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The biocontrol potential of lytic bacteria against cyanobacterial blooms

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Abstract

There is great concern for the widespread increase of cyanobacterial blooms globally. Blooms can be toxin producers and cause serious health implications for wild life and humans. However, even blooms that are not toxin producers can be detrimental to the environment due to biomass increase, which can cause oxygen depletion that lead to fish death.

Our goal was to investigate if cyanolytic bacteria isolated from lakes that experience phytoplankton blooms possessed potential biocontrol properties. From a productive lake (Ekoln, basin of lake Mälaren - Sweden) we managed to isolate cyano-lytic bacteria and test them for potential biocontrol properties against two *Microcystis aeruginosa* strains (PCC 7820 and 7941) using a plaque assay method and liquid culture inhibition test in micro-plates. From the 151 isolated strains of cyano-lytic bacteria, four were selected for further tests to assess lytic ability and one isolate was selected for its capacity to inhibit the activity of other lytic bacteria. The 16S ribosomal ribonucleic acid (rRNA) gene of the five strains were partially sequenced and identified as *Pseudomonas fluorescence*, *Pseudomonas veronii*, *Pseudomonas teessidea*, *Delftia acidovorans* (lytic bacteria) and *Acinetobacter heamolyticus* (inhibitory bacteria). The lytic isolates showed strong lytic ability on solid media and also somewhat reduced cyanobacterial growth in liquid culture over shorter time scales (< 1 week). Inhibition was temporary and this contrasting effect may be attributable to the need for direct contact with the cyanobacteria or a quorum-sensing dependence of the lytic action. We used the terminal-restriction fragment length polymorphism (t-RFLP) method to study bacterial community structure and follow the temporal dynamics of putative cyanolytic bacteria in the lake during the sampling period. Bloom formation was correlated to phosphorus and chlorophyll-a. Only one of the lytic isolates was detected in the lake and this only happened at one occasion, suggesting that they are rare members of the indigenous bacterial community.

Contents

Introduction

Water quality is greatly influenced by anthropogenic activities such as nutrient loadings from agricultural fertilizers, sewage water and waste from industrial and urban sites (Codd, 2000). Biological production is closely related to nutrient stoichiometry, and furthermore, may increase when systems providing enough phosphorus receive bioavailable nitrogen inputs (Schindler *et al*., 1985). Cyanobacteria quickly respond to nutrient inputs by bloom formation and thus can be used as eutrophication indicators in monitoring the effect on environmental change (Paerl *et al*., 2003).

A cyanobacterial bloom is characterized as a growth above the normal average concentration for a given lake. The visible surface blooms occur when cyanobacteria, able to produce gas vacuoles, float to the surface, forming scum. However, contrary to common belief, in order for a bloom to form, there need not be a dense concentration of cells, only a preexisting dispersed population. When the water column becomes calmer, with less mixing, this population concentrates after floating to the surface, forming a bloom (Oliver & Ganf, 2000).

Many bloom-forming cyanobacteria are included in the Harmful Algal Bloom (HAB) category and are known as CyanoHABs. They can occur in freshwater and produce seasonal blooms with accumulation of toxins. Cyanobacterial toxins are capable of causing serious health damage to wild life and domestic animals, which come into contact with or consume contaminated water. Depending on the predominant strain, blooms can produce a variety of different toxins. These include neurotoxins, hepatotoxins and dermal toxins (Carmichael, 2001). There is an increasing concern about the widespread occurrence of toxin producing blooms and its correlation with environmental change. It has recently been indicated that an increase in atmospheric CO_2 and nitrogen availability could potentially cause blooms of cyanobacteria of the genus *Microcystis* to produce higher concentrations of certain variants of the microcystin toxin (Van de Waal *et al*., 2009).

The reason why cyanobacteria are so successful at dominating certain environments is probably due to their great adaptive ability. Some characteristic features of cyanobacteria include the ability to store nitrogen, to switch between photosystem type I

and II, to move through the water column due to buoyancy attributed to gas vacuoles (Whitton & Carr, 1982) and in some blooms cyanobacteria are able to dominate and outcompete other photosynthetic organisms due to efficient CO_2 uptake (Shapiro, 1997). Clearly a long term effort into the control and management of aquatic systems is necessary, however, a short term remediation strategy of CyanoHABs could be an effective solution for small body waters (Sigee *et al*., 1999).

Control of nuisance blooms are mostly aimed at reducing phosphate levels in the surface water and includes chemical and physical treatment strategies. Chemical treatments using aluminum salts and lime have shown positive effect on coagulation and precipitation of phosphorus in the sediment, thus subsequently reducing blooms. However, aluminum salts can be toxic to both cyanobacteria and many other organisms in the ecosystem and also increase the release of intracellular cyanobacterial toxins into the surrounding water (Lam *et al*., 1995). It has also been suggested that internal loading of phosphorus can occur from the sediment, causing eutrophication still occur. Therefore it is important to take these factors into consideration before utilizing chemicals for this purpose. Physical treatments, like aeration of the bottom layers are also used to avoid thermal stratification and release of phosphate from the sediment and such strategies have been successful in oxygen limited lakes (Mason, 2002).

Biological control of blooms has been proposed as an alternative or complement to such nutrient-reduction strategies. It is often suggested to be the least expensive and most specific form of treatment of blooms and typically rely on viral cyanophages, fungal parasites, protozoa grazers and cyanolytic bacteria as control agents. Studies with different cyanolytic agents have shown that bacteria are particularly promising in the control of blooms (Sigee *et al*., 1999). Many different bacterial strains have previously been studied from this perspective (Table 1). The lytic bacteria studied by these authors were tested on different cyanobacterial genera and strains which vary in susceptibility to lytic effects. While some bacteria are able to lyse cyanobacteria that belong to different genera, others possess a more limited host/prey range.

Table 1. Bacteria tested for lytic abilities against cyanobacteria in previous studies.

Author	Lytic bacteria isolated	Classification	Cyanobacteria inhibited
Burnham *et al.*, 1984	Myxococcus xanthus; Myxococcus fulvus	Deltaproteobacteria (Class IV)	Phormidium luridum var. olivaceae.
Choi *et al.*, 2005	Streptomyces neyagawaensis	Actinobacteria (Class I)	Microcystis aeruginosa; Anabaena cylindrica; A. flos-aquae
Ensign & Wolfe, 1965	Cytophaga (Myxobacteriales)	Acidobacteria	Arthrobacter crystallopoietes
Fraleigh & Burnham, 1988	Myxococcus fulvus	Delta proteobacteria (Class IV)	Nostoc muscorum; Phormidium luridium
Kim *et al.*, 2008	Xanthobacter autotrophicus	Alphaproteobacteria (Class I)	Microcystis aeruginosa
Kim *et al.*, 1997	Moraxella sp.	Gammaproteobacteria (Class III)	Anabaena cylindrica
Manage *et al.*, 2000	Alcaligenes denitrificans	Betaproteobacteria (Class II)	Microcystis aeruginosa; M. viridis; and M. wesenbergii
Rashidan & Bird, 2001	Cytophaga sp.	Acidobacteria	Anabaena flos-aquae; Synechococcus leopoliensis; S. elongates; and Anacystis nidulans
Shilo, 1970	Myxobacteria	Delta proteobacteria (Class IV)	Broad testing, included Nostoc sp; Anacystis nidulans
Whyte *et al.*, 1985	Streptomyces achromogenes.	Actinobacteria (Class I)	Anabaena cylindrical, Tolypothrix tenuis
Wright *et al.*, 1985	Bacillus sp.	Firmicutes	Anabaena cylindria; Anacystic nidulans; Gleocapsa alpicola

Studies based on plaque assays show that the abundance of lytic bacteria in lakes correlates positively with chlorophyll levels (Daft *et al.*, 1975). This suggests that they may be efficient biocontrol agents as they are able to maintain viable populations directly in the environment without the presence of a host and that they can respond rapidly to increased prey density, reducing cyanobacterial populations in the process (Rashidan & D.F. Bird, 2001). Cyanolytic bacteria can also be used for more specific and targeted control of certain species of cyanobacteria. This would be an important feature for future use as biocontrol agents as it minimizes the effect on the ecosystem and other organisms (Choi *et al*., 2005).

The goals of this study were 1) to assess if cyanolytic bacteria co-occur in correlation with cyanobacterial blooms, 2) to study when they are present and 3) test if such cyanolytic bacteria could represent potential biocontrol agents. To answer these questions, field studies were carried out to link environmental characteristics to the occurrence of lytic bacteria. Cyano-lytic bacteria were isolated from a productive lake and screened for activity using plaque assays on *Microcystis aeruginosa,* which is a major nuisance bloom-former of global significance. Characterization of selected strains was carried out using molecular (16s ribosomal ribonucleic acid - rRNA) methods. Lytic tests in liquid culture were performed on two different *Microcystis* strains to investigate the dose-response and efficiency of cyanolytic effects and inhibition. We further followed changes in bacterial community composition in response to bloom development in the studied lake. Both, the general community response to bloom development and the presence of specific cyanolytic bacterial populations, were studied using terminal-restriction fragment length polymorphism (t-RFLP) of amplified 16S rRNA.

Materials and Methods

Study site

Water samples were collected from early July to late October of 2010 in Lake Ekoln, which is the northern basin of Lake Mälaren (59º 78'N-17º 63'E), Sweden's third largest

lake. Lake Ekoln is a moderately humic, eutrophic lake (Langenheder *et al*., 2005). It receives urban sewage water through the River Fyrisån, and this is one cause of re-occurring cyanobacterial blooms (Ulen & Weyhenmeyer, 2007). Samples were collected from surface water in heat sterilized plastic bottles and kept dark until further processing in the laboratory (within 3 hours of sampling). *In situ* temperature was measured with a digital thermometer using a cable probe.

Water characteristics

Water pH was measured using a pH meter (Crison Micro pH 2001). Chlorophyll-a (Chl-a) was analyzed by absorbance after ethanol extraction. Pigments from cells were collected by vacuum filtration of 0.5-2 L of lake water through GF/F glass fibre filter with a nominal pore size of 0.7 µm (Whatman, 47 mm Ø) and were analyzed according to International Standard procedures (ISO 10260, 1992).

Dissolved organic carbon (DOC) was analyzed by high temperature catalytic combustion following a previously described method (Bertilsson & Tranvik 2000). Water filtered through 0.7 µm glass fibre filters (see above) was stored frozen until analysis. Sample aliquots of 6 ml were acidified by adding 50 µL of 1.2 M HCL for subsequent analysis of non-purgeable organic carbon after sparging with carbon dioxide-free air using the TOC-5000 carbon analyzer (Shimadzu).

Soluble reactive phosphorus (SRP) was determined by colorimetry following the method of Murphy & Riley (1962). Water colour was measured spectrophtometrically at 436 nm in a 5 cm cuvette (Perkin Elmer Lambda 40 UV/VIS spectrophotometer).

Cultivation

Algae culture

Two *Microcystis aeruginosa* strains from the Pasteur Culture Collection of Cyanobacteria - PCC 7820 and PCC 7941 - were used as experimental models for *in vitro* lytic tests. The strains used were unicellular, planktonic and producers of gas vesicles. The cells do not posses surrounding mucilage, which can be seen in some *Microcystis* strains (Bergey's Manual of Systematic Bacteriology, 2001). PCC 7820 and 7941 are both toxin-producers (Robillot *et al*. 2000 and Birk *et al*. 1988). They were

cultivated in Cyanobacteria BG11 Freshwater media (Sigma Aldrich) at 22 ºC under photosynthetically active radiation (PAR) irradiance of approximately 5 µE m^2s^{-1} (IL1400 radiometer with PAR sensor) with a 12 hour light/12 hour dark cycle.

Isolation of lytic agents

To visually isolate lytic bacterial strains, a "cyanobacterial lawn" (Whyte et al., 1985) was prepared. A volume of 15 ml of each of the axenic cyanobacterial cultures (PCC 7820 & PCC 7941) measuring approximately 800 fluorescence units (approximately $4.8x10^5$ cells ml^{-1}) were filtered (TD-700 Fluorometer, Turner Designs) onto 0.22 µm Polyether sulfonate membranes (Gelman Supor, 47 mm diameter). Different amounts of lake water sample inoculums (1 ml, 0.1 ml and 0.01 ml) were included in each filtration. The filters were transferred to BG11-agarose petri plates sealed with parafilm and incubated under the cyanobacterial growth conditions described above. The filters were monitored for 3 days for clearing colonies, observed under a stereo microscope (Olympus SZ61). Colonies considered lytic were further purified on LB-agar (Luria-Bertani agar medium). The morphology of the isolated colonies was noted.

Lytic test on Cyanobacterial lawn

To confirm that the isolated colonies maintained their lytic abilities they were re-streaked on BG11-agar (1.4 %), when visible colonies formed they were re-streaked the same way for 2 consecutive times. The colonies were then suspended in BG11 liquid media and 5 µl of suspension was dropped onto a "cyanobacterial lawn" in replicates and on two different strains of cyanobacteria (PCC 7820 & PCC 7941) (Adapted from Daft & Stewart, 1971). The "cyanobacterial lawns" were photographed for 3 consecutive days (Canon G9 Zoom lens 6x15 fixed to a Kaiser RS1 Camera stand) so that colony diameter and lytic halo diameter formation could be measured using Image J (public domain – Image Processing and Analysis in Java). The enzymatic activity was then represented as the colony diameter divided by the halo diameter ratio expressed as Enzymatic Index (EI Ø) (Rosato *et al*., 1981).

Lytic test in cyanobacterial liquid culture

The selection of isolates for this test was based on morphologic characteristic; the enzymatic index (EI) from the lytic test on "cyanobacterial lawns"; and on differing

success depending on the cyanobacterial strains (PCC 7820 and PCC 7941). In total 6 isolates were selected: isolate 1 (E52) had the best EI on PCC 7820; isolate 2 (E55) had good EI on PCC 7820 and was isolated from the same strain; isolate 3 (E35) isolate had good EI on PCC 7820 and was isolated from PCC 7941; isolate 4 (E52) had best EI on PCC 7941; the isolate 5 (E10) had good EI on PCC 7941 and was isolated from it; and the isolate 6 (E124) had good EI on PCC 7941 and was isolated from PCC 7820.

The selected isolates were suspended in 2 ml of BG-11 medium and incubated at 27 ºC for 48 hours. The bacterial suspension was then prepared for flow cytometric enumeration according to del Giorgio and collaborators (1996). This enumeration was repeated to assess the average growth expected during the experimental setup. This data was then used to dilute the bacterial isolates to similar cell concentrations for the lytic assay (Sigee *et al*., 1999 and Daft & Stewart, 1971). Estimation of the cyanobacterial cell density was done using the acridine orange direct counting technique with an epifluorescent microscope (Nikon digital camera, DXM 1200).

A MicroWell method was used, adapted from Uchida et al. (1998), 96 well plates (optical-flat bottom black, NUNC) were inoculated with 100 µl of cyanobacteria and 100 µl of the cell suspensions of cyanolytic bacteria in BG-11 media. The isolates were added in triplicates of different dilutions. On each MicroWell plate duplicates of only cyanobacteria and only BG-11 media were maintained as controls. Two experiments were carried out, lytic test 1 with PCC 7820 and PCC 7941, lytic test 2 with only PCC 7941 but with higher concentrations of lytic bacteria. The MicroWell plates were read in the morning and in the afternoon for up to 472 hours for the first and up to 344.5 hours for the second test. Fluorescence was measured in the MicroWell plates in an Ultra 384 Tecan (Tecan Austria GmbH) at 450 nm excitation and 670 nm emission (25 nm bandwidth) and raw signals obtained were corrected by dividing them by the instrument gain.

DNA extraction, PCR and sequencing

From lake waters, cells were collected by vacuum filtration through 0.2 µm pore size, Supor membrane filters (Pall Corporation). Filters were immediately frozen at -80 °C for later processing. Extraction of community deoxyribonucleic acid (DNA) from the membrane filters and from the isolated bacteria was carried out using the PowerSoil DNA isolation Kit (MO BIO laboratories) as recommended by the manufacturer. The molecular size of extracted DNA was determined by agarose gel electrophoresis (1 %) after staining with GelRed (Biotium, California, U.S.A). DNA was detected by transillumination using a cooled CCD camera and Gel-pro Analyzer (3.1 Media Cybernetics).

All isolates from the lytic tests were subjected to polymerase chain reaction (PCR) amplification of the 16s rRNA gene. The bacterial primers 27 forward (27f) (Vergin *et al*., 1998) and 519 reverse (519r) (Lane *et al*., 1985) were used. For community fingerprints with terminal restriction fragment length polymorphism (T-RFLP), DNA from both isolates and lake bacterioplankton was subject to PCR. The primers used were a forward (27f) labeled with the hexachlorofluorescein (hex), fluorescent dye, and unlabeled 519r.

Each 20 µl PCR reaction contained 1x PCR buffer; 0.25 mM dNTP mix; 0.2 µM of each primer; 0.05 U Taq polymerase (Biolin); 1.5 mM MgCl dissolved in Milli Q water. A MyGene Series Peltier thermal cycler (Model MG96+) was used for the amplification step (adapted from Langenheder *et al*., 2005). The amplicons were first purified using a QIAquick PCR purification kit and subsequently quantified using a Quant-iT^{TM} PicoGreen (dsDNA) Reagent Kit (Invitrogen), according to Invitrogen manual instructions. DNA quantification was performed with an Ultra 384 (Tecan Austria GmbH) at 485 nm excitation and 530 nm emission (20 and 25 nm bandwidth respectively).

Amplicons from strains were directly sequenced at The Dept. of Genetics and Pathology sequencing facility (Genome Centre, Uppsala, Sweden) using Sangar sequencing with the ABI Dye Terminator kit on an ABI3730XL sequencer. The operational taxonomic unit (OTU) data matrix was obtained using Sequence Scanner (v. 1.0). Sequences were manually inspected and a phylogenetic tree was constructed using Ribosomal Database Project (RDP). RDP was used to link taxonomy to phylogeny by construction of a Phylogenetic Tree using distance-based method of weighted neighbor-joining or

Weighbor (J. R. Cole *et al*., 2009). The output from RDP was used to draw the tree in Treeview (Page, 1996).

T-RFLP

Amplicons with forward labeled primers, as previously described, were diluted to obtain similar concentrations for all samples. The restriction enzymes used for digestion were HinfI and HaeIII (New England Biolabs.). Samples were incubated for 16 hours under 37 ºC for complete digestion, following product manufacturer instructions. Fragment sizing was performed by Rudebeck laboratories (Genome Centre, Uppsala, Sweden) using an ABI3730XL 96 capillary DNA analyzer in Genescan mode. Fragment analysis was carried out in duplicates with Gene Marker (v. 1.91). The terminal-restriction fragment (T-RF) abundance was expressed as peak area and the categorical operational taxonomic units were manually grouped (bin window 0.5 base pairs). Fragments below 50 base pairs were not analyzed. To distinguish noise from signal the minimal intensity threshold was set for 100 fluorescence units (Schütte et al., 2008).

Data Analysis

A multiple regression and Pearson's correlation tests were used to correlate environmental parameters measured (transformed to achieve normal distribution) to the intensity of the cyanobacterial bloom. Wilcoxon signed-rank test, paired t-test and ANOVA were performed to evaluate the lytic ability of bacterial isolates on cyanobacteria in liquid medium (BioEstat 4.0 statistical software - Ayres et al., 2005). Terminal restriction fragments (TRF's) from both restriction enzymes (HaeIII and HinfI) were used to characterize the environmental sample and a cluster analysis was performed (unweighted pair group average algorithm) using Bray Curtis distance measure with Bootstrap confidence estimation. To evaluate the possible correlation between environmental variables and change in bacterial community structure a canonical correspondence test was performed using PAST (Palaeontological Statistics v. 2.06 , PAST - Hammer et al., 2001)

Results

Phytoplankton bloom characteristics and water chemistry

During the summer sampling period, the collected field data at Lake Ekoln, was used to characterize environmental conditions in relation to the cyanobacterial bloom. A multiple regression was performed showing none of the abiotic variables (pH, DOC, soluble reactive phosphorus (SRP), water temperature) to be significantly correlated to chl-a. A linear regression of DOC (dependent variable) and temperature correlated (R^2 = 48.90 %, F = 10.57, p = 0.0099). Pearson's correlation coefficient, based on log - transformed values of chlorophyll-a and soluble reactive phosphorus (SRP), was inversely correlated, (r = -0.7822 and p = 0.0127, Figure 1), which indicates phytoplankton depletion of biologically available phosphorus during bloom peak.

The range average of water parameters, measured during the whole sampling period, showed highest variations in SRP and chl-a values, the latter was measured from beginning of the bloom until it was visibly undetectable. The temperature was stable during the summer months, ranging between 20.8 and 19.6 ºC from July to August, declining in the months leading from September to late October. The pH did not vary considerably within the lake and during the sampling period (Table 2).

Table 2. Water parameters with average and range values of the sampling period (early July until late October)

parameters	average	range
Chl-a (µg L^{-1})	19.80	2.59-76.59
DOC (mg L^{-1})	12.23	8.00-14.4
pH	7.94	6.65-8.81
Water temperature (ºC)	18.54	7.8-25
SRP (µg L^{-1})	7.93	0.53-29.48

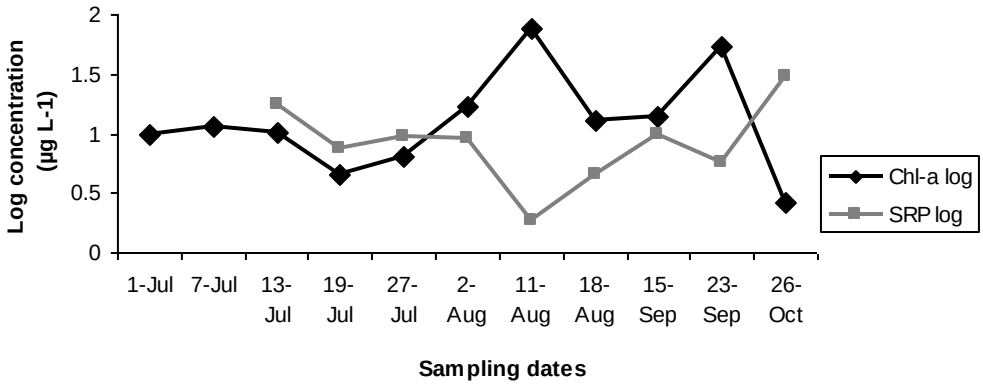


Figure 1. Log plot of chlorophyll-a (µg/l) and soluble reactive phosphorus (SRP) (µg/l), throughout the sampling period. There were two peaks of chlorophyll-a, one in August and another one late September, each with a corresponding decline in soluble reactive phosphorus.

Lytic isolates

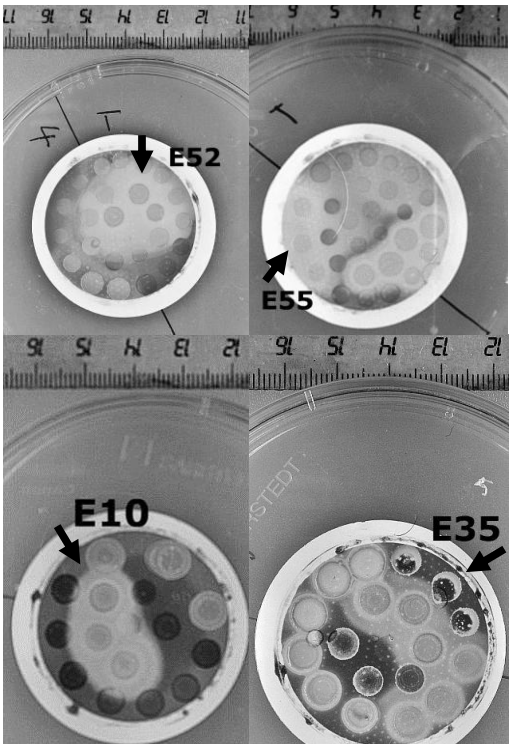
A total of a 151 lytic bacteria were isolated and tested for lytic abilities on cyanobacterial lawns. Among these, different lytic characteristics were recorded and classified depending on colony morphology and lytic ability. Colonies that produced discoloration on the cyanobacterial lawns (yellow, light green, and white) and inhibition halos surpassing colony diameter (diffuse halo and defined halo) were selected for further processing. The isolates with visible lytic halos were only observed on PCC 7941, and were used to calculate enzymatic activity (Enzymatic Index – EI Ø) (Table 3). The lower EI indicates higher lytic activity. The isolates chosen, due to low EI, for further lytic activity studies were E52 (defined halo); E55 (diffuse halo); E35 (on PCC 7941 displaying light green colour under colony with white dots and a white border; on PCC 78201 presents diffuse halo); E10 (on PCC 7941 and PCC 7820 presents diffuse halo); E124 was also subjected to further test but was later proven to be the same OTU as E55 and the data was thus not included. The isolate E141 produced an anti inhibition halo against other bacterial isolates, impeding the effect of the other lytic bacteria on the “cyanobacterial lawn” under the E141 colonies (Figure 2).

Table 3. Lytic activity (Enzymatic Index)

Isolate	Col. diam. mean (mm)	Halo diam. mean (mm)	EI Ø col../halo (diam. - mm)	Lysis halo	Date of isolation
E10	5.72 ± 0.41	12.9 ± 1.57	0.44	diffuse halo	August 3rd
E11	5.63 ± 0.24	9.38 ± 0.86	0.60	diffuse halo	August 3rd
E13	5.92 ± 0.21	9.43 ± 0.51	0.63	diffuse halo	August 3rd
E14	5.73 ± 0.29	9.01 ± 0.49	0.64	diffuse halo	August 3rd
E16	5.85 ± 0.15	13.97 ± 0.69	0.42	yellow	August 3rd
E20	5.72 ± 0.53	15.09 ± 2.99	0.38	diffuse halo	August 3rd
E22	5.57 ± 0.27	3.63 ± 1.09	1.53	diffuse halo	August 3rd
E24	6.75 ± 0.56	8.16 ± 0.05	0.83	defined halo	August 3rd
E27	6.11 ± 0.49	7.84 ± 0.53	0.78	defined halo	August 3rd
E28	6.34 ± 0.19	7.92 ± 0.50	0.80	defined halo	August 3rd
E29	6.57 ± 0.24	8.69 ± 0.15	0.76	defined halo	August 3rd
E30	6.84 ± 0.36	7.49 ± 0.32	0.91	defined halo	August 3rd
E32	6.27 ± 0.43	7.85 ± 0.72	0.80	defined halo	August 3rd
E33	5.81 ± 0.40	8.20 ± 0.93	0.71	defined halo	August 3rd
E34	6.00 ± 0.48	8.91 ± 0.84	0.67	diffuse halo	August 3rd
E36	6.33 ± 0.26	8.13 ± 0.43	0.78	defined halo	August 3rd
E38	5.88 ± 0.31	13.23 ± 0.32	0.44	diffuse halo	August 3rd
E39	5.81 ± 0.40	8.21 ± 0.56	0.71	diffuse halo	August 3rd
E40	5.51 ± 0.85	7.98 ± 0.90	0.69	diffuse halo	August 3rd
E31	5.24 ± 0.49	7.25 ± 0.64	0.72	defined halo	August 3rd
E46	5.64 ± 0.79	12.07 ± 0.76	0.47	diffuse halo	August 3rd
E52	4.85 ± 0.53	20.89 ± 1.05	0.23	defined halo	August 3rd
E55	4.35 ± 0.12	11.76 ± 1.53	0.37	diffuse halo	August 3rd
E57	4.84 ± 0.79	10.45 ± 0.98	0.46	defined halo	August 11th
E61	5.34 ± 0.59	10.83 ± 1.48	0.49	defined halo	August 11th
E77	4.38 ± 0.30	6.72 ± 0.61	0.65	diffuse halo	August 11th
E101	4.26 ± 0.47	7.53 ± 1.15	0.57	defined halo	August 11th
E102	4.16 ± 0.10	8.53 ± 0.53	0.49	defined halo	August 11th
E104	3.85 ± 0.47	9.49 ± 0.53	0.41	defined halo	August 11th
E105	4.92 ± 0.50	8.55 ± 1.59	0.58	defined halo	August 11th
E108	4.67 ± 0.48	7.71 ± 0.31	0.61	defined halo	August 11th
E103	4.53 ± 0.48	8.48 ± 1.87	0.53	defined halo	August 11th

Col. diam: colony diameter; mean (mm): average in millimeters. EI: Enzymatic index.

a.



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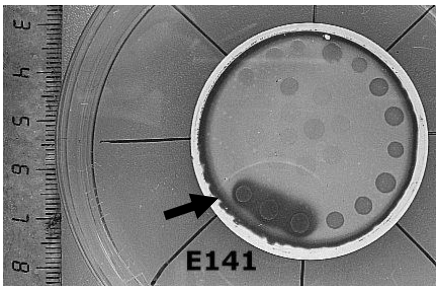

Figure 2. a- Lytic characteristics of isolates dropped in triplicates on to PCC 7941 cyanobacterial-lawn. E52 presents defined halo around colony; E55 presents diffuse halo; E10 shows defined halo; E35 presents light green colour under colony with white dots and a white border. **b-** Isolate E141, on PCC 7941, shows an inhibition halo against other lytic bacteria, maintaining a very green halo around and under the colony.

Lytic test in cyanobacterial liquid culture

There was a strong difference in fluorescence measurements taken in the morning and in the afternoon of the same day. Measurements were higher in the afternoon compared to morning (Wilcoxon signed-rank test $p < 0.0001$). Therefore the mean fluorescence measurements between morning and afternoon were used.

Two lytic tests were performed, the first using both strains of cyanobacteria (PCC 7820 and PCC 7941) and the second with only PCC 7941. Cyanobacterial cell concentrations (cells mL^{-1}) for Lytic tests 1 were: PCC 7820: 3.45×10^5 and PCC 7941: 3.98×10^5 and for Lytic test 2: PCC 7941: 2.35×10^5.

Lytic test 1

Different cell concentrations of lytic isolates were tested (Table 4). For the lytic test 1 the concentration, which yielded higher inhibition when tested with PCC 7820 was 10^6 cells mL^{-1} for all isolates except E141, where the highest inhibition was found with cell contcentrations of 10^4 cells mL^{-1}. When tested with PCC 7941 most isolates presented higher inhibition capacity at 10^4 cells mL^{-1}, except E52 and E55, which were more inhibiting at concentrations of 10^6 cells mL^{-1}. The results for Lytic test 1 can be seen in Figure 3 for tests on PCC 7820 and in Figure 4 for tests with PCC 7941.

Table 4. The different cell concentrations (cells mL^{-1}) of lytic bacteria used in lytic test 1.

Isolate	A	B	C	D	E	F
E52	0.91×10^6	0.91×10^5	0.91×10^4	0.91×10^3	0.91×10^2	9.15
E55	0.99×10^6	0.99×10^5	0.99×10^4	0.99×10^3	0.99×10^2	9.98
E10	0.75×10^6	0.75×10^5	0.75×10^4	0.75×10^3	0.75×10^2	7.51
E35	0.52×10^6	0.52×10^5	0.52×10^4	0.52×10^3	0.52×10^2	5.23
E141	0.23×10^6	0.23×10^5	0.23×10^4	0.23×10^3	0.23×10^2	2.40

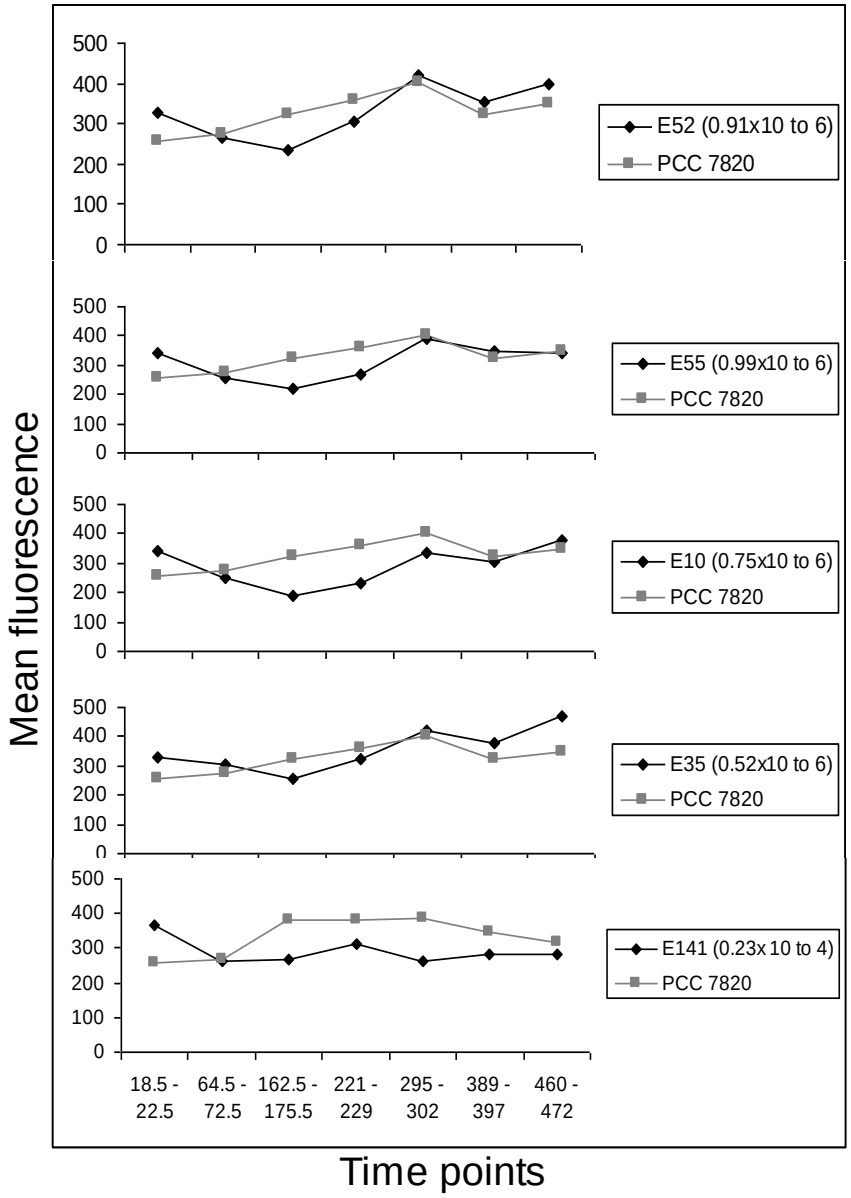

Figure 3. Lytic test 1 measure pattern of the cyanobacteria inoculated with lytic isolates compared to a control without inoculation of lytic strains (PCC 7820). Time points in hour intervals.

The suppression of cyanobacterial growth and biomass development caused by lytic bacteria was compared to the control treatment for each time point. The highest measured suppression of PCC 7820 in lytic test 1 was at time ranging from 162.5-175.5 hours, corresponding to 6 to 7 days after inoculation. The highest suppression against

PCC 7820 reached 41 % and was achieved with E10. This effect was significant (paired t-test $t = 3.05$, $p = 0.0377$, $n = 5$).

The inhibition on PCC 7941 was significantly higher than on PCC 7820 ($p < 0.005$) for all isolates except E10 which exhibited high inhibition on both strains (Figure 4). After 162.5-175.5 hours of incubation, the cyanobacterial biomass assessed with chlorophyll-a was 61 and 64 % lower than references for incubations with E52 and E55, respectively, and this inhibition was significant ($t = 3.59$, $p = 0.0114$ and $t = 5.35$, $p = 0.0.0017$, respectively). The other lytic isolates caused higher inhibition at time points of 389-472 hours, ranging from 44.18 to 55.5 %. Lytic isolate E10 had high suppression on 7941 and the inhibition was 44.18 % lower than references at a time point of 389-397 hours and the suppression was significant ($t = 5.87$, $p = 0.0020$).

Cyanobacteria treated with lytic bacteria typically showed a decline followed by an increase towards the end of the experiment, when inhibition seems to become less effective. This pattern was observed for both cyanobacterial strains PCC 7820 and 7941 in lytic test 1 (Figures 3 and 4). In lytic test 2, this pattern was again apparent when bacteria were incubated with cyanobacterial strain PCC 7941. However there was no increased growth towards the end of the experiment. One possible explanation for this could be that the cyanobacteria in lytic test 1 gain resistance to the lytic bacteria.

We also tested whether isolate E141, which had shown inhibition against lytic bacteria on “cyanobacterial lawns”, were able to cause similar inhibition in liquid culture. In lytic test 1, we inoculated equal amounts of each lytic bacteria and E141 with the cyanobacterial strain. There was no significant inhibition of the lytic activity of isolates E52, E55, E10 or E35 by isolate E141 when tested in cyanobacterial strain PCC 7820. However, where the highest inhibition had been seen previously for E10 with 40.9 % it was reduced to 28.6 %. The presence of E141 did not cause any reduction in E35 inhibition. Using strain PCC 7941 we observed significant reduction in lytic activity for E52 and E55 when E141 was inoculated along with the lytic bacteria ($t = 2.59$, $p = 0.023$, $n = 7$ and $t = 4.22$, $p = 0.0012$, $n = 7$ respectively). For E10, maximum level of inhibition

was lowered from 44.2 % to 19.1 %. For E35 lytic inhibition was observed from 33.5 % to 9.1 %.

In lytic test 2 when we tested for inhibition of lytic activity by the isolate E141, we observed significant inhibition of the activity of all cyanolytic isolates.

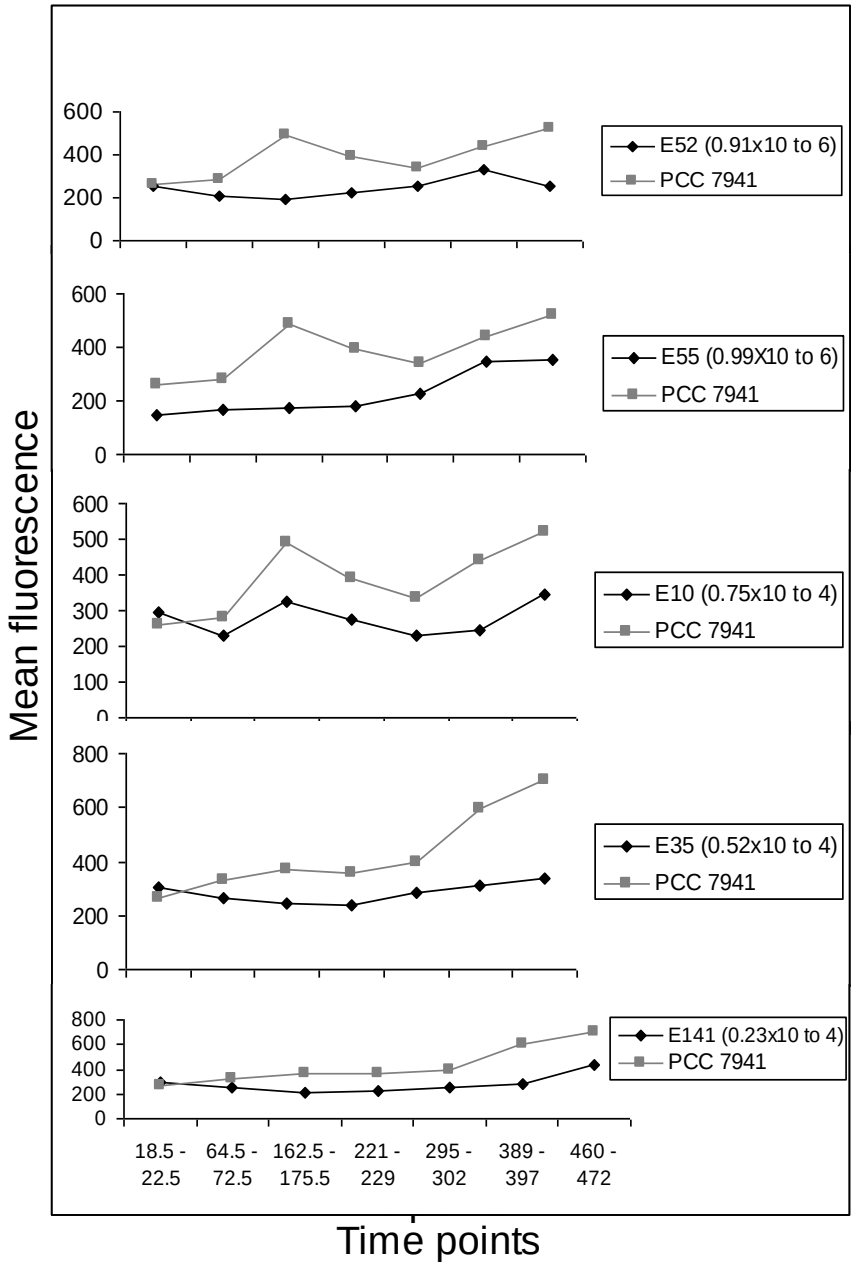

Figure 4. Lytic test 1 measure pattern of the cyanobacteria inoculated with lytic isolates compared to a control without inoculation of lytic strains (PCC 7941). Time points in intervals of hours.

Lytic test 2

For the second lytic test only one cyanobacterial strain was used (PCC 7941). Slightly higher concentrations of lytic bacteria were inoculated to test if there would be significant difference in suppression of cyanobacterial biomass (Table 5). Intermediate concentrations (ca 10^5 cells ml^{-1}) caused the highest inhibition for all isolates. The highest bacterial inoculum (10^7cells ml^{-1}) seemed to have the inverse effect on inhibition, and rather stimulated cyanobacterial growth (Figure 5).

Table 5. The cell concentration (cells ml^{-1}) of the bacterial isolates used in lytic test 2.

Isolate	A	B	C	D	E	F
E52	0.47×10^7	0.67×10^6	0.67×10^5	0.67×10^4	0.67×10^3	0.67×10^2
E55	0.51×10^7	0.77×10^6	0.77×10^5	0.77×10^4	0.77×10^3	0.77×10^2
E10	0.38×10^7	0.43×10^6	0.43×10^5	0.43×10^4	0.43×10^3	0.43×10^2
E35	0.27×10^7	0.21×10^6	0.21×10^5	0.21×10^4	0.21×10^3	0.21×10^2
E141	0.12×10^7	0.44×10^6	0.44×10^5	0.44×10^4	0.44×10^3	0.44×10^2

The highest suppression of cyanobacterial biomass compared to the control was at time point 117-126 hours for E55 and E10. Isolate E52 had the highest inhibition of all the isolates and this was achieved at time point 141-148.5 hours with 33.18 %, and for E35 and E141 this level of inhibition was reached a bit later at time point 261-269 hours. The general inhibition was significant for all isolates (ANOVA $p < 0.05$ and $F > 5.0$) except E141. The highest general inhibition was reached by E52 ($F = 48.91$, $p = 0.0004$) (Figure 5).

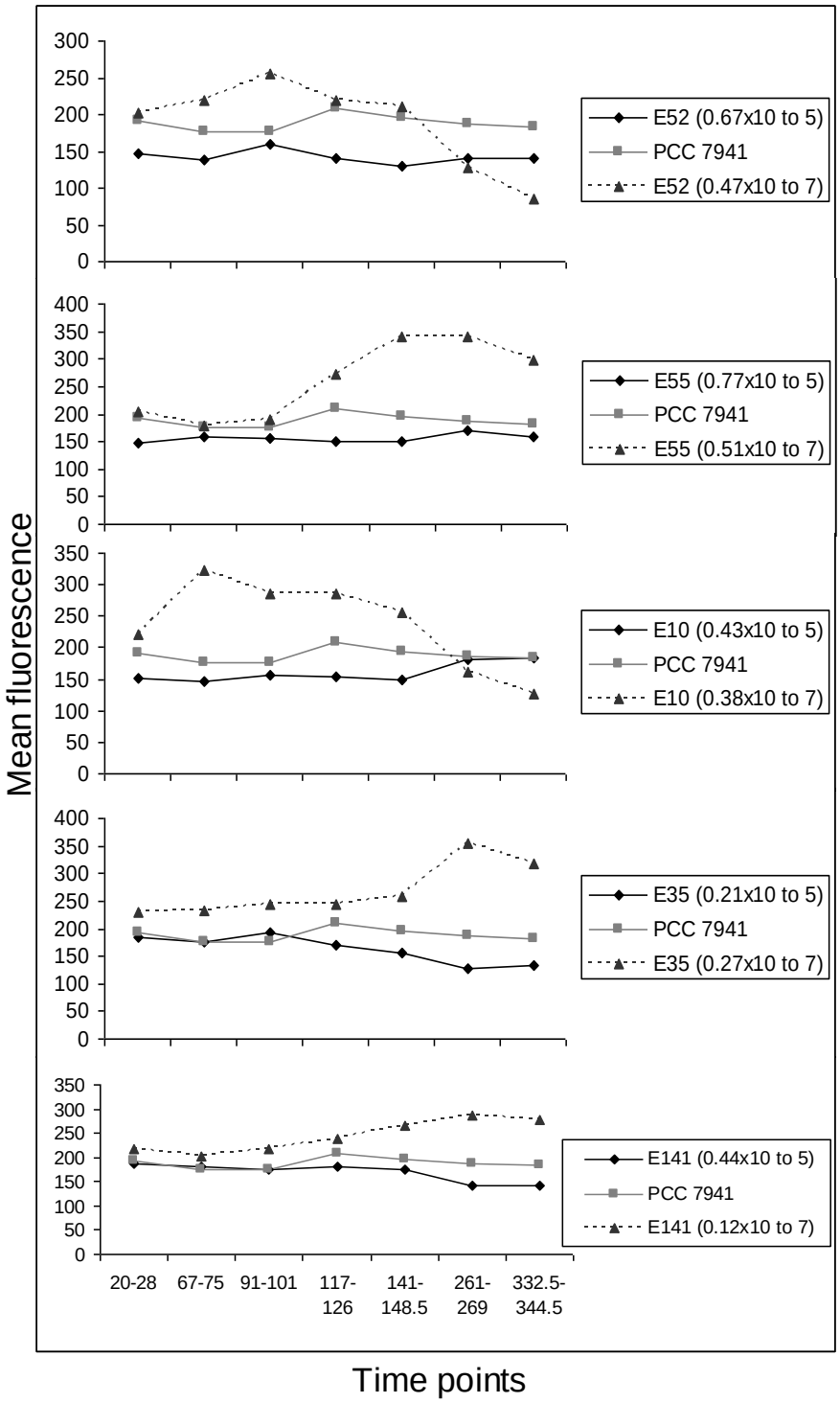

Figure 5. Lytic test 2 measure pattern of the cyanobacteria inoculated with lytic isolates compared to a control (PCC 7941). The dilution with most successful inhibition represented by filled line ($x10^5$) and the highest concentration used, dashed line, which was not the most successful at causing inhibition ($x10^7$).

Sequence data

The sequences obtained were compared to 16s rRNA sequences from the Ribosomal Database Project (RDP). This database provides comparison with sequences that have been put through a screening for anomalies, allowing a selection of good quality reference sequences for comparison and identification. The original sequences are found in the supplemental materials (Table S1).

The isolates were phylogenetically related to known isolated bacteria and their physiological characteristics have already been described by other authors (Figure 6).

Isolate E141, shown to inhibit lytic activity of other bacterial strains, was placed close to *Acinetobacter heamolyticus,* previously described by other authors to possess antibiotic resistance (Guardabassi *et al*., 1999). Isolate E35 and E10 clustered with *Pseudomonas fluorescens* and *Pseudomonas veronii* respectively; many *Pseudomonas* strains have been shown to have potential biocontrol properties against plant root pathogens (O'Sullivan & O'Gara, 1992 and Adhikari *et al*., 2001). Isolate E55 clustered with *Delftia acidovorans* [formerly *Comamonas* (Wen *et al*., 1999)] this type specie has been described to be able to degrade herbicides (2,4-dichloro-phenoxyacetic acid) (Hoffmann, 2003) and other recalcitrant xenobiotic compounds (Schulz *et al*., 2000) .

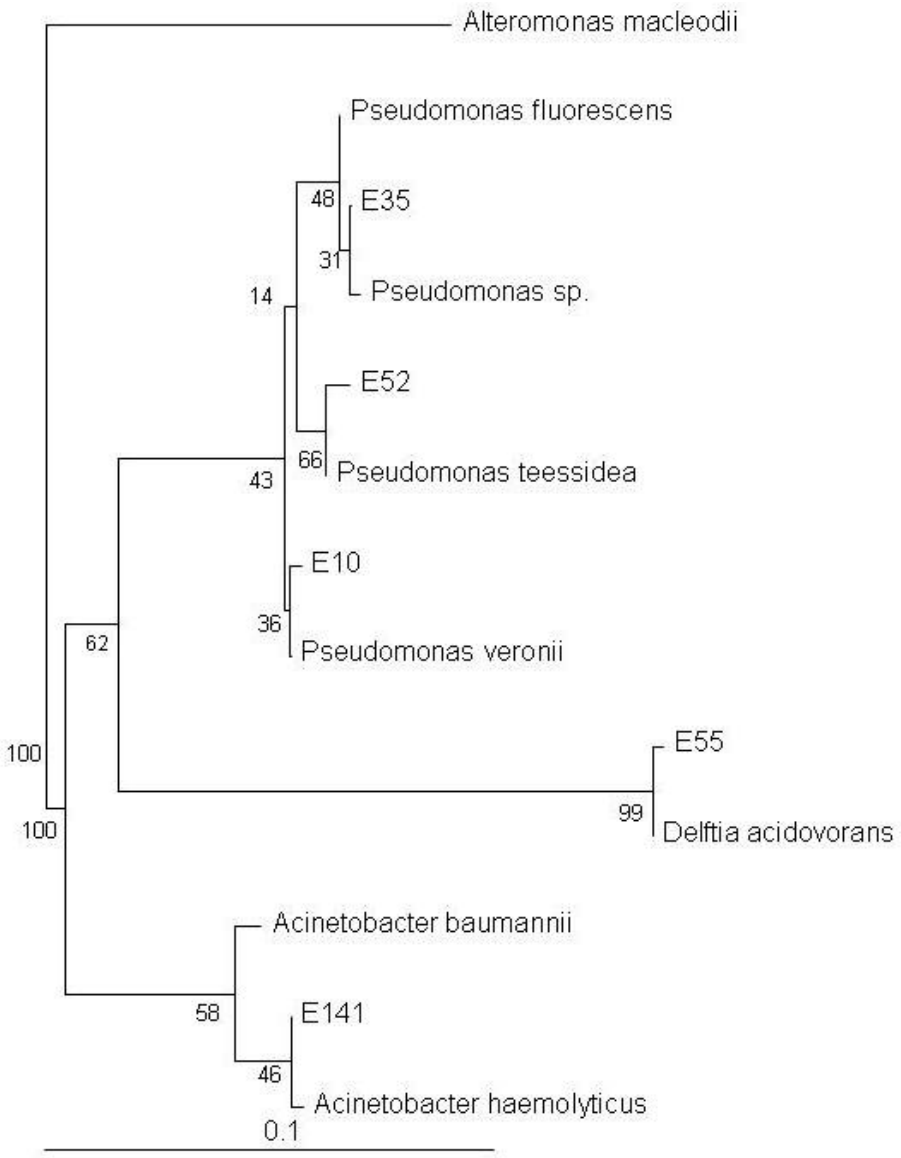

Figure 6. Phylogenetic tree of isolates E52, E55, E10, E35 and E141.

Community assembly analysis

The analysis of the terminal restriction fragments (T-RF's) allows us to carry out a survey on the occurrence of dominant bacterial populations in the lake samples over the study period. A combination of T-RF's from both HaeIII and Hinfl enzymes resulted in a temporal analysis of the change in the bacterial community structure. This change was evaluated against environmental parameters, to investigate a possible correlation, using a canonical correspondence test (PAST - statistical software), the environmental data

was normalized and the two first sampling points (Table 6) were excluded to achieve normalized data of soluble SRP (Figure 7). From the canonical correspondence ordination, the arrow size of SRP and chl-a are the largest and appeared to be the most important environmental variables in determining bacterial community sample distribution.

The arrows point in the direction related to highest change in the environmental variable. The sampling time with highest chlorophyll-a measures was on August 11th (11/8), which, seen in Figure 7 can be placed in the direction of the axis with an increasing chlorophyll gradient. Soluble reactive phosphorus shows a gradient in the opposite direction of Chl-a and has greatest influence where the highest phosphorus and lowest Chl-a values (26/10) are represented. The community distribution can thus be interpreted as indirectly influenced by these two environmental variables (Ter Braak, 1986).

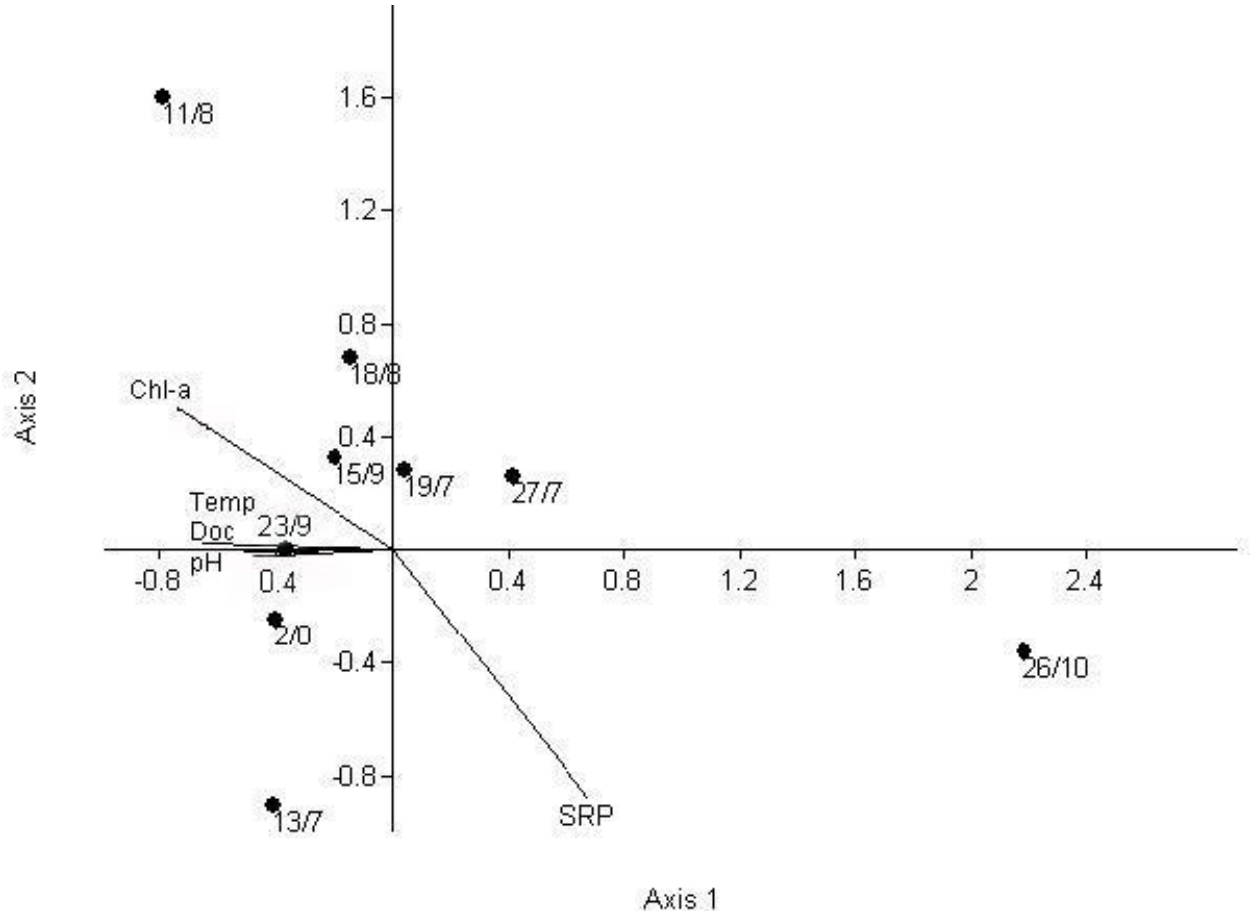

Figure 7. Canonical correspondence with a triplot showing the relation of the bacterial community samples to environmental factors. The arrows for temperature, DOC and pH are overlapping. The dates are shown in the plot as day of the month sampled.

Analyzing the similarity of bacterial communities over time, a cluster analysis showed that the first sampling period (July 1-19) clustered together along with samples obtained between late August and mid-September. These two clusters were clearly separated from the remaining samples which were quite divergent. The cophenetic correlation coefficient was 0.9912 (Figure 8).

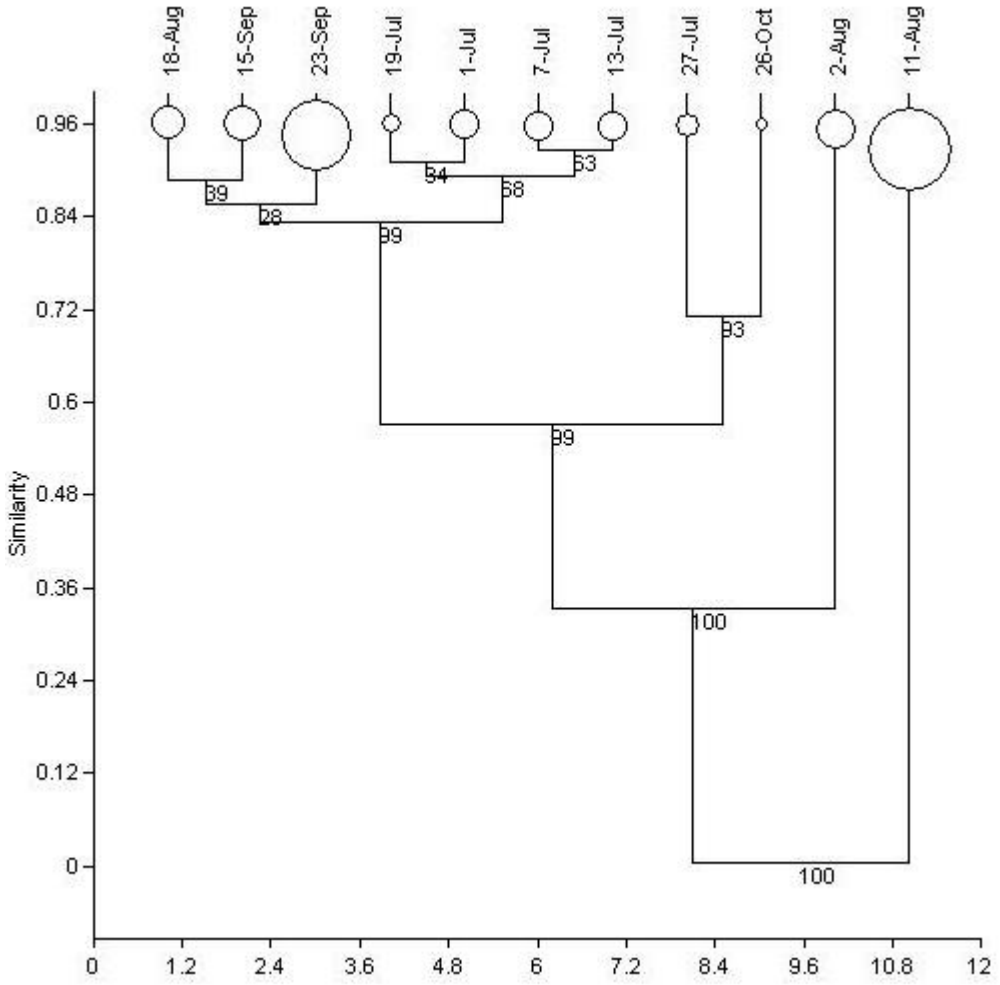

Figure 8. Cluster analysis of TRF's of each sampling time from 1st of July until 26th of October. The size of the circles at the tip of the branches are proportional to Chlorophyll-a levels from sampling points.

From the T-RFLP analysis performed with the isolated bacteria (E52, E55, E10, E35 and E141) it was only possible to putatively identify the fragment size of isolate E55 within the environmental samples. The positive identification (both of the expected T-RF's present) was only found in the sample from the 7th of July (Table 6). We used two restriction enzymes to facilitate the distinction of lytic strains from other bacterial populations (Marsh et al., 2000). Even if the fragment was absent in the analysis it does not rule out the possibility that it exists in the population. It implies that it was not abundant enough to be detected (Schütte *et al*., 2008).

Table 6. Terminal fragment size from two enzymatic digestions.

Fragment length (bp)			
HaeIII	HinfI	Isolate	Sample
180.7	78.3	E52	nd
196.6	94.1	E55	July 7
nrs	111.7	E35	nd
197.3	113.7	E10	nd
251.4	112.3	E141	nd

bp= base pairs; nrs= no restriction site;
nd= not detected in environmental sample.

The peak distribution was compared to the occurrence of the SRP and Chl-a measurements during the bloom together with the total bacterial abundance. The highest peak in chlorophyll occurs closely to a decrease in the number of TRF peaks, which then increase again. The bacterial abundance seems to decrease right after the cyanobacterial bloom formation and correlates strongly with temperature and DOC (Pearson: $r = 0.87$, $p = 0.0005$ and $r = 0.689$, $p = 0.018$, $n = 11$, respectively). The highest numbers of lytic bacteria were isolated on August 11 correlating to the highest peak in measured chlorophyll-a (Figure 9).

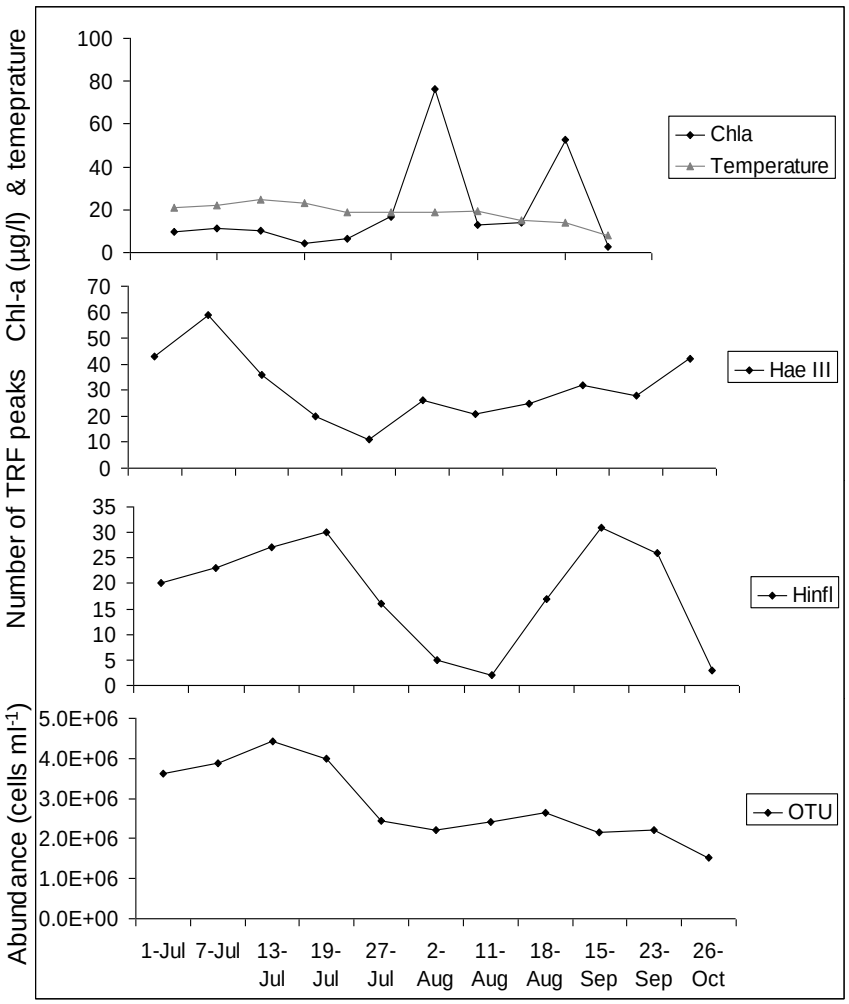

Figure 9. Chlorophyll-a in µg/l and temperature (°C), total peak number of TRF's and cell abundance per ml from first of July to 26 of October.

Discussion

Recovery of cyanolytic bacteria during the sampling period was carried out during cyanobacterial bloom formation. We observed that most lytic bacteria were detected when chlorophyll-a peaks were the highest. Daft and collaborators (1975) suggest that total bacterial abundance can also be generally correlated to cyanobacterial growth, although not as directly as lytic bacteria. In our study we found that total abundance of bacteria was directly correlated to organic nutrients and temperature, this is in

agreement with the hypothesis that bacteria can benefit from the primary production during warmer months.

We used fingerprinting methods and cluster analysis to characterize the change in bacterial communities during the months of bloom formation. This lead us to the conclusion that there is a shift in community structure, which can indirectly be related to the environmental factors. In this study, phosphorus and chlorophyll seemed to have most influence. When studying the detection of the lytic bacteria isolated (E52, E55, E35, E10 and E141) in the environmental samples we observed that the threshold for their detection was below what is considered for T-RFLP analysis. Though we used two restriction enzymes and identified isolate E55 occurring in the environmental sample taken on July 7, there is still the possibility that other lytic bacteria feature the same T-RF size. The restriction site reflects phylogeny to some extent but can be extended to populations, thus including a well characterized isolate can give a better reference perspective (Marsh *et al*., 2000).

The bacteria that were selected for specific lytic tests, in liquid cultures of cyanobacteria, belong to the Phylum *Proteobacteria* of the *Gammaproteobacteria* - Class III (*Acinetobacter heamolyticus; Pseudomonas fluorescence; and P. veronii)* and the *Betaproteopbacteria* – Class II *(Delftia acidovorans*). The occurrence of lytic bacteria in the *Proteobacteria* group agrees with other author's studies on cyanolytic bacteria, however antagonist bacteria seem to have a wide distribution and are not restricted to this group.

The investigation of the lytic bacteria as potential biocontrol (E52, E55, E35, and E10) indicates that the bacteria manifest different lytic activity on the cyanobacterial-lawn and to some extent also in cyanobacterial liquid culture (micro-plate). On the cyanobacterial lawn, inhibition halos produced by some of the lytic bacteria were clearly visible after 48 hours of incubation (E52, E55 and E10). While other bacteria (E35) produced inhibition limited only under the colony; and in one case (E141) other lytic bacteria were inhibited from lysing the cyanobacteria. Isolate E35 (*Pseudomans fluorescence*) might be limited to contact lysis as have been suggested in previous studies (Jung *et al*., 2008).

When tested in liquid culture the bacteria capable of producing inhibition halos were more successful at reducing the *Microcystis* test strains. This can be attributed to the different lytic strategies of bacteria. On a solid media there is greater chance of lysis due to direct contact and close proximity between predator and prey. In liquid culture, the production of an extracellular inhibition compound would be more favorable. The quorum sensing in the bacteria was not studied. However, this mechanism could play a role in the lytic activities of the studied bacteria. Quorum sensing bacteria are able to detect accumulation of autoinducers produced by other bacteria and as these levels build up when populations of the bacteria increase, this will trigger an alteration in gene expression and behavior (Waters & Bassler, 2005). Other strategies can also increase lytic ability, for instance if the bacterium posses gliding ability they can form aggregates and enhance predation on cyanobacteria by secreting lytic enzymes at high concentrations (Fraleigh & Burnham, 1988). In this study only two strains of the same cyanobacterial genus was tested. In lytic test 1 we compared the inhibition success on the two strains. All lytic bacteria except E10 where more successful at inhibition on PCC 7941.

The size of the treatment inoculum was another factor that had an influence on the reduction of cyanobacterial growth. In our study a higher inoculum did not show better inhibition. Instead it sometimes seemed to stimulate cyanobacterial growth. The inoculum of c x 10^5 or c x 10^6 had higher inhibition success compared to c x 10^7. This could be due to nutrient depletion during the experiment that would favor cyanobacteria. PCC 7820 is a facultative phototroph and chemoheterotroph, this could be a reason for its resistance compared to PCC 7941 (Garrity, 2001). Furthermore, Daft & Stewart (1971) showed that lytic bacteria in post-growth stage, although possessing lytic activity, were less efficient in lysing their prey. The growth state of the cyanobacteria can also influence susceptibility to antimicrobial agents. When the change from exponential growth to stationary phase occurs sensibility decreases dramatically, in some cases forming persister cells. The growth rate is decisive in this transition to tolerance (Samuilov *et al*., 2008). Some cyanobacterial strains might be more sensitive to biocontrol depending on the growth rate at the time of inhibition. In field application, one solution to this problem would be to prevent cyano-blooms before they are fully formed

(Sigee *et al*., 1999). Biocontrol would be the best option in this case since applying chemicals or investing in more expensive treatments before the formation of a bloom would be undesired and would encounter institutional reluctance.

The micro-plate method employed here was combined with direct lytic activity analysis performed on cyanobacterial lawns to test for cyanobacterial inhibition. Uchida and collaborators (1998) suggest that a combination of these two techniques yields better quantative and qualitative data. It has further been shown that there is no significant difference in chlorophyll-a measurements between micro-plates and flask cultures used in previous studies (Geis *et al*., 2000). However, previous studies of inhibition in micro-plates applied inhibitory compounds (Uchida *et al*., 1998; Yamamoto *et al*., 1998 and Eisentraeger *et al*., 2003). Therefore the inhibition dynamics of applying directly a biocontrol agent in micro-plates with cyanobacteria cultures are not well documented. Although the inhibitory effect of cyanobacterial microcystin toxins is well established, studies relating to this toxic effect have yet to fully determine if its influence on aquatic bacterial and algal composition reaches the point of altering bacterial community structure (Valdor & Aboal, 2007). Other authors argue that the microcystin toxin itself may not always have an inhibitory effect but that secondary metabolites effectively exert antibacterial properties (Østensvik *et al*., 1998). Many interactions that occur in the environment were not reproduced in this *in situ* experiment. In this sense, in the environment there are bacterial strains able to degrade cyano toxins released from cyanobacterial cell lysis or extracellular secretion, which could benefit from the activity of cyanolytic bacteria. This could explain why lower microcystin concentrations have been detected in the field from what could be expected during a cyanobacterial bloom, this can be due to dilution into the environment coupled with the activity of microcystin degrading bacteria (Christoffersen *et al*., 2002). In our micro-plate assay however, the secondary metabolites and microcystin production was not evaluated. It would be interesting to further examine if these compounds could reduce the effective inhibition of cyanobacteria especially in 96 well micro-plates with a reduced volume of 200 µl.

Conclusion and future perspectives

There was a successful isolation of lytic bacteria from Lake Ekoln during cyanobacterial bloom season. *In situ* measurements detected significant inhibition of two *Microcystis* test strains.

The isolated bacteria are not fastidious and grew within 24-48 hours on rich media such as Luria Bertani (LB) or fresh water media BG 11. This is an important factor when considering field trials, when there usually is limitation in nutrient availability. The bacteria used for biocontrol must be able to sustain growth long enough to cause significant inhibition. In our study highest inoculum did not always confer the best inhibitory results.

Though we found that cyanobacterial strain PCC 7941 had generally a higher growth than PCC 7820, potentially making it more susceptible to bacteriolytic inhibition, there are factors, such as host specificity, that may restrict successful inhibition to certain cyanobacteria. Some bacterial strains are generalists while other are target specific, this could depend from which environment they are isolated and the therein predominant cyanobacterial strain (Daft *et al*., 1975 and Rashidan & Bird, 2001). It would be of interest to test isolated biocontrol agents against cyanobacteria endogenous to the location of isolation, using well described techniques for cyanobacterial isolation and purification (Rippka, 1988). This might produce different results from tests on model strains of *Microcystis aeruginosa*. The next step would be in field, small-scale experiment to develop management strategies for reducing the re-occurrence of nuisance toxin-producing blooms. The advantage of using agents specific to an environment, without genetic modification, or of exotic origin, is that the alteration of the environment is temporary and a short term effect in bloom control, given that the lake assumes its natural conditions after the treatment is discontinued (Sigee *et al*., 1999). Biocontrol with indigenous bacteria do not pose a health threat and are an alternative solution to avoid build up of noxious cyanobacterial blooms before the onset of the summer season.

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References

Adhikari, T. B., Joseph, C. M., Yang, G., Phillips, D. a, & Nelson, L. M. (2001). Evaluation of bacteria isolated from rice for plant growth promotion and biological control of seedling disease of rice. *Canadian Journal of Microbiology*, *47*(10), 916-924. doi: 10.1139/cjm-47-10-916.

Ayres, M., Ayres, J. R., Ayres, D. L., & Santos, A. S. (2005). *BIOESTAT 4.0: Aplicação Estatística nas áreas das ciências biológicas e médicas* (p. 324). Belém:Sociedade Civil Mamirauá.

Bertilsson, S., & Tranvik, L. J. (2000). Photochemical transformation of dissolved organic matter in lakes. *American Scociety of Limnology and Oceanography*, *45*(4), 753-762.

Birk, I. M., Matern, U., Kaise, I., Martin, C., & Weckesses, J. (1988). The peptide toxin of the cyanobacteria Microcystis aeruginosa PCC 7941. *Journal of Chromatography*, *449*, 423-431.

Burnham, J. C., Collart, S. A., & Daft, Melvin J. (1984). Myxoeoccal predation of the cyanobacterium Phormidium luridum in aqueous environments. *Archives of Microbiology*, *137*, 220- 225.

Carmichael, W. (2001). Health Effects of Toxin-Producing Cyanobacteria: "The CyanoHABs." *Human and Ecological Risk Assessment: An International Journal*, *7*(5), 1393-1407. doi: 10.1080/20018091095087.

Choi, H.-jin, Kim, B.-ho, Kim, J.-dong, & Han, M.-soo. (2005). Streptomyces neyagawaensis as a control for the hazardous biomass of Microcystis aeruginosa (Cyanobacteria) in eutrophic freshwaters. *Biological control*, *33*, 335-343. doi: 10.1016/j.biocontrol.2005.03.007.

Christoffersen, K., Lyck, S., & Winding, a. (2002). Microbial activity and bacterial community structure during degradation of microcystins. *Aquatic Microbial Ecology*, *27*(Falconer 1993), 125-136. doi: 10.3354/ame027125.

Codd, G. (2000). Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. *Ecological Engineering*, *16*(1), 51-60. doi: 10.1016/S0925-8574(00)00089-6.

Cole, J. R., Wang, E., Cardenas, J., Fish, B., Chai, R. J., Farris, A. S., et al. (2009). The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids. Res. 37 (database issue): D141-D145; doi:10.1093/nar/gkn879. *Database issue: D141-D145*.

Daft, M. J., McCord, S. B., & Stewart, W. D. P. (1975). Ecological studies on algal-lysing bacteria in fresh waters, *5*, 577-596.

Daft, M. J., & Stewart, W. D. P. (1971). Blue-Green Algae: Isolation of Bacteria-free Cultures from Hormogone-producing Blue-Green Algae. *New Phytologist*, *70*(No. 5), 819-829. doi: 10.1038/1921275a0.

Eisentraeger, A., Dott, W., Klein, J., & Hahn, S. (2003). Comparative studies on algal toxicity testing using fluorometric microplate and Erlenmeyer flask growth-inhibition assays. *Ecotoxicology and environmental safety*, *54*, 346-354. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12651191.

Ensign, J. C., & Wolfe, R. S. (1965). Lysis of Bacterial Cell Walls By an Enzyme Isolated From a Myxobacter. *Journal of bacteriology*, *90*(2), 395-402. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14330733.

Fraleigh, P. C., & Burnham, J. C. (1988). Myxococcal predation on cyanobacterial populations: Nutrient effects. *Limnology and Oceanography*, *33*(3), 476-483.

Garrity, G. M. (2001). *Bergey's Manual os Systematic Bacteriology- The Archaea and deeply branching and phtotrophic Bacteria*. (D. R. (Ed. in Chief) Boone & R. W. (Eds) Castenholz, Eds.) (2nd ed.).

Geis, S. W., Fleming, K. L., Korthals, E. T., Searle, G., Reynolds, L., & Karner, D. A. (2000). Modifications of the algal growth inhibition test for use as a regulatory assay. *Environmental Toxicology and Chemistry*, *19*(1), 36-41. doi: 10.1897/1551-5028(2000)019<0036:MTTAGI>2.3.CO;2.

Giorgio, P. A. del, Bird, David F, Prairie, Y. T., & Planas, D. (1996). Flow cytometric determination of bacterial abundance in lake plankton with the green nucleic acid stain SYTO 13. *Limnology and Oceanography*, *41*(4), 783-789.

Guardabassi, L., Dalsgaard, a, & Olsen, J. E. (1999). Phenotypic characterization and antibiotic resistance of Acinetobacter spp. isolated from aquatic sources. *Journal of applied microbiology*, *87*(5), 659-67. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10594705.

Hammer, Ø., Harper, D. A. T., & Ryan, P. D. (2001). PAST: paleontological Statistics Software Package for Education and Data Analysis. Electronica4(1):9pp.http://palaeo-electronica.org/2001_1/past/issue1_01.htm.

Hoffmann, D. (2003). A transposon encoding the complete 2,4-dichlorophenoxyacetic acid degradation pathway in the alkalitolerant strain Delftia acidovorans P4a. *Microbiology*, *149*(9), 2545-2556. doi: 10.1099/mic.0.26260-0.

International Standard ISO 10260:1992. (n.d.). *Water Quality - Measurements of biochemical parameters - Spectrometric determination of the chlorophyll-a concentration.*

Jung, S. W., Kim, B.-H., Katano, T., Kong, D.-S., & Han, M.-S. (2008). Pseudomonas fluorescens HYK0210-SK09 offers species-specific biological control of winter algal blooms caused by freshwater diatom Stephanodiscus hantzschii. *Journal of applied microbiology*, *105*(1), 186-95. doi: 10.1111/j.1365-2672.2008.03733.x.

Kim, B.-ho, Sang, M., Hwang, S.-jin, & Han, M.-soo. (2008). In situ bacterial mitigation of the toxic cyanobacterium Microcystis aeruginosa : implications for biological bloom control. *Limnology and oceanopgraphy: methods*, *6*, 513-522.

Kim, C.-H., Leem, M.-H., & Choi, Y.-K. (1997). Isolation of bacterium that inhibits the growth of Anabaena cylindrica. *The journal of Microbiology*, *35*(4), 284-289.

Lam, A. K.-Y., Prepas, E. E., Spink, D., & Hrudey, S. (1995). Chemical control of hepatotoxic phytoplankton blooms: Implications for human health. *Water Research*, *29*(8), 1845-1854. doi: 10.1016/0043-1354(94)00348-B.

Lane, D. J., Pace, B., Olsen, G. J., Stahl, D. a, Sogin, M. L., & Pace, N. R. (1985). Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America*, *82*(20), 6955-9. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=391288&tool=pmcentrez&rendertype=abstract.

Langenheder, S., Lindström, E. S., & Tranvik, L. J. (2005). Weak coupling between community composition and functioning of aquatic bacteria. *Limnology and Oceanography*, *50*(3), 957-967. doi: 10.4319/lo.2005.50.3.0957.

Manage, P. M., Kawabata, Z., & Nakano, S.-ichi. (2000). Algicidal effect of the bacterium Alcaligenes denitrificans on Microcystis spp . *Aquatic Microbial Ecology*, *22*, 111-117.

Marsh, T. L., Saxman, P., Cole, J., & Tiedje, J. (2000). Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Applied and environmental microbiology*, *66*(8), 3616-20. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=92192&tool=pmcentrez&rendertype=abstract.

Mason, C. F. (2002). *Biology of Freshwater Pollution* (4th ed.). Pearson, Essex.

Murphy, J., & Riley, J. P. (1962). A modified single solution method for the determination of phosphate in natural waters. *Analytica Chimica Acta*, *27*, 31-36.

Oliver, R. L., & Ganf, G. G. (2000). *The Ecology of Cyanobacteria; Their Diversity in Time and Space.* (pp. 150-194).

Ostensvik, O., Skulberg, O. M., Underdal, B., & Hormazabal, V. (1998). Antibacterial properties of extracts from selected planktonic freshwater cyanobacteria--a comparative study of bacterial bioassays. *Journal of applied microbiology*, *84*(6), 1117-1124. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9717298.

O'Sullivan, D. J., & O'Gara, F. (1992). Traits of fluorescent Pseudomonas spp. involved in suppression of plant root pathogens. *Microbiological reviews*, *56*(4), 662-76. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=372893&tool=pmcentrez&rendertype=abstract.

Paerl, H. W., Dyble, J., Moisander, P. H., Noble, R. T., Piehler, M. F., Pinckney, J. L., et al. (2003). Microbial indicators of aquatic ecosystem change : current applications to eutrophication studies. *FEMS Microbiology Ecology*, *46*, 233-246. doi: 10.1016/S0168-6496(03)00200-9.

Page, R. D. M. (1996). TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, *12*, 357-358.

Rashidan, K. K., & Bird, D.F. (2001). Role of Predatory Bacteria in the Termination of a Cyanobacterial Bloom. *Microbial Ecology*, *41*, 97-105. doi: DOh 10.1007/s002480000074.

Rippka, R. (1988). Isolation and Purification of Cyanobacteria. *Methods in enzymology*, *167*.

Robillot, C., Vinh, J., Puiseux-Dao, S., & Hennion, M.-C. (2000). Hepatotoxin Production Kinetics of the Cyanobacterium Microcystis aeruginosa PCC 7820, as Determined by HPLC−Mass Spectrometry and Protein Phosphatase Bioassay. *Environmental Science & Technology*, *34*(16), 3372-3378. doi: 10.1021/es991294v.

Rosato, Y. B., Messias, C. L., & Azevedo, J. L. (1981). Production of Extracellular Enzymes by Isolates of Metarhizium anisopliae. *Journal of invertebrate pathology*, *38*, 1-3.

Samuilov, V. D., Bulakhov, a V., Kiselevsky, D. B., Kuznetsova, Y. E., Molchanova, D. V., Sinitsyn, S. V., et al. (2008). Tolerance to antimicrobial agents and persistence of Escherichia coli and cyanobacteria. *Biochemistry (Moscow)*, *73*(7), 833-838. doi: 10.1134/S0006297908070122.

Schindler, D. W., Turner, M. A., & Hesslein, R. H. (1985). Acidification and alkalinization of lakes by experimental addition of nitrogen compounds. *Biogeochemistry*, *1*, 117-133. doi: 10.1007/BF02185037.

Schulz, S., Dong, W., Groth, U., & Cook, a M. (2000). Enantiomeric degradation of 2-(4-Sulfophenyl)Butyrate via 4-sulfocatechol in Delftia acidovorans SPB1. *Applied and environmental microbiology*, *66*(5), 1905-10. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=101431&tool=pmcentrez&rendertype=abstract.

Schütte, U. M. E., Abdo, Z., Bent, S. J., Shyu, C., Williams, C. J., Pierson, J. D., et al. (2008). Advances in the use of terminal restriction fragment length polymorphism (T-RFLP) analysis of 16S rRNA genes to characterize microbial communities. *Applied microbiology and biotechnology*, *80*(3), 365-80. doi: 10.1007/s00253-008-1565-4.

Shapiro, J. (1997). The role of carbon dioxide in the initiation and maintenance of blue-green dominance in lakes. *Freshwater Biology*, *37*(2), 307-323. doi: 10.1046/j.1365-2427.1997.00164.x.

Shilo, M. (1970). Lysis of blue-green algae by Myxobacter. *Journal of bacteriology*, *104*(No. 1), 453-461.

Sigee, D. C., Glenn, R., Andrews, M. J., Bellinger, E. G., Butler, R. D., Epton, H. A. S., et al. (1999). Biological control of cyanobacteria: principles and possibilities. *Hydrobiologia*, *395/396*(Suppl. 1), 161–172. doi: 10.1051/animres:19950569.

Ter Braak, C. J. F. (1986). Canonical Correspondence Analysis : A New Eigenvector Technique for Multivariate Direct Gradient Analysis. *Ecology*, *67*(5), 1167-1179.

Uchida, H., Kouchiwa, T., Watanabe, K., Kawasaki, A., Hodoki, Y., Ohtani, I. I., et al. (1998). A coupled assay system for the lysis of cyanobacteria. *Japanese Journal of water treatment biology*, *34*(1), 76-75.

Ulen, B., & Weyhenmeyer, G. (2007). Adapting regional eutrophication targets for surface waters—influence of the EU Water Framework Directive, national policy and climate change. *Environmental Science & Policy*, *10*(7-8), 734-742. doi: 10.1016/j.envsci.2007.04.004.

Valdor, R., & Aboal, M. (2007). Effects of living cyanobacteria, cyanobacterial extracts and pure microcystins on growth and ultrastructure of microalgae and bacteria. *Toxicon : official journal of the International Society on Toxinology*, *49*(6), 769-79. doi: 10.1016/j.toxicon.2006.11.025.

Van de Waal, D. B., Verspagen, J. M. H., Lürling, M., Van Donk, E., Visser, P. M., & Huisman, J. (2009). The ecological stoichiometry of toxins produced by harmful cyanobacteria: an experimental test of the carbon-nutrient balance hypothesis. *Ecology letters*, *12*(12), 1326-35. doi: 10.1111/j.1461-0248.2009.01383.x.

Vergin, K. L., Urbach, E., Stein, J. L., DeLong, E. F., Lanoil, B. D., & Giovannoni, S. J. (1998). Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order Planctomycetales. *Applied and environmental microbiology*, *64*(8), 3075-3078. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=106819&tool=pmcentrez &rendertype=abstract.

Waters, C. M., & Bassler, B. L. (2005). Quorum sensing: cell-to-cell communication in bacteria. *Annual review of cell and developmental biology*, *21*, 319-46. doi: 10.1146/annurev.cellbio.21.012704.131001.

Wen, A., Fegan, M., Hayward, C., Chakraborty, S., & Sly, L. I. (1999). Phylogenetic relationships among members of the Comamonadaceae, and description of Delftia acidovorans (den Dooren de Jong 1926 and Tamaoka et al. 1987) gen. nov., comb. nov. *International Journal of Systematic Bacteriology*, *49*, 567-576. doi: 10.1099/00207713-49-2-567.

Whyte, L. G., Maule, a, & Cullimore, D. R. (1985). Method for isolating cyanobacteria-lysing streptomycetes from soil. *Journal of Applied Microbiology*, *58*(2), 195-197. doi: 10.1111/j.1365-2672.1985.tb01447.x.

Wright, S.J.L. and Thompson, R. J. (1985). Bacillus volatiles antagonize cyanobacteria. *FEMS Microbiology Letters*, *30*, 263-267.

Yamamoto, Y., Kouchiwa, T., Hodoki, Y., Hotta, K., Uchida, H., & Harada, K.-I. (1998). Distribution and identification of actinomycetes lysing cyanobacteria in a eutrophic lake. *Journal of Applied Phycology*, *10*(3), 391–397.

Supplement tables:

Table 1. Sequences from lytic bacteria (E52, E55, E10, E35 and E141)

Sample	Sequence
E52	TGGATTCaGCGGCGGACGGGTGAGTAATGCcTAGGAATCTGCCTGGTAGTGGGGGACAA CGTTTCGAAAGGAACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTcG GGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCA CCAAGGCTACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGAACTGAGACA CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTG ATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGG AGGAAGGGTTGTAGATTAATACTCTGCAATTTTGACGTTACCGACAGAATAAGCACCGG CTAACTCTGTGCCAGCcGCCGCGG
E55	GGTCTTCGGACGCTGACGAGTGGCGAACGGgTGAGTAATaCatcggaacGTGCCCAGTC GTGGGGGATAACTACTCGAAAGAGTagCTAATACCGCATACGATCTGaGGATGAAAGCG GGGGACCTTCGGGCCTCGCGCGATTGGAGCGGCCGATGGCAgATTAGGTAGTTGGTGGG ATAAAAGCTTACCAaGCCGACGATCTGTAGCTGGTCTGAGAGGACGACCAGCCACACTG GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACAATGG GCGAAAgCCTGATCCAGCAATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGC TTTTGTACGGAACGAAAAAGCTTCTCCTAATACGAGAGGCCCATGACGGTACCGTAAGA ATAAGCACCGGCTAACTACGTGCCAGCcGCCGCGGtAAT
E10	gCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAAtGcCTAgGAATCTGCCTGGTAGT GGGGGATAACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGG GGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGAGGT AATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACACTGGA ACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGC GAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTT TAAGTTGGGAGGAAGGGCAGTTACCTAATACGTGATTGTTTTGACGTTACCGACAGAAT AAGCACCGGCTAACTCTGTGCCAGCcGCCGCGGT
E35	TGCAAGTCGAGCGGCAGCACGGGTACTTGTACCTGGTGGCGAGCGGCGGACGGGTGAGT aaTGcCTAGGAATCTGCCTAGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCG CATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATTAGATGAGCCTA GGTCGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTC TGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAG CAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG AAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTG ATTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCcGCCGCGGT AAT
E141	CGAGCGGAGTGGTTGTGCTTGCACAATCACTTAGCGGCGGACGGGTGAGTAAtGcTtag GAATCTGCCTATTAGTGGGGGACAACATTCCGAAAGGAATGCTAATACCGCATACGCCC TACGGGGGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCGGATT AGTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAGGATG ATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAA TATTGGACAATGGGGGGAACCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTT GGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTAgTACTAATACTACTGGATAGTGG ACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCcGCCgcGG