-VF2	flat dots or trichomes	dark green	microscopic	colorless	u	4-10	4-10
<i>N. paludosum</i>	point gelatinous		macroscopic	colorless yellowish	loosely		3-3.5
<i>N. punctiforme</i>	?		microscopic	colorless	very dense		3-4
<i>N. commune</i>	rounded folded tom	olive green brown green	macroscopic	yellow brown	dense	4.5-6	4.5-6
<i>N. microscopicum</i>	rounded oval	olive green brow	macroscopic	yellow	dense		5-8
<i>N. muscorum</i>	rounded spread out	olive green yellow brown	macroscopic	yellow brown	dense	6-8	3.5-4

	heterocytes			akinetes			ecology
	shape	dimension um		shape	surface	dimension um	
CM1-VF10	rounded oval	3-7	2-6	rounded oval	granular yellowish	4-9 4-7	desert soil
CM1-VF14	rounded oval	3-8	2-8	rounded oval	smooth yellowish	6-11 5-11	desert soil
<i>Collema Nostoc</i>	rounded oval	3-6	2-6	rounded oval	smooth granular	4-9 3-7	phycobiont <i>Collema tenax</i> desert soil
JT2-VF2	oval rounded	4-12	4-10	no observed			desert soil
<i>N. paludosum</i>	barrel		3-3.5	oval	smooth	6.8 4-4.5	stagnant water cosmopolitan
<i>N. punctiforme</i>	?	4-6.5		rounded	smooth	5-8 6.5	stagnant water
<i>N. commune</i>	rounded	7	7		smooth	4.5-6 4.5-6	intercellular space in <i>Gunnere</i> soil cosmopolitan
<i>N. microscopicum</i>	rounded	7	7	oval	smooth	9-15 6-7	wet soil
<i>N. muscorum</i>	rounded		6-7	oval	smooth yellow	8-12 4-8	mosses wet soil, mosses cosmopolitan

closer relations). Examples for either the support or the disapproval of the molecular approach could be found. On the one hand, morphological differences within *Microcystis* genus (OTSUKA ET AL. 1998) and within the *Mersimopedia* genus (PALINSKA ET AL. 1996) are not reflected at the 16S rRNA gene level. On the other hand, the unicellular cyanobacteria that are morphologically similar and simple can be phylogenetically different (WARD ET AL. 1992).

The order *Nostocales* is strongly supported monophyletic clade (CASTENHOLZ 1989) but the relationships within the genus *Nostoc* are still unclear. Two distinct branches were found in the 16S rRNA gene analysis in this study. The strains were from the different localities throughout the world. The genetic variability of observed *Nostoc*- species was not congruent with geography. e.g. strains SAG 2028 and ATCC 53789 were in one clade, but strain SAG 2028 was isolated in Dragonsberg Mts., South Africa and strain ATCC 53789 from lichen, Arrow Island, Scotland). The same results were obtained by Boyer et al. (in print) with the genus *Microcoleus* from desert soil. On the other hand, in some cases only geography and ecology of species is in agreement with the genetic analysis (genus *Microcystis* – Li et al. 1997, genus *Prochlorococcus* and *Synechococcus* Rocap et al. 2002).

One of the questions of this study was: “Is phycobiont from *Collema tenax* *Nostoc commune*?” My results did not support this theory, because phycobiont of *Collema tenax* is in the different clade like *N. commune* (strain EV1) and also in the different clade like strain ATCC 53789 isolated from lichen. The similarity between strains EV1 and ATCC 53789 was 98.5% and between strains EV1 and phycobiont *Collema tenax* was 98.1%, despite this fact it could not be said that they are same species (STACKERBRAND & GOEBEL 1994). According the literature many phycobionts from the lichen are designated as *Nostoc* sp. It is clear that a wide range of *Nostoc* species and strains are symbiotically competent in a wide spectrum of plants (ZIMMERMAN & CULLEY 1991, BERGMAN ET AL. 1992, WEST & ADAMS 1997).

JT2-VF2 strain was separated from the other strains of *Nostoc* in the dendrogram. The similarity between this strain and the others was low (< 96%). When sequence similarity is below 97% it is fairly certain that DNA-DNA reassociation is below 70%, and thus the compared strains should be recognized as separate species (STACKERBRAND & GOEBEL 1994).

The rest of the strains in the phylogenetic tree did not create clades and showed that the geographically diverse strains of *Nostoc* are very closely related. Similar findings were reported for *Microcystis* (NEILAN ET AL. 1997) and *Nodularia* (BOLCH ET AL. 1999, LEHTIMÄKI ET AL. 2000).

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Chapter 2

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