University of South-Eastern Norway

Faculty of Technology, Natural Sciences and Maritime Sciences

Master's Thesis

Master's in environmental sciences

Spring 2019

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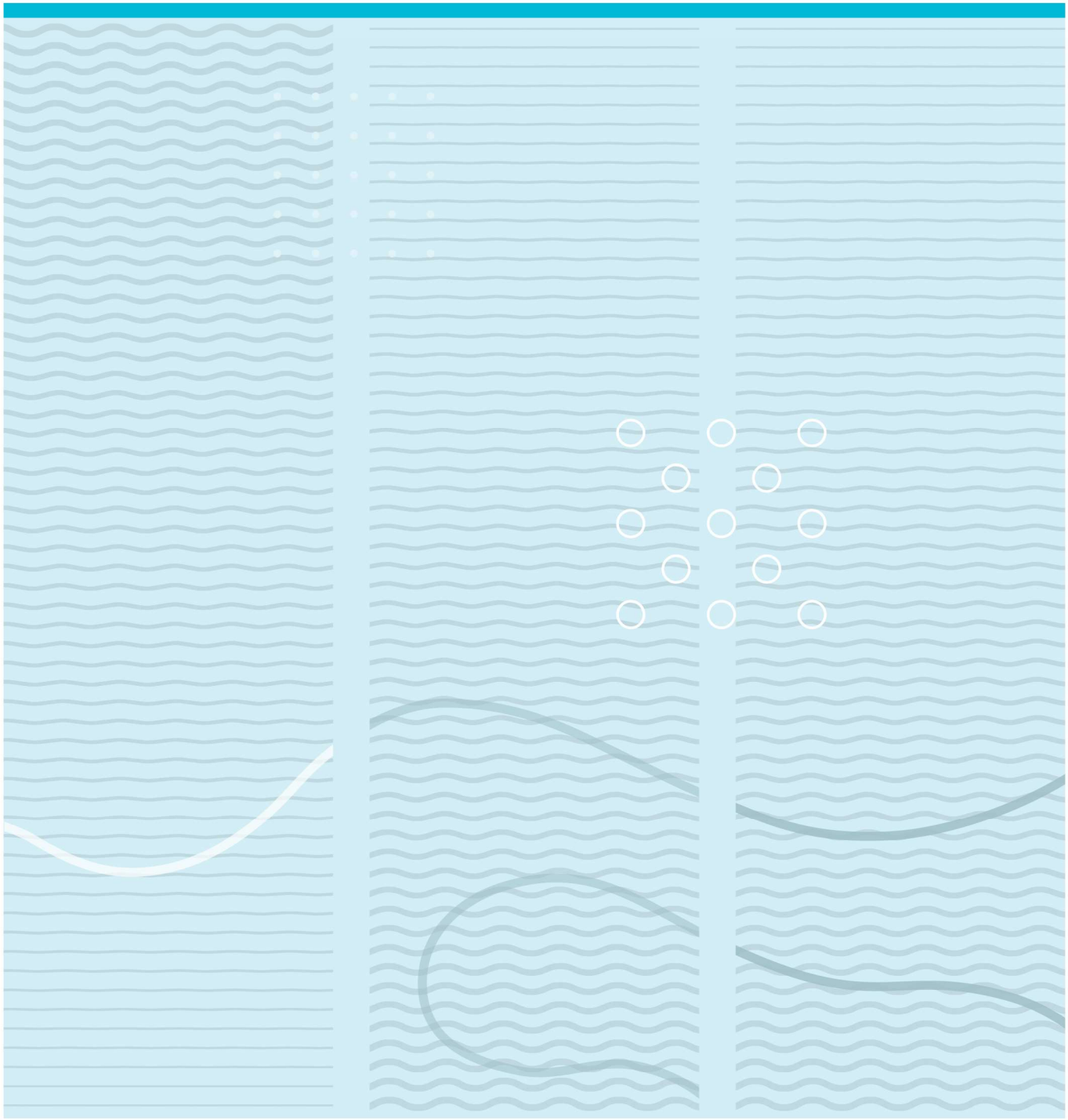
Growth and Toxin production by *Cylindrospermopsis raciborskii* under different light intensities, Phosphate and Nitrates concentrations



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This thesis is worth 60 study points

Abstract

The cyanobacteria species *cylindrospermopsis raciborskii*, that is known for its bloom forming and toxin production (cylindrospermopsin) got a lot of attention by scientist due to its harmful risk to living organism. In this study effects of light intensities, limited nitrogen and phosphorus concentration on the growth, toxin production, heterocyst production and filaments length were examined. The result confirmed that *C. racciborskii* can grow in low light intensity as compared to high light. The optical density result of low and without addition of nitrogen and phosphorus was significantly lower than the higher concentration. The length of the filaments was higher with increase in nitrates and phosphates concentration. The number of the heterocysts was only found when the dissolve nitrogen was in minimum concentration (33mgN/L). While there was no toxin (cylindrospermopsin) found in any sample in all treatments under high and low light intensities. In conclusion *C. raciborskii* can rapidly shows a growth response in high nutrients values.

Keywords: *C. raciborskii*, nitrates, phosphate, optical density, heterocysts, filaments length, cylindrospermopsin.

Contents

Foreword

I am very grateful to the faculty of Department of Environmental Sciences USN – (Universitetet I Sørøst-Norge, campus Bø) to give me opportunity for my master study and providing me funds for my laboratory works. I would like to thanks to my thesis supervisor Synne Kleiven for the support of my master study, her motivation and the best advice that helps me a lot for writing me this thesis and especially allow me to work under her supervision. Thank you for your guidance and help from the start to the end of my thesis.

Besides my supervisor, I am very grateful to Hans Christian Utkilen for providing me literature for my thesis. A special thanks to Karin Brekke Li and Ikumi Umetani for helping and guidance during laboratory work.

My sincere thanks also go to my parents who support me financially for my study and their motivation that helps me a lot to complete my master study. Especial thanks to all my friends and classmates giving me a good company that never feel me that I am far away from my home.

Bø i Telemark, 4.5.2019

Zohaib Hassan

1 Introduction

1.1 Cyanobacteria and the environment

Over the last two decades, the increased occurrence of harmful Cyanobacterial blooms is considered as a serious environmental threat. Because of risks related to human sickness and animal mortality these blooms have attained significant attention in the scientific community (Paerl and Otten, 2013). Moreover, they are disrupting marine ecosystems as well. Same as other prokaryotes, they do not have specialized membrane-bound organelles like nuclei and nor do they sexually reproduce (Echlin, 1966). However, they use chlorophyll-a (Chl-a) to bind light energy to convert inorganic carbon into organic compounds like other eukaryotic photoautotrophs (i.e., higher plants and true algae) and liberating oxygen at the same time. Cyanobacteria possess a unique pigment named phycocyanin (bluish phycobilin). Therefore, they are commonly known as "blue-green algae". They are usually dominating in warm, nutrient-enriched hard waters such as lakes of temperate and subtropical regions (Sommer et al. 1986, Mez et al. 1998).

Another attractive feature of this tiny creatures is nitrogen-fixation, possessing the unique ability to perform oxygenic photosynthesis and fix nitrogen and therefore, referred to as perfect producers. They obtain nitrogen by any of these three sources. They can fix nitrogen gas from the atmosphere (Kumar et al. 2010). Similarly, several cyanobacterial species can either take up carbon dioxide or bicarbonate as substitutable carbon sources depending on availability in the surrounding. Thus, a pathway to acquire these substitutable nutrients that cyanobacteria select affects both its environment and organism (Gundersen and Mountain, 1973). Due to toxic metabolites from the cyanobacteria leaching into the environment, the cyanobacterial blooms have been considered under more attention by the environmentalist (Paerl and Otten, 2013). These harmful metabolites, cyanotoxins have been categorized into four major classes depending upon their mode of action. Class-I consisting of toxins such as nodularin and hepatotoxic microcystin (Carmichael et al., 1988; Zurawell et al., 2005); Class-II the neurotoxic saxitoxins (STX), anatoxin and beta-methylamino-L-alanine a (Devlin et al. 1977), Class-III the cytotoxic cylindrospermopsins (CYN) (Ohtani, et al., 1992) and Class-IV, Lyngbya toxins and dermatotoxic aplysiatoxins (Kato et al, 1974). The cytotoxic

cylindrospermopsin is alkaloid made up of a tricyclic guanidine attached with a sulfonic acid group and hydroxymethyluracil, is of specific concern famous for a human poisoning incident occurred on Palm Island in 1979 (Bourke et al. 1983). High chemical stability and their low degradation efficiency are responsible for high concentrations of CYN in water bodies. Additionally, it causes injury to various organs, including the thymus, liver, heart, and kidney by interfering with multiple metabolic processes at the cellular level (Banke et al. 2001). Among cyanobacteria, a filamentous diazotrophic species in the order of Nostocales is *Cylindrospermopsis raciborskii*. This species was identified as CYN producer for the first time in Queensland Australia in 1979 (Palm Island Mystery Disease), (Hawkins et al. 1985). Now the most well-known bloom-forming cyanobacteria are *C. raciborskii* (Paerl et al. 2011). It is emerging worldwide in phytoplankton communities. These cyanobacterial invasive behaviors are spreads from tropical and subtropical climate to temperate zones. *C. raciborskii* is present worldwide in Africa, Asia, Europe, Australia, New Zealand, South, and North America. CYN producing a strains of *C. racciborskii* only occur in water bodies of Australia, Asia and New Zealand (Stüken et al. 2006).

Although *C. raciborskii* can generate a group of potentially harmful compounds the capability to make them is not universal. Some Brazilian strains produce paralytic shellfish poisoning (PSP) toxins while Australian strains of *C. raciborskii* produce an alkaloid cytotoxin, CYN (Neilan et al. 2003). Toxicity in mice bioassay was observed from strains isolated from Portuguese lakes but they do not create any of the known cyanotoxins (Saker et al. 2003). On the other hand, there is little data on toxin production from the northern hemisphere strains, Recently the CYN concentrations have been correlated with *C. raciborskii* densities in an Italian lake (Messineo et al. 2010). Many factors such as high temperature, a stable water column, high nutrient concentrations (nitrogen (N); phosphorus (P) and organic compounds), low concentrations of available N relative to P (low N:P), high pH, low CO_2 availability and low grazing rates by large zooplankton are considered ideal conditions for the development of cyanobacterial bloom population. Thus, not a single feature is directly responsible for bloom forming action, but all previously cited factors are involved (Mez et al. 1998).

Furthermore, many factors such as wide range of tolerance to key environmental factors, superior competition for resources, high phenotypic plasticity, global warming and eutrophication have been suggested important for inducing cyanobacteria blooms (Mez et al. 1998). Cyanobacteria in natural water bodies are frequently subjected to nutrient deficiency, particularly phosphorus (P), nitrogen and sulfur (Smith 1933), which most likely affects both the metabolism and physiology of these organisms. Phosphorous plays a critical role in determining the primary productivity of the water bodies and is considered as a limiting nutrient in freshwater bodies. Trimbee and Prepas (1987) reported the presence of phosphorous as a good indicator of the total relative biomass of cyanobacteria. Data collected for long-term data in Lake Dianchi (1991–2013) showed that variation in the total phosphorus (TP) concentration affect the phytoplankton biomass considerably both seasonally and inter-annually (Zhou et al. 2016). Chen et al., (2005) reported that a decrease in dissolved phosphorous concentration cause decline of cyanobacterial blooms in water bodies. Dissolved inorganic phosphorus (DIP) is the preferred P source for phytoplankton in comparison to dissolved organic phosphorus (DOP). The reason is direct bioavailability of DIP whereas DOP can also be exploited (Harke et al. 2012).

The other important nutrient for controlling Cyanobacterial growth is Nitrogen. The majority of reported CYN producers belongs to order Nostocales, which could easily fix atmospheric nitrogen and therefore compensate for nitrogen limitation in its surrounding environment (Willis et al. 2016). Under such conditions, one of the primary restriction factors which significantly influence the CYN-producing species growth is inorganic P (Chislock et al. 2014). Literature proposed CYN as an allelopathic compound which can stimulate an alkaline phosphatase activity in surrounding phytoplankton community to provide inorganic P (Bar-Yosef et al. 2010). Still, the understanding regarding the distribution of CYN within the cyanobacterial cells and water bodies needs to be clarified. Past studies showed that the presence of a sulfonic acid group make CYN highly water-soluble and thus could be extensively released in the extracellular environment (Bar-Yosef et al. 2010). Mainly *Aphanizomenon, Anabaena, Anabaenopsis, Raphidiopsis, Cylindrospermopsis, and Planktothrix* constitute the cyanobacterial community in German lakes, and about more than 80% of CYN in total were found

extracellular in 31% of the samples (Rücker et al. 2007). High extracellular portion of CYN (92%–96%) of CYN was observed in the case of *C. raciborskii* (Jiang et al. 2014). Similarly, in a previous study conducted on cyanobacteria, the reported extracellular CYN percentage was 20% during the rapid growth phase which later reached up to 50% in the slow growth phase (Hawkins et al. 2001). However, in another study, Saker and Griffiths, (2000) showed that up to ninety percent of CYN remained in the cell in the exponential growth of 7 cultures *C. raciborskii* strains. Reported literature showed that with the increasing age of cells an increasing CYN release trend was the highest dissolved CYN content was observed in older blooms or in batch cultures of stationary phase (Dyble et al. 2006). Davis et al. (2014) added that CYN release in the water bodies as a result of cell lysis in the period with environmental stress or in the stationary phase of growth.

The actual exposure and resulting effects posed by exposure to cyanotoxins and health risks related to it have still not been conclusively quantified particularly in the human situation. The oral route via drinking water is most likely the most important route for human exposure (Falconer, 1996). Consumption of algal health food tablets and recreational use of lakes and rivers must be considered. Likely during the recreational use of water bodies, the dermal route may play a role (swimming, canoeing, and aerosols, etc.) (Falconer,1996). Further reports of toxic strains in other geographic regions are to be expected. Overall, both climate change conditions and the physiological features of *C. raciborskii* could lead to an overall increase of *C. raciborskii* reports. This is significance because of potential toxic bloom formation of this species. World Health Organization (WHO) has adopted a provisional guideline value of 1.0 μg/L for microcystin-LR in drinking water. Still, other efforts to control water toxicity in drinking water and consequently efforts have been made to provide clean water to mankind.

1.2 Study objectives

C. raciborskii is mostly studied for its invasiveness and toxin production that have major effects on living organisms. *C. raciborskii* is a tropical species but many driving factors such as climate change and nutrient levels changes are promoting its expansion in most parts of the world. Therefore, its necessary to know the influences of their driving factors. The main purpose of this study was to investigate the growth and toxin production by *C. racciborskii* at different nitrate and phosphate concentrations, at high and low light intensities. This study is done to increase the understanding of the behavior of this species and help to predict their future blooms in the nature.

1.3. Literature review

Toxic cyanobacteria bacteria in all types of waters especially water sources used for drinking and recreational purposes, cause serious risks to human health. Still, these issues are neglected and not given the appropriate attention even on a local level. Even wild and domestic animals are facing hazards due to the accumulation of scums of cyanobacteria or blue-green algae accumulating along the shores of lakes and ponds (Carey et al. 2012). Providing safe drinking water to the human population is one of the most critical matters in public health. This chapter highlights the background, geographical distribution and potential toxins of cyanobacteria, particularly related to *C. raciborskii*.

1.4. General morphology and taxonomy of cyanobacteria

An ancient group of organisms commonly knew as “Cyanobacteria” possess vast range of habitats from temporarily frozen ponds to hot springs. They are present in both marine and freshwater environments (Xie et al. 2011). Like eubacteria, these Cyanobacteria also lack nucleus however, they produce oxygen in photosynthesis. Cyanobacteria possess about 150 genera with 2,000 species and at least 40 of which are potential toxin producers according to current taxonomy (Paerl and Otten, 2013). They grow as filaments, in colonies or just as single cells, while nitrogen-fixing special cells called

"heterocyst" are present in some filamentous genera. In the case of filamentous species or *Microcystis sp*, cells growing in colonies may be packed in a mucilaginous sheath which forms free-floating strands or floating mats. An additional feature that many cyanobacterial species have is the presence of gas vacuoles which allow them to maintain their position in the water column and give them a distinctive ecologic advantage compared to other planktonic species (Mez et al. 1998).

1.5. Cyanobacterial bloom formation

The most efficient method for avoiding cyanobacterial toxin contamination in drinking water is the prevention of bloom formation. However, the characteristics which lead to the development of cyanobacterial blooms (cell numbers > 10^6/L), whether of nontoxic or toxic species have yet not been identified satisfactorily. Factors like phosphorus, nitrogen, light, temperature, micronutrients (molybdenum (Mo), iron (Fe)), buoyancy, hydrologic and meteorological conditions, pH as well as alkalinity and the morphology of the water reservoir will all influence the development (Mez et al. 1998).

1.6 Cyanobacterial toxins

Cyanobacteria generate various toxins, commonly known as cyanotoxins, which are further categorized functionally as neuro-hepato and cytotoxins. Additionally, lipopolysaccharides (LPS), as well as secondary metabolites, are produced by cyanobacteria that are potentially pharmacologically useful. According to the chemical structure of cyanotoxins, they are categorized in different groups such as, alkaloids (the neurotoxins, anatoxin, and saxitoxins), cyclic peptides (the hepatotoxins, microcystins, and nodularin) (Painuly et al. 1988).

Cyanobacteria produce toxins, but the environmental conditions under which they produce them largely remain unknown. Although, these toxins are produced is now becoming clearer. Microcystin cyclic small size structure and content of unusual amino acids show that these cyclic peptides are manufactured non-ribosomally rather than

ribosomally (Namikoshi et al. 1990). Peptide synthetase enzymes possess highly conserved structures which are involved in non-ribosomal peptide synthesis. These peptide synthetase coding genes are modular containing information for each single peptide synthetase unit. Search was performed to predict homologous sequences in toxic and nontoxic strains of *M. aeruginosa* by using two conserved sequence motifs of the adenylate-forming domain of peptide synthetases, further it was found that peptide synthesis gene sequences were found only in toxic strains (Carey et al. 2012). Thus, the ability to produce toxins in cyanobacterial strains may primarily depend on the possession and expression of such genes under certain environmental conditions. Nodularins and Microcystins are the most widespread cyanotoxins. They are commonly found in cyanobacterial blooms ranging from oceans to freshwater bodies. Microcystins have been described to belong by the genera *Anabaena, Planktothrix, Microcystis*, *Nostoc,* and *Anabaenopsis*, while *nodularin* is found in *Nodularia* only (Namikoshi et al. 1990).

Numerous cases of animal poisonings have been reported due to *Nodularia spumigena* bloom in Lake Alexandrina, Australia since 1878 (Paerl and Otten, 2013). The death of farm animals or poisonings of dogs swimming in cyanobacterial scum has been documented due to contamination of ponds with cyanobacteria. Cyanobacterial blooms have also been linked with fish kills resulting in economic losses (Toranzo et al. 1990). Different toxin produced by cyanobacteria target on different organs. For example, the major target organ for microcystin toxicity is liver (Toranzo et al. 1990). Studies on pigs and mice have shown that mortality, liver weight, and plasma alanine amino transferase levels are directly associated with loss of body weight when exposed to toxic extracts of *M. aeruginosa* blooms (Carey et al. 2012). Neither lactate dehydrogenase levels nor other organ systems seemed affected. In the case of mice, death occurred within 3 hours due to intrahepatic hemorrhage. e necrosis (Myhre et al. 2018).

Their uptake is linked with adenosine triphosphate (ATP)-dependent transporters as most of these congeners are hydrophilic and generally not able to penetrate vertebrate cell membranes. However, in rat liver, one un-identified multi specific organic anion transporter (or bile acid transporter) was found as the carrier of these cyclic peptides (microcystin) (Trimbee and Prepas, 1987). Therefore, the toxicity of nodularins and

microcystins is restricted to those organs which express the organic anion transporter on their cell membranes, for example, the liver.

1.6.1. Cylindrospermopsin

The main producer of cylindrospermopsin (CYN), an alkaloid cyto and hepato-toxin are *C. raciborskii.* The CYN, a polyketide-derived alkaloid with a central functional guanidinium moiety combined with hydroxymethyluracil attached to its tricyclic carbon skeleton was first toxic compound ever identified (in 1992) for *C. raciborskii* (Poniedziałek et al. 2012). This toxin is stable over a wide range of pH and temperatures and actively released by intact cells to the extracellular environment (Preußel et al. 2009). It has also been found in sediments from approximately 4700 years ago. They can accumulate in the water column (Waters, 2016).

1.6.2. Distribution and potential toxicity

Toxin-producing *C. raciborskii* strains have been reported from New Zealand, Australia (Antunes et al. 2015) and Asia (Lei et al. 2014). However, no Polish or European toxic strain of *C. raciborskii* have been identified as they lack CYN producing or possessor genes involved in their biogenesis (Rzymski et al. 2017). In Serbia alleged production of CYN by *C. raciborskii* have been reported due to the bloom formation in Lake Aleksandrovac, however, this information was not based on a molecular or analytical investigation of the isolated strain (Dorđević et al. 2015).

The first report on the observation of *C. raciborskii* came from the island of Java, Indonesia (1899–1900) but for the first time, it was identified by Woloszynska*. C. raciborskii* usually found in eutrophic lakes is a potent bloom-forming species. Its presence in lowland rivers has also been documented (Soares et al. 2013). By means of morphological features, it can be easily distinguished under a light microscope from other cyanobacteria. It usually possesses solitary and straight trichomes although coiled forms are also identified. So far in Polish lakes, only straight types have been recognized. Cells

are barrel or cylindrical shaped, longer as compare to width, constricted at the cross-walls within the trichome. Presence of an elongated drop-like shape heterocyst at terminal position is a characteristic feature of this species. However, akinetes are formed during unfavorable environmental conditions (Singh, 1962). Except for Antarctica, this species is described in temperate, tropical and subtropical climates in all continents. In Europe first report on the presence of *C. raciborskii* came from Lake Kastoria, (Skuja, 1937). Near the end of the twentieth century, it was hypothesized that this species colonized from Greece and Hungary toward higher latitudes (Singh, 1962). However, in the African continent, the first description dates probably from the end of the nineteenth century (Huber-Pestalozzi, 1938). In 1979 in Australia, in America, it was first informed in1955 and in the Middle East, it was first documented in one Israeli lake. Today *C. raciborskii* is invading around the globe, both in the Southern and Northern hemisphere, in rivers, lakes shallow water bodies and reservoirs.

The STXs are the second important groups of toxins which are identified to be produced by *C. raciborskii*. This type of toxins is produced in South America strains (Rzymski et al. 2017). Their presence is also confirmed in waters of Greece however, molecular and analytical studies conducted on the isolated strain are lacking convincing evidence (Gkelis and Zaoutsos, 2014). STX are also created by genera in Nostocales (Ballot et al. 2010). These alkaloid neurotoxin causes paralytic shellfish poisoning (O'Neill et al. 2016). About 55 analogues have been identified including mono-sulfated, di-sulfated, non-sulfated, decarbamoylated and hydrophobic variants which vary in their toxicity (Wiese et al. 2010). Interestingly, one strain of *C. racciborskii* from Tunisia (Bir M'cherga reservoir) was reported to possess two segments from the *mcy* gene cluster (*mcyA* and *mcyE* genes) involved in the production of MC, a cyclic peptide hepatotoxin. Most recently, the production of MC by a Greek strain of *C. raciborskii* has been demonstrated (Panou et al. 2016).

The lipophilic congeners of PMA (polymethoxy-1-alkenes) are produced by North American strains of *C. raciborskii* which induce toxic and particularly, teratogenic activity in the zebrafish embryo (Jaja-Chimedza et al. 2012). However, up till now, no strains have been screened to produce PMA from other geographical regions. Several *C. raciborskii* strains from Europe were shown *in vitro* and *in vivo* to be toxic but still, no known

cyanotoxins are recognized. Similarly, German isolates extracts were toxic to human hepatoblastoma, human colon adenocarcinoma cells and primary rat hepatocytes (Fastner et al. 2003). A French strain of *C. raciborskii* caused liver damage in rodents (Bernard et al. 2003). Likewise, crude extracts of a Portuguese strain induced Hepatotoxicity as well as neurotoxic effects (piloerection, lethargy, and difficulty in breathing) in mice (Saker et al. 2003). Similarly, strains from Lake Balaton showed toxicity in their extracts in four bioassays: *Daphnia Magna* acute immobilization assay, *Thamnocephalus platyurus* acute lethality test; *Danio rerio* embryo developmental toxicity assay and *D. Magna* feeding inhibition assay (Acs et al. 2013). Recently in frog embryos, the retinoid-like activity provoking teratogenic effects was reported from on strain of Lake Balaton (Smutná et al. 2016). Neuro toxic effects were also reported for the Hungarian strains of *Helix pomata* (Roman Snail) resulted in neurotoxicity including inhibition of the acetylcholine responses. Toxic effects were also reported in strains of *C. raciborskii* from Poland.

1.7. Factors affecting *Cylindrospermopsis raciborskii*

1.7.1. Temperature

Strains of *C. raciborskii* from various parts of the world display wide range of temperature tolerance (Gundersen and Mountain, 1973), and biomass are capable of sustaining at temperatures between 14 to 17°C or even down to 11°C (Piccini et al. 2011). The production of polar carotenoids in this species may be a consequence of adaptation to low temperatures. The highest temperature value where showing net growth of *C. raciborskii* is 35°C (Briand et al. 2002). Modeling data verified that *C. raciborskii* blooms formation are more likely to happen in the temperature's interval between 25–32°C (Recknagel et al. 2014). Higher temperatures are more favorable, which elucidate their regular emergence in tropical regions (Soares et al. 2013). Most probably it is considered as a tropical species, maybe it is said so due to lack of information of other geographical strains.

1.7.2. Water column and light stability

C. raciborskii also possess a high tolerable range of light intensities. Growth has been observed from a few tens to hundreds of µmol photons $m^{-2}s^{-1}$ which constitute a wide range of irradiance values (Mehnert et al. 2010). Several strains are habitat specific and show their appearance as well as dominance at specific light condition (Poniedziałek et al., 2012). Blooms *of C. raciborskii* can even persist at a low light intensity. In the water column, the factor of buoyancy plays an important role for this species to optimize light uptake (Padisák, 1997). However, unlike temperature, solar radiations are not considered a crucial factor in limiting *C. raciborskii* expansion in temperate climates (Poniedziałek et al. 2012). Another study demonstrated that the main factor in the phytoplankton succession was the combined effect of solar radiation, wind and thermal stability of the water column in a subtropical reservoir. Other physiological studies explained the influence of adaptation of *C. raciborskii* to a wider range of light intensities on photosynthetic activity. *C. raciborskii* dark acclimated cells were more productive in moderate irradiance while cells adapted to high irradiance values showed to be more productive (O'Neill et al. 2016). Furthermore, in comparison to *Microcystis* and *Aphanizomenon*, the carotenoid, phycobilin concentrations, and photosynthetic activities were significantly higher for *C. racciborskii*. Regarding both thermal acclimation and photoacclimation of *C. raciborskii* spread to higher latitudes has also been studied.

C. raciborskii is reportedly more susceptible to light stress as compared to the native species *Aphanizomenon gracile* with the light intensities increased at lower temperatures conditions (Mehnert et al. 2012). Under such circumstances, *C. raciborskii* respond with a higher photoprotective carotenoids ratio. This may relate to the distinctive bio-geographical origins of the two species. *C. raciborskii* is mostly adapted to low light conditions encountered in turbid and eutrophic water but still, it is dispersed throughout the water column (Padisák, 1997; Mehnert et al., 2012; Recknagel et al. 2014).

1.7.2. Nitrogen influence

In terms of nitrogen (N) usage, *C. raciborskii* may be considered a generalist because of facultative diazotrophs. These species respond to environmental variations of N element by alternating between dissolved inorganic N assimilation and N_2 fixation (Moisander et al. 2012). The terminal heterocyst cells sole carried out N_2 fixation and permit this species to survive under low dissolved N systems. This trait contributes to the dominance of this species in nitrogen limited lakes as well as in water reservoirs giving this species an ecological advantage (Harris and Baxter, 1996). Laboratory studies have confirmed different preferences for N sources of *C. raciborskii*. Fastest growth rates were observed in the environment with ammonia, then nitrate and at end urea (Harris and Baxter, 1996). Additionally, this invading species was shown to grow faster than cosmopolite *Planktothrix* at high ammonia concentrations (Ammar et al. 2014). For cellular assimilation N_2 is less efficiently used than nitrate, most likely due to cost associated with the establishment of the heterocyst. *C. raciborskii* had fewer heterocysts in reservoirs with high concentrations of nitrate, indicating its preference for nitrate as N source (Briand et al. 2002). However, in tropical reservoirs, the formation of *C. raciborskii* blooms was limited by dissolved inorganic N (Figueredo et al. 2013).

1.7.3. Phosphorus influence

Regarding the usage of dissolved inorganic phosphorus (DIP), the *C. raciborskii* may be considered an opportunistic species. This species possess both high storage capacity and a high uptake affinity for phosphorus. Under fluctuations of P concentrations these characteristics are seemed to be beneficial (Wu et al. 2009). Pulsed additions of DIP and high P storage ability of *C. raciborskii* are known to be the best growth conditions for *C. raciborskii* growth and are considered favorable over stable inputs of DIP. For the akinetes germination, this character seemed to be important (Padisák and Istvánovics, 1997). Phosphorous transformation and its effective uptake are considered superior in *C. raciborskii* as compared to other species of Cyanobacteria such as *Microcystis aeruginosa* and *Aphanizomenon flosaquae* (Wu et al. 2009). The defensive means to overcome P limitation in the environment is an adaption to low ambient DIP concentrations by

increasing alkaline phosphatase (ALP) in *C. raciborskii* which is considered as physiological metabolism regulation (Wu et al. 2009). Moreover, different organic P sources were used by *C. raciborskii* to support its growth. A recent study verified that situations of DIP deprivation caused both an induction of CYN production as well as an up-regulation of DIP uptake machinery in a strain *Aphanizomenon*. Currently, it is not known how much this mechanism is distributed, but various *C. raciborskii* strains produce CYN and may eventually benefit and possess from this approach (Bai et al. 2014).

Sometimes even presence of high concentrations and uptake of P gives no advantage on *C. raciborskii* growth and in this situation the different uptake rates of ammonium acts as deciding factor in the competition between species (Wu et al. 2009). To fully explain the role in the growth of *C. raciborskii* populations, the different strategies to nutrient uptake should be considered. For example, it was recently shown that under both low and high N:P ratio *C. raciborskii* dominates in eutrophic areas. The successful invasion of *C. raciborskii* into temperate waters can be better explained by the synergistic effect of global warming with local nutrient conditions (Bai et al. 2014).

1.7.4. Effect of pH, salinity & CO2

Low salinity conditions with optimal growth in fresh to oligohaline conditions were recognized for *C. raciborskii* (Padisák, 1997). However, high salinity values are observed to be an inhibiting factor for *C. raciborskii* growth (Moisander et al. 2012). This species can grow in slightly brackish waters, specifically under nutrient enrichment conditions (Briand et al., 2002), or elevated concentrations of dissolved minerals (Calandrino and Paerl, 2011). As a result, this species invades eutrophic systems of moderate salinity (Calandrino and Paerl, 2011). As far as pH is regarded, *C. raciborskii* shows more preference for high pH values and its growth was observed between 8.1 and 9.4. More recently, *C. raciborskii* growth was observed in the lakes with pH values between 5.4 and 9.9 with a median value of 8.2 (Bonilla et al. 2012). The pH value is lowered by an increase in the atmospheric partial pressure of CO_2. With an increase in CO_2, the proportion of CO_2 to HCO_3 will increase which effect the pH and in the future may result in a competitive

disadvantage for *C. raciborskii* (Holland et al., 2011). However, compared to the expected effects of global rising temperatures, the influence of CO_2 in the spread of *C. raciborskii* may be negligible. Effects on the ecological performance of this species may not be affected significantly with increasing CO_2 concentrations (Sinha et al. 2012).

1.7.5. Climate change and *C. racciborskii*

C.raciborskii is tropical and subtropical species but its presence is increased in temperate climate region from the last twenty years. (Briand et al. 2002). This is due to an increase in water temperature due to global warming (Wiedner et al. 2007). In harsh condition akinetes (phosphate storage cells) helps the species for survival at lower water temperature and then the again start when the water temperature starts increasing (Sinha et al. 2012). Therefore, long summer and high-water temperature could be the reason for the expansion of *C. racciborskii.*

2. Materials and methods

2.1. Bacterial strain of *C. raciborskii*

The strain of *C. raciborskii* (NIVA-CYA-506) was obtained from Norwegian Institute for Water Research (NIVA) Oslo, Norway which was first isolated from Kazinga Channel Uganda (2004 - 2009).

2.2. Cultural conditions and experiment design

The growth of the strain was maintained in O2 medium (Van Liere and Mur, 1978) at controlled light (10 µE $m^{-2}s^{-1}$) and temperature 25°C in a climate cabinet (Termarks). The culture was shifted into new O2 medium one week before start of the experiment. All experiments were performed at the University of Southeast Norway (USN) campus Bø. 2-L glass vessels having O2 growth medium were used for batch culture of *C. raciborskii* and nearly 10 ml of precultured (one week old) *C. racciborskii* culture was added to each glass vessel as inoculum in all experiments.

The experiments were performed in a climate cabinet (Termaks) with temperature conditions of 25 °C ± 0.1 ° C under two different light conditions one is of relatively high light intensity (65µE$m^{-2}s^{-1}$) and other is low intensity (10µE$m^{-2}s^{-1}$) provided by cool white fluorescent tubes (Philips 36W/33). The vessels were aerated with the help of 1 ml glass pipette passing through Cellulose acetate membrane filter (pore size of 0.2µm, Merck Millipore, Darmstadt, Germany). Following three main experiments were performed.

Table 1. C. racciborskii experimental design of the three experiments.

Experiment 1. Normal 02 medium, Experiment 2. Different phosphate concentration mgP/l. Experiment 3. Different Nitrate concentration mg N/l (H= high, M= medium, L= low and W= without addition in 02 medium).

Culture conditions	**Experiment 1**	**Experiment 2**	**Experiment 3**
Number of glass vessels (2-L)	4	8	8
Addition of *C. raciborskii* per vessel (day 0) from pre-culture	10ml	10ml	10ml
Changes in O2 Medium	Normal O2 medium	Only changes in K_2HPO_4	Only changes in $NaNO_3$
Different Phosphate concentration		H=2 (4.45 mg P/l) M= 2(0.08 mg P/l) L= 2 (0.044 mg P/l) W=2 (0 mg P/l)	----------
Different Nitrate Concentration	----------	----------	H=2 (82.4 mg N/l) M=2 (57.6 mg N/l) L=2 (32.96b mg N/l) W=2 (0mg N/l)
Temperature (˚C)	25˚C ± 0.1 ° C	25˚C ± 0.1 ° C	25˚C ± 0.1 ° C
Light intensities (µE $m^{-2}s^{-1}$).	High Light (65µE m^{-2} s^{-1}). Low light (10 µE m^{-2} s^{-1}).	High Light (65µE m^{-2} s^{-1}). Low light (10 µE m^{-2} s^{-1}).	High Light (65µE m^{-2} s^{-1}). Low light (10 µE m^{-2} s^{-1}).

2.3. Experiment 1

Firstly, the growth response and changes in pH of the *C. raciborskii* culture was investigated under two different light intensities as described above without inducing any change of O2 medium (table 1).

2.4. Growth measurement under different phosphate concentrations

The second experiment was performed under different phosphate concentrations to measure the optical density, toxin analysis and morphology study of *C. racciborskii*. In the treatment without addition of phosphate KCL was added to maintain the ionic strength (Table 1).

2.5. Growth measurement under different Nitrate concentration

The third experiment was conducted under different concentrations of Nitrate in O2 medium. In treatment without (W) NaNO3, the NaCl was used to maintain the ionic strength of the medium for toxin analysis, optical density and morphology study of *C. racciborskii.*

Each treatment in the experiment was performed under high and low light positions in the same cabinet (Termaks) at the same period to evaluate the influence of light intensity on *C. raciborskii* growth.

2.6. Sample preparation for analysis

About 25ml of the sample was collected with the help of 25ml sterilized pipette every second day in a control cabinet. The samples were used for the quantification of *C. raciborskii* growth by measuring optical density, number, and length of filaments, a number of heterocyst per filaments during different treatments, toxins produced, phosphate as well as nitrate analyses and pH measurement. The optical density and pH were measured on the same day. For phosphate and nitrates analyses,15 ml of sample

was filtered by Whatman GF/C filter. For growth quantification of *C. raciborskii,* 5ml sample was preserved in Lugol solution every 2nd day. The Lugol was added until the color of the sample turned to light brown. These samples were placed in the refrigerator under dark conditions at 3°C and analyzed at the end of each experiment. For measuring cylindrospermopsin, 5-ml sample was preserved in glass tubes and frozen (-18 C˚).

2.7. Analytical methods

Perkin Elmer UV / VIS Spectrometer Lambda 25 and 1 cm quartz cuvette (QS) was used to measure optical density (OD) at 740 nm. The pH was measured with PHM210 standard pH meter (meter Lab). Phosphate (mg P/L) was analyzed by Perkin Elmer Lambda 25 through the spectrophotometric method according to NS4724 while the Nitrate (mg N/L) was measured by the DIONEX ICS 1100 (Ion Chromatography-intern method). Cylindrospermopsin concentration was measured by using the ELISA- kit from Abraxis, Biosense Laboratories 520011. The sample size of 5ml was frozen in acid washed tubes. The analysis was performed in accordance with the method described in the cylindrospermopsin Kit.

2.8. Growth Variations Analysis

The changes in the morphology and density of *C. raciborskii* during different experiments such as total number and length of filaments as well as a total number of heterocyst were analyzed using the hemocytometer method as defined by (Lobban et al. 1988) using ptical microscope (Olympus Optical Co-Ltd Japan Model CK2) at a magnification of 400-1000x.

2.9. Statistical analysis

One-way ANOVA was used to test the statistical differences between the different concentration of phosphate and nitrate treatments. *P* value less than 0.05 were considered as a significant.

3.Results

3.1. Effects of light intensity on growth of *C. raciborskii* in standard O2 medium and batch culture

The growth of *C. raciborskii* in low light treatment after the lag phase (day 8) shows a gradual increase in OD_{740nm} reaching stationary phase with OD 0.25 day 20. Maximum OD in high light was recorded to be 0.09 at day 16 (Fig. 3.1(a)). The OD_{740nm} values between the treatment at high light were significantly less than the treatment at low light intensity. The maximum generation time was 2.7 days at high light while the minimum generation time was 2 days at low light intensities (table 3.1). The highest pH was 9.3 under low light and 8.1 in high light intensity (Fig 3.1).

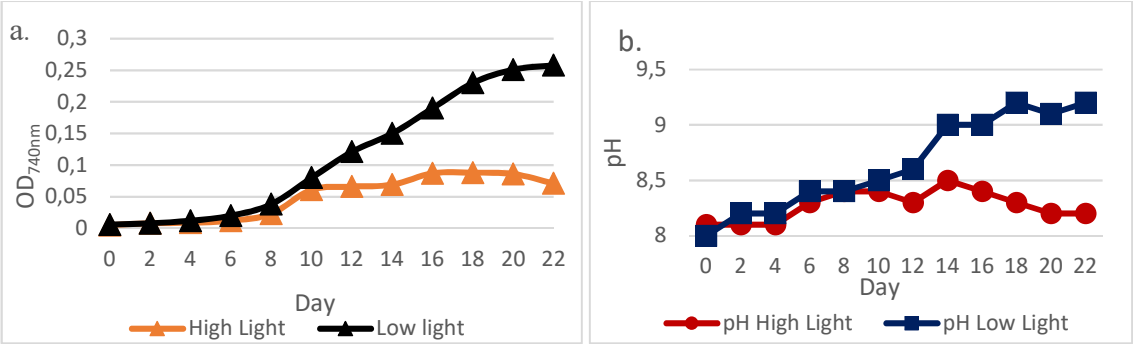

Figure 3.1. *(a) optical density (OD_{740nm}) and (b) pH measurement of C. raciborskii in O2 medium in high light (65µE m^{-2} s^{-1}) and low light intensity (10µE m^{-2} s^{-1}).*

3.2. Effect of light intensity and phosphate concentration on the growth of *C. raciborskii*

An increase in phosphate concentration led to an increased growth rate of *C. raciborskii*. In high light intensity, the generation time with high, medium, and low phosphate concentration was recorded as 1.5, 2.3 and 1.7 days respectively while the longest generation time 3.5 days was noticed without addition of phosphate in O2 medium. Under low light intensity, the shortest generation time was 1.4 days in high, medium and low phosphate concentrations however, without addition of phosphate in O2 medium it was 3.6 days (table 3.1).

Table 3.1. Generation time for C. racciborskii in three different experiments at high (65 µmol photons $m^{-2}s^{-1}$) and low light (10 µmol photons m^{-2} s^{-1}) intensities. Experiment 1 (normal O2 medium), experiment 2 (different Phosphate concentration), experiment 3 (different nitrate concentration), high (H), medium (M), low (L) and without addition (W) in standard O2 medium.

Experiment number	Light intensity	Generation time for (H)	Generation time for (M)	Generation time for (L)	Generation time for without addition (W)
1. Normal O2 Medium	High light	2.7 days			
	Low light	2 days			
2. Different P concentration	High light	1.5 day	2.3 days	1.7 days	3.5 days
	Low light	1.4 day	1.4 day	1.4 day	3.6 days
3. Different N concentration	High light	1.5 day	1.4 day	2 day	3.8 days
	Low light	1.5 day	1.8 day	1.6 day	4 days

The maximum OD_{740nm} was notice 0.8 with the high concentration of P while it was 0.1 with medium, low and without addition of P in the medium (Figure 3.2). There was no significance difference ($p > 0.05$ ANOVA single factors) between the treatments in high and medium phosphate concentration however, it was significant lower ($p < 0.05$) with high low and without addition of phosphate in 02 medium at both high and low light intensities treatments.

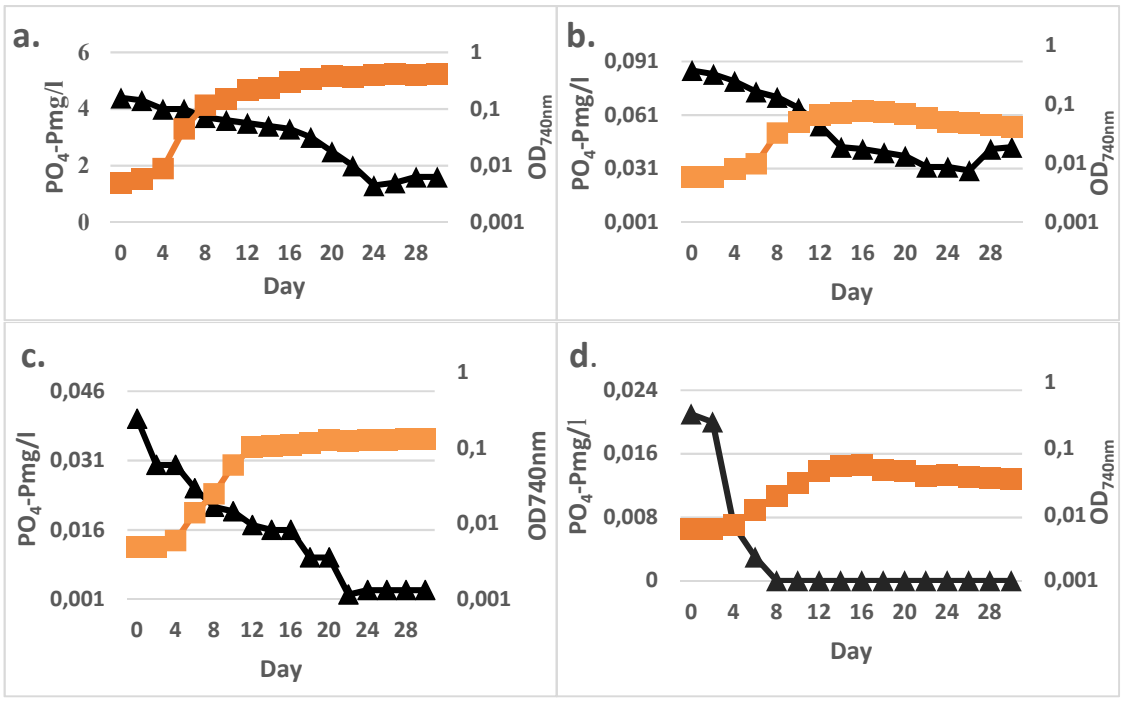

Figure 3.2. Changes in PO_4-P concentration (▲) and optical density (■OD_{740nm})) under high light intensity in batch culture of C. raciborskii. a High, b Medium, c Low and d no phosphate additions.

The maximum optical density (OD_{740nm}) values was 1 ± 0.1 in the treatment with high, medium and low phosphate concentration while the minimum (OD_{740nm}) values was noticed 0.2 in the treatment without addition of phosphate in the O2 medium at low light intensity (Figure 3.3).

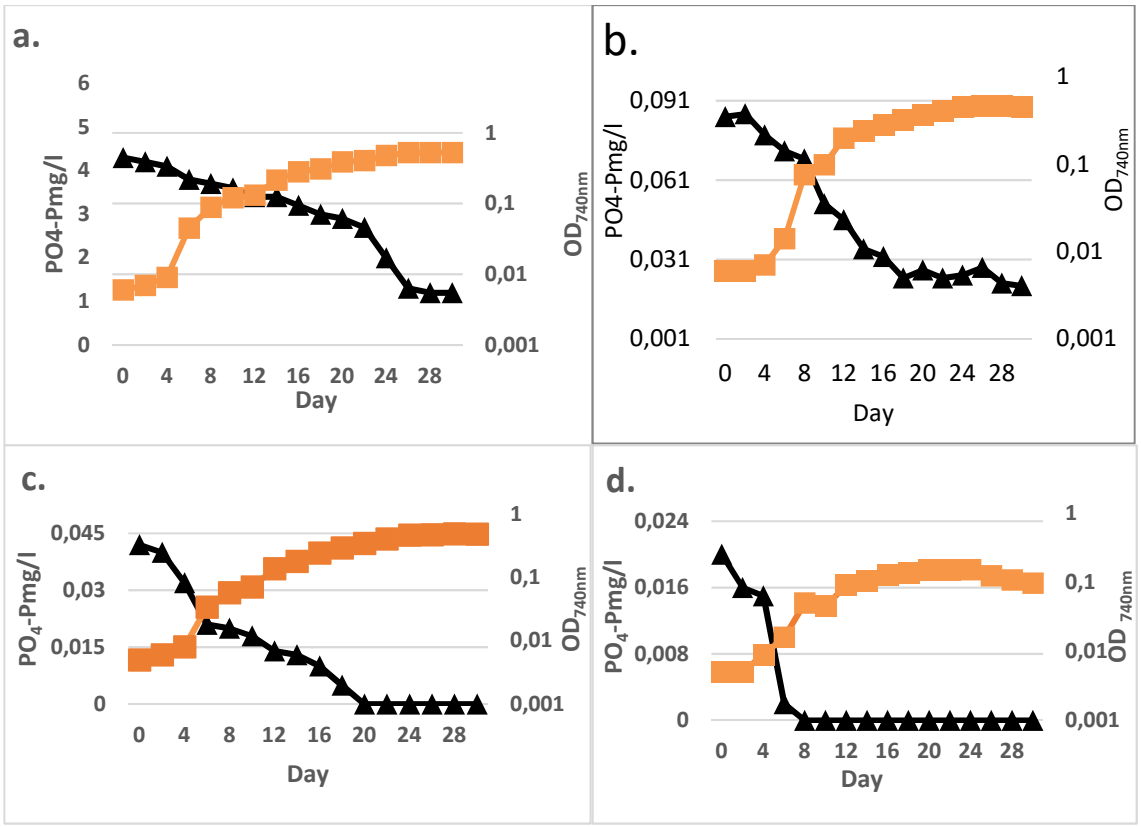

Figure 3.3. Change in PO_4-P (▲) concentration and optical density (■ OD_{740nm}) under low light intensity on the batch culture of C. raciborskii. a (High), b (Medium), c (Low) and d no phosphate additions.

3.3. Effect of phosphate concentrations on filament length and numbers of heterocysts of *C. raciborskii*

Filaments length show an increase after two days and longer filaments length was recorded in the treatments under low light intensities as compared to high light. The largest filament length was recorded to be 0.7mm ± 0.1mm in high P on day 22 while in low and no addition P the longest filament length was 0.3 ± 0.1 mm at day 18 in high light intensity. The largest filaments length in low light intensity was 0.9 ± 0.1mm at high P, 0.7± 0.1mm in medium P, 0.6 ± 0.1mm at low P additions. Overall the largest filaments lengths were noticed in the experiment under low light intensities as shown in Figure 3.4.

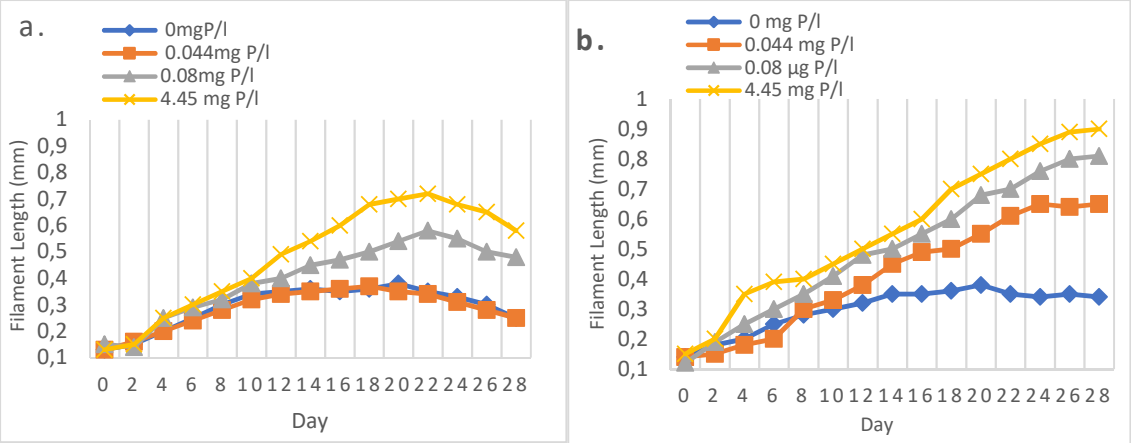

Figure 3.4. Effect of phosphate on filament length (mm) under different phosphate concentrations in High light (a) and Low light intensity (b).

3.4. Phosphate and production of Cylindrospermopsin

Representative samples from lag, exponential and stationary phase were analyzed for the CYN concentration by ELISA. No toxin was found in any samples as all samples were had a concentration less than 0.05 ppb and should be referred as negative according to the ELISA-manual *(Appendix 1).*

3.5. Effect of light intensity and nitrate concentration on the growth and toxin production of *C. raciborskii*

At high light intensity, the shortest generation time during the 40 days experimental growth of *C. raciborskii* was 1.5 ± 0.1 day in high and medium concentration of nitrate while the longest generation time was 3.8 days without addition of nitrate in the treatment in standard O2 medium. Treatments under low light intensity, the longest generation time was 4 days without addition of nitrate however, in the high, medium and low concentration of nitrate in O2 medium it was 1.5, 1.8 and 1.6 day (Table 3.1).

The optical density was same 0.5 in the treatment with high, medium and low concentration of nitrate with no significant difference, while it was 0.03 without addition of nitrate concentration in the O2 medium at high light intensity (Figure 3.5).

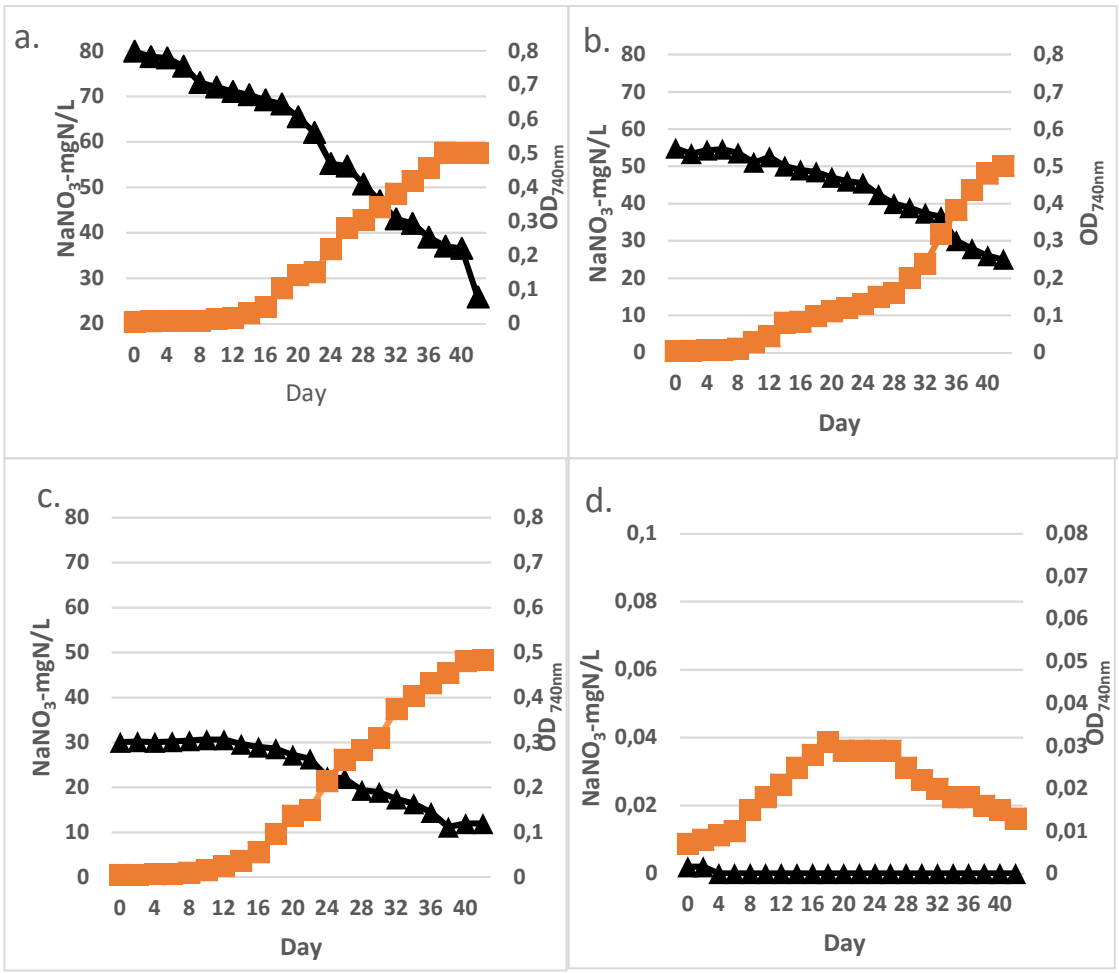

Figure:3.6. Effect of different concentration of $NaNO_3$-mgN/L (▲) high light (65 µmol photon -m-² s-¹) intensity on batch culture growth of C. racciborskii (■OD740nm). High (a), Medium (b), Low (c) and Without adddition (d).

The maximum optical density in high, medium, low and without the addition of nitrates concentration was 0.7, 0.4, 0.3 and 0.03 in the treatment in low light intensity. The $p<$ 0.05 between the high and without addition of phosphate concentration. There was no difference observed between the lag period in high, medium, low and without the addition of nitrate, but the shortest lag phase of two days noticed in the treatment without addition of nitrate in O2 medium at low light intensity (Figure 3.7).

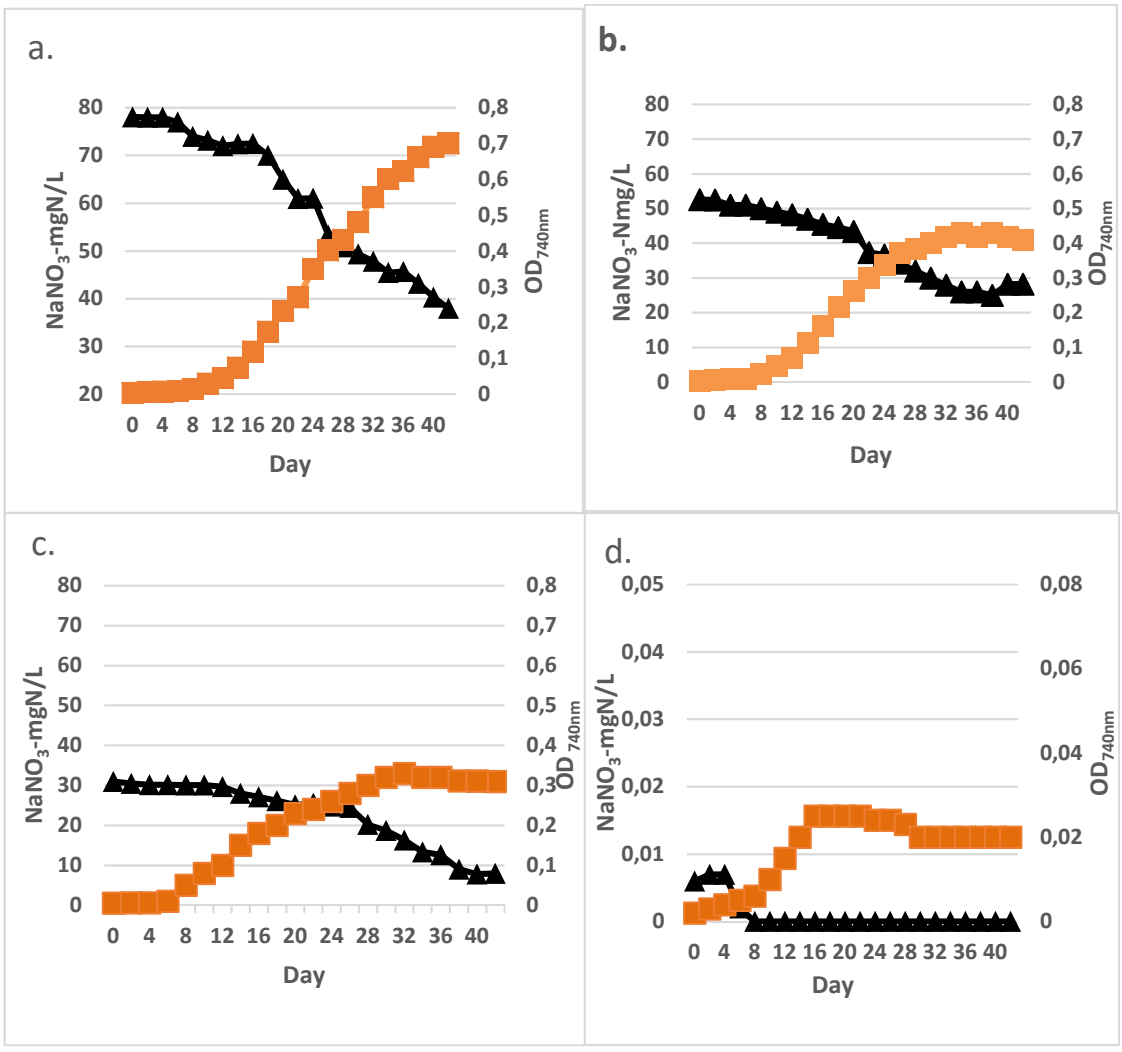

Figure:3.7. Effect of different concentration of $NaNO_3$-mgN/L (▲) low light (10 µmol photon -$m^{-2}s^{-1}$) intensity on batch culture growth of C. racciborskii (■OD_{740nm}). High (a), Medium (b), Low (c) and Without (d).

3.6. Heterocyst analysis:

The numbers of the heterocysts start increasing after two days of transferring the sample in O2 nitrogen-free medium. The heterocyst frequency reached a maximum with low concentrations and without the addition of N in O_2 medium. There were no heterocysts

present in nitrogen concentrations above 33 mg/L in both light intensities. The heterocyst number was high at day 24 at high light $4x10^4$/ml and at day 28 in low light $3.8x10^4$/ml without the addition of Nμg/L. The number of heterocyst at the end of the stationary phase was 40000/mL in the treatment with 33mg N/L both in high and low light intensities (figure 3.8). At Day 10 the color of the nitrogen-free medium changed into yellowish green.

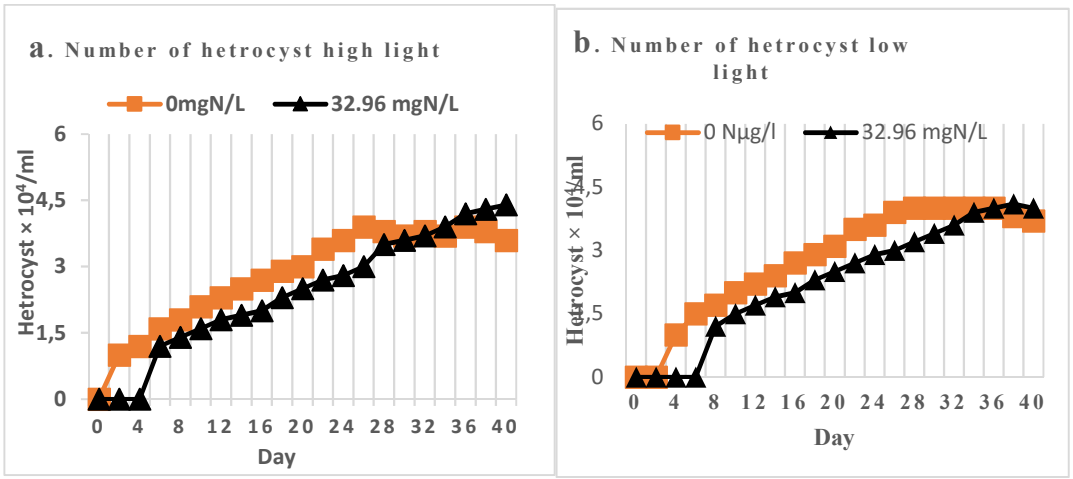

Figure 3.8. Numbers of heterocysts in C. raciborskii in high light (a) and low light (b) intensities during the growth with low and no addition of N concentration.

3.7. Effect of nitrate concentration on filament length

The length of the filaments was higher in high concentration of nitrate than at lower concentrations. Under high light intensity, the highest length of the filament was 0.6mm at high N concentration and 0.5mm at medium and low N. The treatment without any nitrate in the media the longest filament was 0.3mm. In low light intensity with high, medium, low and without the addition of N highest filaments length was 0.8, 0.7, 0.6 and 0.3. respectively (Fig: 3.9).

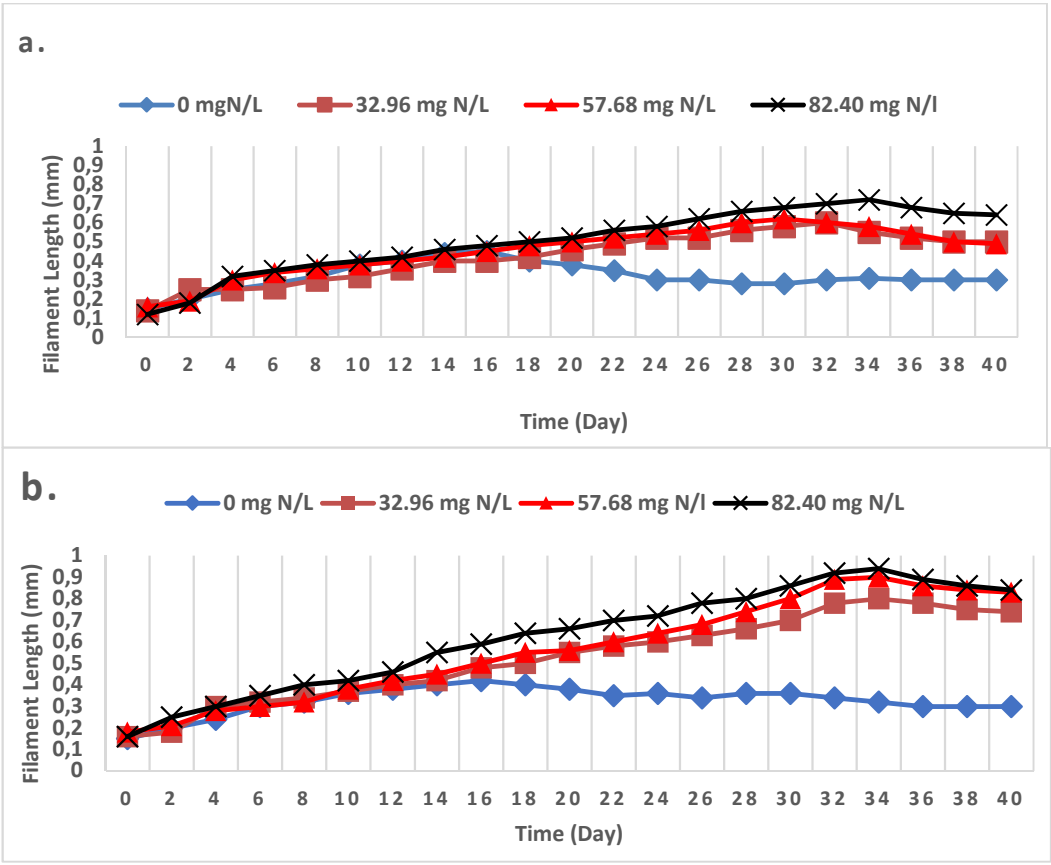

Figure 3.9. Effect of different Nitrate concentration on filament length (mm) in high light (a) and low light (b) intensities.

3.8. Toxin analysis (Cylindrospermopsin) in nitrate concentration in O2 medium

Representative samples from lag, exponential and stationary phase were analyzed for the CYN concentration by ELISA. No toxin was found in any samples as all samples were less than 0.05 ppb and should be reported as a negative according to the according to the ELISA-manual *(Appendix 1).*

4. Discussion

4.1 Effects of light intensity on the growth (optical density OD_{740nm}) of *C. raciborskii*

The P and N-dependent growth, filaments length, toxin yield and number of heterocysts in *C. racciborskii* (NIVA-CYA-506). Result presented in this study showed that *C. raciborskii* showed developed high optical density in the presence of low light as compared to the high light. Results were in accordance with Chapman and Schelske (1997), they recognized that *C. raciborskii* showed more preference to habitat in low salinity and light conditions. A study conducted by Padisák (1997) also showed that mostly cyanobacteria especially the tropical species like *C. racciborskii* can form blooms in low light intensities and in shading environment. It is well known that this is a bloom shading species.

4.2. Effects of phosphate and nitrate on the growth (optical density OD_{740nm}) of *C. raciborskii*

In the absence of phosphate and nitrate, some turbidity was seen in the culture medium which indicated some growth of *C. racciborskii* in the experiments. This could be possibly due to the ability of Cyanobacterial species to store the dissolve inorganic phosphorus and use its stored phosphorus at the time of fluctuation of phosphate concentration in the surrounding environment (Fabbro and Duivenvoorden 1996). According to Wu et al. (2009), *C. raciborskii* species has this characteristic of phosphate storage that helps in the germination of the akinetes (Padisák and Istvánovics 1997). This

is the possible reason for the growth of *C. racciborskii* in the phosphate-free medium. This species has a high affinity for P uptake and effective P transformation as compared to other species such as *Microcystis aeruginosa* and *Aphanizomenon flosaquae* (Wu et al. 2009). The other possible reason could be some P contamination in the culture as it was the previously cultured in O2 medium therefore; a minimum growth in both light conditions was observed.

4.3. Effect of light, nitrate and phosphate on the filament length

The length of the filament increases with increasing N and P concentration. *C. raciborskii* can save resources by adjusting metabolic pathways and reducing high energy consuming physiological process under low nutrients supply, the same response was found in the *Microcystis aeruginosa* (Steffen et al. 2014). Present experimental data showed that reduction in filament length and optical density was observed in high light. According to Shafik et al. (2001), prolonged photoperiod could reduce growth and filament length. Long photoperiod (such as 14 L: 10 D) enhanced the bioproduction of the thylakoids hence caused biosynthesis of chlorophyll in cells (Mitra et al., 2011). Increased chlorophyll leads to increase photosynthesis which resulted in high photosynthesis. Studies suggested that accumulation of materials like glucose and carbohydrates obtained from photosynthesis results in the reduction of photosynthetic activity (Mitra et al., 2011). Therefore, a decrease in photosynthetic activity would reduce the growth rate and filaments length. No formation of any heterocysts was found under both low and high light intensities because of the high concentration of the nitrates used in the media. A batch culture experiment with *Anabaena Variabilis* was done by Ogawa and Carr (1969). They noticed that heterocyst number was lower when NH_4-N was

supplied as a nitrogen source and its formation was high when N_2-N was obtained from the atmosphere.

4.4. Effects of nitrate and phosphate on heterocysts formation and toxin production

Results of Ogawa and Carr (1969) experiments demonstrated that phosphate as well as phosphate-free cultures showed no heterocyst formation in any of the batch cultures. Even under different phosphate concentrations, no heterocyst was found. Similarly, in our study, not even a single heterocyst was observed under high nitrogen concentration. Not all strains *C. racciborskii* can produce the toxin. Results are justified with Sinha et al. (2014) who narrated that the most basic factor involved in the toxicity of *C. raciborskii* is the availability or absence of the CYN cluster gene. Similarly, the literature reported that CYN producing strains of *C. raciborskii* are of Australia, Asian and New Zealand water bodies origins (Stüken et al. 2006). And the *C. racciborskii* strain used in this study was of African origin which is supposed to be non-toxic.

With the increasing N concentration growth also increased with increasing in filament length. It was noticed that after 10th day the color of nitrogen-free medium starts changing to yellow-green that may be due to lack of nitrogen in the medium. The *C. racciborskii* experimental flask containing nitrogen remained green and did not turned to yellow. Similar findings were seen in a study held by Ogawa and Carr (1969), they indicated that the yellowing color of the bacteria is the sign of the nitrogen deficiency. Another experimental data collected by FOGG (1942) showed that the nitrogen fixation by heterocysts could not be possible if there is the presence of enough nitrogen in the environment more than 0.5mg/L. Similarly, a water sample was taken from the basin of Lake Erie in 1967 in which nitrogen was depleted after the spring. From July to

September the nitrates concentration remained almost near to zero. At this time of low nitrate concentration, the area was covered with *Ralfs* and *Anabaena.* They found that in the presence of high nitrates the green algae were high in density while the number of heterocysts was high in low nitrate concentration (Ogawa and Carr 1969).

Biomass of the phytoplankton community and the relative abundance of cyanobacteria have been described significantly in previous reports (Bouvy et al. 1999). In shallow eutrophic lakes, the filamentous cyanobacteria are of importance and dominate the entire water bodies thus affects its quality (Sas, 1989). Similarly, eutrophication and changes in nitrogen to phosphorus (N: P) ratios are directly linked to shifting in phytoplankton composition and abundances, with cyanobacterial dominance particularly during summers the toxic cyanobacterial blooms in drinking water reservoirs have been extensively documented all over the world.

Conclusion

It could be concluded *C. raciborskii* growth and filaments length depends on the availability of N and P concentration as these concentrations increase growth and filaments length also increased. The ecological impact of *C. raciborskii* in terms of its magnitude and consequences in the invaded communities is still not completely clarified. The reports on the occurrence of *C. raciborskii* toxic strains in other geographic regions are expected to come. However, the climate change conditions and ecophysiological features of *C. raciborskii* should lead to an overall increase of *C. raciborskii.* Regular monitoring of reservoirs is carried out to ensure that water quality guidelines are met for the ecosystem and human health. However, monitoring the water quality and supply of safe drinking water demands extensive fiscal and human resources.

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Appendixes

Appendix 1. OD_{740nm} and pH measurements of *C. raciborskii* in normal O2 medium in two light intensities high light and low light. CHL (Control High Light) HL (High Light) CLL (Control Low) LL (Low Light).

Experiment 1	Day	OD_{740nm}	pH	Experiment 1	Day	OD_{740nm}	Ph
CHL	0	0.006	8	CHL	12	0.061	8.6
HL	0	0.006	8.1	HL	12	0.066	8.3
CLL	0	0.005	8	CLL	12	0.105	8.8
LL	0	0.006	8	LL	12	0.121	9.3
CHL	2	0.009	8.1	CHL	14	0.084	8.9
HL	2	0.008	8.1	HL	14	0.069	8.5
CLL	2	0.008	8.2	CLL	14	0.139	9.5
LL	2	0.007	8.1	LL	14	0.15	9.9
CHL	4	0.009	8.5	CHL	16	0.087	8.5
HL	4	0.009	8.1	HL	16	0.087	8.4
CLL	4	0.009	8.1	CLL	16	0.17	9.8
LL	4	0.012	8.2	LL	16	0.19	10.1
CHL	6	0.012	8.4	CHL	18	0.089	8.5
HL	6	0.012	8.3	HL	18	0.088	8.3
CLL	6	0.02	8.3	CLL	18	0.2	9.9
LL	6	0.02	8.4	LL	18	0.23	10.3
CHL	8	0.021	8.2	CHL	20	0.097	8.3
HL	8	0.023	8.4	HL	20	0.086	8.2
CLL	8	0.03	8.3	CLL	20	0.211	10.3
LL	8	0.038	8.4	LL	20	0.251	10.5
CHL	10	0.046	8.3	CHL	22	0.097	8.4
HL	10	0.061	8.4	HL	22	0.071	8.2
CLL	10	0.07	8.6	CLL	22	0.225	10.2

Appendix 2. One-way Annova to test the statistical difference in the optical density in

high and low light intensity experiment one.

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.013728	1	0.013728	3.065889	0.09	4.30095
Within Groups	0.09851	22	0.004478			
Total	0.112238	23				

Appendix 3. Optical Density, pH, phosphate, Cylindrospermopsin, heterocysts and filament length measurements result at high light concentration.

Experiment #2 high light	Day	OD_{740nm}	pH	PO_{-4} P(µg/l)	Cylindrospermopsin (µg/l)	Heterocyst $\times 10^4$/ml	Length of filaments (mm)
H	0	0.006	7.5	4400	0.02	0	0.13
M		0.006	7.8	86		0	00.15
L		0.005	8.2	40		0	0.13
W		0.006	8	21		0	0.13
H	2	0.011	7.9	4300		0	0.15
M		0.013	8.9	84		0	0.14
L		0.019	8.3	35		0	0.16
W		0.008	8.4	15		0	0.15
H	4	0.016	8	4000		0	0.25
M		0.012	8.2	80		0	0.25
L		0.011	8.4	33		0	0.2
W		0.008	8	7		0	0.2
H	6	0.045	7.8	4100	0.03	0	0.3
M		0.018	9	74		0	0.29
L		0.017	9.1	25		0	0.24
W		0.011	8.8	3		0	0.25

H	8	0.118	8.6	3750		0	0.35
M		0.06	8.2	71		0	0.32
L		0.045	7.9	21		0	0.28
W		0.019	9.4	0		0	0.3
H	10	0.151	8.7	3700		0	0.4
M		0.05	8.6	65		0	0.38
L		0.06	8.9	22		0	0.32
W		0.03	8.1	0	0.03	0	0.32
H	12	0.219	8.6	3519		0	0.49
M		0.066	8.4	55	0.02	0	0.4
L		0.102	8.7	17		0	0.34
W		0.045	8.9	0		0	0.35
H	14	0.236	9.2	3450		0	0.54
M		0.066	8.1	43		0	0.45
L		0.108	8.2	20		0	0.35
H	16	0.301	10.2	3300		0	0.6
M		0.078	8.2	42		0	0.47
L		0.109	8.3	16		0	0.36
W		0.057	8.1	0		0	0.35
H	18	0.351	9.8	3100		0	0.68
M		0.067	8.2	40		0	0.5
L		0.119	8.5	10		0	0.3
W		0.48	8.1	0		0	0.37
H	20	0.386	8.8	2500		0	0.7
M		0.07	8.2	38		0	0.58

L		0.127	8.5	6	0.03	0	0.34
W		0.45	8.1	0		0	0.38
H	22	0.395	9.2	2000		0	0.72
M		0.04	8.2	32		0	0.58
L		0.126	8.2	0		0	0.34
W		0.38	8.2	0		0	0.34
H	24	0.398	9.5	1300		0	0.68
M		0.051	8.2	32		0	0.55
L		0.127	8.2	0		0	0.31
W		0.04	8.1	0		0	0.33
H	26	0.410	10	1400		0	0.65
M		0.490	8.2	30		0	0.5
L		0.129	8.4	0		0	0.28
W		0.37	8.3	4		0	0.3
H	28	0.409	8.3	1600		0	0.58
M		0.051	8.3	42		0	0.48
L		0.131	8	3		0	0.25
W		0.36	9.8	2		0	0.25
H	30	0.401	9.4	1650	0.03	0	0.60
M		0.42	8.3	47	0.02	0	0.50
L		0.143	8.9	2	0.02	0	0.25
W		0.34	8.3	3	0.03	0	0.26

Appendix 3. Optical Density, pH, phosphate, Cylindrospermopsin, heterocysts and filament length measurements results at low light concentration.

Experiment #2 low light	Day	OD_{740nm}	pH	PO_4-P(µg/l)	Cylindrospe-rmopsin (µg/l)	Heterocyst $\times 10^4$/ml	Length of filament
H-B	0	0.006	8	4300		0	0.15
M-B		0.005	7.8	85		0	0.12
L-B		0.005	8.2	42		0	0.14
W-B		0.005	7.9	20		0	0.14
	2						
H-B		0.011	8.8	4250		0	0.2
M-B		0.011	8.1	80		0	0.19
L-B		0.011	7.9	38		0	0.15
W-B		0.009	8.8	16		0	0.0.18
	4						
H-B		0.032	8.8	4100		0	0.35
M-B		0.025	8.4	78		0	0.25
L-B		0.028	8.5	32		0	0.18
W-B		0.024	8.4	5		0	0.2
	6						
H-B		0.045	8.9	3860		0	0.35
M-B		0.042	7.9	72		0	0.25
L-B		0.044	8	21		0	0.18
W-B		0.036	8.2	2		0	0.2
	8						
H-B		0.089	9.1	3700		0	0.39
M-B		0.076	9.2	69		0	0.3
L-B		0.058	8.1	19		0	0.2
W-B		0.05	7.5	0		0	0.25

	10						
H-B		0.121	8.8	3650		0	0.4
M-B		0.099	9	52		0	0.35
L-B		0.071	9.1	18		0	0.3
W-B		0.045	8.9	0	0.03	0	0.28
	12						
H-B		0.132	8.7	3400	0.03	0	0.5
M-B		0.199	9.5	46		0	0.48
L-B		0.138	7.5	14		0	0.38
W-B		0.019	8.9	0		0	0.32
	14						
H-B		0.215	9.6	3400		0	0.55
M-B		0.222	9.3	35		0	0.5
L-B		0.181	9.4	13		0	0.45
W-B		0.104	8.7	0		0	0.35
	16						
H-B		0.238	9.9	3250		0	0.6
M-B		0.284	9.6	32	0.02	0	0.55
L-B		0.25	9.6	10		0	0.49
W-B		0.0128	8.5	0		0	0.35
	18						
H-B		0.309	9.7	3050		0	0.7
M-B		0.318	9.4	24		0	0.6
L-B		0.296	9.8	5		0	0.5
W-B		0.136	8.4	0		0	0.36
	20						
H-B		0.391	9.5	3250		0	0.75
M-B		0.365	9.3	27		0	0.68
L-B		0.349	9.8	0		0	0.55
W-B		0.151	8.4	0		0	0.38
	22						

H-B		0.408	9.6	2700		0	0.8
M-B		0.403	9.5	24		0	0.7
L-B		0.408	9.9	0		0	0.61
W-B		0.152	8.3	0		0	0.35
	24						
H-B		0.481	10.1	2000		0	0.85
M-B		0.451	9.9	25		0	0.76
L-B		0.473	10	0	0.03	0	0.65
W-B		0.152	7.9	0		0	0.34
	26						
H-B		0.525	9.9	1300		0	0.89
M-B		0.462	9.7	28		0	0.8
L-B		0.475	10.2	0		0	0.64
W-B		0.123	8.2	0		0	0.35
	28						
H-B		0.528	8.3	1200		0	0.9
M-B		0.532	10.1	22		0	0.81
L-B		0.493	9.6	0		0	0.65
W-B		0.108	10.4	5		0	0.34
	30						
H-B		0.521	10.1	1250	0.03	0	0.7
M-B		0.54	9.8	21	0.01	0	0.8
L-B		0.51	10	3	0.02	0	0.6
W-B		0.97	8.3	4	0.01	0	0.3

Appendix 4. Optical Density, pH, Nitrate, Cylindrospermopsin, heterocysts and filament length measurements result at high light concentration.

Experiment #3 high light	Day	OD_{740nm}	pH	NO3-N(µg/l)	Cylindrospe-rmopsin (µg/l)	Heterocyst $\times 10^4$/ml	Length of filament (mm)
H	0	0.005	8	75900	0.03	0	0.15
M		0004	8	54851		0	0.16
L		0.005	8	30950		0	0.18
W		0.005	8.3	20		0	0.15
H	2	0.006	8.2	75750		0	0.25
M		0.005	8.2	53350		0	0.21
L		0.006	8.1	30500		1	0.18
W		0.005	8.2	17		0	0.2
H	4	0.007	9.1	78400		0	0.3
M		0.006	8.1	54350		0	0.28
L		0.006	8.2	30150		0	0.3
W		0.005	7.5	12		1.2	0.24
H	6	0.007	8.1	76700		0	0.35
M		0.006	8.3	54550		0	0.3
L		0.006	8.1	30140		1.2	0.32
W		0.006	8.2	9		1.6	0.3
H	8	0.007	8.1	73000		0	0.4
M		0.011	8.0	53550		0	0.32
L		0.007	8.0	32150		1.4	0.34
W		0.006	8.1	0		1.8	0.32

H	10	0.013	8.3	67000		0	0.42
M		0.027	8.6	51100		0	0.38
L		0.02	8.2	32600		1.6	0.37
W		0.013	8	0		2.3	0.36
H	12	0.017	8.3	70050		0	0.46
M		0.043	8.4	52500		0	0.42
L		0.029	8.0	31607		1.8	0.4
W		0.018	8.2	0		2.3	0.38
H	14	0.03	8.2	76350	0.02	0	0.55
M		0.08	8.5	50000		0	0.45
L		0.016	9.0	28000		1.9	0.42
W		0.027	8.3	0	0.03	2.5	0.4
H	16	0.049	8.6	75250		0	0.59
M		0.083	8.9	49000		0	0.5
L		0.097	8.8	29150		2	0.48
W		0.031	8.0	0	0.02	2.7	0.42
H	18	0.103	9	68300		0	0.64
M		0.097	8.2	48500		0	0.55
L		0.139	8.9	26250		2.3	0.5
W		0.024	8.3	0		2.9	0.4
H	20	0.142	9.6	53650		0	0.66
M		0.11	8.3	47250		0	0.56
L		0.15	9.2	23350		2.5	0.55
W		0.024	8.2	0		3	0.38
H	22	0.15	9.7	57350		0	0.7

M		0.12	7.4	46000		0	0.6
L		0.18	8.4	23350		2.7	0.58
W		0.02	7	0		3.4	0.35
H	24	0.218	9.5	63200		0	0.72
M		0.13	8.2	48450		0	0.64
L		0.193	8.3	24800		2.8	0.6
W		0.017	8.6	0		3.6	0.36
H	26	0.281		54650		0	0.78
M		0.15	10.2	42400		0	0.68
L		0.226	8	24050		3	0.63
W		0.013	8	0		3.9	0.34
H	28	0.303	9.8	54700		0	0.8
M		0.16	9	3800		0	0.74
L		0.251	8.5	20200		3.5	0.66
W		0.011	8	0		3.8	0.36
H	30	0.341	10.5	47000		0	0.86
M		0.2	9.2	38950		0	0.8
L		0.292	8	18750		3.6	0.7
W		0.01	8.2	0		3.7	0.36
H	32	0.381	9.8	45000		0	0.92
M		0.238	9	40450		0	0.89
L		0.389	8.4	16350		3.7	0.78
W		0.009	8.3	0		3.8	0.34
H	34	0.418	10.2	45350		0	0.94
M		0.318	8	36500		0	0.9

L		0.433	9	13350		3.9	0.8
W		0.009	8	0		3.7	0.32
H	36	0.456	9.7	40000	0.04	0	0.89
M		0.383	10	30000		0	0.86
L		0.491	8	12600		4.2	0.78
W		0.009	9	0		3.9	0.3
H	38	0.501	9.7	38000		0	0.86
M		0.436	9.9	27850		0	0.84
L		0.51	8.6	9600		4.3	0.74
W		0.008	7.8	0		3.8	0.3
H	40	0.50	10.2	36550		0	0.84
M		0.48	10	26000		0	0.83
L		0.525	8.4	7850		4.4	0.74
W		0.005	8.1	0		3.6	0.3
H	42	0.55	10	25850	0.04	0	0.83
M		0.5	9	25100	0.03	0	0.84
L		0.553	9	4300	0.04	4	0.72
W		0.004	8.2	0		3.7	0.3

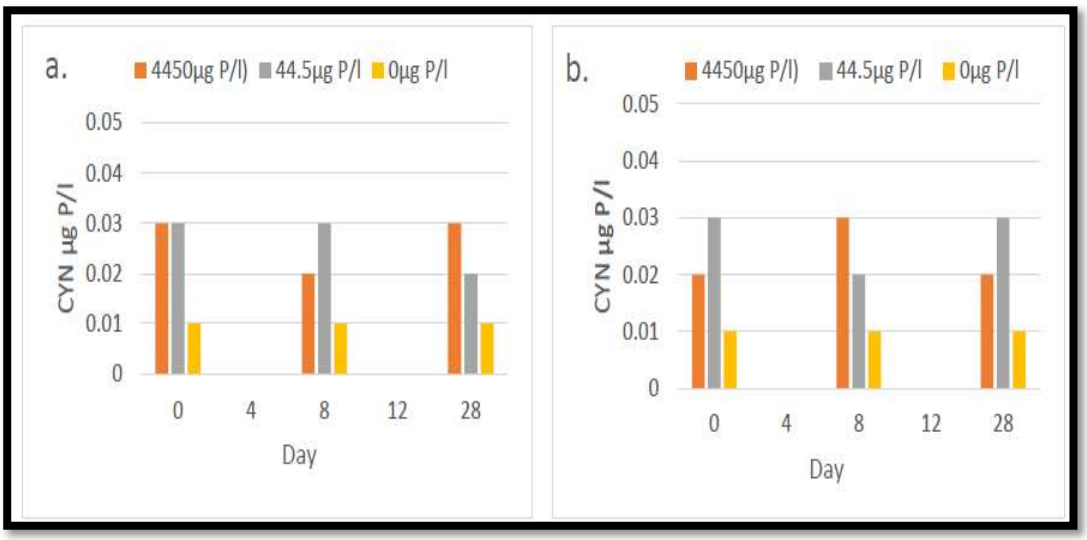
Appendix 5. Optical Density, pH, nitrate, Cylindrospermopsin, heterocysts and filament length measurements results at low light concentration.

Experiment #3 low light	Day	OD_{740nm}	pH	NO3-N(µg/l)	Cylindrospe-rmopsin (µg/l)	Heterocyst /ml	Length of filament
H-B	0	0.004	8	78050	0.02	0	0.12
M-B		0.004	8	52550		0	0.16
L-B		0.006	8	30100		0	0.14
W-B		0.005	8.4	19		0	0.15
	2						
H-B		0.007	8.1	76000		0	0.18
M-B		0.007	8.2	52550		0	0.19
L-B		0.006	8.1	30250		0	0.25
W-B		0.007	8.2	18		0	0.2
	4						
H-B		0.008	8.7	77000		0	0.32
M-B		0.011	8.2	53600		0	0.3
L-B		0.007	8.2	30105		0	0.25
W-B		0.008	8.2	11		0	0.25
	6						
H-B		0.009	8.1	75000		0	0.35
M-B		0.011	8.1	53000		0	0.34
L-B		0.007	8.2	30200		0	0.26
W-B		0.01	8.1	8		1.5	0.28
	8						
H-B		0.015	8.2	77700		0	0.38
M-B		0.024	8.3	55000		0	0.36
L-B		0.011	8.1	32400		1.2	0.3
W-B		0.015	8.2	0		1.7	0.32
	10						

H-B		0.03	8.1	76250	0.03	0	0.4
M-B		0.047	8.5	55100		0	0.38
L-B		0.016	8.1	30200		1.5	0.32
W-B		0.031	8.2	0		2	0.38
	12						
H-B		0.045	8.4	72050		0	0.42
M-B		0.07	8.2	53250		0	0.4
L-B		0.025	8.4	30650		1.7	0.36
W-B		0.034	8	0		2.2	0.4
	14						
H-B		0.75	8.9	72450		0	0.46
M-B		0.113	8.5	51100		0	0.42
L-B		0.037	8.3	29650		1.9	0.4
W-B		0.035	8.5	0		2.4	0.44
	16						
H-B		0.119	9.2	72500		0	0.48
M-B		0.163	9.6	51550		0	0.46
L-B		0.056	8.5	29000		2	0.4
W-B		0.04	8.1	0		2.7	0.45
	18						
H-B		0.175	10	73000		0	0.5
M-B		0.219	10	46650		0	0.48
L-B		0.097	8.3	28650		2.3	0.42
W-B		0.031	8.2	0		2.9	0.4
	20						
H-B		0.233	9.6	72050		0	0.52
M-B		0.263	10.3	43400		0	0.5
L-B		0.138	9.1	25250		2.5	0.46
W-B		0.026	8.3	0		3.1	0.38
	22						
H-B		0.27	9.5	61000		0	0.56

M-B		0.3	10.1	37400		0	0.52
L-B		0.15	8.9	26400		2.7	0.49
W-B		0.025	8.2	0		3.5	0.35
	24						
H-B		0.35	9	61050		0	0.58
M-B		0.338	8.2	39650		0	0.54
L-B		0.214	8.5	22400		2.9	0.52
W-B		0.025	8	0		3.6	0.3
	26						
H-B		0.402	10	53350		0	0.66
M-B		0.372	9	34300		0	0.6
L-B		0.262	8.5	22050		3	0.56
W-B		0.024	8	0		3.9	0.28
	28						
H-B		0.431	9.4	54850		0	0.66
M-B		0.386	9.2	29850		0	0.6
L-B		0.284	8.3	19400		3.2	0.6
W-B		0.024	8.4	0		4	0.3
	30						
H-B		0.480	10	49400		0	0.68
M-B		0.447	9.5	36800		0	0.62
L-B		0.31	8	19050		3.4	0.58
W-B		0.027	8.2	0		4	0.28
	32						
H-B		0.55	9.9	52850		0	0.7
M-B		0.5	8.8	27950		0	0.6
L-B		0.374	8	17050		3.6	0.6
W-B		0.025	8.4	0		4	0.3
	34						
H-B		0.601	9.8	53850		0	0.72
M-B		0.547	8.6	29050		0	0.58

L-B		0.404	9.2	16400		3.9	0.55
W-B		0.022	8.2	0		4	0.31
	36						
H-B		0.623	9.8	50750		0	0.68
M-B		0.572	9.8	12350		0	0.54
L-B		0.432	8.6	14850		4	0.52
W-B		0.018	8.5	0		4	0.3
	38						
H-B		0.662	9.8	45200		0	0.65
M-B		0.648	9.7	18900		0	0.5
L-B		0.455	8.6	11900		4.1	0.5
W-B		0.015	8	0		3.8	0.3
	40						
H-B		0.691	9.5	40350	0.03	0	0.64
sM-B		0.77	9.9	20750		0	0.49
L-B		0.481	8.7	12000		4	0.5
W-B		0.017	8.3	0		3.7	0.3
	42						
H-B		0.7	10.5	38200	0.04	0	0.6
M-B		0.785	9.5	19150	0.03	0	0.45
L-B		0.484	8.6	11000	0.03	3.8	0.48
W-B		0.016	8.4	0		3.6	0.3



Appendix 6. Cylindrospermopsin (CYN µg/l) in different phosphate concentration under two light intensity (a. High Light) and (b. Low Light).

Appendix 7- Standard O2 medium (Van Liere and Mur 1978) O2 medium.

K_2HPO_4	500mg/l
$MgSO_4\ 7H_2O$	25mg/l
$CaCl_2.2H_2O$	50mg/l
Fe- Losning	13mg/l
$NaHCO_3$-losning	10ml/L after autoclaving
Mikroelemetlosning	5ml/L after autoclaving
K_2HPO_4	1ml before autoclaving

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
low P	16	1.324	0.08275	0.00281
High P	16	3.842	0.240125	0.026341

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.198135	1	0.198135	13.59347	0.000896	4.170877
Within Groups	0.437273	30	0.014576			
Total	0.635408	31				

Appendix 8. Statistical difference ANNOVA single factor test for high and allow P

Appendix 9- Statistical significance (*P* values) by one-way ANNOVA under with different

treatment.

Significance differences between OD	High light *P*- Value	Low light *P*- Value
High and low phosphate	0.000896	0.05
High and low nitrogen	0.08	0.01
Trichome length	High light *P*- Value	High light *P*- Value
High and low nitrogen	0.01	0.02
High and low phosphorus	0.07	0.03