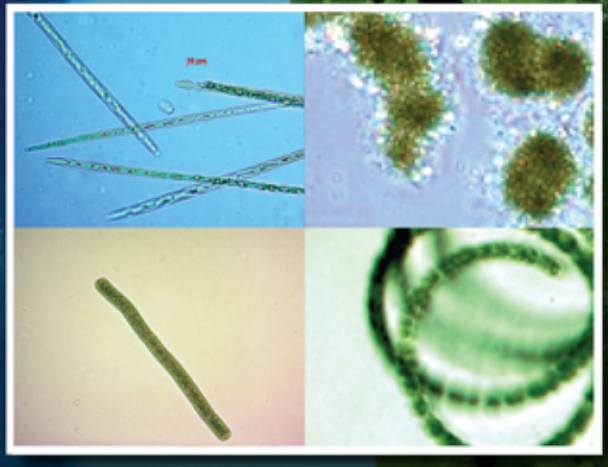
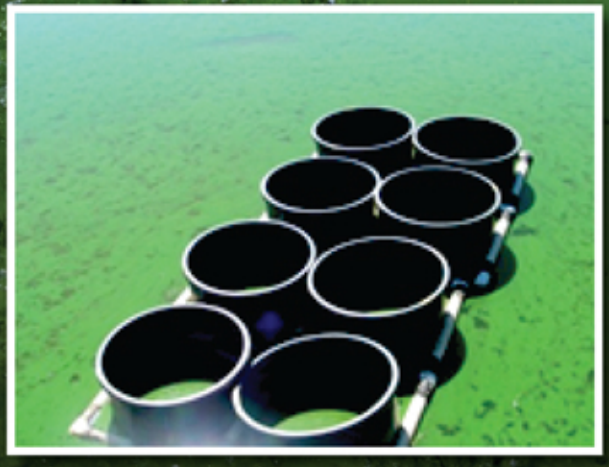
*Bacteriology Research Developments*

# Cyanobacteria

## Ecology, Toxicology and Management





Aloysio da S. Ferrão-Filho
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# CYANOBACTERIA

# ECOLOGY, TOXICOLOGY AND MANAGEMENT

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# CYANOBACTERIA

# ECOLOGY, TOXICOLOGY AND MANAGEMENT

ALOYSIO DA S. FERRÃO-FILHO
EDITOR

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# CONTENTS

# PREFACE

It has been a consensus in the academic circles that global climate changes can alter irreversibly the face of Earth. Along with these changes, aquatic ecosystems may have its dynamic and functioning tremendously affected, with consequences not only for the aquatic communities, but also for the future human generations.

In this context, it is been reported that phytoplankton communities are also changing globally, with cyanobacteria becoming the dominant group of organisms in many latitudes [1-3]. Furthermore, species invasions are another important issue that may be influenced by global warming since tropical species are likely to migrate to temperate regions as temperature increases. An example of invasive cyanobacteria is the species *Cylindrospermopsis raciborskii*, a member of the Order Nostocales, originally described as a tropical/subtropical genus, which has been spreading fast throughout northern Europe and North America [3-5] and South America [6-10]. Its success rely on its ability to adapt to different mixing of water column and light regimes, high phosphorus storage and nitrogen fixing capacity, buoyancy regulation and shade tolerance [1,3,5].

The increase in frequency and duration of cyanobacterial blooms worldwide as a potential consequence of global warming is a phenomenon of great concern as several species are toxin producers. Cyanobacterial toxins, or cyanotoxins as they are commonly known, have been involved in several cases of wild and domestic animal poisoning [11] and also in human intoxication reports, including fatalities [12,13]. These cases can increase if increasing temperatures led to the development of more cyanobacterial toxic blooms, especially in undeveloped countries and semi-arid regions of the world, where treatment plants are not able to eliminate cyanotoxins and drought is a complicating factor.

In more than a century of research about this topic (see the reviews of Codd et al. [14] and Stewart et al. [11]), there are still gaps in the understanding of the role of cyanotoxins (see also reviews of Ibelings and Havens [15] and Ferrão-Filho and Koslowsky-Suzuky [16]). This book was conceived with the intent to bring some insights into this issue and also to discuss some aspects related to the "Ecology, Toxicology and Management" of cyanobacteria. The first two chapters are reviews devoted to the debate of the possible roles of cyanotoxins in species interactions, namely with their potential competitors (i.e. algae, bacteria and other cyanobacteria).

The third chapter is an extensive review on the role of *Nodularia spumigena* in the Baltic Sea food webs, its importance on the nitrogen cycle, the transfer of nodularin in the food web and its role as an anti-grazer defense. In this chapter the authors discuss also the effects of

eutrophication and climate-driven changes in food webs and their effects on toxin production by *Nodularia spumigena*.

The fourth chapter is a review on the studies of ecology of *Cylindrospermopsis raciborskii*, a species with wide occurrence in Brazil [6-10]. In this chapter, using a large database (n=439 samples taken from 51 systems), the authors discuss the possible factors that favor *C. raciborskii* in Brazilian aquatic ecosystems. With those data, the authors show that *C. raciborskii* is able to colonize lakes and reservoirs comprehending a latitudinal gradient from Northern to Southern Brazil, with varying depths, light and mixing regimes and trophic status, emphasizing its high phenotypic plasticity and tolerance to a wide range of environmental conditions.

Chapter 5 (not placed by chance after chapter 4) is a case study of three reservoirs located in the Brazilian semi-arid, a region with great problems of water supply for local populations and with recurrent and long lasting blooms of cyanobacteria, especially of species *C. raciborskii*, *Microcystis aeroginosa* and *Planktothrix agardhii*. The authors show that there is an interchange in the dominance among these three species and that stratification of the water column and low concentrations of nitrogen associated with decrease in water transparency are the main controlling factors of cyanobacterial community in semi-arid ecosystems.

Chapter 6 is also a case study of a the abundance and community structure of pico-phytoplankton, heterotrophic bacteria and virus-like particles (VLP) along a natural, continuous salinity gradient in a South Australian temperate coastal lagoon. The authors used advanced techniques of flow cytometry (FCM) under epifluorescent microscopy (EM) to study these communities, which have been neglected in many studies. Their findings show a significant decrease in the number and species richness of cytometrically-defined sub-populations of heterotrophic bacteria and VLP with increasing salinity. These changes could reflect both a modification of phylogenetic composition and activity level within the bacterial community. In addition, the number and richness of cytometrically-defined picophytoplankton populations also decreased with increasing salinity, affecting both prokaryotic and eukaryotic organisms. Moreover, their results highlight the ability of cyanobacteria to tolerate and acclimate to a wide range of salinity and therefore to colonize extreme environments.

Chapter 7 is a review about the accumulation and phytotoxicity of microcystins (MCs) in vascular plants. The authors approach the uptake and accumulation of MCs in both aquatic and terrestrial plants, giving emphasis to the uptake and biotransformation of MCs in agricultural plants, which are exposed to cyanobacterial toxins through irrigation practices. This is a theme of great importance for risk assessment and public heath, since vegetables can be an exposure route for humans to MCs through their diets.

In chapter 8, the author reviews several aspects related to the health hazard and risk assessment of cyanotoxins, especially related to humans. This chapter also critically approaches some drawbacks in the regulation measures and guidance values for cyanotoxins, pointing out to the need for correct evaluation of risks based on the diversity and quantity of cyanotoxins rather than to rely only on cell numbers, the need to consider carcinogenicity in order to express valuable safety levels, the need for better estimation of covalently-bound MC and the need to take into account the synergistic ability of some of the main toxins to enhance their damage when present together. Moreover, this chapter highlights important management measures to prevention and treatment recommendations after human exposure to cyanotoxins.

The last three chapters are devoted to aspects of management, such as bioremediation, monitoring technologies and ecological control of cyanobacteria. In Chapter 9, the authors show the application of confocal laser scanning and electron microscopic techniques as powerful tools for determining the *in vivo* effect and sequestration capacity of metals in cyanobacteria. Using both natural populations and filamentous cyanobacteria strains isolated from microbial mats in the Ebro Delta, a polluted river in Spain, the authors showed that these microorganisms are able to sequester and accumulate high amounts of lead both externally in extrapolymeric substances and internally, in polyphosphate inclusions. This is particularly interesting from the point of view bioremediation of polluted watersheds and shows indeed that cyanobacteria are not all "bad guys".

In chapter 10, the authors show data from the Pampulha Reservoir, a eutrophic reservoir in Southwest Brazil, with recurrent blooms of cyanobacteria. They used a new technology to monitor chlorophyll concentrations that makes it possible to gather a very large and fine-scale amount of spatial data concerning the subsurface concentrations of this pigment. The proposed method consists of an integrated use of a highly sensitive fluorescent probe, coupled to a high-precision D-GPS that provides geographical coordinates with sub-metric precision. The structure can be easily mounted on every kind of small boat. A new software program was also created, to precisely synchronize the data files delivered by two different devices. The final result is the production of a detailed thematic chart that shows the spatial pattern of chlorophyll in great detail. This makes it possible for local managers to initiate measures to mitigate or even prevent the wider spread of an algae bloom soon after the first signs of this undesired phenomenon are detected. The methodology was validated using traditional measure of chlorophyll-a (Lorenzen method), giving a good correlation.

Finally, chapter 11 is a commentary about "Ecological control of toxic cyanobacterial blooms in aquatic ecosystems". In this short review the authors explore some aspects related to studies using biomanipulation: the manipulation of higher trophic levels (adding piscivores or removing planktivores) to increase the size, abundance, and grazing pressure of herbivorous zooplankton to reduce algal abundance. Based on field experimental evidence, the authors argue that cyanobacteria may serve as a beneficial food resource for zooplankton, that ecological control of cyanobacterial blooms is practical for some systems, and that greater attention should be placed on direct biomanipulation of zooplankton communities (e.g., stocking *Daphnia*) in conjunction with the manipulation of higher trophic levels. They also highlight the need for more data documenting zooplankton-cyanobacteria interactions in tropical freshwater ecosystems, whose biological, chemical, physical, and geological characteristics vary remarkably from their temperate counterparts, which could limit the use of biomanipulation.

It is worth of note that research on cyanobacteria in Brazil has increased in quantity and quality, resulting of a lot of investments from scientific financial agencies in the last decade. Since the incident in Caruaru (Pernambuco State, Brazil), in which several renal patients died after a hemodialysis section and the incontestable evidence of the involvement of hepatotoxins in this event [13], health and environment authorities have paid more attention to the problem of toxic cyanobacterial blooms in water supplies and this has being a driving force in the development of a scientific background for the basis of regulation policies and managements efforts to create safety guidelines for the use of supply water. These efforts have been responsible for the development of several excellence research groups and the publication of a reasonable, high quality literature. Two International Congresses on Toxic

Cyanobacteria have occurred in Brazil, one in 2001 and the other in 2007, both coordinated by Dr. Sandra M.F.O. Azevedo from the Federal University of Rio de Janeiro. She was directly involved, together with her colleague Dr. Wayne Carmichael, in the discovery that MCs was the main causing agent of the "Caruaru Syndrome". She has been responsible for formation of some generations of high qualified Brazilian scientists, to which I am proud to be part of (her second Ph.D. student, from 1994-1998).

I could not forget to acknowledge all the reviewers that contributed to improve the quality of the chapters, giving their precious time. I would like to thank Dr. Marcelo Pelajo (Instituto Oswaldo Cruz, Fiocruz, Brazil), Dr. Maria Carolina S. Soares (Department of Sanitary and Environmental Engineering, Federal University of Juiz de Fora, Brazil), Alice Sato, Silvia Nascimento, Christina W. Castelo Branco and Betina Koslowsky-Suzuky (Department of Natural Sciences, Federal University of the State of Rio de Janeiro, Brazil), Ana Beatriz F. Pacheco and Raquel M. Soares (Institute of Biophysics Carlos Chagas Filho, Federal University of Rio de Janeiro, Brazil), Lúcia Helena S. Silva (Department of Botany, National Museum, Federal University of Rio de Janeiro, Brazil), Renata Panosso (Department of Microbiology and Parasitology, Federal University of Rio Grande do Norte, Brazil), and especially to Dr. Sandra Azevedo for hers valuable comments on the preface.

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*Chapter 1*

# POSSIBLE ROLES OF CYANOTOXINS IN SPECIES INTERACTIONS OF PHYTOPLANKTON ASSEMBLAGES

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## ABSTRACT

Cyanobacteria ("blue-green algae") are among the most well studied organisms; above all because of their ability to produce an extremely diverse array of biologically active metabolites, cyanotoxins among them. It is known that the biosynthesis of cyanotoxins requires a significant amount of energy and nutrient resources of the cyanobacterial cells. With this knowledge, the question that arises is '*what could be the functional role of these "expensive" metabolites in the ecology and distribution of cyanobacteria*'.

One function of the metabolites in question could be that they serve as allelochemicals. Allelopathy between phytoplankton organisms in aquatic habitats is not as well studied as between e. g. vascular plant in terrestrial systems, although it is considered as an important regulating factor of phytoplankton dynamics and community composition. The discussion of cyanotoxins as allelochemicals is a controversial issue. Some authors say that toxins have effect on several organisms, including some that may not be present in the producer immediate environment (toxicity), while allelopathic compounds play role in the interactions between the emitter organisms and their direct competitors or predators. From this point of view cyanotoxins are not specifically allelochemicals. However, a number of observations indicated allelopathic nature of cyanotoxins, so in some cases it is difficult to isolate clearly these two phenomena: toxicity and allelopathy. The issue is further complicated by the following facts: (i) A natural cyanobacterial population is a mixture of toxin producer and non-producer ecotypes in most cases; (ii) the release of cyanotoxins varies in a very wide range, usually they are released in the surrounding water only in small amounts by living cells. Most

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recently new hypotheses arise according to which cyanobacterial secondary metabolites, especially cyanotoxins can serve as signal molecules among the cells within one or different genera. Thus, despite the increscent number of studies the question about the role of cyanotoxins still remained mostly unanswered. This chapter will discuss the existing evidence for the roles of cyanobacterial secondary metabolites, mainly toxins, in the interactions of phytoplankton species; trying to highlight the functions of these compounds in dynamics of phytoplankton assemblages.

## INTRODUCTION

Allelopathy is a manifold ecological/physiological phenomenon also among algal species. Compounds produced by algae may affect other algae in the producer's vicinity; the producer itself (autotoxicity); heterotrophic bacteria living in the producer's vicinity; vascular plants; invertebrates grazing on algae or even vertebrates - although the latter cases lead us to the field of toxicology [1].

Some researchers believe that chemical interactions, particularly allelopathy, play a very important role in competition among phytoplankton species [2]. Others put it more rigorously: it can be assumed that allelopathy among phytoplankton taxa plays an important role in shaping of the community structure and in the regulation of dynamics of water blooms in lakes [3-5]. In streams and rivers the importance of allelopathy is certainly lower, because of strong dilution effects; but it could be still momentous in benthic communities [6]. According to Legrand et al. [2], allelopathy is defined as a phenomenon in that the secondary metabolites produced by one of the species inhibit the growth and other physiological processes of another species. Leflaive and Ten-Hage [6] state that allelopathy is the inhibitory effects of secondary metabolites on the producer's competitors and predators. Toxins, which are also secondary metabolites, are toxic as well to organisms which are not directly related to the producer. The two concepts are not mutually exclusive – allelochemicals also can be found among toxins. The differences between these two not firmly distinguishable groups can be generally summarized as follows: production of allelochemicals is not related to taxa, while production of toxins in freshwater mainly appears among cyanobacteria. The mechanism of action of allelochemicals is widely varied, while toxins are primarily enzyme inhibitors, or acts by binding to membrane receptors. The ecological role of allelochemicals is clear in most cases, while for toxins this question remains rather unclear [6].

There are many possible approaches to study allelopathy, so far there was no standard method. According to one general method, the cell free extract of the donor algal culture is added to another algal culture. Using of fractionated exudates or purified allelochemicals gives the opportunity to gain more exact results about the effects of the compounds [2]. Most allelochemicals have been described from cyanobacteria, but some toxins of marine dinoflagellates and many cyanotoxins also have allelopathic activity. The mechanism of action of allelochemicals is extremely variable. Most of them cause the inhibition of growth (cell division) or cause cell lysis [2]. In the following sections we summarized some general examples for allelopathic activity of phytoplankton species from the results of allelopathy research of the last one and a half decade.

Schlegel et al. [7] studied bioactivity of 198 cyanobacterial strains on *Scenedesmus acutus*, *Coelastrum microporum* and *Monoraphidium convolutum* green algae. Among the

198 cyanobacteria 20 had generalized anti-algae effects; from these 10 belonged to the genus *Fischerella* and 7 to *Nostoc*, most of which are sessil species. Non-toxic (*Anabaena doliolum* auto- and heterotrophic strains, *Synechocystis sp*. and *Nostoc sp*. auto- and heterotrophic strains) and toxic (*Microcytis aeruginosa*, *Anabaena circinalis* and *Nodularia spumigena*) cyanobacterial cultures were also studied. It was found that five of the extracts were toxic to *A. doliolum* auto- and heterotrophic forms, while two extracts caused inhibition only of *A. doliolum* autotrophic strain (i.e. inhibited photosynthesis ) [7].

Not only growth inhibitory, but excitatory effects can also be observed between eukaryotic microalgae and cyanobacteria (although, as outlined above, some authors do not discuss facilitation within the field of allelopathy). Mohamed [8] observed in Egyptian irrigation channels that the presence of the green alga *Spirogyra* favors the development of *Oscillatoria (Planktothrix) agardhii* bloom. Laboratory experiments confirmed their observations: when *O. agardhii* cultures were treated with the extract of *Spirogyra*, growth of the treated cultures increased compared to the untreated cultures [8]. The role of microcystins produced by *O. agardhii* is not clear: it is possible that the toxin causing the death of *Spirogyra* helps increase of cyanobacterial growth by the release of stimulating compound from dead cells. Nevertheless, this assumption is not proven.

Volk [9] studied the effects of 35 algal extracts on the cyanobacterium *Arthrospira laxissima*. Among the tested extracts 12 were highly toxic, 1 was extremely toxic, and interestingly the extract of *Arthrospira laxissima* was toxic to the producer organism itself (autotoxicity) [9].

Gantar et al. [10] tested 8 cyanobacterial and 6 green algae strains. In a total of 182 experiments conducted, 37 cases showed a negative or positive effect. The benthic cyanobacterium *Fischerella sp*. always had a negative impact during co-culturing, but its extracts did not have an inhibitory effect on two cyanobacterial strains. The authors assume that synergistic interactions with certain substances (which are lost during extraction) are involved. The photosynthesis of the green alga *Chlamydomonas sp*. was inhibited from 1 μg $mL^{-1}$ *Fischerella* extract, with 50% inhibition caused by 5 μg $mL^{-1}$ extract. The discoloration and vacuolisation of *Chlamydomonas* cells was observed, the changes in the first two days were significant compared to the control culture. Electron microscopic studies have shown that the thylakoid system is degenerated and that the cell structure disappeared. The ecological significance of bioactive compounds produced by *Fischerella* is that they likely inhibit colonization of other species. The study of one-month old *Fischerella* culture medium showed an amount of bioactive compounds (4 μg $mL^{-1}$), which significantly inhibited the photosynthesis of *Chlamydomonas*. The positive impact of *Chlamydomonas* on the cyanobacterium *Anabaena flos-aquae* strain was likely due to "nutritional supplementation" effect. Gantar et al. [10] showed that some species produce many biologically active substances, but they do not reach the environment until the cells are destroyed (there was no evidence of active extraction), so these cases can not be discussed as real allelopathy [10].

Lopes and Vasconcelos [11] tested the green alga *Chlorella sp*., the heterokont *Nannochloropsis* sp. and the cyanobacteria *Microcystis aeruginosa*, and *Synechocystis salina* with the crude extracts of 18 benthic cyanobacteria. The extracts were prepared with water and with methanol:water mixture; a total of 144 different experimental settings were used. The results are completely diverse, positive and negative effects were detected in both aqueous and methanolic extracts. Among the most interesting results is that the aqueous extract of a *Leptolyngbya* strain (belonging to the order Oscillatoriales) significantly

stimulated the growth of *Microcystis sp*. The negative effects were concentration-and extract-dependent and it changed from strain to strain, whether there was a detectable effect or not. In some cases, the inhibitory effect of extracts was stronger than that of the pure toxins. The authors believed that the stimulatory effects of the extracts were due to their nutrient content. Another explanation of the stimulation could be that the target organism is able to metabolize the allelochemicals. Also, the extracellular phytohormone production by cyanobacteria cannot be excluded, as well as antibacterial and antifungal activity of cyanobacteria which can also favor the growth of other phytoplankton species [11]. It was observed that extracts of closely related Chroococcales species inhibit growth of each other, suggesting that such biochemical interactions are group-specific. In the case of the extracts, it should be noted that synergistic effects also could be possible among the various components. There could be concentration dependent phenomenona and the reactions could be toxin dependent [11]. It can be stated in conclusion, that benthic cyanobacterial species are able to influence the composition of phytoplankton communities, the succession and distribution of phytoplankton taxa.

Factors controlling the production of allelochemicals are poorly known. The production of allelochemicals can be increased under light or nutrient limitation [12-14]. However, for example, the allelopathic relationship between the dinoflagellate *Peridinium gatunense* and the cyanobacterium *Microcystis sp*. seemed to be independent from the amount of available nutrients [15]. The role of temperature in the production of allelochemicals also proved to be significant in some cases: the regulation of antibiotic production of the cyanobacteria *Oscillatoria angustissima* and *Calothrix parietina* seemed to be of paramount importance [16]. It should be noted that pH can also affect the production of allelochemicals: algicid production of the cyanobacterium *Oscillatoria laetevirens* was negatively correlated with pH [13].

Environmental factors affect the amount of intracellular cyanobacterial toxins and toxin release as well. Generally, it can be said that relevant quantities of toxins are released into the environment only during lysis of producer cells (with a few exceptions [17-19]), which is the reason, why these compounds are discussed separately from allelochemicals [6]. The highest intracellular toxin concentration generally can be measured under favorable growth conditions [20]. Toxin production also could be controlled by the presence of competitive species: the anatoxin production of an *Anabaena flos-aquae* strain increased, while production of microcystins by the same species was completely inhibited during treatment with the extract of the green alga, *Chlamydomonas reinhardtii* [21]

The mentioned general examples show that allelopathic activity of phytoplankton species is an important phenomenon, although it is not clear, whether cyanotoxins could be also considered as allelochemicals in nature. The ecological meaning of testing a large set of different strains, without co-occurrence in the same environment is to find reasons (allelochemicals, toxins), why the tested species do not coexist (although, theoretically their coexistence would be possible). In other cases the goal of these kinds of works is not to find an ecological role, but to find compounds which could be employed for biotechnological applications such as algaecides, herbicides, insecticides, or for applications in medicine as antibiotics, immunosuppressant, anticancer, antiviral and anti-inflammatory agents [11]. In the following part of the chapter we summarized current knowledge about the effects of the main cyanotoxin families (peptides, alkaloides) on phytoplankton species, complemented with the data of some minor or newly described compounds.

## EFFECTS OF CYANOTOXINS AND OTHER ALLELOPATHIC COMPOUNDS TO PHYTOPLANKTON SPECIES

As indicated above, allelopathy plays an important role in phytoplankton assemblages. It is not clear, whether several compounds showing allelopathic activity in laboratory, could be also considered as allelochemical in nature; this is especially true to cyanotoxins. Here we summarized the effects of the best known cyanotoxins on phytoplankton species, trying to emphasize how the results of laboratory studies correspond with the naturally observed phenomena.

### Peptides

#### *Microcystins*

The most frequently detected cyanotoxins in water blooms belong to cyclic peptides, within them to microcystins (MCs). MCs were isolated from many unicellular and filamentous cyanobacterial species (without the claim of an exhaustive list, possible producers are: *Microcystis, Anabaena, Oscillatoria (Planktothrix), Nostoc, Anabaenopsis, Hapalosiphon* species [22]). The molecular weight of MCs range ~ 900-1100 Da, they are built up of seven amino acids, L-and D-amino acids among them, while unusual, rare amino acid derivatives can be also found [22]. In eukaryotic cells, MCs are protein serine/threonine phosphatase 1 and 2A inhibitors [23] and can cause oxidative stress by either increasing the amount of reactive oxygen species (ROS) in the cells or by decreasing the antioxidant capacity of cells (e.g., inhibiting enzymes involved in detoxification [24]). Because of this ability they can cause DNA degradation, or cell death respectively [25-28].

Keating has already shown in 1978 that MCs inhibit the growth of certain eukaryotic algae (*Chlamydomonas, Haematococcus, Navicula*, and *Cryptomonas*) [29]. Since then, the effects of different MC variants on several laboratory cyanobacterial and eukaryotic algae strains were studied.

#### Green Algae

A large number of results prove that MCs inhibit the growth and physiological processes of green algae. Escoubas et al. [30] described the inhibition of chlorophyll production in *Dunaliella tertiolecta* cultures treated with MC-LR. Christoffersen [31] observed that MC-LR treatment cause a slight inhibition of growth of *Nephroselmis olivacea*. MC-RR treatment can inhibit or stimulate *Chroococcus minutus* on a light intensity dependent manner. The growth inhibition of *Coelastrum microporum* was observed only at higher MC concentrations [32]. Pietsch et al. [33] described the increase of peroxidase and glutathione synthetase enzyme activity in purified MC-LR-treated cultures of *Scenedesmus armatus*. MC containing cell-free extracts inhibited the glutathione synthetase and photosynthesis of the green alga [33]. Kearns and Hunter observed growth inhibition and cell paralysis in MC-containing extract or purified MC-LR-treated *Chlamydomonas reinhardtii* cultures [21,34]. In the case of *Scenedesmus quadricauda*, it was observed that the larger the cell numbers in MC treated cultures were, the more pronounced the aggregation of four-celled coenobia became. The volume of toxin-treated cells on the 2-4 days was significantly higher than that of control cells. The

autofluorescence of toxin-treated cells increased compared to control cells, not only due to increasing cell volume but also due to the increasing volume of chloroplasts. The chlorophyll content of cells increased significantly during toxin treatments. The increase in autofluorescence and chlorophyll content suggests intensive metabolism; so this phenomenon can be considered as a kind of stress [35].

In contrast with the inhibition phenomena discussed above, the results of Babica et al. [36] showed that some Chlorophyta species is not highly sensitive to MCs. Five green algal species (*Chlamydomonas reinhardtii, Chlorella kesslerii, Pediastrum duplex, Pseudokirchneriella subcapitata, Scenedesmus quadricauda*) were treated with MC-LR and -RR. The growth of *C. reinhardtii, C. kesslerii,* and *P. duplex* was more strongly affected by MC-RR than -LR, although it should be noted that environmentally relevant concentrations (1-10 µg $L^{-1}$) caused no significant differences in the growth of the cultures [36]. 100-5000 µg $L^{-1}$ MCs (-LR and -RR) caused no inhibition of most species, except for *P. subspicata* cultures, where 1000 µg $L^{-1}$ or higher concentrations of MCs caused significant growth inhibition. The lowest sensitivity was observed in the case of *S. quadricauda*; detectable effects appeared only at 25000 µg $L^{-1}$ or higher concentration of MCs [36].

Similar results were obtained when a *Klebsormidium* strain was co-cultured with the cyanobacterium *Geitlerinema splendidum*: although MC was detected in the medium (0.10 µg $L^{-1}$), it did not inhibit the growth of *Klebsormidium* [37]. The green alga was not sensitive even to 50 µg $mL^{-1}$ MC-RR. It is assumed that the resistance of *Klebsormidium* is because this species is phylogenetically close to vascular plants, where the presence of detoxification system is possible [38-40].

Bartova et al. [41] found that neither the *M. aeruginosa* extract nor the cell-free medium, or 300 µg $L^{-1}$ MC-LR and -RR had effect on the growth of *Pseudokirchneriella subcapitata*. The purified MCs caused an increase in glutathione reductase activity, but had no effect on other detoxification enzymes. The *M. aeruginosa* extract had no effect on glutathione S-transferase, while the cell-free medium caused an increase in the activity of the enzyme. The results of short-term tests (3-24 hours) suggested that oxidative stress occured, while the long term (10 days) experiments did not show significant differences from controls [41].

**Dinoflagellates**

Inhibition effects of MCs on the growth and photosynthesis of *Peridinium gatunense* have been reported [42]. Vardi et al. [4] found that the initiating cell numbers of *Peridinium gatunense* and *Microcystis aeruginosa* had great importance in the inhibitory effects. The growth of *P. gatunense* cultures inoculated with 200 cells $mL^{-1}$ were completely inhibited by $10^6$ cells $mL^{-1}$ *Microcystis* even if extra nutrients were added to the cultures. At 5600 cells $mL^{-1}$ *Microcystis* caused "only" 60% inhibition, while 2300 cells $ml^{-1}$ *Microcystis* had barely detectable effects. At 1000 cells $mL^{-1}$ *Peridinium* did not affect the growth of *Microcystis*, whereas higher values did, even at higher nutrient concentrations [4]. The amount of reactive oxygen species (ROS) in *Peridinium* cells significantly increased at least within half an hour after the addition of *Microcystis* cells; it achieved the first peak in the first hour; a second maximum was observed within 24 hours; then the amount of ROS decreased. The results in purified MC-LR-treated *P. gatunense* cultures were very similar with the difference that the amount of ROS reached the second peak much sooner. In contrast, the addition of non-MC-producing *Microcystis* cells to *Peridinium* cultures caused slight increase in ROS, and the second peak was not detected either. The maximum ROS level is also influenced by the age

of the *Microcystis* culture what was added to the *Peridinium* cultures: The addition of older cultures shortened the time between the two peaks and older *Microcystis* cells caused 30% death of *P. gatunense*, while adding younger *Microcystis* culture caused lower mortality [4]. The mitogen activated protein kinase (MAP kinase) family plays a role in sensing cyanobacteria or the cyanobacterial toxin in *P. gatunense* cells, and their associated signal transduction processes affect the accumulation of ROS. Addition of *Microcystis* cells or MCs to *Peridinium* cultures can lead to programmed cell death through the accumulation of ROS; yet, in surviving *Peridinium* cells, it stimulates cell division. This phenomenon was not detected when dinoflagellate cultures were treated with purified MC-LR [4]. The protein kinase (PK) activity of *P. gatunense* increased by adding non-toxic or toxic *Microcystis* cells, culture filtrate or purified MC-LR in the first half hour. Studying the impact of toxic *Microcystis* on PK activity of *Peridinium* cells after 22 hours, it was observed that it decreased to the control levels. Toxic *Microcystis* and its filtrate resulted in a much higher activity than the purified toxin. Towards smaller molecular weight kinases, their activity increased continuosly during all experiments [4].

**Chrysophytes**

Growth stimulation, increased superoxide dismutase (SOD) activity and lipid peroxidation as well as not significant changes in glutathione pool were reported in MC-LR and -RR treated chrysophyte cultures [43]. Cell structure changes (vacuolization, swelling of chloroplasts) and phenomena similar to programmed cell death were also observed. Biodegradation of MCs were also observed and it was showed that the cells do not accumulate the toxin [43].

**Cryptomonads**

Very few data are available in literature about the effects of MCs on cryptomonads, notwithstanding this group is of great importance in the diet of zooplankton; so their sensitivity to MCs could be significant from the point of view of the whole plankton community. MC-RR treatment can cause the inhibition or stimulation of *Cryptomonas ovata* on a light intensity dependent manner. MC-RR inhibited the growth of *Cryptomonas erosa* under low light intensities (4 $\mu E\ m^{-2}\ s^{-1}$) [32].

**Cyanobacteria**

Despite the fact that MCs are known to cause inhibitory effects mostly on eukaryotic protein phosphatases, anticyanobacterial effects are also reported. Singh et al. [44] observed inhibition of growth, photosynthetic oxygen production and $CO_2$ consumption and nitrogenase activity after the treatment of an *Anabaena* strain and a *Nostoc muscorum* strain with 25 $\mu g\ mL^{-1}$ MC-LR. It has been shown that both species take up the toxin in daylight as well as in the dark [44].

Over growth inhibition reduced chlorophyll content, photosynthesis and nitrate reductase activity and changes in carbohydrate and protein content were observed in a MC-RR treated *Synechococcus elongatus* strain [45].

The research results show that the producer organism itself is not sensitive to MCs. Growth inhibition of *M. aeruginosa* cultures was observed only from toxin concentrations of 25000 $\mu g\ L^{-1}$ [36].

It was observed during co-culturing of *Geitlerinema splendidum* with *Nostoc sp*., *Pseudocapsa sp*. and *Scytonema sp*. that although MC was detectable in the medium (0.10 µg $L^{-1}$), the cell number of each species increased. The authors treated these cyanobacteria with the crude extracts of *Oscillatoria sp., Rivularia biasolettiana, Rivularia haematites, Geitlerinema splendidum, Phormidium sp., Tolypothrix distorta* and *Scytonema myochrous*. *Scytonema sp*. was inhibited by all of the extracts except by *R. biasolettiana* extract after the 4-day incubation. In an 8-day exposure, *Oscillatoria* extract also caused inhibition, whereas in other cases "only" reduction of growth was observed [37]. These experiments focused on benthic species, but the secondary metabolites they produce may play a role in the life of phytoplankton (e.g. sedimentation, resting forms).

Interesting results were obtained during the study of the impacts of MCs on filamentous cyanobacteria. In *Trichormus variabilis* 0.67-37 µg $L^{-1}$ MC caused the elevation of heterocyst frequency, while higher concentrations of MCs led to a reduced heterocyst frequency, and inhibition of akinete differentiation. Presumably the inhibition of serine-type proteases was responsible for the defects of cell differentiation. In order to shed light on whether these phenomena are intra- or inter-specific activity of the oligopeptides, or a "side effect" is involved, further studies are required [46].

Thus, research results indicate that the protein phosphatase inhibitory effects also appear in the case of some cyanobacterial enzymes [44,46-49]. It should be noted that some protein phosphatases isolated from *Microcystis* species are resistant to MCs [50]. The toxicity of MCs on microalgae manifests by the interaction with protein kinases and phosphatases [15]; [4], while oxidative stress and the increasing amount of ROS are also involved [51].

**The Possible Role of MCs in Phytoplankton Assemblages**

The results discussed above show that the effect of MCs and related compounds are species-specific - the effect of different MC variants may differ in the same species and different species may respond differently to the presence of the same MC variant. A number of studies do not corroborate the hypothesis that direct allelopathic effect is the natural function of MCs, at least for the direct inhibition of growth [36]. It should be noted that in some of those studies, which showed growth inhibition, higher MC concentrations were applied (10 to 57000 µg $l^{-1}$) than commonly observable in nature (< 10 µg $l^{-1}$). It is therefore questionable whether MCs primarily acts as allelopathic compounds in aquatic ecosystems [52].

Still there are some phenomena referring that MCs take part in interspecific interactions, namely that in which the presence of a certain phytoplankton species or their exudates affect on MC production. The exudate of the green alga *Clamydomonas reinhardtii* inhibited MC production of *Anabaena flos-aquae* [53]. There are opposite examples: MC production of an *Oscillatoria agardhii* strain increased in the presence of *Spirogyra* green algae [8]. Increased MC production of a *M. aeruginosa* strain was observed when cultures were treated with non-toxic *Planktothrix agardhii* culture or its filtrate medium [54]. Vardi et al. [4] observed the increase in the amount of gene product McyB in MC producer organisms in the presence of *Peridinium gatunense* or *Clamydomonas reinhardtii* exudates. Schatz et al. [55] observed that many other stress factors cause this phenomenon. The increase in McyB was also observed by the influence of MCs, microginins or micropeptins. The authors assume that during the lysis of cells in aged cultures the released peptides caused increase in MC production of the surviving cells [55].

The function of MCs in the producer organisms is not clear. Some authors assume that MCs had a function in the cell, which has now lost [56]. In contrast, the high cost of toxin production strengthens the aspects that MCs have a current function as well. One of the most important questions is about whether there is an active release of MCs by the producers or not. In some cases MCs were detected in the cell wall and the capsule surrounding area, which may indicate the active toxin release from intact cells [57]. Rohrlack and Hyenstrand [58] tracked the intracellular fate of MCs in a *M. aeruginosa* strain by radiolabelling. They found that the toxin is not excreted to the extracellular space, independently of the culturing conditions (low or high light intensity). Lack of intracellular degradation suggests that the toxin is not involved in cellular metabolism either [58]. Based on these results the question about the role of MCs remains unanswered, at least for the tested strain.

The high affinity of MCs to iron, copper or zinc ions, suggests that these molecules can function as siderophores [59]. This hypothesis may be supported by the observation that in iron depleted environment higher toxin contents were detected [60]. Otherwise, there are examples for high toxin content at high iron concentrations; in these cases intracellular toxin could have a protective role. High amount of MCs could keep the level of free radical formation low chelating surplus iron in the cells [61].

Sedmak and Kosi [32] assume that MCs have a growth regulator function, which helps in cell division. Treatments with 104-519 μg $L^{-1}$ MC-RR increased cell division in non-toxic *M. aeruginosa* cultures. A similar effect was observed in the cyanobacterium *Chroococcus minutus*, although it should be noted that the activating effect of MC-RR was strongly light dependent, cell division activation effect was detected only at low light intensity (4 μE $m^{-2}$ $s^{-1}$). Increased proliferation was also observed in *Monoraphidium contortum* and *Scenedesmus quadricauda* green algae cultures at the same light intensity, while a strong inhibition was observed in *Cryptomonas* cultures at the end of exposition time. It should be noted that there was activation of cell division for only a few days (up to one week), there was inhibition on subsequent days [32]. The role of MCs in growth and cell differentiation seem to be confirmed by the results of Bartova et al. [46], discussed above.

Some research suggests that the amino acid 3-amino-9-methoxy-2-6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) of MCs is able to bind to thylakoids (where most of the intracellular MCs localized in the cells [57]), thus showing that this may play a role in chromatic adaptation. This is supported by the fact that in some cases, increased expression of genes responsible for MC synthesis was observed at high light intensity [62]. Somehow contradictory to this assumption is the observation of Kardinaal et al. [63] that the non-toxic *M. aeruginosa* strain outgrew the toxic strain, independently from inoculation ratio (1:1 toxic:non-toxic or 9:1 toxic:non-toxic). There were differences between the toxin-producing and non-producing strains, between cultures kept at high light or in the dark, related to photosynthesis, production of amino acids and fatty acids, accumulation or inhibition of proteins involved in other cellular processes and the appearance of different isomers [64].

Sedmak and Elersek [35] treated non-toxic *Microcystis* strains with 1-5 M MC-LR, -RR and -YR variants. The toxin caused the establishment of large, incompact aggregates and the cells increased in volume after the treatment compared to the control cells. If toxin was added to the non-toxic culture only at the 1$^{st}$ day, the cell volume increased by the 8$^{th}$ day and then reduced back to control size. In the case when toxin was added on the 11$^{th}$ day to the previously treated culture, the cell volume increased again. These phenomena are important because the increased cell volume or aggregation both can play a role in the vertical and

horizontal distribution, migration, and can be beneficial to the population. The autofluorescence of toxin-treated cells has increased compared to control cells. This phenomenon was also observed in the case of toxic *Microcystis* strains. It was observed both in non-toxic and toxic *Microcystis* strains that the toxins increased phycocyanin content; however, chlorophyll concentration did not change [35].

These data show that the question about the functions of MCs is still unanswered. Some studies suggest that they play a role in the life of phytoplankton communities and there is no doubt that MCs are one of the most potent toxins in the aquatic ecosystem. Despite of this, many studies suggest that neither toxicity nor allelopathic activity are the reasons why cells produce the toxin. This is supported by the fact that there is not significant release of toxins even in exponential phase, which could really endanger the surrounding organisms. Recent research rather suggests that these toxic, otherwise bioactive compounds play a role in communication between cells [65]. Being still far from fully understood the role of MCs, increasing evidence highlight the intracellular role of MCs during oxidative stress. Further research is needed for the final confirmation or rejection of the allelopathic hypothesis, and new information is necessary from the area of evolution and molecular biology to answer the questions about the role of MCs.

### *Nodularins*

Nodularins are cyclic peptides composed from five amino acids; they are closely related to MCs not only in their structure but also in their mechanism of action: they cause inhibition of serine / threonine phosphatase 1 and 2A in eukaryotic cells. Nodularin production has been reported from the cyanobacterium *Nodularia spumigena* [22]. Like MCs, the toxin occurs primarily intracellularly, it appears extracellularly only in the stationary phase of growth [66].

There are relatively few data in literature about the impacts of nodularins on the members of phytoplankton community. The presence of the cryptomonad *Rhodomonas salina* did not cause any significant difference in toxin production and release of *N. spumigena* [66]. Purified nodularin treatment had no effect on growth of *R. salina*, similarly there was no allelopathic effects detected in other phytoplankton species neither. The results suggest that changes in pH, and pH tolerance of the investigated phytoplankton species regulate their growth, rather than allelopathic activity. *N. spumigena* was able to increase the pH of the environment (which can reach up to 10.6); so it is able to eliminate competing species with narrower pH tolerance [66].

### *Other Peptides*

A large numbers of other linear (aeruginosins, microginins, microcolins, mirabimids, tantazoles, mirabazoles) and cyclic (anabaenopeptins, cyanopeptolins, cyclamides, microviridins, crytophycins) peptides were isolated from planktonic and benthic cyanobacterial strains; however, their functions are largely unknown, especially for their role in phytoplankton assemblages [67]. Here are some summarized results of studies relevant to phytoplankton.

Flores and Wolk [68] isolated a cyclic peptide containing six amino acids, named nostocyclamine from a *Nostoc* strain, which had inhibitory effects on a number of cyanobacteria and several species of green algae (*Nannochloris*, *Ankistrodesmus*, *Scenedesmus obliquus*, *S. subspicatus*).

Todorova et al. [69] isolated a cyclic peptide, named nostocyclamide from a *Nostoc* strain, which proved to be allelochemical against cyanobacteria. From the same strain Jüttner et al. [70] isolated nostocyclamide-M, which was also proved to have anticyanobacterial activity. Three cyclic peptides were isolated from *Stigonema dendroideum*, which were named dendroamides [71]. These cyclic peptides are in the focus of interest because of their ability to reverse drug resistance in tumor cells, their role in the producer organism or to other cyanobaceteria and algae is not known yet [71]. Tenuecyclamides were isolated from *Nostoc spongiaeforme* [72]; raocyclamides from *Oscillatoria raoi*. These oxazole- and thiazole-containing cyclic peptides show cytotoxic activity against sea urchin eggs, their role in the producer organism or to other cyanobaceteria and algae is not known yet [73, 74]. Kasumigamide and microcins were isolated from *Microcystis* species, both showing allelopathic effects against photoautotrophs [75,76]. The cyclic peptide pahayocolid, recently isolated from *Lyngbya* species, also shown algicid activity [10,77]. Anabaenopeptins and planktopeptins have impact on the growth and activation of cell lysis of *M. aeruginosa* [78,79].

## Alkaloids

### *Neurotoxic Alkaloids*

There are three groups of alkaloid type toxic secondary metabolites isolated from cyanobacteria, which are known to be neurotoxic to invertebrates and vertebrates:

1) The anatoxin-a is a low molecular weight alkaloid (165 Da), a secondary amine, and was isolated from *Anabaena flos-aquae, Anabaena* spp. (*flos-aquae – lemmermannii* group), *Anabaena planktonica,* and from *Oscillatoria, Aphanizomenon* and *Cylindrospermum* species; the homoanatoxin-a (179 Da) is a homologue of anatoxin-a and was isolated from an *Oscillatoria formosa* (*Phormidium formosum*) strain [80]. The anatoxin-a is a cholinergic type nicotine antagonist; it binds to nicotine-acetylcholin-receptors, thereby causing $Na^+$ influx, which leads to depolarisation of $Na^+$-$Ca^{2+}$ channels. As a result, the number of activated $Ca^{2+}$ channels increases [81].
2) The anatoxin-a (S) is a cyclic N-hydroxyguanin phosphate ester and it was firstly isolated from the NRC 525-17 *Anabaena flos-aquae* strain. The compound was also detected later in *Anabaena lemmermannii* [82]. Anatoxin-a (S) has no structural variant yet. Similarly to organophosphate insecticides, anatoxin-a (S) inhibits acetylcholinesterase activity [83,84].
3) The saxitoxins are carbamate alkaloid neurotoxins, there are non-sulphates (saxitoxin - STX and neosaxitoxin - NEO), monosulphates (gonyautoxinok - GTX) or disulphates (C-toxins). Decarbamoil variants and more new toxins were also described from some species. Saxitoxins were found in the following cyanobacterial species: *Aphanizomenon flos-aquae, Anabaena circinalis, Lyngbya wollei* and *Cylindrospermopsis raciborskii* [22]. Saxitoxins block the $Na^+$ channels of nerve cell membrane, we found no information, if this effect was observed on unicellular organisms or not.

Relatively few studies deal with the effects of neurotoxic alkaloids on aquatic photosynthetic organisms. Thus, it is not known whether these compounds have a role in phytoplankton communities. It has been shown that anatoxins inhibit the release of photosynthetic oxygen and increase the glutathione S-transferase activity in the aquatic plant *Lemna minor*. The increase of peroxidase activity was also shown in *L. minor* and in the sessile alga *Cladophora fracta* [85]. In the light of the few results about the effects on photosynthetic organism, it can be assumed that neurotoxic alkaloids play a role in protection against grazers and not in the interactions between phytoplankton species.

***Cytotoxic Alkaloids – Cylindrospermopsins***

Cylindrospermopsin (CYN) is a cyclic guanidine alkaloid (415 Da), which was isolated from several species of filamentous cyanobacteria (*Cylindrospermopsis raciborskii, Umezakia natans, Anabaena berghii, Aphanizomenon flos-aquae, A. issatschenkoi, A. ovalisporum, Raphidiopsis curvata,* and *Lyngbya wollei* [86]). Deoxicylindrospermopsin, a structural variant of CYN was isolated from a strain of *C. raciborskii* [87]. The variant 7-epicylindrospermopsin was found in *A. ovalisporum* strain ILC-164 [88]. CYN and its analogues are irreversible inhibitors of protein synthesis; they cause necrosis, atrophy, DNA fragmentation and mutations [89-92]. After MCs, CYN is probably the second most studied cyanotoxin. Despite the fact that, in contrast to MCs, a number of producer actively release the toxin (the extracellular toxin content can reach up to 50% [17-19], there are very few data available in literature on the effects of CYN on phytoplankton species.

In the Lake Kinneret, when the amount of *Aphanizomenon ovalisporum* increased, Bar-Yosef et al. [93] observed also increased alkaline phosphatase (AP) activity. This was partly due to the fact that Pi limitation led to the release of AP by *A. ovalisporum* cells. However, it was also observed that AP activity was higher at the beginning of the water blooms, when there was a lower number of filaments and the amount of polyphosphate bodies were higher in the cells, than in the later stages of the bloom. Fluorescent marking demonstrated that AP originated not from *A. ovalisporum* filaments, but from the cells of the filaments vicinity. Experiments with *Chlamydomonas reinhardtii* demonstrated that threatment with cell-free medium of *A. ovalisporium* or CYN resulted in excretion of AP of the green algal cells, even when phosphate was not limitant in the medium. AP release to the media was greater in *C. reinhardtii* cultures treated with *A. ovalisporum* cell-free medium, than in cultures treated with purified CYN; presumably due to synergistic effects. The authors concluded that CYN with synergistic compounds compel individuals of other phytoplankton taxa living in the community to increase their AP excretion in Pi limited environment. A parallel increase in the Pi uptake rate of *A. ovalisporum* cells leads to an advantage over other cells [93]. These conclusions are not supported by the results of Bácsi et al. [94] which showed the reduction of CYN in *A. ovalisporum* ILC-164 cells. Further, extracellular CYN did not increase under phosphate limited circumstances [94]. Although it is known that the same isolates may behave differently in different laboratories.

***Other Alkaloids***

The compound known as norharmane were isolated from *Nodularia harveyana* showed anticyanobacterial activity to both unicellular and filamentous cyanobacteria [95].

Becher et al. [96] identified nostocarboline from *Nostoc* 78–12A strain, which inhibits the growth of a number of cyanobacteria due to its actions on photosynthesis.

It requires further investigation, to know the effects of alkaloids on phytoplankton species. It is possible that these compounds do not directly impact the species of phytoplankton community, but they have an impact on other organisms, including grazers from zooplankton, providing competitive advantages to the producers.

## Other Toxins/Allelochemicals

In addition to the compounds described above, a number of secondary metabolites with very diverse chemical structures were isolated from cyanobacteria, which are proved to be bioactive against phytoplankton species.

*Fischerella muscicola* produce fischerellin (2-pyrrolidone derivative), which inhibits photosynthesis of other cyanobacteria [97,98]. Interestingly, this compound - as the "classical" cyanotoxins - was detected in the producer cells rather than in the culturing medium [97].

Vepritskii et al. [99] and Gromov et al. [100] isolated the compounds named cianobakterin LU-1 and LU-2 (phenolic amino sugar derivatives) from *Nodularia linckia*. These compounds inhibit the electron transport in PSII, specifically the electron transfer from the secondary quinone-binding site ($Q_B$). It is interesting that LU-1 inhibits the photosynthesis of cyanobacteria and eukaryotic algae, while LU-2 was effective only in case of cyanobacteria. Unlike fischerellins and compounds later isolated from *Scytonema* (also known as cyanobacterins, but with a completely different chemical structure), cyanobacterin LU-1 and LU-2 were described as exometabolites; almost 100% of the total amount was detected in the culturing medium [99,100].

Cyanobacterin (chloro-gamma-lactone) produced by *Scytonema hofmannii* inhibits the PSII-mediated electron transfer. Similarly to fischerellins, this compound was also present in a higher proportion in the cells than in the medium [12,101-103]. It was also found that cyanobacterin inhibits colonization of some diatoms, for example *Nitzschia pusilla* [104,105]. An anticyanobacterial and antialgal metabolic product was isolated from *Oscillatoria laetevirens* after field observations of phytoplankton community. The compound (with 444 kDa molecular weight) presumably acts on the acceptor side of PSII, at a different site than the herbicide DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) [106-109].

Allelopathic activity of fatty acids isolated from *Microcystis* species have also been reported [110,111].

The compound 12-epi-hapalindol E izonitril isolated from *Fischerella sp.* or calothrixin A isolated from *Calothrix sp.* cause the inhibition of protein synthesis and DNA replication [112].

The volatile geozmins and geranylakton inhibit the growth of *Chlorella pyrenoidosa* [113].

The violet pigment nostocine A, produced by *Nostoc spongiaeforme*, is highly cytotoxic to many microalgae [114]. The compound caused the increase of the amount of reactive oxygen species in *Chlamydomonas reinhardtii* [115].

### Phytohormones

Cyanobacterial biomass as well as cyanobacterial extracts has been studied to detect phytohormon production [116,117]. It became obvious that cyanobacteria produce and release compounds acting as phytohormones such as auxins, cytokinins, gibberellins, abscisic acid, ethylene and jasmonates [118], which may affect the phytoplankton species.

Stirk et al. [116] studied the cytokinin and auxin production of 7 green algal species (*Chlorella sp., Scenedesmus quadricauda, Coenochloris sp., Chlorosarcina sp., Tetracystis sp.* and 2 *Chlamydomonas sp.*) and 3 cyanobacteria (2 *Calothrix* strains and *Phormidium animale*). They found that all strains showed remarkable cytokinin-like activity, whereas 7 strains showed a weak auxin-like activity in cucumber cotyledon test.

Sergeeva et al. scanned 34 symbiont (endo- and exosymbionts) and free-living, non-filamentous and filamentous, nitrogen-fixing and non nitrogen-fixing cyanobacteria for auxin production and release. Twenty-one strains showed auxin release; 5 from the 16 free-living strains and 15 from the 18 symbiont species released auxin. More nitrogen-fixing strains were found to release auxin among the free-living strains than non-nitrogen fixing filamentous species or the unicellular ones [119].

Studiing auxin accumulation and release in a free-living and in a symbiotic *Nostoc* strain, it was found that the free-living strain had higher accumulation in the 72$^{nd}$ hour, then accumulation was higher in symbionts on the 1$^{st}$, 2$^{nd}$ and 3$^{rd}$ weeks. Auxin release of symbionts was always higher [119].

Tsavkelova et al. proved the auxin-like activity of *Anabaena, Anabaenopsis, Calothrix, Chlorogloeopsis, Cylindrospermum, Gloeothece, Nostoc, Plectonema,* and *Synechocystis* strains [120].

Hussain et al. showed the production of 5 cytokinins (zeatin cis and trans isomers, zeatin riboside, dihydrozeatin riboside, and zeatin-o-glucoside) from *Anabaena sp., Oscillatoria sp., Phormidium sp., Chroococcidiopsis sp,* and *Synechosystis sp.* [121].

However, further research is needed to respond to the question if the hormone-like compounds also have a role in the interactions of phytoplankton species.

## BACTERIA IN PHYTOPLANKTON ASSEMBLAGES

A large number of cyanobacterial metabolites have antibacterial or antifungal activity. These compounds often have no effect on phytoplankton, but cyanobacteria could gain competitive advantages with the production of these kinds of compounds. Production of these compounds may also be beneficial to the growth of phytoplankton species by reducing the bacterial and/or fungal "preassure". Bacteria generally have higher growth rate and higher nutrient uptake rates than algae, so the presence of antibacterial compounds could be beneficial to the growth of phytoplankton due to the reduction of bacterial cell number [11]. Falch et al. [122] tested 54 types of hydrophilic and hydrophobic compounds containing extracts of 20 laboratory strains of cyanobacteria, of which 78% showed antibacterial and 45% antifungal activity. Antibacterial, antifungal, molluscicid, cytotoxic, anti-inflammatory and antiviral activity of ambigol A and B, isolated from the lipophilic extract of *Fischerella*

*ambigua*, was demonstrated. Tjipanazol C isolated from the same species, showed moderate antibacterial activity [122].

Ueki et al. [123] co-cultured heterotrophic bacteria with *Microcystis*, and found that gene transfer significantly increased in heterotrophic bacteria with the growth of *Microcystis* cell density. The similar phenomenon was observed, when *M. aeruginosa* extracts were added. The increase of gene transfers did not change as much, when the cultures of heterotrophic bacteria were treated with heat-inactivated *Microcystis* cells. It was also found that gene transfer was higher in crude extract treated cultures compared to glucose supplemented ones. It is not clear why these phenomena are relevant for the producer organisms [123].

It should be noted that bacteria may also play a role in the regulation of cyanobacterial water blooms on other ways. Some results suggest that the heterotrophic bacterial communities may play a role in the development of *Microcystis* colonies. When unicellular *Microcystis* cultures were co-cultured with their naturally associated heterotrophic bacteria, colony formation was observed [124]. The specific growth rate, chlorophyll content and the amount of excreted polysaccharides were higher in non-axenic cultures after aggregation than in axenic ones. The biomass of heterotrophic bacteria was also higher in mixed cultures than in axenic heterotrophic bacterial cultures. PCR-DGGE analysis showed that *Porphyrobacter* and Flavobacteriaceae species may be responsible for the aggregation of *Microcystis* cells [124]. The increase of the amount of extracellular polysaccharides helps *Microcystis* cells to remain together and the proliferation of the associated heterotrophic bacteria. These results suggest that heterotrophic bacterial communities may play a role in the development of characteristic *Microcystis* blooms in surface water [124]. Pearl and Millie [125] observed that $CO_2$ fixation rates were higher in *M. aeruginosa* in the presence of heterotrophic bacteria.

## CYANOBACTERIA AS TARGETS OF THE ALLELOPATHIC EFFECTS

Cyanobacteria may be included in phytoplankton community not only as producers of allelochemicals, but also as victims of allelopathic effects. As it is presented above, a lot of cyanobacterial secondary metabolites have anticyanobacterial activity. Additionally, eukaryotic algae also could produce anticyanobacterial compounds.

Studying the impact of the dinoflagellate *Peridinium gatunense* on *Microcystis* showed that above 1000 cells $ml^{-1}$ inoculated *Peridinium* has a significant impact on *Microcystis* cells. *Peridinium* filtrate decreased floating ability of *Microcystis* cells and 75% of *Microcystis* cells were lysed within 24 hours. Treatment with *Chlamydomonas* extract also caused lysis of *Microcystis* cells as well in minor extent [4].

The periphyton (complex community of photoautotrophic and heterotrophic microorganisms) can play an important role in regulating the formation of water blooms [126]. The results show that cyanobacteria responsible for water blooms can be characterized by higher nutrient concentration limits. Below this they can not compete effectively with organisms living in periphyton. According to the observations, periphyton biofilm is able to produce water-soluble allelochemicals, such as indole and 3-oxo-alfa-jonone which significantly inhibit the growth of cyanobacteria. These compounds can damage the thylakoid membranes, thereby inhibiting the transport of electrons in PSII, thus causing inhibition of photosynthesis [126].

The macroalga *Chara vulgaris* also proved to be able to produce allelopathic compounds that may play a role in regulating the growth of cyanobacteria. Zhang et al. [127] found that there is a reciprocal allelopathy between *C. vulgaris* and *Microcystis aeruginosa*. *C. vulgaris* produce (Z,Z)-9,12-octadecadienoic (ODEA, 18:2), tetradecanoic (TDA, 14:0) and hexadecanoic acids (HAD, 16:0), which inhibited the growth of *M. aeruginosa*; among them ODEA seemed to be the most effective. These three fatty acids had synergistic inhibitory effects on the growth of *M. aeruginosa*, i.e. the macroalgae *C. vulgaris* could play a role in bloom formation of *M. aeruginosa* [127].

The so-called "rotten reed solution" (RRS) treatments have shown that such solutions could inhibit the growth of cyanobacteria [128]. The research results indicated that the inhibitory effect varied seasonally. The GC/MS analysis of RRS extracts showed that mainly phenolic compounds (p-coumaric, ferulic, vanilic, sinapic, syringic, caffeic, protocatechuic, and gallic acids) and fatty acids (myristic, palmitic, pelargonic, and stearic acids - released during the decay of reed) were responsible for inhibition. Four phenols (sinapic, syringic, caffeic, and gallica acids) and pelargic acid inhibited the growth of both *Phormidium tenue* and *Microcystis aeruginosa*; while p-coumaric, ferulic, myristic and vanillic acid had species-specific effects. Ferulic, stearic, and palmitic acids did not show any effect [128].

Similar inhibitory effects were observed during treatments with the extract of rice straw as well [129]. Salicylic acid seemed to be the strongest inhibitor in the aqueous extract of rice straw. In addition, similarly to reed, phenolic compounds were responsible for the growth inhibition of *M. aeruginosa* [129].

## POTENTIAL BIOTECHNOLOGICAL APPLICATIONS OF CYANOBACTERIAL SECONDARY METABOLITES

The phenomenon that cyanobacteria themselves could be targets of allelopathic effects could provide an opportunity in the future for biotechnological application of the secondary metabolites discussed in this chapter.

Products with allelopathic activity are used in agriculture (such as green manure in the prevention of soil pests). Not inconceivable that such products may play a role in the future in the regulation of dangerous water blooms [2].

Cyanobacterial compounds with highly specific bactericide, fungicide and/or algaecide activity (such as fischerellins or cyanobacterins) would be applied to prevent the surfaces of in-water-used equipments from the so-called bio-contamination caused by the formation of biofilms [130].

Studies of the allelopathic activity of periphyton showed that the periphyton biofilm may be suitable for controlling cyanobacterial water blooms, which could be a temporary tool for creating favorable conditions for ecosystem restoration [126].

On the basis of past results and future research there would be opportunities to select and use the appropriate species-specific allelochemicals/toxins in the regulation of harmful water blooms or in the limitation of the spread of invasive cyanobacterial species [131].

## CONCLUSION

Allelopathy undoubtedly plays an important role in phytoplankton communities. Although current intensive research has discovered a large number of compounds, certainly more secondary metabolites will be explored in the future. To study the function of the new compounds and the already described ones, and refer them as allelochemicals or toxins is a great challenge for future research. In many cases, the physiological and/or ecological role of the compound is not clear even if the detailed mechanism of action is identified. In the case of a great number of studies appliing exudates or crude extracts of a known toxin producer, the observed effects are related to the toxins, although it is not confirmed until the purified toxin is tested. Therefore it is important for future studies to explore the effects of the purified compounds as well. It is also important to study the regulation of allelochemical/toxin production. These research findings could be complemented by investigating allelopathic phenomena among controlled field conditions and by studying the evolutionary biological aspects of allelopathy. All of these research findings could take us closer to understand the role of this very diverse chemical repertoire in the life of phytoplankton communities.

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*Chapter 2*

# NEGATIVE ALLELOPATHY AMONG CYANOBACTERIA

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## ABSTRACT

Cyanobacteria are well known producers of a wide variety of allelochemicals, which positively or negatively affect sympatric organisms from similar or even different taxons. In the traditional approach for studying allelopathy in water systems, cyanobacteria and photoautotrophic micro-eukaryotes were grouped together under the term of micro-algae. Because these two groups are phylogenetically and phenotypically distinct and the production of allelopathic compounds is often highly species- and even strain-dependent, it is appealing to assess the present available knowledge concerning allelopathy within and among cyanobacteria separately.

In this chapter, information is reviewed about i) cyanobacteria production of alleopathic substances, ii) the chemical nature of these allelopathic secondary metabolites, and iii) the mechanisms of the allelopathic inhibition. Furthermore, (iv) the possibility to use allelopathy to control harmful cyanobacterial blooms is discussed.

## INTRODUCTION

Species composition and biomass of phytoplankton are fundamental in food web ecology. Therefore, many studies on competition among aquatic microbes have traditionally focused on the availability and partitioning of resources and predator-prey interactions, while slightly overlooking their biochemical interplays [1]. Cyanobacteria possess both bacterial and algal characteristics, being unicellular to multicellular prokaryotes and performing oxygenic photosynthesis associated with photosystems I and II [2]. For those reasons, those groups

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belonging to phytoplankton were very often pooled and studied together with other planktonic micro-algae [3,4]. However, based on fundamental differences between cyanobacteria and eukaryotic microalgae (e.g., phylogenetic, genotypic and phenotypic traits); it is interesting to consider cyanobacteria and their biochemical interactions separately.

Cyanobacteria from marine and freshwater habitats produce a wide range of bioactive metabolites. Several of these compounds participate in a complex chemical interaction among photosynthetic organisms, known as allelopathy, and can have either a positive or negative impact on the growth of sympatric species [5]. However, there is a dispute among scientists whether only direct negative biochemical interactions should be included in allelopathy [6].

Here, I focus on negative allelopathic effects of cyanobacteria on cyanobacteria and present an overview of the chemical nature and mode of action of cyanobactericidal compounds produced by cyanobacteria. Furthermore, I discuss the possible use of these substances for biocontrol of harmful cyanobacterial blooms (HCB).

## OUTLINE ABOUT CYANOBACTERICIDAL CYANOBACTERIA

In the last decade, many elegantly written reviews have been presented about algal and cyanobacterial metabolites (allelochemicals, toxins) in freshwater [7,8,9] marine [10] or both water systems [4].

Despite the fact that the majority of studies were carried out on freshwater cyanobacteria, some marine, brackish and soil cyanobacterial isolates have been tested (Table 1). Therefore, it seems relevant that not only freshwater cyanobacteria are subjected to allelopathic challenges. Many other relevant papers concerning the production and effect of algal and/or cyanobacterial allelopathic compounds on algae and/or cyanobacteria have been published.

Despite all those important and interesting studies, a comprehensive view about cyanobactericidal cyanobacteria in marine and freshwater systems is missing.

The first published studies on extracellular metabolites produced by cyanobacteria inhibiting growth of other cyanobacteria or algae appeared in the middle of the twentieth century and were based on field observations and carbon isotope assays [reviewed in 8]. Yet, more intensive and focused research on cyanobacterial allelopathy only started in the 1970s and 1980s, with the isolation and identification of cyanobactericidal compounds such as hapalindole from *Hapalosiphon intricatus* [11], cyanobacterin from *Scytonema hofmanii* [12] and hapalindole A from *Hapalosiphon fontinalis* which have shown fungicidal properties as well [13]. In the following years, additional cyanobacterial genera and their allelopathic potential with target organisms including macro- and micro-algae, angiosperms and cyanobacteria have been studied [6,14]. A comprehensive overview of cyanobactericidal cyanobacteria including the target organisms, the nature of the chemical compound, and its mode of action, is synthesized in Table 1.

Regarding the taxonomical designation, based on the available published information cyanobactericidal allelopathic cyanobacteria belong to four orders (*Chroococcales*, *Nostocales*, *Oscillatoriales* and *Stigonematales*, Table 1), from the seven defined orders (NCBI Taxonomy Browser).

The most diverse order is *Nostocales* with eight different genera, while *Chroococcales* is represented by one genus - *Microcystis* spp. The order with the greatest number of known

allelopathic representatives is *Nostocales* (29 studied isolates), followed by *Stigonematales* (18 isolates), while the least numerous come from *Chroococcales* or *Oscillatoriales* (both 9 isolates studied). According to the traditional classification with morphological distinctions (see Bergey's Manual [15]), cyanobacteria are divided into five subsections (I-V). Members of four from those five subsections are listed in Table 1, the only subsection missing is II.

It can be asked whether some allelopathic cyanobacteria are generalists or specialists with respect to their target range. Here, a cyanobacterium is considered as a generalist when it was shown to lyse or harm hosts across different classes of microbes (not only cyanobacteria). As shown in Table 1, there are some generalists among cyanobacteria that were shown to have a negative impact on a wide spectrum of microbes across different taxa (e.g., *Nostoc* sp. 31, *Calothrix parientina*, *Fischerella muscicola*). The assignment of allelopathic cyanobacteria as specialist (having as a target only cyanobacteria or only some cyanobacterial genera or even species) is difficult, because generally the allelopathic effect has not been tested on a sufficiently wide range of organisms to confirm the specificity of the inhibitory effect.

It is difficult to infer about the ecological significance of cyanobacterial allelopathy, because most studies have been carried out under laboratory conditions. Therefore, caution is necessary when addressing the role of cyanobacterial allelopathy in ecosystems [8]. Nevertheless, as discussed below, the laboratory experiments elucidated modes of action and the structures of allelochemicals and/or potential allelochemicals.

## ALLELOCHEMICALS AND THEIR MODES OF ACTION

Allelochemicals are the chemical effectors of allelopathy [16]. It is challenging to classify a chemical compound as an allelochemical [8]. From nearly 800 diverse bioactive secondary metabolites originated from cyanobacteria [17] only a small portion can be classified as allelochemicals. These chemicals include cyclic and non-cyclic peptides, polyketides, alkaloids, phenols and chlorinated aromatic compounds [8]. The diversity of chemical structures of cyanobactericidal allelochemicals is schematically shown in Figure 1.

Interesting among cyanobactericidal compounds is a chlorinated aromated lacton - cyanobacterin, produced by *Scytonema hofmanni*. It was one of the first allelochemicals isolated and described [12]. Cyanobacterin inhibited the growth of cyanobacteria, algae and plants. This compound seems to be a potent inhibitor of photosynthetic electron transport and acts at a site in photosystem II [18]. Another study assayed an antibiotic also named cyanobacterin LU-1, produced by *Nostoc linckia* [19]. However, except for the name this compound has nothing in common with the previously mentioned cyanobacterin described by Mason et al [12]. Cyanobacterin LU-1 hindered cell division and light-dependent oxygen evolution in *Synechococcus* sp. It was not active against heterotrophic bacteria and fungi, and was not toxic to mice [19]. The most diverse and numerous groups of cyanobactericidal allelochemicals and potential allelochemicals are lipopetides (Figure 1). Microcystins (MCs) belong to a family of cyclic heptapeptides frequently produced by some strains of planktonic bloom-forming cyanobacteria (*Microcystis, Anabaena, Planktothrix*) or terrestrial/benthic cyanobacterial genera (*Nostoc, Hapalosiphon*) [20]. The effect of MCs on cyanobacteria has been characterized in several studies (Table 1), and some implications concerning allelopathic properties of these highly toxic metabolites have been made (reviewed by [21]).

**Table 1. Cyanobacteria targeting cyanobacteria: phylogeny, target, chemical compound, mode of actions and reference**

| Order, family, genus, species, strain Subsections (I-V)[b] | Target | Chemical compound | Mode of action[a] | References |
|---|---|---|---|---|
| Cyanobacteria - no single agent tested | | | | |
| cyanobacterial extracts (F, M[c]) | many cyanobacteria (F, M) | extracts | nd | [52,81] |
| extracts of *M. aeruginosa* and *Aphanizomenon flos-aquae* (both F) | *Synechocystis* PCC6803 (F) | MC-LR | oxidative stress and higher expression of antioxidant genes | [82] |
| cyanobacterial extracts (M, B) *Leptolyngbya* sp. 1 LEGE 06069 (B) | *Synechocystis salina* (M) *M. aeruginosa* LEGE 91094 (F) | crude extracts methanolic extract | nd | [83] |
| Chroococcales Microcystaceae (subsection I) | | | | |
| *Microcystis* sp. (F) | *Synechococcus elongates* (F) | MC-RR | inhib. of PSII, chlorophyl*a*, phycocyanin; changes in protein an carbohydrate concentrations and in nitrate reductase activity; oxidative stress (ROS) | [30,31] |
| *Microcystis* sp. (F) | *Aphanizomenon* sp. (F) | MC-RR | PSII inhib., filament disintegrations, cellular inclusions, damage of cell membranes | [32] |
| *Microcystis* sp. (F) | *Anabaena oscillarioides* (F) | nd | nd | [84] |
| *Microcystis* sp. (F) | *Chroococcus minutus* (F) | MC-RR | growth inhib. under high light intensity | [85] |
| *M. aeruginosa* (F) | *Anabaena* BT1 (Sr), *N. muscorum* (F) | MC-LR | PSII and nitrogenase activity inhib. | [34] |
| *M. aeruginosa* (F) | *Synechocystis* PCC6803(F) | MC-RR | oxidative stress and higher expression of antioxidant genes (ROS) | [33] |
| *M. aeruginosa* (F) | *M. aeruginosa* PCC7806 (F) | MC-RR | growth inhib. at the highest MCs concentrations[d] | [23] |
| *M. aeruginosa* (F) | Oscillatoria angustissima, *Anabaena* PCC7120 (both F) | crude extract (MC-LR?) | growth inhib. | [86] |
| *M. aeruginosa* (F) | *Trichormus variabilis* (F) | nd | inhib. of cell differentiation | [36] |
| Nostocales Nostocaceae (subsection IV) | | | | |
| *Anabaena* sp. (Sr) | *Synechococcus* sp., *Synechocystis* sp. (both F) | nd | nd | [87] |
| *A. torulosa* ASW01028 (F) | *Anabaena cylindrica, Microcystis flos-aquae* (both F) | nd | nd | [88] |

| Order, family, genus, species,strain Subsections (I-V)[b] | Target | Chemical compound | Mode of action[a] | References |
|---|---|---|---|---|
| *A. constricta* (F) | *Arthrospira laxissima, Nostoc carneum, Chroococcus minutus, Synechocystis aquatilis* and *Synechococcus* sp. (all F) | bromoanaindolone | nd | [56] |
| *A. doliolum* (F) | many cyanobacteria (F) | antibiotics | nd | [89] |
| *A. holsaticum*(F) | *Oscillatoria agardhi* (F) | nd | nd | [57] |
| *A. spiroides*(F) | *Microcystis aeruginosa* (F) | spiroidesin | nd | [41] |
| Aphanizomenon elenkinii *(F)* | *Oscillatoria agardhi, Oscillatoria rubescens, Anabaena* sp.(all F) | nd | nd | [57] |
| *Cylindrospermum* sp. (F) | *Anabaena cylindrica, Microcystis flos-aquae* (both F) | nd | nd | [88] |
| *Cylindrospermopsis raciborskii* (F) | *Microcystis aeruginosa, M. wesenbergii* (both F) | nd | PSII inhib. | [71] |
| *Nodularia harveyana* (B) | different cyanobacteria (F) | norhamalane | nd | [52,55] |
| *Nodularia harveyana* (B) | *N. carneun, N. insulare, Arthrospira laxissima, Chroococcus minutus, Synechocystis aquatilis* (all F) | norharmane | nd | [53] |
| *Nostoc* WA 96/19, NSW 95/10 (S and/or F) | *Anabaena doliolum, A. circinalis, Microcystis aeruginosa* (all F), *Nodularia spumigena* (B, M) | nd | nd | [90] |
| *Nostoc* sp.31 (F) | some cyanobacteria (F), algae and rotifer | nostocyclamide | nd | [39,91] |
| | *Anabaena* sp. (F) | nostocyclamide M | nd | [40] |
| *Nostoc* ATCC 29132 (S roots) | many cyanobacteria (F) | antibiotics | nd | [89] |
| *Nostoc* ASW01020 and 1010 (both F) | *Anabaena cylindrica, Microcystis flos-aquae* (both F) | nd | nd | [88] |
| *Nostoc* 78-12A (F) | *M. aeruginosa* PCC7806, *Synechococcus* PCC6911 (F) | nostocarboline and 7 derivates | PSII inhib. | [51] |
| *N. insulare* (S) | *N. carneun, Arthrospira laxissima, Chroococcus minutus, Synechocystis aquatilis* (all F) | 4,4'-dihydroxybiphenyl | nd | [52,53] |
| *N. linckia* CALU 892 (S) | many cyanobacteria (F,S) and algae but not eubacteria | "cyanobacterin" LU-1 | inhib. cell division and $O_2$ evolution | [19] |
| *N. muscorum* (F) | *Anabaena cylindrica, Microcystis flos-aquae* (both F) | nd | nd | [88] |

**Table 1. (Continued)**

| Order, family, genus, species,strain Subsections (I-V)[b] | Target | Chemical compound | Mode of action[a] | References |
|---|---|---|---|---|
| *N. spongiaeforme* TISTR8169 (F) | *Anabaena variabilis*, *A. cylindrica*, *Nostoc commune* (all F) | nostocine A | formation of ROS? | [43] |
| | *Calothrix*169 (F) | nostocine A | na | [42] |
| *Trichormus doliolum* (F) | *Anabaena* PCC7120, P9, *Synechococcus* PCC6911, *Phormidium* SAG 212.80 (all F), *Synechocystis* CB3 (B) and some algae | nd | growth inhib. under light | [92] |
| | *Anabaena* PCC7120, P9 (both F) | nd | PSII inhib. | [93] |
| Rivulariaceae (subsection IV) | | | | |
| *Calothrix elenkinii* (Sr) | *Synechococcus* sp., *Synechocystis* sp. (both F) | nd | nd | [87] |
| *Calothrix parientina* (S) | *Microcystis aeruginosa*, *Synechococcus* sp., *Scytonema hoffmani*, *Phormidium mölle (all F)*, and bacteria, algae and fungi | antibiotics | nd | [94] |
| Scytonemataceae (IV) | | | | |
| *Scytonema hofmanni* (F) | *Synechococcus* sp. (F) | cyanobacterin | PSII inhib. | [12] |
| | *Synechococcus* sp. ATCC 27146 and many other cyanobacteria (F, M) | cyanobacterin | PSII inhib. | [95] |
| | *Anacystis nidulans* R2 (F) | cyanobacterin | PSII inhib. | [18] |
| Oscillatoriales Oscillatoriaceae (subsection III) | | | | |
| *Oscillatoria* sp (F) | *Oscillatoria rubescens*, *Pseudoanabaena galeata* (both F) | nd | nd | [57] |
| *Oscillatoria* sp. (F) | *Anacystis nidulans* UTEX 625, *Plectonema boryanum*, *Phormidium* spp., *Nostoc muscorum*, *Microcystis* sp. (all F) and alga *Chlorella* sp. | nd | PSII inhib. | [69] |
| Order, family, genus, species,strain Subsections (I-V)[b] | Target | Chemical compound | Mode of action[a] | References |
| *Oscillatoria* sp. (F) | *Anacystis nidulans* UTEX 625, some other cyanobacteria (all F), algae and plants | cell free extract (antibiotics) | PSII inhib. | [96] |
| Oscillatoria angustissima *(S)* | *Microcystis aeruginosa*, *Synechococcus* sp., *Scytonema hoffmani*, *Phormidium mölle*(all F), and bacteria, algae and fungi | antibiotics | nd | [94] |
| *Oscillatoria late-virens* (F) | *Microcystis* PCC7820 (F) | antibiotic | PSII inhib. | [97] |

| | | | | |
|---|---|---|---|---|
| *Oscillatoria rubescens*(F) | Aphanizomenon elenkinii *(F)* | nd | nd | [57] |
| Phormidiaceae (subsection III) | | | | |
| *Planktothrix agardhii* (MC producing) (F) | *Planktothrix agardhii* (MC non producing) (F) | MC | growth inhib. under limited nutrients | [35] |
| *Planktothrix rubescens*(F) | axenic *M. aeruginosa* (F) | cyclic peptides[e] | release infectious virus-like particles | [37] |
| | non-axenic *M. aeruginosa* (F) | planktopeptin BL1125 | | [38] |
| *Planktothrix rubescens* (F) | *Planktothrix agardhii* (F) | nd | nd | [1] |
| Stigonematales<br>Stigonemataceae (subsection V) | | | | |
| *Fischerella* JAVA 94/20, NSW 95/10, LOM 95/17, 95/3, 95/9, CAN 96/12, 96/13, VIET 97/2, NT 97/5 (S, F) | *Anabaena doliolum*, *A. circinalis*, *Microcystis aeruginosa* (all F), *Nodularia spumigena* (B, M) | nd | nd | [90] |
| *Fischerella* JAVA 94/20 (S) | *Anabaena doliolum*(F) | 12-epi-hapalindole E isonitrile | nd | [49] |
| *Fischerella* 52-1 (F) | many cyanobacteria (F), algae | nd | nd | [5] |
| *Fischerella* CENA 19 (F) | *Microcystis* spp., *Synechococcus* PCC7942 (both F) | fischerellin A, 12-epi-hapalindole F | nd | [48] |
| *F. muscicola* UTEX 1829 (F) | *Anabaena* PCC7120 and P-9, *Synechococcus* PCC6911 (all F) | fischerellin A | PSII inhib. | [45] |
| *F. muscicola* UTEX 1829 (F) | cyanobacteria (F), some algae but not eubacteria | fischerellin | PSII inhib. | [44] |
| *F. muscicola* UTEX 1829 (F) | *Anabaena* sp P9 (F), algae, plants | fischerellin A | PSII inhib. | [47] |
| | *Oscillatoria* sp., *Anabaena flos-aquae*, *F. muscicola* (all F), bacteria and algae | | | [46] |
| *F. muscicola* UTEX 1829 (F) | many cyanobacteria | antibiotics | nd | [89] |
| *Hapalosiphon intricatus* | *Anabaena* sp.(F) | hapalindole | nd | [11] |
| *Hapalosiphon fontinalis*(S) | cyanobacteria (F) and fungi | hapalindole A | nd | [13] |

[a]nd.- not determined; ?- Authors are not sure about the mode of action; MC- microcystins; PSII inhib. -photosystem II inhibition

[b]traditional taxonomy into subsections according Bergey's Manual.

[c]source of isolation of cyanobacteria: S - Soil, Sr - Soil from rice field, F - Freshwater, M - Marine, B - Brackish and/or Estuarine.

[d]inhibition of *M. aeruginosa* at the highest experimental concentration (25.000 µg $l^{-1}$), no effect at environmentally relevant concentrations (1-10 µg $l^{-1}$).

[e]planktopeptin BL1125, anabaenopeptin B and anabaenopeptin F.

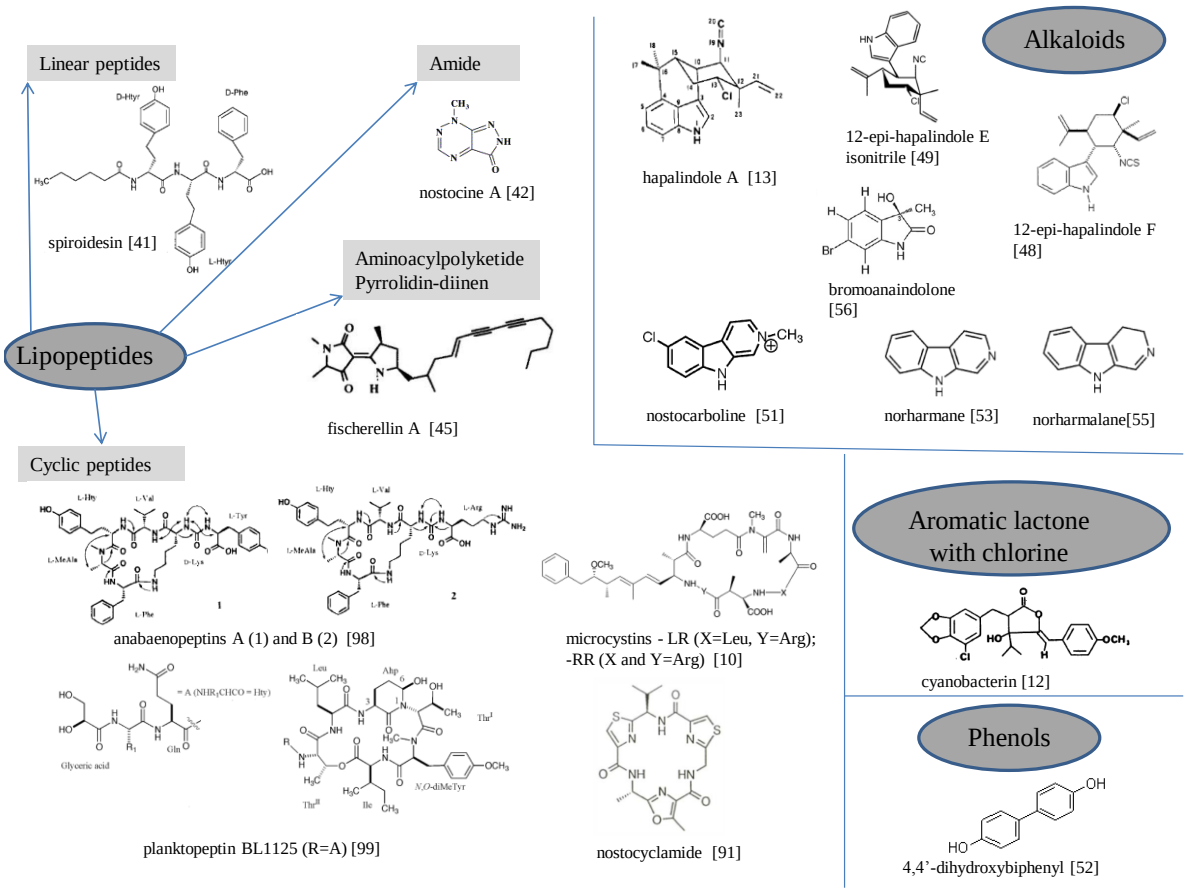


Figure 1. Chemical structures of cyanobactericidal allelochemicals and potential allelochemicals produced by cyanobacteria. References are cited between brackets.

However, the role of MCs as allelochemicals is disputed. Jüttner and Lüthi [22] showed that MCs were not exuded by living cyanobacteria but accumulated inside the cells, primarily bound to proteins, and therefore should not harm other organisms as long the producer is alive. Furthermore, Babica et al. [23] did not detect any effect of MCs on the growth of several chlorophytes using environmentally relevant MCs levels. Though, when high concentrations of MC-RR (for chemical structure see Figure 1), which were far above those found in the environment, were released into the medium, growth inhibition of *M. aeruginosa* (and also chlorophyta) was observed (Table 1). There is not yet a consensus on the biological role of MCs, however one of the suggested roles — defense against zooplankton grazers (which some authors include in the concept of allelopathy) — has been excluded by phylogenetic analysis [8, 24]. Another suggestion is that MCs could play a role as intracellular metal chelators. Humble et al. [25] found that they have moderate metal-binding properties. Schatz et al. [26] proposed a novel possibility: that MCs released into the medium by cell lysis act as info-chemicals to *Microcystis*, since the authors observed that the levels of intracellular MCs increased with the increase of MC concentration in the surrounding medium. Moreover, in the response of *Microcystis* to oxidative stress, MC binds covalently to specific proteins delaying their degradation, which would promote greater cellular resistance to this kind of stress [27]. Sedmak and Eleršek [28] observed morphological and physiological changes such as cell aggregations, increased cell volumes, and overproduction of photosynthetic pigments caused by environmentally feasible concentration of MCs (MC-RR, MC-LR, and MC-YR) in both microcystin-producing and nonproducing *M. aeruginosa* strains. Other recent study has confirmed that MCs stimulates colony formation in some species of the *Microcystis* genus, increasing the production of extracellular polysaccharides by inducing the correspondent gene expression, an effect mediated by its presence in the extracellular medium [29].

What are the effects and modes of action of MCs on other cyanobacteria? Microcystins, mostly MC-RR, caused inhibition of phytosystem II in different cyanobacterial genera [30,31,32]. Hu et al. [30] also observed negative effects of MC-RR on *Synechococcus elongatus* evident from inhibition of chlorophyll-*a* and phycocyanin synthesis, changes in protein and carbohydrate concentrations and in nitrate reductase activity. Results obtained in a subsequent study [31] suggested that oxidative stress manifested by elevated reactive oxygen species (ROS) levels and malondialdehyde content might be responsible for the toxicity of MC-RR to *S. elongatus*. Additionally, the cyanobacterium *Aphanizomenon* sp. when exposed to MC-RR showed morphological changes such as filament disintegration, cellular inclusions, and substantial damage of cell membranes, which resulted in cell lysis [32]. Li et al. [33] confirmed that MC-RR caused oxidative stress in *Synechocystis* sp. and showed an increase in gene expression of antioxidant enzymes that might protect the attacked cells from the oxidative damage. The few publications concerning MC-LR showed that purified toxin from freshwater bloom-forming *M. aeruginosa* inhibited completely the growth of *Nostoc muscorum* and *Anabaena* sp., photosynthetic processes and nitrogenase activity were significantly reduced [34].

Briand and coauthors [35] observed that MC-producing strains of *Planktothrix agardhii* out-competed the non-MC-producing ones, when environmental conditions limited cell growth. This suggested that, under growth-limiting conditions, the benefits of producing MC outweigh the cost. Recently, Bartova et al. [36] tested a semi-purified *Microcystis* extract containing MCs on cell differentiation of filamentous cyanobacterium *Trichormus variabilis*.

As a result, heterocyst and akinete formation significantly decreased. However, the molecular background of this effect remains a mystery. Up to now, the ecological role and bioactivity of MCs in aquatic systems are still uncertain and represent a challenging field for further studies. *Microcystis* produces other secondary metabolites as kasumigamide, microcin, microcarborin A, which may have allelopathic functions [8], but their effect has not been (until now) tested on cyanobacteria. Bloom forming *Planktothrix rubescens* produces three other apparently "non-toxic" cyclic peptides: planktopeptin BL1125, anabaenopeptin B and anabaenopeptin F (Figure 1). Fascinatingly, when these chemicals are released into the water environment, they interfere with the metabolism of *Microcystis* spp. and activate the lytic cycle of temperate cyanobacterial viruses (or virus-like particles) present in the *Microcystis* cell, which causes host cell lysis [37].

These results were confirmed by Sedmak and his colleagues [38] in their subsequent study, where the release of virus-like particles induced by planktopeptin BL1125 were detected and analyzed with transmission electron microscopy and cyanobacterial cytoskeleton immunostaining.

Another cyclic peptide, nostocyclamide, had a pronounced effect on the morphology of *Anabaena*. The filaments became short and contained only a few cells, which were swollen and much wider than normal [39]. Nostocyclamide M has a similar basic structure to nostocyclamide, but the valine moiety is replaced by methionine [40]. Despite the knowledge of its chemical structure and probable biosynthetic pathway, the mechanism of inhibition caused by this allelochemical is not known.

The only linear peptide among cyanobactericidal allelochemicals is spiroidesin (Figure 1). It inhibits the cell growth of *Microcystis aeruginosa* [41], however, the mode of this inhibition remains to be explored.

Freshwater cyanobacterium *Nostoc spongiaeforme* produces a violet pigment with an amide structure - nostocine A, which inhibits a broad spectrum of organisms (some bacteria; algae; plant and animal cells) [42]. Because of this wide growth inhibition effect it was suggested that this compound should be classified as a cyanotoxin. A subsequent study showed that the nostocine A inhibitory effect tends to be stronger towards green algae than towards the tested cyanobacteria. Moreover, this chemical seemed to cause oxidative stress in the target microbes accelerating the generation of ROS such as $O_2^-$ [43].

The secondary metabolite fischerellin (Fs or FsA for fischerellin A), named after its source of isolation – the cyanobacterium *Fischerella muscicola*, strongly inhibited the electron flow in photosystem II in cyanobacteria [44]. The chemical properties of FsA have been described and its inhibitory effects on photosystem II were further tested on cyanobacteria and algae by Hagmann and Jüttner [45]. Srivastavas and colleagues [46,47] continued to assay this allelochemical and discovered that Fs affects the fluorescence transients, as well as $O_2$ evolution by the cyanobacterium *Anabaena* sp. Lately, genes encoding nonribosomal peptide synthetases and a polyketide synthase that may be involved in the biosynthesis of FsA and another allelochemical (12-epi-hapalindole F) produced by a strain of *Fischerella* sp. have been identified [48].

The above mentioned 12-epi-hapalindole F belongs to the second most numerous group of allelochemicals - the alkaloids (Figure 1). This toxic metabolite kills a range of organisms and cell types, including Gram + and Gram - bacteria, cyanobacteria, fungi, eukaryotic green algae, protozoa and mammalian cells [49].

To elucidate the modus operandi of hapalindoles, Doan et al. [50] tested these chemicals on bacteria (*Bacillus subtilis* and *Escherichia coli*) and observed inhibition of RNA synthesis.

Lately, Gantar and colleagues [5] detected ultrastructural damage and phytosynthesis inhibition caused by hapalindoles in the algae *Chlamydomonas* sp. The cellular basis of the allelochemical effect observed upon bacteria and algae, may be the same for cyanobacteria. Blom et al. [51] isolated another alkaloid, nostocarboline, produced by *Nostoc* sp. and detected its algicidal effect. Moreover, seven nostocarboline derivates have been synthesized and their inhibitory effect towards cyanobacteria and algae observed. Because these compounds did not have a negative effect when applied on cyanobacteria in darkness, photosystem inhibition has been suggested as a possible mode of action.

By using a diffusion test in agar, cultures of *Nodularia harveyana* and *Nostoc insulare* were found to strongly inhibit several cyanobacteria [52]. The cyanobactericidal metabolite in the *N. harveyana* medium was identified as the indole alkaloid norharmane and in the *Nostoc insulare* medium, the phenolic compound was determined as 4,4'-dihydroxybiphenyl.

Both isolated compounds exhibited cytotoxicity towards *Arthrospira laxissima, Chroococcus minutes, Nostoc carneum* and *Synechocystis aquatilis* [53]. In a subsequent work, norharmane was also found among the exometabolites of *N. insulare* [54]. A similar compound to norharmane named norharmalane was later isolated from *N. harveyana* culture media [55]. The cellular basis of the negative effect of these three allelochemicals on cyanobacteria needs further research.

Recently, Volk and colleagues [56] identified a new brominated indole alkaloid, designated as bromoanaindolone, isolated from the culture media of the cyanobacterium *Anabaena constricta*. This alkaloid showed bactericidal and cyanotoxic properties. The authors speculated whether this exometabolite may act as an allelopathic substance, and may give an advantage to *A. constricta* in competition with microorganisms in the same habitat, as has previously been proposed for other antimicrobial exometabolites of cyanobacteria [8, 37, 53, 54, 57].

Examination of Table 1 reveals that many studies concerning cyanobactericidal compounds produced by cyanobacteria have been published, but the effective chemicals remain to be isolated and identified, in most cases. Surely, these secondary metabolites have a potential ecological role as allelochemicals, yet more ecologically relevant studies are needed to confirm such a role.

## POSSIBLE USE OF ALLELOPATHIC CYANOBACTERIA IN BIOCONTROL OF CYANOBACTERIAL BLOOMS?

In freshwater, brackish and coastal marine ecosystems, under certain favorable conditions, cyanobacteria can multiply very rapidly and reach visible accumulations of high cell densities. This phenomenon is referred to as "cyanobacterial blooms". Freshwater bloom taxa, members of the orders Nostocales and Chroococcales including *Anabaena*, *Nostoc*, *Aphanizomenon*, *Microcystis* and *Nodularia* species, exhibit severe toxicity to a broad spectrum of organisms including animals (neuro-, cyto- and hepatotoxicity). Blooms of marine cyanobacteria are also becoming an increasingly familiar occurrence, mainly within the tropical and sub-tropical regions. Bloom taxa, members of the orders Chroococcales and

Oscillatoriales, including *Synechocystis*, *Hormothamnion*, *Oscillatoria* and *Lyngbya* species, grow along shallow, sheltered, back reef zones and periodically develop into dense blooms that often wash ashore and accumulate *en masse* (as reviewed in[10]). The occurrence of these harmful cyanobacterial blooms (HCBs) (toxic, food-web altering, hypoxia generating) has become a worldwide problem and represents a serious threat to the use and sustainability of our water resources. Moreover, the frequency and intensity of the HCB formations have increased within the last few decades mainly owing to anthropogenic excessive release of chemicals into the environment resulting in eutrophication of surface waters [21].

The methods and techniques to control these unwanted HCBs can be mechanical (physical), chemical or biological and have been reviewed by many authors [3,58,59,60,61,62,63,64]. Since the release of secondary effective metabolites is widespread and an important mechanism to harm cyanobacteria [58], allelopathy could represent a possible method in biocontrol of HCBs. However, the role of allelopathy in the HCBs is disputed and still an unclear topic. Firstly, how can some species dominate the whole cyanobacterial community? And secondly, what factors affect the dynamics of the aquatic cyanobacterial communities and the formation and disappearance of blooms?

Generally, the persistence of a species depends on its competitive capacity and species succession has often been explained as a consequence of competition. A species that produces allelopathic compounds would have an advantage over its competitors as discussed by Wolfe [65] in the case of algal blooms. Thus, allelopathy, resulting in increased competition, could at least partly explain species succession. To support this statement, several cases have been well described where cyanobacterial and/or algal succession and the formation of blooms were related to the production of allelopathic compounds [66, 67]. In the case of cyanobacteria, Keating [57, 68] combined field observations and laboratory studies and revealed that allelopathic interactions might be implicated in the establishment of successive blooms in a eutrophic lake. Cyanobacteria that were dominant could inhibit both their predecessors and their successors. In rivers and streams, where cyanobacterial exudates may be rapidly carried away by the current, allelopathy may be less important in planktonic communities, although it can still be a factor in benthic communities [7].

Bagchi et al. [69] originally proposed that natural algicides could effectively be applied in order to control HCBs, but until now this possibility is still speculative. Moreover, different opinions have been presented, e.g. Oberhaus et al. [1] suggested that allelopathy could have little effect on competition dynamics at the onset of a cyanobacterial bloom. But the ability to produce allelopathic substances, according to Keating [57], could have a greater influence on bloom maintenance, when biomasses are higher and allelochemical concentrations greater, thus influencing competition with other components of phytoplankton as well as among different strains of the bloom-forming species.

Similarly, Jonsson et al. [70] looked for evidence that allelopathy might explain the initiation of blooms in a meta-analysis of recent experimental work and confirmed that, with few exceptions, allelopathic effects were only significant at very high cell densities, typical of blooms. Therefore, the role of allelopathy in bloom formation is rather doubted due to the lack of experimental support for allelopathy at pre-bloom densities [70].

In summation, different and more or less important ecological roles have been attributed to the production of allelochemicals by cyanobacteria, including phytoplankton succession, bloom formation, resource and interference competition [7], and invasive ecological fitness [71].

In order to understand the ecological significance of allelopathy in aquatic cyanobacterial communities, the environmental factors that may modulate allelopathic events have been characterized in several studies. The presence of competitors [67, 72, 73] and coexisting heterotrophic bacteria that degrade allelochemical substances [74] have been identified as biotic factors. Light intensity [75, 76], temperature [77], nutrient levels, and pH [35, 78, 79] have been shown to control allelochemical production in some cyanobacterial species [17].

How could the cyanobactericidal allelopathy be exploited in the biological control of HCBs?

One can consider two strategies in using allelochemicals in biocontrol of HCBs. The first would be to use the natural indigenous producers of allelochemicals as competitors of cyanobacteria causing HCBs and therefore to create the so called "cyanobacterium vs. cyanobacterium scenario". In this case, the biocontrol cyanobacterium should be specific enough to harm only the wished target causing the HCB. Another partial solution could be to substitute the ecologically harmful cyanobacterium with non-harmful competitors (e.g., toxic *Microcystis* sp. with non-toxic *Microcystis* sp. as showed by Kardinaal et al. [76] in light competition experiments). A further possibility could be to use microbial mutual consortia containing allelopathic cyanobacteria or algae to depress HCBs as well as other microbes (e.g., bacteria) capable to degrade toxins produced by HCBs as presented in the work of Wu and colleagues [80] with periphyton biofilms.

A second approach would be to apply an effective and specific allelochemical on the HCB. This has been the case in most studies where allelopathy has been tested using cell-free manipulations.

A good example of the possible application of cell-free allelochemicals against cyanobacterial blooms was demonstrated by Sedmak et al. [37]. The presence of cyclic peptides planktopeptin BL1125, anabaenopeptins B and F, produced by bloom forming non-toxic cyanobacteria, can provoke lysis of toxic *Microcystisaeruginosa* via the induction of virus-like particles. This effect implies a possible role of these peptides in the natural environment controlling cyanobacterial population density. Therefore, cyanobacteria with lysogenic viruses could act as hot-spots that, in the presence of cyanobacterial cyclic peptides, release numerous infectious particles. The process can be self-augmented with the simultaneous release of additional cyclic peptides from the producing lysogens, starting a forest fire effect that causes the collapse of the HCB [37].

## CONCLUSION

In this chapter, current knowledge has been summarized concerning negative allelopathy among cyanobacteria. The allelochemicals or potential allelochemicals of cyanobacterial origin exert their effects not only on cyanobacteria but also on a wide variety of other organisms. Many of these substances inhibit photosystem II, $N_2$ fixation, or cause oxidative stress in cyanobacteria. Furthermore, an overview of cyanobactericidal allelochemicals was presented. Despite all the studies mentioned, the role played by allelopathy in cyanobacterial ecology is still unclear. It is possible to relate allelochemicals to bloom formation, proliferation, succession or termination events. However, present knowledge is not sufficient to fully disprove or approve such link. Indeed, the production and release of allelopathic

metabolites may play several roles in the biology of cyanobacteria. Interdisciplinary approaches are required, considering both the biological and chemical characters of the allelopathic interactions integrating laboratory and field observations. Better understanding of allelopathy among cyanobacteria requires carefully designed and ecologically feasible experimental work as well as molecular approaches in order to provide further information on chemical structures and modes of action of allelochemicals and their ecological implications.

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*Chapter 3*

# THE BLOOM-FORMING CYANOBACTERIUM NODULARIA SPUMIGENA: A PECULIAR NITROGEN-FIXER IN THE BALTIC SEA FOOD WEBS

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## ABSTRACT

A peculiar feature of the Baltic Sea is the massive summer blooms of cyanobacteria. Various environmental, economic and sanitation repercussions of these blooms have attracted considerable attention among the scientific community, water management agencies and general public. Of particular concern is the increase in frequencies and amplitude of the hepatotoxic blooms of *Nodularia spumigena*. This is a planktonic, toxic, filamentous nitrogen-fixing cyanobacterium capable of forming massive blooms when environmental conditions are suitable. The toxicity of *N. spumigena* is mainly attributed to the production of the hepatotoxin nodularin, which is also a tumor promoter. Furthermore, through its nitrogen fixation activity, *N. spumigena* contributes significantly to the total annual nitrogen load in the Baltic Sea. Here, we highlight the physiological peculiarity of *N. spumigena* related to nitrogen fixation and heterocyst formation and how this may be regulating its ability to form blooms. We also discuss some key molecular and physiological aspects of the toxin production. Furthermore, we highlight the interactions between *N. spumigena* and its grazers in the food webs and potential effects of climate-related factors on these interactions. All these aspects are important to consider if we want to predict consequences of the eutrophication and global change for bloom proliferation and toxin production by *N. spumigena*.

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## INTRODUCTION

The Baltic Sea is the largest brackish water ecosystem in the world. Approximately, 16 million people live in nine countries surrounding the Sea and an approximately 85 million people live in the 14 countries in its catchment.

The Sea is composed of several basins and is connected to the North Sea through the narrow Skagerrak/Kattegat. Due to its semi-enclosed nature, water exchange is limited and there is a salinity gradient stretching from nearly fresh water (< 2) in the Bothnian Bay to > 20 in the Skagerrak. In the Baltic Proper, there is a permanent halocline between 30 m and 80 m that prevents vertical water mixing [1]. The deep water of the Baltic is displaced to a significant degree through non-frequent large inflows from the North Sea, while small but more frequent inflows are generally of minor importance. These peculiarities in the Baltic Sea morphometry and hydrology result in limited water exchange, long residence times, retaining of nutrients and organic matter within the Sea [2,3]. Sedimenting organic matter together with poor mixing generate anoxic bottom areas that have been reported to increase over the last century due to eutrophication and resulted in a severe decrease of macrobenthic communities below the halocline [4].

Starting from the mid-19th century, human population in the region expanded accompanied by expansion of industrial and agricultural activities. The Sea ecosystem changed as a result, with the most prominent anthropogenic impact being eutrophication caused by discharges of nutrients, especially of nitrogen and phosphorus.

Cyanobacterial blooms that occur every summer in the Baltic proper, and the Gulfs of Finland and Riga are one of the main problems caused by the Baltic eutrophication. The blooms affect recreation industry and tourism when blown ashore due to nuisance odors and scums forming under calm conditions. Moreover, the blooms can also be toxic and incidences of animal death and human illness have been reported.

The frequency and intensity of the blooms has increased [5,6], which is of public concern. On the other hand, there is evidence that such blooms have been a natural feature of the Baltic Sea since it became a brackish sea, about 7000 years ago [7].

In addition, the blooms fix nitrogen and add several hundred thousand tonnes of nitrogen to the Baltic Sea each summer, thus adding to its eutrophication [8] and facilitating bottom hypoxia [9]. Therefore, cyanobacteria are of high biogeochemical importance for this region. In general phytoplankton growth in the Baltic proper and the Gulf of Finland are nitrogen-limited except for the nitrogen ($N_2$)-fixing cyanobacteria [8,10,11], which are nitrogen sufficient through their growth period. During the peak of their seasonal development, $N_2$-fixing cyanobacteria suffer phosphorus limitation and slow down their growth to nearly zero [8]. During autumn, the joint-effects of phosphorus limitation, changes in some abiotic factors, such as light intensity, temperature and availability of nutrients and cyanophage attacks cause blooms to collapse [12,13,14].

The filamentous cyanobacteria *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena* spp. are important constituents of the cyanobacterial summer blooms in the Baltic Sea [15, 16]. Together, they can form kilometers-large blooms that are considered to be among the largest bloom-formations in the world. While *Aphanizomenon* is present in the water column during the whole year, *Nodularia* and *Anabaena* are present only during the warm summer period [17, 18].

All species are heterocystous nitrogen-fixers and contribute significantly to the nitrogen input into the Baltic Sea. Phylogenetic studies on planktonic *Nodularia* strains isolated from the Baltic Sea revealed that they all belong to the toxin-producing species *N. spumigena* [19].

To date, *N. spumigena* is the most studied filamentous cyanobacteria in the Baltic. To understand relative importance of various environmental factors on nitrogen fixation and toxin production, these studies have been facilitated by relatively easy maintenance of *Nodularia* strains in laboratory conditions. In comparison to *Nodularia*, the studies on *Aphanizomenon* and *Anabaena* spp. have been limited, which is in part due to the difficulty of isolating their strains.

## NITROGEN FIXATION AND HETEROCYST DIFFERENTIATION BY CYANOBACTERIA

### Nitrogen Assimilation by Cyanobacteria

Cyanobacteria mainly utilize inorganic nitrogen as ammonia, nitrate and nitrogen gas ($N_2$) as a nitrogen source to fulfill their nitrogen requirements [20,21]. Other organic nitrogen sources, such as urea and some amino acids, can also be used by some cyanobacterial species. In cyanobacteria, nitrogen assimilation occurs via the glutamine synthethase/glutamate synthase pathway (known as GS/GOGAT pathway).

Ammonia is generally the preferred source of nitrogen for cyanobacteria because it is assimilated directly via the GS/GOGAT pathway, while other sources need to be first converted into ammonia before they can be assimilated [22]. Glutamate is a major nitrogen donor for the biosynthesis of other nitrogen-containing metabolites, such as nucleotides, amino sugars and amino acids, and is itself a direct precursor of some amino acids and nitrogen-containing compounds such as chlorophyll [22]. A strong regulatory network operates in cyanobacteria at the level of ammonia assimilation and the operation of the GS/GOGAT pathway. To manage nitrogen assimilation efficiently, ammonia inhibits the expression and activity of several proteins involved in the assimilation of other nitrogen sources.

### Biological $N_2$- Fixation and the Nitrogenase Enzyme

Although $N_2$ is the most abundant gas in the earth atmosphere, it is directly available for assimilation only for the $N_2$-fixers, i.e., diazotrophs, such as some cyanobacterial species. By having an access to atmospheric $N_2$ pool, the diazotrophs gain a competitive advantage over the co-existing non-fixing autotrophs.

The reaction is catalyzed by a multimeric enzyme complex, the nitrogenase enzyme, and is highly energy demanding, requiring 16 ATP molecules for every $N_2$ molecule being reduced. The nitrogenase complex is composed of two metalloproteins: the dinitrogenase reductase and the dinitrogenase. The dinitrogenase reductase, also known as the iron protein, is a homodimer with a total molecular weight of approximately 60 kDa and is encoded by the *nifH* gene. The dinitrogenase component of the nitrogenase enzyme, also known as the Mo-Fe

protein, is a heterotetramer with a molecular weight of approximately 220 kDa and is encoded by *nifDK* gene. Both genes (*nifH* and *nifDK*) form one operon, called the *nifHDK* operon, which is highly conserved in cyanobacteria [23, 22].

Nitrogenase is irreversibly inactivated by oxygen [24]. The $N_2$-fixing cyanobacteria, living in aerobic environments and performing oxygenic photosynthesis, have developed strategies to protect the nitrogenase enzyme from the deleterious effect of oxygen. Unicellular and some filamentous cyanobacteria undergo temporal separation between photosynthesis and $N_2$- fixation, with the former performed during the day and the latter during the night [25,26]. Other filamentous cyanobacteria differentiate specialized cells, called heretocysts, for $N_2$- fixation, separating photosynthesis and $N_2$- fixation in space.

## Heterocyst Development

The heterocysts are terminally differentiated cells [27,28].Their sole known function is to spatially protect the nitrogenase enzyme under oxic conditions. They provide micro-anaerobic conditions to the oxygen–sensitive nitrogenase enzyme through the loss of PSII activity, formation of a thick cell envelope and increased rate of respiration [29,30,31]. Heterocysts are formed at semi-regular intervals along the vegetative filaments. The vegetative cells that are to become heterocysts are spatially selected and their pattern and frequency of formation are maintained through the subsequent growth of the filaments in nitrogen-deprived media. The frequency of heterocysts along the filaments varies between approximately 5-10 % depending on species. In some species, heterocysts are almost exclusively formed at the ends of the filaments. Heterocysts, lacking the activity of PSII, depend on neighboring vegetative cells to provide them with the carbohydrate required for their growth and function, while they provide the vegetative cells with fixed nitrogen.

Heterocyst differentiation involves the activation of many genes in a cascade-like mechanism of transcriptional activation [27]. The process has been thoroughly investigated mainly in three species that are considered "model cells": *Anabaena* PCC 7120 strain, *Nostoc punctiforme* and *Anabaena variabilis*. One of the key genes involved in nitrogen metabolism and heterocyst formation is *ntcA*. It encodes the transcription factor NtcA, which belongs to the cyclic AMP receptor family (CRP-family) of bacterial regulators [32]. NtcA activates the transcription of many genes involved in nitrogen metabolism including the *nifHDK* operon [33,34,35] and its activity is required for the development and function of mature heterocysts. NtcA also indirectly activates the expression of *hetR*, the master regulator of heterocyst differentiation. *hetR* encodes HetR, a serine-type protease with DNA binding activity and is expressed early during heterocyst differentiation. HetR is crucial to the differentiation process and mutations in *hetR* block early steps in the differentiation process [36,27]. *hetR* is positively autoregulated and has a positive effect on *ntcA* transcription [37,38].

Addition of an exogenous nitrogen source, such as ammonia, usually stops $N_2$-fixation activity and inhibits heterocyst differentiation [29,20,39]. There have been some reports of heterocyst differentiation and subsequent $N_2$-fixation during nitrogen supplementation but not differentiation that is not associated to $N_2$-fixation activity [40,41,42].The signaling pathway of this response is not fully understood, but the cells seem to sense the carbon/nitrogen balance and redox status of the cells [20,21].

## Nitrogen Fixation and Heterocyst Differentiation in Nodularia Spumigena

Although *N. spumigena* is ecologically important in the Baltic Sea, where it contributes substantially to both primary production and the annual nitrogen input. Few studies have addressed the physiology and genetics of $N_2$- fixation and heterocyst differentiation in this cyanobacterium. To date, most studies have focused on environmental conditions promoting bloom formation in general, and toxin production in particular.

In general, ammonia and nitrate inhibit $N_2$-fixation in cyanobacteria, with nitrate being less effective and strain dependent [43,44,45,46]. This is because, as explained above, ammonia is readily incorporated into the GS/GOGAT pathway while nitrate needs to be first reduced to ammonia before entering the pathway. In the Baltic *N. spumigena* strain AV1, ammonia was found to inhibit $N_2$- fixation activity [14,46,47], but not to sustain growth for a long period of time [46,47]. Indeed, unlike "model" cyanobacteria, there are no reports showing clearly that Baltic strains of *N. spumigena* can grow for several generations by utilizing ammonia in the absence of $N_2$-fixation activity [46]. In addition, it was also found that nitrate does not exert any negative effect on $N_2$-fixation activity [14,46,47]. Accordingly, *N. spumigena* seems unable to utilize neither ammonia nor nitrate to support growth. Thus, the Baltic cyanobacterium *N. spumigena* seems to be an "obligatory" $N_2$-fixer, if we can use such a term in this context. Expression analysis of *nifH* transcription has confirmed the results obtained through measuring growth and $N_2$-fixation activity and showed that expression of *nifH* responds negatively to ammonia supplementation and does not respond to nitrate supplementation in *N. spumigena* stain AV1 [46,47]. In a mesocosm experiment conducted by Vuorio et al. [48], it was demonstrated that *N. spumgiena* enriched with dissolved inorganic nitrogen (DIN) did not show any response while *Anabaena* spp. grew better. In synthesis, it seems that the Baltic Sea *N. spumigena* is not as efficient in DIN uptake/assimilation as other "model" cyanobacteria. This partially explains the observation that blooms of *N. spumigena* in the Baltic Sea develop when nitrogen is limited and are more affected by abiotic factors, such as temperature, salinity and phosphorus level.

Investigations about heterocyst formation and frequency in Baltic Sea *N. spumigena* strain AV1 in the presence of ammonia has revealed that the filaments form heterocysts and maintain heterocyst frequency in the presence of ammonia and in the absence of any detectable $N_2$-fixation activity [46,47]. Moreover, the expression patterns of *ntcA*, the key regulator of nitrogen metabolism, and *hetR*, the master regulator of heterocyst differentiation, were not affected [46]. Thus, Baltic Sea *N. spumigena* strain AV1 exhibits what Vintila and El-Shehawy [46] called "uncoupling" between $N_2$-fixation and heterocyst differentiation. As far as we know, such a behavior was never reported in any of the previously studied heterocystous cyanobacteria.

A detailed proteomic analysis of a Baltic Sea *N. spumigena* strain AV1 during ammonia supplementation has revealed that the cells exhibit low energy metabolism and carbon fixation [49]. The authors suggested that the inefficiency of the Baltic Sea *N. spumigena* in utilizing ammonia as an external nitrogen source could be due to ammonia toxicity that causes damage to PSII [49]. In absence of a repair mechanism, energy production goes down, leading to lower carbon fixation and lower growth rate [49]. It was previously suggested that the toxicity of ammonia to cyanobacteria may be due to rapid photo-damage to PSII [50]. Proteomic analysis thus confirmed the results obtained from the transcriptional analysis, morphological observations, and activity measurements, and showed that *N. spumigena* is not

efficient in utilizing externally supplied ammonia or nitrate. Accordingly, Baltic Sea *N. spumigena* does not replace the energy-intensive $N_2$-fixation by assimilation of DIN and continues to form heterocysts in the presence of DIN and in the absence of any detectable $N_2$-activity.

It is then important to investigate if such "uncoupling" behavior is characteristic to the species *N. spumigena* or only to the strains living in the Baltic Sea. Vintila and El-Shehawy [47] have analyzed the response of three non-Baltic Sea *N. spumigena* isolates (two strains were isolated in Australia and one strain was isolated in Canada) as well as five Baltic Sea isolates. Their results demonstrated that the non-Baltic Sea isolates exhibited a classical response to ammonia supplementation; cessation of $N_2$-fixation activity and loss of heterocysts, while the Baltic Sea isolates exhibited the "uncoupling" response [47].

There is a clear need to analyze more isolates before drawing a general conclusion. However, for now we can ask the question: what makes Baltic Sea *N. spumigena* peculiar? Is it the variations in its genetic background? Is it a genome reduction? Or is it impairment in the regulatory network at the level of nitrogen/carbon metabolism?

Analysis of *hetR* sequence of the Baltic Sea *N. spumigena* strain CCY9141 versus the sequence of the same gene in *Anabaena variabilis*, *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* revealed that there are three amino acids substituted in *hetR* of *Nodularia* versus the other *hetR* sequences. Nevertheless, these amino acids were not shown to be among the ones that are required for *hetR* function in *Anabaena* sp. PCC 7120 [51,46]. Moreover, analysis of *N. spumigena* CCY9141 genome revealed the presence of *amt*, *nir*, *nar* and *nrtP*, which code for ammonium permease, nitrite reductase, nitrate reductase and nitrate/nitrite permease, respectively. Hence the peculiarity of *N. spumigena* is not likely to be due to variations in the genetic background of Baltic Sea *N. spumigena* in regards to *hetR* sequence or as a result of a genome reduction in N uptake/assimilation machinery.

During heterocyst development, DNA-rearrangements take place within the nif-operon. In vegetative cells of *Anabaena* sp. PCC 7120, *Anabaena variabilis* and *Nostoc punctiforme*, the *nifD* gene is interrupted by an excision element that varies in size depending on species [52, 53,54]. During late stages in heterocyst differentiation, site-specific recombination between repeated sequences at the ends of the excision element causes deletion of the element leading to the transcription of *nifH*. Vintila et al. [55] have found that, unlike "model" cyanobacteria, the Baltic Sea *N. spumigena* contains an insertion element in the *nifH* of the *nifHDK* operon and an operating DNA rearrangement mechanism. They showed that this insertion element is present only in the tested Baltic Sea strains that exhibit "uncoupling" between $N_2$-fixation and heterocyst differentiation, while it is absent in the tested non-Baltic Sea strains. Furthermore, they have analyzed NifH protein using Western Blot and 2-D electrophoresis coupled to Maldi TOF/TOF and demonstrated the unusual presence of three NifH protein bands.

Two of these bands seem to be a result of an unknown modification in response to the light/dark growth regime, while the third seems to be a membrane bound and does not respond to the light regime. The presence of two NifH protein forms that are modified have been previously reported in nitrogen fixing cyanobacteria [56,57,58], while membrane bound nitrogenase was not demonstrated. In fact some studies have demonstrated that nitrogenase is randomly distributed in heterocyst [59, 60], nevertheless, localization of nitrogenase close to the membranes that are filling up heterocyst cannot be excluded.

Altogether, the data gathered demonstrate that the regulatory circuit regulating $N_2$-fixation in the Baltic Sea *N. spumigena* seems, at least in some key features, different than what is known to operate in "model" cyanobacteria [5].

## Toxin Production by Nodularia Spumigena

*N. spumigena* produces nodularin, a hepatotoxin and tumor promoter. The toxicity of nodularin is due to the inhibition of the eukaryotic protein phosphatase 1 and 2A [61,62]. Nodularin consists of 3-amino-9-methoxy-2,6,8-trimetyl-10-phenyl-4,6-decadienoic acid (Adda), D-glutamic acid (D-Glu), N-methyldehydrobutyrine (MeDhb), D-erythro-β-methylaspartic acid (D-MeAsp) and L-arginine (L-Arg) [63]. Nodularin is a cyclic pentapeptide and is produced non-ribosomally by a large multi-enzyme complex composed of non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) modules and tailoring enzymes [64,65].The nodularin synthetase gene cluster, or nda-cluster, consists of nine open reading frames (ORFs) that is transcribed from a bidirectional promoter [65]. The ndaCDEF region encodes enzymes that seem to be responsible for the production of Adda, and the ndaFGHAB region encodes the enzymes responsible for peptide synthesis, cyclization and transport [65].

Several studies have investigated the effect of environmental factors such as temperature, salinity, radiation and nutrient concentration on growth and nodularin production by *Nodularia spumigena* [66,67, 68,69,70]. In general, it was found that the production of nodularin is optimum at optimum cell growth [66].

Very few studies have addressed the molecular biology of the nda cluster. Understanding the regulatory mechanism controlling toxin biosynthesis and the up-stream signaling pathway is crucial to a comprehensive understanding of nodularin production [71,72]. Real Time PCR analysis of the nine *nda* genes constituting the nda cluster in *N. spumigena* strain AV1 has revealed that the cluster is constitutively transcribed [71]. The level of transcription decreased in the presence of ammonia and increased under phosphate starvation. The intracellular and extracellular nodularin concentrations during these experiments did not vary significantly [71]. The authors suggested that the cells maintain a threshold level of nodularin inside them. The maintenance of such a threshold level is achieved through 1) the down-regulation (possibly through internal degradation or active transport) of the excess nodularin resulting from the continuous expression of the nda cluster, and/or through 2) a transcriptional regulatory mechanism acting on the level of enzyme activity and regulating nodularin biosynthesis/maturation. Other studies have suggested that most of the nodularin produced remains inside the cells during the exponential phase while it is released during the stationary phase by cell lysis [73,74,75]. Furthermore, Pattanaik et al. [76] have found a significant correlation between intracellular and extracellular nodularin concentrations and suggested that accumulation and release of nodularin are dependent on each other. Basically all the above studies suggest that nodularin remains inside the cells under optimum growth phase during exponential growth; changes of environmental conditions or shifting to a stationary phase of growth causes nodularin to be released to the environment either by positive influence of environmental changes on the active release of nodularin (during exponential growth) or by cell lysis (during stationary growth). During exponential growth, the cells seem to control the internal level of nodularin through internal degradation/active transport, and/or

through regulating enzymatic activity during biosynthesis/maturation. Either mechanisms of control still need to be identified.

Kankaanpää et al. [77] have found that NOD-R (a variant of nodularin) is present as dissolved in the senescent blooms, and is rapidly degraded in the water column suggesting the operation of a microbial biodegradation process. On the other hand, Mazur-Marzec et al. [78] found high concentrations of nodularin surface sediments after months of a cyanobacterial bloom, indicating that natural degradation of nodularin is slow, which also has implications for benthic surface-feeding fauna and bioaccumulation in the food web. Several bacterial species with the ability to degrade microcystin and nodularin have been isolated from freshwater bodies experiencing cyanobacterial blooms [79,80,81]. The genes responsible for the biodegradation process have been identified and characterized in the genus *Sphingomonas* sp. [79]. Degradation rates of microcystin and nodularin vary, most likely, depending on the chemical structure of the toxin variants with microcystin-RR and nodularin showing the lowest rate of degradation [81]. To the best of our knowledge, no bacteria or any other microorganism was isolated from the Baltic Sea with nodularin degrading activity. Furthermore, it was demonstrated that nodularin was degraded under high UVR-R in cellular extract, while intracellular nodularin was resistant to photodegradation [82]. Cyanobacteria produce some compounds, such as mycosporin-like amino acids (MAAs), carotenoids and scytonemin [83] that are known to protect against UV-B damage, as well as enzymes and metabolites that are protecting and repairing against oxidative stress. Interestingly, Ziliges et al. [84] suggested that microcystin-LR functions as a protein-binding peptide that protects some cellular proteins against the harmful effect of oxidative stress caused by high UVR levels.

In conclusion, the slow degradation of nodularin in nature, the protection from photodamage, and the maintenance of an intracellular nodularin level, suggest that nodularin, just like microcystin, may be important to certain biological function(s). So far, studies on the biological function(s) of cyanotoxins have been focused mainly on microcystin and several biological functions have been suggested, yet all of them need to be confirmed by further studying other cyanotoxins especially nodularin which shares structural similarities with microcystin [85].

## Trophic Interactions between Nodularia Spumigena and Grazers

### *Feeding on Nodularia Spumigena: Laboratory and Field Evidence*

Grazing mortality of *Nodularia* is often considered negligible. This, however, is highly debatable, although contribution of the cyanobacterium in the diets of zooplankton and benthic species, rates of consumption and factors regulating grazer-*Nodularia* interactions are still poorly understood.

There is little consensus regarding the most important consumers of *N. spumigena* in the Baltic Sea and the effects that the cyanobacterium exerts on its consumers. Whereas some zooplankton (cladocerans: [86]; copepods: [87]; mysid shrimps: [88,89]) and benthic (ostracods and nematodes [90]) grazers ingest *N. spumigena* with no apparent harm, others show signs of oxidative stress (mussels: [91,92]; fish: [93]), suffer decreased growth (fish: [93]), feeding (amphipods: [94]), offspring production and abnormalities in gonad

development and egg sacks (copepods:[95-98]; amphipods: [94]), and increased mortality (copepods:[96,98]; amphipods: [94]).

The harmful effects of *N. spumigena* on various crustaceans, such as copepods and gammarids [96,98,94] and small fish [100] observed in feeding experiments are related to the presence of the toxin. Some studies have reported no or very little grazing by zooplankton [95,96,88]. However, most of this evidence is derived from feeding experiments that may suffer numerous artifacts arising from lengthy bottle incubations, inadequate food variability in the feeding mixtures, handling stress, and uncertainties with consumption rate estimates based on cell counts, etc. [101,102].

Alternative methods based on stable isotopes, fatty acids and molecular diet analysis [103,89, 87,104] for studying zooplankton feeding have convincingly shown that many Baltic species have relatively high *in situ* grazing and growth rates on toxic cyanobacteria, including *N. spumigena*. These data are particularly valuable because they provide information on naturally occurring consumption and assimilation under ambient conditions. Field observations show that cyanobacteria blooms attract a wide range of small organisms, including metazooplankton [105,106] and may have a positive effect on their nutrition and growth. As a substantial part of the fixed nitrogen is leaking out from *N. spumigena* (Figure 1), the cyanobacterium colonies comprise highly productive microenvironments and an attractive nitrogen microenvironment to be utilized by other organisms in the Baltic Sea during the nitrogen limitation [107]. Indeed, in the Baltic Sea, where nitrogen limitation is common during most part of the growth season, tolerance to nodularin may convey a selective advantage by allowing primary consumers to feed on nitrogen fixing phytoplankton. This would provide otherwise limited nutrient and relaxes intra- and interspecific competition for other preys [89].

From this point of view, Baltic filamentous cyanobacteria, including *N. spumigena*, are an attractive food source as they generally have higher nitrogen content than non-diazotrophic phytoplankton [108]. Moreover, recent reviews (e.g., [109]) suggest that cyanobacteria are a valuable source of a variety of compounds, such as polysaccharides, lipids, proteins, vitamins, sterols, and enzymes, which are in high demand in aquatic food webs. However, filamentous cyanobacteria are generally considered a poor food source compared with eukaryotic marine plants, due to their toxic nature, morphology (i.e., large colonies that are difficult to manage), and inadequate nutritional content [110].

In particular, the lack of essential fatty acids in cyanobacterial cells and the size of their filaments reduce their value to consumers. However, high ingestion of toxic *N. spumigena* [97,111] suggests that the animals might cope with ingested toxins and suboptimal nutrition quality of cyanobacteria in general and *N. spumigena* in particular, when feeding on mixed assemblages [87,111].

The microorganisms colonizing cyanobacteria filaments and relying on nitrogen are likely to complement *N. spumigena* in terms of its nutritional value for the consumers and degrade toxins, at least partially. Therefore, the attractiveness of cyanobacteria as food for mesozooplankton increases in the late stage of the bloom, when the filaments are colonized by heterotrophic bacteria, flagellates and ciliates [106].

By utilizing production of *N. spumigena* and other filamentous cyanobacteria, these grazers enhance secondary productivity of the system and may affect food availability for larval, planktivorous and benthic fish. This, however, would depend largely on the enhanced production that might be derived from cyanobacterial addition to the diet.

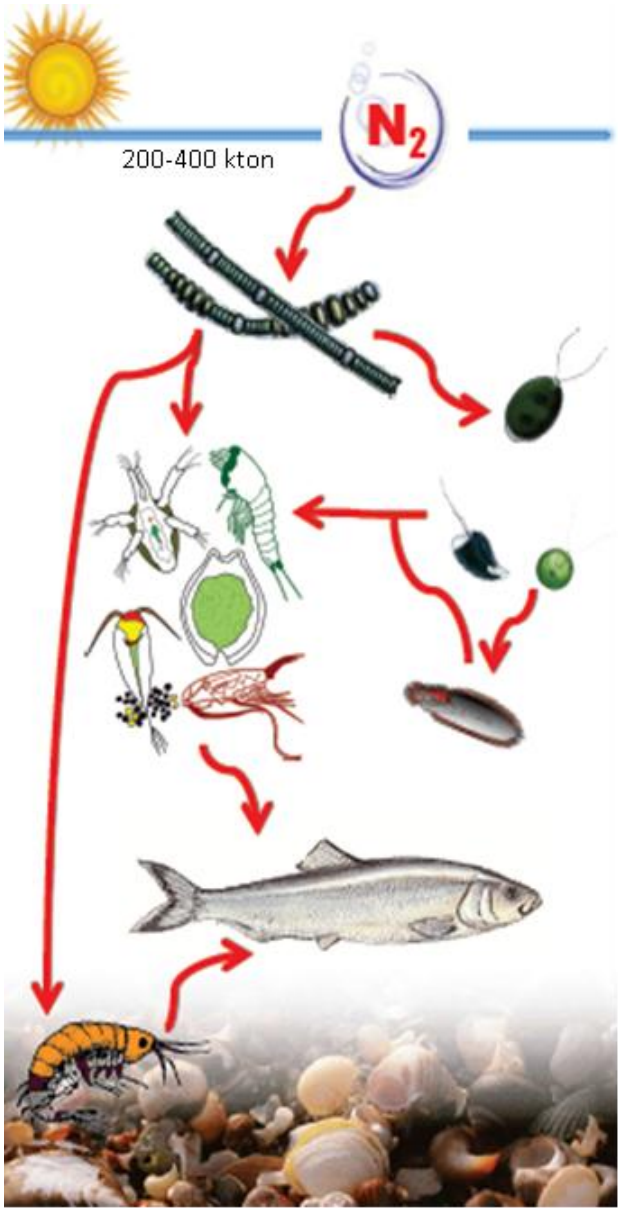


Figure 1. Plausible role of diazotrophic cyanobacteria in maintaining secondary production. In the Baltic Proper, filamentous diazotrophic Nodularia spumigena fix yearly ~200-400 kton N and may play an important role in the N cycle by providing fixed nitrogen to the food webs. Much of the secondary production accompanying blooms is based on consumption of phytoplankton and bacteria other than nitrogen-fixing cyanobacteria. Modified from Larsson et al. [8].

### *Nodularin Transfer in the Food Web*

Most studies have addressed the effects of cyanobacteria on feeding in aquatic animals, whereas linkages between toxin concentrations in consumers and cyanobacteria are less studied. From ingested *N. spumigena*, nodularin is incorporated into consumer' body tissues [97,112,113,114], partly metabolized and partly egested; in case of copepods and mysids, the egested part can be determined by analyzing fecal pellets [115,112]. From zooplankton

grazers, nodularin can be transferred to the higher trophic levels, as demonstrated in mysids [116,], mussels [78], fish [78,117,114,118], and sea birds [119].

Species-specific variations in nodularin content of zooplankton reflect variations in feeding preferences and vertical distributions in the water column. For example, nodularin levels in the copepod *Eurytemora affinis*, which grazes directly on *N. spumigena* [120] and inhabits the surface layers of the water column where cyanobacterial blooms occur, were higher compared to the copepod *Acartia* spp., which can avoid cyanobacteria by selective feeding [97,113]. In zooplankton inhabiting the northern Baltic proper, average concentration of 0.07±0.01 μg $g^{-1}$ wet weight was observed [112], whereas for some species (i.e., onychopod cladoceran *Pleopis polyphemoides*) as much as 2.36 μg $g^{-1}$ wet weight of these toxins were detected [114]. Benthic organisms in the Baltic Sea, such as clams, and deposit feeders like the blue mussel *Mytilus edulis* accumulate the highest concentration of nodularin [121,122]. Bottom feeders, such as flounder, also accumulate substantial amounts of nodularin in their liver [123, 78]. However, there appears to be a biodilution rather than a biomagnification of nodularin as less than 1% is transferred from one trophic level to another due to toxin degradation at every level [124], which is similar to microcystin behaviour in multiple trophic transfers [125].

In addition to direct consumption of *N. spumigena*, aquatic animals can acquire nodularin via other pathways. Karjalainen et al. [126] suggested that metazooplankters may obtain dissolved nodularin directly from the water or via ciliates and microbial loop organisms (bacteria, heterotrophic nanoflagellates), which in turn are able to take up dissolved nodularin. Generally, the microbial loop is an important link in the transfer of dissolved organic matter from bacteria via hetero- and mixotrophic flagellates and ciliates to crustacean zooplankton [127]. During the *N. spumigena* bloom, production of heterotrophic bacteria and picoautotrophs increases significantly [128], partly due to the favorable microenvironments provided by the colonies leaking nitrogen [107] and colonization of the cyanobacterial filaments by microorganisms, mainly bacteria, protozoa and diatoms. Interestingly, Tuomainen et al. [129] reported that *Nodularia* aggregates were colonized by various bacteria, including those whose 16S rDNA sequence have not been yet described. The bacteria-sized organisms in these assemblages are actively grazed by heterotrophic flagellates and small ciliates [130]. Ciliates, in turn, are mainly controlled by metazooplankton. Thus, a bloom of filamentous cyanobacteria forms a rich planktonic community of alternative food for grazers [106], and within that community nodularin can serve as a source of dissolved organic carbon for bacteria and become transferred in the food web via both direct consumption of *N. spumigena* and the microbial loop [111].

***Responses to Grazing and Competitors: Abundance and Toxin Accumulation***

The physiological and population responses of the cyanobacterium in terms of growth and toxin production to grazing pressure is highly relevant in order to understand processes behind bloom formation, propagation, and its effects on aquatic grazers. Any prey discrimination by grazers exerts a selective pressure on phytoplankton species. Indeed, most laboratory feeding experiments with mesozooplankton grazing on a mixture of toxic and nontoxic *Nodularia* show generally low grazing on toxic cyanobacterial strains and preferential feeding on non-toxic strains [88,131]. By selective removal of nontoxic strains and species from cyanobacterial populations, grazers promote competitive abilities and

growth of toxic strains, increase their relative abundance and thus increase average cellular quota of the toxin in the population.

Similar to other toxin producing phytoplankton, allelopathy and inducible defence are the two main mechanisms being considered to explain variations in toxin production in *N. spumigena*. Allelopathy refers to chemical inhibition (or stimulation) of one plant or microorganism species by another; grazer deterrence is also sometimes considered as an allelopathic property [132].

Inducible defences of cyanobacteria against grazing include changes in morphology (e.g. filaments), as well as production of toxins and other chemical substances suppressing growth or activity of grazers. Using algal and cyanobacterial cultures, allelopathic responses of phytoplankton to *N. spumigena*, its cell-free filtrate and pure nodularin have been studied experimentally [133,134,135].

While *N. spumigena* and its filtrate suppressed algal growth, no evidence for nodularin operating as the allelopathic agent has been found using pure nodularin [134,135]. This led Suikkanen et al. [134] to suggest that the allelopathic effects of *N. spumigena* are most probably due to metabolite(s) other than nodularin.

Recent reports have indicated that *N. spumigena* mixed with other algae (*Rhodomonas salina* and *Tetraselmis suecica*) and ambient plankton communities decreased nodularin–cell quota when exposed to copepod grazers [87,120]. This decrease was attributed to the preferential copepod grazing on the algae competing with *N. spumigena*, and thus relieving the cyanobacterium from competition [120]. When the competition was relaxed, so was the need to synthesize nodularin to be used as an allelopathic substance against the competitors. Interestingly, while *N. spumigena* severely inhibited growth rates in *R. salina*, with inhibition being related to the nodularin concentrations, those in *T. suecica* were unaffected.

This supports the view that these allelopathic interactions are species-specific. It is clear that degree of zooplankton grazing on toxic cyanobacteria and induction of toxin production are influenced by many factors, including toxin concentrations, cyanobacteria species, zooplankton species, and various environmental conditions. Therefore, eutrophication- and climate-mediated changes of the phenology, growth performance and species-specific survival of grazers can lead to alterations in toxicity of the cyanobacterial populations.

### *Eutrophication and Climate-Driven Changes in Food Webs and Their Effects on Toxin Production by Nodularia Spumigena*

Most studies investigating toxin production and its cellular quota in *N. spumigena* have focused on the influence of abiotic factors, such as temperature, salinity and nutrient availability. However, these cellular processes may also be affected by grazers and competitors [87,111,120]. Furthermore, these food-web interactions are also potential targets of eutrophication- and climate-driven changes. Recent reviews suggest that projected climate change scenarios will favor cyanobacteria [136], whereas total phytoplankton stocks have declined over the past century, due to both climate change and nutrient misbalance related to eutrophication [137]. This implies a strong probability for an increased dominance of cyanobacteria. In line with this, both frequency and intensity of the cyanobacterial blooms have been increasing during the last few decades in the Baltic Sea [5,6] as well as average abundance of cyanobacteria [138]. Moreover, while species composition of the filamentous diazotrophs remains the same, the ratio of toxic vs. nontoxic cyanobacterial species observed in late summer has increased [139]. This may affect competition between autotrophs and

related toxin production; however, the outcome of these interactions would be highly complex and system-specific. Climate-induced changes in thermal regime of an ecosystem, including higher surface water temperature and prolonged periods of thermal stratification, have been implicated in phenological and structural changes of zooplankton communities [140,141,142].

In the Baltic Sea during the past years, the predicted and observed declines in the relative abundance of the most efficient phytoplankton grazers, such as large copepods, and increase in small-sized zooplankters, such as rotifers and podonids, due to decreased salinities and increased water temperatures [143] may influence top-down control on primary producers. As abundance of small-sized grazers correlate positively with high frequencies and magnitude of the cyanobacteria blooms (Figure 2), the climate-induced changes in zooplankton community structure may further facilitate cyanobacteria dominance.

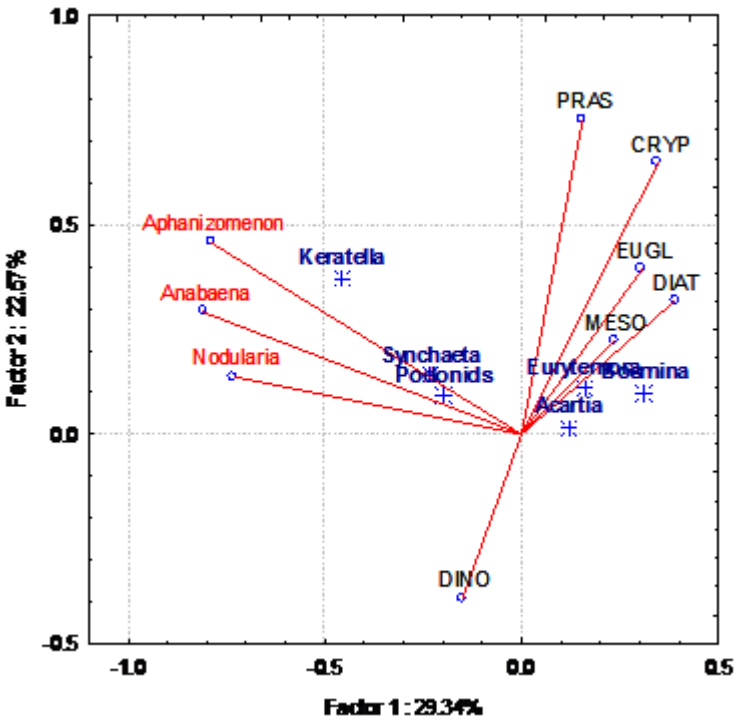


Figure 2. Positive effects of diazotrophic cyanobacteria on abundance of rotifers in a coastal area of the northern Baltic Sea (Askö Biological station) based on the Principal Component Analysis (PCA) of the long-term data for 1976-2010 (June-September, samples were collected bi-weekly and analyzed according to the HeLCOM guidelines). Summer abundances of filamentous diazotrophic *Nodularia*, *Aphanizomenon* and *Anabaena* (Susanna Hajdu, Stockholm Universiy, pers. comm.) correlate positively with population stocks of rotifers (genera *Keratella* and *Synchaeta*) and podonid cladocerans (genera *Podon* and *Pleopsis*). By contrast, none or weak negative correlations are observed between copepods (genera *Eurytemora* and *Acartia*) or larger cladocerans (*Bosmina maritima*) and the cyanobacteria, and neither of other phytoplankton groups have strong correlations with these zooplankters. Data used for the analysis were obtained from the database of the Swedish National Monitoring Programme. Abbreviations: DINO–dinoflagellates, DIAT–diatoms, MESO–Mesodinium spp., EUGL–euglenophyceans, CRYP–cryptophyceans, PRAS–prasinophyceans.

In turn, preferential exclusion of various bacterio- and phytoplankton groups may alter allelopathic interactions between the cyanobacterium and its potential competitors. As a result, changes in nodularin quotas in *N. spumigena* populations might occur. In mixed plankton assemblages, production of nodularin as well as other toxins produced by *N. spumigena* [144] seems to be regulated by complex growth-related mechanisms and environmental chemical cues, including chemicals secreted by competitors and grazers. Therefore, the outcome of competition between the cyanobacterium and other phytoplankton species would depend on the rate of release and lifetime of these water-born chemical cues.

Although high temperatures and high irradiances could increase an active exudation of nodularin during natural blooms [69], nodularin is more likely to breakdown at an increased rate in these conditions [145] and thus weaken the allelopathic signal and chemical communication between the members of the food web.

It has been shown that excreted nodularin is efficiently taken up by organisms of the microbial loop [111] and used as a substrate by bacteria [146]. Also, bacteria can efficiently degrade hepatotoxins in natural waters, and heterotrophic nanoflagellates respond quickly to the bacterial growth [146,147]. These data raise interesting questions about potential effects of decrease in phyto- and bacterioplankton growth driven by increased UVR and temperature in temperate regions on hepatotoxin degradation [147]. The predicted temperature increase will not only act on toxin degradation in the environment, but also on other temperature-dependent processes, such as detoxification in the food chain, bioaccumulation and biotransformation, and, thus, potential human exposure to hepatotoxins accumulated in top consumers. This is particularly relevant for cyanobacterial toxins in commercial fish that can greatly impact the quality of fish for human consumption and even impose legal restrictions on food safety.

## SUMMARY AND FUTURE DIRECTIONS

*Nodularia spumigena* is an important constituent of summer cyanobacterial blooms in the Baltic Sea, which are an annually recurring phenomenon. Moreover, the frequency and intensity of the blooms as well as *N. spumigena* stocks have increased due to the eutrophication. As a nitrogen fixer, *N. spumigena*, contributes substantially to the total annual nitrogen load in the Baltic Sea, which may further enhance eutrophication and negatively affect oxygen regime.

Studies into the $N_2$-fixation and heterocyst formation in *N. spumigena* have revealed that the Baltic Sea *N. spumigena* is an unusual $N_2$-fixer. Filaments respond to ammonia supplementation by ceasing $N_2$-fixation activity while maintaining heterocyst frequency along the filaments. The cells continue to express the master regulators ntcA and hetR and their expression was not significantly affected in response to treatment with ammonia. Furthermore, the cells exhibit lower energy production and carbon metabolism. Accordingly, Baltic Sea *N. spumigena* is unable to replace the energy-expensive $N_2$-fixation by utilization of externally supplied ammonia. These might explain why blooms of *N. spumigena* develop during nitrogen limitation and respond more strongly to other abiotic factors.

Nodularin, a hepatotoxin produced by *N. spumigena*, is toxic for vertebrates and mammals. Transcriptional analysis of the gene cluster responsible for nodularin biosynthesis

combined with toxin measurements revealed that *N. spumigena* maintain a threshold of intracellular nodularin level. Nodularin release to the environment occurs during stationary phase of growth, however environmental factors can affect active release of the toxin during the exponential phase. Metabolization and degradation of nodularin in the environment are facilitated by bacteria, but these are poorly known despite its importance for fauna and accumulation in the food-webs, both pelagic and benthic.

*N. spumigena* is involved in complex food web interactions. In addition to abiotic factors, its propagation and toxin production are affected by grazers and competitors. In turn, growth of some phyto- and bacterioplankton species is affected by the presence and abundance of *N. spumigena*, nodularin synthesis and cellular allocation. Zooplankton grazers can mediate nodularin production by selective removal of phytoplankton altering its community composition and abundance.

Climate-driven changes in food-web structure and interactions may have consequences for frequency and magnitude of *N. spumigena* blooms as well as for production and accumulation of nodularin in aquatic ecosystems. In particular, a predicted stronger stratification due to effects of climate change in the Baltic Sea with increased temperature and increased precipitation and increased UV-B due to ozone losses, imply a continuing future dominance of *N. spumigena* and associated high toxin production. On the other hand, shifts in grazer community structure towards small-sized zooplankters, such as rotifers and small cladocerans, would result from joined effects of increased temperatures, eutrophication and *N. spumigena* blooms.

To understand impacts of global warming on *N. spumigena* blooms and their toxicity, a holistic approach integrating biotic and abiotic aspects of bloom proliferation, function, production and accumulation of hepatotoxins and their effects in the food webs is needed.

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*Chapter 4*

# WHICH FACTORS ARE RELATED TO THE SUCCESS OF *CYLINDROSPERMOPSIS RACIBORSKII* IN BRAZILIAN AQUATIC SYSTEMS?

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## ABSTRACT

Blooms of *Cylindrospermopsis* have become more and more frequent in Brazilian aquatic systems because of its competitiveness in tropical eutrophic systems. Beyond of ecological effects of the blooms, this genus is a potential producer of toxins (cylindrospermopsin/hepatotoxin and saxitoxin/neurotoxin), which cause problems to public health and environmental hazards. *C. raciborskii* is usually described as invasive specie and can represent up to 100% of total algal biomass under certain environmental conditions. This occurrence in tropical systems is usually associated with low light availability and its high affinity for nutrients. In order to evaluate these generalizations, we analyzed limnological data of 51 Brazilian aquatic ecosystems, where *C. raciborskii* occurs. The data base included limnological information of reservoirs and coastal lagoons, comprising a latitudinal gradient from the northeast, south, southeast and central regions of the country. Nutrient concentration showed widely range from oligotrophic to hypereutrophic conditions. In general these systems presented low water transparency (Secchi disk: 0.05 to 1.7m) and the annual average temperature is higher than 22°C. Relative contribution of *C. raciborskii* in these systems ranged from 0.01 to 99% of phytoplankton biomass. The data analysis showed that relative contributions of *C. raciborskii* greater than 80% were associated with high values of temperature, pH,

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alkalinity and conductivity. In general, low DIN concentrations were also related to high percentage contribution. So the success of *C. raciborskii* in Brazilian aquatic ecosystems can be related to its low light requirements, high affinity for ammonium and phosphorus and its ability to tolerate high ionic concentration. Moreover, some of these systems have high ion concentrations which reflect in elevated conductivity values.

## INTRODUCTION

Cyanobacteria are recognized as a critical problem worldwide and their blooms are typical phenomena in eutrophic lakes, often linked to external nutrient enrichment and with subsequent incorporation of cyanotoxins in different trophic levels [1,2]. Among the genera most frequently observed forming blooms, stand out *Microcystis*, *Anabaena* and *Cylindrospermopsis*, described in the literature as potentially producing hepatotoxins and/or neurotoxins. Blooms of toxic species of these genera have been recorded in various Brazilian ecosystems [3-5].

### Distribution of *Cylindrospermopsis raciborskii*

The tropical planktonic genus *Cylindrospermopsis* (Cyanobacteria), belonging to the order Nostocales, has been often incorrectly identified and classified as various other genera [6]. The species was originally described by Woloszynska as *Anabaena raciborskii* in 1912 [7], for a plankton collected in a small pond in Java, Indonesia. In 1923, it was reclassified by Elenkin, as *Anabaenopsis raciborskii* and later as *Cylindrospermopsis raciborskii* by Seenayya and Subba-Raju [8], based primarily on the morphology of trichomes and terminal position of heterocyst.

*Cylindrospermopsis raciborskii* (Woloszynska) Seenayya and Subba-Raju is a planktonic invasive species, which was originally described from tropical regions, but it also proliferates in warmer periods of temperate countries. This species is known by its high competitive ability to expand its range the past decade in diverse aquatic ecosystems, including lakes, reservoirs, rivers and estuaries. Currently, its occurrence has been described in Australia, Europe, South and North America [9-16].

Padisák [13] was the first to propose a spread route of this species, suggesting a scattering center located in Africa, with a greater species diversity of species and another in Australia, where it is speculated that the South American populations have emerged. Particularly in Brazil, the first report of its occurrence was in Itaipu reservoir located in the south region [17], and in Lago Paranoá, a reservoir situated in the Midwest [9]. Since the 90´s, other studies have pointed out the occurrence of *C. raciborskii* in other regions of Brazil. Bouvy et al. [4,18] described the distribution of *C. raciborskii* in reservoirs of Pernambuco State. In the southeast region, the occurrence of this species was described for many reservoirs, lakes and one coastal lagoon [19-22]. New records for the south region describe the occurrence of *C. raciborskii* in a coastal lagoon [23] and in another reservoir [24].

## Ecophysiology of *Cylindrospermopsis raciborskii*

As *C. raciborskii* is usually described as an invasive species, it can represent up to 100% of total algal biomass under certain environmental conditions. This species occurrence in tropical systems is usually associated with low light availability, superior shade tolerance and its high affinity for ammonia uptake and $N_2$ fixation, herbivory resistance, and high dispersal ability and survival under oligohaline or low flow rivers [13,25].

Generally, the ability of regulating fluctuation is more effective during thermal stratification periods. Blooms or dominance of *C. raciborskii* have been related to vertical structure resilience [11,13,26]. However, the occurrence in shallow systems without durable vertical stratification was also recorded in tropical and subtropical environments [4,20,27].

The most common environmental trait associated with the dominance of *C. raciborskii* is the reduced availability of light. It can be considered a shadow species, strategist for low intensity, with ability to grow under limited light availability conditions [13]. Laboratory studies reported $I_k$ values ranging from 15 to 26 $m^{-2}$ $s^{-1}$ for *C. raciborskii* strains of several regions of the world, including Brazil [28,29].

Considering the issues related to nutrient availability and uptake rate of N and P *C. raciborskii* blooms are generally related to low concentrations of dissolved N and P [9,13,20, 30]. Présing et al. [31] reported the half-saturation constant for the absorption of $N\text{-}NH_4$ and $N\text{-}NO_3$ as low as 7 µg N $L^{-1}h^{-1}$ and 1.1–1.4 µg N $L^{-1}h^{-1}$, respectively. Another ecophysiological study demonstrated that the $N\text{-}NO_3$ absorption increases with the concentration of this ion, because its absorption requires less energy than the $N_2$ fixing [28]. In tropical and subtropical regions, N deficiency may be regarded as the principal cause of the dominance of heterocystous cyanobacteria, including *C. raciborskii* (see [32]). However, for shallow lakes of temperate regions, the high affinity of this species by ammonia, and not the N deficiency, has been considered as the major factor for their dominance [13].

The role of phosphorus in the invasion and dominance of *C. raciborskii* is associated with a capacity for high phosphorus storage, rapid uptake of phosphorus and the utilization of pulsed phosphorus supplies [28,33,34]. Low values of Km (1.5–2.5 µg P $L^{-1}$) and $Q_0$ (2.5–3.1 µg P mg $C^{-1}$) were reported for European strains [33], when compared with other algae. Thus, *C. raciborskii* can be pointed as an opportunistic species for phosphorus acquisition.

Temperature has been also considered a major limiting factor for growth of *C. raciborskii* [13,35], with its optimal growth rate being at 25–35°C [30,36-38]. Moreover, these values were recorded using nitrate or ammonium as a source of nitrogen. *C. raciborskii* does not reach the maximum growth rate when only $N_2$ was available [28]. Furthermore, its akinetes germination occurs primarily from 22–23.5°C [13].

## Other Considerations

*C. raciborskii* succession and proliferation in aquatic systems worldwide have been also related with the phenotypic plasticity and biotic interactions. Global warming phenomenon and ability of *C. raciborskii* to tolerate a rather wide range of climate condition were indicated by Briand et al. [30] as main factors responsible for colonization of the species in mid latitudes. However, Figueredo et al. [39] suggested that an allelopathic advantage could explain the geographic expansion of *C. raciborskii*.

Cyanobacteria are generally avoided by zooplankton due its size and low palatability, while chlorophytes and cryptophytes are preferred by zooplankton. However, some studies have shown that filaments of *C. raciborskii* can be preyed [4,40]. More often, the literature indicate the absence of zooplankton grazing on populations of *C. raciborskii* [9,13]. Another important aspect is that *C. raciborskii* can be harmful for zooplankton, due to the effects of toxins on reduced survival and fecundity, in addition to decreased mobility of the organism [41,42]. Beyond of ecological effects of the blooms, this genus is a potential producer of toxins (cylindrospermopsin and saxitoxins), which cause problems to public health and environmental hazards. Only Brazilian strains are known to produce saxitoxins and it is possible that toxins production is related to the presence of some ions in water [43].

In order to evaluate these generalizations, we analyzed data from different Brazilian aquatic ecosystems where *Cylindrospermopsis raciborskii* occurrs. This study focused on *C. raciborskii* occurrence and those factors favoring its dominance in major watersheds of Brazil.

## DATA ANALYSIS

The study area is located in several of the Brazilian aquatic systems, comprehending a latitudinal gradient ranging from 06° 08' S 37° 07' W to 27° 42' S 48° 33' W. From this area we analyzed a database of 51 different aquatic systems, including reservoirs and coastal lagoons, deep and shallow sites, with different trophic states (from oligo-mesotrophic to hypertrophic), from Midwest, Northeast, South and Southeast regions of Brazil. We compiled a database of 439 samples taken from 51 systems where *C. raciborskii* was present in at least one time. The data were obtained from published (scientific articles and thesis) and unpublished (reports) material, in which biomass and relative contribution of *C. raciborskii* were available. The data were extracted directly from tables and from figures, using the software Engauge Digitizer 4.1. The environmental variables were lake area, mean depth, lake volume, water temperature, water transparency (Secchi depth), euphotic zone to mixing zone ratio (Zeu/Zmix), electric conductivity, alkalinity, pH, dissolved oxygen [DO], soluble reactive phosphorus [SRP], ammonium-nitrogen [$NH_4^+$], nitrate-nitrogen [$NO_3^-$], total nitrogen [NT], total phosphorus [TP] and chlorophyll-a. DIN was calculated as the sum of dissolved $NH_4^+$ and $NO_3$. Shade index was estimated as a ratio of depth/Secchi depth (adapted from Scheffer [44]). The biomass and relative contribution of *C. raciborskii* were also analyzed. All the data used were measured at the water surface. All the aquatic systems data were organized and grouped by its watershed, as shown in Table 1 and Figure 1.

We found no significant correlations between any limnological variable (nutrient concentration, temperature, Secchi depth, etc.) and the biomass or the percentage of contribution of *C. raciborskii* in the analyzed database. Thus, we use Principal Component Analysis (PCA) to explore the possible relationships of *C. raciborskii* data with limnological characteristics of the aquatic systems. For the analysis we build a matrix with the values of depth, temperature, Secchi depth, Zeu/Zmix, Shade index, conductivity, alkalinity, pH, DO, SRP, $NH_4^+$, $NO_3^-$, DIN, NT, PT, chlorophyll-a, percentage of *Cylindrospermopsis* contribution (% Cylindro) and biomass of *Cylindrospermopsis* (BiomCyl). The outliers were removed considering 2.5 times the standard deviation as a cutoff.

**Table 1. List of Brazilian aquatic systems with *Cylindrospermopsis* occurrence: geographical coordinates and morphometrical data (nd – not determined). Basins: NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin**

| Systems | Coordinates | Basin | Mean depth (m) | Area ($Km^2$) | Volume ($10^6$ $m^3$) | References |
|---|---|---|---|---|---|---|
| A.R. Gonçalves I, RN | 06° 08' S 37° 07' W | NEAT | 12,3 | 36,7 | 2400 | Costa, [55] |
| Gargalheiras, RN | 06° 26' S 36° 38' W | NEAT | 5,2 | 0,2 | 18,06 | Chellappaand Costa, [56] |
| São José II, PE | 07° 28' S 37° 17' W | SF | 4,0 | 1,21 | 7,15 | Bouvy et al., [18] |
| Araripina, PE | 07° 34' S 40° 28' W | SF | 0,8 | 0,75 | 3,7 | Bouvy et al., [18] |
| Rancharia,PE | 07° 41' S 40° 33' W | SF | 4,8 | 0,32 | 1,04 | Bouvy et al., [18] |
| Barrinha, PE | 07° 43' S 39° 15' W | SF | 5,5 | 0,46 | 1,95 | Bouvy et al., [18] |
| Brotas, PE | 07° 44' S 37° 37' W | SF | 5,0 | 4,67 | 19,64 | Bouvy et al., [18] |
| Rosário, PE | 07° 46' S 37° 28' W | SF | 7,0 | 8,98 | 34,99 | Bouvy et al., [18] |
| Lagoa do Barro, PE | 07° 46' S 40° 23' W | SF | 4,5 | 4,05 | 24 | Bouvy et al., [18] |
| Chinelo, PE | 07° 47' S 37° 47' W | SF | 2,0 | 0,98 | 2,86 | Bouvy et al., [18] |
| Gergelim, PE | 07° 48' S 40° 29' W | SF | 1,2 | nd | 0,03 | Bouvy et al., [18] |
| Barriguda, PE | 07° 51' S 40° 29 W | SF | 5,0 | nd | 1,62 | Bouvy et al., [18] |
| Lopes II, PE | 07° 52' S 39° 56' W | SF | 3,0 | 7,60 | 23,93 | Bouvy et al., [18] |
| Barra, PE | 07° 56' S 38° 17' W | SF | 4,0 | 0,59 | 2,73 | Bouvy et al., [18] |
| Saco I, PE | 07° 56' S 38° 17' W | SF | 2,0 | 6,61 | 36 | Bouvy et al., [18] |
| Jucazinho, PE | 07° 58' S 35° 48' W | SF | 16 | 16,00 | 327 | Dantas, [57, 58] |
| Cachoeira II, PE | 07° 58' S 38° 19' W | SF | 5,0 | 3,90 | 21,03 | Bouvy et al., [18] |
| Custódia, PE | 07° 58' S 38° 19' W | SF | 1,5 | 3,90 | 21,62 | Bouvy et al., [18] |
| Algodões II, PE | 07° 58' S 40° 19' W | SF | 4,3 | 11,58 | 58,48 | Huszar et al., [32] |
| Algodões, PE | 07° 58' S 40° 19' W | SF | 2,2 | 11,58 | 58,48 | Bouvy et al., [18] |
| Jazigo, PE | 07° 59' S 38° 14' W | SF | 4,0 | 4,60 | 15,54 | Bouvy et al., [18] |
| Chapéu, PE | 07° 59' S 39° 34' W | SF | 7,2 | 26,15 | 188 | Huszar et al., [32]; Bouvy et al., [18] |
| Tapacurá, PE | 08° 02' S 35° 09' W | SF | 9,5 | 9,8 | 94,2 | Ferreira, [59]; Dantas,[57, 58] |
| Ingazeira, PE | 08° 03' S 36° 58' W | SF | 5,6 | 1,30 | 4,81 | Bouvy et al., [18]; Huszar et al., [32]; Bouvy et al., [4] |
| Pão de Açúcar, PE | 08° 03' S 36° 58'W | SF | 8,7 | 56,0 | 55 | Huszar et al., [32] |

**Table 1. (Continued)**

| Systems | Coordinates | Basin | Mean depth (m) | Area ($Km^2$) | Volume ($10^6$ $m^3$) | References |
|---|---|---|---|---|---|---|
| Boa Vista, PE | 08° 03' S 39° 03' W | SF | 5,5 | 2,7 | 16,45 | Bouvy et al., [18] |
| DuasUnas, PE | 08° 04' S 35° 02' W | SF | 7,2 | 3,9 | 23,5 | Dantas, [57,58] |
| Cachoeira I, PE | 08° 04' S 37° 13' W | SF | 1,5 | 1,20 | 5,95 | Bouvy et al., [18] |
| Tabocas, PE | 08° 06' S 36° 13' W | SF | 8,6 | 1,6 | 13,6 | Bressan, [60] |
| Entremontes, PE | 08° 13' S 39° 53' W | SF | 6,5 | 46,05 | 339,33 | Bouvy et al., [18] |
| Ipaneminha, PE | 08° 22' S 36° 51' W | SF | 5,0 | 0,67 | 3,6 | Bouvy et al., [18] |
| Mororó, PE | 08° 29' S 36° 56' W | SF | 8,6 | 0,42 | 2,93 | Bouvy et al., [18]; Dantas, [57, 58] |
| Poço da Cruz, PE | 08° 30' S 37° 42' W | SF | 12,0 | 56,10 | 504 | Bouvy et al., [18]; Huszar et al., [32] |
| Arcoverde, PE | 08° 33' S 36° 59' W | SF | 8,0 | 2,00 | 16,8 | Bouvy et al., [18]; Dantas, [57, 58] |
| Venturosa, PE | 08° 34' S 36° 52' W | SF | 2,0 | nd | 0,12 | Bouvy et al., [18] |
| Buíque, PE | 08° 37' S 37° 10' W | SF | 2,5 | nd | 0,11 | Bouvy et al., [18] |
| Iati, PE | 08° 37' S 37° 10' W | SF | 6,0 | 3,05 | 0,14 | Bouvy et al., [18] |
| Itaíba, PE | 08° 55' S 37° 25' W | SF | 5,0 | 0,14 | 0,44 | Bouvy et al., [18] |
| Ipanema, PE | 09° 05' S 37° 09' W | SF | 3,0 | 0,30 | 0,71 | Bouvy et al., [18] |
| Itaparica, BA | 09° 25' S 38° 19' W | SF | 10,4 | 828 | 11000 | Marinho, non-published data. |
| Serra da Mesa, GO | 13° 50' S 48° 18' W | TO | 30,5 | 1784 | 54000 | Marinho, non-published data. |
| Lagoa Santa, MG | 19° 38' S 43° 53' W | SF | 4,5 | 1,31 | nd | Figueredo, [61,62] |
| M. Moraes, MG | 20° 17' S 43° 03' W | PR | 10,0 | 250 | 4040 | Marinho, non-published data |
| Duas Bocas, ES | 20° 18' S 40° 28' W | EAT | 4,3 | 0,51 | 2,0 | Delazari-Barroso, [22] |
| Furnas, MG | 20° 40' S 46° 19' W | PR | 15,7 | 1440 | 22950 | Marinho, non-published data |
| Funil, RJ | 22° 30' S 44° 45' W | EAT | 22,0 | 40 | 890 | Soares et al., [21] |
| Juturnaíba, RJ | 22° 33' S 42° 37' W | EAT | 5,0 | 43,00 | 100 | Marinho and Huszar, [20] |
| Imboassica I, RJ | 22° 50' S 44° 42' W | EAT | 0,7 | nd | nd | Melo, [19] |
| Garças, SP | 23° 39' S 46° 37' W | PR | 4,5 | 0,09 | 0,4 | Tucci and Sat'Anna, [26] |
| Irai, PR | 25º 24' S 49º 06' W | PR | 5,0 | 163 | 58 | Lagos, [23] |
| Peri, SC | 27° 42' S 48° 33' W | SAT | 4,2 | 5,07 | 21,2 | Laudares-silva, [24] |

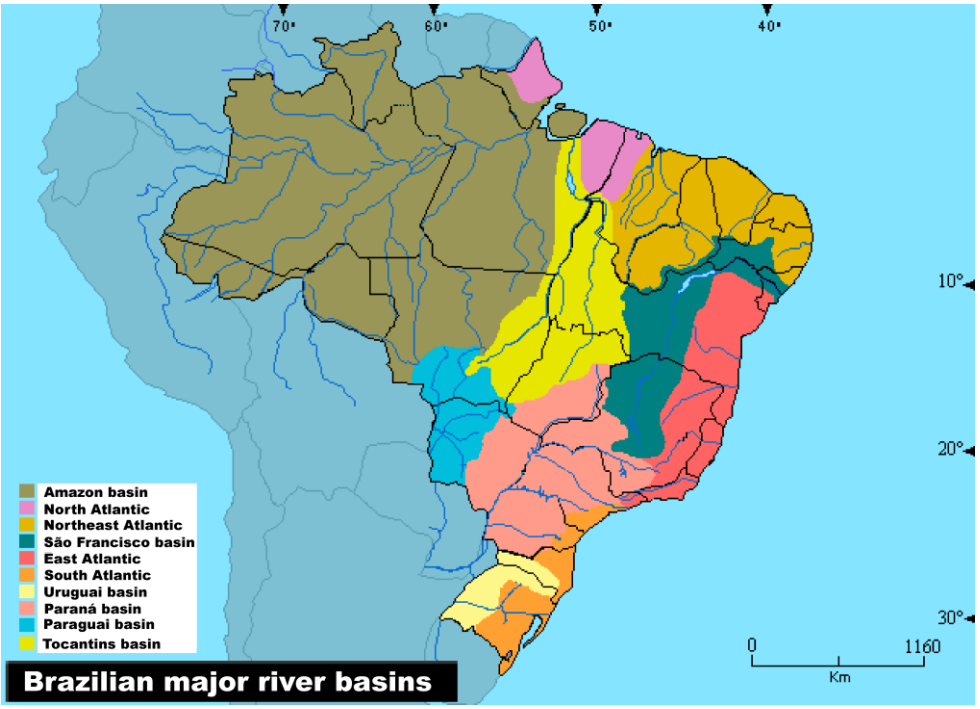


Figure 1. Map with the major Brazilian river basins.

The final matrix had a dimension of 19 variables by 156 samples. Our interest was to discriminate which variables could be considered as major factors in the occurrence and dominance of *Cylindrospermopsis*.

Then, the data of occurrence and biomass were used as supplementary variables in PCA. For further exploration of the analyzed data and of the hypotheses suggested by the ordination results we applied the Loess regression model to fit values of the variables that most contributed to explaining data in the ordination space. All the analysis were run on the software CANOCO version 4.5 [45].

## BRAZILIAN AQUATIC SYSTEMS FEATURES

The most aquatic environments studied was shallow (depth bellow 10m), especially represented by Northeast systems, while deepest water body was Serra da Mesa, reaching up to 100m depth. The East Atlantic Basin (EAT) systems showed a wide variation depth (0.7 – 49.9m), (Figure 2a). The Secchi depth median for the most of systems was 1.0m, while TO basin showed the highest values (Figure 2b). Zeu/Zmix was lower in NEAT and SF basins (Figure 2c) and the shade index ranged from 1.0 to 116.7 (Figure 2d), for all watersheds. It suggests that the most of systems have low light availability, and the highest Zeu/Zmix did not means elevated light availability, because it was represented by the deepest systems from EAT, SAT and TO basins, which also had high shade index values.

The pH values ranged from acid (5.5) to alkaline (9.9), with the most of values (76%) above 7.0 and 25% above 8.0 (Figure 3a).

**Table 2. Mean values and variation intervals of environmental and biotic variables of each basin studied. $Z_{eu}$: $Z_{mix}$: euphotic zone, mix depth ratio; DIN: dissolved inorganic nitrogen; SRP: soluble reactive phosphorous; TP: Total phosphorus. Basins: NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin**

| | Basins | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | NEAT | | | SF | | | EAT | | | PR | | | SEAT | | | TO | | |
| Variables | Mean | Min. | Max. | Mean | Min. | Max. | Mean | Min. | Max. | Mean | Min. | Max. | Mean | Min. | Max. | Mean | Min. | Max. |
| Wat. temp. (ºC) | 27.2 | 27.1 | 27.4 | 27.1 | 22.9 | 32.6 | 26.4 | 20.3 | 31.6 | 23.0 | 15.6 | 29.2 | 22.8 | 17.5 | 28.0 | 28.2 | 26.0 | 30.0 |
| pH | 8.4 | 8.3 | 8.6 | 8.1 | 6.4 | 9.4 | 7.8 | 5.5 | 9.9 | 7.3 | 5.3 | 9.7 | 7.2 | 6.7 | 7.7 | 7.7 | 7.2 | 8.2 |
| Conductivity ($\mu S\ cm^{-1}$) | 286.7 | 263.3 | 317.2 | 1462.6 | 60.0 | 19630.0 | 114.4 | 45.0 | 2000.0 | 46.9 | 34.0 | 141.7 | | | | 95.9 | 86.4 | 107.2 |
| Alkalinity | 97.7 | 95.4 | 101.1 | 1525.1 | 23.7 | 5567.0 | 837.5 | 837.5 | 837.5 | 371.4 | 206.0 | 958.0 | | | | 931.7 | 693.1 | 1262.5 |
| $Z_{eu}$:$Z_{mix}$ | 0.2 | 0.1 | 0.2 | | | | 0.3 | 0.1 | 0.9 | | | | 1.3 | 0.8 | 2.0 | 1.1 | 0.8 | 1.5 |
| DO ($mg\ L^{-1}$) | 7.0 | 6.5 | 7.6 | 7.6 | 3.7 | 14.6 | 8.0 | 0.8 | 14.8 | 6.3 | 0.4 | 11.4 | | | | 6.7 | 4.9 | 8.4 |
| N-NH4 ($\mu g\ L^{-1}$) | 167.0 | 92.8 | 242.3 | 60.8 | 0.0 | 768.0 | 8.6 | 0.4 | 93.4 | 64.4 | 0.0 | 546.0 | | | | 41.6 | 26.8 | 72.4 |
| N-NO3 ($\mu g\ L^{-1}$) | 13.6 | 10.5 | 19.3 | 90.0 | 0.0 | 722.0 | 23.6 | 0.0 | 71.4 | 511.1 | 2.8 | 1570.0 | | | | 119.9 | 3.7 | 456.9 |
| DIN ($\mu g\ L^{-1}$) | 180.5 | 112.1 | 253.2 | 141.6 | 0.0 | 900.0 | 30.0 | 9.3 | 76.9 | 489.2 | 6.8 | 1610.0 | | | | 148.7 | 37.4 | 550.3 |
| SRP ($\mu g\ L^{-1}$) | 4.8 | 4.0 | 6.0 | 62.5 | 0.0 | 1198.3 | 9.6 | 0.00 | 62.5 | 24.8 | 0.93 | 115.7 | | | | 15.6 | 10.60 | 33.1 |
| TP ($\mu g\ L^{-1}$) | 37.5 | 34.1 | 39.7 | 29.8 | 10.1 | 131.5 | 11.5 | 0.8 | 62.5 | 91.4 | 20.0 | 210.0 | 31.8 | 23.3 | 55.9 | 13.7 | 11.7 | 16.4 |
| Chlorophyll-a ($\mu g\ L^{-1}$) | 78.7 | 34.0 | 152.5 | 47.7 | 3.1 | 229.0 | 90.7 | 6.7 | 254.4 | 31.5 | 0.9 | 101.4 | 20.6 | 13.1 | 32.3 | 6.1 | 0.4 | 10.4 |
| C.raciborskii (%) | 41.0 | 14.0 | 90.0 | 40.6 | 0.1 | 98.8 | 28.0 | 0.1 | 90.0 | 12.8 | 0.0 | 78.0 | 73.2 | 45.1 | 83.4 | 72.6 | 7.3 | 94.7 |
| C.raciborskii biomass ($mg\ L^{-1}$) | 3.0 | 1.0 | 7.0 | 12.0 | 0.0 | 280.2 | 3.3 | 0.0 | 22.9 | 1.0 | 0.0 | 22.7 | 24.6 | 9.6 | 36.4 | 0.7 | 0.0 | 1.3 |

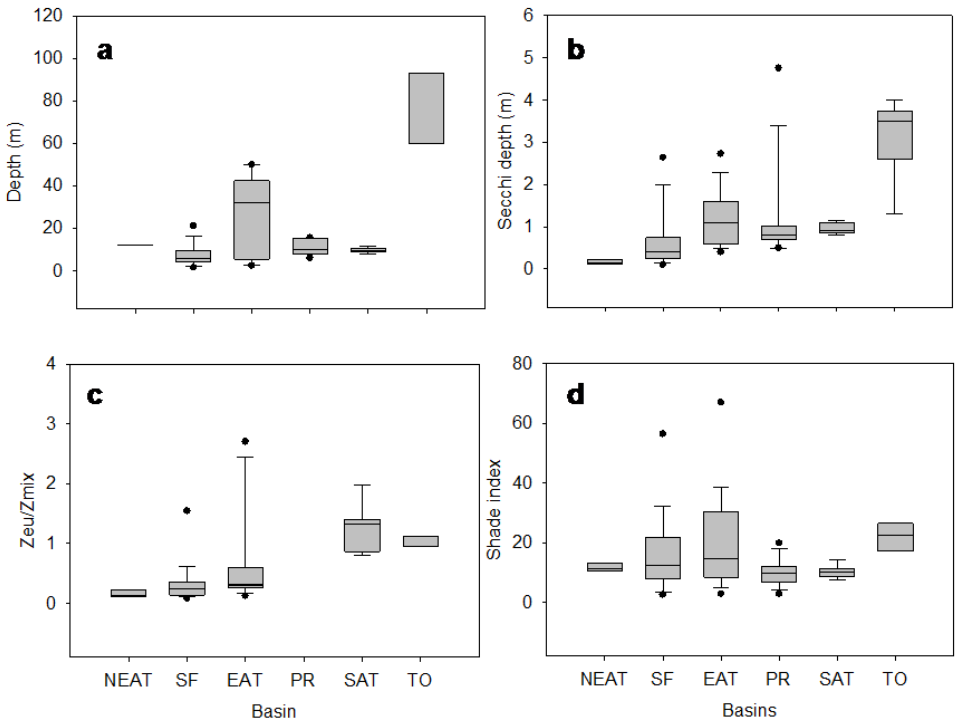


Figure 2. Variation of depth (a), water transparency (Secchi depth) (b), Zeu:Zmix (c) and shade index (d) data, grouped by different watersheds. NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin. In the box whisker plot, the line in each box represents the median, and box, whiskers, and dots comprise 75, 90 and 95% of the data, respectively.

Water temperature was decreasing toward the Northeast to the south, respecting latitudinal gradient. It ranged from 32.6°C in Gergelim reservoir, Pernambuco (SF basin) to 15°C in Mascarenhas de Moraes (PR basin), (Table 2; Figure 3b). It means that, although *C. raciboorskii* shows highest growth rates around 30°C [30], it can also occur in lower water temperatures, but with reduced biomass. Except for Northeastern (NEAT) and TO watersheds, the water temperature varied a little higher than 10°C between minimum and maximum observed in each basin.

The quantity and variability of conductivity and alkalinity values were higher in SF basin reservoirs (1462.6 µS $cm^{-2}$ and 1525.1 µEq. $L^{-1}$, respectively) (Figure 3c,d). Conductivity ranged from 56 to 19630 µS $cm^{-2}$, with high values (>10000 µS $cm^{-2}$) in 2 reservoirs located in SF basin. Alkalinity was also extremely high (>5000 µEq. $L^{-1}$) at the same 2 reservoirs. In general, dissolved nitrogen concentration was reduced in almost all basins, expect for NEAT and PR basins (Figure 4c), although SF basin has also presented maximum values of 900 µg N $L^{-1}$, but it was very infrequent. The NEAT basin showed the highest amount of $NH_4^+$, while PR basin showed the highest values of $NO_3^-$ (Figure 4a,b); other basins had nitrogen concentrations around or bellow 100 µg N $L^{-1}$. $NO_3^-$ concentrations ranged from 2.8 to 1570 µg $L^{-1}$ in PR basin (Table 2). High values of SRP were observed for almost all water bodies,

with overall mean of 22.7 µg $L^{-1}$ (Figure 4d). NEAT watershed was the only basin which presented limited values for phytoplankton growth of phosphorus (< 10 µg $L^{-1}$) [46].

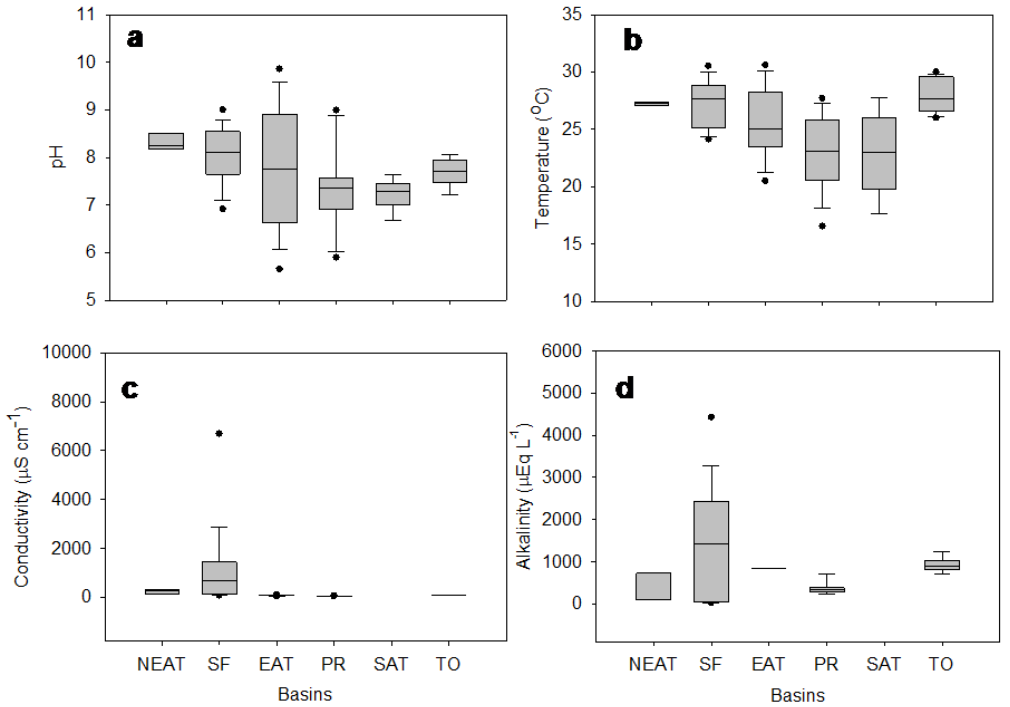


Figure 3. Variation of pH (a), Water temperature (b), Electric conductivity (c) Alkalinity (d) data, grouped by different watersheds. NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin. In the box whisker plot, the line in each box represents the median, and box, whiskers, and dots comprise 75, 90 and 95% of the data, respectively.

The TO and EAT watersheds presented the lowest TP concentrations, while PR basin showed the highest values (Figure 5a). NEAT and TO basins had lowest total phosphorus variations, as a consequence of poorness of data availability. However, PR basin was rich in TP, reaching maximum of 210 µg $L^{-1}$ (Table 2). Considering chlorophyll-*a* concentration as an environmental trophic index, according with Nürnberg [47], all basins showed eutrophic conditions, expect TO basin in which mean chlorophyll concentration mean was low (<10 µg $L^{-1}$). EAT and NEAT watersheds had highest chlorophyll concentrations (mean of 90.7 and 78.7 µg $L^{-1}$, respectively).

## *Cylindrospermopsis raciborskii* in Brazilian Aquatic Systems

*C. raciborskii* was observed in higher biomass in SAT (mean: 24.6 mg $L^{-1}$) and SF (mean: 12.0 mg $L^{-1}$) watersheds. On the other hand, TO and PR basins had lowest *C. raciborskii* biomass (means: 0.7 mg $L^{-1}$ and 1.0 mg $L^{-1}$, respectively). The SF basin had reservoirs with very low biomass of *C. raciborskii* (minimum 0.001 mg $L^{-1}$) and in other

systems it reached 280.2 mg $L^{-1}$ (Figure 6a). In general, biomass values in the other basins (EAT, SAT, PR) varied between 0.001 to almost 40 mg $L^{-1}$ (Table 2).

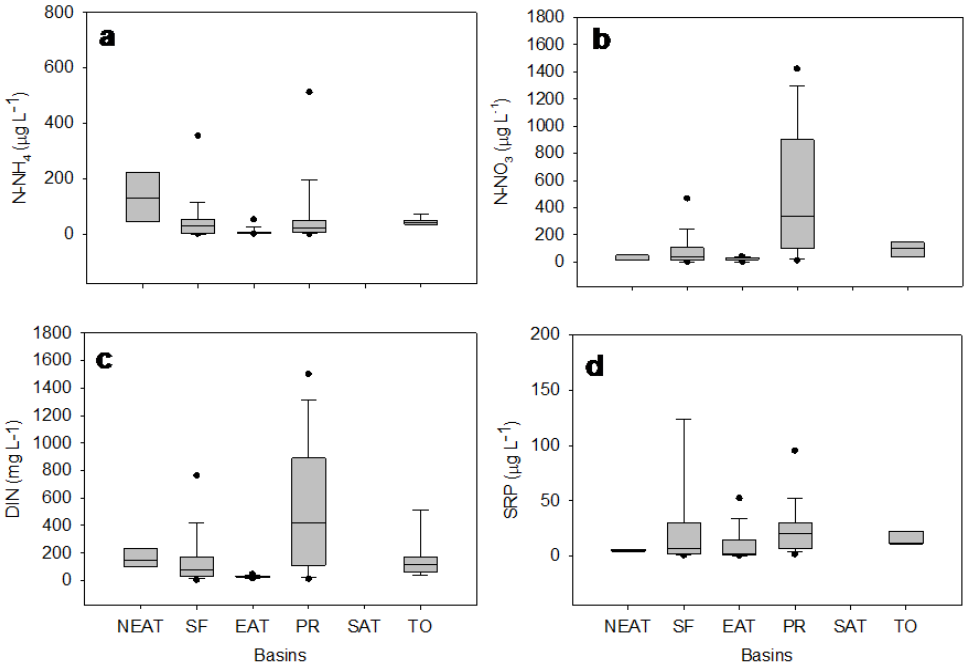


Figure 4. Variation of nitrogen-ammonium (N-NH4) (a), nitrogen-nitrate (N-NO3) (b), dissolved inorganic nitrogen (DIN) (c) and soluble reactive phosphorus (SRP) (d) data, grouped by different watersheds. NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin. In the box whisker plot, the line in each box represents the median, and box, whiskers, and dots comprise 75, 90 and 95% of the data, respectively.

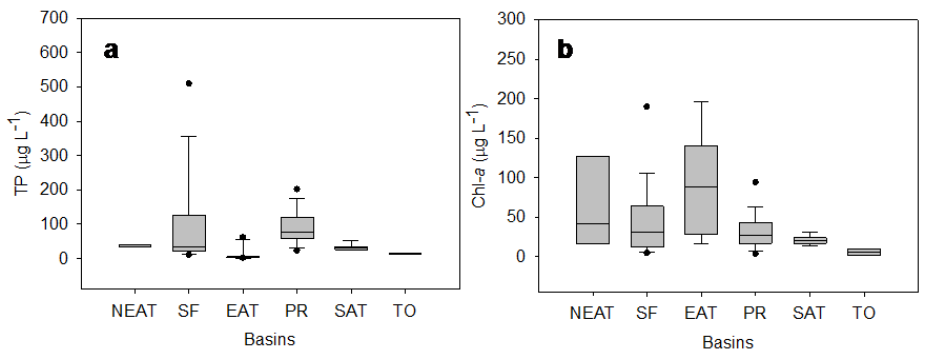


Figure 5. Variation of Total phosphorus (a) and Chlorophyll concentration (b) data, grouped by different watersheds. NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin. In the box whisker plot, the line in each box represents the median, and box, whiskers, and dots comprise 75, 90 and 95% of the data, respectively.

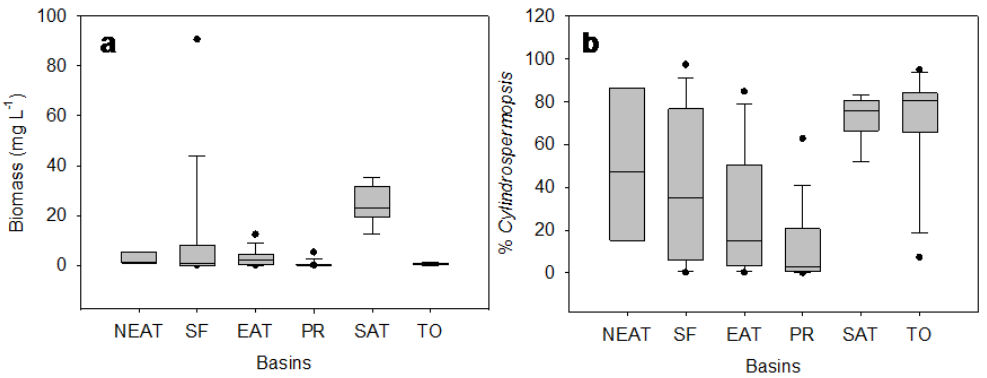


Figure 6. Variation of biomass (a) and percentage of contribution (b) of *C. raciborskii* grouped by different watersheds. NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin. In the box whisker plot, the line in each box represents the median, and box, whiskers, and dots comprise 75, 90 and 95% of the data, respectively.

*C. raciborskii* represented from 0.02 to 99% of total phytoplankton biomass for the entire data set (Table 2). Nevertheless, not always the highest values of relative contribution of this species were accompanied by higher biomass. Despite of NEAT and TO basins have presented low *C. raciborskii* biomass for all systems, its dominance was over 90% in some cases. On the other hand, the *C. raciborskii* contribution at SAT watershed was between 45% and 83%, although its biomass was relatively high varying from 9.6 to 36.4 mg $L^{-1}$.

## RELATIONSHIPS OF *C. RACIBORSKII* AND ENVIRONMENTAL FEATURES

The Brazilian aquatic ecosystems analyzed differed among the watersheds as shown by PCA (Figure 7). The two principal components explained 52.7% of the total variability of the data. The results indicated that the first principal component reflected a gradient of ionic concentration and was related with degree of trophy.

The variables positively correlated with the first principal component (alkalinity, electric conductivity, pH and chlorophyll) showed high values in aquatic systems of the SF watershed.

This was true especially for the smaller and shallower water bodies. Larger aquatic systems, like Itaparica reservoir located in the São Francisco river, are more similar to aquatic systems of EAT watershed.

Water transparency, shade index, temperature, DIN and total phosphorus influenced the formation of the second principal component, which discriminate the systems based on nitrogen and light availability. The second component discriminate the water bodies of PR and SEAT basins. The TO and EAT basins were similar to each other. Water transparency, shade index and temperature were correlated negatively and DIN and total phosphorus positively with the second component (Table 3).

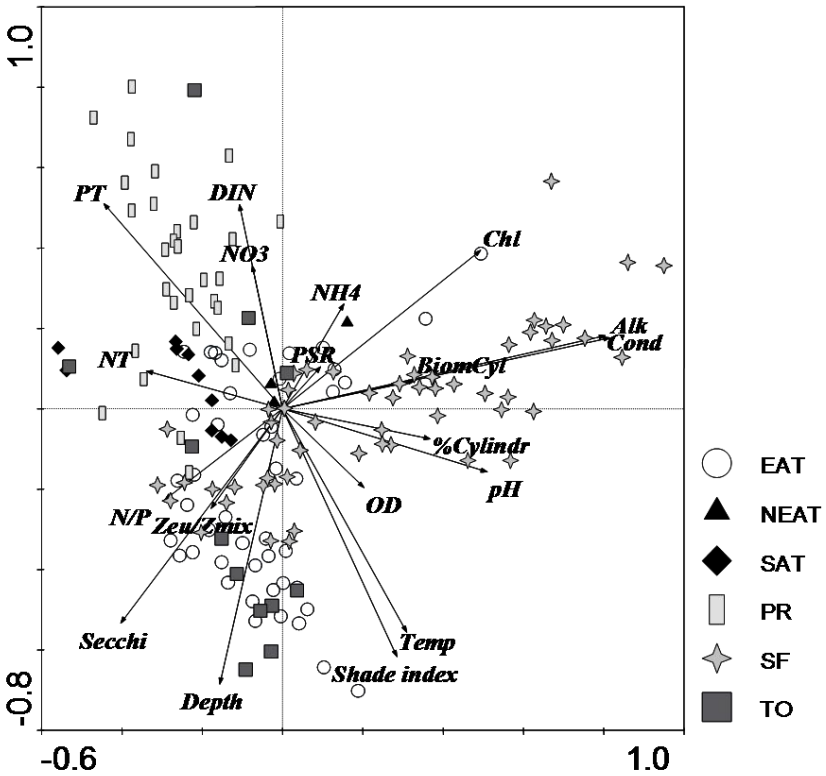


Figure 7. Ordination diagram of the first two axes of PCA. The data points represents samples of the different aquatic systems from the major Brazilian watersheds (NEAT, Northeast Atlantic basin; SF, São Francisco basin; TO, Tocantins basin; PR, Paraná basin; EAT, East Atlantic basin; SAT, South Atlantic basin).

To explore these gradients and better relate it with *C. raciborskii* dominance and biomass we use a generalized Loess regression model to fit values of the variables that most contributed for explaining the data in the ordination space [48]. The trophy gradient was evidenced by chlorophyll concentration which increased in SF basin water bodies (Fig 8a). Higher phytoplankton biomass was not correlated with higher nutrient content, even for TP. This relatively weak TP–Chl relationship in the tropical–subtropical systems could not be attributed to N limitation, and a number of possible mechanisms could explain these differences, but they are not well understood [49].

However, it was noteworthy that almost all aquatic ecosystems of our database (except for PR basin systems) showed a tendency to have values of dissolved inorganic nitrogen of less than 100 µg $l^{-1}$ (Figure 8b). This could be considered as an indicative of a potential limitation by nitrogen [46].

Water transparency and the shade index were used as proxies for light availability, and were related with the gradient observed in PCA. Lower values of water transparency were observed for SF basin water bodies, which presented corresponding high values of shade index (Figures 8c, 8d). Although the systems in EAT and TO basins showed higher values of Secchi depth, they also had high values of shade index. This could be explained by the higher depth of these systems, which results in reduced availability of light in the water column.

**Table 3. Correlations of the limnological variables from the 51 aquatic ecosystems with the first two principal components. Coefficients in boldface were important for the formation of the components**

| Variables | PC 1 | PC 2 |
|---|---|---|
| Depth* | -0.156 | -0.685 |
| Temperature | 0.309 | -0.556 |
| Water Transparency (Secchi depth) | -0.403 | -0.533 |
| Zeu/Zmix* | -0.177 | -0.248 |
| Shade index | 0.285 | -0.616 |
| Conductivity | 0.804 | 0.182 |
| Alkalinity | 0.815 | 0.177 |
| pH | 0.510 | -0.157 |
| OD | 0.203 | -0.196 |
| NH4* | 0.153 | 0.261 |
| NO3* | -0.075 | 0.356 |
| DIN | -0.107 | 0.508 |
| PSR | 0.095 | 0.104 |
| NT* | -0.339 | 0.093 |
| PT | -0.445 | 0.510 |
| N/P* | -0.293 | -0.226 |
| Clorophyll-*a* | 0.494 | 0.394 |
| Percentage of *C.raciborskii** | 0.368 | -0.075 |
| Biomass of *C.raciborskii** | 0.329 | 0.070 |

* Variable was set as supplementary (passive) in PCA, and not influenced the ordination axes, but added afterwards so that their relation to the other samples or variables can be still judged from the ordination diagram.

These gradients observed through PCA analysis can be related with the occurrence of *C. raciborskii* in the 51 Brazilian water bodies considered. The Figure 9 show the fitted values of biomass and percentage of dominance in ordination space of the two first principal components. The response surface is modeled using the generalized loess method, using the locally weighted first-order generalized linear mode.

The highest biomasses were found to the water bodies of SF basin (right in the ordination diagram) and also presented the highest values of dominance (Figure 9a). However, despite aquatic systems of East Atlantic and Tocantins watersheds presented biomass values 4-10 times smaller on average than in SF basin (Table 2), high values of dominance were also

observed. In both situations of high percentage of dominance we found low DIN concentration (Figure 8b), which suggests N limitation.

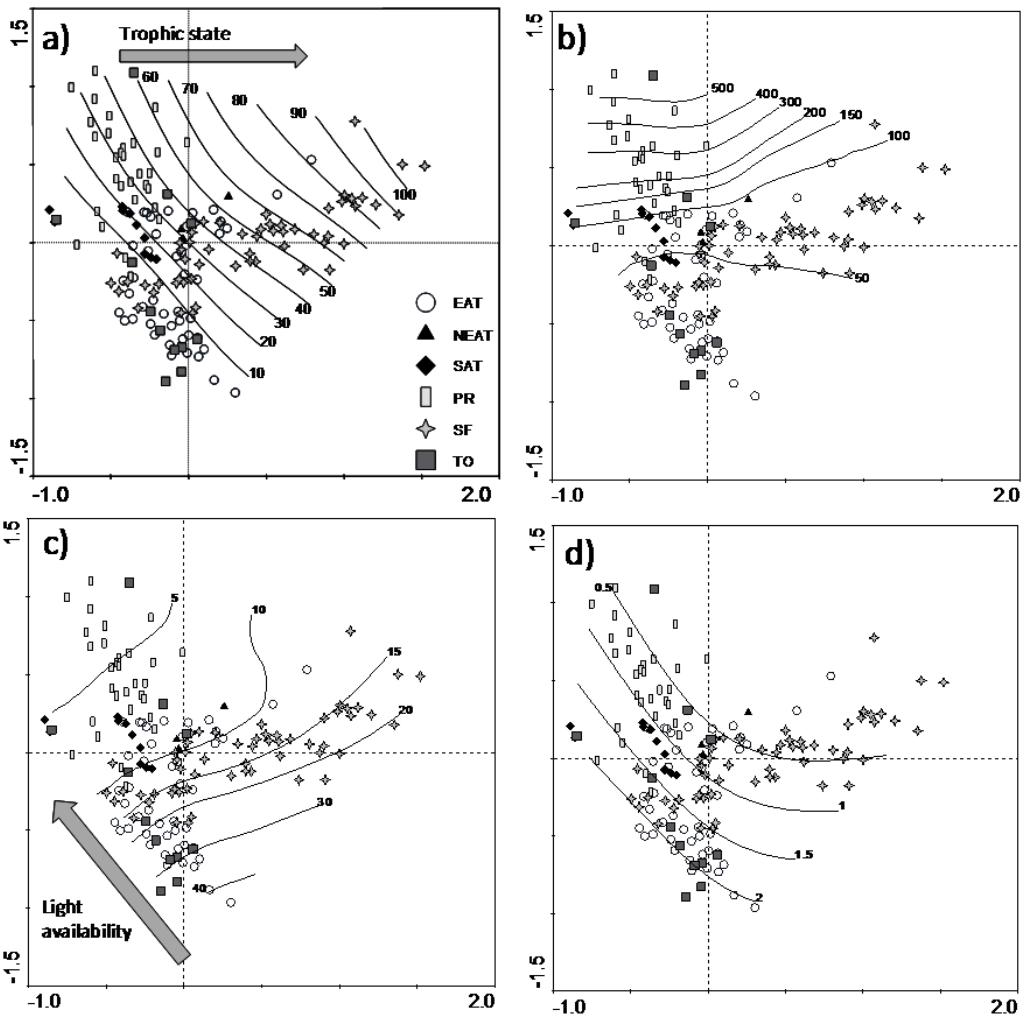


Figure 8. Response surface of a) chlorophyll-*a* concentration, b) DIN concentration, c) Shade index and d) water transparency (Secchi depth) in the ordination plane spanned by the first two PCA axes. The surface is modeled with generalized loess procedure using a first-order regression model.

*Cylindrospermopsis raciborskii* has the capacity to fix atmospheric nitrogen ($N_2$) through heterocytes, conferring a competitive advantage in nitrogen-depleted environments [50].

This ability was used to account for its dominance in tropical or subtropical systems [20, 51, 52]. However, some studies have pointed toward ammonium absorption at low concentrations as being more important than the fixation process [15,25,31].

In a recent study of the potential nutrient limitation from 83 South American shallow lakes on a latitudinal gradient N-fixing cyanobacteria was exclusively found in lakes with DIN concentrations of <100 µg $L^{-1}$[53]. However, N limitation does not necessarily lead to

N-fixing cyanobacterial dominance and tropical or subtropical systems can vary between N limitation, P limitation and co-limitation of N and P [49].

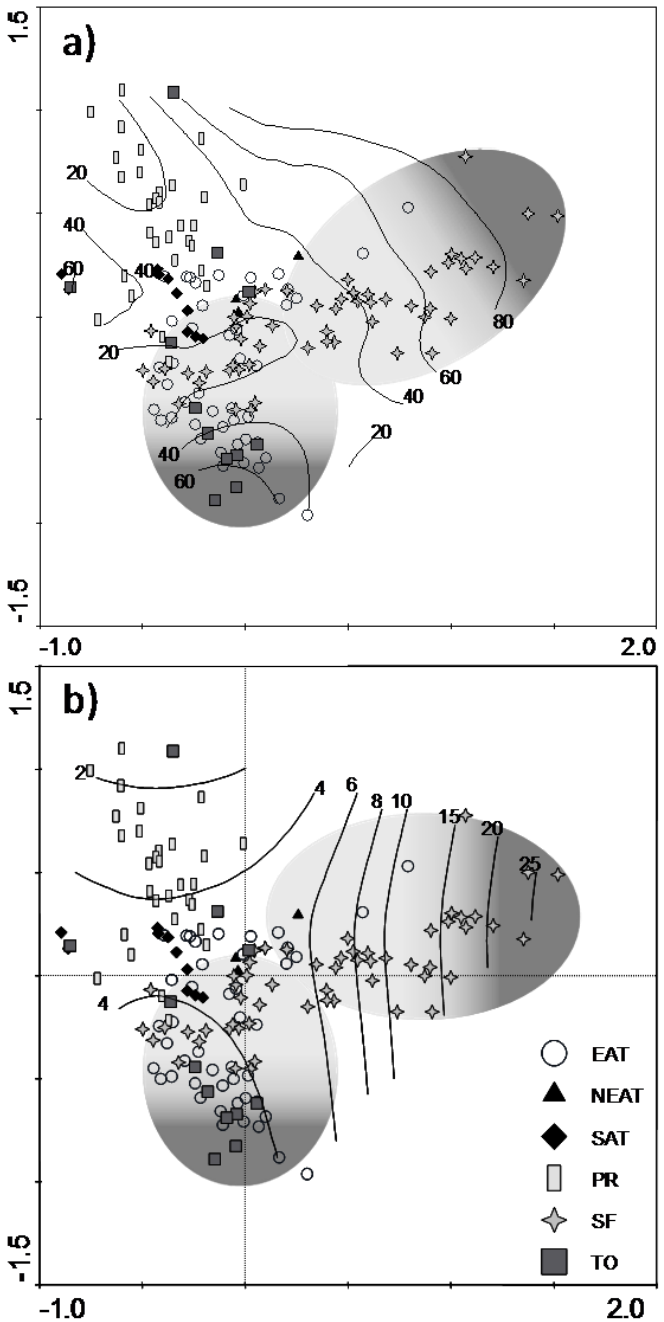


Figure 9. Response surface of a) percentage of dominance and b) biomass of *C. raciborskii* in the ordination plane spanned by the first two PCA axes. The surface is modeled with generalized loess procedure using a first-order regression model.

Besides low nitrogen availability, higher dominance of *C. raciborskii* in Brazilian aquatic systems was also related with low light availability in the small and shallow water bodies of the SF basin. In these systems water transparency (Secchi depth) was very low and shade index elevated (>16). Low transparency is a common feature in shallow environments and caused by sediment resuspension, which is generally accepted as playing an important role in controlling the functioning and structure of shallow lakes [44].

On the other hand, low light availability was also evident for the aquatic systems of EAT and TO basins that showed the highest values of shade index (Figure 8c).

In many of the studies we used to collect the data for this study the authors inferred that low light availability could be an explanation for the dominance of *C. raciborskii* [18, 20, 21, 32]. So it is not surprising to find this relationship for a shadow species [15,28].

In the other extreme of the gradient we have the PR basin systems, in which we found low values of biomass and the lowest percentage of dominance of *C. raciborskii* on average when compared to other watersheds (Figure 9; Table 2). DIN concentration was high (>150) and the proxies for light indicated higher availability when compared with all other watersheds (Figure 7).

## CONCLUSION

In summary, we found *C. raciborskii* in major river basins of Brazil, and its occurrence was not correlated with the size, depth or trophic state of the water body. It occurred not only in eutrophic but in oligotrophic and mesotrophic systems.

The presence of this species with wide distribution in different water bodies of the main Brazilian basins reflects its high phenotypic plasticity and its wide tolerance ranges to key environmental factors [54]. But analysis of the data showed that the highest percentage of contributions and biomass of *C. raciborskii* were related with low DIN (<100µg $L^{-1}$) concentrations and lower light availability.

Therefore, the success of this species in Brazilian aquatic ecosystems can be related to its low light requirements, high affinity for ammonium, capacity to fix atmospheric nitrogen ($N_2$) and its ability to tolerate high ionic concentration.

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*Chapter 5*

# IS CYANOBACTERIAL DOMINANCE IN BRAZILIAN SEMI-ARID RESERVOIRS REGULATED BY ENVIRONMENTAL OR STOCHASTIC FEATURES?

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## ABSTRACT

Cyanobacterial blooms have been associated with eutrophication process in aquatic ecosystems; however, more importantly than identifying the general conditions that determine the cyanobacterial occurrence, is the understanding of the factors that regulate the occurrence of the individual species that can be related with the temporal dynamic of composition and biomass of this group. The neutral model of biodiversity describes how local communities are structured if population dynamics are statistically identical among species in a constant, possibly patchy, environment with random speciation. Our objective was to determine whether cyanobacterial communities exhibit spatial structuring, thus suggesting neutrality, or are structured by local environments features, suggesting that they are under niche-based control. We analyzed the determinants of cyanobacterial community structure across three reservoirs located in Brazilian semi-arid region. Eight components of species variation were separated and identified: pure environmental, pure temporal, pure spatial, spatially and temporally structured environmental variation, spatial-temporal, spatial-environmental, and temporal-environmental component. Our results suggest that the distribution of cyanobacteria community is affect mainly by the environmental component, with an important temporal

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component in species distribution. The finding that temporal configuration, with dominance interchanges among *Microcystis protocystis*, *Cylindrospermopsis raciborskii*, and *Planktotrhix agardii*, was more important than spatial factors, can be related to temperature and water transparence as the main controlling factors of cyanobacterial community in semi-arid ecosystems.

## INTRODUCTION

A major goal in community ecology is to identify the mechanisms that determine species structure and distribution in ecosystems [1]. Ecologists have carried out significant progress in small spatial and temporal scales using reductionist approaches [2]. However, important theoretical and empirics questions remain unanswered such as distribution and abundance between species, the relationship between distribution and species and the variation of diversity among environments [3]. Cyanobacteria are organisms with a large distribution all over the world. Characterized for being cosmopolitan and to inhabit diverse and adverse local, these organisms has been studied widely [4,5,6,7], mainly for being potentially producers of extremely toxic compounds, which can cause problems to public health and damages to the environment [8,9]. The success of cyanobacterial blooms, is usually influenced by multiple factors, such as light, temperature, oxygen concentration, pH, phosphorus and nitrogen concentrations, stability of water column and herbivory, these factors acting synergistically and antagonistically [10]. However, more important than identifying the general conditions that determine the occurrence of cyanobacteria is to understand the factors that regulate the occurrence of the individual species, which can be related with the temporal dynamic of composition and biomass of this group. The neutral model of biodiversity [2] describes how local communities are structured if population dynamics are statistically identical among species in a constant, possibly patchy, environment with random speciation. Even thought the temporal components of ecological variation make the study of community structure highly complex, ecologists usually do not know the fraction of variation on the species data controlled only by environmental or time, or by both sets of variables. In order to help solving this problems Boccard et al. [11] proposed a method of partitioning out the spatial component of ecological variation based on canonical correspondence analyses (CCA) and redundancy analysis (RDA). The authors used constrained and partial canonical techniques [12] and separated the species abundance variation into four independent components: a pure spatial, a pure environmental, an environmental component with spatial structure, an environmental component with temporal structure and an undetermined component. Anderson and Gribble [13] propose an extension to this method including temporal variables in the analyses.

In arid and semi-arid regions the hydrological regime (temporal scale) is considered the key factor driving ecological functioning and biodiversity to aquatic ecosystems [14]. Therefore, the present study describes the seasonal variation in the abundance of cyanobacterial communities from reservoirs located in the Brazilian semi-arid region, and separates and identifies the independent components of the variation by canonical correspondence analysis. Our objective was to determine whether cyanobacterial communities exhibit spatial structuring, thus suggesting neutrality (stochastic), or are

structured by local environmental features, suggesting that they are under niche-based control.

## MATERIAL AND METHODS

### Description of the Study Area

The study was conducted in three reservoirs located in the Paraiba River Basin in a semi-arid region of Brazil. The Poções Reservoir (7°53'38"S, 37°0'30"W) occupies an area of 7.73 $km^2$, with a mean depth of 8m, and an accumulation capacity of approximately $30\times10^6$ $m^3$. The Cordeiro Reservoir (7°47'38.00" S, 36°40'14.04" W) has a capacity of approximately $70\times10^6$ $m^3$, covers 11.23 $km^2$ and has a mean depth of 8,3 m. The Camalaú Reservoir (7°53'33.94" S, 36°50'39.16" W) covers 8 $km^2$, has a capacity of approximately $47\times10^6$ $m^3$ and a mean depth of 12.5m (Figure 1).

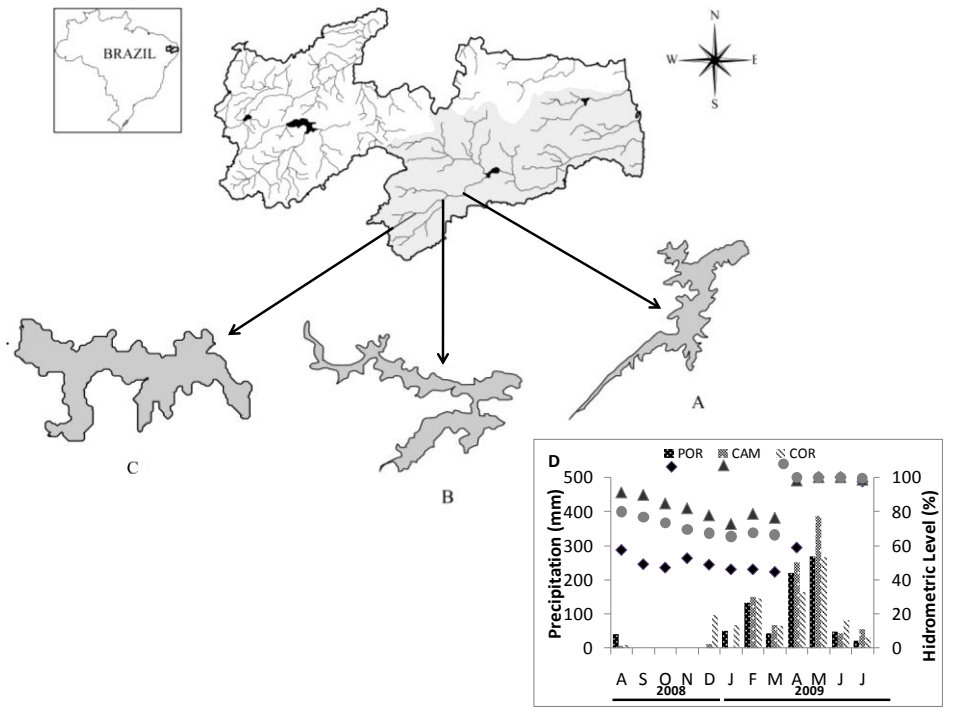


Figure 1. Localization of the study areas: (A) Poções reservoir, (B) Cordeiro reservoir and (C) Camalaú reservoir. The figure 1 D indicated the precipitations and hydrometric level of the reservoirs.

In the region where the reservoirs are inserted the climate is BSwh according to Koppen's classification , semi-arid hot with 7-9 months dry. The temperature averages are between 28 and 31ºC and the precipitations about 700 mm/year. In all reservoirs the dry period occurred between August/2008 to March/2009. In these periods low precipitation was observed (40-280 mm) and the hydrometric levels of the reservoirs were generally low too. In the rainy season, May-July/2009, the precipitation was higher (150-450 mm) and the reservoirs reached

the maximum capacity (Figure 1D). The wind velocity in the region is irrelevant, ranged to 3 and 4m/s. The total annual evaporation varies between 2500 and 3000 mm with decreasing values by west to east.

## Water Sample Collection

Bimonthly samples were performed during a year between August/2008 to July/2009. Environmental variables were measured and phytoplankton samples were taken at four depths, according to light attenuation by Secchi disk (100%, 50%, 1% and 0% of luminosity) on a permanent site in the deepest region of reservoirs. Environmental variables evaluated were: water temperature (digital thermometer with 0.1ºC accuracy), pH (portable membrane pH meter), dissolved oxygen (digital oxymeter) and water transparency (Secchi disk). Water samples were collected in PVC bottles previously cleaned with distilled water and kept in ice for transportation to the lab, where they were frozen and nutrients (ammonium, nitrite, nitrate, total nitrogen, soluble reactive phosphorus and total phosphorus) were analyzed according to APHA [15]. Sub-samples (300 mL) were preserved in Lugol's solution and observed under an inverted microscope using five 25-mL sedimentation chambers for phytoplankton identification and quantification [16]. Phytoplankton genera and species were identified by distinguishing morphological characteristics cited in the literature [17,18,19]. Phytoplankton biovolume was obtained by multiplying population density by individual volume [20]. Zooplankton community was identified and quantified in order to know the pressure of herbivory on cyanobacterial community. Zooplankton samplings were collected through vertical hauls of the entire water column filtering 100 L of the water using plankton nets of 68 µm mesh. Organisms were preserved in 4% formalin. A quantitative analysis of zooplankton was done by counting subsamples, using a Sedgwick-Rafter chamber. Length–weight relationships [21,22] were used to estimate biomass. If possible up to 50 individuals were measured.

## Data Analysis

Canonical correspondence analysis was used for data analysis. Four fundamental matrices were used for the analysis:

- Species matrix – the initial data matrix (72 station-points×94 species) was reduced by dominant species (biovolume more them 50% of total biovolume). The resulting matrix (72 stations×8 species) was analyzed after the log transformation of biovolum data, $y=\ln(x+1)$ to avoid skewness [23];
- Environmental matrix – the 13 quantitative environmental parameters were standardized by $\ln(x+1)$;
- Spatial matrix – the spatial variables was calculated including all terms for a cubic trend surface regression, with x=longitude (centered) and y=latitude (centered) (i.e terms included were: x, y, x2, xy, y2, x3, x2y, xy2 and y2; [24]);

- Temporal matrix – was used a qualitative parameter "time", related with each sampling period (August/08, October/08; January/09; March/09; May/09; July/09), which yielded 6 dummy binary variables (instrumental variables).

Spatial matrices were also constructed for: spatial + temporal variables, environmental + spatial variables and environmental + temporal variables. A series of steps, involving constrained and/or partial CCA, were done using CANOCO (Table 1). For each step, the value of the sum of canonical eigenvalues for analysis was recorded. The proportion of the total variation that this sum represented was then calculated and multiplied by 100 to obtain a value for the explained variation percentage for each step. In addition for each step, unrestricted permutation tests (with 1000 permutations) were done of the overall trace statistic [25]. The values corresponding to the percentage variation for each component of variation was calculated according the method of Anderson and Gribble [13]. The percentage of total explained variation ($\Omega$) is equal to [1]+[7]+[12]. Thus, the unexplained variation, U=(100-$\Omega$). The pure environmental component, E, is equal to [6]; the pure spatial component, S, is equal to [9]; the pure temporal component, T, is equal to [12]; and the spatially and temporally structured environmental variation, STE, is equal [9]+([2]-[7])+([2]-[8])-[2]. Once the value for STE has been obtained, then calculations for remaining three components: spatial temporal component, ST, is equal ([2]-[8])-STE; spatial environmental component, SE, equal to ([1]-[4])-STE; and temporal environmental component, TE, ([1]-[5])-STE. This finishes the analysis, partitioning the variation into eight components, and allows a complete Venn diagram to be drawn for each the data.

**Table 1. Steps in the analysis done using CANOCO**

| Step | Description |
|---|---|
| [1] | CCA of species matrix, constrained by the environmental matrix |
| [2] | CCA of species matrix, constrained by the spatial matrix |
| [3] | CCA of species matrix, constrained by the temporal matrix |
| [4] | CCA of species matrix, constrained by the environmental matrix, with spatial variables treated as covariables |
| [5] | CCA of species matrix, constrained by the environmental matrix, with temporal variables treated as covariables |
| [6] | CCA of species matrix, constrained by the environmental matrix, with spatial +temporal variables treated as covariables |
| [7] | CCA of species matrix, constrained by the spatial matrix, with environmental variables treated as covariables |
| [8] | CCA of species matrix, constrained by the spatial matrix, with temporal variables treated as covariables |
| [9] | CCA of species matrix, constrained by the spatial matrix, with environmental + temporal variables treated as covariables |
| [10] | CCA of species matrix, constrained by the temporal matrix, with environmental variables treated as covariables |
| [11] | CCA of species matrix, constrained by the temporal matrix, with spatial variables treated as covariables |
| [12] | CCA of species matrix, constrained by the temporal matrix, with environmental + spatial variables treated as covariables |

# RESULTS

## Limnological Features

Temperatures remained at approximately 28 °C at all sites. As regards vertical temperature profiles, the reservoirs present different patterns of mixing and stratification.

Poções and Camalaú Reservoirs have a period of stratification during the dry season, in the months of October/08 and January/09, respectively, with isothermal conditions in the water column in the remaining months. Both reservoirs had well-defined stratification with thermocline surface at the beginning of the rainy season. Cordeiro reservoir had two periods of isothermia in the early dry period (Aug/08), and in the beginning of the rainy season (Mar/09) and in the remaining months presented a stratified thermocline (Figure 2).

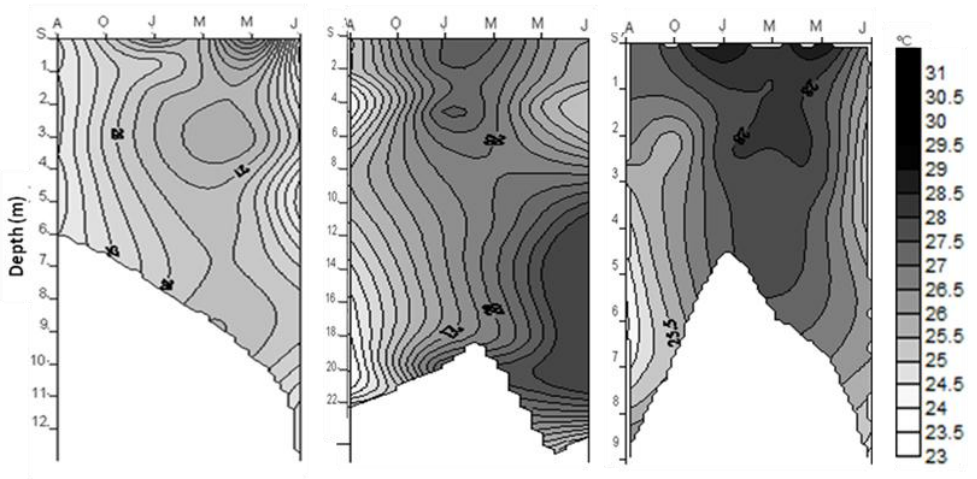


Figure 2. Variation of the water temperature profile in Poções reservoir (Left), Camalaú reservoir (Middle) and Cordeiro reservoir (Right).

The dissolved-oxygen concentrations indicated the existence of oxygenated water columns.

Water pH levels were alkaline to neutral, showing low variation throughout the reservoirs. Light penetration was low in water from Cordeiro and moderate in water from Poções and Camalaú. In all reservoirs nitrate was the most abundant nitrogen form followed by ammonium and nitrite. The SRP concentration was in general only 30% of total phosphorus. The average concentration of total phosphorus was 224.77$\mu gL^{-1}$, 185.13 $\mu gL^{-1}$, 125.19 $\mu gL^{-1}$ in Poções, Cordeiro, and Camalaú Reservoirs, respectively. There were not significant differences in the distribution of the variables in the water column (Table 2).

A total of 18 zooplankton taxa were identified in all three reservoirs studied, belonging to Rotifera (11 taxa), Copepoda (4 taxa), and Cladocera (3 taxa).

Copepods dominated the zooplankton community in the three reservoirs in terms of biomass, making up over 70% of the total biomass, and the genera *Notodiaptomus* and *Thermocyclops* were the most representative. *Moina* and *Ceriodaphinia* were the main genera

of cladocerans observed, and among rotifers, *Brachionus*, *Keratella* and *Filinia* contributed most to the biomass of the group (Table 2).

**Table 2. Limnological variables measured in reservoirs**

| Variables | Cordeiro reservoir | | Camalaú reservoir | | Poções reservoir | |
|---|---|---|---|---|---|---|
| | Mean | ±SE | Mean | ±SE | Mean | ±SE |
| Water Transparence (m) | 0.94 | 0.16 | 1.70 | 1.00 | 1.33 | 0.32 |
| Water Temperature(ºC) | 27.13 | 1.15 | 26.73 | 1.62 | 25.88 | 1.63 |
| pH | 8.03 | 0.49 | 7.51 | 0.56 | 7.98 | 0.43 |
| Dissolved Oxigen (mg $L^{-1}$) | 6.09 | 2.14 | 5.58 | 1.92 | 4.64 | 1.47 |
| N-$NH_4$ (µg $L^{-1}$) | 28.79 | 32.64 | 26.79 | 32.97 | 24.49 | 22.18 |
| N-$NO_2$ (µg $L^{-1}$) | 5.12 | 5.33 | 7.37 | 8.15 | 9.08 | 8.74 |
| N-$NO_3$ (µg $L^{-1}$) | 149.16 | 136.37 | 189.32 | 138.53 | 138.83 | 137.63 |
| SRP (µg $L^{-1}$) | 27.46 | 28.35 | 29.15 | 22.05 | 65.96 | 34.31 |
| Total Phosphorus (µg $L^{-1}$) | 185.13 | 159.31 | 125.19 | 114.32 | 224.77 | 161.51 |
| Total Nitrogen (µg $L^{-1}$) | 3602.44 | 1999.02 | 1622.96 | 1241.50 | 2097.86 | 1740.80 |
| Biomass of Naupliis (µg PS $L^{-1}$) | 5.70 | 8.38 | 5.70 | 8.38 | 7.47 | 2.34 |
| Biomass of Calanoida (µg PS $L^{-1}$) | 8.99 | 14.50 | 8.99 | 14.50 | 8.43 | 5.94 |
| biomass of Cyclopoida (µg PS L-1) | 11.76 | 11.69 | 11.76 | 11.69 | 53.40 | 87.75 |
| Biomass of Rotifera (µg PS $L^{-1}$) | 0.02 | 0.01 | 0.02 | 0.01 | 0.17 | 0.18 |
| Biomass of Cladocera (µg PS $L^{-1}$) | 4.59 | 7.05 | 4.59 | 7.05 | 13.13 | 15.39 |

## Cyanobacterial Community

The phytoplankton community of the three reservoirs is represented by a total of 94 taxa (genus and species) from the following divisions: Chlorophyta (33), Cyanophyta (24), Bacillariophyta (23), Euglenophyta (4), Zygnemaphyceae (5), Oeodogonophyceae (1) and Chlamydophyceae (4). The Cyanophyta group was represented by 24 taxa at Camalaú reservoir, 21 in Poções Reservoir and 17 in Cordeiro Reservoir. The species *Cylindrospermopsis raciborskii* Woloszynska, *Microcystis aeroginosa* Kützing and *Planktothrix agardhii* Gomont were common in all reservoirs. *Sphaerocavum brasiliense* Azevedo and Sant'Anna occurred only in the Cordeiro Reservoir, and *Anabaena circinalis* Rabenhorst occurred only in the Camalaú Reservoir. With regard to the relative abundance of cyanobacteria, the highest biovolume occurred in Cordeiro Reservoir 9.13 $mm^3L^{-1}$ (± 6.36) followed by Camalaú Reservoir 0.47 $mm^3.L^{-1}$ (±0.30) and Poções Reservoir 0.30 $mm^3.L^{-1}$ (±0.29).

The high phytoplankton biomass observed in Cordeiro Reservoir was due to episodes of potentially toxic cyanobacterial blooms, which represented over 65% of the total biomass of

phytoplankton. The blooms were formed by populations of different species of cyanobacteria that alternated in dominance throughout the study; *Microcystis protocystis* (Jan/09), *C. raciborskii* (October/08, Jul/09) and *P. agardhii* (May/09) contributed most to the phytoplanktont biomass in the Reservoir. The dominance of cyanobacteria was alternated with the diatom *Aulacoseira granulata* in August/08 and March/09 (Figure 3A).

In Camalaú Reservoir, the dominance of cyanobacteria was alternated between *C. raciborskii* and *P. limnetica* in August/08, *M. protocystis* was dominant in October/08 and July/09, and *A. circinales* in May/09 (Figure 3B). *A. granulata* was dominant in January/09 and March/09. Cyanobacterial blooms had not occurred in Camalaú and Poções reservoirs, but dominance of cyanobacteria has been alternating with diatoms. The cyanobacteria *P. agardhii* was dominant in Poções in January/09 and March/09, *A. granulata* was dominant in the other months (Figure 3C).

In relation to the species partitioning of ecological variation, the correspondence analysis of the species matrix (CA) pointed out that the sum of all eigenvalues equals 1.241. The percentage of the total explained variation of the species data and the sum of all canonical eigenvalues at each step of the method is presented in Table 3.

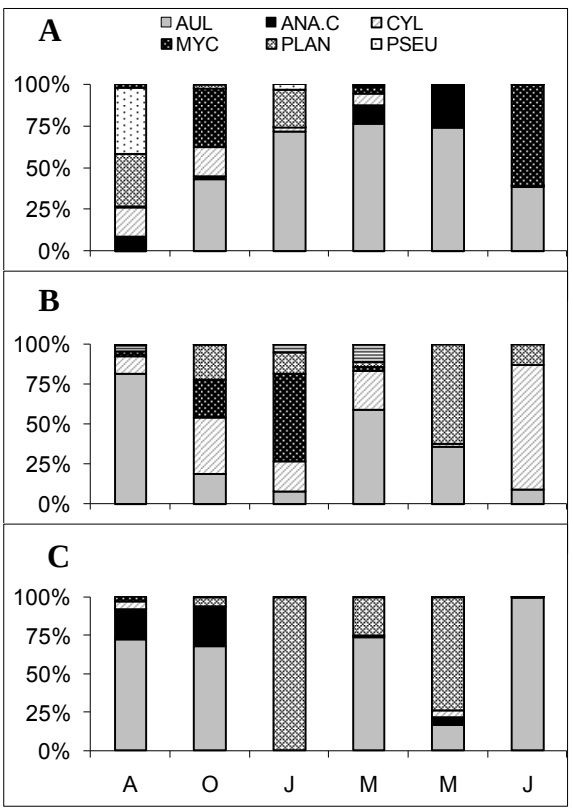


Figure 3. Temporal variation in biovolum of dominant species of cyanobacteria in Cordeiro (A), Camalaú (B) and Poções (C) reservoirs between August/2008 and July/ 2009. AUL= *A. granulata*; ANA.C=*A.circinales*; ANA.A=*A. aphanozomenoide*; PSEU= *P. limnetica*; PLAN= *P. agardhii*; MYC= *M. protocystis*; CYL=*C. raciborskii*.

Figure 4 illustrates the relative importance of the various processes that control the variation of the cyanobacterial community in the reservoirs. The percentage of total explained variation (Ω) was calculated as 75.9%, consequently the unexplained variation, U, was 24.1%. In general, the greatest variation in cyanobacterial community data was explained by environmental component and by the combined temporal/environmental (TE) component (Figure 4). This means that the processes governing the species abundance distribution have a significant temporal structuring component, independent of the environmental variables considered. Also, the major part of the variation explained by the environmental variables is due to non temporal effects, since only a residual amount of the environmental variation is shared by time.

The influence of the environmental factors (pure environmental) on the abundance of the species are represented in Figure 5. The ordination diagram shows the segregation data for Poções Reservoir in a negative axis I, which was associated to higher values of transparency, high biomass of zooplankton and fitoplâncton community (dominated by *A. granulata*).

**Table 3. Summary of constrained and partial canonical correspondence analyses for data on biomass of six species of cyanobacteria**

| Step in analyses | Sum of canonical eigenvalues | Explaned variation (%) | *P* |
|---|---|---|---|
| [1] | 0.71 | 56.97 | 0.006 |
| [2] | 0.23 | 18.45 | 0.002 |
| [3] | 0.38 | 30.46 | 0.002 |
| [4] | 0.56 | 44.96 | 0.01 |
| [5] | 0.51 | 41.18 | 0.002 |
| [6] | 0.39 | 31.51 | 0.002 |
| [7] | 0.08 | 6.45 | 0.002 |
| [8] | 0.17 | 14.02 | 0.002 |
| [9] | 0.05 | 4.35 | 0.002 |
| [10] | 0.18 | 14.67 | 0.002 |
| [11] | 0.32 | 25.95 | 0.002 |
| [12] | 0.16 | 12.49 | 0.002 |

On the negative axis stood sampling units for the Cordeiro Reservoir, was associated with higher temperature, correlated with high biomass of *M. protocystis* and *C. raciborskii*. On the positive axis II is observed segregation of cases relating to Camalaú Reservoir with concentrations of SRP and high biomass of *A. circinales* and *P. agardhii*.

The variation of the species abundance data left after removing the effect of the environmental factors is shown in Figure 6. The ordination diagram shows the temporal species distribution in the reservoirs.

The positive side of axis I distinguishes those species exclusively or predominantly present in dry period from the others present in rain period. Species highly correlated to this axis are *P. agardii* and *C. racibosrkii*, which were abundant in the reservoirs during the dry periods. In the negative side of axis I are placed species particularly numerous in rainy period, such as *A. granulata*. A central group around the intersection of both axes was identified as species of wide temporal distribution, present all the year round, and showing no relation to the canonical axes formed (Figure 6).

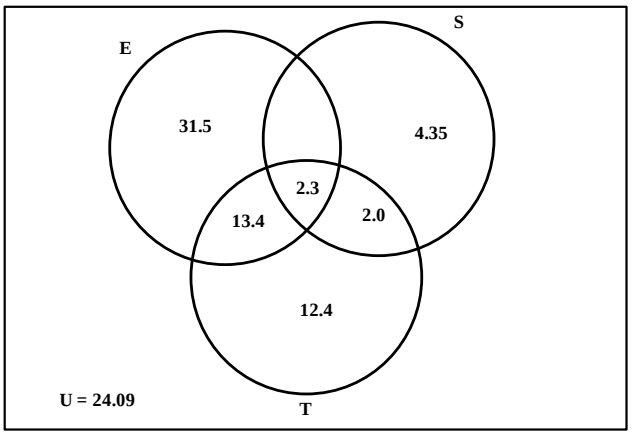


Figure 4. A Venn diagram showing the partitioning of variation (%) according to three sets of independent variables, environmental (E), temporal (T) and spatial (S). The rectangle is the set corresponding to the total variation in the dependent (species) data. Each area of overlap of the three circles represents the intersection of three sets.

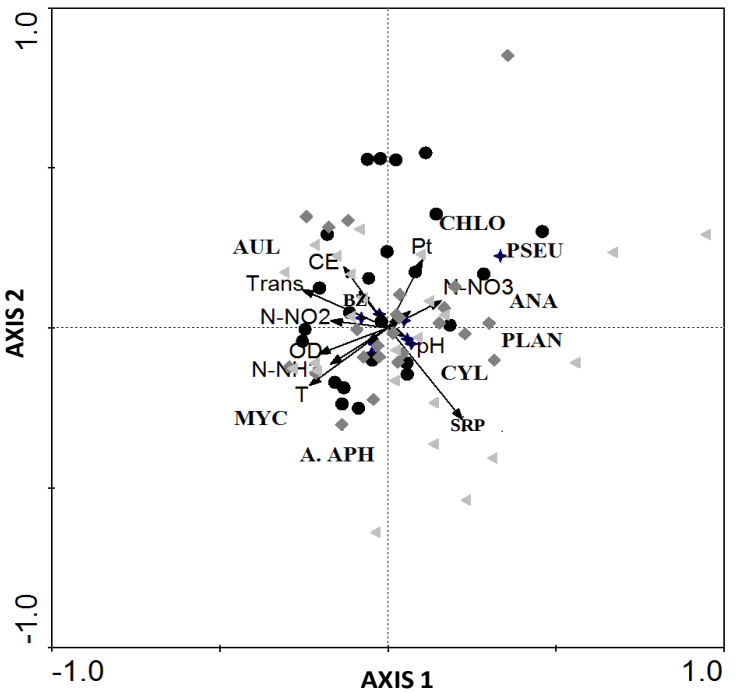


Figure 5. Partial canonical correspondence analysis showing the effect of environmental variables without temporal influence on the species distribution. Species names are *Microcystis protocystis* (MYC), *C. raciborskii* (CYL), *P. agardii* (PLA), *A. circinales* (ANA), *P. limnética* (PSE), *A. granulata* (AUL), *A. aphanizomenoides* (A.APH). The reservoirs are indicated as ( ◂ ) Camalaú, ( ◆ ) Cordeiro and ( ⬬ ) Poções. Arrows show magnitude and direction of the environmental variables water Transparence (Trans), water temperature (T), dissolved oxygen (OD), ammonium (N-NH4), nitrite (N-NO2), nitrate (N-NO3), soluble reactive phosphorus (SRP), total phosphorus (Pt), Zooplankton Biomass (BZ).

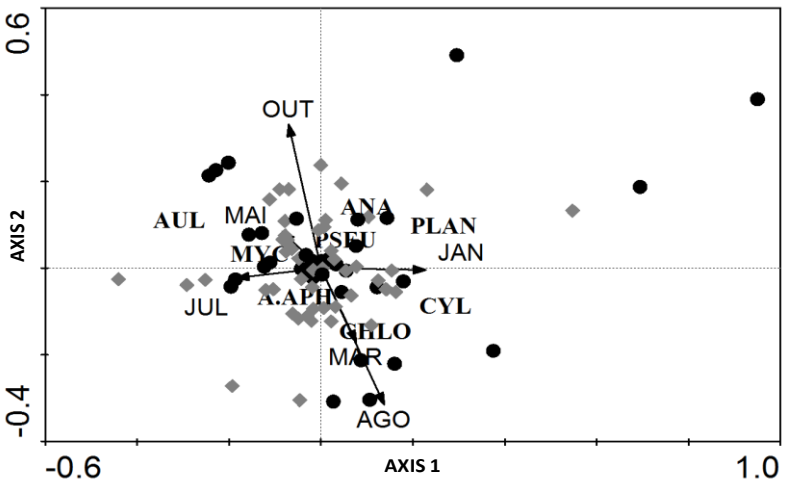


Figure 6. Partial canonical correspondence analysis showing the effect of time on species distribution after removing the effect of environmental factores. Species names are *M. protocystis* (MYC), *C. raciborskii* (CYL), *P. agardii* (PLA), *A. circinales* (ANA), *P. limnética* (PSE), *A. granulata* (AUL), *A. aphanizomenoides* (A.APH). The reservoirs are indicated in ( ● ) Camalaú, ( ◆ ) Cordeiro and ( ◀ ) Poções. Arrows show magnitude and direction of temporal variables.

## DISCUSSION

The variation in cyanobacterial populations structure was similarly attributable to pure environmental factors and temporal factors, suggesting that cyanobacteria might also be under niched-based control. This result is partially in agreement with the notion of high dispersal capacity of small aquatic organisms.

The results show that a large part of the total variation present within the data can be attributed to environmental factors, mainly to temperature and water transparency, as indicated by the correlation analysis. After excluding the effects of environmental variables, a significant temporal structure remained on the species abundance variation. In aquatic ecosystems of semi-arid regions, the hydrological regime is considered the key factor driving ecological functioning and biodiversity [14].

The alternation of dominant species of phytoplankton between Bacillariophyta and cyanobacteria is common in tropical reservoirs [27,28,30,31]. In the reservoirs under study, stratification and water mixture, and the transparency addressed this interchange. Diatoms are reported as well developed in mixing conditions in the water column, since the large silicates frustules make them denser than water, so in stratification conditions they settle faster. Moreover, the constant mixing of the water resuspends phosphate, nitrogen and silicate of the sediment which favors the development of these species [32].

The synergy between some environmental factors, combined with specific adaptations is more plausible to explain the substitution pattern of the main species, and especially blooms of *M. protocystis* and *C. raciborskii* in the reservoirs. The main species of filamentous cyanobacteria have similar characteristics such as size, position, adjustment in the water column and potential to produce toxins. While the large size provides some immunity against herbivores, the presence of gas vacuoles promotes a rapid adjustment of the vertical position.

*A. circinalis*, *C. raciborskii*, *P. agardhii* and *M. protocystis* were dominant during periods of thermal stratification (except in the Camalaú reservoir) associated with high temperature, which was critical to a rapid biomass increase of these species. *C. raciborskii* has been recorded as dependent on physical stability of the water column [33], and the dominance of this species in many systems has been related to periods of stratification [28,34] as happened in the Camalaú reservoir in August/08. The dominance of this species particularly is given by its physiological capacity to grow in low light [28,33].

The stratification of the water column and low concentrations of nitrogen associated with decrease in water transparency give advantages to the dominance of *C. raciborskii* [35]. In contrast, studies in Australian reservoirs attributed the dominance of *C. raciborskii* to its ability to match and store nutrients and to regulate their position in the water column for a better reception of light [36].

The occurrence of *C. raciborskii* with populations of different shapes has been reported in reservoirs in northeastern Brazil [28,30,37] and in Australia [36], but it is not known what leads to the occurrence of coiled trichomes [37]. The absence of the trichomes with heterocytes in *C. raciborskii* even at low concentrations of nitrogen has been also reported [27,28,31]. According to these authors, the absence of heterocytes occurs because these species are well adapted to low light, suggesting good ability to use a better strategy to capture light and fixing nitrogen.

The dominance of *P. agardhii* in the reservoirs can be attributed to thermal stratification of the water column, its affinity for nitrogen, and low concentrations of phosphate. This species is adaptable to develop in phosphorus limiting conditions for other species of cyanobacteria [38]. In Cordeiro Reservoir this species was dominant in May/09 July/09 being replaced by *C. raciborskii*. This change was due to the decreased availability of nitrogen. The presence of the heterocytes in *C. raciborskii* during this period and its ability to fix nitrogen favored the dominance of this species.

The alternation in dominance between *M. protocystes* and *C. raciborskii* that occurred in the Cordeiro Reservoir was also related to the concentration of phosphorus. *Microcystis* absorbs more nitrogen and phosphorus in their metabolic processes than *C. raciborskii*, and develops better in conditions of greater water transparency. Such features were observed in the months of January/09 in Cordeiro reservoir and in October/08 and July/09 in Camalaú reservoir. A similar pattern was also observed in a subtropical eutrophic reservoir [38].

The cyanobacterial dominance in reservoirs has been consequence to low herbivory pressure [27]. On the other hand, Calanoid and rotifer are important components of zooplankton in eutrophic reservoirs in semiarid region, possibly for their capacity to fragment cyanobacterial filaments [28] and use small colonies as a food resource [29]. However no relationship was observed between the zooplankton community and cyanobacterial occurrence in this study.

The influence of all factors regulating the relationship diversity-species abundance is difficult to measure. Many biological interactions should be evaluated only through particular experimental designs. Nevertheless, the present results demonstrate that the dynamics of the cyanobacteria in reservoirs located in Paraíba river basin, in the Brazilian semi-arid, is mainly affect by the environment, with an important temporal component in species distribution and not by stochastic features.

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*Chapter 6*

# PICOPHYTOPLANKTON COMMUNITY STRUCTURE IN A HYPERSALINE COASTAL LAGOON: ROLE OF SALINITY AND LINKS WITH VIRAL AND MICROBIAL COMMUNITIES

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## ABSTRACT

Picophytoplankton (i.e. cyanobacteria and pico-eukaryotes) are abundant and ecologically critical components of the autotrophic communities in the pelagic realm. These micro-organisms colonized a variety of extreme environments including hypersaline waters. However, the distribution of these organisms along strong salinity gradients has barely been investigated. The abundance and community structure of pico-phytoplankton, heterotrophic bacteria and virus-like particles (VLP) were investigated along a natural continuous salinity gradient (18‰ to 155‰) in a South Australian temperate coastal lagoon, using flow cytometry. A concomitant increase in viral and bacterial abundances with salinity was observed from 50‰ to 150‰, where the highest abundances were observed. Highest picophytoplankton abundances were recorded under salinity conditions ranging between 80‰ and 110‰. A viral population, exhibiting flow-cytometric characteristics of picophytoplankton viruses was observed from 50.3‰ to 100‰. Two populations of

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cyanobacteria (likely *Synechococcus* and *Prochlorococcus*) and 5 distinct populations of pico-eukaryotes were identified along the salinity gradient. The picophytoplankton cytometric-richness decreased with salinity and the most cytometrically-diverse community (4 to 7 populations) was observed in the brackish-marine part of the lagoon (i.e. salinity below 35‰). One population of pico-eukaryotes dominated the community throughout the salinity gradient and was responsible for the bloom observed between 80‰ and 110‰. Finally only this halotolerant population and *Prochlorococcus*-like cyanobacteria were identified in hypersaline waters (i.e. above 140‰). The complex patterns described here represent the first observation of cyanobacteria and pico-eukaryotes dynamics along a continuous gradient where salinity increases from 18‰ to 155‰. Although the spatial dynamics observed here are in accordance with the patterns observed previously along discontinuous salinity gradients, the high abundances of pico-phytoplankton as well as the existence of a *Prochlorococcus*-like population in hypersaline waters set this saline lagoon apart from the systems studied previously.

## INTRODUCTION

The ubiquitous distribution of viruses, heterotrophic bacteria, picophytoplankton (i.e. cyanobacteria and pico-eukaryotes) and their importance in terms of biomass and production, make them a critical component of food webs and carbon cycling in marine systems [1-7]. However, most of the investigations concerned pelagic ecosystems and the understanding of the dynamics of these communities in coastal waters and estuaries still need to be documented.

Coastal habitats are characterized by strong environmental gradients, which are likely to be important areas of highly dynamic compositional and functional changes [8]. In particular, important ecological changes such as decreasing biodiversity and increasing dominance of prokaryotes are assumed to occur along salinity gradients [9]. However, little is still known about the effect of salinity on the distribution and community composition of viruses, heterotrophic bacteria and picophytoplankton, and the consequences of shifts in communities' composition on ecosystem functioning along natural salinity gradients.

The dynamics of viruses and heterotrophic bacteria have been mainly investigated along salinity gradientsin estuaries [8,10,11-14]. However, in these studies estuarine salinities did not exceed 50‰, and the dynamics of these organisms under high salinity conditions (i.e. above 50‰) has been mainly conducted in crystallizer ponds from solar salterns [15-19] or in hypersaline lakes (i.e. 0.2‰ to 364‰; [20-24]. Similarly, while the environmental factors controlling the distribution and composition of cyanobacteria and pico-eukaryotes have been extensively reviewed [1,25-27] most of these investigations concerned pelagic ecosystems and the cyanobacteria and pico-eukaryotes communities in coastal waters have still received little attention. Several studies investigated planktonic cyanobacteria and/or eukaryotic picophytoplankton communities in estuaries and bays [e.g. 27-29]. However, in these studies, salinity never exceeds 35‰ and investigations of the dynamics of phototrophic communities under high salinity conditions (i.e. above 35‰) have been restricted to crystallizer ponds andsolar salterns [30-32] and hypersaline lakes [33-35].

In this context, the present chapter investigates the distribution of viral, bacterial and picophytoplankton (i.e. cyanobacteria and eukaryotes) communities along a strong and

continuous salinity gradient in the Coorong, a shallow South Australian coastal lagoon. With salinity gradually increasing from brackish (18‰) to hypersaline (155‰), the Coorong represents a unique model system to investigate the role of salinity in shaping the niche development in viruses, heterotrophic bacteria, and cyanobacteria and pico-eukaryotes. More specifically, given the lack of information related to the dynamics of picophytoplankton, viral and bacterial communities along continuous natural hypersaline gradients, our objectives were (i) to investigate the changes in abundance and diversity of flow cytometrically-defined populations of planktonic viruses, heterotrophic bacteria, cyanobacteria and pico-eukaryotes along the salinity gradient, (ii) to identify the main factors driving their distributions, and (iii) to discuss the potential mechanistic links behind the observed patterns.

# METHODS

## Sampling

The Coorong is a South Australian shallow lagoon, running parallel to the coast over nearly 150 km and separated from the open ocean by a network of sand dunes (Figure 1).

This coastal lagoon forms the Murray Mouth with the lower lakes (Lake Alexandrina and Lake Albert), which is the terminal lake system of the River Murray [36]. The Coorong is characterized by a strong salinity gradient with salinity ranging from *ca*. 20‰ close to the Murray Mouth to more than 150‰ near Salt Creek (Figure 1).

Samples were collected at 20 locations along the lagoon, from the brackish waters near Goolwa (salinity=18‰) to the hypersaline waters near Salt Creek (salinity=155‰; Figure 1) on February 3-4, 2007. At each sampling site, referred to as $S_i$ with $i$ = 1 to 20 (Figure 1), measurements and water sample collection were performed in 50 cm of water (a depth representative of most parts of the lagoon) from (i) the sub-surface waters and (ii) at the water-sediment interface (WSI).

Temperature (ºC), conductivity (mS $cm^{-1}$) and dissolved oxygen concentrations (DO; mg $l^{-1}$) were recorded using a YSI 85 (Fondriest) multiparameter probe. Salinity (‰) was calculated from temperature and conductivity following Fofonoff and Millard [37]. Water samples were collected at each depth using acid-washed 1-liter borosilicate bottles with special care to avoid sediment resuspension.

Dissolved inorganic nutrient concentrations (i.e. ammonium, nitrite, nitrate and phosphate) were determined from 12 ml filtered (Whatman GF/C) water samples. Analyses were performed in the field using a portable LF 2400 photometer (Aquaspex®) according to standard colorimetric methods for $NH_4^+$ (Indophenol blue), $NO_2^-$ (naphtylethylenediamine), $NO_3^-$ (naphtylethylene diamine after zinc reduction) and $PO_4^{3-}$ (ascorbic acid reduction). The instrument resolution was 0.6 µM for ammonium, 0.2 µM for nitrite, 1.6 µM for nitrate and 1.1 µM for phosphate.

Samples (50 to 100 ml) for suspended particulate material concentration (SPM; mg $l^{-1}$) were filtered through pre-combusted (400ºC; 4 hours) and pre-weighted glass-fiber filters (Whatman GF/C), and immediately deep frozen in liquid nitrogen until analysis. In the laboratory, filters were rinsed with MilliQ water, dried at 60°C for 24 h, and reweighed to determine the mass of suspended solid retained on the filter [38].

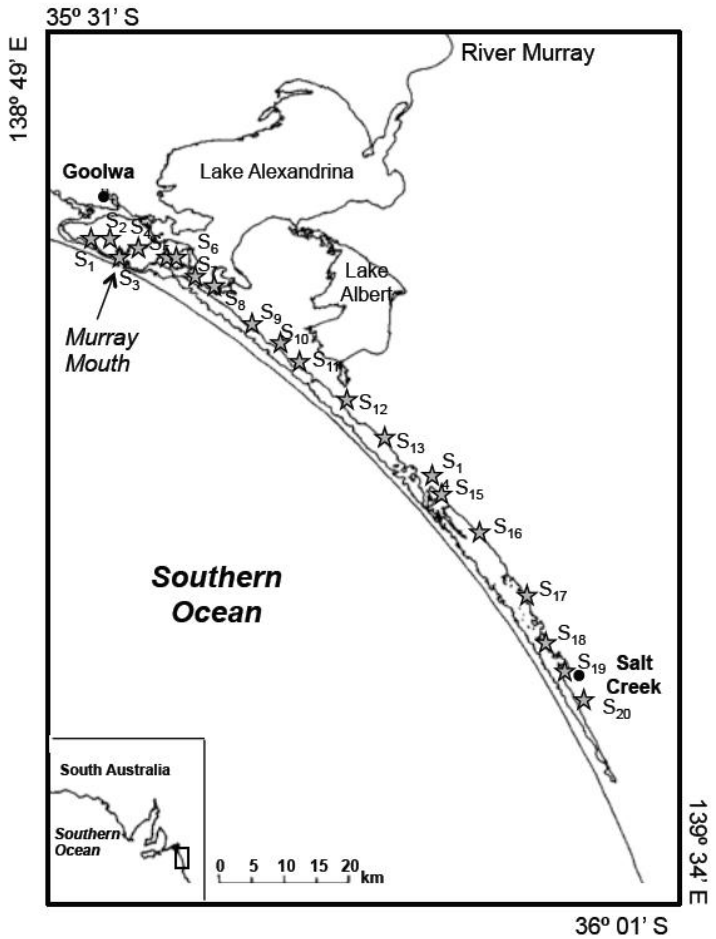


Figure 1. Study area, the Coorong (South Australia). This shallow coastal lagoon, located approximately 90 km south-east of Adelaide in South Australia, is bordered by adjacent fresh water lakes (Lake Alexandrina and Lake Albert) and separated from the open ocean by a network of sand dunes. This coastal lagoon forms with the Murray Mouth with the lower lakes, which is the terminal system of the River Murray. More than 140 km in length, the Coorong is characterized by a strong salinity gradient with salinity values ranging from ca. 20‰ close to the Murray Mouth to more than 150‰ near Salt Creek. Location of the 20 sampling sites (stations $S_1$ to $S_{20}$) are indicated by grey stars.

The bulk phase phytoplankton biomass was estimated through chlorophyll *a* (Chl*a*) concentrations. Samples (50 to 100 ml) were filtered through glass-fiber filters (Whatman GF/C) and immediately deep frozen in liquid nitrogen until analysis. Chlorophyllous pigments were then extracted in 5 ml of methanol in the dark at 4°C during 24 h [39]. Concentration of Chl*a* (µg $l^{-1}$) was determined following Strickland and Parson [40] using a Turner 450 fluorometer previously calibrated with a pure Chlorophyll *a* solution (*Anacystisnidulans* extract, Sigma Chemicals, St Louis).

Two distinct sets of samples (3 × 1 ml) were collected for the identification and enumeration of (i) virus-like particles and heterotrophic bacteria and (ii) picophytoplankton populations by flow cytometry (FCM). FCM analyses were performed using a FACScanto flow cytometer (Becton-Dickinson). VLP, heterotrophic bacteria and picophytoplankton

populations were identified and quantified using the flow cytometry analysis software WinMDI 2.9 (©Joseph Trotter).

## Virus-Like Particles (VLP) and Heterotrophic Bacteria

For the identification and enumeration of VLP and heterotrophic bacteria, samples were collected in triplicate (1 ml) at each sampling station, fixed with 0.5% (final concentration) glutaraldehyde in the dark at 4°C for 15 min, quick frozen in liquid nitrogen and then stored at -80°C until analysis. All samples were processed within 1 month to minimize storage loss [41]. After being quick thawed, samples were diluted (1:10) in 0.2 µm filtered TE Buffer stained with SYBR Green I solution (1:5000 dilution) and incubated at 80°C in the dark for 10 min [42]. Fluorescent beads 1 µm in diameter (Molecular Probes, Eugene, Oregon) were added to all samples, as an internal size and concentration standard. Working bead concentrations were estimated after each flow cytometry (FCM) session under epifluorescent microscopy (EM) to ensure their reliability [43], and all FCM parameters were normalized to bead concentration and fluorescence. Forward-angle light scatter (FSC; 285 V), side-angle light scatter (SSC; 550 V), green (SYBR-I) fluorescence (500 V), red fluorescence (500 V) and orange fluorescence (500 V) were acquired for each sample. Subpopulations of VLP and heterotrophic bacteria were discriminated based on their differences in SYBR-I Green fluorescence and SSC, i.e. as non-overlapping classes of size and green fluorescence [42,44,45]. More specifically, we inferred the presence of bacteriophages and phytoplankton-infecting viruses, and discriminated these two types of virusesusing their differences in side-angle light scatter (related to their size) and green fluorescence (related to their DNA content) following recent works [46,47].

Note that the correspondence between FCM counts and epifluorescence microscopy (EM) counts was investigated using samples collected from 4 stations characterized by increasing salinities, i.e. 27.4‰, 83.0‰, 133.5‰ and 154.8‰ at Stations $S_3$, $S_{13}$, $S_{16}$ and $S_{20}$, respectively (Figure 1). Bacteria and viruses were counted by EM following the SYBR-I Green staining method described in Noble and Fuhrman [48]. Five samples were considered for each site, and for each sample 10 to 20 fields of view were selected randomly; a total of >500 viruses and >500 bacteria were counted on a Leitzdialux 20 EB microscope attached to a Leitz 12 V Quartz lamp at 1000× magnification under blue excitation. No significant differences were found between viral and bacterial counts obtained from FCM and EM (Mann-Whitney *U*-test, $n = 5$, $p > 0.05$) at each of the 4 stations sampled (i.e. $S_3$, $S_{13}$, $S_{16}$ and $S_{20}$). In addition, EM observations did not reveal any specific changes in filamentous bacteria abundances, nor specific changes in bacterial morphologies along the salinity gradient.

## Cyanobacteria and Pico-Eukaryotes

Water samples (1 ml) were collected in triplicate, fixed with 2% (final concentration) of paraformaldehyde, immediately deep-frozen in liquid nitrogen and then stored at -80°C. After being quick thawed, picophytoplankton cells were discriminated and enumerated by FCM according to their specific auto-fluorescence and light scatter properties [45,49]. Forward-angle light scatters (FSC), right-angle light scatter (SSC), and both red and orange

fluorescence were recorded for each sample. As the values of FSC are those most affected by density differences between the sheath fluid and the samples [31], the values of FSC were not used to enumerate cells. Fluorescent beads 1 µm in diameter (Molecular Probes, Eugene, Oregon) were added to all samples as an internal standard. Working beads concentrations were estimated after each FCM session under epifluorescence microscopy to ensure reliability of the beads concentration and all FCM parameters were normalized to bead concentration and fluorescence. *Synechococcus* sp., *Prochlorococcus* sp. and autotrophic pico-eukaryotic cells were discriminated in plots of side-angle light scatter (SSC) versus orange fluorescence (from phycoerythrin) and red fluorescence (from chlorophyll), according to standards protocols [45,49]. *Synechococcus* and *Prochlorococcus* cells were discriminated from autotrophic pico-eukaryotic cells by their flow cytometry scatter signal (SSC) related to their size, and their fluorescence emission when excited by a blue light. Specifically, the phycobilins contained in *Synechococcus* emit a strong orange fluorescence, whereas *Prochlorococcus* harvest light mainly through chlorophyll *a* and *b*, and therefore emit only red fluorescence when excited by blue light [50]. In addition, *Synechococcus* cells are larger than *Prochlorococcus* cells (*ca*. 1 and 0.6 µm in diameter, respectively) [51]. Pico-eukaryotes were identified by their larger size (SSC) and higher red fluorescence. Because, in the absence of genetic fingerprinting the identification of *Synechococcus* and *Prochlorococcus* cannot be warranted *sensu stricto*, the populations exhibiting the flow cytometric signatures of *Synechococcus* and *Prochlorococcus* as reported in the literature were referred to as *Synechococcus*-like and *Prochlorococcus*-like populations.

## Data Analysis

Comparisons between the two sampling depths were conducted using the Wilcoxon-Mann-Whitney *U*-test (*U*-test hereafter). The BIOENV [52] and BVSTEP [53] procedures (PRIMER version 6.0) were used to investigate relationships between environmental variables and picophytoplankton community's composition along the salinity gradient. Both analysis compare rank correlation between the matrices of environmental variables (based on normalized Euclidian distance) and the biotic similarity matrix of picophytoplankton variables (based on the Bray-Curtis similarity) using different permutations of the environmental variables. BIOENV compares different combinations of a specified number of variables, whereas BVSTEP uses a stepwise procedure to identify the best subset of variables.

Spearman rank correlations between the biotic and abiotic similarity matrices were used to identify the best suites of environmental variables that best explained the distribution of picophytoplankton communities along the salinity gradient and the significance of the correlation was determined using a permutation procedure [54]. Environmental variables considered in the BIOENV/BVSTEP analysis for picophytoplankton communities were salinity, [DO], [SPM], $[NH_4^+]$, $[NO_3^- + NO_2^-]$, $[PO_4^{3-}]$ and VLP3 abundances.

As temperature variability between stations was mainly related to the time of the day when the sampling occurred, this parameter was not considered in the analyses. Similarities between stations for picophytoplankton communities along the salinity gradient were inferred through a cluster analysis (i.e. hierarchical agglomeration using complete linkage cluster analysis performed on Euclidian distances) performed on the log (abundance + 1) data matrix [55]. This analysis was performed with STATISTICA version 8 software.

# RESULTS

No significant differences were found between sub-surface and water-sediment interface (WSI) for any abiotic or biotic parameters (*U*-test, $0.10 < P < 0.02$); sub-surface and WSI data were then pooled for further analysis. Besides, this indicates that the water column was well mixed along the lagoon, in accordance with previous results [36].

## Environmental Parameters

Water temperature ranged between 25.2°C and 27.7°C. Salinity increased from 17.7‰ in $S_1$ to 154.8‰ in $S_{20}$ (Table 1). At stations $S_1$ and $S_2$, salinity levels remained below 25.0‰. Salinity then slowly increased from 27.5‰ to 50.3‰ between stations $S_3$ and $S_{11}$. In contrast, salinity sharply increased from station $S_{12}$ to reach 150‰ at station $S_{17}$ (Table 1).

**Table 1. Hydro-chemical parameters along the salinity gradient. Salinity (‰), ammonium [$NH_4^+$] (µM), nitrite + nitrate [$NO_3^- + NO_2^-$] (µM) and phosphate [$PO_4^{3-}$] (µM) dissolved oxygen [DO] (mg $l^{-1}$) and suspended particulate matter [SPM] (mg $l^{-1}$) concentrations observed on the 20 sampling stations ($S_1$ to $S_{20}$). Ammonium, nitrite, nitrate and phosphate measures ranged from 0.6 to 110 µM, 0.2 to 160 µM, 1.6 to 160 µM and 1.1 to 50 µM, respectively. DL: detection limit. R: maximum range. ×: no data available**

| Station | Salinity (‰) | [$NH_4^+$] (µM) | [$NO_3^- + NO_2^-$] (µM) | [$PO_4^{3-}$] (µM) | [DO] (mg $l^{-1}$) | [SPM] (mg $l^{-1}$) |
|---|---|---|---|---|---|---|
| $S_1$ | 17.7 | 91.7 | < DL | 40.0 | 1.72 | 30.0 |
| $S_2$ | 24.4 | 56.0 | < DL | 35.3 | 2.18 | 49.5 |
| $S_3$ | 27.4 | 20.3 | < DL | 32.6 | 5.34 | 42.8 |
| $S_4$ | 32.4 | 22.5 | < DL | 21.6 | 3.74 | 41.5 |
| $S_5$ | 32.8 | 28.3 | < DL | 48.4 | 3.85 | 87.0 |
| $S_6$ | 33.2 | 60.0 | < DL | 20.0 | 3.50 | 56.5 |
| $S_7$ | 33.5 | 14.2 | 1.8 | 4.7 | 5.43 | 89.0 |
| $S_8$ | 35.4 | 26.1 | < DL | < DL | 5.27 | 49.5 |
| $S_9$ | 42.7 | 10.3 | 2.0 | < DL | 5.46 | 96.5 |
| $S_{10}$ | 46.9 | 6.7 | < DL | 3.2 | 4.69 | 126.5 |
| $S_{11}$ | 50.3 | 13.3 | < DL | < DL | 4.17 | 140.7 |
| $S_{12}$ | 67.5 | 16.7 | < DL | 2.1 | 4.69 | 241.2 |
| $S_{13}$ | 83.0 | 48.9 | 2.0 | 9.0 | 5.35 | 192.2 |
| $S_{14}$ | 100.0 | 72.5 | < DL | 17.9 | 5.18 | 287.2 |
| $S_{15}$ | 107.2 | 78.2 | < DL | 11.1 | 4.18 | 377.2 |
| $S_{16}$ | 133.5 | > R | < DL | 16.3 | 2.66 | 459.5 |
| $S_{17}$ | 150.1 | > R | 1.6 | < DL | × | 967.4 |
| $S_{18}$ | 149.2 | > R | < DL | < DL | 2.62 | 660.0 |
| $S_{19}$ | 145.4 | > R | < DL | 3.7 | 2.19 | 566.5 |
| $S_{20}$ | 154.8 | > R | 1.6 | 3.7 | 1.42 | 538.0 |

Ammonium was by far the most abundant form of nitrogen and represented more than 80% of the total inorganic nitrogen pool throughout the salinity gradient (Table 1). [$NH_4^+$] increased with salinity and highest concentrations (i.e. > 110 µM) were observed from 133.5‰ ($S_{16}$). Phosphate concentrations were relatively high below 33.2‰ (i.e. from $S_1$ to $S_6$) with values ranging between 20 to 40 µM, and decrease thereafter to reach very low levels (i.e. <4 µM) between 33.5‰ and 67.5‰ (i.e. from $S_7$ to $S_{12}$). Relatively high concentrations (i.e. 9 to 16 µM) were again observed from 83.0‰ to 133.5‰ (i.e. $S_{13}$ to $S_{16}$) and decreased thereafter to reach very low level (i.e. < 4 µM) in the hypersaline waters of the lagoon (i.e. salinity > 140‰; Table 1).

Dissolved oxygen concentrations ([DO]) remained below 3.0 mg $l^{-1}$ in the brackish (i.e. salinity < 25‰) and hypersaline waters of the lagoon (i.e. salinity > 130‰; Table 1). Highest [DO], ranging between 4.2 mg $l^{-1}$ and 5.5 mg $l^{-1}$, were observed between 27.4‰ and 107.2‰. Concentrations of suspended particulate matter ([SPM]) increased exponentially along the salinity gradient, with values increasing from 30 mg $l^{-1}$ at 17.7‰ to 967 mg $l^{-1}$ at 150.1‰ (Table 1).

## Phytoplankton Biomass

Chlorophyll *a* ([Chl*a*]) concentration increased from 0.4 µg $l^{-1}$ to 14.1 µg $l^{-1}$ with salinity increasing from 17.7‰ to 133.6‰ (Figure 2). [Chl*a*] sharply decreased thereafter, for salinity greater than 140‰, and remained below 4.5 µg $l^{-1}$ in the hypersaline part of the lagoon (i.e. for salinity greater than 140‰; Figure 2).

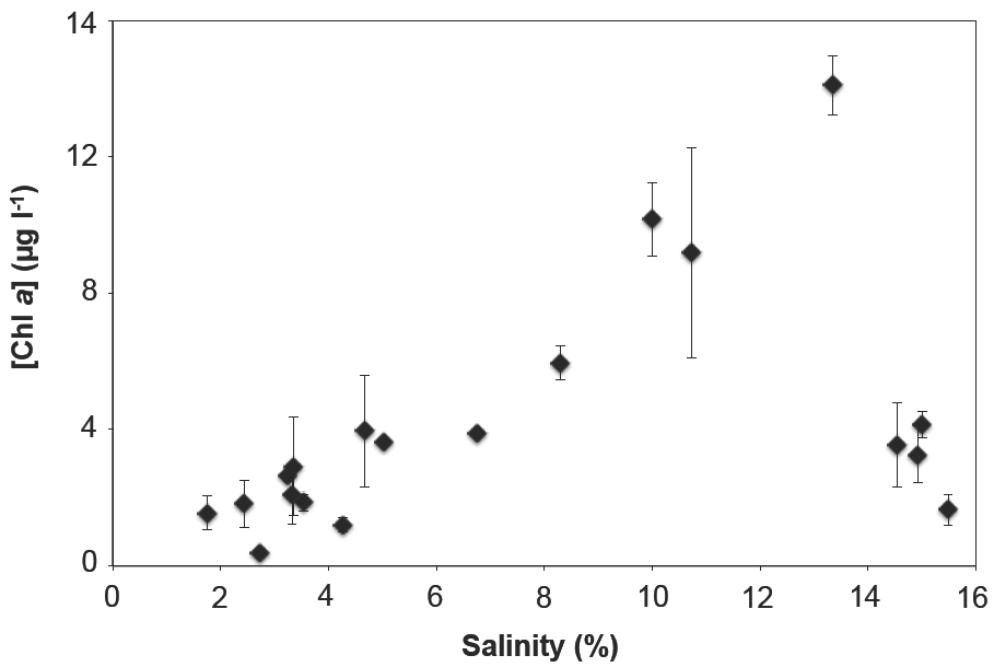


Figure 2. Phytoplankton biomass along the salinity gradient [Chl*a*] (µg $l^{-1}$). The errors bars are the standard deviation.

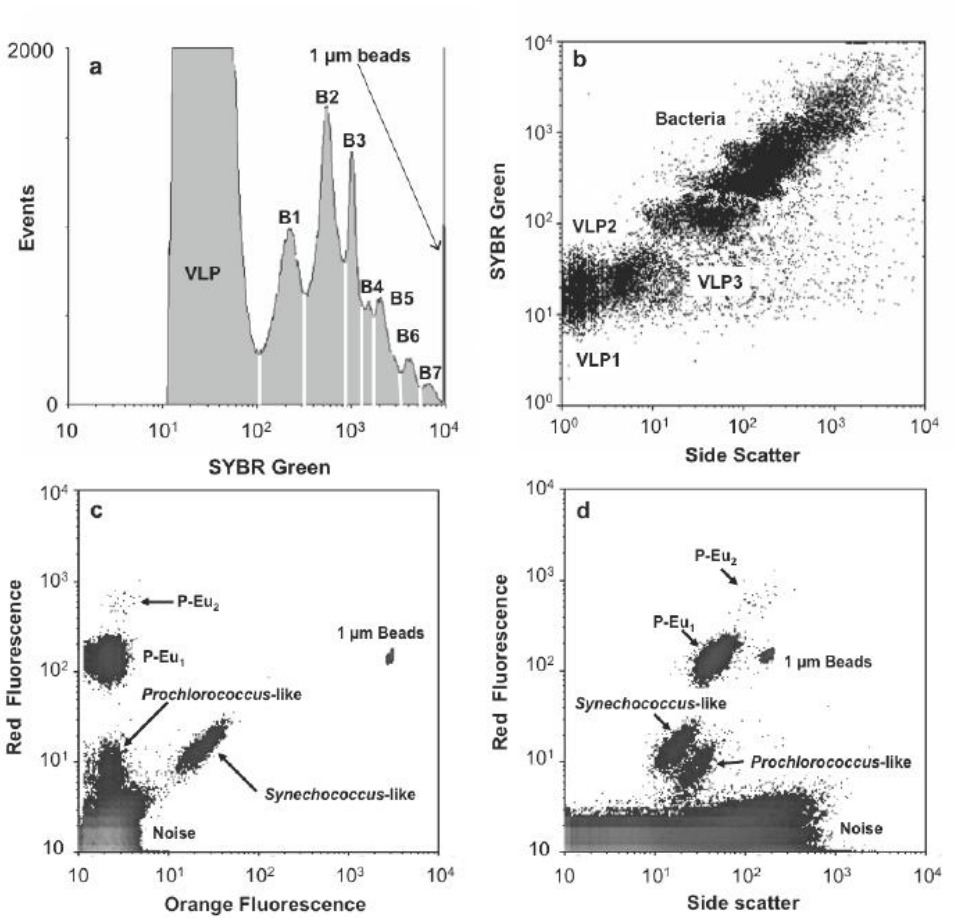


Figure 3. Archetypal signatures of virus-like particles (VLP), heterotrophic bacteria and picophytoplankton populations observed along the salinity gradient. (a) Histogram of green fluorescence showing peaks relating to 7 populations of heterotrophic bacteria with increasing DNA content ($B_1$, $B_2$, $B_3$, $B_4$, $B_5$, $B_6$ and $B_7$). (b) Scatterplot of side-angle scatter (SSC) versus green fluorescence (SYBR Green) showing 3 viral sub-populations: VLP1, VLP2 and VLP3. (c) Scatter plot of orange fluorescence versus red fluorescence and side scatter versus red fluorescence (d) showing: (i) 2 populations of cyanobacteria, one exhibiting fluorescence and side-scatter characteristics of *Prochlorococcus* sp. (referred as *Prochlorococcus*-like) and the second exhibiting fluorescence and side scatter characteristics of *Synechococcus*sp. (referred as *Synechococcus*-like) and (ii) 2 populations of pico-eukaryotes (*P-Eu*$_1$ and *P-Eu*$_2$).

## Virus-Like Particles (VLP)

Three virus-like particles populations were identified along the salinity gradient (Figure 3a,b). The two first populations (VLP1 and VLP2) exhibit the same cytometric signature (SSC and green fluorescence) of viral population observed previously in seawater and identified as bacteriophages [45]. In contrast, the third population (VLP3) exhibits the same SYBR Green fluorescence (related to DNA content) level as VLP2 but a higher side scatter (i.e. size; Figure 3a,b), and hence it could represent a group of phytoplankton viruses [46,47].

Viral abundance ranged between $9.0 \times 10^6$ and $2.5 \times 10^8$ ml$^{-1}$ along the lagoon (Figure 4a). High viral concentrations were observed on both extremes of the salinity gradient: (i) in the estuarine part of the lagoon where salinity remained below 24.4‰ with abundances ranging from $1.3 \times 10^8$ to $1.5 \times 10^8$ ml$^{-1}$ and (ii) in the hypersaline waters where salinity was higher than 145.4‰ ($1.8 \times 10^8$ to $2.5 \times 10^8$ ml$^{-1}$). More specifically, the subpopulation VLP1 was observed in all samples throughout the lagoon representing more than 64% of the viral community from 17.7‰ ($S_1$) to 100.0‰ ($S_{14}$) and was the only sub-population observed for salinity higher than 107.2‰ (from station $S_{15}$).

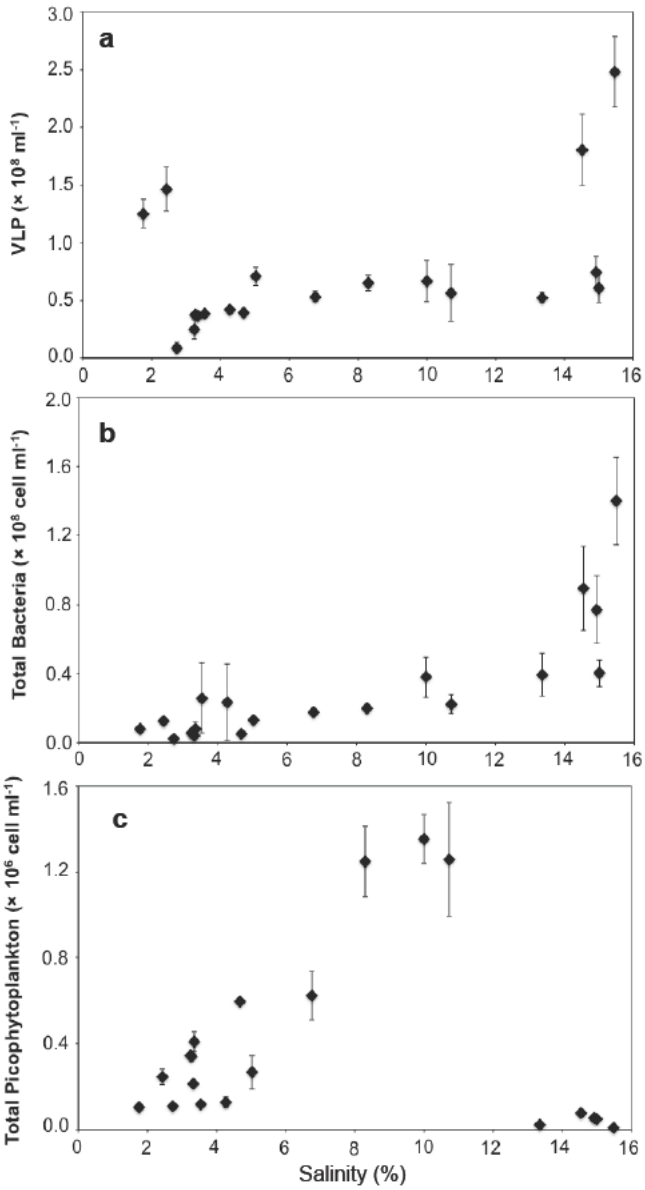


Figure 4. Micro-organisms along the salinity gradient. (a) Abundances of total virus-like particles (VLP×$10^8$ ml$^{-1}$), (b) abundances of total heterotrophic bacteria (×$10^8$ cell ml$^{-1}$) and (d) abundances of total picophytoplankton cells (×$10^6$ cell ml$^{-1}$). The errors bars are the standard deviation.

In contrast, VLP2 was only present from 17.7‰ to 50.3‰ ($S_1$ to $S_{11}$) where this sub-population accounted for 151‰ to 341.2‰ of the total viral abundance. VLP3 was only observed from 50.3‰ to 100.0‰ (i.e. $S_{11}$ to $S_{14}$) and contributed to less than 10% of the total viral abundance (Figure 4a). From station $S_1$ to $S_{14}$, where the 3 VLP populations were present, VLP1 was by far the most abundant population and always accounted for more than 66% of the viral community (Figure 4a).

## Heterotrophic Bacteria

Seven distinct populations of heterotrophic bacteria were discriminated, through their distinct side scatter and green fluorescence flow cytometric signatures (Figure 3a,b). As a consequence, the seven bacterial populations observed here were not classified into the high and low DNA subpopulations previously observed in marine and freshwater systems [56-60], but instead defined as 7 discrete populations following Bouvier et al. [64].

These populations were furthermore identified as non-phototrophic bacterial populations as their size and nucleic acid content were consistent with bacterial-sized organisms, and because they lacked red or orange fluorescence (indicative of chlorophyll or photo-pigment content; [45]). Bacterial abundances ranged from $2.1 \times 10^6$ $ml^{-1}$ at station $S_3$ (S = 27.4‰) to $2.4 \times 10^8$ $ml^{-1}$ at station $S_{20}$ (S = 154.8‰; Figure 4b). High bacterial abundances were observed in the hypersaline part of the lagoon where salinity ranged from 150‰ to 156.5‰ (i.e. $S_{18}$ to $S_{20}$). Three populations, $B_1$, $B_3$ and $B_4$, were by far the most abundant and contributed to more than 70% of the total heterotrophic bacteria abundances (Figure 5b). Whilst $B_1$ and $B_3$ were observed in all samples, population $B_4$ was only observed where salinity was higher than 27.5‰ (i.e. stations $S_3$ to $S_{20}$; Figure 5b). In contrast, populations $B_2$, $B_5$, $B_6$ and $B_7$ were restricted to particular habitats and their relative abundance remained < 25%. Population $B_5$ was observed locally over a large range of salinity conditions from stations $S_3$ to $S_{18}$. Populations $B_2$, $B_6$ and $B_7$ were mainly observed from 50.3‰ to 150‰ (i.e. $S_{10}$ to $S_{18}$; Figure 5b). Population $B_2$ exhibited a particularly restricted distribution as this population was only observed from 107.2‰ to 150.1‰ (i.e. $S_{15}$ to $S_{17}$; Figure 5b). The cluster analysis performed on bacterial abundance discriminated 2 main groups of stations based on their population richness, defined here as flow cytometrically-defined richness (i.e. FCM richness) (Figure 6a): (1) a high FCM richness group (i.e. richness > 4) mainly occurred for salinity ranging from 50.3‰ to 150‰ (i.e. $S_{11}$-$S_{17}$), and (2) a low FCM richness group (i.e. richness $\leq$ 4) comprised of stations where salinity remained below 50.3‰ and stations located in the hypersaline part of the lagoon where salinity was greater than 140‰ (i.e. $S_{18}$-$S_{20}$).

## Picophytoplankton

### *Picophytoplankton Abundance and Community Structure*

Samples were characterized by a highly complex community structure with multiple sub-populations of picophytoplankton throughout the salinity gradient. Two distinct populations of pico-cyanobacteria (*P-Cya*) were observed exhibiting fluorescence and side-scatter characteristics of *Prochlorococcus* sp. and *Synechococcus* sp. (referred hereafter as

*Prochlorococcus*-like and *Synechococcus*-like), and 5 different populations of pico-eukaryotes (*P-Eu*), exhibiting consistently different side scatter (related to size) and red fluorescence (Figure 3c,d).

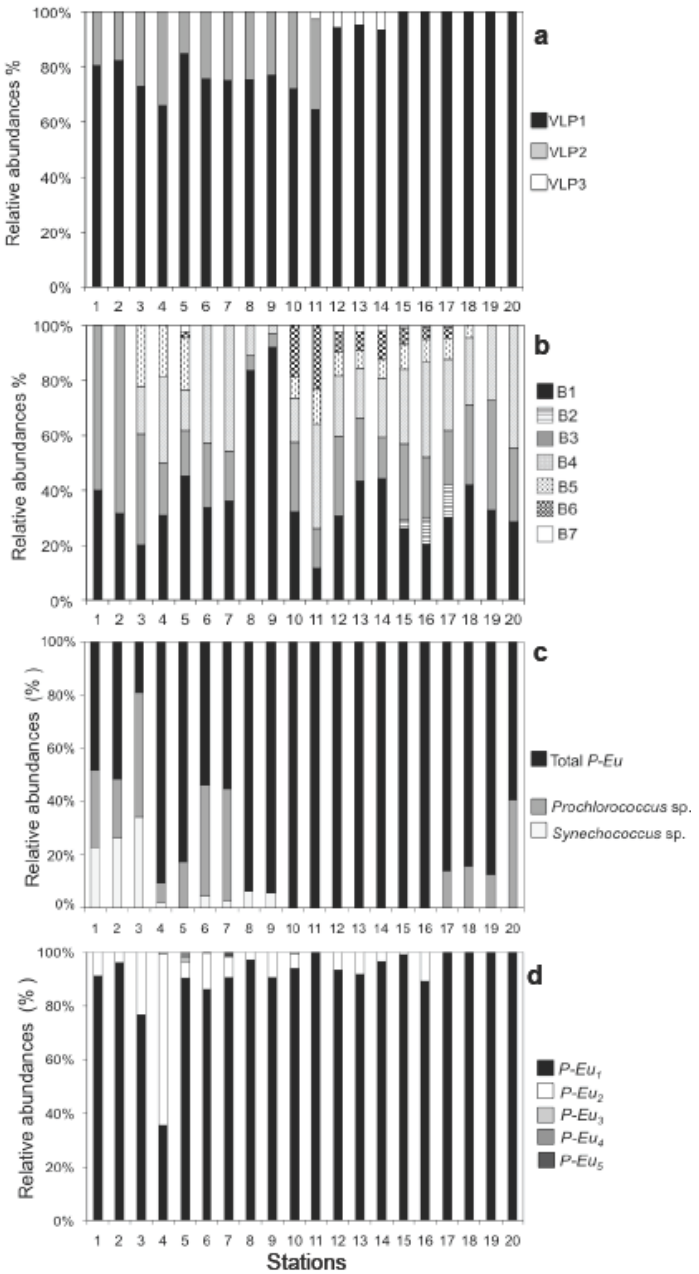


Figure 5. Relative abundances (%) of cytometrically defined different picophytoplankton populations (A and B), heterotrophic bacteria (C) and virus-like particles (D) observed on the 20 sampling stations ($S_1$ from $S_{20}$). (A) Relative abundances of total pico-eukaryotes and cyanobacteria (i.e. *Prochlorococcus*-like and *Synechococcus*-like). (B) Relative abundances of cytometrically-defined different pico-eukaryotes populations (*P-Eu*$_1$, *P-Eu*$_2$, *P-Eu*$_3$, *P-Eu*$_4$ and *P-Eu*$_5$) to the total pico-eukaryotes community.

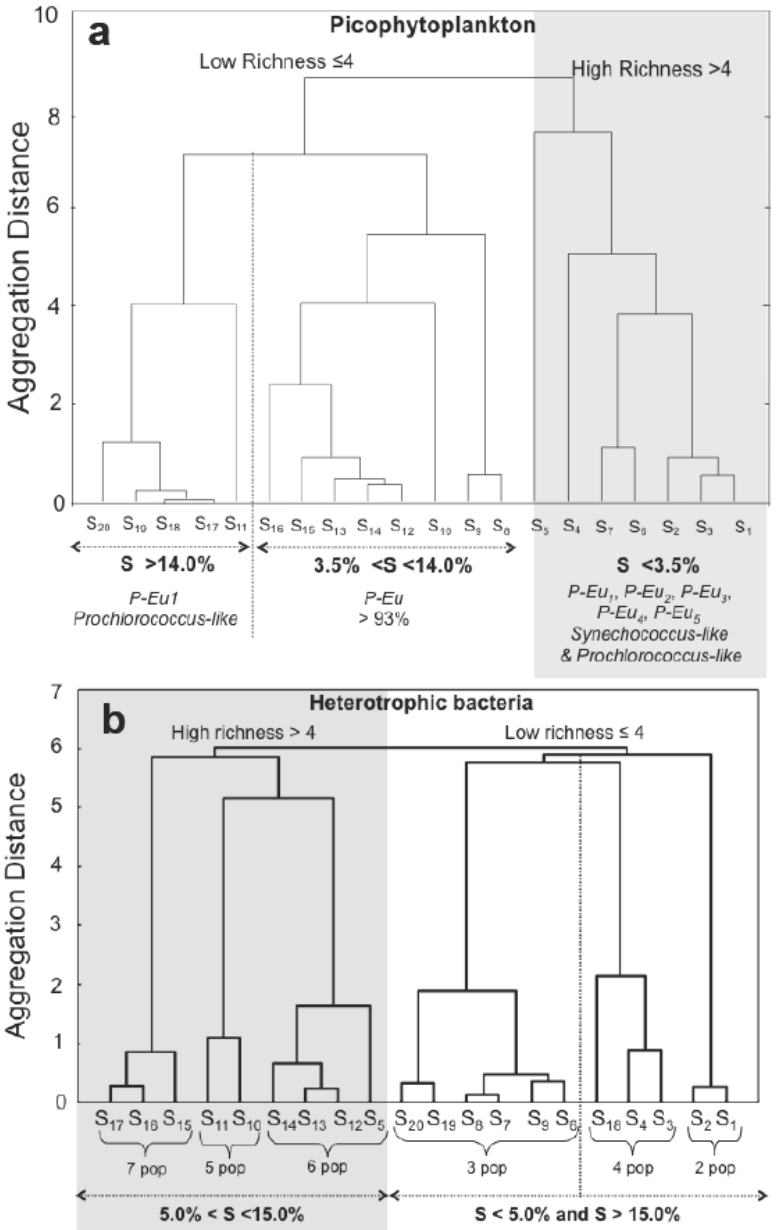


Figure 6. Cluster analysis of heterotrophic bacteria communities (a) and picophytoplankton communities (b) along the salinity gradient. The analysis was performed on the log (abundance + 1). The *x*-axis shows stations $S_1$ to $S_{20}$. (a) Two main groups of stations were discriminated based on their population richness: high richness (grey, >4 populations) for salinity ranging between 50‰ and 150‰, and low richness (white, ≤4 populations) for salinity <50‰ and for salinity >140‰. (b) Two main groups of stations were discriminated based on their cytometrically-defined population richness (grey, >4 populations) for salinity <35‰ where the seven different sub-populations were identified (*P-Eu*$_1$, *P-Eu*$_2$, *P-Eu*$_3$, *P-Eu*$_4$ and *P-Eu*$_5$, *Synechococcus*-like and *Prochlorococcus*-like), and low richness for salinity >35‰ (white, ≤4 populations). In this latter group, two sub-groups of stations where discriminated based on their population composition: a sub-group characterized by a great dominance of pico-eukaryotes (>93%) for salinity ranging between 35‰ and 140‰, and a sub-group where only *Prochlorococcus*-like and *P-Eu*$_1$ occurred from salinity greater than 140‰.

Picophytoplankton abundances were highly variable along the salinity gradient with values ranging between $8.3 \times 10^3$ cells $ml^{-1}$ and $1.4 \times 10^6$ cells $ml^{-1}$ (Figure 4c). Concentrations were relatively low (i.e. $\leq 6.3 \times 10^5$ cells $ml^{-1}$) where salinity remained below 70‰ (i.e. $S_1$-$S_{12}$). High abundances were observed for salinity between 80‰ and 110‰ (i.e. $S_{13}$-$S_{15}$) with values ranging from $1.3 \times 10^6$ cells $ml^{-1}$ to $1.4 \times 10^6$ cells $ml^{-1}$. Above 130‰ (i.e. $S_{16}$-$S_{20}$) picophytoplankton abundance was very low with values remaining below $8.0 \times 10^4$ cells $ml^{-1}$. Except in locations where salinity remained below 30‰ (i.e. $S_1$ to $S_3$), *P-Eu* were by far the most abundant and contribute to more than 54% of the total abundances throughout the salinity gradient (Figure 5c). Furthermore, the picophytoplankton community was only composed of *P-Eu* for salinities ranging from 45‰ to 140‰. Below 30‰ *P-Cya* were much more abundant, contributing from 48% ($S_2$) to 81% ($S_3$) of the total community, with *Synechococcus*-like and *Prochlorococcus*-like populations respectively representing 23% to 34% and 22% to 47% of the total abundances (Figure 5c). The *Synechococcus*-like population was observed from 17‰ to 45‰ ($S_1$ to $S_9$). In contrast, the *Prochlorococcus*-like population was observed for salinity ranging from 17‰ to 35‰ ($S_1$ to $S_7$) and for salinity greater than 140‰ ($S_{17}$ to $S_{20}$).

Five different sub-populations of *P-Eu* exhibiting different flow cytometric side scatter signal (SSC) and fluorescence levels were discriminated (Figure 5d).

These populations consistently exhibited different side scatter and red fluorescence signatures (Figure 3c,d). *P-Eu*$_1$ was by far the most abundant and contributed to more than 70% of the *P-Eu* abundances. *P-Eu*$_2$ was the second most abundant population with relative contribution to the total *P-Eu* only occasionally exceeding 23%. Whilst *P-Eu*$_1$ was identified throughout the salinity gradient *P-Eu*$_2$ was not observed for salinity greater than 140‰; *P-Eu*$_1$ was also the only sub-population observed in these hypersaline waters of the lagoon.

The relative abundances of populations *P-Eu*$_3$, *P-Eu*$_4$ and *P-Eu*$_5$ remained below 2% and were locally observed mostly for salinity ranging from 32‰ and 35‰ (Figure 5d).

The cluster analysis performed on picophytoplankton abundance (Figure 6b) discriminated 2 main groups of stations based on their population richness (defined here as the cytometrically-defined richness, i.e. FCM richness): a high FCM richness group (i.e. richness $\geq 4$) occurred for salinity lower than 35‰ ($S_1$ to $S_7$), and a low FCM richness group (i.e. richness $< 4$) included stations where salinity was greater than 35‰ (Figure 6b).

In the later, two sub-groups of stations were identified: a sub-group where *P-Eu* (*P-Eu1*, *P-Eu*$_2$ and *P-Eu*$_3$) contributed to more than 93% to the total abundances occurring for salinity ranging between 35‰ and 140‰ ($S_8$ to $S_{16}$), and a sub-group characterized by a community composed exclusively by the *P-Eu* sub-population *P-Eu*$_1$ and *Prochlorococcus*-like picocyanobacteria comprising stations where salinity was greater than 140‰ ($S_{17}$ to $S_{20}$).

### *Picophytoplankton and Environmental Variables*

The multivariate correlations analysis, BIOENV, showed that the environmental variable that best explained the picophytoplankton abundance pattern along the lagoon was salinity ($\rho$=0.542; $p$ <0.01; Table 2).

Salinity in combination with ammonium concentrations and abundance of viral population (VLP3) are the best subset of variables explaining the variability of picophytoplankton abundances observed along the salinity gradient (BVSTEP; Table 2).

The Spearman's rank correlation coefficient for this analysis was 0.520 and was statistically significant ($p$<0.01).

**Table 2. Results of the BIOENV and BVSTEP analyses. Combination of the best environmental variables that predict the patterns of picophytoplankton abundances along the salinity gradient. *n*: number of abiotic variables used in the analysis; *ρ*: Spearman rank correlation coefficient; *P*: significance levels; *k*: number of corresponding significant environmental variables. Significant contributing environmental variables were ordered according to the degree of match. "S": salinity; "VLP3": abundances of viral population VLP3 potentially infecting phytoplankton; [$NH_4^+$]: ammonium concentrations**

| | *n* | *ρ* | *P* | *k* | Environmental variables |
|---|---|---|---|---|---|
| BIOENV | 9 | 0.542 | 0.01 | 1 | S |
| BVSTEP | 9 | 0.520 | 0.01 | 3 | S –[$NH_4^+$]- VLP3 |

# DISCUSSION

## Salinity and Viral and Bacterial Abundance

The significant decrease in VLP abundance in the lagoon when salinity increased above 25‰ (Figure 4a) is consistent with the decrease in VLP abundance reported in estuaries from freshwater to marine waters [38, 65]. Note, however, that in the brackish zone of the lagoon (i.e. salinity < 25‰), viral abundance ranged between $1.3 \times 10^8$ and $1.5 \times 10^8$ $ml^{-1}$, whereas at comparable salinities in the Brisbane estuary [38], Tampa Bay [65] or saline Antarctic lakes [66], the maximum VLP concentration did not exceed $1.26 \times 10^8$ $ml^{-1}$. This suggests that salinity may not be the only factor that plays a part in determining VLP abundance in brackish waters; limitation by nutrients such as carbon, oxygen, nitrogen and phosphorus has indeed been shown to indirectly affect viral proliferation through its effects on host metabolism [67], and changes in the phosphate status of water could directly affect viral production [67].

One of the main features of the spatial dynamics of VLP and heterotrophic bacteria along the Coorong lagoon was the very high abundances observed under high salinity conditions, i.e. $2.5 \times 10^8$ and $1.4 \times 10^8$ $ml^{-1}$ above 150‰, respectively (Figure 4a,b). This increase in VLP and bacterial abundances with increasing salinity is congruent with previous observations conducted in salterns under comparable salinity conditions. The maximum abundances we observed were, however, well above values observed in these semi-artificial systems, which never exceeded $4.6 \times 10^7$ $ml^{-1}$ for bacteria and $5.0 \times 10^7$ $ml^{-1}$ for viruses, under similar salinity conditions [15,16,69]. These strong differences in bacterial and viral abundances (5.4 and 2.8 times higher, respectively) highlight the unique properties of these communities found along a strong continuous salinity gradient.

## Salinity and Viral and Bacterial Richness

The increase in viral and bacterial abundances at salinities higher than 150‰, was concomitant to a decrease in the number of FCM subpopulations of heterotrophic bacteria and

VLP. This salinity range was characterized by only one VLP population (VLP1) and 2 to 3 populations of heterotrophic bacteria (Figure 6a). In contrast, the number of FCM subpopulations was higher under less saline conditions, and particularly for salinity ranging between 50‰ and 150‰ (see Figure 6a). The most diversified bacterial community was observed between 50‰ and 150‰ (Figure 6a). This coincided with the maxima of phytoplankton biomass and SPM, DO and ammonium concentrations observed over this range of salinity (Table 1).

The related variety of resources and ecological niches may have then favored the development of highly specialized populations of bacteria (e.g. population B2; Figure 5b) and, therefore, the high bacteria richness observed in this part of the lagoon. However, this cytometric richness could also reflect a high activity level within the bacterial community. The abundance and diversity of resources present in this area may have favored both the activation and growth of bacterial groups present in this part of the lagoon, leading to the observation of additional subpopulations exhibiting high DNA content (e.g. populations $B_5$, $B_6$ and $B_7$).

The increase in salinity above 150‰, the decrease in phytoplankton biomass (Table 1) and the resulting decrease in the diversity of ecological niches may have triggered both the loss of the more specialized groups of bacteria observed previously throughout the lagoon and the decrease in bacterial activity, hence leading to the observed low cytometric bacterial richness. Considering that both the phylogenetic composition and the metabolic activity of aquatic bacterioplankton determine the extent to which they contribute as potential hosts for virioplankton [43,70], the decrease in viral FCM population richness could be explained by the limitation of heterotrophic bacteria richness and activity under high salinity conditions.

These observations are consistent with the hypothesis that the diversity of metabolically active prokaryotes would decrease with salinity [71]. A decrease in prokaryotic and viral diversity has been previously shown in solar salterns [15,18,19,72,73,19] and in hypersaline lakes [e.g. 22,24,74] for salinities >150‰. However, using fluorescent *in situ* hybridization (FISH) and/or denaturing gel gradient electrophoresis (DGGE) with subsequent DNA sequencing, these previous studies did not show a negative correlation between the number of DGGE bands with increasing salinity. Instead, they showed a decrease in the bacterial richness of genera, suggesting an increase in the microdiversity within there maining phylogenetic groups at higher salinities [18,22,24].

## Flow Cytometric Bacterial Populations and Their Role in Ecological Processes

Flow cytometry allows differentiation of subpopulations of bacteria *via* light scattering properties (related to size) and DNA content of individual cells [43,56,58,61,64]. However, the ecological and functional roles of these different subpopulations within bacterioplankton communities remain uncertain [64]. Variations in DNA content have been related to single cell activity with high DNA and low DNA content cells respectively constituting the active cells and the dormant or dead cells [43,56,58,61]. However, there is now evidence that low DNA cells may also be active [62,63]. In addition there are conflicting results concerning the phylogenetic composition of these different fractions. While some studies conclude that these

fractions are phylogenetically different [63], others suggest that the composition varies between different fractions [62].

A recent study does not support any of these scenarios, and suggests that the existence of different FCM subpopulations within bacterioplankton assemblages is the result of complex processes that involve (i) the passage of cells from one subpopulation to another, through activation and growth, inactivation, damage and death, and (ii) the existence of components characteristic of each subpopulation [64]. Consequently, the variability of bacterial cytometric richness observed here along the salinity gradient likely reflects a modification of the phylogenetic composition of bacterial populations as well as their activity level.

In this context, it is likely that distinct populations of heterotrophic bacteria defined through their flow cytometric signatures play different roles in the ecosystem, and may hence be differently impacted by losses related to viral infection and lysis, and microzooplankton grazing. This is supported by a dilution assay [47,75,76] conducted in the Coorong in 2008 at 4 sites characterized by their increasing salinity (i.e. 38‰, 55‰, 112‰ and 131‰) and devoted to assess the relative contribution of microzooplankton grazing and viral lysis to the mortality of each of the bacterial populations defined through their flow cytometric signature as described above (Figure 7). Results unambiguously indicate that each flow cytometrically-defined populations exhibit very specific mortality rates, irrespective of the salinity. This is an indication that distinct flow cytometrically-defined bacterial populations are impacted by microzooplankton grazing and viral infection and lysis in specific ways, hence that they play distinct roles in the functioning of the ecosystem as previously suggested [62-64].

## Autotrophic Biomass along the Salinity Gradient

Both chlorophyll *a* concentrations and picophytoplankton cell numbers exhibited maxima at salinities in the range 110‰ to 130‰ and 80‰ to 110‰, respectively (Figures 2 and 4c). This is consistent with previous observations conducted in solar salterns over comparable salinity ranges [17,31,32,68]. The increase in autotrophic biomass was observed from salinity higher than 70‰ where dissolved oxygen concentrations were relatively high (Table 1). This may be indicative of an enhancement of primary production in this part of the lagoon as previously observed under a comparable salinity range in solar salterns [68]. An increase in primary production may be the result of a change in nutrient availability along the salinity gradient. This is consistent with the results of the BVSTEP analysis, which highlighted a strong relationship between picophytoplankton abundances and ammonium concentrations (Table 2). The high abundances observed from salinity higher than 80‰ could hence be explained by the high ammonium concentrations found in the same part of the lagoon (Table 1). This is congruent with previous work showing the ability of picophytoplankton to efficiently utilize regenerated forms of nitrogen such as ammonium and urea [51,77].

A modification of the light regime along the salinity gradient may also have impacted the pattern of autotrophic organisms observed in the present study. The increase in suspended matter along salinity gradient (Table 1) is indeed likely to impact turbidity leading to a decline in light penetration in the water column in the high salinity area. However, the shallowness of the lagoon, as well as wind mixing and heating convection might prevent light limitation by ensuring sufficient turnover of the water column, as suggested by the absence of vertical stratification observed during our sampling.

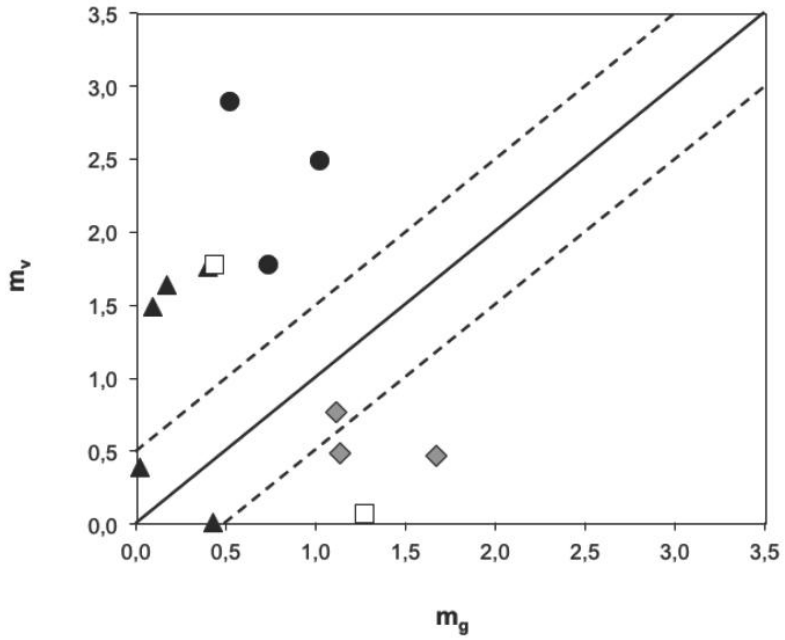


Figure 7. Bacterial mortality due to microzooplankton grazing ($m_g$) and viral lysis ($m_v$). Microzooplankton grazing rates ($m_g$; $d^{-1}$) plotted versus viral lysis rates ($m_v$; $d^{-1}$) for each bacterial sub-population defined through their flow cytometric signature at 4 distinct salinities, i.e. 38‰ (open squares), 55‰ (black triangles), 112‰ (grey diamonds) and 131‰ (black dots). Each symbol represents an individual flow cytometrically-defined bacterial sub-population. The continuous line is the first bissectrix, i.e. $m_g = m_v$, and dashed lines indicates the 95% confidence intervals; modified from Newton [83].

Viruses infecting both components of the picophytoplankton community (i.e. cyanobacteria and eukaryotes) have been previously reported [75,78-80] and the role of viral lysis on picophytoplankton mortality is now well established (e.g. [27,78]). The peak in picophytoplankton abundance, observed for salinity ranging between 80‰ and 110‰, was concomitant to the occurrence of the viral population VLP3 in the water column in this narrow range of salinity (Figure 5b). This suggests a positive correlation between picophytoplankton populations and VLP3, as shown by the result of the BVSTEP analysis (Table 2). The potential role of VLP3 as picophytoplankton infecting viruses is consistent with both the typical cytometric signature of phytoplankton-infecting virus of VLP3 [46,47] and the strong positive relationship consistently observed between virus and their potential hosts [5,7,81,82]. In addition, the abundances of VLP3 recorded in the lagoon ($1.9\times10^6$ - $4.3\times10^6$ VLP $ml^{-1}$), were in the highest range of concentrations previously reported in marine waters, i.e. $>10^5$ VLP $ml^{-1}$ [78].

This suggests an important contribution of viral infection and lysis to phytoplankton losses in this part of the lagoon, in agreement with recent observations of high virally induced mortality of phytoplankton along the lagoon at salinities ranging between 55‰ to 112‰ (Figure 7). These results highlight the role of phytoplankton viruses along the salinity gradient and further work is needed to confirm the identity of this viral population.

Microzooplankton grazing, could also contribute to the observed pattern of autotrophic biomass along the salinity gradient. More specifically, considering the importance of

microzooplankton grazing as a source of nutrients recycling in planktonic systems [77] and the high level of ammonium concentrations observed for salinity greater than 110‰ (Table 1), the sharp decrease in picophytoplankton abundances and phytoplankton biomass observed in the same area (Figure 2 and 4c) may then be the result of an increase in grazing pressure. This is supported by recent observations of high phytoplankton mortality rates in the Coorong due to microzooplankton grazing at 112‰ (i.e.>2.5 $d^{-1}$; [83]), and previous studies reporting high microzooplankton grazing rates for salinity higher than 40‰ [69] as well as high abundances of heterotrophic nano-flagellates up to the highest salinities (i.e.>300‰) [84,85]. The decrease in chlorophyll *a* concentrations, representing the size fraction >1.2 µm, observed for salinity higher than 130‰ may also be the result of an increase in grazing pressure by large metazoans consumers, such as the brine shrimps (i.e. *Artemia* sp.) that were very abundant during the sampling experiment (i.e. 20-50 ind $l^{-1}$; Seuront, unpublished data) and known to survive up to the highest salinities [17,86]. Moreover, picophytoplankton population growth is tightly controlled by fast growing protozoans consumers under high nutrients conditions [85,86] whereas larger cells are temporally and/or locally, protected from predation by slow-growing Metazoa under the same conditions [89,90]. Therefore the decrease in picophytoplankton abundances and phytoplankton biomass (size fraction>1.2 µm) observed in different part of the lagoon (see Figure 2 and 4c) could be explained by a difference in size-related grazing rate along the salinity gradient.

## Salinity and Picophytoplankton cytometric Richness

The picophytoplankton cytometric-richness decreased along the salinity gradient, affecting both prokaryotic and eukaryotic picophytoplankton (Figure 6). The existence of a decreasing trend in the number of phytoplankton species with increasing salinity has previously been observed in solar salterns ponds [17,31,32,34,69] and hypersaline lakes [35,91]. More specifically, a decrease in picophytoplankton cytometric-richness with increasing salinity has been reported in Bras del Port salterns [31].

The most diversified community was observed for salinity lower than 35‰ (Figs. 5 and6). In this habitat, *Prochlorococcus*-like and *Synechococcus*-like populations were abundant and 5 distinct populations of pico-eukaryotes were identified. This high cytometric richness coincided with relatively low total abundance (Figure 4c). Favorable environmental conditions may have led to the establishment of highly diversified picophytoplankton community in this brackish-marine part of lagoon. In contrast, the number of cytometrically-defined populations was limited under higher salinity conditions (Figure 5d) where the highest abundances were observed (Figure 4c). More specifically, the peak in picophytoplankton abundances observed for salinity ranging between 80‰ and 110‰ was largely dominated by the pico-eukaryotes *P-Eu*$_1$ (Figure 5d). This is consistent with previous works reporting the dominance of one population of pico-eukaryote under the same salinity range in solar salterns [31,32,34] and hypersaline lakes [33,35].

The existence of a bloom of pico-eukaryotes, observed in such different saline systems suggests that salinity may be the main factor triggering the dominance of pico-eukaryotes over this particular salinity range. This hypothesis is supported by the results of the BIOENV/BVSTEP analysis (Table 2). The pico-eukaryote *P-Eu*$_1$ may be dominant through a higher tolerance to high salinity and the subsequent decrease in competition within the

reduced picophytoplankton community, may have allowed this salinity-tolerant population *P-Eu$_1$* to grow extensively and flourish in this part of the lagoon. The collapse of the pico-eukaryote bloom observed for salinity greater than 110‰ was followed by an increase in *Prochlorococcus*-like concentration which contributed to more than 40% of the total abundance for salinity greater than 150‰ (Figure 5c). This observation is consistent with many studies highlighting the abundance of cyanobacteria under extreme saline conditions (e.g. [9]). It is also stressed that this is the first report of a *Prochlorococcus*-like population in such highly saline habitat. However, further work is therefore needed to confirm the identity of this population.

The succession of picophytoplankton was only defined in the present work through their flow cytometric signature. Even if flow cytometry is a powerful tool to investigate the composition of picophytoplankton organisms, further work is needed to identify the species succeeding along the salinity gradient, especially considering the diversity existing among cyanobacteria and pico-eukaryotes [2]. However, our results provide new insight into the effect of salinity on pico-eukaryotes and cyanobacteria communities.

## Effect of Salinity on Picophytoplankton Community's Succession

Salinity has been identified as the main factor triggering the succession of pico-autotrophs along the salinity gradient (Table 2). Salinity could act directly on picophytoplankton assemblages by selecting groups adapted to life at a particular salt concentration. Cyanobacteria are known to tolerate and acclimate to high salt concentrations [92]. However, the different groups of cyanobacteria do not exhibit the same tolerance to salinity stress and have been consequently classified into 3 groups, i.e. stenohaline, halotolerant and extremely halotolerant [93]. The decrease in cyanobacteria abundances for salinity higher than 35‰, suggests that the populations inhabiting the brackish-marine part of the Coorong may belong to the stenohaline group with a salinity tolerance range characteristic of estuarine and marine populations. *Synechococcus* species are known to be abundant in transitional and freshwater areas [94] whereas *Prochlorococcus* species are thought to be restricted to marine waters [95]. However, the observations of *Prochlorococcus*-like populations in the Rhône River [96], in the Changjiang river estuaries [28] and in the present work in the low-salinity part of the Coorong tend to challenge this hypothesis.

In contrast, the occurrence and predominance of the pico-eukaryote *P-Eu$_1$* throughout the salinity gradient, suggest that this population may represent halotolerant organisms. The dominance of halotolerant pico-eukaryotes has been previously described for the same salinity range [33-35]. In particular, eukaryotic pico-autotrophs have been shown to be responsible for dense blooms in surface water of Mono Lake in California where salinity was around 85‰ [35]. The organism isolated from this lake and identified as a *Picocystis* spp. has been shown to exhibit high growth efficiency over the 2.0‰-150‰ salinity range, which is consistent with the range of salinity where the population *P-Eu$_1$* was found in the present work. However, this population was identified here through flow cytometry and further work is needed to unambiguously conclude on the identity of this population.

Independent of their intrinsic salinity tolerance, the succession of picophytoplankton organisms along the salinity gradient could be indirectly controlled by salinity. In particular, by controlling the diversity and abundance of microzooplankton and VLP, salinity could exert

a control on the top-down processes including grazing and viral lysis. The presence of a large population of phytoplankton virus up to the highest salinity observed in the present work highlights the necessity to investigate the role of viral infection in regulating the community structure of picophytoplankton along this salinity gradient.

## CONCLUSION

In the literature, microbial community dynamics has been mainly described along discontinuous (i.e. solar salterns, hypersaline lakes) or weak salinity gradients (i.e. estuaries). The present chapter then constitutes the first observation of the joint dynamics of viral, microbial and picophytoplankton communities in a system where salinity continuously ranged from brackish to hypersaline.

High abundances of virus-like particles and heterotrophic bacteria were observed in the hypersaline part of the lagoon. In contrast, the highest picophytoplankton abundances were recorded for salinity ranging from 80‰ to 110‰. This shift from autotrophy to heterotrophy with increasing salinity may have considerable consequences on food web structure and biogeochemical cycles. Salinity was identified as the main factor controlling the picophytoplankton dynamic along the salinity gradient. However, the variability in nutrients availability as well as the intensity of viral lysis and microzooplankton grazing may have also played an important role in structuring the succession of picophytoplankton communities along the lagoon.

As previously shown, important ecological changes such as decreasing biodiversity and increasing dominance of prokaryotes occurred along salinity gradients (e.g. [97,98]). Our findings highlight a significant decrease in the number of cytometrically-defined sub-populations of heterotrophic bacteria and VLP with increasing salinity.

These changes could reflect both a modification of phylogenetic composition and activity level within the bacterial community. In addition, the number of cytometrically-defined picophytoplankton populations also decreased with increasing salinity, affecting both prokaryotic and eukaryotic organisms. Moreover, our results highlight the ability of cyanobacteria to tolerate a wide range of salinity.

The existence of a *Prochlorococcus*-like population in both extremes of the salinity gradient (i.e. low salinity and the hypersaline water) highlights the ability of cyanobacteria to tolerate and acclimate to a wide range of salinity and therefore to colonize extreme environments. However, even if the *Synechococcus*-like and *Prochlorococcus*-like populations identified in the present work exhibited the archetypical flow cytometric signature of *Synechococcus* sp. and *Prochlorococcus* sp., further work is needed (e.g. genetic fingerprinting) to unambiguously identify these populations.

The results obtained in this study nevertheless provide new insight into the potential effects of salinity gradient and perturbations on phytoplankton community shifts.

As these shifts in abundances and richness may ultimately have considerable implications in biogeochemical cycling dynamics along the salinity gradient, the signification of this decrease in cytometrically-defined richness of heterotrophic bacteria and picophytoplankton with increasing salinity warrant further considerations.

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*Chapter 7*

# ACCUMULATION AND PHYTOTOXICITY OF MICROCYSTINS IN VASCULAR PLANTS

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## ABSTRACT

Microcystins contamination resulted from cyanobacterial blooms in eutrophic water has received increasing attention worldwide. Aquatic plants have been shown to absorb microcystins from water and exposure of microcystins has adverse effects on the growth of aquatic plants.

In addition, terrestrial plants could be exposed to microcystins via the use of eutrophic water that may contain cyanobacterial blooms and microcystins from irrigation. Microcystins transporters have not been identified in plants, but microcystins has been detected in both shoots and roots of terrestrial plants, which implicates that microcystins could be absorbed and transported in terrestrial plants.

The absorption, transportation, and metabolism of microcystins in plants will be discussed in the first part of this chapter. Microcystins accumulated in plants can result in phytotoxicity by inducing growth limitation and histological alterations.

Besides of the inhibition of microcystins to phosphatases 1 and 2A, lipid peroxidation and the increase of anti-oxidative enzymes induced by microcystins suggested that oxidative damage might contribute to the phytotoxicity of microcystins in plants.

Our recent study also suggests that nitric oxide is involved in physiological process of microcystins-induced phytotoxicity. The advances in the mechanisms of microcystins-induced phytotoxicity will be reviewed in the second part of this chapter.

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## INTRODUCTION

In recent years, eutrophication of fresh water bodies caused by elevated nutrients inputs has becoming one of the most severe environmental problems worldwide [1,2]. One of the most harmful consequences of water eutrophication is visible cyanobacterial blooms on surface, which is the abundant growth of cyanobacteria including genera such as *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc* and *Nodularia* [3,4].

Cyanobacterial blooms perennially occur in many inland lakes throughout the world, such as Victoria in Africa, Okeechobee in the United States, Taihu in China, and the Baltic Sea in Europe, etc [5]. Besides of the damage of aquatic ecosystem, another extremely dangerous impact of cyanobacterial blooms is the production of toxins.

The most commonly occurring toxins produced by these blooms are microcystins (MCs), which are cyclic heptapeptides and share common structural features, including the Adda side chain and a ring, consisting of five amino acids.

More than 90 isoforms of microcystin have been discovered [6]. The variations occur between different strains of the same genus, as well as between different genera [6]. Among the MCs varieties, Microcystin-LR (MC-LR) is a widely occurring and most frequently studied of the heptapeptide cyanotoxins produced by various bloom-forming cyanobacterial genera [6-8].

Taihu Lake, the third largest freshwater lake in China, has been suffering severe cyanobacterial blooms in the past decade [5,9]. In summers, the blooms covered almost 2/5 of the total area of Taihu Lake. In addition, continuous monitoring has demonstrated that the frequency and area of cyanobacterial blooms in Taihu Lake increased annually, which sharply enhanced concentrations of MCs in the water [9,10].

The concentration of MCs was up to 3.65 μg/mL in eutrophic water bodies in New Zealand between 2001 and 2004 [11]. MCs can be taken up directly by plants from cyanobacterial-blooming water, which in turn induce phytotoxicity by interrupting intrinsically physiological process in plant cells.

## Uptake and Accumulation of MCs in Plants

### *Uptake and Accumulation of MCs in Aquatic Plants*

Aquatic plants exposed to eutrophic water environment have been shown to absorb MCs easily from water. The first report of MCs accumulation in emergent aquatic macrophyte is the detection of MC-LR in the emergent reed plant *Phragmites australis*. MC-LR was rapidly taken up by *P. australis* with clear distribution in the different cormus parts of the plant, which the highest accumulation of MC-LR was detected in the stem, followed by the rhizome [12].

Mitrovic et al. [3] has compared the accumulation rate of MC-LR between two different aquatic plants. *Lemna minor* accumulated microcystin-LR (MC-LR) up to a concentration of 0.2887 ng/mg wet wt. plant material over the 5 days of the experiment, equivalent to an accumulation rate of 0.058 ng/mg/day. However, the accumulation rate of MC-LR in *Wolffia arrhiza* was much lower than that of *L.* minor (0.008 ng/mg/day). This study suggested that the capability of accumulating MC-LR was variable among different aquatic plant species.

MC-RR, another important isoform of microcystin, can be accumulated differentially in the roots and leaves of submerged macrophyte *Vallisneria natans* in both dose- and time-dependent manners. The roots showed higher accumulation of MC-RR than leaves, which indicated that MC-RR could be rapidly absorbed by roots with limited transportation to the shoots of *V. natans* [13].

In addition, aquatic plants seem to selectively accumulate different MCs isoforms from natural eutrophic water. For instance, duckweed *Lemna gibba* could accumulate MC-LR from toxic *Microcystis* culture extracts (including MC-LR, MC-RR, and MC-WR) in dose-dependent manner. However, MC-RR was less accumulated in plants while MC-WR was not detected in plant extracts [14].

Bioaccumulation of MCs are harmful for aquatic plants, but Nimptsch et al. [15] proposed a "Green Live Concept" that MC-LR concentrations from raw lake surface water could be reduced by using the bioaccumulation potential of aquatic macrophytes. The combined macropyhtes consisting of *Lemna* sp., *Myriophyllum* sp., and *Hydrilla* sp. showed the most efficient elimination rate of MCs, reducing an initial MC-LR concentration of 12.1 and 9.2 μg/L to values below the World Health Organization (WHO) guidelines for drinking water of 1.0 μg/L MC-LR in only three days. The field test in a natural outdoor pond system showed that these macrophytes resulted in the decrease of MC-LR concentration by 84%, which suggests that certain macrophyte species could be applied in eliminating MCs in raw lake water.

### *Uptake and Accumulation of MCs in Terrestrial Plants*

Terrestrial plants could be exposed to MCs via the use of eutrophic water that may contain cyanobacterial blooms and toxins from irrigation. The uptake of MCs by agricultural plants could occur via spray irrigation of crop plants when surface water is contaminated with MCs. Crush et al. [16] have tested the uptake of MCs in rape, ryegrass, clover, and lettuce after spraying water containing cyanobacterial cells and MCs collected from nutrient-enriched lake. Among the tested plants, only the edible shoots of lettuce could accumulate MCs at a dangerous level. The average concentration of MCs in lettuce was 0.79 mg/kg DW, which was determined by enzyme-linked immunosorbent assay (ELISA). Health risk evaluation suggested that MCs intake by consuming treated lettuce significantly exceeded the Tolerable Daily Intake (TDI) of 0.04 μg/kg of body weight per day of MC-LR recommended by WHO [4]. This result suggested that irrigation with water containing MCs might pose potential risk to human health by transferring MCs into human body through food chain.

MCs could also accumulate in plants via root irrigation with MCs-contaminated water. Järvenpää et al. [17] have detected the accumulation of MCs in broccoli (*Brassica oleracea* var. italica ) and mustard (*Sinapis alba*) by watering the roots of plant seedlings in an laboratorial study. Plants roots were exposed to extracts from cyanobacterial cells for 19-20 days. Among the four MC variants present in the exposure mixture, only MC-LR was clearly detectable at concentrations ranged from 0.9 to 2.6 ng/g FW. The toxin was found only in the roots of broccoli and mustard, which suggested that these plants may have limited accumulation of MC-LR in shoots in this specific condition. However, the possibility of MC-LR translocation from roots to shoots cannot be excluded.

Peuthert et al. [18] have reported that MCs could be absorbed by roots and be translocated from roots to shoots in seedlings of eleven agricultural plants. Among the tested agricultural plants, the roots of alfalfa and wheat showed especially high uptake values of

MCs. Root-absorbed MCs could be translocated into shoot even though the concentration of MCs in shoots occurred at much lower level than in roots.

In another comparative study, rape accumulated much more MCs than rice. After the exposure of extracts containing MC-LR at the concentration of 3 μg/mL for 10 days, the recovery of MCs were 651±78.71 and 5.4±0.85 ng /g FW in rape and rice, respectively [19].

It has been documented that the shoots of apple (*Malus pumila*) seedling could accumulate considerable MCs as well. After 14 days exposure to the MCs at concentration of 3 μg/mL, the shoots of apple seedling accumulated MCs up to a concentration of 510.23±141.10 ng/g FW, equivalent to an accumulation rate of 36.45 ng/g day [20].

In order to identify the preference of MCs variants accumulation in plants, Saqrane et al. [21] detected the accumulation of six MCs variants in four different plant species using tandem Liquid chromatography-Mass spectrometry (LC-MS). MC-LR and MC-RR were the major MCs variants accumulated in plants after plants exposure to the highest MCs concentration.

Since MCs could be accumulated in terrestrial plants in laboratorial studies, the detection of MCs accumulation in plant samples from natural environment is essential for health risk evaluation of MCs in the food chain. Mohamed et al. [22] described the MCs concentration in ten groundwater wells and tissues of six kinds of vegetable plants irrigated with well waters in Asir region, southwest of Saudi Arabia. All the vegetable samples (including radish, lettuce, parsley, arugula, dill, and cabbage) were collected from farms that used groundwater for irrigation. All vegetable plant samples were found to accumulate MCs in their roots and shoots. The total MCs concentration in plants correlated positively with their concentrations in related well waters ($r = 0.92$). This study provided strong evidence that MCs could enter into food chain by accumulating naturally in vegetables, and thus pose significant threat for human health.

In addition, Chen et al. [23] provided another important evidence that MCs could accumulate in rice grains collected from natural environment, which suggested that cereal could be another common medium for transfering MCs to human body. In this study, rice grain samples were collected from rice fields irrigated with cyanobacterial blooming water from Taihu Lake. MC-LR was detected at 21 of the total 44 sited rice grain samples with a concentration range varing from 0.04 to 3.19 μg/kg DW.

Wannemacher [24] reported that MC-LR is stable even at temperatures up to 300°C in laboratory conditions. Therefore, MC-LR can enter into human body by the consumption of cooked rice containing MC-LR. Risk evaluation suggested that MC-LR intake by consuming the sampled rice did not exceed the TDI recommended by WHO, which suggested that the current accumulation of MC-LR in rice around Taihu Lake may not pose a threat to human health through food chain. However, the possibility of considerable accumulation of MC-LR in rice grains cannot be excluded with the increase of MC-LR concentration in the fresh water of Taihu Lake if continuous pollution aggravates cyanobacterial blooms in Taihu Lake [23].

Some MC variants may cross cell membrane by penetration or diffusion [4], but MC-LR is unable to easily penetrate biological membranes and bioaccumulate in cells due to its high molecular weight (~1000 Da) [25]. However, some kinds of animal cells express specific membrane transporters that contribute to MC-LR accumulation in animals. Some members of the organic anion transporting polypeptide superfamily (rodent: Oatps; human: OATPs) are responsible for the transport of the most common MC variants including MC-LR. Fischer et al. [26] demonstrated that rat Oatp1b2, human OATP1B1, human OATP1B3, and human

OATP1A2 transported MC-LR 2- to 5-fold above water-injected control oocytes by using the *Xenopus laevis* oocyte expression system. Among the tested Oatps/OATPs, Oatp1b2, OATP1B1, and OATP1B3 are responsible for microcystin transport into hepatocytes, whereas OATP1A2 mediates MC-LR transport across the blood-brain barrier. Meier-Abt et al. [27] found that the evolutionarily ancient Oatp1d1, a precursor of the liver-specific mammalian Oatps/OATPs, is able to mediate uptake of MC-LR into skate liver. Until current time, the mechanism of how MC-LR enters into plant cell still remains unclear. And MC-LR transporters have not been identified in plants. Whether plant cells absorb MC-LR through OATPs-like system will be an interesting topic to be investigated further.

***Metabolism and Biotransformation of MCs in Plants***

It has been reported that glutathione (GSH) plays vital role in the detoxification of MCs in animals [28]. Recently, GSH-mediated detoxification process has been proposed in plants as well. The GSH-MC-LR conjugate with mass of m/z 1308, analyzed by HPLC followed by MALDI-TOF mass spectrometry, occurred in aquatic macrophyte *Ceratophyllum demersum* after exposure of 5 μg/L MC-LR for 24 h [29].

Glutathione S-transferase (GST) plays protective roles against phytotoxicity by catalyzing the covalent conjugation of reduced GSH with toxins. Activation of GST by MC-LR was much higher in comparison to the activation by phenobarbital and atrazine, two known activators of GST [30].

Glutathione reductase (GR) contributes to the transformation of oxidized glutathione to reduced GSH. In aquatic macrophyte *C. demersum*, exposure of MC-LR resulted in the increase in the GR activity in a time-dependent manner, which suggested that MC-LR induced the formation of reduced GSH. However, a significant depletion of GSH was detected after MC-LR exposure, which can be explained by the conjugation of GSH with MC-LR resulting from the increase in GST activity [29].

The process of GST-mediated biotransformation of MC-LR was also confirmed in submerged macrophyte *V. natans* (Lour.) Hara. Exposure of MC-LR induced the increase in GST activity and the decrease in reduced GSH concentration in dose-dependent manners [31]. Yin et al. [32] have reported that MC-RR could also induce the depletion of reduced GSH in *Arabidopsis thaliana* suspension cells. Also, GST activity increased significantly in both roots and leaves of rice after exposure of MCs [33].

Pflugmacher et al. [34] investigated the tolerance to MC-LR and GST activities in six spinach variants. Three variants (Ballat, Sharan, and Matador) showed relatively strong tolerance to MC-LR, and GST activities in these three variants were higher than in other variants. These results suggested that MC-LR tolerance in plants probably resulted from the capture of MC-LR by GSH mediated by GST.

In addition, the compartmentation of GST in different organelles may contribute to the plant adaptation to MCs. The activation of the microsomal and cytosolic GST activity in the aquatic plant *C. demersum* was obtained after the exposure to MC-LR with various concentrations.

However, microsomal GST was activated by lower concentrations of MC-LR (0.12 μg/L), whereas the cytosolic GST seems to react to concentrations above 0.5 μg/L [30]. Therefore, the balance between microsomal and cytosolic GST activities is required for plant's adaptation to MC-LR stress.

## MCs-Induced Toxicity in Plants

### *Morphological and Ultrastructural Changes*

MCs-induced growth inhibitory effect has been widely demonstrated in various plant species. Saqrane et al. [21] investigated the inhibitory effect of MCs on the growth of wheat (*Triticum durum*), maize (*Zea mays*), pea (*Pisum sativum*), and lens (*Lens esculenta*). The biomass of all the tested plant seedlings decreased significantly under the MC-LR treatment in dose-dependent manners.

For aquatic plants, it has been reported that MCs remarkably reduced the frond number in *L. gibba* [14], *L. minor* and *W. arrhiza* [3].

Exposure of MC-LR for 16 days resulted in the decreases in fresh weight and shoot length of potato shoot cultures in dose-dependent manners. Potato shoots exhibited 50-100% necrosis on shoot and leaf tissue at MC-LR concentrations of 0.05-5 μg/mL. At lower MC-LR concentrations (0.001-0.01 μg/mL), shoots showed less than 25% necrosis [35].

MC-LR induced obvious necrosis of *Sinapis alba* cotyledons by the observation of tissue anatomy under light microscopy [36].

In a recent study, MCs induced significant decreases in stem length and fresh weight of tomato (*Lycopersicon esculentum*) plants. The inoculation of tomato leaves with the MCs solution induced the appearance of necrosis around the injection areas of microcystins [37].

Irrigation with cyanobacterial crude extracts containing 0.05 μg/L MC-LR for 6 weeks caused obvious morphological changes in MC-LR sensitive spinach variants, such as growth stunt, reduced leaf sizes, leaf chlorosis and yellow leaves [34].

Root treatment with cyanobacteria extracts containing MC-LR (2.22-22.24 μg/mL) for 30 days significantly inhibited the growth of shoots and roots, resulting in the decrease in yield production of leguminous plant alfalfa (*Medicago sativa*). In addition, the number of nodules decreased dramatically with the increase of MC-LR concentration in treatment solution, which indicated that MC-LR significantly affected the symbiotic fixation of atmospheric nitrogen by the root of alfalfa [38].

Lahrouni et al. [39] evaluated the effects of MCs on the growth, nodulation process, and nitrogen uptake of a faba bean (*Vicia faba* L., Fabaceae). MCs showed adverse effect on the total number of nodules on the roots of faba bean. Further data showed that MCs exposure significantly impact nitrogen assimilation by faba bean seedlings inoculated with selected rhizobial strains RhOF6 and RhOF21, while the impact was not significant on beans seedling inoculated with RhOF4. These authors suggested that the behavior of tolerant rhizobia-legumes symbioses may represent potential as a friendly biotechnological approach to remediate MCs contamination in agriculture [39].

MCs also has been proved to inhibit seed germination of several plant species, such as bean [39], tomato [37], alfalfa [1], wheat [40], and rice [19]. Amylase play vital role in catalizing the breakdown of starch into sugars to provide energy during the process of seed germination. MC-LR exposure remarkably reduced the activities of α- and β-amylase in rice seed, which may contributed to the inhibitory effect of MC-LR on rice seed germination (unpublished data).

Rice (*Oryza sativa*) roots can come into contact with MCs if the irrigation water is contaminated by MCs. Chen et al. [19] have reported that the inhibitory effects on rice root length and rice root biomass were significant after the exposure of MC-LR at concentrations of 0.12-3 mg/L while the effects were not significant with MC-LR concentration below 0.12

mg/L. As a monocot plant, rice produces numerous crown roots and lateral roots, both of which are crucial for rice's acquisition of water and nutrients during the growing period [41, 42]. In order to determine the detailed toxic effect of MC-LR on rice root system, Chen et al. [23] evaluated rice root morphogenesis under MC-LR stress. Exposure of MC-LR (0.5-4 μg/mL for 5 days) significantly impeded the rice root morphogenesis by inhibiting root elongation, crown root formation, and lateral root development from primordia.

Máthé et al. [8] has reported that MC-LR inhibited the development of embryogenic calli and the growth of aquatic macrophyte reed (*Phragmites australis*) plantlets. The 50% plantlet growth inhibitory concentration value (IC50) of MC-LR was 12 μg/mL and 36 μg/mL on mineral medium and Murashige-Skoog medium, respectively. Histochemical approaches were used in this study to reveal MC-LR-induced anatomy changes in plant tissues. MC-LR could induce aerenchyma obturation, intense callus formation, root coalescence, and root necrosis in reed plantlets. MC-LR captured the development of lateral root by promoting the lignification in stele cell walls and aerenchyma in the cortex at 5 mm from the root tip [8].

Further study suggested that early lateral root with radial swelling of cells appeared in the elongation and root hair zone from reed plant exposed to MC-LR [7]. MC-LR induced-cytoskeletal changes also included the microtubule disruption in meristems and in the elongation and differentiation zones, which was accompanied by root cell shape alteration. MC-LR with low concentrations (0.5-5 μg/mL) enhanced mitotic index at long-term exposure and induced the increase of the percentage of meristematic cells in prophase as well as telophase and cytokinesis of late mitosis. Root growth stunt induced by high MC-LR concentrations (10-40 μg/mL) resulted from the inhibition of mitosis [7]. MC-LR-induced microtubule alteration also occurred in the shoots of submergent aquatic macrophyte *C. demersum*. Exposure of MC-LR (0.01 μg/mL) for only 4 days significantly inhibited the elongation of *C. demersum* shoot tips, which correlated with the induction of cortical microtubule reorientation from transverse to longitudinal known to induce radial expansion of meristematic cells instead of normal elongation [43].

Jiang et al. [31] investigated the ultrastructural changes of in mesophyll cells of submerged macrophyte *V. natans* under MC-LR stress by using transmission electron microscope (TEM) and scanning electron microscope (SEM). After exposing to 0.1 μg/L of MC-LR for 14 days, only the number of osmiphilic granules increased slightly and a slight plasmolysis could be observed in some cells. The degree of cell and organelle structural damages increased as MC-LR concentrations increased. MC-LR at high concentrations (1, 10, or 25 μg/L) induced a serious of organelle structural changes, such as plasmolysis, mitochondria swelling and cristae vague, karyopyknosis, chromatin condensation, chloroplast swelling with distended or indistinct thylakoid lamellae, and outer membrane disappearance.

Huang et al. [44] has reported the morphological and ultrastructural changes in tobacco BY-2 cells exposed to MC-RR. Exposure to MC-RR at 60 μg/mL for 5 days led to the typical apoptotic morphological changes including chromatin condensation, "half moon" structure, cytoplasm shrinkage, and decreased cell volume, as revealed by fluorescent microscopy, light microscopy, and TEM, respectively.

Exposure to MC-RR at 120 μg/mL for 5 days led to morphological and ultrastructural changes of typical necrosis, such as rupture of the plasma membrane and the nuclear membrane and a marked swelling of cells. These authors suggested that exposure of tobacco BY-2 cells to MC-RR at a lower and higher concentration resulted in apoptosis and necrosis, respectively.

***Physiological and Biochemical Disturbance***

MCs-induced growth stunt of plant shoots has been linked with photosynthesis disturbance. MCs-induced decrease in chlorophyll content has been determined in variant plant species, such as *Solanum tuberosum* [35], *L. gibba* [14,45], *V. natans* [31], *C. demersum* [43], *Z. mays* [21], and *L. esculenta* [21]. However, chlorophyll is not the target of MCs in some plant species. For instance, MCs inhibited the growth of *T. durum* and *P. sativum* without affecting chlorophyll contents [21], which suggested that other factors may contribute to MCs-induced growth stunt.

The physiological state of the photosynthetic apparatus can be determined using the Fv/Fm ratio of the fluorescence measurements, which represents the maximum quantum yield of photosystem II (PSII) [46]. It has been recommended that an Fv/Fm value lower than 0.8 will reveal the plant stress condition, indicating a particular photo-inhibition phenomenon [47]. MCs exposure induced significant reduction in Fv/Fm ratio below 0.8 in tomato and alfalfa seedlings in dose-dependent manners [37,38]. MC-LR treatment reduced Fv/Fm ratio in four tested plant species including wheat, maize, pea, and lens. The Fv/Fm ratio decline was dose- and plant species-dependent, with the most sensitive species being pea. These studies suggest that MCs-induced growth arrest may partially results from the disruption of photosynthetic activity [21].

MCs stress significantly affects the uptake of water and nutrients. In a hydroponic study, the seedlings of runner bean (*S. tuberosum*) grown in the presence of MC-LR (1.12 μg/mL) took up approximately 30% less medium after 18 days than those grown in control medium [35]. Saqrane et al. [21] investigated the mineral contents ($Ca^{2+}$, $Na^{+}$, $K^{+}$, P, N) in several terrestrial plants under MC-LR. MC-LR exposure remarkably induced the increase in overall mineral nutrients accumulation in several plants in dose-dependent manner. These authors suggested that the increase in the uptake of mineral nutrients might result from MC-LR-induced increase in the permeability of plant root cells.

Oxidative stress has been proposed as a major consequence of MCs-induced phytotoxicity. Abiotic stresses lead to the overgeneration of reactive oxygen species (ROS) in plants, which are highly toxic to proteins, DNA, and lipids which ultimately results in oxidative stress [48]. Generally, ROS accumulation can be adequately controlled by the antioxidant systems present in plant cells [49]. However, environmental stress may disturb the homeostasis of ROS and then enhance the production of ROS [50]. Pflugmacher et al. [29] suggested that ROS might occur during the biotransformation process in which MC-LR-GSH conjugate is broken down. The time-course experiment demonstrated that the accumulation of hydrogen peroxide ($H_2O_2$), a variant of ROS, was sharply enhanced in *C. demersum* under MC-LR stress. By the time of 24 hours, the $H_2O_2$ concentration was almost 10-fold higher that the control. The overgeneration of superoxide radical ($O_2^{\cdot}$-), another important ROS variant, was clearly detected in leaf tissues of *V. natans* seedlings under MC-LR stress via spin trapping by EPR spectroscopy [31]. Exposure of rice root to MC-LR significantly induced the accumulation of total ROS as determined by using ROS-specific fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) [23]. Similarly, total ROS level reflected by DCF fluorescent density increased significantly in a time-dependent manner in tobacco BY-2 cells with prolonged stress of MC-RR [50,51].

ROS is able to induce apoptosis via mitochondria-dependent or mitochondria-independent pathway in various cells and tissues, and the loss of mitochondrial transmembrane potential ($\Delta\psi m$) is an early event in mitochondria-mediated apoptosis [53,54].

In MC-RR-treated tobacco BY-2 cells, the application of ascorbic acid (ASA), a major primary antioxidant, prevented the increase in ROS overgeneration, and blocked the decrease in $\Delta\psi m$ and subsequent cell apoptosis [51]. This study suggested that ROS played a critical role in causing a reduction of $\Delta\psi m$ and in cell apoptosis in MC-RR-treated tobacco BY-2 [51].

Overgenerated ROS can cause lipid peroxidation by attacking membrane lipids [55]. In *A. thaliana* suspension cells, MC-RR-induced oxidative stress has been linked to the accumulation of malondiadehyde (MDA), a typical indicator of lipid peroxidation [32]. MC-LR-induced MDA accumulation has been observed in submerged macrophyte *V. natans* [31]. Peuthert et al. [18] has demonstrated that lipid peroxidation is correlated well with the concentration of MCs accumulated in the seedlings of several important crop plants.

MC-LR-induced ROS accumulation is counteracted by anti-oxidative system including a variety of anti-oxidative enzymes and antioxidants. Superoxide dismutase (SOD) constitutes the first line of defense against ROS by scavenging free radicals by means of catalyzing the dismutation of $O_2^{\cdot-}$ into $O_2$ and $H_2O_2$ in plants. In addition, peroxidase (POD) and catalase (CAT) are indispensable defensive anti-oxidative enzymes that remove over-generated hidrogen peroxide by reducing $H_2O_2$ to $H_2O$ [48]. MCs-induced increases in anti-oxidative enzymes in various plant species, such as *Spinacia oleracea* [34], *M. sativa* [38], *L. minor* [3], *L. gibba* [14], *C. demersum* [29], *V. natans* [31], tobacco BY-2 cells [52], *A. thaliana* suspension cells [32]. These studies suggested that MCs-induced ROS burst triggered the expression of anti-oxidative enzymes.

Secondary metabolites play important roles in plant against oxidative injury. Phenols are a diverse class of secondary metabolites in plant resistance to environmental stress [56]. The accumulation of phenolic compounds could be stimulated in *L. esculentum* and *L. gibba* under MCs stress [14,37]. Saqrane et al. [14] suggested that phenolic compounds can be used as indicators of biotic stress induced by cyanobacteria on aquatic plants. Tocopherol system is also able to act as an antioxidant scavenger [57]. Increases in α- and γ-tocopherol also suggested the occurrence of oxidative stress in the spinach variants by exposure to the cyanobacterial crude extract containing MC-LR [34]. Nitric oxide (NO) is a biologically active gaseous molecule and has been related to lateral root growth as well as plant responses to oxidative stress under various environmental stresses [58]. MC-LR exposure of rice root remarkably induced the increase in endogenous NO level as determined using specific fluorescent probe 4.5-diaminofluorescein diacetate (DAF-2DA). The application of sodium nitroprusside (SNP), a donor of exogenous NO, significantly attenuated the MC-induced growth arrest in rice roots [23]. This study suggested a crucial role of NO in regulating plant root development under MC-LR stress. However, the detailed mechanism of NO in MC-LR-induced disturbance in intrinsic physiological process needs to be clarified.

## CONCLUSION

Cyanobacterial blooms caused MCs contamination in eutrophic water. Both aquatic plants and terrestrial plants have high probabilities to come into contact with MCs. The MC-LR transporter in plants have not been identified yet, but the detection MCs accumulation in various plant species suggested that MCs could be taken up and transported in plant tissues.

However, the mechanism of MCs accumulation in plants is largely unknown. MCs could induce severe morphological and ultrastructural damage in plant organs and organelles, respectively. MC-LR-induced phytotoxicity has been closely linked to disturbance in water and nutrients uptake, decline of photosynthesis, oxidative injury, and apoptosis. The molecular mechanism of the involvement of MCs in plant intrinsic physiological process needs to be further investigated in order to provide basis for risk evaluation and safety control of MCs in agricultural environment.

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*Chapter 8*

# CYANOTOXIN HEALTH HAZARD AND RISK ASSESSMENT IN FRESHWATER LAKES

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## ABSTRACT

Cyanobacterial toxins are very toxic for animals and human beings, and are endowed with severe acute and chronic intoxications. It is therefore very important to provide people toxin-free water, or at least guarantee a toxins level below secure threshold limits. If contamination of water can't be avoided, adequate measures have to be taken to avoid intoxication episodes and exposure of humans to toxins. Such measures should be as environmental friendly as possible, but nonetheless effective and definitive. These should include actions to avoid exposure and to minimise contamination of drinking water plants. In addition, since intoxication cases can occur, the best diagnostic and medical treatments have to be addressed, for an adequate first aid intervention. Health hazard from microcystin contaminations in drinking water, food supplements, commercial fish and edible mussels has been studied in several lakes from Northern, Central and Southern Italy, in order to evaluate the human risk resulting from several routes of exposition. Many countries have adopted monitoring programs which take into consideration for the risk assessment only the abundances of cyanobacterial cells.

This view does not take sufficiently into account the relationships between cyanobacterial abundance and cyanotoxin concentrations in water. There is a strong need to homogenize the procedures for risk assessment evaluation and to define threshold limits based on cyanobacterial abundance and/or cyanotoxin concentrations, studying the implications deriving from the presence of risk cofactors like heavy metals or pesticides, too. These aspects are propaedeutic for the preparation of transnational guidelines for risk assessment and management.

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## INTRODUCTION

Cyanobacteria are a widespread group of organisms colonizing all ecosystems. They are common inhabitants of freshwater bodies throughout the world, several of them form blooms that accumulate in surface forming scums; other species can reach very high densities without surface accumulation. Under favourable conditions many species of cyanobacteria may become dominant in the phytoplankton of water bodies, and their cell densities may reach many millions per litre [1].

Cyanobacteria are known to produce several secondary metabolites significant from the public health perspective of acute exposure: lipopolysaccharides [2], and cytotoxic, tumor-promoting and enzyme inhibiting metabolites like cyclic depsipeptides, cyclic peptides (microcystins, nodularins, anabaenopeptins and nostophycins), linear peptides (aeruginosins and microginins) [3-5], neurotoxic alkaloids (anatoxin-a, homoanatoxin-a) and saxitoxins [6-10]. Recently, a neurotoxic non-protein aminoacid (β-N-methylamino-L-alanine, BMAA), widely produced among cyanobacteria [11], has been associated with neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis/parkinsonism-dementia complex [12-16].

Several categories of powerful toxins are unique to this group of organisms with the exception of saxitoxins, which are produced also by some bacteria and dinoflagellates [17,18]. Cyanotoxin poisoning in humans was mainly caused by three toxic groups: microcystins (MCs), cylindrospermopsin (CYN) and anatoxin-a (ANA-a), and occurred through exposure to contaminated drinking water supplies [19,20], recreational waters [1,21,22] and medical dialysis [23].

Cyanobacterial blooms occur worldwide from USA to Australia, causing human health risks and animal deaths as well. In Europe, they have been detected in Belgium, Germany, France, Finland, Norway, United Kingdom, Hungary, Portugal, Spain and Italy [24,25].

The principal woldwide recorded cyanobacterial genera causing freshwater toxic blooms are *Microcystis*, *Anabaena*, *Aphanizomenon*, *Planktothrix*, *Cylindrospermopsis*, and *Nodularia*. Their predominance varies according to latitude, continental climate and nitrogen:phosphorus rates of the lakes: the most common cyanobacterium in European prealpine lakes, representative for volume and depth in this continent, is the filamentous species *Planktothrix rubescens* Anagnostidis and Komarek (previously *Oscillatoria rubescens* DC ex Gomont) [26], which efficiently generates MCs; in warmer latitude Countries, as in South America or Africa, *Microystis aeruginosa*, *Anabaena flos-aquae* and to a minor extent *Cylindrospermopsis raciborskii* are the most common bloom forming toxic species [27].

### Microcystins

Most poisoning by cyanobacteria involves acute hepatotoxicosis caused by a structurally similar group of low molecular weight cyclic heptapeptides, the microcystins (MCs). Microcystins are hepatotoxins [28-33] acting as specific inhibitors of protein phosphatases 1, 2A [31], 3 (MC-LA) [34], 4 and 5 [35] and to a minor extent PP 2B [36]. They dephosphorylate serine and threonine residues in animals and plants. The inhibition of PP1

and PP2A results in an increase of the phosphorylation of proteins in the liver cells, affecting several processes like metabolism, cell contractility, membrane transport, secretion, cell division and gene transcription and translation.

MCs are responsible for liver failure and death in humans [23,29], wild animals, livestock and aquatic life [24,37]. They are also tumour promoters [38-44], endocrine-disruptors [45-49], immunotoxicants [50] and possibly carcinogenic to humans [51]. MCs can induce oxidative DNA damage [52], genotoxicity [53] and cause the activation of proto-oncogenes c-jun, c-fos and c-myc [54]. Indirect evidence supporting tumour promotion of human cancer from MCs exposure comes from the studies of Yu [55], Ueno et al. [41] and Zhou et al. [44] in China, Fleming et al. [43] in Florida, and Svircev et al. [56] in Serbia.

MCs have also cumulative effects, well documented in experimental studies on mice [57]. In these studies, daily sub-acute doses of MCs, intranasally administered to mice for 7-day periods, caused cumulative pathologic effects two times stronger than those produced by the sum of the MCs administered as a single dose. Bioaccumulation has been proposed to be responsible for this effect [58].

Over ninety MCs variants are now described [5], produced by a number of species of not more than ten cyanobacterial genera. From a structural point of view, MCs are a class of monocyclic heptapeptides consisting of D-alanine at position 1, two variable L-amino acids at positions 2 and 4, g-linked D-glutamic acid at position 6, and 3 unusual amino acids; b-linked D-erythro-b-methylaspartic acid (MeAsp) at position 3; (2S, 3S, 8S, 9S)-3-amino-9-methoxy- 2, 6, 8 trimethyl-10-phenyldeca-4, 6-dienoic acid (Adda) at position 5 and N-methyl dehydroalanine (MDha) at position 7.

Recently, the need for a better understanding of congener-specific MC kinetics in general and in humans, has been recognized in order to assess the risk in a chronic exposure setting [59]. Microcystin producing cyanobacterial blooms can be used in dietary supplement production, creating another source of possible human intoxication. In our studies, dietary supplements commercialized in Italy gave microcystin values up to 4.5 μg/g of pills and, for a recommended dosage of 8 pills per day, a consequent total toxin intake (TDI) of 10.4 μg/day is estimated, which is above the recommended WHO TDI of 2.4 μg/day for a healthy adult of 60 kg body weight [60].

When MCs are released into the water during bloom decay, a wide range of aquatic organisms are directly exposed to the toxins in solution. Several large scale fish death outbreaks have been associated to massive occurrence of cyanobacteria in water bodies [61-63]. Studies on fish contaminations have shown species-specific sensitivities to MCs. The uptake of these cyanotoxins in fish results primarily from oral ingestion and, to a minor extent, from absorption via the gill epithelium [64].

MCs can concentrate in various fish tissues [65]. Liver, kidney and gill pathologies are present in fish exposed to these cyanotoxins, due to specific inhibition of protein phosphatases and other downstream hepatotoxic effects causing increased liver enzyme values in the serum. Decreased development of juvenile fish [66] and behavioural changes [67] have been observed after immersion of fish in water containing MCs, this latter effect probably being caused by the ability of the toxins to cross the blood-brain barrier, carried by anion transporting polypeptides [68,69], making brain an important bioaccumulation organ, in some species the second after liver (*Rutilus panosi*) [70].

The toxicity of MCs in fish depends on the balance between accumulation and metabolism [71], and the observed species-specific sensitivities have been interpreted as the result of anatomical, physiological, behavioural differences and detoxification capacities via the glutathione-S-transferase pathway among the various fish orders [69,72,73].

In the past the risk of human consumers of gutted fish was traditionally considered low, because MCs were thought to accumulate mainly in the fish liver. Recent studies, however, detected microcystin concentrations at 337.3 μg/kg (*Tilapia rendalli*) [74], 102 μg/kg (*Oreochromis niloticus*) [75], 96.5 μg/kg (*Hypophthalmichthys molitrix*) [76] and 28 μg/kg (*Oncorhynchus mykiss*) [77] in the muscle tissue of wild or farmed fish (WHO recommended TDI 0.04 μg/kg human body weight/day), indicating that even the consumption of fish muscle might constitute a threat for human health. In Italy, 155 analyzed samples of fish and crustaceans collected in five contaminated lakes gave concentration values ranging from a minimum of 0.21 μg/kg to a maximum of 14.6 mg/kg [78].

MCs can accumulate in fish to levels that although not lethal to the fish, may lead to toxin exposure that are above the recommended limits for human consumption. This potential hazard has led to the development of several extraction techniques and analytical methods to detect MCs in different matrixes and fish tissue.

Several methods have been described in the literature, based on protein phosphatase inhibition bioassay, immunoassays (ELISA), mouse test, HPLC with UV detection. Nevertheless, these methods showed some limitations, because they lack of selectivity in identifying the single cyanotoxins in the sample, and sometimes may also be affected by the presence of endogenous activities (protein phosphatase inhibition bioassay) [79,80]. On the contrary, the sensitivity of HPLC based methods is not recognized as adequate in determining low contamination levels of cyanotoxins.

In our studies different microcystin values were detected in ELISA and triple quadrupole LC-MS/MS analyses of the same samples [78]. Differences in total microcystin concentrations measured by triple quadrupole LCMS/MS or ELISA in fish tissue have been observed in other studies [77,81-83]. LC-MS/MS in our studies only screened for six variants, so it is possible that not all the MCs contained in samples were detected.

For these samples the same extraction techniques were adopted for the immunological and instrumental analyses, in order to perform both kinds of measurement starting from the same extraction efficiency. Yuan et al. [84] provided evidence that the utilized instrumental techniques have basic importance in measurement equivalence (e.g. between ELISA and LC-MS/MS results). The different availability of free and bound MCs to our LC-MS/MS analytical method could explain the differences found in our results.

## Cylindrospermopsin

Poisoning episodes are also caused by cylindrospermopsin (CYN), which is a nephrotoxic, thymotoxic and hepatotoxic sulfated-guanidinium alkaloid with 5-substituted-2,4-dioxypyrimidine (uracil) moiety, (m.w. 415 g/mol) [85,86].

CYN has deserved particular scientific interest in the last decade, because of the spreading of its producing species from tropical to temperate environments, in apparent correlation with the occurrence of global warming phenomena. CYN is produced by the species *Cylindrospermopsis raciborski* [87,88], *Umezakia natans* [89], *Aphanizomenon*

*ovalisporum* [86], *Anabaena bergii* [90], *Raphidiopsis curvata* [91], *Aphanizomenon flos-aquae* [92], *Anabaena lapponica* [93], *Lyngbya wollei* [94] and *Aphanizomenon gracile* [ 95].

The first episode of human poisoning caused by contaminated drinking water was recorded in Palm Island (Australia) in November 1979, when an aboriginal community was affected by an outbreak of hepatoenteritis [96]. The presence of CYN was detected in Europe for the first time in 2002 [97] and in Italy in 2004 [98].

Apart from the liver, effects on kidneys, lung, heart and thymus were also described after CYN poisoning [85]. Cutaneous sensitivity reactions produced by pure toxin or CYN–containing suspensions of *C. raciborskii* [2] and fetal toxicity after exposure in late gestation phase [99] were demonstrated in mouse.

Recently, mutagenicity of CYN was proven *in vitro*, and strong evidences also exist for its carcinogenicity *in vivo*, such as DNA fragmentation and modification [42,100,101] were observed in the liver of treated mice. Also, a range of cytogenetic abnormalities have been observed in human lynphoblastoid cells exposed to CYN, with formation of centromere-negative micronuclei indicating double-stranded DNA breakage [42]. Genotoxic activity is caused by the ability of CYN to induce DNA strand breaks, and loss of the chromosome structure due to the damages to centromere/kinetochore function. Moreover, CYN induces stress responses in human cultured fibroblasts and HepG2 cells that result in the activation of the p53 transcription factor [102].

At low doses CYN suppresses protein synthesis, probably by inhibiting ribosomal translation via binding to a protein associated with the eukaryotic translation system [103,104]. At higher concentrations a much faster toxic process, metabolism dependent, dominates: acute toxicity appears to be modulated by cytochrome p-450-generated metabolites [105].

Environmental toxicity towards amphibians [106] and antibacterial activity [107] has been proven for CYN. Recent evidences have also confirmed that plant growth and metabolism [108] as well as pollen germination [109] and aquatic macrophyte growth [110] are inhibited by CYN, with implications for current spray-irrigation practices.

Biosynthesis of CYN is poorly understood but its chemical structure, and feeding experiments with stable isotopes, suggested that guanidinoacetic acid is the starter unit and indicated involvement of a polyketide synthetase [111]. Other structural variants of CYN have been isolated, the 7-epicylindrospermopsin, with equal toxicity to CYN [86] and the deoxy-cylindrospermopsin [112], recently estimated toxically quite equivalent to CYN [102]. CYN has been shown to be very resistant to biodegradation [113].

CYN presence in Italy appears to be correlated only to the species *A. ovalisporum* and *C. raciborskii*, whereas several other species have been identified as producers in other European countries [93,92]. In the literature, water bodies investigated for CYN occurrence generally hosted only one cyanobacterial species producing the toxin. During a long-term study regarding the Italian volcanic lake Albano, the seasonal presence of the two succeeding cyanobacterial species *A. ovalisporum* and *C. raciborskii* producing the toxin, was for the first time described in our Country [114].

The presence of CYN levels [up to 1.6 μg/L] higher than the proposed guideline of Humpage and Falconer (1 μg/L) [115] in absence of *C. raciborskii* cells, detected in a water sample from a well used as drinking water source for a town close to the lake, showed the ability of these toxins to reach groundwater even in the extracellular form, similarly to MCs.

## Anatoxin-a

Anatoxin-a (ANA-a) is a potent neurotoxic alkaloid first detected in the cyanobacterium *Anabaena flos-aquae* Brébisson ex Bornet et Flahault, and it is perhaps one of the most powerful cyanobacterial toxin [116]. Poisoning episodes by this toxin increased in severity in recent years [21].

ANA-a is a cholinergic agonist alkaloid (a secondary amine) that opens sodium and calcium channels by binding to acetylcholine receptors (nicotinic and muscarinic receptors), depolarizes muscle cells, and evokes contraction at the neuromuscular junction. The inhibition of repolarization, as a result of the degradation resistance of ANA-a to acetylcholinesterase, causes fasciculation and paralysis by overstimulation. Toxicity is quite high, with a LD50 of 200 μg/kg (i.p.-injected mice) [117], and a sufficiently high dose can cause death by suffocation within minutes [116].

ANA-a producing blooms are known to be responsible for mass mortality in wildlife [118] and livestock [119]. Exposure to the toxin causes alteration of heart rate at different developmental stages of zebrafish embryos [120], and i.p. injection to goldfish causes pathology similar to that produced in rodents at similar dose levels [121].

No TDI for ANA-a was fixed by the World Health Organization (WHO) because of the inadequacy of toxicological databases [122]. In addition to ANA-a, homoanatoxin-a and 4-hydroxyhomoanatoxin-a have been described. Some photodegradation stable, non-toxic products of ANA-a, namely dihydroanatoxin and epoxyanatoxin, have also been identified [123] and detected as toxin metabolites in the stomach content of poisoned dogs [124].

## Saxitoxins

Saxitoxins, also known as paralytic shellfish poisons (PSP) or toxins (PST), are produced by several species of bacteria, cyanobacteria and marine dinoflagellates. Especially, the toxic dinoflagellates have caused large numbers of human fatalities through accumulation of saxitoxins in shellfish [125]. Saxitoxins are carbamate alkaloids of which more than 30 chemical analogues have been characterised [18] and 16 have been identified in freshwater cyanobacteria [24]. They belong to three categories: saxitoxins, gonyautoxins (GTXs), and C-toxins, of which the saxitoxins are chemically characterized by the lack of sulphate moieties whereas GTXs and C-toxins possess one and two, respectively. In addition, decarbamoyl derivatives of several saxitoxins (dcSTX) and GTXs (dcGTXs) have been described. All these analogues acts as sodium channel blockers, silencing neurons trough the stop of impulse propagation and causing paralysis of muscles [116].

The first freshwater reports originated from blooms and laboratory cultures of *Aphanizomenon flos-aquae* Ralfs ex Bornet et Flahault from New Hampshire, USA. Two toxins, neoSTX and STX were identified [9,10]. Contrarily to these first reports, the following studies proved that *Aphanizomenon flos-aquae* is not a saxitoxin producer: other species in the genus or samples from mixed populations revealed that another cyanobacterium actually was the toxin producer [126-128]. During an extensive neurotoxic bloom of cyanobacteria in the Darling River, Australia, in 1990-1991, a variety of saxitoxins were detected in bloom samples [129] and in the small intestine of sheep that died after drinking

dam water [130]. The dominant species was *Anabaena circinalis* Rabenhorst ex Bornet et Flahault, and laboratory cultures isolated from the bloom were shown to produce saxitoxins, mainly the C-toxins C1 and C2 [131,132]. In the South-eastern United States, the mat-forming benthic *Lyngbya wollei* (Farlow ex Gomont) Speziale et Dyck was shown to produce saxitoxins [133,134]. The latest observation derives from Brazil, where STX and neoSTX have been found in *Cylindrospermopsis raciborskii* (Wolosz.) Seenayya et Subba Raju [135,136].

Some recent reports of saxitoxin producer *C. raciborskii* strains and environmental samples showed potential neurotoxic effects of cyanobacteria on aquatic organisms, like cladocerans and fish [137,138].

## CORRECT EVALUATION OF RISKS BASED ON THE DIVERSITY AND QUANTITY OF CYANOTOXINS

A few data are available in literature on the European diffusion of the latter two toxin groups [95,139-143], as well as studies on the occurrence and diffusion of cyanotoxins in Italian water bodies [98,144-150].

In Germany ANA-a-producing blooms were detected in 20 water bodies over 3 years, 1995–1997 [142]. In Italy *Anabaena* blooms that are ANA-a producers have been known since 1990 in Mulargia Lake [146], even though ANA-a is not the most frequently detected toxin in Italian lakes.

Since the end of the 1970s, Italian water bodies have shown a noticeable increase in detected algal blooms. In fact, between 1993 and 1999 the number detected grew from 18 a year to 64 a year, one-third of which were toxic [151].

In 1985, for the first time, suspected toxic algal blooms occurring in the Italian artificial lakes of Medio Flumendosa and Mulargia (Sardinia, Western Mediterranean Sea) were investigated by our laboratory. Some analyses on these blooms of *Planktothrix rubescens* ex *Oscillatoria rubescens* [D.C. ex Gomont] Komarek and Anagnostidis evidenced the presence of a hepatotoxic substance, with a LD50 of 100 mg/Kg body weight in mice and with MCs characteristics (i.e. considerable liver damages, pulmonary and kidney haemorrhages)[152].

Since then, reports of toxic blooms continually reached our laboratory, and in 1993 a data base concerning both marine and freshwater algal bloom episodes was set up. The presence of cyanobacterial blooms in drinking and recreational waters required surveys on their occurrence and presence of toxins, in order to avoid human health risks and to provide information for successful restoration programs.

Elevated microcystin concentrations may be found with low cell abundances [77]. Thus, in case of toxin persistence after cell lysis and death, cell counts alone may not give a valuable indication of microcystin concentrations in order to avoid toxicological consequences, as in course of risk assessment evaluation the toxin level has more value than cell number in water control [149,153,154].

CYN is a highly water-soluble molecule, often present in water in large proportions in the extra-cellular fraction, for long periods [100, 113,155-158]. In September 2005, high levels of extracellular CYN were detected in Albano Lake surface water in presence of relatively low cell densities of *A. ovalisporum* (510 cell/mL; 126 μg/L CYN). These findings suggest

avoiding CYN level evaluation based only on cyanobacteria cell weight, as recommended by Spoof et al. [93], to prevent the risk of under-estimate total concentration of the toxin in the water.

In our studies we found that ELISA measurements of total MCs in water may be significantly higher if, instead of analyzing the whole water sample (e. g. after sonication), the intracellular and extracellular compartments are separately analyzed, and the resulting amounts summarized.

The actual Tolerable Daily Intake (TDI) guideline for MC-LR, proposed by WHO in 1998 for an adult of 60 kg b. w. was 0.04 μg/kg body weight/day [1]. The WHO Commission, although being aware of the studies on the chronic and tumour promoting effects of MCs, following the request of the Australian representatives decided not to discuss a guideline for chronic effects [159]. This was also due to the lack of some subsequent, reliable toxicological studies like the 28-day study by Heinze [160], based on administration of MC-LR in drinking water to rats.

In 2006, the American Environmental Agency (USEPA) stated the need for further studies on cancerogenicity in order to express valuable safety levels, but at the same time found that a lot of in vivo and in vitro studies were available to better define the threshold limits for acute poisoning and chronic toxicity effects (i.e. cirrhotic state induction, etc.).

Based on Heinze's [160] and other more recent studies which were fit with the USEPA statistical linear model softwares, USEPA proposed guidelines developed for acute and chronic risk (0.006 and 0.003 microcystin μg/kg b.w./day, respectively)[161], although reputing the available studies not yet adequate to propose a guideline for cancerogenicity. Further, in 2006 the International Agency for Research on Cancer (IARC) classified microcystin-LR as possibly carcinogenic to humans (group 2B).

International TDI levels and elaboration of models to establish human safety levels necessarily take into account toxicological data, but are simply recommendations, even if authoritative; in effect several Countries have made their own guidelines for cyanotoxins [162]. However, the USEPA proposed guidelines that are at present the most up-to-date evaluations in the field for MCs, and in a modern risk assessment their recent values should be considered instead of the older WHO proposed guideline, never more revised since 1999, in spite of the 2006 IARC classification.

Brazilian legislation (Portaria 518/04/MS from the Ministry of Health) has established a requirement for cyanobacterial toxins in drinking water supplies, setting recommended guideline values of 1 μg/L for MCs, 3 μg/L for equivalents of STX, and 15 μg/L for CYN, along with the installation of biomass monitoring programs [162]. Toxicity testing or toxin analysis is required when cell counts exceed 20,000 cells/ml or 2 $mm^3$/L (~2 mg/L) biovolume. However, no distinction is made for the different taxa of cyanobacteria, which can have very different morphology (i.e., single cells, colonial, or filamentous) and biomass, as well as production of different toxic compounds [138].

Based on the No Observed Effect Concentration (NOEC) of 5 mg/kg obtained in mouse bioassays and applying a safety factor of 0.001, Zagatto [135] calculated a Maximum Acceptable Limit (MAL) of *C. raciborskii* biomass for drinking water. Considering an average individual of 70 kg and an Average Daily Ingestion (ADI) of 2 L of water: MAL=(NOEC×0.001)×70/ADI. This resulted in a MAL of 0.18 mg/L, which is approximately 10 times lower than the limit of 2 mg/L (i.e., based on biovolume)

recommended by the Brazilian legislation for drinking water. Based on this result, Zagatto et al. [138] proposed a revision of the legislation regarding safety factors for drinking water supply.

Another serious question for a correct risk evaluation is represented by MC underestimation of instrumental analyses. HPLC or LC/MS conventional methods, based on prior solvent extraction of the analyte, may largely underestimate total MC content because of covalent binding between the toxin and active sites of protein phosphatase, with consequent inability of solvent to extract enzyme-bound toxins, which are slowly released by the liver and are toxically active [163,164]. Conventional methods may underestimate total MCs by orders of magnitude [165,166]

The cyanotoxin risk assessment in drinking water has to take into account the synergistic ability of some of the main toxins to enhance their damage when present together or when assumed for a period (even a short period) of time [57].

For an estimation of the ANA-a human health hazard, an LD50 of more than 5 mg/kg (oral administration) was reported by Fawell and James [167] and an LD50 of 2 mg/kg (intranasal application) by Fitzgeorge et al. [57], was reduced to 0.5 mg/kg in the presence of sublethal doses of MCs.

Assuming that human and rodent toxicity are equal, the toxic uptake by swallowing water would thus be a concentration of more than 500 mg/L for a 10-kg child, with a mean of 100 mL of water swallowed during baths [142]. However, because the intranasal route is probably the most sensitive uptake route for swimmers, the toxic uptake could be lowered to 20 mg/L of aerosol for a 10-kg child, even reducible to 5 mg/L in the presence of sublethal doses of MCs.

In the Fitzgeorge et al.'s study, administration of microcystin-LR in mice at a sub-lethal dose of 31.3 μg/Kg at 30 min. prior to ANA-a exposure of mice via intranasal route, lowered the LD50 for ANA-a by approximately 4-fold. In the same study, repeated daily doses for seven days of a sub-lethal dose producing no apparent increase in liver weight, were shown to produce an accumulative effect and resulted in a final liver weight increase of 75%.

Microcystin-LR LD50 via intratracheal administration proved to be comparable to LD50 of i.p. administration, with the same liver and pulmonary damages [168].

Groundwater can link lakes across the landscape [169]. MCs and CYN from contaminated lakes can percolate in the groundwater towards the pumping stations for drinking water supply, with presence in the water proportional to the duration of the toxic blooms [149,170]. In the district of Haimen, China, MCs were detected in shallow well waters used for drinking purposes [41]. In this case the few but existing data on human intoxications from drinking waters have to be considered in connection with the synergistic ability of microcystin-LR (and possibly of all MCs) to enhance the toxic damage after repeated sub-acute exposure.

Two of these cases occurred during toxic *P. aghardii* bloom episodes responsible for severe outbreaks of acute illness, one in Finland in 1989, were MCs were detected between 0.1 and 0.5 μg/L, and another in Sweden in 1994, with MCs up to 0.82 μg/L [19]. According to these levels, a mean daily exposure should have ranged from 0.2-1 μg/day in the first case, reaching 1.6 μg/day in the second case; both values well below the WHO TDI limit but above the USEPA acute one.

Recent studies on human MCs daily intake and related detected liver damage in China, refer values of 2.2-3.9 μg MC-LR/day per adult [171] and 0.36-2.03 μg MC-LR/day per child [172], values below or around the WHO limit but well above the USEPA chronic TDI limit. These findings lead to consider the USEPA limits for MCs more adequate for a modern risk assessment. A proposed modern calculation for the human hazard ratio divides the ADI (Average Daily Intake) value by the TDI, considering water unsafe for consumption in case of a ratio greater than 1. The ADI can be estimated multiplying the MCs concentration in drinking water by the volume of daily water intake divided by the body weight [173].

Several episodes are known of human gastrointestinal illness, dermatitis, pneumonia and even death in bathers frequenting water bodies affected by toxic cyanobacterial blooms [21,174,175]. Apart from cyanotoxins, cyanobacterial lipopolysaccharide endotoxins (LPS), produced by a wide range of species, are able to induce dermatitis in bathers, inflammatory and gastrointestinal incidents [2,27,176]. The study of Pilotto et al. [175] established a threshold number of 5000 cells/L, above which dermatologic irritations, gastrointestinal and/or influenza symptoms occurred; toxin levels were not determined.

A recent case-report was signaled concerning a nineteen-year-old young man immersed for two hours in an intense scum of *Microcystis aeruginosa*, and developing four hours later flu symptoms, abdominal pain, pneumonia and hepatotoxicosis requiring hospitalization for twenty days [22]. In this case a MC-LR level of 48.6 μg/L in water was detected, and according to WHO theoretical mean levels of water exposure during recreational activities in water (100 mL) [1], the young man was possibly exposed to 4.8 μg of MC-LR. According to these reports, and to the ability of some toxins to resist to degradation for weeks, even when the producing blooms disappeared, a correct risk assessment for bathing waters must take into account both cyanobacterial numbers (regardless to species) for LPS presence, and toxic levels for poisoning risks. The toxic levels are often a sum of several toxins, sometimes from different families (i.e. MCs plus CYN) [114,149], and synergistic [57]; thus, the acute TDI limits for the various cyanotoxins (see above) have to be carefully considered, conveniently lowering the banning limit when these substances are coexisting in water.

## MANAGEMENT AND TREATMENT RECOMMENDATIONS AFTER HUMAN EXPOSURE TO CYANOTOXINS

When intoxication cases occur, the best diagnostic and medical treatments specific for each toxin have to be addressed, for an adequate first aid intervention. Water or food sources found contaminated by cyanotoxins should no longer be consumed, due to the high variability of the toxin levels during the blooms, to the proved synergistic effects of some of them (especially MCs) and regarding chronic effects. These properties make no TDI sufficiently safe and acceptable in the case of daily consumption of contaminated water.

### Microcystin Poisoning

Symptoms of MCs poisoning may take 30 minutes to 24 hours to appear, depending upon the size of the organism affected and the amount of toxin consumed. Gross and

histopathologic lesions caused by MCs are quite similar among species, although species sensitivity and signs of poisoning can vary depending on the type of exposure. One of the earliest effects (15-30 minutes) of MCs poisoning is increased serum concentrations of bile acids, alkaline phosphatase, γ-glutamyltransferase, and AST. MCs symptoms in mammals and other animals may include jaundice, shock, abdominal pain/distention, weakness, nausea/vomiting, severe thirst, rapid/weak pulse, and death. It is likely that the number of incidents with low-level symptoms such as nausea, vomiting, and diarrhea associated with recreational exposure to cyanobacterial toxins are under reported. Death may occur following exposure to very high concentrations within a few hours (usually within 4-24 hours) or up to a few days. Death is due to intrahepatic hemorrhage and hypovolemic shock. In animals that live more than a few hours following high level exposure, hyperkalemia or hypoglycemia, or both, may lead to death from liver failure within a few days [177].

According to the Merck Veterinary Manual [178], surviving animals have a good chance for recovery because the toxins have a steep dose-response curve. Activated charcoal oral slurry is likely to benefit exposed animals, even though therapies for cyanobacterial poisonings have not been investigated in detail. An ion-exchange resin such as cholestyramine has proved useful to absorb the toxins from the GI tract and certain bile acid transport blockers such as cyclosporin A, rifampin, and silymarin injected before dosing of microcystin have been effective in preventing hepatotoxicity in laboratory studies. If silibinin is available, administration of 20 to 50 mg/kg/day intravenously administered has to be considered [177]. A recent study propose the use of the anti-inflammatory drug LASSBio 596 to avoid pulmonary, lung and hepatic inflammation induced by MC-LR [179].

## Cylindrospermopsin Poisoning

Symptoms of CYN poisoning are diarrhoea, abdominal tenderness and pain to palpation.

Monitoring has to be performed of serum enzyme samples (GGT, GTP, ALT, AST, LDH), blood glucose, fibrinogen, and prothrombin time for hepatocellular damage; and serum electrolytes, urea, creatinine and glucose for impending renal failure. The risks are increased serum liver enzyme values in conjunction with liver necrosis and development of hepatorenal syndrome, included danger of hepatorenal infarction. In the follow-up, liver function and structure (with ultrasound) and renal function have to be checked [177].

## Paralytic Shellfish Poisoning

Signs and symptoms of PSP most often occur within 10 to 30 minutes. Problems can include nausea, vomiting, diarrhea, abdominal pain, and tingling or burning lips, gums, tongue, face, neck, arms, legs, and toes. Later problems may include shortness of breath, dry mouth, a choking feeling, confused or slurred speech, and lack of coordination.

In general, supportive measures are the basis of treatment, especially ventilatory support in severe cases. Without supportive treatment, up to 75% of severely affected persons die within 12 hours. When the ingestion of contaminated food is recent, gut decontamination by the gastric lavage and administration of activated charcoal (240 mL water/30 g charcoal) or diluted bicarbonate solution is recommended. In this case, the use of isotonic sodium

bicarbonate as a lavage solution is advantageous because many of the shellfish toxins have reduced potency in an alkaline environment. Nasogastric or orogastric lavage may be performed if the patient presents within 1 hour of ingestion, but this is often unnecessary. However, care must be taken concerning aspiration with the neurologically compromised patient.

Use of anticholinesterase agents are not recommended, and could actually be harmful [125,180-182]. Anticurare drugs were ineffective, while DL amphetamine (benzedrine) was most effective in aiding the artificial respiration and decreasing the recovery period.

The lactic acidosis of unknown origin seen in experimental animals and possibly humans can be treated by assisted ventilation, fluid therapy and periodic monitoring of the blood pH. It is possibly that the fluid therapy will also assist in the renal excretion of toxin [125].

Many endemic areas have traditionally used local treatments with variable success. In the Philippines, a drink of coconut and brown sugar is administered; demonstrations in mice show that these ingredients may have active detoxification substances [183].

Any cases of PSP should be reported to the appropriate public health authorities for follow up to ascertain other cases and to prevent further spread and every effort should be made to obtain contaminated materials and their source.

### Anatoxin-a Poisoning

Symptoms of ANA-a poisoning are myosis, shallow and labored breathing; potentially tingling of lips, tongue, extremities; salivation, tachycardia, possible convulsions and seizures. Cholinesterase activity in whole blood has to be checked. Supportive care (up to stabilisation) is needed, with intragastral intubation with active charcoal slurry; support of respiration and in case of seizures 5 mg diazepam intravenously administered.

Phenothiazines, parasympathomimetics and antihistamines are contraindicated since they have anticholinesterase activity and may potentiate toxicity. Long term treatment and follow-up for anatoxin-a as for organophosphates and organocarbamate pesticides or chemical warfare agents (soman, tabun, sarin) is needed [177].

## CONCLUSION

There is a strong need to homogenize the procedures for risk assessment evaluation and to define threshold limits based on cyanobacterial abundance and cyanotoxin concentrations, studying the implications deriving from the presence of risk cofactors like heavy metals or pesticides, too. These aspects are propaedeutic for the preparation of transnational guidelines for risk assessment and management.

The possible contemporary presence in lake water of several components, such as cyanobacterial toxins and chemical toxic contaminants (i.e. arsenic, as in the Italian Lake Vico) [184] may lead to heavier risk evaluations in respect to a single detected toxin, suggesting also the need to fix lower limits for MCs in case of carcinogen detected presence. Also, the synergy between the different cyanotoxins must be taken into account.

Several systems can eliminate toxins from water plans [162] but the most diffused problems during water supply treatment from eutrophized sources are the periodic high algal biomasses, and the following tryalomethane compound formation if chloride quantities, adequate to toxin destruction, are used. The use of activated carbon filters in the last treatment section of the plant throughout the year, eventually associated to ultrafiltration devices in case of elevated algal biomasses, is the best solution in order to protect the health of the local population. The filters, adequately kept, will remove both exceeding algal biomass and toxins, without the risks of manual dosage that free carbon powder has. Periodic banning and subsequent remediation of eutrophized water bodies are the only short- and long-term ways to avoid human exposure to toxic blooms and solve the environmental contaminations. Specific remediation plans, although sometimes may appear costly and difficult, are the most economical way, over the long period, to restore in the best way the natural environments, wildlife and principally the tourism fluxes which often regulate the local economies.

Lake remediation may generally be focused on minimization of orthophosphate levels. However, this objective is only achieved by changing agronomical practices and setting stricter regulations on waste water releases.

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*Chapter 9*

# CONFOCAL LASER SCANNING AND ELECTRON MICROSCOPIC TECHNIQUES AS POWERFUL TOOLS FOR DETERMINING THE IN VIVO EFFECT AND SEQUESTRATION CAPACITY OF LEAD IN CYANOBACTERIA

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## ABSTRACT

Microbial mats are laminated benthic ecosystems made up of microorganisms having diversely coloured layers due to the photosynthetic pigments of cyanobacteria, algae and purple anoxygenic phototrophic bacteria. For many years, our group of work has been studying the microbial mats in the Ebro Delta (Tarragona, Spain). Despite the fact that the Ebro Delta has been a protected area for years, is currently subjected to anthropogenic pollution. Heavy metal contamination in these environments is very considerable, making the microorganisms in these ecosystems a subject of great interest when analyzing their ability to sequester metals. Our group has optimized different high-resolution microscopy techniques such as Confocal Laser Scanning Microscopy (CLSM), Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM), these last two techniques coupled to an Energy dispersive X-ray detector (EDX). The CLSM coupled to a spectrofluorometric detector (CLSM-*λscan*) was applied in determining the in vivo effect of lead (Pb) in phototrophic microorganisms (tolerance/resistance), and the CLSM and image analysis (CLSM-IA) were used in determining changes in total and individual cyanobacteria biomass. Additionally, the electron microscopic techniques were utilized in determining the ability of these microorganisms to capture metal both externally in

extrapolymeric substances (EPS) and internally, in polyphosphate inclusions (PP). For this purpose, we used different cyanobacteria from Pasteur culture collection (*Oscillatoria* sp. PCC 7515, *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313) and *Microcoleus* sp. isolated from Ebro Delta microbial mats. Pb was selected because the Ebro River is polluted by this metal and also because it is a non-essential toxic metal. An inverse correlation between the mean fluorescence intensity (MFI) and the concentration of the metal used have been demonstrated in all phototrophic bacteria tested in CLSM-*λscan*. On the other hand, the SEM-EDX and TEM-EDX analysis showed that all phototrophic microorganisms have the ability to accumulate Pb in EPS and in PP inclusions. Experiments made in unpolluted and polluted microcosms, demonstrated that cyanobacteria from the polluted microcosm accumulate Pb in PP inclusions, whilst no Pb was detected in the unpolluted microcosm by means of TEM-EDX. Finally, the TEM-EDX analyses spectra from PP inclusions of different cyanobacteria from Ebro Delta microbial mats samples, demonstrated that no type of metal pollution was detected. It can be deduced that this ecosystem was pristine during the sampling procedure. In conclusion, the combination of the techniques outlined here provides valuable information to select cyanobacteria as bioindicators of metal pollution and its potential for bioremediation.

## INTRODUCTION

The Ebro Delta, located at the outfall of the Ebro River (Spain), is the second largest wetland in Spain after the Guadalquivir River marshes and the second biggest in the Mediterranean after the Camargue (France). In 1983, some of the most outstanding natural areas of the Delta were included in the Ebro Delta Natural Park (Parc Natural del Delta de l'Ebre) for their biological and cultural significance. The Ebro Delta is the third largest delta in the Mediterranean: a triangular area of 320 $km^2$ and located on the northeastern coastline of the Iberian Peninsula (0°35'E – 0°56'E; 40°33'N – 40°47'N) [1]. Of its total area, 78 $km^2$ corresponds to the Natural Park (25%), 160 $km^2$ to rice farming (50%) and 85 $km^2$ to orchards and fruit (25%). Different ecosystems such as microbial mats, marshes, salt ponds, dunes and sandy beaches provide habitat for a large number flora and fauna species forming part of the Ebro Delta.

Since 1990, our research team has studied the Ebro Delta microbial mats (Figure 1a). These ecosystems, developed in water-sediment interfaces (Figure 1b), are formed by multilayered benthic microbial communities distributed along vertical micro-gradients of different physical-chemical parameters (Figure 1c). Microbial mats are widely distributed around the world in different environments, such as marine waters [2,3], fresh waters [4], hypersaline ponds [5], estuaries [6], hot deserts [7], hot springs [8,9], soils [10], Antarctic ice ponds [11] and hydrothermal vents in deep oceans [12]. Ebro Delta microbial mats are formed by different microorganisms mainly: cyanobacteria, algae, colorless sulfur bacteria, purple sulfur bacteria and sulfate-reducing bacteria. Among these, cyanobacteria are the most abundant and they are located mainly in the upper layers (green layer) of the mats. These microorganisms are phototrophic gram-negative oxygenic bacteria and they have the capacity to assimilate $CO_2$ being their major photosynthetic pigment, chlorophyll-*a* (chl-*a*), as in algae and plants.

In recent years, microbial mats have also become particularly interesting ecosystems due to their bioremediation capacities. Different authors have demonstrated the significant role of

these ecosystems in the bioremediation of oil-polluted coastal areas of the Arabian Gulf, during the 1991 Gulf War, achieving hydrocarbon degradation in few months [13]. The ability of these ecosystems to sequester heavy metals has also been demonstrated [14]. As mentioned above, the Ebro Delta has been declared a protected area for more than 25 years. Despite this, both the River and the Delta are nowadays subjected to anthropogenic pollution, mainly as a consequence of industrial activities that discharge their waste into the River and the agricultural crops (rice and fruit), which increase the amount of pesticides in the Delta. All these circumstances cause serious environmental effects attributable to the accumulation of the contaminants in sediments, water, soil and biota. The predominant pollutants in these habitats are: herbicides, insecticides, hydrocarbons and heavy metals [15].

Another important source of pollution has been the use of Pb pellets in hunting waterfowl [16]. Nowadays, metals are released from natural and anthropogenic sources (e.g. industry, transport, fossil fuel combustion, the mining industry and agriculture) into natural aquatic environments [17]. For many microorganisms some metals are considered essential because they form part of metabolic processes or the active centers in some proteins, whilst others have always a toxic effect [18].

Different techniques have been employed to treat of metal industrial effluents, usually falling into two broad divisions: abiotic and biotic methods. Abiotic methods include precipitation, adsorption, ion exchange, and membrane and electrochemical technologies.

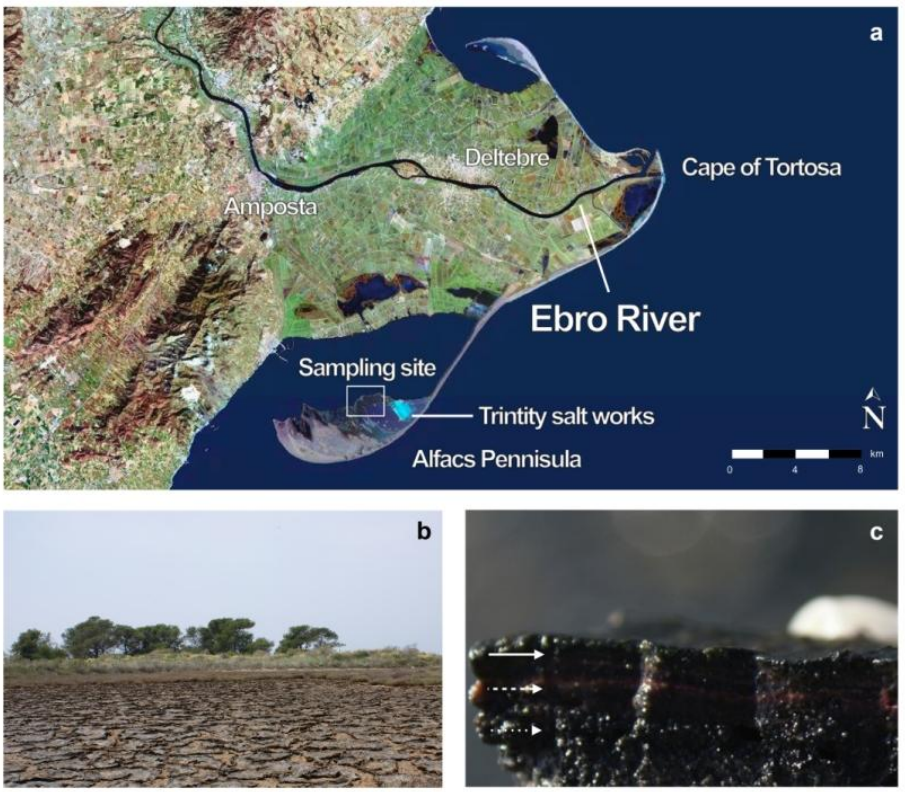


Figure 1. Location of Ebro Delta (a). Ebro Delta microbial mats in a dry season (b). Microbial mat structure: Cyanobacteria, green layer (—); purple reducing bacteria, red layer (----) and sulfate reducing bacteria, black layer (·····) (c).

Regarding biotic methods, there are three main advantages of biological technologies for removing pollutants: biological processes can be carried out *in situ* at the polluted site; bioprocesses are environmentally benign (no secondary pollution is generated) and thirdly, they are cost-effective [19]. Biotic processes are based on the ability of organisms to interact with metals by biosorption, bioaccumulation, biomineralization, bioleaching and enzymatic transformation of metals.

In the face of this problem, studies are being conducted on how heavy metals affect the microbial communities of natural environments [20-25]. On the other hand, many studies have been carried out to demonstrate the capacity of microorganisms to sequester metals [26-30]. Several species of micro and macroorganisms have been used as bioindicators of metal pollution in natural habitats [31-35]. Despite the extensive information mentioned above, nothing is known about the use of phototrophic microorganisms (mainly cyanobacteria) as bioindicators of metal pollution in microbial mats, although they are the most abundant microorganisms in these habitats.

In recent years, our group has optimised high resolution microscopy techniques: Confocal Laser Scanning Microscopy (CLSM) coupled to a spectrofluorometric detector (CLSM-*λscan*), and Scanning Electron Microscopy (SEM) and Transmission Electron microscopy (TEM), both coupled to an energy dispersive X-ray detector (EDX).

CSLM has proven to be a fast and efficient method by which *in situ* observations are performed on a cellular level and without manipulation of the samples to determine the effect of Pb on different photosynthetic microorganisms. This is an outstanding optical microscopy technique because allows accurate and non-destructive optical sectioning giving high-resolution images (optical sections) where out-of-focus views are eliminated. It is also a very interesting technique for observing phototrophic microorganisms (algae and cyanobacteria) because they emit natural fluorescence and staining protocols are not required for this kind of sample, avoiding the need to use of long and exhaustive protocols. By means of this methodology it is easy to differentiate morphotypes of phototrophic microorganisms living in mixed populations. CLSM has made it possible to characterize cyanobacteria in Ebro Delta microbial mats and determine the biomass of different types of cyanobacteria in these habitats at micrometer scale [36,37,38]. On the other hand, a spectrofluorometic detector (*λscan* function) coupled to the microscope allows us to determine the effect of a metal at photosynthetic pigment level [(chl-*a* and phycoerythrin (PE)]and provides an *in vivo* analysis considering the state of the pigments performed in individual cells without manipulating the sample.

On the other hand, SEM and TEM both coupled to EDX have been used to determine the capacity of cyanobacteria to sequester Pb [39,40]. EDX is a technique used for the elemental analysis of a sample. The technique is based on the analysis of X-rays emitted by matter in response to collisions with charged particles. The number and energy of emitted X-rays in a sample can be measured semi-quantitatively by an energy dispersive spectrometer. This detector that may be coupled to both a SEM and a TEM, is connected to a computer with the software INCA v.4.13 (Oxford Instruments, Bucks, England) that generates graphics with different peaks corresponding to each of the chemical elements present in the analized area. Usually the analysis of a sample area takes 60 s. The great advantage of this approach is that the area to be analyzed can be selected by the user.

The aims of this chapter are to summarize the results previously obtained using these methodologies, while new experiments were also performed to determine the efficacy of

these techniques when they are applied both to artificial laboratory ecosystems (microcosms) and to samples that have been directly obtained from the natural environment, including environments polluted with hydrocarbons that can contain heavy metals.

## MATERIAL AND METHODS

### Cyanobacteria and Culture Conditions

For this chapter, different microorganisms have been selected: a) *Microcoleus* sp. isolated from a consortium of microorganisms of Ebro Delta microbial mats, and b) three cyanobacteria (*Oscillatoria* sp. PCC 7515, *Chroococcu*s sp. 9106 and *Spirulina* sp. PCC 6313) chosen from the Pasteur culture collection of cyanobacteria (PCC). *Microcoleus* sp. cultures from Ebro Delta microbial mats were cultivated and maintained in Pfennig mineral medium [41] *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313 strains were grown in BG-11 and ASN III (1:1 v/v) medium [42] and *Oscillatoria* sp. PCC 7515 strain was maintained in MN medium with nitrate ommited [42].

All microorganisms were mantained at 27$^{o}$C and under light conditions (15 μE m-2 s-1). Pb solutions were prepared with $Pb(NO_3)_2$ (Merck KGaA, Darmstadt, Germany) in deionized water and sterilized by filtration in Millex GP 0.22 μm filters (Millipore, USA). Diferent concentrations of Pb from 0.1 to 25 mM, were used in the polluted experiments. All experiments were performed for 9 days in the same above-mentioned culture conditions.

### Microcosms and Natural Environment

Microbial mat samples were collected from the Ebro Delta on October 2009. Samples were taken in 55 mm × 43 mm × 88 mm poly(methyl methacrylate) boxes and transferred to the laboratory. Two microcosm experiments were prepared, one microcosm was used as a control experiment (unpolluted) and the other microcosm was polluted with 0.5 mM Pb solution. The microcosms were maintained in laboratory conditions for 9 days, after which the metal solution was removed from the polluted microcosm. On the other hand, samples direclty taken from Ebro Delta microbial mats were analyzed by TEM-EDX. The protocol is described in the transmission electron microscopy section.

### Confocal Laser Scanning Microscopy

CLSM (Leica TCS SP5; Leica Heidelberg, Germany) was used in order to determine the sensitivity of cyanobacteria to Pb by using the *λscan* function (CLSM-*λscan*). Each image sequence was obtained by scanning the same *xy* optical section throughout the visible spectrum. Images were acquired at the *z* position at which the fluorescence was maximal, and acquisition settings were constant throughout the experiment for all microorganisms studied. The sample excitation was carried out with an Argon Laser at 488 nm ($\lambda_{exe}$ 488) with a step size of between 3 and 4.74 nm for an emission wavelength between 500 and 800 nm. In order

to measure the mean fluorescence intensity (MFI) of the *xy*, CLSM data sets obtained by means of the Leica Confocal Software (Leica Microsystems CMS GmbH) were used. The regions-of-interest (ROIs) function of the software was used to measure the spectral signature. For each sample, 30-70 ROIs of 1 $\mu m^2$ taken from cyanobacteria were analysed.

## Scanning Electron Microscopy

SEM coupled to EDX was used in order to determine the extracellular sequestration of Pb by cyanobacteria. Samples were filtrated in Nucleopore™ polycarbonate membranes (Whatman, Ltd.) and then were fixed in 2.5% glutaraldehyde Millonig's buffer phosphate [43] (0.1M pH 4) for 2 hours and washed four times (15 min) in the same buffer to remove excess of fixative. They were then dehydrated in a graded series (30%, 50%, 70%, 90%, and 100%) of ethanol (5 min) and dried by critical-point (CPD 030 Critical Point Drier, BAL-TEC GmbH D - 58579 Schalksmühle).

Samples were mounted on aluminum metal stubs using a electrically-conductive double-sided adhesive tape and then coated with a 5 μm gold layer (K550 Sputter Coater, Emitech, Ashford, UK) Finally, samples were viewed in a Jeol JSM-6300 (Jeol Ltd., Tokyo, Japan) and in a Zeiss EVO® MA 10 (Carl Zeiss NTS GmbH, Oberkochen, Germany) scanning electron microscopes. An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to the microscopes was used.

## Transmission Electron Microscopy

TEM coulpled to EDX was used in order to analyze the capacity to acumulate Pb intracelullarly in cyanobacteria and to assay if samples directly obtained from mats were pristine or polluted by heavy metals.

Samples were fixed in 2.5% glutaraldehyde Millonig's buffer phosphate [43] (0.1M pH 4) for 2 hours and washed four times (15 min) in the same buffer. Then samples were post-fixed in 1% osmium tetroxide ($OsO_4$) for 2 hours, and washed four times in the same buffer. Then samples were centrifuged in order to obtain a pellet.

They were then dehydrated in graded series (30%, 50%, 70%, 90% and 100%) of acetone and embedded in Spurr resin. Once the samples were included in the resin, a piramidotome was used (TM 60, C. Reichert AG. Wien, Austria) for pyramiding samples and an ultramicrotome (Leica EM UC6 ULTRACUT, Leica Microsystems, GmbH, Heidelberg, Germany) for the ultrathin sections.

To show a better quality image, sections of 70 nm of the samples were mounted on carbon-coated copper grids and stained with uranil acetate and lead citrate according to the method described by [44].

Samples were viewed in a Hitachi H-7000 electron microscope (Hitachi Ltd., Tokyo, Japan). An EDX Link Isis-200 (Oxford Instruments, Bucks, England) operated at 20 kV coupled to the microscope was used.

For TEM-EDX analysis the sections were not stained with lead citrate to avoid elemental substitution during the analysis.

## RESULTS AND DISCUSSION

The aim of this chapter as previously mentioned, has been to use CLSM and electron microscopy techniques to determine how different photrophic microorganisms are affected by metals, as well as their capacity to capture them. Moreover, to prove the effiency of these techniques when they are applied in natural habitats. The methodological optimization of the techniques used and the results obtained are described as follows.

### Methodological Optimization

In the experiments carried out with CLSM-*λscan* to determine Pb sensitivity in photosynthetic microorganisms, the protocol described and optimized by [39] has been followed.

In this chapter, the methodological optimization has basically focused on analysis by EDX coupled with SEM and TEM. The EDX is a technique used for the elemental analysis of a sample. The technique is based on the analysis of X-rays emitted by matter in response to collisions with charged particles coming from an energy beam of the electron microscope. The number and energy of emitted X-rays in a sample can be measured semi-quantitatively by an energy dispersive spectrometer that can be coupled to both SEM and TEM. Concerning EDX, one of the main disadvantages of its use is the possible interaction between metals that may occur at protocol and at sample observation level.

In this case, the concentration of the metal tested, the staining used and the metallization of the samples has been considered. Although in this chapter Pb has been used (as described above), the methodological optimization has been done for other metals, with the aim that these techniques could be used for any type of environmental pollution.

The protocols used to prepare the ultrathin section for TEM include solutions that dye the samples, providing contrast. The most commonly used are uranyl, phosphotungstic acid and lead citrate. The use of lead citrate has therefore been ruled out for evaluating Pb capture both in intracytoplasmic inclusions and in the cytoplasm of the microorganisms, to prevent interference with this metal.

Concerning the preparation of samples for SEM, in order to obtain good contrast with them, metallizing is carried out with a noble metal. Whether the metal used for metallizing the samples, has an energy peak in the element spectrum overlapping the metal to be evaluated has been taken into account. This is why Au has been used, as its energy peak does not overlap either with Pb.

Support grids have also been considered. Given the importance given to the selection of support grids, we also sought to evaluate their composition, considering those that are most frequently used: Cu, Au, Ni and Ti both for SEM and for TEM (Figures 2 and 3).

In the results obtained by SEM, all of the grids only presented the metal peak corresponding to the type of grid, except for Au, which also contains Cu. In this case, this grid must be discarded for studies of Cu pollution. Meanwhile, if the same grids are analysed by TEM, they all present pollution by Cr and Cu, which in this case is attributed to the microscope's specimen holder and they should therefore be discarded in studies of Cr and Cu pollution.

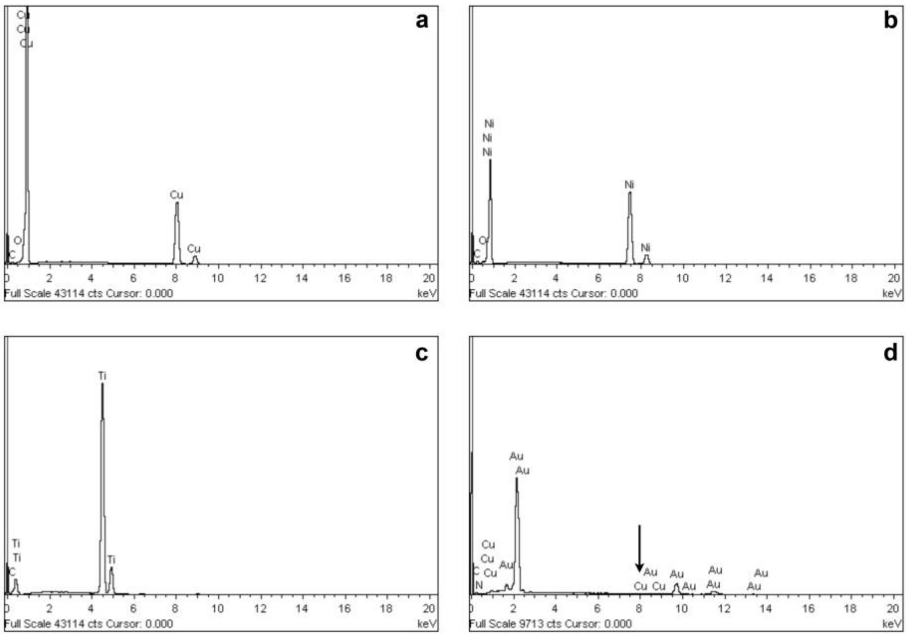


Figure 2. EDX spectra obtained by SEM from Cu (a), Ni (b), Ti (c) and Au (d) grids. Cu peak is indicated by arrow.

In this chapter Pb has selected, therefore any of the grids mentioned above could be used.

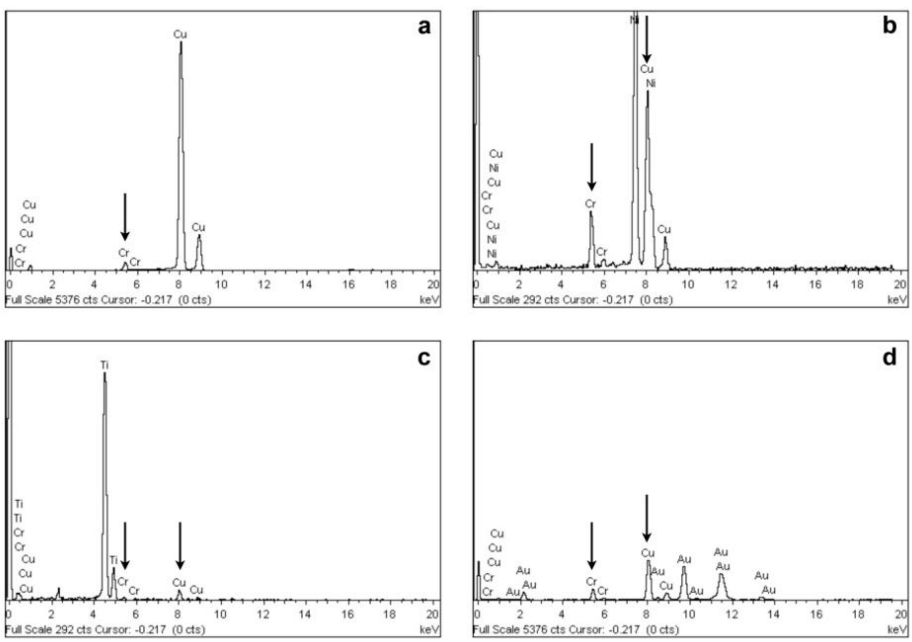


Figure 3. EDX spectra obtained by TEM from Cu (a), Ni (b), Ti (c) and Au (d) grids. Cu and Cr peaks are indicated by arrows.

## Determination of the Effect of Pb (Tolerance-Resistance) on Cyanobacteria Using CLSM-*λscan*

Recently, we have determined the tolerance-resistance of the different cyanobacteria to Pb by means CLSM-*λscan* [39,40]. The state of pigments was considered by means of the maximum fluorescence signal detected at: 575nm (PE) for *Oscillatoria* sp. PCC 7515 [45]; 680 nm (chl-*a*) for *Chroococcus* sp. PCC 9106, 652 nm (chl-*a*) for *Spirulina* sp. PCC 6313 and 655 nm (chl-*a*) for *Microcoleus* sp.

The results obtained demonstrate that in all cases there is an inverse correlation between the mean fluorescence intesity (MFI) and the concentration of the metal used. The minimum metal concentration of Pb with a toxic effect on cyanobacteria were: 0.1 mM for *Oscillatoria* sp. PCC 7515 and *Spirulina* sp. PCC 6313 and 0.5 mM for *Chroococcus* sp. PCC 9106 [40]. In *Microcoleus* sp. the most abundant cyanobacteria in the Ebro Delta microbial mats, the minimun metal concentration with toxic effect was 0.75 mM, these results are shown in Figure 4 . These results demonstrate that *Microcoleus* sp. show greater tolerance to Pb than the other cyanobacteria tested. On the other hand, pigments were degraded when *Microcoleus* sp. cultures were polluted with Pb at the maximum doses used (25 mM) [39]. The same effect was observed in *Chroococcus* sp. PCC 9106 and *Spirulina* sp. PCC 6313 [40].

Moreover, we have assayed the sensitivity of this technique and the results demonstrate that the CLSM-*λscan* is very sensitive because detect changes in in fluorescence intensity at 100 nM (Seder-Colomina et al., in prep.).

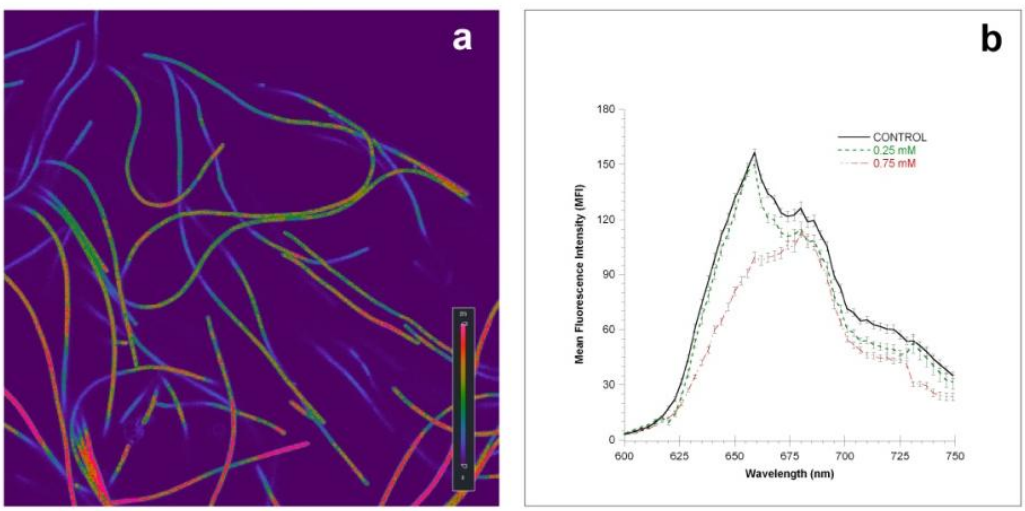


Figure 4. CLSM image from a *Microcoleus* sp. unpolluted culture (a). In this confocal image, the pseudo-colour palette 4 (Leica Aplication Suite, Leica Microsystems CMS GmbH) was used, where warm colours represent the maximum intensities and cold colours represent the low intensities of fluorescence. *λscans* plot from *Microcoleus* sp. cultures polluted with different Pb concentrations (b). 2D plot represent the MFI spectra: emission wavelength, *x* axis; MFI, *y* axis.

## Sequestration of Pb by Cyanobacteria Growing in Cultures by SEM-EDX and TEM-EDX

The capacity of different microorganisms to accumulate Pb both externally in the EPS and internally in intracitoplasmatic inclusions was also determined [39, 40].

### *Extracellular Sequestration of Pb*

All cyanobacteria studied can accumulate Pb in extracellular polymeric substances (EPS) envelopes. Figure 5 shows images of the Pb sequestration in the EPS by the elemental analysis spectra by the dominant cyanobacteria in the Ebro Delta: *Oscillatoria* PCC 7515 (Figure 5b) and *Microcoleus* sp.(Figure 5c).

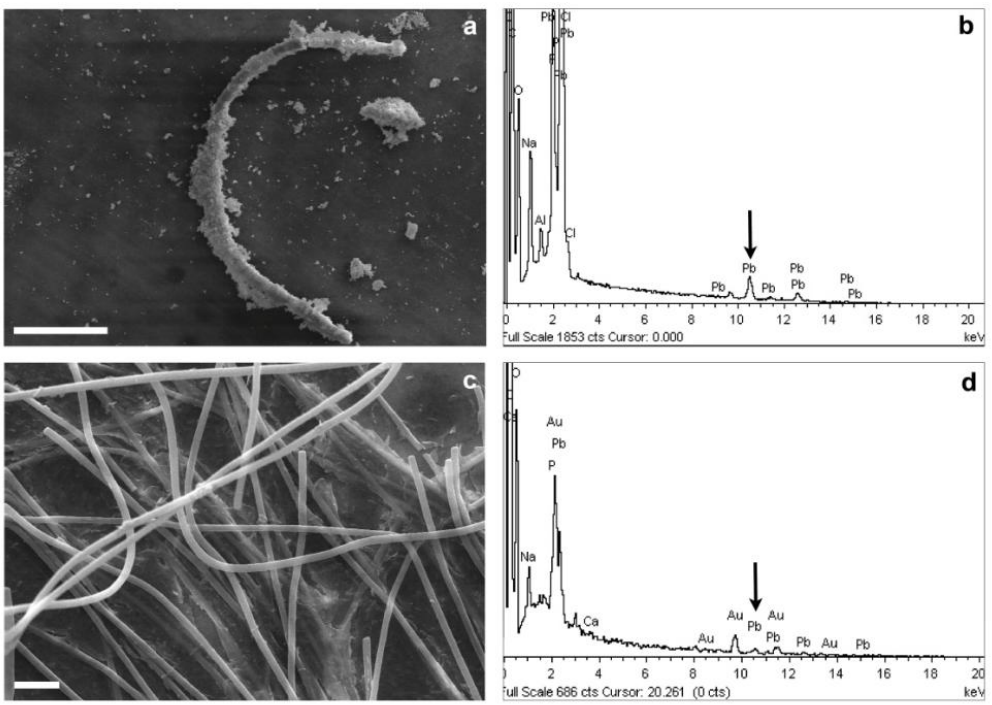


Figure 5. Lead polluted cultures of *Oscillatoria* sp. PCC 7515 (a) and *Microcoleus* sp. (c) by means of SEM-EDX. Scale bars represent 100 μm and 10 μm, respectively. Pb peak is shown in the spectrum obtained by SEM-EDX in *Oscillatoria* sp. PCC 7515 (b) and *Microcoleus* sp. (d).

As has already been said, the most abundant microorganisms in microbial mats are cyanobacteria and the majority of these microorganisms show EPS envelopes that can be present in the form of sheaths or slimes. With respect to the functions the EPS may have, various authors have suggested protection against dehydration and UV radiation, biomineralization, phagocytosis, adhesion capacity to the surrounding substrate and capacity to sequester metals [30]. The latter is also observed in this study.

A tendency to increase the thickness of the EPS in a directly proportional way to the concentration of the metal tested has been observed through the images obtained by SEM and TEM. These results are in agree with those obtained in microbial mats by other authors [46].

***Intracellular Sequestration of Pb***

Ultrathin sections of all the cyanobacteria studied show that these microorganisms can accumulate Pb in PP inclusions. In Figure 6 results obtained from *Oscillatoria* sp. PCC 7515 (Figures 6a and 6b) and *Microcoleus* sp. (Figures 6c and 6d) grown both in microcrocosms are shown. Both cyanobacteria accumulate Pb in said intracitoplasmatic inclusions as shown in the EDX spectral. It has been shown using the EDX spectra that these inclusions are PP because they contain P and also divalent cations of Ca and/or K. The accumulation of metals in these inclusions has also been demonstrated in other microorganisms: algae [47], yeasts [48] and some cyanobacteria [49, 50, 30].

It has been demonstrated that these inclusions are formed due to some kind of stress or the presence of pollutants [51]. The sensitivity of this technique by SEM-EDX and TEM-EDX is 0.1 µM (M. Seder-Colomina et al., in. prep.).

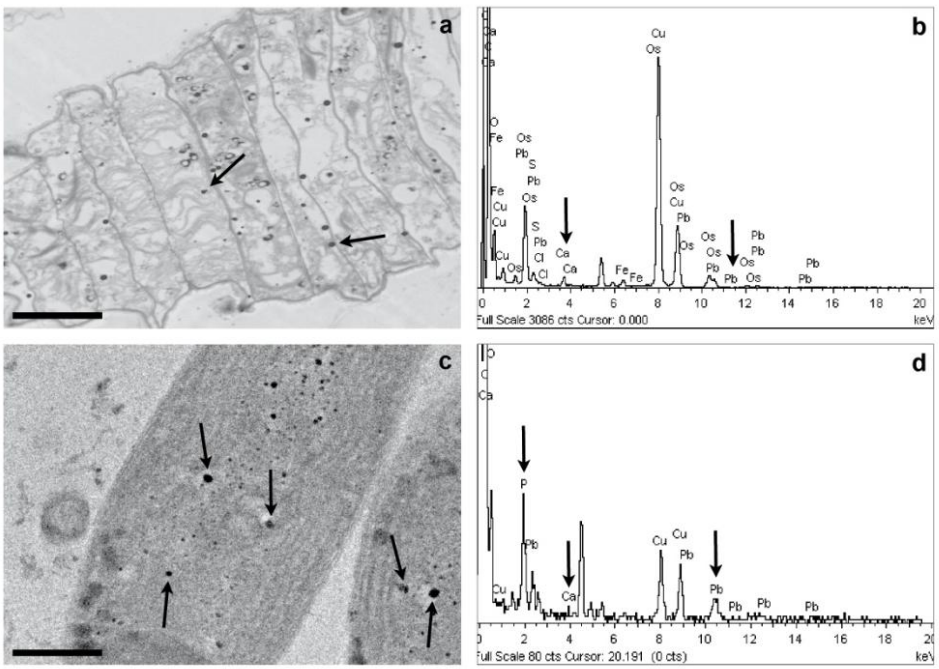


Figure 6. Lead polluted cultures of *Oscillatoria* sp. PCC 7515 (a) and *Microcoleus* sp. (c) by means of TEM-EDX. High electron-dense inclusions from an ultrathin section of *Oscillatoria* sp. PCC 7515 and *Microcoleus* sp. are indicated by arrows. Scale bars represent 1 µm and 0.5 µm, respectively. Spectra from high electron-dense inclusions from *Oscillatoria* sp. PCC 7515 and *Microcoleus* sp. show Pb, P and Ca peaks (arrows).

## Cyanobacteria As Indicators of Pb Pollution

The results obtained in the previous sections lead to the conclusion that all of the essayed cyanobacteria, both those from collection cultures and those from cultures of microorganisms isolated from the natural environment, have the capacity to accumulate Pb extra and intracellularly and especially in the latter case in PP inclusions. The main objective of this

chapter has focused on analysing whether this capacity can also be demonstrated in microcosms and in samples obtained directly from the natural environment.

To do this, as described in the Materials and Methods, samples were taken from the natural environment and two microcosms were prepared, one without pollution and the other polluted with 0.5 mM Pb. Both the results in Figures 7a and 7b corresponding to images obtained using SEM-EDX and those in Figs. 8a and 8b corresponding to images obtained using TEM-EDX show that no Pb was detected in the spectral analysis of the unpolluted microcosm; while in the polluted microcosms Pb sequestration was observed both in EPS (Figures 8c and 8d) and in PP inclusions (Figures 8c and 8d) for the dominant cyanobacterium, in this case *Phormidium*-like.

Meanwhile, as these ecosystems are often also found to be polluted with hydrocarbons and these can contain metals, new experiments were performed by polluting *Microcoleus* sp. cultures (from natural samples) with Maya oil (a sulphur oil).

The aim was to show the reliability of the technique. No Pb was detected in the control experiment with *Microcoleus* sp. (Figure 9a and 9b), while the presence of this metal was observed in the PP inclusions of this cyanobacterium in the polluted culture (Figures 9c and 9d), which shows the mentioned oil contained Pb and that the microorganism was equally able to collect it.

Finally, analyses were made of samples taken directly from the microbial mats of the Ebro Delta using TEM-EDX, with *Microcoleus* sp. selected as the indicative cyanobacterium. The results shown in Figure 10 reveal that no type of metal pollution was detected in the PP inclusions, so in this case the ecosystem can be considered unpolluted by Pb.

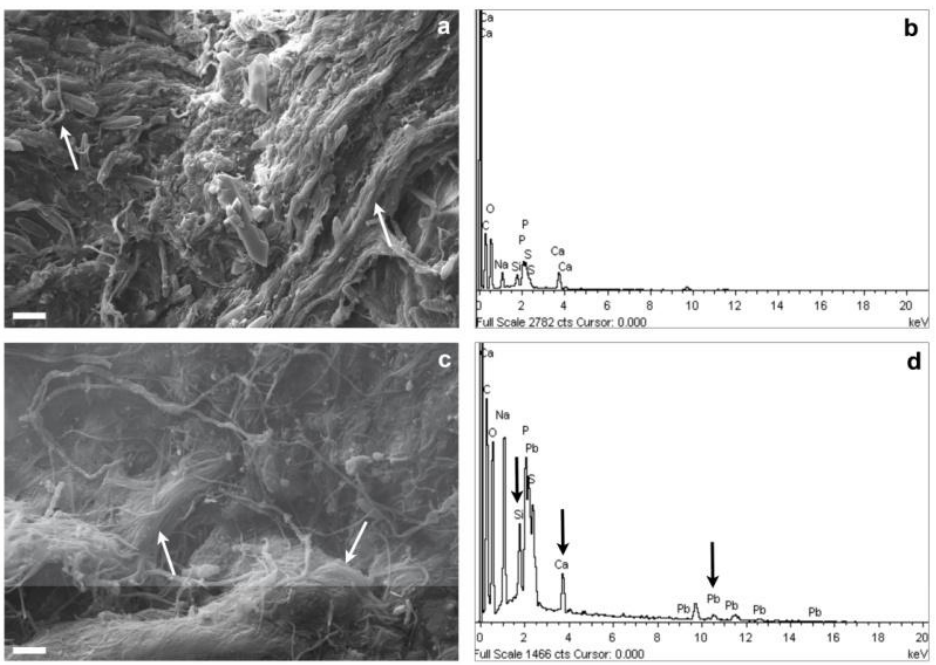


Figure 7. Samples from microcosms unpolluted (a) and polluted with Pb (c) by means of SEM-EDX. Analyses points are indicated by arrows. Scale bars represent 20 μm. Spectra from unpolluted (b) and polluted samples (d). The latter indicating the Pb peaks (arrow).

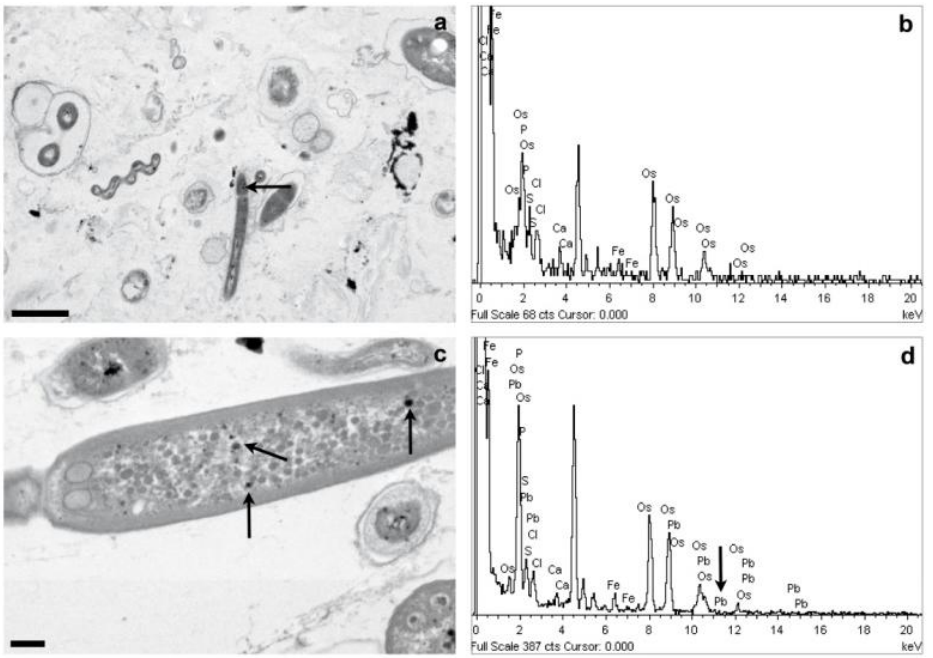


Figure 8. Samples from microcosms unpolluted (a) and polluted with Pb (c) by means of TEM-EDX. Analyses points from high electron-dense inclusions of *Phormidium*-like are indicated by arrows. Scale bars represent 1 and 0.2 μm, respectively. Spectra from unpolluted (b) and polluted samples (d). The latter indicating the Pb peak (arrow).

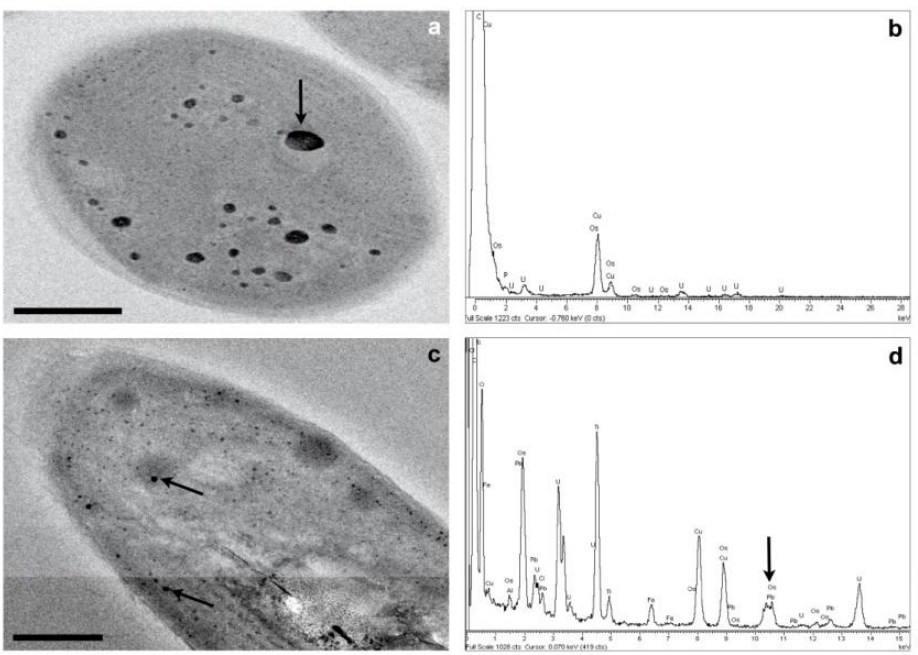


Figure 9. Ultrathin sections of unpolluted cultures of *Microcoleus* sp. obtained from Ebro Delta microbial mats (a) and oil polluted cultures (c) showing a peak of lead in the PP inclusions indicated by arrows (d). Scale bars represent 0.5 μm.

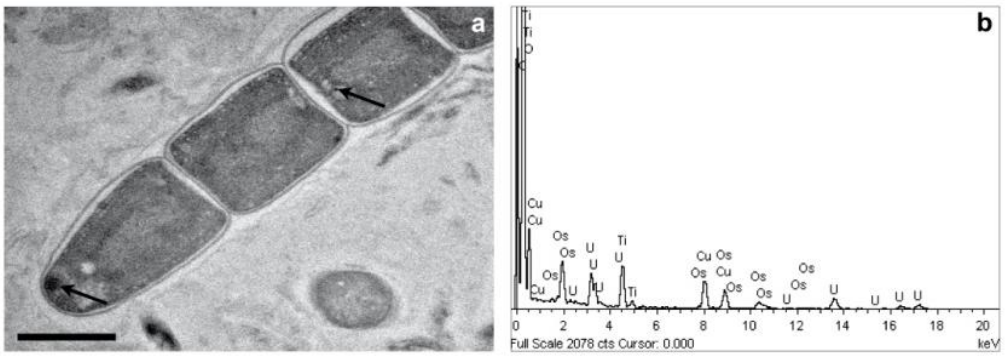


Figure 10. Ultrathin sections of samples obtained directly from microbial mats. PP inclusion analysis does not detect heavy metals, indicating that these mats are unpolluted by Pb. The spectrum also shows the presence of traces of Os and Ur used in the sample staining process, Cu corresponding to the microscope's specimen holder, Ti corresponding to the grid and Si, probably due to the presence of diatoms.

## CONCLUSION

The results obtained in this chapter lead to the conclusion that CLSM-*λscan* is a very useful technique for quickly determining the tolerance-resistance of phototrophic microorganisms in vivo, on a cellular level and without manipulation of the samples. Meanwhile, SEM and TEM coupled to EDX enable the determination of the capacity of phototrophic microorganisms to accumulate Pb extracellularly and intracellularly.

Finally, to summarise, all cyanobacteria can be considered good bioindicators of Pb pollution as they are autochthonous in the natural environment, present high biomass, tolerate high Pb concentrations and are capable of capturing it.

## ACKNOWLEDGMENTS

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*Chapter 10*

# A NEW METHODOLOGY FOR RAPID ASSESSMENT OF SPATIAL DISTRIBUTION OF PHYTOPLANKTON BLOOMS: CASE STUDY IN PAMPULHA RESERVOIR

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## ABSTRACT

Hypertrophic lakes and reservoirs frequently suffer from algal and cyanobacterial blooms. In general, this phenomenon causes a rapid decrease of water quality. Traditional monitoring programs of these systems usually cover only a limited number of sampling points. Furthermore, counting of phytoplankton or laboratory analysis of chlorophyll require time-consuming procedures. Thus, local managers have access to the required information only after the undesirable effects of a bloom have become established. Since these lakes are usually located near urban areas, algae blooms in hypertrophic lakes are prone to affect local human populations in different ways. Thus, the prompt availability of this kind of data is of great importance for managers. We present a new methodology for monitoring chlorophyll-a that makes it possible to gather a very large and fine-scale amount of spatial data concerning the subsurface concentrations of this pigment. The proposed method consists of an integrated use of a highly sensitive fluorescent limnological probe, coupled to a high-precision D-GPS that provides geographical coordinates with sub-metric precision. We developed a structure that can be easily mounted on every kind of small boat. A new software program was also created, to

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precisely synchronize the data files delivered by two different devices. The final result is the production of a detailed thematic chart that shows the spatial pattern of chlorophyll in great detail. This makes it possible for local managers to initiate measures to mitigate or even prevent the wider spread of an algae bloom soon after the first signs of this undesired phenomenon are detected.

## INTRODUCTION

Eutrophication is one of the most important human impacts affecting freshwater ecosystems around the globe. Usually, eutrophication is a result of uncontrolled inputs of external nutrient load (mainly N and P), associated not only with point sources of nutrient inputs but also with disseminated runoff of subterranean waters carrying higher contents of phosphorus, nitrogen (mainly as nitrate) and high concentrations of organic matter. Eutrophication causes a series of well-known ecological effects including increases in the density and biomass of autotrophic organisms, and wide fluctuations of oxygen and pH levels [1,2,3]. Monitoring of freshwater resources affected by eutrophication usually includes a varying array of physical, chemical and biological variables, but most eutrophication indexes are based on a very limited number of variables such as water transparency, phosphorus and chlorophyll-*a* [4].

A recent literature review [5] suggested that there is a scarcity of investigations focused on spatial patterns of eutrophication that support the measurements of these variables, with more detailed biological data. These biological variables include, besides chlorophyll-a, density, biovolume or biomass of bacteria, phytoplankton, zooplankton and fish. The lack of information about spatial patterns and eutrophication results not only from the time-consuming nature of these analyses, but also from the considerable effort required to process and clearly understand all this biological information. Zooplankton, for instance, can show contrasting responses to eutrophication depending on a series of local and regional abiotic and biotic ecological factors [6]. Nevertheless, the comprehension of an ecosystem's functioning and, eventually, the development of the ability to control it depends, to a large extent, on this biological knowledge. The basic problem in ecology is to determine the causes of distribution and abundance of organisms in space and time [7]. Thus, the study of spatial variations of organisms is a central topic not only for the science of limnology, but also for ecology.

Reservoirs are artificial lakes that are distinguished by more intense hydrodynamics and also by constant and intense rates of changes of material and processes with the surrounding terrestrial ecosystems. As a consequence, the spatial patterns of biological variables in these systems often reflect external inputs of materials or energy from the catchment areas.

Phytoplankton populations are typically estimated by measuring chlorophylla-*a*, the primary photosynthetic pigment present in all forms of algae. Currently, chlorophyll-*a* can be estimated by several different methods. The most common methods are all derived from the classical paper of Lorenzen [8]. They are based upon extraction of cell pigments by using an organic solvent followed by spectrophotometric determination of the absorbance at a specific wavelength. This method (and its variations) usually requires time-consuming laboratory procedures. The pigment content can be also estimated by measuring the fluorescence of chlorophyll. This is a phenomenon of some algal producers, which are able to absorb specific wavelengths of light and almost instantaneously emit longer wavelengths of light.

Chlorophyll-*a* naturally absorbs blue light and emits red light. Fluorometers detect chlorophyll by transmitting an excitation beam of light in the 440 nm (blue) range and by detecting the light emitted by the sample in the 680 nm (red) range. Recent advances in water-monitoring techniques are opening new possibilities for the limnologists [9,10,11]. The appearance of a whole generation of multi-probe sondes makes it possible to increase enormously not only the amount of data collected and the accuracy of each single measurement, but also the velocity of data acquisition. These sondes can be programmed to record data at specific dates and depths. The operator can download the data directly onto a laptop computer immediately following the sampling. In some cases, a telemetry kit can be installed on a small floating platform, which is usually placed at the deepest point of a lake. In some cases, the sonde is able to descend and collect information at several points, covering the entire water column of a lake or reservoir. Some of these sondes can be equipped with sensors able to read the in situ fluorescence of planktonic autotrophs [10].

Reservoirs typically exhibit clear and well-defined horizontal and vertical gradients [12,13]. This issue has been addressed by a series of surveys, which usually cover a set of 5-30 different sampling points distributed over the entire lake surface [5,14,15,16,17]. Some investigators have advised, however, that the number of sampling points must be much larger than the set of 5-30 sampling points that are usually taken into consideration [18]. Increasing the number of sampling points will make the data set more representative of the spatial patterns usually present in a reservoir.

The great advantage of the use of these new methodologies for water-quality monitoring is the possibility to acquire large amounts of basic information in a rapid and efficient way. The possibility to use this new kind of information together with conventional data or in computer models, or even using other methodological approaches (i.e., hydrodynamics) makes it possible to perform complex analyses of spatial and temporal data. This obviously opens new perspectives for the advancement of the entire science of limnology.

Since the SCUFA sonde has a fluorescent sensor that can measure chlorophyll-*a* in situ, we maximized the data acquisition of this device by developing a geo-referenced continuous data acquisition procedure. This chapter describes a new system, which consists of coupling a fluorescent SCUFA sonde (Turner, Inc.) directly to a mounting kit that can be adjusted on the side of a small aluminum boat. On the top of this structure, a D-GPS was mounted.

We also compare the data delivered by this system with the data gathered by means of a conventional monitoring program, based on 23 samples collected at different points, covering all the basic compartments of a reservoir. We compare the two methodologies for estimating the spatial pattern of distribution of chlorophyll-*a*. We also provide detailed information about phytoplankton biovolume, with special emphasis on cyanobacteria populations.

## METHODOLOGY

### Study Area

Pampulha Reservoir was constructed in 1938, with the basic purpose of supplying drinking water for the city of Belo Horizonte, the capital of Minas Gerais state ($19^{o}55´09´´S$, $43^{o}56´47´´W$). The continued expansion of the city of Belo Horizonte during the 1960s and

70s rapidly erased the picturesque atmosphere of the lake in the "golden" years of the 1940s and 50s. Two major phenomena, silting and eutrophication, contributed to the worsening of the ecological health of the ecosystem.

Initially, Pampulha Reservoir had a surface area of 2.1 $km^2$ and a volume of 12 million $m^3$. The silting process led to a reduction of more than 15% in lake area and a 23% decrease in lake volume [19,20].

As a result, the reservoir presently has a total surface of only 1.8 $km^2$ and a volume of 9.2 million $m^3$ [21]. The maximum depth decreased from 17.0 to 15.1 m, and the mean depth is now 4.98 m.

Biweekly sampling from 1993 through 1996 revealed steady increases in conductivity, nitrogen, phosphorus and chlorophyll-*a*, and reduction of water transparency (these trends were determined after data treatment that removed the effects of seasonal fluctuations). During those years, and thereafter, recurrent cyanobacteria blooms and outbreaks of the macrophyte *Eichhornia crassipes* were also regularly observed in the lake [22].

The Pampulha ecosystem may be one of the most-studied manmade lakes in Brazil. Several important scientific contributions have been published on this artificial lake, many of them with special emphasis on cyanobacteria [23,24,25,26].

## Data Sampling

A previous investigation examined the basic temporal patterns of seasonal evolution of water quality in this reservoir [27]. This study revealed a clear seasonal pattern characterized by higher nutrient levels (and biomass of producers) during the dry season, which usually extends from May to September.

During the rainy season (the annual rainfall of about 1500 mm is primarily concentrated in the December-February period), the heavy rains lead to large inputs of suspended solids, which cause severe reductions in the density and biomass of all planktonic organisms.

The data used in the present investigation were obtained from two sampling campaigns covering the entire lake, during the dry season, in September 2009 and May 2011. The coordinates and depths of all sampling points on both occasions are listed in Table 1 and the positions of all sampling points in the two campaigns are represented in Figure 1.

In both sample series, a detailed inventory of the water quality was carried out. The following variables were measured in both periods: depth, water temperature, transparency, conductivity, dissolved oxygen, pH, chlorophyll-*a* (Lorenzen method), turbidity, suspended solids, inorganic nitrogen (nitrate, nitrite and ammonium), total nitrogen and total phosphorus.

In May 2011, we programmed an intensive survey aiming to test a new methodology to investigate the subsurface concentrations of chlorophyll-*a* in this lake. A SCUFA Submersible Fluorometer was used for the in situ and in vivo measurements of chlorophyll-*a*. Since we wanted to obtain a spatial pattern of the measurements, we mounted the SCUFA sonde on the side of a small boat. On the top of the same stack where the SCUFA sonde was attached, a D-GPS antenna (Tech Geo, Ltda.) was firmly mounted. This arrangement allowed us to obtain geographical coordinates with high precision, at the same position where the SCUFA sonde was mounted (Figure 2)

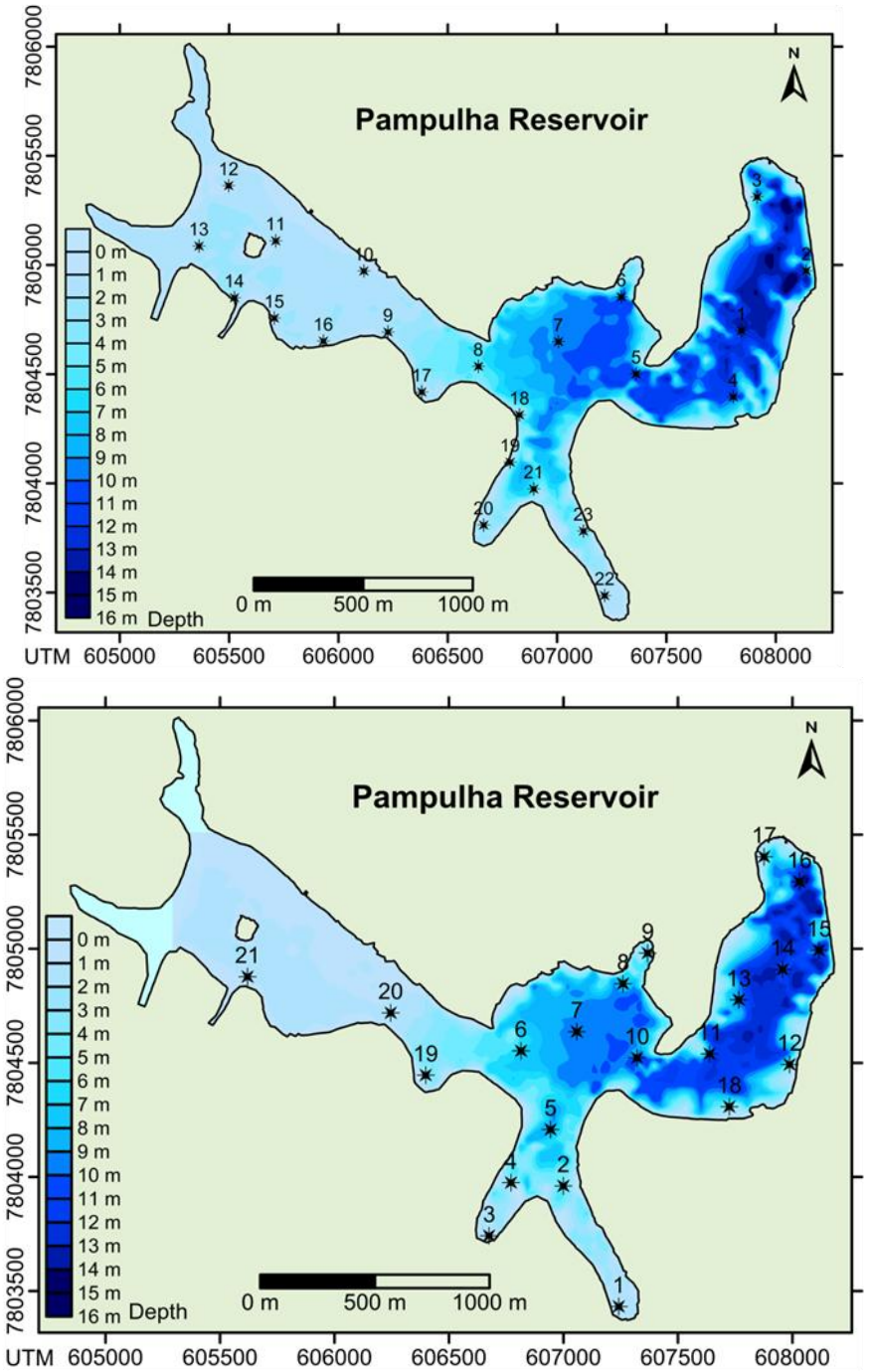


Figure 1. Sampling points used in the study. In 2009 (Top graph), the samples were taken between 11:00 and 17:00 hs, on September 15. In 2011, the samples were taken between 13:29 and 15:55 hs, on May 23 (Bottom graph). In the 2009 sampling, besides measuring physical and chemical variables, we focused on the spatial distribution of phytoplankton and zooplankton. A M.Sc. dissertation was completely devoted to the detailed analysis of this campaign [28].

**Table 1. Water quality variables measured on 15 September 2009 in Pampulha Reservoir, Belo Horizonte, MG**

| Point | Max. depth (m) | Secchi (m) | Temp. (°C) | Cond. (µS $cm^{-1}$) | D.O. (mg $L^{-1}$) | Chl-a (µg $L^{-1}$) | Turbidity (NTU) | Susp. solids (mg $L^{-1}$) | $NO_2$ (µg $L^{-1}$) | $NO_3$ (µg $L^{-1}$) | $NH_4$ (µg $L^{-1}$) | DIN (µg $L^{-1}$) | NIT (µg $L^{-1}$) | PT (µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 14.0 | 0.8 | 22.7 | 399.6 | 6.5 | 51.2 | 37.6 | 53.9 | 686 | 9957.1 | 3561 | 14204 | 4699 | 159 |
| 2 | 9.0 | 0.8 | 23.3 | 385 | 14.5 | 49.5 | 31.8 | 21.9 | 686 | 10067.4 | 3733 | 14486.4 | 1055 | 116 |
| 3 | 8.0 | 1.0 | 23.9 | 385.3 | 7.1 | 61.2 | 56.2 | 40.7 | 725 | 6485.7 | 3585 | 10795.7 | 2603 | 186 |
| 4 | 9.0 | 0.5 | 24.1 | 385.2 | 5.3 | 38.7 | 24.8 | 19.7 | 686 | 8916.8 | 2016 | 11618.8 | 5054 | 126 |
| 5 | 11.0 | 0.5 | 24.6 | 392.5 | 9.1 | 43.7 | 84.7 | 36.9 | 776 | 9539.4 | 1718 | 12033.4 | 3630 | 228 |
| 6 | 10.0 | 0.5 | 23.6 | 386.1 | 5.3 | 47.1 | 129 | 50.7 | 647 | 6213.8 | 3402 | 10262.8 | 2926 | 267 |
| 7 | 9.5 | 0.5 | 25.0 | 391.8 | 8.5 | 53.2 | 104 | 48.3 | 647 | 6666.9 | 1863 | 9176.9 | 6117 | 248 |
| 8 | 6.0 | 0.5 | 25.7 | 396.6 | 8.7 | 55.7 | 130 | 58.2 | 647 | 6308.3 | 1911 | 8866.3 | 10521 | 274 |
| 9 | 1.5 | 0.3 | 25.9 | 403 | 6.3 | 56.6 | 227 | 104.6 | 595 | 2207 | 2040 | 4842 | 238 | 386 |
| 10 | 1.0 | 0.3 | 26.6 | 424 | 5.5 | 41.9 | 175 | 71.4 | 482 | 1364.7 | 4454 | 6300.7 | 4142 | 406 |
| 11 | 1.5 | 0.3 | 26.8 | 462.6 | 6.2 | 43.4 | 179 | 68.4 | 97 | 175.2 | 2093 | 2365.2 | 8099 | 464 |
| 12 | 1.5 | 0.2 | 27.0 | 470.1 | 6.1 | 41.3 | 180 | 74.5 | 49 | 218.6 | 5555 | 5822.6 | 7577 | 544 |
| 13 | 2.0 | 0.1 | 28.3 | 463 | 12.3 | 68.4 | 421 | 188.4 | 65 | 85.9 | 2093 | 2243.9 | 10647 | 1230 |
| 14 | 2.0 | 0.3 | 27.7 | 453.2 | 10.5 | 54.7 | 177 | 80.5 | 159 | 389.8 | 5111 | 5659.8 | 6929 | 451 |
| 15 | 2.0 | 0.3 | 28.2 | 440 | 8.6 | 67.1 | 241 | 109.8 | 307 | 888.1 | 2059 | 3254.1 | 7769 | 341 |
| 16 | 1.5 | 0.3 | 26.4 | 443 | 9.2 | 47.9 | 206 | 79.9 | 211 | 624.5 | 2016 | 2851.5 | 7476 | 300 |
| 17 | 2.0 | 0.3 | 26.9 | 400.2 | 10.4 | 68 | 295 | 116.9 | 581 | 1756.1 | 1930 | 4267.1 | 8430 | 313 |
| 18 | 2.5 | 0.5 | 26.2 | 400.4 | 9.3 | 46.9 | 97.5 | 40.5 | 628 | 6158.6 | 2093 | 8879.6 | 7007 | 370 |
| 19 | 3.0 | 0.3 | 26.9 | 401.8 | 8.3 | 48 | 119 | 47.9 | 610 | 2224.7 | 2059 | 4893.7 | 12025 | 352 |
| 20 | 3.0 | 0.3 | 28.5 | 402 | 8.8 | 70 | 302 | 142.6 | 610 | 2144.4 | 1863 | 4617.4 | 17989 | 337 |
| 21 | 3.0 | 0.3 | 27.6 | 403 | 8.5 | 62 | 66 | 34.9 | 628 | 2354.1 | 1707 | 4689.1 | 6721 | 337 |
| 22 | 2.0 | 0.5 | 27.2 | 396.2 | 9.6 | 59.2 | 50.5 | 28.7 | 610 | 6253.2 | 1953 | 8816.2 | 10115 | 278 |
| 23 | 3.0 | 0.3 | 26.7 | 393.9 | 8.5 | 55.4 | 51.3 | 44.2 | 610 | 5760.6 | 1844 | 8214.6 | 6276 | 368 |
| Mean | 4.70 | 0.42 | 26.08 | 412.11 | 8.40 | 53.53 | 147.19 | 67.98 | 510.52 | 4207.00 | 2637.35 | 7354.86 | 6871.52 | 351.35 |
| Median | 3.00 | 0.30 | 26.60 | 400.40 | 8.50 | 53.20 | 129.00 | 53.90 | 610.00 | 2354.10 | 2059.00 | 6300.70 | 6929.00 | 337.00 |
| SD | 3.90 | 0.21 | 1.68 | 28.08 | 2.26 | 9.39 | 101.48 | 41.24 | 231.79 | 3473.21 | 1151.89 | 3692.08 | 3887.19 | 219.10 |
| Minimum | 1.00 | 0.10 | 22.70 | 385.00 | 5.30 | 38.70 | 24.80 | 19.70 | 49.00 | 85.90 | 1707.00 | 2243.90 | 238.00 | 116.00 |
| Maximum | 14.0 | 1.00 | 28.50 | 470.10 | 14.50 | 70.00 | 421.00 | 188.40 | 776.00 | 10067.40 | 5555.00 | 14486.40 | 17989.00 | 1230.00 |
| N | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 |


Figure 2. The "SCUFA-TECHGEO" system with a fluorescent sonde (SCUFA®, Turner, Inc.) and a D-GPS (Tech Geo®) mounted on the side of a conventional aluminum boat. The D-GPS mounted at the top of the structure and the SCUFA sonde can be finely adjusted at different depths, near the surface of a lake or reservoir (z=0.3m – 0.6 m).

Because *in vivo* chlorophyll analysis is a semi-quantitative measurement, we also performed a calibration procedure prior to the real sampling. The SCUFA Sonde was calibrated using samples collected in the Pampulha Reservoir three days before the real intensive survey on 23 May 2011. A subsurface (z=0.5m) sample of 10 L was taken at the reservoir and immediately transported to the laboratory, located a 5-minute drive from our laboratory at UFMG. A series of 1:5, 1:10 and 1:100 dilution subsamples was performed in triplicate from this sample. From each dilution, two replicate aliquots of 250 ml were filtered onto glass fiber filters of 47 mm diameter, using a low-pressure vacuum pump and the chlorophyll-a concentration was determined using the method of Lorenzen [8], using a 90% acetone solution for extracting the chlorophyll pigments from the phytoplankton cells. These results were used to calibrate the SCUFA sonde.

To obtain an ASCII file XYZ where X and Y are the geographical coordinates (UTM, Zone 23S, Datum WGS 84) and Z is the chlorophyll-a concentration measured by the SCUFA sonde, a PASCAL routine was written in order to synchronize the two different sources of data set precisely according to the local time (GMT+3 h). The internal time clock of the sonde was set to the time of a Garmin GPS, just one hour before each sampling, to avoid internal errors associated with small differences in time measurements. Finally, this XYZ data matrix was processed by the Surfer program version 10.0, using the Kriging interpolation method to generate the thematic chart.

To verify the relationship between the two different methodologies used to estimate the amount of chlorophyll-*a* in the reservoir, we used the linear correlation analysis through software BioStat 5.3. For this analysis, we used only the values of chlorophyll-*a* estimated by the SCUFA method obtained at the points with the same geographical coordinates where the samples of Lorenzen method were analyzed.

The thematic charts of the phytoplankton biovolume as well as the chart of chlorophyll-*a* obtained with the Lorenzen method were constructed in a similar way, but the geographical coordinates were obtained by using a conventional GPS Garmin 76.

## RESULTS AND DISCUSSION

The two sample series considered here (September 2009 and May 2011) cover different but predictable phases of the annual cycle of chemical conditions in Pampulha Reservoir. The rainy season is well defined in the region. In the annual cycle, the last rainfall episodes usually occur in late March.

As a consequence, the samples taken in May reflected the conditions that usually prevail at the beginning of the dry season. According to this seasonal pattern, the most critical environmental conditions are typically observed in September-October, in practically all lakes in this region. Electrical conductivity, for instance, ranged from 385 to 470 µS $cm^{-1}$ with mean of 412 µS $cm^{-1}$ (September). In May, the conductivity decreased, and ranged from 238 to 408 µS $cm^{-1}$ (mean=346 µS $cm^{-1}$).The largest differences between the two sampling periods were observed in the following variables: suspended solids, nitrites, nitrates, ammonium and total nitrogen, all of them with higher levels in September (Tables 1 and 2).

As expected, Pampulha Reservoir showed high levels of chlorophyll-*a* and phosphorus, in both periods. In September 2009 (Table 1) chlorophyll concentrations, as expected, were generally higher, ranging from 38.7 to 70.0 µg $L^{-1}$ (mean=53.5 µg $L^{-1}$). In May 2011 the chlorophyll-*a* concentration ranged from 9.6 to 59.4 µg $L^{-1}$ (Table 2). In September, total phosphorus varied from 116 to 1230 µg P-$PO_4$ $L^{-1}$ (mean=351 µg P-$PO_4$ $L^{-1}$). In May, total phosphorus ranged from 318 to 1341 µg P-$PO_4$ $L^{-1}$ (mean=556 µg P-$PO_4$ $L^{-1}$).

The samples from May and September confirmed the advanced stage of eutrophication of Pampulha Reservoir.

The trophic level of the reservoir was determined by calculating the Carlson Trophic State Index TSI [4], which verifies the degree of trophy of a given water body. This index was calculated using the values of chlorophyll-*a*. Lakes and reservoirs with values lower than 45 are typical of oligotrophic systems, whereas systems with values between 45 and 55 are classified as mesotrophic and systems with TSI higher than 55 are eutrophic. The Pampulha Reservoir was considered hypereutrophic, with TSI values ranging between 61 and 76 [28].

There is a clear spatial pattern of trophy in the reservoir. The trophic condition usually increases from the dam to the reservoir arms, which have tributaries of various sizes, or near Amores Island where inputs from several polluted tributaries grouped into an artificial canal is concentrated. Most chemical and biological variables that are important for eutrophication, including conductivity, phosphorus, nitrogen and chlorophyll-a usually follow this spatial pattern. Therefore, Pampulha Reservoir is an ideal model for testing new tools aiming to improve the available techniques for regular monitoring of eutrophic reservoirs.

**Table 2. Water quality variables measured on 23 May 2011 in Pampulha Reservoir, Belo Horizonte, MG**

| Point | Maximum depth (m) | Temp. ($^oC$) | Cond. ($\mu S\ cm^{-1}$) | pH | D.O. ($mg\ L^{-1}$) | Chl-a ($\mu g\ L^{-1}$) | Susp.solids ($mg\ L^{-1}$) | $NO_2$ ($\mu g\ L^{-1}$) | $NO_3$ ($\mu g\ L^{-1}$) | $NH_4$ ($\mu g\ L^{-1}$) | NIT ($\mu g\ L^{-1}$) | PT ($\mu g\ L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1.0 | 21.4 | 340 | 7.6 | 9.1 | 16.8 | 21.0 | 11.2 | 76.6 | 361.6 | 5994.4 | 526.2 |
| 2 | 4.0 | 22,2 | 346 | 7.6 | 8.5 | 17.8 | 24.3 | 3.3 | 47.2 | 423.4 | 7304.6 | 526.2 |
| 3 | 1.0 | 22.3 | 348 | 7.7 | 8.4 | 40.9 | 39.3 | 16.8 | 111.2 | 383.7 | 7092.3 | 1178.8 |
| 4 | 2.0 | 21.6 | 238 | 7.6 | 8.9 | 16.8 | 20.3 | 2.5 | 49.1 | 399.1 | 2876.9 | 517.9 |
| 5 | 8.0 | 22.3 | 350 | 7.6 | 8.5 | 18.8 | 18.5 | 2.1 | 42.7 | 449.2 | 4114.8 | 522.1 |
| 6 | 6.0 | 22.2 | 350 | 7.4 | 8.7 | 22.1 | 17.3 | 0.45 | 44.6 | 361 | 3587.0 | 459.7 |
| 7 | 8.0 | 22.2 | 351 | 7.5 | 8.6 | 18.2 | 18.8 | 0.61 | 28.0 | 411.7 | 4404.3 | 572.0 |
| 8 | 6.0 | 22.2 | 351 | 7.4 | 8.7 | 18.5 | 19.3 | 0.76 | 47.2 | 356.6 | 4319.4 | 588.6 |
| 9 | 2.0 | 22.2 | 351 | 7.4 | 8.6 | 19.8 | 17.8 | 0.45 | 22.8 | 400.1 | 4443.9 | 505.5 |
| 10 | 10.0 | 21.9 | 350 | 7.3 | 8.9 | 15.2 | 17.5 | 2.8 | 52.9 | 317.5 | 2762.5 | 501.3 |
| 11 | 9.5 | 22.4 | 349 | 7.3 | 8.5 | 14.5 | 13.8 | 0 | 15.2 | 366.3 | 4253.7 | 409.9 |
| 12 | 3.0 | 21.7 | 349 | 7.3 | 9.0 | 18.5 | 13.8 | 0 | 0 | 378.3 | 4073.7 | 401.5 |
| 13 | 8.0 | 22.8 | 348 | 7.3 | 8.2 | 13.9 | 13.0 | 0 | 0 | 338 | 4030.0 | 484.7 |
| 14 | 12.0 | 22.0 | 348 | 7.2 | 8.9 | 13.2 | 12.8 | 0 | 0 | 383.4 | 4600.6 | 364.1 |
| 15 | 11.5 | 22.0 | 348 | 7.1 | 8.9 | 18.2 | 14.5 | 0 | 0 | 409.8 | 4266.2 | 547.0 |
| 16 | 16.0 | 22.1 | 349 | 7.2 | 8.7 | 9.6 | 11.5 | 0 | 0 | 400.1 | 3351.9 | 389.1 |
| 17 | 2.0 | 22.2 | 347 | 7.2 | 8.6 | 11.2 | 11.8 | 0 | 0 | 376.7 | 3543.3 | 364.1 |
| 18 | 3.5 | 22.6 | 349 | 7.3 | 8.5 | 13.5 | 15.8 | 0 | 0 | 338.9 | 2685.1 | 318.4 |
| 19 | 1.5 | 22.9 | 351 | 7.4 | 8.5 | 26.4 | 27.3 | 4.4 | 0 | 348.9 | 2563.1 | 634.3 |
| 20 | 1.0 | 23.3 | 353 | 7.3 | 8.1 | 24.4 | 20.8 | 0.3 | 0 | 320.6 | 3823.4 | 517.9 |
| 21 | 1.0 | 22.4 | 408 | 7.2 | 8.8 | 59.4 | 41.5 | 0.6 | 0 | 525.5 | 3730.5 | 1340.9 |
| Mean | 5.57 | 22.2 | 346 | 7.4 | 8.6 | 20.4 | 19.5 | 2.20 | 25.60 | 383.35 | 4182.0 | 555.7 |
| Median | 4.00 | 22.2 | 349 | 7.3 | 8.6 | 18.2 | 17.8 | 0.45 | 15.20 | 378.30 | 4073.7 | 517.9 |
| SD | 4.42 | 0.4 | 28 | 0.2 | 0.2 | 11.1 | 8.0 | 4.22 | 31.22 | 46.90 | 1269.5 | 249.0 |
| Minimum | 1.00 | 21.4 | 238 | 7.1 | 8.1 | 9.6 | 11.5 | 0.00 | 0.00 | 317.50 | 2563.1 | 318.4 |
| Maximum | 16.0 | 23.3 | 408 | 7.7 | 9.1 | 59.4 | 41.5 | 16.80 | 111.20 | 525.50 | 7304.6 | 1340.9 |
| N | 23 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 | 21 |

Pampulha Reservoir is largely dominated by Cyanophyceae (Figure 3). Nevertheless, during these sampling periods, the phytoplankton community included 128 taxa (23 sampling points), 22 of which were present at all sampling stations. The number of taxa in each algal class was: Bacillariophyceae (25), Chorophyceae (42), Cyanophyceae (20), Euglenophyceae (18), Zygnematophyceae (15), Cryptophyceae (5), Xanthophyceae (2) and Dinophyceae (1). The species richness varied from 46 to 71 species at the individual sampling points. The most frequent members of Cyanobacteria were *Aphanocapsa incerta*, *Cyanodiction* sp., *Merismopoedia tenuissima*, *Microcystis aeruginosa*, *M. protocystis*, *Planktothrix isothrix*, *Raphidiopsis* sp., *Romeria elegans* and *Sphaerocavum brasiliense* [28].

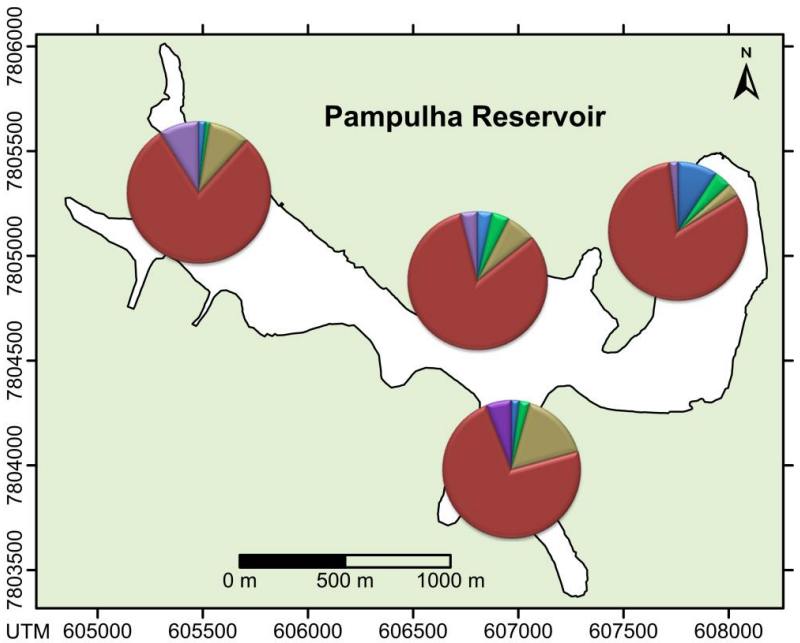


Figure 3. Spatial variation in the relative composition of major groups of hytoplankton of Pampulha Reservoir in September 2009 (in % of total biovolume).

As expected, phytoplankton density increased from the dam area to the riverine regions of the reservoir (Figure 4). The horizontal pattern of biovolume of total phytoplankton clearly reflected the spatial distribution of cyanobacteria, demonstrating the importance of this group of primary producers for the lake. The total biovolume of phytoplankton ranged from 21.53 to 88.93 $\mu m^3 mL^{-1}$ [28].

In addition to eutrophication, other ecological factors may explain the high values of chlorophyll-*a* in this lake. One of these factors is the structure of the planktonic food chain. The high biomass of phytoplankton possibly reflects the low efficiency of algal consumption by zooplankton. A recent investigation [26] demonstrated that the phytoplankton-zooplankton relationships are indeed very weak in the lake. These findings confirm another previous experimental investigation conducted with planktonic organisms from the Pampulha system, which demonstrated that the energy requirements of zooplankton in this lake are not covered by the local producers [29].

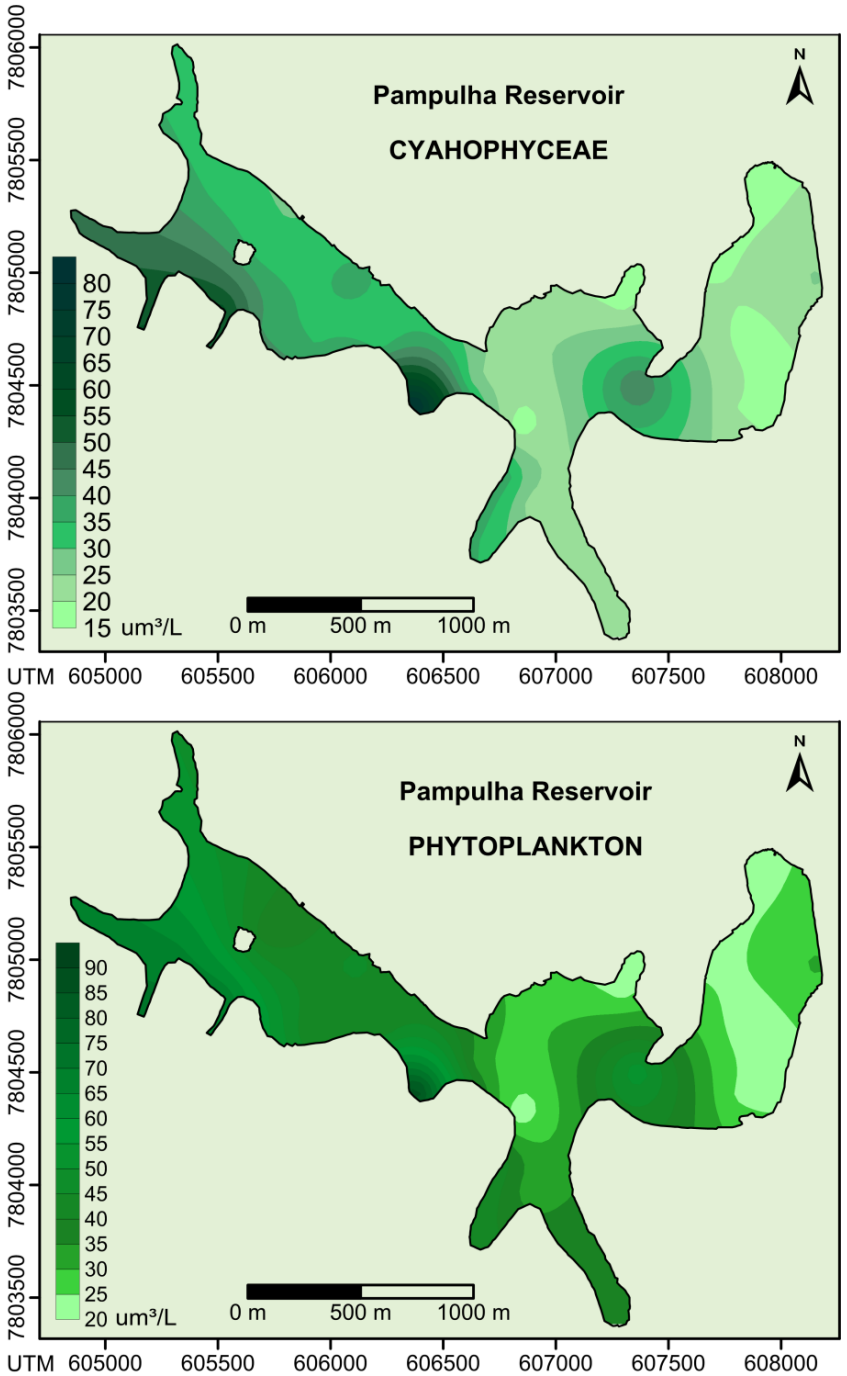


Figure 4. Spatial variation in biovolume ($\mu m^3 mL^{-1}$) of cyanobacteria and total phytoplankton in Pampulha Reservoir on 15 September 2009.

The data from 2009 clearly demonstrate that cyanobacteria form the bulk of the phytoplankton biovolume in this lake (Figure 4). During most of the dry season they form extensive blooms in the lake. It is common to observe the formation of floating algal mats that drift from one arm to another, according to the prevailing winds, one of the major driving

forces of the lake [28]. This trend to increasing dominance of cyanobacteria as the dry season progresses has been well established in the literature for Pampulha Reservoir [22,23,24,25].

The comparison of basic statistics between the two different methods (SCUFA versus Lorenzen) of estimating chlorophyll-a revealed surprisingly similar results (Table 2, Figure 5), with a strong, positive and significant correlation between the different methodologies ($p<0.0001$, $R^2= 0.72$, N= 21).

Nevertheless, the SCUFA measurements of chlorophyll-*a* were somewhat lower and exhibited a wider range of values than those obtained by using the Lorenzen method. The mean ± SD values were 13.9 ± 15.7 µg $L^{-1}$ and 20.4 ± 11.1 µg $L^{-1}$ for the SCUFA and Lorenzen method, respectively. The range of values of Lorenzen determinations was 9.6-59.4 µg $L^{-1}$ whereas the SCUFA measurements ranged between 0.0 and 169.5 µg $L^{-1}$. It is important to keep in mind the enormous difference is the number of measurements obtained with the SCUFA sonde: 5779 single SCUFA measurements against only 21 determinations using the Lorenzen method. Even considering these differences, the difference in SDs between the methods was less than 5.0 µg $L^{-1}$. The thematic charts of both methods are presented in Figure 5.

It is obvious that the SCUFA determinations produced a much more precise and detailed spatial pattern. This spatial fine-tuning provided by the SCUFA data permits spatial patches at the lake surface to be identified with great accuracy, since the error of the D-GPS coordinate determinations is less than 1.0 m. We can see that the spatial heterogeneity of the lake is much more complex than previously thought.

A comparison between the charts of phytoplankton biovolume and chlorophyll-*a* (Figure 5) also serves to highlight the potential use of the new methodology for monitoring chlorophyll-*a*.

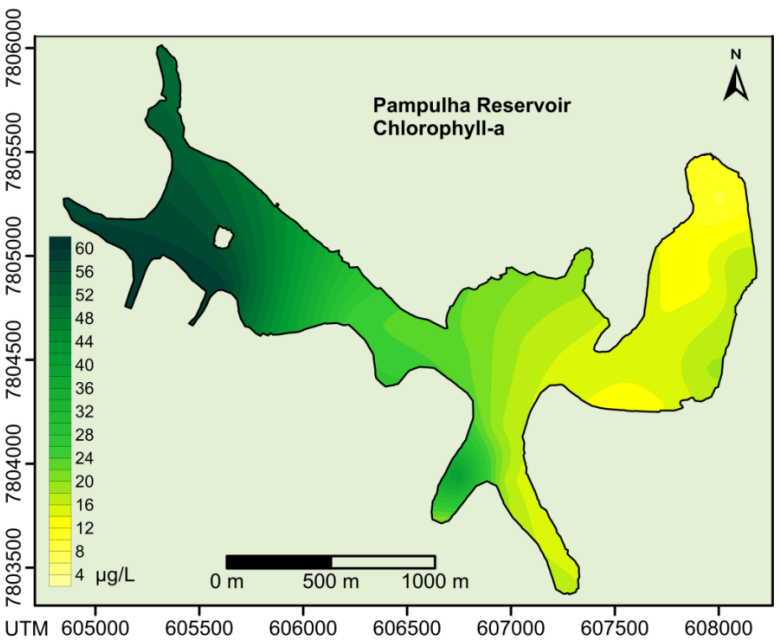


Figure 5. (Continued).

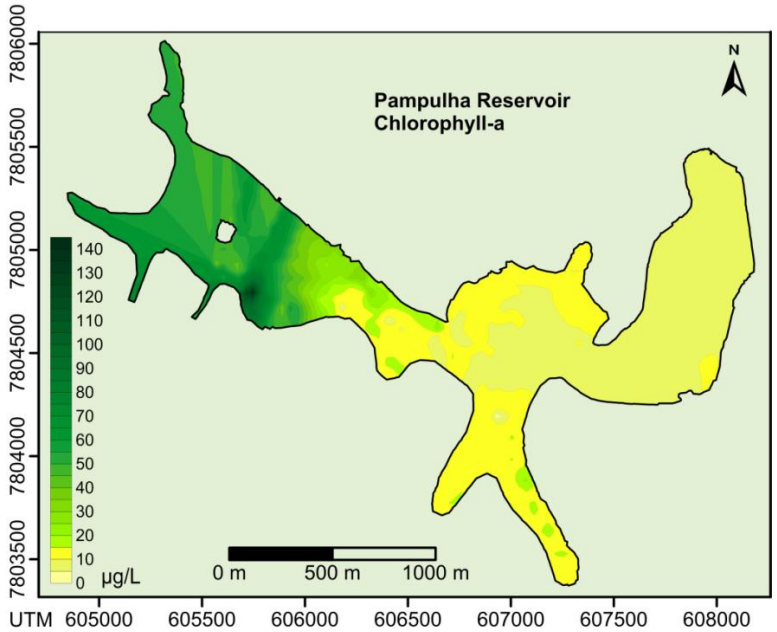


Figure 5. Spatial patterns of chlorophyll-*a* obtained with two different methodologies (Top graph: Lorenzen method, n=21 points; Bottom graph = SCUFA data, n=5779 points) in Pampulha Reservoir (sampling date 23 May 2011).

Both charts show a general pattern for higher concentrations of chlorophyll and phytoplankton toward the upper arms of the reservoir. However, the spatial gradients obtained by the SCUFA method are much more detailed and clear. They pinpoint detailed spatial patches with a high spatial resolution.

Using this kind of methodology, it is now possible to detect any source of nutrient input with an accuracy that is appropriate to the morphometry of the lake. Other potential factors that are important to explain the spatial patterns of chlorophyll, such as wind, currents, or the presence of macrophytes can be studied with this kind of information. Another possibility is the application of this methodology in the regular monitoring, to improve the positioning of the sampling points used for conventional analyses.

We believe that the results presented here may be an important contribution for the automatic monitoring of chlorophyll-*a* in freshwater systems. Monitoring of biological variables that are important for the eutrophication of freshwaters is still very limited. This monitoring is usually carried out by the use of different kinds of specialized and expensive sondes. Some of them are equipped for optical counting of particles (OPCs), or fluorescent sondes that are able to determine the concentration of photosynthetic pigment such as chlorophyll [30].

## CONCLUSION

Biological spatial heterogeneity is at the basis of ecological theory [33]. In the reservoirs, the spatial patterns that are frequently observed may be a result of a complex array of forcing functions such as winds, the influx of water from tributaries, and thermal structure. Spatial

patterns can also result from other powerful ecological constraints such as predation, competition and even parasitism [34]. The spatial complexity of ecosystems may contribute to the stability of the entire ecosystem [35].

The explanation of the real nature of the horizontal patchiness shown in Figure 5 may require a series of additional investigations. One such investigation clearly should be centered on a better knowledge of hydrodynamics, especially the effects of wind on these spatial patterns. The proposed methodology can also open several new prospects to apply all this information for better management of the reservoir itself. Knowing the spatial patterns can help local decision-makers to identify the major external forces that are causing these disturbances, and this information can be used to order possible remediation measures in a logical hierarchy of priorities.

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*Chapter 11*

# ECOLOGICAL CONTROL OF CYANOBACTERIAL BLOOMS IN FRESHWATER ECOSYSTEMS

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## ABSTRACT

Cyanobacterial blooms pose one of the most serious threats to freshwater ecosystems by producing toxic secondary metabolites that can poison aquatic food-webs, pets, livestock, and humans. Consequently, water resource managers routinely employ a variety of strategies aimed at controlling blooms of cyanobacteria, including reducing nutrient inputs, using potent herbicides, disrupting stratification, and shading waterbodies with water-based stains. The role of ecology in cyanobacterial bloom management is poorly understood despite a decades-long history of studies using biomanipulation: the manipulation of higher trophic levels (adding piscivores or removing planktivores) to increase the size, abundance, and grazing pressure of herbivorous zooplankton to reduce algal abundance. Past biomanipulation efforts conducted primarily in temperate systems have provided equivocal results, and the presence of the generalist herbivore, *Daphnia*, seems to be critically important to the success of biomanipulation efforts.

While cyanobacteria are relatively poor quality food for planktonic herbivores including cladocerans, copepods, and rotifers, recent meta-analyses of zooplankton-cyanobacteria studies show that, in general, cyanobacteria can support positive zooplankton population growth and purportedly toxic cyanobacterial secondary metabolites have, if any, ambiguous effects on zooplankton. Furthermore, recent research has shown that freshwater zooplankton, including the cladoceran *Daphnia* and the calanoid copepod *Eudiaptomus*, can adapt to tolerate toxic cyanobacteria in the diet following prolonged exposure to cyanobacterial blooms. Related field experiments clearly show that *Daphnia* can control cyanobacteria when freed from fish predation. In this review, we argue that cyanobacteria may serve as a beneficial food resource for zooplankton, that ecological control of cyanobacterial blooms is practical for some systems, and that greater attention should be placed on direct biomanipulation of zooplankton communities (e.g., stocking *Daphnia*) in conjunction with the manipulation of higher trophic levels. We also highlight the need for more data documenting

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zooplankton-cyanobacteria interactions in tropical freshwater ecosystems, whose biological, chemical, physical, and geological characteristics vary remarkably from their temperate counterparts.

## COMMENTARY

Biomanipulation – the alteration of a food-web to restore ecosystem health – has been well-studied in disparate communities [1,2] since the concept was first introduced by Joseph Shapiro and his colleagues as an approach to manage nutrient-rich freshwater lakes [3]. In lakes, the basic premise of biomanipulation is that secondary consumers (planktivorous fishes) are removed either through the addition of tertiary consumers (piscivorous fishes) or harvesting, which allows for the dominance of large-bodied, generalist zooplankton grazers (e.g., *Daphnia*) [4] to control phytoplankton. When planktivorous fishes are abundant and there is no predation refuge (e.g., oxygenated hypolimnion) for large-bodied zooplankton, less efficient small-bodied zooplankton grazers (e.g., rotifers and herbivorous copepods) typically dominate zooplankton communities thus allowing for the overgrowth of phytoplankton (i.e., algal bloom). Many past studies, conducted primarily in temperate systems, have shown strong correlations between the size structure of zooplankton communities and phytoplankton abundance [5-9]. These data support the notion that predatory top-down forces can have important implications for aquatic communities and ecosystems [10,11]. With that said, fish-centric biomanipulation effects on water quality are typically short-lived (i.e., weeks to months), most obvious in small, easily-managed systems (i.e., ponds), and impacted by resource availability, namely phosphorus and nitrogen [12-15]. For example, a common consequence of excess nutrient loading in lakes is elevated primary production [16] and the promotion of algal blooms [17,18]. Given the complexity of algal bloom dynamics across space and time, we still know very little regarding the relative strengths of top-down (predation) and bottom-up (resources) forces regulating ecosystem function in aquatic systems (but see [19,20]). This is especially true for under-studied subtropical and tropical systems [21-23].

Cyanobacteria (blue-green algae) are one of the primary indicators of poor water quality in lentic systems and have been implicated in the sickness and death of pets, livestock, and humans [24,25]. Cyanobacteria tend to dominate algal communities under nutrient enrichment, low nitrogen-to-phosphorus ratios, elevated temperatures, periods of stagnant or stratified conditions, high zooplanktivory, or a combination of these factors [17,26-31]. Regarding food-web interactions, cyanobacteria are considered to be poor food for grazers relative to other algal taxa, such as flagellates and chlorophytes [32,33]. Mechanisms mediating this distinction include the lack of cyanobacterial fatty acids required by zooplankton, colonial and filamentous morphologies, and intracellular toxins produced by several cyanobacterial genera that may negatively affect zooplankton population growth [33-37].

Of these three primary mechanisms, the role of nutritional deficiencies has shown to be the most robust across studies. For example, von Elert and colleagues [36,38-40] have conducted numerous laboratory-based experiments showing that zooplankton somatic and population growth rates can be enhanced when fed cyanobacterial diets supplemented with lipophilic chemical constituents, such as sterols and poly-unsaturated fatty acids, produced by

some algal taxa (chlorophytes and cryptophytes). Intuitively, large and irregularly-shaped cyanobacterial growth forms (e.g., *Microcystis* colonies or *Anabaena* filaments) should deter grazing by gape-limited zooplankton. However, a recent quantitative literature review of laboratory-based studies showed that filamentous cyanobacteria comprised significantly better diets for freshwater zooplankton relative to diets consisting of single-celled or colonial cyanobacteria [33]. Findings from this meta-analysis may be influenced by the inability of some zooplankton to consume large filaments, thus not ingesting purported intracellular toxins or cyanobacteria lacking essential nutrients. Future experiments should consider using different size fractions of the same phytoplankton diet (i.e., strain) to tease apart the influence of algal size on food quality for zooplankton (see [41,42]). Such an approach would preclude confounding factors related to intra- and interspecific physiological variation in phytoplankton [32,33].

The role of intracellular cyanobacterial secondary metabolites as zooplankton toxins is ambiguous, at best, despite the large zooplankton-cyanobacteria literature. In the same meta-analysis described above, Wilson and colleagues [33] found no clear effects of "toxic" cyanobacteria (defined as cyanobacterial strains shown to produce known toxins, such as microcystin or anatoxin-*a*) on the population growth rates of freshwater cladocerans and rotifers. In other words, zooplankton performed similarly on diets containing toxic or non-toxic cyanobacteria, albeit still worse relative to higher quality diets lacking cyanobacteria. Given that cyanobacteria produce a diverse suite of known and unknown compounds [43,44], it is reasonable to consider that "non-toxic" cyanobacteria (defined as cyanobacterial strains that do not produce known toxins) could produce "toxic" secondary metabolites that are currently unknown. Alternatively, cyanobacterial toxins traditionally identified using bioassays involving rodents may not be toxic to zooplankton. We are aware of only one study which directly tested the effect of one cyanobacterial toxin, microcystin-LR, in the diet on the fitness of a zooplankter, *Daphnia pulicaria* [37]. Data from this study showed that microcystin-LR can be toxic to zooplankton, but that this effect is not universal. For example, one *D. pulicaria* clone isolated from a eutrophic lake that was previously shown to perform well on a diet containing live, toxic *Microcystis* exhibited negative population growth when fed a diet containing lyophilized *Chlorella* (chlorophyte) treated with microcystin-LR. Interestingly, another *D. pulicaria* clone collected from an oligotrophic lake that performed poorly on the same diet containing live *Microcystis* was not affected by microcystin-treated, freeze-dried *Chlorella*. It is unclear what mechanism is driving these patterns, but these data definitely show that *Daphnia* performance on toxin-laced diets were consumer genotype-dependent, albeit not as expected based on their source habitats. Finally, although cyanobacteria are relatively poor food for zooplankton [33], it is imperative to recognize that most zooplankton taxa exhibit positive population growth on diets containing part or all cyanobacteria, regardless of its toxicity or morphology [32], and that the effects of cyanobacteria on zooplankton are likely context specific. Together, these data strongly suggest that the current paradigm describing cyanobacteria as generally harmful to zooplankton may need to be reconsidered.

During cyanobacterial blooms, small-bodied zooplankton tend to dominate plankton communities, and past observational studies have attributed this pattern to anti-herbivore traits of cyanobacteria [35,45,46]. However, planktivorous fish biomass is often positively related to productivity [6]. Thus, alternative explanations for the lack of consumer control of cyanobacteria could include zooplanktivory [47] or synergistic effects of cyanobacterial traits

and consumer control of large-bodied zooplankton [28]. We are unaware of any field empirical tests that have directly studied these hypotheses. Moreover, given that most zooplankton-cyanobacteria studies have been conducted in the laboratory but that the focus of these studies is on dynamics in nature, we encourage a greater emphasis of studies that determine if interactions observed in the laboratory can be extended to the field (see [48]).

Despite strong, but variable, inhibition of *Daphnia* by cyanobacteria in the laboratory, repeated field observations in eutrophic lakes have documented strong suppression of phytoplankton, including cyanobacteria, by *Daphnia* when freed from predation by planktivorous fishes [19,49-52]. Moreover, we have conducted several field experiments of 2-3 months duration (fish-less 144 L enclosures; without or with *Daphnia*) in hypereutrophic aquaculture ponds dominated by various species of cyanobacteria and found consistent, large effects of *Daphnia* on algal abundance (Figure 1, M. Chislock and A. Wilson, unpublished data). One potential explanation for the incongruity between results generated from laboratory and field studies is that laboratory experiments sometimes use *Daphnia* genotypes that are evolutionarily naïve to toxic cyanobacteria or incorporate diets consisting of cyanobacterial genotypes that are especially toxic prey [32,33]. Recent laboratory experiments support this explanation, as *Daphnia* genotypes from eutrophic environments with frequent cyanobacterial blooms are less inhibited by microcystin-producing *Microcystis* than *Daphnia* from oligotrophic environments where cyanobacteria are rare [53,54].

Furthermore, phenotypic acclimation has been observed in *Daphnia* [55], as well as the calanoid copepod *Eudiaptomus gracilis* [56], in response to exposure to sublethal diets of cyanobacteria. Given that some zooplankton can rapidly adapt to tolerate cyanobacteria and their associated toxins, the response of eutrophied systems to abatement efforts may depend not only on the presence of large zooplankton, like *Daphnia*, but also on the role of zooplankton adaptation to cyanobacteria. Available empirical data show that *Daphnia* can suppress cyanobacteria in nature, and *Daphnia* adaptations to toxic cyanobacteria may mediate these interactions. The role of grazer adaptations to better tolerate or avoid harmful prey is under-studied [53-56] but very exciting and could explain some of the variability observed in the zooplankton-cyanobacteria literature [32,33].

While the use of biomanipulation to improve water quality has been well-studied in temperate systems, the potential for top-down control of phytoplankton in subtropical and tropical lakes is less studied (but see [21-23,57]).

Elevated planktivory and temperatures occurring over increased seasonal durations in the tropics can promote cyanobacterial blooms and can extirpate large, competitively superior zooplankton, such as *Daphnia* from lakes [6,28]. It is well-known that species diversity, consumer density, and per capita consumer effects often increase closer to the equator [58-60]. Subtropical and tropical lakes also have more diverse fish communities that are commonly dominated by omnivorous species that consume detritus, phytoplankton, in addition to zooplankton [57].

Consequently, tropical and subtropical communities may be more strongly regulated by complex, web-like species interactions, relative to the chain-like food webs of most temperate lakes [61]. Thus, trophic cascades often documented in temperate lakes may be less common in the tropics. However, this hypothesis is untested, and manipulative field experiments in the tropics are needed to examine the generality of biomanipulation as a tool to improve water quality across systems.

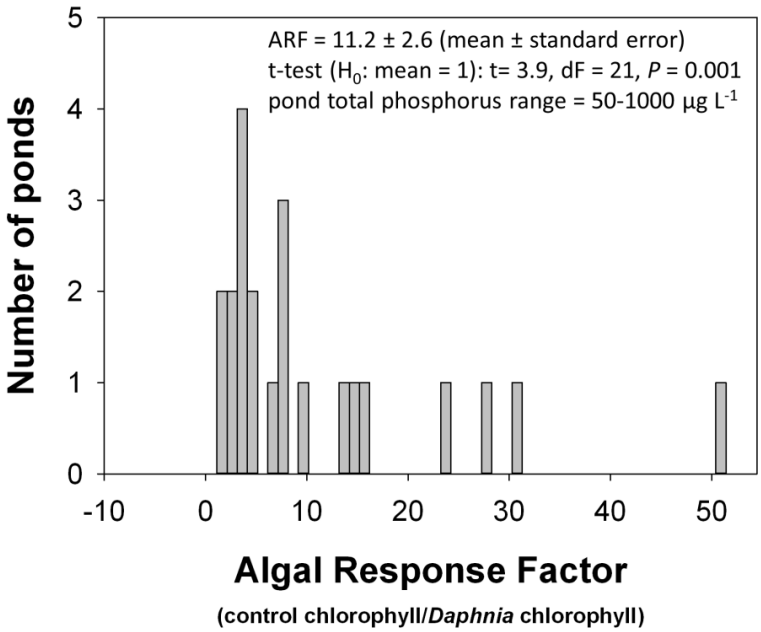


Figure 1. Legend: Histogram of algal response factors (ARF [20]; measured as [(final chlorophyll concentration in control)/(final chlorophyll concentration in treatment)]) in 2-3 months-long field experiments with two treatments (control = no *Daphnia*; treatment = *Daphnia*) conducted in hypereutrophic aquaculture ponds dominated by cyanobacteria (M. Chislock and A. Wilson, unpublished data). An ARF = 1 denotes no difference between final chlorophyll concentrations in the control and treatment. An ARF > 1 shows that the presence of *Daphnia* reduces chlorophyll concentration relative to enclosures lacking *Daphnia* (i.e., control).

Given the influence that predicted climate change and human population growth will have on future water quality and quantity, there is an immediate need by water resource managers to understand how to minimize the intensity and frequency of algal and cyanobacterial blooms. We contend that existing data support the notions that cyanobacteria are not necessarily harmful to zooplankton, that ecological control of cyanobacteria is possible under certain circumstances, and that a more directed focus on the management of large-bodied zooplankton (e.g., *Daphnia*) adapted to cyanobacteria could provide a long-term, sustainable solution to future cyanobacterial blooms in freshwater lakes that contain low densities of planktivorous fishes. We encourage large-scale, field tests of these ideas in the future.

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