NATO Science for Peace and Security Series A:
Chemistry and Biology

Algal Toxins: Nature, Occurrence, Effect and Detection

Edited by
Valtere Evangelista
Laura Barsanti
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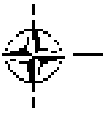

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PREFACE

This volume contains the lectures and seminars given at the NATO Advanced Study Institute on "Sensor Systems for Biological Threads: The Algal Toxins Case", held in Pisa, Italy in October, 2007. The Institute was sponsored and funded by the Scientific Affairs Division of NATO. It is my pleasant duty to thank this institution.

This ASI offered updated information on how far the research on algal toxins has gone in the exploration of structures, biosynthesis and regulation of toxins, and the development of technology for bio-monitoring these compounds.

Algae can form heavy growths in ponds, lakes, reservoirs and slow-moving rivers throughout the world; algae can house toxins which are usually released into water when the cells rupture or die. Hundreds of toxins have been identified so far. Detection methods, including rapid screening, have been developed to help us learning more about them, especially to find out which toxins are a real threat for people and what conditions encourage their production and accumulation. Early detection of algal toxins is an important aspect for public safety and natural environment, and significant efforts are underway to develop effective and reliable tools that can be used for this purpose.

A quick reacting biosensor simple to use has been a goal for many years. There are four main challenges that must be dealt with in the choice of naturally occurring biosensors: identification of a commonly occurring viable biosensor, establishment of a reliable method for monitoring and collecting data on the biosensor, protocols for the analysis and interpretation of the data collected, and packaging the system so that it could move out of the lab environment and into the field. This book will offer updated information on the development of automated systems for in situ detection of harmful algae and their toxin. Moreover, this book will offer a large amount of data by using a multidisciplinary approach strategy, which utilizes not only physical, chemistry and engineering sciences, but also biological information.

The first part of the book deals with a general overview of the toxins and toxicity related to the algal world, whereas the second part deals with uses and applications of the different kind of sensors so far developed. The first part includes an introduction on the main algal features written by our group; than, Professor Whitton describes the diversity of the cyanobacteria,

the algal division that possesses more toxic species, in relation to the environment; Dr. Zaccaroni gives us an overview on the fresh water and marine algal toxins; Professor Graneli and Doctor Fistarol describe the allelophaty phenomenon, i.e. any influence on the growth and development of natural systems produced by the algae metabolites. The first part ends with the description of toxic algal blooms in several European geographical areas by Dr. Congestri, Dr. Rakko and Dr. Bouza.

The second part of the book deals with the review of sensor organisms, the use of biochemical methods and laser Doppler techniques for toxin determination presented by Professor Parshykova; the use of nucleic acid sensor sensors for identification of toxic species illustrated by Dr. Penna and Dr. Dierks; the use of immunological ELISA analyses combined with various electrochemical detection systems to quantify algal toxins tested by Professor Albertano; a review by Dr. Scozzari on sensors based on electrochemical methods, and a gene-engineered yeast usable as biochemical instrument for toxin assessment by Dr. Gonchar.

I do hope this book has caught the spirit in which the ASI was conceived.

Paolo Gualtieri

Director of the ASI "Sensor Systems for Biological Threads: The Algal Toxins Case".

THE WORLD OF ALGAE

LAURA BARSANTI [1], PRIMO COLTELLI [2], VALTERE EVANGELISTA [1], ANNA MARIA FRASSANITO [1], VINCENZO PASSARELLI [1], NICOLETTA VESENTINI [3] AND PAOLO GUALTIERI[1]*
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Abstract: In the following sections of this chapter we will try to give an outline of some algae characteristics and general information on their classification, distribution, structure, nutrition and reproduction. In the last paragraph a short account on the origin of eukaryotic algae is set out.

Keywords: Algae, endosymbiosis theory, pigments distribution

1. Introduction

In the following sections of this chapter we will try to give an outline of some algae characteristics and general information on their classification, distribution, structure, nutrition and reproduction. In the last paragraph a short account on the origin of eukaryotic algae is set out. Some of the most peculiar and interesting features mentioned in these pages will be discussed with more details in the last chapter of the book. Although the term algae has no formal taxonomic standing, it is nevertheless routinely used to indicate a polyphyletic and artificial assemblage, of O_2–evolving, photosynthetic organisms. Then, how can we distinguish algae from plants? The answer is quite easy since the similarities we have listed between algae and plants are much fewer than their differences. Actually, plants exhibit an elevated level of differentiation (e.g. roots, leaves, stems, xylem/phloem vascular network);

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their reproductive organs are surrounded by a jacket of sterile cells; their multicellular diploid embryonic development is dependent on the parental gametophyte for a significant length of time (and this feature is the source of the name embryophytes given to plants); they exhibit tissue-generating parenchymatous meristems at the shoot and root apices which give rise to widely differentiating tissues and, last but not least, all plants have exclusively a sexual reproduction with an alternation between a haploid gametophyte and a diploid sporophyte. Algae do not form embryos, all the cells of their reproductive structures are potentially fertile and sterile cells with protective function are absent; parenchymatous development is present only in some groups and, finally, they exhibit both sexual and asexual reproduction. Moreover, in contrast with the uniformity of vascular plants, algae occur in many dissimilar forms such as microscopic single cell, macroscopic multicellular loose, or filmy conglomerations, matted or branched colonies, or more complex leafy or blade forms. Same environmental pressure led to the parallel, independent evolution of similar traits in both plants and algae, while the transition from relatively stable aquatic environment to a gaseous medium exposed plants to new physical conditions that resulted in key physiological and structural changes necessary to be able to invade upland habitats and fully exploit them.

The profound diversity of size ranging from picoplankton only 0.2–2.0 μm in diameter to giant kelps with fronds up to 60 m in length, ecology and colonized habitats, cellular structure, levels of organization and morphology, pigments for photosynthesis, reserve and structural polysaccharides, type of life history, reflects the varied evolutionary origins of this heterogeneous assemblage of organisms, including both prokaryote and eukaryote species. The term *algae* refers to macroalgae and a highly diversified group of microorganisms known as microalgae. The number of algal species has been estimated at between one to ten million, and most of them are microalgae (Barsanti and Gualtieri, 2006).

2. Classification

No easily definable classification system acceptable to all exists for algae, since taxonomy is under constant and rapid revision at all levels following every day new genetic and ultrastructural evidence. Keeping in mind that the polyphyletic nature of the algal group is somewhat inconsistent with traditional taxonomic groupings, and that taxonomyc opinion may change as information accumulates, we will adopt a tentative scheme of classification mainly based on that of Van Den Hoek et al. (1995), and compared with the classifications of Bold and Wynne (1978), Margulis et al. (1990), Graham and Wilcox (2000), Lewin, 2002 (Table 1).

TABLE 1. Classification scheme of the different algal groups.

Kingdom	Division	Class
PROKARYOTA EUBACTERIA	Cyanophyta	Cyanophyceae
	Prochlorophyta	Prochlorophyceae
EUKARYOTA	Glaucophyta	Glaucophyceae
	Rhodophyta	Bangiophyceae
		Florideophyceae
	Heterokontophyta	Chrysophyceae
		Xanthophyceae
		Eustigmatophyceae
		Bacillarophyceae
		Raphidophyceae
		Dictyochophyceae
		Phaeophyceae
	Haptophyta	Haptophyceae
	Cryptophyta	Cryptophyceae
	Dinophyta	Dinophyceae
	Euglenophyta	Euglenophyceae
	Chlorarachniophyta	Chlorarachniophyceae
	Chlorophyta	Prasinophyceae
		Chlorophyceae
		Ulvophyceae
		Cladophorophyceae
		Bryopsidophyceae
		Zygnematophyceae
		Trentepohliophyceae
		Klebsormidiophyceæ
		Charophyceae
		Dasycladophyceae

3. Distribution and Occurrence

Distribution of algae can be aquatic or subaerial, when they are exposed to the atmosphere rather than being submerged in water. Aquatic algae are found almost anywhere from freshwater spring to salt lakes, with tolerance

for a broad range of pH, temperature, turbidity, O_2 and CO_2 concentration. They can be either planktonic, as is the case of most unicellular species, living suspended throughout the lighted regions of all water bodies including under ice in polar areas, or they can be benthonic, living attached to the bottom or within sediments, limited to shallow areas because of the rapid attenuation of light with depth. Benthic algae can grow attached on stones (epilithic), on mud or sand (epipelic), on other algae or plants (epiphytic) or on animals (epizoic). On the basis of their growth habits, marine algae are designated as supralittoral, when they grow above the high tide-level, within reach of waves and spray; intertidal, when they grow on shores exposed to tidal cycles, or sublittoral, when they grow in the benthic environment from the extreme low-water level to around 200 m deep, in the case of very clear water (Bourelly, 1966, 1968, 1970).

Oceans contain more than 5000 species of planktonic microscopic algae, the phytoplankton, which forms the base of the marine food chain and produces roughly 50% of the oxygen we inhale. However, phytoplankton may become a cause of death when the population increases in response to pollution with nutrients such as nitrogen and phosphate and it's blooms reduce the water transparency, causing the death of other photosynthetic organisms. They are often responsible for massive fish and bird kills, producing poisons and toxins (Tilden, 1935). The cause and effect of pollution and algal blooms, the toxins produced by algae and their outcome as well as the sensors adopted to detect these phenomena will be the issue of the other chapters of this book. The temperate pelagic marine environment is also the realm of giant algae, the kelp. These algae have thalli up to 60 meter long, and the community can be so crowded that it forms a real submerged forest. The kelp not only inhabits temperate waters but may also form luxuriant thickets beneath polar ice sheets, and can survive at very low depth. Internal freshwater environment display a wide diversity of form of microalgae, although not exhibiting the phenomenal size range of their marine relatives. Freshwater phytoplankton and the benthonic algae form the base of the aquatic food chain.

A considerable number of subaerial algae have adapted to life on land. The activities of land algae are thought to convert rock into soil, to minimize soil erosion, to increase water retention and nutrient availability for plants growing nearby. Algae also form mutually beneficial partnership with other organisms. They live with fungi to form lichens, or inside the cells of reef-building corals, in both cases providing oxygen and complex nutrients to their partner, and in return receiving protection and simple nutrients. This arrangement enables both partners to survive in conditions that they could not endure alone.

The different type of habitat colonized by the algae of the divisions is shown in Table 2.

TABLE 2. Distribution of algal divisions. (n.d.: not detected).

Division	Habitat			
	Marine	Freshwater	Terrestrial	Symbiotic
Cyanophyta	yes	yes	yes	yes
Prochlorophyta	yes	n.d.	n.d.	yes
Glaucophyta	n.d.	yes	yes	yes
Rhodophyta	yes	yes	yes	yes
Heterokontophyta	yes	yes	yes	yes
Haptophyta	yes	yes	yes	yes
Cryptophyta	yes	yes	n.d.	yes
Chlorarachniophyta	yes	n.d.	n.d.	yes
Dinophyta	yes	yes	n.d.	yes
Euglenophyta	yes	yes	yes	yes
Chlorophyta	yes	yes	yes	yes

4. Structures

On the basis of thallus structure algae may have a unicellular, filamentous, siphonous or parenchimatous organization. Examples of the distinctive morphological characteristics within different divisions are set forth in Tables 3–5.

TABLE 3. Thallus morphology in the different algal divisions. (n.a.: not available; n.d.: not detected).

Division	Unicellular and non-motile	Unicellular and motile
Cyanophyta	*Synechococcus*	n.d.
Prochlorophyta	*Prochloron*	n.d.
Glaucophyta	*Glaucocystis*	*Gloeochaete*
Rhodophyta	*Porphyridium*	n.d.
Heterokontophyta	*Navicula*	*Ochromonas*
Haptophyta	n.d.	*Chrysochromulina*
Cryptophyta	n.d.	*Cryptomonas*
Dynophyta	*Dinococcus*	*Gonyaulax*
Euglenophyta	*Ascoglena*	*Euglena*
Chlorarachniophyta	n.d.	*Chlorarachnion*
Chlorophyta	*Chlorella*	*Dunaliella*

TABLE 4.Thallus morphology in the different algal divisions. (n.a.: not available; n.d.: not detected).

Division	Colonial and non-motile	Colonial and motile
Cyanophyta	*Anacystis*	n.d.
Prochlorophyta	n.d.	n.d.
Glaucophyta	n.d.	n.d.
Rhodophyta	*Cyanoderma*	n.d.
Heterokontophyta	*Chlorobotrys*	*Synura*
Haptophyta	n.d.	*Corymbellus*
Cryptophyta	n.d.	n.d.
Dynophyta	*Gloeodinium*	n.d.
Euglenophyta	*Colacium*	n.d.
Chlorarachniophyta	n.d.	n.d.
Chlorophyta	*Pseudosphaerocystis*	*Volvox*

TABLE 5. Thallus morphology in the different algal divisions. (n.a.: not available; n.d.: not detected).

Division	Filamentous	Siphonous	Parenchimatous
Cyanophyta	*Calothrix*	n.d.	*Pleurocapsa*
Prochlorophyta	*Prochlorothrix*	n.d.	n.d.
Glaucophyta	n.d.	n.d.	n.d.
Rhodophyta	*Goniotricum*	n.d.	*Palmaria*
Heterokontophyta	*Ectocarpus*	*Vaucheria*	*Fucus*
Haptophyta	n.d.	n.d.	n.d.
Cryptophyta	*Bjornbergiella*	n.d.	n.d.
Dynophyta	*Dinoclonium*	n.d.	n.d.
Euglenophyta	n.d.	n.d.	n.d.
Chlorarachniophyta	n.d.	n.d.	n.d.
Chlorophyta	*Ulothrix*	*Bryopsis*	*Ulva*

Many algae are solitary cells, with or without flagella, hence motile or non-motile. Other algae exist as aggregates of several to many single cells held together loosely or in a highly organized fashion, the colony. In this type of aggregate, cell number is indefinite, growth occurs by cell division of its components, there is no division of labor and each cell can survive on its own. When the number and arrangement of cells are determined at the

time of origin of the colony, and remain constant during the life span period of the individual colony, the colony is termed coenobium; this can or can not be motile.

The characteristic filaments of filamentous algae result from cell division in the plane perpendicular to the axis of the filament, and have cell chains consisting of daughter cells connected to each other by their end wall. Filaments can be simple, or, otherwise, have false or true branching; they may be uniseriate or multiseriate, consisting respectively of a single or multiple layer of cells.

Siphonous algae are characterized by a siphonous or coenocytic construction, consisting of tubular filaments lacking transverse cell walls. These algae undergo repeated nuclear division without forming cell walls; hence they are unicellular, but multinucleate (or coenocytic).

Parenchimatous, as well as pseudoparenchimatous algae are mostly macroscopic with tissue of undifferentiated cells and growth originating from a meristem with cell division in three dimensions. In the case of parenchymatous algae, cells of the primary filament divide in all directions, and any essential filamentous structure is lost. This tissue organization is present in many of the brown algae. Pseudoparenchymatous algae are made up of a loose or close aggregation of numerous, intertwined, branched filaments that collectively form the thallus, held together by mucilages, especially in red algae. Thallus construction is entirely based on a filamentous construction with little or no internal cell differentiation.

5. Nutrition

In consideration of their general definition, most algal groups should be considered photoautotrophs, i.e. depending entirely upon their photosynthetic apparatus for their metabolic necessities, using sun light as source of energy, and CO_2 as carbon source to produce carbohydrates and ATP. Most algal divisions contain colorless heterotropic species that can obtain organic carbon from the external environment, either by taking up dissolved substances (osmotrophy) or by engulfing bacteria and other cells as particulate prey (phagotrophy) (Graham and Wilcox, 2000). Algae incapable of synthesizing essential components such as the vitamins of the B_{12} complex, or fatty acids, and have to import them are defined auxotrophic.

Actually, algae are mixotrophic organisms, that is, they are competent to use a complex spectrum of nutritional strategies, combining photoautotrophy and heterotrophy. The relative contribution of either nutritional strategy within mixotrophic species varies along a gradient from algae whose dominant mode of nutrition is phototrophy, through those for which phototrophy or heterotrophy provide essential nutritional supplements, to those

for which heterotrophy is the dominant strategy. Some mixotrophs are mainly photosynthetic and only occasionally use an organic energy source. Others meet most of their nutritional demand by phagotrophy, but may use some of the products of photosynthesis from sequestered prey chloroplasts (Jones, 2000). Photosynthetic fixation of carbon and use of particulate food as a source of major nutrients (nitrogen, phosphorus and iron) and growth factors (e.g. vitamins, essential amino acids, and essential fatty acids) can enhance growth, especially in extreme environments where resources are limited. Heterotrophy can be important for the acquisition of carbon when light is limiting and, conversely, autotrophy can maintain a cell during periods when particulate food is scarce.

Algae that derive their nutriment from prey, but are capable, in lack of the latter, of sustaining themselves by phototropy are said obligate heterotrophic -it is the case, for example, of *Gymnodium gracilentum*, Dynophyta.

On the other hand, algae that obtain energy primarily from sunlight and carbon from CO_2, but in case of limiting light, are capable of opting for phagotrophy and/or osmotrophy are named obligate phototrophic.

Algae that, depending on the environmental conditions, grow equally well as photoautotrophs or heterotrophs are called facultative mixotrophic, while algae that are mainly photoautotrophs, but require for their survival nutriments obtained via phagotrophy and/or osmotrophy, are named obligate mixotrophic algae.

6. Reproduction

Methods of reproduction in algae may be vegetative by division of a single cell or fragmentation of a colony, asexual by production of motile spore, or sexual by union of gametes. Sexual mode involves plasmogamy (union of cells), karyogamy (union of nuclei), chromosome/gene association, and meiosis, resulting in genetic recombination. Vegetative and asexual modes provide a fast and economical means of increasing the number of individuals and grant the stability of an adapted genotype within a species from a generation to the next, although genetic variability is restricted. On the other hand, sexual reproduction allows variation but is more costly, because of the waste of gametes that fail to mate.

The simplest form of reproduction is by binary fission. In this mode of reproduction, which is, for example, that of *Euglena* (Euglenophyta), the parent organisms divide into two equal parts, each having the same hereditary information as the parents. In unicellular algae cell division may be longitudinal or transverse. The growth of the population follows a typical curve consisting of a lag phase, an exponential or log phase, and a stationary or plateau phase, where increase in density has leveled off.

Another method of asexual reproduction is by formation of zoospores. These, that are typical, for example, of *Chlamydomonas* (Chlorophyta) are flagellate motile spores produced within a parental vegetative cell. Spores that begin their development within the parent cell wall and are released still partially undeveloped, are named aplanospores. The latter have no flagellum, but may develop it, and thus be unrecognizable form zoospores. Finally, spores that are almost perfect replicas of the vegetative cells that produce them but do not develop flagella are named autospores. Examples of autospore forming genera are *Nannochloropsi*s (Heterokontophyta) and *Chlorella* (Chlorophyta). Spores may be produced within and by ordinary vegetative cells or within specialized cells or structures called sporangia.

The reproductive mode of coenobia or colonies is named autocolony formation. This kind of reproduction implies that each cell within the colony can produce a new colony similar to the one to which it belongs. Thus, cell division gives rise no longer to unicellular individuals, but to multicellular groups: a sort of embryonic colony that differs from the parent in cell size but not in cell number. This is the reproductive mode of green algae such as *Volvox* (Chlorophyta) and *Pediastrum* (Chlorophyta).

Vice versa, non-coenobic colonies reproduce themselves via fragmentation: that is filaments break into two or more fragments that develop into new individuals.

Sexual reproduction involves, of course, gamete formation. Gametes may be morphologycally identical with vegetative cells or markedly differ from them, depending upon the algal group. The main difference is obviously the DNA content that is haploid instead of diploid. Different combinations of gamete types are possible. In the case of isogamy, gametes are both motile and indistinguishable. When the two gametes differ in size, we have heterogamy. This combination occurs in two types: anysogamy, where both gametes are motile, but one is small (sperm) and one is large (egg); oogamy, when only one gamete is motile (sperm), and fuses with the other that is non-motile and very large (egg).

Algae exhibit three different life cycles with variation inside the different groups. The main difference is the point where meiosis occurs and the type of cells it produces, and whether or not there is more than one free-living stage present in the life cycle.

In the haplontic or zygotic life cycle the predominant vegetative state is haploid and meiosis takes place upon germination of the zygote. The opposite comes about in the diplontic or gametic life cycle, in which the predominant vegetative state is diploid and meiosis gives rise to haploid gametes. The former mode of reproduction is typical, for example of *Chlamydomona*s (Chlorophyta) and the latter of Diatoms and Fucus (Heterokonphyta). In diplohaplontic or sporic life cycles there is an alternation of generation between

the two phases of haploid gametophyte and diploid sporophyte. The former produces gametes by mitosis and the fomer spores by meiosis. Alternation of generation can be isomorphic – morphologically identical – (as is the case in *Ulv*a (Chlorophyta)) or heteromorphic with predominance of either the sporophyte (e.g. Laminaria (Heterokontophyta)) or the gametophyte (e.g. *Porphiria* (Rhodophyta)).

Under unfavorable conditions, such as shortage of environmental nutrients, limiting light or desiccation, many algal groups produce thick walled resting cells, such as hypnospores, hypnozygotes, statospores, and akinetes. Resting stages represent a survival strategy and resting cells may dwell in sediments for many years, enduring very harsh conditions, and remain viable to assure the continuance of the species. When suitable conditions for vegetative growth are restored, the akinete germinates into new vegetative cells.

7. Origin of Eukaryotic Algae

Cyanobacteria are the living procariotic ancestors of algae. Cyanobacteria evolved more than 2.8 billion years ago and have played fundamental roles in driving much of the ocean carbon, oxygen and nitrogen fluxes from that time to present. The evolution of cyanobacteria was a major turning point in biogeochemistry of Earth. Prior to the appearance of these organisms, all photosynthetic organisms were anaerobic bacteria that used light to couple the reduction of carbon dioxide to the oxidation of low free energy molecules, such as H_2S or preformed organics. Cyanobacteria developed a metabolic process, the photosynthesis, that exploits the energy of visible light to oxidize water and simultaneously reduces CO_2 to organic carbon represented by $(CH_2O)n$ using light energy as a substrate and chlorophyll a as a requisite catalytic agent (Carr and Whitton, 1982). Formally oxygenic photosynthesis can be summarized as:

$$CO_2 + H_2O + \text{light} \xrightarrow{\text{Chlorophyll}a} (CH_2O)n + O_2$$

All other oxygenic producing algae are eukaryotic, that is they contain internal organelles, including a nucleus, one or more chloroplasts (Marin, 2004), one or more mitochondria, and, most importantly, in many cases they contain a membrane bound storage compartment or vacuole.

Historically, the major groups of algae are classified into Divisions (the equivalent taxon in the zoological code was the Phylum) on the basis of pigmentation, chemical nature of photosynthetic storage product, photosynthetic membranes (thylakoids) organization and other features of the chloroplasts, chemistry and structure of cell wall, number, arrangement and ultrastructure of flagella (if any), occurrence of any other special features, and sexual cycles. Recently, all the studies that compare the sequence of

macromolecules genes and the 5S, 18S and 28S ribosomal RNA sequences tend to assess the internal genetic coherence of the major divisions (Cavalier-Smith, 2002; Lewis and McCourt, 2004). This confirms that these divisions are non-artificial, even though they were originally defined on the basis of morphology only (Van den Hoek et al., 1995). Table 6 and 7 attempt to summarize the main characteristics of the different algal divisions.

TABLE 6. The main pigments of the algal divisions.

Division	Pigments			
	chlorophylls	phycobilins	carotenoids	xantophylls
Cyanophyta	*a*	c-Phycoerythrin *c*-Phycocyanin Allophycocyanin	β-carotene	myxoxanthin zeaxanthin
Prochlorophyta	*a, b*	absent	β-carotene	zeaxanthin
Glaucophyta	*a*	Phycoerythrocyanin *c*-Phycocyanin Allophycocyanin	β-carotene	zeaxanthin
Rhodophyta	*a*	*r,b*-Phycoerythrin *r*-Phycocyanin Allophycocyanin	α-, and β-carotene	lutein
Cryptophyta	*a, c*	Phycoerythrin-545 *r*-Phycocyanin Allophycocyanin	α-, β-, and ε-carotene	alloxanthin
Heterokontophyta	*a, c*	absent	α-, β- and ε-carotene	fucoxanthin violaxanthin
Haptophyta	*a, c*	absent	α- and β-carotene	fucoxanthin
Dynophyta	*a, c*	absent	β-carotene	peridinin fucoxanthin diadinoxanthin dinoxantin gyroxanthin
Euglenophyta	*a, b*	absent	β- and γ-carotene	diadinoxanthin
Chlorarachniophyta	*a, b*	absent	absent	lutein neoxanthin violaxanthin
Chlorophyta	*a, b*	absent	α-, β- and γ-carotene	lutein prasinoxanthin

TABLE 7. The main storage products of the algal divisions.

Division	Storage products
Cyanophyta	cyanophycin (argine and asparagine polymer) cyanophycean starch (α -1,4-glucan)
Prochlorophyta	cyanophycean starch (α -1,4-glucan)
Glaucophyta	starch (α -1,4-glucan)
Rhodophyta	floridean starch (α -1,4-glucan)
Cryptophyta	starch (α -1,4-glucan)
Heterokontophyta	chrysolaminaran (β -1,3-glucan)
Haptophyta	chrysolaminaran (β -1,3-glucan)
Dynophyta	starch (α -1,4-glucan)
Euglenophyta	paramylon (β -1,3-glucan)
Chlorarachniophyta	paramylon (β -1,3-glucan)
Chlorophyta	starch (α -1,4-glucan)

Within the algae, different evolutionary lineages are discernable. Three major eukaryotic photosynthetic groups have descended from a common prokaryotic ancestor, through an endosymbiotic event (Patterson, 1999). Therefore these algae possess primary plastid, i.e. derived directly from the prokaryotic ancestor. Other algal groups have acquired their plastids via secondary (or tertiary) endosymbiosis, where a eukaryote already equipped with plastids is preyed upon by a second eukaryotic cell. Endosymbiotic process produced nested cellular compartments one inside the other, which can give information about the evolutionary history of the algae containing them (Keeling, 2002; Dyall et al., 2004).

The three major algal lineages of primary plastids are the Glaucophyta lineage, the Chlorophyta lineage, and the Rhodopyta lineage.

Glaucophyta lineage occupies a key position in the evolution of plastids. Unlike other plastids, the plastids of glaucophytes retain the remnant of a gram-negative bacterial cell wall of the type found in cyanobacteria, with a thin peptidoglycan cell wall and cyanobacterium-like pigmentation that clearly indicate its cyanobacterial ancestry.

Green algae (Chlorophyta) constitute the second lineage of primary plastids. The simple two membrane system surrounding the plastid, the congruence of phylogenies based on nuclear and organellar genes, and the antiquity of the green algae in the fossil record all indicate that the green

algal plastid is of primary origin. In these chloroplasts chlorophyll *b* was synthesized as a secondary pigment and phycobiliproteins were lost. Another hypothesis is that the photosynthetic ancestor of green lineage was a prochlorophyte that possessed chlorophylls *a* and *b* and lacked phycobiliproteins.

The green lineage played a major role in oceanic food webs and the carbon cycle from about 2.2 billion years ago until the end-Permian extinction, approximately 250 million years ago. It was this similarity to the pigments of plants that led to the inference that the ancestors of land plants (i.e. embryophytes) would be among the green algae, and is clear that phylogenetically plants are a group of green algae adapted to life on land. Euglenophyta and Chlorarachniophyta derived from this primary plastid lineage by secondary endosymbiosys; the green algal plastid present in Euglenophyta is bounded by three membranes, while the green algal plastid present in the Chlorarachniophyta is bound by four membrane.

Since that time however, a second group of eukaryotes has risen to ecological prominence; that group is commonly called the "red lineage". The plastids of the red algae (Rhodophyta) constitute the third primary plastid lineage. Like the green algae, the red algae are an ancient group in the fossil record, and some of the oldest fossils interpreted as being of eukaryotic origin are often referred to the red algae, although clearly these organisms were very different from any extant alga (McFadden and van Dooren, 2004). Like those of green algae, the plastids of red algae are surrounded by two membranes. However, they are pigmented with chlorophyll *a* and phycobiliproteins, which are organized into phycobilisomes. Phycobilisomes are relatively large light-harvesting pigment/protein complexes that are water-soluble and attached to the surface of the thylakoid membrane. Thylakoids with phycobilisomes do not form stacks like those in other plastids, and consequently the plastids of red algae (and glaucophytes) bear an obvious ultrastructural resemblance to cyanobacteria.

A number of algal groups have secondary plastids derived from those of red algae, including several with distinctive pigmentation. The cryptomonads (Cryptophyta) were the first group in which secondary plastids were recognized, on the basis of their complex four membrane structure. Like red algae, they have chlorophyll *a* and phycobiliproteins, but these are distributed in the intrathylakoidal space rather than in the phycobilisomes found in red algae, Glaucophyta, and Cyanophyta. In addition, cryptomonads possess a second type of chlorophyll, chlorophyll *c*, which is found in the remaining red lineage plastids. These groups, which include the Heterokontophyta (including kelps, diatoms, chrysophytes, and related groups),

Haptophyta (the coccolithophorids), and probably those dinoflagellates (Dynophyta) pigmented with peridinin, have chlorophylls *a* and *c*, along with a variety of carotenoids, for pigmentation. Stacked thylakoids are found in those lineages (including the cryptomonads) that lack phycobilisomes. The derivation of chlorophyll c containing plastids from the red algal lineage is still somewhat conjectural, but recent analyses of both gene sequences and gene content are consistent with this conclusion.

A few groups of dinoflagellates have plastids now recognized to be derived from serial secondary endosymbiosis (the uptake of a new primary plastid- containing endosymbiont) such as *Lepidodinium* spp. or tertiary endosymbiosis (the uptake of the secondary plastid-containing endosymbiont), such as *Dinophysis*, *Karenia*, and *Kryptoperidinium*.

All of these groups are comparatively modern organisms; indeed the rise of dinoflagellates and coccolithophorids approximately parallels the rise of dinosaurs, while the rise of diatoms approximates the rise of mammals in the Cenozoic (Hackett et al ., 2004). The burial and subsequent diagenesis of organic carbon, produced primarily by members of the red lineage in shallow seas in the Jurassic period provide the source rocks for most of the petroleum reservoirs that have been exploited for the past century by humans.

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CYANOBACTERIAL DIVERSITY IN RELATION TO THE ENVIRONMENT

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Abstract: Cyanobacterial diversity is reviewed to provide background knowledge for researchers concerned with cyanobacterial toxins and toxicity, with emphasis on ecological, morphological and physiological aspects. Ways in which the environment influences cyanobacterial morphology on evolutionary and short-term time-scales are assessed, because lack of understanding about this underlies some of the muddle in current taxonomic approaches. The review then deals with how cyanobacteria respond to their environment, including sensing, N versus P limitation, some less well recognized aspects of P limitation and responses to grazers. The importance of considering flowing water and other types of envirponment besides lakes and reservoirs is stressed. Comment on interactions between cyanobacteria and other organisms includes mention of the use of barley straw in controlling blooms, an approach which has often proved successful in the British Isles and elsewhere in Europe, but less so in the USA. The review ends with how the environment influences toxin production and toxicity of the toxins released. Several topics are suggested where there is a need for research, such as whether the calyptra of some Oscillatoriaceae has a role in sensing the environment, and whether some rice cultivars produce straw useful for controlling blooms.

Keywords: Cyanobacteria, environment, toxins, toxicity, taxonomy, phosphorus limitation, phosphatases, grazers, allelopathy, barley straw

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1. Introduction

The cyanobacteria are photosynthetic prokaryotes found in most, though not all, types of illuminated environment (Whitton and Potts, 2000; Castenholz, 2001). They all have the ability to synthesize chlorophyll a and most can also form the blue accessory phycobilin pigment, phycocyanin; in some cases the red accessory pigment, phycoerythrin, is formed as well. However, several genera do not form the phycobilin pigments, instead possessing chlorophyll b. Among these, *Prochlorococcus* was first reported as recently as 1988 by Chisholm et al., but is now realized to be of major importance in the oceans (Zwirglmaier et al., 2007) and it seems surprizing that it was overlooked for so long. Other important phenomena were also long overlooked, so the reader is encouraged to take a critical approach to the present literature on cyanobacteria.

A number of features favour their success. The temperature optimum for many or most is higher by at least several degrees than for most eukaryotic algae (Castenholz and Waterbury, 1989). Many terrestrial forms tolerate high levels of ultra-violet irradiation (see 2.3), whereas the success of many planktonic forms is favoured by their ability to utilize light for photosynthesis efficiently at low photon flux densities (van Liere and Walsby, 1982). Tolerance of desiccation and water stress is widespread (Potts, 1994) and cyanobacteria are among the most successful organisms in highly saline environments (Bauld, 1981). Photosynthetic CO_2 reduction can sometimes proceed efficiently at very low concentrations of inorganic carbon (Pierce and Omata, 1988). The ability of many species to fix atmospheric nitrogen provides a competitive advantage where combined nitrogen concentrations are low (see 3.2.1). Free sulphide is tolerated by some species at much higher levels than by most eukaryotic algae and H_2S is sometimes utilized as the hydrogen donor during photosynthesis (Cohen et al., 1986). The ability to form gas-vacuoles in some common freshwater plankton species and the marine *Trichodesmium*, and hence increase buoyancy, gives an advantage in waters where the rate of vertical mixing of the water column is relatively low (van Liere and Walsby, 1982).

The fact that some species are highly toxic to humans and some other animals has been reported extensively, though most accounts relate to bloom-forming species in freshwaters and shallow coastal waters (Codd et al., 2005); several chapters in the present book review methods for monitoring these species and their toxins. It is less clear how widespread cyanobacterial toxicity is in other types of environment or how the environment may influence the toxicity of species where toxins can occur. (The terms toxins and toxicity is used here to refer to effects on mammals, including humans, unless other organisms are mentioned specifically).

The aim of this chapter is to provide an environmental perspective for researchers investigating these aspects of toxicity and who are not necessarily specialists in cyanobacteria, though anyone lacking previous experience of the organisms is recommended to read the account by Mur et al. (1992) first of all. Because of the wide range of topics covered, the choice here is highly selective. The chapter starts with a brief overview of cyanobacterial diversity, followed by comments on how the environment influences cyanobacteria, both their long-term evolution and short-term responses, including methods for sensing their environment. This is followed by an account of the environments where toxicity has been reported. Finally, there is an attempt to bring the two subjects together by assessing what is known about the influence of environmental factors on toxins and toxicity. Molecular aspects of cyanobacterial toxins are mentioned only briefly, partly because there are reviews elsewhere (Börner and Dittmann, 2005; Neilan et al., 2007) and partly because the rapid progress in the subject, exemplified by the sequencing of the genome of a toxic strain of *Microcystis aeruginosa* (Kaneko et al., 2007), means that comments are likely to date rapidly.

2. Long-term Responses to Environment

2.1. MORPHOLOGY

Single-celled cyanobacteria range in size from about 0.6 μm to well over 30 μm in their largest dimension. Although most species exceed 2 μm, the upper limit of organisms recognized as picoplankton, it seems likely that, based on world-wide biomass, the majority are below this limit. The picophytoplankton include both *Prochlorococcus* and the phycocyanin-containing *Synechococcus*, which together form the dominant phototrophs of many regions of the tropical and subtropical oceans (Zwirglmaier et al., 2008). In contrast, they are conspicuously absent or rare in polar oceans (Vincent, 2000).

Small-celled cyanobacteria also occur in the plankton of almost all freshwater lakes, (Hawley and Whitton, 1991), though not in the most acidic (Steinberg et al., 1997). Their relative importance is greatest in large, low-nutrient lakes (Callieri, 2007), where the cells are typically larger than the marine forms and sometimes slightly exceed the picoplankton size limit. Unlike those in the open oceans, some of the freshwater picoplankton-size cyanobacteria are joined by mucilage strands or other arrangements (Skuja, 1948). Such organisms typically occur in humus-rich waters and are often overlooked in standard counting procedures. The plankton of small waterbodies is sometimes dominated by slightly larger individual cells, but most

of the larger unicellular forms are aggregated in various ways to form colonies. *Microcystis*, the most notorious of the freshwater toxin-formers, is an example (Visser et al., 2005). There are also many colonial unicellular genera associated with surfaces.

The trichomes of filamentous cyanobacteria come into the diameter range 0.4 μm to 50 μm, but there are a few records of species with trichomes well over 100 μm diameter. Even when they include only a single trichome, filaments may be much wider due to the presence of extracellular polymers organized into firm sheaths. The filamentous forms have been separated in various ways into several orders, with the important split between those without and those with heterocysts, the site of nitrogen fixation in most nitrogen-fixing cyanobacteria of well-oxygenated environments. Phylogenetic studies indicate that the non-heterocystous filamentous forms are highly diverse, but the large number of genes involved in heterocyst development suggests that the main steps in heterocyst evolution occurred only once (Henson et al., 2004).

2.2. TAXONOMY AND NOMENCLATURE

It is important for toxicity and other studies to be able to give meaningful and consistent names to the diversity of organisms found in field and laboratory samples, yet perhaps for more than any other group of phototrophs, this can be a challenge. This is in part because there have been four different approaches to their nomenclature. Although a critical assessment of these approaches lies outside the scope of this chapter, some understanding is needed to interpret the literature on toxicity. One of the approaches, that due to F. Drouet (e.g. 1968) was based on a huge reduction in number of species, with individual species distinguished from one another only by large differences in morphology. This approach has now largely been discarded and is only mentioned here because Drouet's taxonomic names were used for the colour pictures shown by Palmer (1966). This book was distributed widely round the world free of charge and the pictures subsequently reproduced in many editions of *Standard Methods for Water and Wastewater Treatment* published by American Public Health Association and the names given by Drouet have therefore been seen by numerous people in water management.

The classical approach evolved during the 19th century, with much of the morphological diversity of what were then known universally as blue-green algae being recognized at the generic level by the end of that century. However, the information was not really consolidated clearly until Geitler's (1932) flora, which is the best known text of what may be considered as classical taxonomy. While it is essential to use a more up-to-date account

(e.g. Komárek and Anagnostidis, 1999) to describe the diversity of unicellular cyanobacteria in field populations, Geitler (1932) is still a useful source to start to assess the diversity of filamentous forms.

A third approach is that introduced in the 1970s by R. Y. Stanier, who became convinced that these organisms should be treated like bacteria, with isolation and culture of individual cells or filaments (Rippka et al., 1979). It was proposed that their taxonomy should follow the rules of the International Code of Nomenclature of Bacteria (rather than the International Code of Botanical Nomenclature), together with a change in name to cyanobacteria (Stanier et al., 1978). The many subsequent steps towards this have been reviewed by Oren (2004), who stressed the need for botanical and bacteriological taxonomists to use unified rules to describe new taxa. *Bergey's Manual of Determinative Bacteriology* (Boone and Castenholz, 2001) provides the most recent taxonomic account using the bacteriological approach.

The fourth approach is that given in a number of articles by Anagnostidis and Komárek (e.g. 1985) and two books by the same authors (Komárek and Anagnostidis, 1999, 2002). This is essentially an on-going elaboration of the classical system with frequent reconsideration of the importance of various characters and at the same time incorporating as much new information as possible. Whitton's (2002) account for the British Isles was much nearer that of Geitler (1932). A conservative approach was adopted because it seemed best to wait until much more molecular data are available before making a major reconsideration of nomenclature for the whole group. None of the present approaches is ideal for all purposes and the situation can be confusing to non-specialists who are unaware of the situation.

2.3. STRESS

Some of the most important criteria in the 19th century descriptions of genera or species were ones which we now know are responses to stress or limitation, though the original authors had little or no understanding of this. The sheaths which enclose the trichomes of many filamentous forms provide one example. Among the freshwater plankton they occur in only some species. Species on terrestrial surfaces subjected to intermittent drying mostly surrounded by firm sheaths or mucilage, which aid desiccation tolerance (Potts, 1994), though may also have other functions, which are not necessarily the same in different species. In many cases species glide out of their sheath if submerged in liquid, but reform them again under water stress. Species with firm sheaths are much less common in the plankton, although many do have surrounding mucilage.

The brown pigment scytonemin which is present in the sheaths or mucilage protects cells again damage by UVR (ultra-violet radiation) damage (Castenholz and Garcia-Pichel, 2000) and in most cases UVR is required to initiate its synthesis (Garcia-Pichel and Castenholz, 1991).

Heterocyst formation in *Anabaena* occurs when combined nitrogen is absent or low in relation to supply of other key nutrients (Wolk, 1983) and most heterocystous genera probably behave in a similar way, though there are examples such as the hair-forming genus *Calothrix* (Sinclair and Whitton, 1977), where heterocyst formation is less clearly linked to the recent environment.

Genera able to form multicellular hairs or other types of long, markedly tapered trichomes do this under conditions of phosphorus limitation (Whitton, 1988). The extent of tapering in *Aphanizomenon flos-aquae*, which in some cases leads to the formation of hair-like ends to the filaments, is also enhanced by phosphorus limitation (author, unpublished data). In contrast to heterocysts, the formation of multicellular hairs has almost certainly evolved a number of times. Colonial cyanobacteria forming hairs, such as *Calothrix* and *Rivularia*, exist in this condition for most of their growth period (Whitton, 1987), only producing motile hormogonia when exposed to high phosphate. Unfortunately the taxonomy of hair-forming cyanobacteria is confused in accounts based on the Stanier system, because the growth medium used for assays (BG11: Allen and Stanier, 1968) contained such a high phosphate concentration (5 mg L^{-1} P) that morphological features associated with phosphate limitation are seldom seen in culture. Even if a reduced concentration of 1 mg L^{-1} P is used for batch culture studies, this is usually still too high for the characteristic morphology to be seen (author, unpublished data).

The cells of multicellular hairs are highly vacuolated, the vacuoles being formed as a result of the separation of the two thylakoidal membranes (Whitton, 2005). Although the hair cells lose their chlorophyll, the intrathylakoidal vacuoles which sometimes occur in other genera can develop in cells which still contain chlorophyll, though it is unclear whether the particular membranes surrounding the vacuole are still pigmented. Such vacuoles often occur in *Scytonema*, where the cells furthest from the filament apex develop them. They also occur in the terminal regions of some non-heterocystous filamentous forms, which taper towards the apex, as illustrated for *Phormidium autumnale* by Geitler (1932). In both these cases the vacuoles form in cells furthest from the growing region of the filament, suggesting that their presence is a sign that the cell is becoming unhealthy. Liquid (intrathylakoidal) vacuoles often occur in all cells of populatons of the unicellular *Synechococcus aeruginosus* and the filamentous *Oscillatoria bornetii* and *O. bourrellyi*, though *O. bourrellyi* and perhaps also the others

can also exist in a state where the presence of the vacuoles is scarcely visible with the light microscope (Whitton, 2002). In (Lake) Windermere, England, the highly vacuolated form of *O. bourrellyi* used to develop in summer and the less vacuolated form in winter, when ambient phosphate concentrations were much higher than in summer (J. W. G. Lund, pers. comm.). When considered together, these observations suggest it would be worth investigating whether intrathylakoidal vacuole formation is associated with a mechanism for optimizing phosphate uptake under P-limiting conditions.

3. Short-term Responses to Environment

3.1. SENSING THE ENVIRONMENT

An ability to detect and respond to variations in the environment is very important and a range of studies have been reported, mainly with respect to light, nitrogen and phosphorus. but also toxin formation. Although dealing mainly with biochemical and molecular aspects, the review by Mann (2000) provides a broader guide to the literature. Many of the earlier studies were concerned with detection and responses to light of motile filamentous forms gliding on a surface. For instance, Castenholz (1983) concluded that most responses to light occurred only after apparently random movements have resulted in the long axis lying parallel to the light field. However, steering did occur in some articulated trichomes, such as members of the Nostocaceae. More recently it has been shown that a range of receptors exist for sensing light (Mullineaux, 2001), including phytochromes, cryptochromes and rhodopsin (Albertano et al., 2001; Jung et al., 2004).

There has also been considerable interest in chemical signalling. Akinete (spore) formation in an exponentially growing *Cylindrospermum licheniforme* could be induced by cell-free supernatant of an akinete-containing culture (Fisher and Wolk, 1976). The akinete-inducing substance was isolated and its structure partially determined (Hirosawa and Wolk, 1979). Heterocysts, but not active nitrogen fixation, were essential for akinete formation (Van de Water and Simon, 1982). Once sporulation commenced in a culture, the process spread rapidly through the population. Field observations on several *Cylindrospermum* populations growing on sediments indicated that a similar behaviour probably occurred there.

It has long been considered that a range of cyanobacterial responses probably involve chemotaxis (Castenholz, 1983), but the first convincing evidence came from Waterbury et al. (1985) for marine phycoerythrin-containing *Synechococcus* isolates showing swimming motility. These did

not exhibit photokinesis, photophobic or photactic responses and motility was retained in the dark. The swimming behaviour, which was confined to open ocean isolates, showed a marked chemotactic response to various nitrogenous compounds (Willey and Waterbury, 1989). The threshold levels for chemotactic responses were in the range 10^{-9}–10^{-10} M, which could be ecologically significant in the ocean.

The study of chemotaxis in cyanobacteria has in recent years largely focussed on the attraction of hormogonia to potential symbiotic partners. The first full account was for *Nostoc* and the liverwort *Blasia* (Knight and Adams, 1996), but the phenomenon has now been shown for a range of associations (Adams, 2000; Nilsson et al., 2006). The mutual attraction of cyanobacterial cells to each other, such as hormogonial aggregation during colony formation in genera like *Rivularia* (Whitton, 2002), still awaits detailed study.

Strangely, the role of the calyptra and other modifications of the terminal cell of many motile non-heterocystous filamentous forms (Oscillatoriceae) has never been investigated. It is probably the best known morphological structure in cyanobacteria about which nothing is known of its function. However, it seems to the author likely that this may be involved in chemotaxis. The fact that such cells form only at one end of the trichome and that the terminal regions of these trichomes often flex around suggests the possibility of their playing a role in detecting features of their environment such as phosphate or other nutrient gradients. If the calyptra does prove to be an important detector region, perhaps it could be used to incorporate detector molecules for toxins and other compounds.

Many authors have suggested the likelihood that quorum sensing is involved in various cyanobacterial processes (e.g. Mann, 2000). This is partly because of similarities with other bacterial processes or genes where it is known to be involved. For instance, some microcystin related genes show a high similarity to genes in other bacteria which are regulated by a quorum sensing system. However, several studies have failed to find evidence for quorum sensing influencing microcystin formation. Density changes during growth of *Microcystis* do not seem to influence transcription of the microcystin gene cluster (Dittmann et al., 2001; Braun and Bachofen, 2004; Pearson et al., 2004). ^{14}C studies with *M. aeruginosa* PCC 7806 showed that cells were not subject to significant loss processes such as export from the cells or intracellular breakdown, and the authors (Rohrlack and Hyenstrand, 2007) interpreted this as lack of evidence for quorum sensing or other functions requiring export of compounds to the medium, such as protection from epiphytes.

3.2. MORPHOLOGY AND PHYSIOLOGY

3.2.1. *Introduction*

Although morphological characters relating to the ability of a cyanobacterium to withstand stress have evolved over long periods of time, many of these characters are also ones which vary during the growth of individual organisms, as described above for scytonemin, heterocysts and multicellular hairs. The extent of gas vacuole formation and hence buoyancy is another obvious way in which planktonic cyanobacteria can respond to their environment. In general high light or limitation by major nutrients such as nitrogen or phosphorus leads to a decrease in number of gas vesicles per cell and hence a decrease in buoyancy, though there are many factors which may complicate this (Oliver and Ganf, 2000).

In most cases the 'stress' characters start to develop in response to only moderate stress or limitation. The pronounced occurrence of a particular character at a site therefore indicates the likely importance of the relevant stress there. This has been considered most often for the heterocyst, because of the ease with which its frequency can be quantified. Nitrogen scarcity favours the development of nitrogen-fixing cyanobacteria, because of their competitive advantage where the concentration of combined nitrogen is low (Howarth et al., 1988; Kardinaal and Visser, 2005). Nitrogen-fixers in well-oxygenated freshwater and terrestrial environment are likely to possess heterocysts. However, the relationship between heterocyst frequency and the concentration of ambient combined nitrogen may sometimes be obscured by the fact that the same heterocyst persists for much longer periods in the morphologically more complex genera like *Aphanizomenon* and *Gloeotrichia* than they do in *Anabaena*.

Cyanobacterial nitrogen fixation in freshwater and terrestrial environments with low dissolved oxygen is usually carried out by species lacking heterocysts and in most cases there are no morphological features indicating whether or not this ability is likely to occur. In the marine environment heterocystous nitrogen-fixers are largely confined to the upper intertidal zone or shallow brackish waters. The main nitrogen-fixers in the open sea are the unicellular *Crocosphaera watsonii* (Zehr et al., 2007) and filamenttous *Trichodesmium* (Lagomela et al., 2002), which lacks heterocysts, but does possess special cells recognizable with light microscopy.

3.2.2. *N versus P limitation*

The relative importance of particular nitrogen fractions and the environmental N : P ratio have a been suggested to influence the success of particular groups of cyanobacteria. Blomqvist et al. (1994) concluded that non-nitrogen-fixing cyanobacteria are favoured by NH_4-N, as was found for

Oscillatoria (*Planktothrix*) in enclosures (Klemer, 1976), while NO_3-N favours the development of eukaryotic phytoplankton. The importance of the environmental N : P ratio has often been mentioned and Smith (1983), based on data from 17 lakes, suggested that cyanobacteria are generally better competitors for nitrogen than phosphorus and thus in lakes with low total N : total P ratio. However, this interpretation should be treated with considerable caution, because most cyanobacteria have several different strategies for optimizing P acquisition, such as the abilities to store phosphate in polyphosphate granules, to hydrolyze a range of organic phosphates and to adjust rapidly to changes in phosphorus conditions (Whitton et al., 2005).

It is often overlooked that some environments, especially streams, show frequent shifts in conditions and some species appear to be adapted especially to deal with such shifts. For instance, genera which can form both heterocysts and either hairs or long tapered filaments are responding to environments with different nutrient conditions at different stages in their growth cycle. *Rivularia* typically undergoes a relatively short period of nitrogen limitation alternating with a long period of phosphorus limitation (Yelloly and Whitton, 1996; Whitton et al., 1998).

One final point should be stressed, because of its value for monitoring purposes. The shape of a filamentous cyanobacterium can tell a lot about its environment, especially nutrient conditions, even without knowing its name. It seems likely, for instance, that morphologically complex cyanobacteria are associated with environments which show characteristic fluctuations in environmental conditions. Further information about the more recent environment is provided if details of intracellular contents, such as the extent of formation of the various types of storage granule and gas vacuoles, is also included.

In addition to the morphological studies on nitrogen and phosphorus limitation mentioned earlier, much information has accumulated on physiological, biochemical and molecular responses to nutrient status. However, it is not always straightforward to establish whether an organism is N- or P-limited. While it may be relatively easy to do so by a combination of morphological and physiological studies in the more complex filamentous forms, Post (2005) concluded that no available measurement provides an unequivocal answer for marine phytoplankton. Although this viewpoint may seem strict, it is worth listing the three methodological problems Post raised. Knowledge of the concentration of a nutrient such as ammonium in the environment does not provide direct information on its flux into cells. Some potential N and P sources are not covered by standard determinations in water chemistry. Bioassays based on nutrient additions in enrichment experiments have a number of inherent problems, such as the possibility of colimitation by trace metal availability. All these problems can occur in freshwater studies,

especially the failure to include the full range of potential N and P sources in water analyses. Because of this, there has been much interest in finding molecules, especially proteins, that indicate nutrient limitation (Dignum et al., 2005), or genes (Mann, 2000) that respond to nutrient limitation or more directly to particular molecules in the external environment.

3.2.3. *Assessing phosphorus limitation*

The widespread occurrence of phosphate limitation in nature has led to much interest in the various types of strategy used by cyanobacteria to deal with this and the ways it can be investigated (Dignum et al., 2005). The formation of surface phosphatase activities under conditions of moderate phosphorus limitation and its complete suppression under high ambient phosphate concentration has been reported for so many cyanobacteria that it seems save to conclude that this is the typical response (Whitton et al., 2005) and that this is a good way to assess phosphorus limitation. Theoretically, the production rate of derepressible (surface) phosphatases should give the best measurement, but in practice the potential phosphatase activity is normally used. Potential phosphatase activity is usually assayed with a suitable artificial substrate at substrate concentrations near the saturation concentration to allow the reactions to proceed at maximum rate. However, such routine assays cannot be used to make a quantitative estimate of activity in nature, because other factors influence activity (Jansson et al., 1988). A review by Whitton et al. (2005) makes clear how many factors can influence surface phosphatases. There is a need for caution when making comparisons based on potential phosphatase activities and some recent accounts of phosphatase activity of cyanobacteria and other phototrophs in freshwaters need critical re-evaluation. Nevertheless the ELF-97^{TM} phosphate now widely used as a substrate for cell phosphatase activity in fluorescence studies can give some insight in understanding differences within communities and populations (Dignum et al., 2004), though it measures only phosphomonoesterase and not phosphodiesterase or other phosphatase activities.

The overall phosphorus concentration of cells is a more direct measure of potential phosphorus limitation, but quantification is time-consuming and difficult to apply to large numbers of cells in a mixed community. Although it is well known that most cyanobacteria form storage polyphosphate when internal phosphate exceeds a certain concentration (Whitton et al., 2005), the information for particular species or populations is sparse. There are a number of reports for freshwater cyanobacteria differing in their phosphorus content by a factor of 8 or more between phosphorus-replete and phosphorus-limited cultures (e.g. *Calothrix*: Islam and Whitton, 1992). However, the equivalent values for strains of the marine picoplanktonic *Prochlorococcus* and *Synechococcus* lay between 3 and 4 (Bertilsson et al., 2003).

In addition, the phosphorus-limited cultures of these marine cyanobacteria have only about one-third the percentage phosphorus (expressed as mass) of large filamentous cyanobacteria (Whitton et al., 2005). It will remain unclear to what extent these difference reflect the different types of environment or merely the difference in cell size until similar studies have been made on freshwater picoplanktonic cyanobacteria.

3.2.4. *Responses to grazers*

Besides the detection and responses to physical and chemical factors, there are several reports of morphological and other changes in the presence of potential grazers. The first for such a behavioural response in cyanobacteria came from Fialkowska and Pajdak-Stós (1997) in a study on two *Phormidium* isolates from shallow pools. When these were subjected to grazing pressure by the ciliate *Pseudomicrothorax dubius*, both strains showed significant increases in the number of filaments terminating in an empty sheath. This was due to active withdrawal of a trichome inside a sheath when disturbed by grazers. *P. dubius* was unable to ingest trichomes enclosed in a sheath, though *Phormidium* may be less efficient under these conditions, perhaps by reduced nutrient uptake.

Another example of a response to a grazer is that of microcolony formation of a strain of *Cyanobium* sp. from single cells, which was induced by the presence of the photophagotroph, *Ochromonas* sp. DS (Jezberová and Komárková, 2007). Colonies were characterized by hundreds of tubules (spinae), 100 nm to 1 μm long and 63 ± 6 nm wide on the surface of *Cyanobium* cells cultured together with *Ochromonas*. Presumably the responses of *Cyanobium* and the *Phormidium* described above involve the detection of molecules associated with grazer activity.

4. Occurrence of Toxicity

4.1. INTRODUCTION

Some types of toxin have been reported from a range of genera, especially microcystins, though this is in part because of the wide range of microcystins reported (71 in 2005 according to Codd et al.). However, the toxin content can differ between genotypes of the same species, as with microcystins in *Microcystis aeruginosa* (Fastner et al., 1999; Rohrlack et al., 2001) and cylindrospermopsins in *Cylindrospermopsis raciborskii* (Saker and Neilan, 2001). Microcystin-LR is considered to be especially widespread

(Sivonen and Jones, 1999), whereas some toxins have so far been recorded from only one species, such as anatoxin-a(s) in *Anabaena flos-aquae* (Matsunaga et al., 1989) and saxitoxin in one cyanobacterial species, *Cylindrospermopsis raciborskii* (Lagos et al., 1999), though its occurrence in some eukaryotic algae is notorious (see relevant chapters in this book). The toxic amino acid BMAA (β-N-methylamino-L-alanine), which was first found in *Nostoc* isolated from a cycad root, was later found in every cyanobacterial culture screened by Cox et al. (2005) and the authors suggested that it may be produced all groups of cyanobacteria. Surprizingly, their screening programme did not include *Arthrospira* ("Spirulina").

Most cyanobacterial molecules associated with toxicity been considered to be secondary metabolites, rarely being involved in primary metabolism, but having nevertheless in many, if not most, cases evolved to benefit a species. For instance, Osborne et al. (2001) reported that the shallow-water marine *Lyngyba majuscula* had been credited with the production of more than 100 novel secondary metabolites. Studies on the synthesis of toxic cyanobacterial peptides have shown that an enzyme complex (hybrid peptide-polyketide synthetase) directed the production of microcystin and is one of the largest known prokaryote gene clusters (Nishizawa et al., 2000; Christiansen et al., 2003; Neilan et al., 2007). Homologous gene clusters have been found in other genera, such as the nodularin synthetase gene cluster in *Nodularia spumigena* (Moffitt and Neilan, 2004) and the hectorchlorin biosynthetic gene cluster in *L. majuscula* reported by Ramaswamy et al. (2007). It is particularly important to understand gene regulation of products in this organism, because it can form not only a range of allelopathic molecules and ones toxic or carcinogenic to animals, but molecules which may have significant therapeutic effects for human health (Rossi et al., 2001; Gerwick et al., 2001).

4.2. LAKES AND RIVERS

The majority of reports for toxic cyanobacteria in nature are from the plankton of fresh, brackish or coastal marine waters (Codd et al., 2005), but it is unclear how much the worries about toxic blooms have led a frequent assumption that this is the main ecological group where they occur. Benthic cyanobacterial mats in lakes have also been reported to be toxic, causing animal deaths in lowland lakes Scotland (Owen, 1984; Gunn et al., 1992) and alpine lakes in Switzerland (Mez et al., 1997, 1998). Mats of *Oscillatoria limosa* and two other Oscillatoriaceae were responsible for toxicity in the Swiss lakes. Benthic cyanobacterial mats at a hot spring site in Lake Bogora, Kenya, were also been found to be toxic (Krienitz et al., 2003).

There have been an increasing number of reports of the toxicity of cyanobacterial mats in rivers, especially *Phormidium* mats on sediments or floating near the surface if buoyed by gas bubbles. For instance, Wood et al. (2006) found that *Phormidium* mats from R. Hutt and four other rivers on the North Island of New Zealand contained the neurotoxins homoanatoxin-a and anatoxin-a. These toxins were also found in the stomach of a labrador, one of five dogs killed in the R. Hutt. The problem was considered sufficiently severe for access restrictions to be placed on over 60% of river catchments in the western Wellington region of North Island.

The algal communities of several small calcareous rivers in Spain with abundant cyanobacteria were shown to be toxic to benthic macroinvertebrates (Aboal et al., 2000, 2002), although the studies did not include details of the presumed toxins. Toxicity was greatest when the temperature and nutrient concentrations were both low. The authors suggested that the toxicity is a factor enhancing the low diversity of macroinvertebrates at the sites. The colonies of Rivularia, which was usually the dominant in the phototroph community, are known to persist for many months. It seems likely that this is a more general phenomenon. Cyanobacterial colonies such as those of *Rivularia*, which persist for long periods in streams and are relatively little affected by grazing (Whitton, 1987), are likely to be toxic to many potential grazers (Pentecoast and Whitton, 2000). As colonies which persist for long periods often show high surface phosphatase activities (Whitton et al., 2005), it would worth investigating if there is any correlation between surface phosphatase activities and toxin formation.

4.3. EFFECTS ON INVERTEBRATES AND HETEROTROPHIC MICROORGANISMS

Other types of environment for which there are records of toxic cyanobacteria include various examples of close or obligate symbiotic associations. In some cases the evidence for toxicity is only circumstantial. There are reports from several locations of submarine populations of the shrimp *Crangon* living in tubes of the red-coloured cyanobacterium *Lyngbya sordida* (Whitton, 1973). These tubes can persist in environments subject to intense grazing, suggesting the possibility that the *Lyngbya* may be sufficiently toxic to reduce grazing predation on both itself and the shrimp. Similarly, circumstantial evidence suggests that *Prochloron*, a unicellular chlorophyll-b containining cyanobacterium, occurring symbiotically in some tropical ascidian colonies, may protect the host from predation (Hirose and Maruyama, 2004) The evidence for this comes from observations on the ascidian *Diplosoma virens*, where numerous *Prochloron* cells surround copepods which feed on the tunic matrices of the ascidian, yet the *Prochloron* cells are rarely

ingested. The authors concluded that the presumed toxicity of *Prochloron* to organisms other than the host tunicate may be important not only here, but in *Lissoclinum punctatum*, where the *Prochloron* cells are intracellular and previous research (Hirose et al., 1996) led to the conclusion that transfer of photosynthates from the cyanobacterium to the tunicate is the key factor involved in the assocation.

Simmons et al. (2008) concluded that marine invertebrates in general are likely to prove particularly rich in bioactive molecules in a range of microorganisms including cyanobacteria. They suggested that natural selection began as ancient marine microorganisms were required to compete for limited resources. These pressures resulted in the evolution of diverse genetically encoded small molecules with a variety of ecological and metabolic roles. Many of the most biologically active molecules derive from invertebrates richly populated by associated microorganisms.

In addition to toxicity to animals, there are accounts of toxicity to other organisms, including possible allelopathic effects on other cyanobacteria and eukaryotic algae. There have been numerous reports of toxicity of cyanobacterial strains to bacteria and fungi (Patterson et al., 1994). Caution is needed in assessing the results of studies where the strains were non-axenic or had been in culture collections for many years and hence risk of their ability of bioactive molecules being lost altogether, making it hard to assess the ecological significance of the findings.

4.4. ALLELOPATHY

4.4.1. *Cyanobacteria against eukaryotic algae*

Comments that dense blooms of ‘blue-green algae’, usually include only low densities of algae belonging to other phyla go back at least as far as Lefèvre (1932). There were a number of quite detailed studies in the 1960s and 1970s which suggested that, even if the chemical environment was especially favourable for a particular species, antagonistic effects were involved as well. Vance (1965) investigated the situation in Randolph Pond, Missouri, in particular detail. Only six species were consistent and important constituents of blooms, the most important being *Microcystis aeruginosa*, *Coelosphaerium naegelianum* and *Aphanizomenon flos-aquae*. When *Microcystis* was at its maximum, *Aphanizomenon* was hardly detectable, and *vice versa*. Vance commented that it was improbable that the abrupt decline of a bloom can be attributed to nutrient deficiency alone, and produced evidence suggesting that active metabolites may play an important role. Tests with laboratory inocula on isolated sections of the pond showed that *Microcystis aeruginosa* had more inhibitory activity than any other species. Strong

circumstantial evidence for allelopathy playing a role in bloom succession in a eutrophic lake was also provided by Keating (1977).

Interest in the possibility of chemical interactions waned for several decades, but has revived more recently. A great deal more circumstantial evidence has come from field studies and also strong or direct evidence from experimental studies in the laboratory. Figueredo et al. (2007) suggested that part of the explanation for changes in *Cylindrospermopsis raciborskii* populations in a pond in France (Briand et al., 2002) and reservoirs in Brazil (Bouvy et al., 2001) might be due to allelopathy, although none of the original authors included this when interopreting their results. Perhaps the most detailed laboratory study is by Schlegel et al. (1999), where almost all 198 cyanobacterial strains had been isolated recently from S-E. Asia and Australia. Although many of the strains were not axenic, the authors included various studies which make it likely that most, if not all, the observations were due to cyanobacterial allelochemicals. Activity was tested against species of the planktonic green algae *Monoraphidium*, *Scenedesmus* and *Coelastrum*. Ten strains of *Fischerella*, seven *Nostoc* and three *Calothrix* produced antialgal compounds with a broad activity spectrum. The 20 active strains were then tested against one strain of each of three bloom-forming cyanobacteria, *Microcystis aeruginosa, Anabaena circinalis* and *Nodularia spumigena*. Fourteen of the strains active against green algae killed one or more of the bloom-forming strains, the effects being particularly strong with three *Fischerella* strains.

Laboratory studies have led to suggestions that extracellular iron chelators (siderophores) may play a role in competitive success, similar to that known in some bacteria. For instance, Maz et al. (2004) thought it likely that siderophore(s) played a role in the inhibition of *Chlamydomonas reinhardtii* by *Anabaena flos-aquae*, but only when the latter was Felimited. However, allelochemicals inhibiting photosynthetic activities more directly seem likely to be the most important (Figueredo et al., 2007), though their molecular structure remains uncertain (see below). Photosystem II seems to be the target site for cyanobacterial allelochemicals influencing photosynthesis (Smith and Doan, 1999; Sukenik et al., 2002). The possible role of cyanophage in competition between cyanobacterial dominants has not been considered in most studies, so cannot be ruled as an explanation of some past observations.

Cell-free filtrates of a *Cylindrospermopsis raciborskii* bloom and the medium from cultures of four non-axenic strains of *C. raciborskii* from the same lake showed inhibitory effects on photosynthetic activities of *Microcystis aeruginosa* and two widespread planktonic green algae, though not the diatom *Navicula* sp. (Figueredo et al., 2007). Two of the strains showed much stronger inhibitory effects on the test organisms than the others.

As well as the growth conditions of the producer organism, the growth conditions of the test organisms can also be important, as shown for *Anabaena doliolum*, where photoheterotrophically grown cultures were inhibited by *Fischerella* JAVA 94/20 and *Nostoc* NSW, but not heterotrophic cultures (Schlegel et al., 1999).

Various authors have investigated whether microcystins inhibit eukaryotic algae, but a study (Babica et al., 2007) of the effects of microcystin-LR and microcystin-RR on five planktonic green algae showed no significant growth changes at environmentally relevant concentrations (1–10 μg L^{-1}), though there were some effects at much higher concentrations. *Pseudokirchneriella subcapitata* was the most sensitive. The authors included a thorough assessment of previous studies on the effects of microcystins on eukaryotic algae. In some cases microcystins had led to an initial stimulation of growth, followed later by inhibition. However, all reports of inhibition involved concentrations higher than the range considered environmentally relevant. The most sensitive was a unicellular cyanobacterium *Synechococcus elongatus*, where growth inhibition and biochemical parameters related to the antioxidative system were found with exposure to microcystin-RR for 6 days (Hu et al., 2005).

The evidence for allelopathic effects of *Cylindrospermopsis raciborskii* seems so convincing that Figueredo et al. (2007) suggested this may be a partial explanation for its recent spread in temperate lakes (Padisak, 1997; Fastner et al., 2003). As the organism in well-known in tropical and subtropical lakes, its spread has been attributed to climatic warming. However, Figueredo et al. (2007) wonder whether its rapid increase in particular temperate lakes may also be due to the inability of native algae in the lakes to withstand allelochemicals to which they may not have been exposed previously. If so, the competitive success of *C. raciborskii* at a site may decrease when native algal populations acquire improved ability to withstand the allelochemicals.

4.4.2. *Eukaryotic phototrophs against cyanobacteria*

The inhibition of cyanobacterial bloom formation by eukaryotic phototrophs has been suggested in several studies and there are reports of green algae inhibiting other phototrophs in laboratory experiments (e.g. Harris, 1970). Nutrient chemistry is nevertheless usually considered the main factor favouring dominance of the freshwater plankton by green and other eukaryotic algae (Klemer, 1976: see above). However, several submerged vascular plants (e.g. *Myriophyllum spicatum*) produce polyphenolic compounds inhibitory to cyanobacteria (Nakai et al., 2000; Leu et al., 2002). Presumably the presence of dense beds of submerged aquatics would reduce

the likely of blooms occurring in shallow lakes, but the field data supporting this are largely circumstantial.

4.4.3. *Barley straw*

Barley straw is now used widely in the British Isles and some other temperate countries to control the development of cyanobacterial blooms (Welch et al., 1990; Gibson et al., 1990; Martin and Ridge, 1999; Brownlee et al., 2003) and the release of polyphenolics from rotting stems has been suggested to be the main factor involved (Pillinger et al., 1994; Everall and Lees, 1997). Other factors such as microbial activity and increased density of zooplankton grazers have also been suggested. The method has proved rather less successful in North America (e.g. Boylan and Morris, 2003), perhaps due to different barley cultivars or fertilizer application. Although there are conflicting reports on how effective the method is, there is convincing evidence for success in shallow, well aerated waters, when care is taken in applying the straw – sufficient bales sufficiently early for rotting to be well underway by the time a bloom population would normally start to increase – typically, late spring in temperate regions.

Barrett et al. (1999) observed no change in response after the use of barley for six consecutive years. However, several authors have raised the possibility that continued use may lead to more sensitive species or strains being replaced with less sensitive ones. The author has observed at a very shallow water site on the Isle of Sheppey, UK, where straw bales have been used for a number of years, that there has been a change from dominance by *Oscillatoria agardhii* and *O. redekei* to one by *Anabaenopsis*. The shift from non-N_2-fixers to a N_2-fixer might of course have been due to other factors. Repeated addition of organic matter to a shallow water might itself lead to changes in water chemistry, such a shift in the N : P ratio.

An experimental study with decaying rice straw showed inhibition of cyanobacterial growth and nitrogen fixation, apparently due to phenolic compounds (Rice et al., 1980). Anecdotal reports from deepwater rice farmers in Bangladesh to the author also indicate that leaving rice straw to rot on soils in autumn decreases winter growths of cyanobacteria. Perhaps the straw of some rice cultivars could be used to control cyanobacterial blooms in warmer regions in a manner similar to barley in temperate regions.

5. Influence of Environment on Toxins and Toxicity

There is considerable evidence for some toxins that the concentrations present in a population or individual culture can change markedly. For instance, the monitoring of microcystin concentrations in lakes has shown a high variability in space and time (Kardinaal and Visser, 2005). Cox et al.

(2005) concluded that BMAA production and storage is a function of growth conditions and/or life cycle stage. They quoted results for *Calothrix* and *Nodularia spumigena* cultures, where initial samples showed no BMAA, but quantifiable amounts later. Detailed results obtained by Ballot et al. (2004) on the range values for microcystins (assayed as microcystin-LR equivalents) and anatoxin-a present in the cyanobacterial populations in two Rift Valley Lakes are of particular interest, because L. Sonachi was dominated by *Arthrospira fusiformis* and L. Simbi co-dominated by it. A monocyanobacterial strain of *A. fusiformis* isolated from L. Sonachi was shown to produce microcystin-YR and anatoxin-a. As *A. fusiformis* is closely related to *A. platensis*, the source of the health food marketed as Spirulina (Vonshak and Tomoselli, 2000), it is especially important to establish what factors can influence toxin production in this genus.

Most studies on the influence of environmental effects have dealt with *Microcystis aeruginosa.* Mez et al. (1998) summarize the conclusions from various studies, but it is difficult to generalize. The most convincing conclusion is about light, with intensity having a positive effect on growth rate and toxin concentrations assayed in several laboratory studies (Watanabe and Oishi, 1985; Van der Westhuizen and Eloff, 1985; Van der Westhuizen et al., 1986; Utkilen and Gjølme, 1992), which taken together give evidence for a correlation between growth rate and light intensity on *M. aeruginosa*. Light has also been shown to have an effect on the transcription of genes for microcystin biosynthesis (Kaebernick et al., 2000). The situation is complicated by the fact that light can determine which microcystin variants are actually produced (Rohrlack and Hyenstrand, 2007). *Microcystis* PCC 7806 produced mainly (D-Asp3) microcystin-LR in the light, but switched to microcystin-LR in the dark. Interpretation of results in nature also requires understanding of the effects of environmental factors. For instance, Sedmank and Kosi (1998) found marked effects of microcystins at very low light intensity (4 μmol photon m^{-2} s^{-1}).

The responses to other environmental factors cases have led to even more contradictory conclusions, but the results can often be interpreted differently from the ways the authors did, especially in studies on nutrients, so it is well worth reading the original papers in full. As shown in several reports mentioned above and a study of *Cylindrospermopsis raciborskii* (Saker and Neilan, 2001), stressful conditions sometimes provide a stimulus for production of toxins and allelochemicals.

6. Concluding Comments

Information about the relative importance of the environment and genotypic differences is essential for predicting future changes in the toxicity of

species at a site. Field studies on toxicity can provide quantitative data for statistical purposes and insight on factors difficult to assess in the laboratory, such as possible interactions with cyanophage, bacteria and zooplankton. Experimental studies in the laboratory on the influence of the environment can help interpretation of the results of quantitative field data, but in the long term it is important to understand how toxin synthethis is regulated at the biochemical and molecular levels, since this may provide clues on the cellular functions of the toxins (Börner and Dittmann, 2005). The rapid progress in molecular studies means it is now worth relooking more critically at the results of previous field and laboratory studies.

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TOXICITY OF FRESH WATER ALGAL TOXINS TO HUMANS AND ANIMALS

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Abstract: Algae and cyanobacteria are responsible of the presence of toxins in fresh waters. Algae are considered less dangerous than cyanobacteria, because even if they can proliferate quite intensively in eutrophic fresh waters, they rarely accumulate to form dense surface blooms like blue-green algae do. Thus the toxins they produce do no accumulate to levels high enough to become hazardous to human and animals health.

Cyanobacteria both planktonic and benthic species, can instead form huge agglomeration close to the shore, which can become very dense and concentrated. This material can take a long time to disperse and so become a risk for human health and mainly for animals, which can easely enter in contact with poisoned water. Lots of blue-green algae species have been found to produce toxins, and some authors assume that it could be prudent to assume that any cyanobacterial population can have a toxic potential. At present known toxins are classified as neurotoxins (anatoxin-a, anatoxin-a(s) and saxitoxin), cytotoxin or cylindrospermopsin and microcystins or nodularins. Anyway, starting from existing studies, it seems likely that other unidentified toxins exists.

Neurotoxins act by blocking neuronal signal transmission with two main mechanisms: anatoxins act as acetylcholine mimic (anatoxin-a) or as cholinesterase inhibitor (anatoxin-a (s)), causing an organophosphate like syndrome,

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while saxitoxin acts by blocking the sodium channel, thus disrupting sodium balance into nerve cells. Despite their high toxicity, their only occasionally are responsible of human intoxication, while neurotoxicity may be experienced by livestock and pets, that can drink polluted water or ingest scum material.

Cytotoxin is an alkaloid that blocks protein synthesis by binding to DNA or RNA. It's responsible for cytogenetic damages *via* DNA strand breaks and loss of whole chromosomes (aneuploidy) and has proved to be potentially carcinogenetic. Finally, it was found that cylindrospermopsin toxicity is associated with significant losses of glutathione and depletion of glutathione results in cell death. The fall in glutathione levels is due to an inhibition of the final common pathway of glutathione synthesis, and this in turn contributes to cylindrospermopsin cytotoxicity, as lower cell glutathione levels predispose to cylindrospermopsin toxicity. Numerous reports involving the poisoning of farm and wild animals following drinking water from lakes and ponds containing surface scum from cyanobacterial blooms exist, most have been documented in Australia. Australia was also interested by human poisoning episodes via drinking water, with patients escaping death only through skilled and intensive hospital care.

Microcystins are the most frequently occurring and widespread of the cyanotoxins; they act by blocking protein phosphatases 1 and 2a, causing toxicity at the hepatic level, as they use bile carrier to pass through cell membranes. Microcystins toxicity is greater after intraperitoneal injection, but also intranasal exposure showed high toxicity, being this uptake route relevant for water sports activities (i.e. waterskiing). Nodularins toxicity has shown to be cumulative, as a single oral dose resulted in no hepatic damage, while the same dose applied daily over several days caused hepatic injury. Microcystins have also shown to be tumor-promoting agents, as they can increase the incidence of hepatic tumors in human too.

Concerning Fresh Water Algae, their toxicity is considerably lower than that of cyanobacteria, because algae do not have effective mechanism of accumulation and the toxic potency of their toxins is several order of magnitude lower than that of cyanotoxins. Available data reports about dinoflagellates within or related to the *Peridinium* genus as potential producers of toxins (ichtyotoxins). Ichtyotoxins caused fish kills and have shown to have an algicidal effect on the cyanobacteria *Microcystis aeruginosa*. It is

possible that toxic blooms of dinoflagellates in freshwater occur more frequently than reported and that they affect the biota in those habitats. Toxins production is considered as a defensive strategy for dinoflagellates from fish larvae preying. In human some allergic reaction has been reported after *Uroglena* spp. and *Gonyostomum semen* exposure, but clear toxic episode was reported.

Keywords: Cyanobacteria, toxins, syndromes, therapy

1. General Characteristics of Fresh Water Toxins Producing Organisms

Freshwater algal toxins are produced by both algae and cyanobacteria (also called blue-green algae). Toxins are secondary metabolites of normal metabolism of the algae and of cyanobacteria, which present a different degree of toxicity: the less toxic induce dermatitis, the most dangerous are hepatotoxic. Generally speaking, algae produce less potent toxins and are rarely responsible of toxic episodes (WHO, 2003).

The algae that produce toxins are small unicellular autotroph organisms, which perform photosynthesis and organication. Cyanobacteria are organisms which have intermediate characteristics between algae and bacteria. So they are photosynthetic, but their cellular structure is similar to that of bacteria, i.e. they lack a cellulose outer wall, do not reproduce sexually and do not have membrane-bound nuclei or specialized organelles. They posses an accessory pigment, phycocyanin, unique to cyanobacteria, which has a bluish color, thus cyanobacteria are also called blue-green algae (Echlin, 1966; Kotak et al., 1995; Rapala and Sivonen, 1998).

Bloom formation is eased by a stable water column, warm waters, high nutrient concentrations, high pH, low CO_2 and low grazing rate by zooplankton (Zurawell et al., 2005).

Usually algae do not produce blooms, while blue-green algae produce important blooms. This differential behavior is partly responsible of the different toxicity and dangerousness of the two groups. Indeed, by not accumulating in the environment to form blooms, algae do not produce toxins amounts high enough to threaten human, livestock or wildlife health. On the contrary, blooms formed by cyanobacteria produce not only cells accumulation, but also an increase in toxins concentrations to levels hazardous to humans or livestock (WHO, 2003).

Main exposure way is through recreational and drinking waters, and absorption ways are ingestion, contact and inhalation. Human activities, causing

eutrophication, are among causes responsible for the increase in proliferation of both algae and cyanobacteria, by increasing the concentration of nutrients necessary for their development and growth. Cells proliferation highly impacts the quality of waters even when no evident bloom is formed.

At present little knowledge is available on toxins of algal origin, even if some evidence exists of toxicity of algae to humans and fish, due to exposure to *Peridium polonicum* (WHO, 2003).

Much more information exist on cyanobacteria toxins, which have been chemically characterized and for which producing organisms have been identified. At present 46 species of toxins producing blue-green algae have been identified, and 60% of studied strains have proved to contain toxins.

First evidence of cyanobacterial intoxications dates up to 1878, when a livestock poisoning in Australia was attributed to blue-green algae presence and to toxin production. "…Symptoms-stupor and unconsciousness, falling and remaining quiet, as if asleep, unless touched, when convulsions come on, with head and neck drawn back by rigid spasm, which subsides before death. Time- sheep, from one to six or eight hours; horses, eight to twenty-four hours; dogs, four to five hours; pigs, three or four hours" (Francis, 1878).

All this considering, present chapter will focus on cyanobacteria toxins.

Cyanobacteria include both pelagic and benthic species, which form two different kind of aggregates.

Pelagic species can float in water column, vertically migrating depending on temperature, light and nutrient availability. These species contain intracellular gas vesicles which help the cells in buoyancy and in maintaining their position at desired depth in water column. In order to move through water column, blue-green algae change the dimensions and the number of vesicles. It has been observed that positive buoyancy is obtained by forming proteinaceous gas containing vesicle. Conversely, reduction of buoyancy result from polysaccharide accumulation and increased cellular turgor pressure; polysaccharide act as ballast molecules, while increased pressure induces collapse of vesicles (Reynolds and Walsby, 1975; Kromkamp, 1987; Humphries and Lyne, 1988; Walsby, 1994; WHO, 2003).

Vesicles dimensions are also adapted to atmospheric conditions, in particular to wind and waves, and are slowly adapted to weather changes. This can lead to an increase of surface concentration of cells and to the formation of floating scums when weather condition turn to less windy (Chorus and Bartram, 1999; WHO, 2003) (Fig. 1).

These scums can be redispersed by wind and waves action, which can also lead to persistence of scums themselves, by accumulating them to the

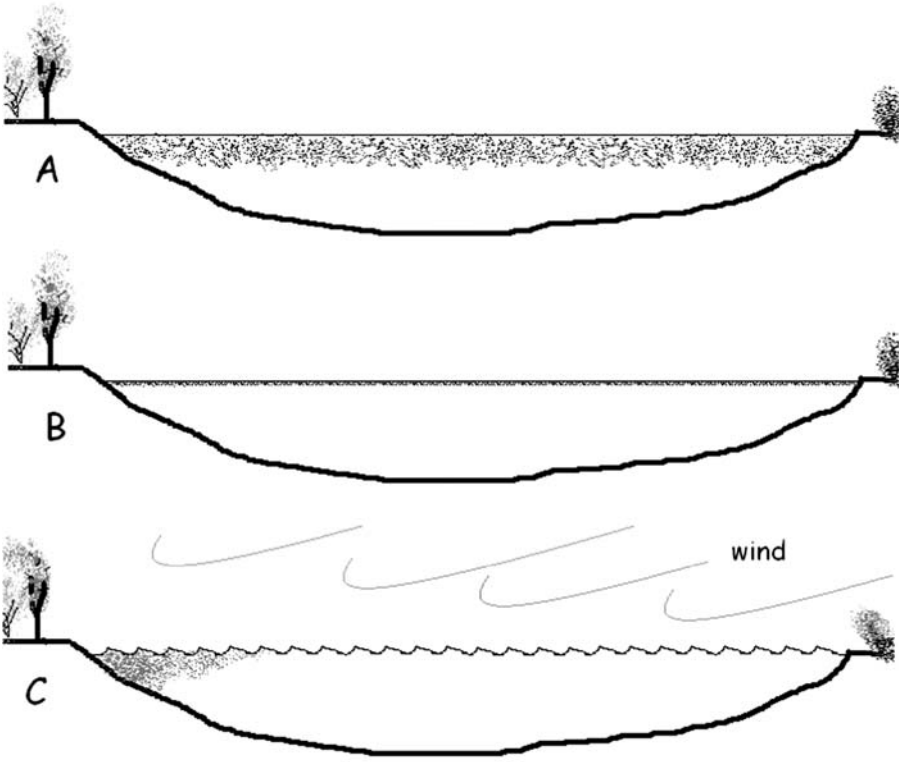

Figure 1. Scums formation. A: cyanobacteria float in calm water; B: water conditions change and cells concentrate on water surface; C: wind accumulate cyanobacteria to the shore.

shore. Wind and waves can also produce a slow dispersal by shore washing or cell disintegration, causing the release of toxins in the environment and potentially increasing toxicity of water (WHO, 2003) (Fig. 2).

Benthic species growth on different substrates (as mud or rocks) and form mats on it, which can then be washed by waves to the shore. When reaching the shore, they can be scavenged by dogs, livestock and wildlife, thus causing toxicity. These cyanobacteria species have a smaller impact on human health with respect to pelagic species, because they have a smaller chance to be ingested, as they can be easily seen and avoided by man (Edwards et al., 1992; Mez et al., 1997; Mez et al., 1998).

A major hazard for humans occurs when mats do not reach the surface. Mats form when water is extremely clear, as light should reach the bottom in order for blue-green algae to replicate, and thus water is perceived by users as safe because it does not present “floating pollution” (WHO, 2003).

Three main classes of cyanotoxins exist:

– Microcystins or nodularins
– Neurotoxins
– Cytotoxin or cylindrospermopsin.

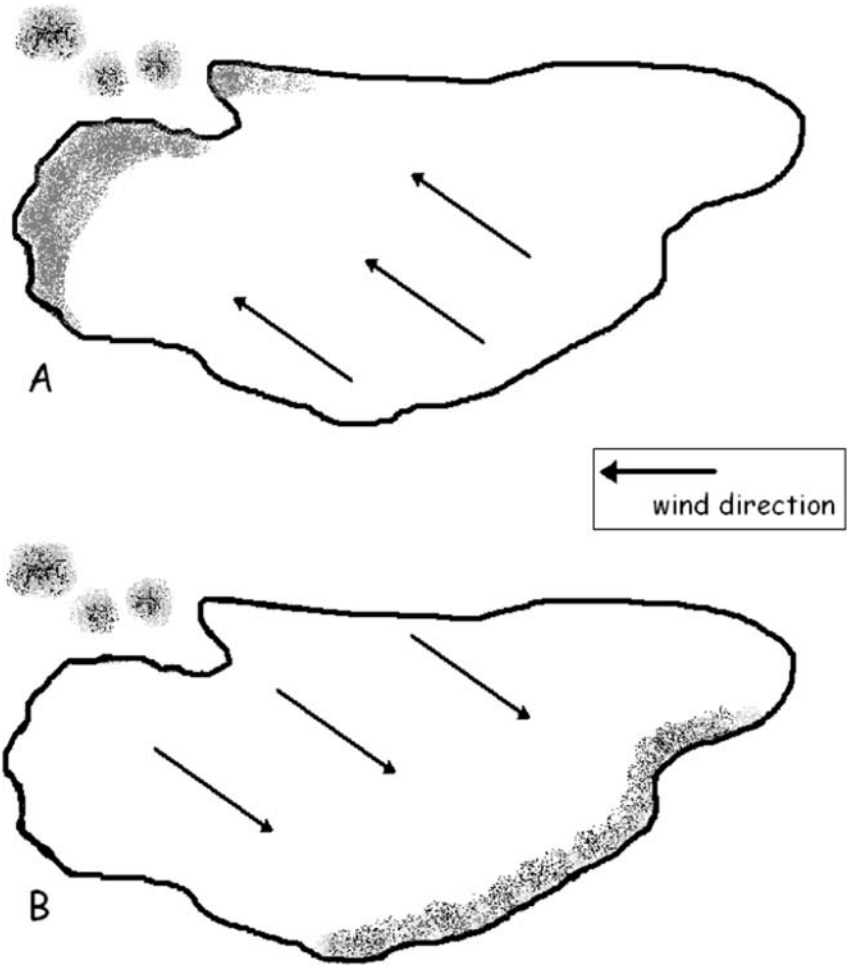

Figure 2. Scums movements depending on weather conditions. A: wind accumulate cells in two different, discontinuous parts of the pond; B: wind direction changes and a uniform scum forms on the other side of the pond.

Laboratory studies have underlined that probably other unidentified toxins exist, starting from the toxic effects observed in cells cultures and fish eggs exposure trials, which could not be ascribed to know toxins.

Most species have been found to produce both microcystins and neurotoxins; wild populations have been proved to be a mixture of toxic and non-toxic strains, so that not always the presence of a potentially toxin-producing species coincides with real presence of the toxin(s).

These toxins have different mechanisms of action, which will be described in following sections. Briefly, they can be resumed as follow:

- Microcystins block protein phosphatases thus acting as cytotoxic compound, being liver their main target (they are indeed considered as HEPATOTOXIC compounds);
- Neurotoxins act by blocking neuronal signalling;
- Cytotoxin blocks protein and glutathione synthesis.

2. Effect of Cyanobacteria on other Species

Cyanobacteria have a potential impact zooplankton, invertebrates and vertebrates, man included (Fig. 3).

2.1. ZOOPLANKTON

Herbivorous plankton feeds on phytoplankton, including blue-green algae.

If any accumulation of toxins or toxic effect occurs at this level, causing an alteration in planktonic populations, a disruption in trophic food chain can occur.

A direct inhibition on zooplankton growth has been observed after exposure to toxic cyanobateria or their toxins. Lots of studies have focused on mycrocystins, which block phosphatases activity in cladocerans and copepods. This inhibition lead to a reduction of grazing and an increased rate of rejection of food. A reduction of reproductive capacity, of growth, of individuals' dimensions and of survivor was observed (Arnold, 1971; Nizan et al., 1986; Vanderploeg et al., 1990; Reinikainen et al., 1994; DeMott and Dhawale, 1995; Weithoff and Walz, 1995; Kurmayer and Jüttner, 1999; Thostrup and Christoffersen, 1999).

The intensity of the effect depends on the species and the strain studied, on the stage of development of zooplankton and on environmental conditions, like temperature or food. The inhibitory effect produce a reduction of competitive capacity of affected species with respect to more resistant

species (Lampert, 1982; Fulton and Paerl, 1988; DeMott, 1989; DeMott et al., 1991; Hietala et al., 1995; Sartonov, 1995; Weithoff and Walz, 1995; Gilbert, 1996b; Gilbert, 1996a; Hietala et al., 1997; Claska and Gilbert, 1998; DeMott, 1999).

Some adaptive mechanism to cyanobacteria presence have been observed in zooplankton species, i.e. the selective grazing on phytoplankton, avoiding toxic species, registered in copepods. In these species a certain degree of inhibition in feeding rate and a high physiological sensitivity to the toxins have been observed, while no such mechanisms seems to exist in daphnids, which completely stop feeding and which seem to be more resistant to toxins (DeMott and Moxter, 1991; DeMott et al., 1991; Larson and Dodson, 1993).

Some "passive selection" mechanism have also been observed, i.e. a strict correspondence between mouth and prey size in rotifers and cladocerans, which impede these species to feed on colonial and filamentous blue-green algae, having them bigger dimensions (Hanazato, 1991; Kirk and Gilbert, 1992; Weithoff and Walz, 1995).

Other defensive mechanisms observed are the modulation of filtrating rate depending on presence or absence of toxic strains and the modification of the range of vertical migrations, in order to avoid the areas where toxic species are present (Forsyth et al., 1990; Berthon and Brousse, 1995).

Indirect effect have also been observed, directed to an alteration of zooplankton's habitat, thus reducing its fitness. The algicides property of cyanobacterial toxins can indeed destroy the algae zooplankton feeds on. This pesticide-like action of toxins acts on both micro- and macro algae, reducing food availability and the quality of zooplankton's habitat (Kirpenko, 1986; Bagchi et al., 1990; Chauhan et al., 1992; Gilbert, 1994; Schriver et al., 1995).

Direct or indirect effect of cyanotoxins on zooplankton can disrupt also the structure and the dynamic of planktonic and higher communities. Indeed, a reduction or an alteration of planktonic population can cause a deficit or an unbalance in food availability for higher levels of trophic chain (i.e. fingerling, whose first developmental stages are highly food-selective), increasing physiological stress and sensitivity to toxins themselves (Gilbert, 1990; Kotak et al. 1996b; Mayer and Wahl, 1997; Thostrup and Christoffersen, 1999; Thompson et al., 2000; Jarvis et al., 2006).

By feeding on cyanobacteria, zooplankton can accumulate toxins in their body and start the bioaccumulation process along food chain. Indeed, in many cases the amount present in their body is not high enough to cause direct toxicity for predators, but this residues can be accumulated in target

organs and reach, at higher trophic levels, toxic concentrations (Watanabe et al., 1992; Laurén-Määttä et al., 1995; Kotak et al., 1996b). Thostrup and Christoffersen (1999) have shown that *Daphnia* can accumulate up to 24.5 μg/l of toxin/g dry weight; this amount is not toxic to fish which prey on *Daphnia*, but they can accumulate the toxins in their body.

Finally, it has been observed that selective filtering activity by zooplankton can induce a selection of blue-green algae present in a pond, causing an increase and dominance of toxins strain and species with respect to non toxic ones. Kurmayer and Jüttner (1999) consider that some co-evolutive biochemical phenomenon could exist between cyanobacteria and grazing zooplankton, which allows blue-green algae to "use" the selective pressure exerted by zooplankton to eliminate other primary producers competitors.

2.2. MOLLUSKS AND CRUSTACEAN

In an exposure study in pulmonate mollusks it was observed that toxins concentration was dependent on the levels of cyanotoxin in phytoplankton and not on that in water, thus demonstrating that tissue content originates from food consumption more than from water filtration (Zurawell et al., 1999).

Lots of species have proved to be able to concentrate toxins in their body, i.e. *Anodonta* spp., demonstrating how these organisms represent a first and fundamental step for the accumulation and transfer of cyanotoxins along food chain. Indeed, mollusks present main food source for crustacean, amphibian, birds and human (Andrasi, 1985; Eriksson et al., 1989; Novaczek et al., 1991; Falconer et al., 1992; Prepas et al., 1997; Watanabe et al., 1997; Williams et al., 1997; Amorim and Vasconcelos, 1999).

Crustacean can accumulate cyanotoxins in their tissues too, in particular in hepatopancreas, by both food ingestion and water filtering. These species have proved to be little or no sensitive to the toxins, thus they can accumulate high amounts of these metabolites and become, like mollusks, an important source of pollution and of intoxication for higher levels of trophic chain, man included (Liras et al., 1998; Saker and Eaglesham, 1999; Humbert et al., 2001).

2.3. FISH SPECIES

Effect of cyanotoxins on fish species are both indirect and direct. Indirect effects are those acting on zooplankton, thus reducing food availability, as already mentioned.

Direct actions of blue-green algae toxins includes the anoxia induced by blooms proliferations, which can cause massive fish deaths.

Direct toxicity have been described in various fresh water species, i.e. carp and trout, where liver, kidney and gills were the target organs, following controlled conditions exposure (Carbis et al., 1996; Kotak et al., 1996a; Bury et al., 1997). Little information is available concerning toxicity in field condition, even if some gills lesions have been described in trout, *Abramis brama* and *Rutilus rutilus* (Eriksson et al., 1986; Rodger et al., 1994). Anyway these lesion are quite general, and can not be directly related to toxins exposure.

In one case only a direct correspondence between toxin exposure and toxic syndrome has been proved; indeed, salmon exposed to microcystin develop the so called Net Pen Liver Disease (NLPD), which have a certain importance because of the huge economical loss it can cause (Andersen et al., 1993; Humbert et al., 2001).

2.4. TERRESTRIAL VERTEBRATES

Lots of toxic episodes have been described in pet and livestock, as well as in wildlife.

As already said, the first report about livestock intoxication dates up to 1878, in Australia (Francis, 1878). After this report, lots of episodes were registered, mainly interesting dog, sheep and cows. These intoxication are worldwide distributed, as cases were registered in Australia, USA, South Africa and Europe. Most of reports are concerning domestic animals, also because of their economical value, but some case has been registered also in wildlife, like white rhinoceros, birds, skunks, minks (Soll and Williams, 1985; Carmichael and Falconer, 1993; Bell and Codd, 1996; Chorus and Bartram, 1999; Saker et al., 1999; Carmichael, 2001; Pitois et al., 2001; Briand et al., 2003; Codd et al., 2003; Krienitz et al., 2003; van Apeldoorn et al., 2007).

2.5. HUMANS

Symptoms and toxicity of each single toxin to human will be described in more detail in following paragraphs.

In this section, a rapid overview of some important toxic episodes will be given, in order to give an idea of how diffuse and dangerous these toxins can be. Generally speaking, human intoxication are due to ingestion of toxic cyanobacteria or of water contaminated with the toxins (Table 1).

TABLE 1. Possible exposure routes for cyanotoxins (from Codd et al., 1997).

Way of exposure	Kind of exposure
Skin contact	Toxic scum or mat material
	Raw water containing toxic blooms or free toxins
	Treated water containing toxic blooms or free toxins
Drinking water	Accidental ingestion of toxic scum
	Raw water containing toxic blooms or free toxins
	Treated water containing toxic blooms or free toxins
Inhalation	Toxins during water-sports, showering or work practices
Food consumption	Shellfish or finfish if containing toxins
	Plant products if containing toxins
Haemodialysis	Using water containing free toxins

First report on human intoxication by cyanotoxins dates up to 1931, when in Ohio (USA) drinking water treatment with copper sulphate in order to destroy an algal bloom caused the death and lysis of cyanobacterial cells, thus releasing toxins INTO water. Water consumption caused the intoxication of thousands of people (Tisdale, 1931).

In following years other episodes were reported in Zimbabwe (1966) were children were affected, Australia (1983), Brazil (1993), where more than 2000 people were affected and close to 100 died (mainly children). In 1996 a serious and deadly episode occurred in Brazil, where more than 100 dialysed persons were affected, and 47 of them died due to the use of contaminated water for dialysis (Zilberg, 1966; Bourke et al., 1983; Falconer et al., 1983; Teixeira et al., 1993).

All these findings and reports made the OMS fix a maximum tolerable level in water of 1μg microcystin equivalent/L for the hepatotoxic toxins.

Allergic reactions to algae and cyanobacteria are frequently reported on the level of anecdotal evidence from eutrophic bathing waters and they are considered relatively common but are little studied (Yoo et al., 1995).

Pronounced skin reactions in response to a bloom of *Uroglena* spp. were observed in a small number of bathers, especially under bathing suits where cells accumulated and partially disrupted during swimming (Chorus, 1993). Frequently, divers complain of dermal reactions to algal material accumulating under their wet suits, which tend to act as a strainer which lets out water, but collects algae between skin and suit. Pressure and friction between fabric and skin leads to cell disruption, liberation of content, and intensified dermal exposure not only to algal cell wall material, but also to substances otherwise largely confined within the cells.

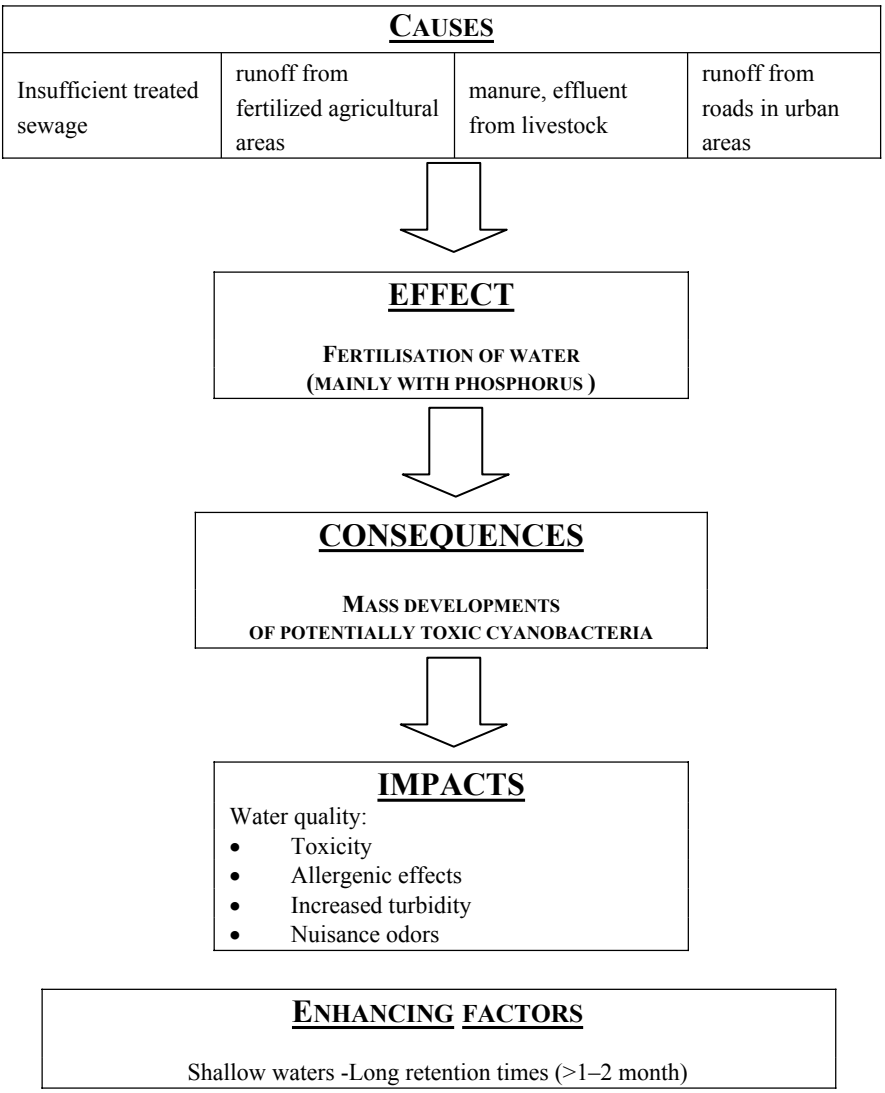

Figure 3. Scheme of the effects of cyanotoxins in aquatic organisms.

It is important to note that allergic reactions are not confined to cyanobacteria. The substances which provoke these reactions are likely to be others than the cyanobacterial toxins. However, allergic reactions require elevated cell densities in bathing water, and in freshwaters, mass developments are most frequently due to cyanobacteria. Further, other groups of algae do not accumulate as surface scums and therefore their metabolites will not occur in comparably high concentrations.

Algae have caused irritative coughs in personnel and patients of a physiotherapeutic unit supplied with coarsely filtered surface water with which it performed underwater massage treatment. As an example, in October 1986, a water body was found to contain 4600 to 58000 cells/ml of the desmid *Staurastrum gracile,* a species that was not effectively eliminated by the filter, and has strong cell walls lined with spine and hook-like structures which may well cause irritations of mucous membranes (Naglitsch, 1988). Whilst this incident may be more a curiosity than a serious health threat, it does highlight the benefit for management of regular microscopic examination of bathing and therapeutical waters in order to recognize algae as a potential cause of health reactions.

3. Microcystins/Nodularins

Microcystins and nodularins are among the most frequently occurring and widespread cyanotoxins.

They are produced by various species, like *Microcystis aeruginosa*, *Anabaena* spp., *Nodularia* spp., *Planktothrix* and *Nostoc* (Fig. 4).

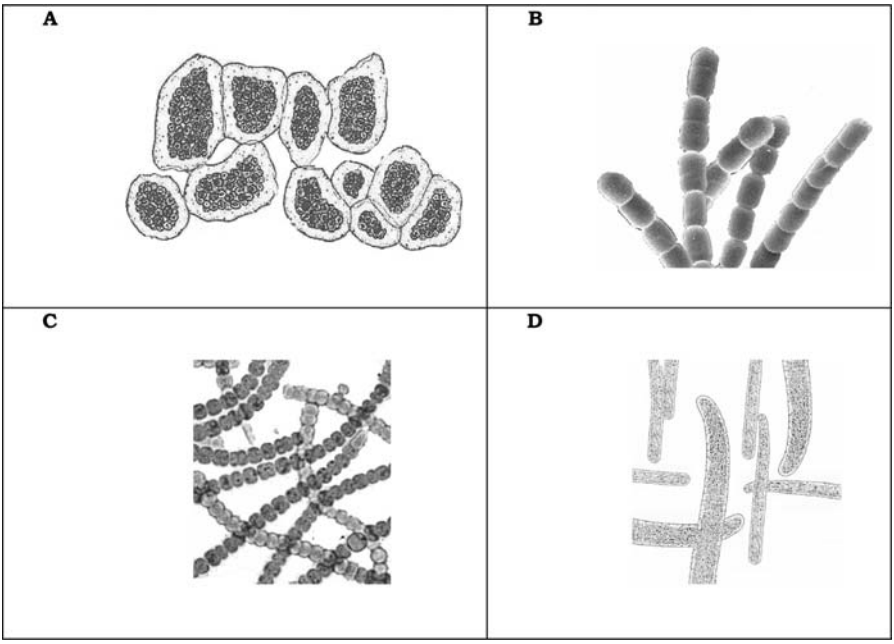

Figure 4. Microcystins and nodularins producing cyanobacteria (A *Microcystin*, B *Anabaena*, C *Nostoc*, D *Planktothrix*).

3.1. CHEMICAL STRUCTURE

These toxins are classified as cyclic peptides with low molecular weight.

Microcystins are cyclic heptapeptides characterized by a molecular weight of 800–1100 and by the presence of particular amino acids, which can be only found in these toxins (Fig. 5):

- N-methyldehydroalanine (Mdha)
- 3-amino-9-methoxy-2,6,8-trimethyldeca-4,6-dienoic acid (Adda)

The most common amino acidic sequence observed is the following:

(D) Ala- (L) X- (D) MeAsp- (L) Z- Adda- (D) Glu- Mdha[1]

Nodularins are cyclic pentapeptides whose molecular formula is:

dhBut- (D) MAsp- (L) Arg- Adda- (D) Glu[2]

Due to all possible amino acidic combinations more than 60 different microcystins exist, while only 6 variants of nodularins have been identified (Table 2).

The analysis of different cyanobacterial microcystins producing strains revealed that in many cases a single strain can produce more than a single toxin. On the contrary nodularins producing strains usually synthesizing only one variant of the toxin.

The molecular structure of microcystins and nodularins make them very stable and resistant to many eukaryotic and bacterial peptidases. Anyway they can be broken by aquatic bacteria living in the rivers and reservoirs where cyanobacteria develop. The degradation of toxins requires a lag phase, where little or no loss of cyanotoxin occurs, lasting from two days up to three weeks, followed by an active degradation process, which lead to the removal of up to 90% of total toxins within 2–10 days. Environmental conditions and initial microcystins concentrations have proved to condition the duration of these two phases. Generally speaking, bacteria can degrade with high efficiency microcystins, but not nodularin (Jones et al., 1994; Rapala et al., 1994; Cousins et al., 1996; Lahti et al., 1997a; Lahti et al., 1997b).

[1] MeAsp: D-erythro-β-methylaspartic acid
[2] dhBut: N-methyldehydrobuthyrine

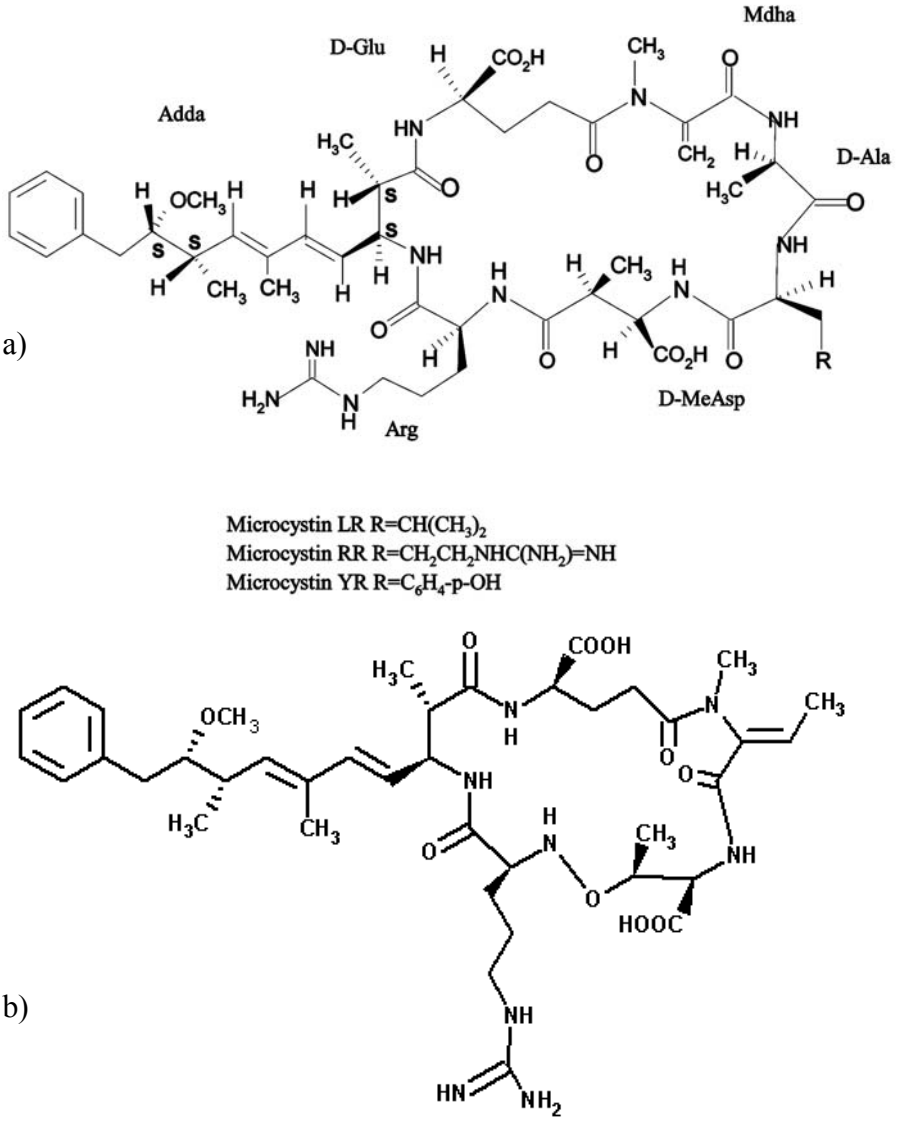

Figure 5. Chemical structure of microcystins (a) and nodularins (b).

TABLE 2. List of identified variants of microcystins, their molecular weight and toxin producing species (Chorus and Bartram (1999) modified).

Microcystin	M.W.	Cyanobacterium
Mcyst-LA	909	*M. aerug., M.vir.*
Mcyst-LAba	923	*M. aerug.*
Mcyst-LL	951	*M. aerug.*
Mcyst-AR	952	*M.* spp.
Mcyst-YA	959	*M. aerug.*
[D-AspDha]Mcyst-LR	966	*M. aerug., Anab.*
[D-AspDha]Mcyst-EE(OMe)	969	*Anab.*
Mcyst-VF	971	*M. aerug.*
[D-Asp]Mcyst-LR	980	*A. flos-aq., M. aerug., M.vir., O. agard.*
[Dha]Mcyst-LR	980	*M. aerug., Anab., O. agard.*
[DMAdda]Mcyst-LR	980	*M.* spp.*, Nostoc*
[Dha]Mcyst-EE(OMe)	983	*Anab.*
[D-AspDha]Mcyst-E(OMe)E(OMe)	983	*Anab.*
Mcyst-LF	985	*M. aerug.*
Mcyst-LR	994	*M. aerug., A. flos-aqu., M. vir.*
[D-AspD-Glu(OCH_3)]Mcyst-LR	994	*A. flos-aq.*
[(6Z)-Adda]Mcyst-LR	994	*M. vir.*
[Dha]Mcyst- E(OMe)E(OMe)	997	*Anab.*
[L-Ser]Mcyst-LR	998	*Anab.*
Mcyst-LY	1001	*M. aerug.*
[L-Ser]Mcyst-EE(OMe)	1001	*Anab.*
[D-AspSer]Mcyst-E(OMe)E(OMe)	1001	*Anab.*
Mcyst-HiIR	1008	*Microcystis*
[D-AspADMAdda]Mcyst-LR	1008	*Nostoc*
[D-Glu(OCH_3)]Mcyst-LR	1008	*A. flos-aq., M.* spp.
[D-AspDha]Mcyst-RR	1009	*M. aerug., M.vir., O. agard.*
[D-AspADMAddaDhb]Mcyst-LR	1009	*Nostoc*
[L-MeSer]Mcyst-LR	1012	*M.* spp.
[Dha]Mcyst-FR	1014	*M.* spp.
[L-Ser]Mcyst-E(OMe)E(OMe)	1015	*A.* spp.
[ADMAdda]Mcyst-LR	1022	*Nostoc*
[D-AspADMAdda]Mcyst-LHar	1022	*Nostoc*
[D-Asp]Mcyst-RR	1023	*M. aerug., Anab., O. agard.*
[Dha]Mcyst-RR	1023	*M. aerug., Anab., O. agard.*
Mcyst-LW	1024	*M. aerug.*
Mcyst-FR	1028	*M.* spp.
Mcyst-M(O)R	1028	*M.* spp.
[Dha]Mcyst-HphR	1028	*Anab.*

(Continued)

[D-AspDha]Mcyst-HtyR	1030	*Anab.*
[Dha]Mcyst-YR	1030	*M. aerug.*
[D-Asp]Mcyst-YR	1030	*M.* spp.
Mcyst-YM(O)	1035	*M. aerug.*
[ADMAdda]Mcyst-LHar	1036	*Nostoc*
Mcyst-RR	1037	*M. aerug., M.vir., Anab.*
[(6Z)-Adda]Mcyst-RR	1037	*M.vir.*
[D-Ser 1 ADMAdda]Mcyst-LR	1038	*Nostoc*
[ADMAddaMeSer]Mcyst-LR	1040	*Nostoc*
[L-Ser]Mcyst-RR	1041	*Anab., M. aerug.*
[D-AspMeSer]Mcyst-RR	1041	*O. agard.*
Mcyst-YR	1044	*M. aerug., M.vir.*
[D-Asp]Mcyst-HtyR	1044	*A. flos-aq.*
[Dha]Mcyst-HtyR	1044	*Anab.*
Mcyst-(H4)YR	1048	*M.* spp.
[D-Glu-$OC_2H_3(CH_3)OH$]Mcyst-LR	1052	*M.* spp.
[D-AspADMAdda.Dhb]Mcyst-RR	1052	*Nostoc*
Mcyst-HtyR	1058	*A. flos-aq.*
[L-Ser]Mcyst-HtyR	1062	*Anab.*
Mcyst-WR	1067	*M.* spp.
[D-Asp.ADMAdda.Dhb]Mcyst-HtyR	1073	*Nostoc*
[L-MeLan]Mcyst-LR	1115	*M.* spp.

3.2. MECHANISM OF ACTION

Microcystins and nodularins enter the organism by using bile acid transport system of hepatic and intestinal cells; they can be thus accumulated in liver, gut and kidney (Fig. 6).

An alternative way of absorption for these toxins is intranasal way, which is particularly important when microcystins and nodularins are released in the environment after cell lyses. This way of absorption gives great concern for recreational waters: inhalation of droplets and spray formed by water sport activities can lead to a high absorption of toxins by human.

They mainly effect hepatocytes, where they inhibit protein phosphatases (PP) by covalently binding to the enzyme in the case of microcystins, and with a non-covalent bond for nodularin. This binding blocks proteins dephosphorilation, affecting cytoskeleton proteins too. As a consequence of PP inhibition, the most affected cytoskeleton components are polymers known as intermediate filaments and microfilaments. Their formation and

structure is a balance between addition and loosing of subunit in intermediate filaments and of association and dissociation in microfilaments. This balance is controlled by the action of PP, which remove phosphate groups, and protein kinases (PK) which add phosphate groups. Thus the inhibition of PP increase the rate of phosphorilation and consequently of subunit loss and dissociation (Carmichael, 1994).

As a consequence of exposure to hepatotoxins, intermediate filaments, at first, and microfilaments loose their organization and cytoskeleton shrinks. This causes the withdrawal of fingerlike projections which allow hepatocyte to interact with neighboring cells, breaking the cell's contact with the other hepatocytes and with sinusoidal capillaries. Thus induced alterations causes a reorganization of cells structure, with the appearance of swallowing of cells, cells retraction and overposition. Cells detach from vessels and vessels themselves loose their organization (Fig. 6). This general alteration lead to blood accumulation in liver, hemorrhage and death of the organism within a few hours up to a few days (Carmichael, 1994).

An additional target of microcystin is β-subunit of ATP synthase, causing mitochondrial apoptotic signaling, but only at high concentrations.

Finally, it has been proved that microcystins enhance oxidative stress. The exposure to the toxins induces formation of reactive oxygen species, loss of mitochondrial membrane potential, mitochondria permeability, transition and activation of calpain and Ca^{2+}/calmodulin-dependent protein kinase II. All of these effects lead to cells apoptosis (Mikhailov et al., 2003).

Radicals formation induces alteration of cytoskeleton structures and LDH leakage.

A protective role was defined for glutathione: binding to glutathione increase water solubility and excretion of the toxins. Protective action is exerted by linking to the same microcystin molecule moiety which binds to protein phosphatases, namely at the Mdha. Thus any phenomena which causes glutathione depletion limits the detoxifying capacity of the organism and/or the prevention of oxidative damage (Runnegar et al., 1987; Hermansky et al., 1991; Pace et al., 1991; Gehringer, 2003).

On the long term, microcystins and nodularins have proved to act as tumor promoters. Indeed, PP and PK not only influence cell structure, but also regulate cell proliferation, by promoting cell division cycle (PK) and inhibiting cell division (PP). Thus PP blocking stimulate cell division; if such a phenomena appears after carcinogenic mutation, it facilitates the development of tumors, by enhancing mutated cells proliferation; thus cyanotoxins should be classified as tumor PROMOTORS and not as tumor INDUCERS (Carmichael, 1994; Sivonen and Jones, 1999).

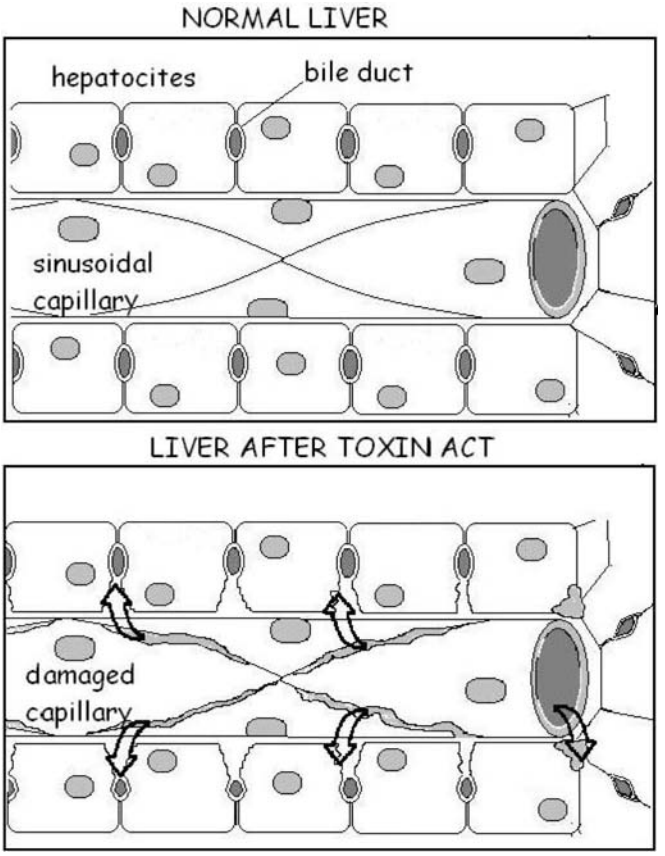

Figure 6. Mechanism of action of microcystins.

Toxicity has proved to be cumulative, due to the irreversible covalent bond microcystin-PP, which increase in number following a higher and/or longer exposure to the toxin. Thus the cells damage becomes more severe with increasing dose and/or exposure time.

It has been observed that microcystins and nodularins, once solubilised in water, adopt a chemical shape that is fundamental for the interaction of the toxins with PP. Indeed, the molecule maintains a saddle-shaped motif, with the free carboxyl groups of D-MAsp and D-Glu projecting laterally and the Adda moiety extending posteriorly from the rigid cyclic backbone. This structure allows the Adda moiety to fit to the hydrophobic groove at the active site; the two carboxylate group and a carbonyl oxygen at the metal-binding site; the L-Leu side chain at a tyrosine at the edge of the C-terminal groove near the active site (Bagu et al., 1995; Goldberg et al., 1995).

3.3. SYMPTOMS AND TREATMENT OF TOXICOSES IN HUMANS

Syndromes observed after ingestion (via drinking or recreational waters) or breathing of cyanotoxins vary depending on the amount of toxin introduced (Carmichael, 2001).

Observed symptoms following the ingestion of small amounts of microcystins and/or nodularins can be ascribed to hepatitis, renal and intestinal failure. They include weakness, anorexia, headache, gastric ache, pallor of mucous membranes, vomiting, painful hepatomegaly, urinary bleeding, cold extremities, diarrhea. This syndrome requires hospitalization of affected people in order to recover (Turner et al., 1990; Carmichael, 1992; Carmichael, 1994; Carmichael, 1997; Carmichael, 2001).

Chronic exposure to low levels of toxins induces chronic alteration of liver and digestive tract, with necrosis, progressive cellular fibrosis and leucocytes infiltration of damaged tissues (Carmichael et al., 1988; Yu, 1989; Carmichael and Falconer, 1993; Carmichael, 1994; Harada et al., 1996; Ueno et al., 1996).

In more severe cases, where exposure is to high levels of toxins, death can occur, due to intrahepatic hemorrhage and hypovolaemic shock, within few hours after exposure (Carmichael, 1992; Carmichael and Falconer, 1993; Carmichael, 1994; Carmichael, 1997; Carmichael, 2001).

As already explained, chronic and sub-chronic exposure to hepatotoxins seems to be tumor promoting, as has been proved in China by an epidemiological study. In that research a elevated correlation between liver hepatocellular carcinoma incidence and microcystins presence in drinking water was observed. Microcystins were considered as a co-factor for tumor appearance, together with Aflatoxins B1 and hepatitis B virus. The removal of both three co-factors reduced liver cancer incidence (Yeh, 1989; Yu, 1989; Zhu et al., 1989).

Direct contact with blue-green algae blooms induces gastritis (due to occasional ingestion of cyanobacteria), acute dermatitis and hay-fever-like symptoms, i.e. rhinitis, conjunctivitis and asthma, following skin and nasal contact. Many of affected people give positive skin test against algal extracts, suggesting an allergic mechanism at the base of the adverse reaction (Billings, 1981; Carmichael et al., 1985; Codd and Bell, 1985).

Lots of episodes have been reported concerning exposure to hepatotoxins by drinking or recreational waters.

The first case reports dates up to 1844 in London, when a woman presenting severe abdominal pain eliminated *Oscillatoria* shred per rectum (Farre, 1844).

The first case of massive intoxication due to microcystins was reported by Tisdale (1931) and refers to a bloom of *Microcystis* sp. in Ohio and

Potomac rivers, which caused the intoxication of thousands of persons consuming rivers' waters.

In following years, lots of episodes were reported in USA, Australia, South America and Africa.

In 1966 gastroenteritis was observed in children living in Harare, Zimbabwe, following *Microcystis* blooms in a water supply reservoir (Zilberg, 1966).

In 1974, pyrogenic reactions, i.e. chills, fever, myalgia, nausea, vomiting and hypotension, were observed in 23 patients of a private dialysis clinic near Washington, USA. High levels of toxins were detected in potable water supply and coincided with a blue-green algae bloom. The outbreak stopped when the cyanobacteria count declined (Hindman et al., 1975).

In 1983 in Armindale, Australia, water supply ponds were treated with copper sulphate (1 ppm) in order to eliminate a *Microcystis* bloom. Following the termination of the bloom a toxic episode characterized by increased liver enzyme activity, namely c-glutamyl-transferase, was observed (Gilroy et al., 2000; Rao et al., 2002).

The two most lethal poisonings attributed to cyanobacteria in drinking water occurred in Brazil. The first one occurred in 1993 and concerned a massive *Anabaena* and *Microcystis* bloom in Itaparica Dam, where 2000 cases of gastroenteritis resulting in 88 deaths, mostly children, were reported (Rao et al., 2002).

In 1996, an second outbreak of acute liver failure at a haemodialysis centre in Caruaru occurred. 116 out of 131 patients experienced visual disturbances, nausea and vomiting after routine haemodialysis treatment. Subsequently, 100 patients developed acute liver failure, and of these 76 died. Following studies led to the conclusion that the major contributing factor to death of the dialysis patients was intravenous exposure to microcystins, specifically microcystin-YR, -LR and -AR with dialysis water (Carmichael et al., 2001; Azevedo et al., 2002).

Man can also be exposed orally via algal health food products. These products are potentially hazardous if they contain any of the toxigenic species or strains of cyanobacteria. Many of these products contain *Aphanizomenon flos-aquae*, a blue-green alga that is harvested from Upper Klammath Lake in southern Oregon, USA. Because *M. aeruginosa* coexists with *A. flos-aquae*, it can be collected inadvertently resulting in microcystin contamination of blue-green algae health products.

Dermal exposure may occur during recreational use of water bodies and during showering and can cause blistering of lips and allergic reactions, like contact dermatitis, asthma, hay fever and conjunctivitis (Rao et al., 2002). In the UK in 1989, 10 out of 20 army recruits developed vomiting, diarrhoea,

central abdominal pain, blistering of the lips and sore throats after swimming and canoe training in water with a dense bloom of *Microcystis* spp. Two of the recruits developed pneumonia attributed to the aspiration of *Microcystis* toxin and needed hospitalization and intensive care. The severity of the illness appeared to be related to the swimming skills and the amount of water ingested (Chorus and Bartram, 1999).

Epidemiological evidence of adverse health effects after recreational water contact was established in a prospective study involving 852 participants. Results showed an elevated incidence of diarrhoea, vomiting, flusymptoms, skin rashes, mouth ulcers, fevers, eye or ear irritation within 7 days following exposure. Symptoms increased significantly with the duration of water contact and cell density of cyanobacteria (Chorus and Bartram, 1999).

Twenty-six cases with skin diseases and multiple systemic symptoms associated with exposure (some via drinking water) to river water or rain water were reported in Australia during 1991–1992. The water was stored in open tanks and contained *Anabaena* blooms (WHO, 1998). Illness in humans associated with inhaling microcystins had been reported. The intranasal route appeared to be as toxic as the intraperitoneal route. Therefore, the risk posed by inhaling microcystins during showering should be of concern (Duy et al., 2000).

No specific treatment exists for cyanobacteria intoxications.

Therapy is basically symptomatic, aimed at maintaining and restoring organism functions. In some occasions antihistaminic drugs and cortisone have been proved to be helpful in allergic reactions following skin contact with blooms or scum.

In mice antioxidant administration, i.e. carotenoids, seems to have a protective role against more severe intoxication. Anyway, this protective action was seen only if antioxidants were administered BEFORE microcystin exposure, thus this treatment seems to have no therapeutic application (Negri and Jones, 1995).

An inhibition of microcystins action on cell cultures, namely on morphological alterations, has been proved by cyclosporine A and rifampicine (Dawson, 1998). No application to humans is actually considered.

3.4. TOXICOSES IN ANIMALS

Microcystin are responsible for intermittent, but repeated cases of poisoning in wild and domestic animals. They were attributed as the cause of death of cattle, geese, sheep, pigs, horses, dogs, cats, squirrels, poultry, waterfowl and birds. In both wild and domestic animals, hepatoxicosis was seen. The signs of hepatoxicosis included weakness, reluctance to move about, anorexia, pallor of extremities and mucous membranes, and mental

derangement. Death occurs within a few hours to a few days and is often preceded by coma, muscle tremors and general distress. Death is believed to be the result of intrahepatic haemorrhage and hypovolemic shock (Duy et al., 2000).

A water bloom of *N. spumigena* in Lake Alexandrina, Australia, which is a shallow lake at the termination of the River Murray, caused numerous livestock deaths in 1878 and was the first scientifically documented case of cyanobacterial intoxication (Falconer, 2001).

In 1963 in Rugen, Germany, 400 ducks were affected by toxins from *N. spumigena*; in 1974–1975, 34 sheep and 52 lambs in South Western and Western Australia were affected and in 1975 30 dogs became sick and 20 died at the Danish coast of the Baltic Sea. At the Swedish, German and Finnish coast of the Baltic Sea, nine dogs, 16 young cattle and one dog plus three puppies, respectively, were affected by toxins from *N. spumigena* in 1982, 1983 and 1984. In 1990 in Wilhelmshafen, Germany, two dogs became sick by toxins from *N. spumigena* and were sacrificed (Duy et al., 2000).

Lots of Flamingos die-off have been reported in both Europe and Africa.

Over the 1990s, episodic mass mortalities of Lesser Flamingos (*Phoeniconaias minor*) have occurred at Kenya's Rift Valley saline, alkaline lakes. Causative agents were three cyanobacterial toxins: microcystin-LR, microcystin-RR and anatoxin-a. These toxins were present at concentrations high enough to have caused the bird's death. The presence of anatoxin-a was consistent with observations of staggering and convulsions in the flamingos before death and with opisthotonus postmortem (Codd et al., 1999).

In 2001, flamingos mass mortalities in southwest Spain have involved wild and captive birds; at least 579 of 943 greater flamingo (*Phoenicopterus ruber*) chicks died, together with a mixed population of other water birds at the Doñana National Park lagoon. Microcystins were identified as the likely cause of this death event in Spain, based on the presence of microcystin-producing cyanobacteria and microcystins in the water and crop contents, postmortem examination of livers and the elimination of alternatives (Codd et al., 2003).

An acute mortality of ten adult captive Chilean flamingos (*P. chilensis*) occurred at SeaWorld, Orlando, Florida, USA, in 2001. The *P. chilensis* deaths were also attributed to microcystin-LR and microcystin-LA, based on poisoning signs, postmortem examination of organs, toxin concentrations in gastrointestinal contents and pond water and elimination of alternatives (Codd et al., 2003).

Other wildlife species affected by hepatotoxins are white rhinoceros, skunks and mink, as well as waterfowl, fish and muskrats (Soll and Williams, 1985; Eriksson et al., 1986; Carmichael, 1992; Bury et al., 1997).

4. Neurotoxins

Neurotoxins are alkaloids typical of cyanobacteria, produced by the genera *Anabaena*, *Planktothrix* and *Aphanizomenon* (Fig. 7).

These toxins are divided in two main classes, anatoxins and saxitoxins. Being saxitoxins produced also by marine algae, they will be discussed in the chapter concerning marine algal toxins, and present section will focus only on anatoxins.

4.1. CHEMICAL STRUCTURE

Among anatoxins, three molecules are known: anatoxins-a, omoanatoxin-a and anatoxin-a(s), which, despite similar names, are chemically unrelated.

Anatoxin-a is a low molecular alkaloid, a secondary anime, 2-acetyl-9-azabicyclo(4-2-1)non-2-eme, with a molecular weight of 165 Da (Fig. 8).

Omoanatoxin-a is an anatoxin-a homologue, characterized by a propionyl group at C-2 instead of acetyl group, weighting 179 Da (Fig. 8).

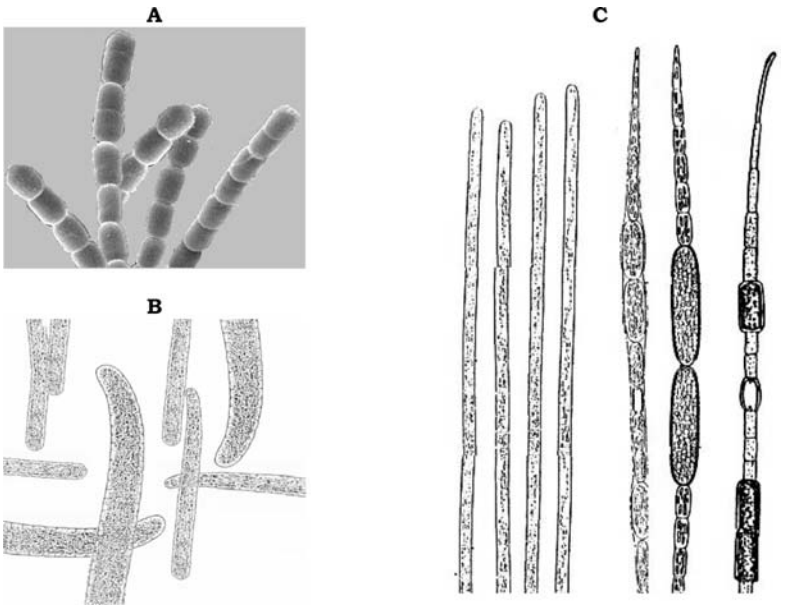

Figure 7. Neurotoxins producing algae (A – *Anabaena*, B – *Planktothrix,* C – *Aphanizomenon*).

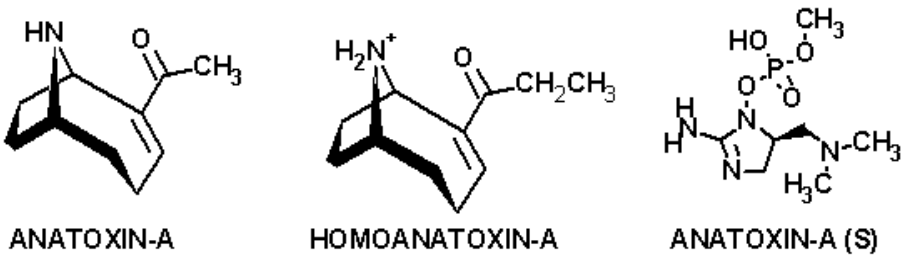

Figure 8. Chemical structure of neurotoxins.

Anatoxin-a was the first cyanotoxin to be chemically and functionally characterized and is at present well known.

Its ionization state is pH-dependent and exists in the protonated form at neutral and acidic pH. It is highly soluble in water and is polar, and easily degraded by sunlight to non-toxic compounds. Omoanatoxin-a presents similar characteristics.

Anatoxin-a(s) is considered a unique phosphate ester of a cyclic N-hydroxyguanidine structure, with a molecular weight of 252 Da.

4.2. MECHANISM OF ACTION

Neurotoxins target the neuromuscular system, paralysing peripheral, skeletal and respiratory muscles with different mechanisms.

Anatoxin-a is a cholinergic agonist binding to nicotinic receptors, causing a depolarizing neuromuscular blockade (Carmichael et al., 1975; Spivak et al., 1980; Aronstam and Witkop, 1981; Spivak et al., 1983).

When acetylcholine is released by neurons that impinge on muscle cells, it binds to postsynaptic receptor. As it attaches to the receptor the Na^+ ion channel opens, triggering the ionic movement that induces muscle cells to contract. Soon after the enzyme acetylcholinesterase degrades acetylcholine, preventing an overstimulation of the muscle cells and allowing the channel to close and the receptor to get ready to respond to new signals (Fig. 9) (Carmichael, 1992).

Anatoxin-a binds in an irreversible way to nicotinic acetylcholine receptor behaving as agonist of acetylcholine, but it can not be degraded by acetylcholinesterase. Thus the binding induces a permanent opening of Na^+ channels, which causes inflowing of the ion and a continued action potential generation, overstimulating muscle cells (Fig. 10).

Anatoxin-a(s) is at present one of the few naturally occurring organic phosphate which acts like synthetic organophosphorous insecticides (OP).

Briefly, the toxin interacts with acetylcholinesterase and blocks the enzyme by irreversibly binding to esterasic active site, impeding cleavage and recycling of acetylcholine (Fig. 11).

Its toxicity to mice is 10 times higher than that of anatoxin-a and about 20 times that of some very potent synthetic OP, i.e. di-isopropyl-fluorophosphate (DFP) (Carmichael et al., 1990).

4.3. SYMPTOMS AND TREATMENT OF TOXICOSES IN HUMANS

No acute intoxication have been reported in humans, because aspect and odor of waters affected by blooms makes them undesirable for consumption. A possible way of exposure is through recreational and water-sport use, via occasional ingestion or inhalation. In these occasions, exposure to sublethal amounts can be expected. Following these exposures, recovery appears to be complete and with no chronic effects following recovery (Kuiper-Goodman et al., 1999; WHO, 2003).

By interfering with acetylcholine metabolism all neurotoxins induce a constant opening of sodium channel and a persistent stimulation of post-synaptic neurons. Consequently symptoms which appears following intoxication are similar.

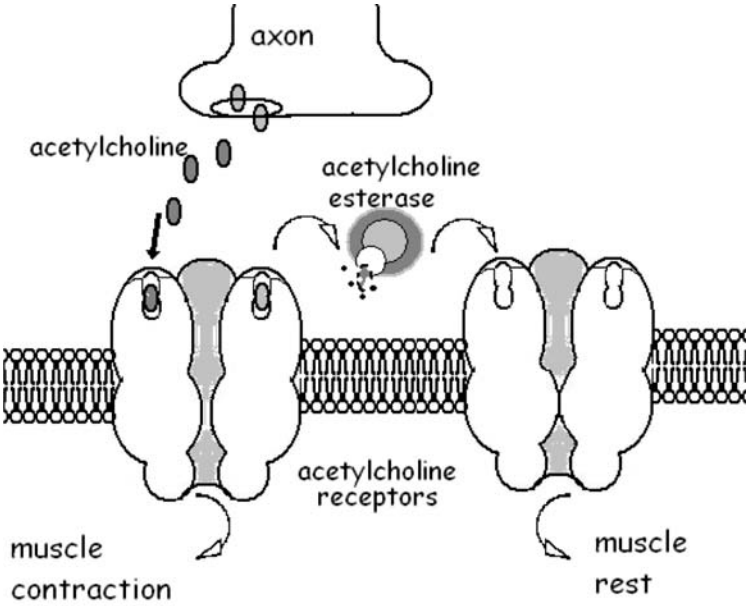

Figure 9. Scheme of action of acetylcholine.

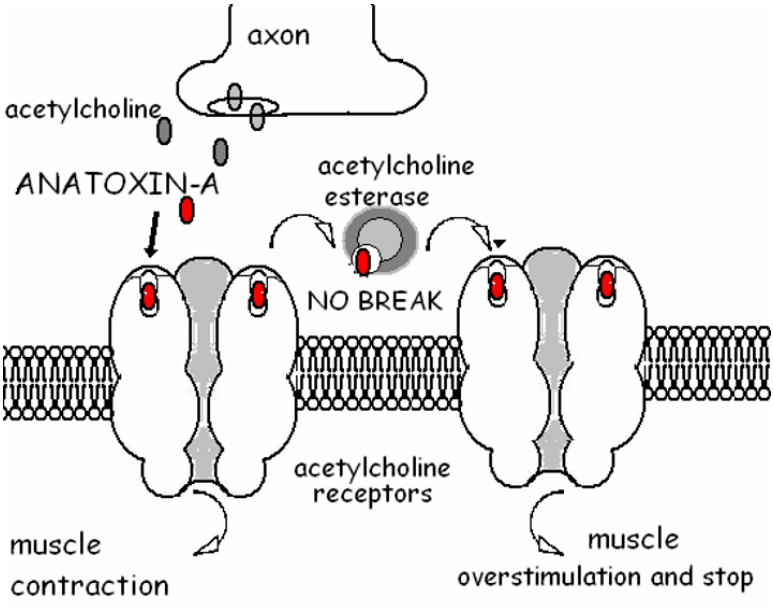

Figure 10. Scheme of action of anatoxin-a.

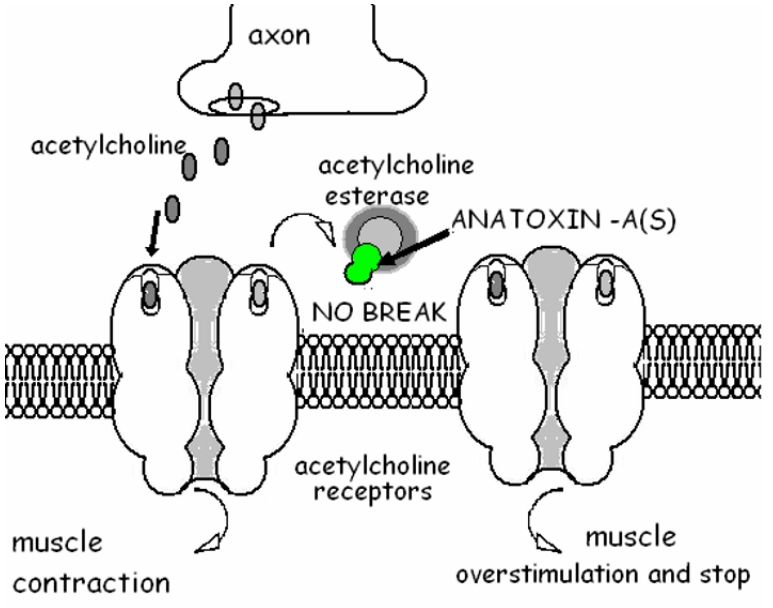

Figure 11. Scheme of mechanism of action of anatoxin-a(s).

Staggering, muscle fasciculation, reduced movement, gasping respiration, bruxism, cyanosis and convulsions appear (Carmichael, 1992; Hunter, 1998; van Apeldoorn et al., 2007).

The distinguishing symptoms between the two group of neurotoxins are:

– Rigid neck contracture in birds for anatoxin-a
– Intense salivation (from which the "s" of the toxins comes from) and mucous nasal discharge for anatoxin-a(s).

Symptoms are similar to those observed in OP intoxication, due to the anticolinesterasic activity.

No specific treatment exists for anatoxin-a intoxication, even if it has been proved in mice and rats that artificial respiration can help in recovering animals, together with lavage and instillation of activated charcoal (Carmichael et al., 1975; Beasley et al., 1989).

On the contrary, treatment of anatoxin-a(s) intoxication can be efficiently performed by applying the same drugs used for OP intoxication, i.e. atropine (Mahmood and Carmichael, 1986; Beasley et al., 1989).

4.4. TOXICOSES IN ANIMALS

Animals as livestock have a high probability of getting intoxicated by neurotoxins, as they can drink high amounts of contaminated water and gather scum material in their fur and ingest it through grooming with the tongue.

Anatoxin-a causes death within minutes to a few hours depending on the species, the amount of toxin ingested, and the amount of food in the stomach. Clinical signs of poisoning follow a progression of muscle fasciculations, decreased movement, abdominal breathing, cyanosis, convulsions and death. In addition, opisthotonus (rigid, "s"-shaped neck) is observed in avian species. In the wild larger animals collapse and sudden death is observed (Carmichael, 2001).

Documented cases of animal fatalities due to the consumption of water with anatoxin-a included cattle in Finland and Canada, and dogs in the USA, New Zealand, Scotland and Ireland. The onset of the symptoms is very rapid (Chorus and Bartram, 1999; Duy et al., 2000; Hamill, 2001; Briand et al., 2003; Furey et al., 2003).

Over the 1990s, episodic mass mortalities of Lesser Flamingos (P. minor) have occurred at Kenya's Rift Valley saline, alkaline lakes. Analyses of flamingo carcass livers and cyanobacterial samples from Lakes Bogoria and Nakuru demonstrated three cyanobacterial toxins in dead flamingo livers: microcystin-LR and microcystin-RR and anatoxin-a.

Toxins were present at concentrations sufficient alone to have caused the birds death, that of anatoxin-a being consistent with observations of

staggering and convulsions in the flamingos before death and with opisthotonus postmortem (Codd et al., 2003). Further research with materials from Lake Bogoria Lesser Flamingos has identified also the microcystins and anatoxin-a in bird stomach, intestine contents and faecal pellets (Krienitz et al., 2003).

Anatoxin-a(S) was responsible for the death of dogs, pigs and ducks in the USA (Briand et al., 2003). Animal poisoning due to anatoxin-a(s) was seen in Denmark (Kaas and Henriksen, 2000).

5. Cylindrospermopsin

Cylindrospermopsin is the only representative of the toxin class of cytotoxin.

It is produced by *Cylindrospermopsis raciborskii*, a tropical cyanobacteria, *Anabaena bergii*, *Aphanizomenon* sp., *Umezakia natans*, *Raphidiopsis curvata* and, for German waters, *Aphanizomenon flos-aquae*. All of these cyanobacteria are filamentous organisms closely genetically related. Most known organism is *Cylindrospermopsis raciborskii*, who is worldwide distributed. It has been observed that this blue-green alga is spreading in cooler areas of the Northen Emisphere; whether this is a result of global warming or of the distribution of more cold-tolerant strains is not yet clarified (Padisak, 1997; Lagos et al., 1999; Griffiths and Saker, 2003; Falconer, 2005; Gugger et al., 2005) (Fig. 12).

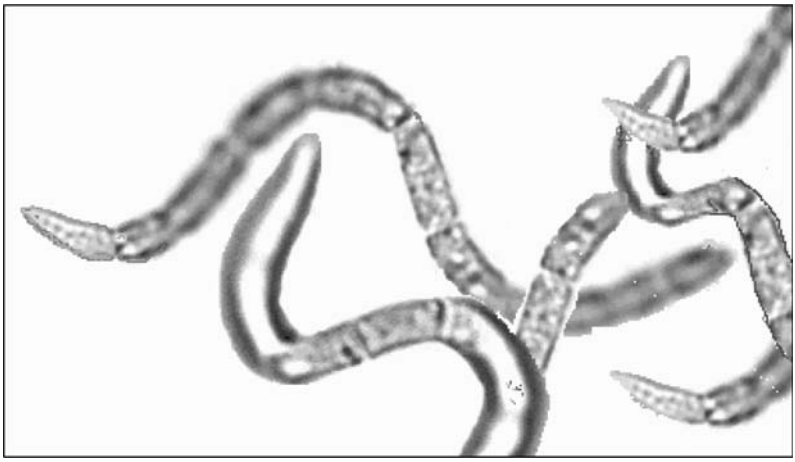

Figure 12. Cylindrospermopsis.

C. raciborskii does not form surface scums, while it forms dense bands well below the lake surface in clear stratified lakes during summer. Thus it is not perceived as a danger by reservoir managers. When present in shallow

mixed lakes, filaments distribute throughout the water column, which assumes a general discoloration. In any case, the toxin content is high, as it leaks from cells in normal conditions; i.e. in *A ovalisporum* blooms around 80% of toxin was in free solution. Thus normal treatment procedure does not satisfactorily remove the toxins (Shaw et al., 1999; Fabbro and Andersen, 2003; Falconer and Humpage, 2006).

5.1. CHEMICAL STRUCTURE

Cylindrospermopsin is an alkaloid containing a tricyclic guanidine combined with hydroxymethyl uracyl (Fig. 13). The hydroxyl bridge can bring two different epimers, cylindrospermopsin and 7-epicylindrospermopsin, which are both two naturally occurring and equally toxic. The molecule, because of its negatively charged sulfatic group and of the positively charged guanidine group, is highly water soluble (Banker et al., 2001; Looper et al., 2005; White and Hansen, 2005; Falconer and Humpage, 2006).

An additional, naturally occurring variant is 7-deoxy-cylindrospermopsin, co-existing with cylindrospermopsin in drinking waters. Its toxicity is still under evaluation, but it is probably as toxic as cylindrospermopsin (Banker et al., 2001; Looper et al., 2005; White and Hansen, 2005; Falconer and Humpage, 2006).

Basic for toxicity seems to be the uracil group: the loss of this group, indeed, effectively removes toxicity (Banker et al., 2001).

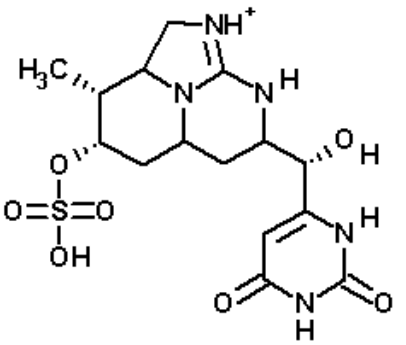

Figure 13. Chemical structure of cylindrospermopsins.

5.2. MECHANISM OF ACTION

Cylindrospermopsin is a general cytotoxin that blocks protein synthesis, and the first clinical symptoms of poisoning are kidney and liver failure (Chorus and Bartram, 1999; Carmichael, 2001). By the oral route, cylindrospermopsin

can cause gastroenteritis through injury to the gut lining, hepatitis from injury to liver cells, renal malfunction from cell injury to the kidneys and haemorrhage from blood vessel injury (Duy et al., 2000).

One of the effects induced by cylindrospermopsin is the activation of cytochrome P-450 (CYP450), mechanism considered of primary importance for toxicity.

A study in rat hepatocytes cultures revealed that protein synthesis inhibition is rapid and irreversible. Indeed, no synthetic activity was observed up to 18 hours after suspension of exposure of cells.

This observation lead to consider protein synthesis inhibition unlikely to be the major toxic insult, as cytochrome P-450 inhibitors that attenuated cylindrospermopsin toxicity, did not protect against the impairment of protein synthesis (Froscio et al., 2003).

Further studies had underlined four main steps in the toxic process, all depending on cytochrome P-450 activation. In the initial phase, ribosomes detach from the membranes of the rough-surfaced ER and accumulate into the cytoplasm of hepatocytes. This process is accompanied by condensation and reduction in the size of nucleoli.

The second phase, which begins 24 h after exposure, is correlated with membrane proliferation. In this phase the amount of total P-450 is greatly decreased in hepatic microsomes.

The third phase is characterized by fat droplets accumulation in the central portion of hepatic lobules, probably induced by free radicals induced by liver injury.

Finally severe liver necrosis occurs (Duy et al., 2000).

Protein synthesis inhibition affects also glutathione synthesis, which is under CYP450 control, but this does not lead to an increase in oxidative stress, thus suggesting that this should not be considered a primary toxicity mechanism (Runnegar et al., 1995).

CYP450 seems to be important also for genotoxic and carcinogenic effect associated to toxin exposure.

Indeed, DNA fragmentation observed in a number of in vitro assay systems seems to require metabolism of the toxin, which is regulated by CYP450 activity. Indeed, active oxidation operated by CYP450 produces genotoxic compounds which induce DNA breaking.

In vitro results seems to be confirmed by epidemiological studies: following a poisoning which occurred in 1979 affecting a group of children, a monitoring study began aimed at verifying any increase in cancer incidence. After more than 20 years of follow-up, a trend toward an increase in gastrointestinal cancer was observed, even thought results obtained were not statistically significant, due to the small number of cases followed (Humpage et al., 2005; Falconer and Humpage, 2006).

5.3. SYMPTOMS AND TREATMENT OF TOXICOSES IN HUMANS

Toxicity symptoms are characterized by hepatitis, renal and intestinal lesions, vomiting, headache, abdominal pain, hematuria, glycosuria and proteinuria, constipation followed by bloody diarrhea and electrolytic unbalance (Bourke et al., 1983).

As for microcystin, treatment is symptomatic and aimed at sustaining and restoring body functioning. It should be remembered, anyway, that protein inhibition is irreversible, thus recovery require long, intensive and specialized hospital cares.

In Australia, in 1979 a cyanobacterial bloom in the drinking water reservoir resulted in complaints from the water consumers of bad taste and odour from the drinking water. Controlling water authorities terminated the bloom by copper sulphate addition to the reservoir. Shortly after copper dosing of the reservoir, children were brought into the hospital with an unusual hepatoenteritis, initially showing acute tender liver enlargement, constipation, vomiting and headache. This was followed by bloody diarrhea and loss of protein, electrolytes, glucose and ketones through the urine, with varying severity of dehydration. Severe cases were flown to the regional hospital, where they received intensive care with intravenous therapy. A total of 140 children and 10 adults received hospital treatment. The clinically most serious cases occurred among the Aboriginal population of Palm Island, off the Queensland coast of Australia in 1979. The intoxication was firstly named “Palm Island Mystery Disease”, because no aetiological agent was identified. Further investigations allowed the isolation of cylindrospermopsin derived from *C. raciborskii*, in water, which showed similar toxicity in animal studies to that in the children reported above (Byth, 1980; Hawkins et al., 1985; Falconer, 2001; Falconer and Humpage, 2006).

5.4. TOXICOSES IN ANIMALS

In August 1997, a farm dam at McKinlay in northwest Queensland, Australia, contained an algal bloom which was identified as a monoculture of *C. raciborskii*. Cylindrospermopsin was detected in material harvested from the dam and in a pure culture of an isolate from the bloom. An extract of this material was lethal to mice 24 h after an i.p injection of 153 mg/kg bw. On a cattle property near the dam three cows and ten calves died. One animal showed signs of staggering and weakness before its death. Abdominal and thoracic hemorrhagic effusion, hyperemic mesenteries and pale and swollen liver were found at necropsy, with nothing abnormal observed in the brain, lungs, spleen or kidney. Histopathology of the liver from a calf

carcass showed signs similar to the known toxicological effects of cylindrospermopsin in mice, i.e. extensive areas of hepatic degeneration and necrosis, with only isolated areas of intact hepatocytes remaining; deposits of fibrous tissue were common throughout the liver (Saker et al., 1999; Briand et al., 2003).

6. Lipopolysaccharides (LPS)

Endotoxic lipopolysaccharides (LPS) are common compounds of the outer cell walls of cyanobacteria and Gram negative bacteria. They have been well characterized in Gram-negative, pathogenic bacteria, but their chemical structure is known for cyanobacteria too.

They consist of lipid A, core polysaccharides and of an outer polysaccharide chain. This chain is composed by a great variety of log chain unsaturated fatty acids and hydroxyl fatty acids and lacks phosphate.

The backbone sugar seems to be glucosamine, presenting variable amounts of 2-keto 3-deoxyoctonate (KDO), galactoses and heptoses (Martin et al., 1989) (Fig. 14).

LPS cause fever in mammals and are involved in septic shock syndrome, which can aggravate toxicant-induced liver injury.

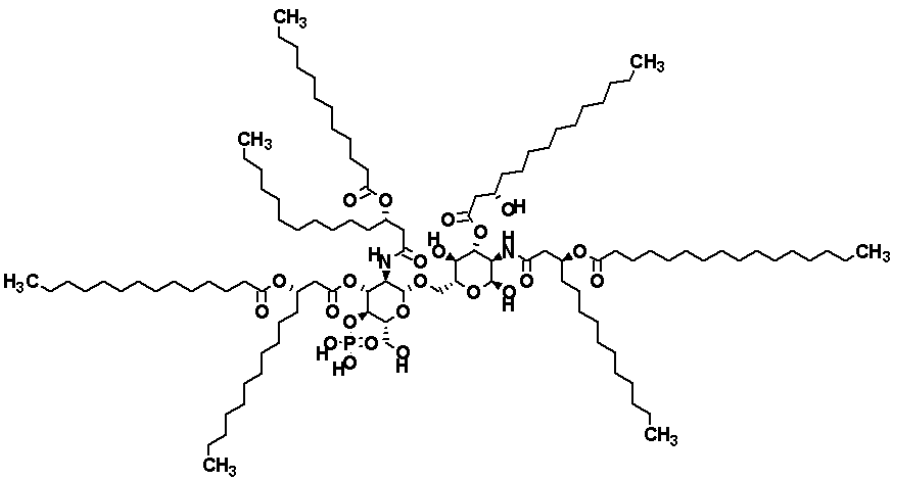

Figure 14. Lipopolysaccharides chemical structure.

This irritating action is mainly due to the fatty acid component of the molecule and is achieved by the releasing of inflammatory mediators, i.e. tumor necrosis-factor α, interferon-γ, interleukin 1 and 6, leukotrienes, prostanoids, nitric oxide. Antibody production is induced by the *O*-antigen.

They can also alter the detoxification systems of the organisms by inhiboting the glutathione S-transferase genes and in fish they induce an osmotic imbalance by stimulation of water ingestion.

Cyanobacteria LPS are less toxic than those of pathogenic Gram-negative bacteria, but could be implicated in human health problems encountered as a result of bathing for example.

7. Bioactive Compounds

Cyanobacteria not only produce toxic compounds which can be lethal to humans and animals, but are known to produce several other bioactive compounds, some of which are of medical interest. These molecules have proved to be active against algae, bacteria, fungi and mammalian cell lines, thus can potentially have a wide range of applications (Carmichael, 1992).

Cyanobacteria have been found to be a rich source of biomedically interesting compounds and therefore screening programmes for new bio-activities are underway (Kashiwagi et al., 1980; Rinehart et al., 1981; Mason et al., 1982; Patterson et al., 1984; Flores and Wolk, 1986; Cannell et al., 1988; Gerwick et al., 1989; Schwartz et al., 1990).

Cyanobacteria are known to produce antitumour, antiviral, antibiotic and antifungal compounds. Of the cyanobacterial extracts screened by a Hawaiian research group, 0.8 per cent showed solid tumour selective cytotoxicity (Moore et al., 1991). Depsipeptides (peptides with an ester linkage) called cryptophycins isolated from a cyanobacterium, *Nostoc* sp. strain GSV 224, are promising candidates for an anticancer drug (Trimurtulu et al., 1994). Recently, several new cyclic or linear peptides and depsipeptides from cyanobacteria have been characterised. Some are protease inhibitors, but the biological activity of the others remains to be characterised (Namikoshi and Rinehart, 1996). Many of the cyanobacterial bioactive compounds possess structural similarities to natural products from marine invertebrates.

It has been observed that bioactive compounds discovered in cyanobacteria are not duplicate structures of known “natural” drugs, as the rate of rediscovery in blue-green algae is very low, as compared with that in actinomycete, rating up to 95% (Patterson et al., 1991; Carmichael, 1992).

Chemically, these compounds include acutiphycins, indolcarbazoles, mirabilene isonitriles, paracyclophanes, scytophycins, tantazoles, tolytoxin, toyocamycin and tubercidin, which have mainly extracted from freshwater and terrestrial cyanobacteria (Patterson et al., 1991; Carmichael, 1992).

8. Cyanobacteria as Food Supplements: Problems Related

Cyanobacteria are at present considered as good food supplements, as they can increment the amount of vital nutrients intake, i.e. amino acids, tace minerals, omega-3 and -6 fatty acids, β-carotene and vitamins. This supplementation seems to improve memory and attention, increase energy and immune status and give relief to exhaustion nervousness, depression and premenstrual syndrome. Blue-green algae extracts are also used in the treatment of Attention Deficit Disorder in children (Dittmann and Wiegand, 2006).

Anyway, such products should be carefully checked, as the species which are used for their production, i.e. *Spirulina* and *Aphanizomenon*, can be contaminated by toxin producing strains and species, or can themselves produce the toxins, as has been proved for *Spirulina*, wich can produce anatoxin-a (Kozlowsky-Suzuki et al., 2003). Gilroy et al. (2000) showed for example that 72% of blue-green algae products analysed presented levels of microcystins higher than safe level fixed by WHO.

Great attention should be paid in using such products, especially in children, as the risk increases depending on doses and duration of exposure to contaminating toxins. Children, due to their lower body weight, are exposed to higher amount of toxins/kg b.w., so they are particularly at risk.

Additionally, the established WHO limit for microcystin has been based on acute testing in animals and does not considers the tumor-promoting capacities of the toxin. An additional uncertainty factor was included, leading to a lower guideline value of 0.3 μg/l (Kankaanpaa et al., 2002).

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TOXICITY OF SEA ALGAL TOXINS TO HUMANS AND ANIMALS

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Abstract: Marine algal toxins are responsible of more than 60000 intoxication/year, with an overall mortality of about 1.5%. Human intoxications are due to consumption of seafood and respiratory exposure to aerosolized toxins. Algal toxins are also responsible for extensive die-offs of fish and shellfish, as well as mortality in seabirds, marine mammals and other animals depending on marine food web. Lots of information are available concerning acute intoxications, while little is known about environmental health effects of chronic exposure to low levels of algal toxins. Toxins are produced by two algal groups, dinoflagellates and diatoms, representing about 2% of known phytoplankton species (60–80 species out of 3400–4000) and can reach humans directly (via consumption of shellfish) or through food web transfer to higher trophic levels (zooplankton and herbivorous fish). Most toxins are neurotoxins and all are temperature stable, so cooking does not ameliorate toxicity in contaminated seafoods; five seafood poisoning syndromes exists: paralytic shellfish poisoning, neurotoxic shellfish poisoning, ciguatera fish poisoning, diarrhetic shellfish poisoning, and amnesic shellfish poisoning.

Paralytic Shellfish Poisoning (**PSP**) is caused by the consumption of molluscan shellfish contaminated with a suite of heterocyclic guanidines collectively called saxitoxins (STXs), causing almost 2,000 cases of human poisonings per year, with a 15% mortality rate. In addition to human intoxications, PSP has been implicated in deaths of birds and humpback whales. STX elicits its effects by inhibiting sodium channel conductance and there-by causing blockade of neuronal activity, mainly at the peripheral

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nervous system level, where its binding results in rapid onset of symptoms (less than 1 hr) that are classic for PSP: tingling and numbness of the perioral area and extremities, loss of motor control, drowsiness, incoherence, and in the case of high doses, respiratory paralysis.

Neurotoxic Shellfish Poisoning (NSP) generally results from consumption of molluscan shellfish contaminated with brevetoxins (PbTx), a suite of nine structurally related ladderlike polycyclic ether toxins. Brevetoxins bind with high affinity sodium channel altering the voltage sensitivity of the channel, resulting in inappropriate opening of the channel under conditions in which it is normally closed, and inhibiting channel inactivation, resulting in persistent activation or prolonged channel opening. Symptoms of NSP include nausea, tingling and numbness of the perioral area, loss of motor control, and severe muscular ache. NSP has not been documented as a fatal intoxication in humans. *Gymnodinium breve* red tides are also frequently associated with massive fish kills. The extreme sensivity of fish may result from lysis of cells passing through the gills, with direct transfer of toxin across the gill epithelium. *G. breve* was also responsible of a manatees die-off in Florida concurrent with a persistent red tide. The demonstration of brevetoxin immunoreactivity in lymphoid tissue of the manatees raises the possibility of immunosuppression as a second mode by which brevetoxin exposure may affect human health, particularly in individuals with chronic exposure to aerosolized toxin during prolonged red tide incidents.

Ciguatera Fish Poisoning (CFP) is another seafood intoxication caused by ladderlike polyether toxins, primarily attributed to the dinoflagellate, *Gambierdiscus toxicus*, which produces a precursors to ciguatoxin which is biotransformed to ciguatoxins and bioaccumulated in the highest trophic levels. Large carnivorous fishes associated with coral reefs are a frequent source of ciguatera. Baracuda, snapper, grouper, and jacks are particularly notorious for their potential to carry high toxin loads; however, smaller herbivorous fishes may also be ciguatoxic, particularly when viscera are consumed. CFP is estimated to affect over 50,000 people annually and is no longer a disease limited to the tropics because of travel to the tropics and shipping of tropical fish species to markets elsewhere in the world; outbreaks are sporadic and unpredictable at others. The symptoms of ciguatera vary somewhat geographically as well as between individuals and incidents and may also vary temporally within an area, but they generally include

early onset (2–6 hr) gastrointestinal disturbance–nausea, vomiting, and diarrhea–and may be followed by a variety of later onset (18 hr) neurologic sequelae such as numbness of the perioral area and extremities, reversal of temperature sensation, muscle and joint aches, headache, itching, tachycardia, hypertension, blurred vision, and paralysis. Ciguatera on rare occasions can be fatal. A chronic phase may follow acute intoxication and can persist for weeks, months, or even years.

Diarrhetic Shellfish Poisoning (**DSP**) is a comparatively milder seafood intoxication that consists of rapid onset (3 hr) gastrointestinal symptoms such as vomiting and diarrhea that generally resolve within 2–3 days. The diarrhetic shellfish toxins (DTX) are a class of acidic polyether toxins consisting of at least eight congeners including the parent compound, okadaic acid. Okadaic acid, DTX-1, and DTX-2 are the primary congeners involved in shellfish poisoning, with the other congeners believed to be either precursors or shellfish metabolites of the active toxins. The DTXs are inhibitors of ser/thr protein phosphatases. Ser/thr protein phosphatases are critical components of signaling cascades in eukaryotic cells that regulate a diverse array of cellular processes involved in metabolism, ion balance, neurotransmission, and cell cycle regulation. Diarrhea associated with DSP is most likely due to the hyperphosphorylation of proteins, including ion channels, in the intestinal epithelia, resulting in impaired water balance and loss of fluids. Okadaic acidlike polyether toxins have been identified as tumor promotors, thus raising the question of what effect low levels of chronic exposure to DSP toxins may have on humans as well as wildlife such as marine turtles.

Amnesic Shellfish Poisoning (**ASP**) is the only shellfish intoxication caused by a diatom (*Pseudo-nitzschia spp.*). The first recorded occurrence of ASP was in Prince Edward Island, Canada in 1987 when approximately 100 people became ill and several died after consuming contaminated mussels. The toxic agent involved in the outbreak was identified as domoic acid. Domoic acid is a water-soluble tricarboxylic amino acid that acts as an analog of the neurotransmitter glutamate and is a potent glutamate receptor agonist. The symptoms of ASP include gastrointestinal effects (e.g. nausea, vomiting, diarrhea) and neurologic effects such as dizziness, disorientation, lethargy, seizures, and permanent loss of short-term memory. Persistent activation of the kainate glutamate receptor results in greatly elevated intracellular Ca^{2+} through cooperative interactions with *N*-methyl-d-aspartate

and non-*N*-methyl-d-aspartate glutamate receptor subtypes followed by activation of voltage dependent calcium channels. Neurotoxicity due to domoic acid results from toxic levels of intracellular calcium, which leads to neuronal cell death and lesions in areas of the brain where glutaminergic pathways are heavily concentrated. The CA1 and CA3 regions of the hippocampus, an area responsible for learning and memory processing, are particularly susceptible. However, memory deficits occur at doses below those causing structural damage. Domoic acid has been identified as the causative agent in the mass mortality of pelicans and cormorants in Monterey Bay, California, in 1991 and in the extensive die-off of California sea lions in the same region in 1998. In both instances the vector for toxin transfer was anchovy.

Pfiesteria Piscicida, a fish-killing dinoflagellate first identified in aquaculture tanks in North Carolina, has been linked to fish kills in the mid-Atlantic region of the United States and is characterized by the presence of open, ulcerative lesions. *Pfiesteria* has been termed an "ambush predator" because it is believed to release a toxin that narcotizes or kills fish and then phagocytizes the sloughed tissue from its prey. *Pfiesteria* has been linked to a human intoxication syndrome, with symptoms that include fatigue, headache, respiratory irritation, skin lesions or burning sensations on contact, disorientation, and memory loss. The toxins responsible for fish lethality or neurologic symptoms have not yet been identified. There is currently no evidence that toxicity is transferred through food.

In conclusion we can state that marine algal toxins impact human health through seafood consumption and respiratory routes. The apparent increase in their occurrence over the past three decades has raised alarm and lead to the establishment of algal and toxin monitoring programs which will assist in providing time series needed to assess interannual and long-term variability in algal and toxin occurrence.

Keywords: Algae, toxins, syndromes, treatment

1. General Characteristics of Producing Organisms

Marine algal toxins are produced by phytoplankton, phytobenthos and bacteria, and are also called phycotoxins.

Phycotoxins are secondary metabolites produced by dinoflagellates and diatoms, which present pharmacologically active compounds which can be harmful to aquatic flora and fauna. Their role is both important for normal physiology of the cell and for the defense against external environmental insults, namely predators (Amzil et al., 2001; Quod et al., 2001).

Marine toxins are not dangerous *per se*, but they became an hazard when dinoflagellates and diatoms proliferate, under particular environmental conditions, i.e. eutrophication, and toxins can accumulate along different steps of trophic chains, particularly mollusks and fish. In these case, the so called HARMFUL ALGAL BLOOMS (HABs) occur, causing a great increase in cells and toxins concentrations (Smayda, 1997; van Dolah, 2000).

Phycotoxins have an great and important toxicological role as they produce a huge number of human illness linked to seafood consumption and contaminated aerosol inhalation. They are also responsible for massive die-off of fish, shellfish and marine vertebrates (van Dolah, 2000).

Generally speaking they are responsible for acute intoxications, which are well known from the toxicological, chemical and etiological point of view, while little is known concerning chronic exposure to low levels of toxins (Landsberg, 1996; Burkholder, 1998; Edmunds et al., 1999; Landsberg et al., 1999).

More than 3000 dinoflagellates and diatoms species are known at present, but only 2% of then (about 60–80 species) have proved to be toxic or harmful. This little group of species, anyway, is responsible for about 60000 human intoxication/year, 1.5% of them fatal. Fatalities are generally linked to ingestion of saxitoxins, tetrodotoxin and, in rare cases, ciguatera and domoic acid (Landsberg, 1996; Burkholder, 1998; Edmunds et al., 1999; Landsberg et al., 1999).

Incidence of HABs has increased in recent years, both in frequency and in geographical distribution (Fig. 1). Causes of this expansion are various, and include on one side the increased awareness concerning the issue and the establishment of monitoring, surveillance and research programs on toxins. This lead to a faster and more detailed identification of blooms and toxic episodes (Anderson, 1989; Smayda, 1990; Hallegraeff, 1993).

On the other side, human activities can directly and indirectly contribute to this expansion. Ballast waters transport or shellfish transplantation can directly act by easing the transfer of toxic, non indigenous species from side to the other of the world. Local and regional environmental changes, i.e. eutrophication and pollution, and/or climate variations at the local or global scale can indirectly act by inducing algae proliferation, thus increasing toxins concentrations (van Dolah, 2000)

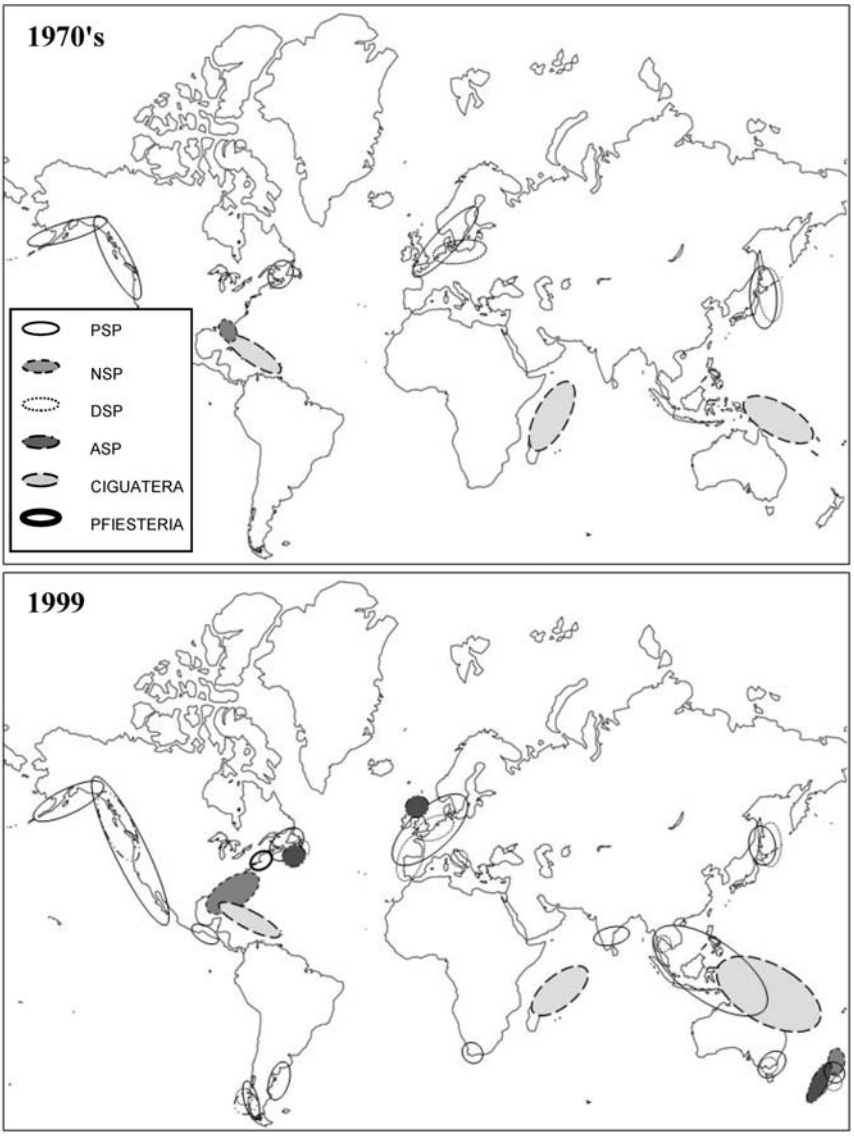


Figure 1. Time changes in HABs distribution (from van Dolah (2000), modified).

Algal blooms can be classified following various criteria: 1) the kind of bloom formed; 2) the chemical structure of the toxin; 3) the solubility in solvents; 4) the syndrome they induce.

Starting from the **kind of bloom** formed, 4 groups have been identified, which are more or less dangerous to humans and /or animals (Andersen, 1996):

– Blooms of species which produce basically harmless water discolorations, with the result that the recreational value of the area decreases due to low visibility of the water and eventually, under exceptionally weather conditions in sheltered bays, the blooms can grow so dense that they cause escape reactions and indiscriminate fish kills and kills of benthic invertebrates due to oxygen depletion. Species forming this kind of bloom are *Noctiluca scintillans, Ceratium* spp, *Prorocentrummicans, Heterocapsa triquetra*, *Skeletonema costatum*, *Trichodesmiumerythraeum*, *Eutreptiella* spp., *Phaeocystis pouchetii*, *Emiliania Huxley*, *Mesodinium rubrum*.

– Blooms of species which produce potent toxins which accumulate in food chains and cause a variety of gastrointestinal and neurological illnesses m humans and other higher animals such as. *Alexandrium tamarense, Alexandrium funndyense, Gymnodinium catenatum, Pyrodinium bahamense* var. *compressum*, *Dinophysis fortii, Dinophysis acuminata, Dinophysis acuta, Dinophysis norvegica*, *Pseudo-nitzschia multiseries, Pseudo-nitzschia pseudodelicatissima, Pseudo-nitzschia australis*, *Gambierdiscus toxicus*, *Gymnodinium breve*, *Anabaena flos-aquae, Nodularia spumigena* can produce these blooms.

– Blooms of species which, in most cases are non-toxic to humans but harmful to fish and invertebrates (especially in intensive aquiculture systems) e.g. by intoxication, damaging or clogging of the gills or other means. Examples of producing species: *Alexandrium tamarense, Chaetoceros convolutus, Gyrodinium aureolum, Chrysochromulina polylepis, Prymnesium parvum, Heterosigma akashiwo, Chattonella antiqua, Aureococcus anophagefferens, Phiesteria piscimortuis, Nodularia spumigena.*

– Blooms of species which produces toxins which are toxic to humans and which are transported by air in aerosols from the bloom area to the coast. *Gymnodinium breve, Pfiesteria piscicida*.

There is no general rule to define harmful concentrations of cells in an algal bloom, the concentration in a HAB is species specific.

Some algae cause harm at low concentrations, with no discoloration in the water, e.g. *Alexandrium tumarense* where PSP toxins are detected in

shellfish at concentrations below 10^3 cells/L, whereas other algae cause harmful effects when they occur in higher in higher concentrations, with discoloration of the water as a result, a "red tide". For example *Gyrodinium aureolum* kills fish and benthic animals at concentrations higher than 10^7 cells/L (Andersen, 1996).

Five main classes of toxins have been identified starting from their **chemical structure**:

1. Amino acid-like compounds (domoic acid and derivatives)
2. Purine derivatives (saxitoxins and derivatives)
3. Cyclic imines (spirolides, gymnodines and pinnatoxin A)
4. Linear and macrocyclic non-azotated polyethers (okadaic acid, pectenetoxins, azaspiracid, primnesines)
5. Trasfused polyehters (brevetoxins, yessotoxins, ciguatoxins).

All the toxins can be classified starting from their **solubility** in water and organic solvents:

1. Hydrophilic compounds (saxitoxins, domoic acid, tetrodotoxin)
2. Lipophilic compounds (okadaic acid, brevetoxins, ciguatoxins)

Finally, most known classification is that starting from the **syndrome** they induce. Starting from this principle 5 different syndromes can be identified:

1. **Diarrhetic Shellfish Poisoning** (DSP) is caused by a group of toxins, represented by okadaic acid, and is characterized by gastrointestinal symptoms (nausea, diarrhea, vomiting, abdominal pain) which following chronic exposure can evolve in digestive system tumors.
2. **Paralytic Shellfish Poisoning** (PSP) is caused by saxitoxins and is characterized by gastrointestinal and neurological symptoms, with nausea, vomiting, diarrhea, tingling or numbness around lips, gradual and more and more severe paralysis, respiratory difficulty, death through respiratory paralysis. It can cause death in humans.
3. **Amnesic Shellfish Poisoning** (ASP) toxin is domoic acid, and main sign of this syndrome is loss of short term memory, accompanied by gastrointestinal and neurological symptoms.

4. **Neurotoxic Shellfish Poisoning** (NSP) toxin is brevetoxin, and typical signs of toxicity are tingling and numbness of perioral area, loss of motor control and severe muscular ache. It is also responsible for some irritative episodes following exposure through contaminated aerosol.
5. One last syndrome is named ciguatera and is due to **ciguatoxin**. Together with tetrodotoxin, this is the only toxin transmitted by fish and not by shellfish. Typical symptoms are diarrhea, abdominal pain, nausea, vomiting, and lots of neurological signs. It can rarely cause death in humans.

When describing a syndrome, different toxins can be included as etiological agent, and that's the criteria followed in present chapter; it should be noted anyway that in some cases these toxins have only chemical similarity to the main toxin, causing the syndrome, and nay have a different action on humans and animals. So in the group of diarrheic toxins yessotoxins is included, even if its main action is at the neurological level.

Many other toxins have been studied and new ones are discovered in recent years, ut they are not included in a precise syndrome: tetrodotoxin, palitoxin, Pfiesteria toxins among the others. These toxins will be described in next sections.

Lots of studies have been conducted to define ideal conditions for algal growth and toxin production, but no clear scenario has been identified (Quod et al., 2001).

One of main question concerning phycotoxins is if they are produced by algae themselves or by symbiotic bacteria. In some species the production of toxins seems to be independent of bacteria presence, i.e. in *Prorocentrum lima*, producing okadaic acid. Studies conducted on saxitoxins production lead to no conclusive result, as these toxins have been found in autotrophous dinoflagellates, fresh water cyanobacteria, macrophytes and some bacteria. Finally it has been proved that tetrodotoxin is produced by symbiotic bacteria, which can be found in various aquatic and terrestrial organisms (Oshima et al., 1984; Scheuer, 1996; Shimitzu, 1996; Dantzer and Levin, 1997; Gallacher and Smith, 1999; Ritchie et al., 2000; Quod et al., 2001).

2. Role of Phycotoxins in Marine Environment

Lots of toxins producing algae contain very potent active principles, showing specific biological activity, which are thought to have a physiological or a defensive role. Indeed a certain correlation was observed between the

production of diarrhetic toxin and photosynthetic activity. Finally, a positive correlation was found between chlorophyll and diarrhetic toxins. An additional factor in favor of this hypothesis is the fact that almost all species are strict of facultative photosynthetic organisms. Okadaic acid has been found to be located into chloroplasts of some dinoflagellates (Zhou and Fritz, 1994; Morton and Tindall, 1995; Wright and Cembella, 1998; Barbier et al., 1999; Quod et al., 2001).

All these data lead to the hypothesis that at least some phycotoxins can modulate photosynthesis.

Other toxins are thought to have a physiological role: saxitoxins seem to be important n chromosome organization, due to their localization close to the nucleus and paralyzing toxins are thought to act also ad pheromones (Anderson and Cheng, 1988; Wyatt and Jenkinson, 1997; Cembella, 1998).

Phycotoxins have proved to have some antibacterial and antifungal activity; these activities are thought to allow dinoflagellates to inhibit growth of competitors like bacteria and fungi, as well as other algal species development. This inhibitory action was observed in various species of *Prorocentrum*, *Amphidinium* and in *Gambierdiscus toxicus* (Nagai et al., 1990; Lewis and Holmes, 1993; Nagai et al., 1993).

A defensive role of phycotoxins against herbivores has also been considered, acting through the alteration of ionic channel functioning , as shown with some studies demonstrating a reduction in grazing activity from macro- and micro- zooplankton.

This inhibiting activity, called allelopathy, has been studied and observed in vitro against other dinoflagellates or microalgae, even if no real evidence exist witnessing an allopathic action in the wild (Elbrächter, 1976; Kayser, 1979; de Jong and Admiraal, 1984; Yasumoto et al., 1987; Gentien and Arzul, 1990; Scheuer, 1990; Rausch de Traubenberg and Morlaix, 1995; Windust et al., 1996; Paul, 1997; Windust et al., 1997; Wright and Cembella, 1998; Sugg and Van Dolah, 1999).

The impact of toxins on and their bioaccumulation along food chains depends on the characteristics of trophic chains themselves and on environmental conditions (Fig. 2).

Thus temperate and tropical ecosystems differ greatly. Indeed, tropical waters are generally olygotrophic and blooms which develop are usually reduced as, affecting very complex ecosystems. These blooms can form a mosaic in close areas: so some zones are toxic, and a close one is not. In these ecosystem fish species, feeding on algae, are responsible for toxicity (Bourdeau et al., 2001) (Fig. 3).

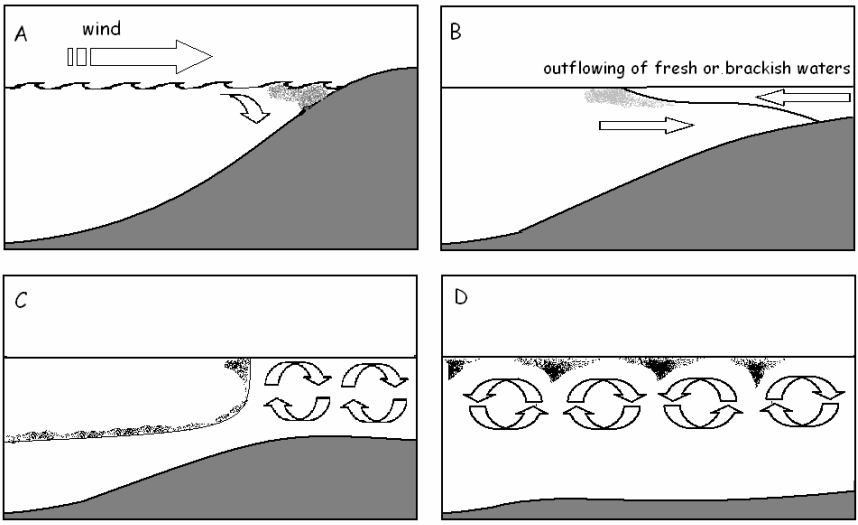


Figure 2. Blooms formation in coastal areas.

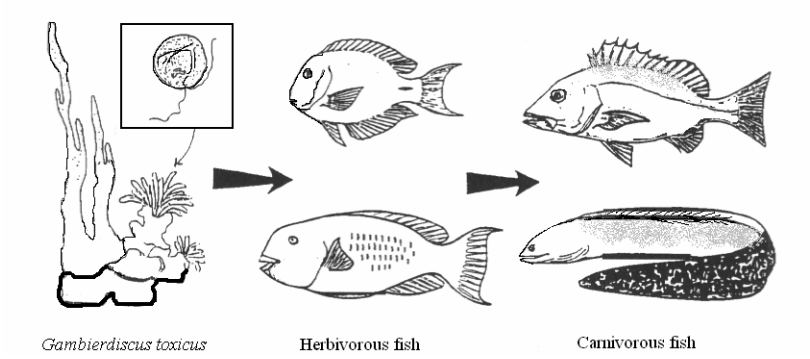


Figure 3. Transfer of phycotoxins along tropical food chains.

In temperate waters, which are more eutrophic, basic step of trophic chain are filtering organisms, like mussels. Only in some case fish, at different levels of trophic chain, can be directly interested by toxicity and accumulation of toxins, for direct contact with the poison or by ingestion of producing algae. These fish species are generally planktophagous species, living in packs (Bourdeau et al., 2001).

In these areas, phycotoxins can affect all levels of food chains.

It is recognized that harmful algae and their toxins can influence ecosystems from both the top-down (i.e. affecting predators and influencing grazing) and from the bottom-up (i.e. affecting plankton and benthic communities). Acute or chronic exposure to HABs and their toxins, either directly or through the food web, puts these populations at increased risk (White, 1980; White, 1981; Ives, 1985; Geraci et al., 1989; Gosselin et al., 1989).

Acute or chronic exposure to HABs and their toxins, either directly or through the food web, place certain populations at increased risk (Fig. 4). Microalgal toxins and their chronic effects need to be recognized as major threats to animal health, sustained fisheries, endangered species, and ecosystems. Long-term effects of biotoxins on the health of aquatic animals include increased susceptibility to disease, immunosuppression, abnormal development, and the induction of tumors. Animals at all trophic levels that are exposed to biotoxins in the long term through their diet may die or display impaired feeding and immune function, avoidance behavior, physiological dysfunction, reduced growth and reproduction, or pathological effects.

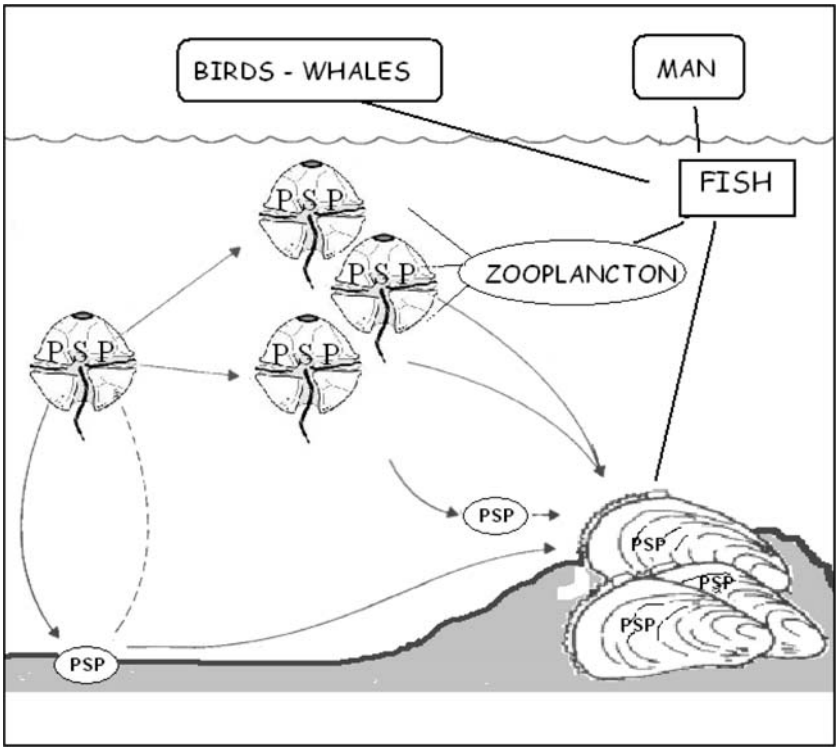


Figure 4. Accumulation and transfer of phycotoxins along food chain. The example of PSP.

2.1. ZOOPLANKTON

In many occasions zooplankton can feed on toxic dinoflagellates without any adverse effect.

When some effect occurs, it is usually a sub-lethal one, like a reduction in feed consumption. This alteration can be seen as the appearance of an avoiding behavior, so that some species avoid grazing on toxic dinoflagellates, while others feed on them without any problem.

Other effects observed are regurgitation of food, tachycardia, uncontrolled motor activity or reduced motility. Some authors think that long term exposure to low levels of toxins can induce reduction of growth rate and motor inhibition, which make copepods more sensible to predation, also easing accumulation along food chain (White, 1981; Hayashi et al., 1982; Boyer et al., 1985; Watras et al., 1985; Huntley et al., 1986; Gill and Harris, 1987; Ives, 1987; Sykes and Huntley, 1987; Uye and Takamatsu, 1990; Anderson and White, 1992).

2.2. FISH SPECIES

Phycotoxins can have a direct effect on fish species, causing larval and adult massive death. Anyway they can also have some important effect linked to long term accumulation of the toxins, turning them poisonous for consumers, being them humans or animals.

A real accumulation of toxins hardly occurs, as the toxicity of phycotoxins to fish is quite high, so in many cases fish die before they can accumulate discrete amounts of toxins. When accumulation occurs, liver and digestive tract are main target of accumulation. In the case of paralyzing toxins, altered swimming, equilibrium loss and complete immobility have been observed; if fish survive, recovery is complete (White, 1980; White, 1984; Carreto et al., 1993).

Being toxins stored in liver and digestive tract, consumption of whole fish, as happens in Borneo and Philippines, can produce deadly episodes, as registered in past years (Maclean, 1979; Maclean, 1989).

Many fish species have proved to accumulate toxins in their body: mackerels, *Sardinella* sp., *Mugil* and *Sillago*; one of the most known species which are able to accumulate toxins are puffer fish, which can stock tetrodotoxin in their viscera. Some doubt exist regarding brevetoxins capacity of accumulate; poisoning episodes in marine mammals seems to confirm the transfer of brevetoxins via plankton-eating fish (Beales, 1976; Estudillo and Gonzales, 1984; Bourdeau et al., 2001).

All specie able to accumulate toxins seems to have some adaptation to the poison; it the case of puffer fish and tetrodotoxin. Indeed, puffer fish have developed resistance to the toxin by a mutation of proteic sequence of sodium channel, which is the target of the toxin (Nakamura et al., 1984).

2.3. SEABIRDS

Lots of reports exist regarding seabirds die-off following contaminated fish eating.

Cormorants, terns, pelicans are among species more frequently affected by paralyzing toxins and domoic acid. Interestingly, different sensitivity was observed among species, cormorants being more sensible than others.

In a toxic episode concerning pelicans and cormorants, anchovy, which were responsible for the transfer of domoic acid to birds, showed no toxic symptoms, showing how in many cases blooms con be underestimated, if not correctly monitored (Coulson et al., 1968; Armstrong et al., 1978; Anderson and White, 1992; Fritz et al., 1992; Anderson, 1994b).

2.4. MARINE MAMMALS

Various episodes, reported since ‘80s, witness for toxicity of phycotoxins to marine mammals and their transfer along food chains. Whales die-off was observed in USA following an *Alexandrium tamarense* bloom, transfer agent being mackerel who fed on the dinoflagellates and who accumulated saxitoxins in liver and kidney. Interestingly, no toxin was found in muscle of contaminated fish. Some concern exist regarding saxitoxins as real causative agent of the die-off, as the levels found were below the toxic threshold defined for humans. Anyway, it has been thought that chronic exposure to lower doses could lead to accumulation of toxic levels (Geraci et al., 1989; Anderson, 1994a; Anderson. 1994b).

Brevetoxins have been considered responsible for a die-off of dolphin in USA during 1987–88. Two possible way of absorption have been considered: 1) intoxication following toxic aerosol inhalation; 2) continuous absorption of low levels of toxin accumulated by natural preys of dolphins (menhadens and mackerels). Absorbed doses were considered as non toxic per se, but they probably caused a defedation of animals, which then experienced bacterial or viral secondary infections (Gerlach, 1989; Anderson and White, 1992; Van Dolah et al., 2003).

The toxin was also responsible for some important die-off of manatees following *Gymnodium breve* blooms. Again, contaminated aerosol inhalation or toxin ingestion have been considered as death cause. In a case reported in 1982, tunicates were considered as transfer organisms, while in a second case, dating 1996, little or no tunicates were found in gastric content of manatees (Freitas et al., 1996; Bossart et al., 1998; Landsberg and Steidinger, 1998).

Domoic acid was responsible of sea lions intoxication in California in 1998, anchovies being the transfer organisms. Anchovies contained the highest amounts of toxin in their internal organs, while sea lions had highest levels in faeces (Lefebvre et al., 1999; Scholin et al., 2000).

Not all marine mammals experience accidental or passive intoxication by phycotoxins. Indeed, it has been observed that sea otter seems to be able to distinguish between contaminated and non contaminated parts of preys, as they discard flesh and siphon of contaminated animals. The observation that sea otters are absent in areas containing saxitoxins at level higher than toxic threshold seems to confirm this hypothesis (Kvitek et al., 1991; Patyten, 1999).

3. Diarrhetic Shellfish Poisoning

Diarrhetic toxins include various toxins: dynophisistoxins, whose principal compound is **okadaic acid** (OA), which is responsible for the syndrome, **pectenotoxins** (PTX) and **yessotoxins** (YTX).

This group is an example of a set of molecules grouped together because of their physico-chemical characteristics, even if biological effects are completely different. Due to these toxicological differences, it is now considered the possibility of modifying their classification, using toxicological criteria (Amzil et al., 2001).

DSP is a relatively recent discovered syndrome, but it is considered that it should exist since long time: gastroenteric symptoms indeed could have lead to the attribution of the syndrome to bacterial or viral infections, leading to ad underestimation of its incidence.

Poisoning follows ingestion of mussels containing *Dynophisis* spp. and *Prorocentrum* spp.; it has been observed that very low *Dynophisis* concentrations (50 cells/L) could lead to mussels contamination and toxicity (Kat, 1983; Marcaillou-Le Baut et al., 2001) (Fig. 5).

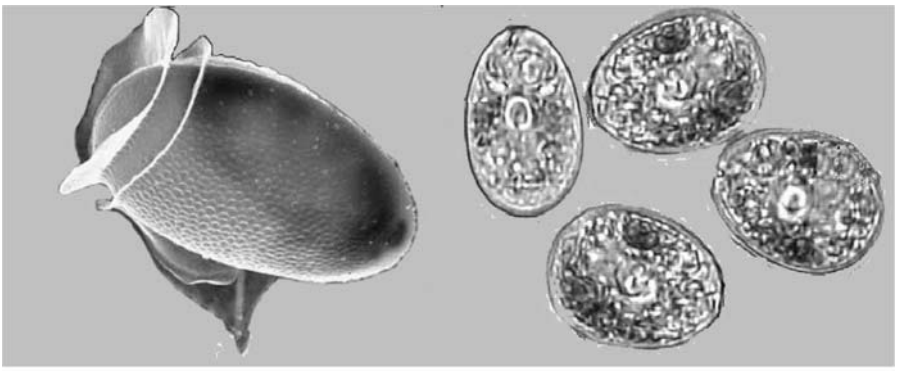


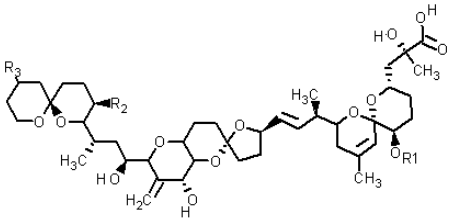
Figure 5. Diarrhetic toxins producing organisms (A *Dynophisis* spp. and B *Prorocentrum* spp).

3.1. CHEMICAL STRUCTURE

3.1.1. *Okadaic acid*

Okadaic acid is a liposoluble cyclic polyether with a carboxylic function (Fig. 6). Various derivatives exists of OA, which originate by modification of OA structure: DTX1 is a methyl derivatives, and DTX2 is an isomer of OA (Draisci et al., 1996; Quilliam, 1998; Van Egmond et al., 2004).

Some more toxins have been identified, originating from the acylation of the okadaic acid molecule, leading to the formation of DTX3, named, acyl-ester. These acyl-esters probably are metabolic derivatives, as they are only found in mussels' digestive gland (Hu et al., 1995c; Windust et al., 1997; Barbier et al., 1999).



Okadaic acid (OA)	R1=H	R2=Me	R3=H
dinophysistoxin 1 (DTX 1)	R1=H	R2=Me	R3=Me
dinophysistoxin 2 (DTX 2)	R1=H	R2=H	R3=Me
dinophysistoxin 3 (DTX 3)	R1=acyl	R2=Me	R3=Me

Figure 6. Okadaic acid and its derivatives molecular structure.

3.1.2. *Yessotoxins*

These toxins are sulphated polyether compounds; two main molecules have been identified; yessotoxin and 45-hydroxy-yessotoxin.

YTX is main toxin in Adriatic Sea mussels, other homologues were identified: homo-YTX, 45-hydroxyhomo-YTX, carboxy-YTX and adriatoxin (Lee et al., 1989; Ciminiello et al., 1997; Satake et al., 1997a; Satake et al., 1997b; Ciminiello et al., 1998; Tubaro et al., 1998; Yasumoto and Satake, 1998; Ciminiello et al., 2000) (Fig. 7).

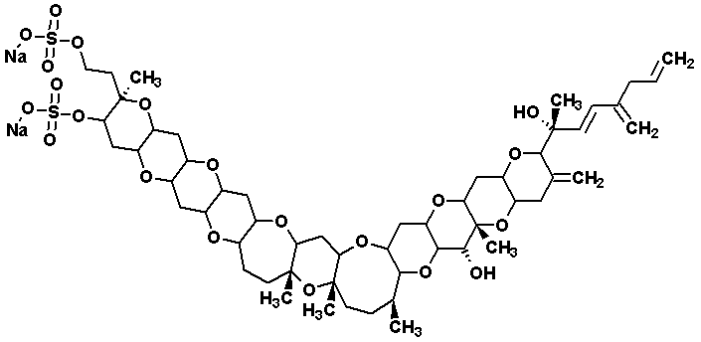


Figure 7. Yessotoxins chemical structure.

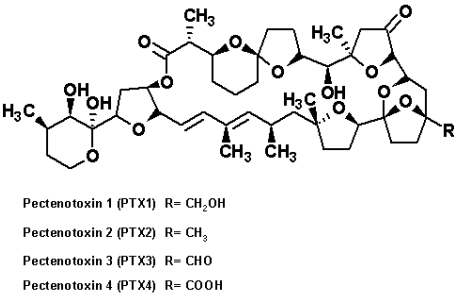


Figure 8. Chemical structure of pectenotoxins.

3.1.3. *Pectenotoxin*

Pectenotoxins (PTXs) are a group of cyclic polyether macrolide sharing the same basic structure. Actually, eight different PTXs (PTX1 to 7 and PTX10) and two new derivatives of PTX2 (PTX2 seco-acid and 7-epi-PTX2 seco-acid) have been described and characterized mainly in shellfish. PTX2 is suspected to be the precursor toxin of the whole PTXs through biotransformation processes which take place in the digestive glands of bivalves

(Lee et al., 1989; Yasumoto et al., 1989; Draisci et al., 1996; Suzuki et al., 1998; Suzuki et al., 2001) (Fig. 8).

3.2. MECHANISM OF ACTION

3.2.1. *Okadaic acid*

Okadaic acid is an inhibitor of protein phosphatases (PP), which induce dephosphorylation of proteins by protein kinases (PK). The accumulation of phosphorilated proteins lead to tumor promotion and contraction of smooth muscles. This last effect is responsible for diarrhea and abdominal pain which are among principal symptoms (Puiseaux-Dao et al., 2001).

It has been observed that only some PP are inhibitied, namely serine/ threonin PPs 1, 2A, 4, 5 and 6, while it seems that the different conformation of PP 2B and 7 makes them particularly resistant: the binding to these PP is probably partially obstructed by the catalytic part of the.

For the non-competitive interaction of OA with PP chemical structure maintenance is mandatory, as loss of carboxyl group, esterification or reduction to okadaol make the toxin no more toxic enzyme (Bialojan and Takai, 1988; Walter and Mumby, 1993; Honkanen et al., 1994; Takai et al., 1995; Dawson and Holmes, 1999).

Binding to catalytic unit is reversible, but break of the bound is very slow.

Hyperphosphorilation induced by okadaic acid occurs in any kind of cell and targets not only serine and threonin, but also tyrosine. Proteins affected are those of cytoskeleton, those involved in signal transduction, transcription and in gene expression (Afshari, 1994; Sawa et al., 1999; Puiseaux-Dao et al., 2001).

Okadaic acid can also alter cell morphology, induce apoptosis and cell death and modify cell physiology: alteration of ions current across membrane, of glucose balance, of resorption of glucocorticoids receptors, increase in T3 secretion (Shibata et al., 1982; Hescheler et al., 1988; Haystead et al., 1989; Ozaki and Haraki, 1989; Mironov and Lux, 1991; Chiavaroli et al., 1992; Neumann et al., 1993; Wang et al., 1993; Arufe et al., 1999; Galigniana et al., 1999).

Diarrhea, one of the main symptoms of DSP, is due to hyperphosphorilation of intestinal epithelia, with the loss of intestinal structure and of *villi*; this expose superior part of intestinal crypt cells and produce an important loss of water (Edebo et al., 1988; Lange et al., 1990; Yuasa et al., 1994; Tripuraneni et al., 1997; Puiseaux-Dao et al., 2001).

3.2.2. *Yessotoxins*

Mechanism of action of these toxins is not known. Toxicological studies have shown that YTXs do not induce sodium channel activation, while and increase in intracellular calcium following intra-cytoplasmatic reserves depletion and extra-cellular calcium use. This effect, coupled with cardiac lesions linked to degeneration of capillary endothelial cells, mitochondria and swelling of cardiac cells, make these toxins more similar to maitotoxins than to domoic acid (Aune, 1989; Alfonso et al., 2000; Puiseaux-Dao et al., 2001).

3.2.3. *Pectenotoxin*

PTXs do not inhibit protein phosphatases nor induce diarrhea in mammals. Most toxicological data available on PTXs (both *in vivo* and *in vitro*) have been obtained with PTX1, showing liver damage following intraperitoneal injection in mice and morphological changes in freshly prepared hepatocytes. Highest lethality for PTX2 with respect to all other PTXs further supports the hypothesis of PTX2 as the parental compound of PTX group. Thus, successive oxidation of substituent in C18 in the digestive glands of bivalves would diminish the toxicity of PTXs. PTX2 has been proven to induce lethality of brine shrimp (*Artemia salina*), as well as cytotoxic activity against several human cell lines, although significant differences were observed in the relative LC_{50} values obtained for each of them (Terao et al., 1986; Sasaki et al., 1998; Suzuki et al., 1998; Hori et al., 1999; Eaglesham et al., 2000).

Apoptosis induction by PTXs has also been proved. Primary cultures of rat and salmon hepatocytes exposed to PTX1 in the micromolar range showed rapid apoptotic changes, but no further studies concerning apoptotic activity of PTXs have been carried out in human cells. No additional data are available on acute and chronic effects of PTXs, and the exact mechanism of action of these toxins is currently unknown.

3.3. SYMPTOMS AND TREATMENT IN HUMANS

3.3.1. *Okadaic acid*

Symptoms of DSP appear within 4 hours after ingestion of contaminated mussels and include vomiting, diarrhea, abdominal pain. More rarely neurological appears.

Recovery is complete within 3 days and there seems to be no long term effect and no deadly episodes (Marcaillou-Le Baut et al., 2001).

3.3.2. *Yessotoxins*

Yessotoxins have not been associated with human poisoning, but only in animals (Marcaillou-Le Baut et al., 2001).

3.3.3. *Pectenotoxins*

Pectenotoxins poisoning have been reported in human since 1997. Symptoms registered are nausea, vomiting and diarrhea. Treatment of toxicosis is similar to that of DSP (Marcaillou-Le Baut et al., 2001).

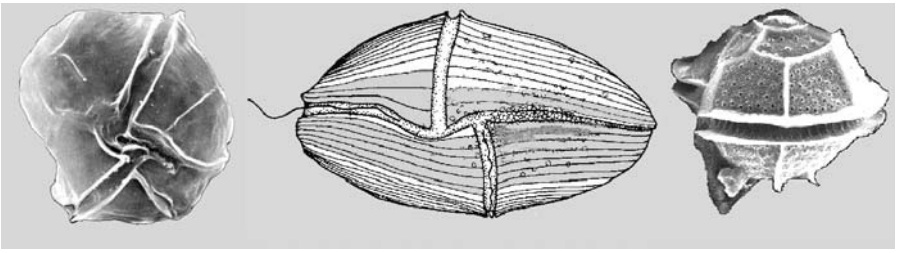
4. Paralytic Shellfish Poisoning (PSP)

This syndrome is caused by **saxitoxins** (STX), a group of toxins including about 20 different molecules.

STX was one of the first marine toxins recognized as responsible for human intoxications, the first report dating up to 1798, even if PSP symptoms were attributed to saxitoxins only after 1920.

Saxitoxins are responsible for about 2000 human cases/year, with a mortality rate ranging from 15 to 50% (van Dolah, 2000; Marcaillou-Le Baut et al., 2001).

The name of the toxin comes from the mollusk in which it was firstly identified, *Saxidomus giganteus*. It is produced by both temperate and tropical dinoflagellates of the genera *Alexandrium*, *Gymnodium* and *Pyrodinium* (Fig. 9).



A B C

Figure 9. Saxitoxins producing organisms A. *Alexandrium* sp., B. *Gymnodium* sp C. *Pyrodinium sp*.

This is one of the few toxins which are produced by both marine and fresh water (cyanobacteria) organisms, even if no report of intoxication exists for fresh water sources.

The major transvector for the toxins are bivalve mollusks, even if also crabs and snails feeding on coral reef seaweeds seem to be able to accumulate them (Fig. 4).

4.1. CHEMICAL STRUCTURE

Saxitoxins are tricyclic, substituted alkali, hydro-soluble, thermo-stable and resistant to acidic environment (Fig. 10). Alkaline pH or oxidizing compounds can inactivate the toxins, which are all derivatives of two basic molecules, saxitoxin and neosaxitoxin, which undergo sulphatation at different sites of their molecules; the chemical characteristics of these molecules are resumed in Table 1 (Amzil et al., 2001).

At physiological pH, to functional groups, the 1,2,3- and the 7,8,9-guanidinic group, present a positive charge, which give the molecules their water solubility characteristics.

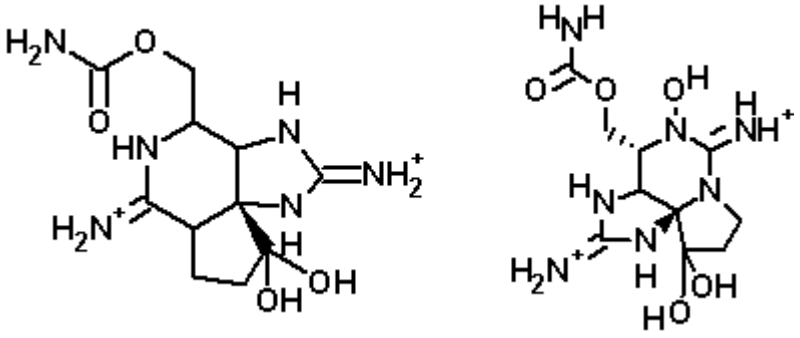


Figure 10. Saxitoxins and neosaxitoxins molecular structure.

Saxitoxins are grouped in 3 classes, based on their toxicity:

- Carbamates derivatives: saxitoxin, neosaxitoxin, GTX1-GTX4;
- N-sulphocarbamoyl derivatives: B1, B2, C1-C4;
- Decarbamoyl derivatives (dc-derivatives)

Decarbamoyl derivatives have an intermediate toxicity between carbamate (highly toxic) and N-sulphocarbamoyl compounds.

TABLE 1. Structure of saxitoxins (from Amzil et al. (2001), modified).

R1	R2	R3	R4	Carbamates toxins	N-sulphocarbamoyl toxins	Decarbamoyl toxins
H	H	H		STX	B1	dcSTX
H	H	OSO3		GTX2	C1	dcGTX2
H	OSO_3	H		GTX3	C2	dcGTX3
OH	H	H		NEO	B2(GTX6)	dcNEO
OH	H	OSO3		GTX1	C3	dcGTX1
OH	OSO_3	H		GTX4	C4	dcGTX4

4.2. MECHANISM OF ACTION

Being polar molecules, STX can not cross blood-brain barrier and thus they affect peripheral nervous system by targeting voltage-dependent sodium channels, which regulate action potential propagation along neurons (Fig. 11). Indeed, these trans-membrane proteins, responsive to membrane potential, control ions movement across the membrane of nervous cells (Hines et al., 1993; Gessner et al., 1997; Andrinolo et al., 1999).

Sodium channel is composed by 3 sub-unit, α, β-1 and β-2, α sub-unit presenting most of the functional properties.

Sub-unit α is composed by 4 repeated domains, numbered I to IV, eah containing 6 membrane-spanning regions, labeled S1 to S6. S4 is a highly conserved region acting as voltage sensor. When transmembrane voltage stimulates S4, it moves towards the extracellular side of the membrane, opening the channel. Another important part of the channel is the amino acidic sequence connecting domains III and IV, which is responsible for channel inactivation (closure) after prolonged activation (West et al., 1992; Catterall, 2000; Goldin et al., 2000; Yu and Catterall, 2004).

Saxitoxins binds to site 1 of the channel, an amino acid sequence negatively charged placed in the external part of the membrane connecting S5 and S6. Specific amino acids have been identified as responsible of the binding of the toxin to the channel. This binding cause a complete block of the sodium channel preventing the ions from passing into the neurons. The block of inward flow of sodium impedes the release of neurotransmitters at the synaptic level, and this causes paralysis of muscle cells (Evans, 1972; Catterall et al., 1986; Kao, 1986; Puiseaux-Dao et al., 2001).

A first hypothesis considered the toxin acting like a stopper on the pore. More recent studies anyway have clarified that there in no direct action on the pore; the toxin binds to an external site to the channel, close but not

inside the pore (Fig. 12). The interaction of only part of the toxin molecule and/or an alteration of the channel structure induced by the toxin are considered more probable (Puiseaux-Dao et al., 2001).

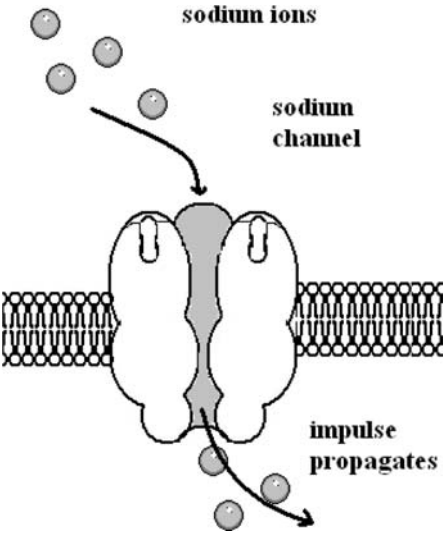


Figure 11. Sodium channel functioning.

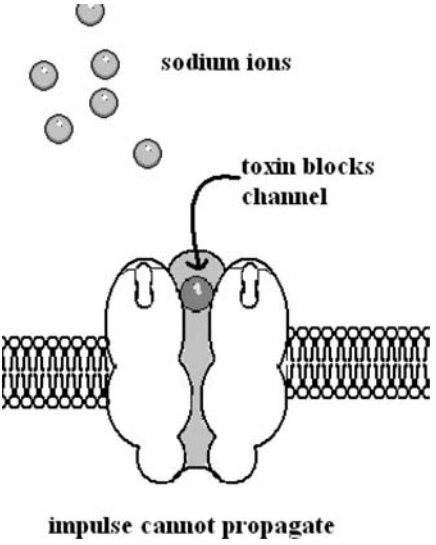


Figure 12. Saxitoxins mechanism of action of sodium channel.

4.3. SYMPTOMS AND TREATMENT IN HUMANS

PSP, s the oldest known intoxication and one of the most dangerous for humans, with a high rate of mortality.

Native populations of Canada perfectly knew the existence of the toxin and prohibited consumption of mussels coming from contaminated areas, which were considered as a food taboo (Marcaillou-Le Baut et al., 2001).

It is a worldwide distributed poisoning, with cases reported for North and South America, Europe, Africa and Asia. It is commonly thought that it is indeed more probable that the toxin or the dinoflagellates have not been detected or searched for.

Symptoms observed during PSP poisoning are characteristic, easy to recognized and impossible to be confused with allergy and viral or bacterial pathologies.

First symptoms appear 5 to 30 minutes after ingestion of mussels and develop following a precise sequence in few hours. The severity of signs depends on the dose ingested and on individual sensitivity.

Usually, recovery is complete in few days, even if in more severe intoxication death can occur following respiratory paralysis. Symptoms have been classified following the severity of intoxication, and are resumed in Table 2.

Even if it is a well known toxin, no antidote has been found for its treatment.

Most efficient treatment is a symptomatic one, including gastric lavage and active charcoal or alkaline dinks administration, which favor the inactivation of the toxins and their elimination with urine. Indeed, their clearance via kidney is rapid, close to 24 hours (Hines et al., 1993; Gessner et al., 1997; Andrinolo et al., 1999).

Forced ventilation is useful in more severe intoxications, when respiratory paralysis occurs, as it can counteract paralysis.

4.4. TOXICOSES IN ANIMALS

Saxitoxins have been implicated in a mass mortality episode of humpback whales which occurred during late 1987-beginning of 1988 in Massachusetts. During an *A. tamarense* bloom, whales were forced to feed on mackerels, as their natural preys, sand lance, was largely absent from the affected area. Mackerels fed on *A. tamarense* and were found to contain a mean concentration of 80 μg/100 g tissue, which were deadly toxic for whales. Indeed, short after feeding on fish, humpback whales were found

dead, without any sign of emaciation (blubber was abundant) or starvation (stomachs contained digested fish). Estimated dos absorbed by whales was 3.2 μg/kg b.w., well below toxic threshold defined for humans. Geraci et al. (1989) consider two possible mechanisms as responsible for apparent higher sensitivity of cetaceans to saxitoxins: 1) approximately 30% of the whales body weight is blubber, into which the water-soluble STXs would not partition, thus being more highly concentrated in metabolically sensitive tissues; 2) the diving physiology of whales concentrates blood to the heart and brain and away from those organs required for detoxification, further concentrating neurotoxins in sensitive tissues (Ridgway and McCormick, 1971; Geraci et al., 1989; Haya et al., 1989; Levin, 1992; Haulena and Heath, 2001; Van Dolah et al., 2003).

TABLE 2. PSP signs classification.

Severity of intoxication	Symptoms
Mild	Mouth paresthesia which can expand to the whole face and neck, to fingers and ears. Nausea, headache, vomiting
Severe	Paresthesia expand to arms and legs. General sensation of numbness, muscular weakness and floating sensation. Altered speech, dysarthria, severe ataxia, motory incoordination. Some respiratory problem appears.
Extreme	Peripheric paralysis, including respiratory paralysis which can lead to death if not rapidly trated.

Another important episode, which had a great importance also from the conservation point of view, is the one affecting a monk seal (*Monachus monachus*) population in Mauritania during 1997. Close to 70% of the total population of monk seals died following an *A. minutum*, *G. catenatum* and *D. acuta* bloom. Affected seals showed lethargy, motor incoordination, paralysis, symptoms who could be ascribed to STX intoxication. Lungs of dead animals showed severe respiratory distress and congestion, and viscera of the animals contained up to 12 μg/100 g liver and 3 μg/100 g brain of decarbamoyl saxitoxins. Again, observed levels were below the toxic threshold for humans, but a higher sensitivity should be considered also in this species (Osterhaus et al., 1997; Hernandez et al., 1998; Osterhaus et al., 1998; Forcada et al., 1999).

Long term effect of STX were also considered, as toxin presence con alter the distribution of predator species, as hypothesized for sea otters (*Enhydra lutris*).

Alaskan populations of sea otters, consuming 20–30% of their body weight as bivalves, avoid eating butter clams during *Alexandrium* blooms. Butter clams can highly accumulate STX in the siphon and can retain it for more than 1 year, probably as a defensive system against predation.

Sea otters seems capable of distinguish between toxic and non-toxic clams and areas, and feed only on healthy mussels in safe areas. This hypothesis was confirmed by a feeding study on caged sea otters. If animals were fed with contaminated clams, they reduced the rate of consumption and selectively discarded most of toxic tissues, i.e. siphons and kidney, thus reducing the risk of intoxication (Kvitek and Beitler, 1991; Kvitek et al., (1991).

Seabirds were also affected by saxitoxins: Nisbet (1983) reported of a massive die-off of common terns in Massachusetts which occurred in 1978. Transfer species was found to be sand-launce, the terns' principal food. Interestingly, it was observed that almost all tens that died were pre-laying females, while all other animals recovered after vomiting. No breeding alteration was observed, while an apparent age-dependent sensitivity was observed, as highest mortality was for 3 years-old females.

5. Neurotoxic Shellfish Poisoning

Neurotoxic Shellfish Poisoning is a little common intoxication, which has not been documented as a fatal intoxication in humans, and results from consumption of molluscan shellfish contaminated with **brevetoxins**. Interestingly, intoxications have been observed not only as a consequence of mussels ingestion, but also after inhalation of contaminated aerosol. Its toxicological importance is more related to massive fish death.

The producing organism is *Gymnodium breve*, which differs from other dinoflagellates because it is an unarmored dinoflagellates; the lack of an external shell make this microalga easily lysed in turbulent waters (Fig. 13). The lysis allows the toxin to be released IN water, making aerosol and droplets potentially toxics (Amzil et al., 2001).

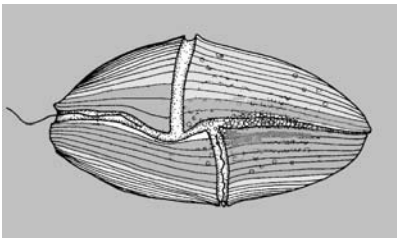


Figure 13. Gymnodium breve.

5.1. CHEMICAL STRUCTURE

Brevetoxins (PbTX) are liposoluble, ladder-like polycyclic ether toxins, classified into two types based on their backbone structure: PbTXB and PbTXA, counting up to 12 different molecules (McFarren et al., 1965; Lin et al., 1981) (Fig. 14).

The two families are composed by molecules counting 10 or 11 cycles. Some of these compounds are thought to be produced in mussels by metabolic processes (Table 3).

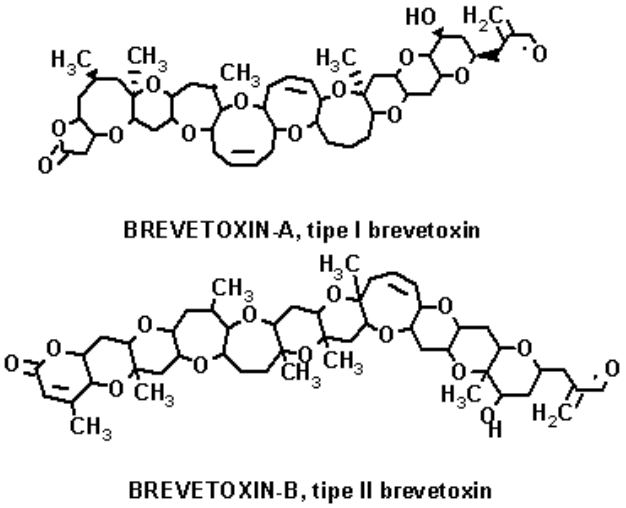


Figure 14. Molecular structure of PbTXA and B.

TABLE 3. Different molecular structure of type A and type B brevetoxins derivative.

Type A	R	Type B	R
Brevetoxins-1	$CH_2C(=CH_2)CHO$	Brevetoxins-2	$CH_2C(=CH_2)CHO$
Brevetoxins-7	$CH_2C(=CH_2)CH_2OH$	Brevetoxins-3	$CH_2C(=CH_2)CH_2OH$
Brevetoxins-10	$CH_2C(CH_3)CH_2OH$	Brevetoxins-9	$CH_2C(CH_3)CH_2OH$
		Brevetoxin-8	CH_2COCH_2Cl

5.2. MECHANISM OF ACTION

PbTX acts by binding with high affinity to voltage-dependent sodium channel. Brevetoxins bind to site 5 of the channel (for sodium channel description see PSP paragraph), altering bio-physic properties of the channel itself (Bidard et al., 1984; Poli et al., 1986; Lombet et al., 1987; Baden, 1989; Dechraoui et al., 1999; Puiseaux-Dao et al., 2001).

They induce and increase in membrane sodium permeability and a shift of activation potential toward more negative values.

It has been supposed that this action results from the intercalation of brevetoxins backbone (whose length is similar to that of membrane lipidic double layer) between transmembrane domains of nervous and muscular cells, provoking spontaneous and/or repeated discharges which appears at very low action potential levels. Discharges are generally followed by a block of excitation, due to important and prolonged membrane depolarization (Huang et al., 1984; Atchison et al., 1986; Sheridan and Adler, 1989; Gawley et al., 1995; Jeglitsch et al., 1998).

Hyperexcitation lead to an initial increase in neurotransmitters secretion, followed by a decrease and an inhibition due to overstimulation, Ca^{2+} being not essential for the release: it has been observed that Na^{+} alone can activate neurotransmitters liberation (Molgo et al., 1990; Molgo et al., 1991; Molgo et al., 1993; Meunier et al., 1997).

Finally, an increase in cell volume was observed. Two different mechanism have been considered:

6. In mielinic nervous fibers sodium ions entrance alters osmotic balance, causing water entrance into the cell and swallow of cell itself. A 50% hyperosmotic external solution can counteract this increase in volume.
7. In motor neurons terminal synaptic vesicles fuse with terminal axon body, allowing vesicular membrane incorporation after neurotransmitter release induced by the toxin. Hyperosmotic solutions only partially counteract this effect (Puiseaux-Dao et al., 2001).

5.3. SYMPTOMS AND TREATMENT IN HUMANS

Two different poisoning have been identified in humans: “indirect” intoxication by ingestion of contaminated mussels and poisoning for direct contact.

In the first one symptoms are both neurological and gastro-intestinal and appear within 1–3 hours after mussels ingestion.

Neurological syndrome includes paresthesia of area around the mouth, the face and throat, muscular ache, ataxia, inversion of thermal perception, bradycardia, midriasis.

Gastro-intestinal syndrome includes abdominal pain, nausea, diarrhea.

Recovery is complete within 24 and 48 hours and no fatality has ever been recorded.

It has been observed that local anaesthetics, calcium administration and hyperosmotic solutions are useful in poisoning treatment, as they counteract brevetoxins action on membrane.

Following direct contact with contaminated aerosol, respiratory tract inflammation (cough, burning sensation) and conjunctivitis appear (Pierce, 1986; Morris, 1991; Marcaillou-Le Baut et al., 2001).

5.4. TOXICOSES IN ANIMALS

Brevetoxins were considered as responsible agent in many poisoning of cetaceans, like manatees and bottlenose dolphin but also of sea turtles (Landsberg and Steidinger, 1998; Van Dolah et al., 2003).

Die-off on manatees have been linked to NSP since 1965 in Florida, but various episodes have occurred in following years (1982 and 1996) in the same area. Timing of mortality events coincided with the presence of *K brevis* blooms and was often associated with fish and seabirds die-off.

Affected animals showed disorientation, inability to submerge or to maintain horizontal position, listlessness, flexing of the back, lip flaring and labored breathing. The only histological lesions observed were cerebral ones, while no other lesion was observed. The analysis of stomachs content showed a high amount of seagrasses and filter feeding tunicates; no measurable PbTX levels were found in tunicates (Layne, 1965; O'Shea et al., 1991).

In 1996 histopathological analysis of tissues showed consistent, severe congestion of nasopharyngeal tissues, bronchi, lungs, kidney and brain; hemorrhage of lungs, liver, kidney and brain were observed, whereas gastrointestinal tract showed no lesions. The presence of PbTX in lymphocytes and macrophages of affected tissues support the hypothesis that toxic effects in manatees is not due to acute neurotoxic effects alone, but rather may have resulted from chronic inhalation (Bossart et al., 1998).

First report of dolphin die-off due to NSP dates up to 1947, in Florida. Brevetoxins were also proposed as a causative agent in an unprecedented mortality of over 740 bottlenose dolphins in 1987 in New Jersey. Most

of stranded dolphins showed a wide range of pathological signs, involving chronic physiological stress, including fibrosis of the liver and lung, adhesion of abdominal and thoracic viscera, and secondary fungal and microbial infections associated with immune suppression (Gunter et al., 1948; Geraci, 1989; Tester et al., 1991; Mase et al., 2000).

Another bottlenose dolphin poisoning occurred in 2000, involving 120 animals, in Florida, coinciding with episodic peak of *K. brevis*. Stranded animals were in good physical condition, but histopathological examination showed significant upper respiratory tract lesions, with lymphoplasmacytic oropharyngitis and tracheitis, as well as lymphoplasmacytic interstitial pneumonia and lymphoid tissue depletion. PbTX was not found in spleen or lung, differently from what observed in manatees, whereas it was found in stomach content and liver. In these two organs PbTX3 was found; considering that PbTX2 is main product of K. brevis, it is thought that it is metabolized in the fish or that this metabolite is selectively retained by dolphins.

At present no lethal dose for dolphins and marine mammals has been determined, nor an acute or chronic adverse effect level (Van Dolah et al., 2003).

6. Amnesic Shellfish Poisoning (ASP)

Amnesic Shellfish Poisoning is a singular intoxication, as it is the only one not caused by dinoflagellates, but by a diatom, namely *Pseudo-nitzschia pungens*, which produce **domoic acid**. It is one of the deadly poisoning, even if fatalities are more rare than with STX and tetrodotoxin (Fig. 15).

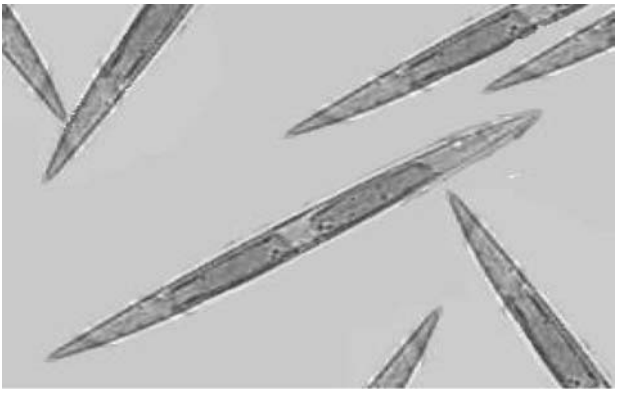


Figure 15. Domoic acid producing *Pseudo-nitzschia pungens*.

6.1. CHEMICAL STRUCTURE

Domoic acid (DA) is a tricarboxylic amino acid which was firstly isolated by red alga *Chondria armata domoi*. Side chain of the molecule contains two ethylenic bounds (Takemoto and Daigo, 1958; Amzil et al., 2001) (Fig. 16).

Domoic acid is thermo-stable, water soluble and instable in acidic environment; it has been shown that temperatures higher than 50°C (cooking temperatures) can transform domoic acid in epidomoic acid, which is the real responsible, together with isodomoic acid D, E and F, of ASP (Fig. 16). Anyway, several congeners of DA have been identified (Amzil et al., 2001).

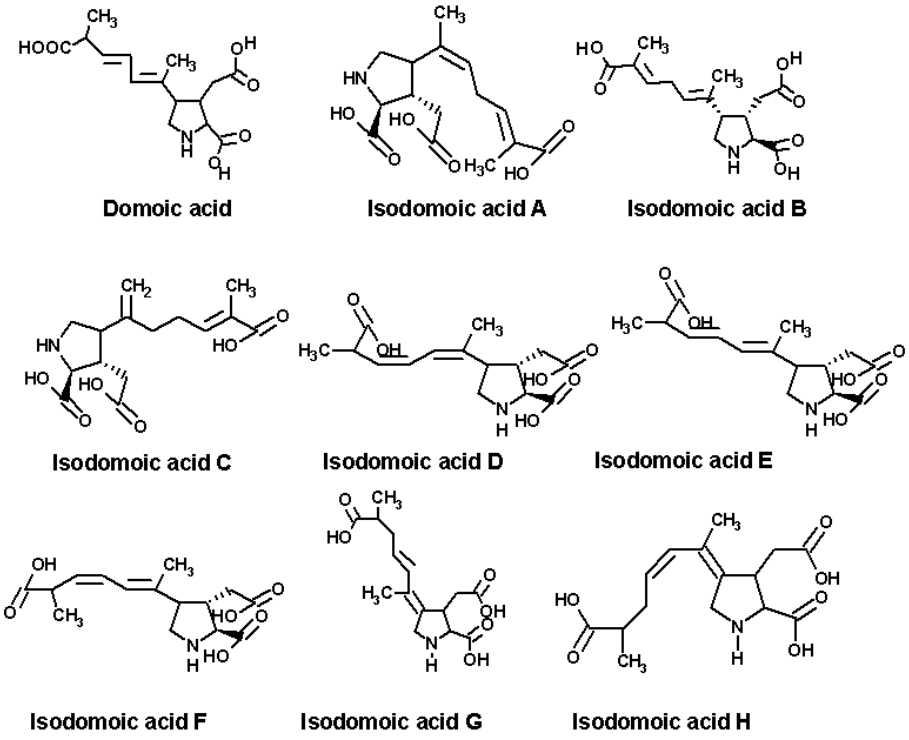


Figure 16. Chemical structure of domoic acid and its derivatives.

Chemical structure of domoic acid is similar of that of the endogenous neurotransmitter glutamate and of the excitatory neurotoxin kainic acid; this similarity is responsible for the mechanism of action of the toxin.

6.2. MECHANISM OF ACTION

Domoic acid acts at the central nervous system level. It is absorbed by gastrointestinal tract and slowly reaches CNS. As already said, it shares chemical similarity with glutamate and kainic acid, and its mechanism of action is based on binding to kainate and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtypes of glutamate receptors, while it does not interact with N-methyl-D-aspartate (NMDA) receptors (Debonnel et al., 1989).

Interestingly, the binding affinity of domoic acid to kainate receptor is higher than that of endogenous agonists (domoic acid>glutamate>AMPA).

Main difference among domoic and kainate acid and glutamate is the fact that while the action of glutamate rapidly disappears, avoiding over-stimulation of nervous cells, desensitation of receptors following exogenous agonist binding is negligible or absent, and when it occurs it is very slowly, that causing a continuous stimulation of the nervous cells (Puiseaux-Dao et al., 2001).

The interaction with the receptor lead to the opening of voltage-dependent calcium channels, allowing entrance of Ca^{2+} in the cell, as well as of other ions, i.e. Na^{+}.

The toxic effect resulting from this influx is at first an increase in nervous cells excitation. This effect is due to the disinhibition of some neural circuits, as domoic acid can inhibit GABA (inhibiting mediator) liberation in hippocampal areas through the activation of protein kinase (PK) C. Subsequently, high levels of calcium cause cell death and lesion in various cerebral areas, especially in areas where glutaminergic pathways are heavily concentrated. These receptors are preferentially distributed in CA1 and CA3 areas of hippocampus, which are responsible for learning and memory. These lesions can be responsible of main effect of domoic acid, complete and permanent loss of short-term memory. Anyway, it has been observed tat memory deficits occur at levels well below those causing structural damage (Cunha et al., 2000; Quintela et al., 2000; Puiseaux-Dao et al., 2001).

In vivo and in vitro studies have shown that DA activates AMPA/kainate receptors in striatum system, which cause release of excitatory amino acids activating NMDA receptors, finally leading to cell death (Larm et al., 1997; Puiseaux-Dao et al., 2001).

6.3. SYMPTOMS AND TREATMENT IN HUMANS

Diatoms were recognized as causative agents of ASP only in recent times, as they were not considered as an hazard for human health.

In 1987, over 100 people showed poisoning sings which could not be ascribed to any of known syndromes. Following researches made it possible to identify DA as active principle and *P. pungens* as producing organism. Interestingly, both two were already known, as domoic acid was used in Japan as vermifuge and the diatom was known, but no correlation among the two was ever seen before.

Poisoning symptoms appears rapidly, from 15 minute to 38 hours from mussels ingestion. After close to two days, some neurological alteration appears, presenting a different degree of severity.

Symptoms include gastrointestinal signs, e.g. nausea (77% of cases), vomiting (76%), diarrhea (42%), abdominal pain (51%), and neurological signs: dizziness, disorientation, lethargy, seizures, permanent loss of short-term memory.

Recovery occurs in a period ranging from one day to 4 months.

In 1987 outbreak, 4 out of the 100 people affected died after seizures appeared.

The analysis of brain of dead people revealed necrotic lesions and/or neuronal loss mainly at the hippocampal and amigdala areas, confirming the toxic effect of DA.

At present, no antidote exist for the treatment of poisoning, and all cares are symptomatic (Marcaillou-Le Baut et al., 2001).

6.4. TOXICOSES IN ANIMALS

Domoic acid has been identified as causative agent in pelicans and cormorants mass mortality in California in 1991 and in various and extensive die-offs of sea lions in the same region in 1998, 2000, 2006 and 2007.

Affected birds exhibited neurological symptoms similar to those reported in experimental animals, i.e. scratching and head weaving. In all instances the vector for toxins transfer was anchovy, but the toxin producing organism was a different member of *Pseuda-nitzschia* genus. At present, more than seven species are recognized as domoic acid producers (Work et al., 1993).

The first confirmed domoic acid poisoning in marine mammals occurred in sea lions in California in 1998. All animals were in good nutritional condition and displayed clinical symptoms, predominantly neurological: head weaving, scratching, tremors and convulsions. Affected animals were mainly adult females, 50% of them pregnant. Abortion was observed and

some pups born during the episode died. Highest levels of DA was found in urine and feces. Sea lions which died within 24 hours of stranding presented histologic lesions of brain, mainly neuronal necrosis, more severe in hippocampus and dentate gyrus. Heart was also affected, presenting myofiber necrosis and edema. Another similar episode occurred in 2000. In the same area and the same periods a die-off of sea otters was observed as well (Lefebvre et al., 1999; Gulland, 2000; Scholin et al., 2000; Bargu et al., 2002; Silvagni et al., 2005; California Wildlife Center, 2006).

Exposure to domoic acid has been proved also for whales, even if no clear toxic event was reported in these species. Lefebvre et al., 2002) found domoic acid in faeces and food (krill, anchovies and sardines) of whales.

7. Ciguatera Fish Poisoning (CFP)

Ciguatera Fish Poisoning is a well known poisoning linked to fish consumption, which was firstly described in 1555 by sailors in Caribbean areas. In 1866 Poey defined this intoxication "ciguatera" from the Cuban common name of a mussel known to cause the intoxication, "cigua" (Marcaillou-Le Baut et al., 2001; Puiseaux-Dao et al., 2001).

At present, the term ciguatera is used to describe both the poisoning and the phenomena affecting marine environment and leading to the poisoning itself, namely coral reef degradation.

Producing organism was found to be *Gambierdiscus toxicus* which produce **ciguatoxins** (CTX) (Fig. 17). Other toxins are included in the group of ciguatoxins, namely **maitotoxins** (MTX), which anyway have never been linked to toxic episodes in humans. Like ciguatoxins, maitotoxins are produced by *G. toxicus*.

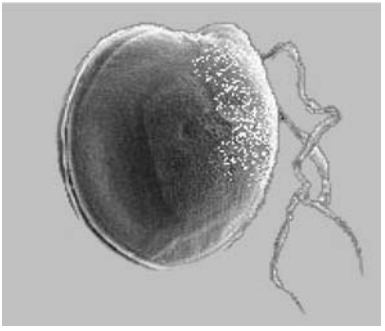


Figure 17. Gambierdiscus toxicus.

7.1. CHEMICAL STRUCTURE

7.1.1. *Ciguatoxins*

Ciguatoxins are highly stable toxins, as cooking, freezing and salting do not reduce their toxicity. They are soluble in organic polar solvents and in water (Fig. 18).

The study of molecular structure of the toxins lead to the identification of two different toxins, one from the Pacific area (P-CTX1) and one from the Caribbean (C-CTX1), which differ for the number of C in the molecules (60 in P-CTX1 and 62 in C-CTX1) and stability in acidic environment: P-CTX1 is acid labile, while C-CTX1 is stable in acidic pH (Vernoux, 1988) (Table 4).

Both two groups of toxins belong to polycyclic polyethers.

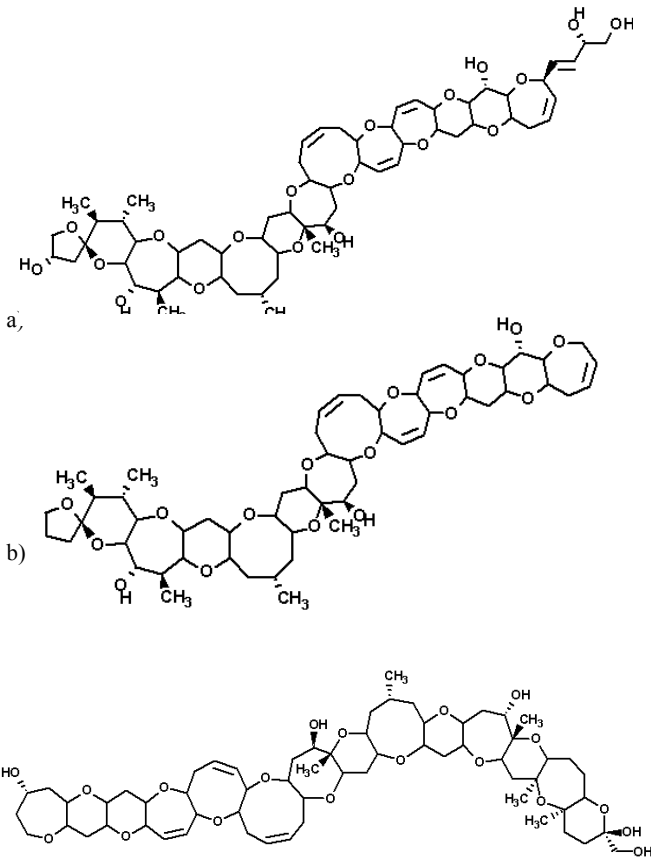


Figure 18. Molecular structure of CTXs: a) P-CTX; b) P-CTX3; c) C-CTX.

TABLE 4. Comparison of some characteristics of CTXs.

	C-CTX	PCTX
Vector fish	*Caranx latus*	*Gymnothorax javanicus*
Molecular formula	C62H92O19	C60H86O19
LD50	3,6 µg/kg	0,35 µg/kg
Number of cyclic ethers	14	13
Terminal cycle	C56 hemyacetal	C52 spiroacetal

These polyethers are chemically little reactive, and it has been found that side chains not involved in cycles formation are the reactive part of the molecules. A reduction in toxicity has been observed following chemical binding of hydroxyl function with various compounds or the formation of double bonds (Yasumoto and Oshima, 1979).

Among Pacific toxins, 9 different structure have been found, while 2 only have been identified for Caribbean group.

These 11 toxins are grouped in 3 types, and it has been found that some of them are conformation epimers at the spyro-acetalic or hemiacetalic asymmetric carbon (Table 5).

TABLE 5. Known chemical structure of CTXs divided by type.

Type	Toxins with known structure	
I	P-CTX1	
	P-CTX2 and P-CTX3	C52 epimers
	P-CTX4A and P-CTX4B	C52 epimers
II	P-CTX3c and P-CTX3B	C49 epimers
	P-CTX2A1	
	51 hydroxy-CTX3C	
III	C-CTX1 and C-CTX2	C56 epimers

One probable explanation for this huge variability in structural characteristics is metabolism: the accumulation along one or more steps of food chains of “basic” toxins make them experience some biotransformation which oxidize the molecule and make it more polar. Although metabolism is conceived to reduce toxicity, it has been seen that oxidized ciguatoxins are more toxic than parent compounds (Puiseaux-Dao et al., 2001).

7.1.2. *Maitotoxins*

Maitotoxins are another group of toxins produced by *G. toxicus* which have never been associated to CFP, as they have never been isolated in flesh

of fish, but only in viscera of herbivorous fish (Yasumoto et al., 1976; Yasumoto et al., 1984).

They are highly hygroscopic polycyclic polyethers, characterized by the absence of 2 sulphated groups (Fig. 19).

Despite molecular similarity with CTXs, their effects are different and no spontaneous or chemical transformation produced CTXs starting from MTXs (Puiseaux-Dao et al., 2001).

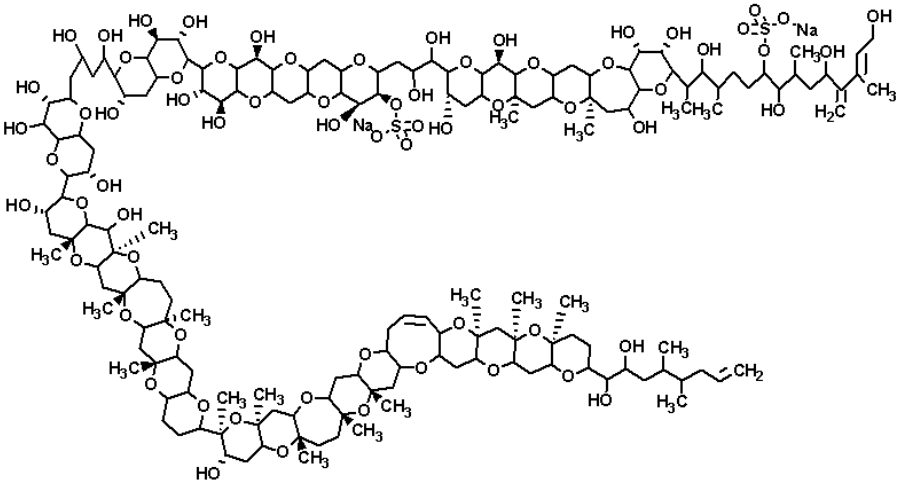


Figure 19. Chemical structure of maitotoxins.

7.2. MECHANISM OF ACTION

7.2.1. *Ciguatoxins*

Ciguatoxins share their mechanism of action with brevetoxins, because they bind to site 5 of voltage-dependent sodium channels. They activate the sodium channel, alter the selectivity of the channel for Na^+, delay or block channel inactivation and activate the channel at more negative membrane potential. This last action persistently increases membrane permeability to sodium.

All the aspects of mechanism of action have already been described in brevetoxins section.

7.2.2. *Maitotoxins*

Maitotoxins can not cross cell membrane and act by hydrolyzing membrane phosphoinositides and phospholipids, inducing calcium influx and the consequent increase in Ca^{2+} cytosolic concentration, and depolarizing all neural

and neuroendocrine membranes, causing an increase in neurotransmitters and hormones Ca-dependant liberation.

Phosphoinositides and phospholipids hydrolysis has proved to be dependant on extracellular calcium and is catalyzed by phospholipase C at high doses and by phospholipase A at low doses (Sladeczek et al., 1988; Gusovsky et al., 1989; Choi et al., 1990; Gusovsky and Daly, 1990; Lin et al., 1990; Meucci et al., 1992; Bressler et al., 1994).

7.3. SYMPTOMS AND TREATMENT IN HUMANS

7.3.1. *Ciguatoxins*

CFP is characterized by a sequence/association of neurological, gastrointestinal and cardio-vascular symptoms, presenting a high variability. Some occasional fatalities have been observed, following severe intoxications.

Although it can affect all ages, a certain, higher sensitivity seems to exist for the age range 30–49, interestingly, some occasional episode of toxin transfer through breast feeding was observed (Marcaillou-Le Baut et al., 2001).

In "classical" syndrome incubation lasts from 3 to 8 hours, even if in severe poisoning the delay time is 1 hour and in mild intoxication it can reach 12–20 hours (Bagnis, 1967; Bagnis et al., 1979).

The prodromic phase of ciguatera lasts about 2 hours and is characterized by gastrointestinal alterations involving face congestion, headache, salivation, and by neural symptoms: numbness of face, tongue and extremities (Pearce et al., 1983).

In the second phase, digestive symptoms are predominating, while neurological and, only in more severe cases, cardiac signs are only occasional (Bourdeau and Bagnis, 1989).

Gastroenteric signs are more intense than in starting phase, precocious and constant and include:

- Abdominal pain
- Vomiting
- Diarrhea.

Neurological signs are progressive and include:

- Tingling
- Burning or electric discharge sensation

- Thermal sensation inversion, which is an indicative, even if not specific, sign of the intoxication.
- Pruritus. This is another frequent and typical sign. It is a tardive sign, starting from extremities and diffusing to the all body, which is quite persistent and can also become permanent. A certain increase in the disturb has been observed at night, causing insomnia in affected people (Bourdeau and Bagnis, 1989).

Interestingly, pruritus can last for weeks after the poisoning has solved, and can be re-evoked by fish consumption (Bourdeau and Bagnis, 1989).

Some more occasional neurological signs can be recorded:

- Mydriasis or strabismus
- Paresis or ataxia of legs
- Articular and muscular pain.

These signs too can be re-induced even years after the poisoning by consumption of reef fish.

Finally, vertigos, teeth pain and cutaneous rashes have been recorded (Marcaillou-Le Baut et al., 2001).

Cardiovascular signs are recorded only in severe intoxication and include:

- Irregular heart beats
- Bradycardia
- Complete alteration of cardiac regulation.

As a general rule, gastroenteric and cardiovascular signs solve in a few days, while neurological signs last for weeks or more.

CFP has a very low mortality rate (0,1–4,5 %) and death occurs after respiratory failure or collapse (Marcaillou-Le Baut et al., 2001).

There seems to be a different trend in clinical symptoms between males and females, males showing more frequently gastro-intestinal signs and females muscular pain (Bagnis et al., 1979).

There is also a different profile in symptoms presentation between Pacific and Caribbean toxins, P-CTX being more neurological and C-CTX more gastroenteric.

A certain spontaneous detoxification has been observed in humans, but it is assumed to take long time, even if it has never been estimated. This can easily lead to accumulation phenomena in humans, and continuous consumption of little toxic fish can thus induce poisoning, similarly to consumption of one single, highly toxic fish (Lawrence et al., 1980; Chan, 1998).

Diagnostic of intoxication is based on clinical signs and on epidemiological data as well as on information obtained from the patient (recent fish consumption, species eaten, etc.) and on exclusion of other pathologies, i.e. bacterial and viral infections, other toxins, allergies (Marcaillou-Le Baut et al., 2001).

The treatment is symptomatic and includes:

Gastroenterical symptoms

– Spasmolitics
– Anti-diarrhoic drugs
– Anti emetics
– Gastric lavage

Neurological symptoms

– Calcium gluconate
– Vitamin B

Cardiovascular signs

– Atropine
– Cardiovascular analeptics

Pruritus

– Corticoids
– Anti histaminic drugs.

Mannitol has proved to have some efficacy against nervous signs in the treatment of poisoning, the most rapidly it is administered the best the effect. It probably acts by counteracting neuronal edema induced by Na entrance in neurons, followed by water influx in the cell (Palafox et al., 1998).

7.3.2. *Maitotoxins*

Maitotoxins poisoning is characterized mainly by neurological symptoms, including, in order of importance:

- Altered thermal perception
- Muscular pain
- Pruritus
- Urinary problems
- Articular pain
- Increased transpiration.

Gastrointestinal signs are quite rare (Marcaillou-Le Baut et al., 2001).

7.4. TOXICOSES IN ANIMALS

Evidence of the involvement on CTX in the morbidity and/or mortality of marine mammals remains speculative.

One interesting case is that concerning decline in population of monk seal in Hawaii. Population decline occurred has been primarily attributed to the poor survival rates among juveniles and pups and to slower growth rate of juvenile. It is yet unclear which are the reasons of this mortality rate, but it has been hypothized that ciguatera could play a role.

Indeed, preliminary survey of known prey fish species showed some positivity to the toxin; anyway no clear connection between CTX presence and population decline (Craig and Ragen, 1999; Van Dolah et al., 2003).

8. Tetrodotoxin

Consumption of fish contaminated by **tetrodotoxin** (TTX) or its derivatives is responsible for one of the most severe intoxication, as close to 60% of affected people die within 4 to 6 hours after ingestion.

Recent researches have provided strong evidence of the bacteriological origins of TTX*:*

- Puffer fish grown in culture do not produce tetrodotoxin until they are fed tissues from a toxin producing fish.
- The blue-ringed octopus found in Australian waters accumulates tetrodotoxin in a special salivary gland and infuses its prey with toxin by bite. This octopus contains tetrodotoxin-producing bacteria.
- Xanthid crabs collected from the same waters contain tetrodotoxin and paralytic shellfish toxin.

It is now clear that marine bacteria have long been in mutualistic symbiosis with marine animals and it is now known that the related toxins tetrodotoxin and anhydrotetrodotoxin are synthesized by several bacterial species, including strains of the family *Vibrionaceae*, *Pseudomonas sp*., and *Photobacterium phosphoreum*. Puffer fish took advantage of a single point mutation in their sodium channel receptors which rendered these fish immune from the effects of TTX. Following herbivores grazing, marine invertebrates and vertebrates accumulate these bacteria, provide them with a suitable host environment, and in return receive the protection of marine biotoxins, compliments of the prokaryotes (Johnson, 2002).

8.1. CHEMICAL STRUCTURE

TTX is a positively charged guanidinium group and a pyrimidine ring with additional fused ring systems, which contain hydroxyl groups which must certainly help stabilize the TTX-sodium channel binding complex at the aqueous interface (Johnson, 2002) (Fig. 20).

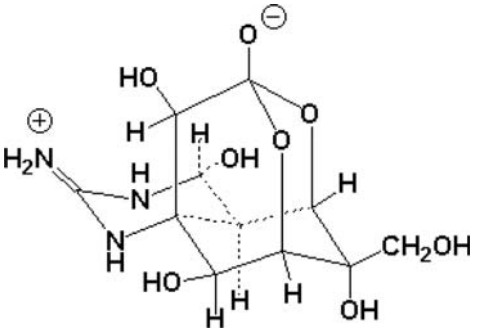


Figure 20. Chemical structure of TTX.

8.2. MECHANISM OF ACTION

TTX is an especially potent neurotoxin, specifically blocking voltage-gated sodium channels on the surface of nerve membranes. The TTX-Na Channel binding site is extremely tight (K_d = 10^{-10} nM). It is proposed that this binding results from the interaction of the positively charged guanidino group on the tetrodotoxin and negatively charged carboxylate groups on side chains in the mouth of the channel. TTX mimics the hydrated sodium cation, enters the mouth of the Na^+-channel peptide complex, binds to a peptide glutamate side group, among others, and then further tightens it

hold when the peptide changes conformation in the second half of the binding event. Following complex conformational changes, TTX is further electrostatically attached to the opening of the Na^+ gate channel (2d event occurs *in vivo* as the dehydration of the aqueo-sodium complex).

TTX's tenacious hold on the Na^+-channel complex is further demonstrated by the occupancy time of TTX v. hydrated-Na^+ at the complex. Hydrated sodium reversibly binds on a nanosecond time-scale, whereas TTX binds and remains on the order of tens of seconds. With the bulk of the TTX molecule denying sodium the opportunity to enter the channel, sodium movement is effectively shut down, and the action potential along the nerve membrane ceases. A single milligram or less of TTX is enough to kill an adult.

It has been proved that TTX does it not poison the host as the sodium ion channel in the host is different than that of the victim and is not susceptible to the toxin. Indeed it has been demonstrated for one of the puffer fish that the protein of the sodium ion channel has undergone a mutation that changes the amino acid sequence making the channel insensitive to tetrodotoxin. The spontaneous mutation that caused this structural change is beneficial to the puffer fish because it allowed it to incorporate the symbiotic bacteria and utilize the toxin it produces to its best advantage. A single point mutation in the amino acid sequence of the sodium-ion channel in this species renders it immune from being bound and blockaded by TTX (Johnson, 2002).

8.3. SYMPTOMS AND TREATMENT IN HUMANS

The first symptom of intoxication is a slight numbness of the lips and tongue, appearing between 20 minutes to three hours after eating poisonous pufferfish. The next symptom is increasing paraesthesia in the face and extremities, which may be followed by sensations of lightness or floating. Headache, epigastric pain, nausea, diarrhea, and/or vomiting may occur. Occasionally, some reeling or difficulty in walking may occur. The second stage of the intoxication is increasing paralysis. Many victims are unable to move; even sitting may be difficult. There is increasing respiratory distress. Speech is affected, and the victim usually exhibits dyspnea, cyanosis, and hypotension. Paralysis increases and convulsions, mental impairment, and cardiac arrhythmia may occur. The victim, although completely paralyzed, may be conscious and in some cases completely lucid until shortly before death. Death usually occurs within 4 to 6 hours, with a known range of about 20 minutes to 8 hours.

From 1974 through 1983 there were 646 reported cases of fugu (pufferfish) poisoning in Japan, with 179 fatalities. Estimates as high as 200 cases per year with mortality approaching 50% have been reported. Only a few cases have been reported in the United States, and outbreaks in countries outside the Indo-Pacific area are rare. Sushi chefs who wish to prepare fugu, considered a delicacy by many in Japan, must be licensed by the Japanese government.

The comparative toxicity of TTX is summarized by William H. Light. "Weight-for-weight, tetrodotoxin is ten times as deadly as the venom of the many-banded krait of Southeast Asia. It is 10 to 100 times as lethal as black widow spider venom (depending upon the species) when administered to mice, and more than 10,000 times deadlier than cyanide. It has the same toxicity as saxitoxin which causes paralytic shellfish poisoning A recently discovered, naturally occurring congener of tetrodotoxin has proven to be four to five times as potent as TTX. Except for a few bacterial protein toxins, only palytoxin, a bizarre molecule isolated from certain zoanthideans (small, colonial, marine organisms resembling sea anemones) of the genus *Palythoa*, and maitotoxin, found in certain fishes associated with ciguatera poisoning, are known to be significantly more toxic than TTX. Palytoxin and maitotoxin have potencies nearly 100 times that of TTX and Saxitoxin, and all four toxins are unusual in being non-proteins. Interestingly, there is also some evidence for a bacterial biogenesis of saxitoxin, palytoxin, and maitotoxin....[i]n living animals the toxin acts primarily on myelinated (sheathed) peripheral nerves and does not appear to cross the blood-brain barrier" (Johnson, 2002).

9. Pfiesteria and Estuary-Associated Syndrome

Possible estuary-associated syndrome (PEAS) is known to occur in brackish coastal waters along the mid-Atlantic coast of the U.S. and has been reported in a few scattered locations worldwide as well. This syndrome seems to be associated to *Pfiesteria* and *Pseudopfiesteria* exposure (Fig. 21), even if no clear connection between algae and syndrome have been found yet (Duncan et al., 2005).

At present the toxins responsible for fish lethality or neurologic symptoms has not yet been identified. Anyway, Moeller et al., 2001 have isolated and partially purified water soluble toxins contained in water from toxic *Pfiesteria* cultures.

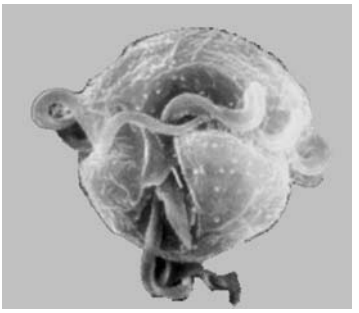


Figure 21. Pfiesteria piscicida.

As well as for the toxins chemical structure, the exact mechanism of action of *Pfiesteria* has not been identified.

Exposure to *Pfiesteria* toxins in the air, water, or fish at the site of an outbreak can cause skin and eye irritation as well as short-term memory loss, confusion, and other cognitive impairments in people. No toxic activity has been detected in shellfish harvested from sites of *Pfiesteria* blooms.

The reported human health effects (e.g. respiratory irritation, skin rashes, and possible neurocognitive disorders) from exposure to natural waters in the mid-Atlantic states are still being assessed. Illness from consuming shellfish and fish in areas of *Pfiesteria* and *Pseudopfiesteria* occurrence are unknown.

Pfiesteria and *Pseudopfiesteria* are associated with fish kills in mid-Atlantic states. Recent studies suggest that fish kills are due to: 1) motile cells attaching to fish and feeding on fish skin cells which could allow invasion of pathogens or weaken the animal's immunity; and/or 2) undescribed, water-soluble and lipid-soluble bioactive fractions being released to the environment. There are published studies purporting different causes and different pathways.

Interestingly, in the laboratory, toxicity of water in an aquarium is rapidly lost within a day if the fish that were provided as food are removed.

Pfiesteria piscicida has a very complex life cycle, and at certain stages of this cycle has a lethal toxic effect on fish, as demonstrated by massive fish kills in North Carolina estuaries and in the Chesapeake Bay (Birkhauser et al., 1975; Ashton et al., 1977; Andersen et al., 1993; Burkholder et al., 2001a; Samet et al., 2001). The organism's polymorphic life cycle (Figure 22) consists of three distinct life-form stages—flagellated, amoeboid, and encystedthat live in bottom sediment s or as free-swimming organisms in

the water column. These stages involve at least 24 size, shape and morphotypic variants, ranging from 5 to 450 μm in size. The stages include rhizopodial, filose and lobose amoebae; toxic and non-toxic zoospores (asexual flagellated spore); cysts of various structure; and gametes (mature sexual reproductive cells having a single set of unpaired chromosomes). Under laboratory conditions in the presence of live fish, its sediment-dwelling amoeboid and resting stages transform rapidly into free-swimming flagellate stages in response to unknown chemical cues secreted or excreted by fish. The induced (excysted) flagellate stages swarm into the water column and become toxic during their continued exposure to the fishderived (sometimes shellfish-derived) chemical stimulants.

The toxic zoospores gather together, alter their random swimming pattern into directed movement, doubling their swimming speed in the process, and commence predatory behavior directed toward targeted fish. The toxic zoospores produce a neurotoxin of unknown structure, soluble in water, and which may be liberated as an aerosol under some conditions. Fish are first narcotized by the toxin, die suddenly, and slough off tissues, which the attacking zoospores consume by sucking out the cell contents through the attached peduncle. The zoospores sometimes ingest other microscopic plant and animal prey at the same time. During this killing period, the zoospores reproduce both asexually (mitotic division) and by producing gametes that fuse to produce toxic planozygotes (actively swimming offspring formed by sexual reproduction, i.e. the union of two gametes). The presence of live fish is required both for completion of the sexual cycle and for toxin induction. Upon fish death or their retreat, the toxic zoospores and planozygotes transform into (mostly) nontoxic amoeboid stages that gather onto the floating fish carcasses on which they feed for extended periods, and follow the sinking fish remains to the bottom sediments. Not all toxic zoospores and planozygotes transform into amoebae. Some encyst and sink into bottom sediments; a lesser number revert to non-toxic zoospores that remain in the water column. The proposed 24 stages of the complex life cycle are based on laboratory observation (Samet et al., 2001).

It has been observed that the morbidity of *Pfiesteria* is dependent on food availability: *Pfiesteria* blooms were stimulated by inorganic nutrient enrichment (Burkholder et al., 1992; Glasgow et al., 1995; Burkholder et al., 2001b), and transition to a toxic stage was associated with the presence of fish tissues or secretions (Burkholder and Glasgow, 1997; Marshall et al., 2000). The fish appeared narcotized, displaying lethargic behavior, a poor fright response, lesions, hemorrhage, and ultimately death. Water samples

indicated that *P. piscicida* was present at concentrations ranging from 600–35,000 cells/ml in waters ranging in temperature from 9–31°C and in salinity from 0–30 psu during fish kills. Much lower concentrations of *P. piscicida* were found only hours after fish kills due to *P. piscicida*'s encystment following lack of food (due to fish death) and settlement into sediment (Burkholder et al., 1992).

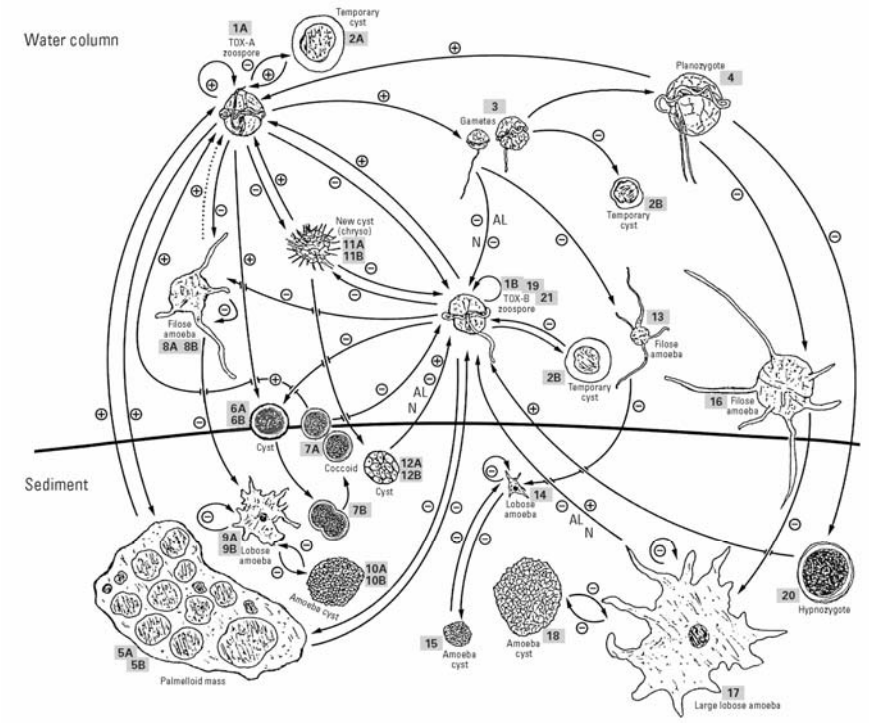


Figure 22. Pfiesteria piscicida life cycle. The pathways indicate the presence (+) versus the absence (–) of live finfish; AL = presence of cryptomonads and certain other algal prey; N = nutrient enrichment as organic and/or inorganic N and P; S = environmental stressor such as sudden shift in temperature or salinity, physical disturbance, or prey depletion. Dashed lines = hypothesized pathways. Stages have been conservatively numbered to facilitate description. TOX-B: zoospores temporarily non-toxic in the absence of live fish prey; TOX-A: zoospores which produce toxin when sufficient live fish are added. Zoospores can transform to filose and lobose amoebae. Planozygotes can transform to larger filose and lobose amoebae. Small filose and lobose amoebae can also be produced by gametes (Burkholder et al. (2001a), modified).

10. New toxins

10.1. AZASPIRACIDS

Azaspiracids are polycyclic polyethers molecules with 40 carbon atoms, with a different level of methylation, presenting a carboxylic acid and a cyclic imine function and a unique cyclic structure (Satake et al., 1998; Ofuji et al., 1999). Some human intoxication has been reported in France, Netherland and Italy (McMahon and Silke, 1998). The producing organism is *Protoperidinium crassipes*, which distributes uniformly in the mussels body (Figs. 23 and 24).

Poisoning is characterized by symptoms similar to DSP (diarrhea, nausea and vomiting) and recovery occurs in 3–5 days.

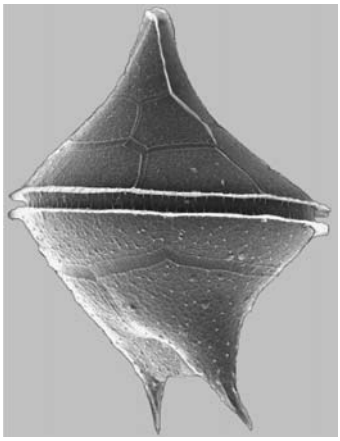


Figure 23. *Protoperidinium crassipes*.

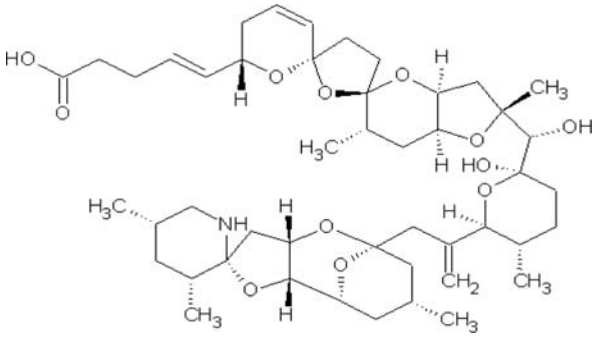


Figure 24. Chemical structure of azaspiracids.

In animals a neurotoxic syndrome is observed, causing death at low doses in two to three days due to progressive paralysis; high doses are lethal in few minutes, and death occurs for paralysis and violent seizures.

Histopathological analysis of rats administered oral or intraperitoneal toxin showed degeneration and desquamation of gut. This phenomena can recover after exposure ends, but recovery times are longer that for OA. Some pancreatic, thymus and cardiac necrosis has been observed, as well as lipidic accumulation in liver (Ito et al., 2000).

10.2. SPIROLIDES

Spirolides (from A to F) are macrocyclic polyethers produced by *Alexandrium ostenfeldii* (Fig. 25). At present their mechanism of action has not yet been clarified, but they seems to affect central nervous system (Hu et al., 1995a; Hu et al., 1995b; Hu et al., 1996a; Cembella, 1998; Cembella et al., 2000).

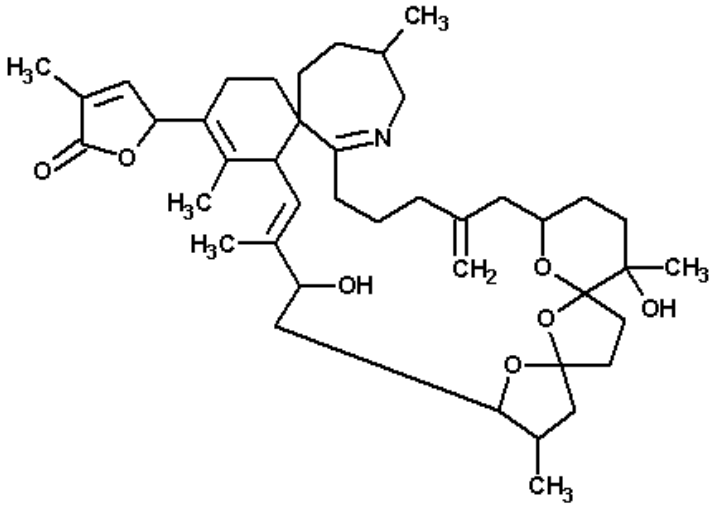


Figure 25. Spirolides chemical structure.

10.3. GIMNODINE

An ichthyotoxic toxin is **gymnodine**, produced by *Gymnodium mikimotoi*; its mechanism of action is not known, but the toxin is not haemolytic, cytotoxic and does not activate ionic channels (Seki et al., 1995; Miles et al., 1999).

10.4. PROROCENTROLIDES

Prorocentrum lima and *P. maculosum* produce not only okadaic acid, but other macrocyclic polyethers named **prorocentrolides** and **prorocentrolides** B, more polar than OA. The toxicological and pharmacological activities are not well defined, but it is clear that they have no inhibiting activity on phosphatases (Torigoe et al., 1988; Hu et al., 1996b).

10.5. COOLIATOXIN

Cooliatoxin is produced by *Coolia monotis*; it is a polyether considered as a monosulphated analogue of yessotoxins. The effect of cooliatoxin in animal is similar to that of yessotoxins, even if the delay time for symptoms appearance is longer (Holmes et al., 1995).

The respiratory distress responsible for animal's death is not a result of direct inhibition of phrenic nerve or of diaphragm activity, but is caused by an initial stimulation followed by block of non-myelized nerves activity, which induce a transitory contraction of smooth muscles and a positive inotrope effect on cardiac muscle (Holmes et al., 1995).

10.6. ZOOXANTELLATOXINS

Zooxantellatoxins are macrocyclic polyethers produced by symbiotic dinoflagellates *Symbiodinium*. Their cellular effects are similar to those of maitotoxins and are controlled by extracellular calcium:

- Increase in cytosolic concentration of calcium
- Hydrolysis of phoshoinositide via phospholipase C and of phospholipids, leading to arachidonic acid liberation
- Muscular contraction
- Sodium influx and potassium efflux from cell (Rho et al., 1995; Rho et al., 1997; Moriya et al., 1998)

As for maitotoxins, two mechanisms of action have been considered for zooxantellatoxins:

- Calcium channels activation dependent on membrane potential
- Activation of non-selective cationic channels

11. Toxicoses Due to "Unsual" Vectors

Some fish and marine vertebrate species are responsible for "unusual" syndromes, generally called "**sarcotoxisms**", which sometimes are caused by a combination of two or more toxins. Toxic compounds produced by benthic and/or epiphytes can affect various trophic pathways, depending on the complexity of affected ecosystem; this effect is thus maximal in tropical areas, where biodiversity if very high (fig. 4).

A summary of poisoning is given in table 6.

TABLE 6. General characteristics of sarcotoxisms.

Name	Species	Clinical characteristics	Toxin
Clupeotoxism	Herrings, anchovies, sardines	Short incubation time, digestive syndrome, pruritus, tachycardia, vertigos, cyanosis. Coma and death are not so rare	Palitoxine? Mixed toxins?
Allucinatory episodes	Acanthuridae Mugilidae, Mullidae, Estraciontidae, Pomacentridae, Serranidae, Siganidae, Sparidae	Short incubation time. Allucinations, vertigos, behaviour alterations, motory incoordination	Various, unknown toxins
Charcarotoxisms	Various shark species	Both nervous and digestive signs	Mixed toxins
Scombroidotoxism	Scrombridae, tuna, bonitos, mackerel	Short incubation time, rapid evolution, nervous and digestive signs. Regression within 8-12 hours	Histamine and biogenic amines
Chelonitoxism	Sea turtles	Digestive syndrome which can also be fatal	Unknown toxins Lyngbyatoxin?

Some of these poisoning are described in following paragraphs.

11.1. LYNGBYATOXIN AND APLYSIATOXIN

The marine cyanobacterium *Lyngbya majuscula* has been implicated in acute adverse health effects in humans over the last forty years with symptoms including dermatitis involving itching, rash, burning blisters and deep desquamation and, respiratory irritation and burning of the upper gastrointestinal tract on ingestion. It has been shown that the toxins **lyngbyatoxin A** (LA) and **debromoaplysiatoxin** (DAT) isolated from *L. majuscula* samples in Australia and USA are at least partially responsible for these symptoms. It should be noted that *L. majuscula* has been found to grow in more than 98 locations around the world in tropical, sub-tropical and temperate climates (Grauer and Arnold, 1961; Mynderse et al., 1977; Solomon and Stoughton, 1978; Cardellina et al., 1979; Fujiki et al., 1985; Izumi and Moore, 1987; Anderson et al., 1988; Marshall and Vogt, 1998; Osborne et al., 2001a; Osborne et al., 2001b).

An association was found between the level of water exposure and reporting of symptoms. Those with higher exposures were more likely to report skin and eye symptoms, which previously have been implicated with exposure to *L. majuscula*. Toxins of L. majuscula have been demonstrated both anecdotally and experimentally to be fast acting, thus eliminating the variable of duration of exposure (Grauer and Arnold, 1961; Solomon and Stoughton, 1978; Serdula et al., 1982; Izumi and Moore, 1987; Marshall and Vogt, 1998).

Episodes of human intoxications due to sea turtles consumption have been known in some Indo-Pacific areas (Halstead, 1970; Champetier De Ribes et al., 1998). The detection of lyngbyatoxin A in meat of turtles lead to the identification of this toxin as the causative agent of intoxication (Yasumoto (1998). Symptoms were characterized by inflammation of oral and esophageal area, difficulty of swallowing, acute gastritis, papule of the tongue, mouth ulcers, weakness, tachycardia, headache, dizziness, fever, salivation, stinking breath. In Madagascar some death in youth and newborn (due to ingestion of toxins with breast milk) were observed. *Lyngbya majuscola* has been proved to produce also **aplysiatoxin**, which induce hypersecretion of mucous from the caecum and large intestine, bleeding from the small intestine and death; the toxin has proved to be a tumor promoter by activating protein kinase C (Mynderse et al., 1977; Fujiki et al., 1981; Ito and Nagai, 1998; Ito and Nagai, 2000).

Lyngbiatoxin has been considered as a promoting agent for fibro-papillomatosis in sea turtles: tumor promoting agents have been shown to enhance viral synthesis, to enhance oncogene-induced transformation of

cells and to reduce immune responses by suppression of the immune-surveillance mechanism. All these actions ease the occurrence of fibro-papillomatosis (Arthur et al., 2008).

11.2. CARCHAROTOXIN

The consumption of sharks' flesh can sometime be the origin of human intoxications, which are recorded since 1993. These intoxications are characterized by neurological signs, mainly ataxia, while gastro enteric symptomatology is quite rare. This poisoning was for long time considered a form of ciguatera. Recent identification of **carcharotoxins** proved that these toxins have no similarity to ciguatoxins (Habermehl et al., 1994; Boisier et al., 1995).

It should be remembered that sharks can anyway contain more than one toxin, ciguatoxins included in Table 7.

TABLE 7. Shark intoxication: syndromes, sources and species involved.

Syndroms	**Source**	**Species involved**
Sarcotoxismes	Ciguatera	*Carcharinus galeocerdo* *Isurus sp.* *Prionace glauca*
Neurosensorial disturbs	Not known	*Somniosus* *Squalus*
Carcharotoxisme	Not known	*Carcharinus leucas* *Carcharinus ambonensis*
Toxicoses	Pollutants Ciguatera Neurosensorial problem Vitamin A	*Prionace* *Sclyliorhinus* *Sphyrna* *Carcharhinus* *Carcharodon*

11.3. PALITOXINS

Clupeotixism is a poisoning characterized by a high rate of fatalities following to sardines, herrings and anchovies consumption. Causative agent for the poisoning was found to **palitoxin**, a toxins produced by *Ostreopsis* spp.; indeed, the dinoflagellates was found in viscera of toxic fish (Usami et al., 1995; Molgò et al., 1997).

Being palitoxin isolated from other organisms than dinoflagellates, i.e. marine coelenterates, sea anemones, crabs and other marine invertebrates, the algal origin of the toxin is under discussion (Moore and Scheuer, 1971; Hirata et al., 1979; Beress et al., 1983; Mahnir and Kozlovskaja, 1992; Gleibs et al., 1995; Yasumoto et al., 1997).

Palitoxin is a polyhydroxylated complex molecules which is constant despite the producing organism (Quod et al., 2001).

Palitoxin was for long time considered as a form of ciguatera, but nowadays clinical symptoms allows to differentiate among the two poisoning (Puiseaux-Dao et al., 2001).

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THE ROLE OF ALLELOPATHY FOR HARMFUL ALGAE BLOOM FORMATION

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Abstract: Strong evidence has accumulated on the last years that some algal species are able to kill not only their grazers but also other algal species, a process called allelopathy. Killing the nutrient-competing phytoplankton species enable these species to freely utilize limiting resources such as nitrogen and phosphorus. While for some algal species, like e.g. the flagellate *Prymnesium sp.,* the allelochemicals seem to be the same substances as their toxins, for some other algal species they are not. *Alexandrium* spp. are among the latter case: their internal toxins (such as saxitoxins) are not able to inhibit the growth of other algal species. However, these species by producing other substances than their internal toxins also cause allelopathic effects. Emphasis is placed here on the flagellate species *Prymnesium parvum*; which is not only able of allelopathy but mixotrophy as well. Mixotrophy, i.e. the capability to ingest bacteria, other algae and even potential grazers, also contributes to the bloom-forming ability of *Prymnesium* spp. Allelopathy, mixotrophy and grazer deterrence increase dramatically when *Prymnesium* spp. cells are grown under N or P deficiency, and so does toxicity, but decrease in intensity or cease completely if cells are grown with high amounts of N and P in balanced proportions. *Prymnesium* filtrates from nutrient deficient cultures have almost the same strong effect on grazers and other plankton cells as *Prymnesium* cells grown together with their target. It seems that toxin production in *Prymnesium* spp. works not only as a defense mechanism, but also, by killing competitors, improve the algae competitive ability under conditions of severe nutrient depletion. We

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can assume thus that a consequence of the increased input of N and P to aquatic ecosystems is provoking an unbalanced nutrient situation for *Prymnesium* spp., as well as many of the other HAB species producing toxins, to growth but ideal to produce toxins instead.

Keywords: HABs, allelopathy, phytoplankton, nutrients, toxins

1. Defining Allelopathy

Harmful effects of plants on other plants or crops dates back at least 2 millennnia, while experimental work, testing allelopathy, started in the late eighteenth century (Willis, 1985). Phytoplankton allelopathy was probably first observed in 1917 by Harder (Inderjit and Dakshini, 1994) when he reported auto-inhibition in the freshwater cyanobacteria *Nostoc punctiforme*. In 1937 the term allelopathy was coined by Molisch (Rizvi et al., 1992) to describe both detrimental and beneficial biochemical interaction between all classes of plants (including microorganisms). The term has evolved since then, including and excluding organisms. We propose to use a definition of allelopathy that is based on Rice's concept (Rice, 1984), but updating the definition along with the taxonomic classification to: "any direct or indirect, harmful or beneficial effect by plants, protists (e.g. microalga, ciliates), bacteria, or viruses on another trough the production of chemical compounds that leak into the environment". Keating (1999) has also suggested using the term allelochemistry, which we believe is more appropriate, since allelopathy evokes only negative effects (pathos = to suffer). In this paper we use the term allelopathy in the context of the negative/positive effect of allelochemicals produced by certain algae on other algal groups/species; i.e. grazer deterrence and or predator avoidance are not discussed. We consider allelopathy as competition strategy, while grazer deterrence and or predator avoidance are toxic effects.

2. Allelopathy in Aquatic Environments

Although phytoplankton allelopathy was observed nearly a 100 years ago by Harder (Inderjit and Dakshini, 1994), only in recent years research on the subject has gained momento (Fistarol et al., 2003; Granéli and Johansson, 2003; Gross, 2003; Legrand et al., 2003; Skovgaard et al., 2003; Fistarol et al., 2004a, 2004b; Suikkanen et al., 2004; Kubanek et al., 2005; Granéli and Pavia, 2006; Granéli and Hansen, 2006; Tillmann et al., 2007; Granéli and Weberg, 2008). It seems that the main reason for the increase in the

interest on phytoplankton allelopathy isbecause most of the phytoplankton species producing allelochemicals are harmful to the marine ecosystems where they occur.

Harmful algal blooms (HABs) have increased worldwide in waters ranging from fresh to coastal estuarine and marine waters (Anderson, 1989; Smayda, 1990; Hallegraeff, 1993; Van Dolah, 2000), causing enormous impacts in the aquatic ecosystems they occur (Maestrini and Granéli, 1981; Granéli and Hansen, 2006). Fish kills are usually the first direct effect seen from the impact of such blooms. The most deleterious impacts occur when they affect entire ecosystems, causing death of phytoplankton, zooplankton seaweeds, and shellfish. The toxins produced by such blooms are secondary metabolites released in the water with capability to punctuate holes in the cells membranes of the target. Blooms caused by high discharges of nitrogen and phosphorus to the coastal areas, which produces high algal biomass that when decomposing causes oxygen depletion in the water column and mostly in the bottom waters, also cause devastating consequences to the benthic community (Allen et al., 2006). However, the difference between these two types of bloom affecting the aquatic ecosystems is that, in the first case, the cause of the deleterious effect on the flora and fauna is the result of a direct action of the chemicals liberated by the HA-species into the water on the other organisms, and not oxygen deficiency provoked by the decomposing bloom. It seems logical to assume that the effect on the entire ecosystem from the action of released chemicals is a secondary effect, while the main purpose of these chemicals would be to kill/inhibit the growth of the other phytoplankton species competing with the HA-species (allelopathy) and also to decrease losses by killing their grazers (grazer deterrence).

Both laboratory and filed work have demonstrated allelopathic effects of several phytoplankton groups. Here we are going to discuss some of the most important findings of the latest works, emphasizing the results of our group, and also the allelopathic effect of the flagellate species *Prymnesium parvum* which is not only able of allelopathy but mixotrophy as well.

3. Testing Allelopathy in Aquatic Systems

To mimic natural conditions when testing phytoplankton allelopathy, an important detail that should be considered is the amount of allelochemicals to which the target species will be exposed. This can be done by having the allelopathic species at natural cell concentrations, and by exposing the target organisms to a continuous addition allelochemicals. Experiments involving just a single addition of allelopathic filtrate to a target species have demonstrated that the allelopathic effect may be lost some time after the exposure, either due to the degradation of the chemicals by light or bacteria,

or the recovery of the target species (Gleason and Paulson, 1984; Windust et al., 1997; Suikkanen et al., 2004; Fistarol et al., 2005). Several studies demonstrated that the toxins produced by some microalgae (e.g. prymnesins produced by *Prymnesium parvum* and nodularin produced by *Nodularia*) can be inactivated, for example by light through photodynamic and/or photooxidative processes, and are sensitive to temperature (Reich and Parnas, 1962; Twist and Codd, 1997; Kvernstuen, 1993 *cited in* Larsen and Bryant, 1998; Fistarol et al., 2003). Some toxins can also be degraded by bacteria (e.g. microcystin, Christoffersen et al., 2002, Hagström et al., 2007). The same degradation processes could occur to allelochemicals. Allelochemicals may also be removed from the system by, for example, binding to cell membranes (Tillmann, 2003). All these results indicate that allelochemicals are not persistent, and thus, one filtrate addition may not be representative of natural conditions. Under natural conditions, allelochemicals are presumably constantly released to the environment. Therefore, experiments using repeated filtrate additions probably give a better representation of natural conditions. In most studies on phytoplankton allelopathy, only one filtration addition is usually employed, and thus the allelopathic effect may have been underestimated. Repeated filtrate additions demonstrate an increase in the allelopathic effect compared to one filtrate addition (Suikkanen et al., 2004; Fistarol et al., 2005).

4. Allelochemicals – Which Substances are they?

The allelochemicals are for the great majority of the algal species unknown, however some allelochemicals have been identified (see Table 1) (Granéli and Weberg, in press). The strongest allelochemicals however, have haemolytic capacity, perforating holes on the cell membranes of other algal species, as in the case of *C. polylepis* and *P. parvum* (Johansson and Graneli, 1999a, 1999b; Schmidt and Hansen, 2001; Fistarol et al., 2003; Fistarol et al., 2004a). As a consequence, there are associated fish-kills, as the delicate gills cell membranes are affected as well by the action of the haemolytic compounds (Igarashi et al., 1995, 1999). The majority of the allelochemicals however, have a mild mode of action, as e.g. inhibition of photosynthesis and growth (Legrand et al., 2003). Studies that have tested if some known algal toxins (e.g. okadaic acid –OA, paralitic shelfish posion – PSPs- toxins, nodularin) caused allelopathic effects have shown a negative result, i.e. the algal toxins were not the compounds causing allelopathy (Sugg and Van Dolah, 1999; Tillmann and John, 2002; Fistarol et al., 2003; Suikkanen et al., 2004, 2006).

TABLE 1. Allelopathic harmful algae species, their allelochemicals and allelopathic effect.

Species	Allelochemicals[a]	Reference
Bacillariophyceae		
Pseudo-nitzschia pungens	U	(Legrand et al., 2003)
Skeletonema costatum	U	(Yamasaki et al., 2007)
Coscinodiscophyceae		
Rhizosolenia alata	U	(Legrand et al., 2003)
Cyanophyceae		
Anabaena sp.	U	(Suikkanen et al., 2005)
A. cylindrica	EP	(Legrand et al., 2003)
A. flos-aquae	HX, A, M, U	(Murphy et al., 1976; Kearns and Hunter, 2001; Legrand et al., 2003)
A. lemmermannii	U	(Suikkanen et al., 2004)
Aphanizomenon sp.	U	(Suikkanen et al., 2005)
A. flos-aquae	U	(de Figueiredo et al., 2004; Suikkanen et al., 2004; Suikkanen et al., 2006)
A. gracile	U	(Legrand et al., 2003)
Cylindrospermopsis raciborskii	U	(Figueredo et al., 2007)
Gomphosphaeria aponina	U	(Legrand et al., 2003)
Hapalosiphon fontinalis	Hapalindole A	(Moore et al., 1984)
Fischerella sp.	U	(Bagchi and Marwah, 1994)
Fischerella muscicola	Fischerellin	(Gross et al., 1991; Legrand et al., 2003)
Microcystis sp.	U, Microcystin	(Sukenik et al., 2002; Vardi et al., 2002)
Nodularia spumigena	U	(Suikkanen et al., 2004; 2005; Suikkanen et al., 2006)
Nostoc sp.	U	(Schagerl et al., 2002; Legrand et al., 2003)
Nostoc spongiaeforme	Nostocine A	(Hirata et al., 1996; Hirata et al., 2003)
Oscillatoria sp.	FA	(Chauhan et al., 1992)
Oscillatoria spp.	U	(Legrand et al., 2003)
Oscillatoria laetevirens	U	(Ray and Bagchi, 2001)
Dinophyceae		
Alexandrium catenella	U	(Arzul et al., 1999)
A. minutum	U	(Arzul et al., 1999; Fistarol et al., 2004b)

(Continued)

TABLE 1. *(Cont.)*

Species	Allelochemicals[a]	Reference
A. ostenfeldii	U	(Tillmann et al., 2007)
A. tamarense	U	(Arzul et al., 1999; Fistarol et al., 2004a; Fistarol et al., 2004b; Wang et al., 2006)
Amphidinium klebsii	U	(Sugg and VanDolah, 1999)
Ceratium sp.	U	(Legrand et al., 2003)
Coolia monotis	U	(Sugg and VanDolah, 1999; Legrand et al., 2003)
Gambierdiscus toxicus	U	(Sugg and VanDolah, 1999)
Karenia brevis (Gymnodinium breve)	U	(Kubanek et al., 2005)
K. mikimotoi (Gymnodinium mikimotoi)	U	(Uchida et al., 1999; Fistarol et al., 2004a)
Ostreopsis lenticularis	U	(Sugg and VanDolah, 1999)
Peridinium aciculiferum	U	(Rengefors and Legrand, 2001)
Prorocentrum lima	U	(Sugg and VanDolah, 1999)
Prymnesiophyceae		
Chrysochromulina polylepis	U	(Myklestad et al., 1995; Schmidt and Hansen, 2001; Fistarol et al., 2004a)
Phaeocystis pouchetii	U, PUA	(Hansen et al., 2004; Hansen and Eilertsen, 2007; van Rijssel et al., 2007)
Prymnesium parvum	U, Prymnesin	(Igarashi et al., 1998; Fistarol et al., 2003; Granéli and Johansson, 2003; Barreiro et al., 2005; Fistarol et al., 2005)
Raphidophyceae		
Chattonella antiqua	U	(Matsuyama et al., 2000 cited in Gross, 2003)
Heterosigma akashiwo	U	(Matsuyama et al., 2000 (cited in Gross, 2003; Pratt, 1966; Yamasaki et al., 2007)

a) A anatoxin, EP extracellular peptides, F fatty acids, HX hydroxamates chelators, M microcystin, OA okadaic acid, PUA polyunsaturated aldehyde, U unknown. Source: Granéli and Weberg, (in press)

5. Abiotic and Biotic Factors Regulating Allelopathy

Many abiotic and biotic factors have been investigated in the context of toxin production, however, as allelopathy is a relatively new topic of research in

aquatic ecosystems, fewer studies and information exists. There are to our knowledge only a handful of published studies showing how the production of an allelochemical is influenced by abiotic or biotic factors. This is no surprise since most allelochemicals have not yet been isolated and structurally determined (Table 1) (Granéli and Weberg, in press). Furthermore, potentially allelopathic compounds have been characterized from intracellular extracts but these cannot be regarded as a allelochemicals until a mode of release into the surrounding environment and a correlation to allelopathic effect have been shown (Willis, 1985). However, some work on factors affecting allelopathic effects rather than quantifying the produced allelochemicals has been done and are presented.

5.1. INFLUENCE OF ABIOTIC FACTORS

5.1.1. *Light*

Allelopathic compounds released by some phytoplankton species seem to be effective only in a relative short time period. Cell-free filtrates of *Prymnesium parvum* added to cultures of *Thalassiosira weissflogii*, *Rhodomonas cf. baltica* and *Prorocentrum minimum* had a great negative impact on cell numbers, but within a few days the exposed species' began to recover (Graneli and Johansson, 2003; Suikkanen et al., 2004; Fistarol et al., 2005). However, when exposed to repeated additions of cell-free filtrate no recovery was possible (Fistarol et al., 2003; Suikkanen et al., 2004; Fistarol et al., 2005). Similar findings were observed for the dinoflagellate *Scrippsiella trochoidea* which managed to recover from *Alexandrium ostenfeldii* exudates (Tillmann et al., 2007). These findings suggest that one or several mechanisms reduce the allelopathic effect. Exposure from UV light at 255 nm and visible light between 400 and <520 nm completely inactivated extracellular ichthyotoxins from *P. parvum* within 90 min (Parnas et al., 1962). Another prymnesiophyte, *Phaeocystis pouchetii*, enhanced its haemolytic activity when incubated at higher light intensities (van Rijssel et al., 2007), thus showing a positive stimulation from light.

5.1.2. *Temperature*

Not much is known how changes in temperature affect phytoplankton allelopathy. An example is that the haemolytic activity of *Phaeocystis pouchetii* increased in higher temperatures going from 4°C to 15°C (van Rijssel et al., 2007). On the other hand, raising the culturing temperature from 14°C to 20°C of *Alexandrium tamarense* gave no difference in allelopathic effect to *Scrippsiella trochoidea* or *Heterocapsa triquetra* (Fistarol et al., 2004a).

5.1.3. *pH*

Coastal surface waters may reach high pH levels (Pegler and Kempe, 1988; Emery, 1969 cited in Hinga, 1992, Hansen, 2002) and the high pH can be confounded with the effect seen from allelopathic interactions. For example, raised pH level reduced motility of the dinoflagellate *H. triquetra* when mixed with low cell densities of *C. polylepis* (Schmidt and Hansen, 2001). Increasing the pH from 8 to 9 resulted in more than double numbers of non-motile *H. triquetra* cells. The toxicity of high-density cultures of *C. polylepis* increased from pH 6.5 and leveled out at pH 8 rendering circa 90 percent of the *H. triquetra* cells non-motile. So, in these studies the authors found that high pH have a negative effect on the motility of *H. triquetra*, and together with the allelochemicals increased allelopathy even further (Schmidt and Hansen, 2001). Similar results have also been found for the freshwater cyanobacterium *Oscillatoria laetevirens*, i.e. the production of algicides almost four folded when pH was elevated from 8 to 9 and at neutral or lower pH the algicide was found in lower concentrations (Ray and Bagschi, 2001).

5.1.4. *Nutrients*

Toxicity in phytoplankton usually increases under nutrient limitation (Edvardsen et al., 1990; Reguera and Oshima, 1990; Granéli and Johansson, 2003; Granéli and Flynn, 2006). If the algae need nitrogen in their toxins (as saxitoxins, nodularin, domoic acid e.g.), this will happen only under phosphorus limitation (excess nitrogen in the medium). For the algae which toxins do neither contain P nor N, toxicity increases under both P and N limitation (Johansson and Granéli, 1999a, 1999b; Granéli and Flynn, 2006). This indicates that the motive behind the increased toxicity is actually stress, for not having enough of the limiting nutrient to provide for cell division.

There are very few studies on allelopathy in aquatic systems dealing with nutrient limitation. Granéli and Johansson (2003) found that starved *Prymnesium parvum* cells from N or P increased dramatically their production of allelochemicals while and NP sufficient conditions allelopathy was nearly nil. Thus, this results support the assumption that production of allelochemicals by algae is similar to their production of toxins, i.e. physiological stress is the cause for it.

The target species also respond differently to the allelochemicals if they are grown under nutrient sufficient or deficient conditions. *Thalassiosira weissflogii* grown under N and P limitation was significantly more sensitive to *Prymnesium parvum* allelochemicals than when it was grown under sufficient nutrient conditions. (Fistarol et al., 2005). Target organisms would

be sharing the same stress environment as the allelopathic species, and it has been suggested that stress would make them more sensitive to allelochemicals (Einhellig, 1995; Tang et al., 1995; Reigosa et al., 1999; Fistarol et al., 2005; Granéli and Pavia, 2005; Granéli and Hansen, 2006). Thus, under stress conditions the allelopathic effect may be higher due to both the increase in the production of allelochemicals and in the sensitivity of the target species, increasing the competitive advantaged of allelopathic algae.

5.2. INFLUENCE OF BIOTIC FACTORS

5.2.1. *The organisms involved*

The main factor influencing allelopathic interactions are the organisms involved. The allelopathic effect depends on both the allelopathic (i.e. donor) species and the target species (Figure 1). Usually, allelopathic species affect several, but not all target species, and target species are sensitive to several, but not to all allelochemicals (Fistarol et al., 2003; Fistarol et al., 2004; Suikkanen et al., 2004; Fistarol et al., 2005). Furthermore, the allelopathic effect also depends on the cell concentration of the donor and the target species (Schmidt and Hansen, 2001; Tillmann and John, 2002; Tillmann, 2003).

The different effect of allelopathic species on different target organisms, as well as the different responses of target organisms to allelochemicals from different allelopathic algae are reported in Fistarol et al. (2003, 2004a and 2005), Suikkanen et al. (2005). For example, in Fistarol et al. (2003 and 2004a), it was found that diatoms (in Figure 1, it could be, e.g. target 1) can be highly inhibited by *P. parvum* (e.g. allelopathic species, AS, 1), but they are only moderately inhibited by *A. tamarense* filtrate (e.g. AS 2). *A. tamarense,* on the other hand, has a higher effect on nanoflagellates (e.g. target 2) than on diatoms. The differentiated effect of allelopathic species on different targets, as well as the resistance shown by some target species, might have implications for the evolution of aquatic microalgae. Evolutionary constrains will be discussed further along in the text.

Since allelopathy is mediated by chemicals released into the medium, its effect depends on the cell concentration of the allelopathic organism. Increasing the allelopathic effect with increase in the cell concentrations has been shown for some phytoplankton groups, e.g. prymnesiophytes, and dinoflagellates (Schmidt and Hansen, 2001; Tillmann and John, 2002; Tillmann, 2003). Tillmann (2003) also demonstrated that an increase in the cell concentration of the target organism decreases the effect (in this case death of *Oxyrrhis marina*). Since the death is caused by lysis of *O. marina*, the author suggests that the toxic compounds are removed from the system when they bind to the cell membrane of the target organism.

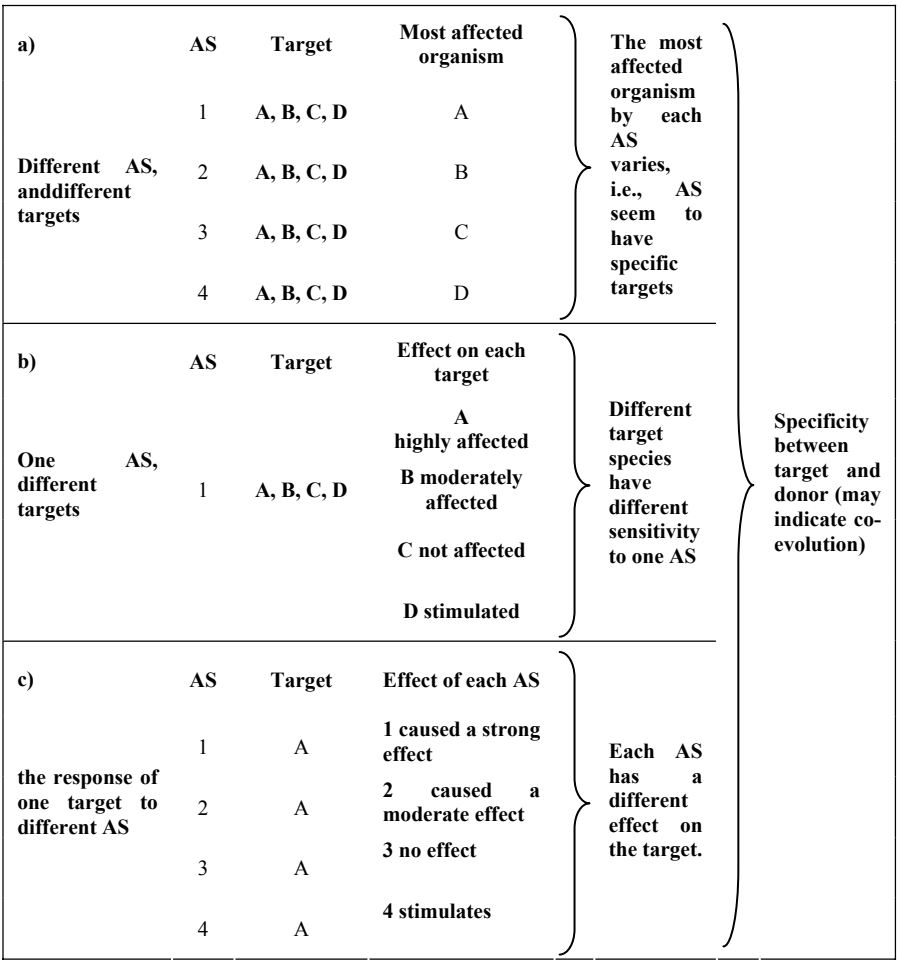

Figure 1. Comparison between the effects caused by different allelopathic species (AS) on one or several target organisms. The most affected organism by different allelopathic species varies (a). An allelopathic species will have different effects on each target (b). The same target organism can be highly affected by one allelopathic species and stimulated by another (c).

There are about 40 harmful phytoplankton species known to exhibit allelopathy (see Table 1). While in marine end estuarine waters the majority of the allelopathic species are found among the dinoflagellates, in fresh-water they are found among the cyanobacteria (Table 1). Nevertheless, it is among the flagellates that are found the allelopathic species producing the allelochemicals with the strongest negative impact on the other algal groups. Blooms of species such as *Chrysochromulina polylepis* and *Prymnesium parvum* are known for killing fauna and flora on the places they occur (Granéli and Johansson, 2003; Legrand et al., 2003).

5.2.2. *Effect of the growth phase*

The intensity of the allelopathic effect depends on the growth phase of the allelopathic species. Schmidt and Hansen (2001) and Suikkanen et al. (2004) demonstrated that allelopathic effect is caused by cells that are growing exponentially, that these effects decrease in the stationary phase, and that senescent cells do not cause allelopathic effects. Since allelopathy is a form of interference competition, it makes sense that the allelopathic species would be more allelopathic during exponential growth, while the cells can benefit from their effects, indicating that these compounds are important to the ecology of allelopathic species.

5.2.3. *Influence of bacteria*

Although most experiments on allelopathy of phytoplankton species were done with non-axenic cultures, it has been demonstrated that the bacteria present in the cultures are probably not responsible for the observed allelopathic effects (Suikkanen et al., 2004; Tillmann and John, 2002).

Tillmann and John (2002) eliminated the influence of bacteria present on *Alexandrium* spp. cultures by removing the bacteria through 0.2 μm membrane filters. They observed that the allelopathic effect of *Alexandrium* spp. did not alter, eliminating therefore the possible bacterial effect.

Suikkanen et al. (2004), tested if bacteria present in the non-axenic cultures of *Nodularia spumigena, Anabaena lemmermannii* and *Aphanizomenon flos-aquae* caused any allelopathic effect. The authors found that bacteria alone caused no effect on the target organism, indicating that the allelopathic effects they observed were indeed caused by cyanobacteria.

Although specific tests should be done for each allelopathic species, the examples above suggest that are indeed the phytoplankton species that cause the allelopathic effects and not bacteria.

6. Ecological Implications of Phytoplankton Allelopathy

Allelochemicals have been suggested to influence phytoplankton competetion, succession, and bloom formation or maintenance (Pratt, 1966; Keating, 1977; Rice, 1984 and references within; Lewis, 1986; Wolfe, 2000; Rengefors and Legrand, 2001; Vardi et al., 2002; Legrand et al., 2003; Fistarol et al., 2003, 2004; Suikkanen et al., 2005). It has been proposed that changes in plankton community structure are caused by the differential effect of allelochemicals on different targets (Mulderij et al., 2003). Target organisms may be completely eliminated, inhibited, resistant to the allelo-chemicals, or be stimulated (Fistarol et al., 2003, 2004a; Suikkanen et al., 2004).

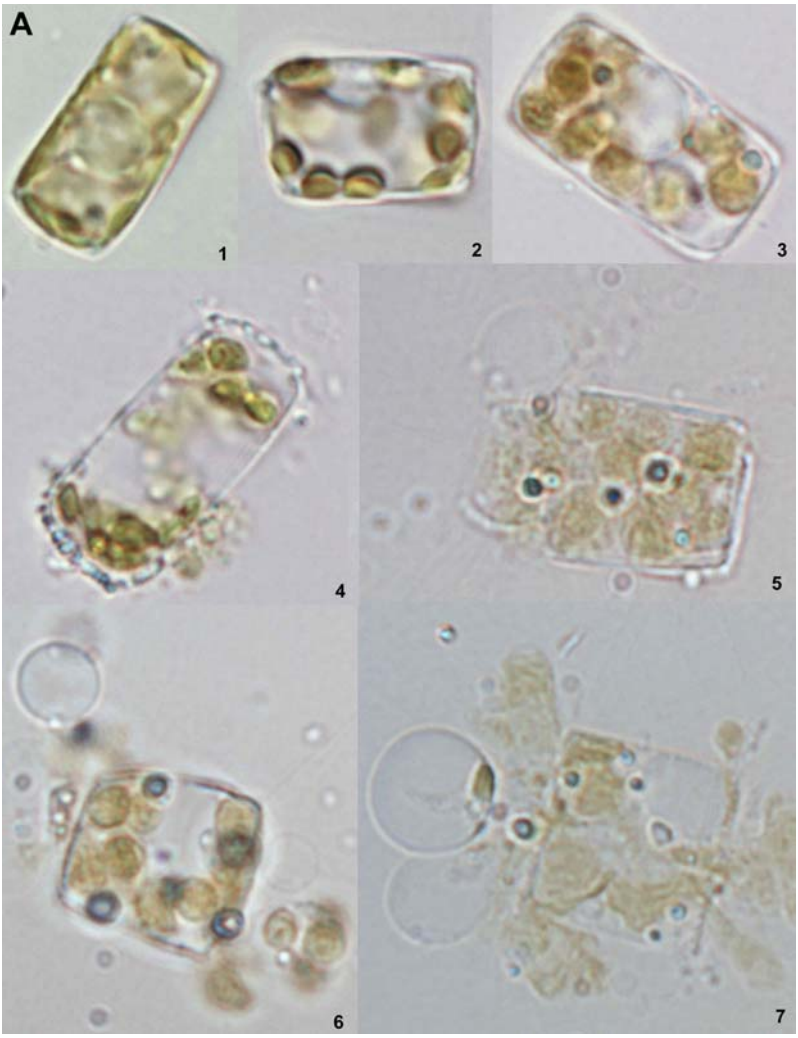

Figure 2. Different stages of cells of *Thalassiosira weissflogii* exposed to *P. parvum* filtrate. The first picture of the sequence shows a normal cell that has not been exposed to filtrate. The pictures are from different cells. Pictures 2 and 3 were after 1 h exposure, picture 4 and 5 after 5 h, and pictures 6 and 7 after 20 h. However, cells completely lyzed could already be observed after 5 h of exposure.

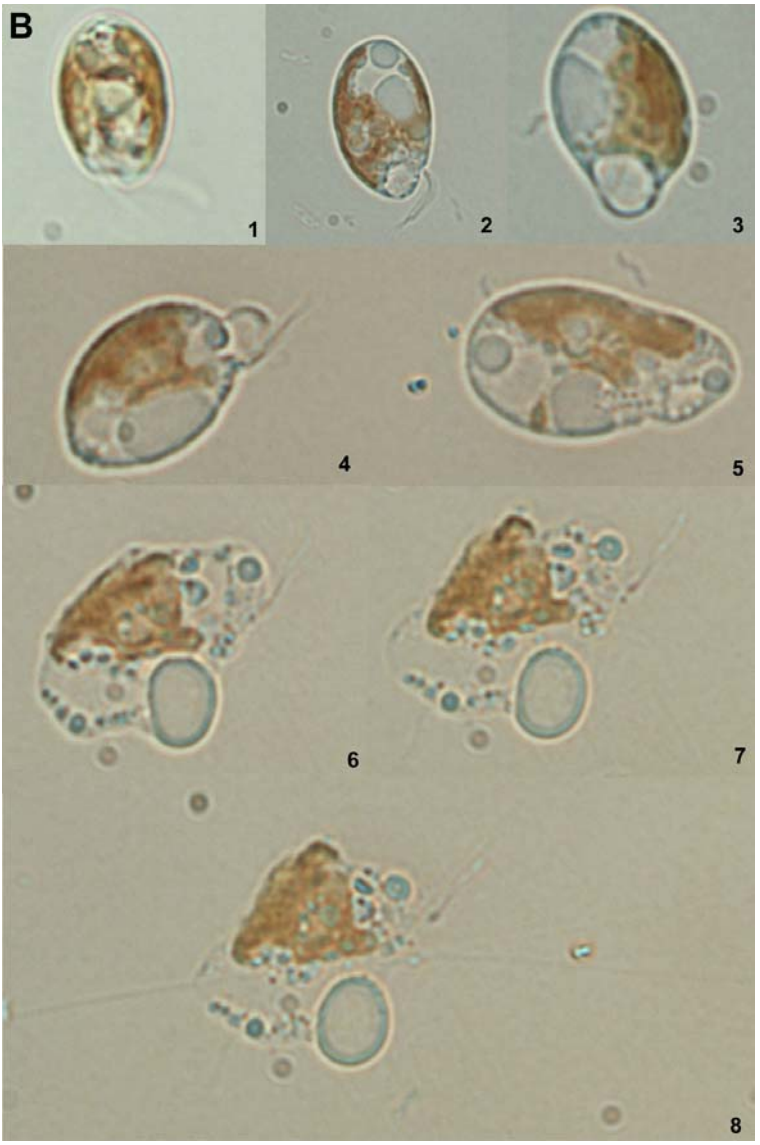

Figure 3. Different stages of cells of *Rhodomonas* sp. exposed to *P. parvum* filtrate. The first picture of the sequence shows a normal cell that has not been exposed to filtrate. The pictures are from different cells, except the three last *Rhodomonas* sp. picture that are all from the same cell. Except for the first *Rhodomonas* sp. picture, all other pictures were taken after 20 h of exposure, and the last three pictures were taken in an interval shorter that 2 min. However, as in the case of *T. weissflogii,* cells completely lysed could be found after 5 h of exposure. Thus, although observations have shown that one cell goes through all the stages depicted in the pictures, some cells are affected before the others.

Selective promotion or inhibition of the growth of individual species will influence succession and competition in aquatic environments.

It is hypothesized that the basic function of allelopathy is to give the donor organism a competitive advantage (Legrand et al., 2003), and the direct effect of allelopathy is shown by killing possible competitors. The fact that *Prymnesium parvum* (Fistarol et al., 2003) had a higher effect on diatoms (predecessor group under natural succession) than over cyanobacteria (group that usually blooms after the highest densities of P. parvum) indicates the potential competitive use of allelochemicals. The effect of *P. parvum* on a natural plankton community (Fistarol et al., 2003) shows how allelopathy can give a competitive advantage to an allelopathic species. The compounds excreted by P. parvum completely eliminated some of the competing phytoplankton groups and kept the biomass of the other groups at low levels (i.e. organisms that were not killed had a lower growth rate, and phytoplankton primary production decreased). Extrapolated to a natural situation these results suggest that *P. parvum* would have less species to compete with, and the ones remaining would show a deficient physiological state.

Usually the lethal effect of allelochemicals involves the lysis of the target organism. This is especially striking when the allelopathic algae caused a strong negative effect, as in *Prymnesium parvum*. Figure 2 and 3 shows different stages of cells of the diatom *Thalassiosira weissflogii* and the cryptophyceae *Rhodomonas* sp., respectively, after exposure to *P. parvum* filtrate. The figures shows how the cells change: how they start to lose pigmentation, that the cytoplasm seems to aggregate in vacuoles, that the cells start to blister, and finally that lyses occurs.

7. Allelopathy and Mixotrophy

Resource competition and grazing are traditionally the main mechanicsms used to explain phytoplankton population dynamics, and allelopathy is rarely taken into account. However, allelopathy, and also mixotrophy, have been shown to affect aquatic communities (Keating, 1977; Vardi et al., 2002; Fistarol et al., 2003, 2004a). It is very likely that these two strategies complement each other. Besides killing possible competitors, allelochemicals may give a further advantage to mixotrophic species, which can use the allelochemicals to help to obtain food mixotrophically, as in the case of *Prymnesium parvum* (Skovgaard and Hansen, 2003; Skovgaard et al., 2003; Tillmann, 2003). Allelochemicals can immobilize the prey, which are then are attacked, or alternatively the allelochemicals can lyse the cells which then release organic material (Skovgaard and Hansen, 2003; Skovgaard et al., 2003; Tillmann, 2003).

8. Conclusions: Evolutionary Aspects

Allelopathy affects phytoplankton succession and competition because it gives a competitive advantage to the allelopathic species and/or the resistant targets organisms. This fact has evolutionary implications because it may favor the selection of the resistant organisms, as it occurs with toxins released by microalgae species, which cause selective pressure on herbivorous organisms (by selecting the resistant ones) (Hairston et al., 2001). Both the tolerance showed by some organisms, and the fact differential effect of allelochemicals on each target species (Fistarol et al., 2003, 2004a; Suikkanen et al., 2004) suggest that co-evolution must be occurring. Furthermore, Fistarol et al. (2004b) shows an example of a behavioral strategy that could be used as a defense mechanism. The tolerance to allelochemicals shown by some target species is especially significant when it is found in successor algae, which then can achieve dominance over the allelopathic algae, as in the case of the resistance of cyanobacteria to *Prymnesium parvum* (Fistarol et al., 2003). Keating (1977) also showed that the phytoplankton groups succeeding the allelopathic species were positively affected, and achieved dominance over the predecessor allelopathic organisms.

Though allelopathic organisms might be good competitors under chemical interactions, they often are poor competitors for nutrients (Huntley et al., 1986). Thus, they will only dominate when their ecophysiological characteristics (Smayda, 1997) make them good competitors, causing a fluctuating selection, where the best competitor under certain conditions will dominate when these conditions occur, but it will be replaced when conditions change.

Enough evidence has been provided showing the ecological importance of phytoplankton allelopathy. The success of a certain microalgae species and the composition of aquatic communities is dependent on a series of abitic and biotic factors, and it is important to consider them all when studying aquatic systems.

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CHECKLIST OF PHYTOPLANKTON ON THE SOUTH COAST OF MURCIA (SE SPAIN, SW MEDITERRANEAN SEA)

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Abstract: The taxonomic structure of phytoplankton populations of southern coast of Murcia region were studied from January 2006 to January 2007. A total number of 243 taxa were identified: diatom was the most diverse algal group with 134 taxa (55%), followed by dinoflagellates with 67 (28%) and coccolithophorids with 33 taxa (14%). The groups represented by lowest number of species were *Chrysophyceae* with 6 (2.5%), *Eustigmatophyceae* with 1 (0.4%) and *Euglenophyceae* with 1 (0.4%). Only a floristic report has been already published for northern part of Murcian region (Ros and Miracle, 1984) and this is the first report of phytoplankton on the southern coast of region. A similar qualitative composition of the phytoplankton but with higher values has been found in a recent study in Alborán Sea (Mercado et al., 2005).

Keywords: Phytoplankton; checklist; SW Mediterranean Sea; Murcian coast

1. Introduction

Algae play an important role in the ecological balance of marine ecosystems. The continuity of marine biota depends on the photosynthetic activity of these organisms. Because of their rapid growth and short, simple life cycles, algae are potential indicators of water quality. Although diverse studies describe the structure of the communities of phytoplankton in western Mediterranean

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sea (Margalef, 1969; Delgado, 1990; Claustre et al., 1994; Fiala et al., 1994; Videau et al., 1994; Estrada et al., 1999; Vila et al., 2001a, 2001b; Vila and Masó, 2005; Mercado et al., 2005; Reul et al., 2005), only a report about a point of the Murcian coast located in the northern part of the region has been published (Ros and Miracle, 1984).

The increase of water demand has promoted the building of several desalinization plants that may contribute to the rise salinity. The warming climate with the increase of temperature, salinity, and nutrient concentrations, will probably change the taxonomic structure of phytoplankton communities and may favour the formation of HABs (Béthoux, 1989; Gómez, 2003).

The scope of this paper is to present a checklist of phytoplankton on the southern coast of Murcia region in order to have the reference conditions prior to the installation of a seawater desalination platform offshore.

2. Materials and Methods

The Mediterranean Sea is an oligotrophic semi-enclosed basin with a natural eastward decrease in productivity (Sournia, 1973). The studied area is influenced by Cartagena anticyclonic eddy, characterized by a low chlorophyll concentration and complex hydrology (Prieur and Sournia, 1994), with high biodiversity of flora and fauna and with ecosystems considered highly vulnerable (Calvín et al., 1998).

Monthly samples were collected from January 2006 to January 2007 at station P2 (Mazarrón 2). The studied station is situated 2,5 Km offshore at Mazarrón Bay (37º32'55''N, 4º46'7''E) in the southern part of the region. Samples derived from sampling at different seasons were collected in stations P2 (Mazarrón 2), P3 (Mazarrón 1), P4 (Calnegre) and P5 (Percheles), there

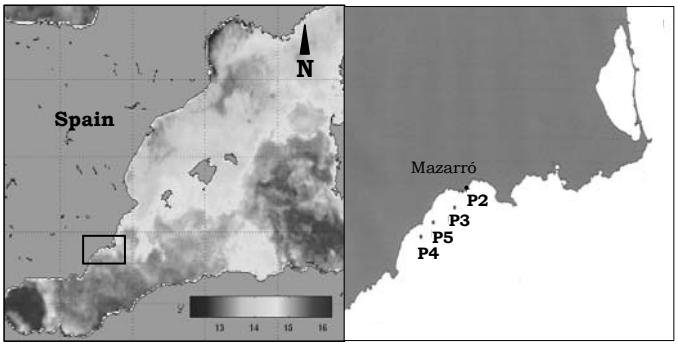

Figure 1. Study area along the Murcian coast showing the sampling stations.

were not significant differences between them. By this reason we refer only to P2 station (Mazarrón) (Fig. 1).

Samples were collected with a Niskin bottle (KC Denmark) at 5, 25 and 40 m depth and subsamples were fixed with formaldehyde, glutarahaldehyde and Lugol's iodine solution. Fresh and fixed samples were examined by light (OLYMPUS BX50) and scanning electron (JEOL-6100) microscopy after critical point drying for qualitative analysis.

The species richness was represented as the total number of taxa presented within the samples. The taxonomic identification of phytoplankton was carried out to the highest level possible using floras of Balech (1988), Cros and Fortuño (2002), Horner (2002), Delgado and Fortuño (1991), Hallegraff et al. (2003), Matsuoka and Fukuyo (2000), Ojeda (2006), Ricard (1987), Round et al. (1990), Sournia (1986), Tomas (1997), Witkowski et al. (2000).

3. Results and Discussion

Two hundred forty three taxa (117 genera) were identified in the phytoplankton of the south coast of Murcia belonging to *Bacillariophyceae*, *Dinophyceae*, *Haptophyceae*, *Dictyochophyceae*, *Chrysophyceae*, *Cryptophyceae*, *Ebriidae*, *Eustigmatophyceae* and *Euglenohyceae*. Table 1 presents a list of the identified taxa in samples with optical and electronic microscopy.

Bacillariophyceae were the most diverse algae group with 137 taxa (56%), followed by *Dinophyceae* with 64 (26%), *Haptophyceae* (mainly coccolithophorids) with 33 taxa (13%), and *Dictyochophyceae* with 4 (2%). The groups represented by the lower number of species, less than 1%, were *Chrysophyceae*, *Cryptophyceae*, *Ebriidae*, *Eustigmatophyceae* and *Euglenohyceae*.

The most diversified genera of diatoms were *Chaetoceros* sp. (27), *Thalassiosira* sp. (12) and *Ceratium* (10), *Protoperidinium* (7), *Prorocentrum* (6) and *Gonyaulax* (6) of dinoflagellates, while *Syracosphaera* (6) and *Umbilicosphaera* (3) were the most diversified coccolithophorids (Figs. 2–5).

Diatoms have been mostly investigated and thus comparable throughout the Mediterranean. The number of diatoms determined in different parts of the Mediterranean mostly varies between 107 and 183 (Viličić et al., 2002 and references therein). The greatest number of diatoms (400) is listed in the northern Mediterranean (Travers and Travers, 1973) and (518) in the eastern Adriatic Sea. Ros and Miracle (1984) reported 144 diatoms in a station of the northern coast of Murcia.

The most productive and studied areas on Spanish Mediterranean coasts are Catalan-Balearic and Alborán Seas that are associated to front and

upwelling features respectively. A similar qualitative composition of the phytoplankton but with higher values have found in a recent study during eight years on Alborán Sea (Mercado et al., 2005) and on the north coast of Murcia (Ros & Miracle, 1984). Mercado et al. (2005) described 180 diatoms, 118 dinoflagellates and 31 coccolithophorids. Ros & Miracle (1984) cited 144 diatoms, 126 dinoflagellates and 17 crysophytes. A common feature on the south coast of Spain (Alborán Sea) is a gradient of diatoms diversity from western to eastern related with the ecological gradient between Atlantic and Mediterranean waters (Delgado, 1990; Rivera, 2004).

Gómez (2003) reported 604 species of free-living planktonic dinoflagellates in the western Mediterranean Sea where the Algerian sub-basin (22%) showed the lowest number of species. We found many species of dinoflagellates that have not been cited in Algerian sub-basin, but this report considered only studies of African coast to elaborate the checklist of Algerian subbasin.

Species diversity of phytoplankton is difficult to compare due to different methodologies of determination to elaborate the checklist (optical and

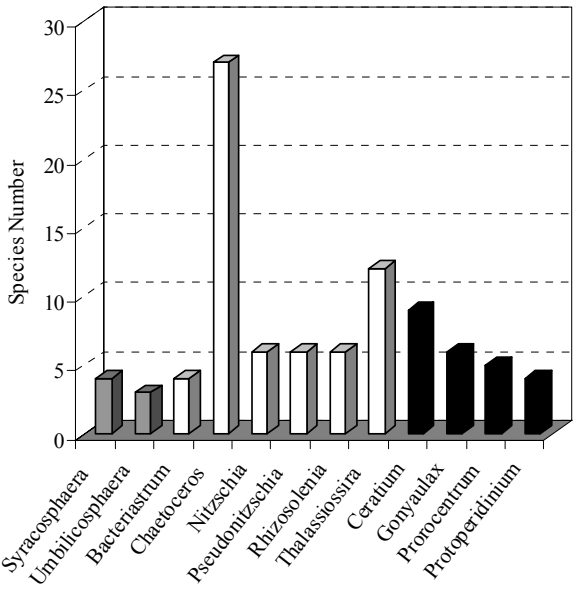

Figure 2. Species number of most diversified genera of main taxonomic groups.

electronic microscopy), sampling periods (days or weeks for single oceanographic surveys *versus* annual samplings) and the different depths of samplings.

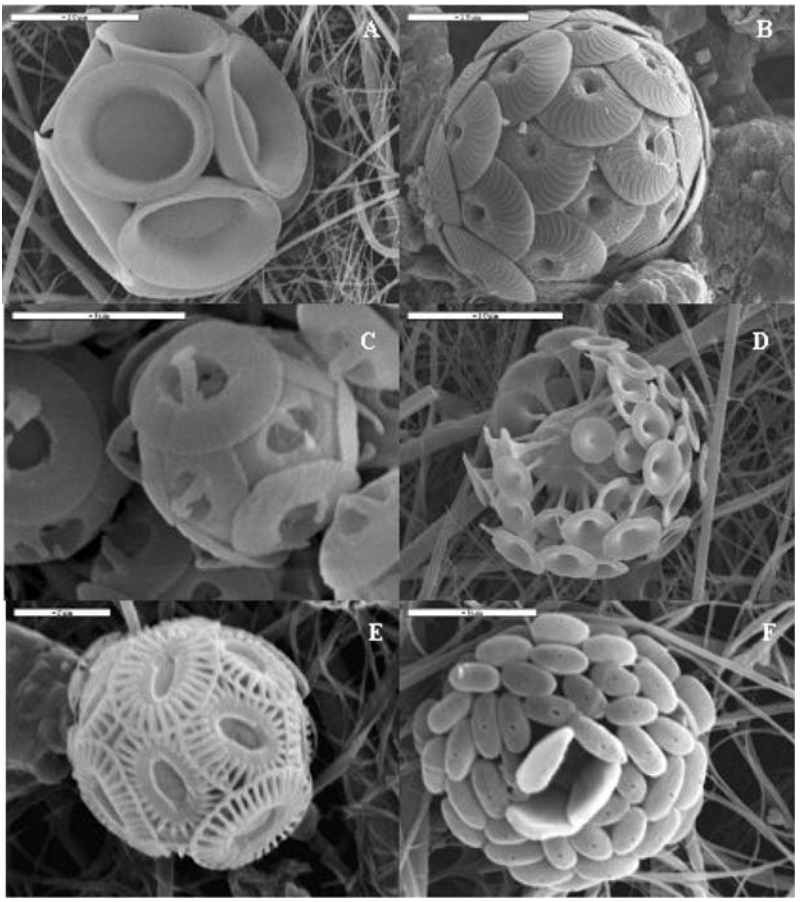

Figure 3. Scanning electronic microscope photographs of the coccolithophorids: 1. *Pontosphaera syracusana* Lohmann, 2. *Calcidiscus leptoporus* (Murria et Blackman) Loeblich et Tappan, 3. *Gephyrocapsa oceanica* Kampter, 4. *Discosphaera tubifera* Murria et Blackman, 5. *Emiliania huxleyi* Hay and Moler, 6. *Algirosphaera robusta* (Lohmann) Norris. Scale bars: A, B, D: 10 µm; C, F: 5 µm; E: 2 µm.

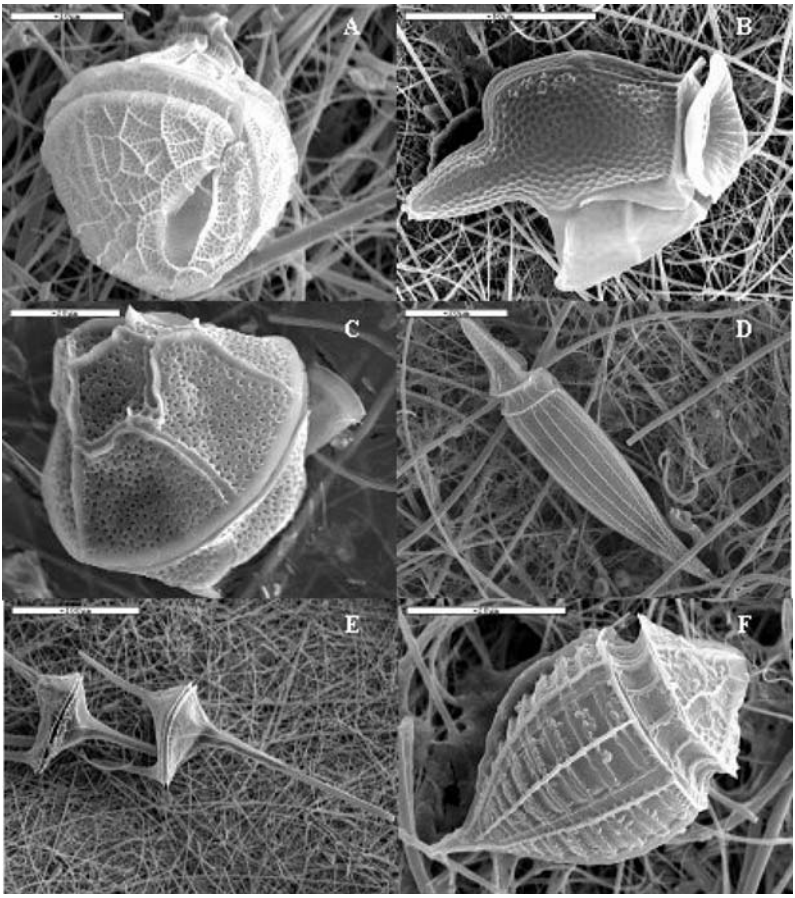

Figure 4. Scanning electronic microscope photographs of the dinoflagellates: A. *Gonyaulax* cf. *scrippsae* Kofoid, B. *Dinophysis caudata* Saville-Kent, C. *Triadinium polyedricum* (Pouchet) Dodge, D. *Oxytoxum longiceps* Schiller, E. Chain of *Ceratium candelabrum* (Ehrenberg) Stein, F: *Corythodinium tesselatum* (Stein) Loeblich & Loeblich. Scale bars: A: 10 µm; B: 50 µm; C, D, F: 20 µm; E: 100 µm.

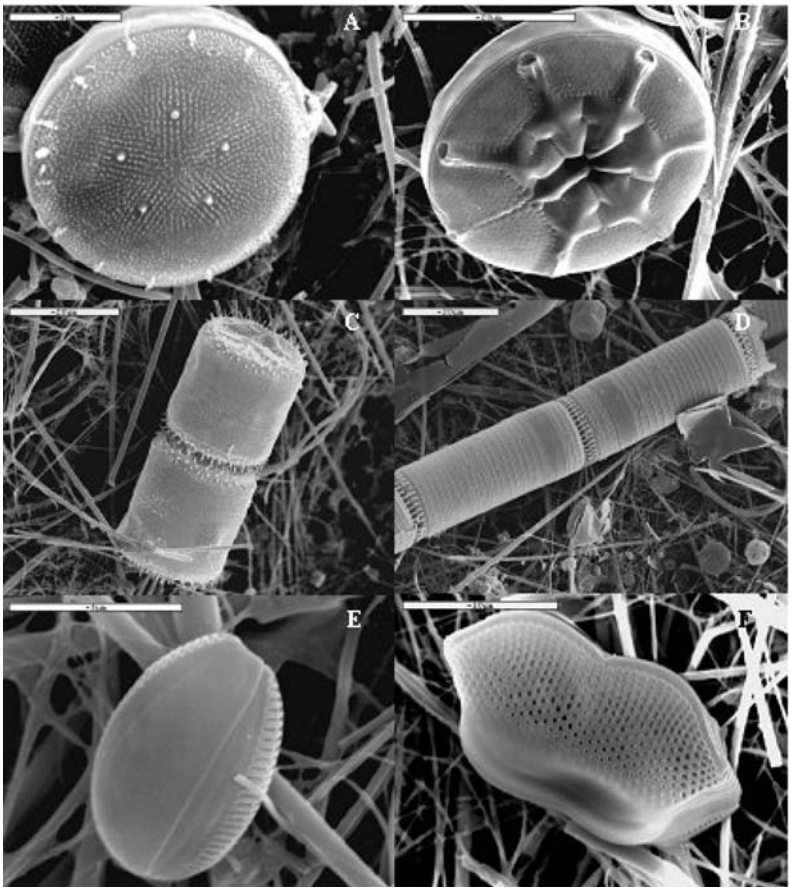

Figure 5. Scanning electronic microscope photographs of the diatoms: A. *Thalassiosira mediterranea* (Schröder) Hasle, B. *Asteromphalus parvulus* Karsten, C. *Lauderia annulata* Cleve, D. Chain of *Detonula pumila* (Cleve) Grunow, E. *Amphora* cf. *coffeaformis* (Agardh) Kützing, F: *Nitzschia panduriformis* var. *continua* Grunow. Scale bars: A, E: 5 µm; B, C, D: 20 µm; F: 10 µm.

TABLE 1. List of taxons of phytoplankton determined on the south coast of Murcia.

HAPTOPHYCEAE
Acanthoica quatrospina Lohmann
Anoplosolenia brasiliensis Lohmann
Algirosphaera robusta (Lohmann) Norris
Alisphaera sp.
Alisphaera unicornis Okada et McIntyre
Calcidiscus leptoporus (Murria et Blackman) Loeblich et Tappan
Calciosolenia murayi Gran
Calytrosphaera sp.
Ceratolithus sp.
Cyrtosphaera lecaliae Kleijne
Daktylethra pirus (Kamptner) Norris
Discosphaera tubifera Murria et Blackman
Emiliania huxleyi Hay & Moler
Florisdosphaera sp.
Garderia corolla (Lecal) Kleijne
Gephyrocapsa oceanica Kampter
Gephyrocapsa ornata Heimdal
Helicosphaera carteri (Wallich) Kamptner
Helladosphaera cornifera (Schiller) Kamptner
Palusphaera vandeli Lecal
Phaeocystis sp.
Pontosphaera syracusana Lohmann
Rhabdosphaera clavigera Murray & Blackman
Syracosphaera anthos (Lohmann) Janin
Syracosphaera histrica Kamptner
Syracosphaera molischii Schiller
Syrascosphaera noroitica Knappertsbusch
Syracosphaera prolongata sensu Heimdal et Gaarder

(Continued)

Syracosphaera pulchra Lohmann

Umbellosphaera tenuis (Kamptner) Paasche

Umbilicosphaera sp.

Umbilicosphaera sibogae var. *sibogae* (Weber-van Bose) Gaarder

Umbilicosphaera sibogae var. *foliosa* (Kamptner) Okada et McIntyre

BACILLARIOPHYCEAE

Achnanthes cf. *placentuloides* Guslakov

Achnanthes cf. *brevipes* var. *pabula* Hustedt

Amphora cf. *coffeaformis* (Agardh) Kützing

Amphora cf. *turgida* Gregory

Amphora sp.

Ardissonea cf. *cristalina* (Agardh) Grunow

Asterionellopsis glacialis (Castracane) Round

Asteromphalus parvulus Karsten

Bacteriastrum cf. *elongatum* Cleve

Bacteriastrum delicatulum Cleve

Bacteriastrum furcatum Shadbolt

Bacteriastrum hyalinum Lauder

Biddulphia sp.

Biremis sp.

Ceratulina cf. *dentata* Hasle

Ceratulina cf. *pelagica* (Cleve) Hendey

Chaetoceros affinis Lauder

Chaetoceros atlanticus Cleve

Chaetoceros cf. *brevis* Schütt

Chaetoceros cf. *lauderi* Ralfs in Lauder

Chaetoceros compressus Lauder

Chaetoceros convolutus Castracane

Chaetoceros curvisetus Cleve

Chaetoceros danicus Cleve

(Continued)

TABLE 1. *(Cont)*

Chaetoceros densus Cleve
Chaetoceros decipiens Cleve
Chaetoceros denticulatus Lauder
Chaetoceros diadema (Ehrenberg) Gran
Chaetoceros holsaticus Schuett
Chaetoceros didymus Ehrenberg
Chaetoceros lorenzianus Grunow
Chaetoceros peruvianus Brightwell
Chaetoceros pseudocurvisetus Mangin
Chaetoceros radicans Schütt
Chaetoceros rostratus Lauder
Chaetoceros similis Cleve
Chaetoceros simplex Ostenfeld
Chaetoceros socialis Lauder
Chaetoceros tenuissimus Meunier
Chaetoceros teres Cleve
Chaetoceros tetrastichon Cleve
Chaetoceros vixvisibilis Schiller
Chaetoceros sp1.
Chaetoceros sp2.
Chaetoceros sp3.
Chamaepinnularia alexandrowiczii Witkowski
Cocconeis cf. *scutellum* Ehrenberg
Cocconeis cf. *haunensis* Witkowski
Cyclotella litoralis Lange-Syvertsen
Cyclotella striata (Kützing) Grunow
Cylindrotheca closterium (Ehrenberg) Reimann et Lewin
cf. *Cymatosira* sp.
Dactyliosolen cf. *blavyanus* (Peragallo) Hasle

(Continued)

Dactyliosolen cf. *fragilissimus* (Bergon) Hasle

Dactyliosolen cf. *phuketensis* (Sundström) Hasle

Detonula cf. *confervacea* (Cleve) Gran

Detonula pumila (Cleve) Grunow

Diploneis sp.

Diploneis bombus Ehrenberg

Diploneis decipiens var. *paralella* Cleve

Diploneis cf. *ovalis-eliptica* Hilse

Dytilum brightwellii (West) Grunow

Entomoneis sp.

Eucampia sp.

Eucampia zodiacus Ehrenberg

Eucampia zodiacus cf. *cylindricornis* Syvertsen

Ethmodiscus sp.

Falcula sp.

Fallacia hyalinula (De Toni) Stickle & Man

Fragilariopsis rhombica (O'Meara) Hustedt

Guinardia delicatula (Cleve) Hasle

Guinardia striata (Stolterfoth) Hasle

Hemiaulus hauckii Grunow

Hemiaulus sinensis Greville

Lauderia annulata Cleve

Leptocilyndrus danicus Cleve

Leptocilyndrus mediterraneus (Peragallo) Hasle

Leptocilyndrus minimus Gran

Licmophora sp.

Lioloma cf. *delicatulum* (Cupp) Hasle

Mastogloia sp.

Melosira cf. *nummuloides* Agardh

Meuniera membranacea (Cleve) Silva

(Continued)

TABLE 1. *(Cont)*

Minidiscus triocularis (Taylor) Hasle
cf. *Nanoneis hasleae* (Norris)
Navicula sp.
Navicula cf. *korzeniewskii* Witkowski
Navicula cf. *halinae* Witkowski
Nitzschia sp.
Nitschia compressa (Bailey) Boyer
Nitschia cf. *bicapitata* Cleve
Nitzschia closterium (Ehrenberg) Smith
Nitzschia longissima Brébisson
Nitzschia panduriformis var. *continua* Grunow
Nitzschia cf. *sicula* (Castracane) Hustedt
Nitzschia cf. *reversa* Smith
Odontella mobiliensis (Bailey) Grunow
Oestrupia sp.
Placoneis cf. *gastrum* Cox
Pleurosigma normanii Ralfs in Pritchard
Porosira cf. *pentaportula* Syvertensen & Lange
Pseudo-nitzschia cf. *australis* Frenguelli
Pseudo-nitzschia cf. *delicatissima* (Cleve) Heiden
Pseudo-nitzschia cf. *fraudulenta* (Cleve) Hasle
Pseudo-nitzschia cf. *lineola* (Cleve) Hasle
Pseudo-nitzschia subcurvata (Haslle) Fryxell
Pseudo-nitzschia sp.
Pseudosolenia cf. *calcar-avis* (Schultze) Sundström
Proboscia alata (Brightwell) Sundström
Rhizosolenia castracaeni Peragallo
Rhizosolenia imbricata Brightwell
Rhizosolenia fragilisima Bergon

(Continued)

Rhizosolenia setigera Brightwell

Rhizosolenia sp.

Rhizosolenia striata Greville

Rhopalodia cf. *musculus* (Kützing) Müller

Skeletonema pseudocostatum (Medlin) Zingone et Sarno

Stephanopyxis sp.

Stephanopyxis palmeriana (Greville) Grunow

Synedropsis hyperborea (Grunow) Hasle

Suriella brebissonii Krammer & Lange-Bertelot

Suriella sp.

Thalassiosira cf. *anguste-lineola* (Schmidt)

Thalassiosira cf. *allenii- diporocyclus* Hasle

Thalassiosira cf. *oestrupii* var. *veurickae* Fryxell

Thalassiosira binata Fryxell

Thalassiosira cf. *ecchinata* Semina

Thalassiosira leptopus Grunow

Thalassiosira mediterranea (Schröder) Hasle

Thalassiosira punctigera (Castracane) Hasle

Thalassiosira rotula Meunier

Thalassiosira tenera Proschkina-Lavrenko

Thalassiosira sp1.

Thalassiosira sp2.

Thalassionema nitzschioides (Grunow) Mereschkowsky

DINOPHYCEAE

Achradina pulchra Lohmann

Alexandrium sp.

Alexandrium affine (Inoue & Fukuyo) Balech

Alexandrium cf. *concavum* (Gaarder) Balech

Amphidinium lacustre Stein

Amphidinium sphenoides Wulff

(Continued)

TABLE 1. *(Cont)*

Amphidinium sp.
Amylax sp.
Centrodinium sp.
Ceratocorys horrida Stein
Ceratium azoricum Cleve
Ceratium candelabrum (Ehrenberg) Stein
Ceratium declinatum (Karsten) Jörgensen
Ceratium furca (Ehrenberg) Claparède & Lachmann
Ceratium fusus (Ehrenberg) Dujardin
Ceratium cf. *incisum* (Karsten) Jörgensen
Ceratium macroceros (Ehrenberg) Vänhoffen
Ceratium massiliense (Gourret) Jorgensen
Ceratium teres Kofoid
Ceratium trichoceros (Ehrenberg) Kofoid
Corythodinium tesselatum (Stein) Loeblich & Loeblich
Dinophysis acuminata Claparède & Lachmann
Dinophysis caudata Saville-Kent
Heterocapsa sp.
Heterodinium cf. *dispar* Kofoid & Adamson
Histioneis longicollis Kofoid
Histioneis cf. *cymbalaria* Stein
Goniodoma polyedricum (Pouchet) Jorgensen
Goniodoma sphaericum Murray & Whitting
Gonyaulax sp.
Gonyaulax pacifica Kofoid
Gonyaulax polygrama Stein
Gonyaulax cf. *scrippsae* Kofoid
Gonyaulax cf. *spinifera* (Claparède & Lachmann) Diesing
Gonyaulax cf. *verior* Sournia

(Continued)

Gotoius abei Matsuoka

Gymnodinium cf. *catenatum* Graham

Gymnodinium impudicum Fraga & Bravo

Mesoporos perforatus (Gran) Lillick

Nematodinium sp.

Noctiluca scintillans (Macartney) Kofoid & Swezy

Ornithocercus magnificus Stein

Ostreopsis cf. *ovata* Fukuyo

Oxytoxum longiceps Schiller

Oxytoxum cf. *ovale* Schiller

Oxytoxum scolopax Stein

Phalacroma rotundatum (Claparède & Lachmann) Kofoid & Michener

Podolampas spinifera Okamura

Podolampas palmipes Stein

Prorocentrum balticum (Lohmann) Loeblich

Prorocentrum compressum (Bailey) Abé ex Dodge

Prorocentrum micans Ehrenberg

Prorocentrum balticum (Lohmann) Loeblich

Prorocentrum gracile Stein

Prorocentrum triestinum Schiller

Protoceratium aerolatum Kofoid

Protoperidinium sp.

Protoperidinium crassipes Kofoid

Protoperidinium cerasus (Paulsen) Balech

Protoperidinium divergens (Ehrenberg) Balech

Protoperidinium mediterraneum (Kofoid) Balech

Protoperidinium biconicum Dangeard

Roscoffia sp.

Scrippsiella cf. *trochoidea* (Stein) Loeblich

Spiraulax sp.

Triadinium polyedricum (Pouchet) Dodge

(Continued)

TABLE 1. *(Cont.)*

DICTYOCHOPHYCEAE
Dictyocha fibula Ehrenberg
Dictyocha cf. *speculum* Ehrenberg
Dictyocha staurodon Ehrenberg
Octatis cf. *octanaria* (Ehrenberg) Hovasse
EBRIIDEA
Hermesinum adriaticum Zacharias
CHRYSOPHYCEAE
Meringosphaera mediterranea Lohmann
CHRYPTOPHYCEAE
Cryptomonas sp.
EUSTIGMATOPHYCEAE
Nannochloropsis sp.
EUGLENOPHYCEAE
Eutreptiella sp.

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TOXIC *PSEUDO-NITZSCHIA* POPULATIONS FROM THE MIDDLE TYRRHENIAN SEA (MEDITERRANEAN SEA, ITALY)

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Abstract: Several diatoms of the genus *Pseudo-nitzschia* are able to produce domoic acid (DA), a neurotoxic aminoacid responsible for Amnesic Shellfish Poisoning (ASP) in humans and animals worldwide. *Pseudo-nitzschia* spp. are widespread along Italian coast, however current knowledge of their diversity, temporal succession and potential toxicity is limited to restricted areas. Blooms of these diatoms have been increasingly recorded along the Latium region coast (Middle Tyrrhenian Sea, Mediterranean Sea) in the last decades prompting investigation of *Pseudo-nitzschia* diversity and potential toxicity in natural communities from coastal stations over one year. Results of the taxonomical analysis are here reported together with distribution data, at species complex resolution, that were collected during a regional long-term program. Transmission electron microscopy on acid cleaned frustules ascertained the presence and identity of eight *Pseudo-nitzschia* species, six of which are known ASP producers. One species, *P. inflatula*, is reported for the first time from Italian waters. Distributional data evidenced coexistence of different species during blooms, seasonal cycles and inter-annual variability, abundances of the genus reached 10^7 cell/l^{-1}.

Keywords: *Pseudo-nitzschia* spp., ultrastructure, domoic acid, distribution, Middle Tyrrehnian Sea

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1. Introduction

Needle-shaped, raphid diatoms belonging to the planktonic marine genus *Pseudo-nitzschia* H. Peragallo have been shown to be the causative agent of Amnesic Shellfish Poisoning, ASP, in humans (Bates et al., 1989) and for extensive sea fauna deaths in the last decades (Scholin et al., 2000; Shumway et al., 2003). Eleven *Pseudo-nitzschia* species are currently known to produce domoic acid, DA, (Lundholm and Moestrup, 2007) a neurotoxic aminoacid that accumulates through foodweb impacting marine organisms, human consumers, causing serious neurological disorders, and ultimately ecosystems and economy. The presence of these widespread, chain forming microalgae has frequently been recorded in Italian coastal waters, particularly following 2001, when a national environmental monitoring program was started at 500 m offshore stations using light microscopy to identify phytoplankton. Intense proliferations of *Pseudo-nitzschia* were also repeatedly observed during long term programs carried out along Latium, Campania and Apulia region coasts (Congestri et al., 2004; Caroppo et al., 2005; Congestri et al., 2006; Zingone et al., 2006). Strains of two species, *Pseudo-nitzschia galaxiae* and *P. multistriata*, isolated from the Gulf of Naples, proved to be toxic (Sarno and Dahlman, 2000; Cerino et al., 2005). However, DA was never detected in mussels collected from Italian sites although it has been reported for French Mediterranean (Amzil et al., 2001) and Greek stocks (Kaniou-Grigoriaou et al., 2005).

To date, knowledge of *Pseudo-nitzschia* occurrence, seasonal dynamics and potential toxicity in Italian waters is restricted to few areas. Spot studies on *Pseudo-nitzschia* diversity and sporadic (non-mandatory) control of DA in seafood contribute to a limited awareness of the real risk of contamination and human poisoning. This is mostly due to the need of a very high level of taxonomical expertise to detect the subtle ultrastructural differences between species and harmful and non-harmful members of this genus. Frustule details are crucial for identification and only visible with transmission electron microscopy (TEM), but TEM equipment is not routinely used during algal surveillance programs. Consequently, monitoring of phytoplankton to protect human health and fisheries during a *Pseudo-nitzschia* bloom is time consuming and this prevents in many cases early warning procedures aimed to mitigate potential danger. Difficulties in assessing true *Pseudo-nitzschia* identity and the considerable interest in having fast and accurate response on its potential toxicity all over the world has led to the development of molecular tools to assist identification at the species level. DNA probes, targeting specific rDNA sequences of DA producers, were designed to discriminate between species of *Pseudo-nitzschia* (Miller and Scholin, 1996; 1998; Scholin et al., 1996). However this approach gave controversial results

when applied to natural communities, preventing a clear-cut separation between specimens (Parsons et al., 1999; Orsini et al., 2002). It is also known that the potential to produce DA varies across clones of the same species and with cell nutrient status and life cycle (Pan et al., 1996; Bates et al., 1998), leading to a possible lack of correlation between the abundance of potentially toxic *Pseudo-nitzschia* registered in the water samples during conventional cell counts, or by means of probes, and their toxicity. This involves the risk that countermeasures may be taken in presence of non-toxic populations or, alternatively, that very rare but extremely toxic organisms are overlooked.

To meet the need of monitoring *Pseudo-nitzschia* spp. dynamics and their potential toxicity along Latium region coast, a 2-phase approach that coupled fine ultrastructural examination of field samples and "real time" toxin analysis, using Screen Printed Electrodes (SPEs, Congestri et al., submitted), was applied on samples collected over one year. Direct detection of DA presence in "critical" (comprising potential toxin producers) phytoplankton matrices with SPEs allow an optimised and reliable monitoring tool to respond to safe food resources demand, making also possible the traceability of toxin along the food chain. This approach minimises probability of seafood contamination by development of prevention strategies based on early warning measures and contingency plans (Palleschi et al., 2002, Albertano et al., this volume).

Here we report on the identity of *Pseudo-nitzschia* populations in samples collected over the year of study together with *Pseudo-nitzschia* distribution data, at species complex resolution, that were gathered during a regional long term program based on light microscopical analysis of phytoplankton. Parallel toxin analysis using SPEs and validated by means of HPLC techniques gave positive results that are reported elsewhere (Congestri et al., submitted).

2. Materials and Methods

The taxonomical investigation was conducted on concentrated phytoplankton collected fortnightly at 6 stations along the Latium region coast over one year (January 2001–December 2002). Samples were obtained by horizontal net (20 μm-mesh) tows. Aliquots were fixed in 2.5% glutaraldehyde for electron microscopical analysis, others opportunely treated and stored for DA determination by HPLC and SPEs (Micheli et al., 2004; Congestri et al., submitted). Measurements of cell and frustule dimensions and evaluation of cell and chain shape were mainly based on light microscopical (LM) analysis of uncleaned material using a Zeiss Axioskop microscope at 40 and 100 × magnification. Ultrastructural details as density of striae (rows

of perforations, poroids, across the valve thickness) and fibulae (bridges of silica between portions of the valve margin that bears the raphe, adjacent fibulae are separated by interspaces) presence of a central larger interspace, poroid structure and arrangement and also girdle band features, were examined on acid (HNO_3 and H_2SO_4) cleaned samples, air-dried on formvar-coated copper grids, using a Zeiss CEM 902 transmission electron microscope, TEM, at 80 KV.

3. Results and Discussion

3.1. MORPHOLOGICAL AND ULTRASTRUCTURAL DIVERSITY OF *PSEUDO-NITZSCHIA* ALONG THE LATIUM COAST

Morphometric traits as frustule shape, size of apical and transapical axes (the valve length and width respectively, when measurements are performed in valve view), and morphology of frustule apices together with the degree of cell end overlap in the stepped colonies, contributed to primary, gross distinction of a number of different *Pseudo-nitzschia* "morphotypes" in the samples. Analysis of data gathered during LM investigation in the light of following taxonomical revision of the genus, especially of the *Nitzschia delicatissima* complex *sensu* Hasle (1965), allowed to distinguish between six different morphospecies with light microscopy.

Colonies of linear cells with rounded apices in valve view and with distinctive sigmoid valve ends in girdle view, of approximately 50 μm in length and 3–3.2 μm in width, were rather easily attributed to *Pseudo-nitzschia multistriata* (Takano) Takano (Fig. 1 a, b).

Chains of lanceolate cells of about 20 μm in length, with a central swelling, in valve view, visible with LM (Fig. 1 c, d), and formerly attributed to *Pseudo-nitzschia prolongatoides*, have been assigned to *P. galaxiae* Lundholm et Moestrup following Lundholm and Moestrup (2002). Field specimens and strains from the Gulf of Naples have been shown to undergo dramatic mor-phological alternation during the seasonal cycle, with dimension of apical axis ranging between 10 and 80 μm and visible change in frustule shape, from linear cells, typical of the larger stages, to rostrate solitary smaller individuals (Cerino et al., 2005). We did not observe *P. galaxiae* in cleaned material under TEM, but the morphometric features of the colonial forms observed during cell counts unequivocally point to *P. galaxiae*. In any case, the presence of this species could have been overlooked during monitoring activities based on LM, as the larger stages could be confused with *P. delicatissima sensu* Hasle (1965).

Gross morphological features revealed during LM analysis allowed to attribute fusiform cells of about 5–6 μm in width and 76–114 μm long to the *Nitzschia seriata* complex *sensu* Hasle (1965). This morphotype, readily distinguishable in valve view had pointed apices with an overlap of adjacent cell endings in the colonies of about 1/6–1/8 of cell length (Fig. 1 e, f).

TEM investigation ascertained its identity as *Pseudo-nitzschia fraudulenta* (Cleve) Hasle. Fibulae and striae were approximately equal in numbers, 19–22 in 10 μm, with fibulae aligned with valve interstriae. Each stria had two or three rows of poroids, there were 5–6 poroids in 1 μm. Poroid vela consisted of hymenate sectors variable in number, usually 4 or 5. Mantle was three poroid high (Fig. 2 a-f).

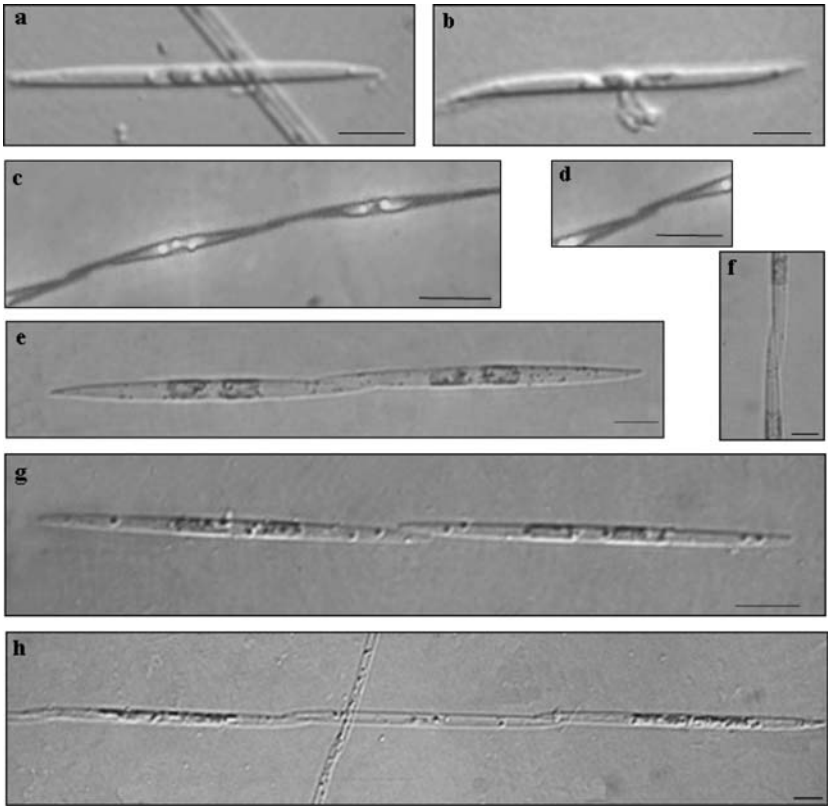

Figure 1. LM micrographs. *Pseudo-nitzschia multistriata* in valve, **a**. and girdle view, showing valve sigmoid shape, **b**. Chain detail of P. galaxiae, **c**. note the central swelling and overlap of adjacent valve ends, **d**. Girdle view of two lanceolate cells of *P. fraudulenta* in chain, **e.** the overlap of pointed apices in the close-up, **f**. *Pseudo-nitzschia delicatissima sensu* Hasle (1965) a short overlap of truncated valve apices was visible in girdle view of the linear valves, **g**. Chain of linear valves tapering towards pointed apices identified as *P. pseudodelicatissima sensu* Hasle (1965)**, h**. Bars =10 μm.

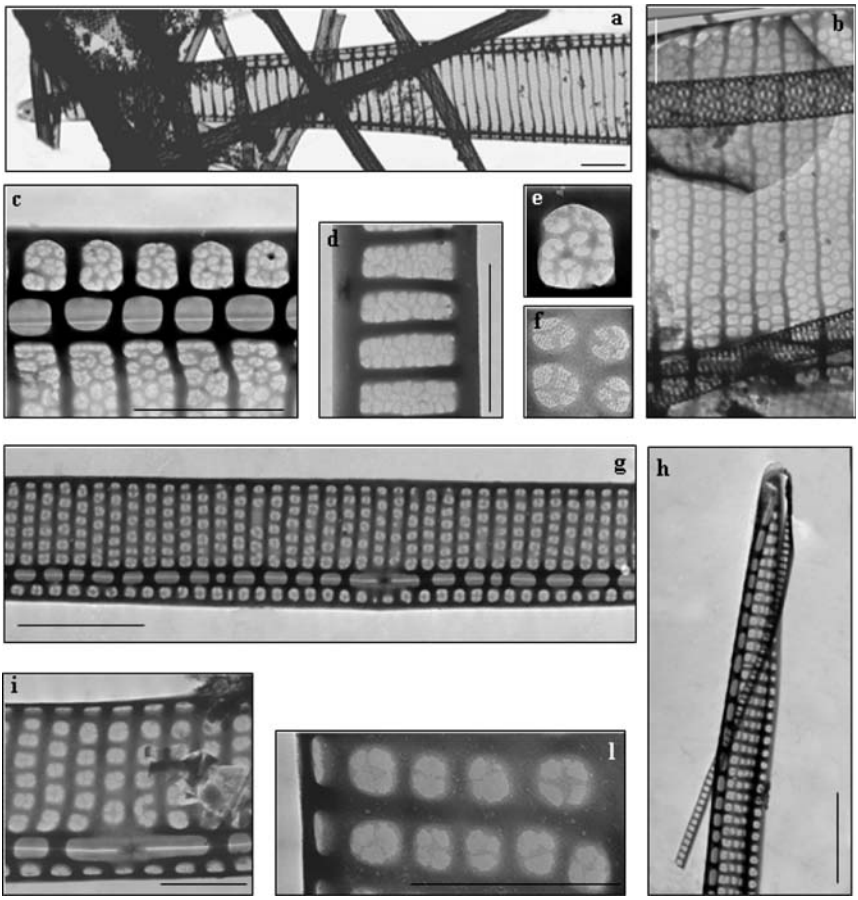

Figure 2. TEM micrographs of *Pseudo-nitzschia fraudulenta*, a-f, and *P. inflatula*, g-l. Tip of fusiform valve showing bi- and triseriate striae, **a**. that were clear at a greater magnification, **b**. Valve and mantle (proximal) poroid structure in **c**. mantle poroid detail in **e**. Girdle band, with the same pattern of striation as the valve, is shown in **d**. hymen of girdle band poroids in **f**. Central part of the valve showing the central larger interspace and the central nodule, of which a close up is shown in **i**. uniseriate striae of the valve and proximal mantle are also visible as well as a slight swelling in the middle, **g**. Valve inflation was more evident at the valve end, **h**. Variation in hymen structure of round to square poroids are shown in **l**. Bars = 2 (a, b, g, h) and 1 μm (c, d, i, l).

Valves of *Pseudo-nitzschia inflatula* (Hasle) Hasle were only distinguished during TEM analysis. The gross morphology of this species is very similar to that of *P. pseudodelicatissima sensu* Hasle (1965). During LM examination of samples, linear cells, 80–100 μm long, with pointed apices in both views were referred to as *P. pseudodelicatissima* long morphotype, although the latter cells were wider, 1.5–2 μm, than those labelled as *P. pseudodelicatissima* during LM investigation. TEM analysis clearly revealed

the presence of inflations in the middle and close to the apices of the valves that together with the following features: presence of uniseriate striae, 30–34 in 10 μm, a number of 16–20 fibulae in 10 μm, the central larger interspace and round to square poroids, 4–5 in 1 μm, with the hymen divided into two large perforated parts separated by narrow strips of silica, led to ascribe this morphotype to *P. inflatula* (Fig. 2 g-l). This is the first report of this species in Italian waters. *P. inflatula* has been recently reported from the Mexican Pacific (Hernandez-Becerril and Diaz-Almeyda, 2006) and formerly by other authors from different locations, leading to consider its distribution to be cosmopolitan.

Linear, narrow cells about 50–70 μm long and 0.6–1.8 μm wide, tapering towards the apices that had cut-off ends, with a very short cell overlap in chains, were attributed to *Pseudo-nitzschia delicatissima sensu* Hasle (1965) during LM observation (Fig. 1 g). TEM analysis of cleaned valves revealed ultrastructural details that fitted the original description of *P. delicatissima* by Hasle (1965), as biseriate striae, 32–40 in 10 μm, of triangular to exagonal small poroids, approximately 8–9 in 1 μm, with finely hymenate vela along with the presence of the central larger interspace, 18–26 fibulae in 10 μm and mantles one poroid high (Fig. 3 a-e). In any case, a degree of variability was observed in our samples concerning poroid structure and arrangement and this was also recorded for cultured and field material from the gulf of Naples, in fact combined morphological, molecular and mating studies showed the presence of five distinct lineages within what was considered to be *P. delicatissima* (Orsini et al., 2004; Amato et al., 2005). Following taxonomical re-assessment of this species, based on ultrastructural features and genetic traits, highlighted the existence of a complex of three different species that incorporated the variability registered in populations previously attributed to *P. delicatissima* from diverse areas of the world (Lundholm et al., 2006).

The data in our hands, based only on morphology and ultrastructure of specimens, did not conform unequivocally to any of the two newly described species and to the third emended taxon *P. delicatissima sensu stricto*. The attribution to *P. decipiens* Lundholm et Moestrup or to *P. delicatissima sensu stricto* remained ambiguous. Latium specimens differed from the two taxa for the shape of poroids, that were rather irregular, not only exagonal. The range of the transapical axis and densities of fibulae and poroids of our material resembled those of *P. delicatissima sensu stricto,* but the number of the striae was more similar to *P. decipiens.*

Linear, to almost linear cells of 60–100 μm in length and 1.3–2.8 μm wide, tapering towards the pointed apices, both in valve and girdle view,

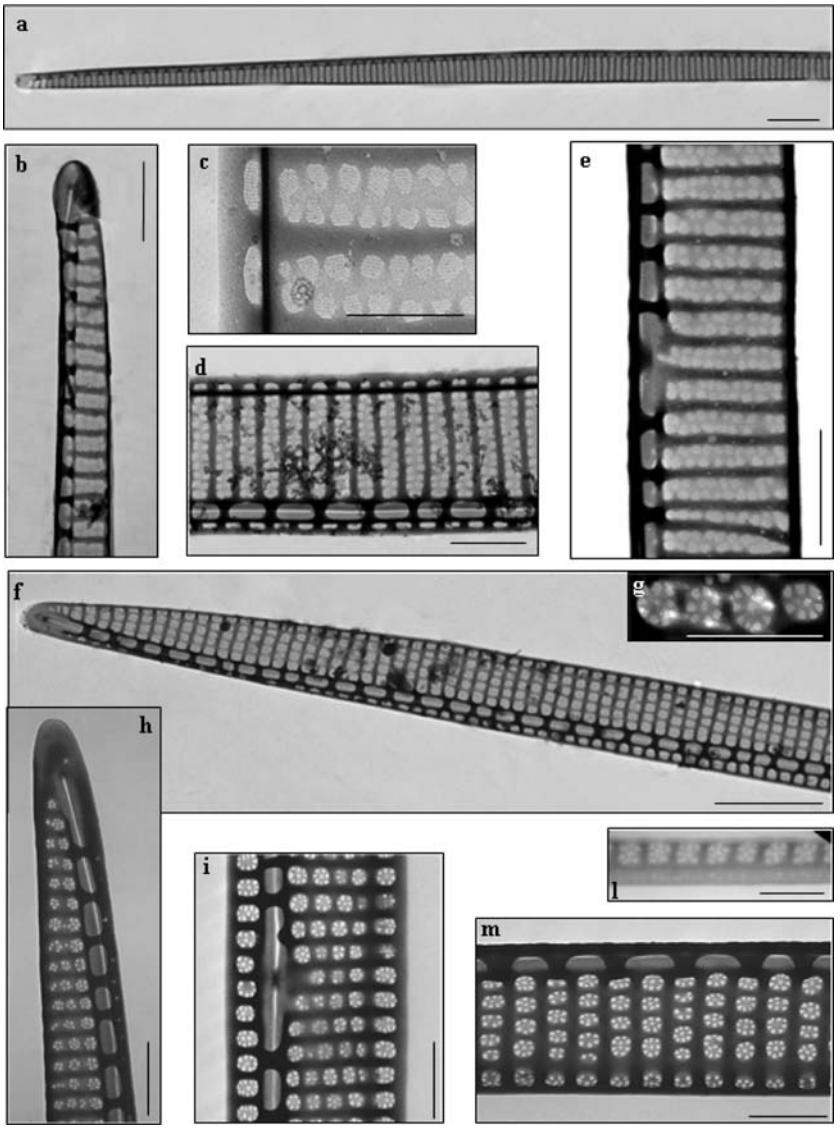

Figure 3. TEM micrographs of *Pseudo-nitzschia decipiens/delicatissima*, a-e, and *P. calliantha*, f-m. Part of the linear valve showing cut-off end, **a**, shown in more detail in **b**. Biseriate striae were visible on the valves, **c** and **d**, finely hymenate vela of triangular to exagonal poroids in d. Both mantles are shown in d and the central larger interspace with a central nodule in **e**. Tip of one valve, **f**, showing pointed apex in **g**. The central larger interspace and central nodule are visible in **i**, as the finely structured uniseriate striae, with hymen sectors arranged in a flower fashion, **m** and **g**; the same velum pattern was observed in the girdle bands, l. Bars = 2 (a, f), 1 (b, d, e, g, h, i, l, m) and 0.5 μm (c).

and with short end overlap in the chains, were ascribed to *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle during LM observations (Fig. 1 h). TEM analysis of frustules featuring these morphometry and gross morphology showed that most of the valves could be attributed to the newly described species *P. calliantha* Lundholm, Moestrup et Hasle. Recently, uncertainty regarding delineation of the species *P. pseudodelicatissima* and *P. cuspidata* (Hasle) Hasle led to the revision, based on ultrastructural and molecular data, of the complex *Pseudo-nitzschia pseudodelicatissima/cuspidata* which outcome was the description of two new species *P. calliantha* and *P. caciantha* Lundholm, Moestrup et Hasle and the emendation of *P. pseudodelicatissima* and *P. cuspidata* (Lundholm et al., 2003). Valves identified as *P. calliantha* during TEM investigation were 80–100 μm long and 1.3–2.0 μm, with 33–40 striae and 18–23 fibulae in 10 μm and the central larger interspace corresponding to 5 striae. Striae were uniseriate, round to square poroids, 4–5 in 1 μm had vela consisted of a central unperforated or hymenate part surroundded by five to eight hymenate sectors arranged in a circle, resembling a flower, valve mantle was one poroid high (Fig. 3 f-m).

Wider lanceolate valves, approximately 2.1–2.8 μm in width and 60–75 μm long were seldom observed in the cleaned material. Morphometry, poroid structure and cingular band features led to a tentative attribution to *P. caciantha* Lundholm, Moestrup et Hasle. The numbers of striae and fibulae were slightly lower, 28–32 and 16–18 in 10 μm, respectively in these specimens and poroid hymen was distinctively different, divided into four-five sectors. Mantle was 1–2 poroids high and the first cingular band, the valvocopula, two poroid wide and three to five poroid high, had 36–40 striae in 10 μm, the other cingular bands appeared shorter (Fig. 4 a-b').

A third, rare, lanceolate morphotype, 60–90 μm long, differed from *P. caciantha* in valve width, 1.5–1.9 μm, with much denser fibulae, 22–23, and striae 36–40 in 10 μm. Each stria had one row of poroids, 4–5 in 1 μm, and the hymen was distinctively divided in two (bipartite) perforated parts (Fig. 4 c-e).

This morph was tentatively attributed to *P. pseudodelicatissima/cuspidata* complex (Lundholm et al., 2003) as the definitive delineation of the two emended species remained unclear also after detailed morphological and phylogenetic analysis. In any case, more recent work reported the two above mentioned species as separated based on valve morphometry, slight differences in valve and band striation and density of fibulae (Lundholm et al., 2006), our specimens more closely resembled *P. cuspidata* (Hasle) Hasle *emend.* Lundholm, Moestrup et Hasle.

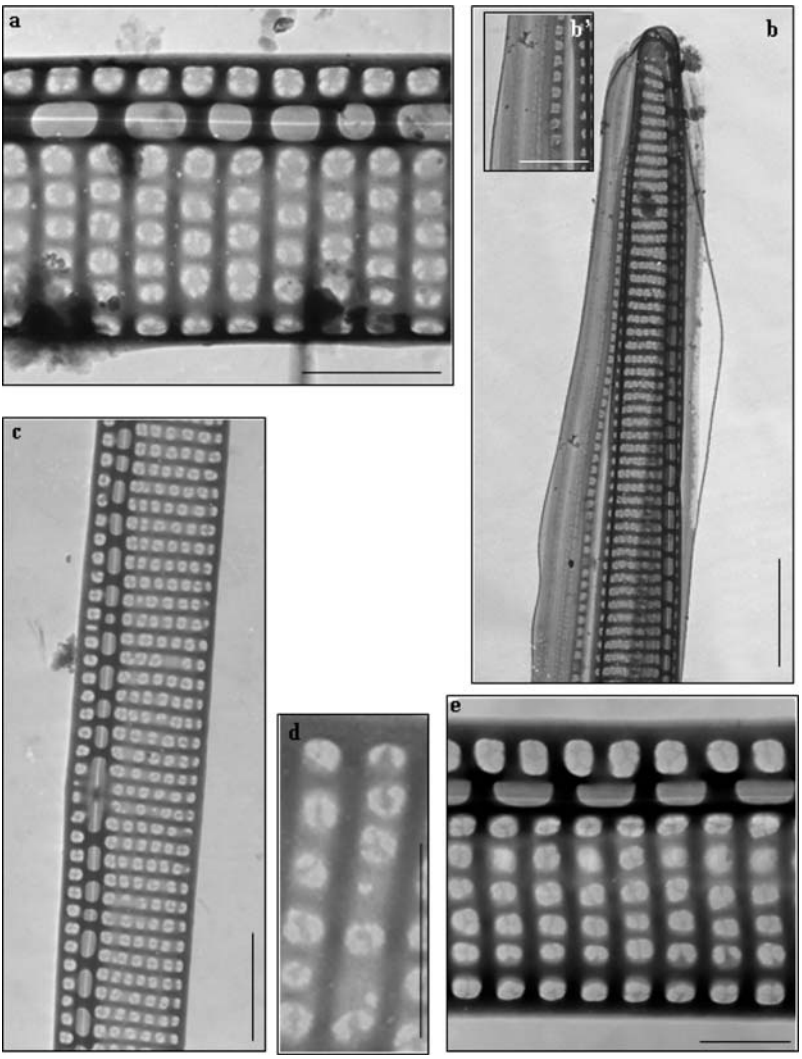

Figure 4. TEM micrographs of *Pseudo-nitzschia caciantha*, **a-b**' and *P. pseudodelicatissima/ cuspidata*, **c-e.** Part of valve showing uniseriate striae of poroids showing unperforated central part of the vela surrounded 4–5 irregular arranged hymenate sectors **a**. Valve end with cingular bands, **b** and **b**'**,** striation of the valvocopula and pattern of the other bands is visible in **b**'. Central part of the valve, **c**, poroid structure different from the former morphospecies is also visible, poroids appeared primarily bipartite, **e**, although a degree of variation was observed, **d** and **e**. Bars = 2 (b), 1 (a, b', c, e) and 0.5 μm (d).

3.2. *PSEUDO-NITZSCHIA* DISTRIBUTION ALONG THE LATIUM REGION COAST, TOXIC AND DOMINANT TAXA

There were eight different *Pseudo-nitzschia* morphotypes distinguished by TEM in the samples collected at the six coastal stations monitored from January 2001 to December 2002. Six species *Pseudo-nitzschia calliantha, P. cuspidata, P. decipiens/delicatissima, P. fraudulenta P. galaxiae* and *P. multistriata* are potential DA producers (Lundholm and Moestrup 2007). *P. inflatula* had never been recorded in Italian waters yet.

Long term data of coastal phytoplankton collected from 1997 at the same stations, except for the one located at the island of Zannone, allowed to identify a total of 374 taxa, 33 of which are toxic. Distribution patterns evidenced inter- and intra-annual variability of populations, with sharp temporal fluctuations of total phytoplankton abundances that generally peaked in summer. This reflected influence from inland input and the anthropogenic impact (Congestri et al., 2006). In accordance, blooms of *Pseudo-nitzschia* as a genus occurred in spring-summer, different species coexisted during blooms and the more southerly stations exhibited maximum abundances, at the species/morphotype level, in 2003. *P. calliantha* and *P. decipiens/ delicatissima* peaked in March (1.3 and 1.5 × 10^6 cell l^{-1}), *P. fraudulenta* in June (4.4 × 10^6 cell l^{-1}) as *P. galaxiae* (2 × 10^6 cell l^{-1}), but in 2002, while *P. multistriata* (6.5 × 10^6 cell l^{-1}) in August.

Further investigation on phytoplankton structure over the long-term study using the Sanders index (Sanders, 1960) was carried out to test dominance of individual taxa at the different sampling stations during the year of maximum abundances (2002–2003). This highlighted a degree of temporal and spatial variability in the seasonal cycles of the different species. *P. galaxiae* was more correlated with the RMB (Ladispoli, Rome) station, largely influenced by the Tiber River run-off while *P. decipiens/delicatissima* dominated at south, at LTD (Rio Martino, Latina) and at LTE (Monte D'Argento, Latina). At this latter station *P. decipiens/delicatissima* was associated with the complex of morphotypes labelled as *P. pseudodelicatissima sensu* Hasle (1965) (Congestri et al., 2006). Rather more diversified patterns of the same species were recorded in the Gulf of Naples (Zingone et al., 2006), although higher numbers of *P. fraudulenta* and *P. multistriata* were recorded in Latium. Conversely, in the Southern Adriatic Sea *P. calliantha* was strongly correlated to winter water conditions, while the species identified as *P. delicatissima sensu* Hasle (1965) had a broader seasonal distribution and appeared independent from major environmental constraints (Caroppo et al., 2005). Other investigation on *Pseudo-nitzschia* in the Northern Adriatic waters confirmed a winter pattern, with occurrence and maximum abundances of the whole genus in winter (Socal et al., 1999).

4. Conclusions

Monitoring activities of phytoplankton require accurate identification of microalgae coupled to fast toxicity assays for contingency plans to be developed allowing prevention or mitigation of human health and economy impact. This study ascertained the identity of *Pseudo-nitzschia* populations, the occurrence in critical numbers of potentially toxic species and their recurrent blooms along Latium region coast. Long-term data provided knowledge of seasonal patterns of the species distinguishable in LM, highlighting periods of maximum abundances when attention should be paid in order to predict outbreaks and mitigate potential toxicity. To date, along Italian coast potentially toxic species are more widespread than toxicity events, which points at a very high risk that toxicity may suddenly arise in some areas, just as a consequence of the increased exploitation of marine resources. In this scenario, fast and reliable tools, as the Screen Printed Electrodes, SPEs, should be introduced in routine monitoring procedures for the detection of algal toxins in diverse matrices in order to track toxins path and fate through the food chains (Albertano et al., this volume).

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ALGAL BLOOMS IN ESTONIAN SMALL LAKES

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Abstract. The database of the Centre for Limnology includes phytoplankton material from 500 lakes: this represents approximately 21% the total number of the lakes in Estonia. A phytoplankton biomass value greater than 8 mg L^{-1} was set as the limit criteria for further investigation. Blooms of cyanobacteria have been the most frequent and intensive: 20 different species of cyanobacterial blooms in different lake types have been detected in approximately 15% of the Estonian lakes. Until 1970s, the most widely distributed phytoplankton mass occurrences were caused by genera *Microcystis* and *Anabaena.* After that period changes in plankton communities took place and chroococcal cyanobacteria were replaced by filamentous forms, e.g. *Planktothrix*, *Aphanizomenon* and *Limnothrix*. From that period onward cyanobacterial biomasses have diminished, probably because of a decreased nutritient load from agriculture. Raphidophyte *Gonyostomum semen* was firstly recorded in the beginning of 1980s and up to now this species has been found in more than 80 lakes, which belong to three different limnological type: dystrophic, semidystrophic and oligotrophic. In addition, blooms of chrysophytes, dinoflagellates, cryptophytes and chlorophytes were also documented.

Keywords: Estonian lakes; algal blooms; cyanobacteria; *Gonyostomum semen*.

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1. Introduction

There are about 2300 small lakes with an aquatory greater than 1 hectare in Estonia which cover 4.8% of its territory (Tamre, 2006). The two largest lakes Peipsi and Võrtsjärv (Figure 1) together make up for 87% of the area of lakes. Roughly 50% of lakes are less than 3 hectares in size; only 45 lakes have a surface area of more than 100 hectares. Most of the lakes are shallow: only 46 of them reach a depth of 15 or more metres (the deepest is L. Rõuge Suurjärv with a depth of 38 m). According to the limnological typology elaborated by Mäemets (1974; 1976) eight lake types can be found in Estonia: oligotrophic (8% of the lakes), semidystrophic (6%), dystrophic (6%), eutrophic+hypertrophic (36–37%), mixotrophic (36–37%), siderotrophic (0,2%), halotrophic (1,4%) and alkalitrophic (2,6%). Each type can be characterized by a specific complex of fauna and flora and a certain matter and energy circulation. This typology is based on the natural accumulation type, very similar to the principles of Water Framework Directive of EU (2000) and differs in principle from the typology that can be found in the literature and which is based on the trophic state.

1.1. ALGAL-BLOOMS IN ESTONIAN LAKES

Algal blooms have been a regular phenomenon in Estonian small lakes. Probably these blooms are as old as the formation period of lakes, which started about 12000–13000 years ago when the continental ice retreated from the present territory of Estonia. However, these blooms are reported to have increased in frequency, biomass, and duration in recent decades, presumably in response to anthropogenic eutrophication (Ott and Kõv, 1999). The principal factors influencing the lakes have been (1) diffuse and point source nutrient load from agriculture, (2) sewage water from rural areas, (3) industrial waste water, (4) drainage and soil amelioration, (5) airborne pollution from industrial areas (Laugaste, 1994, Mäemets *et al*., 1994). The most intensive nutrient load originated from agriculture which culminated in the years 1985–1988, contributed about 50–60% of the total nutrient loading to Estonian lakes. From the late 1980s this loading decreased due to a significant drop in the use of mineral fertilizers (Loigu, 1993). After that period the ecological status of many lakes started to improve rapidly. The improvement however is not relevant to all the lakes, mainly because of a higher internal nutrient load.

1.1.1. *Cyanobacteria*

Cyanobacterial blooms (biomass >8 g L^{-1}) have been detected in approximately 15% of the Estonian lakes. The blooms are caused by 20 different

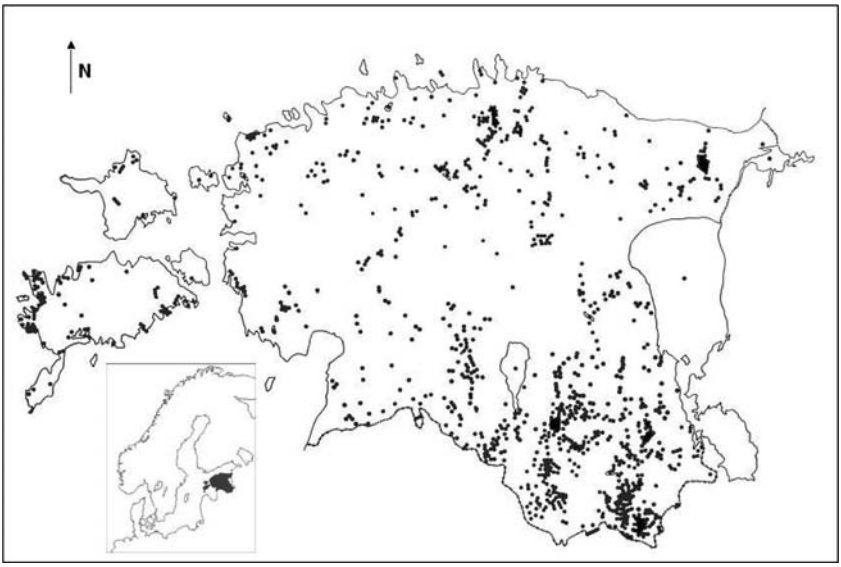

Figure 1. Location of Estonian lakes (Kask, 1979).

species in different lake types, even in oligotrophic lakes. Cyanobacterial blooms were recorded for the first time in Estonian lakes at the beginning of the 20th century (Olli, 1996). These blooms typically occurred in midsummer and dominant species were *Anabaena lemmermanni*, *A. flos-aquae* ja *Microcystis aeruginosa*. In the 1950s when more comprehensive studies of Estonian small lakes began, the same genera were found widely distributed in the plankton of mesotrophic and eutrophic lakes. Species from genera *Microcystis* (*M. aeruginosa*, *M. wesenbergi*, *M. flos-aquae*, *M. botrys*) and *Anabaena* (*A. lemmermanni*, *A. flos-aquae*, *A. hassali*, *A. circinalis*) were widely distributed approximately up to the late 1960s. Since the 1970s with the increase in nutrient load, the species composition changed – the abunance of large colonies of *Microcystis* and some species of *Anabaena* (*A. flosaquae*, *A. hassali*, *A. lemmermanni*, *A. circinalis*) decreased, while some filamentous cyanobacteria such as *Pseudanabaena limnetca*, *Planktothrix agardhii and Limnothrix redekei* have expanded in abundance and distribution considerably (Ott and Kõv, 1999). Moreov er, in opposition to the period prior 1970, the frequency of autumn blooms increased, especially in stratified lakes. These blooms were induced mainly by nutrient enrichment (predominantly phosphorus) from the anoxic bottom layers in correspondence to the autumn turnover (Milius and Pork, 1977). Due to the replacement of the large colonies by remarkable smaller filaments, the biomass values started to decrease after 1970s. Nevertheless, some species, like *P. agardhii* and species belonging genera *Anabaena*, have quite large dimensions, forming the greatest biomasses of phytoplankton (Table 1). In addition, it was

also shown that *P. agardhii* formed the highest biomass in meta- and hypolimnion in strongly stratified lakes during the vegetation period (Laugaste, 1991; Kangro *et al*., 2005) and *P. rubescens* during winter under the ice (Laugaste, 2006). Biomass concentration was still very high (>30 mg L^{-1}) at the beginning of 1990s in several lakes and dropped permanently from the middle of the decade up to now. Concentrations higher than 10 mg L^{-1} have almost never been found. Dominant species are *Limnothrix planctonica*, *Aphanizomenon yezoense*, *A. gracile* and *Planktolyngbya limnetica*. Biomass and distribution of *P. agardhii* have diminished due to the decrease of external nutrient loading since the beginning of 1990s (Ott *et al*., 2002).

The abundance of species from genera *Microcystis* has changed – *M. aeruginosa* has decreased, while *M. botrys* increased. Probably this change was not so obvious because in previous investigation periods the *M. botrys* was confused with several other species from *Microcystis* genera, mainly *M. aeruginosa* (Cronberg and Komarek, 1994). *Microcystis* species inhabits mainly small unstratified or weakly stratified lakes.

TABLE 1. 5 highest recorded cyanobacterial biomasses in Estonina natural lakes. DEP-soft-water mixotrophic; HY– hypertrophic; HM – hard-water mixotrophic; HE – hard-water eutrophic.

Lake	Type	Surface area, ha	Maximum depth, m	Biomass, mg*L^{-1}	Species	Year
Rummu	DEP	50	2,8	888	*Anabaena lemmermanni*	1985
Verevi	HY	12,6	11	499	*Planktothrix agardhii*	1985
Konsu	HM	139,2	10,2	507	*Planktothrix agardhii*	1982
Peta	HE	3,6	25	205	*Planktothrix agardhii*	1975
Ruusmäe	HY	4,8	11,6	190	*Anabaena spiroides*	1990

There has been very little information available about the real toxicity of cyanobacterial blooms in Estonia because of the scarce work in this field. Basically, in small lakes, microcystins are produced by several bloom forming cyanobacterial genera including *Microcystis*, *Anabaena* and *Planktothrix* (Table 2). There is lack of evidence that toxins have caused the death of cattle, wildlife, and present hazards to human health via drinking water or accidental ingestion of cyanobacterial bloom material. However, skin irritation and allergic reactions in swimmers exposed to cyanobacterial blooms have been reprorted several times.

1.1.2. *Raphidophytes*

There are two species in Estonian lakes belonging to raphidophyte genera – *Gonyostomum semen* and *G. palludosum*. Both species are uncommon and no blooms have been documented.

TABLE 2. Concentrations of microcystins found in the water of some Estonian small lakes (Taner *et al*., 2005).

Sampling point	Sampling date	Dominant species***)	Concentrations of microcystins µg/l****)				
			Mcd mRR	McRR	McY R	Mcd mLR	McL R
Pihajärv*)	3.07.01	*M. botrys*, *M. viridis*, *M. wesenbergii*, *M. flos-aquae*	na	na	na	na	1.1
Ähijärv*)	5.07.01	*M. flos-aquae*, *Radiocystis geminata*	na	na	na	na	0.1
Narva reservoir.**)	6.09.02	*A. circinalis*, *M. viridis*	na	na	na	na	67
Pihajärv**)	25.06.04	*M. botrys;* *M. viridis;* *M. flos-aquae;* *M. wesenbergii*, *A. lemmermannii*	11.5	8.3	2.0	1.8	3.2
Pihajärv*)	25.06.04	*M. botrys;* *M. viridis;* *M. flos-aquae;* *A. lemmermannii*	0.53	0.44	0.09	0.05	0.2

Notes:

*) mean water samples from the open area of the lake from the depth 30–50 cm

**) water sample from the region of inshore accumulation of cyanobacterial mass

***) A.- *Anabaena*; M. - *Microcystis*

****) na – not analysed

G. semen was found at first at the beginning of 1980s in some soft-water forest bog and dystrophic lakes where the total alkalinity (HCO_3^-) is below 60 mg L^{*-1} (Ott and Kõiv, 1999). In the beginning of the 1990s *G. semen* formed a great biomass in dystrophic lakes and was dominant also in some semidystrophic, soft-water mixotrophic and in two formerly oligotrophic lakes (Laugaste, 1992). This species was not found during the earlier investigation period (up to1980s). It is well known phenomenon that after fixation of sample with Lugol solution *G. semen* cells break immediately therefore making their recognition rather complicated (Hongve *et al*., 1988): this might have been the main reason why the presence of *G. semen* has been often overlooked. This, however, is not case of the samples collected before the 1980s as re-examination of many of these old samples has proved lack of remnant cells.

On the other hand widening may be connected with changes in water chemistry. Cronberg *et al*., 1988 reported that the main reason for the expansion of *G. semen* in Swedish forest lakes was related to the acidification. On the contrary no drastic decrease of pH in Estonian small lakes has been observed. Estonia has carbonate-rich soils and therefore lakes are well-buffered and, moreover, acidification is inhibited by alkaline air pollution (Õlk, 1989), which neutralizes the effect of acid rains. It is more probable that wider distribution and increased biomass of *G. semen* in lakes is related to increasing nutrient content (mainly phosphorus) during the past decades (Nõges and Laugaste, 2002). Positive correlation between higher phosphorus levels and biomass has also been reported by Rosén (1981, cit. Nõges and Laugaste, 2002).

Up to now *G. semen* has been found in more than 80 lakes, most of which belonging to bog and forest dystrophic or mixotrophic lakes, but also in two oligotrphic lakes in the 1990s (Nõges and Laugaste, 2002). The highest biomasses were reported by the same authors in dystrophic lakes with extremely dark water (Secchi depth 1 m; COD_{Cr} 60–100 mg O L^{-1}). The highest biomass concentration 100 mg L^{-1} was recorded in 1991 in brown-water lake (lake Orava Mustjärv) near the town Põlva in the south-eastern part of Estonia. The occurrences and higher biomasses correlated positively with increasing phosphorus level in the lakes with moderate pH (7). It is also showed that *G. semen* can exploit nutrient resources from deeper water layers (hypolimnion) as well. Studying Finnish dark-water lakes, Salonen and Rosenberg (2000) found the highest biomass near the surface during the daytime and in the hypolimnion during night. They pointed out that diurnal vertical migration of *G. semen* was directly related to taking up and storage of soluble reactive phosphorus (SRP). This gives an important competitive advantage over the other algae and explains the dominance and high biomasses of *G. semen* in lakes. However, beside vertical distribution, simultaneous horizontal distribution has also been observed in one Estonian oligotrophic lake – Nohipalu Valgjärv (soft water, surface area 6,3 ha, maximum depth 11,7 m) – in August 2007. (Figure 2). This horizontal patchiness was probably due to concentration of cells in upwind areas that has been already described for other downward-migrating algae, e.g. dinoflagellates (Heany, 1976). As there may be differences in horizontal and vertical distribution of cells, it may be sometimes overlooked already in the sampling phase particularly in brown-water lakes.

However, *G. semen* has spread not into some semidystrophic lakes in Estonia that exhibit nearly neutral pH, but in those lakes where the bicarbonate content is over the 60 mg L^{-1}. Its fast expansion in Estonian lakes

Figure 2. Bloom of *Gonyostomum semen* in lake Nohipalu Valgjärv in august 2007.

remains still unclear, but nutrient load seems to be one of the main factors that may have enhanced it. According to the data of biomass from Estonian lakes, *G. semen* usually reaches its biomass maximum on July or August but it has also been observed in plankton from May to September. Blooms of *G. semen* were considered to be non-toxic but, may result unpleasant for swimmers by provoking mucilage and skin irritation thereby lowering recreational value of lakes.

1.1.3. *Other algae*

Sometimes not regular phytoplankton mass occurrences have been noticed by other algal groups. Higher biomasses have been observed among chrysophytes, dinoflagellates, cryptophytes and chlorophytes. The annual maximum of chrysophytes is usually in spring, after the ice break. Dominating species during that period belong to the genera *Uroglena*, but rarely the biomass exceeds a value greater than 8 mg L^{-1}. In midsummer higher biomass values may originate from *Mallomonas caudata* and in 2007 at first recorded by small-celled *Chrysococcus* sp. in oligotophic soft water lake Viitna Pikkjärv (surface area 16,3 ha, maximum depth 7 m) (Rakko, 2007). The greatest

recorded biomass values are 35 mg L^{-1} in the Lake Kallete in 1975 (hard water, surface area 8 ha, maximum depth 6,2 m, dominant *Peridinium willei*) and 20 mg L^{-1} in the lake Vagula in 1989 (hard water, surface area 518 ha, maximum depth 11,5 m). In the latter, higher biomass values of *C. hirundinella* have been reported several times. Cryptophytes may have higher biomasses usually under the thermocline in stratified lakes (Laugaste, 1991) or in nutrient rich shallow lakes. The greatest recorded values are 30 mg L^{-1} in hypertrophic Lake Kooraste Linajärv in 1975 (soft water, surface area 2,7 ha, maximum depth 12,7 m). Approximately 30–40 dominating chlorococcal species were recorded in highly eutrophic lakes. The highest biomass values, in the range of 40–78 mg L^{-1}, were recorded several times in the lake Harku in 1976–1990, suffered from sewage water, (hard water, surface area 164 ha, maximum depth 2,5 m, dominant *Scenedesmus quadricauda,*). Also, in other hypertrophic lake Partsi Kõtsijärv (suffered from flax retting), high biomass (35 mg L^{-1}) was recorded in summer in 1975 (soft water, surface area 3,4 ha, maximum depth 5 m, dominant *Tetraëdron minimum*).

2. Conclusions

The database of phytoplankton allows to follow long term dynamics and phytoplankton mass occurrences in Estonian lakes. The highest values were documented during Soviet time (1970–1980s), when extensive agriculture prevailed. The main dominating species belong to cyanobacteria. During the past deacades water blooms caused by chroococcal species (mainly Microcystis) have been substituted by filamental species from Nostocales. Among the other algae, *Gonyostoum semen* has become an important dominating species in soft water lakes. In the lakes, suffered from easily decomposing organic matter, chlorococcal species can prevail. Generally, the ecological status of Estonian lakes has improved since 1990s. The status of the lakes depend more from weather in different years and less from nutrient loading.

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COMPARATIVE ESTIMATION OF SENSOR ORGANISMS SENSITIVITY FOR DETERMINATION OF WATER TOXICITY

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Abstract: It was established that bioassay is convenient first test for estimation of toxicity in screening of water-bloom material and laboratory cultures or cell extracts. It is discussed the advantages and difficulties at using as bioassay the organisms from different systematic groups. It is determined lethal doses for checking of natural algae toxins activity.

Keywords: Water "blooming", toxicity, bioassay, lethal dose

1. Introduction

Algae are the main primary producers of organic material and oxygen. They are utilizers of CO_2 as well in continental reservoirs as in World Ocean. Due to the high photosynthetic potential they synthesize up to 74% organic substances in water ecosystems or 25% of total production, which is formed on the Earth (Considine, 1984).

Algae export into environment the significant part of organic substances that are produced in process of vital activity. The portion of these substances in the general cells balance is very valuable and makes about 30% of the all daily oxygen balance or 40% pure daily production of photosynthesis

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(Kirpenko, et al., 1977). Chemical composition of metabolites is varied essentially. Among exometabolites are identified aminoacids and peptides, compounds of polysaccharide nature, essential oils, aldehydes, organic acids, terpenes, vitamins, compounds of polyphenolic and indolic nature, antimicrobial components and others compounds with high biological activity (Sirenko and Kozitskaya, 1988). It was detected substances with allergenic, mutagenic and cancerogenic effects, antihormonic, plant growth regulation, bactericidic, insecticydic, fungicydic and algicidic activity.

Algae toxicity is bring to the attention at estimation of water quality, functional activity of hydrobionts and products, which are received from it. For example, it is carried out a great bulk of investigations on studying of algae toxins effect on fishes and also organisms-filtrators in the context of negative action of *Microcystis aeruginosa* on speed of zooplankton filtration and lowering of speed the feed consumption in 10–15 times (Sirenko and Parshykova, 1988). An important value has a search among algae toxins – compounds with high biological activity, which may be used as medicines (Carmichael, 1980). Fulfillment the complex of above mentioned researches promoted to essential widening the list of toxic algae flora representtatives, deciphering the chemical nature of separate toxins, showing up the factors, which are contribute to strengthening or weakening of water toxicity. Thus, investigation of microscopic algae toxic exometabolites and preparative, purification of its compounds has as well theoretical as practical significance.

Goal of our experiments was analyzed of perspectives for using of different organisms (warm-blooded, hydrobionts, microorganisms) for estimation of biological activity of algae toxins.

2. Difficulties in Determination of Biological Activity of Algae Toxins

Taking into consideration that algae develop in close association with other macro- and microorganisms in reservoirs, it is very important to study the chemical nature of metabolites that produce such associations into environment. An additional point to emphasize is that biologically active compounds which are produced by algae-microorganisms associations can create conditions for formation of free-radical processes which will reinforce the toxicity or to be of its reason. Localization of biologically active compounds causes essential troubles in establishment of water microorganisms' toxicity too. There are 2 types of algae toxicity: endogenous and exogenous.

Endogenous toxicity is caused, on one side, by organism genotype and metabolic processes, on other side, by population, allelopatic and adaptive mechanism of cells regulation. In all cases it is connected with accumulation in cells the biologically active substances, which are formed as a result of cells ability to biosynthesis of special metabolites. Determination of chemical nature for compounds of this group is very difficult. It is conconnected as well with its lability, low concentration and small specificity, as a result of demonstration of toxicity for various compounds. For example, for cells of different living organisms (such as animals, plants and microorganisms) the intracellular oxygen may be toxic due to its superoxides, O_2, HO_2, hydrogen peroxide, OH-radical forms. The oxygen toxicity can show up because of degradation of subcellular structures, inactivating of enzymes and formation of intracellular lipid peroxides (Sirenko and Parshykova, 1988).

Exogenous toxicity links compounds which production by algae, postlethal degradation of organic matter and adaptive provision to influence of foreign chemical ingredients. For example, *Microcystis aeruginosa* toxicity sometimes is explained by necessity to accumulation of soluble organic compounds in cultural medium. It is obligatory condition for growth of young cells that feed on heterotrophic on earlier stage of ontogenesis. In this case toxicity can be considered as biological appliance for supporting of population (Sirenko and Parshykova, 1988).

3. Studying of Biological Activity of Algae Toxins

It is well known, that algae toxins are found out in representatives of *Cyanophyta* (*Cyanobacteria*), *Dinophyta*, *Chlorophyta* and *Chrysophyta*. Among of *Cyanobacteria* toxins the microcystins and nodularine are most investigated in many countries of the world. There are 4 groups of algae toxins by effect of human: hepatotoxins, dermatoxins, cytotoxins and neurotoxins (Carmichael, 1988). Comparative estimation of biological activity for natural algae toxins testifies that metabolites of *Cyanobacteria* are on intermediate position between toxins of microorganisms, amphibious animals and toxins of higher plants or mushroom origin (Table 1).

It was registered, that lethal dose (LD_{50}) for Cyanobacteria are: for anatoxin – A/S (producer of *Anabaena flos-aquae*) –20 μg/kg, nodularine (*Nodularia spumigena*) and microcystine LR (*Microcystis aeruginosa*) –50, anatoxin – A (*A. flos-aquae*) –200 μg/kg of animal body. It is significantly

TABLE 1. Comparative toxicity of different biological toxins (Carmichael, 1992; Carmichael, 1994; Carmichael, 1997).

Toxins	Producer, source	Usual name	Lethal* dose LD_{50}
Botuline toxin	*Clostridium botulinum*	Bacteria	0.00003
Tetanus toxin	*Clostridium tetani*	Bacteria	0.0001
Ricin toxin	*Ricinus communis*	Plant	0.02
Diphtheria toxin	*Corynebacterium diphtherial*	Bacteria	0.3
Cocoy toxin	*Phyllobates bicolor*	Frog toxin	2.7
Tetrodotoxin	*Arothron meleagris*	Fish	8
Saxitoxin	*Aphanizomenon flos-aquae*	Cyanobacteria	9
Cobra toxin	*Naja naja*	Snake poison	20
Nodularine	*Nodularia spumigena*	Cyanobacteria	50
Microcystin-LR	*Microcystis aeruginosa*	Cyanobacteria	50
Anatoxin-A/S	*Anabaena flos-aquae*	Cyanobacteria	20
Anatoxin-A	*Anabaena flos-aquae*	Cyanobacteria	200
Curare	*Chrondodendron tomentosum*	Brazilian plant	500
Strychnine	*Strychnos nox-vomica*	Plant	500
Amatoxin	*Amanita sp.*	Mushroom	200–500
Muscarine	*Amanita muscaria*	Mushroom	1100
Fallotoxin	*Amanita sp.*	Mushroom	1500–2000
Potassium cyanide			10000

*Lethal dose in μg on 1 kg of body weight: intraperitoneal injection for mice and rats.

less in comparison with alkaloid curare (Brazilian plant *Chrondodendron tomentosum*) –500, muscarine (mushroom *Amanita muscaria*) –1100 μg/kg and even with potassium cyanide (LD_{50} – 10000 μg/kg).

As it may be seen from Figure 1, investigations, which were carried out in many countries, allowed analyzing the experience of isolation, studying and estimation of *Cyanobacteria* toxicity. Worlds search for new and more sensitive separation and detection methods for phycotoxins seems partly a genuine desire to limit the negative consequences of these poisons in our food and water supplies plus scientists' inherent drive to always develop something "bigger and better" or, in keeping with the proper context, "more rapid and more sensitive". This is also true in the progression of techniques for analysis of the cyanotoxins.

For estimation the biological activity of *Cyanobacteria* toxins in cells biomass, water samples and animal tissues traditionally have been used bioassay, chemical methods and immunological technologies. Bioassay is

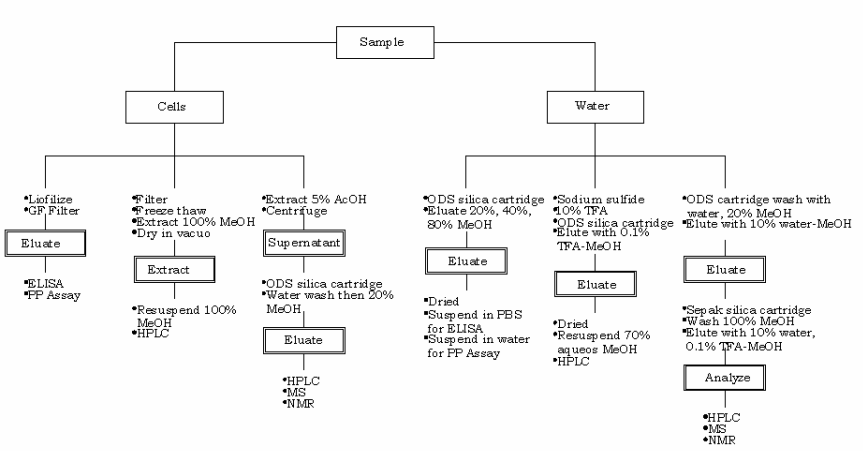

Figure 1. Flow chart for the isolation, detection and analysis of microcystins and nodularines based upon screening method (Carmichael, 1994; Carmichael, 1997):GF – glass fiber; PP – protein phosphotase inhibition; MS – mass spectroscopy; PBS – phosphate buffered saline; TFA – threefluoroacetic acid; ODS – octadecylsilanized; HPLC – high-performance liquid chromatography; NMR – nuclear magnetic resonance; ELISA – enzyme-linked immunosorbent assay.

first test for estimation the toxicity in screening water-bloom material and laboratory cultures or cell extracts and more fast than chemical and immunological methods. As a bioassay may be used organisms from different systematic groups such as warm-blooded animals, invertebrates, microorganisms and others.

4. Toxicological Experimentes on Warmblood Animals

Received experimental data testifies (Kirpenko, et al., 1977) that white mice, rats, chickens, guinea-pigs, cats and rabbits are sensitive to toxic metabolites of *Cyanobacteria*. In many manuscripts (Sirenko and Kozitskaya, 1988; Carmichael, 1980; Carmichael, 1988; Carmichael, 1994; Carmichael, 1997) was discussed the level of sensitivity of different living organisms because are known facts about absence of intoxication symptoms after entering of *Cyanobacteria* toxins to test-organisms. It is possible that contradiction of information about sensitivity to algae toxins for different animals may be connected with character of substances entering and differences in its biological activity. Some authors belief that toxicity of

Cyanobacteria toxins may appear only after parenteral entering. It was shown by other scientists that manifestation of toxic effect of *Cyanobacteria* possible after enteral introduction. However, toxicity of *Cyanobacteria* metabolites is decreased in this case (Kirpenko, et al., 1977).

At Ukraine presence of toxic metabolites of *Cyanobacteria* in cells and water filtrate was discovered on white mice and rats after peroral introduction. By this way algae toxins may detect its unfavorable action in water consumption for drinking aims.

It is well known that toxic metabolites are produced by specific strains of *Cyanobacteria* and more intensive toxins emission in places of its accumulation. In this connection toxicity were used main agents of water "blooming" in different world reservoirs – *Microcystis aeruginosa* and *Aphanizomenon flos-aquae*. It was estimated toxicity of *Cyanobacteria* cells depends on different states of biomass such as tinned biomass or preserving by drying, after boiling, freezing, ultrasound treatment (50 kHz) and during different time of destruction process (Table 2).

Received experimental data testifies that in fresh and dried cells biomass of *Cyanobacteria* toxic compounds were presented. Samples with domination of *M. aeruginosa* (97%) had higher toxicity than with *Aph. flos-aquae* (96%). Water filtrates of algae were toxic too but with low degree (1.76>1.21) than cells biomass.

Table 2 indicates, that after freezing algae cells biomass with further thaw or ultrasound treatment algae toxicity was increased (2.2 >1.68). Boiling of algae cells decreases the toxic properties of *Cyanobacteria* (1.96 >1.72) but don't effect on water filtrates toxicity.

Toxicity of algae biomass and water filtrates is decreased during extraction process, especially after increasing of its terms (1.92 >1.12). These experiments verify that white mice and rats are very sensitive to algae toxins.

So, toxicity of algae biomass may be increased after effect of thermal and mechanical factors (such as freezing with further thaw, treatment of ultrasound and other). It caused the formation of great quantity of degraded algae cells. Boiling of algae biomass decreases the cells toxicity but don't effect on toxicity of water filtrates. This fact are interested from two points of view. At first, it indicates on possibility of strengthening the toxicity of natural algae populations under effect of extreme factors, which always takes place under natural conditions (freezing, mechanical influence in passing of hydrotechnical constructions, pumps during transfer and so on). At second, it throws light on contradiction in toxicity estimation for the same algae strains under different conditions.

TABLE 2. Semilethal doses (mg/kg) of water filtrates and Cyanobacteria biomass for different animals (biomass content – 0,316 g/ml by dry matter) (Kirpenko, et al., 1977).

Probated patterns	Using animals	LD_{50}
TINNED BIOMASS		
Microcystis aeruginosa **biomass**	Mice	7.5 ± 0.6
M. aeruginosa **biomass**	Rats	8.0 ± 0.7
M. aeruginosa **water filtrate**	Mice	10.4 ± 1.1
M. aeruginosa **water filtrate**	Rats	14.3 ± 1.6
Aphanizomenon flos-aquae	Rats	10.5 ± 1.2
Aph. Flos-aquae **water filtrate**	Rats	15.2 ± 1.8
FROZEN BIOMASS		
M. aeruginosa **water filtrate**	Mice	3.9 ± 0.4
M. aeruginosa **biomass**	Mice	3.6 ± 0.3
M. aeruginosa **biomass**	Rats	3.7 ± 0.3
M. aeruginosa **water filtrate**	Rats	5.4 ± 0.5
Aphanizomenon flos-aquae	Rats	10.1 ± 1.1
Aph. Flos-aquae **water filtrate**	Rats	9.7 ± 0.9
BIOMASS AFTER BOILING		
M. aeruginosa **water filtrate**	Rats	8.5 ± 0.8
M. aeruginosa **biomass**	Rats	21.6 ± 1.9
M. aeruginosa **biomass**	Mice	8.3 ± 0.8
M. aeruginosa **water filtrate**	Mice	7.3 ± 0.7
Aphanizomenon flos-aquae	Rats	21.6 ± 1.8
Aph. Flos-aquae **water filtrate**	Rats	10.3 ± 1.2
BIOMASS WORKED UP WITH ULTRASOUND (50 kHz, 20		
M. aeruginosa **water filtrate**	Mice	9.7 ± 1.2
M. aeruginosa **biomass**	Mice	4.3 ± 0.4
M. aeruginosa **water filtrate**	Rats	5.6 ± 0.5
M. aeruginosa **biomass**	Rats	6.0 ± 0.6
BIOMASS DURING DESTRUCTION PROCESS		
M. aeruginosa **fresh biomass**	Mice	8.6 ± 0.8
M. aeruginosa **fresh biomass**	Rats	7.4 ± 0.6

(Continued)

TABLE 2. *(Cont)*

TWO DAYS LATER		
M. aeruginosa **biomass**	Rats	12.5 ± 1.3
M. aeruginosa **water filtrate**	Rats	16.9 ± 1.5
M. aeruginosa **biomass**	Mice	10.2 ± 1.1
M. aeruginosa **water filtrate**	Mice	14.0 ± 1.4
FIVE DAYS LATER		
M. aeruginosa **biomass**	Rats	22.0 ± 2.6
M. aeruginosa **water filtrate**	Rats	25.0 ± 3.4
M. aeruginosa **biomass**	Mice	16.1 ± 1.8
M. aeruginosa **water filtrate**	Mice	23.0 ± 2.4

5. Using of Invertebrates for Algae Toxins Indication

High biological activity of algae toxins, complexity and bulkiness of physical and chemical methods of its detection, possibility of cumulating effect display demand the elaboration of quick induction methods of presence of algae toxins in water environment. It is possible to presence the plankton of *Crustacea* (such as *Daphnia*, *Cyclops*) among the biological indicators that are perspective for the usage (Fig. 2).

Figure 2. Daphnia magna.

Test with *Daphnia magna* st. is sensitive and quick methods for determination of the toxic metabolites of algae in natural conditions. It is shown (Braginskiy et al., 1987), that under concentration in water the colony of *Microcystis aeruginosa* in the limits of 875000 per liter destruction of *Daphnia* was started in 16 hours (15%). *Daphnia* destructed completely in 48 hours. Under the concentration of *M. aeruginosa* within the limits of 11075000 cells per liter the complete destruction in 4 hours has been observed.

The obtained results show that the *Daphnia magna* could be quite sensitive biological test for the presence of *Cyanobacteria* toxic metabolites in water. Considering that experimental results obtained on *Daphnia* showed the good correlation under control with warm-blooded animals in sharp experiment test with *Daphnia* could be used as the express-method for toxicity determination. It is considered that under the usage of biological tests like *Daphnia* to determine the toxicity of unknown samples one has to mention that their destruction could be caused not only by toxic metabolites, but also by the products of algae disintegration and also by own animals excrements especially under prolonged experiments in closed volume of environment.

The check of biological activity of algae toxins preparations allocated by Ukrainian scientists due to *Daphnia* rest showed the complete destructtion of *Daphnia* was observed under concentration of algae toxin 0.0015 –0.0045 mg/l in 4 hours (Table 3).

TABLE 3. *Daphnia magna* st. survival (% to control) under different algae toxins concentrations (Kirpenko, et al., 1977).

Microcystin concentration, mg/l	Survival under different expositions, hours					
	2	4	8	16	24	48
0.0045	36	0	0	0	0	0
0.0015	70	0	0	0	0	0
0.00015	100	100	100	100	90	80
0.00004	100	100	100	100	100	66

Another fractions of metabolites extracted from *Cyanobacteria* showed less activity to *Daphnia magna*. So the algae toxin 7 (Kirpenko, et al., 1977) caused the complete destruction of *Daphnia* under concentration 0.21 mg/l in 16 hours. The other preparations obtained on the base of natural water samples gathering in spots of “blooming” water with the domination of *M. aeruginosa* also shown less activity (Fig. 3). Algae toxins 6 and 8 were purified from different samples of natural *Cyanobacteria* population. Algae toxin 6 contains 0.029 μg/ml, algae toxin 8 – 0.008 μg/ml of toxic compounds.

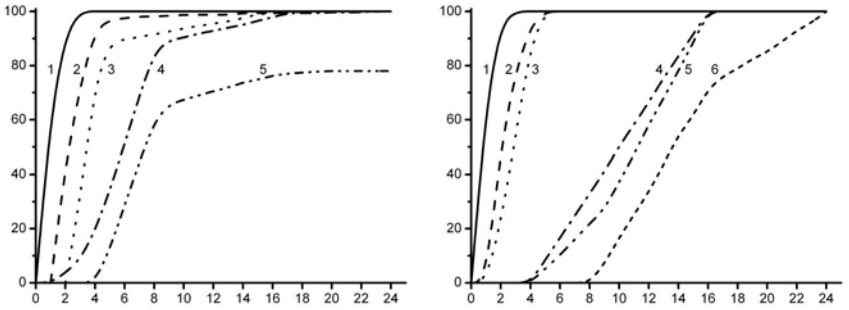

Figure 3. Influence of algae toxins 6 and 8 on *Daphnia survival:*
1 – concentration 7.25 mg/l; 2 – 1.75; 3 – 0.87; 4 – 0.44; 5 – 0.11 mg/l.
1 – concentration 6.7 mg/l; 2 – 3.35; 3 – 1.67; 4 – 0.84; 5 – 0.42; 6 – 0.21 mg/l.

The limitation for this method usage may be some statements namely daphnia test could be non-specified if using of natural water samples. In this case *Daphnia* may be destructed under another chemical substances exposition. Moreover the test cannot be used always as the *Daphnia* are presented in biotopes only in a short period of time. Beside this in the moment of high intensity of water "blooming" with the *Cyanobacteria* the *Daphnia* usually leave the place with concentration of sestone and it is quite difficult to determine them in water basin in this period.

6. Effect of Algae Toxins on Bacterial Flora

While searching of biological indicators for express-estimation of quantity and activity of algae toxins in water surrounding the significant attention attract the microorganisms. The collective of the Ukrainian scientists has executed a complex of experimental works on studying of algae toxins antimicrobial action as the metabolites of algae with the highest biological activity that play an essential role in formation of algobacterial cenosis (Kirpenko, et al., 1977). The spectrum of antimicrobial action was studied on next pure cultures of microorganisms: *Shigella flexneri*, *Escherichia coli*, *Proteus vulgaris*, *Staphylococcus aureus* and others. For investigations had been used algae toxins with maximal biological activity that have been collected in spots of water "blooming". The sensitivity of microorganisms to algae toxins and its bactericidal and bacteriostatic concentration of algae toxin were established by method of serial cultivations in liquid cultural medium. For bacteriostatic concentration we accepted that least concentration of preparation at which the full growth inhibition of microorganisms

culture was marked. As bactericidal we considered that least quantity of substance that caused absence of microorganisms growth under sowing on the agar with one loop. The account was made in 48 hours after cultivation in thermostat. Bactericidal and bacteriostatic concentrations of algae toxin preparation are established by a method of serial cultivations on meat-peptone broth (pH 7.2).

TABLE 4. Bactericide and bacteriostatic concentrations of algae toxin 1* during studying by method of serial cultivations (Kirpenko, et al., 1977).

Testing microorganism	Concentration, %	
	Bactericide	Bacteriostatic
Escherichia coli	10^{-5}	10^{-7}
Shigella flexneri	10^{-6}	10^{-7}
Salmonella Paratyphi B	10^{-5}	10^{-7}
Salmonella Typhimurium	10^{-5}	10^{-7}
Staphylococcus aureus	10^{-4}	10^{-5}
Staphylococcus weismani	10^{-4}	10^{-5}
Enterococcus	10^{-4}	10^{-5}
Mushrooms gen. *Candida*	10^{-5}	10^{-6}

*Algae toxin 1 was purified from natural samples of *Cyanobacteria*, contains 0.1 μg/l of toxic compound.

As it is shown on the Table 4, *Escherichia coli*, *Shigella flexneri* and *Salmonella* are the most sensitive to algae toxins of *Cyanobacteria*. While studying the mechanism of action of this algae toxin on microbial cell the researches of dehydrogenase activity of microorganisms under effect of the allocated preparation were carried out Table 5.

TABLE 5. Algae toxins effect on dehydrogenase* activity of Staphylococcus aureus cells

Dehydrogenase	Time of methylene-blue discoloration, s			
	Algae toxin concentration, %			
	Control	$1 \cdot 10^{-5}$**	$1 \cdot 10^{-6}$	$1 \cdot 10^{-7}$
Glucose dehydrogenase	20.2	–	70.0	34.2
Glycerin dehydrogenase	24.5	–	65.7	26.0
Succinic acid dehydrogenase	312.5	–	562.5	320.0

*Dehydrogenase activity was measured with time (s) of methylene-blue discoloration.

**Under algae toxin concentration $1 \cdot 10^{-5}$ % methylene-blue didn't become colorless during 3 days.

As dehydrogenase inactivation leads to infringement of exchange processes, that in turn suppresses ability to live of the cell, the specified parameter can be used for biological testing of physiological activity of substance, including algae toxins. As it is shown on Table 5 the introduction of algae toxins into cultural environment caused the inhibition of microorganisms dehydrogenase that surely inhibit its duplication and caused dying off. The maximal oppressing effect on studied dehydrogenase of *Staphylococcus aureus* has algae toxin in concentrations $1 \cdot 10^{-5}$ and $1 \cdot 10^{-6}$%.

Therefore, one from ways of negative influence of algae metabolites on bacterial cell is dehydrogenase inhibition, which has very important role in energy processes of microbial cell. In one's turn, dehydrogenase inhibition and further test-culture extinction may be an index for indication of biological activity in water environment.

It is interesting to note that *Cyanobacteria* cells may be perspective sensor for definition of biological activity of non-algae toxins. A hypothesis on the appearance and persistence of natural foci of *Cholera* based on ecological and bioenergetics features of the process was developed by I. Brown and L. Sirenko (1997). In next years this hypothesis received experimental verification. At the sacrifice of ability of various bacteria, including the genus *Vibrio* and many *Cyanobacteria* species to perform energy coupling, depending on external conditions by means of two cycles (the proton and sodium cycles). Induction of the sodium cycle of energy coupling increases the resistance of bacteria to various environmental factors, such as high concentrations of sodium, alkaline pH, and high proton conductance of coupling membranes and probably the virulence of these vibrios. In this case development of *Cyanobacteria* in an aquatic environment enriched with Na^+ accelerates alkalization of the medium and stimulates the development of the community of *Cyanobacteria* with *Vibrio cholerae*, an autochthonous inhabitant of saline water bodies and marine shallow waters. Salinization of water bodies accelerates their blooming and enriches them with soluble organic matter, a substrate for vibrios inhabiting the biotope. Further propagation of *Cholera* infection is related to eating of heat-untreated hydrobionts from "blooming" of water bodies.

Thus, possibility of sharp growth of cholera epidemic in many districts of Earth may be forecasted with high precision by intensity of water "blooming".

7. Conclusions

1. Algae cells egest into environment an essential quantity of organic compounds of different chemical nature and biological activity in process of

its vital functioning. Most essentially on all links of ecosystem influence compounds, which have toxic properties, inasmuch as they in a great deal determine metabolites effect on forming of quantitative and qualitative indices of hydrobiocenoses. In this connection using the biological production of water ecosystems on further trophic levels, including human, as higher link of biocenotic chain, needs a great attention to water quality and estimation of its possible toxicity.

2. Range of toxic influence for metabolites, which were extracted from algae samples essentially varies depending on algological composition, vitality degree for cells of main producer of toxic compounds.
3. During preliminary stage of organic matter extinction its toxicity with respect to warm-blooded organisms usually strengthens. However, in this case determinative is don't only increasing of algae toxins production, but also presence of toxic products as a result of albuminous compounds extinction.
4. Testing of different regimes for algae biomass detoxication – boiling, strong acid hydrolysis, treatment by adsorbents (surfactants and ion-changing pitchesми) testifies about possibility of carrying out the detoxication with valuable decrease of toxicity. Choice of detoxication regime is determined by character of further using of product and demands to its security.
5. LD_{50} of algae toxin during peroral introduction to warm-blooded animals usually is about 10–20 mg/kg, during intraperitoneal introduction – less than 1 mg/kg.
6. Among perspective sensors for determination of biological activity for algae toxins may be used biological organisms of different systematic groups: warm-blooded and invertebrate animals (for example, *Daphnia magna* st.), bacterial microflora and so on.
7. Toxic-biological methods of detection *Cyanobacteria* toxins, which were described above, may be used advisably under presence of toxin within the bounds of 10^{-3}–10^{-4} mg/l. Researches will be began from *Protozoa* (for example, determination of concentrations for *Daphnia* death).
8. Advantage of using the bioassays for toxicity determination is moderate cost of method and possibility of receiving the quick answer about qualitative and quantitative toxin present. Weakness of bioassay method is impossibility to determine low concentration of algae toxin, especially in drinking water, and failure to disjoint it sharply between closely related algae toxins, which were produced by different algae strains.

9. Maximum permissible concentration of *Cyanobacteria* toxin in water is about 0.0000006 mg/l. Higher concentrations are evidence of taking special prohibitive measures necessity.

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BIOCHEMICAL METHOD FOR QUANTITATIVE ESTIMATION OF CYANOBACTERIA TOXINS

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Abstract: It is shown the possibility of using the model reaction with cholinesterase inhibition for qualitative and quantitative determination of Cyanobacteria toxic metabolites in natural waters and algae cells. It was established that changes of cultural medium pH may be registered by spectrophotometric method with using of bromine thymol blue indicator or by electrometric method with indexes of pH-meter. Depending on degree of cholinesterase inhibition the different quantity of organic acid is evolved. It is determined that method differs from other biochemical methods by rapidity and may be used under field conditions.

Keywords: Cyanobacteria, toxins, cholinesterase, nature water

1. Introduction

Water "blooming", which is induced by mass reproduction of some algae species, is most wide-spread case of biological contamination. This phenomenon is registered as well in the majority of eutrophic freshwater reservoirs (such as lakes, ponds, estuaries), as some seas (Azov, Black, Baltic) and separate part of oceans. Water "blooming" in freshwater is caused by mass reproduction of *Cyanobacteria* but well known also cases of water "blooming"

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which are caused by reproduction of *Chlorophyta*, *Diatomea*, *Dinophyta* and *Chrysophyta (*Canter-Lund and Lund, 1995; Sirenko and Gavlilenko, 1978). Water "blooming" in sea-water (what is called as "red tides") is caused by different species of *Diatomea* algae. During recent years "red tides" were registered in some freshwater reservoirs (for example, Biva lake in Japan).

Microcystis aeruginosa Kuetz. emend. Elenk., *Aphanizomenon flos-aquae* (L.) Ralfs., *Anabaena flos-aquae* (Lyngb.) Breb. are main agents of water "blooming" among *Cyanobacteria*. These species are characterized by essential toxicity. Taking into consideration the negative effect of *Cyanobacteria* toxins and its high biological activity, methods of identification, preparative purification and its quantitative estimation were elaborated in many countries of the world (Figure 1) (Harada, 1996).

By and large methods of detection and quantitative determination of *Cyanobacteria* toxins may be divided by convention on the 3 groups (Kirpenko et al., 1977; Carmichael, 1997):

Toxicity (mice)					●						
Screening ELISA								●	●	●	
Phosphatase assay								●	●	●	
FID-GC, HPLC(FL)						●					
NMR	●										
MS, MS/MS			●	●							
TLC/FAB					●						
Identification LC/MS (FAB)					●						
LC/MS (ESI)							●				
micro LC/MS (FAB)						●	●				
HPLC (photodiode array)					●	●	●				
HPLC (CE)-linked phosphatase assay						●	●				
Separation and HPLC (UV)						●					
Quantification micro LC/MS (FAB, SIM)								●			
HPLC (FL, CL)									●	●	
	1mg		1μm			1ng				1pg	

Figure 1. Summary of screening, identification, separation and quantification techniques available for *Cyanobacteria* toxins (Harada, 1996). FL – fluorescence; FAB – fast atom bombardment; ESI – electron spray ionization; CE – capillary electrophoresis.

- physics-chemical methods (relieving of UV, IR-spectrum, electron spin resonance, chromatography, disk-electrophoresis in polyacrylamide gel, high-voltage electrophoresis) which are used for identification of chemical nature for preparative purified compounds;
- enzyme and immunological reactions for detection and quantitative estimation of toxins in water and biological medium (determination of *Cyanobacteria* toxins presence by cholinesterase activity, tripsin, ELISA method);
- toxicology-biological methods (estimation of acute toxicity on warm-blooded animals and hydrobionts, using of insulated organs and mitochondria, tissue cultures and others experimental materils).

The aim of our work was acquaintance with experience of Ukrainian scientists by studying of toxicity and elaboration the method of quantitative estimation of presence and activity of *Cyanobacteria* toxins in water by reaction with cholinesterase.

2. Chemical Nature of Cyanobacteria Toxins which were Used for for Elaboration of Reaction with Cholinesterase

It is well known that *Cyanobacteria* toxins are complicated many-component organic compounds by its chemical constitution. It is shown the empiric composition and structural formulae for many toxins in the literature (Carmichael, 1994; Carmichael, 1997; Sirenko and Kozitskaya, 1988). Some of *Cyanobacteria* toxins were synthesized. However, nature of the majority of *Cyanobacteria* toxins don't elucidate because of difficulties of its purifycation and identification. In connection with heterogeneity of *Cyanobacteria* toxins is more rationally to use complex approaches for qualitative and quantitative its studying in water and biological mediums.

For elaboration of biochemical method had been used *Cyanobacteria* toxin which was isolated from natural populations (Sirenko, L. et al., 1995; Kirpenko, 1996). It is white fine crystallized material, soluble in water up to concentration of 1%. Solutions of toxins have no color, smell and optically transparent. This compound withstand sterilization by boiling and autoclaving. Toxins are soluble in organic solvents (such as butyl, ethyl, methyl alcohols), but from these solvents toxins were very difficult to crystallized. Form of crystals after crystallization from ethanol are presented at Figure 2.

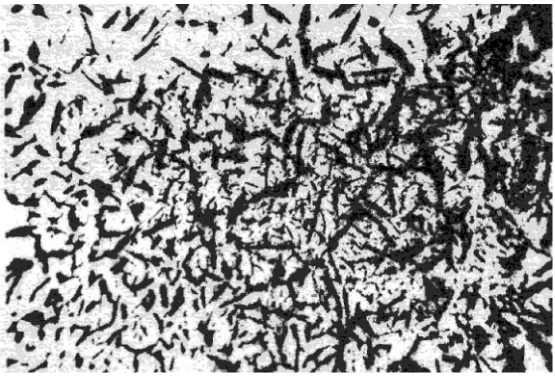

Figure 2. Form of toxin crystals during crystallization from ethanol (Kirpenko et al., 1977).

Molecular weight of more active toxins fraction is about 19000. Toxin has 5–7 characteristic spots with Rf 0.12; 0.24; 0.43; 0.56; 0.73; 0.93; 1.14 at thin layer chromatography with using of standard plates "Silufol" UV-254 and next systems of solvents: isopropyl alcohols ($CH_3CH(OH)CH_3$): acetic acid : water in proportion (60:1:40).

It were detected 5 fractions at electrophoretic separation of toxin in boratic buffer (pH 8.4) with using of 600 V and 20mA during 4 hours. There were up to 56% of protein, 4% peptides, more than 1% carbohydrates and about 0.3% of reduction compounds in toxic substance of algae.

It were detected 16 aminoacids in toxin hydrolisate after hydrolysis by proteolitic enzymes. It were leucyne (13.9±1.6), phenylalanine (4.6±0.27), valine + methionine (4.1±0.41), tyrosine (6.3±0.36), proline, alanine (8.4±0.73), threonine (14.9±1.27), glutamine acid (15.1±0.92), glycine + serine (7.9±5.6 and 13.3±0.8), asparaginic acid (5.4±0.27), arginine (5.1±0.32), hystidine (2.4±0.73) and lysine (2.8±0.73). It was found the $1–2 \cdot 10^{16}$ free radicals in the toxin composition with using of electron spin resonance method (Malyarevskaya et al., 1986).

Solution of *Cyanobacteria* toxin in distilled water had the next maximum of absorption: 187, 189, 192, 193 nm for UV-areas and 2950, 2850, 1412, 730, 680 sm^{-1} in IR-areas. Toxin was fractionated into 6 components with relative electrophoretic mobility (REM)) in zones 0.09; 0.12; 0.17; 0.25; 0.44; 0.80 after using of disk-electrophoresis in polyacrylamide gel. There are 4–5 acid components with close and identical mobility in its structure. It is found the specific proteins in zones with REM near 0.80–0.91 for most toxic preparations.

Cyanobacteria toxin (which is used for elaboration of this method) was depolarized neuromuscular blocking agent and high cumulative by toxic-pharmacological action. Anticholeneesterase acting were dominated after sharp poisoning by toxin.

3. The General Principle of Model Reaction with Cholinesterase Inhibition

All main elements of model reaction *in vitro* were based on experimental data by Martin and Chatterjee (1969) (Sirenko et al., 1975) connected with inhibition of acetylcholinesterase by algae toxins. This method was modified by group of scientists (Kirpenko et al., 1976; Sirenko et al., 1997) and had been used for qualitative and quantitative estimation of toxic metabolites in water and algae cells.

The principle of reaction lies in the fact that cholinesterase interact with substrate (acetylcholine or butyrilcholine) and to decompose its into choline and acetic or butyric acids by next scheme:

$$^{-}HO(CH_3)_3N^{+}CH_2CH_2OOCCH_2 \xrightarrow{\text{cholinesterase}} \text{acetylcholine}$$

$$HOCH_2CH_2N^{+}(CH_3)_3OH^{-} + CH_3COOH \quad \text{(acetic acid)}$$
$$\text{choline} \qquad\qquad CH_3CH_2CH_2COOH \quad \text{(butyric acid)}$$

Changes of cultural medium pH are due to this reaction. This changing may be registered by spectrophotometric method with using of bromine thymol blue indicator or by electrometric method with indexes of pH-meter. Depending on degree of cholinesterase inhibition the different quantity of organic acid is evolved.

With aim of this reaction in mind to water sample with inferred present of toxin added enzyme of cholinesterase with well-known activity and fixed quantity of acetylcholine. Toxin quantity are determined by calibration curve. Experiments were demonstrated that this method of *Cyanobacteria* toxic metabolites estimation has high sensitivity. It makes possible to estimate qualitative and quantitative content of biologically-active metabolites in experimental samples. Method differs from other biochemical methods by rapidity and may be used under field conditions (Kirpenko, 1996; Sirenko et al., 1975). However, for its widespread use take into account that phenols, heterocyclic amines which formation in large quantities in the breakdown of organic compounds of algae may inhibition of cholinesterase too.

4. The Sampling of Water for Toxin Determination

Samples of water for estimation of algae toxin content were withdrawn from reservoirs or at water-purifying stations on different phases of cleaning by standard method. Volume of water samples was 0.5 l. Selected samples are placed to glass capacities with neutral reaction. Plastic capacities for drinking water or food products may be used too. Plastics and polyethylene of other quality may leached different chemical compounds into water. It makes difficult the receiving of analyze results.

All taking samples of water must be labeled with indication of day and place of sampling, information about function of water. Water samples for toxicology-hygienic analysis must be treated during 10–12 hours after selection. Otherwise these samples necessary must be preserved in refrierator at temperature 4°C but no more than 2–3 days (Malyarevskaya et al., 1986).

5. Preparing of Necessary Reagents

1. Standard solution of cholinesterase in borate buffer: 0.5 g of borax ($Na_2B_4O_7$), 0.65 g of boric acid (H_3BO_3) and 0.02 g of cholinesterase is dissolved in 1 l volumetric retort. Activity of cholinesterase is measured in International units (U) (at temperature 25°C in 1 mg of dry preparation) is 0.4–0.8 U.

   Activity of cholinesterase in absolute units is characterized by quantity (in mg) of butyrilcholineiodide cloven by 1 mg of dry enzyme preparation for 1 hour at 20°C. Following a recommendations from Enzyme Commission of International Biochemical Union the unit of enzyme activity (U) is taken to be equal to its quantity which catalyzes the transformation of 1 μM substrate during 1 min at 25°C under optimal pH value and substrate concentration.

   Method for determination of initial cholinesterase preparation activity is based on titration with butyric acid which is evolved after butyril-cholineiodide hydrolysis at 25°C and pH 8.0.

   It is profitable to use solution with preparation concentration of 0.09 mg/l if level of cholinesterase cleaning the unknown. It is necessary to prepare more concentrated solution if the reaction proceeds slowly. Solution must be diluted if the reaction occurs rapidly.

   Cholinesterase preparation weighing on analytical balance with an accuracy of ±1% for determination of its activity. Then dissolved in 200 ml of saline solution. It is appropriate to prepare such concentrations of preparations for analyze (mg/ml): 1.5; 0.5; 0.2; 0.09; 0.07; 0.05; 0.03; 0.01 (Malyarevskaya et al., 1986).

24 ml of cholinesterase with fixed concentration adds to reaction capacity at 25°C. Then pH of cultural medium increases up to 8.5 with using of 0.1 n NaOH. 1 ml of butyrilcholiniodide solution adds to this mixture. If pH will decrease up to 8.0 it is necessary to add such quantity of 0.1 n NaOH that pH is increased up to 8.1. It is noted the level of alkaline in microburette after this manipulation. It is necessary to turn stop-watch at pH 8.0. Then the solution of NaOH adds during 3–5 minutes with such speed that pH-meter pointer deflects from pH 8.0 no more than 0.5 point to the left or to the right.

It is established the level of alkaline in microburette after necessary time and ending of titration. Cholinesterase content is determined in International units by formulae:

$$\mathbf{A} = \frac{P_1}{\mathbf{t \cdot P_2}},$$

where
- P_1 – quantity of butyrilcholiniodide which was hydrolyzed (μM);
- t – time (min);
- P_2 – quantity of cholinesterase which used for 1 determination (mg).

It is determined that 1 ml of 0.1 n NaOH represents the hydrolysis of 100 μM of butyrilcholineiodide.

Value of P_2 can be deduced from concentration (mg/ml) and volume of cholinesterase (24 ml of saline solution), which used for 1 determination by next formulae:

$$\mathbf{P_2} = \frac{\mathbf{100 \cdot K}}{\mathbf{C \cdot 24}},$$

where
- K – correction factor on titre of 0.1 n NaOH;
- C – concentration of cholinesterase, mg/ml.

Precision of method is ±1.5%. Solution of cholinesterase must be kept in refrigerator at 4°C.

2. Solution of acetylcholine is prepared by dissolving of 0.2 g in 10 ml of distilled water immediately before using.
3. Bromine thymol blue soluble indicator is prepared by dissolving of 0.1 g in 100 ml of distilled water. Solution is kept in refrigerator.
4. Standard of coloring for spectrophotometry: 1 g of acetous copper ($Cu(CH_3COO)_2$) is dissolved in 19 ml of distilled water under heating up to 40–50°C. To chilled solution it is flushed 2.5 ml of 0.1 n $KMnO_4$ and 1 ml of ethyl alcohol. Mixture is stirred intensively under heating on

water bath up to 50°C during 15 minutes. Then it is endured 2 hours for stabilization of coloring under room temperature. Work solution is obtained by mixing of 1 ml standard solution with 3.5 ml of distilled water. Main standard solution is kept under darkness in vessel with sealing plug.

5. Butyrilcholineiodide (recrystallized) – $C_9H_{20}O_2NJ$
6. Magnesium chloride ($MgCl_2$), pure for analysis
7. Sodium hydroxide – NaOH, 0.1 n
8. Tetraboric potassium (borax) – $Na_2B_4O_7$, pure for analysis
9. Boric acid – H_3BO_3, pure for analysis
10. Distilled water
11. Saline solution: 6 g NaCl, 3 g $MgCl_2$, 0.1 g borax potassium are dissolved in 1 l of distilled water.
12. Solution of butyrilcholineiodide in saline solution with concentration of 200 mg/ml (20%) is prepared before using (Malyarevskaya et al., 1986).

6. Construction of Calibration Curve for Estimation of Toxin Quantity

For estimation of toxin quantity by inhibition of cholineesteraze activity with using of pH-meter or spectrophotometer it is necessary to pursue its preliminary calibration for transformation of equipment indices into units of toxin concentration (Kirpenko et al., 1977; Kirpenko, 1996; Martin and Chatterjee, 1969).

Calibration of instruments is carrying out with using of solution well known concentration of compounds by standard method. For preparation of calibration curve used series of toxin solutions from 0.1 to 10^{-12} mg/l. These solutions are prepared step by step after dilution of initial solution. 0.1–0.5 ml of extracted toxin solution under different concentration is flushed into test-tube. Then 1–2 ml of cholinesterase solution in borate buffer with pH 8.3–8.4 is added into experimental test-tube. Mixture is incubated during 10 min under temperature 37°C during 20 minutes. In the control test-tube is flowed the distilled water instead of toxin solution. After first incubation in test-tube it is necessary to add 0.1 ml of bromine thymol blue and 0.2 ml of acetylcholine solution. The optical density of solution is determined by using of cuvette in 0.5 cm thick at 680 nm by spectrophotometry after second incubation (20 min). As the control had been used standard of coloring.

Computation of cholinesterase change is produced by subtraction of optical density or pH of experimental solution from control value, which are always greater, than with algae toxin.

Construction of calibration diagram is executed by put aside abscissa axis concentration of spectrophotometrized solutions in μg (mg) on ml (l). Aside ordinate axis are put values of optical density for spectrophotometrized solutions (control – experiment). Obtained calibration diagram is used for estimation of toxin concentration in investigated solutions on the base of values of its optical density or indices of pH change.

7. Qualitative Estimation of Cyanobacteria Toxic Compounds in Natural Water

0.1–0.5 ml of experimental water samples added into test-tubes (3 parallel). Then it is necessary to add 1–2 ml of cholinesterase solution in borate buffer. Mixture is incubated during 20 minutes under temperature 37°C. In control test-tube add of distilled water instead of experimental solution. After incubation 0.1 ml of bromine thymol blue indicator add into investigational test-tube. After second incubation with acetylcholine (during 20 minutes) is determined optical density of solution with using of Spectrophotometer SF-46 and cuvette in 0.5 cm thick. Investigational length of wave is 680 nm.

Computation of cholinesterase change is produced by subtraction of optical density or pH of experimental solutions from control value, which are always greater, than with algae toxin.

On the base of calibration curve is calculated the quantity of toxin in water by the formulae:

$$T = \frac{a \quad V}{V_1 \cdot V_2},$$

where
- T – toxin quantity in investigational water samples, mg/ml;
- a – quantity of toxin on calibration curve, mg;
- V – volume of investigated water sample, ml;
- V_1 – volume of water for determination of toxin, ml;
- V_2 – total volume of mixture for carrying out the reaction, ml.

Thus, reaction with cholinesterase may be used as express-reaction of qualitative and quantitative estimation of toxin in water. Obtained information allows to judge about water quality on different stages of its purification or biological medium, which is used for cultivation of other hydrobionts.

However, above mentioned method isn't suitable for quantitative estimation of algae toxin for *Cyanobacteria,* which is accumulated in tissues of hydrobionts and animals. For these purposes using of immunological methods is more perspective. With the help of specific antiserum, which is obtained by toxin immunization (as example, for guinea-pigs), allows to receive reliable and precise data about toxins concentration in water and other biological objects, which may accumulate toxins in own organisms.

8. Conclusions

Algae toxin oppresses oxidative-restoration enzymes and cholinesterase, increasing of aldolase activity under chronic poisoning. Destruction of carbonic and protein exchanges is consequence of enzyme activity changing. It causes accumulation of unoxidated products of carbonic exchange, strengthening of ATP consumption and inhibition of its restoration. It has a negative effect on energy balance of cells and causes pathologic changes in its functional activity.

By this means action of *Cyanobacteria* toxic metabolites may be determined by inhibition of enzyme activity which catalyzes the different stages of metabolism in organism. It was proved that *Cyanobacteria* toxins may be regarded to enzyme poisons. Such method in algae toxins pharmacodynamics is highly efficient because it combines a great group of toxic compounds with different properties. It is evident that inactivation of enzyme systems plays decisive role in pathogenesis of intoxication (Kirpenko, 1996).

Proportional biochemical methods for quantitative determination of *Cyanobacteria* toxins by inhibition of cholinesterase reaction can be used for mono- and mixed algae cultures. Toxicity is decreased under algae cultivation in algologically pure cultures. Conversely, toxicity is increased under algae cultivation in mixed cultures. It was registered that toxicity may be greatly increased after joint development of species-antagonists (for example *Microcystis* and *Aphanizomenon*, *Microcystis* and *Anabaena*, *Aphanizomenon* and *Anabaena*). It was shown that sensitivity of this method achieved to 10^{-12} mg/l.

This biochemical method can be carried out in field conditions. The restriction of this biochemical method using can be promoted by strengthening of destruction processes in cells. At these processes decomposition of algae biomass are intensified considering that Phenols and heterocyclic amines which are formed after destruction processes can oppress cholinesterase too. In this case it is advantageous to use the complex estimation of the qualitative and quantitative content of *Cyanobacteria* toxins, supplemented biochemical investigations by the performance of immunological tests.

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USING OF LASER-DOPPLER SPECTROMETRY FOR DETERMINATION OF TOXICITY DEGREE OF CHEMICAL AND NATURAL COMPOUNDS

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Abstract: It is presented the results of using of laser-doppler spectrometry method for estimation of toxicity level as well synthesized (on example, $K_2Cr_2O_7$) as natural biologically active substances (Emystime C). As the test-objects had been used 3 species of *Chlamydomonas*: *Chl. reinhardtii* Dang., *Chl. aculeata* Korschikoff in Parscher and *Chl. pitchmannii* Ettl. It is shown the high sensitivity of laser-doppler spectrometry method and perspectives of its using for determination of toxicity for different exometabolites caused by plants, mushrooms and microorganisms into environment.

Keywords: Laser-doppler spectrometry, microalgae, speed of movement, energy consumption, potassium dichromate, growth regulator

1. Introduction

Changes of physiological reactions of plant and animal organisms are widely used for studying of toxicity degrees in reservoirs and soils. The answering reaction of living organisms (as well single-cells as multi-cells) conducts on

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cell level under influence of extreme factors of environment. In all probability mechanism of one-typical nonspecific answering reaction on the impaired influence during evolution process in cells was elaborated. Invertebrates (such as *Daphnia magna* Straus, infusoria *Tetrahymena pyriformis* W.) and high water plants (such as *Elodea canadensis* L., *Vallisneria spiralis* L.) very often used as the primary, non-expensive and sensitivity test-objects. In the first case the mortality of infusoria or dead of more than 50% of Daphnia from starting quantity (during 96 hours exposition) comparison with control are estimated for water toxicity degree (Braginskiy, L.P. et al., 1987). The speed of cytoplasm movement in cells of high water plants is main physiological reaction for vital activity of object (Smirnova and Sirenko 1993). It associated with that mobility of intracellular structure is as well function of intensity of energy process that caused in cell as characteristic of aggregative state of cytoplasm (its viscosity). Considerable and short-term stimulation of oxidative-restoration process with the generation of energy (for example, increasing of respiration intensity) may be happen under the effect of individual toxic mixtures. This process results in increasing of cytoplasm movement, which with time have drained the cell supply of energy and caused of its death. All organisms may perish after blocking of photosynthetic processes.

The movement of green algae (such as Dunaliella salina Teod., D. viridis L., Euglena gracilis Klebs, Pedinomonas tennuissima M., *Chlamydomonas reinhardtii* Dang. and other) is of frequent use in biotesting of sea and freshwater reservoirs among microscopic organisms (Barsanti and Gualtieri, 2006; Massjuk et al., 2007, Novikova et al., 2007). The reason is that movement of living, nondestructive algae cells are connected with main fundamental processes of vital activity of organisms such as photosynthesis, energy transformation, transfer the compounds into membranous structure of cell. Studying of movement cells has a direct relation to revealing of general principles for regulation as well intracellular metabolism processes as ontogenesis, embriogenesis and morphogenesis. Studies of peculiarities of algae movement are definitely of interest for ecology and geography of algae. In particular, outecology allow specifying characteristics of some species concerning their reactions to considerable amount of parameters (for example, reaction to light, temperature, content of cultural medium) and determine optimum, maximum and minimum values of acting factors of environment. It may help to estimate the toxicity of biology-active compounds as well of experimental plants as other organisms which entering into environment. In this connection the origination of demand in elaboration and using quick, sensitivity and reliable methods for estimation of

parameters and speed of movement of different algae species as the test-objects.

The aim of our investigations was elucidation of possibility to using of laser-doppler spectrometry (LDS) method for express-estimation of toxicity degree of chemical and natural compounds.

2. Material and Methods

As the test-objects have been used algological and bacterial pure cultures of mobile algae from *Chlorophyta* genus *Chlamydomonas* that were selected from freshwater biotopes: *Chlamydomonas reinhardtii* Dang. (from collection of Oak Ridge National Laboratory, USA); *Chl. Aculeate* Korschikoff in Parscher, *Chl. pitchmannii* Ettl. (from SAG collection from University of Gettingen, Germany). The algae were grown in Erlenmeyer retort with using of the similar cultural medium for different species of *Chlamydomonas* (Sirenko et al., 2005) at temperature $20 \pm 2°C$ and lighting 4500–5000 lux. Duration of light and dark periods were 12/12 hours. For experiments have been used the cultures on the logarithmic and stationary growth phase. There were two series of experiments for determination of sensitivity of LDS method. Chemical and natural compounds that used in industrial and biotechnological processes had been used in these experiments.

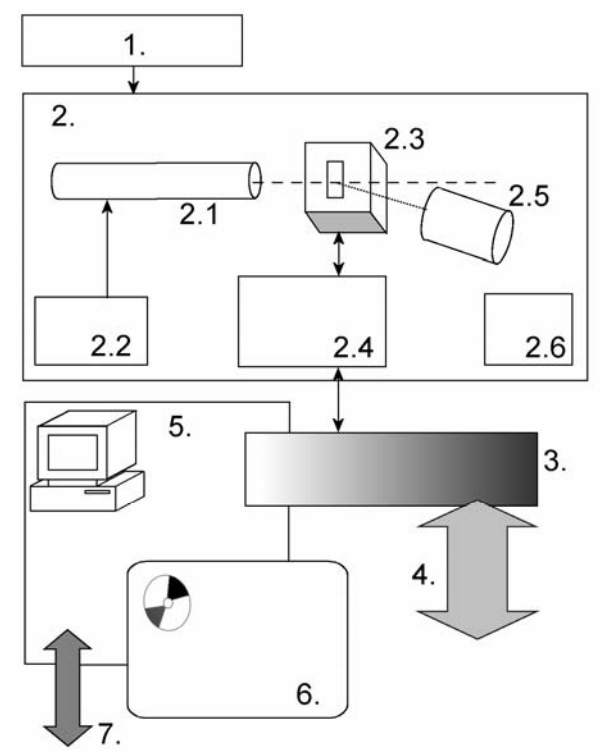
Series 1. – $K_2Cr_2O_7$ was used as toxic compound. Using concentration were 0.05; 15.0; 45.0 and 75.0 mg/l. Determination of experimental indices of algae development were provided during 1, 7 and 14 days of cells contact with potassium dichromate.

Series 2. – Growth regulator with wide spectrum of action (Emystime C) had been used in this series. Emystime C is exometabolites of mushrooms-epiphytes that were cultivated in root system of medical plant. Emystime C added in concentration 0.005; 0.013; 0.025; 0.05; 0.1; 0.15 and 0.25 mg/ml to cultural medium. Estimation of cell development indexes was provided after 1, 2, 7, 14, 21 and 28 days.

Laser-Doppler Spectrometer (Vlasenko et al., 1992) produced by Molfar Instruments was used for control of vital activity of test-object. Figure 1 shows a block diagram of information-measuring systems on the basis of Laser-Doppler spectrometer. LDS makes it possible to estimate the speed of cells motion (μm/s), energy consumption for cells movement (relative units), part of living and dead algae cells (%) as well as the reactions under the effect of different groups of possible toxicants. 300–500 μl of algae suspension with cell concentration from 0.5 to 200 billion cells per 1 ml

were placed in a cuvette. Measurements for 1 sample lasted up to 3 minutes. Measurement error does not exceed 3% for all parameters.

Changes of speed *Chlamydomonas* cells movement and its consumption of energy had been proved in 10 times repetition. For this aim it was registered averaged experimental data. Methodical aspects for procedure of energy consumption by algae cells are presented in our last paper (Novikova et al., 2007).



1. Electronic module of a network or autonomous power supply.
2. Laser-optical measuring module.
   2.1. Laser (He-Ne, 632.8nm). 2.2. Laser power supply unit. 2.3. Temperature-controlled zone of measurement. 2.4. Electronic control of thermal stability. 2.5. Photodetector. 2.6. Electronic amplifier.
3. Multi-channel analog-digital signal converter.
4. Universal interface with auxiliary and other specialized measuring systems of monitoring.
5. Computer.
6. Specialized software.
7. Channels of exchange and connection of a complex with standard networks.

Figure 1. Laser-doppler spectrometer.

Number of algae cells was defined with using of Goryaev chamber on MBI-1 microscope. Size of cell surface (μm^2) and volume of microalgae (μm^3) were calculated by suitable formulae (Parshykova et al., 2006) with using of ocular-micrometer indices. Concentration of algae cells may be estimated by LDS method (in relative units) too. It is necessary to pursued calibration of laser-doppler spectrometer for receiving this parameter in absolute units. With this aim used parameters must be received by hard calculation of cell number with Goryaev chamber.

Control of algae pigment complex is carried out by chlorophyll *a* content with using of extraction or non-extraction methods. For measurement of pigment concentration by extraction (spectrophotometry) method have been used Spectrophotometer SF-46 with regard to UNESCO work group recommendation (Musienko et al., 2001). Method of differential fluorimetry of native algae cells was used for non-extraction estimation of pigment concentration. In these experiments have been used the Planctofluorometer FL 300 3M, which was elaborated by specialists from Krasnoyarsk State University (Gold et al., 1984, Gold et al., 1996). At parallel it is determined the ΔF indices which characterized by intensity of fluorescence before and after addition of symazine as inhibitor of electron transport of photosynthesized cells. ΔF index characterizes the level of vital activity of algae by magnitude of its potential photosynthetic activity (Parshykova et al., 2001).

Methods of statistic analysis have been used for mathematical treatment of receiving experimental data (Lapach et al., 2000). Conclusions were formulated on the base of Student criterion with confidence probability $P = 0.95$.

3. Results and Discussion

3.1. SERIES 1. CHEMICAL SYNTHESIZED REAGENT – $K_2CR_2O_7$

Receiving experimental data testifies that cells of different species of *Chlamydomonas* differ by speed of cell movement and energy consumption up to beginning of experiments. Cells of *Chl. aculeata* (52.60 ± 3.87 μm/s) had the highest speed of movement in comparison with *Chl. pitchmannii* (43.51 ± 2.74 μm/s) and *Chl. reinhardtii* (34.93 ± 2.45). Energy consumptions for movement realization was higher in cells of *Chl. aculeata* (17.67 ± 2.15 rel. units) in comparison with other objects (*Chl. pitchmannii* (6.51 ± 0.68) and *Chl. reinhardtii* (3.61 ± 0.27) (Figure 2).

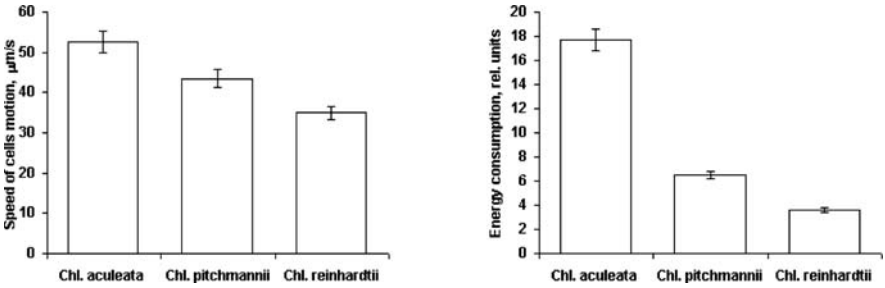


Figure 2. Initial (without addition of reagents) speed of cells motion and energy consumption for different species of *Chlamydomonas*.

Different species of *Chlamydomonas* are specific by answer reaction after 1 contact day with $K_2Cr_2O_7$. *Chl. aculeata* cells had more resistance to presence of $K_2Cr_2O_7$ in cultural medium (Figure 3). These cells don't feel toxicity of Cr (VI) that was in content of potassium dichromate. Speed of *Chl. aculeata* cells motion was decreased only at maximal experimental concentrations of $K_2Cr_2O_7$ (45 and 75 mg/l).

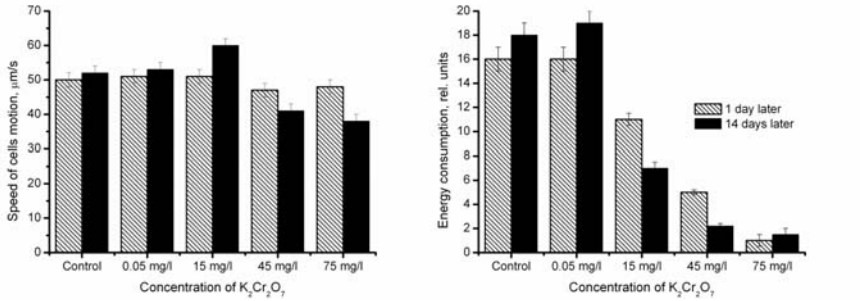


Figure 3. Effect of potassium dichromate on speed of motion and energy consumption for *Chlamydomonas aculeata* cells.

The speed of cells motion is kept on the level of control for limit-permissible concentration of $K_2Cr_2O_7$ (0.05 mg/l) after 14 day of contact. The speed of cells movement to begin to decline at maximal experimental concentrations 45 and 75 mg/l (on 28–34%). It testifies about increasing of toxicity level of $K_2Cr_2O_7$ at rising of the contact time for *Chlamydomonas* cells with toxicant. It is significantly that energy consumption for cells motion was gradually reduced beginning with concentration of 15 mg/l after 1 day of contact with $K_2Cr_2O_7$. It was noted that stimulation of energy consumption of *Chl. aculeata* cells at more low concentration (0.05 mg/l)

after 14 days of contact in comparison with control. It testifies that algae cells were sensitive to $K_2Cr_2O_7$ toxicity and increased the consumption of energy on its movement. In this connection is good reason to believe that mobility of algae cells can helps to survived under effect of unfavorable chemical factors of water environment. It must be emphasized that such protective mechanism can help in survive of microalgae cells only up to fixed concentration of toxic compounds. In experimental cells had been registered sharp decreasing the consumption of energy on further increasing of toxicant concentration. It may be connected with damaged of photoreceptor apparatus under higher concentrations. We observed the similar reaction in *Chl. aculeata* cells at concentration of $K_2Cr_2O_7$ 15 mg/l after 14 days of contact. It was registered the sharp decreasing the consumption of energy (at 88–91% for concentrations 15 and 75 mg/l) under increasing of acting concentration above 15 mg/l. It testifies about dying away of microscopic algae cells.

Chl. reinhardtii cells had medium degree by resistance of potassium dichromate toxicity from experimental species of *Chlamydomonas* (Figure 4). Cells of this species increased speed of movement beginning from concentration of 15 mg/l after 1-day contact with $K_2Cr_2O_7$. The limit-permissible concentration of $K_2Cr_2O_7$ don't influence on speed of cells motion but maximal experimental concentration (75 mg/l) makes *Chl. reinhardtii* cells immovable after 1 days of contact. The speed of cells movement was perceptibly decreased at concentrations 45 and 75 mg/l (in medium on 79–85%) after 14 days of contact in comparison with control.

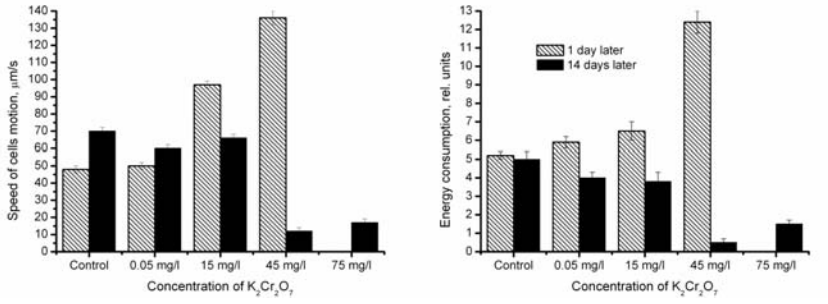


Figure 4. Changes in speed of motion and energy consumption for *Chlamydomonas reinhardtii* cells under influence of $K_2Cr_2O_7$.

The consumption of cell energy increased at rise of toxicant concentration after 1 contact day with $K_2Cr_2O_7$. It was registered the maximal increasing of the consumption of cell energy for concentration of 45 mg/l (on 235%) in comparison with control. Maximum experimental concentration (75 mg/l) is appears lethal for *Chl. reinhardtii* cells under 1 contact day after addition of $K_2Cr_2O_7$ into cultural medium. It was registered the successive decreasing

the consumption of energy on 20–91% at concentrations 0.05 and 75 mg/l in comparison with control (Figure 4).

Chl. pitschmannii cells are the least resistance to presence of $K_2Cr_2O_7$ in cultural medium (Figure 5).

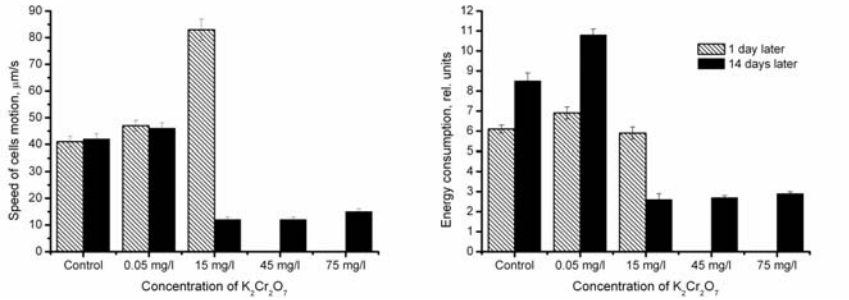


Figure 5. Changes in speed of motion and energy consumption for *Chlamy-domonas pitchmannii* cells under presence of different concentrations of potassium dichromate in cultural medium.

Algae cells of this species lost of mobility at concentrations of reagent 45 and 75 mg/l after 1 day of contact with potassium dichromate. It was registered the increasing of speed of movement and the consumption of energy under decreasing of $K_2Cr_2O_7$ concentration to 0.05 mg/l. It testifies about its capacities to survive. It is interesting to note that at $K_2Cr_2O_7$ concentration 15 mg/l was registered the sharp increasing of speed of movement (on 100%) after 1 contact day in comparison with control. Lethal concentration of $K_2Cr_2O_7$ for *Chl. pitchmannii* cells was 45 mg/l after 1 day of contact with it. It is noted that the consumption of energy for *Chl. pitchmannii* cells at maximum experimental concentrations of $K_2Cr_2O_7$ were registered at level of 2.65–2.81 relative units. It testifies about possible damage surface membrane – plasmalemme by $K_2Cr_2O_7$. Such membrane is found as well under hydroxyproline cell walls that are surrounded of *Chlamy-domonas* cells as cover of filaments.

As illustrated of experimental data the least resistance to $K_2Cr_2O_7$ species of *Chlamydomonas* (*Chl. reinhardtii* and *Chl. pitchmannii*) had as well less cells number in suspension as lower temps of quantity growth (Table 1).

It is evident that biomass factor or total number of cells in liquid volume is of considerable importance in determination of toxicity level of $K_2Cr_2O_7$ and changes of physiological state of algae. The greater is the biomass of algae the feeble is inhibition influence of $K_2Cr_2O_7$ on the physiological state of algae.

Obtaining experimental data connection with toxicant effect of photo-synthesis productivity (Figure 6) and dynamic of photosynthetic pigments content confirmed main conclusions that had been used with LDS method.

Levels of *Chlamydomonas* cells resistance to addition of $K_2Cr_2O_7$ into cultural medium are arranged in the next order: *Chl. aculeata* > *Chl. reinhardtii* > *Chl. pitchmannii*.

TABLE 1. Changes of cell number (mln. cells/ml) species of Chlamydomonas under influence of different concentration of $K_2Cr_2O_7$.

Variant of experiment	*Chl. aculeata*		
	initial	1 day later	14 days later
Control	3.61 ± 0.28	3.87±0.26	6.60±0.45
0.05 mg/l		3.46±0.23	6.19±0.47
15 mg/l		5.43±0.39	2.31±0.16
15 mg/l		5.09±0.34	4.35±0.31
75 mg/l		3.98±0.29	3.62±0.28
Variant of experiment	*Chl. reinhardtii*		
	initial	1 day later	14 days later
Control	1.01 ± 0.08	1.02±0.09	1.19±0.11
0.05 mg/l		0.67±0.06	0.46±0.04
15 mg/l		0.84±0.08	0.80±0.07
15 mg/l		0.62±0.05	0.49±0.05
75 mg/l		0.60±0.06	0.46±0.04
Variant of experiment	*Chl. pitchmannii*		
	initial	1 day later	14 days later
Control	0.82 ± 0.07	0.82±0.06	0.85±0.09
0.05 mg/l		0.76±0.07	0.29±0.03
15 mg/l		0.52±0.05	0.41±0.04
15 mg/l		0.51±0.05	0.44±0.05
75 mg/l		0.45±0.04	0.31±0.03

By this means the complex using traditional (microscopic analyses and spectrophotometry) methods of analyze for physiological state of micro-algae and LDS method are supported the sensitivity of this method and assurance of receiving information. It is significant that LDS is efficient method for determination of species specificity of answer reaction for different species of one microalgae genus.

3.2. SERIES 2. NATURAL GROWTH REGULATOR – EMYSTIME C.

Much attention is given to search and elaboration of effective growth regulator of cells in connection with intensive development of algae biotechnologies. In current time the practical choosing of growth regulator of

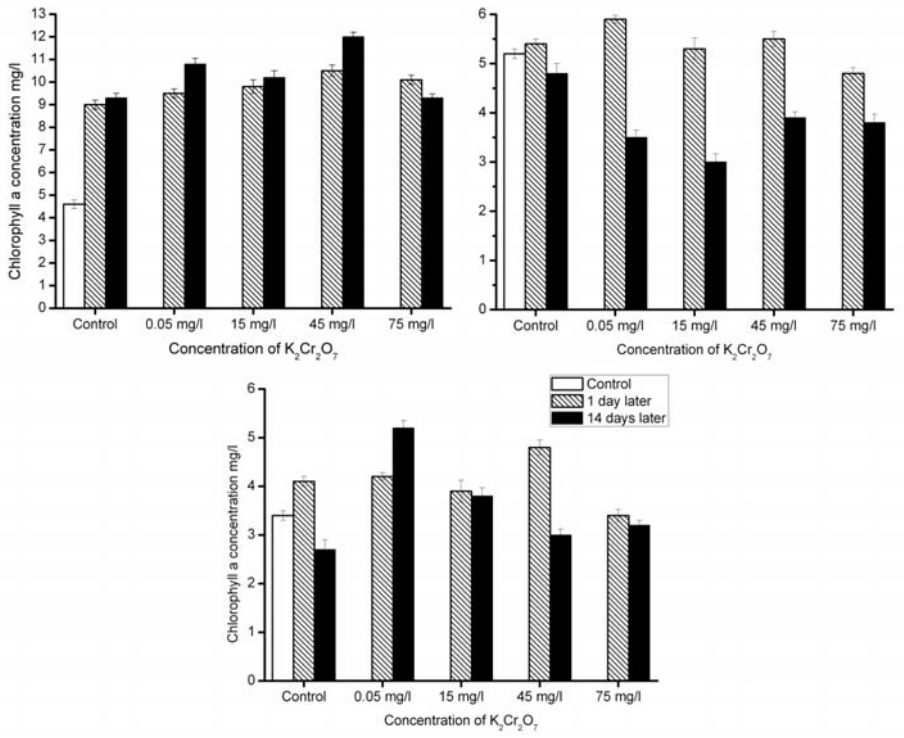


Figure 6. Influence of potassium dichromate on dynamics of chlorophyll a content for different *Chlamydomonas* species: A – *Chl. aculeata*; B – *Chl. reinhardtii*; C – *Chl. pitchmannii*.

microalgae in intensive cultivation isn't large and limited by derivates of β-indolylacetic acid, hyberillines, cytocynines and other very expensive compounds. In this connection scientists carried out experiments in receiving of natural phytohormone on the base of plants, mushrooms and microorganisms. LDS method had been used for express estimation of toxicity and efficiency natural biologically active compounds.

It was shown that addition of Emystime C into cultural medium essentially stimulated activity in cells motion (Figure 7). Stimulation effect was of greater intensity than higher using concentration of Emystime C. It was noted increasing of speed of cell movement and it's the consumption of energy during all time of observations.

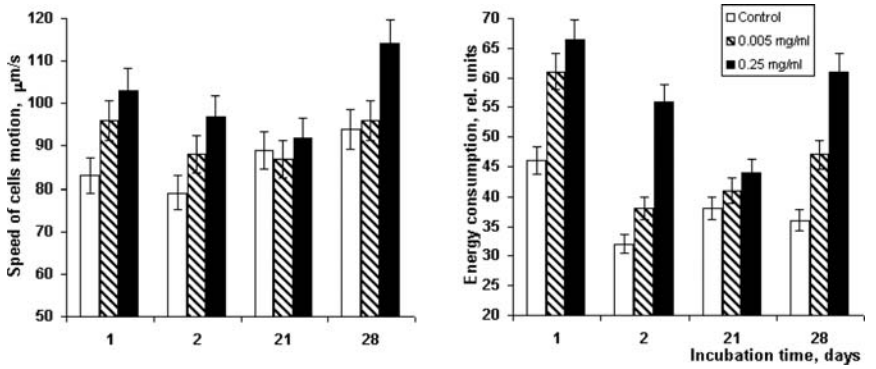


Figure 7. Changes in speed and energy consumption for *Chlamydomonas reinhardtii* cells under addition of Emystime C.

Changes the speed of test-object movement and increasing of its energy consumption at first hours of contact the algae cell with growth regulator which have been estimated by LDS method are supported by strengthening of cultures growth by dynamic of chlorophyll *a* concentration. As illustrated Figure 8 temps of chlorophyll *a* growth in experimental variants are changed after addition of growth regulator into cultural medium. Then chlorophyll *a* content rised at increases of duration of cells contact with Emystime C and depends from its concentration.

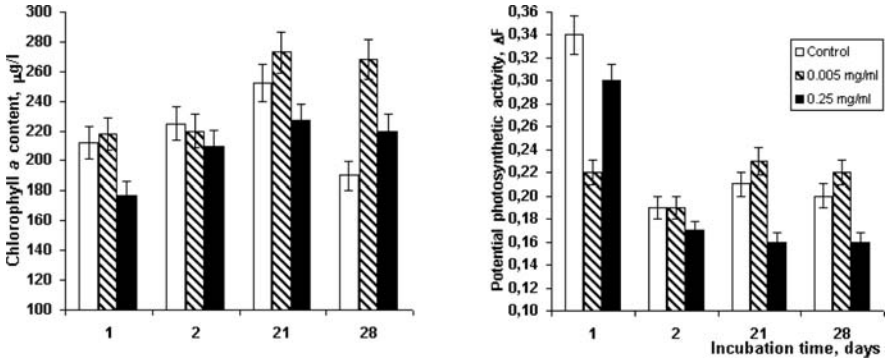


Figure 8. Influence of Emystime C on chlorophyll a content and its photosynthetic activity.

Emystime C concentration of 0.005 mg/ml had maximal stimulation effect on the dynamic of chlorophyll biosynthesis. The higher concentration

didn't demonstrate stimulation effect at first time. When duration of contact time is increased higher concentrations had positive effect on chlorophyll biosynthesis. Potential photosynthetic activity of algae (ΔF) changed too under effect of Emystime C. The decline of ΔF is registered at Emystime C concentration of 0.25 mg/ml. This is due to the fact of increasing of optical density for culture in experimental variants. The reason has to do the self-shading cells in cultures and impairment of light regime.

Cells numbers of Emystime C for terms of optimal work of photoreactor are tabulated in Table 2. Referring to it, cells number increasing and at 7 days was more than control on 47% for Emystime C at concentration of 0.005 mg/ml. Self-shading of cells have clearly defined on 28 day of contact of test-object with growth regulator in laboratory experiments.

TABLE 2. Changes is cells number under influence of growth regulators.

Variant of experiment	Number of cells, mln/ml			
	initial	1 day later	2 days later	7 days later
Control	6.72 ± 0.35	5.58 ± 0.28	6.20 ± 0.32	6.65 ± 0.34
Emystime C				
0.005 mg/ml		4.56 ± 0.19	8.82 ± 0.42	9.80 ± 0.46
0.25 mg/ml		4.50 ± 0.18	4.78 ± 0.21	5.02 ± 0.22

Factor of self-shading is removed at mass (industrial) cultivation of algae. The way to do this is through photobioreactor construction, intensity of its lighting and regime of light delivery to algae suspension (for example, by special optical fibers). Regular removal of algae biomass is of considerable importance in elimination of self-shading. Considering that the main goal of our investigations was possibilities of LDS method using we didn't conducted of regulation of self-shading of algae cultures.

Results of investigations are confirmed that natural growth regulator Emystime C are influence positively on speed of cells movement for algae test-cultures, its consumption of energy, the pace of chlorophyll *a* accumulation and photosynthetic activity of organism. This suggests the possibility of Emystime C at mass cultivation of algae.

4. Conclusions

By this means LDS method may be recommended for express estimation of toxicity as well chemical synthesized as natural biological-active substances, which are produced by microorganisms, plants and mushrooms into environment. LDS method may be used for biotesting investigations because makes it

possible receiving of spatiotemporal characteristic of unfavourable state of water quality (for example, with the availability of water "blooming" spots, caused by mass reproduction of toxic algae species) on the different parts of water objects or in recervoirs as a whole. LDS method may be used for operative revealing of local contamination sourses, for mapping zones of its distribution and determination of more ecologically unfavourable part of water area. It is evident that LDS method have the benefits in comparison with other methods which using in for natural water monitoring.

LDS method with standard set of toxicants may be successfully used control of sewage quality before it's entering to water reservoirs by industrial plants.

High sensitivity of LDS method makes possible to extend the range of search for most perspective and selective acting of growth regulator for microalgae and accelerates its choosing for practical using at mass (industrial) algae cultivation. It is shown that addition of growth regulator in special choosing concentration for every algae species into cultural medium will increase temps of growth biomass and efficiency work of photobioreactors.

5. Acknowledgements

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PCR TECHNIQUES AS DIAGNOSTIC TOOLS FOR THE IDENTIFICATION AND ENUMERATION OF TOXIC MARINE PHYTOPLANKTON SPECIES

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Abstract: Molecular based techniques applied to the marine environment for the rapid, specific and sensitive detection of target toxic microalgae have been developed and performed in many laboratories worldwide. Further, a need to quantify species specific target HAB (Harmful Algal Bloom) cells in natural assemblages avoiding the time consuming traditional methods of the microscopy is basically required. Here, a brief review of the quailtative and quantitative PCR-based methods applied for the monitoring of HAB species in field samples from the Mediterranean Sea is presented.

Keywords: HAB species, Mediterranean Sea, molecular techniques, PCR, primers, rRNA genes

1. Introduction

During these last decades numerous phylogenetic studies on marine phytoplankton at genus, species and population levels permitted to achieve relevant information on the existing relationships among them. Most of these

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V. Evangelista et al. (eds.), *Algal Toxins: Nature*, *Occurrence*, *Effect and Detection*.

The harmful phytoplankton constitute a group that holds sanitary, ecological and economic implications towards the coastal environments, the phylogenetic studies used the ribosomal genes (rRNA) that included a huge sequence dataset useful to assess the genetic variability within the genes or non coding regions to design oligonucleotide primers and/or probes taxon specific for the HAB micro-organisms to be examined (Scholin et al., 1996; Simon et al., 2000; John et al., 2005). The molecular assays for the identification of the toxic marine phytoplankton taxa are strictly related to the level of genetic variability within a species or genus and/or a fine grouping as among populations within a species. Molecular species is defined based on the molecular divergence or accumulated mutations among single individuals or individual groups in selected coding or non coding regions of nuclear and/or organelle genomes (Palumbi, 1992; 1994). This concept is well visualized by a phylogenetic analysis where each clade is evolutionary distinct also by its ancestor and descendent even if it may be not reproductively isolated (Gosling, 1994; Avise, 1998). Another important consideration has to be taken care on the molecular species concept applied to the marine phytoplankton, as the potential conflict with the morphological speciation concept; based on several studies it well established that different morphotypes of the *Alexandrium tamarense* species complex belong to the same geographic ribotypes (Scholin et al., 1994; John et al., 2003); within some diatom species of the genus *Pseudo-nitzschia* high genetic variation was found among spatial and temporal series collected isolates (Orsini et al., 2004; Evans et al., 2004; Lundholm et al., 2006).

Within the coding or non coding regions of the nuclear genome once assessed the level of genetic variation at which the potential discrimination of taxa (genus, species, toxic or no toxic isolates) have to be done, appropriate molecular markers can be selected and used in the molecular assay. The choice of the molecular marker in these assays is of great importance since it reflects species specific differences in polymorphism between populations of a species within its geographic range. In fact, the genetic probes for *Pseudo-nitzschia* species designed from ribosomal gene sequences of isolates collected over a limited geographical area did not match with isolates of the same species elsewhere (Parson et al., 1999; Orsini et al., 2002). The biodiversity in marine systems is higher respect to that of terrestrial and freshwater ecosystems due to the high dispersal and great population sizes; in fact, a large portion of species exists as a planktonic phase, and the marine microorganisms are subjected to environmental factors responsible of the species adaptive strategies (Avise, 1998; Feral, 2002).

human health and mariculture facilities due to the consequences of recurrent harmful microalgal occurrences or blooms. The adverse effects of HABs include toxin production, fish gill clogging, oxygen depletion with ipoxia or anoxia events and unpleasant water quality.

2. Detection and Monitoring of HABs

The Harmful Algal Blooms are increasing worldwide (Graneli and Turner, 2007). This may be attributed to many reasons, including the increased utilization of coastal waters for aquaculture, eutrophication and/or unusual climatological conditions, the movement of resting cysts caused either by ships' ballast waters or the translocation of shellfish stocks, overfishing and also an increased scientific interest regarding harmful species. The design of harmonized global detection and monitoring approaches is relevant and an early warning system for detection of HAB species is needed. This effort will be facilitated by the implementation of official monitoring procedures using novel innovative technologies, including also molecular and toxin detection. Molecular-based methods for detecting HAB species are used routinely in many laboratories around the world. No single type of molecular probe or assay strategy appears as the "best". Indeed, some HAB species can be detected using a variety of probes. The choice of probes/techniques for a given species seems to follow personal references, technical background and available laboratory equipment. These innovative molecular methods for detection and monitoring of HABs may be applied together with traditional methods or constitute alternative methodologies to traditional ones (Round Table Detection and Monitoring of HABs, XI Int. Conf. Harmful Algal Blooms, Cape Town, 2004). Current methods depend on microscopy, toxin and pigment analyses, which are time consuming and require considerable expertise and skill. The molecular techniques present many advantages including being more rapid, more specific at the species and population level, may require a minor level of expertise in the routine laboratory procedures respect to expertise needed to discriminate key morphological features indicative of HAB species, and they can be applied in the screening of numerous field samples (Anderson, 1995; Scholin et al., 1999; Bowers et al., 2000; Penna and Magnani 2000; Litaker and Tester, 2002; Medlin et al., 2006).

3. Nucleic Acid Extraction

Genomic DNA of microalgal clonal cultures and field samples is extracted and purified using a variety of commercial extraction kits as the Dynabeads DNA DirectTM Kit (Dynal, A.S., Oslo, Norway), DNeasy Plant Kit (Qiagen, Valencia, CA, USA), Dinoflagellate Isolation Kit (Diatheva, Fano, Italy). The efficiency of extraction and purification of genomic DNA from field samples is relevant for the PCR-based assay when the target cells are at very low concentration. The efficiency is related to the lysis process of cellular material and purification is based on the removing potential inhibitors and recovering target DNA. The substances such as poly-phenols, poly-saccharides, humic acids, clays, heavy metals can act as inhibitory agents on the PCR reaction. These substances have to be eliminated from the sea-water samples during the genomic DNA extractions otherwise the subsequent PCR reactions may be compromised. Several attempts to eliminate the effects of those compounds can be applied. Effects of inhibitors on PCR can be reduced by adding bovine serum albumin (BSA) or dimetylsulphide (DMSO) to PCR and sometime it is also useful to dilute the DNA template,.

4. PCR Techniques

4.1. QUALITATIVE PCR AND OLIGONUCLEOTIDE PRIMER DESIGN

To analyze the genetic variability it is necessary to select the appropriate molecular technique and the DNA or RNA target regions that can discriminate at the required hierarchical level (Hershkovitz and Lewis, 1996).

PCR amplification technique is fundamental for the genetic identification and characterization of microorganisms (Monis et al., 2005) and widely used in many areas of research for cost and ease of use. In these last decades, PCR based techniques were successfully employed for the genetic characterization of many taxa of harmful phytoplankton species in the marine environments (Bolch, 2001; Connell, 2002; Godhe et al., 2002; Vila et al., 2005; Penna et al., 2007).

Primers are short synthetic oligonucleotides ranging between 10–30 (bp) and containing 40–60% G/C content. They have to be complementary to target sites on the template DNA. A list of essential requisites has to be taken into consideration for the optimal primer design:

- **a)** primers should have G or C, or CG or GC at the 3' termini to increase the efficiency of priming;

- **b)** self complementary as the formation of hairpins (secondary structures) inside the primer should be avoided. A 3èn d hairpin with a ΔG of –2kcal/mol and an internal hairpin with ΔG of –3 kcal/mol is tolerated (Fig. 1);
- **c)** the 3²ends of primers should not be complementary to avoid primer dimer formation;
- **d**) cross dimers between sense and antisense primers should be avoided. A 3'end cross dimer with a ΔG of –5 kcal/mol and an internal cross dimer with a ΔG of –6 kcal/mol is tolerated (Fig. 2);
- **e)** melting temperatures (Tms) are preferred to be in the range of 52–58°C;
- **f)** primer annealing temperature (Ta) is the primer melting temperature of the DNA-DNA hybrid stability. Too high Ta will give insufficient primer-template hybridization resulting in low PCR product amounts. Too low Ta can give aspecific products caused by a high number of mismatches;
- **g)** optimal lengths of primers should be recommended in the range of 18–22 bp to avoid the missing of base or mistakes during the dNTP incorporation;
- **h)** it should be avoided an imbalanced distribution of G/C and A/T rich domains in the primers;
- **i)** the amplified product length for standard PCR can be in the range of 100–500 bp;
- **l)** primer can be located at the 5' and 3' ends, and generally the sequence close to the 3' end is known to show higher confidence and preferred most frequently, because this is the end of the primer that is extended by the DNA polymerase;
- **m)** primers have to be designed with high specificity in the target gene to avoid cross homology with other DNA sequences in the PCR mix. Commonly, primers are designed and sequences are analyzed in silico using BLAST tool or others, to check for the specificity.

Primer design is achieved using software platforms such as Oligo (www.oligo.com), CLCbio (www.clcbio.com), Primer Premier (www.premierbiosoft.com/primerdesign/).

4.2. DESIGN OF PHYTOPLANKTON TAXON SPECIFIC PRIMERS

The identification of harmful algal species is done using different genetic markers coupled with molecular assays. Ribosomal RNA (rRNA) genes,

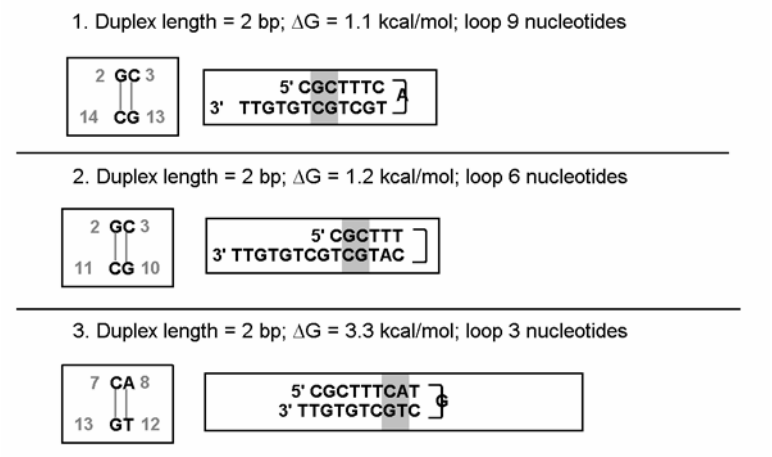

Figure 1. Hairpin formation.

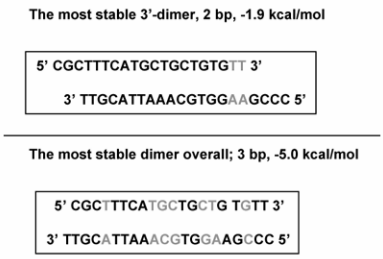

Figure 2. Cross dimer formation.

including the SSU (Small Subunit), D1/D2/D3 regions of LSU (Large Subunit), 5.8S, and the non-coding Internal Transcribed Spacer (ITS) and External Transcribed Spacer (ETS) regions, are traditionally employed and a huge sequence database for many phytoplankton taxa is currently available in Genbank (http://www.ncbi.nlm.nih.gov/), EMBL (http://www.ebi.ac.uk/) and DDBJ (http://www.ddbj.nig.ac.jp/). Ribosomal genes can cover a number of addressed questions for the phytoplankton genetic diversity characterization (Guillou et al., 2002; John et al., 2005). In fact, conserved and variable regions are present within ribosomal genes: highly conserved regions such as SSU are preferentially used for the discrimination above species level for taxonomy and phylogeny purposes (Edvardsen et al., 2003; Lange et al., 2002); whereas, moderately conserved or variable regions such as LSU and ITS, respectively, are used for the discrimination between species for taxonomy, phylogeny and detection purposes (Adachi et al., 1996; Saito et al., 2002; Sako et al., 2004; Penna et al., 2005a). A series of molecular

techniques are employed to investigate the genetic diversity at different taxonomic levels (Table 1).

TABLE 1. Molecular techniques, marker and their application for the genetic characterization in marine phytoplankton.

Technique	Marker	Application	Discrimination level	Reference
RFLP (Restriction Fragment Length Polymorphism)	Variable regions	Polymorphism study of cleaved DNA fragments	Among strains, populations	Scholin et al., 1994
AFLP (Amplified Fragment Length Polymorphism)	Variable regions	Polymorphism study of amplified and cleaved DNA fragments	Among strains, populations	John et al., 2004
RAPD (Random Amplified Polymorphic DNA)	Variable regions	Polymorphism study of amplified DNA fragments	Among strains, populations	Bolch et al., 1999
PCR and **real time PCR** (Polymerase Chain Reaction)	Target DNA regions	Taxonomy, Phylogeny, diagnostic detection	Genus, species	Godhe et al., 2002; Galluzzi et al. 2004; Bowers et al., 2000; Penna et al., 2007
DGGE (Denaturing Gradient Gel Electrophoresis)	Ribosomal genes	Polymorphism study of amplified DNA fragments	Among strains, populations	Van Hannen et al., 1998; Larsen et al., 2001
TSA-FISH (Tyramide Signal Amplification-Fluorescence Hybridization)	Ribosomal genes	Diagnostic detection	Genus, species	Miller and Scholin, 2000; Not et al., 2004
SHA (Sandwich Hybridization Assay)	Ribosomal genes	Diagnostic detection	Genus, species	Scholin et al., 1999; Tyrrell et al., 2001
DNA ARRAY with fluorescence	Ribosomal genes	Diagnostic detection	Taxon specific, Genus, species	Metfies and Medlin, 2004
Electrochemical detection	Ribosomal genes	Diagnostic detection	Taxon specific, Genus, species	Metfies and Medlin, 2005

In particular, the target 5.8S rDNA and ITS regions are useful in developing genus and species specific primers (Fig. 3); the high variable ITS1 and ITS2 regions together with the more conserved 5.8S rDNA sequences proved robust among target genera and species, allowing discrimination of the inter-specific variability in environmental samples containing mixed phyto-plankton populations (Penna et al., 2007).

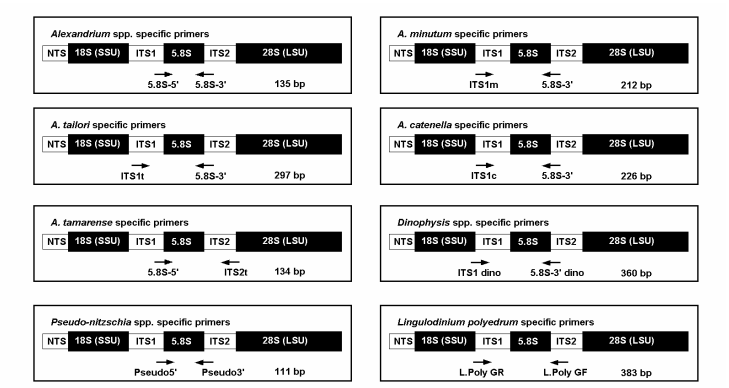

Figure 3. Harmful phytoplankton taxa oligonucleotide primer locations.

We report the design of genera and species-specific primers in the 5.8S rDNA-ITS regions (Table 2). To design the couple of primers for each specific taxon is essential carrying out the multiple alignments of the several sequences within each species or genus. Sequence alignment can be done on CLUSTAL W program (http://www.ebi.ac.uk/Tools/clustalw/).

A PCR based assay of natural samples always has to include a positive control containing the plasmid cloned target fragment-taxon specific to test that all reagents are working properly; a negative control with no DNA added to test for the potential contamination of the PCR reagents with extraneous template DNA; and a spiked control containing known amount of plasmid DNA added together with DNA of field samples to assess inhibition of the DNA polymerase caused by contaminants.

The PCR products are resolved on 1.8% (w/v) agarose gel, 0.5X TBE (Tris/Borate/EDTA) buffer gel and are visualized by standard ethidium bromide staining under UV light, along with a molecular weight standard. The presence or absence of a appropriate size base pair band indicates if specific harmful taxa are present in the sample.

TABLE 2. Oligonucleotide primers targeting the ITS-5.8S rDNA regions of different Harmful Algal Bloom genera and species of the Mediterranean Sea*.

Target taxa	Forward primer (5'– 3') Reverse primer (5' – 3')	Size bp	Primer location
Alexandrium spp.	F'- GCAADGAATGTCTTAGCTCAA	135	5.8S (5'→3')
	R'- GCAMACCTTCAAGMATATCCC		5.8S (3'←5')
Dinophysis spp.	F'- GCACGCATCCAAYTATCCATAAC	360	ITS1 (5'→3')
	R'-CATACAGACACCAACGCAGG		5.8S (3'←5')
Pseudo-nitzschia	F'-CGATACGTAATGCGAATTGCAA	111	5.8S (5'→3')
spp.	R'-GTGGGATCCRCAGACACTCAGA		5.8S (3'←5')
Ostreopsis spp.	F'-AAAACGATATGAAGAGTGCAGC	92	5.8S (5'→3')
	R'-CCAGGAGTATGCCTACATTCAA		5.8S (3'←5')
A. catenella	F'-AGCATGATTTGTTTTTCAAGC	226	ITS1 (5'→3')
	R'-GCAMACCTTCAAGMATATCCC		5.8S (3'←5')
A. minutum	F'-CATGCTGCTGTGTTGATGACC	212	ITS1 (5'→3')
	R'-GCAMACCTTCAAGMATATCCC		5.8S (3'←5')
A. tamarense	F'- TGTTACTTGTACCTTTGGGA	134	5.8S (5'→3')
	R'- ACAACACCCAGGTTCAAT		ITS2 (3'←5')
A. taylori	F'-TGGTGTTTGAATGCGGTTGT	297	ITS1 (5'→3')
	R'-GCAMACCTTCAAGMATATCCC		5.8S (3'←5')
Fibrocapsa	F'-GCAGAGTCCAGCGAGTCATCA	180	5.8S (5'→3')
japonica	R'-TAATATCCCAGACCACGCCAGA		ITS2 (3'←5')
Coolia monotis	F'-ATAAGTTCAACATGTGATGA	121	5.8S (5'→3')
	R'-CATATCTTCAAGCATATCC		5.8S (3'←5')
Lingulodinium	F'-ATGTGTTCTCATCGGATGTTG	383	ITS1 (5'→3')
polyedrum	R'-CACAGTACCGCTGCCACTTAAA		ITS2 (3'←5')
Protoceratium	F'-TGCTGATTGCCATCTATCTT	382	ITS1 (5'→3')
reticulatum	R'-CAGAAGCGCGTTAAACAG		ITS2 (3'←5')
O. ovata	F'-CAATGCTCATGTCAATGATG	210	ITS1 (5'→3')
	R'-CCAGGAGTATGCCTACATTCAA		5.8S (3'←5')
O. cf. *siamensis*	F'-TGTTACCATTGCTGAGTTTG	223	ITS1 (5'→3')
	R'-CCAGGAGTATGCCTACATTCAA		5.8S (3'←5')

Degenerate code D = A/G/T; M = A/C; Y = C/T; R = A/G

*modified from Penna et al. (2007)

5. PCR Detection Assay

Generally, once an assay is developed, it should be assessed using as many related microorganisms as possible to test the specificity. Potential cross

reactivity with other organisms in the sample should be also investigated when the assay is applied to natural populations in the seawaters. The other important requisite is the range of sensitivity of a molecular assay; it is relevant to assess the limit of detection for the assay especially when the molecular method is compared with the traditional ones for the detection of HAB phytoplankton species. In fact, the PCR based technique gave higher positive detection of target species than microscopy methods; target phytoplankton cells can be detected in environmental samples containing mixed phytoplankton population even at low cell concentration or below the detection limit of the microscopy and when the target taxon is not a dominant component of the phytoplankton community (Galluzzi et al., 2004; Penna et al., 2007).

5.1. SPECIFICITY OF THE PCR BASED ASSAY

As previously mentioned, the specificity of the primers is first evaluated in silico using the BLAST tool. Then, the specificity can be assessed in vitro by the PCR amplification of genomic DNAs purified from non target microalgal clonal strains together with target species of clonal cultures (Penna et al., 2007). Afterwards, the primers specificity has to be tested in natural seawater samples. The validation of taxon specific primers consists in the absence of non-target genomic DNA fragment amplifications in all these assays (Fig. 4).

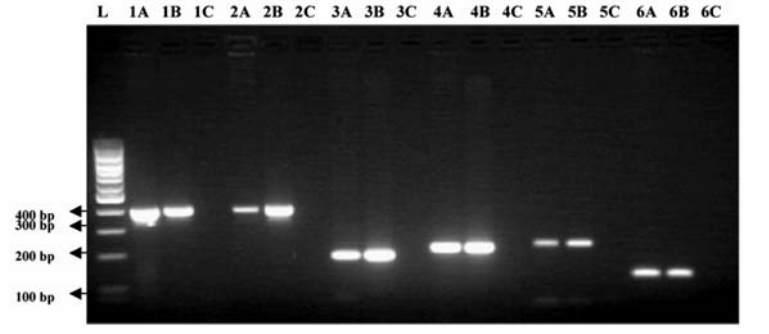

Figure 4. Genus and species specific PCR based amplification assay. Genomic DNAs (1 ng) of target HAB phytoplankton in the presence of non target background DNAs were amplified with specific primers for *Lingulodinium polyedrum* (1A), *Protoceratium reticulatum* (2A), *Fibrocapsa japonica* (3A), *O. ovata* (4A), *O.* cf. *siamensis* (5A), *Coolia monotis* (6A). L, size standards; lane A, genus or species specific PCR product; lane B, positive control (103 copies of plasmid containing cloned amplified fragments of genus or species ITS-5.8S rDNA); lane C, negative control containing mixed background DNAs of different clonal culture samples (modified from Penna et al., 2007).

5.2. SENSITIVITY OF THE PCR BASED ASSAY

The sensitivity of the PCR assay can be tested spiking serial dilutions of genomic DNA of target phytoplankton taxa, as 100, 10 and 1 pg of genomic DNA, in the presence of background net seawater DNA and unrelated DNA in the mixture. Further, the PCR sensitivity can be assessed using serial dilutions of a plasmid containing the target sequence of the taxa to be examined, as 10^4, 10^3, 10^2 and 10 copies of plasmid DNA.

The PCR assay is usually sensitive enough to detect 10 copies of plasmidic DNA and 1 pg of genomic DNA. The presence of background DNA doesn't affect the sensitivity of the PCR assay. A PCR sensitivity test using genus and species-specific primers is illustrated in Fig. 5.

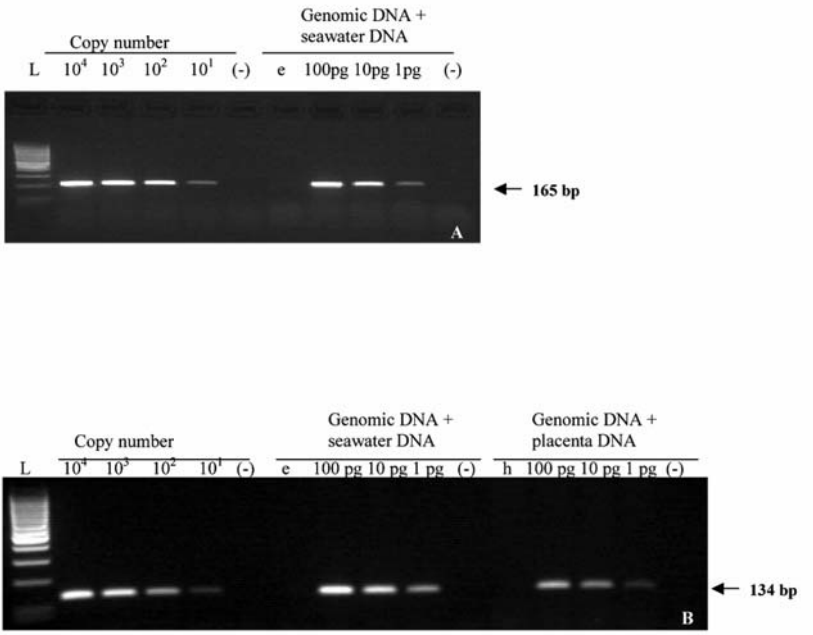

Figure 5. Sensitivity of the PCR based amplification assay to target HAB genera and species. Sensitivity of the PCR was tested both with plasmidic DNA containing the ITS-5.8S rDNA and genomic DNA of the target genus and species. Plasmidic DNAs were serially diluted from 104 to 10 copies per reaction tube. Genomic DNA of *O. ovata* CNR-D1 (A) and *A. tamarense* CNR-ATA1 (B) are serially diluted from 100 pg to 1 pg with 25 ng of background DNA of a net concentrated seawater sample and with 500 ng of background DNA of human placenta. (e), genomic DNA of net seawater. (h), genomic DNA of human placenta. (-) negative control with sterile water (modified from Penna et al., 2007).

5.3. DETECTION OF POTENTIALLY HARMFUL PHYTOPLANKTON GENERA AND SPECIES IN NATURAL SEAWATER SAMPLES IN THE MEDITERRANEAN SEA

PCR amplifications were carried out on many lugol fixed natural seawater samples collected along the coastal areas of the Mediterranean Sea, including Italy, Spain and Greece, to detect the presence of the four potentially HAB genera and ten species, as listed in Table 2. The bucked or net seawater samples contained mixed phytoplankton populations with target, potentially HAB taxa ranging from amounts of $5.0 \cdot 10^1$–$1.0 \cdot 10^3$ of total concentrated cells to bloom densities, such as $\geq 1.0 \cdot 10^4$–$1.0 \cdot 10^6$ of total concentrated cells to be processed in the PCR assay. In other seawater samples, potentially HAB target cells were below the detection limit of light microscopy (<10 cells L^{-1}). Genomic DNAs purified from seawater samples were promptly amplified by PCR using the genus and species – specific primers. The PCR amplified fragments of expected size for each genus and species were detectable in all samples examined containing the target taxa, confirming the amplificability of the genomic DNA and the applicability of the PCR based assay to environmental samples.

Furthermore, the PCR detection analyses were compared with microscopy counts. The PCR analysis of potentially harmful phytoplankton taxa gave positive results even when target cells were not observed in the field samples using microscopic determinations. Further, the PCR based assay allowed the identification of HAB species in seawater samples when uncertain species-specific identification of cells was obtained by light microscopy and calcofluor epifluorescence examinations.

5.4. DETECTION OF POTENTIALLY HARMFUL MICRO-PHYTOBENTHOS IN BENTHIC ASSEMBLAGES IN THE MEDITERRANEAN SEA

PCR analyses were also applied to environmental samples containing benthic and epiphytic assemblages of microalgae communities to detect the presence of the two potentially toxic *Ostreopsis* species, *O. ovata* and *O.* cf. *siamensis*, and *C. monotis* in different coastal areas of N Tyrrhenian, Ionian and Catalan Seas. PCR analyses detected the presence of the genus *Ostreopsis* in all examined samples, while *O.* cf. *siamensis* was present in in Tossa de Mar (Catalan Sea, Spain) and Siracusa (Ionian Sea, Italy), and *O. ovata* was detected in Genova (N Tyrrhenian Sea, Italy). These PCR detections were in agreement with microscopy identification of the genus *Ostreopsis*, while the identification at the species level was determined only by PCR analysis. *C. monotis* was detected in Porto Torres (Italy) and Genova (Italy) field samples in agreement with microscopy detections.

5.5. COMPARISON OF THE PCR ANALYSES AND MICROSCOPIC DETERMINATIONS

Molecular determinations by PCR based assay are always carried out together with microscopy analyses of the phytoplankton and microphytobenthos target taxa present in the natural samples. Harmful target genera and species are indentified and counted under an inverted light microscope at a magnification of x 200–400. Then, PCR detections of the target species are compared with the microscopy analyses of the same natural samples. Based on numerous environmental samples analyzed by PCR assay and optical microscopy for different harmful taxa, the PCR based technique provide higher positive detection of target species than microscopic methods; target cells can be detected in environmental samples containing mixed phytoplankton populations even at low cell concentration or below the detection limit of the microscopy and when the target species is not a dominant component of the natural plankton or phytobenthos community. These higher frequencies of positive detection events are registered for all harmful taxa examined until now (Fig. 6). The higher percentage of the qualitative detection of harmful taxa by PCR technique when compared to light microscopy

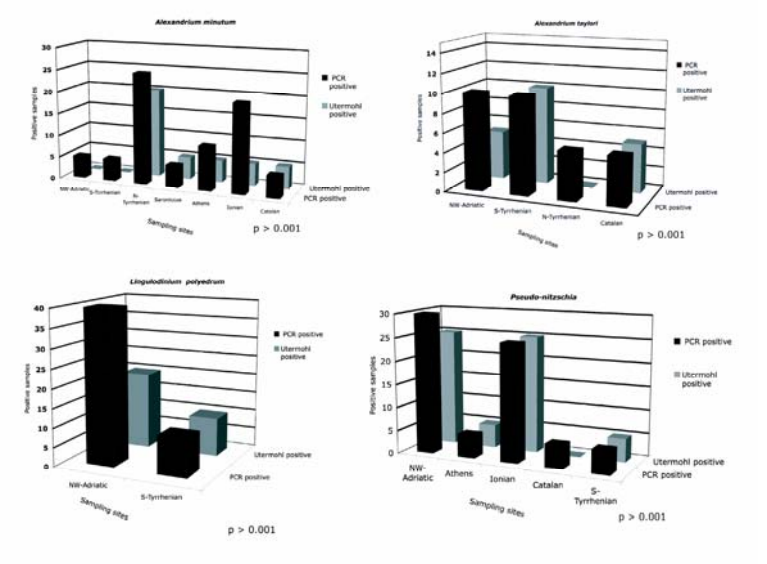

Figure 6 . Comparison of positive samples by PCR and microscopy methods for some HAB taxa detection. Net seawater samples were collected along coastal sites of the Mediterranean Sea during the period of 2002–2005.

could depend on the different sample volume processed in both methods; in the microscopic method only 5–10 ml of net sample was settled in the counting chamber to avoid the overlapping of the target phytoplankton cells; this limits the microscopic method to the processed volume of sample, potentially loosing the effective target cell number to be counted when the target harmful species are a minor component of the phytoplankton community. In the genomic DNA extraction, the entire net sample can be processed, but only a small fraction is used as DNA template in the PCR reaction. This can be balanced by the fact that the target sequence is usually rDNA, which is expressed in multiple copies in the genome (Galluzzi et al., 2004).

In the case of the microphytobenthos toxic species of *Ostreopsis* spp., the species-specific identification of *O. ovata* and *O.* cf. *siamensis* can be confirmed only by the PCR analysis, due to the extreme variability of the morphological and morphometric features within this genus (Penna et al., 2005b).

6. Quantitative Real-time PCR

6.1. BASIC PRINCIPLE OF THE REAL-TIME PCR ASSAY

Quantitative real-time PCR is a very sensitive method, which has been used in the last years to detect and quantify various phytoplankton species in environmental samples (Bowers et al., 2000; Popels et al., 2003; Galluzzi et al., 2004; Coyne et al., 2005). In the real-time PCR assay, a target gene is amplified with specific primers and product formation is monitored after each cycle (in real-time) by measuring a fluorescence signal. If the efficiency of the reaction remains constant, the increase in fluorescence observed during the reaction will be proportional to the starting quantity of the target molecule. The fluorescence can be generated by using an intercalating fluorescent dye (e.g. SYBR Green) or a number of alternative probe systems (e.g. TaqMan®, molecular beacons, etc.). The use of intercalating dyes is the cheaper and simplest approach and consists in adding the fluorescent dye directly to the PCR mixture. These dyes bind to double stranded DNA and, following a conformational change, emit fluorescence (Fig. 7A). SYBR Green-based assays are very sensitive, but it is noteworthy that any double stranded DNA can be detected using this dye, including primer dimers or non-specific amplified sequence, leading to false positive results. For this reason, the primer's specificity is crucial. Moreover, multiplexing is usually not feasible.

The TaqMan® approach is based on the use of oligonucleotide probes complementary to a sequence located between the two primers used for PCR amplification. The TaqMan® probe possess a fluorescent reporter dye and a quencher dye conjugated at the 5' and 3' ends. Due to the close proximity of these two dyes, the fluorescence of one (the reporter) is quenched by the other through fluorescence resonance energy transfer. During the extension step of the PCR, the DNA polymerase (which possesses a 5'–3' exonucleolytic activity) encounters the bound TaqMan® probe and degrades it. This degradation results in the separation of reporter from quencher and an increase in fluorescence emission can be observed (Fig. 7B). This approach is less subject to false positives than the intercalating dye method, but it is more expensive. Furthermore, it is possible to carry out multiplex PCR by choosing appropriate fluorophores.

Molecular beacons are oligonucleotide probes that can adopt a stem-loop structure by the annealing of complementary sequences at its 5' and 3' ends. The sequence in the loop is the sequence complementary to the target region. Similar to the TaqMan® probe, a fluorophore and a quencher are covalently linked at each end of the molecule. In the "closed" conformation, fluorophore and quencher are into proximity and the fluorescent signal is absent. When the probe loop anneals to its target sequence forming a probe-target hybrid (with greater stability than the stem structure) the fluorophore and the quencher are separated from each other and a fluorescent signal can be observed (Fig. 7C). This signal is proportional to the amount of DNA amplified.

The fluorescence signal, generated either by intercalating dyes or fluorescent probes, is measured by the real-time PCR instrument at the end of every cycle. The instrument also normalizes the fluorescence signal of the

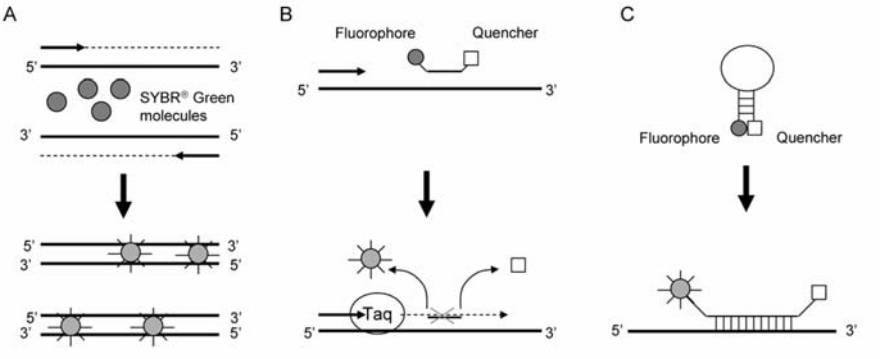

Figure 7. Chemistries used in real-time PCR assays. A) SYBR Green molecules; B) Taqman® probe; C) Molecular beacons (see text for details). (from Galluzzi et al., 2007).

reporter dye by the fluorescence signal of the passive reference dye (ROX) to obtain a ratio defined as the normalized reporter signal (Rn). The fact that DNA amplification is measured in real-time inside the reaction tube makes the method a "closed-system" and limits problems associated with carry-over contaminations (Raoult et al., 2004).

The C_t value (threshold cycle) is defined as the number of cycles required for the fluorescence to cross a fixed threshold above the baseline. Due to the fact that the increase in fluorescence and the starting amount of the target molecule are strictly related, the amount of target sequence in an unknown sample can be calculated by plotting its C_t value on a standard curve generated using the target sequence cloned into a plasmid, or using the DNA extracted from a known number of cultured cells. When a plasmid is used as a standard, it is essential to know the amount of target gene per cell in order to allow the determination of the cell number in the field sample. This implies certain work on laboratory cultures to optimize the method for each target species or strain. An example of standard curve generated with plasmid containing the target sequence is shown in Fig. 8.

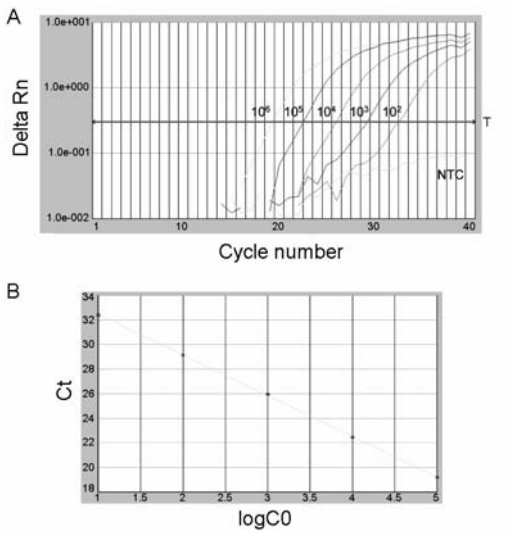

Figure 8. A) Example of amplification plots for a standard curve generated with different amounts of plasmid molecules (from 106 to 10 copies) containing the target sequence. The cycle number is plotted vs the Delta Rn, which represents the normalized reporter signal (Rn) minus the baseline signal established in the early PCR cycles. B) Calibration curve plotting log starting copy number vs Ct. NTC, no template control; T, threshold (from Galluzzi et al., 2004).

6.2. APPLICATIONS OF THE REAL-TIME PCR BASED ASSAY

Real-time PCR based assays have been developed for the monitoring of a number of toxic phytoplankton species. Also, several real-time PCR protocols have been optimized for the quantitative approach using different standard curves (generated using plasmid dilutions or axenic cultures), different DNA template preparation (purified DNA or cell lysates), different real-time PCR chemistries (intercalating dyes or fluorescent probes), and different target DNA sequences.

Recently, a real-time PCR assay based on the use of a TaqMan probe has been developed for the specific detection and quantification of *Cryptoperidiniopsis brodyi* (a *Pfiesteria*-like dinoflagellate) in environmental samples (Park et al., 2007). The assay has been developed against the ITS2 ribosomal DNA region. The choice of this variable rDNA region ensured the species-specificity of the assay. This assay was used in conjunction with the real-time PCR assays specific for *Pfiesteria piscicida* (Bowers et al., 2000) and *Pfiesteria shumwayae* (Zhang et al., 2005) to investigate the temporal variations of *C. brodyi*, *P. piscicida*, and *P. shumwayae* abundance in a coastal region of Tasmania.

Several species of *Alexandrium* have been detected and quantified using real-time PCR using either SYBR green or TaqMan chemistries. Assays were developed for *A. minutum* (Galluzzi et al., 2004), *A. fundyense* (Dhyrman et al., 2006), *A. catenella* (Hosoi-Tanabe and Sako, 2005) and *A. tamarense*, both for vegetative cells (Hosoi-Tanabe and Sako, 2005) and cysts (Kamikawa et al., 2005).

Other toxic species have been the objective of the real-time PCR-based assay development: these include *Aureococcus anophagefferens* (Popels et al., 2003), *Karenia brevis* (Casper et al., 2004), *Cochlodinium polykrikoides*, *Karenia mikimotoi*, *Heterocapsa circularisquama*, *Chattonella antiqua*, *C. marina, C. ovata* (Kamikawa et al., 2006), *Heterosigma akashiwo* (Coyne et al., 2005; Kamikawa et al., 2006), *Chattonella subsalsa* (Coyne et al., 2005) and *Prymnesium parvum* (Galluzzi et al., in press).

Real-time PCR multiplexing and multiprobing have also been investigated. Handy et al. (2006) designed and evaluated a TaqMan®-based real-time PCR assay for single tube detection of three raphidophyte species (*Chattonella* cf. *verruculosa, C. subsalsa* and *Heterosigma akashiwo*). Environmental samples containing these raphidophytes have shown to be successfully multiplexed or multiprobed with only minimal losses in sensitivity.

In principle, the real-time PCR technique can be applied to any phytoplankton species having DNA sequence data available and informative at the species or strain level, so that primers and/or probes can be

designed. However, due to the possibility of variations of target gene (mostly rRNA genes) copy number among different strains, and in order to verify the primers specificity, the method needs to be tested and optimized with the local phytoplankton population in a geographical area to be investtigated.

The main advantages of the real-time PCR application in phytoplankton monitoring concern specificity, sensitivity and applicability to preserved environmental samples. Sample preservation is often necessary, but the use of fixatives may cause the morphology distortion of some phytoplankton species, making more difficult to distinguish them from closely related species using a microscope. The development of a quantitative real-time PCR-based assay may help to overcome this kind of problems. Moreover, the general high sample throughput of this method may reduce working time compared to the microscope-based methods.

Concerning the costs, the real-time PCR instruments are becoming to be affordable also for small research groups and are now quite common in molecular biology-equipped laboratories. Moreover, the consumable cost per sample has been estimated in about $4.0 to $6.0 (Handy et al., 2006; Galluzzi et al., in press) depending on the method/master mix/chemistry, which makes the real-time PCR a potential affordable method for monitoring applications.

Generally, only one species or strain at the time can be analyzed in a quantitative approach, unless a multiplex reaction is performed. However, although multiplexing and/or multiprobing are powerful tools for molecular investigations of specific groups of toxic algae, their development and validation can be difficult and expensive (Handy et al., 2006).

7. Conclusion

Based on different studies, the specificity, sensitivity and feasibility of the PCR detection of several potential harmful phytoplankton taxa in environmental samples have been demonstrated (Guillou et al., 2002; Godhe et al., 2002; Connell, 2002; Galluzzi et al., 2005). In most of these studies the molecular markers are the ribosomal genes as target regions for the primer design. Further, the PCR method revealed higher detection efficiency than microscopic analysis, giving the higher positive percentage values of noxious target species presence in seawater samples and benthic assemblages.

PCR based technique coupled with quantitative real-time PCRs could represent well set up methods for the detection of the phytoplankton cells at the pre-bloom levels necessary to predict species-specific potential bloom sites, to determine potential environmental factors that influence a bloom, and to evaluate potential sources of species-specific low-level cell

inoculations through natural or anthropogenic transport mechanisms in coastal seawaters.

Quantitative real-time PCR method is still at the developmental stage and its application for monitoring of harmful algae requires validation in the area to be investigated due to the possible variability in terms of target gene copy number in different phytoplankton populations within the same species.

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DETECTION OF PHYTOPLANKTON WITH NUCLEIC ACID SENSORS

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Abstract: A potential hand-held biosensor system for the *in-situ* analysis of toxic algae was developed during the EU-project ALGADEC. Identification of toxic algae is based on molecular probes that specifically target its rRNA. 17 taxon specific probe sets were developed for harmful algae that occur in three different coastal areas in Europe. A sandwich-hybridization and two labelled probes are used to detect the rRNA. A capture probe, immobilised on the biosensor, binds to RNA-strands isolated from the target organism. A second digoxigen-labelled probe binds also to the RNA-strands. After incubation with an antibody-enzyme complex directed against digoxigenin, a substrate is added and a redox-reaction takes place. The resulting electrical current is measured and the amount of bound rRNA is proportional to the electrical current. The adaptation to the sensor and the probe specificity tests were done using laboratory strains with closely related species to avoid false positives and to guarantee that only desired strains are detected. The signals from the different probes are recorded by a microcontroller unit. If a PC is connected to the system, an easy to operate software visualizes process data, graphic results, and the measured values will be stored on the hard disc. The main steps of the analysis process are executed automatically in the measurement device. Only a manual filtering, including a lysis procedure has to be done before the automatic measurement. The portable ALGADEC device is also capable to operate as a stand-alone system with

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A bloom is regarded as a sudden increase in the microalgal population activated by suitable growth conditions so that concentrations of 10^4–10^5 build in keypad, display, power supply and memory card. In a new project, the ALGADEC device shall be further automated, optimized and implemented into a FerryBox system.

Keywords: Biosensor, microalgae, ALGADEC, sandwich hybridisation, FerryBox

1. Introduction

Microalgae are the major producers of biomass and organic compounds in the oceans because of their photosynthetic activity and they represent the base of the aquatic food chain. About 5000 species of marine microalgae are known to date (Sournia et al., 1991) and some 300 species can proliferate in such high numbers that they discolour the surface of the sea (Daranas et al., 2001; Hallegraeff, 2003) as a so-called bloom (Figure 1).

Figure 1. Bloom of *Noctiluca scintillans* in October 2002, Leigh, New Zealand (photo: Miriam Godfrey)

cells per liter can be reached for a certain period of time (Masó and Garces, 2006). A particular species or a group of species can dominate the bloom (Masó and Garces, 2006). The proliferation of microalgae is a normal event and can be beneficial for aquaculture and fisheries operations. However, it can also have a negative effect if the bloom consists of harmful algae and thus cause severe economic losses to aquaculture, fisheries and tourism (Hallegraeff, 2003). About 200 noxious microalgal species and 97 toxic species (mainly dinoflagellates) are known to have the potential to form Harmful Algal Blooms (HABs) (Zingone and Enevoldsen, 2000; Moestrup, 2004). The impact of HABs is defined by the concentration of harmful species, even the most toxic species must occur with a minimum cell concentration to exert a harmful effect (Zingone and Enevoldsen, 2000). HABs have occurred throughout recorded history and are natural phenomena. However, in the past decades, the public health and economic impacts appear to have increased in frequency, intensity and geographic distribution (Daranas et al., 2001; Hallegraeff, 2003). This can possibly be explained by an increase of scientific awareness and by increased aquaculture. The increase of fish and shellfish farming has been observed worldwide and consequently, the reports of harmful algae and human illnesses rise. Additionally some algal blooms appear to be stimulated by eutrophication activated by domestic, industrial and agricultural wastes. Also, climatological conditions can have an effect on the spatial distribution of a species.

1.1. AQUACULTURE AND HARMFUL ALGAL BLOOMS

Shellfish production and mariculture experience a worldwide expansion, especially in the Asia-Pacific region where seafood products are consumed in large amounts. In Europe, Spain, France, Italy, Denmark and the Netherlands are the main shellfish producers, with a total production of about one million tonnes in 1997. In 1998, worldwide production of mariculture fish was about 0.7 million tonnes (Rensel and Whyte, 2003).

Shellfish, such as bivalve molluscs, gastropods, crabs and lobsters, accumulate phycotoxins by direct filtration of the algal cells or by feeding on contaminated organisms. Toxin accumulation rates as well as the rates of toxin loss by filter-feeding shellfish from toxic algae are toxin- and species-specific (Fernández et al., 2003). Consequently, the duration of market closure depends on these rates. For example, in 1984, the Swedish mussel industry was shutdown for almost a year because of DSP toxins (Hallegraeff, 2003) that resided in the mussels depurated at slow rate (Svensson and Förlin, 2004). High economic losses to aquaculture were caused by fish killing

microalgae in the last decades. A massive bloom of *Chrysochromulina polylepsis* occurred in 1988 in the Skagerrak, the Kattegat, the Belt and the Sound between Denmark, Norway and Sweden and caused the deaths of 900 tonnes of fish, including cod, salmon and trout (Hallegraeff, 2003).

2. Monitoring of Phytoplankton

Monitoring programmes along the coastlines all around the world include, in the majority of cases, a surveillance for potentially toxic algal species (identification and quantification) and the monitoring of toxin content in shellfish. HAB monitoring programmes, e.g., GEOHAB, aim to prevent intoxication of humans and animals through the consumption of contaminated seafood as well as the protection of humans from algal toxins delivered via sea spray or direct contact. The damage of living resources, such as shellfish and fish, as well as the economic losses to fisherman, aquaculturists and the tourist industry should be minimized (Andersen et al., 2003). In addition, water temperature, salinity, nutrients, chlorophyll, water stratification, current circulation and other parameters are also observed for bloom prediction.

Microscope-based methods can identify and quantify microalgae at the species or genus level. However, the identification of unicelluar algae requires a broad taxonomic knowledge, because toxic and non-toxic strains can belong to the same species and thus are morphologically identical (e.g., *Alexandrium tamarense* species complex, (John et al., 2005). Consequently, an improved monitoring, rapid detection and enumeration of toxic algae is crucial because monitoring methods based on light microscopy are time-consuming and costly if many samples need to be processed. In the past decade, a variety of molecular methods have been adapted for the detection of harmful algae.

2.1. PROBE DEVELOPMENT

Molecular probes are widely applied for the identification of micro-organisms. The small and the large subunit ribosomal RNA genes are the usual targets for probes, because of their high target number in the cell. More or less conserved regions in these genes make it possible to develop probes that are specific at different taxonomic levels (Groben et al., 2004). Oligonucleotide DNA probes have usually a length of 18-25 base pairs and can be designed using the probe design option in ARB software package (Ludwig et al., 2004). It is necessary that probe specificity is extensively tested to eliminate false positives. The probes must be tested so that close phylogenetic neighbours (clade tests) and probe neighbours (probe tests), who are species

with close target sequence but who are phylogenetically unrelated do not bind to the probe. Additionally, a BLAST search (Altschul et al., 1990) should be conducted to test the overall specificity of the probes against all publically available sequences not included in the ARB database.

Probes for several toxic algae (e.g., *Alexandrium minutum* and *Gymnodinium catenatum*) have been developed for the detection with sandwich hybridization formats (Diercks et al., 2008c). Developed probes need a regular review for their specificity, because new sequences are added to the available online genetic databases daily. This cross check will help to determine if there is any cross reactivity with other marine organisms.

2.2. SANDWICH HYBRIDIZATION

The principle of the sandwich hybridization (SHA) was described by Dunn and Hassel, 1977; Zammatteo et al. (1995) and Rautio et al. (2003) (Dunn and Hassel, 1977; Zammatteo et al., 1995; Rautio et al., 2003) and represents a DNA probe-based method for rapid target identification that uses two oligonucleotide probes targeting ribosomal RNA (rRNA). A capture probe bound to a solid surface immobilizes the target ribosomal RNA and forms a hybrid complex with a second signal probe (Figure 2). An antibody-enzyme complex binds to the signal moiety of the signal probe and reacts with a substrate forming an electrochemical current (Metfies et al., 2005) in case of the biosensor.

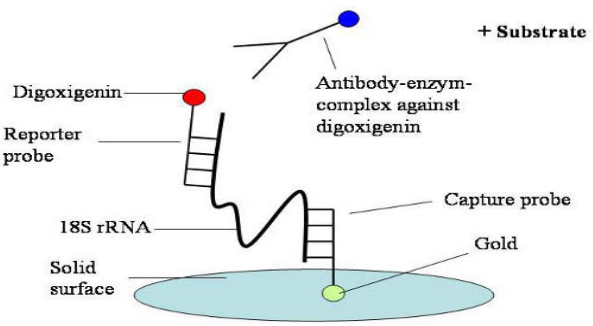

Figure 2. Principle of sandwich hybridization.

3. Biosensor

Biochemical recognition with signal transduction for the detection of specific molecules is combined on electrochemical biosensors. The detection component (e.g., probe sequence, antibodies, enzymes) catalyzes a reaction with

or specifically binds to the target of interest. A transducer component transforms this detection event into a measurable signal such as an electrical current.

Biosensor types comprise optical, bioluminescent, thermal, mass and electrochemical recognition (Gau et al., 2005). A specific detection of targets in a complex sample is possible, as a result of this various sectors, such as clinical diagnostic, environmental monitoring, biothreat detection and forensics, apply single electrode sensors as well as arrays (Berganza et al., 2006; Lermo et al., 2006; Taylor et al., 2006). Arrays of electrodes enable a simultaneous detection of multiple species with different molecular probes (Dock et al., 2005; Farabullini et al., 2007). The *in-situ* use of biosensors circumvents the need to return samples into the laboratory and thus rapid identification of aquatic microorganisms is possible. The identification of microorganisms, as well as physical and chemical measurements of the environment, are important for the understanding of coastal dynamics and processes that can impact marine ecosystems, such as the introduction and spreading of microbial pollutants and the initiation of HABs (Lagier et al., 2005).

3.1. SINGLE ELECTRODE MEASURING DEVICE

A biosensor in combination with a hand held device was introduced by Metfies et al. (2005). This first prototype was used in combination with sandwich hybridization for the detection and identification of the toxic dinoflagellate *Alexandrium ostenfeldii* (Metfies et al., 2005). Metfies et al. (2005) showed that the signal intensity is proportional to the rRNA concentration applied to the sensor with a detection limit of ~100 ng/μL RNA for the first prototype. A second prototype (Figure 3), the PalmSens (Palm Instruments, Houten, Netherlands), has been extensively used to improve the biosensor system (Diercks et al., 2008b). The protocols and electrochemical readings of the measuring device are simple and easy to use, even for laypersons. Single electrode sensors can be produced very cheaply and coated in advance of use with capture probes. The long term storage of 12 months enables mass production and introduction to the market (Diercks et al., 2008a).

The electrochemical detection method with the hand held device and biosensors is a rapid method to detect toxic algae in a water sample. However, the method for the use of the prototypes for single electrode sensors contains a lot of manual steps, such as the isolation of RNA from the algal cells.

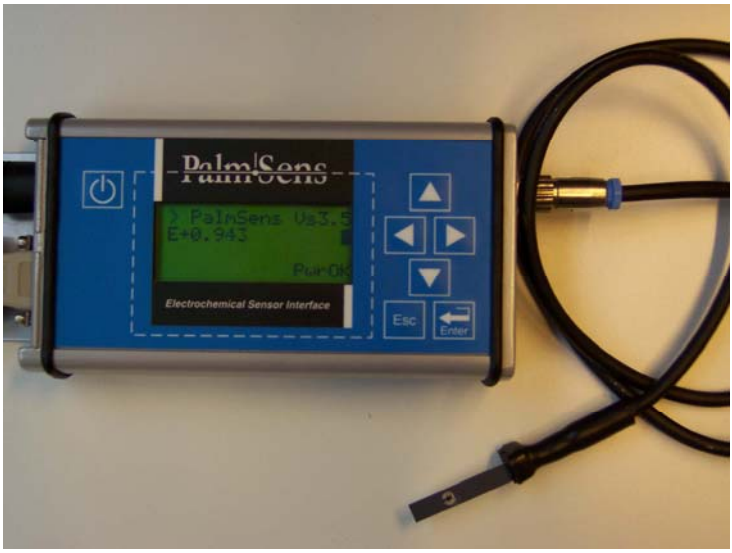

Figure 3. Handheld PalmSens device from Palm Instruments.

The manual isolation of RNA is currently the limiting factor of the system, because the concentration and quality of the RNA required is high. Single electrode sensors can only detect one species per working electrode and additionally a positive and a negative control has to be carried out. The ribosomal RNA concentration per cell has to be determined for each target species. Additionally, a calibration curve must be developed for each new probe set in order to determine the signal intensity at different RNA concentrations. Using the obtained information on the curve, the electrical measurement of the hand held device can be related to cell numbers, e.g., in field samples.

3.2. MULTIPROBE CHIPS

As mentioned above, arrays of electrodes facilitate a simultaneous detection of multiple species with different molecular probes. This system is beneficial for the observation of phytoplankton communities, which consist of different species. The temporal and spatial variability in species composition in the sea is substantial and therefore a simultaneous detection of multiple species is important.

A system with two major parts was developed during the EU-project ALGADEC: a multiprobe biosensor and semi-automated device (see chapter 3.3). The present multiprobe chip consists of a disposable ceramic sensor chip with 16 gold electrodes (Figure 4) (Diercks et al., 2008a).

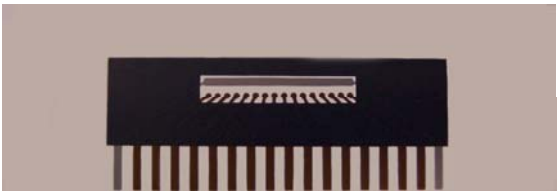

Figure 4. Multiprobe chip with 16 gold working electrodes and gold stems.

This multiprobe chip can be used for the simultaneous detection of 14 different targets plus two controls and thus for the detection of species compositions in harmful algal blooms. Signal transmission between the electrodes was assessed and no tranmission was determined. This is an important attribute for the coating of the 16 working electrodes with different species probes and false positive signals must be avoided (Diercks et al., 2008a). Automated spotting of the multiprobe chips with DNA probes is possible using a piezo spotter with image recognition. The automated spotting will achieve a regular signal formation and increase the sensitivity of the system.

The goal of ALGADEC was the detection of the different toxic algae species in three different areas in Europe: Skagerrak in Norway, the Galician coast in Spain and the area of the Orkney Islands in Scotland. Thus, probe sets for different toxic algae (e.g., *Alexandrium minutum*, *Pseudo-nitzschia* sp., *Dinophysis* sp.) have been developed and tested (Diercks et al., 2008c).

3.3. ALGADEC DEVICE

A portable semi-automated device was developed in the ALGADEC project (Figure 5) (Diercks et al.). This device enables the electrochemical detection of toxic algae in less than two hours. The main steps of the probe to target hybridisation and analysis process are executed automatically in the measurement device. A manual filtering and a lysis procedure has to be done before the automatic measurement. And prior to measurement, the one-way multiprobe chip has to be inserted into the flow cell unit.

The ALGADEC device contains reservoirs for antibody, substrate and washing buffers. The signals from the different working electrodes are recorded by a microcontroller unit whilst the electrochemical reaction takes place on the chip. If a PC is connected to the system, an easy to operate software (Figure 6) visualizes process data, graphic results, and the measured values will be stored on the hard disc.

Figure 5. Portable ALGADEC device in waterproof case.

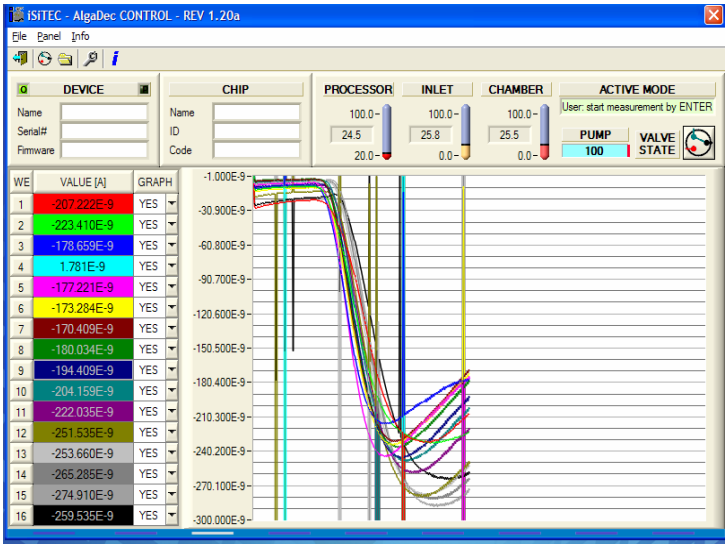

Figure 6. Software of the ALGADEC device.

A flowchart visualizes the status of the valves and temperature of the inlet and the hybridization chamber (Figure 7).

The device can be used by laypersons because of the development of a lysis protocol that eliminates the need for manual RNA isolation. The multiprobe

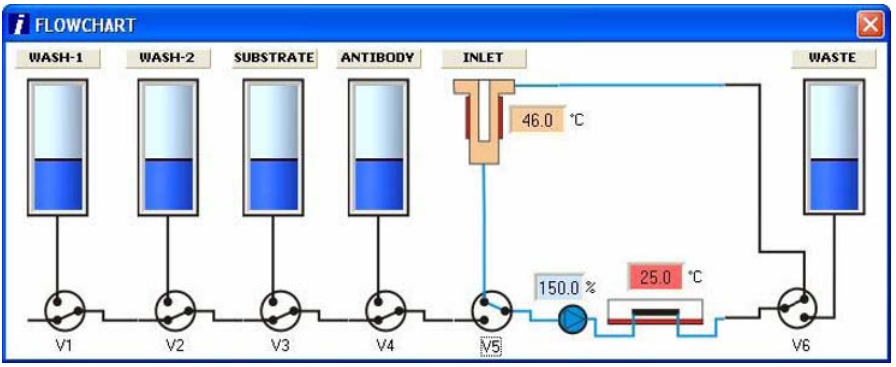

Figure 7. Flowchart for status visualization.

chip and the ALGADEC device can be used as a stand-alone system in the field or aboard ships with build in keypad, display, power supply and memory card. A waterproof case protects the system and allows the use under heavy conditions.

4. Future Research

4.1. SYSTEM IMPROVEMENTS

The sensitivity of the system has to be optimised and the detection limit must be reduced, because when a cell count of the toxic algal cells is reached, then fisheries are closed. An inrease of sensitivity and regular signal formation can be achieved by an automation of spotting of the multiprobe chips with DNA probes. Thus, different DNA probes i.e., species can be spotted onto a chip and area specific chips can be developed. 17 specific DNA probe sets for toxic algae have already been developed (Diercks et al., 2008c) and new probe sets for other toxic can be developed and need to be adapted to the chips. Furthermore, the sensors must be calibrated for each DNA probe set to allow a conversion of the electronic signal with the help of custom-made software into the concentration of toxic cells.

The biosensor system can be simplified by an an automated water sample filtration, because a manual filtering and a lysis procedure is still required. However, with this adaptation it would be possible to integrate this system into a buoy. Additionally the biosensor can be adapted to other fields, e.g., medical diagnostics.

4.2. IMPLEMENTATION OF THE BIOSENSORS INTO THE *FERRYBOX* SYSTEM

The biosensor will be implemented into a *FerryBox* system at Helgoland in the North Sea. The current "German *FerryBox*" consists of a fully automated flow-through system (Figure 8) with different sensors and automatic analysers, e.g., water temperature, salinity, turbidity, dissolved oxygen, pH, nutrients (ammonium, nitrate/nitrite, phosphate, silicate) and the algal online analyser (Petersen et al.).

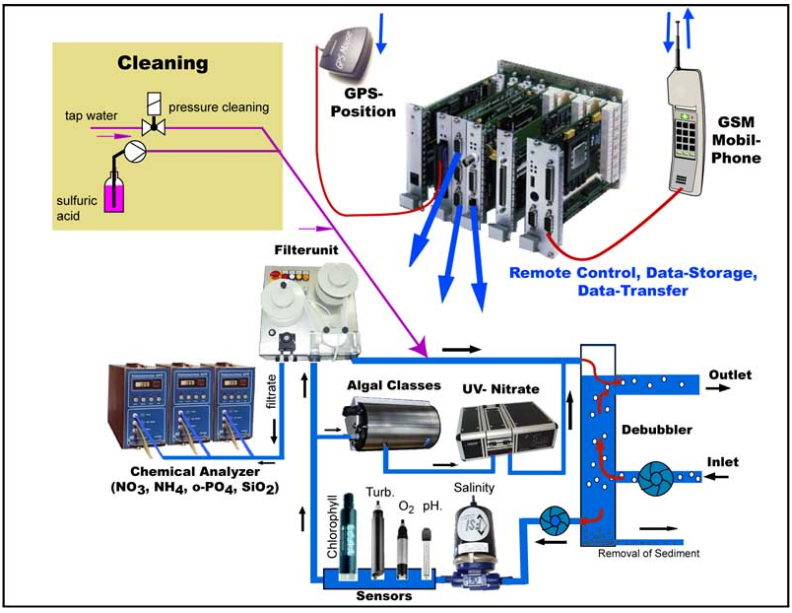

Figure 8. Flow chart of the FerryBox System.

The Algal online analyser (AOA, bbe-moldaenke, Germany) measures the chlorophyll fluorescence of the main algal groups. Five different algal groups are detected with the algal online analyser:

– Chlorophyceae
– Cyanophyceae
– Bacillariophyceae
– Dinophyceae
– Cryptophyceae

However, it is not possible to differentiate Bacillariophyceae and Dinophyceae (Figure 9) and to identify species.

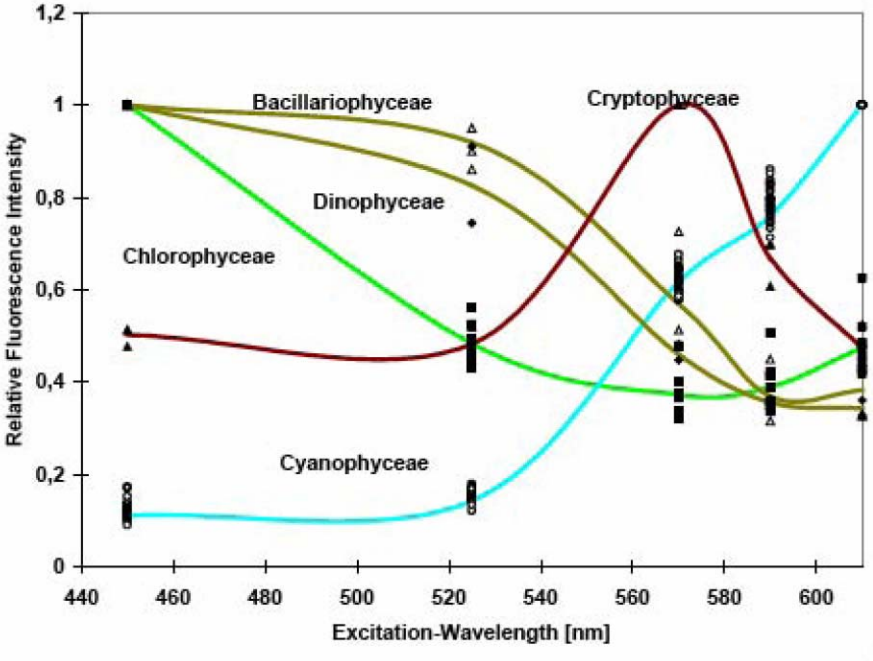

Figure 9. Relative chlorophyll fluorescence intensity of the different algal groups.

The biosensor system will be used to identify species or clades with the help of clade level or species level probes. For this system reusable multi-probe chips will be developed, so that the chip must not be changed and a continuous flow through is possible. Thus, an automated screening of biodiversity and species composition during the course of the year will be possible.

5. Conclusions

A portable semi-automated device was developed in the EU project ALGADEC that automatically processes the main steps of the probe to target hybridisation and facilitates the electrochemical detection of toxic algae in less than two hours. A multiprobe chip for the simultaneous detection of 16 different target molecules was developed. The multiprobe chip in combination with the ALGADEC device is an autonomous and portable system and thus can be used as a stand-alone system in the field and aboard ships. Furthermore, the device can be used by layperson because a manual RNA isolation is now no longer required. 17 probe sets for toxic algal species were designed and can be used for the detection using the semi-automated device and the multiprobe chips. The ALGADEC device will contribute to monitoring programs to provide an early warning system for

the aquaculture and tourist sectors who are most affected by toxic algal blooms.

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The authors would like to thank all partners from the EU-Project ALGADEC for excellent cooperation and valuable discussions in the development of the multiprobe chips and the ALGADEC device. Sonja Diercks was supported by the EU-project ALGADEC (COOP-CT-2004-508435-ALGADEC) of the 6th Framework Programme of the European Union and the Alfred Wegener Institute for Polar and Marine Research.

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DEVELOPMENT OF SENSORS TO TRACE TOXINS FROM DINOFLAGELLATES AND OTHER ALGAE TO SEAFOOD

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Abstract: Sensor technology based on immunological ELISA analyses combined with various electrochemical detection systems is being developed to quantify phycotoxins in algae and seafood. The use of disposable screen-printed electrodes for the immunosensor development is illustrated. Laboratory responses on contaminated mussels were obtained by domoic acid and saxitoxin sensors with detection limit of 5 and 0.1 ng/ml respectively. Application to algal extracts was also performed to detect domoic acid concentration in phytoplankton populations along Latium (Middle Tyrrhenian Sea, Mediterranean Sea) coast.

Keywords: Phycotoxin traceability, domoic acid, saxitoxins, immunosensors

1. Introduction

In recent years, marine food and environment resources have been threatened worldwide by the massive development of several harmful phytoplankton species which contaminate shellfish and other marine

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products causing animal mortality and severe human illness, and endangering ecosystem quality (Zingone and Enevoldsen, 2000; Scholin et al., 2000; Shumway et al., 2003; Van Dolah et al., 2003).

Increasing number of reports pointed to health risks connected to seafood consumption along Mediterranean coasts and lagoons (Taleb et al., 2001; Vila et al., 2001; Turki, 2004; Blanco et al., 2005; Turki and Balti, 2005; Illoul et al., 2007; Mikhail et al., 2007; Turki et al., 2007).

Blooms of Diarrhoeic Shellfish Poison (DSP) species are a common phenomenon in the Adriatic and the Central-Southern Tyrrhenian Sea. Extended stretches of coast are frequently interested by the massive presence of DSP algae (*Dinophysis* spp. and *Phalachroma* spp.) and most rarely by Paralytic Shellfish Poison (PSP) producers (Ciminiello et al., 2000; Zingone et al., 2006). On the contrary, PSP-producing *Alexandrium* spp. cause severe problems in coastal areas of the Ionian (Giacobbe et al., 2004) and North Adriatic Sea (Honsell et al., 1996). In addition, *Gonyaulax spinifera, Lingulodinium polyedrum* and *Protoceratium reticulatum* have been recently shown to be responsible of yessotoxin production and accumulation in shellfish along North Adriatic coast (Ciminiello et al., 2003; Riccardi et al., 2007), while pectenotoxins have been detected both in algae and mussels (Draisci et al., 1999).

To understand the risk connected to the development of these harmful algal blooms (HABs), the structure and dynamics of marine phytoplankton has been recently assessed along Latium coast since 1997 to 2006 (Congestri et al., 2006). In this study 33 potentially toxic species, out of 374 microalgae present in the natural populations, have been identified with light and electron microscopy (Fig. 1).

Sixteen thecate (Congestri et al., 2004a) and at least six naked dinoflagellates were recorded together with six *Pseudo-nitzschia* species, the latter being responsible for massive annual blooms (up to 10^7 cells l^{-1}) (Congestri et al., this volume). There were also sporadic brown tides due to *Fibrocapsa japonica* intense growth during summer. Bloom periodicity and cell abundances highlighted that spring and summer were the most critical for toxicity events (Bianco et al., 2006).

Furthermore, recurrent summer blooms of the dinoflagellates *Ostreopsis ovata* and *Coolia monotis* (Fig. 2) along with *Prorocentrum lima* started to affect rocky, sheltered environments along Italian coast and prompted monitoring of toxic microphytobenthos (HBABs) from 2003 (see www.bentoxnet.it, Congestri et al., 2006; Ingarao et al., 2007). However, the production of phycotoxins has being seldom recorded in phytoplankton communities from the Central Mediterranean, and data on accumulation in mussels, fish and other animals are still scarce (Azmil et al., 2003; Kaniou-Grigoriadou et al., 2005).

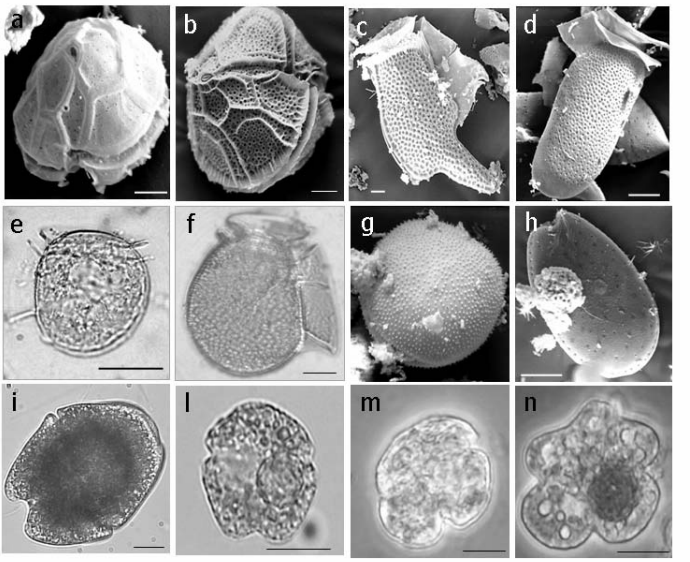

Figure 1. SEM micrographs of *Alexandrium minutum* a, *Lingulodinium polyedrum* b, *Dinophysis caudata* c, *D. sacculus* d, *Prorocentrum minimum* g and *P. lima* h, LM micrographs of *Dinophysis rotundata* e, *D. fortii* f, *Akashiwo sanguinea* i, *Karenia* cf. *bicuneiformis* l, *K.* cf. *mikimotoi* m, *K.* cf. *papilionacea* n. Late spring-summer occurrence of these species was observed along Latium coasts, distribution was patchy and densities never exceeded 10^5 cell l^{-1}. Highest abundances were recorded for *Alexandrium minutum* (9.3×10^4 cell l^{-1}) at one station in May 2005, while at the same site *Lingulodinium polyedrum* peaked in June (5.8×10^4 cell l^{-1}), *Dinophysis* spp. had low densities, *D. caudata* showed maximum values (2.3×10^4 cell l^{-1}) in July 2003, and other species were present with densities around 10^2 cell l^{-1}. Among unarmoured taxa *Karenia* cf. *mikimotoi* had the highest abundances (4.6×10^4 cell l^{-1}) in May 2004 at a southern site. Bars = 4 μm (g); 5 μm (a); 10 μm (b, h, i, l, m, n); 20 μm (d, e, f); 50 μm (c).

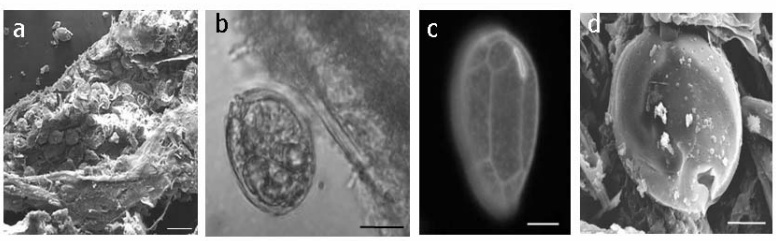

Figure 2. Mucilaginous benthic aggregates mostly comprising of *Ostreopsis ovata* (up to 7.5 × 105 cell g-1 fresh weight, 5 × 105 cell l-1) (a, c) and *Coolia monotis* (b, d), present with lower densities, developed on living organisms (c) and rocky substrates (c) regularly from 1999 to 2006 in the southern Latium coast. The first bloom event also coincided with mass mortality of zoobenthos. Using LC-MS/MS methods a palytoxin analogue was detected in mucous aggregates dominated by *Ostreopsis ovata*. Bars = 10 μm (b, d, e, f, g); 30 μm (c).

To face the very high risk of seafood toxicity, fast analytical procedures based on the use of electrochemical disposable immunosensors for the detection of phycotoxins were recently developed and applied directly in shellfish samples following easy extraction procedures (Palleschi et al., 2003, Micheli et al., 2004). Analogous approach was developed to quantify toxins in algae (Micheli et al., 2003). These studies mostly focused on domoic acid (DA) and saxitoxin (STX). DA is a potent water-soluble acidic amino acid neurotoxin that causes the human Amnesic Shellfish Poisoning (ASP) syndrome, while the water-soluble and thermostable tetrahydropurine compound STX is responsible of the Paralytic Shellfish Poisoning (PSPs). These toxins accumulate in the digestive glands of shellfish without causing any apparent toxic effect on the molluscs. Conversely, human consumption of a sufficient amount of seafood contaminated by DA and STX (both odourless and tasteless) can result in mild to severe neurological symptoms and in rare cases, death.

2. Methodological Approach and Results

The construction of electrochemical immunosensors coupled to electrochemical techniques as differential pulse voltammetry (DPV) and chronoamperometry for the detection of DA and STX in algal and seafood extracts is illustrated.

The analysis involves the use of disposable screen-printed electrodes (SPEs) for the immunosensor development based on a "competitive test" (Friguet et al., 1985). The support of the immunochemical chain was the screen-printed electrode (SPE) (Fig. 3), produced by a thick-film technology that combines the easiness of use and portability with simple and inexpensive fabrication techniques. Application of the immunoelectro-chemical ELISA (immunosensor), allows the selectivity of immunological analysis to be combined with the sensitivity of the electrochemical detection (Del Carlo et al., 1997; Santandreu et al., 1998; Warsinke et al., 2000).

In the case of domoic acid, the toxin was conjugated to bovine serum albumin (BSA-DA) and coated onto the SPE. Incubation with the sample (or standard toxin) and the anti-DA antibody followed (Fig. 4A). An anti-goat IgG-alkaline phosphatase conjugate was used for signal generation, measured with DPV. For the detection of STX, the "direct format" (Fig. 4B) was used with the immobilisation of the primary antibody (against the toxin) onto the electrode and the competition between the free (STX) and labeled toxin with enzyme (STX-HRP) was then carried out. The enzymatic product was detected by Chronoamperometry.

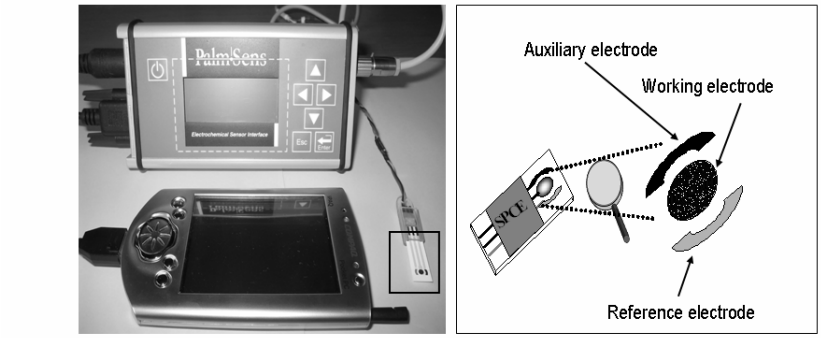

Figure 3. Screen-printed electrode with PalmSens, portable electrochemical instrumentation.

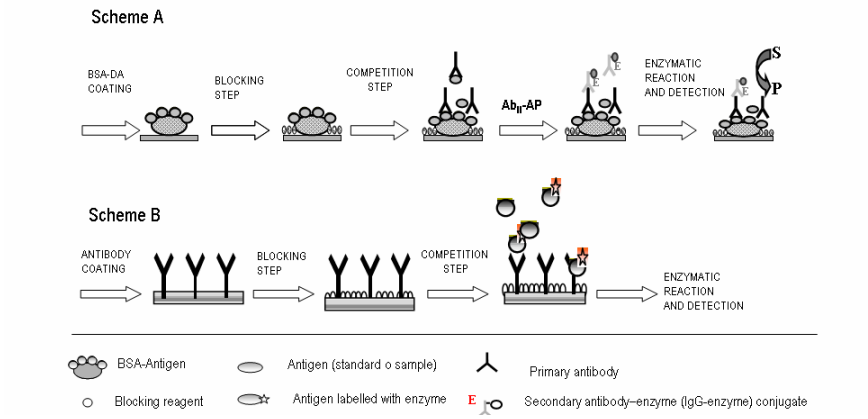

Figure 4. Schemes of indirect A and direct B competitive ELISA formats used for SPE-immununosensors.

DA results showed a detection limit of 5 ng/mL with a working range between 5–70 ng/mL of DA (Fig. 5A), while a detection limit of 0.1 ng/mL and a working range of 0.1–10^3 ng/mL was obtained for STX (Fig. 5B).

The suitability of the assays for the quantification of these toxins in mussels was also evaluated. Samples were spiked with toxin standard before and after the sample treatment to study the extraction efficiency and the matrix effect, respectively. After treatment, samples were analysed at 1:1000 v/v dilution in PBS for STX and 1:250 v/v in PBS-M (phosphate saline buffer pH 7.4 + CH_3OH 10%) for DA to minimize the matrix effect and to detect the regulatory limit for both toxins in mussels as given by the Food and Drug Administration (www.fda.org, Compliance Programme 7303.842, Guidance Levels, Table 3, p. 248).

The reliability of the immunoassays for the determination of the DA and STX in spiked samples was demonstrated by comparison of the data with the fully validated confirmatory HPLC results (Micheli et al., 2004). Very

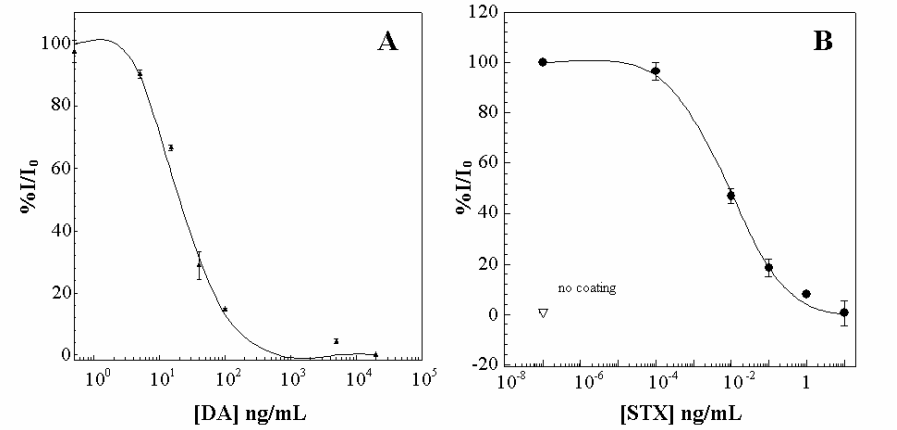

Figure 5. Calibration curves of SPE-immunosensors for DA (5 ng/ml) A and STX (5–70 ng/ml) B.

good recoveries (90–110%) were obtained for both toxin, demonstrating the suitability of the proposed assays for accurate determination of the both toxin concentrations in mussel samples.

2.1. APPLICATION TO ALGAE

The method for DA was adjusted to test natural algal matrices. Extracts were obtained from net samples collected fortnightly during one year at six stations along Latium (Italy) coast, and analysed by using SPEs immunosensors and HPLC. About 22% of samples were positive for DA and results of the two assays showed a RE = ± 20% (Micheli et al., 2003). Countings at the species level in surface water samples allowed to put in relation high abundance of *Pseudo-nitzschia* spp. to the summer peaks of DA observed at some stations (Congestri et al., 2004b).

3. Conclusions

Bloom events of toxic algal species may result in extensive and unprecedented closures of shellfish harvesting areas to prevent poisoning syndromes in human consumers. *Karenia* and *Ostreopsis* blooms may affect residents in coastal areas by inducing respiratory irritation in beach-goers. Bacterial decomposition of dead animals and algal cells may cause anoxia of bottom water, which may spiral into multiple species unusual mortality events in thousand square-miles of sea-bottom. Preliminary estimates of the economic impact due to HABs along US coasts accounted for more than 80

million dollars per year in 1987–2000. These estimates included public health costs of illness (45%), lost revenues of commercial fisheries (46%), local recreation and tourism impacts (5%), and coastal monitoring and management expenditure (4%) (Jewett et al., 2007).

Furthermore, the worldwide increase in coastal algal blooms is currently regarded as one of the first biological signs of global warming of ocean waters, thus continuous monitoring activities will be crucial for the understanding of aquatic processes and climate change.

In this scenario, environmentally sound techniques to reduce HABs impacts should be based on effective methodologies for rapid field detection of phycotoxins to develop early warning systems, response plans, and methods to reduce public health, ecological, social, and economic impacts of HABs.

There is, therefore, a critical need for cost-effective and user-friendly monitoring tools that can be used by tribes, local environmental groups, and state agencies to monitor toxins concentrations.

Competitive immunoassays for DA and STX are indeed a functional strategy useful to support surveillance activities of both phytoplankton and seafood. The rapid and accurate identification of phycotoxins in a working range that is comparable to that of conventional methods and the detection limit suitable for "on-site" monitoring allow successful application on algae and mussel matrices. Electrochemical SPE-immunosensors may presently contribute to the development of early warning protocols for routine application. Further studies on different animal matrices might reveal the fate of phycotoxins and their amplification along the whole food chain and thus contribute to assess the extent of the impact of toxic bloom events on environmental and human health.

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RECOMBINANT FORMALDEHYDE DEHYDROGENASE AND GENE-ENGINEERED METHYLOTROPHIC YEASTS AS BIOANALITYCAL INSTRUMENTS FOR ASSAY OF TOXIC FORMALDEHYDE

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Abstract: Recombinant yeast clones, originated from the recipient *Hansenula polymorpha* strains NCYC 495 and CBS 4732, resistent to elevated concentrations of formaldehyde in a medium (up to 15–20 mM) and overproducing a homologous NAD- and glutathione-dependent form-aldehyde dehydrogenase, were constructed. Optimal cultivation conditions for the highest yield of the enzyme were established. A simple scheme for the isolation of formaldehyde dehydrogenase from the re-combinant strains was proposed, and some characteristics of the purified enzyme were studied. Enzymatic and biosensoric methods for formaldehyde assay based on the formaldehyde dehydrogenase and the constructed recombinant cells were developed. The reliability of the developed analytical approaches was tested on real samples of waste waters, pharmaceuticals, formaldehyde-containing industrial products, and vaccines. The comparison of formaldehyde content values obtained by the use of biosensors (enzyme and cells-based), enzymatic

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methods and two routinely used chemical ones (chromotropic acid and 3-methyl-2-benzothiazolinone hydrazone) showed a good correlation between these approaches.

Keywords: Yeast *Hansenula polymorpha* (*Pichia angusta*), gene engineering, formaldehyde dehydrogenase, enzymatic assay, biosensor, formaldehyde

1. Introduction

Formaldehyde (FA), extremely toxic agent, is found in more than 2000 commercial products. This compound is widely used as building block for the synthesis of many organic chemicals and materials, for example, phenol-, urea- and melamines-derived polymeric resins. Humans are exposed to FA from a variety of sources. The major source of atmospheric FA is related to combustion processes – forest fires, automobile exhaust, photooxidation of hydrocarbons. In water, FA is also formed by the irradiation of humus substances by sunlight (Liteplo et al., 2002; National Research Council, 1982). Additional exposure to FA emissions comes from its use as an embalming fluid in anatomy labs, morgues, and its use as a fumigant and sterilant (Liteplo et al., 2002). FA is used as a preservative in the production of vaccines instead of harmful merthiolate that can cause neurodevelopmental disorders including autism and autism spectrum disorders (Offit and Jew, 2007; Geier and Geier, 2004), in the production of different consumer goods: detergents, soaps, and shampoos (Gerberich and Seaman, 1994). Resin treated fabric, rugs, paper, *etc*. and materials such as particle board and plywood which contain resin adhesives and foam insulation release FA which may build up in homes and occupational atmospheres (Liteplo et al., 2002; National Research Council, 1982). Recently, a new health risk factor associated with FA has been revealed. Some advanced technologies of potable water pre-treatment include the ozonation process during which FA is generated as a result of reaction of ozone with humus traces (Schechter and Singer, 1995). At the same time, FA is a natural metabolite of all living organisms. It has been found in fruits, vegetables, flesh, and biological fluids of human origin (Gerberich and Seaman, 1994). In extreme cases, some frozen fish, especially of *Gadoid* species, can accumulate up to 200 mg of FA per 1 kg of wet weight due to the enzymatic degradation of a natural fish component – trimethylamine oxide (Rehbein, 1995; Pavlishko et al., 2003).

FA is emitted by bacteria, algae, plankton (Liteplo et al., 2002). Data on the aquatic toxicity of FA are numerous. The most sensitive aquatic effects identified were observed for marine algae. FA concentration of 0.1 and 1

mg/L in water caused 40–50% mortality after 96 h in day-old zygotes of *Phyllospora comosa*, a brown marine macroalgae endemic to south-eastern Australia. Total (100%) mortality resulted from exposures to 100 mg/L for 24 h and 10 mg/L for 96 h. The 96h no-observed-effect concentration and lowest-observed-effect concentration of 7-day-old embryos of the same species were reported as 1 and 10 mg/L, respectively, indicating that older organisms are more tolerant to FA. Concentrations of 0.1, 1, and 10 mg/L also reduced germination and growth rates of the zygotes and embryos. Freshwater algae may be slightly more tolerant of FA, based on a cell multiplication inhibition test. The toxicity threshold was 0.9 mg FA/L. The toxicity of FA for fish is also variable: the most sensitive freshwater fish were fingerlings of striped bass *Roccus saxatilis* (Liteplo et al., 2002).

FA has a negative influence on human's health, especially on the central nervous, blood and immune systems. It is a potent nasal irritant, causes stunted growth, blindness and respiratory diseases. Release of FA vapors in mobile homes has been associated with headache and pulmonary and dermal irritation (Ellenhorn and Barceloux, 1988). The relation of chronic respiratory symptoms and pulmonary function to FA in homes was studied in a sample of 298 children (6–15 years of age) and 613 adults. The permissible level of FA in industrial areas is set to 0.5–2.0 ppm. Significantly greater prevalence rates of asthma and chronic bronchitis were found in children from houses with FA levels 60–120 ppb than in those less exposed, especially in children also exposed to environmental tobacco smoke. In children, levels of peak expiratory flow rates decreased linearly with FA exposure, with the estimated decrease due to 60 ppb of FA equivalent to 22% of peak expiratory flow rates level in nonexposed children. The effects in asthmatic children exposed to FA below 50 ppb were greater than in healthy ones. The effects in adults were less evident: decrements in peak expiratory flow rates due to FA over 40 ppb were seen only in the morning, and mainly in smokers.

FA is classified as a mutagen and possible human carcinogen (Feron et al., 1991), one of the chemical mediators of apoptosis. These considerations are sufficient to demonstrate the necessity for FA control in consumer goods, environment, as well as in biological samples. Control of pollutants and toxic compounds is of great importance for all countries, and such control requires the development of simple, cheap, sensitive, and selective methods for FA analysis. Among them, enzymatic and biosensor-based approaches are the most promising due to high selectivity and sensitivity. The existing enzymatic methods for FA assay are laborious, not enough selective and specific, and the corresponding kits are still unavailable at the world market place (Ho and Richards, 1990; Patent, 2002). Recently, we have described the fabrication and properties of a reagentless bienzyme amperometric biosensor based on alcohol oxidase/peroxidase in combination

with an Os-complex modified electro-deposition paint (Smutok et al., 2005). Although the developed biosensor showed good sensitivity for the detection of FA, the poor selectivity of the used biological recognition element, alcohol oxidase, application of these sensors is limited.

To solve this problem, we have developed new analytical approaches for FA assay based on NAD^+- and glutathione-dependent formaldehyde dehydrogenase (FdDH) isolated from recombinant yeast cells-overproducing FdDH.

FdDH, a key enzyme of FA metabolism in microorganisms, is proposed to be used for bioanalytical purposes (Ben Ali et al., 2006). The broad use of FdDH in analytical practice is hampered by insufficient activity of the commercial preparations of the enzyme from *Pseudomonas putida* and *Candida boidinii*, as well as by relatively high costs of the enzymes' preparations isolated from the wild type strains (Sigma-Aldrich Catalogue, 2007).

In this paper, we describe:

- construction of the recombinant yeast strains, originated from the recipient thermotolerant methylotrophic yeast *Hansenula polymorpha*, overproducing a homologous thermostable FdDH;
- optimization of the cultivation conditions for the gene-engineered strains, the procedure of enzyme isolation from the recombinant cells, some characteristics of the purified FdDH;
- development of enzymatic FdDH-based approach for FA assay;
- development of biosensoric methods for FA assay, based on the use of the recombinant FdDH and gene-engineered yeast cells over-producing this enzyme;
- comparison of the developed bioanalytic approaches, namely bio-sensoric (FdDH- and cells-based) and enzymatic ones, with standard chemical methods for FA assay in real samples: wastewater, some industrial goods, pharmaceuticals, and vaccines.

2. Materials and Methods

2.1. MICROBIAL STRAINS, MEDIA, CULTIVATION CONDITIONS, AND PREPARATION OF CELL-FREE EXTRACTS

The following strains were used in the present study: *Hansenula polymorpha* 356 (*leu2*) line DL1, *H. polymorpha* NCYC 495 (*leu*1-1) and *H. polymorpha* CBS 4732 (*leu*2-2*)*. For vector construction, gene *FLD1 H. polymorpha* and plasmid pYT1 were used (Baerends et al., 2002; Demkiv et al., 2005).

Yeast cells were cultivated in flasks on a shaker (200 rpm) at 28°C until the middle of the exponential growth phase (~24 h) in a medium containing (g/L): $(NH_4)_2SO_4$ – 3.5; KH_2PO_4 –1.0; $MgSO_4 \times 7H_2O$ – 0.5; $CaCl_2$ – 0.1; yeast extract – 0.5 with the supplement of standard microelements (Demkiv et al., 2005). As carbon sources, 1% methanol, 1% glucose, or 1% ethanol were used. For cultivation of the strains *leu 1-1* and *leu 2-2*, leucine was added up to 40 mg/L.

After washing, the cells were suspended in 0.05 M K, Na-phosphate buffer, pH 8.0 (PB), containing 0.4 mM PMSF and 1.0 mM EDTA, frozen, and kept at –20°C. To obtain the cell-free extract (CE), cells were disrupted with glass beads (d = 0.45 – 0.5 mm) in a planetary disintegrator at 1000 rpm (r = 10 cm) at 4°C for 6 min. The cell debris was removed by centrifugation at 15000 rpm (r = 8 cm) for 40 min and the supernatant (CE) was used for testing and isolation of the enzyme.

2.2. CONSTRUCTION AND SELECTION OF FDDH OVER-PRODUCING RECOMBINANT STRAINS

To construct strains, over-producing thermostable NAD^+- and glutathione-dependent FdDH, the *H. polymorpha FLD*1 gene with its own promoter (Baerends et al., 2002) was inserted into the integrative plasmid pYT1 (Demkiv et al., 2005) containing the *LEU2* gene of *Saccharomyces cerevisiae* (as a selective marker). The constructed vector was used for multi-copy integration of the target gene into the genome by transformation of *leu 1-1* (Demkiv et al., 2005) and *leu 2-2* recipient cells (both *leu* allels are complemented by *S. cerevisiae* gene *LEU2*). The transformation was performed using three different methods: electroporation (Delorme, 1989), lithium chloride method (Ito et al., 1983), and protoplasting procedure (Hinnen et al., 1978).

Selection of FdDH-overproducing strains was carried out by leucine prototrophy and simultaneously by the resistance to elevated concentrations of FA in the medium (up to 20 mM). Finally, FdDH specific activities were tested in cell-free extracts (CE) of the selected FA-resistant Leu-prototrophic transformants. Activity of FdDH was determined by the rate of NADH formation monitored spectrophotometrically at 340 nm (Schutte et al., 1976). One unit (1 U) of the enzyme activity was defined as the amount of the enzyme which forms 1 μmole NADH per 1 min under standard conditions of the assay: 25°C, 1 mM FA, 1 mM NAD^+, and 2 mM glutathione in PB (50 mM PB, pH 8.0). Instant activity of FdDH (A) was calculated as a difference between A^{+FA} (in the presence of FA) and A^{-FA} (without addition of FA). Protein concentration was estimated by Lowry method.

2.3. ISOLATION AND PURIFICATION OF FDDH

The stable recombinant strain Tf 11-6 was chosen as FdDH over-producer shown to have the highest activity of FdDH (up to 4.0 μmoles·min^{-1}·mg^{-1} protein) in cell-free extract (Demkiv et al., 2005).

FdDH was isolated from the cell-free extract (CE) by two-step column chromatography on anion-exchange sorbent DEAE-Toyopearl 650 M (Demkiv et al., 2007a). On the first step, CE was applied to column, equilibrated by PB, pH 8.0. The fraction of unabsorbed proteins, which contained FdDH, was diluted by water (1:3), Tris-base was added for adjusting pH to 8.8, and the final solution was applied to the same column (the second step), previously washed by 1 M NaCl and equilibrated by 40 mM Tris-buffer, pH 8.8 (TB). Enzyme was eluted by 0.1 M NaCl in the initial buffer (TB), and the specific activity of FdDH was assayed in each fraction. The concentration of protein was determined by the Lowry method. The fractions of eluate with the activity of the enzyme higher than 10 U·mg^{-1} were combined, then dithiothreitol (DTT) – up to 2 mM, and ammonium sulfate (up to 80% saturation; pH 8.0; at 0°C) were added. After incubation at 0°C for 1 h, the enzyme was collected by the centrifugation (15000 g, r_{av} = 8 cm, 30 min, 4°C), and pellet was resuspended in a minimal volume of ammonium sulfate solution (80% saturation) in 40 mM PB with 1.0 mM EDTA, 2 mM DTT and kept at –10°C.

2.4. ELECTROPHORESIS

The molecular weight of the FdDH subunits was calculated from the electrophoretic mobility values of FdDH and a set of standard proteins after SDS-gel electrophoresis in 5–20% polyacrylamide gel (PAAG). SDS-PAAG was stained with Coommassie Blue R-250. To visualize enzyme bands in native PAAG (Maidan et al., 1997), we used a modified mixture for FdDH assay: 1.0 mM FA, 1.0 mM NAD, 2.0 mM glutathione, 0.05 mM nitrotetrazolium blue (NTB), and 0.003 mM phenazine methosulfate (PMS) in 50 mM PB, pH 8.0. The PAAG was washed with water after the appearance of blue-violet FdDH bands.

2.5. METHODS OF FA ASSAY

2.5.1. *Chemical methods*

The assay was performed by two methods using chromotropic acid (Polska Norma PN-71 C-04568, 1988) and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) (Sawicki et al., 1961).

2.5.2. *Enzymatic methods*

2.5.2.a. FdDH-based method "Formatest"

0.5 ml of model and real samples (water for the blank sample) were treated at room temperature by 0.5 ml FdDH-containing reagent of the following composition: 23 mU/ml FdDH, 0.63 mM glutathione, 0.31 mM NAD^+, 0,2 mM NTB, 0.024 mM PMS, and 0.01% Triton X-100 in 50 mM PB, pH 8.0. Reaction mixture was incubated for 30 min at room temperature. To terminate the reaction, 3 ml 0.3 M HCl was added, and optical density of the sample at 570 nm was measured. FA content was calculated from the calibration curve.

2.5.2.b Oxidase/peroxidase-based method "Alkotest"

Assay was performed as described by us (Gonchar et al., 2005), using yeast alcohol oxidase capable to oxidize FA by O_2 to formic acid and H_2O_2.

2.6. CONSTRUCTION OF FDDH- AND CELLS-BASED AMPEROMETRIC BIOSENSORS

2.6.1. *Electrodes*

Graphite rods (type RW001, 3.05 mm diameter) from Ringsdorff Werke (Bonn, Germany), sealed in glass tubes by means of epoxy glue and used as working electrodes, were polished with emery paper of decreasing size. Platinisation of the graphite electrodes (for enzyme-based biosensors) was carried out in a 6 $mg \cdot ml^{-1}$ solution of hexachloroplatinum(IV)-acid-hexahydrate in HPLC-grade water by cyclic voltammetry (0.4 to –0.6 V at a scan rate of 10 $mV\ s^{-1}$, 3–4 potential cycles). After platinisation, the electrodes were rinsed with 0.2 M phosphate buffer, pH 8.2. The properties of amperometric biosensors were evaluated by means of constant-potential amperometry in a three-electrode configuration with a Ag/AgCl/KCl (3 M) reference electrode and a Pt-wire counter electrode.

2.6.2. *Immobilization of FdDH by entrapment within the polymer layer of a cathodic electrodeposition paint*

2 µl of FdDH suspension (15 $U \cdot ml^{-1}$) and 2 µl of cathodic paint (*CPOs*) were mixed and dropped onto the surface of a platinised graphite electrode. In a miniaturized electrochemical cell the cathodic paint was precipitated using a potentiostatic pulse sequence with pulses to a potential of –1200 mV for 0.2 s and a resting phase at a potential of 0 mV for 5 s (Ngounou et al., 2004). At the applied cathodic potential, water is reduced at the electrode surface leading to an increase of the pH-value in a diffusion zone in

front of the working electrode surface. Subsequently, the cathodic paint is deprotonated imposing a significant change in its solubility which leads to the precipitation of the polymer on the electrode surface simultaneously entrapping the enzyme.

2.6.3. *Entrapment of FdDH and NAD^+ within an Os-complex modified cathodic electrodeposition paint*

2 µl of 25 mM NAD^+, 2 µl of FdDH suspension (15 $U \cdot ml^{-1}$) and 2 µl of Os-complex modified cathodic paints were dropped on the surface of a platinized graphite electrode, *CPOs* was electro-precipitated and enzyme with NAD^+ were co-entrapped within the polymer film. After the immobilization procedure, the electrodes were rinsed with 0.02 M PB, pH 8.2.

2.6.4. *Co-entrapment of glutathione and covering of the sensing layer by a Nafion membrane*

On the top of a *1CPOs-NAD^+ -FdDH*-modified electrode, 3 µl of a 50 mM neutralized to pH 8.0 solution of reduced glutathione were dropped. After drying (2–4 min), 5 µl of 1% Nafion solution in ethanol (neutralized) were dropped on the sensor surface. The Nafion membrane was allowed to dry for 20 to 25 min at a temperature of +4 ^oC.

2.6.5. *Immobilization of recombinant FdDH-producing yeast cells*

2 µl of FdDH suspension (15 $U \cdot ml^{-1}$) was put on the surface of graphite electrode. On the top of a *Cells*-modified electrode, 3 µl of a 50 mM neutralized solution of reduced glutathione and 2 µl of 25 mM NAD^+ were dropped. After the immobilization procedure, the electrodes were rinsed with 0.02 M PB, pH 8.0. After drying (2–4 min), 5 µl of a 1%neutral Nafion solution were dropped on the sensor surface. The Nafion membrane was allowed to dry for 20–25 min at +4°C. Phenazine methosulfate was used as a free-diffusing redox mediator for cells-based biosensors. 10 ml of a 1 mM solution of the mediator in 20 mM PB, pH 8.2 was added to the electrolyte solution. In these experiments, the glass cell was wrapped with aluminium foil as phenazine methosulfate is light sensitive.

2.6.6. *Amperometric measurements*

Amperometric measurements were carried out using an Autolab PGstat12 potentiostat (Eco Chemie, Utrecht, Holland) controlled by the GPES4.9 software. Amperometric experiments were performed in steady-state mode using a standard cell with 10 ml volume at 25°C under continuous stirring. After 20 min of the background current stabilizing, the experiments were started by addition of the sample aliquots. In the course of the experiments,

the modified electrodes were stored in 20 mM PB, pH 8.0 at 4°C. All measurements were repeated at least 3 times.

2.7. CONSTRUCTION OF FDDH-BASED POTENTIOMETRIC BIOSENSORS

2.7.1. *Insulating/semiconductor (IS) structures preparation*

A pH-sensitive insulator–semiconductor (IS) transducer ($Si/SiO_2/Si_3N_4$) made in the Institute of Microtechnology of University of Neuchatel (Switzerland) was used. The studied $Si/SiO_2/Si_3N_4$ structures were based on a p-type silicon substrate, 400 μm thickness, with 10 Ohm/cm resistance, covered with a 50 nm layer of thermally grown silicon dioxide and a 100 nm layer of silicon nitride prepared using a low pressure chemical vapour deposition (LPCVD) technique at 750°C. The Ohmic contact was obtained using deposition of indium/gallium alloy on the silicon unpolished face. In our work, we have used the $Si/SiO_2/Si_3N_4$ structures as physical transducers due to their good stability and low hysteresis properties.

Substrates were treated in a sulfochromic mixture and rinsing with ultra-pure water to increase the number of free active sites on the surface of Si_3N_4 (silanol and silylamine sites). After the cleaning process, the substrate was placed at room atmosphere at 70°C during 10 min to remove water.

2.7.2. *Transducer functionalization*

20 μl of the mixture containing 5 mM PB, pH 7.25; 50 mg/ml serum bovine albumin, 0.1 M NAD^+ (neutralized), 20 mM reduced glutathione (neutralized), and 36 U/ml of recombinant FdDH was dropped on the surface of the structure $Si/SiO_2/Si_3N_4$ ($1cm^2$) and spinned (v = 2300 rpm) for 5 min. The sample was treated in glutaraldehyde vapours during 20 min, dried in air during 20 min and, finally, covered with 1% Nafion (neutralized). After 20 min of drying, the bio-functionalised structure was washed several times with 5 mM PB, pH 7.25 and stored in this buffer at 4°C before using.

2.7.3. *Capacitance measurements*

To examine the response of the FdDH-based biosensor, capacitance measurements were performed in a three-electrode electrochemical cell (Pt-counter, Ag/AgCl electrode as reference and the bio-functionalized $Si/SiO_2/Si_3N_4$ structure as a working electrode). The cell was connected to impedance analyzer Voltalab 40 (Radiometer Analytical SA Villeurbane, France). The capacitance–voltage measurements were carried out at DC voltage was swept from −0.5 to 2 V and an AC voltage was superposed

with a frequency of 10 KHz and a signal amplitude of 10 mV. All of these experiments were performed in darkness at room temperature.

2.8. STATISTICS

Statistic treatment of the data and the level of correlation between experimental values have been calculated using computer program *Origin 6.0 and Microsoft Excel*.

3. Results and Discussion

3.1. YEAST ENGINEERING FOR OVERPRODUCING FORMALDEHYDE DEHYDROGENASE (FDDH)

As already reported (Demkiv et al., 2005), the *FLD1* gene from *H. polymorpha* with its own promoter has been re-cloned into *LEU2*-containing plasmid pYT1 devoid of the ARS to be used for multi-copy integration of the gene into chromosomes of the recipient strain *leu1-1* (Fig. 1). The transformation by electroporation have been done by linear and cyclic forms of the plasmid, and 50 clones among Leu^+-transformants with a higher resistance to formaldehyde have been selected and tested by their FdDH activity in CE. The stable recombinant strain Tf 11-6 with the highest FdDH activity up to 5.0 U/mg was chosen and characterized (Demkiv et al., 2005).

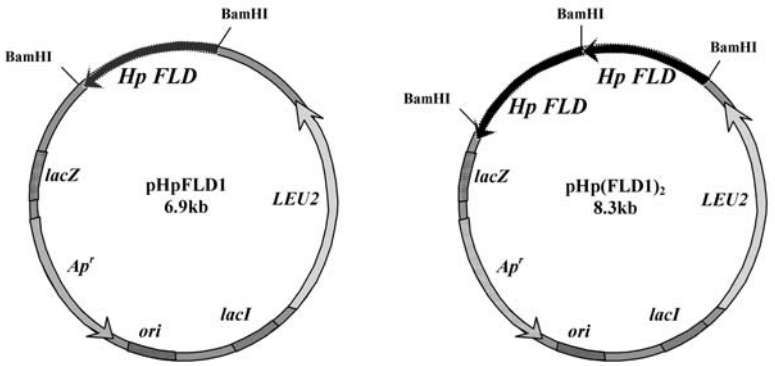

Figure 1. The structure of plasmids as the vectors in construction of H. polymorpha FdDH over-producers.

Similar experiments were carried out using strain *leu 2-2* as a recipient for the transformation by *FLD1*-containing plasmid pYT1. Different methods for the transformation were used (see 2.1) to select new integrative clones by the phenotypes of enhanced resistance to FA and increased FdDH synthesis. All selected strains (approximately 80), isolated by LiCl-method, had the highest resistance to FA (up to 15 mM on a methanol medium) and two clones, Tf 22-142 and Tf 22-126, were resistant up to 20 mM FA (on plates). The tested integrants grew better and were more resistant to elevated FA content in the growth medium in comparison with the recipient strains both on the plates (in solid) and in the liquid medium. The selected strains were considered to be not only a good source for FdDH production, but also the perspective organisms for the environmental bioremediation technologies.

3.2. OPTIMIZATION OF CULTIVATION CONDITIONS FOR FDDH-OVERPRODUCING STRAINS

To optimize the cultivation conditions for the highest enzyme yield, the influence of a growth medium composition on FdDH level for two the best strains, Tf 11-6 and Tf 22-142, was investigated (Fig. 2). As shown in Fig. 2A, FdDH activity in CE was dependent on a carbon source. The only cultivation on 1% methanol provided a considerable level of the enzyme synthesis for the tested strains, both recipient cells and their transformants. Addition of FA to methanol medium additionally stimulated synthesis of FdDH. As shown in Fig. 2B, FdDH specific activities in CE of Tf 11-6 and Tf 22-142, cultivated on the methanol medium supplemented by 10 мМ FA, were found to be 7.0 and 8.5 U/mg, respectively, that is more than twice higher compared to the activity of FdDH of the cells cultivated without addition of FA.

3.3. FDDH PURIFICATION AND CHARACTERIZATION

For enzyme isolation (Demkiv, 2007a), cells of the recombinant overproducer strain Tf 11-6, cultivated in 1% methanol medium in the presence of 5 mM FA, were used. Some physico-chemical characteristics of the purified FdDH are shown in Figure 3. The molecular mass of FdDH subunit, estimated by SDS-electrophoresis, was shown to be about 40 kDa (Fig. 3A), that is the same found for *C. boidinii* – 41 kDa (Melissis et al., 2001). In Fig. 3B the evalution of pH-optimum and pH-stability of the enzyme (incubation in the appropriate buffer at room temperature during 60 min) is presented. pH-optimum was found to be in the range of 7.5–8.5, and the highest stability of FdDH was observed at pH 7.0–8.5.

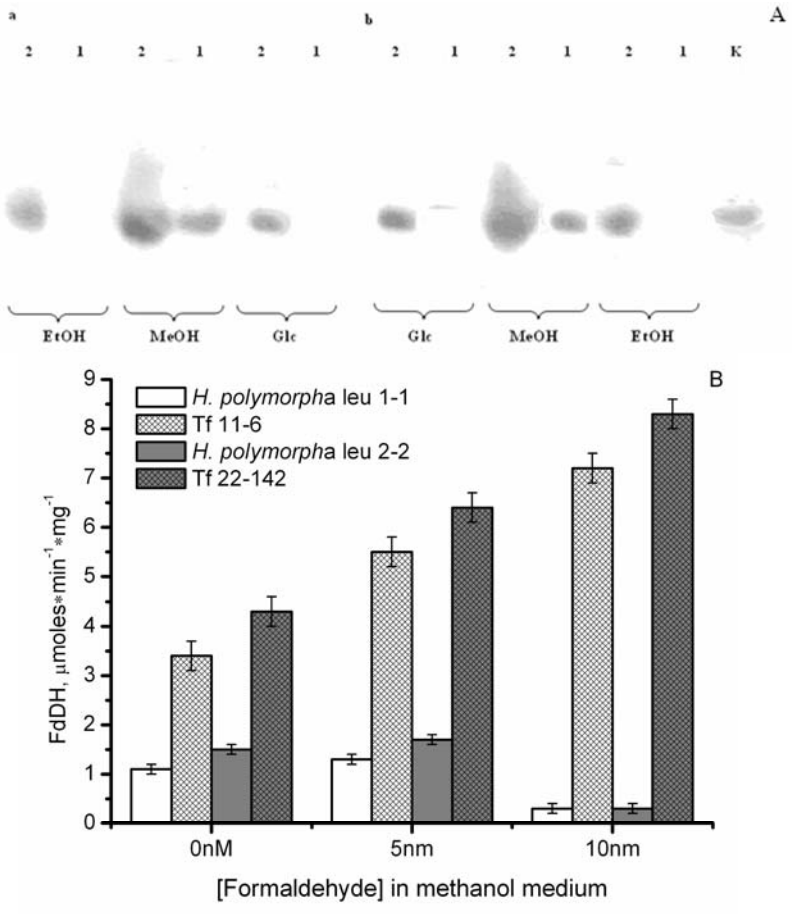

Figure 2. FdDH activity in CE of the recipient and recombinant strains cultivated in different media. A. Vizualization of FdDH activity in 8% PAAG after native electrophoresis of CE. Carbon sources in a growth medium: 1% ethanol (EtOH), 1% methanol (MeOH) or 1% glucose (Glc). Each CE sample contained 0.1 mg protein: a) 1 – *leu1-1* (0.01 U for EtOH and Glc, 0.15 U for MeOH); 2 – Tf 11-6 (0.12 U for EtOH, 0.35 U for MeOH, and 0.10 U for Glc); b) 1 – *leu2-2* (0.01 U for Glc, 0,07 for EtOH and 0.15 U for MeOH); 2 – Tf 22-142 (0.12 U for EtOH, 0.40 U for MeOH, and 0.11 U for Glc); K – 0.01 mg of purified FdDH preparation (0.17 U). B. FdDH activity in CE of cells cultivated in a medium containing 1% methanol and different concentrations of FA, mM.

Optimal temperature for the enzyme activity is 50℃, at 65℃ enzyme retained about 60% of its highest activity, that is equal to the level of FdDH activity at 30℃ (Fig. 3C). The thermostability of the enzyme (Fig. 3C) is

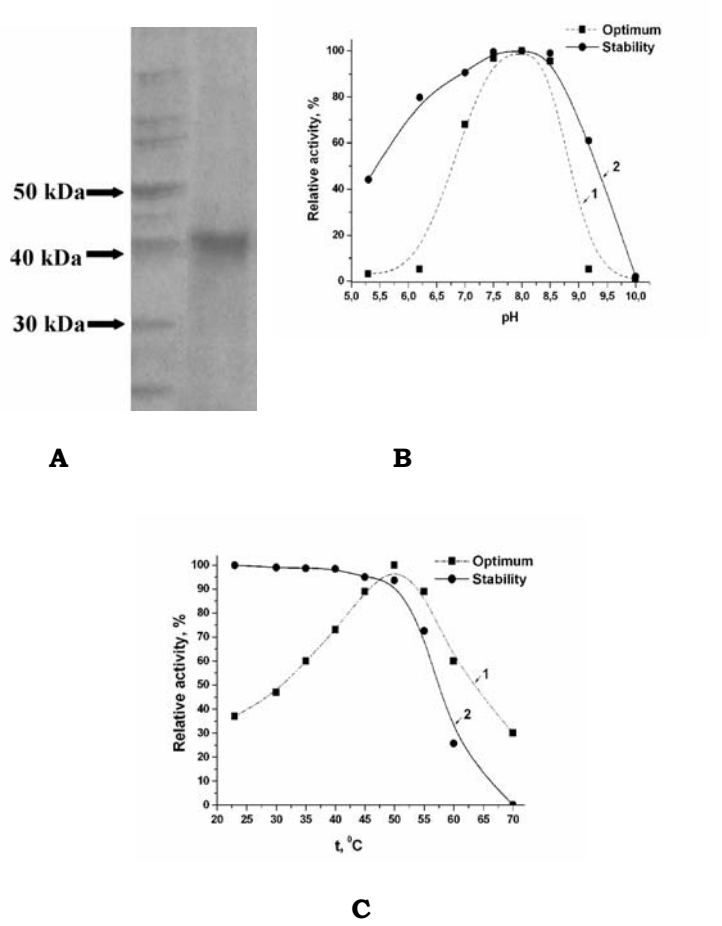

Figure 3. Some characteristics of the purified FdDH preparation (17 U/mg). A. Estimation of M.m. of the enzyme subunit (kDa) in 5–20% SDS-PAAG: 1 – protein standards; 2–5 μg FdDH. B. pH-optimum (1) and pH-stability (2) of enzyme preparation. (2) FdDH was incu bated at different buffers (for pH 5.25–8.0 – in 50 mM PB, pH 9.2–10.0 – in 50 mM borate buffer) during 60 min and then tested for a residual activity at standard assay conditions. C. Temperature optimum (1) and thermostability (2) of FdDH. (1) – Before FdDH adding, reaction mixture was pre-incubated for 10 min at a fixed temperature (23°C, 30°C, 35°C, 40°C, …70°C); then FdDH was added and its activity was determined in thermostated cuvette at the same temperature for at least 5 min; (2) – FdDH solution in PB, pH 8.0 was heated during 10 min at fixed temperature (23°C, 30°C, 35°C, 40°C, …70°C), cooled and tested for a residual activity at standard assay conditions (25°C).

apparently high allowing the usage of FdDH for bioanalitical purposes, namely, for FA assay in food products, waste-water, and pharmaceuticals, as well as for biotransformation of FA to formic acid.

It was reported that the predicted *FLD1* gene product (Fld1p) is a protein of 380 amino acids, 41 kDa (Baerends et al., 2002). Taking into account that the molecular weights of native FdDH from various methanol-utilizing yeasts were estimated to be from 80 to 85 kDa (Table 1), the isolated by us thermostable NAD^+- and glutathione-dependent FdDH from the gene-engenered thermotolerant methylotrophic yeast *H. polymorpha* is supposed to be dimeric. As shown in Table 1, values of the Michaelis-Menten constant (K_M) for FA and NAD^+ calculated for this enzyme are close to K_M for the wild-type enzyme.

TABLE 1. Some characteristics of the purified FdDH from the different yeasts.

Strains	M.m, kDa		K_M, mM			Reference
	enzyme	subunit	FA	GSH	NAD^+	
Candida boidinii	80	40	0.25-0.29	0.13	0.025–0.09	Schutte, 1976;
Pichia pastoris	84	39	0.43	0.48	0.24	Allais, 1983
H. polymorpha, wild type strain	82	40.6	0.21	0.18	0.15	Dijken, 1976;
H. polymorpha, recombinant strain		40	0.18		0.21	This paper

3.4. APPLICATION OF FDDH FOR ENZYMATIC ASSAY OF FA

FdDH preparation isolated from the recombinant strain of the yeast *H. polymorpha* with the specific activity 17.0 units per mg of protein at 25°C (about 27 $U \cdot mg^{-1}$ at 37°C, see Fig. 3C) was proposed to be used for the enzymatic assay of FA. In methylotrophic yeasts, NAD^+- and glutathione-dependent FdDH catalyzes the oxidation of FA to formic acid under simultaneous reduction of NAD^+ to NADH. The proposed enzymatic method is based on the photometric detection of colored product, formazan, formed from nitrotetrazolium blue in reaction coupled with FdDH-catalyzed oxidation of FA in the presence of an artificial mediator, PMS (Demkiv et al., 2007a).

The assay was performed in a mode of incomplete conversion of the analyte (approximately, 10%), using a limited concentration of the enzyme (23 mU/ml) in the reagent. These conditions are economic and reasonable,

because of a high content of FA in the tested samples. At conditions of complete oxidation of FA (excess of the enzyme), sensitivity of assay was determined to be 2.5 μM (in final reaction mixture) or 20 μM – in the tested samples. The reliability of the developed method "Formatest" was tested on the real waste-water samples containing FA. As shown in Table 2, the comparison of FA content values obtained by FdDH-based method and by two routinely used chemical ones (chromotropic acid and MBTH), showed a good correlation between both approaches. Only in some cases (samples DK5 and DK7) with a lower FA content, the difference between the compared methods is higher than 15%–41% and 26%, respectively. A relatively high difference is also observed between two chemical methods for the mentioned above samples –37% and 21%. This can be explained by a higher error in measurement of low optical density values obtained for samples with a low FA content. On the other hand, it is worth to emphasize that the used chemical approaches are not free from possible mistakes due to interfering effects of the co-impurities, usually present in the real samples, for example, phenol which is an attendant pollutant of FA-containing wastes (Polska Norma PN-71 C-04568, 1988).

Thus, we can conclude that analytical data obtained by FdDH-based method are more reliable than chemical ones. Due to this very important analytical feature of the enzymatic method, it can be recommended for practical application instead of chemical ones which are labour- and time

TABLE 2. Comparison of different methods for FA assay (mg/L) in real samples of waste-water (DK), pharmaceutical (Formidron), disinfectant (Descoton forte) and industrial product (formalin)

Samples	Chemical methods		Enzymatic methods	
	Chromotropic acid	MBTH	«Formatest»	«Alcotest»
DK 1	9.3 ± 0.61	9.56 ± 0.51	7.89 ± 0.59	9.6 ± 0.45
DK2	8.7 ± 0.50	8.06 ± 0.32	6.66 ± 0.26	8.12 ± 0.2
DK3	7.2 ± 0.33	7.84 ± 0.36	6.88 ± 0.41	8.00 ± 0.44
DK4	7.1 ± 0.36	6.3 ± 0.46	7.58 ± 0.32	6.86 ± 0.3
DK5	1.65 ± 0.35	1.2 ± 0.15	2.32 ± 0.08	1.97 ± 0.12
DK6	4.64 ± 0.24	4.99 ± 0.059	5.73 ± 0.32	5.60 ± 0.28
DK7	1.62 ± 0.17	1.96 ± 0.20	2.47 ± 0.15	2.19 ± 0.20
Formidron	107.70 ± 12.2	107.1 ± 8.3	96.60 ± 7.31	97.5 ± 9.3
Descoton forte	44.4 ± 7.82	49.22 ± 1.83	42.05 ± 4.02	45.93 ± 3.36
Formalin	420 ± 24	378 ± 21	405 ± 21	–

consuming: need distillation of the samples or performing standard addition test (in the case of phenol contamination).

3.5. CONSTRUCTION OF FA-SELECTIVE BIOSENSORS

3.5.1. *FdDH-based capacitance biosensor*

FdDH was tested as a FA-recognising element coupled with semiconductor-based structure $Si/SiO_2/Si_3N_4$ as a transducer (Ben Ali et al., 2007). The bio-recognition element had a bi-layer architecture and consisted of FdDH, cross-linked with albumin, and two cofactors (NAD and glutathione) in the high concentrations (first layer); the second layer was a negatively charged Nafion membrane which prevented a leakage of negatively charged co-factors from the bio-membrane. Changes in capacitance properties of the bio-recognition membrane were used for monitoring FA concentration in a bulk solution. It has been shown that FA can be detected within a concentration range from 10 μM to 25 mM with a detection limit of 10 μM (Fig. 4).

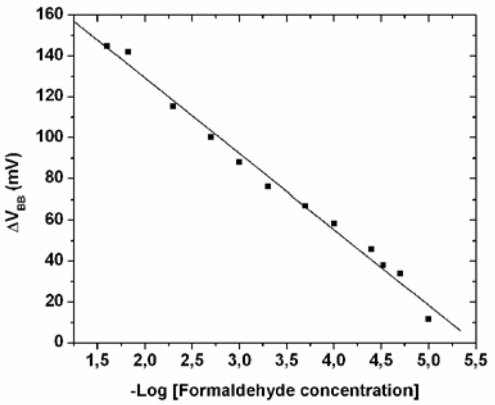

Figure 4. Response of bio-functionalized Si/SiO2/Si3N4 structure for formaldehyde (in logarithms of the molar concentration) in the tested solution.

3.5.2. *FdDH- and cells-based amperometric biosensors*

In the physiological electron-transfer pathway, the electrons are transferred from FA via intermediate hydroxymethylglutathione to the active centre of FdDH under simultaneous reduction of NAD^+ to NADH. For the design of an electron-transfer pathway for the immobilised FdDH as a bioselective element of the sensor, the enzymatically generated NADH has to be reoxidised additionally at the electrode surface using a suitable redox mediator (Fig. 5).

The recombinant yeast cells *H. polymorpha* and FdDH isolated from these cells were used as biorecognition elements of amperometric biosensors. Electron transfer between the immobilized bioelement and graphite electrode was established using different mediators. The best mediators for enzyme biosensor were shown to be positively charged cathodic electrodeposition paints modified with Os-bis-N,N-(2,2'-bipyridil)-dichloride ($[Os(bpy)_2Cl_2]$) complexes. Among five tested Os-containing redox polymers of different chemical structure and properties, complex *1CPOs* of osmium-modified poly(4-vinylpyridine) with molecular mass 60 kDa containing diaminopropyl groups was selected as optimal. Polymer layer simultaneously served as a matrix for keeping the negative charged low-molecular cofactors, glutathione and NAD^+, in the bioactive layer. For cells-based biosensors phenazine methosulfate (free-diffusing redox mediator) exhibited the best electron transfer characteristics.

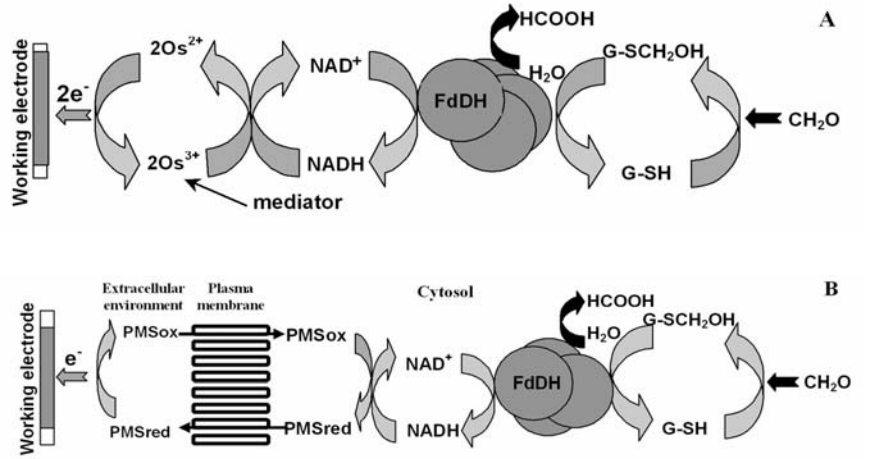

Figure 5. Schematic representation of the electron pathway for the FdDH-based amperometric biosensor for FA detection (A) and the scheme of intracellular red-ox reactions coupled with electrochemical oxidation of the mediator PMS for cells-based sensor (B).

For construction of the envisaged FA biosensor we proposed a sophisticated sensor architecture aiming on the secure fixation of all sensor components in a bioactive layer on the transducer surface. Especially, the sensor architecture was designed to prevent any leakage of the low-molecular and free-diffusing cofactors of the enzyme thus enabling FA determination without addition of the cofactors to the analyte solution (Fig. 6). In the optimized biosensor's construction, platinised graphite electrode was used as a transducer and $[Os(Me_2bpy)_2Cl_2]$-modified positively charged cathodic paint *1CPOs* was found to be the best redox mediator, as well as a good matrix for enzyme or cells electrodeposition and for holding enzyme's cofactors, glutathione and NAD, in a bioactive layer. Covering of the biolayer

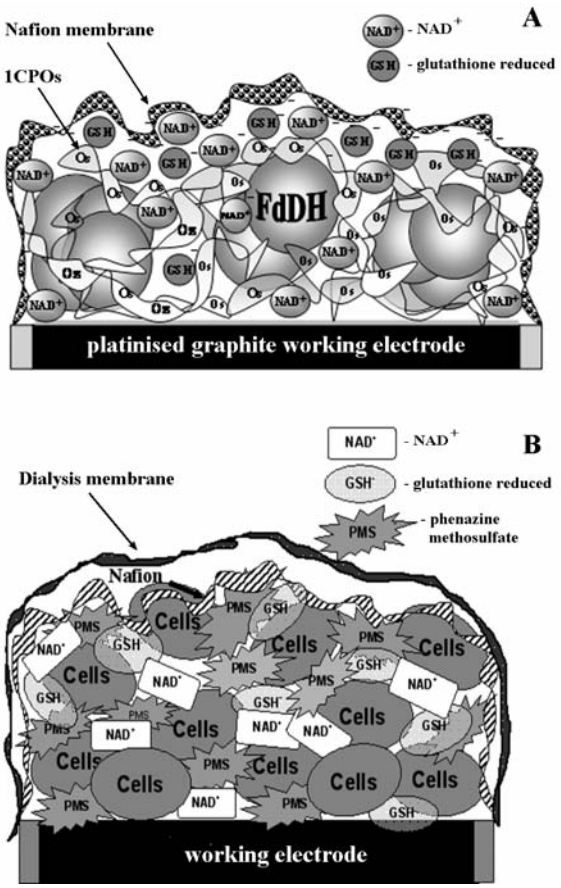

Figure 6. Architecture of FdDH- (A) and cells-based (B) amperometric biosensors.

by a negatively charged Nafion membrane additionally prevents the co-factors from a leakage, as well as contributes to the enhanced stability of the sensor.

Bioanalytical characteristics of the constructed biosensors (Table 3) were studied in detail: kinetics, dynamic and linear range, selectivity, dependence of sensors output on temperature. For enzyme-based biosensor the maximum current value was 250 ± 5.25 μA and the apparent Michaelis-Menten constant (K_M^{app}) derived from the FA calibration curves was 120 ± 5.3 mM with a linear detection range for FA up to 20 mM. For cells-based biosensor, the maximum current value was 1.07 ± 0.04 μA and the apparent Michaelis-Menten constant (K_M^{app}) derived from the FA calibration curves was 20.1 ± 1 mM with a linear detection range for FA up to 8 mM. The

optimal pH-value for the developed biosensors was in the range of 7.6 to 8.3 with an optimal temperature between 45–50℃, due to a higher thermostability of the used enzyme and thermotolerance of recombinant yeast cells. The bioanalytical properties of the developed biosensors were evaluated specifically aiming at an improved long-term operational stability of the sensor. A novel biosensor demonstrated a good sensitivity, high selectivity to FA, and a good stability. A typical response of the developed *1CPOs-NAD*$^+$*-FdDH*-modified electrode towards FA and the linear range are shown in Fig. 7.

Another amperometric biosensor developed by us in a cooperation with scientific group headed by Prof. E. Czöregi (University of Lund, Sweden) was a bi-layer bi-enzyme sensor based on diaphorase and FdDH together with $[Os(4,4'\text{-dimethylbipyridine})_2Cl]^{+/2+}$ (PVP-Os). The sensorarchitecture comprised a first layer containing diaphorase from *Bacillus stearothermophilu* cross-linked with the PVP-Os redox polymer. On the top, a second layer was formed by additional cross-linking of FdDH with poly(ethylene glycol) (400)diglycidyl ether. The sensor architecture was optimised with respect to efficient electron transfer and stability of the enzyme(s). Bioanalytical basic characteristics of the biosensor, polarized at +180 mV vs. NHE, are presented in Table 3.

TABLE 3. Bioanalytical characteristics of developed biosensors (FdDH-based and recombinant cells-based)

Characteristics	FdDH-based biosensor			Cells-based biosensor
	Mono-enzyme	Bi-enzyme (+diaphorase)	Mono-enzyme	
Type of signal detection	Amperometric		Capacitance	Amperometric
Detection limit, mM	0.003	0.032	0.01	0.11
Linear range, mM	up to 20.0	0.05–0.5	0.01–25	up to 8.0
I_{max}, μA	250±5.25	0.18	–	1.07 ± 0.03
Sensitivity	358 $A \cdot m^{-2} \cdot M^{-1}$	22 $A \cdot m^{-2} \cdot M^{-1}$	31 mV/decade	5.1 $A \cdot m^{-2} \cdot M^{-1}$
Reference	Demkiv et al., 2007b	Nikitina et al., 2007	Ben Ali et al., 2007	Paryzhak et al., 2007

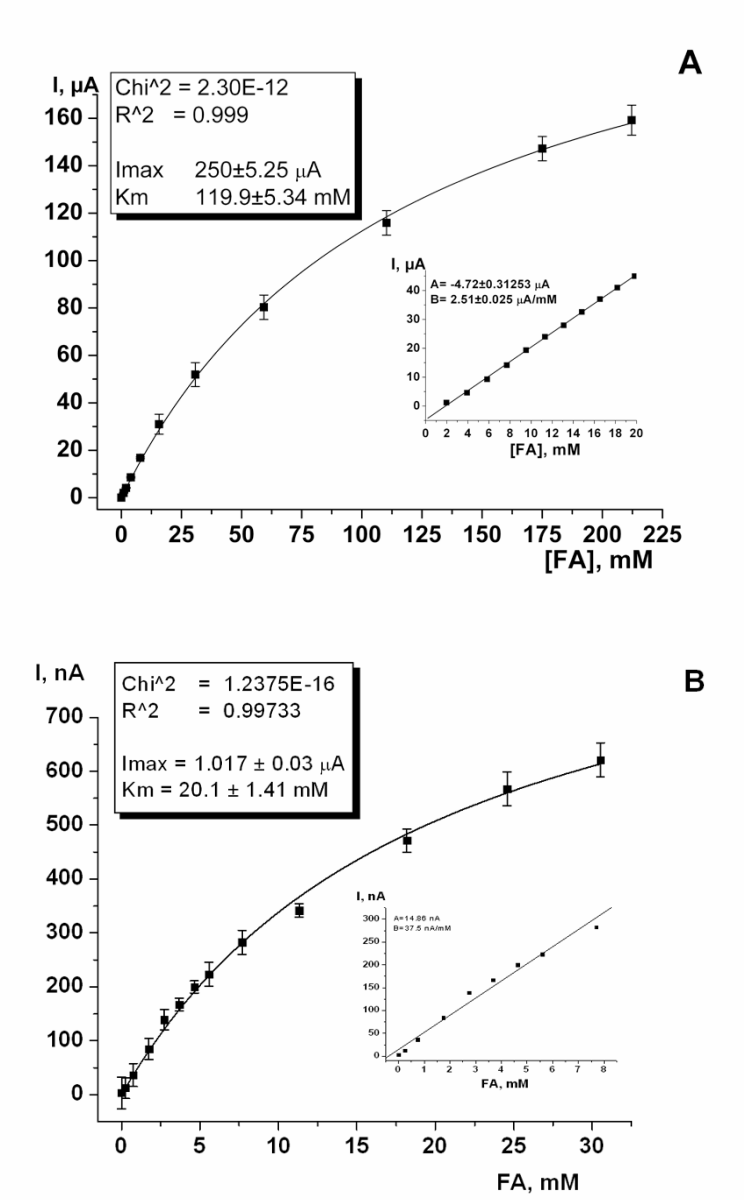

Figure 7. Chronoamperometric determination of FA, using a 1CPOs-NAD+-FdDH-GSH-Nafion -based biosensor (A) and Cells-Tf11-6-NAD+-GSH-Nafion-modified graphite electrode (B) and linear concentration range for biosensors.

3.6. APPLICATION OF AMPERMETRIC BIOSENSORS FOR FA-MONITORING IN REAL SAMPLES

The constructed amperometric biosensors revealed a high selectivity to FA (100%) and a very low cross-sensitivity to other structurally similar substances: butyraldehyde (0,93%), propionaldehyde (1,89%), acetaldehyde (5,1%), methylglyoxal (9,12%) (Paryzhak et al., 2007). These sensors were applied for FA testing in some industrial goods: Formidron, Descoton forte, formalin and rabbit vaccine against viral hemorrhage. A good correlation was observed between the data of FA testing (Table 4) by the biosenor's approaches (FdDH and cells-based), proposed enzymatic method Formatest and standard chemical methods.

TABLE 4. FA content in real samples determined by different methods: chemical (MBTH, Chromotropic acid), enzymatic (FdDH-based) "Formatest", and biosensor approaches (FdDH-based and recombinant cells-based sensors)

Sample/ Method	FA molar (mole/L) concentration, M±m				
	Chemical methods		FdDH-based methods		
	MBTH	Chromotropic acid	Formatest	FdDH-biosensor	Cells-biosensor
Formidron	1.64 ± 0.61	1.48 ± 0.26	1.53 ± 0.31	1.57 ± 0.13	1.48 ± 0.06
Descoton forte	3.57 ± 0.30	3.59 ± 0.44	3.25 ± 0.8	3.61 ± 0.13	3.29 ± 0.12
Formalin	12.6 ± 0.73	14.0 ± 0.81	13.5 ± 0.7	13.6 ± 0.6	13.82 ± 0.54
Rabbit vaccine against viral hemorrhage	0.038 ± 0.003	0.029 ± 0.005	0.042 ± 0.004	0.041 ± 0.005	0.042 ± 0.002

4. Acknowledgements

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ELECTROCHEMICAL SENSING METHODS: A BRIEF REVIEW

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Abstract: Electroanalytical methods are often seen as an effective tool to study chemical and biological systems. This chapter gives an overview of the most common electrochemical sensing techniques, their basic working principles and their typical configurations, paying a brief look to some more recent developments. Due to the huge extension of the subject and to the very large amount of the possible applications, what is given here is a synthesized review of the most general families of methods regarding aqueous solutions, together with a few particular application examples. In the most recent developments, the combination of electroanalytical techniques with different sensing methods, and the usage of signal processing techniques for pattern recognition applied to the electroanalytical data series, emerged in the scientific literature, as pointed out in the text.

Keywords: Electrochemistry, liquid, characterization, water monitoring

1. General Concepts About Electrochemical Techniques

Electrochemical methods are probably among the oldest measurement techniques, and are also comprising a very wide range of analytical possibilities, enhanced by the recent technological developments in signal processing and front-end electronic systems.

Electrochemistry implies the transfer of charge between an electrode and a liquid or solid phase. Both the electrode reactions at the interface and the electrical conduction in the bulk solution participate to this process.

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In the absence of convection, the transfer of charge carriers may be due to a concentration gradient of electroactive species (diffusion current) or to the presence of an electric field (migration current). Mass transport by migration is what mostly happens in the bulk solution and is generally responsible for its electrical conductivity.

The diverse kinds of electrochemical sensors can be split into the following main classes:

- *potentiometric*, implying a pure voltage measurement;
- *amperometric*, where current in a closed loop involving two electrodes is the measured variable;
- *conductimetric*, where the conductance of an electrochemical cell is determined.

1.1. ELECTROCHEMICAL CELLS

Most of the applications can be reduced to a two-electrodes or a three-electrodes cell scheme, as shown in Figure 1. A signal source (generator) is connected between a pair of electrodes and, in the most generic approach, both a current and a voltage measurement are performed.

In a two-electrodes arrangement (Figure 1a) a Working Electrode (WE) is coupled with a non-polarizable Reference (REF); a difference of electrical potential is measured between the WE and the well-defined potential of the REF electrode.

Amperometric measurements are performed by using an excitation signal coming from a generator, and by measuring the resulting current in the loop closed by the cell.

Potentiometric measurements are performed in zero-current conditions, thus removing the generator and the current measurement device from the scheme, and by measuring the voltage across WE and REF with a device having a high input impedance, to minimize the contribution of the ohmic potential drop to the total difference of potential.

In a three-electrodes arrangement (Figure 1b) the current loop comprises a large auxiliary electrode (AUX), having a small charge-transfer resistance, in order to overcome the said ohmic potential drop, due to the current flowing through the cell. This configuration is very general, works also with solutions having poor electrical conductivity, and is the one typically used in laboratory instruments based on *voltammetry*.

Voltammetry is a group of electroanalytical methods in which information about the analyte is derived from the measurement of a current flowing through a polarized WE, with an imposed voltage between WE and REF. That is, the WE potential (calculated with respect to REF) is forced

to adhere to a predetermined program or waveshape, and the current *i* is measured as a function of the applied potential, or as a function of time. The electronic device that implements such concept is called *potentiostat*.

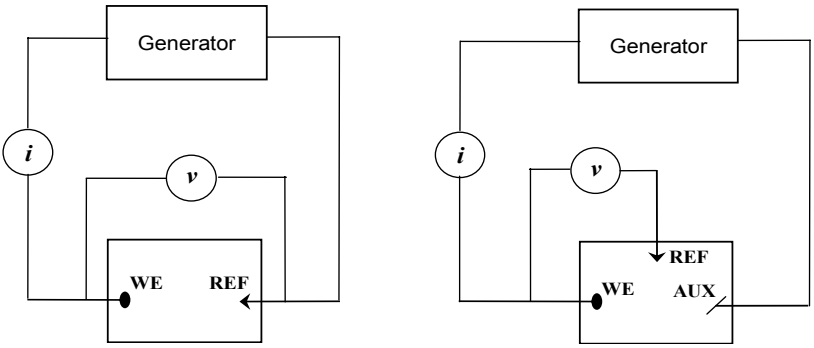

Figure 1. a) Two-electrodes cell configuration; b) Three-electrodes cell configuration.

1.1.1. *Electrical equivalent cell models*

The equivalent circuit of a cell is a schematic diagram, made with concentrated parameters elements, having an electrical behavior equivalent to the one exhibited by the cell. The intrinsic non-linearity of such systems and their complex behavior make the even most reliable model of the electrochemical mechanisms only partially applicable. In particular, the model discussed here can be complicated by additional components, in order to better fit a real behavior, leading to a larger network where also non-linear components and frequency-dependent impedances may be present.

A passive equivalent circuit, made of resistors and capacitors, can be a suitable electrical model for small amplitude sinusoidal excitation signals, at a given frequency. According to the simplest approach almost universally presented in the literature, the components that contribute to the total impedance of the cell are:

- the solution resistance, R_Ω,
- the double-layer capacitance, C_d,
- the faradaic impedance, Z_f.

The double-layer capacitance is due to the ionic charge accumulated on the solution side of a polarized interface (electrode); an ideally non-polarized interface would have a perfectly resistive behavior, instead.

The faradaic impedance Z_f involves interfacial effects, and it is seen as an electrical bypass to the double-layer capacitor; it is due to the electrolytic

reactions and it has a remarkably non-ideal behavior, characterized by a strong dependency upon frequency.

The behavior of Z_f as a function of frequency carries out meaningful chemical information, that can be extracted with more or less direct methods. Typical techniques for the measurement of the faradaic impedance vs. frequency rely on *ac voltammetry* or *polarography* methods, where a relatively small amplitude ac signal (generally a few mV peak) is superimposed to a voltage ramp in a three-cell embodiment. An explanation of how the potentiostat works in this configuration is given in paragraph 1.3.2.

In Figure 2 the equivalent circuit of an electrochemical cell, in the form discussed here, is shown. The faradaic impedance is often reduced to a pure resistance, the *charge transfer resistance* R_{ct}, which shorts the double layer capacitor C_d, as it is shown in Figure 3a. A capacitive element is often added to the network, either in a series (R_s, C_s) or in a parallel (R_p, C_p) configuration, as it is shown in figure 3b.

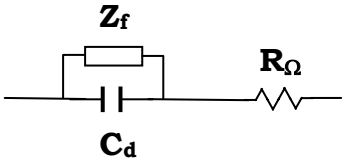

Figure 2. Equivalent circuit of an electrochemical cell.

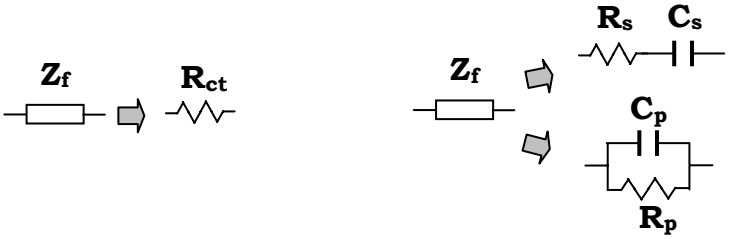

Figure 3. a) Purely resistive faradaic impedance. b) Complex faradaic impedance (series and parallel topology).

Again, while R_Ω and C_d have a basically linear and almost pure electrical behavior, Z_f is strongly dependent upon frequency. According to a wide literature available, the faradaic impedance is related to the kinetic parameters of the electrode reactions. Z_f can thus be seen as the combination of a mass-transfer impedance and a contact resistance; its amplitude and phase angle take information about the concentration of the electroactive species

and the charge transfer resistance (Delahay and Reilley, 1954; Bard and Faulkner, 1980, 2000).

1.2. POTENTIOMETRIC SENSORS

1.2.1. *Working principle*

Electrodes for potentiometry are based on the interface between two electrolyte phases, which are put into contact. A non-polarized interface (selective or not) exhibits an half-cell potential, that is related to the activities of the species involved into the equilibrium of the electrochemical potentials. The half-cell potential (or formal potential) is defined as the electrical potential measured with respect to a standard hydrogen reference electrode (Bard and Faulkner, 1980, 2000; Ives and Janz, 1961; Janata, 1989).

The electrochemical potential in a location P, can be defined as (Butler, 1926; Guggenheim, 1929; Guggenheim, 1930):

$$\overline{\mu_i^{\alpha}} = \mu_i^{0\alpha} + RT \ln a_i^{\alpha} + z_i \cdot F \cdot \phi^{\alpha} \tag{1}$$

where:

z_i is the charge of species i.

F is the Faraday's constant,

ϕ^{α} is the electrical potential at the location P for phase α,

$\mu_i^{0\alpha}$ is the standard chemical potential of the species i in phase α,

a_i^{α} is the activity of the species i in phase α.

Non-polarized interfaces reach equilibrium conditions that are governed by activities of the ions; the difference in electrical potential, that makes the electrochemical potential constant across the interface, depends on the said activities as explained by the Nernst equation, put into the following form:

$$\pi = \pi^0 + \frac{RT}{z_i F} \ln \frac{a_i^{\alpha}}{a_i^{\beta}} \tag{2}$$

where:

π is the interfacial potential difference,

π^0 is the standard interfacial potential difference, measurable with equal activities in phase α, β,

a_i^{β} is the activity of the species i in phase β.

The two phases α, β may represent the sample phase and the electrode phase, according to the described approach. Thus, π is the voltage measurable across a semipermeable membrane between the sample and an unpolarized reference. It is now clear how selective membranes, that respond in a Nernstian fashion to specific electroactive species, can be the fundamental building block of Ion-Selective Electrodes (ISE). In this context, ion activity can be measured with a two-electrodes configuration, where the WE (often called *indicator*) is actually a reference electrode interfaced to the sample via a specialized membrane and a reference solution in between, having the analyte at a fixed activity.

1.2.2. *Ion-selective electrodes*

Ion-selective electrodes are based on an ion selective membrane that interfaces an internal reference to the external environment (that is, the sample). Glass membranes are probably the most commonly used; in fact, they have been developed and used since the beginning of the 20th century, especially for the measurement of pH. Other ion-selective membranes, both solid-state and liquid, have been developed and are commercially available; the usage of some particular kinds of membranes, such as lipid/polymer ones and chalcogenide glasses, is still matter of research, especially for the development of arrays of sensors.

When the ion selective membrane uses a glass/electrolyte interface, the whole cell is configured in the following way:

$$\text{Ag/AgCl/HCl } (c)\text{/Glass membrane/SAMPLE//Reference} \qquad (3)$$

where:

/ denotes the interfaces,

// denotes the junctions,

c is the concentration of the reference solution.

The internal reference in this example is of the Ag/AgCl kind; the external reference electrode can be of the same kind, but not necessarily.

In the case of pH electrodes, the membrane essentially responds to H^+ ions, and is formed by a dry bulk and two thinner dry layers that come into contact with the liquid phase. In the bulk structure only mobile cations present in the glass, such as Na^+ or Li^+, are substantially responsible for electrical conduction. Deep explanations about the principles and construction of pH electrodes can be found in the literature cited in the references (Bates, 1973; Garrels , 1967).

In addition, there's a wide availability of sources of information about theory and construction of the ISEs, such as the book edited by H. Freiser (1978).

1.2.3. *Semiconductor field-effect sensors*

A typical issue in the usage of glass electrodes lies in their high output impedance, that is, the need for the voltmeter device, placed between the ISE and the REF electrode, to drain the smallest possible current from the measurement loop. In fact, typical impedance values for commercial combined pH electrodes (embedding their REF) are in the order of the hundreds of MOhms.

The front-end electronic component typically used in the electrometers for potentiometry is a kind of FET (Field-Effect Transistor) that may be realized in different ways, according to the particular technology employed. In general, the active building-blocks of the amplifier and buffer stage will be one or more FETs, with insulated gates in bipolar or MOS technology; such stage has to process the weak signal coming from the electrodes and adapt its impedance level to the input of data acquisition and display devices, connected to the output of this buffer/amplifier stage.

The idea to embed an ion-sensitive capability into a FET device comes from the early 70s, and has generated the first integrated chemical sensor. CHEMFETs (CHEMically sensitive FETs) can be mainly split in two categories:

- ISFETs, based on an ion-sensitive membrane on behalf of the metallized gate of standard FETs,
- ENFETs, based on a chemically selective enzyme layer.

To avoid confusion, it must be clarified that some authors consider ENFETs a mere kind of ISFETs, and do not use the term CHEMFET.

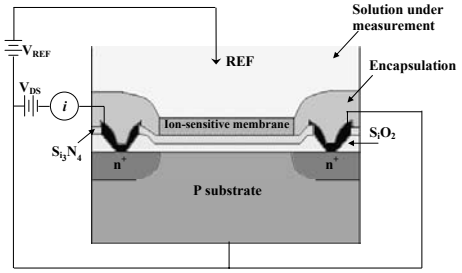

Figure 4. Structure of a CHEMFET/ISFET device. Electrical connections are shown.

The structure and a simplified schematic diagram for potentiometric measurements performed by using an ISFET device are shown in Figure 4. The V_{REF} generator provides the right Gate voltage to polarize the FET for a correct modulation of the channel in the desired working region. Channel conductance is a function of the ion activity because of the electric field generated by the charges accumulated on the membrane; thus the observable

is the current *i*, which is also a function of the Drain-to-Source voltage (V_{DS}). Among the various sources of information which are available, good explanations abut the working principles of CHEMFETs can be found in the book by Jiri Janata (1989).

1.3. VOLTAMMETRY

1.3.1. *Working principle*

The electrochemical behavior of a system can be determined by imposing known varying potentials between WE and REF, and recording either the current-voltage or the current-time curve, that is obtained in each experiment.

The saturation level of the current in the *i-v* curve, due to the mass-transport limitation, carries information about the concentration and nature of the electroactive depolarizing species involved in the process, that is, those involved in the interfacial charge transfer across the double layer.

The different decomposition potentials, diffusion coefficients and mass-transfer limits, that are exhibited by electroactive species in a solution, generate different contributions to the complex curve (voltammogram) obtained; different kinds of voltammetry are classified according to the waveform of the excitation signal.

In this framework, a known technique to get a complete electrochemical signature of a system consists into applying a series of potential steps (actually rectangular pulses) having different amplitude, always recording the current vs. time curve. But this represents just one of the possibilities.

There's a wide range of possible implementations of measurement methods based on voltammetry, according to the type and material of the working electrodes (i.e. solid metal microelectrodes, dropping mercury, planar rotating disks, carbon electrodes, etc.), and according to the particular electrochemical experiment, which depends on the program imposed by the generator. There's a huge quantity of useful readings to get a deep knowledge about polarographic methods and more recent applications of voltammetry; among the many available, we can mention the excellent textbook by Bard and Faulkner (Bard and Faulkner, 1980, 2000) and others cited in the references (Janata, 1989; Bond, 1980; Macdonald, 1977).

Under the theoretical point of view, the difference of electrochemical potential between two points (A,B) of a solution can arise because of:

- a difference in the concentration of electroactive species over the distance A to B,
- an electric field with a non-zero component along the direction A-B.

These two phenomena contribute to the mass transport by diffusion and migration, respectively; the flux of charged species transported can be expressed in terms of current density.

For non convective mass transfer, in the proximity of an electrode, the electroactive substance is transported by both the processes previously described (migration and diffusion).

In this case, the general flux equation is given by:

$$J_j(x) = -D_j \nabla C_j - \frac{z_j \cdot F}{R \cdot T} \cdot D_j \cdot C_j \cdot \nabla \phi \tag{4}$$

where:

D_j is the diffusion coefficient of species j,

C_j is the local concentration of species j.

Stirred solutions, which are mostly used in practice, involve forced convection. In this case, a model has been proposed (Bard and Faulkner, 1980, 2000), where it is assumed that convection maintains the concentrations of all the species uniform and equal to the bulk values, till a certain distance δ from the electrode. Within this layer having thickness δ, it is assumed that there's no solution movement, thus mass transfer is thought to be due by diffusion only.

1.3.2. *The potentiostat*

The basic component of the measurement system is called *potentiostat*. A potentiostat is a device which injects current into the AUX electrode, closing the current loop via the working electrode WE, in order to impose a known difference of potential between WE and a reference electrode REF. Such voltage has to be measured with a high impedance differential amplifier, to make negligible the current going through REF.

The functional diagram of a potentiostat to make voltammetric measurements with a three-electrodes cell is shown in Figure 5. The voltage imposed across WE and REF is supposed to be determined by a function generator placed at the input of the potentiostat; the excitation function corresponds to the $v_e(t)$ signal indicated in the diagram. The experimental observable, that is obtained by each measurement session, is the response of the system potentiostat-cell to the excitation signal (voltage) imposed, that is, the loop current i_{WE}(t). Under the hypothesis that all the current losses in the system can be neglected, i_{WE}(t) is equal to the output current of the control amplifier (1).

The potential on the WE is tied to the analog ground by the I/V converter (3); the differential electrometer (2) is connected to the negative input

of the control amplifier (1) and closes the feedback loop, which ensures that the voltage difference between the reference electrode REF and the WE is equal to the voltage set by the input signal $v_e(t)$. All this is true until both the voltage assumed by the auxiliary electrode AUX and the current which flows through it, lie in the output swing capability of (1).

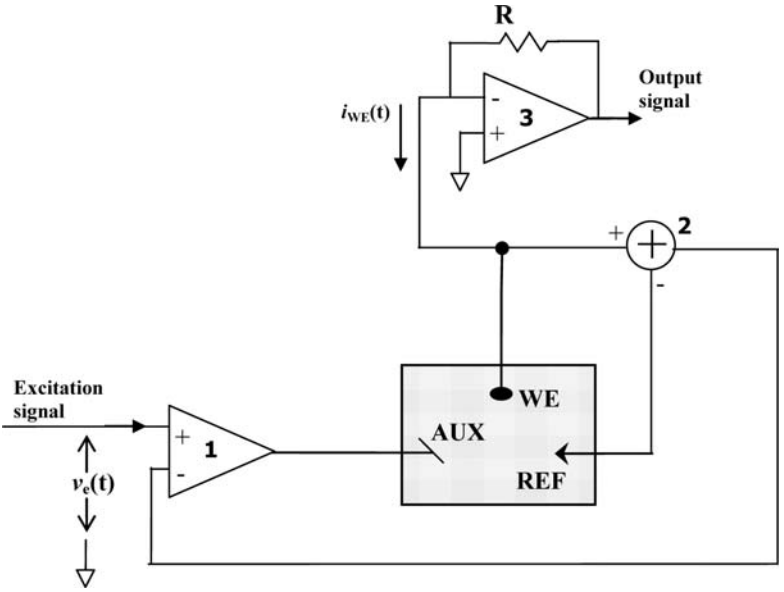

Figure 5. Simplified block diagram of a potentiostat in a three-electrodes configuration.

Automatic measurements are often performed by controlling the potentiostat with a Personal Computer and an adequate piece of software. Different kinds of working electrodes have been presented in the literature and are also commercially used, depending on the different measurement requirements, and can also be automatically switched.

Fundamental parameters, such as the number and type of electrodes, the shape of the excitation waveforms, and the duration and amplitude of such waveforms can usually be changed, by configuring the measurement device accordingly.

Many different measurement schemes can be obtained by combining different excitation functions and data recording criteria; we can mention here linear sweep chronoamperometry, where current is recorded as a function of time with a linear (ramp) excitation, and pulse polarography, where a current-potential response is obtained, according to the varying amplitude

of the potential steps applied. Even if the terms seem quite exchangeable in the literature, according to a IUPAC recommendation, the term 'polarography' should be used only with liquid renewed working electrodes, such as the dropping mercury ones (DME).

Ramp signals and rectangular pulses may also have small amplitude alternating signals superimposed; such small amplitude potential changes give the possibility to work in a region where the current is dominated by electrode kinetics, while large amplitude steps go directly to the mass-transfer controlled region. Deep explanations of this family of methods can be found in a rich literature available (Bard and Faulkner, 1980, 2000; Janata, 1989; Bond, 1980; Macdonald, 1977), going outside the aim of this chapter.

1.4. CONDUCTIMETRY

Conductance and impedance measurement techniques play a significant role in the arena of electrochemical methods. The current flowing in a solution under the influence of an electric field is due to the migration of ions; individual ionic currents are proportional to the strength of such field, and are determined by the balance between the frictional drag and the said electric field, which bounds the terminal velocity of each ionic species, thus its associated current density.

Conductance, which is the reciprocal of resistance, is generally defined for a given volume, delimited by parallel current flow lines, as:

$$G = \sigma \frac{S}{L} \tag{5}$$

where:

σ is the electrical *conductivity* of the medium,

S is the cross-sectional area of two finite surfaces normal to the flow lines,

L is the path length between the two said surfaces.

G is expressed in Ω^{-1} (or Siemens), while σ, which is an intrinsic property of the solution, is expressed in Ω^{-1} cm^{-1}.

The contribution of each ionic species in the solution to the total conductivity is called *transference number*, and is defined in the following way:

$$t_i = \frac{u_i C_i |z_i|}{\sum_j u_j C_j |z_j|} \tag{6}$$

where:

i, j are the subscripts denoting the generic i, j ion species,

u_i is the mobility of the generic i-th ion species,

C_i is the concentration of the generic i-th ion species,

$|z_i|$ is the normalized charge for the generic i-th ion species.

In most of the applications, the conductivity of a solution is measured in a two-electrodes cell configuration, with two identical metal electrodes; conductance is usually measured between the two terminals, either by measuring the current flowing at a known imposed voltage, or by measuring the potential drop at a known injected current, finally, various versions of bridge configurations have been seen in the literature.

In order to avoid the effects of polarization and of faradaic processes on the measurement (represented in the model by the double-layer capacitor and the faradaic impedance), periodic and alternating excitation signals are generally chosen. Both pure sinusoidal signals and rectangular pulses have been proposed in the literature, with different approaches as to sampling, rectification and filtering of such signals; even other waveshapes have been experimented, such as triangular ones (Luce, 1986).

Typical probes for measuring the conductivity of a solution are made by two facing Pt plates, coated with a colloidal deposit of “platinum black”, confined into an electrically isolated environment in order to bound the cell volume precisely. The advantage of platinum black lies in its large effective wet surface with respect to its actual geometry, thus limiting the effects of adsorption due to fast electrode reactions that may happen during each half-cycle of the excitation signal.

Other materials, such as titanium cladded with platinum, have been proposed (Iwamoto, 1993); also, graphite electrodes are available on the market and carbon-filled polymers are proposed for conductivity measurements in microdevices(Baldock et al., 2003).

There are alternatives to the two-electrodes approach used in practice, such as a four-electrodes configuration, where current is injected through a pair of electrodes and voltage is measured across the other two electrodes with an high input impedance device, being substantially prevented from interfacial effects due to the transit of current. In addition, non-contact inductive sensors are sometimes used in the industry, but their description goes beyond the scope of this chapter.

2. Recent Developments and Applications

2.1. IMPEDANCE MEASUREMENTS

The measurement of a purely dissipative (real) impedance parameter of a fluid, such as the conductivity, represents a limited source of information with respect to its complete behavior as a function of frequency. In fact, depending on the frequency content of the excitation signal used, the electrical behavior of the circuit under measurement (the cell) changes in a way that makes a purely resistive model unsatisfactory.

When dealing with conductivity measurements, the excitation waveforms and frequencies are selected in order to extract information about the real part of the impedance under measurement, trying to work in a region where undesirable effects (i.e. polarization and faradaic processes) are negligible.

Characterization and quality control of organic matter and materials, instead, get the information from the whole impedance, often denoted by the frequency behavior of a sample; amplitude and phase information are often put in terms of concentrated parameters of an equivalent electrical circuit.

Examples of recent applications of impedance measurement techniques regard the usage of faradaic impedance spectroscopy for the development of enzyme sensors, immunosensors and DNA sensors; in this case, the faradaic impedance spectroscopy has been proposed in association with chronopotentiometry as a mean to follow biocatalytic precipitation processes in biosensing applications (Alfonta et al., 2001).

Other examples of biosensing transduction by using impedance spectroscopy techniques may regard the detection of bacterial cells by directly measuring the impedance of the bacterial cell suspensions (Yang, 2008), and some studies for the detection of enzyme activity by the degradation of the gelatine coating of interdigitaded electrodes, indirectly affecting the measurable impedance (Saum et al., 1998).

There are also recent theoretical works, which look for a deeper knowledge about the impedance measurement of an electrochemical cell and its fundamental mechanisms. The study of non-linear impedance spectroscopy, due to second-order effects (Mishuk et al., 2002), gives a good example of such basic research, which is still fully alive.

2.2. POTENTIOMETRIC MEASUREMENTS

A good part of the development of new transducers still relies on potentiometric techniques; one of the major application frameworks, that populated some literature in the last fifteen years, consists into the development of sensor arrays for the characterization of liquids.

In this context, data fusion between different sensor modalities and feature extraction techniques applied to multisensor data, have been frequently proposed and studied in the recent literature. There are a number of applications where an automatic (and fast) quality assessment of a resource attracts scientific and industrial interests; among the many which have been proposed we can mention: food and beverage quality tests, pollution monitoring of surficial and ground water, drinking water quality tests and classification, including water distribution networks.

The fundamental idea that lies behind such devices is their capability to provide an aggregate of chemical information, useful for characterizing the liquid being measured, or, to provide alarms upon the detection of changes. Sometimes, especially in the most recent literature, this class of devices is referred to as "electronic tongue" or "e-tongue" devices. This is the wet-counterpart of the e-nose concept, which has been very much explored in the scientific literature, especially in the early 90s.

Several chemometric methods, based on the multicomponent analysis of signals generated by an array of cross-sensitive potentiometric sensors, have been proposed. Sometimes, these are also combined with completely different sensor modalities, such as spectrophotometry and/or gas chromatography of the gases separated in the head space of an equilibrium cell.

An example of an array of screen-printed electrodes made of carbon paste on a polymeric substrate is given by Lvova et al. (2002); other examples about the usage of non-specific arrays of sensors can be found in the last ten years (Ciosek et al., 2004; Legin et al., 1999). Both the latter two examples propose arrays of Ion-Selective Electrodes as a sensory interface to the liquid under measurement, making use of custom-made selective membranes, commercial electrodes and also solid-metal electrodes. Good results in terms of discrimination capability have been shown in the classification of fruit juice, wine and water, in the experiments which have been published.

Data fusion between potentiometry, gas chromatography and spectrophotometry is presented by Rodriguez-Mendez et al. (2004) for the characterization of wines, while a general approach for the combination of liquid-phase information (electronic tongue) and vapor-phase information (electronic nose) is proposed by Winquist et al., (1999), where the liquid-phase device is based on voltammetry, as it will be explained in the next paragraph.

2.3. VOLTAMMETRY

Voltammetry has been recently proposed as a possible tool for the characterization of liquids with a non-conventional chemometric approach. This mature sensor technique, with the aid of a suitable signal processing methodology, has proven to be an interesting option for the continuous monitoring of a process, thanks to its very low analytical limits and to the large amount of data that it is possible to obtain, particularly with "pulse polarography" experiments. Both the ability to classify samples and the ability to detect changes in a solution under measurement have been investigated in recent works.

The usage of pulse voltammetry, with a series of rectangular pulses modulated by a ramp, has been presented in different contexts (Winquist et al., 1999; Scozzari et al., 2005) as an efficient sensor technique for automatic classification/characterization purposes. In fact, some recent developments (Robertsson and Wide, 2005; Scozzari et al., 2007) are concerning signal processing aspects, in the framework of pattern recognition techniques for automatic monitoring systems. In the works cited, focus has been given to the drinkable water distribution surveillance and to food processing applications.

This kind of measurement approach is often catalogued in the literature as belonging to the family of e-tongue devices, this time relying on voltammetry as a sensor technique.

The large multivariate space (Scozzari et al., 2007), generated by the time series of the current signals acquired, requires a suitable processing in order to extract the useful information; such processing steps may be resumed in:

- reduce the dataset with the minimum loss of information,
- extract the salient features from the reduced data-set to make a correct characterization of the sample.

The performance of such systems is generally expressed in terms of:

- capability to discriminate between different classes of liquids (i.e. different brands of bottled mineral water),
- capability to detect changes in the electrochemical behavior of a solution (change detection, i.e. due to pollution).

The latter being particularly interesting for what regards the detection of pollutants, poisons, or simply any alteration in a process under monitoring.

Promising results have been published, essentially in the area of water quality assessment and beverage industry, by using pulse voltammetry with a set of solid metal switched working electrodes, made by different metals (Krantz-Rulcker et al., 2001; Scozzari, 2007). Also, an application example

regarding the quality control in the production of food for children has recently been proposed (Robertsson and Wide, 2005).

In the framework of possible applications to drinkable water monitoring, the capability to detect changes in water due to slight levels of pollution by organics and pesticides have been investigated (Scozzari, 2007), showing detectible limits compatible with drinkable water regulations.

Finally, methods based on polarography and chronoamperometry are still matter of research; as an example, the development of biosensors cited in paragraph 2.1 combines impedance spectroscopy with chronoamperometry (Alfonta et al., 2001), in order to determine the charge-transfer resistance in the measurement of the faradaic impedance.

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ODDITIES AND CURIOSITIES IN THE ALGAL WORLD

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Abstract: The term *algae* refers to a polyphyletic, non-cohesive and artificial assemblage, of O_2–evolving, photosynthetic organisms. The profound diversity of size, shape, habitat, metabolic traits and growth strategies makes this heterogeneous assemblage of both prokaryotic and eukaryotic species an almost unlimited source of curious and unusual features. Algae display an incredible adaptability to most environments, and provide an excellent system for testing hypotheses concerning the evolution of ecological tolerance. In fact, they are not limited to temperate waters, but can survive at very low depth and very low irradiance, and thrive beneath polar ice sheets. Upon adaptation to life on land, algae have colonized such surprising places, as catacombs, tree trunks, hot springs, and can also resist desiccation in the desert regions of the world. Moreover, relations between them and other organisms, which include competition within and between species for space, light, nutrient or any limiting source, are based on a variety of associations, which includes epiphytism, parasitism, and symbiosis. Algae can share their life with animals, growing on sloth hair, inside the jelly capsule of amphibian eggs, upon the carapaces of turtles or shells of mollusks, camouflaging the dorsal scute of harvestmen. They can also light up the sea at night, and cause infections in animals and humans.

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1. Introduction

The term *algae* has no formal taxonomic standing, nevertheless it is routinely used to indicate a polyphyletic, non-cohesive and artificial assemblage, of O_2–evolving, photosynthetic organisms (with several exceptions of colorless members, which are undoubtedly related to pigmented forms). No easily definable classification system acceptable to all exists for algae, since taxonomy is under constant and rapid revision at all levels following every day new genetic and ultrastructural evidence. Keeping in mind that the polyphyletic nature of the algal group is somewhat inconsistent with traditional taxonomic groupings, though they are still useful to define the general character and level of organization, and aware of the fact that taxonomic opinion may change as information accumulates, the tentative scheme reported in Table 1 of chapter one of this book is adopted (Barsanti and Gualtieri, 2006).

This review is meant to discuss these diverse organisms from a different perspective by focusing on some of their more unusual characteristics or oddities. The oddities we will deal with are quite diverse, since we came across them studying the algal world in its different aspects. The world of any organism results from the interaction of both abiotic (physiochemical) and biotic factors. Among the major physiochemical factors affecting algae are light, temperature, salinity, nutrient availability. Among biotic factors are relations between algae and other organisms, which include competition within and between species for space, light, nutrient or any limiting source, and a variety of associations, which includes epiphytism, parasitism, and symbiosis. The interactions between these different variables can both hide and reveal odd traits of algae.

2. Occurrence and Distribution

Algae can be aquatic or subaerial, when they are exposed to the atmosphere rather than being submerged in water. Aquatic algae are found almost anywhere from freshwater springs to salt lakes, with tolerance for a broad range of pH, temperature, turbidity, O_2 and CO_2 concentration. They can be planktonic, as are most unicellular species, living suspended throughout the lighted regions of all water bodies including under ice in polar areas. They can be also benthonic, attached to the bottom or living within sediments, limited to shallow areas because of the rapid attenuation of light with depth.

Benthic algae can grow attached on stones (epilithic), on mud or sand (epipelic), on other algae or plants (epiphytic) or on animals (epizoic). In the case of marine algae, other terms can be used to describe their growth habits, such as supralittoral, when they grow above the high tide-level, within reach of waves and spray; intertidal, when they grow on shores exposed to tidal cycles, or sublittoral, when they grow in the benthic environment from the extreme low-water level to around 200 m deep, in the case of very clear water.

Dealing with subaerial algae, oddities do not lack; upon adaptation to life on land, algae have colonized such surprising places, as hot springs, catacombs, and they can also be found embedded within desert crust or sharing their life with animals.

2.1. LOW DEPTH

The depth record is held by dark-purple crustose coralline algae collected at 268 meters, where the faint light is blue-green and its amount is approximately 0.0005% of the surface value (Markager and Sand-Jensen, 1992; Gattuso et al., 2006). Considering that "full sun" (i.e. irradiance in the middle of the day) shines approximately 10^4 mol photons m^{-2} $year^{-1}$ on the earth surface (Littler et al., 1985; Spalding et al., 2003), 0.0005% is equivalent to a photon flux of only 5 mol photons m^{-2} $year^{-1}$ during full sunlight at its maximum zenith. At these depths the red part of the sunlight spectrum is filtered out from the water and not enough energy is available for photosynthesis. These red algae are able to survive in the dark blue sea because they possess accessory pigments, including carotenoids, xantophylls and phycobiliproteins, that absorb light in spectral regions different from those of the green chlorophylls *a* and *b.* This absorbed light energy is channeled to chlorophyll *a*, which is the only molecule able to convert sunlight energy into chemical energy (Barsanti et al., 2007). Due to the presence of these pigments, the green of their chlorophylls is masked and these algae look dark purple.

Also green algae occur to depths comparable with those of red algae. In the temperate western North Atlantic, green algae such as *Derbesia marina* (Chorophyta) may occur with red algae in the deep clear waters of the continental shelf to at least 30 m, (Sears and Cooper, 1978; Lewbel et al., 1981). In southern California, an encrusting *Codium* sp. (Chorophyta) was found at 91 m (Lissner and Dorsey, 1986). The occurrence of these green algae in deep water is not surprising, because many deep-water green algae, including the Derbesiales and Codiales, have been found to contain siphonaxanthin, a carotenoid capable of absorbing the green light prevalent in deep waters (Yokohama et al., 1977; Yokohama, 1981). *Palmophyllum umbracola,* one

of the deepest occurring Chlorophyta (70 m), (Hanisak and Blair, 1988), also possesses an unidentified orange pigment (Nelson and Ryan, 1986) that may be siphonaxanthin.

2.2. COLD ENVIRONMENTS

Other unusual and extreme habitats for algae are represented by very cold environments, such as platelet ice layer, the snowfield and the glaciers, (Leya et al., 2000). Algal cells adapting to cold temperatures have to take care of the following three processes: (1) changing the ratio of saturated and unsaturated fatty acids in the lipids of the cell membranes to maintain membrane fluidity (Morris et al., 1981; Roessler 1990), (2) adjusting the permeability of the cell membrane for water to react to osmotic changes of the outer medium (Kawecka, 1986), and, (3) when exposed to freezing temperatures, reducing the water content of the cell interior to prevent ice crystal formation (Bunt, 1968; Giese, 1973).

The platelet ice layer consists of a lattice of flat, disk-shaped ice crystals that accumulate to a thickness of up to several meters under sea ice located adjacent to floating ice shelves (Dayton et al., 1969, Smetacek et al., 1992). Because it is submerged beneath 1–2 m of consolidated ice, irradiance reaching the platelet ice is first attenuated by the overlying snow cover, sea ice crystals and brine inclusions, particles, and soluble material (Robinson et al., 1995), resulting in a light field that is reduced in magnitude and restricted in spectral distribution (Palmisano et al., 1987a; SooHoo et al., 1987; Arrigo et al., 1991; Robinson et al., 1995). Nevertheless, reports of high algal production and biomass accumulation in the platelet ice of the Weddell and Ross Seas (Antartica) (Bunt and Lee, 1970; Grossi et al., 1987, Smetacek et al., 1992, Arrigo et al., 1995) attest to the success of the platelet ice microalgal community at growth in these low light conditions. *Phyllophora antartica* (Rhodophyta) is a representative alga in this environment. The structure of the platelet ice layer provides a large surface area for the attachment and growth of microalgae (Arrigo et al., 1993). In addition, the fixed position of the platelets provides a stable substrate that isolates the algae from vertical mixing and holds them in a higher and less variable light field than that available within the underlying water column. Vertical compression of the biomass also reduces the fraction of PAR absorbed by the ice/water milieu of the platelet layer and increases the fraction absorbed by the algae for use in photosynthesis (Arrigo et al., 1993). The algae inhabiting the platelet ice layer are extremely shade adapted, typically attaining maximum photosynthetic rates at an irradiance lower than 500 mol photons m^{-2} $year^{-1}$ (Palmisano and Sullivan 1985; Palmisano et al., 1987b; Arrigo et al., 1993; Robinson et al., 1995).

The successful exploitation of the Antarctic platelet ice layer by sea ice microalgae for growth results from a unique combination of physical properties and biological adaptations, (Cota, 1985; Dieckmann et al., 1992; Lizotte and Sullivan 1992; Lizotte et al., 1998; Thomas and Dieckmann, 2002). Adaptive features include pigments, polyols (sugar alcohols, e.g. glycerin), sugars and lipids (oils), mucilage sheaths, motile stages and spore formation. Moreover, since the algae living in regions that receive full solar exposure are adapted to high UV light environment, they can augment their UV protection capacity especially during summer months by producing secondary metabolites including phenylpropanoids, carotenoids, xantophylls, and mycosporine amino acids (Bidigare et al., 1993; Ryan et al., 2002).

True snow algae are defined as those that grow and reproduce wholly within the water retained by snow during snowmelt. During summer months large blooms occur, which can reach cell concentration of 10^6 cells mL^{-1} and color whole snow banks red, orange, green or gray depending on the species and habitat conditions. Most snow algae belong to Chlorophyta, such as *Chloromonas rubroleosa* and *Chlamydomonas nivalis*. These algae color the snow red, due to the excess of carotenoids and xantophylls (Müller et al., 1998; Hoham and Blinn, 1979; Kol and Eurola, 1974; Thomas, 1972; Kol, 1969). Species from other algal groups are also important and the dominant alga in many of the glaciers around the world is the saccoderm desmid *Mesotaenium berggrenii* (Chlorophyta), an alga that colors the snow grey, due to its iron tannin compounds, (Ling and Seppelt, 1990; Yoshimura et al., 1997).

Snow algae go through a complex life history, involving motile vegetative stages that undergo syngamy and thick-walled resting spores and zygotes. The latter allow them to survive the time when the snow has completely melted, and probably to be spread by wind (Muller et al., 2001). Many of these algae possess vegetative and or motile cells that are usually green in color and immotile spores or cysts that may be red, orange or yellow green in color. The green vegetative cells color the snow green, whereas the red and orange snow are generally caused by the spore stages though some snow algae may be red-pigmented in their vegetative state. The spores usually have thick walls and large amounts of lipid reserves, polyols and sugars. They are able to withstand sub-zero temperatures in winter and also high soil temperatures and desiccation in summer, which would kill normal vegetative cells. The motile stages enable them to re-colonize the snow from germinating spores left behind on the soil as well as to position themselves at the optimum depth for photosynthesis in the snow/ice column. The cells of some species also secrete copious amounts of mucilage which enable them to adhere to one another and to snow crystals and prevent the cells from being washed away by melt water. The mucilage also forms a protective

coat and delays desiccation, and it may have an additional function as an UV shield. A few species are common worldwide, but others are restricted to either the Northern or Southern Hemispheres.

Snow algae sustain a highly diverse microbial community on snow fields and glaciers, which is composed of bacteria, heterotrophic flagellates and ciliates. These organisms in turn, sustain a community of cold-adapted animal species, such as midges, copepods and snow fleas on Himalayan glaciers, and ice worms and collembolas on North American glaciers (Aitchison, 2001; Kikuchi, 1994; Kohshima, 1984; Goodman, 1971). Blooms of snow algae can reduce the surface albedo, (i.e. the ratio of reflected to incident light, see later) of snow and ice, and largely affect their melting, (Takeuchi et al., 2001; Kohshima et al., 1993).

The ecology of snow algae is important for understanding the glacial ecosystem since they can be used as indicators to date ice cores drilled from glaciers. Their biomass and community structure inside ice cores may also provide information on the paleo-environment (Yoshimura et al., 2006).

2.3. HOT SPRINGS

Another example of ecological diversification of algae is the colonization of alkaline hot spring habitats across western North America, Asia, Africa, and possibly Europe by members of the genus *Synechococcus* (Cyanophyta), (Castenholz, 1996). Hot spring outflows typically exhibit marked temperature gradients, and microbial communities containing *Synechococcus* generally develop in these systems at temperatures between about 45°C and 73°C, which is the thermal maximum for photosynthetic life (Brock 1967, Castenholz, 1999; Brock, 1978). Studies on the behavior of *C*-phycocyanin from *Synechoccocus lividus* showed that purified *C*-phycocyanin is stable up to least 70°C and it is highly aggregated with identical spectroscopic behaviors at 20°C and 70°C (Edwards et al., 1996; Edwards et al., 1997). For these characteristics, it is termed temperature resistant protein (Samsonoff and MacColl, 2001).

The biology of acid hot springs is quite different from that of the neutral/alkaline springs. Though most thermo-acidophiles are prokaryotes (Archea and Bacteria), photosynthetic prokaryotes, such as cyanobacteria, are completely absent from acid water even when the temperature has dropped to quite low values. The photosynthetic microorganisms of such acid water are rare kinds of eukaryotes found no where else on earth, the Cyanidiales, a group of asexual unicellular red algae (Cinilia et al., 2004). The main representative in hot acid springs is *Cyanidium caldarium* (Rhodophyta), an organism of unusually evolutionary ancestry, which can grow even when the acidity is close to zero and maintain its intracellular

pH at close to neutral. How this important physiological resistance is achieved is still not understood, though a strong proton pump or a low proton membrane permeability are possibilities. The upper temperature limit for *Cyanidium* is about 56°C, lower than that of the cyanobacteria as would be expected since *Cyanidium* is a eukaryote. As in the case of *Synechococcus,* C-phycocyanin from *Cyanidium* fits the characteristics of a temperature-resistant protein. Its hallmark for stability is to remain inflexible toward structural change over a wide range of temperatures from 10°C to 50°C. The protein denatures irreversibly at the temperature at which the alga is no longer viable between 60°C to 65°C, (Eisele et al., 2000).

2.4. CATACOMBS

Different species of terrestrial epilithic cyanobacteria, such as *Leptolyngbya* sp. and *Scytonema* sp., occurring in Roman hypogea, inside St. Callistus and Priscilla Catacombs, live under extremely low photon fluxes, 3–30 mol photons m^{-2} $year^{-1}$ (Albertano et al., 2000), similarly to deep-water algae. At this extremely low photon fluxes, these algae can grow because of the presence of phycobyliproteins organized in phycobilisomes in the thylakoid membranes inside the cell that transfer their absorbed extra energy to chlorophylls. They sense the light direction by means of a photoreceptive apparatus that is located in the apical portion of the tip cell, which is composed by a carotenoid-containing screening device and a light detector based on rhodopsin-like proteins, (Albertano et al., 2001).

2.5. DESERT CRUSTS

Crusts can be defined as microbiotic assemblages formed by living organisms and their by-products, creating a complex surface structure of soil particles bound together by organic material. Some crusts, are characterized by their marked increase in surface topography, often referred to as pinnacles or pedicles (Anderson and Rushefort, 1977). Other crusts are merely rough or smooth and flat (Johansen, 1993). The process of creating surface topography, or pinnacling, is due largely to the presence of filamentous cyanobacteria and green algae. These organisms swell when wet, migrating out of their sheaths. After each migration new sheath material is exuded, thus extending sheath length. Repeated swelling leaves a complex network of empty sheath material that maintains soil structure after the organisms have dehydrated and decreased in size (Belnap and Gardner, 1993; Barger et al., 2006).

Algal crusts of desert regions have been suggested to retard soil erosion (Booth, 1941; Fletcher and Martin, 1948; Shields and Durrell, 1964; Metting

1981; Mucher et al., 1988; Liu and Ley, 1993; Johansen, 1993; St. Clair et al., 1986), which generally includes rain and wind erosion. Although the erodibility of soil with and without crusts has been quantified (Booth 1941; Loope and Gifford, 1972; Brotherson, and Rushforth, 1983; Gillette and Dobrowolski, 1993; Maxwell and McKenna Neuman, 1994; Liu et al., 2001), only few studies have been focused on the specific effects of different algae in stabilization of sand dunes. The recovery rate of cryptogamic crusts (i.e. a thin crust made up of mosses, lichens, algae, and bacteria) in natural and artificial conditions has been examined (Belnap, 1993; St. Clair et al., 1986), as well as the effect of the wind regime (including wind force and types), moisture, crust development, soil texture, vegetation coverage, season and human activity on algal crust integrity, (Belnap and Gillette, 1997,1998; Brotherson and Rushforth, 1983, Dong et al., 1987, Hu et al., 1991, Liu et al., 1994, Williams et al., 1995). Most algal crust formation in arid area is initiated by the growth of cyanobacteria during episodic events of available moisture with subsequent entrapment of mineral particles by the mechanical net of cyanobacterial filaments and glue of extra-cellular slime, (Johansen 1993; Belnap and Gardner, 1993). Algal crusts are critical to the ecosystem in which they occur. Evidence has shown that they may play important roles in the stabilization of soil surfaces and the improvement of soil structure, contributing significantly to soil fertility of these regions through such processes as nitrogen fixation, excretion of extracellular substances, and retention of soil particles, organic matter and moisture (Hu et al., 2002; Li et al., 2002; Barger et al., 2006). Nitrogen fixation by cyanobacteria and lichens (due to their symbiotic cyanobacteria) comprising the crusts is the primary source of nitrogen input in many of the arid ecosystems on a worldwide basis. The fascinating points herein are why and how the algal crusts, only a few millimeters thick, play such important roles, and how the relevant organisms survive and even flourish in such a harsh environment with extreme desiccation, strong radiation, and large fluctuation of temperature (Evans and Johansen, 1999; Wynn-Williams, 2000).

Systematic investigations of algal crusts conducted in the Tengger Desert (China) have provided data on the vertical microdistribution of cyanobacterial and algal species within samples aged 42, 34, 17, 8, 4 years (Hu et al., 2003). This vertical distribution was distinctly laminated into an inorganic-layer with few algae (0.00– 0.02 mm,), an algae-dense-layer relatively compact and densely inhabited with algae (0.02–1.0 mm) and an algae-sparse layer (1.0–5.0 mm). Due to extremely high irradiation, the surface of the inorganic layer was the harshest microenvironment of the desert crusts, and therefore was colonized only with *Scytonema javanicum,* and *Nostoc flagelliforme* (Cyanophyta), desiccation-tolerant species possessing high UV screening pigments. These two heterocystous, diazotrophic species were the only algae

found at the depth of approximately 0.02–0.05 mm, while around 0.05–0.10 mm, the coccoid green alga *Desmococcus olivaceus* (Chlorophyta), characterized by strong resistance to stressful environments, was the dominant species. The biggest algal biovolume was present at 0.10–0.15 mm, with the dominance of *Microcoleus vaginatus* (Cyanophyta), a sheath-forming and polysaccharide-excreting cyanobacterium capable to stabilize sand grains. The diversity of algal species was the largest in all the crust samples at the depth of approximately 0.15–0.50 mm. Filamentous (*Anabaena azotica, Phormidium tenue, Lyngbya cryptovaginatus*) and unicellular cyanobacteria (*Gloeocapsa* sp., *Synechocystis pevalekii*) unicellular coccoid green algae (*Chlamydomonas* sp., *Chlorococcumhumicola*, *Chlorella vulgaris* and *Palmellococcusminiatus*), diatoms (*Naviculacryptocephala*, *D. vulgare* var. *ovalis* and *Hantzschia amphioxys*) and euglenoids (*Euglena* spp.) were present. Within the range of approximately 0.50–1.00 mm there were much more green algae and euglenoids than in the other strata. Because the upper 1 mm of the crust was the euphotic zone (i.e. the zone where enough light penetrates for photosynthesis to occur), more than 96% algal bio-volume was distributed in this algae-dense-layer (0.02–1.0 mm). By the algae-sparse-layer, dramatically reduced irradiance was inadequate for efficient photosynthesis and therefore this layer had only about 4% of the total algal bio-volume. At 1.0–2.0 mm, species usually found were *L. cryptovaginatus*, *M. vaginatus*, *P. tenue, S. pevalekii*, *N. cryptocephala* and *H. amphioxys*. At the depth of approximately 2.0–3.0 mm, *P. tenue* was the dominant species together with diatoms, mainly *N. cryptocephala* and *H. amphioxys*. At the depth of approximately 3.0–4.0 mm only diatoms were present. Existence of a diatom layer at the crust base might be the result of downward seepage of water and the high motility of these algae.

The older the crusts, the nearer to the surface were *Nostoc* sp., *Chlorella vulgaris, Microcoleus vaginatus, Navicula cryptocephala* and fungi, which might be less resistant to the surface stresses. This might reflect the slow but effective process of algal crust development at early stages, and this process might be beneficial to the transformation from algal crusts to lichen crusts at later stages in consideration of the integration of cyanobacteria and green algae, such as *Nostoc*, with fungi to form lichen, (Hu et al., 2003).

3. Animal-Algae Interactions

Algae are involved into complex relationship with very different animals. They have been reported to grow epizoic on sloths, polar bears, seals, frogs and salamanders, artropods and turtles. In the case of the tree sloths, *Bradypus* sp. and *Choloepus* sp., the algae effectively turn these animals green, giving them excellent camouflage among the leaves. The camouflage is crucial to

the sloth's survival, because its inability to move quickly makes it an easy target for predators such as the harpy eagle (*Harpia harpyja*). Among the many odd features of these interesting animals, perhaps the oddest of all is their hair which, with its peculiar structure and its algal presence, is unlike the hair of any other mammal (Gilmore et al., 2001). During the dry season, the hair of sloths usually has a dirty brown coloration, but during long period of rain it may show a very appreciable greenish tinge brought about by the increased presence of symbiotic algae. The algae may already be present in the hair of juveniles only a few weeks old and they could provide camouflage for the sloths while obtaining shelter for themselves. The algae have a distinct distribution patterns in *Choloepus* and *Bradypus*, lying longitudinally along the grooves in the former and in short lateral tongue or lines in the latter. Algae representing four phyla have been cultured from *Bradypus*, these being Chlorophyta, Chrysophyta, Cyanophyta, Rhodophyta, (Thompson, 1972). The algae found on the coat of *Bradypus tridactylus* lie between the cuticle scales (Aiello, 1985) and the hair changes with age in apparently all species of *Bradypus*. Young hairs are white, gray, brownish or black and do not possess the deep cracks seen in older hairs. The first traces of algae appear on these young hairs as tiny dots or extremely narrow transverse lines. Older hairs have larger, wider algal colonies and obvious deep transverse cracks. When wet, these cracks close considerably, but when dry give the effects of beads on a string. The oldest hairs are badly deteriorated with the spongy cuticle worn off on one side exposing the full length of the cortex. In the older hairs living algae are absent. It was suggested (Aiello, 1985) that either the algae colonize the very narrow cracks in young hairs or the algae themselves initiate the cracks. The hair of all the three *Bradypus* species (*B. tridactylus, B. variegatus* and *B. torquatus*) readily absorbs water, but those of *Choloepus* do not. Lack of healthy algal colonies has been observed in *Bradypus* kept in captivity; since they do not survive long under these conditions, algae have been suggested to provide nutrition or a particular trace element essential for the health of the animals (Aiello, 1985).

Polar bears (*Thalarctos maritimus*) normally have creamy-white fur, presumably an adaptation for camouflage in a snowy environment. However, cases are been reported of polar bears kept in captivity in different American zoos, which turned green as result of algae growing on their fur (Lewin and Robinson, 1979). The coloration was particularly evident on the flanks, on the outer fur of the legs and in a band across the rump. This coloration was clearly attributable to the presence of algae inside the hairs, specifically in the hollow medullae of many of the wider (50–200 μm), stiffer guard hairs of the outer coat. The thinner (less than 20 μm) hairs of the undercoat, which were not hollow, were colorless. The fact that some of these lumina were in connection with the external air or water could explain how the

algal cells could have entered the hairs in the first place, and how exchange of O_2 and CO_2 and uptake of water and mineral salts would be facilitated and could permit growth of the algae if suitable illuminated. Such a habitat has certain advantages, being warm and protected from most kind of potential predators. The algae isolated from the polar bear hairs and cultured under controlled conditions were identified as cyanobacteria, (Lewin and Robinson, 1979). Unidentified green algae are also known to color green the fur of the monk seal (*Monachus* sp.), refer to http://www.pifsc.noaa.gov/psd/mmrp/brochure.pdf).

Another mutualistic association with algae occurs in some amphibians. All amphibians lay eggs with a jelly capsule, although the form and thickness of the capsule vary widely (Duellman and Trueb, 1986; Salthe, 1963). Some amphibians, including the spotted salamander *Ambystoma maculatum* and the pickerel frog *Rana palustris*, embed their eggs in large masses of relatively firm jelly, which are attached to vegetation in ponds. Other amphibians, including the wood frog *Rana sylvatica* and the spotted marsh frog *Lymnodynastes tasmaniensis*, lay their eggs in masses that float at the surface of the pond or are loosely attached to vegetation. The jelly capsule, at least in aquatically developing amphibians, protects the eggs from predators (Ward and Sexton, 1981). It also resists exchange of respiratory gases. A further factor in gas exchange in amphibian egg masses is that many are colonized by symbiotic algae. For example, virtually all *Ambystoma maculatum* egg masses in the wild are inhabited by algae (Gatz, 1973). The algae were first noted by Orr (1888), who speculated that they must have considerable influence on the respiration of the embryos. It is well established that this relationship is symbiotic. The alga *Oophila ambystomatis* (Chlorophyta) is found exclusively in amphibian egg masses, mostly in those of *Ambystoma maculatum*, but also in those of *R. sylvatica* and some other species (Gilbert, 1942), and derives its name from the association. The benefit to the algae may be higher CO_2 or ammonia concentrations found inside egg capsules; algal growth is much greater in the presence of embryos than after the embryos had been removed from the jelly (Gilbert, 1944). The amphibian embryos also benefit, having higher hatching success and shorter developmental times when reared with algae than without (Gilbert, 1944) and, in egg masses with algae, higher hatching success in light than in darkness (Breder, 1927; Gilbert, 1942). The basis for the beneficial effect of the algae on the embryos was uncertain until the work of Pinder and Friet (1994). These authors concluded that symbiotic algae in *A. maculatum* egg masses produce more O_2 than they consume, making the egg mass hyperoxic in light. Because O_2 diffusing in from the water is consumed before reaching the centre of the egg mass, O_2 produced by local algae may be the only source of O_2 for innermost late-stage embryos.

Algae are also important for tadpoles of other amphibians, establishing an ecologically important mutualism that is conditional and provide partner species with novel options for adjusting to changing environment (Hay et al., 2004). It is well know that organisms reaching their critical thermal maximum (CTM, the minimal high deep-body temperature that is lethal to an animal) are incapable of escaping the lethal conditions (Freidenburg and Skelly, 2004). This holds especially true for aquatic organisms in thermally uniform systems, which have no refuge from heat stress; further, temperature increases within such systems decreases the concentration of the necessary gases oxygen and carbon dioxide, (Wu and Kam, 2005). Aquatic organisms that are stressed for these gases for respiration and photosynthesis would benefit from fortuitous mutualistic interactions in which the "by-product" gases evolved by metabolism can be absorbed reciprocally. Observations were conducted on numerous tadpoles of the dwarf American toad, *Bufo americanus charlesmithi* in a shallow temporary pool subjected to extended exposure to solar radiation, located in Ashley County (Arkansas, USA), (Tumlison and Trauth, 2006). The water became very warm by mid-afternoon, and some of the tadpoles possessed an atypical greenish coloration. The tadpoles were late stage, and some of them exhibited well-developed legs. Microscopic examination of live tadpoles from the pool revealed cluster of biflagellated green algae identified as *Chlorogonium* (Chlorophyta) scattered as greenish blotches over the skin, (Nozaki et al., 1998). Individuals of this alga were observed actively flagellating to maintain a position oriented to the skin of the tadpole. The distribution of the alga generally followed the pattern of cutaneous blood vessels on the dorsal surfaces of the legs, tail, and lateral body wall.

The high CTM of toads help them survive in warmer conditions and shortens the time required for development, thereby promoting metamorphosis prior to desiccation of the habitat, (Noland and Ultsch, 1981). Rates of oxygen consumption in tadpoles increase with higher temperatures, but water at higher temperature holds a lower concentration of gases (Ultsch et al., 1999). Although tadpoles are tolerant to warmer temperatures, the O_2 deficits can lead to respiratory distress and death. Under conditions of low O_2, tadpoles of some species can supplement oxygen intake by gulping air, but the late development of the lungs precludes this in *Bufo*, (Duellman and Trueb, 1994). Consumption of O_2 increases sharply prior to metamorphosis. Thus, the warmer water contains less O_2 at a time when more may be needed. Even after acclimatization to warmer temperatures, the CTM of tadpoles of most anuran species is 38–40°C, with a few exceptions above 41°C in species that develop in xeric (with very little moisture) or tropical habitats. On the other hand, the rate of photosynthesis tends to increase with increases in temperature up to an optimum, after which it decreases rapidly,

partly limited by the availability of inorganic carbon. Growth rate of algae slows in stagnant cultures because the rate of diffusion of CO_2 from the air become limiting, partly because CO_2 diffuses 10^4 times faster in air than in water. Green algae (Chlorophyta, including *Chlorogonium*) tend to dominate in temperatures of 15–30°C, but are replaced by cyanobacteria above 30°C, (DeNicola, 1996). Thermophilic algae thrive best in waters rich in CO_2, where conditions necessary to maintain high rates of photosynthesis are met, (Fogg, 1969). The pattern of association and distribution of *Chlorogonium* over the skin of tadpoles allow maximum potential for uptake of otherwise limiting CO_2 released via cutaneous respiration by tadpoles. The relatively small size of *Chlorogonium* specimens also could indicate stress. The mean length of the cells taken from the tadpoles was 13.4 μm (range 7–22 μm), and width ranged only between 1.5–3 μm. The normal measurements from species known to occur in the United States ranges from 19–59 μm in length and 5–18 μm in width. Smaller cells result in a higher surface/volume ratio, which could help maximize absorption in CO_2-limited environment.

The CTM at which tadpoles of *Bufo americanus* could survive independently is 39.5°C. In a heat-stress-inducing environment, however, the CTM could be expanded by over 4°C (to about 43°C) in the presence of a photosynthetic, mutualistic alga such as *Chlorogonium*. Considering these phenomena, it is hypothesized that the *Chlorogonium* and tadpoles are exhibiting a facultative symbiosis in which tadpoles gain O_2 produced via photosynthesis adjacent to the skin, and concomitantly *Chlorogonium* receive the metabolic CO_2 evolved from the tadpoles, (Tumlison and Trauth, 2006). Similar algal accumulations have been found on tadpoles of gray tree frogs (*Hyla versicolor*) and cricket frog (*Acris crepitans*) at other locations within Arkansas (USA), (Tumlison and Trauth, 2006).

Arthropods are also good host for algae. Cyanobacteria were reported to grow epizoically on the dorsal scute of the harvestman *Neosadocus* sp. (Arachnida, Opiliones), in the Cardoso Island, southeast Brazil. The epizoic algae almost fully covered the harvestmen's back, giving the animals a greenish coloration contrasting markedly with the brownish body and appendages. The growth of the algae did not affect behavior and locomotion of the animals, which would benefit from the presence of the photosynthetic organism by being camouflaged and thus protected from visual diurnal predators, (Machado and Moreira Vital, 2001).

The occurrence of carapacian algae on turtle is a common phenomenon often reported in literature. Among them the green algal genus *Basicladia* Hoffman and Tilden (Chlorophyta) contains species which are specifically epizoic on carapaces of turtles or shells of mollusks, (Normandin and Taft, 1959; Neil and Ross Allen, 1954; Ernst and Norris, 1978). Closely related

to *Cladophora*, *Basicladia* is mainly distinguished by its epizoic nature. Five species are recognized, and all are known only from freshwater turtles or snails. *Basicladia crassa* and *B. chelonum* have been reported from freshwater turtles in several states of the Rocky Mountains. Outside of the United States, *B. sinensis* was described from the back of a turtle brought from China to an aquarium in California, and *B. ramulosa*, an exceptionally large species, is known from Australian turtles. The fifth taxon, *B. vivipara* is known only from the freshwater snail, *Viviparus malleatus* Reeve. In 1975, *B. crassa* was reported for the first time from Virginia on the carapace of a red-bellied turtle *Chrysemis rubriventris*. The algae were restricted to the turtle carapace, where they formed a wide band thickly covering the marginals and ventral half of the pleural scutes, but thinning as it extended dorsally. The rugose carapacial surface of this turtle is well suited for the attachment of algal rhizoids, but the basking habit of the turtle may account for absence of most carapacial algae since they are subject to increased desiccation and solar radiation above the water level. Also *C. rubiventris* sheds the epidermal scutes of its carapace periodically, thus freeing itself of any algae attached. However, it is not uncommon to find "moss-back" red-bellies in the spring, especially just after emergence from hibernation when air temperature are still too cool for much basking. *B. crassa* and *B. chelonum* are able to survive periods of basking desiccation and even more heat than the turtles themselves. It is possible that repeated exposure to the sun's ultraviolet rays and the drying effect through frequent basking associated with the grazing action of herbivorous fish or amphipods would at least limit the growth of these algae, (Ernst and Norris, 1978).

Algae can also establish pathogenic association with both animals and humans. Among the genera most intensely investigated is *Prototheca*, (Di Persio, 2001). These algae are unicellular, spherical to oval in shape, ranging from 3 to 30 μm in diameter. *Prototheca* species are closely related to the green alga *Chlorella* (Chlorophyta), but lack chloroplasts and possess a two-layered, instead of three-layered cell wall (Joshi et al., 1975); they are heterotrophic and require external source of organic carbon and nitrogen (Koenig and Ward, 1983). The life cycle is similar to that of algae of the genus *Chlorella*; reproduction is asexual by internal septation and irregular cleavage, with subsequent rupture and release of 2–16 autospores through a characteristic split in the cell wall of the parent cell. Released autospores then go on to develop into mature cells (Pore, 1998a). The taxonomic status of the genus *Prototheca* has changed during the last decades and currently the following four species are assigned to this genus: *P. zopfii, P. wickerhamii, P. stagnora, P. ulmea* and *P. blashkeae* (Roesler et al., 2006). A fifth species, *P. moriformis* is not generally accepted (Kruger, 1894; Pore, 1985; Ueno et al., 2003). Only two of these species have been documented

to cause infections in humans and animals (Pore, 1998b), i.e. *P. zopfii* and *P. wickerhamii*.

These algae are globally ubiquitous (Pore et al., 1983) and can be isolated from various reservoirs, such as environment, animals and food. Typical sources of *Prototheca* species are the slime flux of trees, fresh and marine waters, soil and sewage, stables and animal buildings, excrement, various animals (cattle, deer, dogs), and food items such as butter, potato peels, bananas, cow's milk (Pore et al., 1983; Pore, 1985; Pore, 1986).

In 1952, *P. zopfii* was first identified as a pathogen of bovine mastitis associated with reduced milk production characterized by thin watery secretion with white flakes (Lerche, 1952). While in the past only sporadic cases of protothecal mastitis have been observed, this form of mastitis now occurs endemic in the most countries of the world (Hodges et al., 1985; Costa et al., 1996; Aalbaek et al., 1998; Janosi et al., 2001; Moller et al., 2007). This infection represent a serious problem since the affected animals must be culled from their herds to halt transmission of the disease (Cunha et al., 2006).

Prototheca produce disease also in humans, and the clinical conditions caused by this alga are generally referred to as protothecosis (Thiele and Bergmann, 2002). The first case of human infection was diagnosed in 1961 in Sierra Leone on a rice farmer; it took the form of a verrucose foot lesion from which *P. zopfii* was isolated as etiological agent. Over the following years, the number of documented cases of protothecosis rose continuously, with about four new cases being diagnosed every year over the past decade (Lass-Florl et Mayr, 2007). Three clinical forms of human protothecosis have been described: cutaneous/subcutaneous infections, olecranon bursitis, and disseminated or systemic protothecosis. Over one-half the documented cases of protothecosis concern cutaneous or subcutaneous manifestation, which are often preceded by skin or wound infections (Thiele and Bergmann, 2002). The incubation time for protothecosis is not generally known, but in situations where documented trauma is believed to be the cause (the algae penetrate the skin following posttraumatic damage) the incubation period has been approximately 2 weeks. The lesions are slow to develop and do not usually resolve spontaneously. They can be eczematoid or ulcerative (Krcmery, 2000), are present mainly in exposed areas, such as the extremities and the face; they generally remain localized, though patients with cellular immunodeficiency show a trend toward dissemination (Iacoviello et al., 1992). Infection of the bursa subcutanea olecrani are generally preceded by injuries or grazing of the elbow; signs and symptoms appear gradually several weeks following the trauma and include mild induration of the bursa accompanied by swelling, tenderness, erythema and production of serosanguineous fluid (Lass-Fllorl and Mayr, 2007).

Only a few cases of systemic disease have been reported. Most infections are likely due to traumatic implantation of organisms, but a few cases of opportunistic infection have also been reported. Arthropod bites were thought to facilitate transmission of this organism in at least one case (Wirth et al., 1999). Disseminated protothecosis occurs in immuno-compromised individuals whose resistance has been weakened by long period of treatment with glucocorticoid steroids (e.g. after transplant or chemotherapy), or suffering from diseases such as diabetes, systemic lupus erythematosus, malignancy, or renal failure. A few cases of protothecosis in patients with AIDS have been described, but HIV disease is not a primary predisposing form of immunosuppression (Laeng et al., 1994). The organs most commonly affected in dissemination are the skin, subcutaneous tissue, gut, peritoneum, blood.

Protothecosis as also been diagnoses among other very different species such as dogs, cats, sheep, deer, Atlantic salmon, carp, and flying foxes, (Thiele and Bergman, 2002).

Despite its non-photosynthetic, obligate heterotrophic nature, *Prototheca* is known to have retained a plastid with starch granules; recent data indicate that several metabolic pathways (e.g. carbohydrate, amino acid, lipid, and isoprenoid) are located in this non-photosynthetic plastid. The reconstructtion of this complex metabolic network could represent a new approach in the treatment of protothecosis (Borza et al., 2005).

4. Dimensions

Growth of algae is possible over quite wide ranges of forms, sizes and relative proportion of the parts. Among giant algae, giant kelp *Macrocystis pyrifera* (Pheophyta) is a marine alga found along the Pacific coast of North America from central California to Baja California, (North, 1971). It has one of the highest growth rates of all macroscopic photoautotrophs (30–60 cm d^{-1}; Gerard, 1982) and can grow up to 60 m long. This alga forms aggregations known as kelp forests and the fronds form a dense canopy at the surface. Therefore, light attenuation is high inside the kelp forest. The irradiance at 20 m depth could be <1% of the incident light at the surface (Dean, 1985). Therefore, photosynthetic tissue of a single organism is exposed to a large gradient of light quantity and quality, (Colombo-Pallotta et al., 2006).

The genus name *Macrocystis* means “large bladder” and it contains at least two recognized species: *Macrocystis pyrifera*, or giant bladder kelp, sometimes referred to as the sequoia of the sea; and *Macrocystis integrifolia* the small perennial kelp. In the Northern hemisphere it occurs only along the Pacific coasts of Canada, the United States and Baja California. Populations

of *Macrocystis* in the North Pacific extend from Alaska to localities of cool, up welled water in Baja California. The kelp beds along the Pacific coast are the most extensive and elaborate submarine forests in the world. The genus is best developed as the species *Macrocystis pyrifera* from the southern California Channel Islands to northwestern Baja California.

Macrocystis plays an important role in the marine environment by providing food and habitat for a wide range of marine invertebrates and fishes in southern California. Forests of giant kelp may support millions of individual organisms and more than 1,000 species of marine plants and animals, (Hepburn and Hurd, 2005). Kelp begins life as a microscopic spore that grows into a tiny male or female plant called a gametophyte. These plants produce eggs and sperm, which fertilize and grow to form the large plants (sporophytes), which in turn release many new spores to start the process over again. The minimum amount of time needed to complete the *Macrocystis* life cycle is believed to be 12 to 14 months although in the environment, grazing by animals and shading by other plants would affect this rate of development (Dayton, 1985).

The average kelp plant is capable of releasing trillions of spores a year. Few, if any, of the billions of spores produced by a single mature *Macrocystis* kelp plant ever make it to adult gametophytes due to burial by sand or mud (sedimentation), competition for limited space with other plant or animal species, the lack of light at the ocean floor due to absorption by the water or shading by kelp and other plant species, nutrient limitation, and the effects of animals which graze on the tiny plants. Only 1 in 100,000 young kelp plants need to mature to reestablish the kelp beds. As the fertilized eggs develop into microscopic sporophytes, they must avoid shading and overgrowth by other organisms; grazing by small echinoids, gastropods, microcrustaceans and the bat star (*Patiria*) as well as being buried and abraded by sediments, (Harrold and Reed, 1985).

Although giant kelp plants are perennial, the individual fronds only survive for about 6–9 months. Fronds of mature kelp plants become senescent and deteriorate about 6 months after they are produced. Mature fronds continually develop, then die and break away in a process known as sloughing, giving way to the new fronds shooting up from the holdfast. Although the individual fronds only survive for about 6 months, individual blades last only about 4 months, (Lobban and Harrison, 1994).

At the other extreme dimensions, there are eukaryotic algae with a diameter of <2–3 μm, the so-called "picoeukaryotes". Up to now the smallest of the small eukaryotic phytoplankton is considered *Ostreococcus tauri* (Chlorophyta) (Derelle et al., 2006). The size of this organism is about that of a typical bacterium. Its genome is equally remarkable for its small size and extreme compactness; it is also unexpectedly complex and provides a

fascinating glimpse into the genetic makeup and metabolic potential of the smallest known eukaryote at the base of the marine food chain (Archibald, 2006).

Ostreococcus tauri, together with its close relatives, has become the focus of concerted efforts to understand the global distribution and ecological significance of eukaryotic picoplankton. *Ostreococcus tauri* was first discovered in 1994 in France's Thau lagoon, a shallow offshoot of the Mediterranean Sea known for its oyster farming. Barely 1 μm in diameter and practically invisible under the light microscope, *O. tauri* was detected by flow cytometry and hailed as the "smallest eukaryotic organism" (Courties et al., 1994). Its ultrastructure proved to be shockingly simple: *O. tauri* cells lack flagella and a cell wall and contain one mitochondrion, one chloroplast, a single Golgi apparatus, and a nucleus containing a single nuclear pore. Molecular data (Guillou et al., 2004) indicate that *O. tauri* belongs to a class of green algae called Prasinophyceae, a lineage thought to be of key importance in elucidating the earliest events in the evolution of chlorophyll b-containing organisms. *Ostreococcus tauri* appears to be ubiquitous in coastal waters and in the open ocean, and its minimal cell structure and high growth rate have made it a promising model picoeukaryote.

The complete genome sequence weighs 12.56 Mbp and is composed of 20 linear chromosomes, making it among the smallest, although not the smallest, nuclear genome of a free-living eukaryote characterized thus far (that honor belongs to the 9.2-Mbp genome of the fungus *Ashbya gossypii* (Dietrich et al., 2004). Derelle et al. (2006) raise the possibility that chromosome 2 is a sex chromosome, however meiosis has never been observed in *O. tauri*, although the presence of a near-complete set of meiotic genes encoded in its genome suggests that sex is at least a possibility (Ramesh et al., 2005).

What does such a small genome reveal about the cell biology and metabolism of this tiny alga? The genome possesses complete or nearly complete gene sets for proteins involved in cell division, starch metabolism, and nitrogen assimilation, as well as a diverse set of transcription factors and proteins with putative kinase- and calcium-binding domains. As expected, a complete suite of enzymes essential for carbon fixation and the Calvin cycle are present, as is a complex gene family encoding prasinophyte-specific light-harvesting antenna proteins. Most unexpected is the presence of genes implicated in C4 photosynthesis. C4 carbon fixation is an improvement over the simpler and more ancient C3 carbon fixation strategy used by most algae and plants. C4 photosynthesis is thought to have evolved multiple times from C3 ancestors. Although timing is uncertain, it is currently thought to have first evolved 24–35 million years ago in relation to environmental pressures (e.g. declining atmospheric CO_2) (Giordano et al., 2005; Sage,

2004). Both C3 and C4 methods overcome the tendency of ribulose-1,5-biphosphate carboxylase-oxygenase (rubisco, the first enzyme in the Calvin cycle) to waste energy by using oxygen to break down carbon compounds to CO_2. C4 plants separate rubisco from atmospheric oxygen, fixing carbon in the mesophyll cells and using oxaloacetate and malate to ferry the fixed carbon to rubisco and the rest of the Calvin cycle enzymes isolated in the bundle-sheath cells. The intermediate compounds both contain four carbon atoms, hence the name C4. This process has evolved repeatedly in higher plants as an adaptation to environmental stress (e.g. drought and low CO_2 concentrations) and involves modifications to leaf structure and altered biochemistry (Sage, 2004). The existence of C4 photosynthesis in phytoplankton is controversial, but *O. tauri* appears to possess the right combination of enzymes in the right cellular locations to drive such a process, and genes putatively encoding all enzymes required for C4 photosynthesis were identified in its genome. Much experimentation will be required to determine whether C4 photosynthesis actually occurs in the tiny cells of *O. tauri*, but it is significant that its genome does not encode any obvious "carbon-concentrating mechanism" (CCM) genes comparable with those in *Chlamydomonas* or common to organisms that actively or passively enhance inorganic carbon influx.

Despite its energetic cost, if *O. tauri* is capable of C4 photosynthesis, it could constitute a critical ecological advantage in conditions of high cell density and low CO_2 levels typical of phytoplankton blooms, especially when competitors have lower CCM efficiencies or no CCM at all.

A very interesting algal feature as to relative proportions of the parts can be found in the algae belonging to the family Characeae and expecially in *Chara corallina,* (Yamamoto et al., 2006*)*. The main axis of this alga consists of regularly alternating discoidal nodal and long cylindrical internodal cells that elongate up to 20 cm with a diameter of 1 mm. These cells are so large that microelectrodes can be easily inserted for electrophysiological studies. These cells contain well over a thousand nuclei, which are produced by the replication of a single original nucleus by a process that does not involve the typical mitotic apparatus. This high number of nuclei is presumably required to balance the large increase in cell volume, which is mediated by development of a large internal vacuole. The cytoplasm nearest the central vacuole of these cells is an ideal site for visualizing cytoplasmic streaming resulting from microfibrillar activity (Northnagel and Webb, 1982). Such streaming is necessary to achieve mixing and long distance transport of cell constituent in long cells having large cytoplasmic volume, which cannot rely on simple diffusion for the transportation and distribution of essential molecules throughout the cell. Probably owing to this large size, cytoplasmic streaming in characean algal cells is very fast (about 70 μm s^{-1}

at 20°C). The cytoplasm flows in a direction nearly parallel to the long axis of the cylindrical internodal cell. It goes up along the inner surface of one hemicylinder to the upper node, turns and comes down along the other hemicylinder to the lower node, and then turns and goes up again. There exist areas between the two hemicylinders where the direction of flow reverses (neutral zones).

It is known that this cytoplasmic streaming is generated by the sliding movement of a myosin along the actin cables fixed on the surface of chloroplasts lining the cytosolic face of the cell membrane (Kamitsubo, 1966; Kachar and Reese, 1988; Yamamoto et al. 1994; Kashiyama et al., 2000). Myosin from *Chara corallina* is very fast, moving actin filaments at 40–50 μm/s in the in vitro motility assay. This sliding velocity is about 10 times faster than that of the fast skeletal muscle myosin (6 μm/s in the rabbit). *Chara* myosin has shown to possess unique kinetic features suited for this fast movement, namely, a dramatic acceleration of ADP release by actin and extremely fast ATP binding rate (Ito et al., 2003; Ito et al., 2007).

5. Bloom-linked Phenomena

In general, the growth rate of a population of algae would be proportional to the uptake rate of one limiting factor (i.e. factor available in the smallest quantity with respect to the requirement of the alga). When the limiting factor is a nutrient, nutrient-limited growth is usually modeled with a Monod (or Michaelis-Menten) equation:

$$\mu = \mu_{max}[LN]/([LN]+ K_m)$$

where μ is the specific growth rate of the population as a function of [LN]; [LN] is concentration of limiting nutrient; μ_{max} is maximum population growth rate (at "optimal" conditions) and K_m is the Monod coefficient, also called the half-saturation coefficient because it corresponds to the concentration at which μ is one-half of its maximum. When the concentration of limiting nutrient [LN] equals K_m, the population growth rate is $\mu_{max}/2$.

As [LN] increases, μ increases and so the number of cells (algal population) increases. Beyond a certain [LN], μ tends asymptotically to its maximum (μ_{max}), and the population tends to its maximum yield. If this concentration is not maintained, rapidly primary productivity returns to a level comparable to that prior to the nutrient enrichment. This productivity variation produces seasonal blooming (Barsanti and Gualtieri, 2006).

Normal becomes abnormal when there is a continuous over-stimulation of the system by excess supply of one or more limiting nutrients, which leads

to intense and prolonged algal blooms throughout the year. The continuous nutrient supply sustains a constant maximum algal growth rate, (Lapointe, 1997; Riegman et al., 1992). Therefore, instead of peaks of normal blooms, followed by periods when phytoplankton is less noticeable, continuous primary production occurs (See images of abnormal algal blooms that were recently reported in the news off Canada's west coast at http://www-sci.pac.dfo-mpo.gc.ca/osap/data/SearchTools/SearchSatellites_e.asp), (Kutser et al., 2006). In this process, the enhanced primary productivity triggers various physical, chemical and biological changes in autotroph and heterotroph communities, as well as changes in processes in and on the bottom sediments and changes in the level of oxygen supply to surface water and oxygen consumption in deep waters. Blooms can result in a series of undesirable effects. Excessive growth of planktonic algae increases the amount of organic matter settling to the bottom. This may be enhanced by changes in the species composition and functioning of the pelagic food web by stimulating the growth of small flagellates rather than larger diatoms, which leads to lower grazing by copepods and increased sedimentation. In areas with stratified water masses, the increase in oxygen consumption can lead to oxygen depletion and changes in community structure or death of the benthic fauna. Bottom dwelling fish may either die or escape. Harmful algal blooms may cause discoloration of the water, foam formation, death of benthic fauna and wild or caged fish, or shellfish poisoning of humans, (Sellmer et al., 2003; Smayda, 1990; Van Dolah, 2000; Carmichael, 2001; Heil et al., 2005).

Under bloom conditions, single cell level features such as the carbonate covering structures of Haptophyta (coccoliths) and the luminescent organelles of Dinophyta (scintillons) give rise to impressive phenomena that will be described in the following.

5.1. MILKY WATERS

Some marine organisms combine calcium with carbonate ions in the process of calcification to manufacture calcareous skeletal material. Calcium carbonate ($CaCO_3$) may either be in the form of calcite or aragonite, the latter being a more soluble form. After death, this skeletal material sinks and is either dissolved, in which case CO_2 is again released into the water, or it becomes buried in sediments. This bound CO_2 is thus removed from the carbon cycle.

The calcification process can be summarized by the following reaction:

$$Ca^{2+} + 2HCO^{-}_{3} \leftrightarrow CaCO_3 + CO_2 + H_2O$$

The amount of CO_2 taken up in the carbonate skeletons of marine organisms has been, over geological time, the largest mechanism for absorbing CO_2. At present, it is estimated that about $50*10^{15}$ tonnes of CO_2 occurs as limestone, $12*10^{15}$ tonnes in organic sediments, and $38*10^{12}$ tonnes as dissolved inorganic carbonate.

Among the marine organisms responsible for calcification, coccolitophores play a major role, especially *Emiliania huxleyi,* (Jordan and Chamberlain, 1997). When the blooms of this haptophyte appear over large expanses of the ocean (white water phenomenon), myriad effects on the water and on the atmosphere above can be observed. Although each cell is invisibly small, there can be as many as a thousand billion billion (10^{21}) of them in a large bloom, and the population as a whole has an enormous impact. *E. huxley* blooms are processed through the food web, with viruses, bacteria and zooplankton all contributing to their demise and decomposition. Some debris from the bloom survives to sink to the ocean floor, taking chemicals out of the water column. While they live and when they die, the phytoplankton cells leak chemicals into the water. A bloom can be thought of as a massive chemical factory, extracting dissolved carbon dioxide, nitrate, phosphate, etc. from the water, and at the same time injecting other chemicals such as oxygen, ammonia, DMS (dimethyl sulfide) and other dissolved organic compounds into the water, (Gabric et al., 2001; Lomans et al., 2002). At the same time, this chemical factory pumps large volumes of organic matter and calcium carbonate into the deep ocean and to the ocean floor, (Fasham, 2003; Sarmiento and Gruber, 2004). Some of this calcium carbonate eventually ends up as chalk or limestone marine sedimentary rocks, perhaps to cycle through the Earth's crust and to reappear millions of years later as mountains, hills and cliffs, (Anbar and Knoll, 2002). Coccolithophorids are primarily found at low abundance in tropical and subtropical seas, and at higher concentrations at high latitudes in midsummer, following diatom blooms, (Iida et al., 2002). Hence, export of inorganic carbon by diatoms in spring at high latitudes can be offset by an efflux of carbon to the atmosphere with the formation of coccolithophore blooms later in the year.

Coccolithophores influence regional and global temperature, since they can affect ocean albedo (i.e. a ratio of scattered to incident electromagnetic radiation power) and ocean heat retention, and causing a sort of greenhouse warming effect. Coccoliths do not absorb photons, but they are still optically important since they act like tiny reflecting surfaces, diffusely reflecting the photons (Fukushima et al., 2000).

A typical coccolith bloom (containing 100 mg m^{-3} of calcite carbon) can increase the ocean albedo from 7.5% to 9.7% . A global satellite study by Brown and Yoder (1994) detected an annual area of blooms of $1.4*10^6$ km^2;

if each bloom is assumed to persist for about a month, then this annual coverage will increase the global annual average planetary albedo by

$$(9.7–7.5)*(1/12)*(1.4/510) = 0.001\%$$

where $510*10^6$ km^2 is the surface area of the Earth.

This is a lower bound on the total impact, because sub-bloom concentration coccolith light scattering will have an impact, over much larger areas (estimated maximum albedo impact = 0.21%). A 0.001% albedo change corresponds to a 0.002 W m^{-2} reduction in incoming solar energy, whereas an albedo change of 0.21% causes a reduction of 0.35 W m^{-2}. These two numbers can be compared to the climate forcing due to anthro-pogenic addition of CO_2 since the 1700's, estimated to be about 2.5 W m^{-2}. Coccolith light scattering is therefore a factor of only secondary importance in the radiative budget of the Earth. However, this scattering causes more heat and light than usual to be pushed back into the atmosphere; it causes more of the remaining heat to be trapped near to the ocean surface, and only allows a much smaller fraction of the total heat to penetrate to deeper in the water. Because it is the near-surface water which exchanges heat with the atmosphere, all three of the effects just described conspire to mean that coccolithophore blooms may tend to make the overall water column dramatically cooler over an extended period, even though this may initially be masked by a warming of the surface skin of the ocean (the top few meters), (Tyrrell, 2002).

As above said, coccolithophores are unique in that they take up bicarbonate (HCO_3^-), with which to form the calcium carbonate of their coccoliths. There are three forms of dissolved carbon in seawater: CO_2, HCO_3^- and CO_3^-; and carbon can shift very easily from being in one of these dissolved forms to being in another. How much of the total carbon is in each form is determined mainly by the alkalinity and by the water temperature. When the seawater carbon system is perturbed by coccolithophore cells removing HCO_3^- to form coccoliths, this causes a re-arrangement of how much carbon is in each dissolved form, and this rearrangement takes place more or less instantaneously. The removal of 2 HCO_3^- molecules and the addition of one CO_2 molecule changes the alkalinity and this indirectly causes more of the dissolved carbon to be pushed into the CO_2 form. Although the total dissolved carbon is obviously reduced by removal of dissolved carbon (bicarbonate ions) into solid calcium carbonate, yet the total effect, paradoxically, is to produce more dissolved CO_2 in the water. In this way, coccolithophore blooms tend to exacerbate global warming (by causing increased atmospheric CO_2), rather than to ameliorate it, as is the case when dissolved CO_2 goes into new organic biomass (Chuck et al., 2005). However, additional properties

of coccoliths may make the situation yet more complicated. Coccolith calcite is rather dense (2.7 kg per liter compared to seawater density of 1.024 kg per liter), and the presence of coccoliths in zooplankton faecal pellets and 'marine snow' (the two main forms in which biogenic matter sinks to the deep ocean) causes them to sink more rapidly. Slow-sinking organic matter may also adhere to the surfaces of coccoliths, hitching a fast ride out of the surface waters. If organic matter sinks faster then there is less time for it to be attacked by bacteria and so more of the locked-in carbon will be able to escape from the surface waters, depleting the surface CO_2. This co-transport of organic matter with coccoliths has been suggested to offset the atmospheric CO_2 increase that would otherwise be caused, and make coccolithophore blooms act to oppose global warming, rather than to intensify it (Neil, 2001).

5.2. BIOLUMINESCENCE

The phenomenon of dinoflagellate bioluminescence has been observed by sailors from the days of the earliest voyages. That each fleck of the sparkling luminescence present in the sea was the flash from a single creature of minute size rather than the phosphorescence of a chemical substance was known before the end of the eighteenth century (Harvey, 1952, 1957). The light emitting properties of some of the dinoflagellates became apparent because these organisms were on occasion so very plentiful. One of the earliest to be recognized was *Noctiluca* (Baker, 1753; de Quatrefages, 1850) because of its large size and the great brightness of its luminescence, as well as its common occurrence along the shores of Europe. The "red tides" of *Gonyaulax polyedra* on the west coast of the United States and the extremely bright luminescence which accompanied them led to the identification of this dinoflagellate as luminescent (Kofoid, 1911). Moreover, these microalgae are considered responsible for luminous trails observed around moving ships, dolphins and breaking waves (Rohr et al., 1998, 2002).

Among the relative few species, the luminescence of which has already been well established, there are: Fragilidium heterolobum, Gonyaulax catenella, Gonyaulax hyaline, Gonyaulax polyedra, Gonyaulax sphaeroidea, Lingulodinium polyedrum, Noctiluca miliaris, Peridinium brochi, Peridinium conicum, Peridinium depressum, Peridinium pentagonum, Pyrodinium bahamense, Pyrocystis lunula, Pyrocystis noctiluca (Sweeney, 1963).

Three stimuli have been observed to trigger the phenomenon of bioluminescence:

- mechanical stimuli – when shear forces, such as those caused by the stirring of water from the wake of a boat, a swimming fish or a breaking wave, deform the cell membrane, the dinoflagellates responds in less than 20 ms with a short flash lasting approximately 100 msec. Deformation

of the cell surface by forces as small as 10^{-6} dyne can trigger dinoflagellate luminescence (Hickman et al., 1980; Widder and Case, 1981; Cussatlegras and Le Gal, 2004). The total mechanically stimulated bioluminescence has been measured in different species and varies from 10^8 photons $cell^{-1}$ in *Gonyaulax* sp. (Seliger et al., 1969) to $6*10^{10}$ photons $cell^{-1}$ in *Pyrocystis* sp. (Swift et al., 1985; Batchelder et al., 1992). The light emission is in the blue wavelengths, with the maximum centered around 473–478 nm (Swift et al., 1973; Hastings and Morin, 1991).

- chemical stimuli – reducing the pH of their external medium by adding acid can cause some dinoflagellates to glow continuously.
- temperature stimuli – some species of dinoflagellate, such as *G. polyhedra*, will be induced to glow if the temperature is lowered.

Whatever the stimulus, the mechanism by which dinoflagellates emit light, though not completely understood, is always the same, with both a electrical and a chemical component to the initiation of a flash. The electrical process consists in an action potential during which the inside of the vacuolar membrane becomes hyperpolarized (it has more negative voltage with respect to resting potential). This sets up the conditions for the chemical reaction. The actual chemical reaction by which light is produced involves a substrate called luciferin and an enzyme called luciferase, which are sequestered into out-pocketings of the vacuolar membrane called scintillons. The cellular localization and ultrastructural features of these subcellular organelles have been elucidated using immunocytochemical techniques (Nicolas et al., 1987a). These small (about 0.5 μm) spherical organelles, about 400 per cell, (Fogel and Hastings, 1972; Nicolas et al., 1987b; Fritz et al., 1990) have a specialized dense matrix and are topologically a part of the cytoplasmic compartment, since as above said, they occur as evaginations protruding into the cell vacuole. The action potential extrudes hydrogen ions into the scintillons and lower their pH from 8 to 6. Under these acidic conditions, luciferin is released from its binding protein and is thus activated. Luciferase catalyses the oxidation of luciferin, resulting in light and an intermediate called oxyluciferin. Energy in the form of ATP must be provided to the system to regenerate luciferin.

The most studied of the many bioluminescent dinoflagellates is *Gonyaulax polyedra*. Because its luciferin reacts with the luciferases of all dinoflagellates tested so far, it is likely to be representative of the group at large. The structure of dinoflagellate luciferin, determined from *Pyrocystis lunula*, shows no similarity to any other luciferin (Nakamura et al., 1989). It is a linear tetrapyrrole probably derived from chlorophyll and very sensitive to autoxidation. The site of oxidation on the chromophore depends on whether

the reaction is luciferase-catalyzed, and luminescence accompanies only the enzymatic reaction. The reaction product is not fluorescent, in contrast to unoxidized luciferin, which fluoresces brightly with a spectrum matching that of the bioluminescence (λ_{max} about 470 nm) (Hastings, 1978; Wilson and Hasting, 1998). This paradox is not yet resolved. One possibility is that the bioluminescence is emitted by an excited transient intermediate, as in the bacterial reaction. Another is that an excited state formed in the reaction transfers its energy to still unreacted luciferin. However, studies indicate that only one luciferin molecule is required for light emission and the bioluminescence intensity in the in vitro reaction decays monoexponentially. Two proteins are involved in *Gonyaulax* bioluminescence. One is a luciferin binding protein (LBP), a dimer of two identical 75.5 kDa subunits, which sequesters luciferin at a physiological pH, protecting it from autoxidation, and releases it as the pH drops to 6 (Morse et al., 1989). The other, a luciferase (LCF) (137 kDa), is inactive at pH 8 and becomes active exactly in the pH range at which the LBP makes luciferin available for the reaction. The two proteins and luciferin are tightly packaged inside the scintillons (Nicolas et al., 1991, Desjardins and Morse, 1993).

Bioluminescence is an expression of circadian rhythmicity, a phenomenon regulated on a daily cycle. In the absence of light, dinoflagellates exhibit peaks and valleys of bioluminescence. However, the biological clock can be 'entrained' by light exposure, shifting the peaks of luminescence to different times of day. Circadian control of cellular processes represents an adaptive advantage for dinoflagellates because they are vertical migrators in the water column. By keeping time, they can anticipate sunrise and be poised to start photosynthesizing at the surface as soon as light is available.

Luminescence in *G. polyedra* and other dinoflagellates is regulated by an endogenous circadian clock, and is maximum during the dark (night) phase with light emission that can be 100 times brighter at night than during the day, (Johnson and Hastings, 1986). Remarkably, both luciferase and luciferin-binding protein in *G. polyedra* are destroyed at the end of the night phase and then synthesized again in the next cycle. Moreover, the scintillons themselves are broken down and reformed each day (Fritz et al., 1990; Suk Seo and Fritz, 2000); the circadian cycle may actually be viewed as a daily differentiation of certain cellular processes. Synthesis and destruction is not the only mode of regulation, even in dinoflagellates. For example, in *Pyrocystis*, the amount of luciferase remains constant over the cycle (Knaust et al., 1998), but its cellular location and responsiveness change from night to day (Widder and Case, 1982).

Bioluminescence represents a community beneficial gain (Burkenroad, 1943; Esaias and Curl, 1972; Abrahams and Townsend, 1993). In this way, the "burglar alarm" hypothesis states that dinoflagellates which sense a

predator emit a flash to alarm a secondary predator that will spot and eat the first predator. In this way, dinoflagellate bioluminescence increases predation on crustacean grazers by fishes, hence reducing the grazing pressure on dinoflagellates.

6. Conclusions

In this review are described some oddities and curiosities of the algal world; however, algae should not be considered only as curious creatures with marginal roles in specific niches of the environment, since they do possess specific and important roles in global ecology and in the dynamic equilibrium of the biosphere. They have played key roles in shaping Earth's biogeochemistry and contemporary human economy, and these roles are becoming ever more significant as human impacts on ecosystems results in massive alteration of biogeochemical cycling of chemical elements. Just think about the Earth's initial atmosphere, 80% N_2, 10% CO/CO_2, 10% H_2, (by volume): no free O_2 appeared until the development of oxygenic photosynthesis by cyanobacteria, transforming the atmosphere composition in the actual 78% N_2, 21% O_2, 0.036% CO_2 and other minor gases. Or the petroleum and natural gas we consume as fuels, plastics, dyes, etc… in our everyday life; these fossilized hydrocarbons are mostly formed by the deposition of organic matter consisting of the remains of several freshwater marine microalgae. These remains contain bacterially- and chemically resistant, high aliphatic biopolymers (algaenans) and long-chain hydrocarbons that are selectively preserved upon sedimentation and diagenesis and make significant contribution to kerogens, a source of petroleum under appropriate geochemical condition. Moreover, we are still using the remains of calcareous microorganisms, deposited over millions of years in ancient ocean basins, for building material. Diatomaceous oozes are mined as addictives for reflective paints, polishing materials, abrasives and for insulation. The fossil organic carbon, skeletal remains, and oxygen are the cumulative remains of algae export production that has occurred uninterrupted for over 3 billions years in the upper ocean.

Algae have been utilized by man for hundreds of years as food for humans, fodder, remedies and fertilizers. Ancient records show that people collected macroalgae for food as long as 500 BC in China and one thousand of years later in Europe, whereas microalgae such as *Arthrospira* have a history of human consumption in Mexico and Africa. In the fourteenth century the Atzecs harvested *Arthrospira* from Lake Texcoco and used to make a sort of dry cake called *tecuitlatl*, and very likely the use of this cyanobacterium as food in Chad dates back to the same period, or even earlier, to the Kanem Empire (Ninth century).

Nowadays, algae are commercially exploited for the extraction of hydrocolloids, as food for humans and animals, fertilizers, cosmetics, nutraceutical and farmaceutical. Moreover, as million years ago, they are still important for the biogeochemical cycling of the chemical elements they uptake, assimilate, and produce such as Carbon, Oxygen, Nitrogen, Phosphorus, Silicon and Sulfur. Surging oil prices and shortage of biofuel feed stocks are reviving interest in making fuel from algae that could serve as both a viable energy source and a carbon sink. They are able to produce high level of hydrocarbons, offering orders of magnitude greater resource potential for natural oils than any terrestrial crop. In addition, algae consume carbon dioxide as they grow, so they could be used to capture CO_2 from power stations and other industrial plants that would otherwise go into the atomsphere (Haag, 2007).

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