

## Chapter 2

# Biological Toxins from Marine and Freshwater Microalgae

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**Abstract** In the last decades the increased occurrence of intoxications caused by biological toxins produced from marine and freshwater microalgae has underlined their relevance as emerging risks for food safety. Biological toxins from algae (i.e. saxitoxin, brevetoxin, okadaic acid, domoic acid) are recognised as a major threat for human and animal health, especially where Harmful Algal Blooms phenomena develop. Many of these toxins are responsible for severe illness or death, mostly related to consumption of seafood contaminated by toxic algae. The present book summarises current knowledge and perspectives for future research on marine and freshwater algal toxins. Specific topics are: overview of the different species producing toxins, their survival strategies in the environment; typologies of toxins, their chemical structure and mechanisms of actions; methods currently in use for their monitoring; emerging issues and future outlooks for their control. The importance of biotoxin monitoring in the framework of the European Marine Strategy Framework Directive is also discussed.

**Keywords** Brevetoxin • Ciguatoxin • Cylindrospermopsin • Domoic acid • Harmful algal bloom • Microcystin • Okadaic acid • Poisoning • Saxitoxin • Yessotoxin

### Abbreviations

ASP	Amnesic Shellfish Poisoning
AOAC	Association of Official Analytical Chemists
AZA	Azaspiracid
AZP	Azaspiracid Shellfish Poisoning
BMAA	$\beta$ -Methylamino-L-Alanine
PbTx	Brevetoxin
CYN	Cylindrospermopsin
BIOTOX	Development of Cost-Effective Tools for Risk Management and Traceability Systems for Marine Biotoxins in Seafood
DSP	Diarrhetic Shellfish Poisoning
DST	Diarrhetic Shellfish Toxin
DiCANN	Dinoflagellate Categorisation by Artificial Neural Network
DTX	Dinophysistoxin

DA	Domoic Acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EFSA	European Food Safety Authority
EU	European Union
GEOHAB	Global Ecology and Oceanography of Harmful Algal Blooms
GES	Good Environmental Status
HAB	Harmful Algal Bloom
HPLC	High-Performance Liquid Chromatography
LD <sub>50</sub>	Median Lethal Dose (50 % of the population)
LOQ	Limit of Quantification
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
MSFD	Marine Strategy Framework Directive
MALDI-TOF	Matrix-assisted Laser Desorption/Ionisation Time-of-Flight
MCY	Microcystin
MCY-RR	Microcystin-RR
MW	Molecular Weight
NSP	Neurotoxic Shellfish Poisoning
N	Nitrogen
OA	Okadaic Acid
PLTX	Palitoxin
PSP	Paralytic Shellfish Poisoning
PTX	Pectenotoxin
P	Phosphorus
PCR	Polymerase Chain Reaction
PSU	Practical Salinity Unit
Q-TOF	Quadrupole-Time-of-Flight
STX	Saxitoxin
SPX	Spirolides
USA	United States of America
YTX	Yessotoxin

## 2.1 Toxin-Producing Microorganisms

### 2.1.1 *Generalities on Phytoplankton and Toxic Species: Spatial and Temporal Distribution—Environmental Drivers*

Phytoplankton comprises unicellular or colonial, microscopic organisms (microalgae) that inhabit many aquatic ecosystems. Algae are autotrophic organisms, which are able through the photosynthetic process to convert carbon dioxide and water

into sugars for the cell metabolism, using the energy from sunlight. They are subdivided into 10 groups (Bold and Wynne 1985): *Cyanophyta*, *Prochlorophyta*, *Chlorophyta*, *Charophyta*, *Euglenophyta*, *Phaeophyta*, *Chrysophyta*, *Pyrrophyta*, *Rhodophyta*, *Cryptophyta* and another group including other species.

Within the planktonic food web, phytoplankton occupies the first trophic level with a key role as primary producer of organic matter. The phytoplankton community inhabiting aquatic environments undergoes seasonal changes characterised by the succession of different unicellular or colonial taxa (Bruno 2000).

The temporal variability in the composition of the phytoplankton community is associated with spatial variability. Along the water column, phytoplankton species are differently distributed depending on the different tolerance of their photosynthetic pigments to light wavelength spectra and on the ability to move towards zones more enriched in nutrients. *Cyanophyceae* are more frequent on surface layers as their coloured pigments protect chlorophyll from ultraviolet (UV) denaturation, while Dinoflagellates inhabit shallow, weakly lighted, environments. At intermediate depths, *Chlorophyceae* are dominant with the exception of lakes in spring and autumn, and spring waters where *Crysophyceae* are prevalent. Close to the thermocline layer, Diatoms predominate.

Of the approximately 5,000 species of identified microalgae, about 300 are able to develop under massive growth, producing 'red-water' phenomena, whose occurrence was reported thousands of years ago. In some cases, the proliferation of planktonic algae (the so-called 'algal bloom') is a real benefit for aquaculture. Many species can create extended marine algal blooms named 'red tides': sometimes, they do not constitute a hazard to human health. Red tides are produced when layers of deep water, rich in nutrients, overlap layers of warmer surface water, due to solar heating or due to surface freshwater supplies. In eutrophic environments, algal blooms involve one or two phytoplanktonic species, which represent 80–90 % of the total biomass. In oligotrophic environments, seasonal blooms also occur but they are never mono-specific. These phenomena occur when warmer surface water overlaps deeper water rich in nutrients; under these conditions, rapidly growing algae consume nutrients from the surface waters, leaving those present under the pycnocline. Motile algae arrive to this layer, where they produce blooms that move towards the surface during the day to capture light and heat (Bruno 2000). Algal species quickly exhaust the nutrients of the surface layers with a rapid growth, leaving those in the colder layer below the pycnocline.

Some algae species such as Dinoflagellates, able to migrate vertically even with higher speeds of 10 m/day, can reach this layer where they find optimal conditions of temperature and nutrients for their growth. Those algal species that can compete successfully for available growth-limiting nutrients have the potential to become dominant and produce blooms (Granéli et al. 2008). In some cases, however, algal blooms are recognised dangerous agents because of the ability of modifying the visual appearance of water. In addition, they can be considered foam-producing organisms and able to cause toxic effects, with possibility of death, on the human population and fish (ICES 1984). For these reasons, these life forms are considered Harmful Algal Blooms (HAB). An approximate number of 75 species, mostly

represented by dinoflagellates and diatoms, inhabit both marine and freshwater ecosystems. They are recognised as toxic substance producers since they produce biotoxins (phycotoxins) that include the most powerful non-protein toxins known to date. In fact, the blooms of toxic microalgae occurring on the coasts have been responsible for die-offs of wild animals, livestock and pets. The consumption of shellfish, fish or water contaminated by algal toxins has been associated with very serious cases of poisoning in humans and negative effects on aquatic environments.

According to the produced effects, the species involved in outbreaks of toxic algal blooms can be distinguished in three main groups (Bruno 2000):

- 1st group. Species that cause water colouration only, resulting in a decrease of the water transparency, and which may exceptionally grow causing some episodes of fish and invertebrate mortality, related to oxygen consumption during their decomposition. Species of dinoflagellates and diatoms belong to this group
- 2nd group. Species which produce powerful toxins that accumulate along the trophic web and can cause effects in upper consumers (animals and humans); dinoflagellates belonging to *Alexandrium*, *Gymnodinium*, *Dinophysis*, *Prorocentrum* and diatoms belonging to the genus *Pseudo-nitzschia* are included in this group
- 3rd group. Species that are not toxic to humans but are noxious to fish and invertebrates (i.e. *Gyrodinium aureolum*, *Chaetoceros convolutus*, *Nodularia spumigena*, *Chattonella* spp). In addition, some toxic species spread their toxins through the production of aerosols reaching the coasts (i.e. *Gymnodinium breve* and *Ostreopsis* spp).

Phytoplankton life forms can cause many problems in Europe and worldwide (Anderson 1989). This bloom results in severe economic and sanitary consequences when waters are used for recreational and productive purposes (tourism, fisheries and aquaculture). Algal toxins have negative impacts on the health of marine organisms, such as fish, shellfish and crustaceans; moreover, they are concentrated in seafood products through water filtration mechanisms and become dangerous to human health when contaminated seafood are consumed. Negative impacts and correlated mechanisms are extremely different. Many of these impacts are characterised by chemical–ecological interactions mediated by secondary metabolites of various bioactivity, as shown by their diverse structural classification and the range of receptors and metabolic processes affected (Cembella 2003). Many biologically-active molecules, including dangerous toxins for animal life, can be chosen as good indicators (Codd 2000). Toxic microalgae are common only among the dinoflagellates, diatoms and cyanobacteria (Katircioğlu et al. 2004).

In marine environments, the division of *Pirrhophyta* includes the greatest number of algal species currently known as producers of toxins or harmful substances: two classes of *Dinophyceae* and *Desmophyceae* are considered. The toxic species such as *Dinophysis*, *Gymnodinium*, *Peridinium* and *Gonyaulax* belong to the class of *Dinophyceae*, while *Prorocentrum* belongs to the class of *Desmophyceae*. Many toxins are produced by dinoflagellates, but also some diatoms are also toxic.

In freshwater environments, toxic algal species mostly belong to *Cyanophyceae*. They are a well-defined group of prokaryotic organisms, concerning about 150 genera and over 2000 species (van den Hoek et al. 1995). These algae have a photosynthetic apparatus similar in structure and function to that of chloroplasts of eukaryotes, thanks to the presence of chlorophyll *a* responsible for oxygenic photosynthesis. *Cyanophyceae* show a great diversity in morphology, structure and functions, and phenotypes; these species are represented by complex populations (ecotypes) which express particular genotypes. The toxic species are about 40; the ability to produce toxins has an important taxonomic significance (Skulberg and Skulberg 1985).

*Cyanophyceae* are ubiquitous organisms present in aquatic environments with wide salinity ranges and temperature up to 73–74 °C, in soil, in rocks; some genera are able to fix atmospheric nitrogen through heterocysts and members of symbiotic relationships. Many filamentous and unicellular species are motile, through mucilage or filaments. Cytoplasm shows some gas vacuoles that regulate their floating over water surface. There are three orders: *Chroococcales*, *Chamaesiphonales*, *Oscillatoriales*; the first and the third include some toxin-producing species.

Many species belonging to Cyanobacteria are responsible (O’Neil et al. 2012) for HAB in freshwater, estuarine and marine environments. The incidence of cyanobacterial blooms has been observed with high results in different aqueous environments (Carmichael 2008; Paerl 2008; Paerl and Huisman 2008; Paul 2008). Some new cyanobacterial toxins, such as  $\beta$ -methylamino-L-alanine (BMAA), have been isolated, as well as new genera of toxin-producing cyanobacteria (Brand 2009; Cox et al. 2005, 2009; Kerbrat et al. 2011).

## 2.1.2 Major Toxic Algal Species: An Overview

### 2.1.2.1 Diatoms—*Pseudo-nitzschia* spp

*Pseudo-nitzschia* was observed with other diatom species in certain Italian and Spanish areas (Quijano-Scheggia et al. 2005; Totti et al. 2000). The production of toxin (domoic acid) was found in different Mediterranean areas (Azmil et al. 2001; Kaniou-Grigoriadou et al. 2005), possibly in association with two *Pseudo-nitzschia* microorganisms (Cerino et al. 2005; Orsini et al. 2002).

The abundance and distribution of toxic *Pseudo-nitzschia* species (particularly *P. calliantha* and *P. delicatissima*, two potential ‘Amnesic Shellfish Poisoning’ toxin producers) was studied in Italian waters (Caroppo et al. 2005). *P. calliantha* showed a stronger seasonal distribution and was correlated with winter water conditions than *P. delicatissima*, which in turn exhibited a broader temporal distribution and appeared independent from major environmental constraints. *Pseudo-nitzschia* spp have been detected in diverse environments such as high-nitrate and low-chlorophyll regions (open ocean), but also in fjords, gulfs and bays; the same thing may be observed when speaking of produced toxins because of the well known stability.

### 2.1.2.2 Dinoflagellates

#### *Alexandrium* spp

The genus *Alexandrium*, including the species *A. minutum*, *A. catenella*, *A. tamarense* and *A. taylori*, is recognised to be responsible for different toxic episodes in many Mediterranean areas (Giacobbe et al. 2007; Penna et al. 2005; Vila et al. 2001, 2005).

Basically, *Alexandrium* is well known because of repeated observations in many different ecosystems (Anderson et al. 2012a). In addition, the specificity of *Alexandrium* species is correlated with the production of three different toxin groups, in spite of the multiplicity of nutritional exigencies. By the hygienic viewpoint, *Alexandrium* blooms have been considered as one of the most important topics when speaking of HAB-related toxin episodes. As a result, a notable amount of scientific literature is available at present with relation to different aspects, including also effects on the environment.

#### *Dinophysis* spp

Being a cosmopolitan genus, *Dinophysis* spp has been considered as one of the main problems for shellfish aquaculture in many European Countries and other regions because of the production of okadaic acid and pectenotoxins, powerful lipophilic toxins. The first report on *Dinophysis* species concerned (Caroppo et al. 2001) the composition and spatio-temporal distribution in the oligotrophic waters of the southern Adriatic coasts (Apulia, Italy).

*Dinophysis sacculus*, *D. fortii*, *D. caudata*, *Phalacroma rotundatum* and *P. mitra* are potentially producers of diarrhetic shellfish poisoning; these dinoflagellates were associated with mixing conditions, low water temperatures and high nutrient inputs; significant correlations of these *Dinophysis* species with chlorophyll *a* were found (Caroppo et al. 2001). These and other important results have been obtained recently (Reguera et al. 2012) in spite of known culture difficulties.

#### *Pyrodinium bahamense*

*Pyrodinium bahamense*, belonging to the family *Gonyaulacaceae*, is a tropical euryhaline dinoflagellate found mainly in the Atlantic ocean, particularly in marine waters that have more than 20 Practical Salinity Units (PSU) of salinity—the amount of dissolved salts in water—and are warmer than 20 °C. The optimal salinity is considered to be around 35 PSU. The cultivation of *P. bahamense* is explained by its specific nutrition needs. It shows optimal growth and chlorophyll levels when nitrogen levels in its environment are greater than 100 µM. Nitrogen is considered an important factor for the synthesis of toxins in *P. bahamense*.

This organism displays bioluminescence when agitated, glowing red due to its pigments. *Pyrodinium*—a mono-specific species with two varieties—was first discovered in 1906 in waters around New Providence Island in the Bahamas. These organisms are a major cause of seafood toxicity and cause of paralytic shellfish poisoning, especially in Southeast Asia and in Central America.

### *Karenia brevis*

*Karenia brevis*, a microscopic and unicellular marine dinoflagellate common in the Gulf of Mexico, is considered responsible for red tides in Florida and Texas. This life form is correlated with the detection of brevetoxins, powerful compounds that can cause gastrointestinal and neurological problems in other organisms and are responsible for large die-offs of marine organisms and seabirds. *K. brevis* is unarmored and does not contain *peridinin*. The region around southwest Florida is one of the major hotspots for red tide blooms where *K. brevis* grow to very high concentrations and the water can take on a reddish or pinkish colouration.

### *Gambierdiscus toxicus*

Taxonomy, geography, ecophysiology and toxicology of *Gambierdiscus* have been recently reviewed (Parsons et al. 2012). This life form is normally correlated with the production of certain gambiertoxins. *Gambierdiscus toxicus* was originally described from the French Polynesia, while *G. beliseanus* is described from coastal waters of Belize. Three additional *Gambierdiscus* species have been isolated from French Polynesia: *G. polynesiensis*, *G. pacificus* and *G. australes*.

The global distribution of 10 *Gambierdiscus* species has been documented recently (Litaker et al. 2010), with most species found in the Atlantic being distinct from those in the Pacific region. New species of *Gambierdiscus* have been detected in European Atlantic waters and in the Mediterranean Sea.

*Gambierdiscus* is likely to grow in shallow water habitats (<50 m) where annual temperatures range between 21 and 31 °C (optimum between 25 and 29 °C), with high, stable salinities, light levels 10 % of incident light and adequate substrate (algae, biofilms). *Gambierdiscus* prefers low light intensities. Many researchers found a positive correlation between *Gambierdiscus* spp cell abundance and water temperature. Toxin production may also be affected by increasing temperatures. On the other hand, *Gambierdiscus* cells appear to grow poorly in low salinity waters preferring a salinity range of 28–35 °C.

### Prymnesiophytes—*Prymnesium*

*Prymnesium* organisms have been studied because of two main features at least (Granéli et al. 2012):

- (a) These life forms, in particular *P. parvum*, are able to create remarkable blooms in many water areas
- (b) The presence of *Prymnesium* organisms can influence the survival and the reproductive cycle of other life forms such as other algae, fish and plankton because of the production of peculiar haemolytic toxins
- (c) Finally, life forms such as *P. parvum* are reported to use water-soluble organic compounds when available. This possibility gives these microorganisms higher survival chances.

#### Dinoflagellates—*Coolia monotis*

*Coolia monotis* is a benthic Dinoflagellate belonging to the family *Ostreopidaceae*. It is a euryhaline species, growing at a salinity range of 20–50 ‰ and surviving in a range of 15–60 ‰, with mean values of around 35 ‰ (Aubert and Aubert 1986). Optimal temperatures for growth is 25 °C and irradiance values are 30–100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ ; in these conditions, the duplication time of *Coolia* is 3–4 days. *Coolia* produces aggregates due to the release of mucous substances; these compounds could have antifungal properties (Pearce et al. 2000). This species is of particular interest since, although it is a cosmopolite organism (Bruno et al. 1997), it has been recently included within the five genera of benthic dinoflagellates which are involved in *ciguatera* episodes in tropical and sub-tropical areas. Although no direct relationships of *Coolia monotis* and toxic phenomena have not been fully demonstrated, some studies have shown the production of a toxin, derived from yessotoxin, called cooliatoxin (Holmes et al. 1995).

This species is reported to be toxic on *Dunaliella salina* (Donner et al. 2000). It may cause effects similar to those observed with maitotoxin; the toxic effect, however, is pH- and temperature-dependent; the pH should be between 8 and 9. In addition the organism is inhibited by a temperature of 40 °C.

#### Dinoflagellates—*Ostreopsis* spp

The genus *Ostreopsis* consists of benthic/epiphytic dinoflagellates living in tropical and sub-tropical waters. Salinity is reported to have an optimum of 32 ‰ and pH between 8 and 8.15. Nine species of *Ostreopsis*—*O. siamenses*, *lenticularis*, *heptagona*, *mascarenensis*, *ovata*, *labens*, *marinus*, *beliseanus* and *caribbeanus*—at least have been reviewed for their morphometric characteristics and habitats (Faust 1999; Faust et al. 1996).

*Ostreopsis* spp have been the object of several studies during the last decades due to its relationships with *Ciguatera* (Holmes et al. 1995). Although the dinoflagellate *Gambierdiscus toxicus* is the only species recognised to be responsible for this phenomenon, some studies performed along the Puerto Rico coast have shown the production of toxic compounds from some dinoflagellates, mostly *O. lenticularis* (Tosteson et al. 1998).



The production palytoxin has been reported in the Mediterranean Sea by *O. ovata* which usually lives on the surface of red and brown macroalgae (Ciminiello et al. 2009). Blooms of this alga have sometimes been associated with mortalities of benthic organisms and respiratory problems in swimmers or people who were close to the affected area. It is not known if the toxins produced by *O. ovata* or other organisms associated to it may accumulate along the food chain, and whether its accumulation can result in a significant health risk.

Since 1998, summer algal blooms of *O. ovata* were recognised to be an emerging problem in the Apuan benthic seawaters (Tuscany, Italy), inducing heavy consequences on benthic communities—like molluscs, coelenterates and echinoderms—and evident alterations of water quality (Sansoni et al. 2003). Other health problems—respiratory illness and fever in about 200 hospitalised people following inhalation of marine aerosols—were reported in northern Italy. As the exposure to produced toxins—palytoxins (PLTX) compounds (Botana et al. 2013)—occurs through respiration, the banning of coastal areas to recreational activities is not a good tool to reduce toxic risks.

The importance of *Ostreopsis* spp is strictly correlated with the geographical distribution. Originally, these life forms were reported in the Mediterranean Sea and in selected areas only (Durando et al. 2007). However, climatic modifications—thermal values above all—and remarkable salinity values have been considered the main reasons for the broadened spreading of *Ostreopsis* spp in other extra-European areas. The diffusion of PLTX is the natural consequence.

Significant abundances of *O. ovata* bloom have been reported (Accoroni et al. 2011, 2012) in the northern Adriatic Sea in September ( $1.3 \times 10^6$  cells/g corresponding to  $63.8 \times 10^3$  cells/cm<sup>2</sup>) and on hard substrata (rocks) than on seaweeds. Hydrodynamism played a major role in *Ostreopsis* blooms, as higher abundances were observed in sheltered sites compared with exposed ones (Totti et al. 2010). Temperature and nutrients did not seem to cause an important effect on *O. ovata* blooms, as this species peaked when temperature values were decreasing. High levels of toxins were recorded in natural samples by high resolution liquid chromatography-mass spectrometry. High total toxin contents were demonstrated (up to 75 pg/cell) including putative palytoxin and ovatoxins; episodes of death of both benthic invertebrates (limpets, sea urchins and mussels) and macroalgae were commonly observed during algal blooms.

### *Gymnodinium sanguineum*

*G. sanguineum* is a toxic Dinoflagellate which may give red tides, causing sometimes a decrease in the abundance of herbivorous zooplankton and of its filtration rate (Fiedler 1982).

*Glenodinium cf. foliaceum*

*G. foliaceum* is a toxic Dinoflagellate species known since 1960. It caused frequent intoxications following the ingestion of the mollusc *Cardium edule* in the lagoon of *Obidos* (Portugal), where a bloom of this dinoflagellate produced red-brown tides. The same species was also found in high numbers in British brackish waters, including also other areas such as: the Baltic Sea, United States of America (USA) and the Mediterranean Sea (Dodge 1982). It can be distinguished from *Protoperidinium* spp due to its preference for brackish habitats and the presence of green-brown chromatophores inside the cell.

*Prorocentrum minimum*

*P. minimum* is a tectate dinoflagellate sometimes associated with fish mortalities (Rabbani et al. 1990); the related toxicity has been demonstrated for *P. minimum* var. *maria lebouriae* (Okaichi and Imatomi 1979).

This microalgal species is common in many coastal and estuarine areas worldwide (Hajdu et al. 2005). The origin of *Baltic P. minimum* is unclear; it could have been transported by ballast water or spread successively by currents from the Skagerrak into the brackish Baltic Sea.

Since the early 1980s, blooms of *P. minimum* have been reported from many eutrophic coastal areas of the Baltic Sea. A laboratory experiment (Hajdu et al. 2000) revealed that the species had optimum growth at 15 PSU, but it could also grow well at salinity below 5 PSU. The growth rate ranged from 0.13 to 0.6 m per day below 10 PSU. Another species belonging to the genus *Prorocentrum* is *P. lima*, a cosmopolitan species that is toxic since related to diarrhoeic shellfish poisoning (DSP) phenomena (Sechet et al. 1998); it is epiphytic-benthonic and is frequently associated with macroalgae.

*Cyanobacteria**Cyanobacteria*—*Microcystis*

*Microcystis* is reported to be a powerful bloom-producing microorganism with a worldwide distribution in freshwaters, with the exception of some inhospitable areas (Fristachi et al. 2008). Very frequent in China lakes (*Taihu*), it is present as aggregated single-cell coccoid genera (O'Neil et al. 2012; Paerl and Otten 2013).

The importance of these life forms is strictly correlated (Fristachi et al. 2008) with:

- (a) Survival and growth rates when certain environmental conditions are assures. In detail, *Microcystis* can survive well in warm waters, with abundance of bioavailable substances and the concomitant presence of carbon dioxide. This substance is abundant in warm ecosystems
- (b) The diversification of toxins. These organisms can synthesise a group of powerful molecules, including microcystins (MCY), anatoxin-a and BMAA. Actually, certain *Microcystis* cells are not able to synthesise MCY.

#### *Cyanobacteria*—*Planktothrix rubescens*

Formerly known as *Oscillatoria rubescens*, *P. rubescens* is a filamentous cyanobacterium. In lakes, the presence of these algae is constant throughout the year; in summer, however, this species—due to its photosynthetic pigments and its needs of low temperature—is distributed preferentially into the deepest part of the lake, while in winter—under conditions of low temperature and light radiation- moves to the surface resulting in conditions favourable for toxic blooms. The related growth depends mostly on the availability of nitrogen compounds and to a lesser extent of phosphate, water temperature and light. Usually, this alga is transported from already contaminated sites by water birds that transport it as spores. It is able to produce several toxins called microcystins which yield hepatotoxic, carcinogenic and gastrointestinal effects.

#### *Cyanobacteria*—*Anabaena*

*Anabaena* is a filamentous, heterocystous *Cyanobacteria* genus. Generally, this type of organism is considered ubiquitous (freshwater environments) and able to grow with low nitrogen and carbon dioxide sources (O'Neil et al. 2012). The importance of *Anabaena* is correlated with the production of MCY, anatoxins, cylindrospermopsin (CYN) and a saxitoxin (STX) where possible.

#### *Cyanobacteria*—*Cylindrospermopsis*

*Cylindrospermopsis* is a solitary, filamentous diazotroph cyanobacterium, which in the last decade has expanded its geographical range across every continent, except Antarctica (O'Neil et al. 2012). The structure of its cylindrospermopsin was determined in 1992. Other cyanobacteria, including *Umezakia natans*, *Aphanizomenon ovalisporum* (Carmichael 2001), *Lyngbya wollei*, *Raphidiopsis mediterranea*, and *Anabaena lapponica* were found to be capable of producing CYN (O'Neil et al. 2012).

### *Cyanobacteria*—*Nodularia*

*Nodularia* is a filamentous, heterocystous *Cyanobacteria* genus, causing blooms in brackish waters worldwide (O'Neil et al. 2012) especially in the Baltic Sea

*N. spumigena* was the species responsible for the first bloom of a toxic cyanobacterial species reported in the world. Morphological features—like the presence of gas vesicles, the dimensions and shapes of vegetative cells, heterocytes, akinetes, the size and shape of trichomes—did not accurately differentiate *Nodularia* strains in the Baltic Sea. Historically, the first bloom episode correlated to a cyanobacterial species has been ascribed to a *Nodularia* organism. This life form, *N. spumigena*, produces nodularin which can promote liver tumours and act directly as a liver carcinogen, due to inhibition of protein phosphatases.

### *Cyanobacteria*—*Lyngbya*

*Lyngbya* sp is a filamentous, non-heterocystous *Cyanobacteria* genus (O'Neil et al. 2012). Actually, different organisms belonging to this species are reported at present: they can produce different toxins and show very dissimilar attitudes when speaking of environmental adaptability. The freshwater species *L. wollei* is capable of producing saxitoxin as well as cylindrospermopsin.

One of the most known life forms of this species, *L. majuscula*, was first reported in Hawaii, USA, during the 1950s to the 1970s, but also during the late 1990s in Australia, in Moreton Bay, Queensland as well as near Perth and Broome. From the health viewpoint, severe illnesses have correlated with the action of *L. majuscula* on professional fishermen (O'Neil et al. 2012). Blooms have also been reported in Florida as well as active spots in the Caribbean and the South Pacific.

This cyanobacterium produces several demotoxic alkaloids, neurotoxins, and bioactive compounds. Toxins associated with *L. majuscula* include lyngbyatoxin-A and debromoaplysiatoxin, causing asthma-like symptoms and severe dermatitis in humans.

In addition to previously reported bioactive compounds toxic for fish and invertebrates, new microcolins, lyngbyamides and barbamides have been identified (Liu et al. 2011).

### *Cyanobacteria*—*Oscillatoria*

*Oscillatoria* sp is a filamentous, non-heterocystous cyanobacterial genus commonly found in watering-troughs waters, and is mainly blue-green or brown-green. This organism uses photosynthesis to survive and reproduce. Each filament of *Oscillatoria* consists of trichome which is made up of rows of cells.

### *Cyanobacteria*—*Trichodesmium*

*Trichodesmium* spp is a group of colonial non-heterocystous filamentous cyanobacterium species belonging to *Oscillatoriales*. Basically, these organisms are

reported to be strong bloom-producing agents. Moreover, they are able to grow in many environmental marine ecosystems including tropical waters. In addition, *Trichodesmium* spp need low available nutrient substances but waters have to be clear enough to allow light penetration. These life forms synthesise water-soluble toxins with consequent aggressive action on other marine competitors (O'Neil et al. 2012).

Interestingly, different substances have been correlated with these organisms, including palytoxin, microcystin (MCY)-LR and a MCY-like cyclic peptide (Kerbrat et al. 2011).

*Synechococcus* is a cosmopolitan open ocean cyanobacterium, but it also forms harmful blooms covering vast areas in Florida Bay, USA. *Synechococcus* blooms are also known to inhibit zooplankton grazing (O'Neil et al. 2012).

### 2.1.3 Harmful Algal Blooms: Occurrence and Causes

HAB—well known because of the adverse action on human and wildlife health—are increasing in frequency and intensity worldwide (Hallegraeff 1993; O'Neil et al. 2012; Van Dolah 2000). These phenomena afflict most temperate and tropical coastal nations; their frequency and negative impacts on fisheries have increased markedly in the last decades since 1970 (Van Dolah 2000). Blooms of *Cyanophyceae*—mostly due to *Microcystis aeruginosa*, *Oscillatoria rubescens* and *Anabaena flos-aquae*—have recently been recorded in freshwaters: Australia, Japan and South Africa (O'Neil et al. 2012).

The widespread occurrence of HAB both in marine and freshwaters has resulted in a growing public interest for their negative interferences on economic activities related to the use of marine resources (fishing, mariculture and tourism) and for their negative implications on biodiversity and ecosystem health (Botana 2014; Cabado and Vieites 2012; Evangelista et al. 2008; Rossini 2014). Simultaneous advances in the monitoring and surveillance have also contributed to increasing records of HAB (Pitcher 2012).

Since selected groups of phytoplankton species occur regularly in the presence of precise chemical and physical conditions, several different models have been suggested to explain species occurrence and abundance (Margalef 1978; Reynolds 1988; Reynolds and Smayda 1998). On the other hand, the role of endogenous regulation in species timing has been considered only marginally (Eilertsen and Wyatt 2000; Garrison 1981). Actually, many other conditions and factors should be taken into account (Garcés et al. 2002; Zingone and Wyatt 2005; Zingone et al. 2001).

In addition, it should be noted that bacteria may play a role in the population dynamics and toxicity of harmful algae (Doucette et al. 1998). Bacteria have been reported to be involved—directly or indirectly—in the production of biotoxins, the promotion or inhibition of the growth of HAB species and the stimulation or inhibition of phytoplankton.

Although natural transport and dispersal mechanisms and natural habitat extensions, driven by environmental change, may contribute to increased HAB observations, anthropogenic activities—including the eutrophication of coastal systems and the translocation of some species—are considered to be main drivers of HAB events. Climate changes caused by global warming consequent to the greenhouse effect and to the hole in the ozone layer, as well as the increased anthropic influence on aquatic ecosystems have been recognised as the main factors involved in the outbreaks of HAB. Proliferations of toxic microalgae have increased with the rise of surface water temperature up to 2° above the seasonal average values. Increasing outbreaks of HAB along Mediterranean coasts suggest that this ecosystem is changing towards conditions typical of tropical and sub-tropical regions (Giacobbe 2008).

Among the causes invoked to explain the expansion of HAB (GEOHAB 2001), several factors have to be highlighted, including also increased nutrient inputs and surface water temperatures due to changing global climate. The general increase in water eutrophication and phytoplankton blooms is usually associated with the massive input of nitrogen and phosphorus (Granéli et al. 2008). The nutrient enrichment of seawater caused by organic wastes released from anthropic activities (i.e. sewages, agriculture, fertilisers) stimulates the algal proliferation. On the other hand, the spreading of toxic species may be explained by the occurrence of natural mechanisms, increased aquaculture activity, improved analytical methods which determine the discovery of new toxins and toxic events (Hallegraeff 1993). Blooms of algal exotic species have also increased due to the spreading of resting/dormant stages in ballast waters, as well as due to transfers of shellfish stocks (Garcés et al. 2001). Ballast waters and the progressive tropicalisation of Mediterranean waters have favoured the dispersion of toxic species from a geographic area to others, leading to the increase of the episodes of HAB.

The frequency and magnitude of processes (i.e. eutrophication and climate changes) that may promote the proliferation of cyanobacterial HAB are expected to increase in next future (Anderson et al. 2002; O'Neil et al. 2012). While some cyanobacterial blooms are associated with eutrophication—being cyanobacteria highly competitive for inorganic phosphorus (P) and able to acquire organic P compounds—other genera appear dependent on the reduced abundance of P and inorganic nitrogen (N). The role of eutrophication in HAB is controversial. The importance of life cycles in this context should be considered (Zingone et al. 2001).

Globally, climate changes can modify all the aquatic ecosystems. Several examples concern the augment of thermal values, the modification of ocean flows, the increased rate of photosynthetic processes, etc. (Hallegraeff 2010). On the other hand, more research is needed when speaking of the correlation between cyanobacteria and the amount of carbon dioxide (Katırcıoğlu et al. 2004).

However, the increasing uncertainty in progressing from climate change to ocean response to biological impact leads to considerable doubt on the ecosystem scenario development (Hallegraeff 2010). Climate simulation scenarios are affected by uncertainties; the comprehension and predictions of how climate may select for HAB are still limited by the scarcity of long-term records of algal blooms required

for assessment of past climate variability on HAB at least. The prediction of phytoplankton community responses and more specifically the response of HAB to climate change, requires accurate forecasts of these environmental and ecological parameters (Garcés et al. 2001).

Many research programs have focused on the monitoring and prevention of HAB phenomena. First, a recent international project has studied the correlation between the abundance of certain HAB species and specific factors such as interactions between different organisms, the availability of nutrients and other ecological agents (GEOHAB 2001). In more recent years, many researches have focused on HAB with the specific objective of developing new assays for rapid detection of biotoxins and of advanced early warning tools for the detection of shellfish toxins as well as of decontamination methods for toxic shellfish.

The recent episodes of blooms due to tropical species typically have highlighted the need to undertake more scientific, organisational and institutional initiatives when speaking of management and protection of aquatic resources.

Different coastal monitoring programmes have been performed in Italy, for the Adriatic Sea and then for all other Regions having shorelines. Lines and protocols of intervention and monitoring have been set up in cooperation among the Italian Ministry of Environment and Health, Local Regional Agencies for Environmental Protection and Research institutes (University, National Research Council) involved in the field of environmental and human protection.

The extensive monitoring for *Alexandrium* spp in Mediterranean coastal waters—including the Catalonia and Balearic Islands (Spain), Aegean regions (Greece), Sardinia and Sicily (Italy)—indicated that major HAB emergencies are in the Catalan area which is characterised by the highest nutrient load, especially nitrogen, and marked human activities along the coast (Giacobbe et al. 2006). The same thing was observed in the Catalonia region and other areas (Luglié et al. 2004).

## 2.2 Typologies of Toxins, Mechanisms of Action and Syndromes

### 2.2.1 *Typologies of Toxins: Chemical Structure*

Damages caused to humans by algal blooms are primarily derived from the production of toxins and to a lesser extent from the effects of dermal sensitisation in relation to cell membranes or volatile substances present in aerosols on the surface of the blooms.

Basically, shellfish toxins are subdivided in two groups depending on the affinity to water or lipids. Table 2.1 shows most important molecules with relation to water affinity or lipophilic attitude. In addition, these toxins are associated with six different syndromes.

**Table 2.1** Groups of marine toxins and correlated syndromes (Botana 2014; Gerssen et al. 2010; van Dolah 2000)

Toxin group	Toxin sub-group	Syndrome
Hydrophilic toxins	Domoic acid	Amnesic shellfish poisoning (ASP)
	Saxitoxins	Paralytic shellfish poisoning (PSP)
Lipophilic toxins	Brevetoxins	Neurotoxic shellfish poisoning (NSP)
	Okadaic acid	Diarrhetic shellfish poisoning (DSP)
	Dinophysistoxins	
	Pectenotoxins	
	Yessotoxins	
	Azaspiracids	Azaspiracid shellfish poisoning (AZP)
Non-regulated lipophilic toxins	Spirolides	–
	Gymnodimines	–
	Pinnatoxins	–
	Ciguatoxins	Ciguatera fish poisoning

The severity of effects produced by toxins depends on the different solubility of these compounds (in water or lipids); modality of exposure, mode of action and the different susceptibility of species are important. Consequently, concentrations below a threshold may result only in a weak physiological or behavioural response if organisms are able to cope with high toxin levels. On the other hand, high concentration of toxins may have lethal effects.

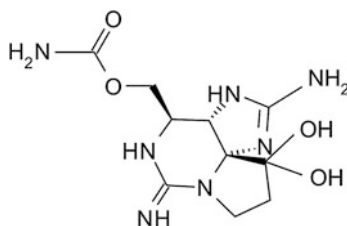
A group of different experts has formulated an indication that toxins are classified according to their chemical characteristics into: saxitoxins (STX), okadaic acid (OA) and dinophysistoxins (DTX); pectenotoxins (PTX); yessotoxins (YTX); brevetoxins (PbTx); domoic acid (DA), azaspiracids (AZA), spirolides (FAO/IOC/WHO 2005).

A synthesis of main syndromes associated with marine toxic algae is reported in Table 2.1.

### 2.2.1.1 Saxitoxins

STX (Fig. 2.1) are the most common toxins. These are a group of around 30 water-soluble, hydrophilic, tetrahydropurine neurotoxins produced by dinoflagellate species belonging to *Alexandrium* spp., *Gymnodinium catenatum* and *Pyrodinium bahamense* (Halstead 2002; Mitrovic et al. 2004). The STX family includes different chemical compounds, divided into subgroups: saxitoxin, neosaxitoxin, tetrodotoxin and gonyautoxins (from 1 to 4, B1 and B2, C1 to C4). Most of these molecules are metabolites of saxitoxin and neosaxitoxin. In general, they are responsible for PSP. Saxitoxins (carbamate alkaloid neurotoxins), a variegated group of chemical compounds, are produced by different freshwater cyanobacteria (Codd 2000; Codd et al. 1999).





**Fig. 2.1** Chemical structure of saxitoxin (STX). BKchem version 0.13.0, 2009 (<http://bkchem.zirael.org/index.html>) has been used for drawing this structure

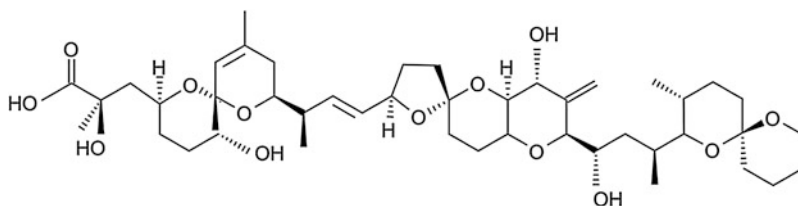
These heterocyclic guanidine compounds interfere with the production of action potentials of some types of excitable cells, causing inhibition in nerve conduction. The block of the influx of sodium ions into the cells (and therefore of the conduction of neuronal axons) occurs through the bond on the receptor exterior to the sodium channel by negatively charged amino acids on the alpha subunit II. The biosynthesis of the toxin occurs through the pathway of methylenetetrahydrofolate glycine-serine—and of the ornithine-arginine—with closure of the imidazole ring.

PSP-producing species have been found in the Adriatic Sea (Honsell et al. 1992); Tyrrhenian Sea (Carrada et al. 1991) and in Cape Peloro Lakes (Giacobbe and Maimone 1994), although the detection of saxitoxins has been also reported in extra-european Countries also (Gerssen et al. 2010). The median lethal dose at which 50 % of the population responds ( $LD_{50}$ ) for adult men is 1–2 mg, orally. Saxitoxin persists in the environment and should be subject to biological magnification. STX has been found in bottom sediments, within resting cysts and can accumulate in the hepatopancreas, adductor muscle and viscera of marine molluscs (like *Mytilus galloprovincialis*, *M. californianus* and *Patinopecten yessoensis*), but also of freshwater molluscs (*Corbicula sandai*). The toxin can accumulate in *Tindinnids*, crustaceans like *Tigriopus californicus* and *Cancer anthonyi* larvae (Bruno 2000). Subsequently, the accumulated toxin is metabolised and the shellfish are decontaminated, but the metabolites retain their toxic properties. The mantle or the kidney remain toxic for up to six months after intoxication while the hepatopancreas detoxifies itself much earlier. Toxins cause a species-specific toxic effect in the same shellfish: it is manifested by altered shell activity, decreased or increased oxygen consumption, altered heart rate, reduced production of fine linen, altered filtration and nutrition rates.

At present, different measures have been taken into account with the aim of protecting human health from PSP within the EU (EFSA 2009a).

### 2.2.1.2 Diarrhetic Shellfish Toxins

Diarrhetic Shellfish Toxins (DST) are a family of lipid-soluble toxins which includes okadaic acid (OA, Fig. 2.2) and its congeners, dinophysistoxins (DTX 1–4); DTX are polyethers derived from a fatty acid with 38 carbon atoms. These compounds are



**Fig. 2.2** Chemical structures of okadaic acid (OA). BKchem version 0.13.0, 2009 (<http://bkchem.zirael.org/index.html>) has been used for drawing this structure

isolated from algae of genera *Dinophysis* and *Prorocentrum*, and are responsible for DSP (Landsberg et al. 2005; Lee et al. 1989; Yasumoto et al. 1978). At present, it has to be highlighted that OA toxins are repeatedly found in many organisms and various geographical areas with notable amounts (Gerssen et al. 2010). DSP-producing algae have been found in all the world. In Italy, these have been found in the Adriatic Sea (Tubaro et al. 1992) and in brackish waters of Sicily (Giacobbe et al. 1995).

DST include also YTX and 45-hydroxyessotoxin, produced by the dinoflagellate *Protoceratium reticulatum*. These toxins may accumulate in shellfish which may be contaminated even with a few hundreds of cells per liter.

The so-called pectenotoxin group, including PTX from 1 to 7, is also considered in the DST category.

YTX and PTX, included into the group of DSP toxins due to their frequent co-occurrence with OA and DTX analogues, do not cause diarrhoea; they should be removed by DSP group (Gerssen et al. 2010). With relation to YTX, a remarkable differentiation has to be considered from the chemical viewpoint. However, four toxins only are been found repeatedly with respect to the total number of similar YTX; some of these molecules have been reported in Europe only (Gerssen et al. 2010). Yessotoxin is a polyether disulphate that exerts its toxic effects on cells of the cardiac muscle, causing a marked cytoplasmic oedema. It has a high toxicity, with a LD<sub>50</sub> of 100 µg/kg of body weight in mouse. For this reason, the current EU limit is 1 mg/kg; however, YTX have not been recognised responsible for human illnesses at present (Gerssen et al. 2010).

On the other side, 15 different PTX toxins have been studied. Apparently, these toxic molecules are generally found in Europe. For this reason, PTX should be considered in the OA group with relation to European legislation. However, the EFSA has recently recommended a different classification (and different safety limits) for PTX (EFSA 2008a, b, c, 2009b; Gerssen et al. 2010).

### 2.2.1.3 Brevetoxins

Brevetoxins (PbTx) are lipophilic, complex, neurotoxic polycyclic ethers, produced by the dinoflagellate *Karenia (Ptychodiscus) brevis* (previously *Gymnodinium breve*) which may be differentiated in two main types (Baden 1989). They cause NSP (Gerssen et al. 2010).

Because of the localisation of NSP in extra-European Countries, this danger has been considered with the determination of safe limits by the Food and Drug Administration, while a scientific opinion is available in Europe (EFSA 2010c).

PbTx may spread through release into the water after cell lysis, or stored in animal tissues or inhaled by the simple breath of aerosol during the algal blooms. They are eliminated through the urine and faeces. Brevetoxins can cause widespread death of fish and even of marine mammals (*Tursiops truncatus*) due to biological magnification.

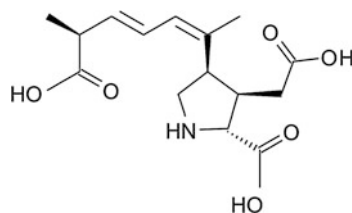
#### 2.2.1.4 Domoic Acid

Domoic acid (DA) is produced by certain diatom species (Sect. 2.1.2.1). This molecule is a cyclic amino acid (Fig. 2.3): a specific, high affinity- glutamate antagonist at the neuronal level. LD<sub>50</sub> is ranged between 35 and 70 mg/kg in rats (Iverson et al. 1989). However, human toxicity was observed in Canada after the consumption of molluscs (*Mytilus edulis*) containing an amount of 1 mg/kg of DA; most people died, whereas those surviving had permanent neurological damages (Perl et al. 1990). The depuration time of molluscs is more than 18 days in uncontaminated waters. At present, ASP has been studied in Europe also (Gerssen et al. 2010). For this reason at least, a permitted level of 20 mg DA/kg shellfish has been defined by the European Legislation (EFSA 2009c). However, new norms may be expected in future.

#### 2.2.1.5 Ciguatoxins

Nonprotein- ciguatoxins are produced by *Gambierdiscus toxicus* and *Ostreopsis lenticularis*, tropical benthic dinoflagellates (Bagnis 1968). Other species which contribute to this toxin are *Coolia*, *Prorocentrum* and *Amphidinium*.

Chemically, they are cyclic polyethers which are soluble in lipids (ciguatoxins) or in water (maitotoxins); they act as exciting agents causing repeated activation of nerve axons (Holmes et al. 1991). The binding site is the same as that of brevetoxins. Ciguatoxins have a LD<sub>50</sub> of 0.45 mg/kg of body weight in mouse. Ciguatoxins



**Fig. 2.3** Chemical structure of domoic acid (DA). BKchem version 0.13.0, 2009 (<http://bkchem.zirael.org/index.html>) has been used for drawing this structure

accumulate through the trophic web, whereas maitotoxins remain located in the digestive tract of plankton-feeding fish (Halstead 2002). An opinion on ciguatoxins has been reported by EFSA (EFSA 2010a).

#### 2.2.1.6 Azaspiracid Shellfish Poisoning (AZP)

AZP is correlated with 24 different toxins but only three molecules—azaspiracid-1, -2 and -3—can be defined the most important menaces for human and animal health (Gerssen et al. 2010). Generally, AZP is reported in Europe and in American Countries.

At present, European legislation has modified the previous safety limit for azaspiracid-1 and other toxins: 30 µg azaspiracid-1 equivalents/kg shellfish instead of the previous amount

#### 2.2.1.7 Spirolides and Gymnodimines (Cyclic Imines)

Spirolides (SPX) and gymnodimines are considered powerful toxins because of rapid lethal effects on test animals. Chemically, it can be affirmed that these molecules are cyclic imines. In addition, the detection of SPX is not circumscribed to European Countries only: however, the importance of these substances in the EU legislation (EFSA 2010b) has to be noted. On the other side, gymnodimines appear to be reported in Oceania only and shellfish from New Zealand.

#### 2.2.1.8 Palytoxin

PLTX toxins appear similar because of the general structure: an extended compound ranging from 2,659 to 2,680 Da. Interestingly, these molecules have hydrophilic attitudes and lipidic affinity at the same time (Botana et al. 2013). A Scientific opinion on PLTX has been recently reported by the EFSA (EFSA 2009d).

#### 2.2.1.9 Aplysiatoxins

Aplysiatoxins are bis-lactones of organic acids produced by tropical marine *Oscillatoriaceae* belonging to *Lynbya majuscula*, *L. gracilis*, *Schizothrix calcicola* and *Oscillatoria nigroviridis*.

These species can grow as epiphytes on macroalgae like *Acanthophora spicifera*, *Laurencia intermedia* and *L. okamurai*. Aplysiatoxins may reach humans through the biological magnification performed by herbivorous fish and molluscs

(*Siganus fuscescens*, *Aplysia kurodai*). There are at least 12 compounds belonging to aplysiatoxins, including indole alkaloids (such as lyngbyatoxin a) and polyacetates (such as aplysiatoxin), which may cause epithelial and gastrointestinal cancers.

### 2.2.1.10 Cyanotoxins

Cyanotoxins are composed of different molecules—anatoxins, microcystins and saxitoxins—produced by cyanobacteria (Sivonen and Jones 1999).

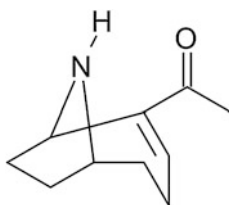
MCY and cylindrospermopsin (CYN) are natural hepatotoxins produced by cyanobacteria (blue-green algae) that grow worldwide in eutrophic freshwaters and cause animal and human water-based toxicoses (Sivonen and Jones 1999). Cyanotoxins fall into three broad groups of different chemical structure (Katircioğlu et al. 2004): cyclic peptides, alkaloids and lipopolysaccharides. On the other side, potent neurotoxins are anatoxin-a ( $C_{10}H_{15}NO$ ), molecular weight (MW): 165 Da, (2) anatoxin-a(s) ( $CHN_4O_4P$ ), MW = 252 Da, and (3) saxitoxins.

Microcystins, produced by genera *Anabaena*, *Microcystis*, *Nostoc* and *Oscillatoria*, are monocyclic hepta-peptides with MW of about 800–1,000 Da. These molecules are constituted by a blocking carbohydrate, seven amino acidic residues and a methylamine. They differ according to two L- amino acids, and more than 60 variants of the first isolated toxin (microcystin-LR) are known (Codd 2000). They show potent hepatotoxicity and activity as tumour promoters.

Nodularin, produced by *Nodularia spumigen*, a brackish water cyanobacterium, is a cyclic pentapeptide structurally very similar to microcystins.

With concern to the health importance of CYN, the chemical compound—a cyclic guanidine alkaloid—has been associated with outbreaks of human sickness and cattle mortality: a certain carcinogenic activity has been reported (Katircioğlu et al. 2004).

In relation to neurotoxins, anatoxins are non-sulphated alkaloid toxins of freshwater cyanobacteria and act as neurotoxins. Anatoxin-a (Fig. 2.4) is an alkaloid with a median lethal dose (at which 90 % of the mouse population responds) of 0.3 mg/kg (Metting and Pyne 1986; Mitrovic et al. 2004). Other important neurotoxins are anatoxin-c and Homoanatoxin-a (Skulberg 1999).



**Fig. 2.4** Chemical structure of a neurotoxic alkaloid: anatoxin-a. BKchem version 0.13.0, 2009 (<http://bkchem.zirael.org/index.html>) has been used for drawing this structure

Prymnesin is an ichthyotoxin produced by *Prymnesium parvum*, a brackish-water golden-brown phytoflagellate (Metting and Pyne 1986).

The presence and correlated health risks of the neurotoxic compound, BMAA, has been recently described (Cox et al. 2003, 2005, 2009). BMAA has been found in remarkable amounts (fish and shellfish tissues) in Northern European waters (Jonasson et al. 2010).

### 2.2.2 Mechanisms of Action of Algal Toxins

The most part of marine algal toxins cause changes in the concentration of sodium and calcium ions. As a result, they interfere with electrical transmission.

Saxitoxins are responsible for the Paralytic Shellfish Poisoning (PSP). In other words, neurotransmission is blocked (Gerssen et al. 2010). The primary route of clearance is via the urinary tract, both in humans and in animals.

DST are inhibitors of serine and threonine phosphatases PP1 and PP2A that are key components of cellular processes related to metabolism, ion balance, and neurotransmission. Diarrhoea originates mostly from the hyperphosphorylation of proteins in the intestinal epithelia.

The tolerance limit set for OA is 40 µg/100 g of shellfish, while the analogous limit for dinophysistoxin-1 is 36 µg/100 g of mollusc.

Brevetoxins, which cause NSP, bind to the sodium channel but in a site different from saxitoxin. In brief, by this mechanism, PbTx alter membrane properties of excitable cells, leading to membrane depolarisation and subsequent disruption of cardiac functions (Baden 1989). PbTx are potent neurotoxins and hemolysins: they can cause depolarisation of smooth muscles in bronchial and tracheal tissues.

DA, which causes ASP, is a neurotoxin. It can produce neurological lesions with symptoms such as memory loss, neuro-excitatory and neuro-degenerative effects in mammals (Bruno 2000).

Spirolides—cyclic imines produced by *Alexandrium ostenfeldii* and *A. peruvianum*—are ‘fast-acting’ algal neurotoxins isolated from shellfish and plankton samples off the eastern shore of Nova Scotia, Canada (Landsberg et al. 2005). The molecular basis for these effects may, at least in part, be due to the recently reported antagonist actions of spirolides at nicotinic-type and muscarinic-type acetylcholine receptors (Munday et al. 2012).

The known activity of PLTX causes different symptoms in animals and humans. This type of human poisoning is called palytoxicosis or clupeotoxicosis. Debromoaplysiatoxins bind stably to the membrane receptor of protein kinase C. These toxic compounds have also been isolated from other *Oscillatoriaceae* (Chorus and Bartman 1999). Finally, anatoxin-a, homoanatoxin-a and anatoxin-a(s) are powerful neurotoxins.

### 2.2.3 Syndromes Caused by Algal Toxins

The main human poisoning syndromes associated with the consumption of shellfish have been reported, depending on the type of symptoms, such as paralytic (PSP), diarrheic (DSP), neurotoxic (NSP), and amnesic (ASP) poisoning, respectively (Botana 2008; van Dolah 2000). AZP is also reported.

The ingestion of edible molluscs containing toxins can cause the onset of very serious symptoms in humans, sometimes with fatal outcome. Algal toxins for the major part are thermally stable and are known for their effects on the nervous system, which often happen within 1 h from food consumption.

Symptoms due to algal toxins can be summarised as follows (Bruno 2000):

- (1) PSP caused by Saxitoxins. PSP is a life-threatening syndrome; the symptoms are purely neurological with rapid onset. Acute intoxication with early symptoms includes tingling of the extremities and perioral area, loss of motor control, incoherence and death by respiratory paralysis
- (2) DSP caused by *Dinophysis* toxins diarrhoea, vomiting, etc. However, no human intoxications by PTX have been reported yet
- (3) NSP caused by brevetoxins. NSP is responsible for an intoxication syndrome with both neurological and gastrointestinal symptoms. NSP toxins can cause symptoms such as dry cough, wheezing, watery eyes, paresthesias, myalgia, vertigo, headache, nausea, diarrhoea, abdominal pain, bradycardia and dilated pupils (Baden 1983), asthma, etc. Exposure to brevetoxin aerosols can result in conjunctival irritation, rhinorrhea and broncho-constriction; asthmatic people may have wheezing (Baden et al. 1982). After exposure to brevetoxin aerosols during a *K. brevis* bloom, inflammatory responses with upper and lower respiratory symptoms have been reported (Backer et al. 2003). NSP syndromes are endemic in Florida and in the Gulf of Mexico
- (4) ASP caused by DA. ASP can be a life-threatening syndrome characterised by a variety of acute symptoms—vomiting, nausea, diarrhoea, hallucinations, confusion, disorientation, breath difficulties, short-term memory loss and in severe cases, death—which may be both neurological and gastrointestinal, and take place within 24 or 48 h of ingestion
- (5) *Ciguatera* fish poisoning by Ciguatoxins. This syndrome is generally confined to tropical and subtropical seas. Since the ciguatoxins include multiple toxic components in fish, several and different symptoms have been identified in humans (Backer et al. 2003). They occur 8–48 h after consumption of the contaminated food (neurological symptoms: paresthesias, myalgia, arthralgia and inversion of sensitivity to heat; gastrointestinal disorders; heart disorders like bradycardia and hypotension; weakness and skin reactions; psychiatric disorders). Usually, the consumption of toxic herbivorous fish is reported to be linked with gastrointestinal or neurological problems, while toxic carnivorous fish may produce cardiovascular and neurological symptoms (Bagnis 1968). Interestingly, different cooking or freezing methods do not lead to toxin inactivation

- (6) Symptoms caused by AZA. Toxic effects observed during AZP intoxication are gastrointestinal disorder, diarrhoea and abdominal cramps
- (7) Symptoms caused by spirolides. Exposure to spirolides in mice induces a number of symptoms that vary with survival time, such as piloerection, ataxia, ophthalmia, abdominal muscle spasms, hyperextension of back, and tail whipping. In terms of toxicity, the lethal dose of desmethyl-spirolide C delivered intraperitoneally to mice was reported to be 40 µg/kg
- (8) Symptoms caused by PLTX. These toxins, inhaled by aerosol formed in the presence of high cell concentrations and waves, may cause respiratory infections and conjunctivitis; in other cases, dermatitis and alterations of body temperature were reported. Another disorder is leukocytosis with additional symptoms with nausea and headaches
- (9) Symptoms by aplysiatoxins. These toxins cause an acute toxic effect which consists of strong itching following contact with algae, that evolves within 3–8 h into a strong erosive dermatitis with erythema on the skin exposed to the toxins action
- (10) Symptoms caused by microcystins and nodularins: nausea, vomiting, intestinal pains within 3–4 h of water ingestion, later fever, acute headache, muscular pains, hepatic damages, death in some immunosuppressed individuals. Microcystins produced by *Cyanophyceae* have a direct effect on humans or animals, who have a contact with lake water during algal blooms
- (11) Symptoms by anatoxins: diarrhoea, vomiting, nausea, no sensitivity; paralysis, breath difficulties, death.

## 2.3 Brief Notes on Detection Methods

### 2.3.1 Analytical Methods for the Determination of Algal Toxins

The Regulation (EC) No. 853/2004 prescribes—Annex III, Section VII, Chapter V (2)(c) and (e)—that live bivalve molluscs commercialised for food purposes have to comply with strict toxin levels (maximum amounts) such as 800 µg/kg for PSP-associated toxins.

The main analytical procedures used for the examination of poisoning syndromes are examined in this section. When speaking of PSP, the following methods are used:

- (a) Biological Test
- (b) Liquid chromatography/mass spectrometry
- (c) Test of sodium channels for cell viability.



For DSP, the following methods are used:

- (a) Cytotoxicity test
- (b) Bioassay
- (c) Liquid chromatography high resolution
- (d) Immunoassay
- (e) 'Ion-spray Liquid Chromatography-Mass Spectrometry'
- (f) Test of phosphatase inhibition
- (g) Thin-layer chromatography.

For NSP the following methods are mentioned:

- (a) Biological Test on mouse
- (b) Test radioimmunoassay
- (c) Test of sodium channels for cell viability
- (d) Competitive bound to sodium channels
- (e) Enzyme-Linked ImmunoSorbent Assay (ELISA) with immunoelectrochemical biosensors
- (f) High-performance liquid chromatography (HPLC).

When speaking of ASP, the following methods are used:

- (a) Bioassay
- (b) Capillary Electrophoresis
- (c) Liquid chromatography high resolution
- (d) Immunochemical tests
- (e) Mass spectrometry
- (f) Bound to competitive receptors
- (g) Thin-layer chromatography.

For the *Ciguatera* syndrome, the following methods are mentioned:

- (a) Biological Test on mouse
- (b) Liquid chromatography
- (c) Test of sodium channels for the cell viability
- (d) Competitive binding of sodium channels
- (e) ELISA
- (f) Latex agglutination test.

In relation to EU official testing methods for the detection of toxins in shellfish, a recent overview has been made available; interestingly, some alternative methods have been also described (Gerssen et al. 2010). The Reader is invited to consult this and other documents with concern to the detection of above mentioned toxins in these products. With concern to alternative methods, polymerase chain reaction (PCR) is one of the last research lines (Penna et al. 2007; Perini et al. 2011).

In brief, EU protocols often consider mouse bioassays or rat bioassays (RBA) when speaking of the detection of common toxins according to the Commission Regulation (EC) No. 2074/2005 (Gerssen et al. 2010).

With the exclusion of bioassays and competitive microplate receptor-binding assays, other commonly used analytical methods are based on biomolecular techniques (EFSA 2010a), immunoassays such as ELISA tests (Gerssen et al. 2010) and chemical approaches. The last group of systems—HPLC, liquid chromatography-mass spectrometry (LC-MS) methods, capillary and high-performance capillary electrophoresis—is very interesting. Some examples of chromatographic methods are reported below.

Total MCY levels released by the cyanobacterium *Planktothrix rubescens* were measured (Messineo et al. 2006) in lake Albano (central Italy) by liquid chromatography-tandem mass spectrometry (LC-MS). MC levels up to 14.2 µg/l were found, with high concentrations in summer at a 20–25 m-depth. The intracellular toxin content varied between 1.5 (surface, January 2004) and 0.21 pg/cell (surface, May 2004). Six different MC were detected, the most abundant being two desmethyl-MC-RR isomers.

A ‘liquid chromatography/electrospray ionisation- quadruple time-of-flight’ method was firstly described (Ferranti et al. 2009) to analyse MCY in freshwaters and allowed the characterisation of a novel variant of microcystin-RR (MCY-RR).

In a water reservoir in southern Italy where a bloom of *Planktothrix rubescens* occurred (Ferranti et al. 2013), cyanobacterial toxins were used using ‘matrix-assisted laser desorption/ionisation time-of-flight’ (MALDI-TOF) mass spectrometry and liquid chromatography coupled to quadrupole-time-of-flight (Q-TOF) tandem mass spectrometry. MALDI-TOF mass spectrometry was employed for rapid screening over a wide mass range; liquid chromatography coupled to Q/TOF tandem mass spectrometry allowed for molecular structure confirmation of each single cyanotoxin. Microcystins, anabaenopeptins and aeruginosins were the main cyanotoxins identified by liquid chromatography coupled to Q/TOF tandem mass spectrometry.

A LC-MS method was developed in the multireaction monitoring mode (Bogialli et al. 2006a): microcystin extraction was performed with a sorbent (Carbograph 4) cartridge. As Cylindrospermopsin is a highly polar compound that is scarcely retained by any sorbent material, 0.5 mL of filtered lake water was directly injected into the LC column. Limit of quantification (LOQ) of the five microcystins were within the 2–9 ng/L range, whereas the LOQ of cylindrospermopsin was 300 ng/L. Two demethylated forms of MCY-RR and one demethylated variety of microcystin-LR were found. Demethylated MCY-RR is known to be even more toxic than MCY-RR towards zooplanktonic grazers.

LC/MS/MS toxin analyses were performed on surface water samples collected from 28 Italian lakes (Messineo et al. 2009). The most widespread species associated with toxin production belonged to the genera *Microcystis*, *Planktothrix* and *Anabaena*. Values up to 226.16 ng/mL were recorded for microcystins (sum of all variants), upto 126 ng/mL for total cylindrospermopsin and to 100 µg/g (dry weight) for anatoxin-a.

With concern to the detection of toxins in fish fillets, a simple, specific, and sensitive procedure for determining six cyanotoxins (microcystins-RR, -LR, -YR, -LA, -LW and nodularin) in fish muscle tissue was reported (Bogialli et al. 2005).

This method was based on the matrix solid-phase dispersion technique with heated water as extractant followed by liquid chromatography tandem mass spectrometry (LC-MS/MS) equipped with an electrospray ion source. The limits of quantification were estimated to range between 1.6 and 4.0 ng/g.

The identification of anatoxin-a in water and fish fillets was reported by LC-MS/MS with electrospray ionisation (Bogialli et al. 2006b), with limits of quantification estimated to be 13 ng/l and 0.5 ng/g in water and fish fillet, respectively.

Liquid chromatography coupled to electrospray ion trap mass spectrometry was described for determination of CYN in freshwater and fish muscle (Gallo et al. 2009). This method became highly selective and reliable in unambiguous identification of CYN in water and cyanobacteria extracts from Lake Averno, near Naples (Italy), with limits of quantification which were 0.10 ng/mL in freshwaters and 1.0 ng/g in fish muscle, respectively.

CYN produced by the cyanobacteria *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* was detected by Messineo et al. (2010) in lake Albano (Italy) by LC-MS/MS and ELISA immunoassay, showing extracellular superficial values ranging from 2.6 to 126 µg/L, and water column values ranging from 0.41 to 18.4 µg/L. Moreover, CYN was detected in tissues from two *Salmo trutta* trouts (up to 2.7 ng/g).

Microcystin detection was reviewed in contaminated fish from Italian lakes using ELISA and LC-MS/MS analysis (Bruno et al. 2009). As a result, 87 % of the analysed extracts of tissues (muscle, viscera and ovary) were positive for the presence of microcystins. ELISA test results were from 3 to eightfold higher than calculated concentration by LC-MS/MS analyses. The rapid screening and accurate mass-based identification of cyanobacteria biotoxins were easily afforded by MALDI-TOF/MS, spanning over wide molecular mass ranges. Nevertheless, accurate structure characterisation of all compounds was attained only studying their own fragmentation patterns by LC-Q-TOF-MS/MS. This hybrid mass spectrometry detector was highly sensitive, selective and repeatable in measuring the characteristic ions from each cyanotoxin studied; this technique was successfully employed in confirming known toxins, as well as in elucidating the molecular structure of several new compounds never described previously. On the other hand, ion trap and triple quadrupole LC-MS/MS offered high repeatability and sensitivity for identifying targeted known compounds, such as some microcystins, but could fail in detecting the presence of structural modified derivatives, or less abundant molecules. As a result, hybrid MS/MS detectors giving full details about the molecular structure of many different biotoxins represent the most modern approach for ‘profiling’ contamination levels and assessing the risk deriving to the consumers, both through freshwaters and foods. More recently, it was highlighted (Bruno 2013) that there is a strong need to homogenise the procedures for risk assessment evaluation and to define threshold limits based on cyanobacterial abundance and/or cyanotoxin concentrations, studying implications deriving from the presence of risk cofactors like heavy metals or pesticides, too. These aspects are needed for the preparation of transnational guidelines for risk assessment and management.

### 2.3.2 *Automatised Methods for the Detection of HAB Species*

A new method for the continuous monitoring and control of HAB species—the ‘Dinoflagellate Categorisation by Artificial Neural Network’ (DiCANN) system—has been developed (Culverhouse et al. 2001). This software is able to detect and identify automatically all species belonging to *Dinophysis*-type DSP.

The software is also able to identify other dinoflagellates like *Prorocentrum lima*, *Ceratium* and *Protoperdinium* (Culverhouse et al. 2001); it may be implemented in future for the identification of other planktonic taxa.

Another system, the HAB Buoy, has also been released (Cabrini et al. 2010); this, automatised system, very innovative, allows the in situ detection of toxic algae in 5 s only. HAB Buoy is composed by a near-infrared light source, a flow cell, an objective and a digital camera connected to a dual-processor computer equipped with 2 GB of random access memory (RAM). The HAB Buoy can be remote controlled through a Wireless Local Area Network. At present, this system is available as a prototype which needs further tests before its commercialisation.

## 2.4 Emerging Issues and Perspectives for Future Research

The understanding of toxic water blooms and extensive intoxications needs a new synthesis of the relevant knowledge in several fields (biology, ecology, chemistry and epidemiology) in order to address issues related to water quality preservation, nature conservation, planning and improvement of the physical environment. There is an emerging evidence that several biotoxins produced from a variety of microalgae—including blue-green algae—are chemically similar and cause analogous physiology and toxicological effect (Katircioğlu et al. 2004).

As future perspectives in the field of algal toxins are concerned, monitoring and control of HAB phenomena requires interdisciplinary research approaches. This strategy is strongly recommended as a challenge for future research. Priority to the advancement in both control and prevention strategies should be given for the safeguard of the coastal marine environment and public health.

The transfer of phycotoxins through aquatic food webs is an important aspect of HAB dynamics, which affects multiple trophic levels (Doucette et al. 2006). In addition to direct effects of toxic algal blooms on humans (which are ascribable to toxins that are transmitted to humans through the food chain), indirect effects on the environment also occur in association with some toxic algal blooms. These effects mainly relate to the both sessile (barnacles, bivalves, gastropods) and mobile epibenthos (echinoderms, cephalopods, small fish).

Trophic linkages between HAB and their ecosystems, and the relevant effects of HAB on aquatic organisms have been recently (Landsberg 2002). Ecological and hygienic-sanitary impact of HAB on the trophic web concerns several levels:

- Copepods, for example, show physiological reactions to toxic species, among which disequilibria in the process of grazing and emission of faecal pellets, with severe consequences in the trophic fluxes and in the interactions between water column and sediments (Sykes and Huntley 1987)
- Molluscs, like bivalves, which feed on phytoplankton through filtration, may accumulate toxins. Different species have different susceptibility to these toxins. In the Adriatic Sea, *Mytilus galloprovincialis* was more able to accumulate DPS and PSP toxins compared to other bivalves (Poletti et al. 1995).

In situ and laboratory observations have shown that many invertebrates (molluscs and worms) apparently are unaffected by algal toxicity even at high cell concentrations ( $10^5$ – $10^7$  cells/l). However, death may be caused by oxygen depletion due to the high algal concentration rather than due to direct neurotoxic effects (Steidinger et al. 1973). Conversely, toxic algae may exert both direct and indirect neurotoxic effects on *Teleostea*; mortalities of fish feeding on plankton have been recorded during blooms of some Dinoflagellates like *Alexandrium* spp. and *Pyrodiscus breve*, following the ingestion of toxins causing neuromuscular lesions (Steidinger et al. 1973). Direct effects have also been recorded on fish of commercial interest (anchovies, herrings, salmons, etc.) which are susceptible to PSP-like toxins that accumulate into their tissues reaching concentrations dangerous for human consumption (White 1984). Significant decreases of fish larvae and juveniles have been found in association with blooms of the dinoflagellate *Gyrodinium aureolum* (Potts and Edwards 1987).

From a public health point of view, damages produced by Dinoflagellates toxic to fish have been recorded in many Countries worldwide. Food intoxications following consumption of seafood contaminated by toxic algae have been reported since the 1970s, even in Italy since 1968 (Viviani 1992).

In the context of seafood safety and health preservation, some aspects that deserve particular attention are those related to the mechanisms toxins uptake, accumulation and detoxification performed by molluscs (Asakawa et al. 2006; Blanco et al. 2003; Chen and Chou 2002; Choi et al. 2003; Ichimi et al. 2001). Several examples can be reported here.

Comparing the detoxification mechanisms of toxic scallop (*Patinopecten yessoensis*) and clam (*Saxidomus purpuratus*), it was shown (Li et al. 2012) that the biotransformation of toxins was species-specific. The reductive enzyme was more active in clams than in scallops and that an enzyme in scallops is more apt to catalyse hydrolysis of both the sulphonate moiety at the *N*-sulphocarbamoyl of C toxins and the 11-hydroxysulphate of C and GTX toxins to produce metabolites.

With reference to domoic acid, little knowledge is available about the accumulation of this toxin in these molluscs. Domoic acid was found in the digestive gland of common cuttlefish with the highest values during spring and summer months (Costa et al. 2004), periods when *Pseudo-nitzschia* occur in the plankton; domoic acid was also found in branchial hearts of the same species during *Pseudo-nitzschia* blooms (Costa et al. 2005), suggesting the degradation and biotransformation of the

toxin. PSP toxins were also found in the digestive gland of common octopus during blooms of *Gymnodinium catenatum* (Costa et al. 2009).

In order to explore adverse effects on benthic invertebrates produced by algal toxins, Gorbi and coworkers have recently investigated in mussels *Mytilus galloprovincialis* the effects produced on immunological, histological and oxidative parameters by exposure to *Ostreopsis* cf. *ovata* (Gorbi et al. 2013). A clear involvement of the immune system of mussels was observed with a significant decrease of granulocytes, phagocytosis activity and lysosomal membrane stability in haemocytes, after both 7 and 14 days of exposure to *O. cf. ovata*. A decrease of the digestive gland wall thickness and of neutral lipid levels, the dilatation of tubules and haemocytes infiltration into the digestive gland were found in exposed mussels; a possible inhibition of the feeding activity, with a consequent induction of autophagic phenomena and utilization of stored reserve products such as neutral lipids was suggested. Antioxidant parameters revealed a limited role of *O. cf. ovata* to induce oxidative stress, except for a slight increase of catalase, glutathione reductase and glutathione peroxidases activities, and a significantly higher capability to neutralise peroxy radicals in mussels exposed for 14 days. The observed effects on the general health status of exposed mussels should be adequately considered when assessing the ecological relevance of algal blooms.

As an output of the ‘Development of cost-effective tools for risk management and traceability systems for marine biotoxins in seafood’ (BIOTOX) Project (2005–2008), a review of ‘European Shellfish producing countries and EU Food Safety legislation on monitoring and control of Bivalves’ was produced, including an overview of industry practice on shellfish toxin control by industry case studies. The detection and depuration methods developed during BIOTOX will be incorporated into ‘Hazard Analysis and Critical Control Points’ verification procedures in Europe for the standardised monitoring, depuration and traceability of biotoxins in shellfish. The risk management practices of European Member States were harmonised to identify potential trade barriers and assist the industry in reaching its full commercial potential.

Within the recent Marine Strategy Framework Directive (MSFD, European Directive 2008/56/EC), some criteria are indicated in the Decision 2010/477/EU for the achievement of Good Environmental Status (GES) inherent to the descriptors listed in Annex I to the Directive. With respect to the Descriptor 5 ‘Eutrophication’, the EU Commission Decision 2010/477/EU on criteria and methodological standards on GES of marine waters requires that: ‘*it is minimised eutrophication of human origin, in particular its negative effects, such as loss of biodiversity, ecosystem degradation, harmful algal blooms and oxygen deficiency in bottom waters*’.

The Decision indicates that the assessment of eutrophication in marine waters must take into account the assessment of coastal and transitional waters under the Directive 2000/60/EC (Annex V, points 1.2.3 and 1.2.4) and related guidelines, so as to ensure comparability, taking into consideration the information and knowledge gathered and approaches developed in the framework of regional sea conventions.

The assessment must combine the information on the level of nutrients and those relating to a number of primary and secondary effects which are relevant from an ecological perspective.

For each criterion, the Decision identifies a number of indicators that can be used for the purpose of description and subsequent assessment of GES, as follows (Criterion 5.2. Direct effects of nutrient enrichment):

- Indicator 5.2.1. Chlorophyll concentration in the water column
- Indicator 5.2.2. Water transparency related to increase in suspended algae, where relevant
- Indicator 5.2.3. Abundance of opportunistic macroalgae
- Indicator 5.2.4. Species shift in floristic composition such as diatom to flagellate ratio, benthic to pelagic shifts, as well as events of toxic algal blooms, due, for example, to cyanobacteria, caused by human activities.

Kalogerakis and coworkers suggest that the link between GES and seafood safety is explicit in MSFD Descriptor 9 in the future monitoring of HAB: contaminants in seafood must not exceed relevant standards (Kalogerakis et al. 2014). According to these Authors, real-time status information of the marine environment and seafood safety is urgently needed by stakeholders (seafood and aquaculture industries, policy makers) to respond timely to rapidly occurring phenomena such as harmful algal blooms (HAB). Thus, it is important to develop new innovative sensors for in situ detection of known and newly identified HAB species and the detection of marine biotoxins. The development of better operational model systems will allow the prediction of early warning proxies/indicators to assess the development and spread of HAB. The development of efficient end-user interfaces for governance, farmers, fishermen, and the general public for access to, for example, simulation and forecast results, toxicity information and advice regarding HAB is important. Finally, the development of new methods and technologies for prevention and controlling of HAB, like nanotechnology, marine ecosystem models, natural and artificial upwelling, are important elements of an integrated management strategy for HAB.

The assessment of phytoplanktonic abundance and composition will be performed within the Proposal of guidelines for 'Marine Strategy Framework Directive' monitoring plans'. Particular interest has also been given to the detection of toxic algae and related biotoxins (e.g. PSP, ASP, DSP, YTX, AZA) already considered within Regulations (EC) Nos. 853/2004 and 854/2004, for the safety of shellfish production areas.

The standard monitoring of shellfish farms for the presence of harmful algae and related toxins usually requires the microscopic examination of phytoplankton, bioassays and toxin determination by HPLC. Microscopy procedures are time-consuming and require taxonomic expertise, thus limiting the number of specimens that can be analysed. Molecular biology techniques have great potential in the detection of target organisms in field samples.



Toxins that have affected mainly the Mediterranean Sea over the last two decades are OA and some of its derivatives, yessotoxins and saxitoxins; the presence of DA has been sporadically detected, but at a lower concentration compared to the tolerance limits prescribed by the current law (Ciminiello et al. 2009).

In the northern Adriatic Sea, a general oligotrophication has been reported (Mozetič et al. 2010); nevertheless, some HAB phenomena have been reported in the last 6 years, probably because of nutrient inputs from the *Po* river, as shown by the Report for Year 2011 on the Environmental quality of seawaters in Emilia Romagna (ARPA Struttura Oceanografica Daphne—Emilia Romagna Region 2012).

The biological complexity of toxic algae needs to be matched with appropriate complexity in the representation of environmental and ecological parameters to allow either short-term regional predictions or long-term predictions of their response to global climate changes (Pitcher 2012).

The prediction of HAB is highly desirable for the management of their impacts; nevertheless, only a few HAB species can be predicted with any success to date, although many of them are regular components in the seasonal succession of phytoplankton. Model development is fundamental to achieve HAB prediction. Through retrospective and predictive calculations, models may be used to analyse, synthesise and test understanding of the dynamics of HAB in complex systems (Pitcher 2012).

Attention should also be paid to the development of advanced technologies for the mitigation of HAB events. It has been suggested (Paerl and Otten 2013) that the proliferation of cyanobacterial HAB in a wide range of aquatic ecosystems is favoured by multiple factors such as anthropogenic nutrient loading, rising temperatures, enhanced vertical stratification, increased residence time and salination, etc. The reduction of nutrient inputs is the most obvious target expected to play a key role in any cyanobacterial HAB mitigation strategies in both freshwater and marine environments.

The reduction of phosphorus is an effective means of reducing cyanobacterial dominance in aquatic, and especially freshwater, ecosystems. However, nitrogen reductions are also needed, especially in eutrophic lakes, rivers, estuaries and coastal waters. A management priority is to establish N and P input thresholds, below which cyanobacterial HAB can be controlled in terms of magnitude, temporal and spatial coverage. For this reason, total nutrient loads and concentrations need to be considered in the management of cyanobacterial HAB. The ratios of N to P inputs should be considered when developing these thresholds. For example, total molar N:P ratios above 15 do not favour cyanobacterial HAB dominance. Nutrient inputs may be point or non-point source. Point sources (i.e. wastewater, industrial effluents) are often associated with well-defined discharge sites; therefore, they are relatively easy to control. The remaining major challenge concerns the control of non-point sources, which frequently are the largest sources of nutrients; therefore, their controls are likely to play a critical role in mitigating cyanobacterial HAB. Nutrient management strategies may also include the removal of nutrients from receiving waters after their discharge, by dredging sediments, harvesting



macrophytes that have assimilated nutrients, and in some cases stocking and then removing higher trophic level consumers (finfish and shellfish) to eliminate nutrient-containing biomass. Some of these treatments, however, can have negative effects: sediment dredging can disrupt important bio-geochemical processes in surface sediments and benthos and lead to enhanced mobilisation of previously retained nutrients. Also, disturbance of the sediment meso- and micro-fauna, as well as microbial communities, can disrupt nutrient, oxygen and carbon cycling to the detriment of ecosystems undergoing mitigation and restoration.

Beneficial effects are expected from manipulating physical factors that are known to play key roles in cyanobacterial HAB competition versus other eukaryotic phytoplankton. For example, devices favouring vertical mixing or breaking down stratification have proved to be effective in controlling outbreaks and persistence of cyanobacterial HAB. The increase of flushing rates with nutrient-low waters, and thereby the decrease of water residence times, can be effective in reducing or controlling cyanobacterial HAB. However, these devices may exert some applicability in small lakes or reservoirs, while they are not suitable over large areas and volumes. In most of the cases, nutrient input reductions, combined with physical controls, are the most effective, simple and economically feasible management strategy.

As nutrient controls can be expensive, alternative nutrient removal strategies may be required, such as cultivation and stimulation of macrophytes, stocking of herbivorous (and specifically cyanobacteria consuming) fish and shellfish species.

Anderson and coworkers have reviewed different approaches adopted by countries and commercial enterprises worldwide to monitor and manage HAB in coastal waters (Anderson et al. 2012b). This result is typically achieved through the establishment of programmes for toxin and cell detection (and quantitation) in water, aerosols, shellfish, fish, etc.; the development of bloom forecasting and early-warning capabilities as well as medical intervention and therapeutic strategies; and the development of bloom prevention and mitigation strategies. Owing to the complexity and diversity of HAB phenomena, many challenges are associated with these activities.

In conclusion, despite significant advances in research, commercial biotechnological applications for in situ monitoring of marine ecosystems are still not available for most groups of toxins. Even if biosensors or technologies are sufficiently sensitive to comply with the regulatory limits, few of these methods have been validated and/or accepted as alternative to the mouse bioassays. Biosensor technologies offers several advantages over analytical methods and animal bioassays, including speed, ease of use and low cost, no ethical issues related to the use of laboratory animals. In most cases, these techniques would be good tools to be used as screening methods in order to reduce the number of animal bioassays. This is reported as an important challenge for future development in Marine Biotechnology for protection and management of marine ecosystems (Marine Board-European Science Foundation 2010).

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