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Can tropical bloom-forming algae thrive in Nordic waters?

A case study using the invasive and highly toxic cyanobacterium Cylindrospermopsis raciborskii

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Abstract

Global environmental change is getting more intense, and marine life is also under a turnaround that could possibly alter human society in a large scale as well. This study is done to get new information about habitat stressors for newly intruding exotic bloom forming cyanobacterium species *Cylindrospermopsis raciborskii* that is expected to spread to the Nordic. This algae is found to be highly toxic and difficult for the original algae species to rival with once it has been establishing its new areas.

This study was done cultivating four strands of *C. raciborskii*, captivated from different places at different times during past few decades, on a temperature gradient and results implicated that there are regional variations in cold tolerance for this species and specially newest and northernmost strain. Further research on molecular level on its adaptative strategies is needed to fully understand the efficiency and possibly estimating the time line of it's spreading for the best results for preparedness.

Language: English Key words: algae, cyanobacterium, invasive alien species, aquatic ecology, temperature, environmental stressor

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Abbreviations

C. raciborskii* – cyanobacterium *Cylindrospermopsis Raciborskii

EPSAG - Abteilung Experimentelle Phykologie und Sammlung von Algenkulturen, department of experimental phycology and collection of algae cultures, Germany

HAB – Harmful algal bloom

IAS – Invasive alien species

BG +N – BG11 growth media, normal nitrogen (N) concentration

BG –N – BG11 growth media without nitrogen

NIVA – Norsk Institutt for Vannforskning, Norwegian water research institute

SLU – Sveriges lantbruk universitet, Swedish university of agricultural sciences

WHO – World Health Organization

1 Introduction

Invasive Alien Species (IAS) are widely spread all over the world (Bax et al., 2001). They are often highly adaptive, have several competitive traits with which to supersede local species and tend to alter the local ecological balance, but it is not always clear which mechanisms and environmental stressors eventually cause one species to overpower the old ones. Global warming has a tremendous effect on this shift and warming waters allow many kinds of migration patterns (Beaugrand, 2015). While change in population sometimes can be of a commercial benefit, it usually poses a grave threat for local biodiversity and long-term sustainability in ways that can't yet really be foreseen, even for human population. According to Molnar et al. (2008), algae are the third largest group of marine invasive species.

The cyanobacterium *Cylindrospermopsis raciborskii* is found at increasing frequency in temperate zones (Sinha, 2012) and the species has been found as far north as Germany (Mehnert *et al.,* 2010) and Poland. Evidence of its germination and light sensitivity has already been found, but it's tolerance for rather cold water remains unstudied. Therefore in this study I aim to to gather more information about the equatorial originating, bloom forming cyanobacteria *C. raciborskii*s possible survival in the area of Nordic countries.

In this study I wanted to test cold tolerance of *C. raciborskii* by comparing four strains isolated from different locations to see if some of them has developed an adaptive strategy for their changing environments to see possible indications on its survival further north than it's now known area of distribution. My null-hypothesis during this experiment was that these strains would have similar temperature tolerance. I was also assuming that low traces of toxin would be found, in which case the mitigation would need to be taken very seriously in order to avoid severe environmental changes.

2 Problem description/Background

2.1 Theoretical background

Harmful Algal Blooms (HAB) have long been an annual nuisance in both Nordic marine and fresh waters. They contribute to eutrophication, in which excessive nutrients from urban areas encourage the growth of phytoplankton that eventually sink to the bottom of lake or sea. Large amounts of decomposing phytoplankton can under some circumstances exhaust all oxygen and can cause massive death in local fish populations. They also block the light-passing water layers and harmfully shade and inhibit the normal functions of other organisms. Pets have been reported dying after swimming in waters with algal blooms, and rashes are common in people (Chorus and Bartman, 1999). Water treatment plants are incapable of processing too high concentrations of toxicity in waters (WHO 2015). As ordinary algae can have adverse effects, if replaced by even more toxic species, HAB would pose an even bigger threat. By estimation there are about 150 known genera of cyanobacteria, 40 of which are known to possess the ability to form toxins (Saker et al., 1999) as their secondary metabolite, and this often gives these species tremendous competitive advantage. Cyanobacteria can produce liver- and neurotoxins. Algae scum are claimed to be muddy and unwanted both commercially and by people living by the sea – besides of causing chemical harm, scum also physically tend to clog pipes, motors and such, as well as smell bad and attract harmful animals when washed out onto the shore.

Cyanobacteria, procaryotes that are able to perform photosynthesis and synthesize chlorophyll, are most usually found in the coastal waters of inland lakes and seas. They are ecologically categorized as algae. Cyanobacteria are widely concidered to have created the Earth's atmospheric oxygen 2.4 billion years ago (Hamilton et al., 2015). Most cyanobacteria prefer warm temperatures of approximately 20°C, they increase in numbers in parallel with rising warmth. Usually their biomass can propagate at any warm period after spring, but often reaches its peak in late summer.

Despite common belief, cyanobacteria are not only found in warm waters. While some specific cyanobacteria are predominant in all-year ice capped polar lakes and streams, cyanobacteria are also common in partly-icy regions, alpine glaciers in temperate zones and in tundra (Christmas et al., 2015). For example, cyanobacteria are the most common colonizers of newly exposed rocks after the receding of a glacier, melt streams and ponds, which offer a chemically rich growing base (Crevecoeur et al., 2015). To be able to live in colder climates, the ecophysiological characteristics of a species need to include an ability to grow over wide range of temperatures and also to tolerate stronger salt concentrations which tend to occur in waters near ice sheets.

Cylindrospermopsis raciborskii has gained considerable attention because it has spread its geographical distribution over the last decades. Reportedly highly toxic (de la Cruz et al., 2013) it is also allelopathic, meaning that it produces biochemicals which alter its biological environment (Leão, 2012). As for traits contributing to toxicity tend to vary along the long process of evolutionary adaptation, they are still subjects of ongoing discussion. *C. raciborskii* can be difficult to detect as it doesn't form surface blooms (Hawkins et al., 2001). This might be due to gas vacuoles that give it ability to move between water columns, and often reported short morphotype in cell filaments (Padisák, 1997).

A study of Noyma et al. (2015) shows that *C. raciborskii* is not tolerant for UVA/UVB radiation. Physical characteristics are drawn from cyanobacteria in general by Christmas et al. (2015) but in entirety it is not yet known how fast such changes could occur in *C. raciborskii*. Changes on *C. raciborskii* successfully undergoing such adaptive changes needs to be studied on a molecular level. It will also become increasingly important to study specimen quite recently isolated from the wild to accurately follow these changes. It might also be crucial to monitor strains from different bioecological locations separately, because of physical changes of individual coping strategies (Wilk-Woźniak et al., 2016) can differ a lot as also changes in lineages history can give a lot of behaviour of certain specimen (Thomas et al. 2016). Zinser et al. (2007) found out about sp. *Prochlorococcus*, different physical variations have developed different ecotype preferences and this is really probable for *C. raciborskii* also. There is evidence indicating that while low temperatures don't completely prevent vital functions, they are strongly limiting and it is impossible to deduct which specimen would adapt. Compared to other phototrophs, cyanobacteria seems to be rather rare on melting snow banks as their growth rate is slower (Vincent, 2000). While it can be difficult to predict the possible.

Bonilla et al. (2016) points out in their research, water transparency and clarity are intensely important factors and often overlooked when determining desired actions in water management and as their research indicates, *C. raciborskii* can often be more dominant in waters that have good light conditions even if the temperature wouldn't be seen as optimal.

Cyanobacteria are generally represented very diversely. Unicells and filament can be found both solitary and forming groups, and these can branch and divide uniformly or in an unexpected manner. They can emerge with or without gas-vacuoles that help them regulate their buoyancy and move trough water columns and some of cyanobacteria also has nutrients-fixing heterocysts. These characteristics are all known features of *C. raciborskii*, strains used in this experiment also differed from each other: some ended up growing long leashes while others stayed relatively uniform and short throughout the experiment.

2.2 IAS and water management

For example WHO has created guidelines (WHO 2015) for monitoring measures so that risk associated with cyanobacteria in the water supply can be minimized, but addressing the need or EIA hardly has an impact on its own. As for Marine Strategy Framework Directive is currently being re-evaluated by European Parliament before its second cycle will be launched in 2018, the studies done have for most parts to do with the chemical baggage in oceans. While agricultural and other urban hotpoints are recognized as sources of increased algae masses, monitoring programs for this kind on environmental change are also difficult to design and maintain, as environmental change for a large part is a global phenomenon and as Douvere (2008) writes, monitoring should, as every marine spatial planning means, be carried out as continuously ongoing for the best results. Even if policy making always, to some extent, has to live with uncertainty and be prepared to make decisions based on best knowledge available, more extensive experimental studies in the field of marine invaders are needed. According to Bax et al. (2001) controlling of marine invasions needs to tackle the problem at an early stage, targeted in a specific way and be well reported.

3 Materials and methods

The experiment was conducted at the facilities of Sveriges lantbruksuniversitet, Uppsala, Sweden during autumn 2016 and took total of 19 days.

3.1 Cyanobcteria strains used

Four different strains (Figure 1) was used in this experiment. The one most resembling the original forms of *C. racibosrkii* for its environmental conditions is from Uganda. Three strains were from continental Europe: One from Germany and two from the same lake in Hungary, isolated at different decades. I wanted to use strains collected at different dates but also from varying locations to see if there would be any variation due to regional factors.

Place in temp. gradient	Strain name	Origin	Year isolated
A	19F6	Germany	2004
B	NIVA506	Uganda	2004
D	NIVA399	Hungary	1996
E	NIVA255	Hungary	1984

Figure 1: Strains used

The German strain was sourced from EPSAG - Abteilung Experimentelle Phykologie und Sammlung von Algenkulturen, department of experimental phycology and collection of algae cultures. It was isolated from lake Melangsee in 2004.
Rest of the strains were ordered from NIVA - Norsk Institutt for Vannforskning, Norwegian water research institute. The strain from Uganda was isolated in 2004 from Kazinga channel, and Hungarian samples were isolated in 1984 and 1996, both from lake Balatong.

It is common for *C. raciborskii* filaments to be of varying lengths even in the same population (Hawkins *et al.,* 2001). Of strains used in this experiment this was also noticeable, especially in all NIVA samples which did grow a lot during the span of three weeks of measuring – the German isolate was considerably shorter even in the end. Figure 2 shows photos of these strains before the start of the experiment.

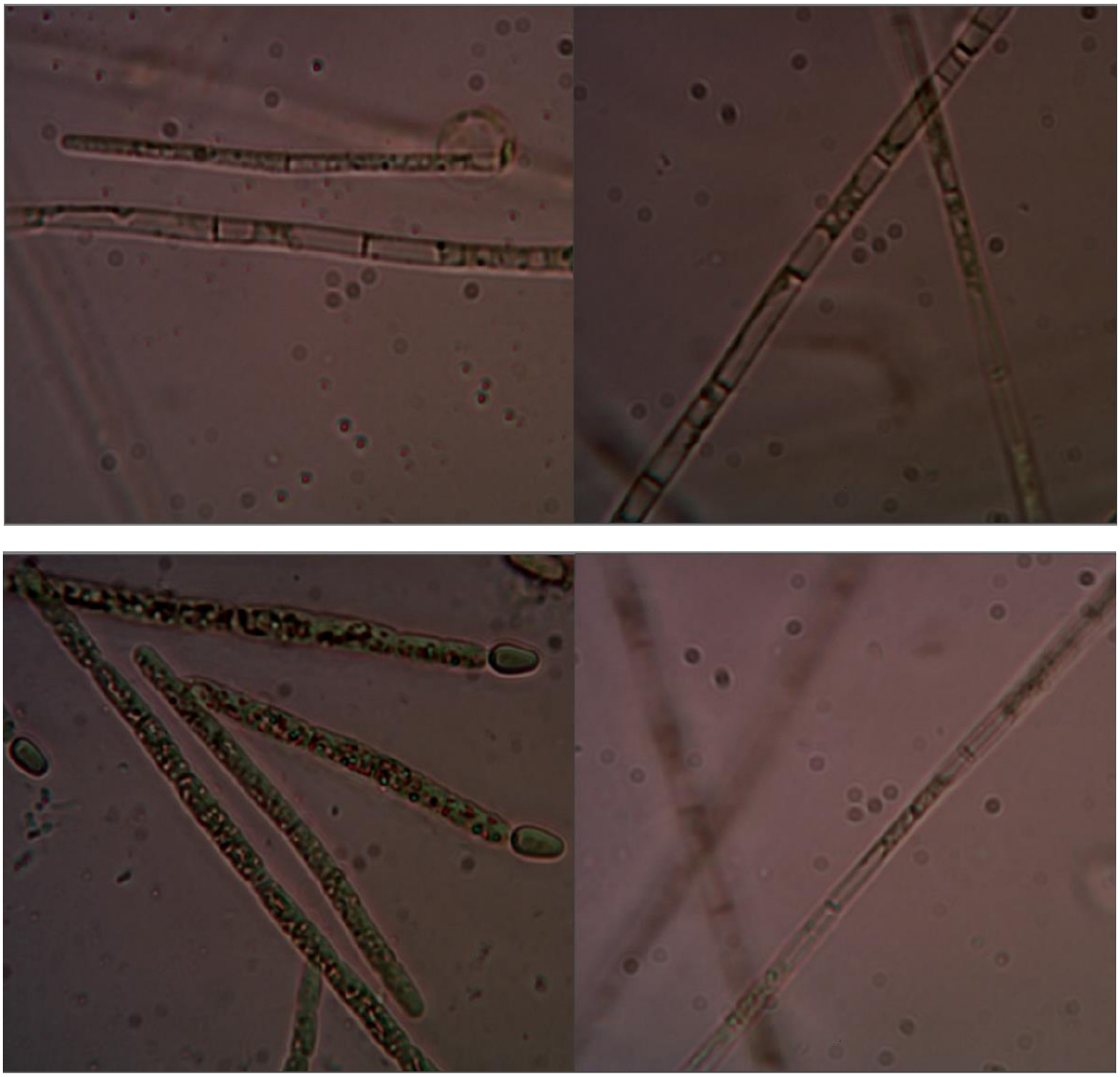

Figure 2: Strains photographed in microscope before the experiment. First row: Niva255, Niva399, second row: 19F6, Niva506.

3.2 Solutions used and strain division

The experiment was executed using BG11 growth media (Appendix 1).

The German strain originally arrived in a culture media called MIII. Samples from Hungary and Uganda were in Z8 medium. Gradually transferring them into BG11 took 7 weeks. During the preparation period BG11 solution was made both with high concentration of inorganic nitrogen (+N) and without nitrogen (-N version), because a species like *C. raciborskii* with ability to fix nitrogen by itself sometimes grow better in low N

concentrations. I wanted to test how different the responses would be. The experiment itself was carried out using 1:1 of these solutions, so the concentration of nitrogen was a little smaller than in the normal BG11.

During these seven weeks the cells were aerated daily using a shaking table and transferred into the new media once a week. While transferring, samples were also gradually divided into more bottles, as the aim was to increase the volume to be able to have enough cells for the experiment that needed 25 bottles of each strain, each with about 25 ml liquid. The dividing was only commenced taking into account the volume rather than exact cell count. Sterile autoclaved test tubes and disposable pipettes and gloves were used all the time.

During the experiment bottles contained a total of 25ml of liquid: 3ml isolate, 11ml BG11 - N + 11ml BG11 +N. It is clear that the amount of cells was not equal, but in statistical calculations done later on, the peak value was proportioned to the first reading, and because the aim was to measure the increase in biomass over time, the initial biomass did not have to be exactly the same. The caps of any bottle were never opened during the experiment in order to avoid contaminating the samples.

3.3 Equipment used

For all four strains 25 test tubes were set in the aluminium chamber with a temperature gradient (Figure 3). Two water thermostats control water temperature at both ends of the structure and between them is a solid aluminium block, which has five rows of holes drilled all the way through. The gradient is formed by pumping water of different temperatures from the thermostats through drilled holes in the aluminum block at each the end of the block. At the cool end of the gradient water with 5 ^{o}C were cooling and at the warm end water with 35 ^{o}C were heating the block resulting in a gradient for the test tubes of 9.3 ^{o}C to 34.1 ^{o}C. Underneath the block is an empty space of about 1 cm, and then an acrylic plexi glass as a bottom so that it is easy to alter for any desired filtration level of lights directed from below. This structure is tightly insulated from the sides and the top so that the changes in room temperature will not disrupt the temperature gradient.

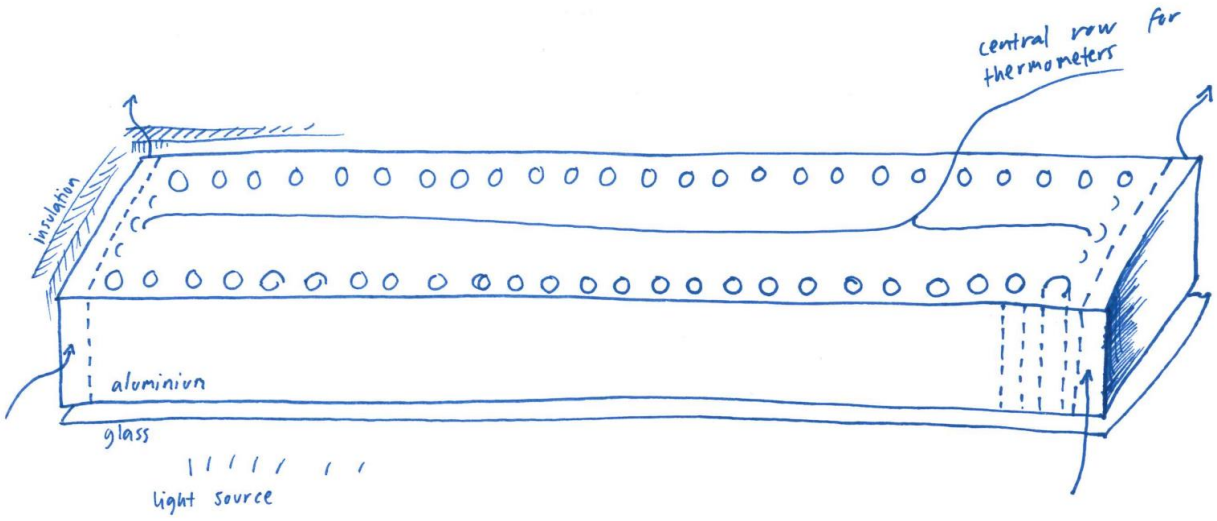

Figure 3: The temperature gradient

As there were four strains in the experiment, the center row was used for water temperature recordings. In this row, five thermometers were placed evenly distributed in test tubes filled with water and the temperature were checked during the cell count daily during the experiment.

Before the experiment, the luminosity was checked in every test tube, that were filled with 20 ml of distilled water each. The probe was inserted at the bottom of a tube so that it touched the glass. The photon flux was set to be between 30 and 34 μ E m^{-2} s^{-1} by adjusting thin layer of paper evenly between the plexi glass.

Each test tube was individually placed in the TD700 fluorometer, which I used to detect the amount of raw chlorophyll. No amount of sample or liquid was removed during the experiment. The machine was calibrated by using a dense culture of *C. raciborskii* to set a maximum value that was not going to be exceeded during the experiment. In order to avoid any factors most commonly giving false readings I let the sample settle down after shaking it so there were no notable turbulence, bubbles or any additional turbidity in the sample bottles. The culture media BG11 has a pH of 7-8, which does not cause distortion of wavelengths (Turner Designs, 2002).

3.4 Data analysis

The analysis is done with the following growth calculation formula, where **k** is the growth rate.

$$\text{Biomass} = \exp^{(k*\text{time})}$$

The growth curve for each isolate was calculated using linear modeling. For those strains having inconstant/discontinuous slopes an alternative slope was also included in the data. At first, value 0 was used for strains having a negative slope, but to get more realistic calculations, their original *(k*time)* value was used.

Logaritmic slope was chosen because the number of new cells is proportional to the already existing population size. This is of a prevalence method to show data in many ecological and bacteriological cases.

For every stain 25 different isolates were plotted into the same graph to create a curve that shows maximum fluorescence at a selected day, in this case 6^{th} day was used, (y-axis) versus temperature (x-axis) and also to get a graph for maximum growth rate (y-axis) versus temperature (x-axis).

3.5 Disposing of materials

It is of importance to mention that any materials used in contact with *C. raciborskii* were autoclaved before disposing of. This includes solutions, for it is not in any way desired that this organism is let out in the natural system because of this study.

4 Results

4.1 Light conditions

The light conditions, as seen on figure 4, were somewhat constant on strains B, D and E, and did vary a little more for A. Median luminosity for strain A was 32.9, for B 32.1, for D 32.2 and for E 32 µ E m^{-2} s^{-1}.

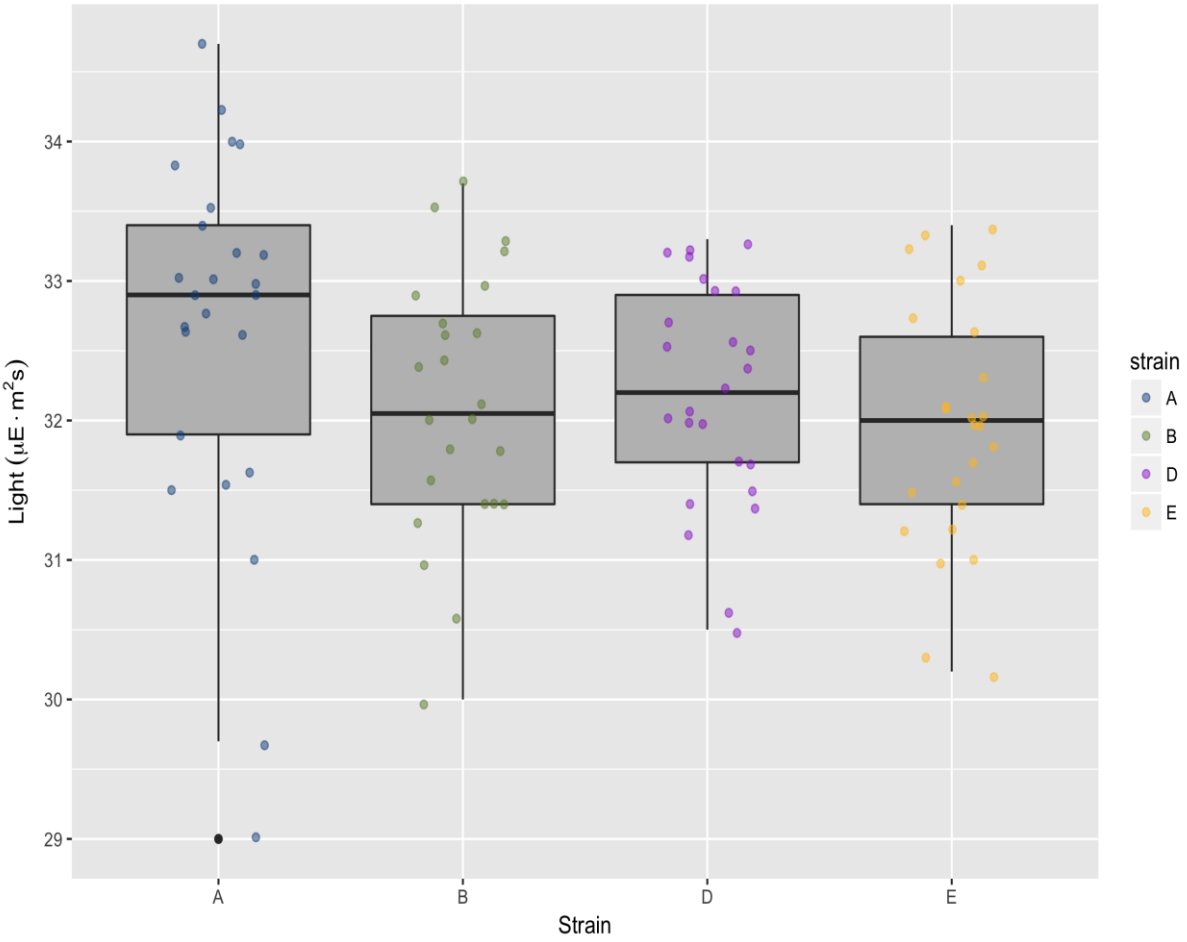

Figure 4: Dots correspond the actual readings on each experiment row, median is shown as thick solid lines and the grey boxes show interquarlile range.

4.2 Temperature conditions

Figure 5: Temperatures on a gradient for each row

The temperature gradient was even between all four strains tested, as seen in figure 5. The temperatures were measured troughout the experiment to make sure that constant light would not alter the features of the insulated aluminium. The temperature didn't raise during the experiment more than a few decimals.

4.3 Growth rate

The growth rates in contrast to temperature gradient can be seen in figure 6. As ideal, bacterial cultures grow exponentially.

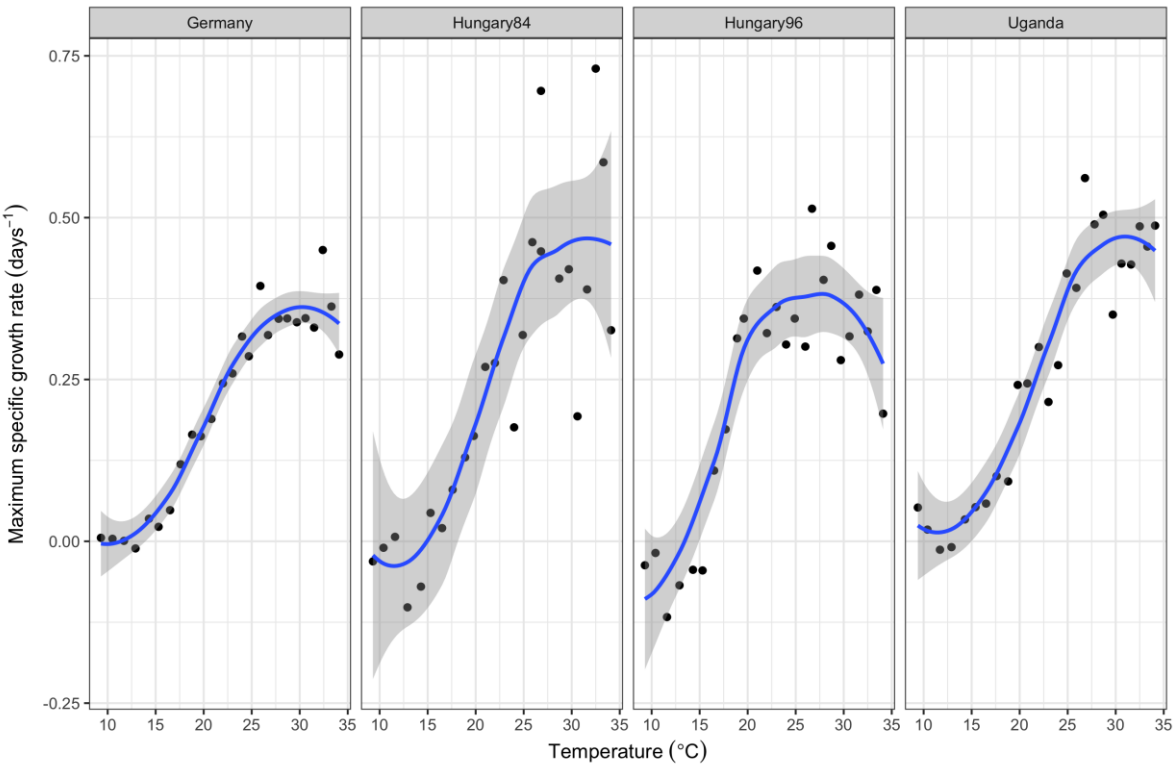

Figure 6: Growth rates on a temperature scale

There was considerably lot of variation in growth rate of NIVA255, the one from Hungary from 1984. Some of these isolates did grow at much faster pace and some began to decrease early on. Both Hungarian strains also had few isolates that began with slightly higher cell count on bottles which were placed too cold for their optimum, and them declining fast caused a slight negative slope at the beginning of the curve. The German and Ugandan strains do have a somewhat more uniform growthline However, the German strain didn't do that well at the warmest temperatures. The Hungarian96 NIVA399 was the second to prefer mid-temperatures.

For example the increase in growth for every strain at the point 0.1 (German strain 18, Hungarian84 18, Hungarian96 17, Ugandan 19) suggests that their growth has begun relatively similarly – the difference between these strains is more about how soon and at which conditions they reach peak of abundance.

4.4 Toxins

When these strains were tested for toxins in the laboratory of Livsmedelsverket, the Swedish national food agency, after this experiment, the toxin cylindrospermopsin were only found in the strain from Uganda. This was studied using mass spectrometry.
There is indication that toxicity can vary between strains, and also between clones of the same isolate. In addition, some strains produce three or more toxins with the relative proportions being influenced by environmental factors: light, pH, nitrate, phosphate, metal ions and temperature and it is entirely possible that some other strain of *C. raciborskii* from these same locations would be toxic.

5 Discussion

All of the strains tested reacted somewhat similar to the change in the temperature, which tells that there isn't such a big adaptive means for the cold in *C. raciborskii* yet. How ever, as seen on the growth figure (figure 6) the main difference between these strains can be seen on the conditions their peak of abundance is reached. The two strains isolated from the wild merely recently (19F6 in 2004 and NIVA399 in 1996) reflect preference towards mid-temperatures instead of the warm. According to this data it would be fairly possible that this species could invade into Nordic countries in the near future as it clearly is adapting to adjust its vital functions for shorter suitable growth period in a cool temperature. First a viable spreading platform just needs to be introduced.

The reason for decline in the growth rate after day six and onwards, depending on the strain, could be explained by many factors. One potential reason in that the abundance of the cells in biomass creates unfavourable conditions through the lack of lighting – self shading. The shape of bottles this experiment was carried out in, might be another potential reason. An Erlenmeyer flask would offer a more realistic shape for algae to grow in and it is also easier to aerate than a thin culture tube, in which the air circulation and the air usable for the test specimen is much more limited.

The strain from Uganda has a fairly small window of opportunity for growth. It is not guaranteed that the strain used was of the right species, although it was supplied by a venearable laboratory. Let us assume that it was the right species – perhaps this would indicate *C. raciborskii* has at some stages on its evolutionary phases been creating resting

stages easily. It could be that all the other isolates also created such resting stages when circumstances became too difficult, this strain just clearly did that at different phase. As for this strain was collected from the wild more recently than NIVA255 or NIVA399, from Hungary, we can't draw conclusions of an evolutionary reasoning in this matter. As the evolutionary history of adaptation to ecological changes correlates relatively well to how species adapt to thermal changes, and in that light it would be good to see strains from one lineage to be tested over longer time period.

As it is known that *C. raciborskii* can often be found at lower water layers, it would be interesting to know how it manages in a conditions with less light. In this study the cells fell on the bottom of test tubes, which after all was the nearest possible location to the constant light source. The range of desired light conditions for *C. raciborskii* would be good to determine in the context of Nordic day light. UVA/UVB intolerance is a trait could be argued to become a limiting factor in the north because of a thinning ozone layer, but it has widely been discussed that the sun is angled too low in the Nordic to have any effect at all. One interesting fact, that is not yet studied, is how fast an adaptive strategy evolves. There is evidence of four mechanisms for cyanobacteria to cope with radiation: avoiding exposure, to create second metabolites that act as a filter, capability to rejuvenate the biological damage or even creating new DNA information.

To gather evidence of immediate consequences of *C. raciborskii* as an invader, testing the effects of it on local phytoplankton communities in cold waters will also offer vital information about the strategies this species might have. As for its allelopathic traits produce chemical that can either kill or limit growth of competitors and phytoplankton that are substantial for regional ecological stability and in a food chain. And which might not have resistance or adaptive strategy against allelochemicals of *C. raciborskii*, its spreading would be assumed. But as these strains were tested non-toxic, and there are many evidence in the scientific literature that any local factors have their impact on said toxicity, we can't for sure tell if it in fact would overrule other cyanobacteria. It might be possible that *C. raciborskii* would at first spread to the Nordic only in tiny and harmless colonies and only after establishing its niche it would have the possibility to produce toxins.

There are many questions raised for future research. In the light of this study I underline the importance of marine- and water management means as it clearly is nothing more than a matter of time until *C. raciborskii* will enter the Nordic waters and quite possibly is here to stay after its arrival.

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Appendix 1.

BG11 media

$NaNO_3$	1.5 g
K_2HPO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.075 g
$CaCl_2 \cdot 2H_2O$	0.036 g
Citric acid	0.006 g
Ferric ammonium citrate	0.006 g
EDTA (disodium salt)	0.001 g
Na_2CO_3	0.02 g
Trace metal mix A5	1.0 ml
Agar (if needed)	10.0 g
Distilled water	1.0 L

The pH should be 7.1 after sterilization

Trace metal mix A5:

H_3BO_3	2.86 g
$MnCl_2 \cdot 4H_2O$	1.81 g
$ZnSO_4 \cdot 7H_2O$	0.222 g
$NaMoO_4 \cdot 2H_2O$	0.39 g
$CuSO_4 \cdot 5H_2O$	0.079 g
$Co(NO_3)_2 \cdot 6H_2O$	49.4 mg
Distilled water	1.0 L