Universidade de Aveiro Departamento de Biologia
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Catarina Isabel Prata Pereira Leitão Churro

Algicidas Naturais Contra Microalgas Nocivas: avaliação do potencial da bacilamida como meio de prevenção de florescências de cianobactérias.

Natural Algicides Againts Harmful Microalgae: screening the bacillamide potential as a prevention tool for cyanobacteria blooms.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia. Trabalho realizado no Laboratório de Microbiologia e Ecotoxicologia do Instituto Nacional de Saúde Dr Ricardo Jorge, sob orientação de Paulo Baptista Pereira, Investigador Auxiliar, e de António Calado, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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Palavras-chave

Florescências algais nocivas (FANs), Algicidas, Bacilamida, Triptamina, Cianobactérias, Microalgas, *Bacillus sp.*, controlo selectivo, IC50.

Resumo

O desenvolvimento de florescências de microalgas e de cianobactérias em ambientes aquáticos, marinhos e de água doce, pode ter consequências nefastas para os ecossistemas e para a saúde animal e humana. A prevenção e o combate à formação destas florescências envolve frequentemente a aplicação de agentes químicos com propriedades algicidas. Contudo, estes agentes são, na sua generalidade, inespecíficos, actuando não apenas sobre os organismos alvo mas também sobre a restante comunidade fitoplanctónica.

A bacillamida é um composto natural recentemente isolado a partir de uma bactéria associada ao declínio de florescências de dinoflagelados marinhos. A sua toxicidade natural contra estas microalgas, associada a um baixo custo de produção em laboratório, apontam para a possível utilização deste composto como agente algicida no combate a proliferações algais nocivas.

Neste trabalho avaliou-se o efeito da bacilamida e de um conjunto de compostos análogos no crescimento de diferentes estirpes de cianobactérias e de microalgas em cultura, com o objectivo de testar a aplicabilidade destes compostos na prevenção e no combate ao desenvolvimento de florescências. As culturas, inoculadas em microplacas de 96 pocetos, foram expostas a uma série crescente de concentrações de cada um dos compostos testados e incubadas numa câmara de culturas durante 216h. O crescimento algal foi seguido ao longo do período de incubação através da medição diária da densidade óptica (450nm) nos pocetos. As percentagens de inibição do crescimento foram calculadas a partir das curvas de crescimento obtidas em cada condição e as concentrações inibitórias IC50-216h foram calculadas por regressão das curvas de inibição através de análise de probits. A selectividade na acção tóxica de cada um dos compostos testados para as diferentes espécies foi estimada por comparação das concentrações inibitórias IC50-216h obtidas nas diferentes culturas. Para além dos efeitos no crescimento, os efeitos na morfologia e ultrastrutura foram analisados por observação das células expostas ao microscópio óptico e ao microscópio electrónico de transmissão. Os resultados obtidos revelaram que as cianobactérias tóxicas *Microcystis aeruginosa*, *Aphanizomenon gracile*, *Anabaena circinalis* e *Anabaenopsis circularis* são mais susceptíveis à bacilamida do que as algas verdes *Ankistrodesmus falcatus* e *Scenedesmus obliquus*. Assim, um tratamento com bacilamida a 80$\mu g.L^{-1}$ deverá afectar o crescimento destas cianobactérias sem afectar o desenvolvimento das algas verdes.

Resumo

Contudo, outras cianobactérias, tais como, *Nodularia spumigena*, *Leptolyngbya* sp. e *Plankthotrix rubescens*, exibiram níveis de tolerâncias à bacilamida semelhantes aos obtidos para a maioria das algas eucarióticas testadas. Resultados semelhantes foram obtidos com a bromobacilamida e a clorobacilamida. A metoxibacilamida, por seu lado, revelou-se particularmente tóxica para as cianobactérias *Anabaena* e *Aphanizomenon* (IC50-216h= 42 e 85 $\mu g.L^{-1}$, respectivamente) em concentrações que não afectaram o crescimento de nenhuma das restantes culturas testadas. A fluorbacilamida e a iodobacilamida revelaram um espectro de acção tóxica mais amplo contra cianobactérias, afectando o crescimento destes organismos em concentrações mais baixas (IC50-216h = 20-40 $\mu g.L^{-1}$) do que os restantes análogos testados. No entanto, a estas concentrações, estas bacilamidas afectaram também a maior parte das algas eucarióticas testadas, revelando-se portanto inespecíficas na sua acção tóxica. De todos os compostos testados, a triptamina (percursor da bacilamida) revelou ser o mais tóxico, afectando o crescimento da maioria das cianobactérias testadas em concentrações muito inferiores (IC50-216h = 1,1-4,1 μL^{-1}) às necessárias para afectar o crescimento das clorófitas (IC50-216h = 6,9-10,2 μL^{-1}). As diatomáceas foram contudo igualmente afectadas pela triptamina (IC50-216h = 0,5-2,5 μL^{-1}) pelo que a sua aplicação em ambientes co-habitados por estes dois tipos de organismos fitoplanctónicos pode resultar em efeitos nocivos para ambos. Por outro lado a cianobactéria *Planktothrix rubescens* revelou-se bastante mais resistente à triptamina que as restantes cianobactérias testadas. As diferentes sensibilidades exibidas pelas diferentes culturas cianobacterianas a cada um dos compostos testados, demonstram que as bacilamidas e a triptamina não devem ser considerados algicidas de largo espectro contra cianobactérias. Contudo a aplicação de distintas bacilamidas no combate ao desenvolvimento de determinadas espécies cianobacterianas em particular, pode revelar-se eficaz desde que o tipo e concentração de bacilamida aplicada não afectem as restantes microalgas que compõem a comunidade fitoplactónica. A decisão sobre o tipo e quantidade de bacilamida a aplicar na prevenção do desenvolvimento de uma dada fluorescência deve portanto ter em conta não só a espécie cianobacteriana dominante mas também considerar a composição relativa de toda a comunidade fitoplanctónica, de modo a não afectar os organismos benéficos para o equilíbrio e produtividade do ecossistema.

Keywords Harmful Algal Blooms (HABs), Algicides, Bacillamide, Tryptamine, Cyanobacteria, Microalgae, *Bacillus sp.*, selective control, IC50.

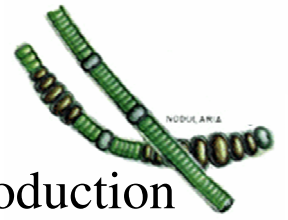
Abstract

Cyanobacterial blooms are of major concern to environmental and human health as they cause water deteoration and produces neuro and hepatotoxins. Bacillamide, a natural algicide produced by the marine bacteria *Bacillus* sp., and several newly synthesized analogues and also its precursors, were screened for selective antialgal activity against different cyanobacteria and eukaryotic algae. Its selective natural toxicity towards algal bloom-forming species and its possible low-cost synthetic production in the laboratory makes it one of the most promising compounds to pursue for use as a selective algicide against harmful algal blooms. A rapid 96-well microplate bioassay was used for screening selective antialgal activity of newly synthesized bacillamides and related compounds against different cyanobacterial and microalgal cultures. Aliquots of exponential growing stock cultures were inoculated in triplicate into the microplate wells previously filled with serial dilutions of each compound in the culture medium. The plates were steadily incubated in an algal culture chamber and daily optical measurements were used to estimate cultures growth over a 216h period. Inhibition values (%) were calculated from the estimated growth curves and inhibitory concentrations IC50-216h were obtained from the sigmoidal inhibition curves fitted by probit regression analysis. The effects of bacillamide and tryptamine on cell morphology and ultrastructure were also analysed by light and transmission electron microscopy. Results showed that the toxic cyanobacteria *Microcystis aeruginosa*, *Aphanizomenon gracile*, *Anabaena circinalis* and *Anabaenopsis circularis* were much more sensitive to bacilamide then the chlorophytes *Ankistrodesmus falcatus* and *Scenedesmus obliquus*. However, clear signs of morphological and ultrastructural changes induced by bacillamide could be observed on both cyanobacteria and chlorophytes. Other cyanobacteria, namely the Nostocales *Nodularia spumigena* and the Oscillatoriales *Leptolyngbya* sp. and *Plankthotrix rubescens*, exhibit higher tolerances to bacillamide, similar to the ones shown by different non-toxic algae. Thus, the use of bacillamide to control/remediate cyanobacterial blooms, should take into account the species composition in the phytoplankton community in order to avoid noxious effects on harmless phytoplankton. Among the other derivates tested, Fluor- and Chlorine-bacillamide showed similar results, while Metoxi-bacillamide seemed much less effective and Iodine-bacillamide strongly affected the growth of all the algae tested with no apparent selectivity. Concerning the bacillamide precursors, tryptamine showed much higher toxicity than bacillamides towards all the test organisms, while the 2-acethyl-1,3-thiazole-4-carboxyic acid had no growth effects on both the cyanobacteria and the microalgae screened.

Table of Contents

List of Tables

List of Figures



Introduction

Harmful Algal Blooms

Massive developments of phytoplanktonic organisms in marine and freshwater environments are frequent worldwide. Toxic phytoplankton blooms are responsible for wildlife and human health hazards, causing economic losses on fisheries, aquaculture and recreational activities (Anderson *et al.* 2002, Codd 1999, Hallegraeff 2003). During a bloom high cell concentrations are found, with low species diversity (Fryxell & Villac 1999). Most algal groups have representatives of harmful species, ranging from dinoflagellates, diatoms, haptophytes and raphydophytes to Cyanobacteria (Hallegraeff 2003). Table I describes some toxic episodes associated with harmful algal blooms (HABs) showing the diversity of harmful species involved.

Table I: Case histories of human illnesses and poisoning episodes associated with harmful algal blooms.

Taxonomic Group	Organism	Habitat	Harmful Effect	Case histories	References
Bacillariophyceae	*Pseudo-nitzschia* spp.	Marine	Domoic acid production (ASP-Neurotoxin)	Human poisoning thru shellfish consumption 3 deaths and 105 cases of acute intoxication in Canada (1987).	Bates *et al* 1989
	Chaetoceros spp.	Marine	Non toxic. Fish gill damaging	Mortality of farmed fish on Pacific Coast of North America (1987-1989).	Horner *et al* 1990
Cyanophyceae	*Nodularia spumigena*	Brackish	Nodularin (Hepatotoxin)	Few Dog deaths in Gulf of Finland (1984).	Smayda 1990
	Trichodesmium erythraeum	Marine	Non Toxic. Anoxia conditions.	Massive bloom, 27 km long with fish mortality costing a loss of 1.16 millions of euros in Gulf of Thailand (1983).	Suvapepun 1989
	Microcystis aeruginosa (*)	Freshwater	Microcystins production (Hepatotoxin)	Tabocas reservoir, Caruaru, Brazil 50 deaths and 51 cases of illness in an dialysis clinic (1996).	Jochimsen *et al* 1998
Dinophyceae	*Pyrodinium bahamense*	Marine	Paralytic shellfish poisoning toxins (PSP-Neurotoxin)	Human poisoning by shellfish consumption 26 deaths and 175 became will in Guatemala Coast (1987)	Rosales-Loessener *et al* 1989
Haptophyceae	*Chrysochromulina polylepis*	Marine	Toxic. Fish gill membrane damaging	Massive bloom with mortality of 800 tons of fish in Skagerrak-Kattegat between Denmark, Norway and Sweden (1988).	Lindahl & Dahl 1990
Raphydophyceae	*Chattonella antiqua*	Marine	Toxic. Fish gill damaging.	Mass mortality of yellowtail fish about 14.000×10^{-3} were lost worth over 500 million euros, in Seto Inland Japan (1972).	Okaichi 1989

(*) Microcystins intoxication probably by *M. aeruginosa.*

In freshwater systems, Cyanobacteria, also called blue-green algae, are the most problematic bloom-forming organisms. They may produce hepatotoxins (microcystins,

nodularins and cylindrospermopsins), neurotoxins (anatoxins and saxitoxins), cytotoxins and irritant compounds (lipopolysaccharides-LPS) (Apeldoorn *et al.* 2007, Codd 1999, Haider *et al.* 2003, Long & Carmichael 2003). Some of these toxins can also act as cancer promoters (Kuiper-Goodman *et al.* 1999, Falconer *et al*. 1999). Unlike marine toxins that usually affect humans through the ingestion of contaminated shellfish, public health concerns over cyanobacteria intoxication address different forms of exposure: direct contact, ingestion, haemodialysis with contaminated water, and ingestion of dietary supplements (Haider *et al.* 2003, Hallegraeff 2003, MacPhail & Jarena 2005). Cyanobacterial blooms significantly deteriorate the quality of drinking water and cause problems in water supply storage facilities (Hrudey *et al*. 1999). The cyanobacterial genera accounting for the most common toxic blooms are, in freshwater, *Microcystis*, *Anabaena*, *Aphanizomenon*, *Planktothrix* and *Oscillatoria* (Haider *et al*. 2003), while in marine waters the genera *Lyngbya*, *Nodularia*, *Aphanizomenon*, *Trichodesmium*, *Schizothrix* and *Umezakia* are the most frequently reported (Long & Carmichael 2003).

Some cyanobacteria may produce volatile compounds, namely geosmin and 2-methyl isoborneol (MIB), which introduce "earthy" and "musty" tastes and flavours into water and fish, rendering them non-potable and unmarketable for humans. Off-flavour problems cost losses of millions of dollars every year in catfish production in United States (Schrader & Dennis 2005, Schrader *et al.* 2003, Schrader *et al.* 1998). Geosmin and MIB production are common in *Oscillatoria*, *Pseudanabaena*, *Anabaena*, *Aphanizomenon*, *Lyngbya*, *Microcystis*, *Oscillatoria*, *Phormidium* and *Schizothrix* (Falconer *et al.* 1999, Sabater *et al.* 2003, Schrader *et al.* 2003, Zimba *et al.* 1999).

Having a world wide distribution, cyanobacterial blooms are perceived to be increasing in the last decades due to anthropogenic causes. The excessive release of nutrients from domestic, industrial and agricultural activities leads to the eutrophication of surface waters, creating opportunities for cyanobacterial development (Babica *et al.* 2006, Lürling & Roessink 2006, Ma 2005, Powell *et al.* 1991).

Cyanobacterial occurrence in continental Portuguese waters is common and toxic blooms are a recurrent phenomenon that raises public health concerns (Vasconcelos 1999). *Microcystis*, *Anabaena, Aphanizomenon, Cylindrospermopsis* are the most problematic genera found (Figueiredo *et al.* 2006, Saker *et al.* 2004, Pereira *et al.* 2004, Vasconcelos 1999).

Research in harmful algal blooms involves understanding the ecophysiology of harmful algae, bloom dynamics, conditions that promote blooms, production of toxins and their impact in human and wildlife health, and measures that can be taken to either prevent or mitigate bloom formation and toxin production (McPhail & Jarema 2005).

Cyanobacterial bloom ecology and ecophysiology

Although optimal cyanobacterial growth rates in general are exceeded, for optimum light conditions, by several eukaryotic microalgae (Reynolds 1984, p. 194-196), cyanobacteria possess some ecostrategies that allow them to outcompete other phytoplankton organisms and become dominant. They generally require lower light intensities for growth when compared with other algae, which provides competitive advantages in lakes which are turbid due to growth of other phytoplankton. Cyanobacteria also have a higher affinity for phosphorous and nitrogen uptake and they have a substantial storage capacity for phosphorous. Some genera like *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia* and *Nostoc* have specialized cells (heterocysts) for nitrogen fixation and blooms of these genera can often be related with periodic nitrogen limitation (Briand *et al*. 2003, Mur *et al.* 1999, Sunda *et al.* 2006). The success of some cyanobacteria is also due to the presence of gas vacuoles that provide buoyancy regulation. During stratified conditions cyanobacteria can migrate through the water column, accessing light at the surface layers and nutrients near the sediment (Mur *et al.* 1999). Cyanobacteria can also produce active substances that inhibit the growth of competing algae and grazers that feed upon them (Figueredo *et al.* 2007, Sunda *et al.* 2006, Vance 1965).

Bloom Treatment and Mitigation

Many attempts and research studies have been made in the past decades in order to find the most adequate approaches for cyanobacterial bloom control and management (Chorus & Mur 1999). Table II summarizes some of the restoration techniques that have been applied in freshwater lakes outlightning the major advantages and disadvantages for each strategy.

Table II: Restoration techniques currently applied in for Harmful bloom prevention and treatment

Applicability	Treatment Process	Advantages/Disadvantages	References
To prevent cyanobacteria blooms directly in field lakes.	Abstraction of hypollimnetic water rich in P by surface currents.	●It can break the cycle of enhanced sediment accumulation of total phosphorous. ●It flush more phosphorous out of the system than the sediments can accumulate each year. ●Efficient if enough water flow is present in the lake upper currents. ● Ecological consequences if all hypolimnion is removed. ●Hypolimnion removed water must be treated for phosphorous precipitation.	Chorus & Mur 1999
	Artificial destratification with bubble plum aerators	●Disrupts the possibility of cyanobacteria to migrate in the water column. ●Mixing improves development of other taxa such as diatoms. ● Only possible in deep water lakes, 20 m depth may be require. ● At least 80% of the water volume should be mixed. ●The costs for destratification vary according the situation, large systems can cost several hundred thousand euros.	Chorus & Mur 1999 Heo & Kim 2004
	Sediment dredging	● Useful in lakes that need sediments cleaning. ● Costly and could result in exposition of hazardous wastes. ● Only for small water bodies.	Chorus & Mur 1999
Avoid offtake of contaminated waters.	● Barriers to restrict scum movement ● Collection of scum ● Offtake by bank filtration ● Offtake structures with multiple offtake depths.	● Requires machinery and time consuming. ● Cell damage can occur. ● Dependent upon local soils characteristics and microbial activity. ● Not all reservoirs offtake are equipped properly.	Hrudey *et al* 1999 Grützmacher *et al* 2002

In general, most of these approaches rely on knowledge about cyanobacterial growth requirements by selectively reducing a limiting nutrient, which is in most cases phosphate. Most of these treatments require time and expensive machinery to produce satisfactory results (Chorus & Mur 1999).

Other approaches for bloom control rely on the use of algicides. Chemical treatments with, e.g. potassium permanganate, chlorine, simazine, atrazine, endothall, diquat, paraquat, diuron, sodium hypochlorite, and phosphorous precipitation with flocculants such as

aluminium sulphate, ferric salts, and ferric aluminium sulphate have been applied in lake waters. Oxidants like potassium permanganate and chlorine, and photosystem II inhibiting algicides such as atrazine, simazine and diuron are toxic to all phytoplankton and will reduce the production of dissolved oxygen by photosynthesis, which can cause mortality of aquatic organisms. Furthermore, oxidants and coagulants are skin irritants and handling should be taken carefully (Boyd & Massaut 1999, Schrader *et al*. 1997, Schrader *et al*. 1998, Tang *et al.* 1997). Paraquat and diquat are toxic to fish and mammals and their efficiency depend on light, endothall is also non selective (Schrader & Tucker 2003, Schrader *et al*. 1997, Schrader *et al*. 1998). In general, such methods are not ecologically appropriate because they are toxic to all aquatic life, they are not stable and are difficult to handle (Boyd & Massaut 1999, Chorus & Mur 1999). Among all the algicides used for short-term control of algae, copper sulphate and copper chelates have been the most commonly and widespread algicides used because they are cheap, relatively safe and easy to apply. The only algicide, other than diuron, registered for use in food-fish production ponds is copper (Hrudey *et al.* 1999, Schrader *et al.* 1997, Schrader *et al.* 2003). However, the efficiency of copper sulphate is sometimes not satisfactory, because fish kills sometimes occur following copper treatment and a variety of aquatic species including all the phytoplankton community can be affected, causing subsequent water quality deterioration (Hrudey *et al.* 1999, Oliveira-Filho *et al.* 2004, Li & Hu 2005). Furthermore, toxic copper deposits may accumulate in the sediments and repeated treatment may induce appearance of unpleasant copper resistant cyanobacteria species (Chorus & Mur 1999, Garcia-Villada *et al.* 2004, Roussel *et al.* 2007, Costas 2004).

The use of algicides to combact algal problems might involve the risk of cell lysis and the consequent release and dispersion of intracellular toxins and odour compounds to the surounding medium (Hrudey *et al.* 1999, Etchegary *et al.* 2004, Lam *et al.* 1995). An algicide should therefore be applied in early stages of bloom development, when cell densities are low, to minimize the liberation of high concentrations of toxins commonly associated with dense blooms (Hrudey *et al.* 1999). Furthermore a suitable algicide should be chemically stable; its degradation products should be non-toxic and it should affect only target organisms, thereby leaving the good functioning of the ecosystem unaffected.

Natural compounds generally degrade faster and have less probability to cause perturbations in natural environments then synthetic compounds (Boyd & Massaut 1999). Thus, natural based algicides are a potentially good alternative to synthetic algicides.

Biological Control Agents

Allelopathy refers to the chemical inhibition of a species by another (Cembella 2003, Granéli & Hansen 2006). In aquatic environments the chemical interaction between organisms is not a new issue (Smayda 1997). Allelopathic agents are produced by higher plants, aquatic macrophytes, fungi, bacteria and algae (Cembella 2003, Weir 2004).

Much efford has been focused recently on the identification of organisms capable of selective inhibition of algal growth. Submerged macrophytes *Myriophyllum verticillatum*, *M. spicatum*, *Ceratophylum demersum*, *Stratiotes aloides*, *Elodea canadensis*, *E. nuttallii* have all been shown to be more effective against cyanobacteria and diatoms then against chlorophytes (Hilt *et al.* 2006, Körner & Nicklisch 2002, Mulderij *et al.* 2005, Erhard & Gross 2006). Some plants used in oriental medicine have been reported to inhibit the growth of dinoflagellates (Zhou *et al.* 2007). Several macroalgae (*Ulva pertusa*, *U. linza* and *Corallina pilulifera*) have been shown to inhibit the growth of dinoflagellates and crytophytes (Jin *et al.* 2005, Nan *et al.* 2004, Jeong *et al.* 2000). Several cyanobacteria have been shown to inhibit chlorophytes, diatoms, crytophytes and other cyanobacteria (Bagchi *et al.* 1993, Chauhan *et al.* 1992, Suikkanen *et al.* 2004). Other examples include dinoflagellates that affect *Microcystis aeruginosa* (Wu *et al.* 1998), fungi that lyse cyanobacteria and chlorophytes (Redhead & Wright 1978, Safferman & Morris 1962), and viruses that infect *Phaeocystis globosa* and *Heterosigma akashiwo* (Baudoux & Brussaard 2005, Nagasaki *et al.* 1999).

Bacteria may also play an important role in bloom dynamics (Jasti *et al.* 2005, Imai *et al.* 1998, Mayali & Doucette 2002). Several studies have been made on this subject and algicidal bacteria have been isolated. Some of these bacteria are species specific in algicidal activity and may be useful for the selective control of blooms (Jasti *et a*l. 2005, Imai *et al.* 1998, Lovejoy *et al.* 1998). These algicidal bacteria are included in the groups: *Cytophaga* (Doucette *et al.* 1998, Imai *et al.* 2001, Mayali & Doucette 2002), *Shewanella* (Hare *et al.* 2005), γ and δ-proteobacteria (Lovejoy *et al.* 1998, Kim *et al.* 2007, Kang *et al.* 2005, Kato *et al.* 1998, Walker & Higginbotham 2000, Caiola & Pellegrini 1984), Flavobacteria and Bacilli (Skerratt *et al.* 2002). However, opinions concerning the benefits

resulting from addition of probiotics are contradictory (Boyd & Massaut 1999, Vershuere *et al.* 2000). Addition of living organisms to natural environments or biomanipulation of parts of the food web can disrupt the entire ecosystem with disastrous consequences (Jeong *et al.* 2000, Vershuere *et al.* 2000).

Isolation of the inhibitory compounds produced by these organisms can be a more effective and safe way to control blooms, but this is not always an easy task. Rice straw and barley straw for example, have proven to inhibit algal growth in laboratory and field experiments (Park *et al.* 2006, Erverall & Lees 1995), but the isolation of the inhibitory compounds from barley has been unsatisfactory, since the inhibitory effect is likely to be a synergistic effect of the various compounds (Ball *et al.* 2001, Ferier *et al.* 2005). However, in some cases the metabolites with inhibition activity towards algae have been discovered, their chemical structure elucidated and biological activity tested (Schrader *et al.* 1998a). Table III presents a list of natural-based compounds isolated from different organisms and reported to have a strong potential for the environmentally friendly control of harmful algae and cyanobacteria.

Table III: Natural compounds with algal inhibition potential.

Organism	Species	Compound	Affected Algae	Unaffected Algae	Reference
Marine sponge	*Aaptos aaptos*	Aaptamine	Cyanobacteria: *Oscillatoria perornata*	Chlorophytes: *Selenastrum capricornutum*	Nagle *et al.* 2003
Plants	*Pragmites communis*	Ethyl-2-methylacetoacetate	Cyanobacteria: *M. aeruginosa* IC50 0.79 mg.L^{-1} Chlorophytes: *Chlorella pyrenoidosa* IC50 0.49 mg.L^{-1}	Chlorophytes: *Chlorella vulgaris*	Li & Hu 2005
	Oryza sativa (Rice)	Palmitoleate	Cyanobacteria: *M. aeruginosa*	---	Chung *et al.* 2007
	Polygonatum odoratum var. *pluriflorum*	L-2-azetidinecarboxylic acid (AZC)	Cyanobacteria: IC90 0.2-6.3 µM *M. aeruginosa* *Anabaena affinis* Dinoflagellates: *Cochlodinium polykrikoides*	Chlorophytes: *Chlorella vulgaris* *Scenedesmus* spp.	Kim *et al.* 2006
	Zea mays and *Secale cereale* (Gramineae)	Hydroxamic acids	Chlorophytes: *Chlorella xanthella*	---	Bravo & Lazo 1996
	Ruta graveolens (Roots)	Rutacridone epoxide	Cyanobacteria: *Oscillatoria perornata* IC50 9x10^{-3} µM	Chlorophytes: *Selenastrum capricornutum*	Mecpagala *et al.* 2005
	Tannin extracts	9,10-anthraquinone Anthraquinone-59 Anthraquinone-19	Cyanobacteria: IC50 5-6 µg.ml^{-1} *Oscillatoria perornata* *O. geminata* *Cylindrospermopsis* spp. *Planktothrix agardhii*	Chlorophytes: *Selenastrum capricornutum* Cyanobacteria *Microcystis aeruginosa*	Schrader *et al.* 2000 Schrader *et al.* 2003
	Juncus effusus	Dihdrophenanthrenes, Tetrahydropyrenes and Phenathrenes	Chlorophytes: *Selenastrum capricornutum*	---	Dellagreca *et al.* 1996 Dellagreca *et al.* 2001
Macroalgae	*Dictyota menstrualis* (Brown alga)	Dicthol B acetate	Cyanobacteria: *Oscillatoria perornata* IC50 2.23 µM	Chlorophytes: *Selenastrum capricornutum*	Nagle *et al.* 2000 Nagle *et al.* 2003
Fungi		Lysine Lysine copper Lysine malonate	Cyanobacteria: *Microcystis* spp. Inhibitory at 0.5-20 mg.L^{-1}	Cyanobacteria: *Oscillatoria rubescens* *Phormidium tenue* Diatoms: *Melosira granulata* *Cyclotella meneghiniana* Chlorophyceae: *Scenedesmus acutus* *Pediastrum duplex*	Hehmann *et al.* 2002 Kaya *et al.* 2005
	Clonostachys rogersoniana	Clonostachysins A and B	Dinoflagellates: *Prorocentrum micans* IC50 30 µM	Cyanobacteria: *Oscillatoria amphibia* Chlorophytes: *Brachinomas submarina* Diatoms: *Skeletonema costatum*	Adachi *et al* 2005

Table III (Continue): Natural compounds with Algal inhibition potential.

Organism	Species	Compound	Affected Algae	Unaffected Algae	Reference
Bacteria	*γ-Proteobacterium*	Prodigiosin pigment	IC50 5.0-12.5 $\mu g.ml^{-1}$ Dinoflagellates: *Cochlodinium polykrikoides* *Gymnodinium impudicum* *Alexandrium tamarense* *Heterocapsa circularisquama* Raphidophyceae: *Heterosigma akashio*	---	Nakashima *et al.* 2006
	Pseudomonas sp.	Harmane Norharmane	Cyanobacteria: Inhibition at 30 $\mu g.disk^{-1}$ *Anabaena cylindrica* *A.variabilis* *Oscillatoria agardhii* *Anacystis marina* *M. aeruginosa* *M. Viridis*	Chlorophytes: *Chlorella vulgaris* *Chlamydomonas tetragama*	Kodani *et al.* 2002
	Vibrio sp.	β-cyanoalanine	Cyanobacteria: *Oscillatoria amphibia* *Synechococcus* sp. *Entophysalis deusta* *M. aeruginosa* *M. viridis*	Chlorophytes: *Brachiomonas submarina* *Nannochloris* *Chlorella saccharophila* Dinoflagellates: *Prorocentrum micans* Diatoms: *Skeletonema costatum* *Asterionella glacialis* *Ditylum brighteelli* *Nitzschia palea*	Yoskiwa *et al.* 2000
	Pseudoalteromonas sp.	Protease	Diatoms: *Skeletonema costatum*	---	Lee *et al.* 2000
	Pseudomanas aeruginosa	Rhamnolipids	IC50 0.4-3.0 $mg.L^{-1}$ Raphidophyceae: *Heterosigma akashiwo* *Dinoflagellates:* *Prorocentrum dentatum*	Dinoflagellates: *Gymnodinium* sp.	Wang *et al.* 2005
	Bacillus brevis	Gramicidin	Cyanobacteria: *Plectonema boryanum*	---	Reim *et al.* 1974
	Bacillus cereus	'Unidentified compound'	*Cyanobacteria:* *Microcystis aeruginosa* *M. viridis*	---	Nakamura *et al.* 2003
	Bacillus sp.	3-methyl-1-butanol	Cyanobacteria: *Anabaena variabilis*	---	Wright *et al.* 1991
	Bacillus sp.	Bacillamide	LC50 3.2-50.2 $\mu g.ml^{-1}$ Dinoflagellates: *Cochlodinium polykrikoides* *Alexandrium catenella* *Gyrodinium impudicum* *Prorocentrum micans* *Scrippsiella trochoidea* Raphidophyceae: *Chatonella* sp. *Heterosigma akashiwo*	LC50 > 100 $\mu g.ml^{-1}$ Diatoms: *Chaetoceros affinis* *Skelatonema costatum* Chlorophyceae: *Chlorella ellipsoidea* *C. Vulgaris* Cyanobacteria: *Anabaena variabilis* *Microcystis aeruginosa*	Jeong *et al.* 2003

(---) Not mentioned or not tested.

Table III (Continue): Natural compounds with Algal inhibition potential.

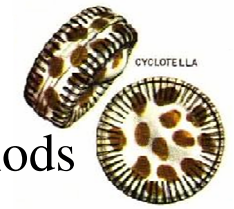
Organism	Species	Compound	Affected Algae	Unaffected Algae	Reference
Cyanobacteria	*Nodularia harveyana* *Nostoc insulare*	Norharmane	LOEC 8-32 μg.ml^{-1} **Cyanobacteria:** *Anabaena constricta* *A. cylindrica* *Oscillatoria brevis* *Anabaenopsis siamensis* *Nostoc carneum* *Nostoc insulare* *Arthrospira laxissima* *Chroococcus minutus* *Synechocystis aquatilis* *Synechococcus* spp.	**Chlorophytes:** *Coelastrum astroideum*	Volk 2005 Volk 2006 Volk & Furkert 2006 Volk & Mundt 2007
	Nodularia harveyana	Norharmalene	*Nostoc carneum* *Arthrospira laxissima* *Chroococcus minutus* *Synechocystis aquatilis* *Synechococcus* spp	---	Volk 2006
	Nostoc insulare	4,4'-dihydroxybiphynyl	LOEC 16-56 μg.ml^{-1} **Cyanobacteria:** *Anabaena constricta* *A. cylindrica* *Oscillatoria brevis* *Anabaenopsis siamensis* *Nostoc carneum* *Arthrospira laxissima* *Chroococcus minutus* *Synechocystis aquatilis* *Synechococcus* spp *Nostoc insulare* (self inhibition 80 μg.ml^{-1})	**Chlorophytes:** *Coelastrum astroideum*	Volk 2005 Volk 2006 Volk & Furkert 2006 Volk & Mundt 2007
	Fischerella spp. *Fischerella muscicola*	Fischerellin A, B	**Cyanobacteria:** *Anabaena* variabilis *Phormidium* sp. *Microcystis aeruginosa* *Synechococcus* sp. *Synechocytis* sp.	**Chlorophytes:** (More tolerance) *Ankistrodesmus falcatus* *Scenedesmus obliquus* *S. Falcatus* *Nannochloris* spp.	Gross *et al.* 1991 Papke *et al.* 1996 Etchegaray *et al.* 2004
	Fischerella sp.	12-epi-hapalindole	**Cyanobacteria:** *Microcystis aeruginosa* *Synechococcus* sp.	---	Etchegaray *et al.* 2004
	Microcystis aeruginosa	Kasumigamide	**Chlorophytes:** *Chlamydomonas neglecta* LOEC 2 μg.ml^{-1}	---	Ishida & Murakami 2000
	Nostoc sp.	Nostocarboline	IC50 2.1-29.1 μM **Cyanobacteria:** *Microcystis aeruginosa* *Synechococcus* sp. **Chlorophytes:** *Kirchneriella contorta*	---	Blom *et al.* 2006
	Oscillatoria sp.	Long chain fatty acids	**Cyanobacteria:** *Anacystis nidulans* *Scytonema hofmonii* *Microcystis aeruginosa* *Synechococcus* sp. *Anabena* sp. *Scytonema* spp. *Phormidium* spp. *Fischerella* sp. **Chlorophytes:** *Scenedesmus obliquus* *Spirogira* sp. *Tetraspora* sp.	**Cyanobacteria:** Anabaena spiroides	Chauman *et al.* 1992
	Oscillatoria late-virens	'Antibiotic'	**Cyanobacteria:** *Microcystis aeruginosa*	---	Bagchi *et al.* 1993

Some of these compounds can have undesirable side effects. Examples include the rutacridone epoxide having a direct-acting mutagen effect (Meepagala *et al.* 2005), the aaptamine which is cytotoxic to mammalian cells (Nagle *et al*. 2003), the norharmane that showed a co-mutagenic and cytotoxicity activity in eukaryotic cells (Volk 2005, Volk & Mundt 2007) and anthraquinones that have a direct toxicity to fish (Schrader *et al.* 1998). These substances are of little use as agents to control blooms.

Recently Jeong *et al.* (2003) reported that bacillamide, a novel algicide from the marine bacterium *Bacillus* sp. SY-1, had a selective activity against the harmful dinoflagellate *Cochlodinium polykrikoides*. *Bacillus* species are saprobic, spore-forming, non-pathogenic gram-positive bacteria normally found in air, water, dust, soil and sediments (Farzanfar 2006, Morikawa 2006). They produce a wide variety of secondary metabolites with antimetabolic activities (Jing & Jianming 2004, Morikawa 2006, Nakamura *et al.* 2003). Bacillamide is obviously derived from tryptophan by decarboxylation, the resulting amine having further reacted with 2-acetylthiazole-4-carboxylic acid. The last compound was discovered in nature in 1990 and has been found widely distributed in members of eukaryotes, archaebacteria and eubacteria. Its possible role as a yet unrecognized cofactor has been suggested (White 1990). More recently two different bacillamides were isolated, one from the bacterium *Microspora aerata* from the Antarctic and the other from *Bacillus endophyticus* from a Bahamian hypersaline microbial mat (Ivanova *et al*. 2007, Socha *et al*. 2007). The possibility of a widespread distribution of bacillamide, coupled with its selective natural toxicity towards algal bloom-forming species and its possible low-cost synthetic production in the laboratory, makes it one of the most promising compounds to pursue for use as a selective algicide against HABs.

Recent studies have shown that certain aminoacids may be selective in causing growth inhibition to cyanobacteria, while others do not affect growth (Hehmann *et al.* 2002). Differential responses have been attributed to different permeabilities and uptake processes of these species to the different compounds (Hehmann *et al.* 2002).

In this work, different bacillamide analogues derived from a common synthetic pathway, as well as related aminoacids (tryptamine and 2-acetyl-1,3-thiazole-4-carboxylic acid) have been tested in terms of selective toxicity towards different species of cyanobacteria and eukaryotic algae, with the aim of assessing their usefullness for the practical control of harmful blooms in marine and freshwater environments.



Tested Compounds

The compounds tested for biological activity were bacillamide, 5-fluor-bacillamide, 5-clorine-bacillamide, 5-methoxy-bacillamide, 5-bromo-bacillamide, and the bacillamide precursors tryptamine and 2-acetyl-1,3-thiazole-4-carboxilic acid (ATCA). The chemical properties of each tested compound are shown in Table V. All these compounds were sinthesized in the organic chemistry laboratory, CQFB-REQUIMTE and SINTOR-UNINOVA, FCT-UNL campus (Figueira *et al.* 2005), and received in a pure state as white/yelow cristal powders. Stock solutions for use in the bioassays were obtained by diluting 16 mg of each bacillamide-compound in 50 ml of culture media (final concentration 320 $\mu g.ml^{-1}$). For tryptamine and for ATCA, 8 mg were diluted in 50 ml of culture medium to yield a concentration of 160 $\mu g.ml^{-1}$. The tryptamine solution was further diluted 1:4 to obtain a stock solution with a final concentration of 40 $\mu g.ml^{-1}$. All stock solutions were sonicated on ice for 3 min in 10-second pulses and filtered through sterile 0.22 µm-pore Milipore membrane filters. The pH of the stock solutions was checked using a digital pH meter. Stock solutions were maintained at 4ºC and warmed up to room temperature before use.

Table IV: Chemical properties of Bacillamide and related compounds used in growth inhibition assays.

Compound	Molecular weight ($g.mol^{-1}$)	Solution proprieties		
		Solubility	Color	PH (23ºC)
Bacillamide	313,37	Low	White	7,69
5-metoxi-bacillamide	329,37	Low	White	7,98
5-clorine-bacillamide	347,82	Very low	yellow	7,71
5-fluorine-bacillamide	331,37	Very low	yellow	7,98
5-iodine-bacillamide	439,27	Very low	yellow	7,74
5-brome-bacillamide	392,07	Very low	yellow	7,91
2-acethyl-thyazol-4-carboxilic	171,17	High	No-color	7,91
Tryptamine	160,22	High	No-color	7,91

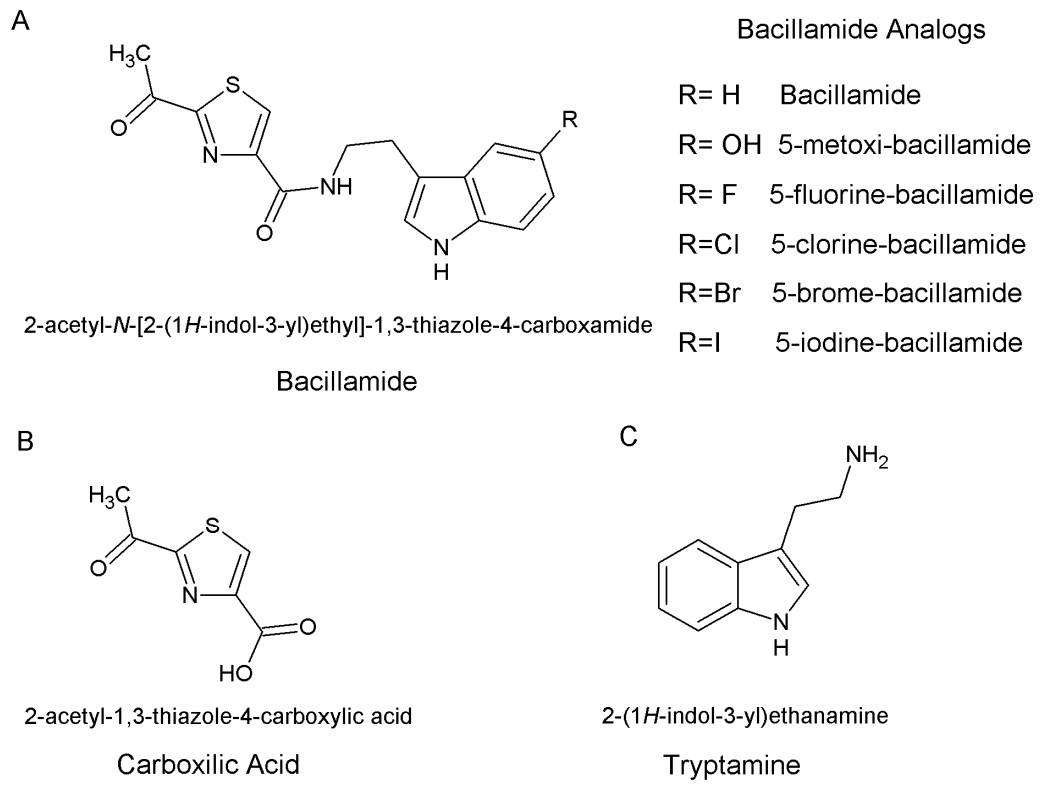


Figure 1: Chemical structure of the screened bacillamides (A) and Bacillamide precursors Tryptamine (B) and 2-acetyl-1, 3-thiazole-4-carboxilic acid (C).

Test organisms and Culture conditions

Species belonging to seven phytoplankton groups were bioassayed, representing a wide range of taxonomy, morphology, physiology and ecological relevance. Strains were selected based on their availability in the culture collection and their ability to produce rapid and uniform growth under essay conditions. Strain information (including source) is given in Table IV. All strains were maintained as steady-state stock cultures in a culture chamber programmed for a 16:8 h light:dark cycle with a light intensity of 36 µmol photons. $m^{-2}.s^{-1}$, and a constant temperature of 20 ± 1 ºC. Inocula from stock cultures growing on 20 mL T-flasks were regularly transferred to new media in order to provide a continuous source of cells growing at a fairly constant rate. Freshwater and marine algae were cultivated in Z8 medium (Skulberg & Skulberg 1990) and f/2 medium at 27 psu (Guillard & Ryther 1962) respectively. Freshwater and marine diatoms were grown in Z8 and f/2 (32 psu) media supplemented with silica. *Nodularia spumigena* was cultivated in f/2 medium with salinity adjusted to 15 psu. Composition of the media used is given in Appendix I.

Table V: List of species used in the bioassays**.**

Class	Order	Species	Strain	Origin	Toxicity	Habitat
Cyanophyceae	Chroococcales	*Microcystis aeruginosa*	LMECYA87	Magos reservoir, Santarém 1998 Portugal	Microcystins 0,33 µg.mg -1 DW	Freshwater
			LMECYA159	Magos reservoir, Santarém 2003 Portugal	Microcystins 1,32 µg.mg -1 DW	
	Nostocales	*Aphanizomenon gracile*	LMECYA40	Crato reservoir, Portalegre 1996 Portugal	PSP 24,28 mole.mg^{-1} DW	
		Anabaena circinalis	LMECYA123C	Montargil reservoir, Ponte de Sor 2000 Portugal	Not determined	
			LMECYA126			
		Anabaena sp.	LMECYA182	Vitonogales, Guadiana river, 1999 Spain	Not determined	
		Anabaenopsis circularis	LMECYA206	Nafarros pound, Sintra, 2003 Portugal	Not determined	
		Nodularia spumigena	—	North Sea, Skagerrak, 2000 Denmark	Not determined	Brackish water
	Oscillatoriales	*Leptolyngbya* sp.	LMECYA173	Hydrothermal pound, Portugal	Not determined	Freshwater
		Planktothrix rubescens	LMECYA203	Beliche reservoir, Algarve 2005 Portugal	Microcystins 3,42 µg.mg -1 DW	
Chlorophyceae	Chlorococcalles	*Ankistrodesmus falcatus*	LMECHL003	—	"Non toxic"	Freshwater
		Scnedesmus obliquus	LMECHL001	—	"Non toxic"	
	Volvocales	*Chlamydomonas* sp.	109	—	"Non toxic"	Marine
Prasinophyceae	Chlorodendrales	*Tetraselmis suecica*	—	—	"Non toxic"	Marine
Bacillariophyceae	Bacillariales	*Phaeodactylum tricornutum*	121	—	"Non toxic"	Marine
	Biddulphiales	*Chaetoceros danicus*	—	Cascais, 2006 Portugal	"Bloom forming non toxic"	
		Cyclotella sp.	—	S. Domingos reservoir Peniche, 2007 Portugal	"Non toxic"	Freshwater
Haptophyceae	Pavlovales	*Diacronema* sp.	68		"Non toxic"	Marine
Eustigmatophyceae	*	*Nannochloropsis* sp.	—	—	"Non toxic"	Marine
Dinophyceae	Gymnodinialcs	*Amphidinium carterae*	295	Óbidos Lagoon, 1986 Portugal	Not determined	Marine

Growth inhibition bioassays

A rapid 96-well microplate bioassay developed by Schrader *et al.* (1997) for screening selective algicide compounds against microalgae and cyanobacteria was used in this study with slight modifications. Figure 2 shows one of the microplates prepared for this study and illustrates the experimental design used in the bioassays. The peripheral wells of the plates (first and last row and first and last columns) were filled with 200 µL of culture medium. No cultures and no tested compounds were added to these peripheral wells. Stock solutions of each compound were micropipetted asseptically into the second row of the plate wells (200 µL per well). One of the wells from this second row (well B8) was filled with 200 µL of culture medium with no tested compound added (control). Subsequent wells initially containing 100 µL of culture medium were used for the serial dilution (1:2,

v/v) of the test compounds (and control) from this second row of wells. Aliquots (100 µL) of late exponential growing stock cultures were added to the wells of the microplates at three different concentrations. Three replicates were used for each concentration and control. The total assay volume in each well was 200 µL. Final yields of bacillamides in the wells were from 160 $\mu g.ml^{-1}$ in the second row to 5 $\mu g.ml^{-1}$ in the 7^{th} row. Final yields of 2-acetyl-4-carboxilic acid were from 80 $\mu g.ml^{-1}$ in the second row to 2,5 $\mu g.ml^{-1}$ in the 7^{th} row. Final yields of tryptamine were from 20 $\mu g.ml^{-1}$ in the second row to 0,625 $\mu g.ml^{-1}$ in the 7^{th} row. Wells from the 8^{th} column of the microplate (with no tested compounds added) were used as controls (Fig. 2).

The plates were sealed with perforated adhesive scotch tape to reduce evaporation and allow gas exchanges. Sealed plates were placed in the culture chamber under the same light and temperature conditions described for stock-culture maintenance.

Optical densities of each well were measured daily for 9 days at 450 nm using a microplate absorbance reader multiskan ascent Thermo Labsystems. In addition, all plates were examined daily with the inverted microscope to check for contaminations and to verify the activity of mobile organisms (flagellates and gliding cyanobacteria).

Standard curves relating spectrophotometric absorbance readings at 450 nm with cell concentration determined by counting were obtained for each cultured organism (Appendix III). Counting was performed in a Sedgwick-Rafter chamber under 200-400x magnification — 100 μm^2 were counted. These curves were used to determine cell concentrations based on spectrophotometric readings of the wells during the growth periods.

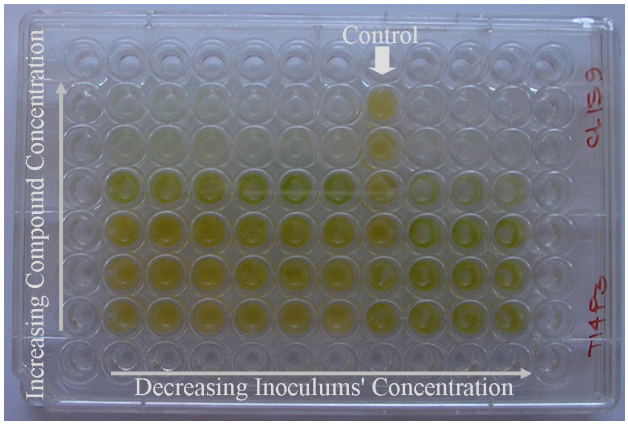


Figure 2: Experimental design of 96well microplate used in dose-responses bioassays.

Mean values and the coefficient of variation (standard deviation/mean) of optical density measurements from three replicates at each concentration and controls were calculated and used to estimate cultures growth over a 216-h period.

Growth inhibition values (%) were calculated after integrating the areas of the growth curves obtained for each of the experimental and control conditions —

$$A = t_1 x\ (N_1-N_0)/2 + (t_2-t_1)x(N_1+N_2-2N_0)/2 + (\ldots..) + (t_n-t_{n-1})x(N_{n-1}+N_n-2N_0)/2$$

Where: A= Integral area of the growth curve

t_1 is the time between the start of the assay and the first measurement (24h)

t_n is the time between the start of the assay and the n^{th} measurement

N_0 is the optical density of the well at time 0

N_1 is the optical density of the well at time t_1 (24h)

N_n is the optical density at time t_n

— by using the following equation:

$$I_{Ai}\ (\%) = 100\ X\ (Ac - A_i)/A_c$$

Where: A_{Ai} = the percentage growth inhibition obtained with the concentration i;

A_i = the integral area of the growth curve obtained with the concentration i;

A_c = the integral area of the growth curve obtained for the control

Estimations of LOEC (Lowest-Observable-Effect-Concentration) were performed using graphs obtained by plotting the % inhibition values against logarithmic concentrations of tested compounds. The IC50 values (i.e. the concentrations of tested compounds that inhibited growth by 50% relative to the controls) were obtained from the sigmoidal inhibition curves fitted by probit regression analysis (SPSS for Windows v.1.3). The dose-response equations were X^2-tested for 95% confidence. IC50s were used to calculate differential specificity values (DS= IC50 for Algae / IC50 for Cyanobacteria).

Algicidal *versus* algistatic properties of the compounds.

To differentiate between algistatic and algicidal properties of tested compounds, samples of 25 µL were removed from apparently inhibited cultures, and added to new microplate wells previously filed with 175 µL of fresh culture medium. The new plates were then incubated in the culture chamber under the light and temperature conditions previously described. Optical measurements were taken in time spaces of 72h for 216h. When growth was observed, the concentration of the tested compound to which the organism had been

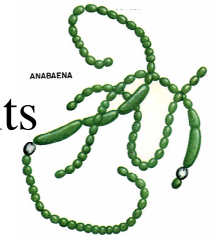
previously exposed was considered to be algistatic. When no growth appeared on the subculture, the concentration of the tested compound and treatment time was considered to be algicidal (Fitzgerald 1964).

Cell ultrastructure analysis

Two strains were selected for ultrastructural studies: *Aphanizomenon gracile* LMECYA40, and *Ankistrodesmus falcatus* LMECHL003. Cells were treated with bacillamide (0-control, 40 and 80 $\mu g.ml^{-1}$) in 24-well tissue culture plates for 144h. Plates were inspected daily under the inverted microscope and samples (3 ml) for transmission electron microscopy (TEM) studies were taken at 72h and 144h of treatment. The samples were centrifuged at 3500 rpm for 10 min. For the cyanobacterial strain, gentle vacuum was applied to force the gas vacuole-filled filaments to sediment.

For TEM preparation, samples were prefixed in 2.5% glutaraldehyde in culture medium 30 min at 20ºC. They were then transferred to 2.5% glutaraldehyde in 0.1M cacodylate buffer and 2.5mM $CaCl_2$ overnight at 4ºC. Postfixation was done in 1% buffered osmium tetroxide for 3h and cells were then washed three times in 0.1M cacodylate buffer for 10 min. To increase cell contrast, they were incubated with 1% uranyl acetate for 1h in the dark and rinsed with distilled water for 10 min. Cells were dehydrated in a graded ethanol series (30%, 50%, 70%, 95%, 20 min each and absolute ethanol 3x20 min) at 4ºC, with a final step with propylene oxide, for 10 min. After dehydration, cells were embedded in Spurr's low-viscosity epoxy resin overnight, placed in Beem capsules and polymerized at 60ºC for 24h. Silver-grey sections were cut in a Leica ultracutR ultramicrotome with a Diatome diamond knife. Sections were collected on 150-mesh formvar coated grids and double stained with 2% uranyl acetate (40 min in the dark) and lead citrate (5 min). Sections were air dried and examined under a transmission electron microscope (Philips Morgagni 268D). Micrographs were acquired with a Megaview III CCD.

Results



Effect of bacillamide on the growth of cyanobacteria and eukaryotic algae.

The growth curves obtained for each test organism grown under different bacillamide concentrations are shown in Fig. 3. Most strains seem to have adapted quite well to the growing conditions in the microplate. Control treatments showed a clear exponential growth phase starting from the first or the second day of the test period and reaching considerable densities at the end of the 216-h period. Some strains exhibited a typical sigmoid growth curve reaching the start of the stationary growth phase at the end of the experiment while others showed a continuous steady growth throughout the whole period.

Differences between the test treatments and the appropriate control treatment started to be noticed within the first days of incubation for most cyanobacteria, namely the Chroococcales and the Nostocales. For these strains, inhibition of growth became clear with bacillamide treatments starting from 20-160 $\mu g.mL^{-1}$ and showed an apparent dose-dependent relationship. Concentrations of bacillamide above 80 $\mu g.mL^{-1}$ completely prevented the growth of *Microcystis aeruginosa*, *Anabaena circinalis*, *Anabaenopsis circularis* and *Aphanizomenon gracile* cultures. Other strains within the Oscillatoriales seemed to be quite insensitive to bacillamide concentrations up to 160 $\mu g.mL^{-1}$. Bacillamide also affected the growth of the freshwater and marine diatoms and the growth of the haptophyte *Diacronema* sp. in a dose-dependent manner. On the other hand, the freshwater and marine Chlorophyceae and the marine cyanobacteria *Nodularia spumigena* were only slightly affected by bacillamide in concentrations up to 160 $\mu g.mL^{-1}$. The marine algae *Tetraselmis suecica* and *Nannochloropsis* sp. were not affected by bacillamide.

Table VI presents the bacillamide LOEC and IC50 values estimated at 216-h for each of the tested organisms. Bacillamide was clearly more toxic towards the cyanobacteria *M. aeruginosa*, *A. circinalis* and *A. gracile*, as shown by the lower LOEC and IC50 values, than to other cyanobacteria including *Anabaena* sp., *A. circularis*, and *Leptolyngbya* sp.. Based on LOEC values, these freshwater cyanobacteria were more sensitive than the freshwater Chlorophyceae *Ankistrodesmus falcatus* (Table VII). However, bacillamide is

even more toxic to the diatom *Cyclotella* sp. than to most of the freshwater cyanobacteria. On the other hand, the toxic cyanobacterium *Planktothrix rubescens* was not affected by bacillamide even at the highest concentration screened (160 $\mu g.mL^{-1}$).

By comparing the LOEC and the IC50 values within the marine organisms, the cyanobacteria *Nodularia spumigena* was found to be more sensitive to bacillamide than both the chlorophyte *Chlamydomonas* sp, the eustigmatophyte *Nannochloropsis* sp. and the prasinophyte *T. suecica*. However, bacillamide was not selectively toxic toward this marine cyanobacterium as shown by the low differential selectivity obtained for *N. spumigena* relative to the marine diatom *P. tricornutum* and the marine haptophyte *Diacronema* sp. (Table VIII). In fact, the marine diatom *P. tricornutum* was amongst the organisms most clearly affected by bacillamide in a dose-dependent manner as shown in the graphs of Fig. 3. Bacillamide affected both freshwater and marine diatoms at doses similar to the ones that affected the most sensitive cyanobacteria.

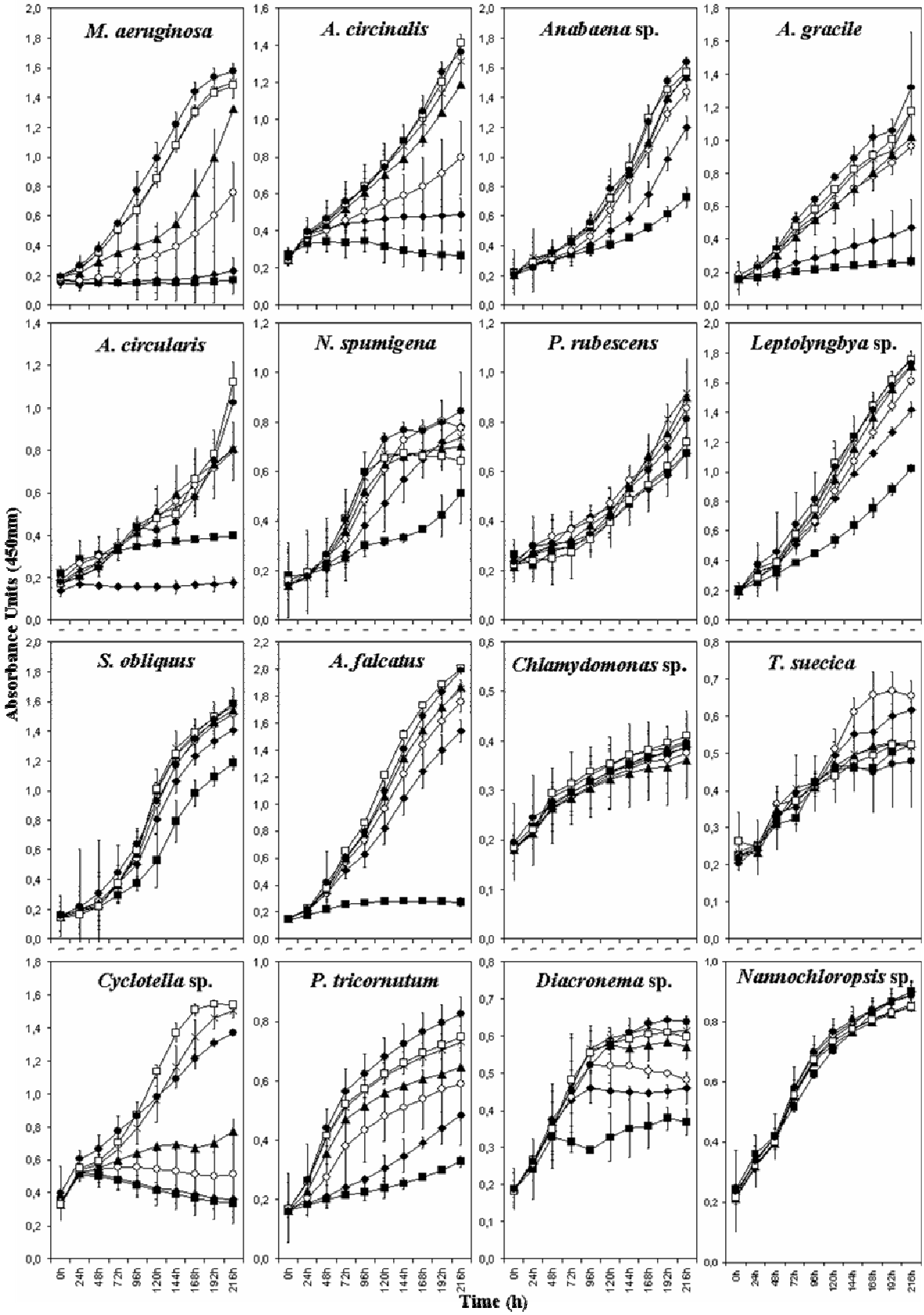


Figure 3: Growth curves obtained for cyanobacteria and microalgae exposed to different bacillamide concentrations. Values represent means from triplicates and bars represent correspondent variation coefficients (standard deviation/mean values). (●)Control, (□)5µg.ml^{-1}, (*)10µg.ml^{-1}, (▲)20µg.ml^{-1}, (○)40µg.ml^{-1}, (♦)80µg.ml^{-1}, (■)160µg.ml^{-1}.

Table VI: LOEC-216h and IC50-216h of bacillamide for each of the cyanobacteria and microalgae screened. Marine species are marked with (*). The right column indicates the range of IC50 values within a 95% confidence interval.

	LOEC	IC50-216h	
M. aeruginosa	20	29	(23,8-35,8)
A. circinalis	20	69,2	(57,9-83,8)
Anabaena sp.	40	117	(100,4-140,0)
A. gracile	10	57,6	(46,7-71,4)
A. circularis	80	146,7	(131,4->160)
N. Spumigena *	80	116,8	(98,6-144,1)
P. rubescens	160	>160	>160
Leptolyngbya sp.	40	140,1	(119,9->160)
S. obliquus	>160	>160	>160
A. falcatus	80	101	(91,8-113,3)
Chlamydomonas sp.*	>160	>160	>160
T. Suecica *	>160	>160	>160
P. Tricornutum *	20	70,3	(55,2-91,6)
Cyclotella sp.	10	59,8	(39,8-90,9)
Diacronema sp.*	40	133,6	(116,4-157,7)
Nannochloropsis sp.*	>160	>160	>160

Table VII: Differential selectivities (DS) of bacillamide towards freshwater organisms. DS values are expressed as IC50-216h of algae / IC50-216 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria. Asterisks (*) - Differential selectivity could not be determined since no growth inhibition was observed for the algae even at the highest concentration screened.

		Algae		
		S. obliquus	*A. falcatus*	*Cyclotella* sp.
Cyanobacteria	*M. aeruginosa*	*	3,48	0,76
	A. circinalis	*	1,46	0,32
	Anabaena sp.	*	0,86	0,19
	A. gracile	*	1,75	0,38
	A. circularis	*	0,69	0,15
	P. rubescens	*	0,62	0,14
	Leptolyngbya sp.	*	0,72	0,16

Table VIII: Differential selectivities (DS) of bacillamide towards marine organisms. DS are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria. Asterisks (*) - Differential selectivity could not be determined since no growth inhibition was observed for the algae even at the highest concentration screened.

	Algae				
	Chlamydomonas sp.	*P. tricornutum*	*Diacronema* sp.	*Nannochloropsis* sp.	*T. suecica*
N. spumigena	*	0,602	1,144	*	*

Effect of OH-bacillamide on the growth of cyanobacteria and eukaryotic algae.

The growth curves obtained for each test organism grown under different OH-bacillamide concentrations are shown in Fig. 4. In general, the growth obtained in the control treatments were comparable to the ones exhibited in the previous test performed with bacillamide. However, for most cultures, the effects of OH-bacillamide on cultures growth were quite different from the ones obtained with bacillamide, when appropriate concentrations are compared. Thus, for the cyanobacteria *M. aeruginosa*, *A.circularis*, *P. rubescens* and *Leptolyngbya* sp. and for the chlorophytes *S. obliquus* and *A. falcatus*, no growth inhibition was observed with OH-bacillamide treatments even at the highest concentration screened. The marine prasinophyceae *T. suecica* also showed not to be affected by OH-bacillamide and for the diatom *P. tricornutum* mild inhibitory effects could only be observed at bacillamide concentration above 80 $\mu g.mL^{-1}$. Interestingly, the growths of *Chlamydomonas* sp and *Nannochloropsis* sp., which were not affected by bacillamide treatments in the previous test, were now clearly inhibited by OH-bacillamide in a dose-dependent manner. The LOEC-216h and the IC50-216h values of OH-bacillamide for each of these strains are shown in Table IX. Within the freshwater species, LOEC results show that *Anabaena* spp. and *A. gracile* were the only cyanobacteria found to be more sensitive to OH-bacillamide than the chlorophyceae *S. obliquus* and *A. falcatus*. For cyanobacteria, differential specificity could not be determined since no growth inhibition was observed even at the highest concentration screened for most of the algae.

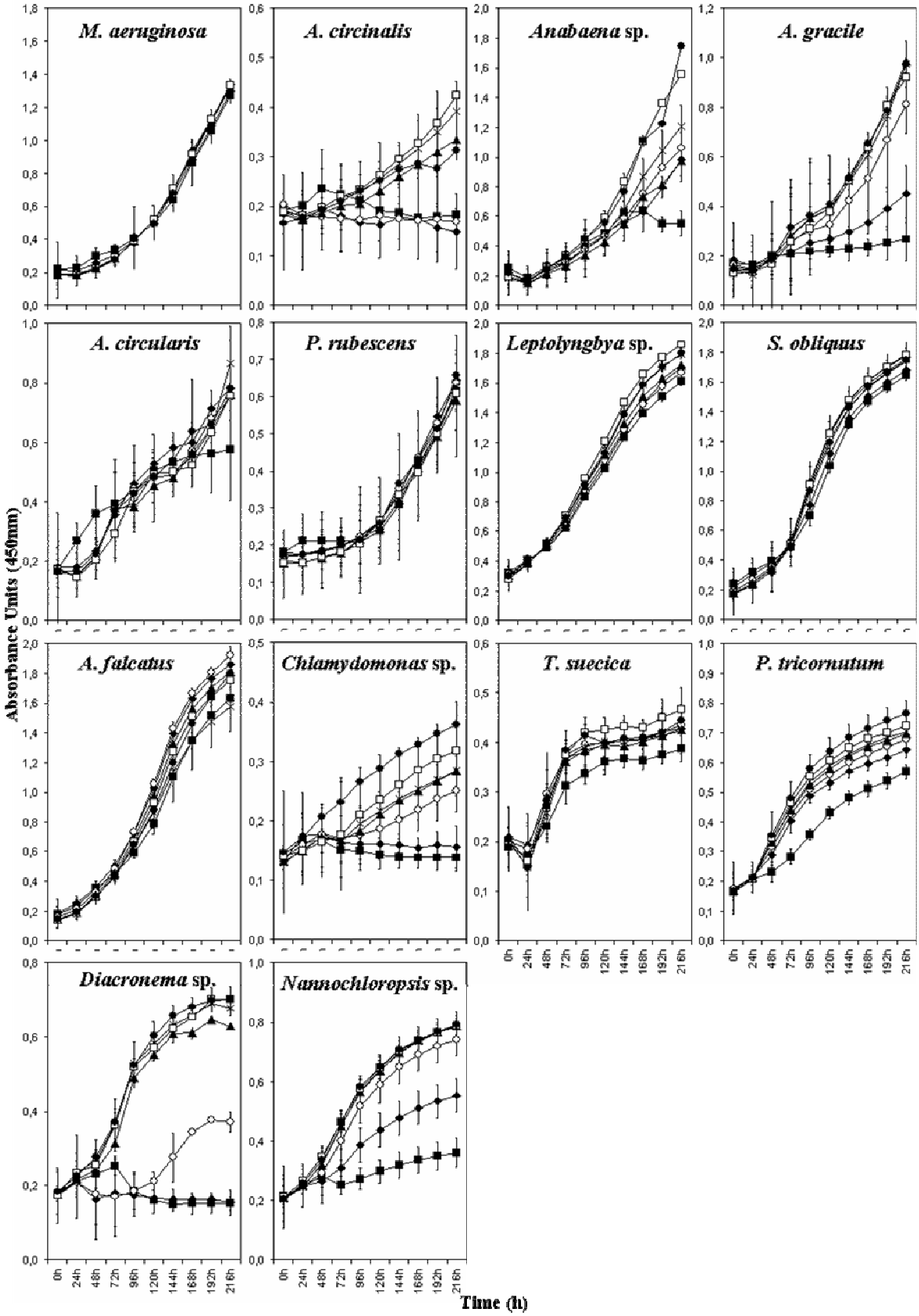


Figure 4: Growth curves obtained for cyanobacteria and microalgae exposed to different OH-bacillamide concentrations. Values represent means from triplicates and bars represent correspondent variation coefficients (standard deviation/mean values) (●)Control, (□)5µg.ml^{-1}, (*)10µg.ml^{-1}, (▲)20µg.ml^{-1}, (○)40µg.ml^{-1}, (♦)80µg.ml^{-1}, (■)160µg.ml^{-1}.

Table IX: LOEC-216h and IC50-216h of OH-bacillamide for each of the cyanobacteria and microalgae screened. Marine species are marked with (*). The right column indicates the range of IC50 values within a 95% confidence interval.

	LOEC	IC50-216h	
M. aeruginosa	>160	>160	>160
A. circinalis	20	42,1	(--)
Anabaena sp.	10	115,5	(85,2->160)
A. gracile	40	85,2	(70,7-104,6)
A. circularis	>160	>160	>160
P. rubescens	>160	>160	>160
Leptolyngbya sp.	160	>160	>160
S. obliquus	>160	>160	>160
A. falcatus	>160	>160	>160
Chlamydomonas sp.*	<5	39,3	(26,4-55,6)
*T. suecica**	160	>160	>160
*P. tricornutum**	80	>160	(146,5->160)
Diacronema sp.*	40	>160	(--)
Nannochloropsis sp.*	40	104,2	(94,5-115,8)

Effect of Br-bacillamide on the growth of cyanobacteria and eukaryotic algae.

Br-bacillamide seems to affect the growth of all the cyanobacteria in a dose-dependent manner (Fig.5). The lowest IC-50-216h of Br-bacillamide was obtained for the Nostocalles *Anabaena circinalis*, *Anabaenopsis circularis* and *Aphanizomenon gracile* (table XI). Based on IC50-216h values, Br-bacillamide was far more toxic to these cyanobacteria than to the chlorophytes *S. obliquus* and *A. falcatus*, as indicated by the high differential selectivity values (Table XII). However, IC50-216h values obtained *M. aeruginosa*, *Anabaena* sp. *Leptolyngbya* sp. and *P. rubescens* were similar to the ones obtained for the freshwater chlorophyceae and differential selectivity of Br-bacillamide towards these cyanobacteria was low (Table XII). Within marine microalgae, IC50s were always above 150 $\mu g.mL^{-1}$.

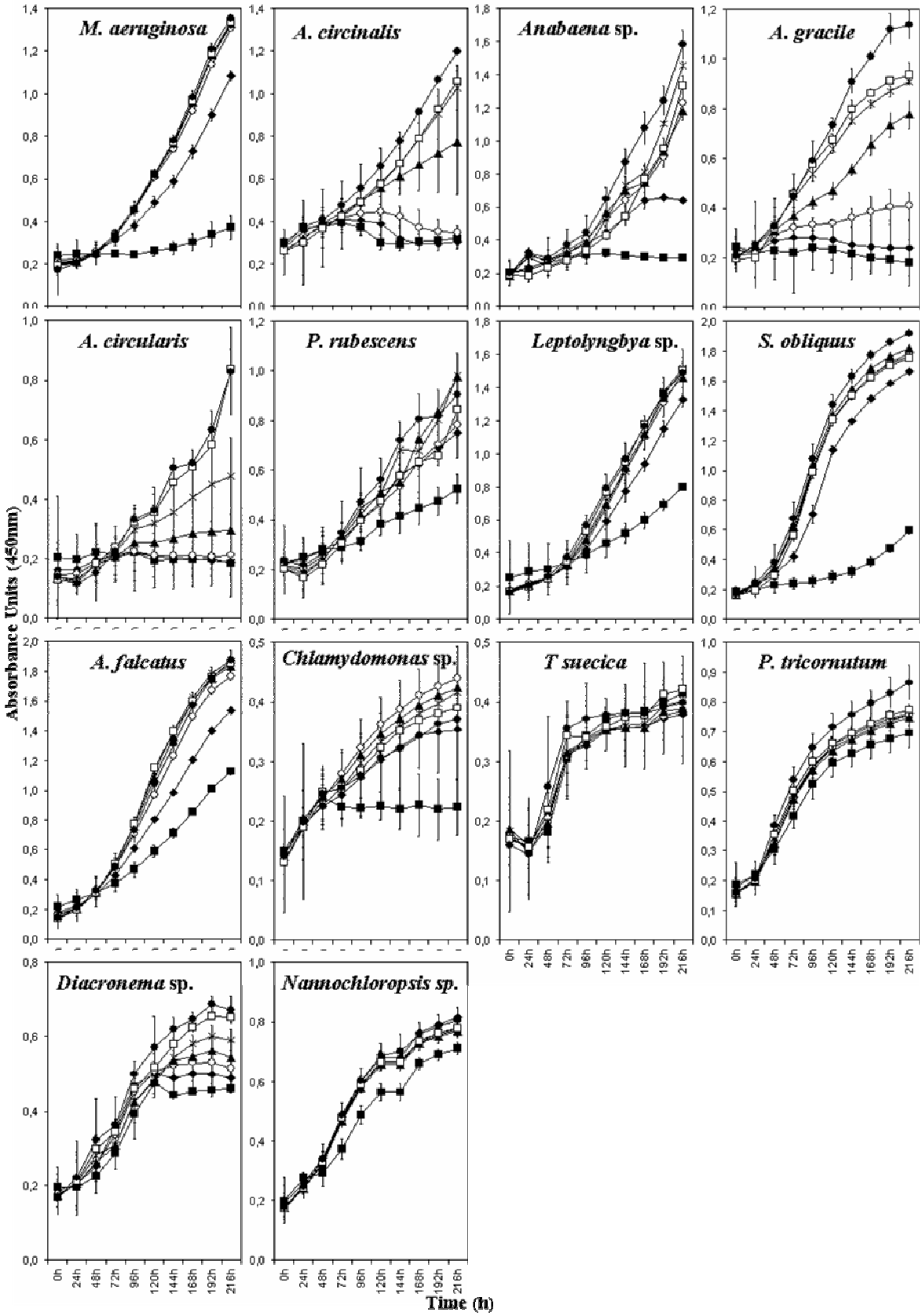


Figure 5: Growth curves obtained for cyanobacteria and microalgae exposed to different Br-bacillamide concentrations. Values represent means from triplicates and bars represent correspondent variation coefficients (standard deviation/mean values). (●)Control, (□)5µg.ml^{-1}, (*)10µg.ml^{-1}, (▲)20µg.ml^{-1}, (○)40µg.ml^{-1}, (♦)80µg.ml^{-1}, (■)160µg.ml^{-1}.

Table XI: LOEC-216h and IC50-216h of Br-bacillamide for each of the cyanobacteria and microalgae screened. Marine species are marked with (*). The right column indicates the range of IC50 values obtained for a 95% confidence interval.

	LOEC	IC50-216h	
M. aeruginosa	80	93,1	(87,2-99,6)
A. circinalis	≤5	52,3	(37,1-72,7)
Anabaena sp.	≤5	124,6	(101,8->160)
A. gracile	5	25,7	(19,1-32,9)
A. circularis	10	34,8	(14,4-65,5)
P. rubescens	20	146,5	(119,9->160)
Letolyngbya sp.	80	120	(102,9-145,8)
S. obliquus	80	106,1	(95,3-119,3)
A. falcatus	80	142,9	(130,0-159,5)
Chlamydomonas sp.*	>160	154,2	(142,1->160)
T. Suecica *	>160	>160	>160
P. Tricornutum *	160	>160	>160
Diacronema sp.*	10	153,9	(123,5->160)
Nannochloropsis sp.*	160	>160	>160

Table XII: Differential selectivities (DS) of Br-bacillamide towards freshwater organisms. DS values are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria.

		Algae	
		S. obliquus	*A. falcatus*
Cyanobacteria	*M. aeruginosa*	1,14	1,54
	A. circinalis	2,03	2,73
	Anabaena sp.	1,29	1,75
	A. gracile	4,13	5,56
	A. circularis	3,05	4,11
	P. rubescens	0,72	0,98
	Leptolyngbya sp.	0,81	1,09

Effect of Cl-bacillamide on the growth of cyanobacteria and eukaryotic algae.

The growth curves obtained for each test organism grown under different Cl-bacillamide concentrations are shown in Fig. 6. The effects of Cl-bacillamide on cyanobacteria and freshwater microalgae were similar to the ones obtained with Br-bacillamide in the previous test. However, Cl-bacillamide seems to be more effective for marine species than its bromo-substituted analogue, as shown by the lower LOEC and IC50 values (Table XIII). Among freshwater cyanobacteria, *P. rubescens*, *Leptolyngbya* sp. and *M. aeruginosa* were less sensitive to Cl-bacillamide than the other screened strains. The IC50-216h of Cl-bacillamide towards these cyanobacteria were always above 118 $\mu g.mL^{-1}$, while for the other freshwater cyanobacteria IC50-216h values were always bellow 56 $\mu g.mL^{-1}$. Based on IC50-216h values, Cl-bacillamide was 2 to 5 times more toxic to these cyanobacteria than to chlorophytes (Table XIII). Thus, when applied at appropriate concentrations (up to 70 $\mu g.mL^{-1}$) Cl-bacillamide can selectively inhibit the growth of some freshwater cyanobacteria without affecting the growth of chlorophytes. However, treatments with Cl-bacillamide at $5\mu g.mL^{-1}$ or above are expected not only to affect the growth of sensitive cyanobacteria but also to affect the growth of the freshwater diatom *Cyclotella* sp. to the same or even at a higher extent (Table XIV). Thus, Cl-bacillamide is not expected to selectively inhibit the growth of cyanobacteria, especially if centric diatoms are also present (Table XIV).

The marine cyanobacterium *N. spumigena* was clearly sensitive to the lowest Cl-bacillamide concentrations screened ($5\mu g.mL^{-1}$), though LOEC values for this cyanobacterium were similar to the LOECs obtained for other marine algae (Table XIII). As shown by the low differential selectivity values (Table XV), Cl-bacillamide treatments in marine environments seem to affect more effectively, *in vitro*, the growth of most eukaryotic algae than the growth of cyanobacteria.

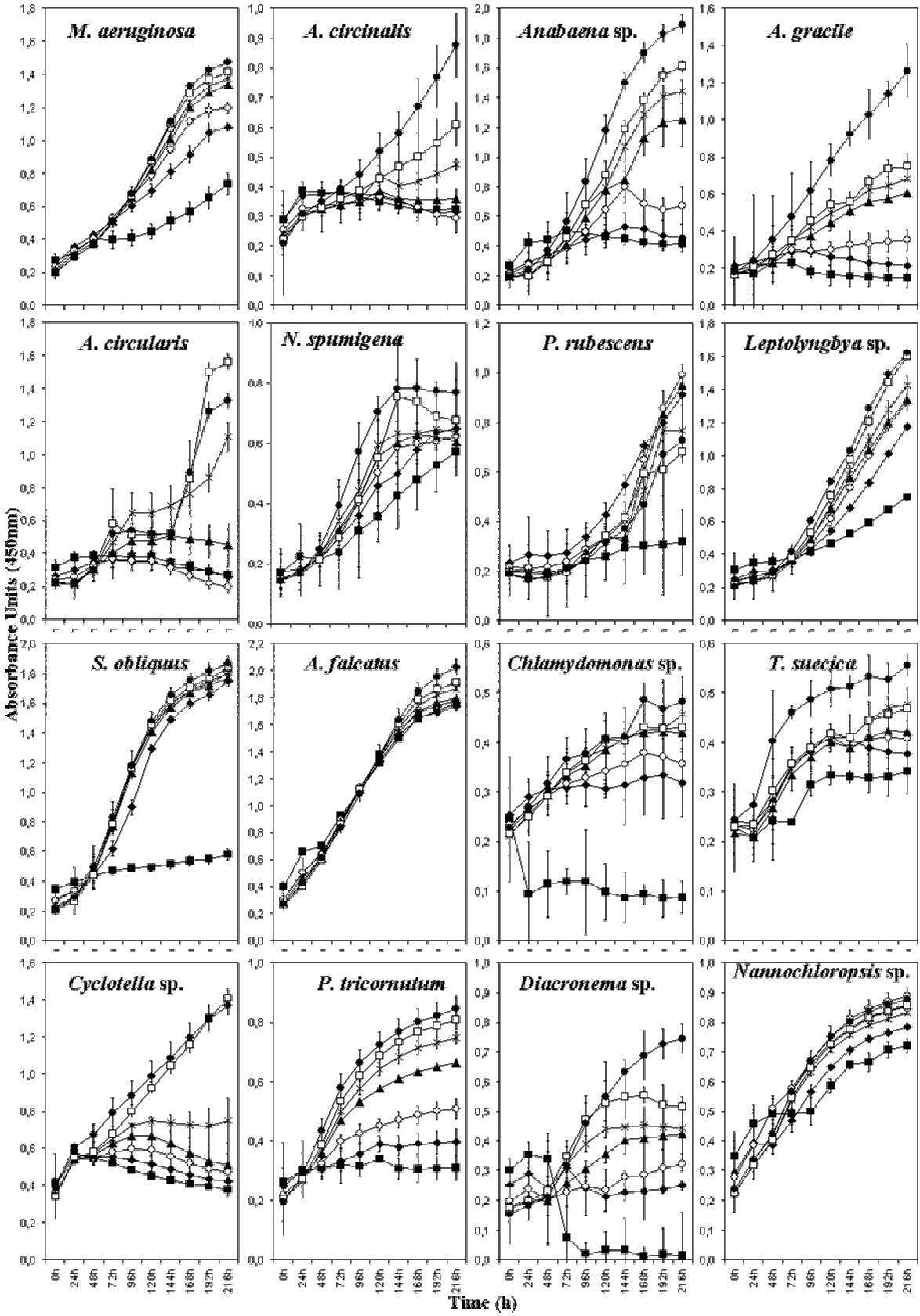


Figure 6: Growth curves obtained for cyanobacteria and microalgae exposed to different Cl-bacillamide concentrations. Values represent means from triplicates and bars represent correspondent variation coefficients (standard deviation/mean values). (●)Control, (□)5µg.ml^{-1}, (*)10µg.ml^{-1}, (▲)20µg.ml^{-1}, (○)40µg.ml^{-1}, (♦)80µg.ml^{-1}, (■)160µg.ml^{-1}.

Table XIII: LOEC-216h and IC50-216h of Cl-bacillamide for each of the cyanobacteria and microalgae screened. Marine species are marked with (*). The right column indicates the range of IC50 values obtained for a 95% confidence interval.

	LOEC	IC50-216h	
M. aeruginosa	20	118	(108,1-130,1)
A. circinalis	<5	28,4	(<5-57,3)
Anabaena sp.	<5	55,9	(37,7-78,9)
A. gracile	<5	20,8	(14,5-27,5)
A. circularis	10	43,7	(14,5-27,5)
N. Spumigena *	<5	124,6	(99,5->160)
P. rubescens	80	150,1	(119,7->160)
Leptolyngbya sp.	10	99,1	(84,4-119,2)
S. obliquus	80	110,6	(100,8-122,2)
A. falcatus	>160	>160	>160
Chlamydomonas sp.*	20	54,8	(47,6-63,9)
T. Suecica *	<5	85	(63,6-120,1)
P. Tricornutum *	10	56,1	(43,9-72,6)
Cyclotella sp.	<5	37,4	(16,6-58,3)
Diacronema sp.*	<5	20,7	(15,6-27,8)
Nannochloropsis sp.*	80	153,8	(135,7->160)

Table XIV: Differential selectivities (DS) of Cl-bacillamide towards freshwater organisms. DS values are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria. Asterisks (*) - differential selectivity could not be determined since no growth inhibition was observed for the algae even at the highest concentration screened.

		Algae		
		S. obliquus	*A. falcatus*	*Cyclotella* sp.
Cyanobacteria	*M. aeruginosa*	0,94	*	0,32
	A. circinalis	3,89	*	1,32
	Anabaena sp.	1,98	*	0,67
	A. gracile	5,32	*	1,8
	A. circularis	2,53	*	0,86
	P. rubescens	0,74	*	0,25
	Leptolyngbya sp.	1,12	*	0,38

Table XV: Differential selectivities (DS) of Cl-bacillamide towards marine organisms. DS are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria.

	Algae				
	Chlamydomonas sp.	*P. tricornutum*	*Diacronema* sp.	*Nannochloropsis* sp.	*T. suecica*
N. spumigena	0,44	0,45	0,17	1,23	0,68

Effect of Fl-bacillamide on the growth of cyanobacteria and eukaryotic algae.

The growth curves obtained for each test organism grown under different Fl-bacillamide concentrations are shown in Fig. 7. Differences between the test treatments and the appropriate control treatments were quite similar to the ones obtained in the previous tests performed with Cl- and Br-bacillamide. However, for the freshwater *S. obliquus* and for the marine *Chlamydomonas* sp., *T. suecica* and *Nannochloropsis* sp., no growth inhibition was observed with Fl-bacillamide, even at the highest concentrations screened. The cyanobacteria, the haptophyceae (*Diacronema* sp.) and the diatoms (*Cyclotella* sp. and *P. tricornutum*) were the most affected by Fl-bacillamide. Within the cyanobacteria, the Chroococcales and the Nostocales were more sensitive to Fl-bacillamide than the Oscillatoriales, as shown by LOEC-216h and IC50-216h values (Table XVI). Within the diatoms, the freshwater *Cyclotella* sp. was more sensitive to Fl-bacillamide than the marine *P. tricornutum*.

Based on IC50-216h, Fl-bacillamide is more toxic to freshwater cyanobacteria than to the freshwater chlorophytes. However, the selectivity values relative to *Cyclotella* sp. (Table XVII) suggest the possibility of deleterious effects on diatoms from Fl-bacillamide treatments.

Nodularia spumigena was far more sensitive to Fl-bacillamide than most of the other marine organisms (Table XVI). However, Fl-bacillamide was not selectively toxic toward this marine cyanobacterium as it also affects the marine haptophyte *Diacronema* sp. (Table XVIII)

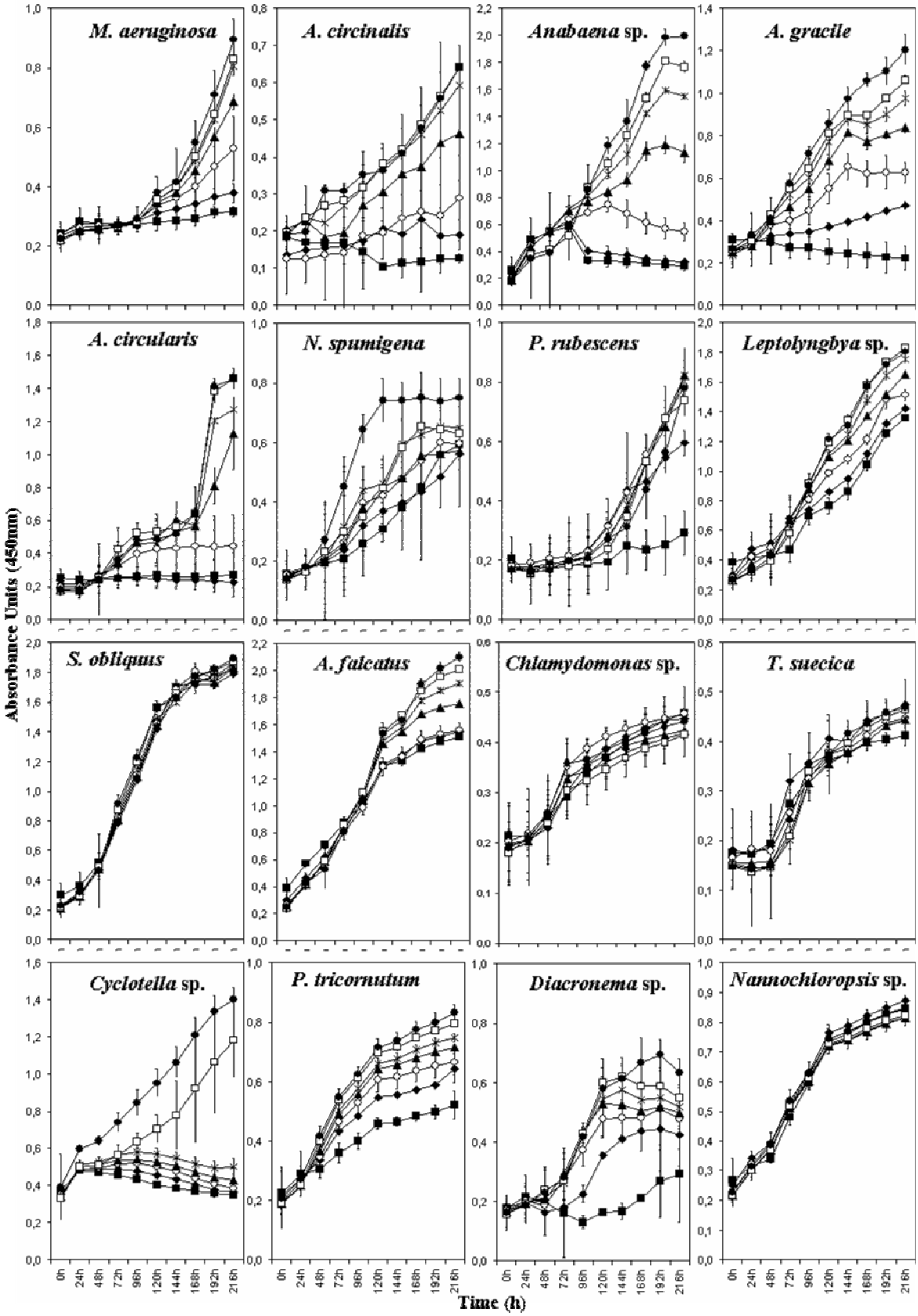


Figure 7: Growth curves obtained for cyanobacteria and microalgae exposed to different Fl-bacillamide concentrations. Values represent means from triplicates and bars represent correspondent variation coefficients (standard deviation/mean values). (●)Control, (□)5µg.ml^{-1}, (*)10µg.ml^{-1}, (▲)20µg.ml^{-1}, (○)40µg.ml^{-1}, (♦)80µg.ml^{-1}, (■)160µg.ml^{-1}.

Table XVI: LOEC-216h and IC50-216h of Fl-bacillamide for each of the cyanobacteria and microalgae screened. Marine species are marked with (*). The right column indicates the range of IC50 values obtained for a 95% confidence interval.

	LOEC	IC50-216h	
M. aeruginosa	20	80,5	(66,5-99,2)
A. circinalis	20	39,5	(30,1-54,2)
Anabaena sp.	20	61,1	(47,4-61,1)
A. gracile	20	42,9	(32,2-40,2)
A. circularis	10	41,5	(--)
N. Spumigena *	<5	89	(61,4-144,1)
P. rubescens	80	128	>160
Letolyngbya sp.	40	145,4	(145,1-126,7)
S. obliquus	>160	>160	>160
A. falcatus	20	>160	(157,4->160)
Chlamydomonas sp.*	>160	>160	>160
T. Suecica *	>160	>160	>160
P. Tricornutum *	10	134,3	(117,4-157,9)
Cyclotella sp.	<5	21,8	(<0,625-42,4)
Diacronema sp.*	10	71,8	(60,8-86,4)
Nannochloropsis sp.*	>160	>160	>160

Table XVII: Differential selectivities (DS) of Fl-bacillamide towards freshwater organisms. DS values are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria. Asterisks (*) - differential selectivity could not be determined since no growth inhibition was observed for the algae even at the highest concentration screened.

		Algae		
		S. obliquus	*A. falcatus*	*Cyclotella* sp.
Cyanobacteria	*M. aeruginosa*	*	*	0,27
	A. circinalis	*	*	0,55
	Anabaena sp.	*	*	0,36
	A. gracile	*	*	0,51
	A. circularis	*	*	0,53
	P. rubescens	*	*	0,17
	Leptolyngbya sp.	*	*	0,15

Table XVIII: Differential selectivities (DS) of Fl-bacillamide towards marine organisms. DS are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria. Asterisks (*) - differential selectivity could not be determined since no growth inhibition was observed for the algae even at the highest concentration screened.

	Algae				
	Chlamydomonas sp.	*P. tricornutum*	*Diacronema* sp.	*Nannochloropsis* sp.	*T. suecica*
N. spumigena	*	1,51	0,81	*	*

Effect of I-bacillamide on the growth of cyanobacteria and eukaryotic algae.

I-bacillamide clearly affected the growth of all cyanobacteria and eukaryotic algae screened in this assay (Fig. 8). A few strains seemed to recover from an initial period of lethargy and reassume growth within a few days after the onset of the exposure period (e.g. *N. spumigena* and *A. falcatus*). However, I-bacillamide treatments of 160 $\mu g.mL^{-1}$ completelly suppressed growth of most organisms. The freshwater chlorophyte *A. falcatus* and the cyanobacteria *P. rubescens* and *Leptolyngbya* sp. were the least sensitive to I-bacillamide, showing LOEC values of 80 $\mu g.mL^{-1}$ (Table XIX). For all the other tested strains the inhibitory effects of I-bacillamide started to be noted at 5 to 40 $\mu g.mL^{-1}$. In general, I-bacillamide was more toxic to most cyanobacteria and microalgae than the other tested bacillamides. However cyanobacteria were often equally or less sensitive to I-bacilamide then other microalgae and differential selectivity towards cyanobacteria was low (Tables XX, XXI).

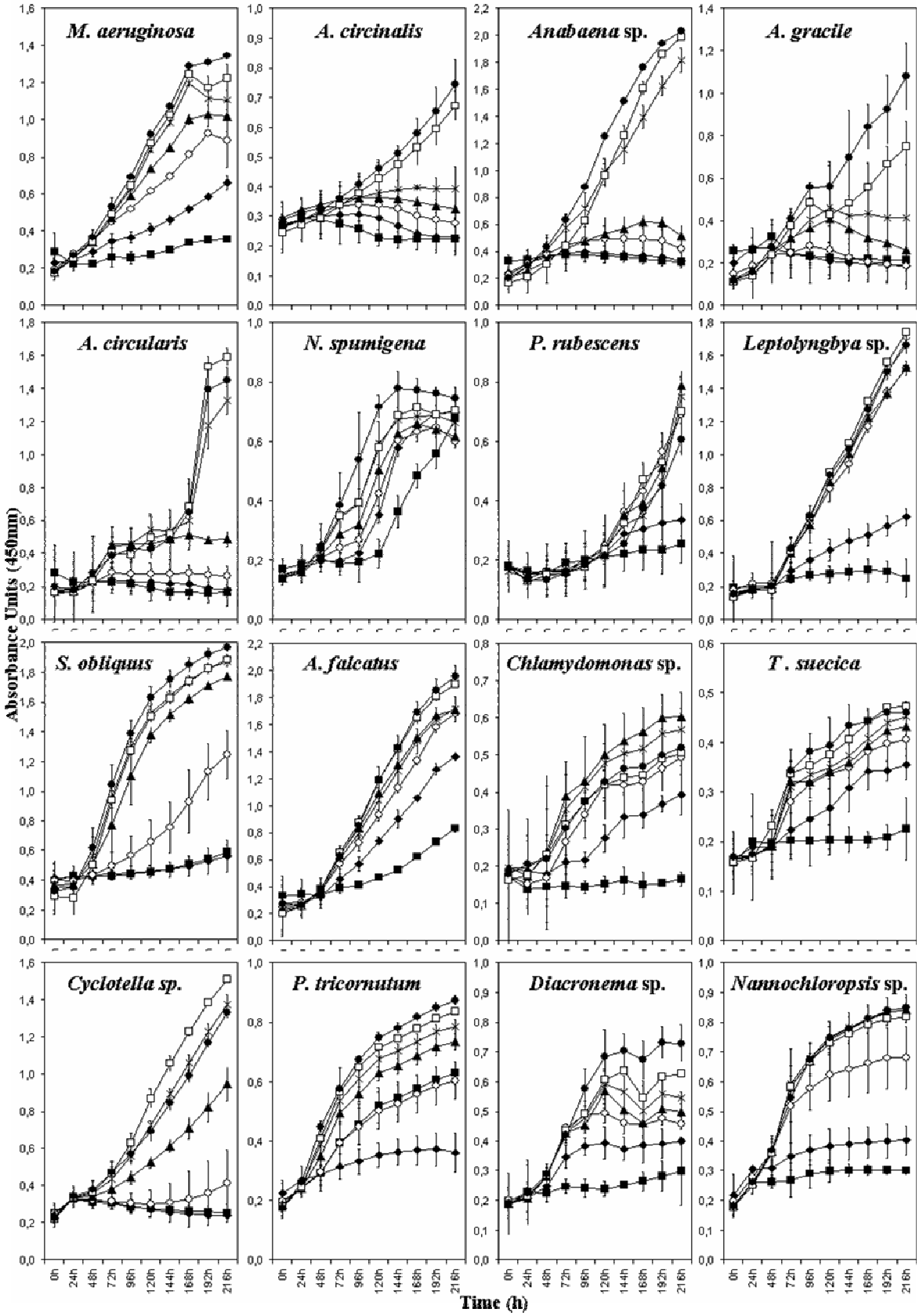


Figure 8: Growth curves obtained for cyanobacteria and microalgae exposed to different I-bacillamide concentrations. Values represent means from triplicates and bars represent correspondent variation coefficients (standard deviation/mean values). (●)Control, (□)5µg.ml^{-1}, (*)10µg.ml^{-1}, (▲)20µg.ml^{-1}, (○)40µg.ml^{-1}, (♦)80µg.ml^{-1}, (■)160µg.ml^{-1}.

Table XIX: LOEC-216h and IC50-216h of I-bacillamide for each of the cyanobacteria and microalgae screened. Marine species are marked with (*). The right column indicates the range of IC50 values obtained for a 95% confidence interval.

	LOEC	IC50-216h	
M. aeruginosa	10	52,6	(44,8-62,5)
A. circinalis	10	21,5	(11,6-33,4)
Anabaena sp.	10	32,1	(12,2-61,1)
A. gracile	<5	19,7	(12,9-27,1)
A. circularis	20	60,1	(48,1-78,8)
N. Spumigena *	<5	110,8	(91,1-141,2)
P. rubescens	80	122,6	(75,7-97,4)
Letolyngbya sp.	80	85,5	(75,7-97,4)
S. obliquus	20	47	(24,6-90,4)
A. falcatus	80	97,4	(86,9->110,2)
Chlamydomonas sp.*	40	73,8	(65,9-83,8)
T. Suecica *	10	102,2	(89,6-118,3)
P. Tricornutum *	10	108,6	(73,4->160)
Cyclotella sp.	10	45,6	(1,78->160)
Diacronema sp.*	<5	20,7	(55,2-71,6)
Nannochloropsis sp.*	40	93,4	(77,9-114,1)

Table XX: Differential selectivities (DS) of I-bacillamide towards freshwater organisms. DS values are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria.

		Algae		
		S. obliquus	*A. falcatus*	*Cyclotella* sp.
Cyanobacteria	*M. aeruginosa*	0,89	1,85	0,59
	A. circinalis	2,18	4,53	1,43
	Anabaena sp.	1,46	3,03	0,96
	A. gracile	2,38	4,94	1,56
	A. circularis	0,8	1,6	0,5
	P. rubescens	0,38	0,79	0,25
	Leptolyngbya sp.	0,55	1,14	0,36

Table XXI: Differential selectivities (DS) of I-bacillamide towards marine organisms. DS are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria.

	Algae				
	Chlamydomonas sp.	*P. tricornutum*	*Diacronema* sp.	*Nannochloropsis* sp.	*T. suecica*
N. spumigena	0,66	0,98	0,57	0,84	0,92

Effect of bacillamide percursors (tryptamine and 2-acetyl-1,3-thiazole-4-carboxylic acid - ATCA) on the growth of cyanobacteria and eukaryotic algae.

The preliminary assays for screening the effects of each bacillamide precursor on culture growth were performed using concentrations of tryptamine and ATCA equivalent to their correspondent molarities screened in bacillamide assays (80-2.5 $\mu g.mL^{-1}$ for tryptamine and ATCA). From these preliminary tests (data not shown) it soon became evident that ATCA exerted no inhibitory effects on cyanobacteria and eukaryotic algae, even at the highest concentrations screened. On the other hand, tryptamine was found to be much more toxic, clearly suppressing growth of most cyanobacteria and eukaryotic algae at the lowest concentration screened. In the following tests, tryptamine was diluted 1:4 and screened within a range of 0.625 $\mu g.mL^{-1}$ to 20 $\mu g.mL^{-1}$, in 2-fold concentration increments. Figure 9 shows the growth curves attained by each test organism under these tryptamine treatments. When compared with appropriate control treatments, most cultures exposed to tryptamine reduced their growth in a clear pattern of dose-response relationship. The LOEC-216h and the IC50-216h values obtained for tryptamine (Table XXII) were much lower than the ones obtained for the different bacillamides screened in the previous assays. Tryptamine treatments of 20 $\mu g.mL^{-1}$ completely prevented the growth of most organisms. However a few strains (eg. *Diacronema* sp. and *A. falcatus*) were able to recover from growth inhibition and resume exponential grow after an initial period of lethargy.

Based on IC50-216h values tryptamine was far more toxic to most freshwater cyanobacteria than to the chlorophytes *S. obliquus* and *A. falcatus*, as shown by the high differential selectivity values (Table XXII). However the comparison of the IC50-216h between freshwater cyanobacteria and the freshwater diatom *Cyclotella* sp. revealed a low differential value of tryptamine for these organisms (Table XXII).

Concerning the marine species, tryptamine showed to be selectively toxic towards the cyanobacteria N. spumigenawhen compared to most eukaryotic algae. However, Diacronema sp. was also greatly affected by tryptamine (Table XXIII).

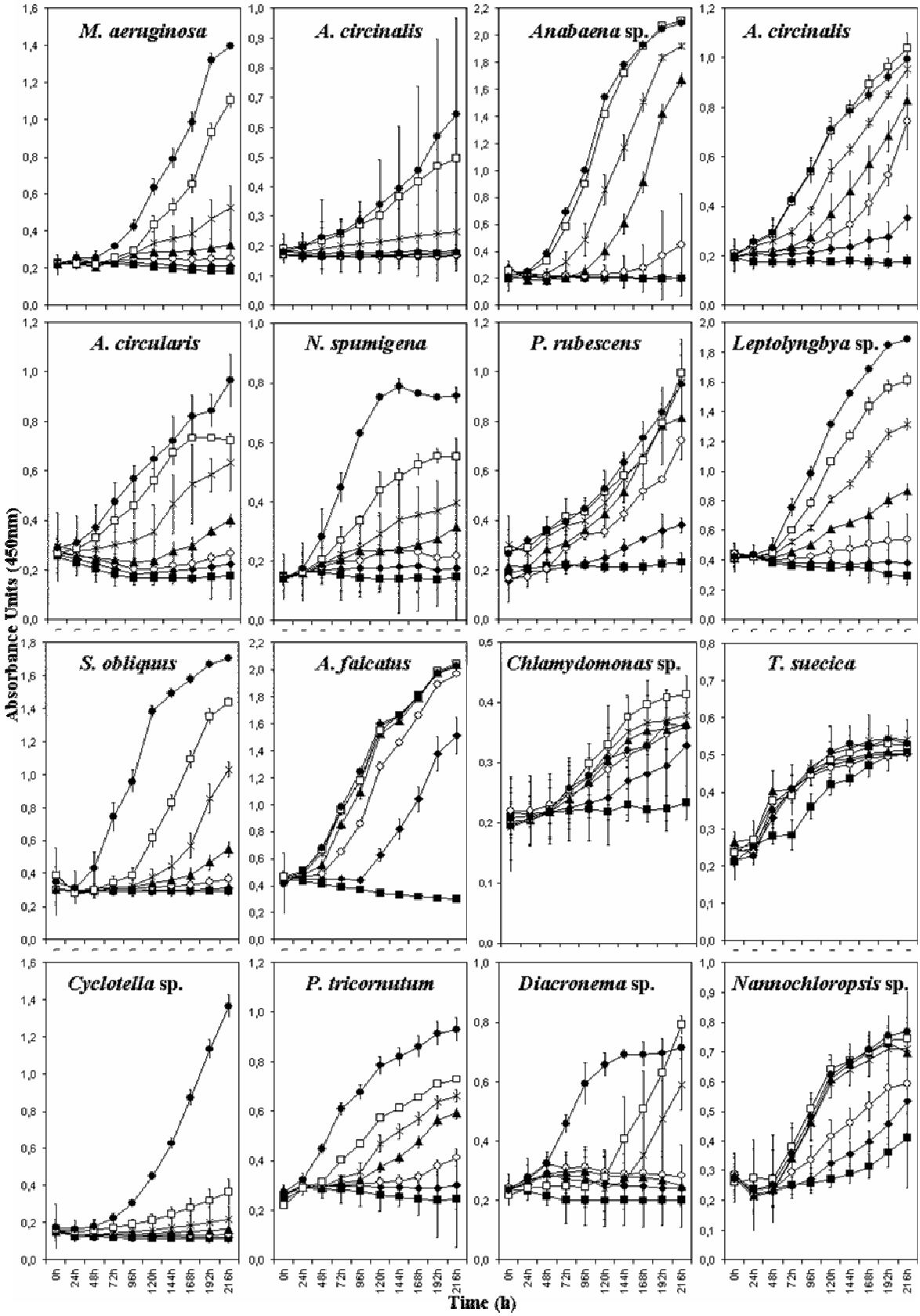


Figure 9: curves obtained for cyanobacteria and microalgae exposed to different tryptamine concentrations. Values represent means from triplicates and bars represent correspondent variation coefficients (standard deviation/mean values). (●)Control, (□)0,625µg.ml^{-1}, (*)1,25µg.ml^{-1}, (▲)2,5µg.ml^{-1}, (○)5µg.ml^{-1}, (♦)10µg.ml^{-1}, (■)20µg.ml^{-1}.

Table XXII: LOEC-216h and IC50-216h of tryptamine for each of the cyanobacteria and microalgae screened. Marine species are marked with (*). The right column indicates the range of IC50 values obtained for a 95% confidence interval.

	LOEC	IC50-216h	
M. aeruginosa	<1,125	1,1	(--)
A. circinalis	1,25	1,25	(--)
Anabaena sp.	1,25	2,36	(--)
A. gracile	1,25	4,1	(3,4-5,1)
A. circularis	<0,625	1,13	(1,0-1,3)
N. Spumigena *	<0,625	1,18	(--)
P. rubescens	5	9,12	(--)
Letolyngbya sp.	<0,625	1,64	(1,2-2,1)
S. obliquus	<0,625	6,9	(--)
A. falcatus	5	7,7	(7,2-8,2)
Chlamydomonas sp.*	5	10,2	(8,6-12,2)
T. Suecica *	20	>20	>20
P. Tricornutum *	<0,625	2,5	(<0,625-7,2)
Cyclotella sp.	0,625	0,5	(0,41-0,59)
Diacronema sp.*	<0,625	1,09	(<0,625-2,4)
Nannochloropsis sp.*	5	6,64	(3,7-14,1)

Table XXIII: : Differential selectivities (DS) of tryptamine towards freshwater organisms. DS values are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria.

		Algae		
		S. obliquus	*A. falcatus*	*Cyclotella* sp.
Cyanobacteria	*M. aeruginosa*	6,27	7	0,45
	A. circinalis	7,26	8,1	0,53
	Anabaena sp.	2,92	3,26	0,21
	A. gracile	1,68	1,88	0,12
	A. circularis	6,11	6,8	0,44
	P. rubescens	0,76	0,84	0,05
	Leptolyngbya sp.	4,21	4,69	0,31

Table XXIV: Differential selectivities (DS) of tryptamine towards marine organisms. DS are expressed as IC50 of algae / IC50 of cyanobacteria. Lower selectivity value = lower selective toxicity towards cyanobacteria.

	Algae				
	Chlamydomonas sp.	*P. tricornutum*	*Diacronema* sp.	*Nannochloropsis* sp.	*T. suecica*
N. spumigena	9,28	2,11	0,92	5,62	16,9

Relationship between the amount of cells and the amount of chemical needed to inhibit growth.

The IC50-21h values of bacillamide towards different cyanobacteria and microalgae were directly related to the amount of cells in the culture inoculums (Table XXV). Thus the amount of chemical needed to prevent growth depended on the initial cell densities of the test organisms.

Table XXV: Effect of the inoculum concentration on the IC50-216h values of bacillamide towards different cyanobacteria and microalgae. C1 – undiluted culture; C2 – culture diluted by 1:2; C3 – culture diluted by 1:4.

	IC50-216h C1	IC50-216h C2	IC50-216h C3
A. circinalis	123	69	61
Anabaena sp.	123	117	96
N. Spumigena	116	108	37
Letolyngbya sp.	180	153	140
P. Tricornutum	83	70	60

Algicidal *versus* algistatic properties of the compounds.

To differentiate between algistatic and algicidal properties of the screened compounds, cultures previously exposed to compounds were transferred to new compound-free culture media and incubated for another 216h. Fig. 10 presents the growth curves obtained by cultures previously exposed to I-bacillamide and subsequently transferred to new cultured media. When no growth appears on the subcultures, the concentration of the chemical and treatment time is considered algicidal. When growth is observed on previously inhibited cultures, the concentration of the chemical is considered algistatic. Thus, I-bacillamide can be considered as an algicide agent to *P. rubescens* and *Leptolyngbya* sp. at concentrations of 160 $\mu g.mL^{-1}$, i.e., though this concentration inhibited growth of these cyanobacteria (see Fig. 8, page 48), they all resumed growth after being transferred to new I-bacillamide-free culture medium. For *A. circinalis*, I-bacillamide acted both as an algistatic at 10 $\mu g.mL^{-1}$ concentrations and as an algicide when concentrations were at 20 $\mu g.mL^{-1}$ or above. For *Anabaena* sp., I-bacillamide was algistatic at 20 $\mu g.mL^{-1}$ and algicidal at 40 $\mu g.mL^{-1}$ or above. For *A. gracile,* algistatic activity was observed at 10 and 20 $\mu g.mL^{-1}$ of I-bacillamide, whereas higher concentrations showed an algicidal activity.

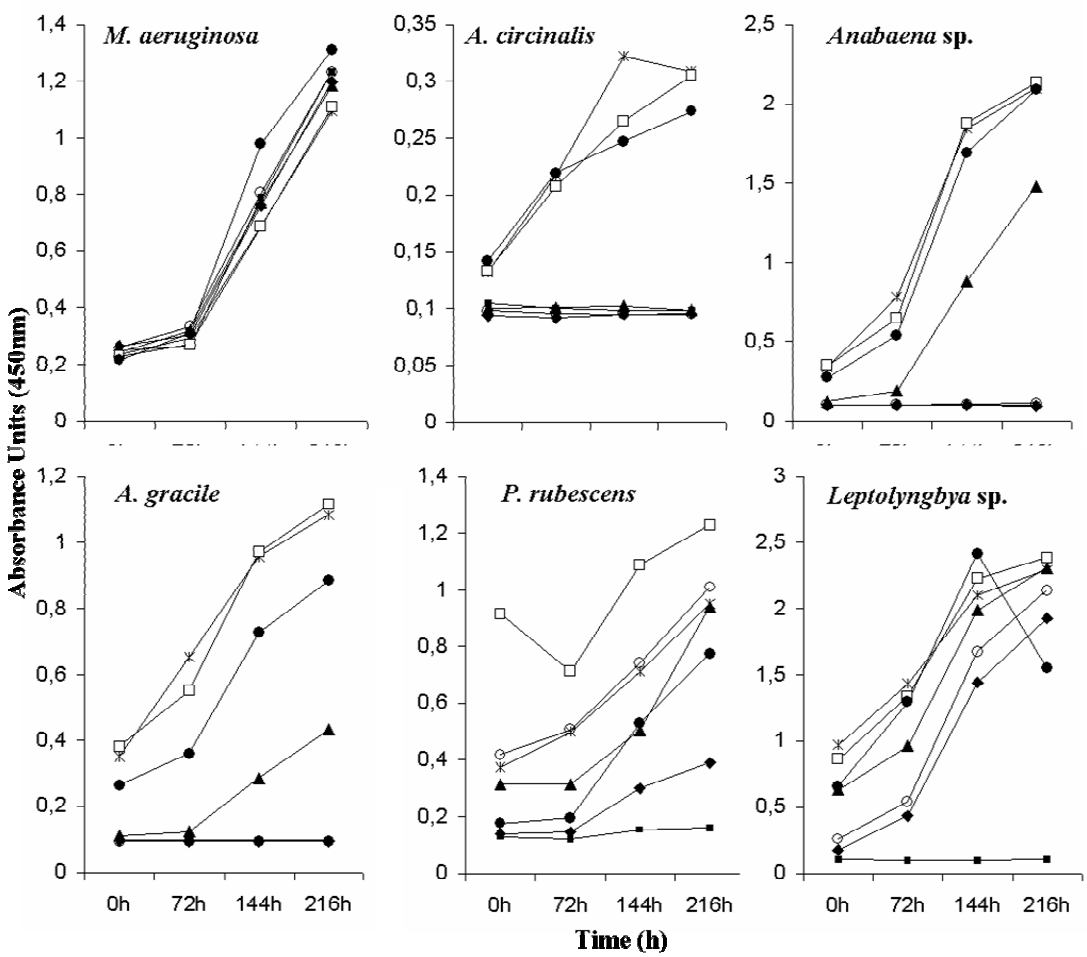


Figure 10: Growth curves obtained by cyanobacteria cultures grown in I-bacillamide-free culture media after being exposed to I-bacillamide for 216h. (●)Control, (□)5µg.mL^{-1}, (*)10µg.mL^{-1}, (▲)20µg.mL^{-1}, (○)40µg.mL^{-1}, (♦)80µg.mL^{-1}, (■)160µg.mL^{-1}. Data represent mean values from triplicates; variation coefficients never exceeded 0,2 (20%).

For the eukaryotic microalgae, all I-bacillamide treatments that inhibited growth proved to have algistatic properties, i.e. growth was resumed in all the apparently inhibited cultures. Note the recovery of *P. tricornutum* and *Diacronema* sp., two species greatly affected by I-bacillamide (Fig. 11).

Tryptamine was found to be either algistatic or aligicidal towards cyanobacteria depending on the concentrations used and on the species screened (Fig. 12). For *M. aeruginosa* tryptamine showed algistatic properties at 2,5 to 10 µg.mL^{-1}. For *Anabaena* sp. all the treatments that suppressed growth (10 and 20 µg.mL^{-1}) were algicidal. On the contrary, tryptamine treatments that inhibited growth of *A. gracile*, *P. rubescens* and *Leptolyngbya* sp. were algistatic. (Fig. 12).

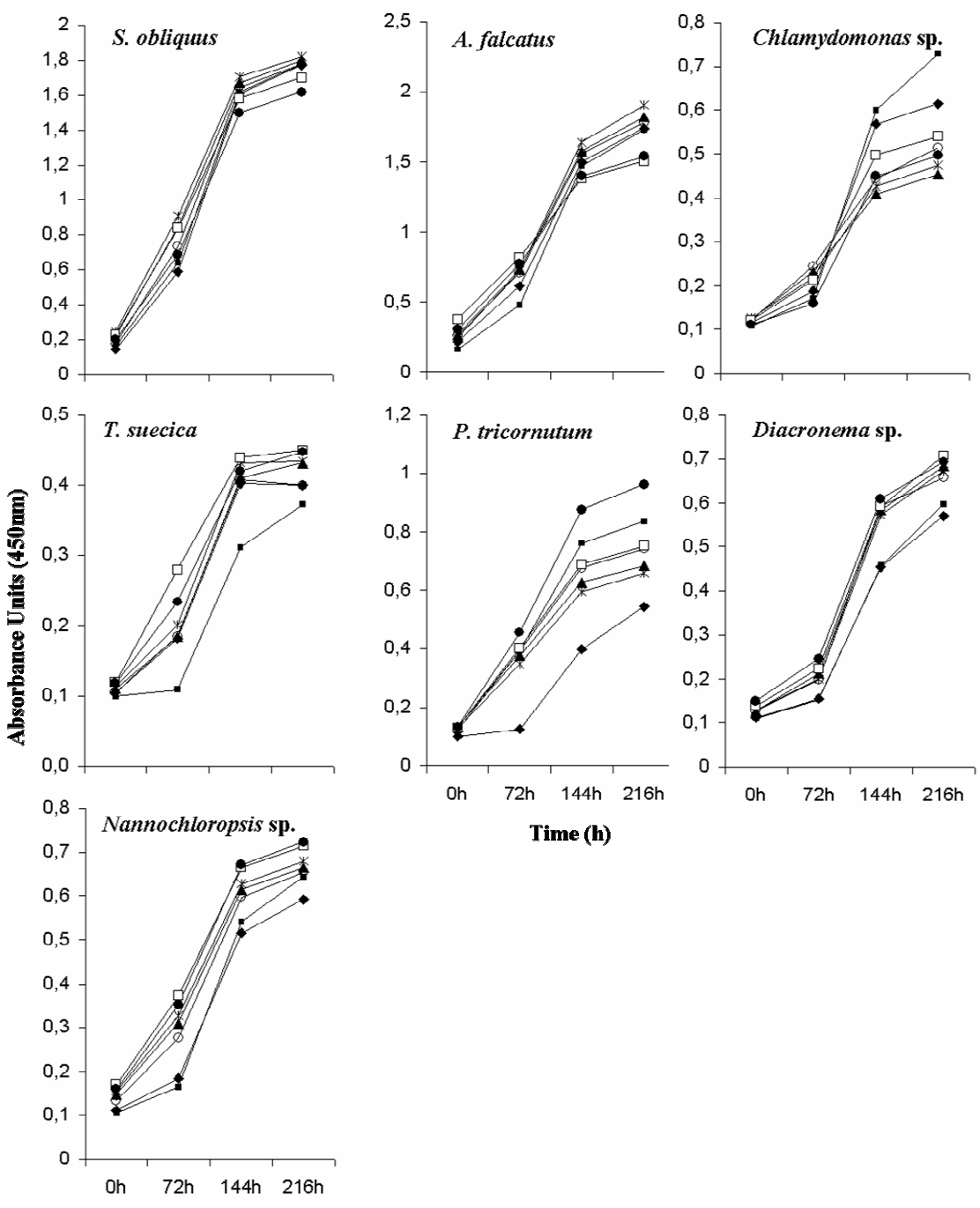


Figure 11: Growth curves obtained by algae cultures grown in I-bacillamide-free culture media after being exposed to I-bacillamide for 216h. (●)Control, (□)5µg.mL^{-1}, (*)10µg.mL^{-1}, (▲)20µg.mL^{-1}, (○)40µg.mL^{-1}, (♦)80µg.mL^{-1}, (■)160µg.mL^{-1}. Data represent mean values from triplicates; variation coefficients never exceeded 0,2 (20%).

Concentrations 10 and 20 µg.ml^{-1} acted as algicidal for *A. circinalis* and *Anabaena* sp.. However, concentrations that affected the growth of *M. aeruginosa*, *A. gracile*, *P. rubescens* and *Leptolyngbya* sp. proved algistatic (Fig. 12).

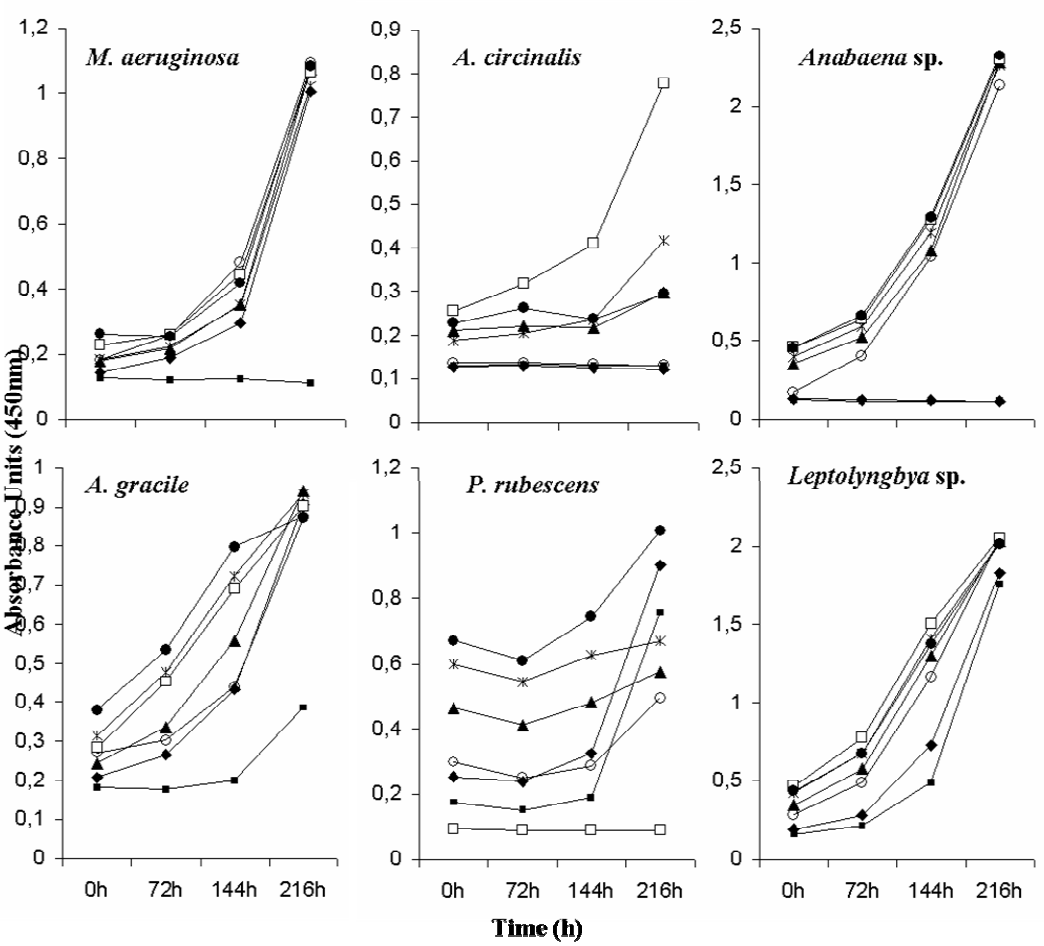


Figure 12: Growth curves obtained by ctanobacteria cultures grown in tryptamine-free culture media after being exposed to tryptamine treatments for 216h. (●)Control, (□)0,625µg.ml^{-1}, (*)1,25µg.ml^{-1}, (▲)2,5µg.ml^{-1}, (○)5µg.ml^{-1}, (♦)10µg.ml^{-1}, (■)20µg.ml^{-1},.Data represent average values (n=3), coefficient of variation that never exceed 18%.

For the eukaryotic microalgae, all the cultures previously inhibited by tryptamine reassumed exponential growth after being transferred to new tryptamine-free culture media, except the haptophyte *Diacronema* sp. (Fig. 13). Cultures from this species previously exposed to 10 to 20 µg.ml^{-1} of tryptamine failed to grow in the new culture medium. For all the other marine and freshwater algae, tryptamine showed to be algistatic regardless the concentrations used (Fig. 13).

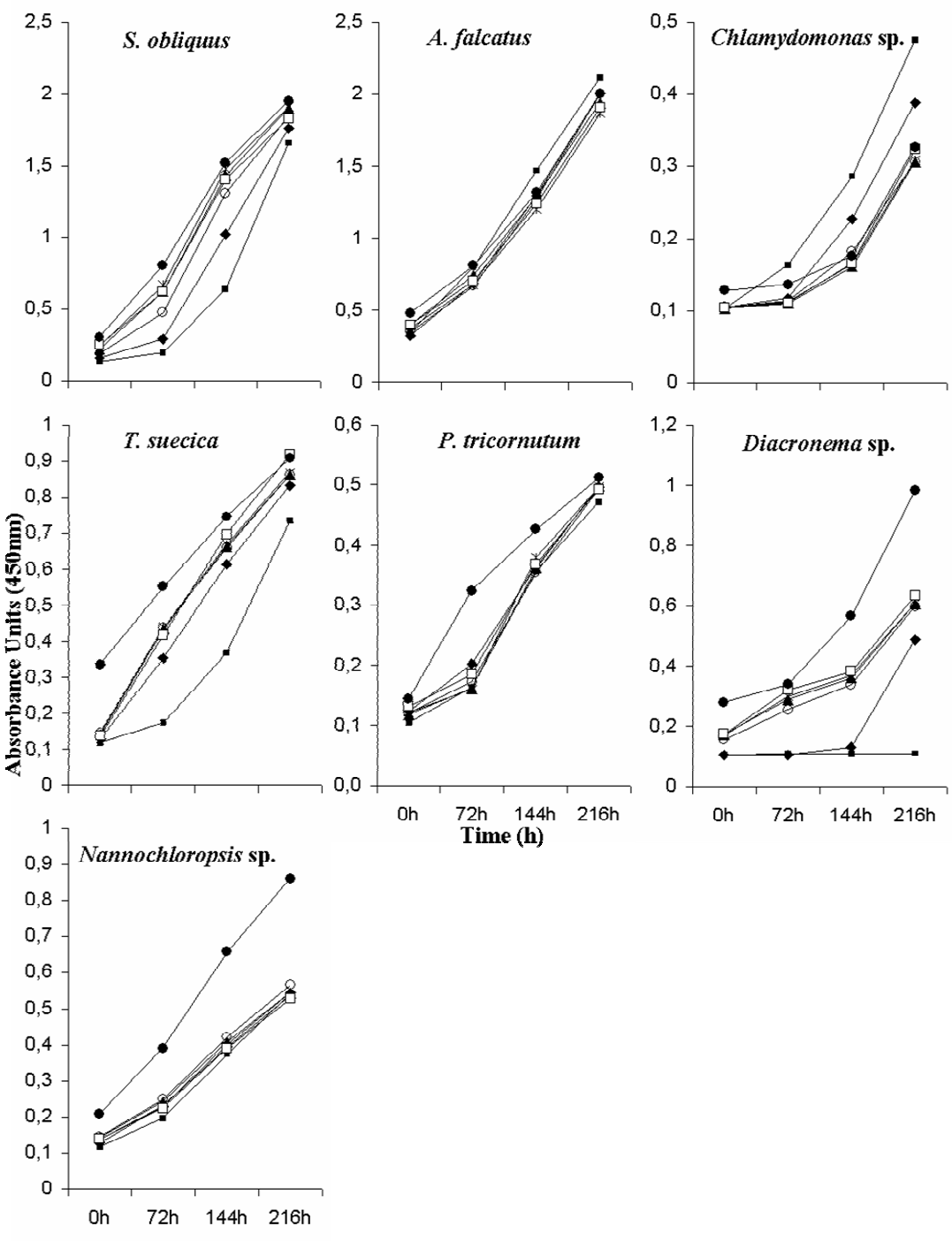


Figure 13: Growth curves obtained by cultures grown in tryptamine-free culture media after being exposed to tryptamine treatments for 216h. (●)Control, (□)0,625µg.ml^{-1}, (*)1,25µg.ml^{-1}, (▲)2,5µg.ml^{-1}, (○)5µg.ml^{-1}, (♦)10µg.ml^{-1}, (■)20µg.ml^{-1},.Data represent average values (n=3), coefficient of variation that never exceed 18%.

Light microscopy and ultrastructural analysis of exposed organisms.

Figure 14 shows optical photomicrographs of unstained living cells of *A. gracile* and *A. circinalis* taken from the control and from bacillamide treated cultures after a 72h exposure period. Cells exposed to increasing amounts of bacillamide showed a gradual dose-dependent bleaching appearance, lacking the numerous gas vesicles and cytoplasmatic inclusions clearly visible in the control treatments. Heterocysts could be seen in both treated and untreated cultures, though showing a more translucid appearance in the exposed cultures.

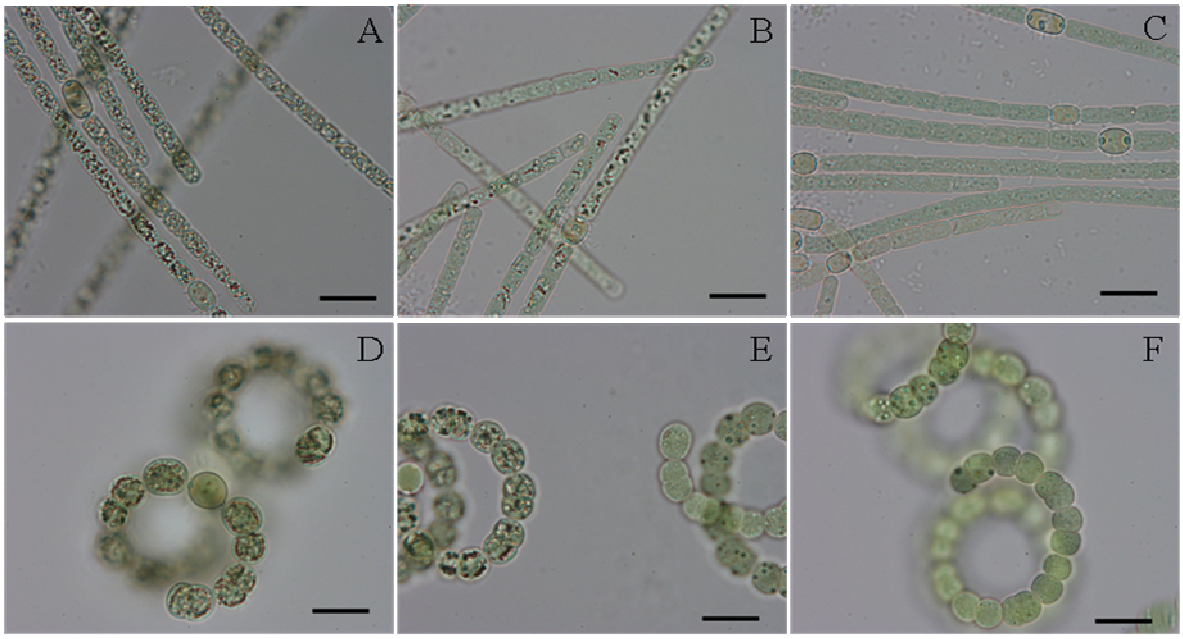


Figure 14: Optical microscopy, phase contrast photographs of *Aphanizomenon gracile* (top) and *Anabaena circinalis* (down) cultures, exposed to 0 µg.mL^{-1} (left), 40 µg.mL^{-1} (middle), and 80 µg.ml^{-1} (right) of bacillamide, for 72h. Scale Bar 10 µm.

These effects were even more pronounced (at lower doses) after a 216h exposure period and were shared among the other screened cyanobacteria (data not shown). For *Planktothrix rubescens* the oscillatory capability of movement seen in the controls was lost after treatments with bacillamide.

Among the chlorophytes, the most visible effects of bacillamide treatments on the morphology of *A. falcatus* were changes in cell shape, more than changes in cell contents.

Whereas unexposed cells Fig. 16A appeared elongated, with a prominent chloroplast, bacillamide treated cells became typically deformed and swollen, as shown in Fig. 16B.

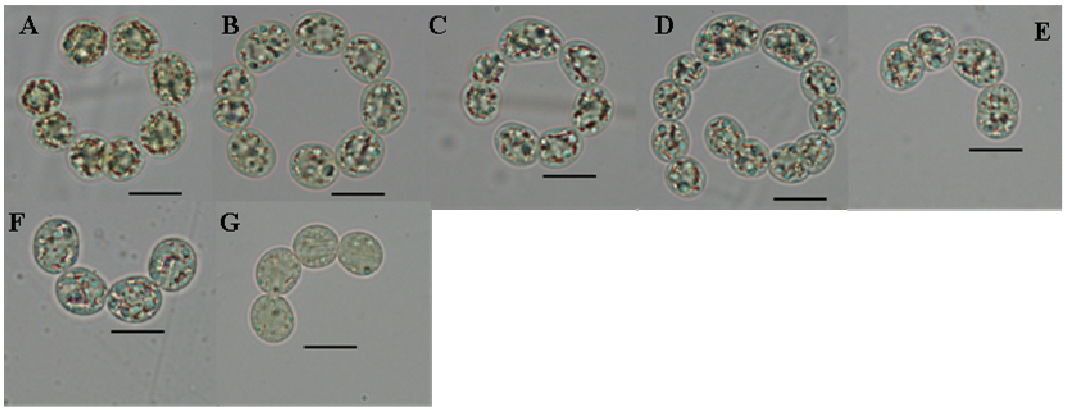


Figure 15: Effect of Bacillamide in the serial concentrations used after 216 hours of exposure time on ***A. circinalis***. It can be seen the gradual loss of 'celular content' and loss of what appears to be the gas vacuoles (arrow). (A) 0 control, (B) 5µg.ml^{-1}, (C) 10 µg.ml^{-1}, (D) 20 µg.ml^{-1}, (E) 40 µg.ml^{-1}, (F) 80 µg.ml^{-1}, (G)1600 µg.ml^{-1} . Scale bar 10µm.

Within diatoms, the effects of bacillamide treatments on cell morphology were less visible. Clearer to the observer was the apparent fragility of the treated cells of *Cyclotella* sp., which easily lysed soon after being exposed to the microscope incident light for a few seconds (Fig. 16C and D). No visible effects were observed between control cells and exposed cells of the other algal groups tested.

Figure 16: Optical microscopy, phase contrast photographs of *Ankistrodesmus falcatus* (top) and *Cyclotella* sp. (down) from control (left) and bacillamide treated (right) cultures. Scale bar 10µm.

The visual effects of tryptamine on the morphology of cyanobacteria and microalgae were, in general, similar to those observed for bacillamide, i.e. cells appeared progressively bleached or deformed, buoyancy was lost and filaments became shorter and apparently broken (Fig. 17 E and F).

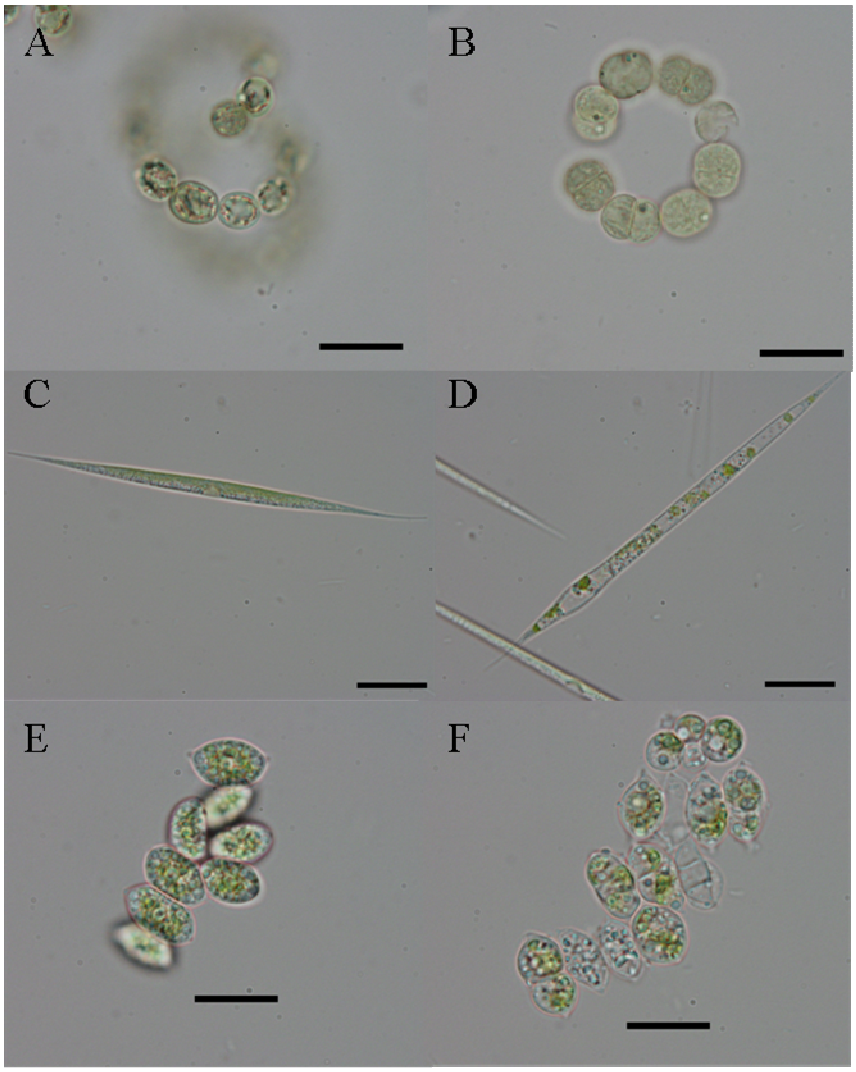


Figure 17: Effect of Tryptamine on cells of Chlorophyceae and cyanobacteria**.** Light microscopy, phase contrast micrographs of**:** ***A. circinalis*** (0 µg.ml^{-1} control) (A) and after treatment with tryptamine 10 µg.ml^{-1}(B), all after 216 hours, ***A. falcatus*** (0 µg.ml^{-1} control) (C) and after treatment with tryptamine 20 µg.ml^{-1} (D), ***S. obliquus*** (0 µg.ml^{-1} control) (E) and after treatment with tryptamine 20 µg.ml^{-1}(F). Scale bar 10µm.

In order to evaluate the bacillamide effect at an ultrastructural level, cells were analysed by transmission electron microscopy. The results for *A. gracile* are presented in Fig. 18. Control cells showed the typical cyanobacterial ultrastructure. Distinctive characters as the three-layered Gram-negative cell wall, absence of nucleus and membrane-bound organelles and photosynthetic lamellae composed of single thylakoids, could be observed. The cells presented numerous gas vacuoles occupying large part of the cytoplasm, and cellular inclusions such as carboxysomes (Polyedral bodies), cyanophycean granules, lipid droplets and polyphosphate bodies. No differences were observed in the control cells at 72 and 144 hours of incubation (Figure 18 and B).

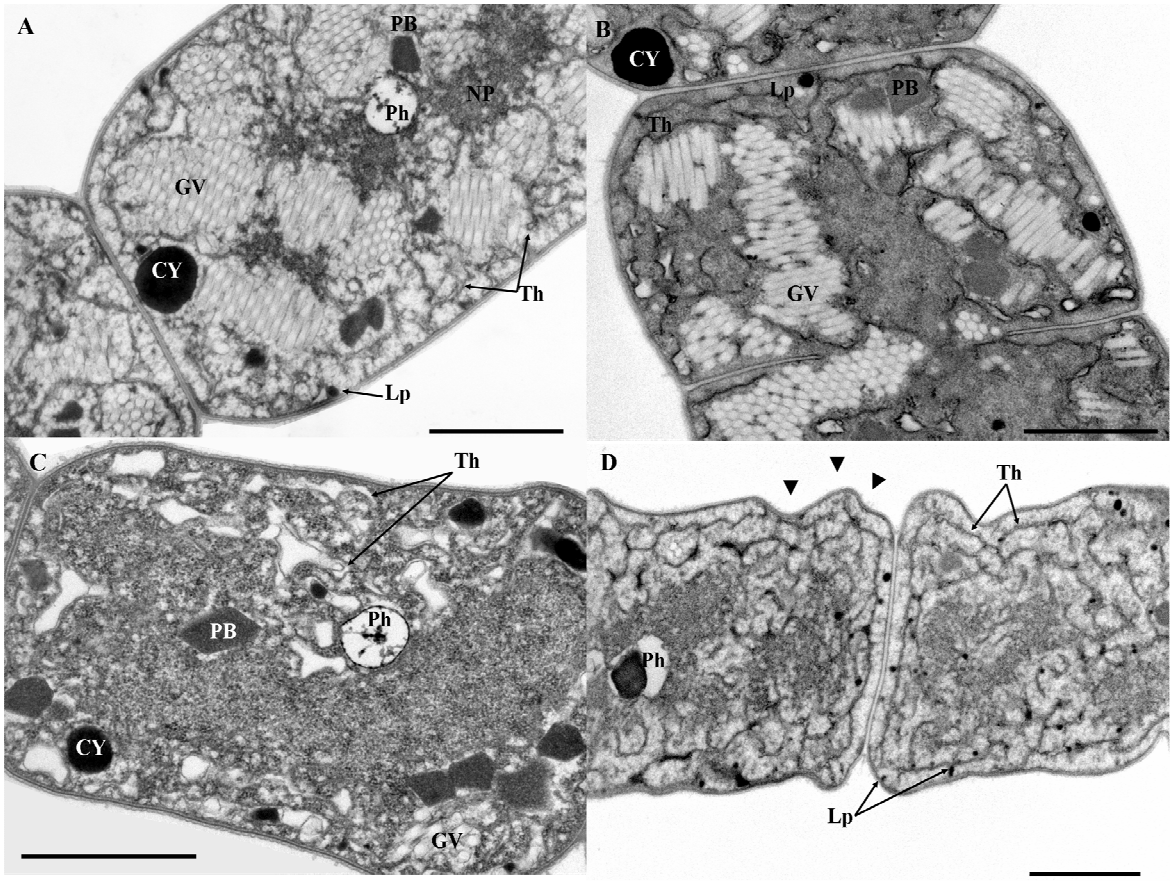


Figure 18: Transmission electron micrographs of *Aphanizomenon gracile*. A and B, control cells showing a typical cyanobacterial ultrastructure after 72 and 144 hours of incubation respectively. Thylakoid membranes (Th). Polyhedral bodies (PB). Polyphosphate bodies (Ph) are observed in the nucleoplasmatic area (NP). Cyanophycin granules (CY) near the thylakoid membranes and cell periphery. Numerous gas vacuoles (GV) occupying a large part of the cytoplasm. C and D, cells exposed to 40 $\mu g.ml^{-1}$ of bacillamide at 72 h and 144 h of incubation, respectively. Notice the increase on intrathylakoidal space and reduction of gas vacuoles in C. After 144h of exposure (D), notice the distortion of cell wal (arrows), the increase in lipid grains near the thylakoids and the absence of gas vacuoles. Scale bar 1µm.

Figure 18C shows a bacillamide treated cell after 72 hours of exposure to 40 μg.ml^{-1}. There was a drastic reduction of gas vacuoles, an increase in intrathylakoidal space and a slight distortion of the cell wall. After 144h of exposure (Fig. 18D) a more severe cell wall distortion was observed, as well as an increase in lipid droplets near the thylakoid membranes and the absence of gas vacuoles and intrathylakoidal spaces.

Control cell heterocysts showed the normal organization, with an elaborate membrane system and typical 'honeycomb' concentration of lamellae in the polar regions (Figure 19A). The heterocysts of cells exposed 72h to 80 μg.ml^{-1} bacillamide showed large vacuolizations localized in the middle of the cell.

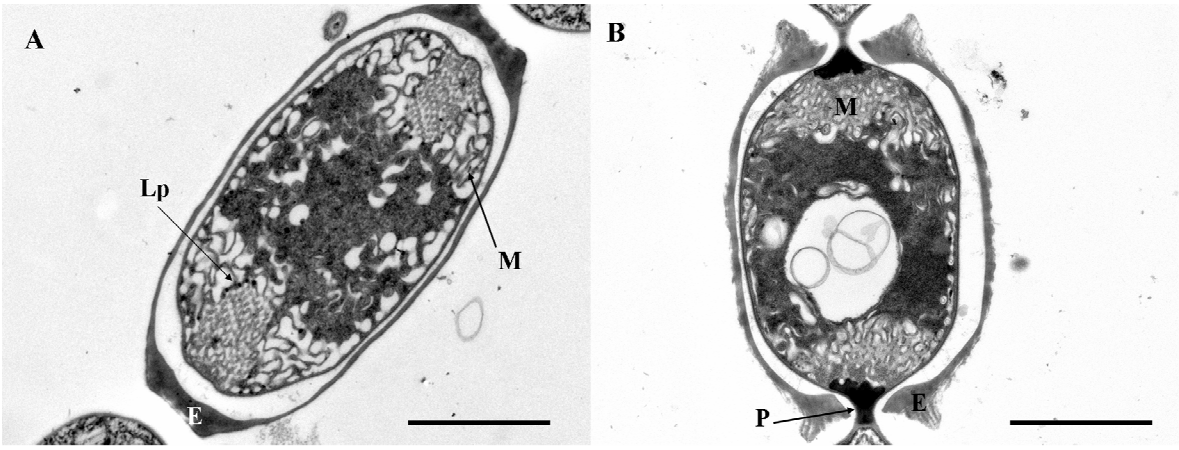


Figure 19: *A. gracile* heterocysts displaying an elaborated membrane system (M), pore channel to the adjacent cell (P) and the multilayered envelop (E). (A) Control cell after 72 hours of incubation. (B) Heterocyst treated with 80 μg.ml^{-1}. Scale bar 2μm.

The bacillamide-induced ultrastructural changes in the Chlorophyceae *A. falcatus* are presented in Fig. 20. Control cells (Fig. 20A and B), with a typical chlorophycean chloroplast with thylakoids grouped in lamellae, intraplastidial starch grains and the typical cell wall of these cocooid green algae, which have three layers with a thicker middle layer. No differences in control cells after 72 and 144 h incubation time were observed.

Although after 72h of exposure to 40 g.ml^{-1} no differences were noted in the ultrastructure of the cells, with increasing exposure times augmented numbers of lysosome-like structures were noted (Fig. 20D). Increased bacillamide concentrations and exposure time also induced an increase in lipid droplets (Fig. 20E) that at higher concentrations appear as large coalescent globules. Furthermore, a progressive swelling of the cells could also be observed (Fig. 20F).

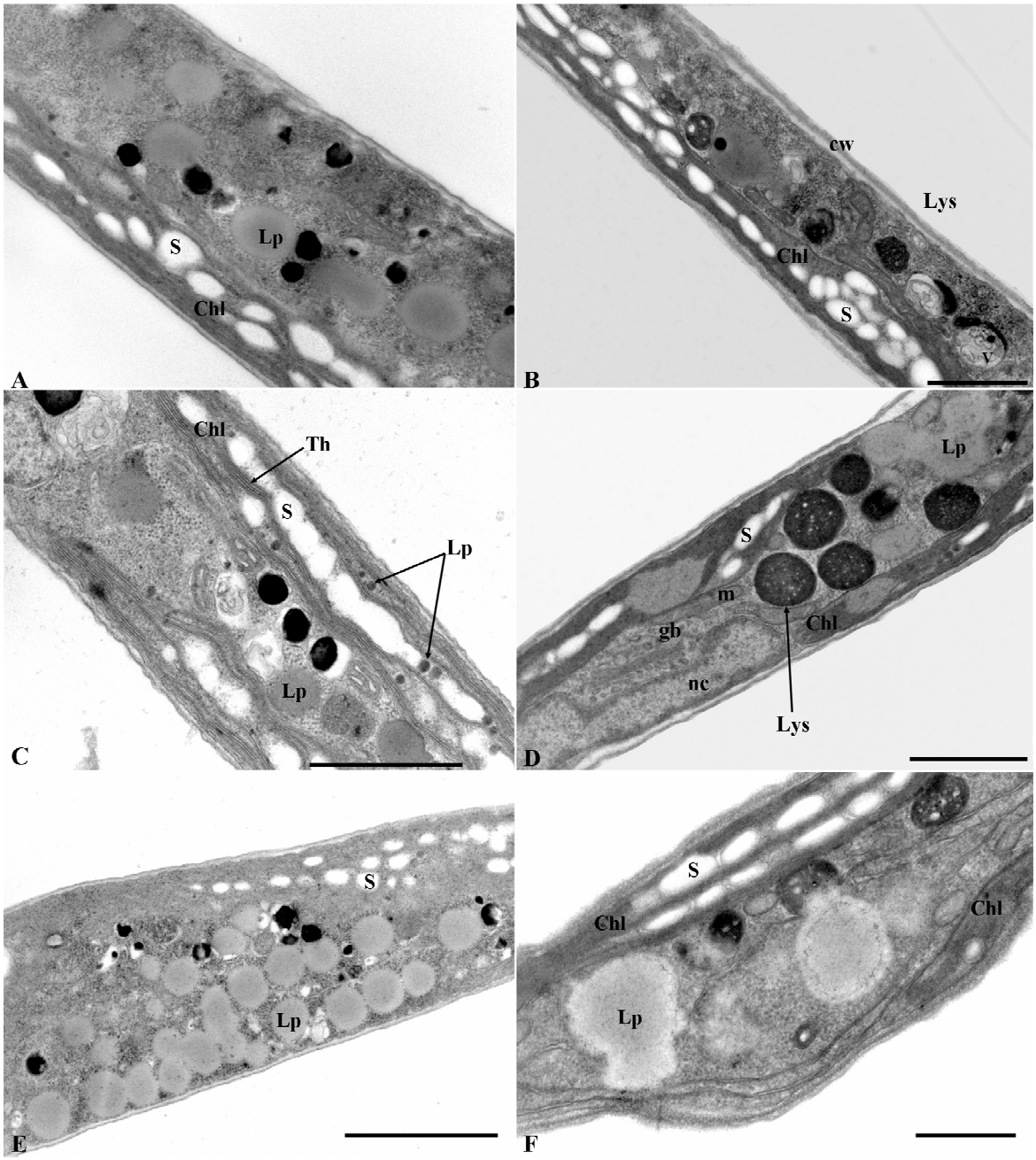
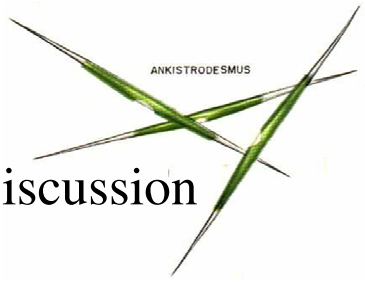


Figure 20: Transmission electron micrographs of *Ankistrodesmus falcatus*. A and B, control cells after 72 and 144 hours of incubation respectively, Scale bar 1µm. The cells show the typical organization, with a prominent chloroplast (Chl) with grana thylakoids (Thy) and starch grains (S). Cell wall (CW). Autolysosomes (V). Primary lysosomes (Lys). Lipid droplets (Lp). C, bacillamide-treated cells with 40 $\mu g.ml^{-1}$ after 72 h of exposure (Scale bar 1µm), notice the increase on intrathylakoid lipid droplets. D, after 144h of exposure with 40 $\mu g.ml^{-1}$ (Scale bar 1µm), notice the lysosome like structures. Golgi body (gb). Mitochondria (m). Nucleus (nc). E, bacillamide-treated cells with 80 $\mu g.ml^{-1}$ after 72 h of exposure (Scale bar 2µm), notice the increase of lipid droplets that are largely dispersed in the cytoplasm. F, after 144 h of exposure with 80 $\mu g.ml^{-1}$, cells became swollen (Scale bar 0,5µm).



Discussion

Excessive algal growth in surface sources of drinking water, such as lakes and reservoirs, is responsible for filter-clogging, undesirable taste and odor, disinfection-by-product formation and toxin production. Although various methods are currently being used to control algal blooms, their successes are limited. Reduction of external nutrient loads, hypolimnion aeration, artificial destratification, biomanipulation, use of macrophyte and microphyte extracted bioactive compounds are some of the methods that have been tried. Despite all these efforts, excessive phytoplankton growth remains a major problem in many lakes and reservoirs (Chorus & Mur 1999).

Reduction of external nutrient inputs, hypolimnion aeration and artificial destratification, are probably the most adequate and environmental friendly counter-measures for long-term reduction of cyanobacteria and microalgae proliferations. However, these methods may not always produce the expected outcome in a short-time period and generally require high economic and energetic inputs (Chorus & Mur 1999).

An alternative approach for the direct elimination of harmful phytoplankton involves the application of synthetic algicides. Though these chemical agents are effective in reducing harmful species, they have a broad-spectrum toxicity towards phytoplankton which can result in the death of the entire phytoplankton community and subsequent water quality deterioration. Moreover, their lengthy persistence in the environment generates concerns about environmental safety and toxicity from residuals formed as breakdown products may have a negative impact on fish, mammals and other microorganisms in the aquatic ecosystem.

Ideally, methods of phytoplankton control or treatment in aquatic environments should result in the complete removal of the target organisms while maintaining the other species at sustainable and productive levels.

Biological controls in the form of viruses or bacteria usually take advantage of species-specific interactions naturally occurring in aquatic environments. It has been demonstrated

that many genera of marine and freshwater bacteria have specific algicidal effects and are associated with the termination of harmful blooms in natural environments. However, the implications of either a species introduction and/or enhancement in abundance of an indigenous species are highly controversial issues that require considerable background research before actual implementation. Though worth exploring, these methods may have the potential of disastrous environmental consequences (Vershuere *et al.* 2000).

The application of natural compounds isolated from algal growth-inhibiting organisms may constitute, on the other hand, a more environmentally benign means of selectively controlling harmful phytoplankton.

Bacillamide isolated from the marine bacterium *Baccilus* sp SY-1, was recently reported to have a high selective activity against the dinoflagellate *Cochlodinium polykrikoides* (Jeong *et al.* 2003). The authors have raised the possibility of using this compound as a natural algicide for bloom control in natural environments. However, the high costs involved in the culturing of the productive organism and in the purification of its bioactive compound could be limiting.

Different bacillamide analogues and related aminoacids were tested in the present work in terms of selective toxicity towards different cyanobacteria and eukaryotic microalgae. Their easy, low-cost synthetic production in the laboratory put them amongst the most promising compounds to pursue as selective algicide agents for the practical control of harmful blooms.

In order to screen the different compounds at several different concentrations in a range of different cyanobacteria and algae cultures we chose to apply a 96-well microplate bioassay previously developed by Schrader *et al.* (1997). This bioassay permits several conditions to be screened simultaneously in a single culture plate, with results being obtainable within a few days and the selectivity of the compounds easily determined. The method proved to be much less space and time consuming than classic methods performed in tubes or flasks and based on direct cell counts. However there were some drawbacks that should be noted. Thus, the method was not adequate for testing some species, namely the motile dinoflagellates *Amphidinium carterae* which did not grow in the plate wells. Some problems were also noted with filamentous species such as *Planktothrix rubescens*, *Anabaena circinalis* and *Nodularia spumigena* which tended to clump in the plate wells after the first 2 days, causing irregular absorbance readings. These problems tend to be

overcome to some extent by plate shaking before optical readings. But some strains of *Microcystis aeruginosa* had to be excluded from the screening tests as they formed dense indissoluble colonies. For all the other test organisms a good linear relation between cell number and optical densities was obtained.

Bacillamides were toxic to different species of cyanobacteria in a dose-dependent relationship. The Oscillatoriales were in general less sensitive to most bacilamide analogues than the Chroococcales and the Nostocales. The reason for such different sensitivities might be due to the growth habits of the Oscillatoriales screened. As mentioned before, both *P. rubescens* and *Leptolyngbya* sp. tended to aggregate forming bundles of dense, tightly packed filaments. It is conceivable that the filaments on the outer surface of the colonies might have protected the cells in the inner part of the colonies from the action of bacillamides. *Nodularia spumigena* also showed a higher tolerance to most bacillamides then the other Nostocales, perhaps through a protective effect of the abundant mucilage layer.

Different sensitivities among the different types of cyanobacteria indicate that bacillamides can hardly be considered as broad spectrum cyanobacterial algicides. However, different bacillamide analogues might be of use in selectively controlling the growth of particular species of cyanobacteria. Bacillamide itself showed to be far more toxic to *Microcystis aeruginosa*, *Anabaena circinalis* and *Aphanizomenon gracile* than to the chlorophytes *Scenedesmus obliquus* and *Ankistrodesmus falcatus*. Thus, bacillamide treatments of up to 80 $\mu g.mL^{-1}$ are expected to suppress the growth of these cyanobacteria without affecting growth of the green algae. Bromo- and chlorine-bacillamides, when applied in the same concentrations, also prevented the growth of the cyanobacteria *Anabaena circinalis, A. gracile* and *Anabaenopsis circularis* with no significant consequences to the above-mentioned chlorophytes. Flourine- and iodine-bacillamides showed a much wider-spectrum activity against cyanobacteria, though the latter affected also the chlorophytes and thus showed a much lower selectivity in its toxic action. On the other hand, metoxi-bacillamide (OH-bacillamide) proved to be quite specific towards *Anabaena* and *Aphanizomenon* at concentrations apparently non-toxic to any of the other freshwater organisms screened.

Taken together, these results indicate that the decision on the type of bacillamide to be used for the selective control of freshwater cyanobacteria should depend on the relative

composition of the phytoplankton community in each particular situation. In cases where *M. aeruginosa* is found as the most abundant species, treatments with either bacillamide or Fl-bacillamide would be more adequate if co-occurring chlorophytes were not to be affected. To control *A. gracile*, treatments with either metoxi-, bromo- or chlorine-bacillamides would be preferable given their higher differential selectivities towards this species relative to freshwater chlorophytes. Concerning the *Anabaena* spp. more satisfying results would be expected from applying chlorine- or fluor-bacillamides, though these cyanobacteria showed some intra-genera variation in sensitivities to bacillamides.

Regardless of the particular species of cyanobacteria we might be focusing on, special attention should also be payed to diatoms as these proved to be quite as sensitive to most bacillamide analogues as the most affected cyanobacteria. In temperate regions, diatoms and cyanobacteria usually tend to colonize eutrophic and hypertrophic freshwater systems in a seasonal succession. Cyanobacteria often dominate the summer phytoplankton and as winter approaches, the increasing turbulence and decreasing light leads to their replacement by diatoms in association with rapidly growing small flagellates (Chorus & Bartram 1999). Those can in turn be followed by green algae in late spring and early summer and then by species which cannot easily be eaten by zooplankton, such as dinoflagellates, desmids and large yellow-green algae in autumn. In cases where such a pattern of succession is followed, the use of bacilamides in the early stages of the cyanobacteria dominant season are not expected to affect diatom populations to a great extent. However, if seasonable differences and other environmental factors are not great enough to induce phytoplankton succession, the use of bacillamide in mixed communities of cyanobacteria and diatoms will most probably affect both.

Within marine species, *Nodularia spumigena* was equally affected by bacillamide, Cl-bacillamide Fl-bacillamide and I-bacillamide. However, the IC50-216h obtained for this cyanobacterium where quite high (89-124 $\mu g.mL^{-1}$) and when compared to other marine algae, the screened bacillamides showed differential selective values too low to be pursued for the practical control of harmful blooms of this particular species.

To predict the effectiveness of a chemical for controlling a particular problem, it is essential to know whether the chemical acts as an algicide or as an algistatic. Differentiation between these types of action will provide valuable information on whether the chemical must be in constant contact with the algae to prevent further growth

(algistatic) or if after a sufficient treatment time the algae will have absorbed enough chemical so they will eventually die. Results obtained in this work showed bacillamides to act either as algicide or algistatic agents, depending upon the concentrations added. The need to know what type of action bacillamides will have at a particular concentration towards a particular species will therefore be important when a method of application must be selected. Occasional "shock" treatments with high bacillamide concentrations should be sufficient to effectively control problematic growth of undesirable cyanobacteria. However such treatments, when applied to well established blooms of toxic cyanobacteria can probably lead to massive cell lysis with consequent risks of toxins release to the extracellular media. Algistatic concentrations, when applied before cyanobacteria communities turn into massive proliferations will prevent the development of blooms as long as sufficient concentrations of the chemical are maintained at all times.

The amounts of bacillamides required to inhibit the growth of each species tested was related to the amount of cells initially present in the inocula. This must be kept in mind when considering the amounts of chemicals to be applied to control an algal problem, and stresses the importance of preventive measures in controlling the growth of cyanobacteria before they turn into a major problem in the aquatic medium.

In this study, the initial cell densities, as measured by optical density readings, were kept as similar as possible for all species to properly compare both the sensitivities to each test compound and the toxicities of the different compounds towards each test culture. The concentration of each bacillamide required to inhibit growth of the most sensitive species were significantly higher than those reported in the literature for other natural compounds with algicidal properties. Studies involving the use of aaptamine, ethyl-2-methylacetoacetate, palmiolate, AZC, rutacridone epoxide, anthraquinone and its analogues, dichtol B acetate, harmane and norharmane, β-cyanoalanine, gramicidin, 4,4'-dihydroxybiphynil, fisherellins, 12-epi-hapalindole, kasumigamide, nostocarbolines and many others, report much higher toxicities of these natural compounds against cyanobacteria (see Table III, page 20 for references). Some of the compounds listed were not yet tested for their selectivity on a wide, representative range of other organisms. Others were shown to have noxious, undesirable side effects such as high mutagenic and/or cytotoxic activities on fish and mammalian cells (Meepagala *et al.* 2005; Nagle *et al*. 2003; Volk 2005; Volk and Mundt, 2007; Schrader *et al.* 1998). Synthetic production of

bacillamides has been accomplished only very recently and no studies on toxicity towards other organisms have been made yet. Nevertheless, the high concentration of bacillamides needed to prevent cyanobacterial growth (IC50s-216h = 30-100 $mg.L^{-1}$) may limit the usefulness of these compounds for large water bodies.

Tryptamine showed a much higher toxicity towards cyanobacteria and eukaryotic microalgae than all of the bacillamides screened in this study. Tryptamine is synthesized by decarboxylation of trypthophan to yield indole alkaloid secondary metabolites. Aromatic amino acids like tryptophan are required as building blocks for protein synthesis and for the production of a large variety of secondary metabolites including quinones, indole derivates and alkaloids (Guillet *et al.* 2000). In plants, bacteria and algae, aromatic aminoacids like tryptophan are biosynthesized through the shikimate and chorismate pathway. This pathway is down-regulated by a feedback process in which the amino acid biosynthesis is repressed by the amino acids themselves or by their derivate products (Stryer 1988, Nelson & Cox 2000, Perley & Stowe 1966, Hamill *et al.* 1970, Guillet *et al.* 2000). In previous studies with the bacterium *Escherichia coli*, tryptamine was found to block tryptophan biosynthesis via tryptophan synthetase inhibition, suppressing the growth of the bacteria (Freundlich & Lichstein 1961). Thus, the growth inhibition effects of tryptamine observed in our experiments could have resulted from the inhibition of tryptophan production by tryptamine. Given that tryptamine cannot be transformed into tryptophan nor be incorporated into proteins (Majerfeld *et al.* 1970), the process will lead to the breakdown of the protein biosynthesis, which in turn will lead to growth inhibition. Studies on the production of aminoacids in the cyanobacterium *Spirulina platensis* reported that some species overcome the toxicity of the aminoacid analog by overproduction of the essential aminoacid. The hight amino acid content in the cellular pool may reduce the uptake of the analog into the cell (Riccardi *et al.* 1981), a mechanism perhaps related to the tolerance exibited by some strains of cyanobacteria to tryptamine, in our study.

Tryptamine is also a known precursor of the phyto-hormone indole-3-acetic acid (IAA). In plants IAA has its major effect on cell growth by influencing wall extensibility, stimulating ethylene production and causing cells to secrete H^+, leading to a raise in the pH and to the consequent loosening of membranes, regulating plant activities such as phototropism, fruit development and root iniciation (Salisbury & Ross 1992). In cyanobacteria and other

microalgae and macroalgae the production of IAA is also well established, but its role is not yet fully elucidated (Serggeeva *et al.* 2002, Lijun 2006). Ahmad & Winter (1968, 1970), reported that IAA might either promote cyanobacterial growth in small concentrations (10^{-9} and 10^{-10} M) or inhibit growth at higher concentrations (>10^{-4}M). The same authors showed that tryptamine had a similar effect, stimulating the growth of cyanobacteria at low concentrations (10^{-6} and 10^{-9} M) while being inhibitory at higher concentrations (10^{-3} and 10^{-4}M). In our work, tryptamine was found to inhibit cyanobacterial growth when applied at concentrations 4,0x10^{-6} to 1,25 x10^{-4} M, which partly agrees with the result obtained by Ahmad & Winter (1968, 1970). However, lower tryptamine concentrations were not screened by us, and the growth enhancement effect described by those authors could not be confirmed by our results.

The major effects of bacillamide on the ultrastructure of cyanobacteria cells were: i) the increase in lipids droplets near the tylakoids and the cell wall membranes; ii) the reduction and collapse of gas vesicles; iii) the distortion of cell wall membranes and iv) the vacuolization of heterocysts. This wide range of toxic effects on cell structure suggests a rather unspecific toxic action of the compounds towards the sensitive organisms.

In general, the action of a biocide may result either from physiochemical interaction with target structures, from specific reactions with biological molecules or from a disturbance of a selected metabolic or energetic processes (Denyer 1990). In our work, the rapid recovery of growth of previously inhibited cultures after being transferred to new culture media, suggests that the observed effects could be due to the feedback inhibition of metabolic pathways which, in turn, could be resumed upon the removal of the inhibiting compound. Interestingly, *Ankistrodesmus* cells exposed to bacillamides and tryptamine showed an increase in the number of lysossome-like particles when compared to unexposed cells (especially within the first 72h after treatment). The presence of such structures in treated cells might be viewed as an attempt of the organism to detoxify or metabolize the toxic agent. Recovery should then be possible as long as the concentration and the exposure time to the compound are sufficiently low to allow cells to repair and recover from damages. While studying the effects of lysine in the growth of *M. aeruginosa*, Hehmann *et al.* (2003) found that this amino acid affected the cell membranes and caused gas vacuoles to disappear. The authors presumed that those effects resulted from the feed-back inhibition of biosynthetic enzymes. While we can only speculate about the mechanisms underlying

the toxic action of bacillamides and tryptamine on cyanobacteria and microalgae, it is possible to admit that growth inhibition by these compounds might be the result of analogous mechanisms.

Conclusion

The use of bacillamide to control cyanobacterial proliferation is probably restricted to small water bodies and aquaculture ponds due to the large concentrations needed to prevent cyanobacterial growth (20-100 $mg.L^{-1}$) requiring the use of large amounts of the compound. It may have potential as an alternative to synthethic algicides in shallow lakes and where aeration of the water column is not possible.

Tryptamine seems a promising agent to selectively control cyanobacteria in freshwater and coastal marine waters; at concentrations of 1-5 $mg.L^{-1}$, it appears potentially useful for preventing the growth of cyanobacteria and inducing a shift from cyanobacterial domination to green algae domination. The effective concentrations are similar to those used for synthethic algicides.

The effectiveness of both bacillamide and tryptamine as a general cyanobacterial algicide is, however, limited as they were not selective against the cyanobacteria *P. rubescens* and *Leptolyngbya* sp.; examination of the phytoplankton community is always needed before rational decisions concerning the eventual application of these compounds can be made.

In order to evaluate bacillamide and tryptamine for use as general selective algicide further studies need are needed, namely for determining the environmental fate of these compounds and their breakdown products; toxicological tests need to be performed with other aquatic organisms that may become exposed; antimicrobial activity, as well as cytotoxic and mutagenic activity, need to be determined.

Although the need for a selective algicide is widely acknowledged, the costs of producing a new aquatic algicide are potentially high and manufacturers need to be confident on a large public acceptance of the product (Gibson 1990). The present work is but a small step on the search for a compound that will meet the requirements for use in sound environmental management.

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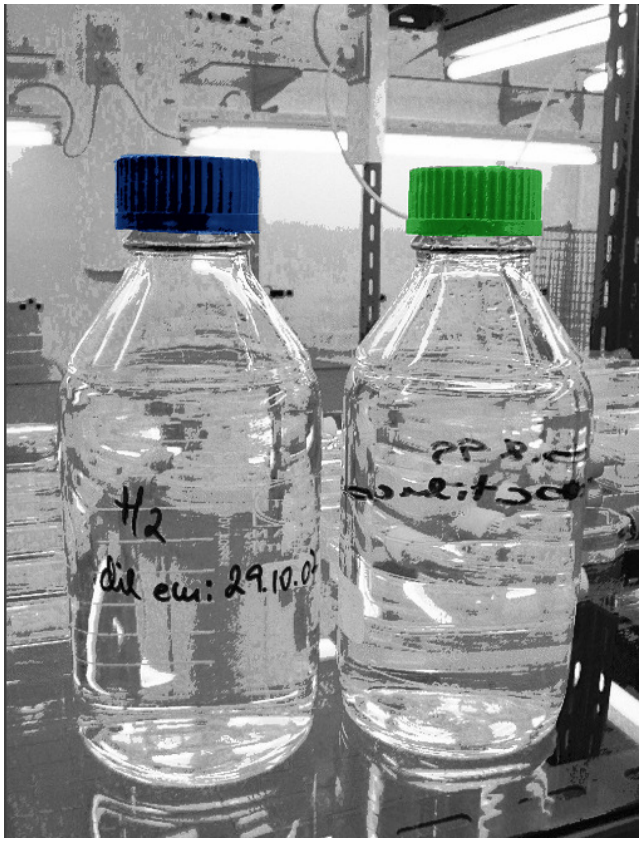
Appendix I
Culture Media Composition



Recipe of f/2 culture medium

(Guillard & Ryther 1962)

Table I: Medium composition used to culture marine Algae used in the experiments. Quantities added to 950ml of filtered seawater at 32psu.

Quantity	Compound	Stock Solution	Molar Conc. in final medium
1ml	$NaNO_3$	75g/L dH2O	$8{,}83x10^{-4}$
1ml	$NaH_2PO_4 \cdot H_2O$	5g/L dH2O	$3{,}63x10^{-5}$
1ml *	$Na_2SiO_3 \cdot 9H_2O$ *	30g/L dH2O *	$1{,}07x10^{-4}$
1ml	f/2 trace metal solution	(see recipe below)	---
0,5ml	f/2 vitamin solution	(see recipe below)	---

* Silicate can be deleted if is not required by the alga.

Table II: f/2 Trace Metal Solution. Quantities added to 950ml of filtered seawater at 32psu.

Quantity	Compound	Stock Solution	Molar Conc. in final medium
3,15 g	$FeCl_3 \cdot 6H_2O$	---	$1x10^{-5}$
4,36 g	$Na_2EDTA \cdot H_2O$	---	$1x10^{-5}$
1ml	$CuSO_4 \cdot 5H_2O$	9,8 g/L dH_2O	$4x10^{-8}$
1ml	$Na_2MoO_4 \cdot 2H_2O$	6,3 g/L dH_2O	$3x10^{-8}$
1ml	$ZnSO_4 \cdot 7H_2O$	22 g/L dH_2O	$8x10^{-8}$
1ml	$CoCl_2 \cdot 6H_2O$	10 g/L dH_2O	$5x10^{-8}$
1ml	$MnCl_2 \cdot 4H_2O$	180 g/L dH_2O	$9x10^{-8}$

Table III: f/2 Vitamin Solution. Quantities added to 950ml of filtered seawater at 32psu.

Quantity	Compound	Stock Solution	Molar Conc. in final medium
1ml	Vitamin B_{12}	1,0 g/L dH_2O	$1x10^{-10}$
10ml	Biotin	1,0 g/L dH_2O	$2x10^{-9}$
200mg	Thiamine·HCl	---	$3x10^{-7}$

For brackish water organisms, salinity as adjusted at 15 psu. Combine 400 ml of seawater with f2 medium and add 600 ml dH_2O.

Recipe of Z8 culture medium

(Skulberg & Skulberg 1990)

Table IV: Medium composition used to culture cyanobacteria and freshwater Algae used in the experiments. Quantities added to 1000ml of deionized water.

Quantity	Compound	Stock Solution
Solution A 10ml	$NaNO_3$	46,7g/L ddH_2O
	$Ca(NO_3)_2 \cdot 4H_2O$	5,9 g/L ddH_2O
	$MgSO_4 \cdot 7H_2O$	2,5g/L ddH_2O
Solution B 10ml	K_2HPO_4	3,1g/L ddH_2O
	Na_2CO_3	2,1g/L ddH_2O
1ml	Micronutrients solution	(see recipe below)
10ml	Fe-EDTA solution	(see recipe below)

Table V: Z8 Gaffron micronutrients Solution. Quantities added to 1000ml of deionized water.

Quantity	Compound
3,1 g	H_3BO_3
0,22 g	$ZnSO_4 \cdot 7H_2O$
2,23 g	$MnSO_4 \cdot 4H_2O$
0,033 g	$Na_2WO_4 \cdot 2H_2O$
0,054 g	$VOSO_4 \cdot 6H_2O$
0,146g	$Co(NO_3) \cdot 6H_2O$
0,088 g	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
0,474 g	$Al_2(SO_4)_3K_2SO_4 \cdot 2H_2O$
0,198 g	$NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$
0,083	KI
0,119	KBr
0,037 g	$Cr(NO_3)_3 \cdot 7H_2O$
0,154 g	$Cd(NO_3) \cdot 4H_2O$

FeEDTA solution:

Made in two solutions

Solution C- 2, 8 g $FeCl_3$ in 100ml 0, 1 HCl

Solution D- 3,9 g EDTANa2 in 100ml 0,1 NaOH

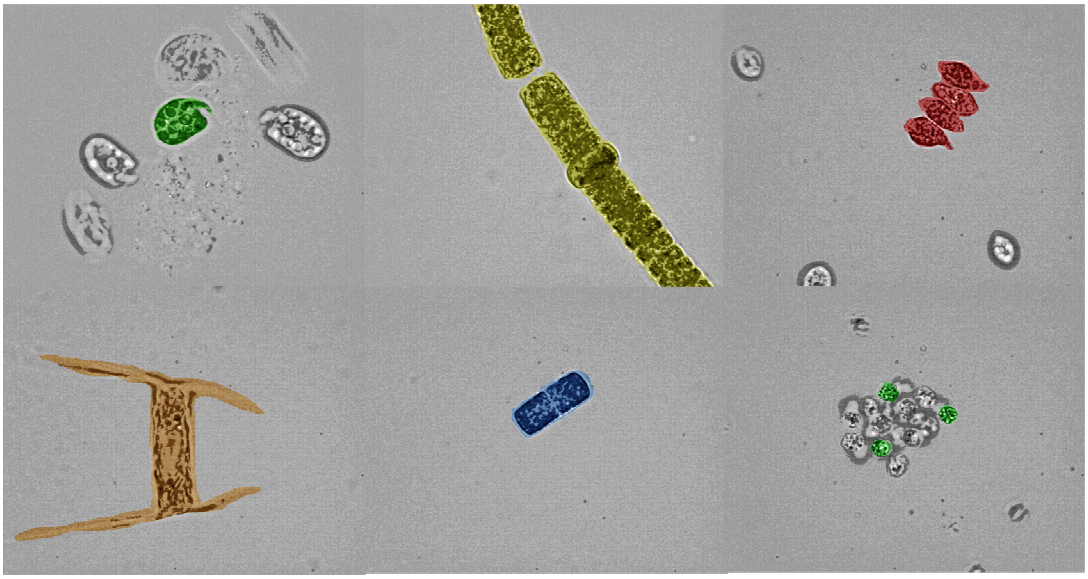
10ml of C plus 9,5ml of D plus 1000ml of deionized water.

Silicate Solution:

$Na_2SiO_3.9H_2O$ -30g/L dH_2O

For culture of freshwater diatoms silicate solution (1ml) was added to Z8 medium (1000ml).

Appendix II
Algae Spectra



The most suitable wavelength for monitoring growth was at 450nm, based on the maximum absorption peak for the wavelengths available in the spectrophotometer.

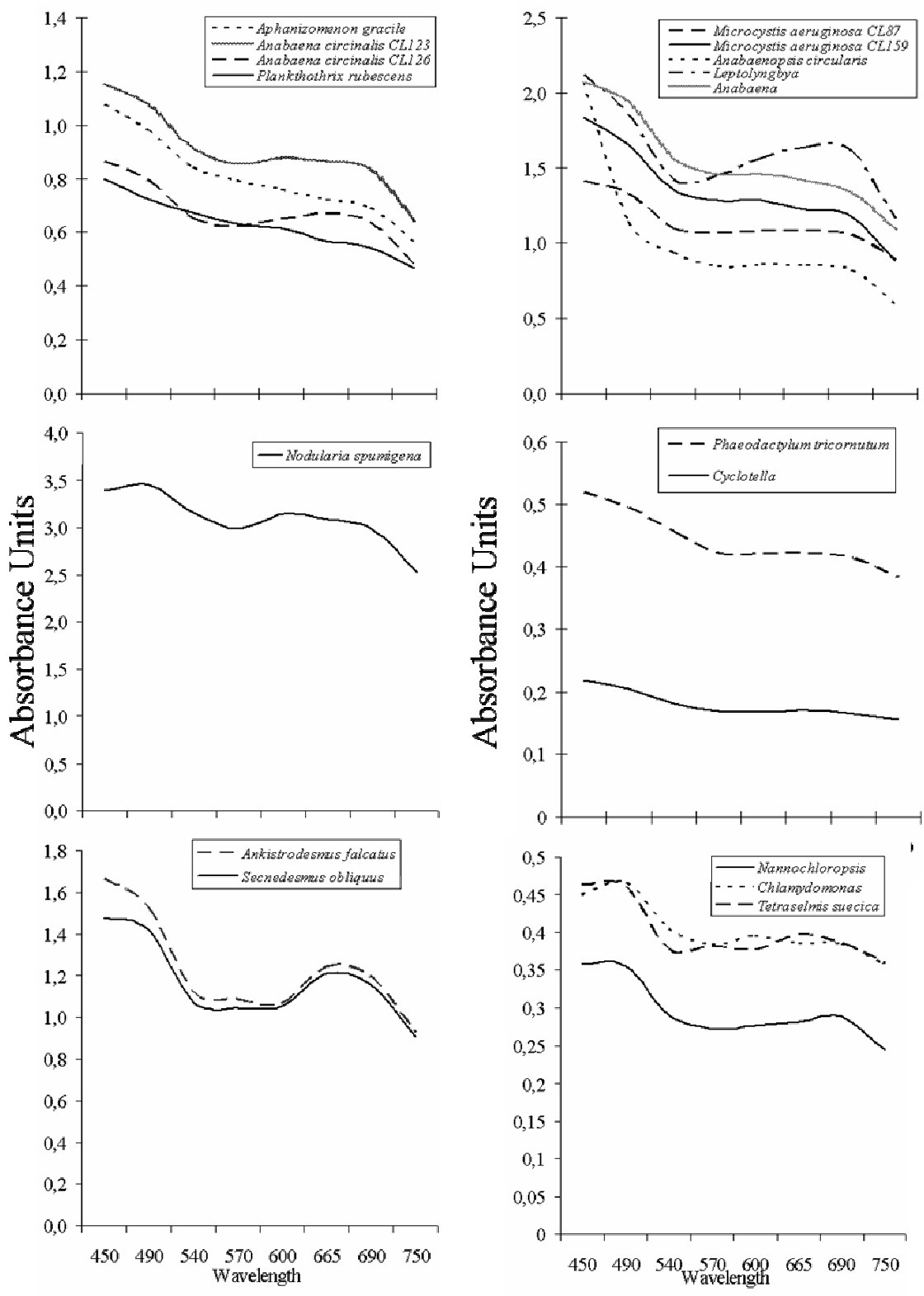
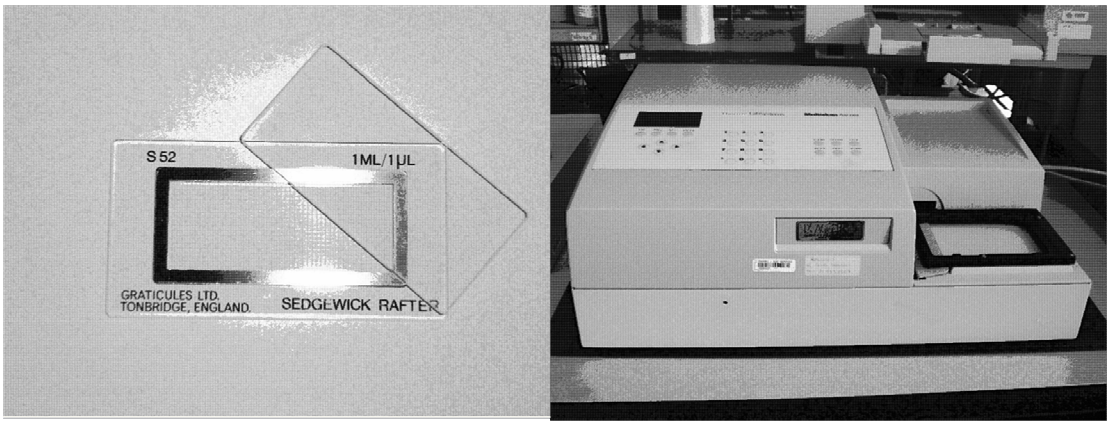


Figure 1: Algae Spectra based on the wavelengths of the spectrophotometer ELISA reader.

Appendix III
Calibration Lines



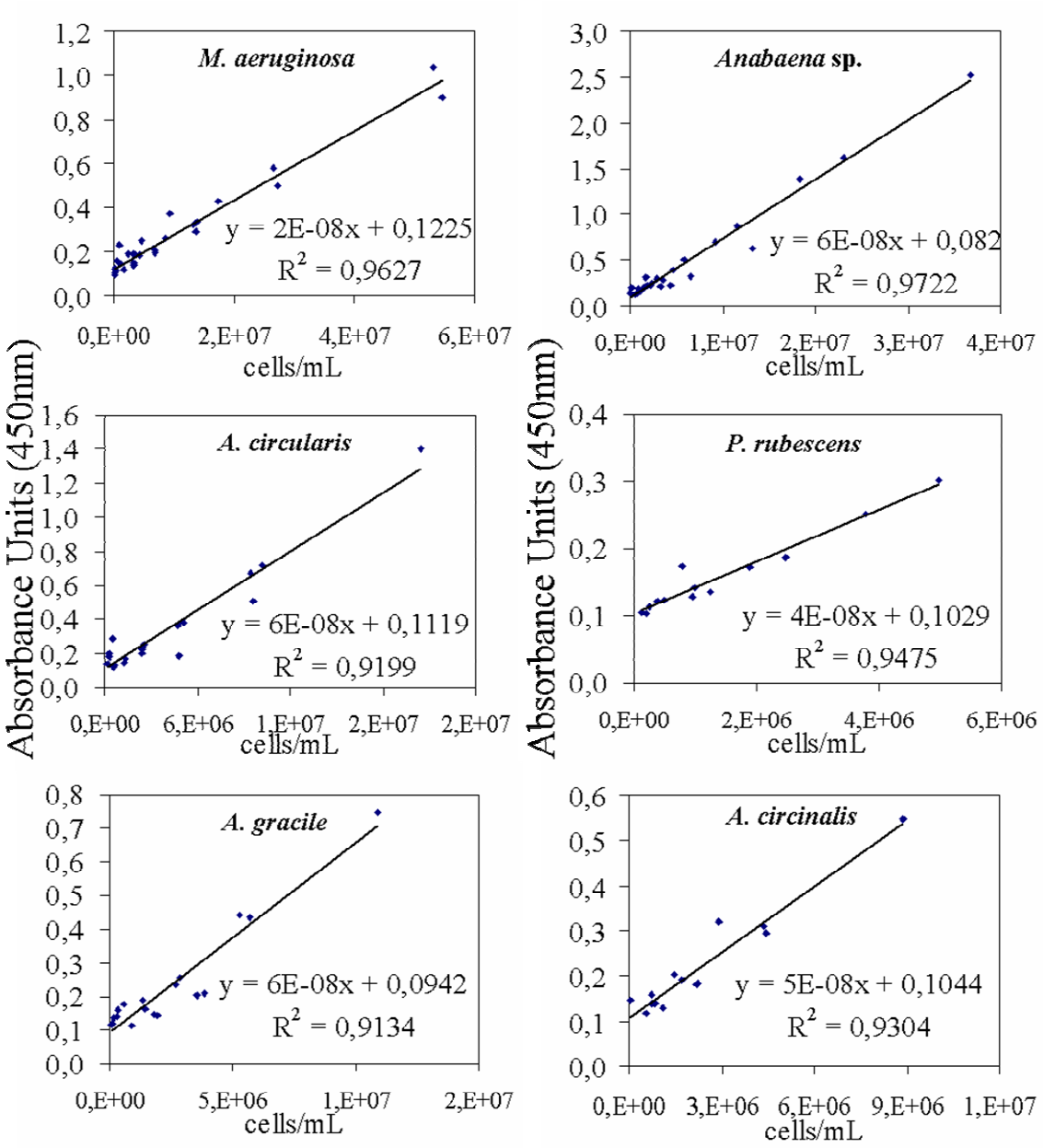


Figure 2: Calibration lines showing the linear proportionality between optical densities and cell number of Cyanobacteria.

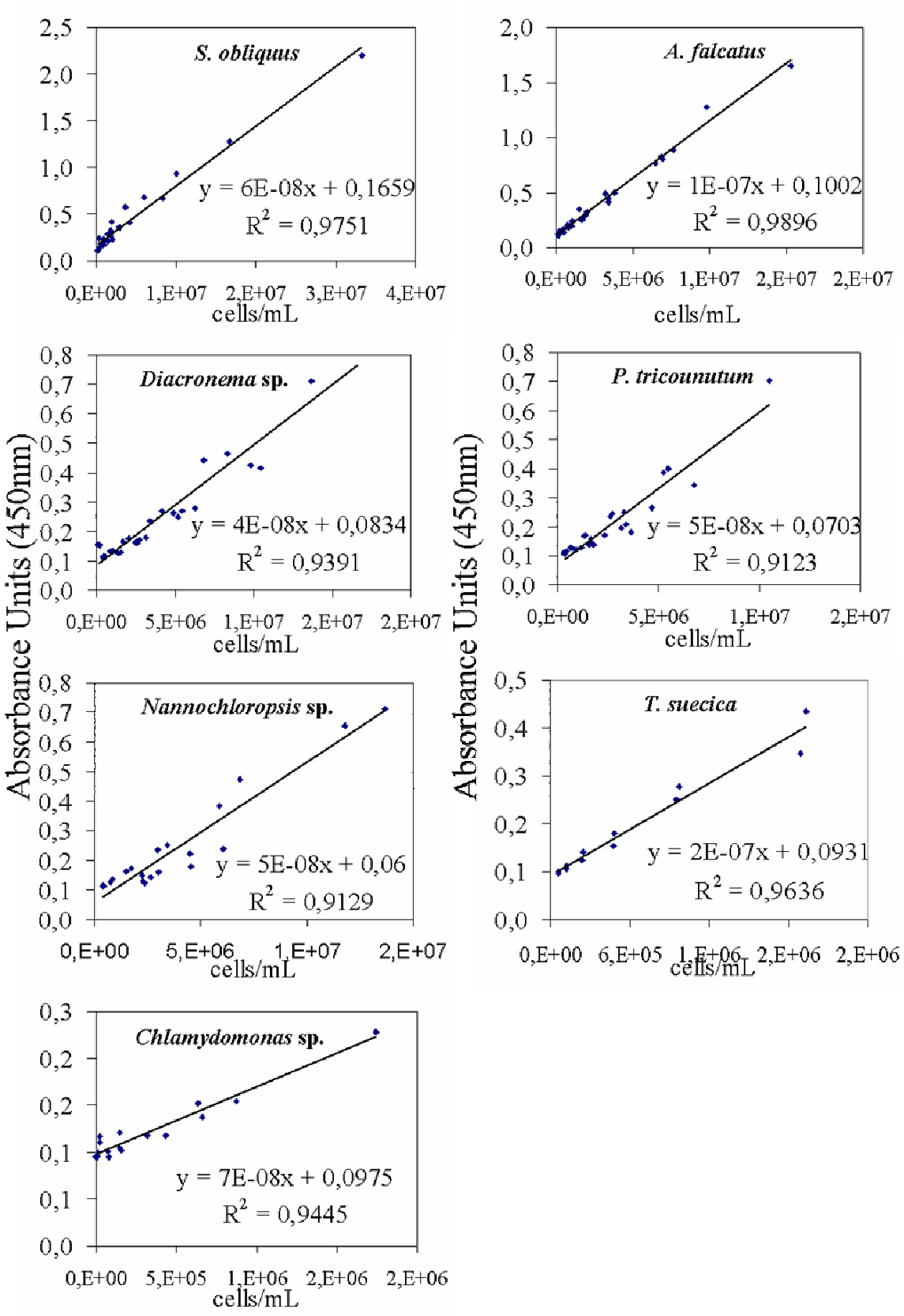


Figure 3: Calibration lines showing the linear proportionality between optical densities and cell number of Algae.