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Harmful algae and their potential impacts on the coastal ecosystem: growth and toxin production dynamics

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1. Introduction

1.1. Harmful Algal Blooms

Coastal systems around the world have suffered a variety of environmental problems, including loss of seagrass habitats, coral reef degradation or destruction, loss of quality of coastal waters for recreational use, deaths of marine mammals, red tides, fish kills, and outbreaks of shellfish poisonings. Some of the problems cited above can be attributable to what is called harmful algae blooms (HABs).

The term 'Harmful Algal Blooms' (HABs) covers a heterogeneous set of events that share two characteristics: they are caused by microalgae and they have a negative impact on human activities. Despite these common features, HABs are very diverse in terms of causative organisms, dynamics of blooms and type of impact.

A phytoplankton bloom, also known as a 'red tide' due to the water discoloration, is a sudden increase in the population of a microalgae that has encountered suitable conditions for growth, and that, together with their adaptive strategies (i.e. migration, active swimming) and the appropriate physical conditions, can reach concentrations of 10^4–10^5 cell L^{-1} during certain period of time (commonly 1–3 weeks). A proliferation like this can be characterised by the occasional dominance of a particular species (monospecific bloom) or group of species.

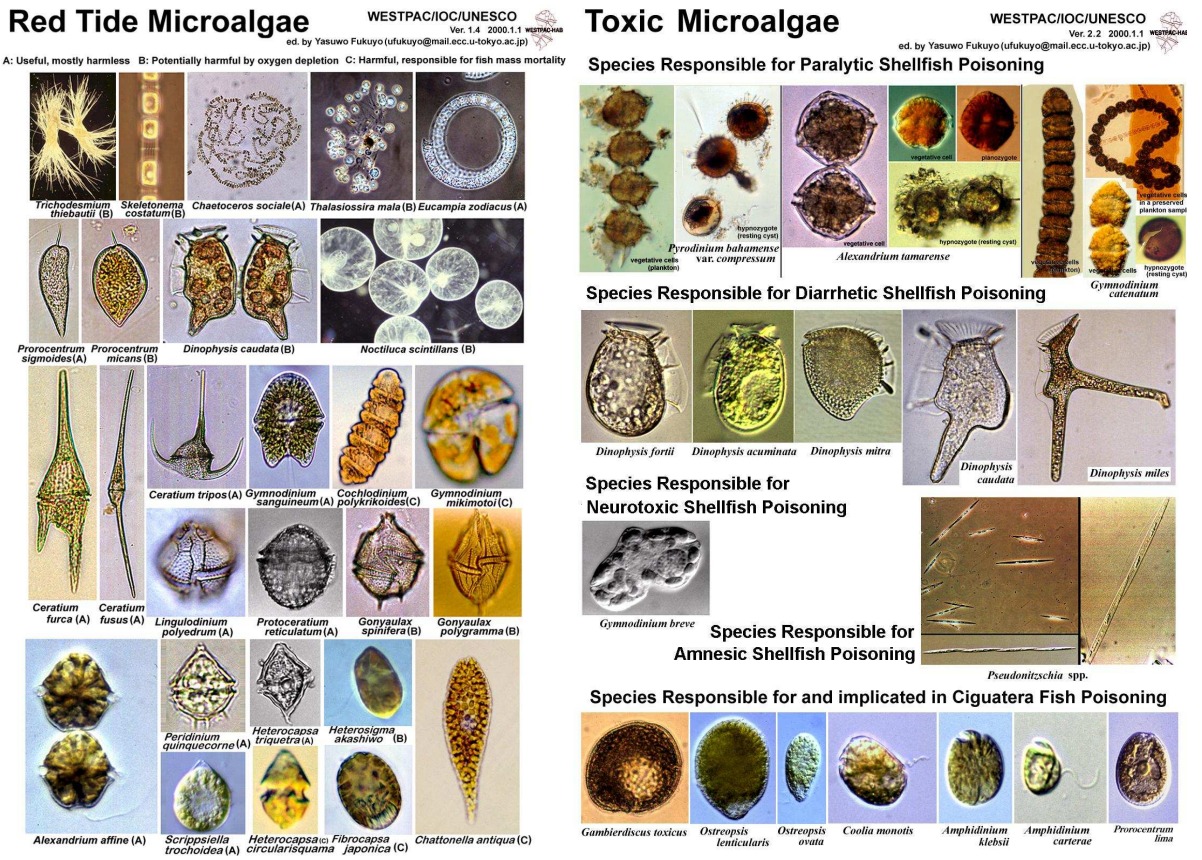
Harmful species belong to six algal groups (diatoms, dinoflagellates, haptophytes, raphidophytes, cyanophytes, and pelagophytes, Fig. 1.1) and these differ greatly in terms of morphological, physiological and ecological characteristics (Zingone and Enevoldsen, 2000; Garcés et al., 2002).

The list of microalgal species that are potentially involved in HABs comprises about 80 toxic species and about 200 noxious species out of an approximate total of 4000 marine planktonic microalgae described thus far (Sournia, 1995). Of these, only around 80 (mainly dinoflagellates) have the potential to produce toxins (Zingone and Enevoldsen, 2000; Smayda and Reynolds, 2003). This list has increased remarkably in recent years because of new cases of harmful events, the development of scientific research in the field, and enhanced human interactions with the coastal zone; however, several thousand phytoplankton species are still undescribed (Andersen, 1992).

Flagellate species account for 90% and, among flagellates, dinoflagellates stand out as a particularly noxious group. They represent the 75% (45-60 taxa) of all harmful algal bloom (HAB) species.

Harmful algal taxa may be nonmotile or motile; pica-, nano-, or larger sized; photoautotrophic, mixotrophic, or obligate heterotrophs; siliceous or nonsiliceous species, etc., and have diverse modes of inimical action.

Fig. 1.1- Some species of microalgae that form red tide or toxic outbreaks (from Fukuyo).



Toxicity and other negative effects caused by harmful algae are therefore not limited to a single algal class or to a few genera, but are distributed among several taxonomic groups. Similarly, the high taxonomical diversity of harmful algae results in a variety of toxins and relative mechanisms of action at different levels of the trophic chain. As shown in Fig. 1.2, they have some of the most complex structures known in nature, ranging from low to high molecular masses and from very polar to highly lipophilic; they possess multi-functional characters and high degrees of chirality, and cause many different types of toxic effects. Ecological requirements and bloom dynamics also vary considerably from one species to another; moreover the taxonomic diversity of HAB species complicates monitoring operations, which require a high degree of specialized expertise. Another implication of the taxonomic diversity of HAB species is that the production of toxic substances or other

offensive or disturbing features has been selected as an advantageous character more than once in the evolution of these microorganisms.

1.2. The diversity of negative effects

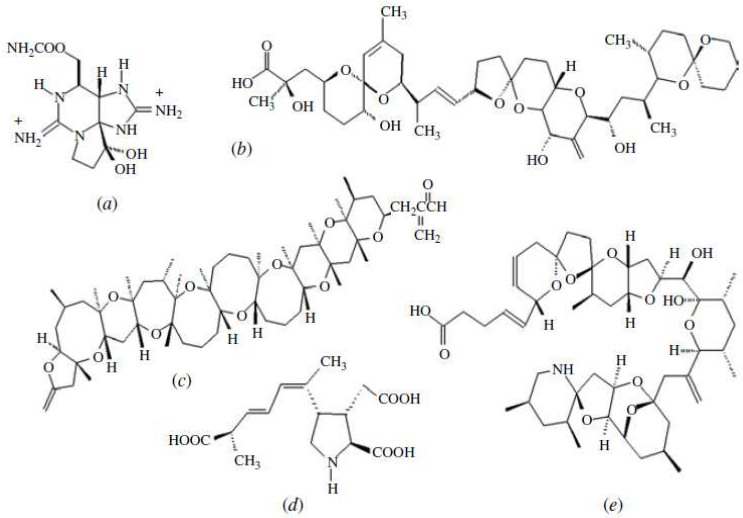
Five major human toxic syndromes caused mainly by the consumption of bivalve molluscs contaminated by algal toxins are reported:

1. **Paralytic shellfish poisoning (PSP)**: PSP toxins are collectively called saxitoxins (STXs) and at least 21 analogues of these cyclic guanidines are known in shellfish, with saxitoxin (Fig. 1.2a) being the most common toxin. STXs exert their effect by a direct binding on the voltage-dependent sodium channel blocking the influx of sodium and the generation of action potentials in nerve and muscle cells, leading to paralysis (Narahashi, 1988). Dinoflagellates that produce STXs belong to three genera (*Alexandrium*, *Gymnodinium* and *Pyrodinium*).
2. **Diarrhoetic shellfish poisoning (DSP)**: DSP toxins were originally divided into three different structural classes: (a) okadaic acid (OA) (Fig. 1.2b) and its analogues, dinophysistoxins (DTXs), (b) pectenotoxins (PTXs) and (c) yessotoxins (YTXs)(Yasumoto et al., 1985). However, YTXs have now been excluded from the DSP classification because they are not orally toxic and do not induce diarrhoea (Ogino et al., 1997; Aune et al., 2002). The mechanism of action of the OA group toxins is via inhibition of serine-threonine protein phosphatise 2A (PP2A) (Bialojan and Takai, 1988), which plays important roles in many regulatory processes in cells. OA probably causes diarrhoea by stimulating phosphorylation of proteins that control sodium secretion in intestinal cells (Cohen et al., 1990). DSP toxins are produced by the dinoflagellates *Dinophysis* spp. and *Prorocentru*m spp. and their toxin profiles can vary within a single species (Murakami et al., 1982; Yasumoto et al., 1980; Fernández Puente et al., 2004).
3. **Neurotoxic shellfish poisoning (NSP):** NSP is a illness caused by the consumption of bivalve molluscs contaminated with neurotoxins that are produced by the marine dinoflagellate *Karenia brevis* (formerly known as *Gymnodinium breve* and *Ptychodiscus brevis*) (Baden, 1983; Steidingen and Baden, 1984). Brevetoxin (Fig. 1.2c) and its analogues can also affect finfish, aquatic mammals and birds and this topic has been recently reviewed (Furey et al., 2007; Watkins et al., 2008). The symptoms of NSP include gastroenteritis and neurological problems (Baden, 1983). The mode of action of brevetoxins is by receptor binding to the sodium channels which control the generation

of action potentials in nerve, muscle and cardiac tissue, enhancing sodium entry into the cell. This leads to the incessant activation of the cell which causes paralysis and fatigue of these excitatory cells (Dechraoui et al., 1999).

4. **Amnesic shellfish poisoning (ASP)**: Domoic acid (DA) was identified as the causative toxin (Fig. 1.2d) (Wright et al., 1989) and marine diatoms of the *Pseudonitzschia* spp. were identified as the source of this toxin (Bates et al., 1989). In addition to gastrointestinal disturbance, unusual neurological symptoms, especially memory impairment, were observed.
5. **Azaspiracid shellfish poisoning (AZP)**: AZP is the most recently discovered toxic syndrome from shellfish consumption and several analogues belonging to this new class of toxins were identified in contaminated mussels (Satake et al., 1998; Ofuji et al., 1999, 2001). More than 20 analogues of azaspiracid AZA1 (Fig. 1.2e) have been identified in shellfish (Ofuji et al., 1999, 2001; James et al., 2003; Rehmann et al., 2008), which complicates the regulatory control of these toxins as most have not yet been toxicologically evaluated. Cytotoxicity studies using neuroblastoma cells showed that AZA1 disrupts cytoskeletal structure, inducing a time- and dose-dependent decrease in F-actin pools. A link between F-actin changes and diarrhoeic activity has been suggested and this may explain the severe gastrointestinal disturbance in AZP outbreaks. Azaspiracids have been identified in two dinoflagellates, *Protoperidinium crassipes* (James et al., 2003) and a new species, *Azadinium spinosum* (Tillmann et al., 2009).

Fig. 1.2 - Structures of the most abundant toxin responsible for each of the five shellfish toxic syndromes; (a) saxitoxin (PSP), (b) okadaic acid (DSP), (c) brevetoxin (NSP), (d) domoic acid (ASP), (e) azaspiracid (AZP) (from James et al., 2010).



In terms of harmful effects, we can consider two types of causative organism: the toxin producers and the high-biomass producers. Some HAB species are related to both characteristics.

High biomass blooms may cause significant ecological problems and harmful effects in the biota of the region (anoxia, community and food-web changes) as well as great economic problems connected to the deterioration of the coastal recreational waters (e.g. discoloration, odour). In some cases, the algal bloom is beneficial for aquaculture and wild fisheries operations; however, algal blooms can have a negative effect, causing severe economic losses to aquaculture, fisheries and tourism operations and having major environmental and human health impacts.

Toxin events can result from very low concentrations of the causative organism. Moreover, in the case of toxic events co-occurring with high-biomass, levels of toxicity which are considered dangerous have often risen before the bloom is conspicuous due to discoloration or cell counts.

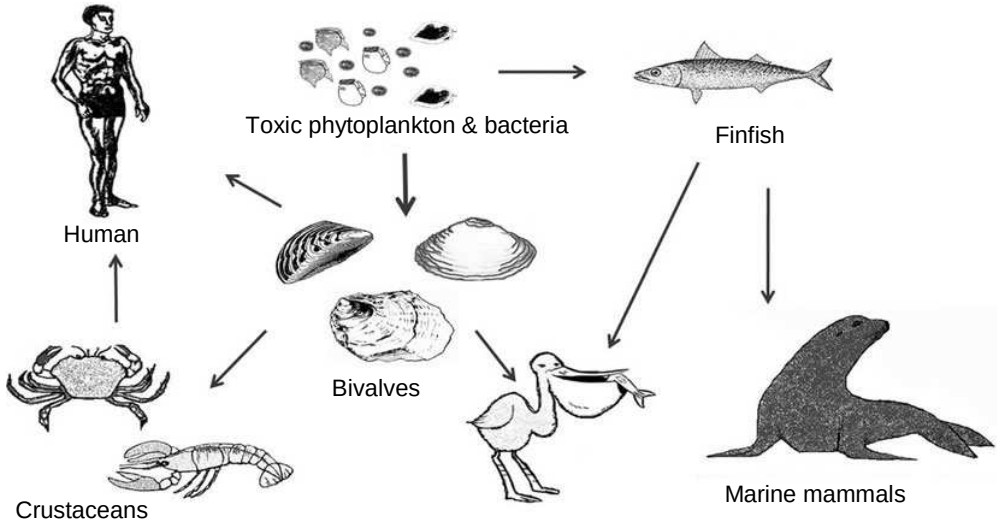
Both high biomass and toxic events can also be associated with the mortality of fish, and different causes are associated with these mortalities (low oxygen levels, direct toxicity, haemolysis, and mechanical damages). In many cases, fish mortality events are related to the fact that fish cannot escape from the cages.

Four categories of deleterious effects related to marine microalgae have been identified:

1. **Risks for human health**: some algal toxins, different in structure and toxicity (Fig. 1.2), reach humans through specific vector organisms that accumulate these noxious substances in their gastrointestinal tract or body tissues (Fig. 1.3). The most toxic algal species are recorded among dinoflagellates, but a number of diatoms and cyanobacteria also produce neurotoxic substances that can endanger human health. Four main harmful effects on humans are associated with the toxic producers: (i) consumption of toxic shellfish that have accumulated phytoplankton toxins filtered from the water, (ii) consumption of tropical fish that accumulated phytoplankton toxins (ciguatera), (iii) respiratory problems due to inhalation of aerosols from sea water that contains toxic species, and (iv) skin irritations due to allergy-like reactions. Toxins can accumulate in many species of bivalve molluscs as well as in some gastropods, crabs and fish; the characteristics of toxification and de-toxification vary remarkably from one vector to another (Shumway et al., 1995). Currently, approximately 2000 cases of intoxication (with a 15% mortality rate) in humans due to consumption of toxic shellfish or fish are

registered annually (Hallegraeff et al., 1995, 2003). However, this is probably an underestimation because many cases, particularly those involving gastrointestinal symptoms, are incorrectly diagnosed. Although many affected areas are nowadays well controlled thanks to monitoring, the occurrence of toxic events in new areas and the lack of control in others pose a serious risk to human health and life.

Fig. 1.3 - The toxin cycle: diagram illustrating the interrelationships between harmful algae and shellfish, finfish, birds and mammals (from James et al., 2010).



2. **Impact on living marine resources**: besides the cases cited above, in which harmful algae may indirectly impinge upon the exploitation of marine resources, several kinds of harmful algae may directly affect wild and cultivated fish or marine invertebrates that are valuable seafood. Most mass mortalities of cultivated fish around the world are generally caused by such ichthyotoxic species as the raphidophytes *Heterosigma akashiwo* and *Chattonella marina*, a number of dinoflagellates and some prymnesiophytes. Harmful algae usually affect fish and other invertebrates by producing toxins, but mucilage-producing or spine-bearing algae may cause mechanical clogging or lesions of the gills. A reduction in oxygen and hydrogen sulphide production can also cause mass mortalities of huge proportions of commercially valuable species. Oxygen depletion can be due to high respiration by algae (at night or in low light during the day) but more often is caused by bacterial respiration during decay of the bloom. Essentially non-toxic bloom formers can sometimes evoke major ecosystem impacts, and unsightly dead fish, slime and foam deter tourism and recreational activities. Whales and porpoises can also become victims when they receive toxins through the food chain via

contaminated zooplankton or fish (Geraci et al., 1989). Poisoning of manatees by dinoflagellate brevetoxins contained in salps attached to seagrass and of pelicans and sealions by diatom domoic acid contained in anchovies has also been reported. Some algal species can seriously damage fish gills, either mechanically or through production of haemolytic substances, whereas other kill fish algae through the production of extracellular neurotoxins. Whereas wild fish stocks have the freedom to swim away from problem areas, caged fish appear to be extremely vulnerable to such noxious algal blooms.

3. **Impact on tourism and on the recreational use of coastal areas**: a requisite for the exploitation of tourism and of the recreational resources in coastal areas is a high quality of the environment, i.e. unaltered seawater colour, transparency, smell. Algal blooms may cause intense discolorations of seawater from golden brown to emerald green, from reddish to whitish, which earned them the name of 'red tides'.
4. **Damage to the marine ecosystem**: a number of HABs affect organisms that may have no commercial value but nonetheless are functional components of the marine ecosystem. The co-occurrence of several conditions is required to generate these situations, i.e. non-limiting light and nutrient supplies and local physiographic and oceanographic conditions that entrap the bloom, thereby amplifying the effects of biomass degradation. Anoxia is not the only mechanism whereby HABs exert a deleterious effect on different components of the coastal ecosystem. They also reduced grazing by microzooplankton and decimated eelgrass beds through severe light attenuation (Cosper et al., 1989). In other cases, specific toxins seem to affect a single population of marine mammals, fish, birds, or other organisms.

1.3. Bloom dynamics

The dynamics of harmful algal blooms cannot be elucidated by research on one or a few organisms; it is in fact difficult to define one kind of coastal area which is more prone to HABs, even when a single species is considered. Indeed, HABs may occur in a variety of areas and hydrographic conditions, including upwelling regions, pristine coastal waters with moderate nutrient levels, and coastal areas affected by shelf-scale circulation.

The concept of high abundance is implicit in the term 'bloom' defined also as a 'significant population increase' (Smayda, 1997), which leads to a peak. The magnitude of this peak is

specific for each species and may vary considerably in space and time, being dependent on environmental conditions. In this sense, a bloom does not imply a high biomass.

Because of the difficulty in detecting low concentrations of planktonic algae, it is not always possible to pre-alert to the harmful events, especially in the case of these extremely noxious species.

On the diametrically opposed end of a scale of danger are species that are noxious only when they reach very high concentrations.

The diversity of bloom dynamics is another aspect to be taken into account. Different interacting physical, chemical and biological factors can trigger a bloom of a species but in different habitats (Zingone and Enevoldsen, 2000). Each species has different adaptive strategies and responds in different ways to the variability of the environment and its changes (Fig. 1.4). It could be argued that any phytoplankton species can exert a negative impact when it persistently dominates phytoplankton populations. Moreover, the expansion of the geographical distribution of a species could be due to an anthropogenic introduction, a natural phenomenon (i.e. currents), or it could be that the species was already in the area but at undetectable concentrations (the case of the NSP producer *Karenia brevis* in New Zealand waters (Hallegraeff et al., 1995)) or present in the distant pass (Dale et al., 1993). Genetic analysis is therefore essential to confirm the invasion of alien microalgae in different coastal areas affected by ballast water or the transfer of shellfish stocks, i.e. *Alexandrium* species expansion in Tasmanian Waters (Scholin, 1996).

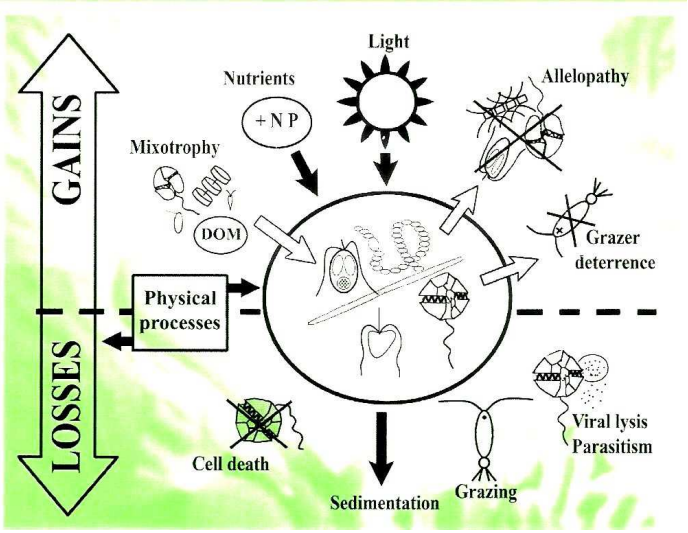


Fig. 1.4 – Factors affecting harmful algae gains and losses. On the upper half the gains include their intrinsic ability to utilize inorganic and organic compounds (mixotrophy), nutrients from anthropogenic origin, and under adverse conditions release allelochemical compounds that kill other algae (allelopathy) or their grazers. On the lower half the losses the harmful algae might suffer: in this case no blooms will be formed or damage to the environment will occur (from Granéli and Turner, 2006).

1.3.1. Human impact

There are still doubts on the extent to which human impact may contribute to HABs; however, factors related to human activities (Fig. 1.5) can be summarised:

(i) the increase in geographical ranges of harmful species distribution due to human induced transport of resting cysts results from movement of shellfish stocks or ballast waters and floating plastic;

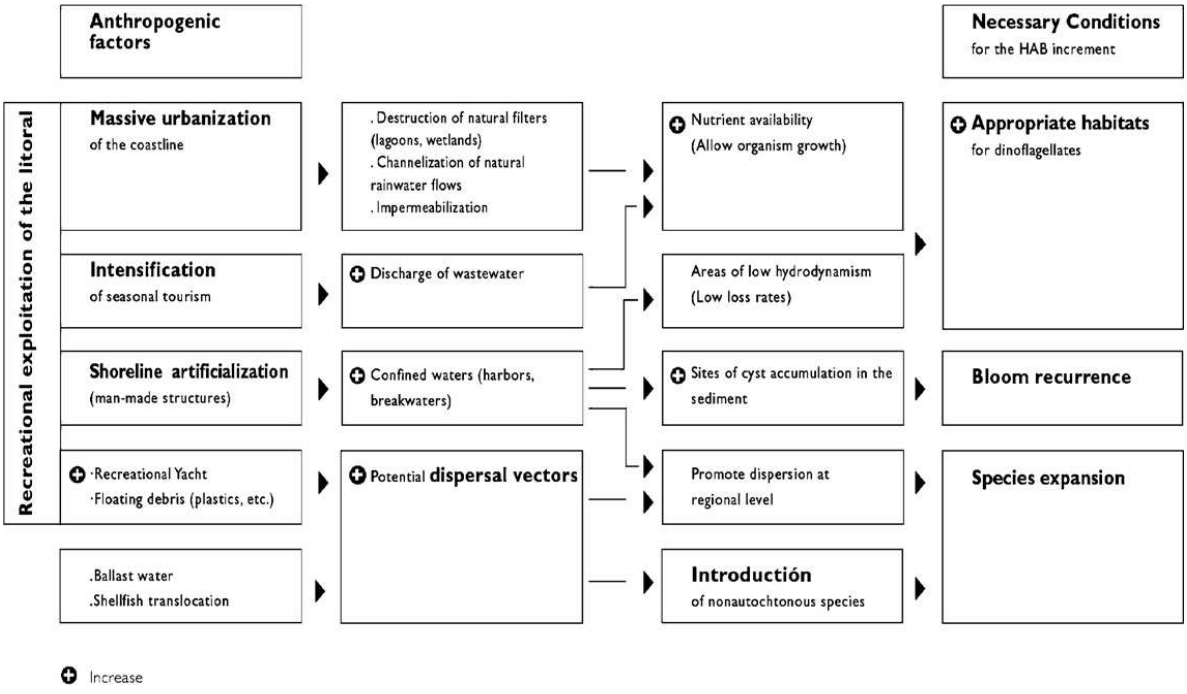
(ii) stimulation due to the over-enrichment of coastal waters (eutrophication);

(iii) human-induced climatic change has also been noted;

(iv) the increase of confined bodies water in coastal areas due to the exploitation of the coastline (coastal development) has been suggested in the Mediterranean Sea (Garcés et al., 2002; Vila et al., 2001);

(v) the decreasing biomass of filter feeding organisms, due to overfishing, or changing environmental conditions.

Fig. 1.5 - Scheme of anthropogenic factors related with recreational activities promoting HAB increase (from Action plans and measures for an integrated control of Mediterranean recreational waters in relationship with harmful algae blooms. http://www.icm.csic.es/bio/projects/wscalvia).



Anthropogenic changes in the ratio of nutrient availability could be one of the key factors in some areas. Coastal zones around the world are in fact subjected to increasingly nutrient

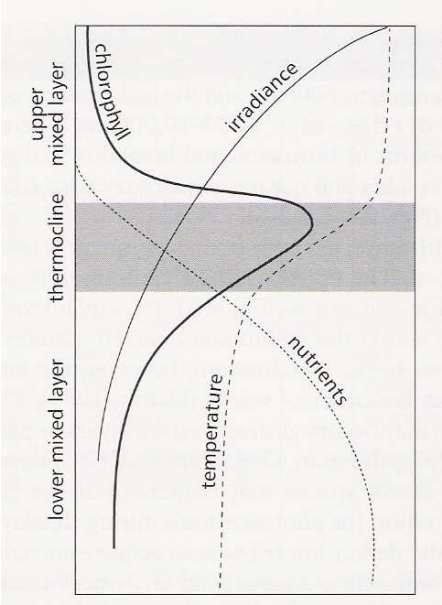
inputs as related to human activities including direct discharges and via rivers, ground water and atmospheric deposition. Anthropogenic sources of nutrients include fertilisers, combustion of fossil fuels, discharge of human waste, and the consequences of animal production and contribute to non-point sources of nutrient loading (Nixon, 1995; National Research Council, 2000). There are well-established positive relationships among nutrient loads in marine systems, and phytoplankton primary production, and fisheries yield (Rabalais and Nixon, 2002). This over-enrichment leads to diverse impacts including increase turbidity with a subsequent loss of submerged aquatic vegetation, oxygen deficiency, disruption of ecosystem functioning, loss of habitat, loss of biodiversity, shifts in food webs, and loss of harvestable fisheries and HABs (National Research Council, 2000; Rabalais and Nixon, 2002).

It is considered in a global context that point sources such as urban waste water and industrial discharges are less important nutrient contributors than non point-sources, which are very difficult to evaluate.

1.3.2. Environmental factors

The taxonomic diversity of HAB species suggests that each species is adapted to some set of preferred environmental conditions or, in ecological terms, to a defined niche.

Conceivably, once the ecological requirements for each species are known, it would be easy to predict its occurrence (Richardson, 1997).



As a result, the capability of a species to cope with environmental conditions apparently not matching its optimal ranges is notably expanded and its occurrence is more difficult to predict (Fig. 1.6). Bloom dynamics are controlled internally through behavioural adaptation. Adaptive strategies such as mobility behaviour (phototaxis, vertical migration, swimming patterns, and aggregation) and life cycle strategies (which include temporary phases and resting cysts) interact with the surrounding environment in development and maintenance of blooms.

Fig. 1.6 – Generic vertical profiles of irradiance, nutrients concentration, temperature and chlorophyll through the water column.

Vertical migration is a mechanism to seek nutrients, but is also a determining factor in the spatial distribution of the population (Prego, 1992; Liu et al., 2002) as well as an adaptation to minimize population loss.

A large part of HABs species research has been dedicated to toxin production and factors that control it. The issue gets more complicated since environmental conditions (light, temperature, salinity and nutrients) can increase or diminish the capability of some species to produce toxins (Cembella, 1998). It has also been said that the same species can be toxic in one area causing very harmful effects, not toxic in other areas, or lose their capability of producing toxins in culture. This statement must be treated with caution; in fact, it may be due to confusion in the identification of the species.

Links between HABs and nutrient loading are complex and imply an understanding of the physiological requirements and the mechanisms of nutrient acquisition by each species. Nutrient assimilation depends on a variety of factors such as the nutritional preferences, uptake capabilities or nutritional status.

Flagellates, including dinoflagellates, have considerable nutritional strategies such as: (i) low nutrient affinity (Smayda, 1997), (ii) preference for reduced N forms (i.e. ammonium and urea) (DeYoe and Suttle, 1994), and (iii) mixotrophy (the ability to acquire N and C via particle ingestion or by the uptake of dissolved organic compounds) (Carlsson et al., 1998; Lomas and Glibert, 1999a,b; Berg et al., 2002, 2003).

Diatoms have an advantage, with respect to flagellates, at low nutrient conditions. A key element in the context of HABs is silica (Smayda, 1997), an element required by diatoms in their walls, as their growth ceases when Si becomes depleted. The ratios N:Si and P:Si have increased substantially in coastal areas affected by human stresses (Justic et al., 1995; Olivos, 2000) and this would favour non-diatom species including several harmful/toxic species (Smayda, 1989).

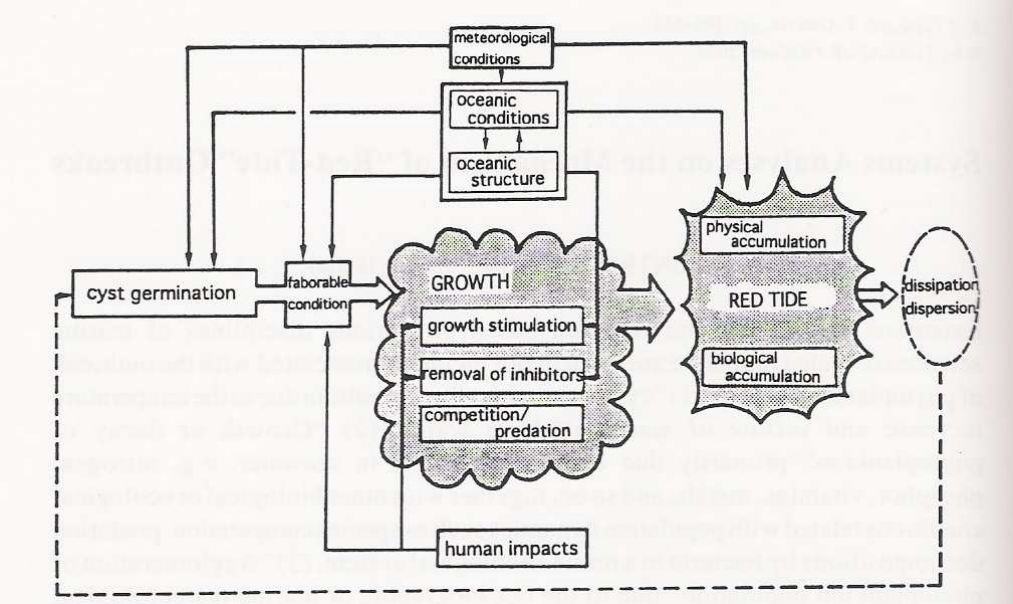
All these cases indicate that complex mechanisms regulate the interactions among different components of the trophic web and that a lowering in diversity of algal population in time and space may pose a serious risk to coastal ecosystems.

1.3.3. Life cicles

Numerous phytoplankton species have complex life cycles, i.e. alternation of stages that differ in terms of physiology, motility, resistance to adverse conditions and life styles. Non motile, benthic resting stages are widely distributed among HAB dinoflagellates and raphidophytes,

and may play a significant role in bloom initiation by inoculating seed populations when conditions are favourable (Fig. 1.7).

Fig. 1.7 - A conceptual diagram representing processes of red-tide outbreaks (from Okaichi, 2003).



At the end of a bloom, cyst formation may represent a mechanism whereby organisms are rapidly removed from the water column so as to prepare the `seed bank' to inoculate the next bloom (Wyatt and Jenkinson, 1997). Within this already complex framework, it appears that encystment rates, dormancy length, and cyst germination triggers can be species-specific and at times strain-specific (Fig. 1.8). The transitions between different life stages of a species are presumably controlled by the interplay of endogenous factors with the physical and chemical environment, but the role and relative importance of these factors for different species are poorly known (Anderson, 1998).

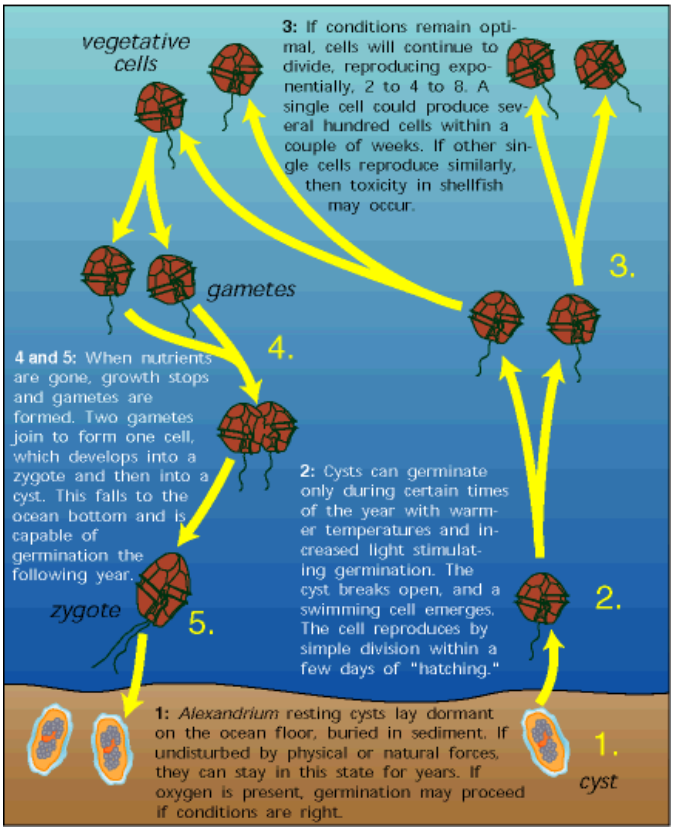
1.3.4. Role of bacteria

Bacterial–algal interactions play a role in dynamics of HABs. This is an ongoing area of research, and efforts are addressed to isolating and identifying the bacteria associated with the species during bloom conditions (Alavi et al., 2001; Hold et al., 2001; Tobe et al., 2001; Vasquez et al., 2001; Biegala et al., 2002; Uribe and Espejo, 2003).

One of the hypotheses explaining the increase in toxic events is that toxic production can provide advantages over other organisms of the community. “Are toxin-producing strains more successful than non-toxic ones?’’ Toxins production has been associated with

allelochemical and allelopathic activities, which inhibit the growth of co-occurring phytoplankton species (Arzul et al., 1999), and act as a deterrent to grazers (Turner and Tester, 1997). Toxin production could thus be a developed adaptation to offset the negative effects of interspecific competition in nutrient limitation conditions, by increasing grazing pressure on non-toxic competitors (Guisande et al., 2002).

Fig. 1.8 - Life cycle of a harmful algae, e.g. *Alexandrium* sp. (Illustration by Don Anderson, Woods Hole Oceanographic Institution).



1.4. HABs and eutrophication

The increase of HAB records in coastal waters around the world has often been associated with nutrients derived from anthropogenic activities (Hallegraeff, 1993; Smayda, 1989). Eutrophication is one of several mechanisms by which harmful algae appear to be increasing in extent and duration in many locations. Although important, it is not the only explanation for blooms or toxic outbreaks. Nutrient enrichment has been strongly linked to stimulation of some harmful species, but for others it has not been an apparent contributing factor.

The term ‘eutrophication’ was formerly used mostly in reference to the natural aging of lakes wherein a large, deep, nutrient-poor lake eventually becomes more nutrient-rich, more

productive with plant and animal life, and slowly fills in to become a pond, then a marsh (Wetzel, 1983). More recently, the term has been used to refer to cultural or accelerated eutrophication of lakes, rivers, estuaries, and marine waters, wherein the natural eutrophication process is advanced by hundreds or thousands of years by human activities that add nutrients (Burkholder, 2000). Nixon (1995) defined eutrophication as 'the process of increased organic enrichment of an ecosystem, generally through increased nutrient inputs'.

Two nutrients in human-derived sources, phosphorus (P) and nitrogen (N), are of most concern in eutrophication. In freshwaters, P is the least abundant among the nutrients needed in large quantity (macronutrients) by photosynthetic organisms, so it is the primary nutrient that limits their growth (Schindler, 1977). P can also limit or co-limit algal growth in estuarine and marine environments that are sustaining high N inputs (Rudek et al., 1991; Fisher et al., 1992). In many temperate and polar coastal marine waters, N is the most important nutrient that limits primary production of photosynthetic organisms (Dugdale and Goering, 1967; Glibert, 1988). N is often the nutrient that first limits primary production at the estuarine interface between marine and freshwater habitats. In lower estuaries both N and P can colimit phytoplankton production (Rudek et al., 1991; Fisher et al., 1992). Other nutrients such as silicon (Si) and iron (Fe) also can significantly influence the outcome of species dominance and the structure and abundance of phytoplankton communities under cultural eutrophication (Heckey and Kilham, 1988; Wilhelm, 1995).

In the laboratory it is easy to demonstrate that algal growth increases with increasing nutrient availability; however, there is scarce evidence for a direct relationship between the spread of harmful events and eutrophication in the sea. High biomass blooms obviously require high nutrient levels, but a high nutrient supply may not necessarily favour harmful species. Also, specialized behaviours, like facultative or obligate mixotrophy (Stoecker, 1999; Granéli and Carlsson, 1998) or the capability of vertically migrating to nutrient-replete water column layers (Hasle, 1950; Villarino et al., 1995), represent very successful adaptations to cope with the vertical segregation of light and nutrients in stratified waters. Moreover, at the ecosystem level, complex trophic relationships, including microbial interactions and excretion from grazers, may enhance nutrient availability and growth rates under apparently oligotrophic conditions (Goldman et al., 1979). Linkages between HABs and eutrophication have been noted within the past two decades (e.g., Officer and Ryther, 1980; Lam and Ho, 1989; Smayda, 1989, 1990; Riegman, 1995; Richardson and Jorgensen, 1996; Richardson, 1997). Coastal waters are receiving massive and increasing quantities of industrial, agricultural, and sewage effluents through a variety of pathways (Vitousek et al., 1997). In many urbanized

coastal regions, these anthropogenic inputs have altered the size and composition of the nutrient pool which may, in turn, create a more favourable nutrient environment for certain HAB species.

The impact of high nutrient supplies and of massive inputs of inorganic and organic compounds in coastal systems undoubtedly merits detailed investigation. In fact, besides the cases of high biomass blooms leading to hypoxic or anoxic events, other negative effects of eutrophication might be worth assessing, e.g. the loss of phytoplankton diversity and shifts in the specific composition of microalgal communities. These changes could influence the fate of primary production and modify the shape of the trophic web in coastal areas, with grave consequences for fisheries and for the exploitation of natural resources.

Despite our increased understanding of the pathways by which nutrients are delivered to ecosystems and the pathways by which they are assimilated differentially by different groups of species, the relationships between nutrient delivery and the development of blooms and their potential toxicity or harmfulness remain poorly understood. Many factors such as algal species presence/abundance, degree of flushing or water exchange, weather conditions, and presence and abundance of grazers contribute to the success of a given species at a given point in time. Similar nutrient loads do not have the same impact in different environments or in the same environment at different points in time.

Many sources of nutrients can stimulate harmful algal blooms, including sewage and animal wastes, atmospheric deposition, and groundwater inflow, as well as agricultural and other fertilizer runoff; yet another source is the growing aquaculture industry in many coastal areas. Nonpoint sources of nutrients (from agricultural activities, fossil-fuel combustion, and animal feeding operations) are often of greater concern than point sources because they are larger and more difficult to control basis (National Research Council, 2000).

HAB species, like all plant-like organisms require certain major and minor nutrients for their nutrition, and these can be supplied either naturally from freshwater and marine biogeochemical processes or through human activities such as pollution. These nutrient sources include dissolved inorganic and organic compounds of various types, as well as particulate nutrients in the form of other organisms or detritus. Nutrients can stimulate or enhance the impact of toxic or harmful species in several ways. At the simplest level, harmful phytoplankton may increase in abundance due to nutrient enrichment, but remain in the same relative fraction of the total phytoplankton biomass. Even though non-HAB species are stimulated proportionately, a modest increase in the abundance of a HAB species can cause it to become noticeable because of its toxic or harmful effects. Specific algal species or species

groups have numerous physiological adaptations that permit them to exploit nutrients differentially (Smayda, 1990, 1997; Anderson et al., 2002; Smayda and Reynolds, 2003). A more frequent response to nutrient enrichment occurs when a species or group of species begins to dominate under the altered nutrient regime.

Some generalities are beginning to emerge with respect to the preference of many bloom-forming species for specific forms of nutrients, as well as the tendency for some blooms to occur when the ratios of nutrient availability or supply are altered. The concept is based largely on the nutrient ratio hypothesis (Tilman, 1977; Smayda, 1990, 1997) which argues that environmental selection of phytoplankton species is associated with the relative availability of specific nutrients in coastal waters, and that human activities have altered these nutrient supply ratios in ways that change the natural phytoplankton community composition and possibly favour harmful or potentially toxic forms. Perhaps the clearest demonstration of the effect of altered nutrient supply ratios involves the stimulation of non-diatom species following changes in the availability of N or P relative to silicate. Diatoms, the vast majority of which are harmless, require silica in their cell walls, whereas most other phytoplankton do not. Since silica is not abundant in sewage effluent but N and P are, the N:Si or P:Si ratios in some lakes, rivers, estuaries, and coastal waters have increased over the last several decades (Schelske et al., 1986; Smayda, 1989, 1990; Rabalais et al., 1996). In theory, diatom growth will cease when silica supplies are depleted, but other phytoplankton classes can continue to proliferate using the excess N and P.

An understanding of physiological responses is further complicated by the fact that the rate of nutrient supply will not necessarily correlate with the rate of nutrient assimilation by the algae, as the latter is controlled by nutritional preferences, uptake capabilities, and physiological or nutritional status. The response by either the total phytoplankton community or individual species within the community also depends on many factors, including interactions with grazers and physical forcing such as turbulence. Grazers may inhibit the development of phytoplankton biomass through their feeding, while at the same time, enhance the regeneration of nutrients through their release and excretion. This in turn will alter the balance of reduced versus oxidized forms of N (Glibert, 1998).

In addition, the assimilation of nutrients by phytoplankton depends on environmental factors such as light, temperature, and water column stability with different environmental effects having differential impacts on different nutrient substrates. The uptake of ammonium and urea are usually thought to be less light dependent than the uptake of nitrate (MacIsaac and Dugdale, 1972; Fisher et al., 1982), and the temperature dependence of ammonium uptake

may also differ from that of nitrate (Lomas and Glibert, 1999a). In recent years, the physiological strategies by which different groups of species acquire their nutrients have become better understood. Rapidly growing marine diatoms have been highly correlated with large and/or frequent additions of nitrate, in part because they have physiological adaptations which allow them to exploit nitrate-rich conditions (Takahashi et al., 1982; Goldman, 1993; Lomas and Glibert, 1999a, b, 2000). Microflagellates, including dinoflagellates, are most frequently associated with low nitrate concentrations, higher ammonium, urea, or dissolved organic nitrogen (DON) supply, and consistent physiological preference for reduced N forms (e.g. Berg et al., 1997; Carlsson et al., 1998; Lomas and Glibert, 1999b). Harmful estuarine dinoflagellates tend to occur in waters that have seasonally high phosphate and nitrate, as well as high DOC and other organic nutrient forms (Burkholder and Glasgow, 1997; Burkholder et al., 1997, 2001a,b; Magnien et al., 2000; Glasgow et al., 2001; Glibert et al., 2001).

Moreover, there is evidence that nutrients can play a major role in the regulation of toxicity in some HAB species, and this can have significant implications to toxin monitoring programs and public health decisions. In some cases, toxicity can increase or decrease dramatically depending on the limiting nutrient. Saxitoxin production by *Alexandrium tamarense* can be 5-10-fold higher in P-limited versus N-limited cells (Boyer et al., 1987; Anderson et al., 1990). Likewise, domoic acid production by *Pseudo-nitzschia multiseries* is inversely correlated with the ambient Si concentration in batch culture (Pan et al., 1996a). In that study, cells began accumulating this toxin only when the division rate declined as a result of partial or total depletion of silica; when cultures were N-limited no toxin was produced, and toxin production was greatly enhanced under P-deficient conditions in continuous cultures (Pan et al., 1996b). For other HAB species a similar picture emerges: toxin production varies significantly with different degrees and types of nutrient limitation.

It is important to recognize that the impacts of nutrient loading depend on many factors, from the species composition and nutritional state of the organisms at the time of the loading, to the physical features of the environment at that point in time, as well as the existence of grazers. Similar nutrient loads will not necessarily have the same effect on a different environment, or on the same environment at a different point in time. Although there have been many successes in relating nutrient quantity and composition to outbreaks of HABs, in general the relationships between nutrient delivery and the development of blooms of many HAB species, and between nutrient enrichment and the potential toxicity of blooms or outbreaks of those species, remain poorly understood.

1.5. Growth dynamics

Phytoplankton growth is classically measured as a whole community response, with chlorophyll used as an index of abundance against which rate processes are normalized.

In reality, community growth is only one of three different, concurrent growth modes which characterize phytoplankton population dynamics: cellular growth, population growth, and community growth.

Cellular growth is the active, basic growth unit. It is the outcome of coupled physiological processes under genetic and multifactorial control, particularly irradiance and nutrient levels.

Population growth is the environmentally modified outcome of cellular growth, the recruitment term; it is also the bloom unit. Population growth is dependent upon the cellular growth rates, but the factors regulating cellular and population growth rates are not identical. Grazing and advection, for example, influence population growth rate, but are irrelevant to cellular growth rates. Population growth rates of a given taxon are always lower than its cellular growth rates. Population expansions, in fact, result from divisions of individual, free-living cells. Red tide species are commonly assumed to be relatively slow growing, with blooms resulting from low grazing pressure and (or) physical accumulation. The early experimental literature generally suggests that daily growth rates of dinoflagellates are $k = 0.3$ day^{-1}, or a generation time of 3 days. Reviews of some of the phytoplankton growth literature (Banse, 1982; Furnas, 1990; Tang, 1995) confirm that diatom growth rates are generally much higher than those obtained for dinoflagellates based on equivalent body mass.

Community dynamics are a consequence of cellular and population growth, not the determinant of the latter. The community is an assemblage of multiple, concurrent species' blooms equal to the total number of taxa present, each in different bloom cycle stages, and each regulated by different combinations of growth factors.

1.6. Affinity coefficient K_s and nutrient acquisition

Red tide blooms occur at both extremes of the nutrient gradient: in oligotrophic and nutrient-enhanced habitats.

Nutrient availability influences nutrient uptake rates, and sets the biomass carrying capacity and, therefore, cellular, population, and community growth rates. Growth-strategy species presumably have elevated maximal uptake rates (Vmax), an adaptation to ensure high velocity uptake of ambient nutrients and pulsed accretions from grazer excretions, transient physical advections, or other opportunistic encounters. Storage-strategy species build up intracellular

storage pools for future growth. In growth-strategy species, such a capability would be advantageous in nutrient-depleted habitats.

Of the three strategies, affinity adaptation has received the most attention. Since affinity-adapted species have low K_s constants, their selection is hypothesized to be favoured in seasons and regions of chronically low nutrient supply rates, where they are expected to outcompete higher K_s species.

Margalef (1978) concluded that diatoms collectively have high K_s coefficients and dinoflagellates lower ones. He reasoned that turbulence, elevated nutrients, and high K_s species should co-occur, because turbulence augments nutrient levels leading to prominent spring and upwelling blooms of diatoms.

The K_s data and their phylogenetic patterns suggest a paradox: collectively, HAB species lack the expected high affinity for nutrient uptake thought to be essential for their frequent bloom occurrences in nutrient-depleted waters.

Flagellates generally have evolved four major adaptations to offset the ecological disadvantages of a high K_s:

1. Nutrient-retrieval migrations

Eppley and Harrison (1975) sketched the basic mechanism of a vertical nutrient-retrieval strategy: diel migration of nutrient-depleted dinoflagellates into NO_3-rich subsurface layers would access nutrients needed to outcompete diatoms and favour red tide blooms. Nutrient-retrieval migrations into cooler, deeper layers would both increase affinity, i.e. lower K_s and increase Vmax:K_s (magnitude depends on the degree of vertical thermal change), and facilitate uptake by the cells now exposed to elevated NO_3 concentrations.

2. Mixotrophic nutrition

Utilization of dissolved organic and particulate nutrients would help offset the ecological disadvantages of a high K_s in an oligotrophic niche. About half of all dinoflagellate taxa are obligate heterotrophs; some have evolved elaborate phagotrophic-feeding mechanisms activated by chemodetection of prey. Almost all HAB taxa are obligate phototrophs; however, their use of supplemental nutritional modes is thus of great interest. Phototrophic flagellates collectively exhibit two primary nutrient supplement strategies: uptake of dissolved organic substances (= osmotrophy) and ingestion of particulate matter (= mixo-phagotrophy).

3. Allelochemically enhanced interspecific competition

Allelochemical will refer to chemically regulated interspecific competition, and allelopathic to antipredation regulation. Phycotoxins (i.e. saxitoxin, brevetoxin, domoic acid, okadaic acid) are distinguished from allelopathic and allelochemical secondary metabolites because of probable differences in their biosynthesis, chemical nature, and specific effects. Phycotoxins and secondary metabolites produced by the same species of certain dinoflagellates and raphidophytes have similar antimicrobial activity (e.g. Nagai et al., 1990). A major difference between phycotoxins and allelo-metabolites is that phycotoxins can be vectored through the food web, accompanied by broad-based trophodynamic effects, whereas allelochemicals are usually directly targeted (Smayda, 1992).

4. Allelopathic antipredation defence mechanisms

Blooms of *Chattonella antiqua, C. marina, C. subsalsa,* and *H. akashiwo* have chronically devastated fish farms in the Seto Inland Sea (Okaichi, 1989). Allelopathic induction of fish avoidance responses is also known. There is considerable evidence, therefore, that HAB flagellates have a diverse allelopathy against a broad spectrum of microbial, zooplanktonic, nektonic, and benthic taxa and of potential significance in natural population dynamics. Poulet et al. (1994) have generalized from experimental evidence that diatoms have evolved an allelopathic, antipredation strategy to reduce copepod population levels by inhibiting their reproductive success.

1.7. Phycotoxin biosynthesis

From a molecular physiological and chemical ecological perspective, toxin classification based upon structural homology presumably reflects shared elements of biosynthetic pathways (Wright and Cembella, 1998). Most if not all polyether phycotoxins are produced via polyketide pathways (Shimizu, 1996), in which acetate units are added sequentially from acetyl-CoA within a pathway regulated by polyketide synthases (PKS). The classical approach is to provide an isotopic enrichment of low-molecular-weight putative precursors (e.g. ^{13}C-acetate) and then follow the incorporation pattern into the target phycotoxin by Nuclear Magnetic Resonance (NMR) spectroscopy. This approach has provide structural elucidation and plausible biosynthetic schemes for key phycotoxins, including saxitoxin and analogues from *Alexandrium tamarense* (Shimizu, 2000), brevetoxins from *Karenia brevis* (Shimizu, 1996), sulphated dinophysistoxins from *Prorocentrum maculosum* (Macpherson et al., 2003) and domoic acid from *Pseudo-nitzschia pungens*. Since many phycotoxins are

derived via polyketide metabolism regulated by PKS, this enzyme complex merits particular attention in gene expression studies. Putative PKS genes have been reported from the diniflagellates *Gymnodinium catenatum, Amphidinium operculum, Prorocentrum lima, Karenia brevis* (Snyder et al., 2005) and *Alexandrium ostenfeldii* (Cembella et al., 2004). However, no definitive attribution can be made regarding their role in biosynthesis of polyketide toxins.

1.8. HABs and climatic fluctuations

It is not possible to demonstrate a worldwide increase in HABs because we are unable to detect long-term trends of algal blooms due to the lack of time series at the correct scale (Wyatt, 1995). However, at a local scale there are numerous examples of HABs in areas where they were previously unknown. It is not clear as to how much of the increase reflects heightened scientific awareness and scrutiny of coastal waters and seafood quality versus an actual increase in the number, severity, or frequency of outbreaks (Anderson, 1989). Many new bloom species are believed to reflect the discovery of hidden flora populations (Smayda, 1989) which had existed in those waters for many years, but which had not been detected or recognized as harmful until the advent of more sensitive toxin detection methods or an increase in the number and training of observers (e.g., Anderson et al., 1994).

The passive introduction of species from other sites by means of natural (currents, storms) or man-mediated events (e.g. ballast waters, keels, mussel strains transport) has been implicated in variations in the geographic range of a species, which has also been supported by the absence of cyst records prior to certain dates (McMinn et al., 1997). Sediment investigations on resting cysts provide further information on planktonic organisms with benthic stages, and may lead to the detection of rare species that eluded planktonic sampling (Montresor et al., 1998). Similar investigations on fossilized resting cysts provide data on the distribution of a species over a wider time scale, which covers different hydrographic situations alternating over the geological periods in given areas.

Climatic variations can also affect the spatial distribution of a species. Temperature variations not only directly affect the geographic range and the magnitude of certain blooms, but can also induce changes in circulation patterns, prolong stratification periods and cause variations in the physical structure of the water column that can favour particular species, including dinoflagellates and their potentially harmful representatives (Yin et al., 1999; Fraga and Sanchez, 1985; Tester et al., 1993).

The issue of a global increase in harmful algal blooms has been a recurrent topic in recent years. Four explanations for this apparent increase in algal blooms have been proposed:

(i) increased scientific awareness of toxic species;
(ii) increased utilization of coastal waters for aquaculture;
(iii) stimulation of plankton blooms by cultural euthrophication and/or unusual climatological conditions;
(iv) transportation of dinoflagellate resting cysts either in ships' ballast water or associated with translocation of shellfish stocks from one area to another.

1.9. Coastal waters for aquaculture

As a consequence of the increased overfishing problems in coastal waters, more and more countries are looking towards aquaculture as an alternative. Aquaculture operations act as sensitive 'bioassay systems' for harmful algal species and can bring to light the presence in water bodies of organisms not previously know to exist there. There is no doubt that the growing interest in utilizing coastal waters for aquaculture is leading to greater awareness of toxic algal species.

A more widespread problem for fish farmers is the production by various algal groups of fatty acids or galactolipids which damage the epithelial tissues of the gills. Fish death may be caused by capillary haemorrhage, dysfunction of gas exchange at the gills, suffocation from an overproduction of mucus, or even from secondary infection of the damaged tissue (Yang and Albright, 1992).

In experimental assay systems these substances destroy red blood cells and therefore have been provisionally termed 'haemolysins' (Yasumoto et al., 1990). Algal species as diverse as the raphidophytes *Heterosigma akashiwo*, *Chattonella antique* and *C. marina*, the prymesiophytes *Chrysochromulina polylepis* and *Prymnesium parvum*, and the dinoflagellate *Karenia mikimotoi* have been implicated.

In countries that pride themselves on their disease- and pollution-free status for aquaculture, every effort should be made to quarantine sensitive aquaculture areas against the unintentional introduction of non-indigenous harmful algal species. Furthermore, no aquaculture industry can avoid having to monitor for an increasing number of harmful algal species in the water column and for an increasing number of algal toxins in seafood products.

1.10. Algal cysts in ballast water

The geographic range of microalgal species can expand depending on natural factors (climate change, catastrophic storm events, ocean currents, transport of spores via wind or bird feet) or human-mediated vectors. Cargo-vessel ballast water was first suggested as a vector in the dispersal of non-indigenous marine plankton some ninety year ago. However, in the 1980s the problem of ballast-water transport of plankton species gained considerable interest when evidence was brought forward that non-indigenous toxic dinoflagellate species had been introduced into sensitive aquaculture areas of Australian waters, with disastrous consequences for commercial shellfish farm operations (Hallegraeff and Bolch, 1992). One single ballast tank was thus estimated to contain more than 300 million toxic dinoflagellate cysts which could be germinated into confirmed toxic cultures. Impacts on shellfish and finfish aquaculture operations may result in cases of toxin-producing microalgae.

Another vector for the dispersal of algae (especially their resting cysts) is with the translocation of shellfish stocks from one area to another. Viable transport of dinoflagellate cells and cysts of *Pfiesteria piscicida, P. shumwaye, Karenia brevis, K. mikimotoi, Alexandrium monilatum, A. tamarense* and *Prorocentrum minimum*, after passage through the digestive tract of shellfish, have also been demonstrated (Scarratt et al., 1993).

The most effective measure to prevent the spreading of dinoflagellate cysts via ship's ballast water would be to avoid ballasting during toxic dinoflagellate blooms in ports. Other options using heat, electrical shock or chemical treatment (chlorine, hydrogen peroxide) of ballast water, either in hold or in onshore facilities, have also been explored. Minimizing the risk of ballast water introductions by microalgae and their cysts represents a very significant scientific and technological challenge, which cannot yet be adequately achieved with best currently available technologies and will be high on the research and development in the future.

1.11. Management perspectives

Harmful algal blooms are apparently an antithesis to the concept of 'health of the ocean'. However, we have argued that in most cases HABs do not damage marine ecosystems, nor impair their sustained biological functioning. Though extremely dangerous for human health and deleterious for the commercial exploitation of coastal areas, these blooms are natural phenomena with barely evident negative effects on coastal oceans. On the other hand, the health of the oceans and the sustainable development of coastal marine ecosystems can be

endangered by human activities that reduce habitat and species diversity. Correct management is required to mitigate the threat posed by HABs to the economic development of coastal areas and to human health, and ad hoc procedures designed to prevent harmful events or to accelerate their termination are the ultimate goal for HAB management.

Harbours may have low flushing rates and are also situated in populated areas, which, in general, mean high nutrient contents. They are also subjected to a high traffic of commercial vessels that are known to be important vectors in the translocation of resting cyst of harmful phytoplanktonic species, and thus act as reservoirs for resting cysts (Garcés et al., 2004).

Since the monitoring of toxin producing species has mainly been associated with shellfish farming, and moreover, the risk of toxin contamination could become even greater in areas not subject to legislation of local/regional aquaculture activities, to reduce impacts in human health and economic activities, the following elements should be taken into consideration:

(i) reliable monitoring networks and databases that allow for the analyses of the expansion of these organisms,

(ii) established channels of exchange of information among scientific and environmental managers,

(iii) outreach and education programs and easy access for users to monitoring networks results (e.g. Institution's WEB sites),

(iv) implication of the medical sector (the consequences in public health are underestimated, necessity of epidemiological studies).

At present, capillary monitoring seems to be the only tool we have to protect ourselves and food resources of marine origin from the deleterious effect of HABs. The key to predict HAB phenomena is to identify and quantify adaptations of HAB species that lead to their selection, in particular hydrodynamic and ecological conditions. Thus, the central research problem and a challenge for biological oceanographers is to understand the critical features and mechanisms underlying the population dynamics of HAB species.

Another gap in our knowledge of phytoplankton, including potentially harmful species, are the long-term trends of occurrence and abundance of these organisms, which are related with climatic factors and with their influence on coastal hydrography.

This would provide the data needed to detect trends and patterns of occurrence and distinguish the effects of natural variability from those of anthropogenic modifications of the environment.

Finally, newly discovered toxins should be characterised, and detection and analytical methods devised in order to clarify the different types of toxins.

1.12. Mediterranean HABs

Dense blooms of phytoplankton are a widespread phenomenon of the global coastal ocean.

In contrast to large-scale blooms that are dominated by mesoscale circulation, Mediterranean HABs are a more localized phenomenon commonly related to areas of constrained dynamism, such as bays, lagoons, ports, beaches and estuaries (Garcés et al., 1998, Vila et al., 2001, Lopez-Flores et al., 2006). In these areas, enhanced growth of phytoplankton not only leads to a perceivable water discoloration along the shoreline but also to a deterioration in water quality. Other unprecedented ecological effects in the Mediterranean, such as fish kills (Garcés et al., 2006) and risks to human health (Penna et al., 2006), have been attributed to toxic algal proliferations in recent years.

Despite the fact that in some cases the proliferation of algae may have a natural origin, it is considered that coastal blooms are an emerging problem that could be related to nutrient enrichment of coastal waters (Duarte et al., 2000). Intensive urbanization and recreational use of coastal watersheds has resulted in a remarkable increase in sources of nutrients along the Mediterranean coasts. This cultural eutrophication generates a contrast between coastal waters and the open ocean where, owing to summer stratification and nutrient depletion, oligotrophic conditions prevail in the upper layer. Nutrient-rich coastal environments of the Mediterranean Sea and, in particular, semi-enclosed areas with low turbulence levels constitute a new and unique environment for which several phytoplankton species with harmful effects may become dominant.

Even though most of the factors involved in the Mediterranean nearshore algal outbreaks are known, the mechanisms that underpin their occurrence are not yet well established. Terrestrial nutrient loads, toxin production, species diversity, grazing pressure, life cycles and strategies, physical transport, mixing and other factors have all been used to explain the onset and evolution of phytoplankton blooms. It is therefore challenging to understand how all of these different factors combine to stimulate and govern outbreaks.

Various external and/or internal factors have been proposed in attempts to explain changes in growth rates of phytoplankton populations:

1. Intraspecific genetic variability changes over time within the same population are possible (Orsini et al., 2002). This suggests that only a fraction of the resident

population is responsible for the bloom; furthermore, differences among growth rates of the different strains are possible.

2. Excretion of chemical signals produced by the organisms could be responsible for the different physiology.
3. More attention has been devoted to the effect of environmental factors (such as irradiance, nutrients, turbulence, vitamins and, particularly, temperature) on growth (Guillard, 1973 and references therein). Temperature is known to alter the enzymatically regulated processes of most organisms, and hence should not be disregarded as a triggering factor of the previously mentioned mechanisms. Indeed, seasonal temperature variations are known to play a major role in the regulation of growth rates of coastal communities in the Mediterranean Sea.

It is generally recognized that there have been more coastal algal blooms, often of greater geographic extent and/or longer duration, with more toxic species observed, more fisheries affected, and higher associated costs from HABs in the past decade than in previous decades (Anderson, 1989; Smayda, 1990; Hallegraeff, 1993; Glibert et al., 2001; Anderson et al., 2002).

However, results from laboratory studies are not sufficient to predict the succession of phytoplankton species and blooms of specific harmful organisms in the sea. Indeed, the net growth performance of a species is affected by complex interactions with other organisms, which are scarcely reproducible in laboratory experiments. These include negative interactions, such as grazing, competition, and viral infections, and a positive feedback from predator's excretion, bacterial nutrient regeneration and viral lysis.

2. Aim of the thesis

The main objective of the present thesis was the study of some harmful algae species which are present in Italian waters and cause blooms, leading to consequences for human health, coastal ecosystem, fishery and tourism. In particular, the attention was put on a raphidophyte (*Fibrocapsa japonica*) and a benthic dinoflagellate (*Ostreopsis* cf. *ovata*), which was studied in laboratory surveys using batch cultures.

The first part of this thesis (chapter 3) was focused on the Adriatic *F. japonica*, through the evaluation of its toxicity for different organisms, from bacteria to crustaceans and fish, as its toxic effects for animals, including species important for fishery, and humans were still unknown. The aim was also to understand the toxic mechanism, basing on the different hypothesis postulated in previous works and performed with strains from different regions worldwide.

Moreover, a chemical characterization and quantification of the fatty acids content, involved in the toxic mechanism of this species, was performed. Finally, as brevetoxins were considered as one of the main factors responsible for the toxicity of the raphidophytes, sensitive liquid chromatography-mass spectrometry (LC-MS) methods for the analysis of brevetoxins in algal extracts were developed and used to investigate the brevetoxins production in some Adriatic raphidophytes, including *F. japonica*.

A second part of this thesis (chapter 4) regarded the dinoflagellate *O.* cf. *ova*ta, with the aim to evaluate its growth and toxicity variations in response to environmental variables. In particular, several strains isolated in recent years in Italian coastal areas were grown at different environmental conditions to investigate the optimal growth and to understand how the growth parameters could affect the presence, proliferation and toxicity of *O.* cf. *ovata* in these areas. This species, in fact, showed a relevant variability in the proliferation along the Mediaterranean coasts, reporting different environmental parameters and toxic effects during its blooms. Effect of growth temperature were evaluated using different isolates, namely a Tyrrhenian (from Latina) and two Adriatic (from Ancona and Bari) strains; while a detailed study on the effects of salinity and nutrients concentration was performed using the Adriatic strain. Toxicity was investigated either through bioassays, such as *Artemia* sp., sea bass ichtyotoxicity and haemolysis assay, and through LC-MS analysis to quantify the toxin content.

Both the toxins produced by *O.* cf. *ovata* and brevetoxins, which are supposed to be produced by *F. japonica*, are polyketides. Little is known about the toxin biosynthetic pathways in the

dinoflagellates; however, recent data from the dinoflagellate *Karenia brevis* indicated polyketide synthases (PKSs) as enzymes probably involved in the brevetoxin biosynthesis. These enzymes are multi-domain complexes that structurally and functionally resemble the fatty acid synthases (FASs). To date, approximately 25 species of dinoflagellates have been found to produce polyketides, but no information exists on PKS proteins of other toxic dinoflagellates. Thus, the third part of this thesis (chapter 5) was focused on the study of the expression and localization of PKSs in *F. japonica* and *O.* cf. *ovata*, using antibodies developed against *K. brevis* PKS proteins with the aim to understand the involvement of PKS proteins in the toxin production.

3. The Raphidophyte *Fibrocapsa japonica*

The Raphidophyceae *Fibrocapsa japonica* Toriumi and Takano is one of the most recurrent harmful algal bloom (HAB) species along the coast of the Adriatic Sea. *F. japonica* blooms have been related to massive fish mortality in the Seto Inland Sea (Japan) (Iwasaki, 1971; Okaichi, 1972, 1989; Toriumi and Takano, 1973); since 1990 this microalga has frequently been observed also in European coastal waters (Vrieling et al., 1995) where its involvement in toxic events has been sometime reported (European Commission, 2003), although without the support of direct evidence. A number of laboratory studies were thus made to evaluate possible toxic effects of European strains. At first, Khan et al. (1996) analyzed the toxin composition of a *F. japonica* strain isolated from the Dutch part of the North Sea in 1993 and five neurotoxic components, corresponding to brevetoxins, were identified. Other studies conducted on a German strain reported toxic effects on *Artemia salina* nauplii, inhibition of *V. fischeri* bioluminescence and haemolysis of human red blood cells; the presence of brevetoxins was excluded while the three haemolytic compounds in the methanol extract were identified as polyunsaturated fatty acids (PUFAs) (Fu, 2003; Fu et al., 2004a). Another toxicological study on *F. japonica* found that a German strain produced one or more bioactive compounds, having a light-dependent inhibitory effect on *Vibrio fischeri*'s luminescence (van Rijssel et al., 2008). These authors supposed that haemolytic compounds and PUFAs, which are also excreted into the medium, or their combination, could inhibit bioluminescence.

Studies on toxicity where performed also on strains from different geographical areas, sometimes with high variability (Guidi-Rontani et al., 2010). Although in Japan this species is regarded as toxic, in the near Korea, in New Zealand and in the North Sea no *F. japonica* blooms have been associated with fish mortality (Cho et al., 1999a,b), an aspect that contrasts with the results of experimental studies performed on isolates from New Zealand, South Carolina and Germany, which evidenced mortality effects on fish (Khan et al., 1996; Bridgers et al., 2004). Different effects of the New Zealand and Japanese isolates on *Artemia salina* were described by Rhodes et al. (1993), the first being non-toxic and the second one causing acute distress to *Artemia*. Cho et al. (1999b) tested the same *F. japonica* strains for neurotoxicity, by mouse bioassay and neuroblastoma assay and both were non-toxic to mice. A recent comparative study on the haemolytic activity of cell extracts of USA, Japan, Australia, New Zealand, the Netherlands and Germany strains was conducted by de Boer et al. (2009). They found that between 7% and 89% of the haemolytic activity was attributed to

the PUFAs and that other light-dependent haemolytic compounds were mainly involved in the activity of those strains with the highest haemolysis values.

Despite the attention to their toxicity, the toxic mechanism of the raphidophytes is still unclear. PUFAs were mostly claimed as the cause of haemolytic effects and fish mortality together with reactive oxygen species (ROS) production (Mostaert et al., 1998; Marshall et al., 2002; Fu et al., 2004a,b). Marshall et al. (2003) investigated the ichthyotoxicity of *Chattonella marina* and postulated that free fatty acids in combination with high levels of ROS damaged gill membranes, resulting in fish mortality. Fish exposed to *Chattonella* sp. reported a decrease in oxygen partial pressure of arterial blood, as earliest physiological disturbance, while ROS produced by this flagellate may induce excessive secretion of mucus on gill surface; thus the toxic effect of *Chattonella* sp. on fish may be due to a decrease of O_2 transfer, resulting in asphyxia (Oda et al., 1997). In addition, the lipooxygenase-promoted oxidative cleavage of PUFAs can generate highly reactive aldehydes. One of these secondary oxidation products is malondialdehyde (MDA), which has been inferred to have mutagenic and cytotoxic effects. Therefore, MDA is considered a good tracer of the involvement of free radical damage in pathologies associated with oxidative stress (Seljeskos et al., 2006).

Since the toxicity of *F. japonica* is still under debate and its effects highly dependent on the strains, the aim of this study was 1) to define the potential toxicity of Adriatic strains which were never investigated before and 2) to understand the involvement of fatty acid and ROS in toxicity. The first purpose was achieved by analyzing the presence of toxic compounds previously found in different strains (brevetoxins, PUFAs, ROS); the second one by performing toxicological assays with various *F. japonica* cellular, subcellular and extracellular fractions and through fish assays and subsequent water and fish gill analyses.

3.1. Resting cysts

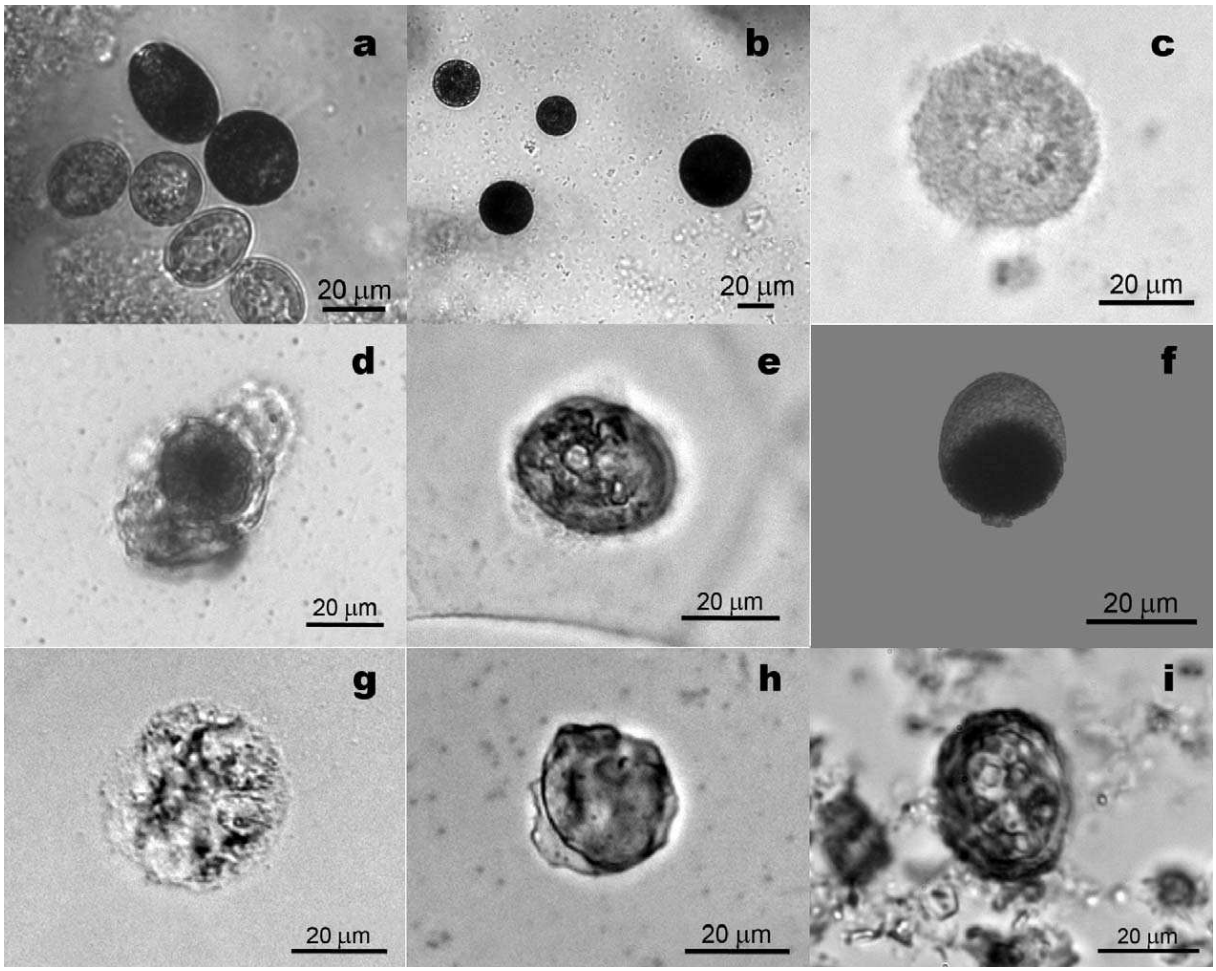
The production of resting cysts has been particularly studied for dinoflagellates under many aspects, such as encystment (Anderson, 1980; Blanco, 1995; Kremp and Heiskanen, 1999; Garcés et al., 2004), dormancy (Anderson and Keafer, 1987; Montresor and Marino, 1996; Figueroa et al., 2008) and germination (Binder and Anderson, 1987; Figueroa et al., 2006; Blanco et al., 2009), which have already been clarified. Cyst strategy allows the survival of species through adverse conditions and it contributes to increase their spatial distribution (Anderson and Wall, 1978; Steidinger and Haddad, 1981; Hallegraeff, 1993), as they can reside in the sediment for a long time and germinate when the environmental conditions become favourable. Consequently, resting cysts play an important role in the life cycle of

many microalgae. The capability of producing resting cysts is also known for several Raphidophyceae, such as *Chattonella* spp., *Heterosigma akashiwo*, *F. japonica* and *Gonyostomum semen*. In *Chattonella*, it is known that encystment may be affected by different factors, such as nutrient depletion, adherence to solid surfaces and low light irradiance (Imai, 1989, 1990; Nakamura and Umemori, 1991; Edvardsen and Imai, 2006). In *Chattonella antiqua* and *C. marina* small pre-encystment cells are produced after meiosis in nutrient-depleted conditions, and then they can change into resting cysts under low light conditions (Imai et al., 1998). Smayda (1998) described two distinct benthic overwintering stages in *H. akashiwo*: non-motile vegetative cells encapsulated by mucilage and smaller resting cysts. In the freshwater species *G. semen*, Figueroa and Rengefors (2006) recently described the entire life cycle, which includes the formation of both asexual and sexual cyst stages. On the contrary, knowledge of the life cycle of *F. japonica* is limited. The first report about *F. japonica* cysts is relative to sediments collected in the Inland Sea of Japan (Yoshimatsu, 1987). The cysts of *F. japonica* were described as spherical, 15-20 mm diameter, brown coloured, smooth and without any particular paratabulation or ornamentation, and lacking a red pigmented body and black spots; these cysts were frequently observed adhering to diatom frustules (Yoshimatsu, 1987). Subsequently Matsuoka and Fukuyo (2003) confirmed this description, reporting a similarity with cysts of *Chattonella* spp. In experimental conditions, cysts of *F. japonica* were observed only once by de Boer et al. (2004), who reported that they were embedded in a brown biofilm; each cyst seemed to have a membrane-like surface over a smooth scale-like inner layer. Moreover, empty cysts resembled the statospores of chrysophyte microalgae. These are the only existing descriptions of *F. japonica* cysts and there are only a few published images to support studies on their identification. In addition, while it is well-known that the cysts of *F. japonica* have dormancy periods of 2–3 months in sediments, their role in bloom dynamics is unclear, because of the scarcity of eco-physiological studies on this species (Edvardsen and Imai, 2006).

Cucchiari et al. (2010) reported for the first time *F. japonica* cysts abundances in natural sediments in the Mediterranean Sea. PCR-based assay confirmed the presence of *F. japonica* cysts in the sediment samples which were analyzed under the microscope and helped in the species-specific identification. The study demonstrated the presence of these cysts in several stations of the Marche region, both in areas where blooms occurred and where vegetative cells were never observed in the water column. Different morphotypes (Fig. 3.1) were observed for *F. japonica* cysts: a first morphotype was characterized by a rounded shape, brown-black colour and was covered by a brown biofilm; while the second was from spherical to slightly

pear-shaped, smooth on the surface and showed inner spots of a pale colour, similar to descriptions reported in literature (Yoshimatsu, 1987; Matsuoka and Fukuyo, 2003; de Boer et al., 2004).

Fig. 3.1 - Different morphotypes of *Fibrocapsa japonica* cysts. Preliminary cysts formed in shadow and optimal temperature (a) and with dark and low temperature treatment (b); (c) preliminary cyst after disruption; (d) cyst type-1: round-shaped, brown-black coloured and covered by a mucilaginous mat; (e) cyst type-2: smooth on the surface and with inner grains of a pale colour; (f) cysts resembling chrysophyte statospores; (g) cyst type-1 after crumbling; (h) cyst type-2 after crumbling: a rigid covering appears; (i) cysts of *Fibrocapsa japonica* observed in natural sediments (from Cucchiari et al., 2010).



3.2. Experimental section

3.2.1. Chemicals and reagents

Tetrahydrofuran (THF) was purchased from Fluka; acetonitrile; 5,8,11,14-eicosatetraenoic (arachidonic) acid; 5,8,11,14,17-eicosapentaenoic acid (EPA); nonadecanoic acid; 9-

hexadecenoic (palmitoleic) acid; 9,12-octadecadienoic (linoleic) acid; 9,12,15-octadecatrienoic (linolenic) acid; brevetoxin 2, ammonium iron (II) sulphate hexahydrate; 2,6-Di-*tert*-butyl-4-methylphenol; *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA-TMCS 99:1); chloroform; $CDCl_3$; Na_2EDTA; pyridine; D-sorbitol; 1,1,3,3-tetraethoxypropane 97%; 2-thiobarbituric acid (TBA) and xylenol orange were purchased from Sigma-Aldrich; $Na_2HPO_4.2H_2O$; ethanol 99.8%; methanol 99.9%; sulphuric acid 96% and trichloroacetic acid (TCA) were purchased from Carlo Erba. All chemicals were used without further purification.

3.2.2. Algal cultures

Fibrocapsa japonica cells were isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from blooms which occurred in 2004 and 2006 in Emilia-Romagna and Marche region (Italy) having cell concentrations in the order of 10^7. After initial growth in microPlates, cells were kept in sterile Erlenmeyer flasks sealed with cotton at 20°C, during a 16:8 h light–dark period at about 100-110 μmol photons m^{-2} s^{-1} from cool white lamps (McLachlan, 1973).

F. japonica strains were cultured in f/2-Si medium (Guillard, 1975) made with natural seawater, adjusted at a salinity of 35 through the addition of distilled water, and kept under the temperature and the light conditions described above. Several of the reported analyses and assays were conducted using different Adriatic strains isolated in 2004 and 2006 near Riccione (Emilia Romagna, Italy) and in Palombina (Marche, Italy) and since no significant differences were evidenced, the results reported in this paper refer to the strain FJAP0603 isolated in 2006 from Palombina. The growth of this strain had been previously characterized (Cucchiari et al., 2008); the cultures used in the present work showed a slightly lower growth rate of 0.68 and mean cell biovolumes of 3470 ± 1200 μm^3 measured in the stationary phase.

Few experiments were conducted with cell in the exponential phase but most cultures were collected during the stationary growth phase (day 12-18); differences in cell numbers used for the different tests are due to the fact that cultures were sometimes concentrated in order to reach higher cell amounts. Cell counts were made in settling chambers following Utermohl's method (Hasle, 1978).

A number of different microalgae were used in the different experiments for comparisons or as controls. These were: *Alexandrium lusitanicum* E. Balech, *Gonyaulax fragilis* (Schütt) Kofoid, *Karenia brevis* (C.C. Davis) G. Hansen & Ø. Moestrup, *Phaeodactylum tricornutum*

Bohlin, *Protoceratium reticulatum* (Claparède & Lachmann) Butschli, *Scrippsiella* sp., *Skeletonema marinoi* Sarno & Zingone; their origin, strain number and culture conditions are reported in Table 3.1.

Table 3.1 - Species, strains, isolation details and culture conditions of microalgal species used for the experiments.

Species	Strain	Isolation details	Temperature (°C)	Salinity (psu)	medium
Fibrocapsa japonica	FJAP0603	Palombina (AN), Italy	20	35	f/2-Si
Alexandrium lusitanicum	ALA9501	Trieste, Italy	20	25	f/2-Si
Gonyaulax fragilis	GFA0201	Cesenatico (FC), Italy	20	30	GP
Karenia brevis	CCMP2281	Gulf of Mexico, Florida, USA	20	35	f/2-Si
Phaeodactylum tricornutum	PTN0301	North Sea, Holland	20	35	f/2
Protoceratium reticulatum	PRA0414	Cesenatico (FC), Italy	20	35	f/2-Si
Scrippsiella sp.	SCA9701	Lido di Dante (RA), Italy	20	30	f/2-Si
Skeletonema marinoi	CCMP2497	North Adriatic Sea, Italy	20	35	f/2

3.2.3. Sample extraction and fractionation

Stationary phase *F. japonica* and *K. brevis* cultures (1.5-5 mL) were filtered under vacuum using two different overlapping filters (GF/C Whatman, 1.2 μm and Millipore, 0.45 μm), that were extracted in a Soxhlet apparatus with methanol-chloroform (50:50). The solution was concentrated to dryness in a rotavapor at 40°C. The extract was left to cool at room temperature and weighed, then it was dissolved in methanol (5 mL) and stored at -20°C. An aliquot of *F. japonica* extract (3 mL) was dried, resuspended in 50% methanol and passed

through a C18 cartridge (Waters SEP-PAK Vac. 20 cc, 5 g). Fifty-four different fractions were collected by elution with a gradient of aqueous methanol, from 0% to 100% in steps of 10%, 5 mL each. The eluates were separately evaporated to dryness under vacuum at 40°C, resuspended in 500 µL methanol and stored at -20°C.

After Gas Chromatography-Mass Spectrometry (GC-MS) analyses, fractions with similar composition were combined into five final fractions (A, B, C, D, E). Each fraction was dried in a rotavapor at 40°C and weighed (Table 3.2).

Table 3.2 – Combined fractions of *F. japonica* extract obtained after C18 SPE fractionation.

Fractions	**Eluent (**H_2O/CH_3OH)	**Weight (mg)**
A	20/80	1.8
B	20/80-10/90	6.8
C	10/90	5.6
D	10/90-0/100	6.8
E	0/100	2.8

3.2.4. Extracellular fatty acids extraction

F. japonica (1540 mL) and *P. tricornutum* (1500 mL) stationary phase cultures were filtered by gravity through a GF/F Whatman filter (0.7 µm) to remove algal cells from the medium, that was subsequently stored at -20°C. Frozen medium was lyophilized and treated according to Bligh and Dyer (1959) with a mixture of 100 mL methanol, 50 mL chloroform, and 40 mL water, sonicated for 10 min and shaken overnight for maximum extraction of fatty acids. The mixture was vacuum-filtered using a Buchner funnel through Whatman (hardened 50) filter paper. After filtration the supernatant was centrifuged at 10000 x *g* at 8°C, added with 50 mL chloroform and 50 mL water; after separation, the methanol-water layer was extracted again with 50 mL chloroform, and the extracts were combined. The solid residue was added again with water and chloroform as described above. Finally, the combined chloroform layers were dried, re-dissolved in methanol (1.5 mL) and stored at -20°C.

3.2.5. Gas Chromatography-Mass Spectrometry (GC-MS) analysis

One or two hundred microlitres of each *F. japonica* fraction, of algal extract and of the extracted medium were dried in a rotavapor at 40°C and then added with 100-150 μL Tri-Sil reagent (BSTFA-TMCS 99:1) at 70°C for 30 min to convert alcohols and carboxylic acids into the corresponding trimethylsilyl (TMS)-ethers and esters, respectively.

GC-MS analysis was performed on a GC Agilent Technologies 6850 Network GC System equipped with a capillary column SUPELCO SPB-5 (poly[5%diphenyl/5%dimethyl]siloxane) (30 m × 0.25 mm × 0.25 μm film thickness), coupled to an Agilent Technologies 5975 inert Mass Selective Detector operating in electron impact ionization (70 eV).

The oven temperature program was: 50°C for 5 min, heating to 310°C at a rate of 10°C min^{-1}, and hold at 310°C for 15 min; injector temperature was 250°C and the transfer line 280°C. High purity helium was used as a carrier gas at a flow rate of 1 mL min^{-1}. A split 1:10 injection mode was applied. Identification of compounds was done by comparison of their retention times and of mass spectra with authentic standards (Sigma-Aldrich) when possible, or by comparison of their mass spectra with Nist libraries.

Calibration curves were produced from standard solutions (four different concentrations from 7 to 26 μg L^{-1}) for the following acids: palmitoleic acid (R^2 = 0.9768), linoleic acid (R^2 = 0.9907), linolenic acid (R^2 = 0.9955) and eicosapentaenoic acid (R^2 = 0.9946). Nonadecanoic acid was used as the internal standard. Response factors (RF) obtained from the calibration curves were used for the quantification of fatty acids in the algal extracts and extracellular media: for 16:1n-7, 16:1 isomer and 16:2 was used the RF of palmitoleic acid (16:1n-7); for 18:1n-9, 18:1n-7 and 18:2n-6 the RF of linoleic acid (18:2n-6); for 18:3n-6, 18:3n-3 and 18:4n-3 the RF of α-linolenic acid (18:3n-3); for 20:4n-6 and 20:5n-3 the RF of eicosapentaenoic acid (20:5n-3). RF of 1 was applied for saturated fatty acids.

3.2.6. Liquid Chromatography-Mass Spectroscopy (LC-MS) analysis

LC-MS analyses of algal extracts and of *F. japonica* fractions were performed by using an Agilent Technologies HP1100 coupled to an Agilent Technologies MSD1100 single-quadrupole mass spectrometer equipped with an API-ES chamber. LC separations were performed on a ZORBAX-Eclipse XDB-C8 Agilent Technologies column with column temperature of 40°C. The mobile phase consisted of water (A) and acetonitrile (B) in binary system, with 0.1% acetic acid as an additive. The column gradient was 35% B for 2 min, a linear gradient to 80% B at 30 min, 95% B at 35 min, hold at 95% for 15 min, return gradient

to 35% B at 60 min, and hold for 10 min before the next injection. Sample injection volume was 5 μL.

The mass spectrometer with an API-ES interface was operated in positive or negative mode. Tune settings were: drying gas temperature 350°C; drying gas flow 11.5 mL min^{-1}; capillary voltage 4500V in positive mode and 3500V in negative mode. Mass spectra of the analytes were acquired in full scan mode in the range *m/z* 50-2600.

For brevetoxin analyses *F. japonica* extracts were compared with those of *K. brevis*, a species known to produce brevetoxins. The extracts were prepared either as described above or according to the method described by Abraham et al. (2006).

3.2.7. NMR spectroscopy analysis

One or two hundred microlitres of *F. japonica* extract or fraction were dried in a rotavapor at 40°C, dissolved in $CDCl_3$ (0.7 mL) and transferred into an NMR tube. ^1H NMR spectra were recorded at 600 MHz with a Varian INOVA 600 spectrometer and compared with a PbTx-2 standard.

3.2.8. *Artemia* sp. assay

The *Artemia* sp. assay was performed using the IRSA-CNR (2003) procedure consisting in both a short- and a long-term test.

Short-term test. It was performed by incubating 10 nauplii in 1 mL sample (put into 24-wells plate) for 24 h and applied to five different kinds of samples: (I) “live cells”, (II) “lysed cells”, (III) “extracellular medium” all tested at several concentrations in triplicate, (IV) “combined algal fractions (A-E)” and (V) “fatty acid standards (18:3n-3, 20:4n-6, 20:5n-3)”. For “live cells” samples, culture aliquots of *F. japonica* and of different microalgae (*A. lusitanicum*, *P. reticulatum*, *G. fragilis*,) used for comparisons, were added to the wells before nauplii addition. *P. tricornutum* was used as control. Some assays were conducted with concentrated cultures obtained by filtering 1.5 L of *F. japonica* stationary phase culture by gravity through a GF/C Whatman filter (1.2 μm), in order to reach a high cell density. “Lysed cell” samples were obtained by sonicating the algal cultures for 3 min and “extracellular medium” samples by filtering culture aliquots by gravity through a GF/F Whatman filter (0.7 μm) to remove algal cells. “Algal fraction” samples were obtained by dissolving each dried sample (0.4-1.6 mg) in 2 mL (for duplicate) of seawater at salinity 35 and by sonicating for 3 min. Seawater or f/2 medium at the same salinity were used as controls.

Long-term test. It was performed with three different kinds of samples: “live cells” consisted of *F. japonica* culture, “lysed cells” and “extracellular medium” from *F. japonica* and *P. tricornutum* cultures were obtained as already described for the short-term test. The assay was carried out inside beakers by incubating 10 nauplii in 35 mL of each sample added with 5 mL *P. tricornutum* culture to feed the nauplii. Five different concentrations, obtained through dilution with seawater, and a blank (seawater) were used in triplicate in each experiment. Effects on the organisms were checked every 24 h up to 96 h.

3.2.9. *Vibrio fischeri* bioassay

Toxicity to the marine bacterium *Vibrio fischeri* was measured as inhibition of bioluminescence using Microtox® equipment and consumables, in accordance with the 90% basic test for pure compounds protocol (Azur Environmental, 1998). The different algal cultures (1.5-3 L) were collected in the stationary phase and filtered under vacuum using two different overlapping filters (GF/C Whatman, 1.2 μm and Millipore, 0.45 μm), that were extracted in a Soxhlet apparatus with Methanol-Chloroform (50:50). The solution was concentrated to dryness in a rotavapor system at 40°C. The extract was left to cool at room temperature and weighed. *F. japonica* extracts were obtained from 3 L cultures collected at cell concentration of about 50 x 10^3 cell mL^{-1}. Each sample for the bioassay was prepared adding 5 ml of diluent solution (to perform 5 trials of 1 ml each) to a fraction of 5.8-7 mg of dried algal extract or by adding 3 mL of diluent solution (for 2-3 trials) to an aliquot (0.5-3.2 mg) of the dried algal fraction, obtained as explained above, and sonicating for 20 min. The endpoint used to establish the concentration-response relationship was the bioluminescence of the bacteria, measured at each concentration as the ratio between the light emission after 15 min of exposure and the emission at time 0, expressed as a percentage of the same ratio in the control:

$$100\frac{I_{15}/I_0}{I_{15}^c/I_0^c}$$

where: I_0: light emission at time 0; I_{15}: emission after 15 min, I^c: emission of the control treatment.

3.2.10. Erythrocyte lysis assay (ELA)

ELA was carried out in conformity with Eschbach et al. (2001). Aliquots from stationary phase cultures of *P. tricornutum* (5 mL) and *F. japonica* (15 mL) were centrifuged at 3000 x *g*

for 15 min at 4°C so that growth medium was discarded; algal pellets were resuspended in ELA buffer (5 mL), obtained according to the method, and sonicated for 1 min on ice. Blood samples were obtained from adult carps (*Cyprinus carpio*) kept at the Faculty of Veterinary; the fish were previously anesthetized with Fenox ethanol (0.02%) then 1 mL blood was drawn from the caudal fin, added with 1 drop eparin and kept refrigerated. To obtain the erythrocytes it was centrifuged at 2000 x *g* for 15 min at 4°C; the pellet was washed twice with ELA buffer and resuspended in the same to a final concentration of 10^7 cells mL^{-1}. The absorption of ELA buffer (blank), of the algal extracts (background value) and of the completely lysed erythrocytes (maximum absorbance value) was measured at 540 nm. The test was conducted by incubating 1 mL of erythrocytes with 1 mL of algal extract at 15°C for 20 hours, in the dark. After incubation, samples were centrifuged at 2000 x *g* for 5 min, to pellet the intact erythrocytes, and their absorbance measured at 540 nm. Two replicates of 6 different sample concentrations (dilutions 1:2), were used in each experiment. Several experiments were conducted and reported similar results. A solution of saponin standard (2 mg mL^{-1} in assay ELA buffer) was used as reference compound, in a concentration series of 1-20 μg mL^{-1}.

3.2.11. Fish bioassay

Sea basses (*Dicentrarchus labrax*) and sea breams (*Sparus aurata*) used for the assays were obtained from the hatchery at Valle Ca' Zuliani (Pila di Porto Tolle, Rovigo, Italy). Sea breams were used only in preliminary experiments lasting 7 days to confirm the low toxicity of *F. japonica* in at least two different species. After the transfer, the juvenile fish (1.0 ± 0.2 g) were kept in a 60-70 L aquarium, aerated by a small dispenser (Hailea) and acclimated at room temperature and salinity 35 for two-three weeks. For the experiments, smaller aerated tanks filled with 1.5 L solution were used and kept at 20°C, in a chamber with a 16:8 h light–dark period. Duplicate tanks contained the same volumes (1.5 L) of either seawater, f/2 medium or algal cultures (in addition to *F. japonica*, *P. tricornutum* and *S. marinoi* were used as controls, and the toxic *K. brevis* for comparison). H_2O_2 was added in seawater at two final concentrations (44 and 88 μM) which were daily monitored and adjusted when necessary. Four juvenile fish were put into each tank and observed for up to 16 days; starting at day 5 they were fed on fish fodder once a day. Fish were considered dead when gill opercular movements ceased. The assays were repeated three times.

3.2.12. H_2O_2 analysis

Hydrogen peroxide produced by *F. japonica* cells in culture and during the fish assay was measured by the method described by Bellincampi et al. (2000). H_2O_2 concentration in the filtered culture medium was measured by the FOX1 method, based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange. This method is extremely sensitive and used to measure low levels of water-soluble hydroperoxide present in the aqueous phase. To determine the H_2O_2 concentration, 500 µL of the incubation medium were added to 500 µL of assay reagent. Absorbance of the Fe^{3+}-xylenol orange complex was detected after 45 min at 560 nm. Standard curves of H_2O_2 were obtained for each independent experiment by adding variable amounts of H_2O_2 to 500 µL of basal medium mixed to 500 µL of assay reagent. Data were normalized and expressed as fmol H_2O_2 per cell.

3.2.13. HPLC determination of malondialdehyde (MDA)

Solutions: 500 mg L^{-1} 2,6-Di-*tert*-butyl-4-methylphenol (BHT) in methanol; 20 mM tetraethoxypropane (TTEP) in ethanol; 28 mM 2-thiobarbituric acid (TBA), in distilled water; 6% TCA solution with 1.34 mM EDTA, in distilled water; Na_2HPO_4 buffer, 5 mM with 0.7% THF, pH 7.0 in distilled water.

Free MDA reacts with TBA to form the pink condensation product MDA-TBA that can be measured spectrophotometrically (λ_{max} = 532 nm). The analysis was adapted for HPLC to separate MDA from TBA byproducts (Draper et al., 1993; Espinosa-Mansilla et al., 1993) as described by Rijstenbil and Gerringa (2002), so that chromatograms show a single well defined peak of the MDA-TBA adduct, with no interference. A calibration curve was made with TTEP diluted in TCA solution, at concentrations of 0, 2.5, 5, 10, 15 µM; MDA-TBA is stoichiometrically formed from TTEP in an acidic environment. The standard curve generated with TTEP standard from 0 to 15 µM resulted linear, with a correlation coefficient R^2 = 0.9755.

Sea bass gills were extracted from the frozen fish, which had been immediately weighed and put at -80°C after death. Fish gills used as controls were obtained from live fish, which had been exposed to *P. tricornutum* or seawater, sacrificed for the experiment and kept at -80°C until analysis. Gill homogenates were prepared by mixing 100-350 mg of fish gills with 1 mL cold TCA solution and 40 µL BHT solution to prevent autoxidation and sonicating on ice for 3 min. After sonication the homogenate was kept in a heating cabinet at 100°C for 30 min and then centrifuged at 14000 x *g* in a refrigerated centrifuge for 20 min at 4°C. Sample

supernatant (~0.9 mL), a volume of standard, or pure TCA solution (blank) was transferred in a 2-mL Eppendorf with a small hole in the lid, and a same volume of 28 mM TBA solution was added; the mixture was vigorously agitated on a vortex, and placed at 100°C for 30 min. After cooling, samples were immediately used for analysis.

HPLC analysis was performed using an HPLC system (Perkin Elmer) consisting of a 250 binary LC pump equipped with a C18-column (Luna, 5 μm, 250 x 4,6 mm) and series 200 Diode Array Detector; 20 μl of each blank, standard or sample were injected. The MDA–TBA complex was eluted with an isocratic mixture of 85% 5 mM sodium phosphate buffer (pH 7) containing 0.7% THF, and 15% acetonitrile at a flow rate of 1 mL min^{-1}. MDA concentrations are expressed in μmol MDA kg^{-1}.

3.2.14. Data analysis

The 50% effect concentration (EC_{50}) of each sample for the *V. fischeri* assay was estimated by fitting the experimental concentration-response curves to a logistic model:

$$y = \frac{a}{1+\left(\frac{x}{EC_{50}}\right)^{b}}$$

where: y = endpoint value; x = substance concentration; a = expected endpoint value in absence of toxic effect; b = slope parameter. The parameters of the equation, including the EC_{50}, were estimated using the non-linear regression procedures implemented in Statistica (Statsoft, Tulsa, OK, USA). An independent estimate of EC_{50} was obtained for each of the experiments.

Values for H_2O_2 and MDA levels were compared using an ANOVA. The assumption of homogeneity of variances was tested by Cochran's C test. When required, data were transformed to attain homogeneity of variances. Whenever a significant difference for the main effect was observed ($P<0.05$), a Newman-Keuls test was also performed.

3.3. Toxicity evaluation of *Fibrocapsa japonica* from the Northern Adriatic Sea

3.3.1. *Artemia* sp. assay

Short-term test. Since in preliminary tests with live cells no toxic effects were recorded at any *F. japonica* concentration reached in culture, either in the exponential or in the stationary

phase, cultures were successively concentrated in order to reach a higher cell number (max concentration: 321.71 x 10^3 cells mL^{-1}). After 24 h in *F. japonica* concentrated culture the nauplii appeared inactive but still alive, while those incubated with *P. tricornutum* displayed a normal motility. The *Artemia* sp. assay is generally based on the evaluation of a mortality effect on nauplii; however in our tests the organisms rather than dying showed abnormal movements, including swimming slowly, rotating fast around one fixed point or immobilization (organisms still alive but unable to swim). The inactivation percentage was directly related to the algal cell density. The EC_{50} value obtained considering the immobilization percentage was 241.91 ± 49.21 x 10^3 cells mL^{-1}. The inactivation effect produced by *F. japonica* was compared with the effect exerted by different microalgae; thus, the harmful species *A. lusitanicum* (max concentration: 32.92 x 10^3 cells mL^{-1}), *P. reticulatum* (max concentration: 11.61 x 10^3 cells mL^{-1}) which produce saxitoxins and yessotoxins, respectively, and *G. fragilis* (max concentration: 5.36 x 10^3 cells mL^{-1}), which releases large amounts of polysaccharides, were used for comparison. Contrarily to *F. japonica* all these species caused nauplii mortality after 24 h. The EC_{50} values obtained were 15.10 ± 8.81, 5.93 ± 0.97, and 0.81 ± 0.28 x 10^3 cells mL^{-1}, respectively.

The *Artemia* sp. assay was also performed with: *F. japonica* lysed cells, extracellular medium, the five combined fractions (A-E) from the extract fractionation and three fatty acid standards at different concentrations. The first two kinds of samples did not affect nauplii viability, while the mortality results obtained with the latter two are shown in Table 3.4. As reported, the fractions B and C were the most toxic; both these fractions contained PUFAs and, in particular, fraction C presented high levels of 20:5n-3 (EPA), 20:4n-6 (AA), 18:4n-3 (OTA), and 18:3n-3 (Table 3.3). When three of these PUFAs were tested separately as purified standards, the relationship between mortality effect and concentration was different for the various compounds; in particular, EPA (20:5n-3) appeared as the most toxic, causing 80% nauplii mortality at 50 μg mL^{-1}.

Long-term test. Contrarily to what observed in the short-term test, a lethal effect of *F. japonica* live cells on *Artemia* sp. was observed in the long-term assay, even after 24 h exposition. In this assay, the same number of *Artemia* nauplii were incubated in a high volume (35 mL) of culture (max concentration: 80 x 10^3 cells mL^{-1}) thus being exposed to a lower cell concentration per mL but to a higher total cell number than in the short-term assay. The EC_{50} values (Fig. 3.2A) showed an increase in mortality with time, reaching a value of 26.42 ± 1.24 x 10^3 cells mL^{-1} after 96 h, when no survivors were present in the two highest concentrations tested (Fig. 3.2B).

Table 3.3 - Composition (%) of *F. japonica* fractions obtained after C18 SPE fractionation.

	% Composition of *F. japonica* fraction				
	A	**B**	**C**	**D**	**E**
12:0		1.7	0.2		
14:0		2.3	33.3	DNQ	DNQ
16:0		DNQ	0.6	75.9	DNQ
16:1			2.7		
16:2			0.5		
18:0		2.3	0.5	2.9	DNQ
18:1				21.2	
18:2n-6			2.4		
18:3n-6 (γ)			2.6		
18:3n-3 (α)			5.0		
18:4n-3		7.9	8.5		
20:4n-6			6.4		
20:5n-3			36.8		
β sitosterol					99.9
arabinofuranose	99.9	18.7			
D-ribofuranose	DNQ	2.4			
D-eritrotetra furanose		55.4			
D-fructose		3.9			
inositol		5.4	0.2		
glicerol			0.4		

DNQ = Detected Not Quantified

The diatom *P. tricornutum*, used in the assay as a control, did not show toxic effects on *Artemia* sp. even at a high cell density (3.92×10^6 cells mL^{-1}) and the same result was reported when testing the filtered algal media of the two microalgae used or the f/2 medium alone. Sonicated *P. tricornutum* and *F. japonica* cultures were also tested in order to evaluate the effects of dead and lysed cells. On testing 35 mL of lysed *F. japonica* cells at the same maximum concentration used before, nauplii mortality occurred more quickly than with live cells, while *P. tricornutum* did not exert any lethal effect (data not shown). A summary of the results obtained in the various toxicological assays is reported in Table 3.5.

Table 3.4 - The effect on *Artemia* nauplii of the different algal fractions and fatty acid standards after 24 h (short-term test).

		conc (µg/mL)	% mortality
***F. japonica* fractions**	A	300	35
	B	800	100
	C	500	100
	D	400	0
	E	400	0
control	seawater	-	0
PUFAs	18:3n-3	100	10
	18:3n-3	500	90
	20:4n-6	10	10
	20:4n-6	143	50
	20:5n-3	50	80
	20:5n-3	500	90

Fig. 3.2 – A) The 50% effect on *Artemia* nauplii (EC_{50}) due to *Fibrocapsa japonica* live cells during a long-term test (24-96 h). Each value is expressed as mean ± SE. EC_{50} values considered mortality effects on organisms. B) Relationship between the concentration of *F. japonica* and the effect on *Artemia* nauplii, after a 96 h exposure (long-term test).

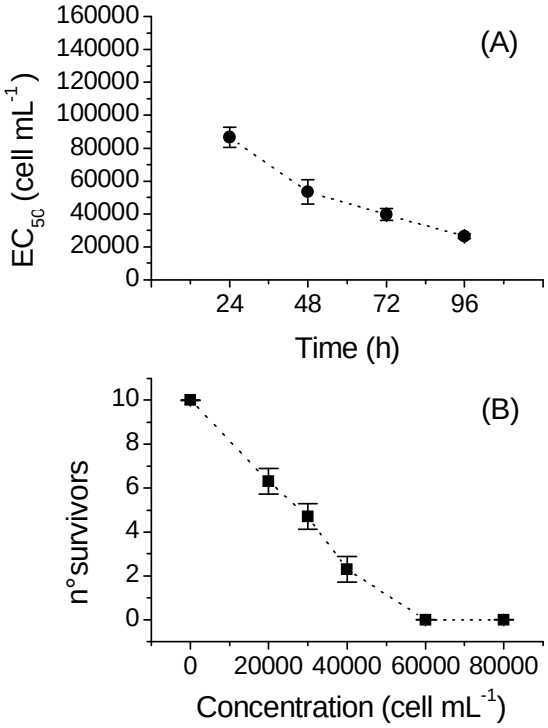


Table 3.5 – Summary of the toxicological assays with *F. japonica* performed in this study, reporting sample characteristics, the effect obtained and relative EC_{50} values. Y or N: presence or absence of a toxic effect.

Test	Sample	Growth phase	Toxic effect	EC_{50} (cell mL^{-1}) FJAP0603
Artemia sp. (short-term)	medium	exponential / stationary	N	
	live cells	exponential	N	
	live cells	stationary (concentrated)	Y	241 910
	lysed cells	exponential / stationary	N	
Artemia sp. (long-term)	medium	stationary	N	
	live cells	stationary	Y	26 420
	lysed cells	stationary	Y	nd
Vibrio fischeri	extract	stationary	Y	1 030
ELA	lysed cells	stationary	Y	5 190
Sea bass	live cells	stationary	Y	nd

nd = not determined

3.3.2. Bacteria bioluminescence

The marine bacterium *V. fischeri* exposed to *F. japonica* extract (max concentration: 1.04 mg mL^{-1}) showed inhibition of the natural luminescence (Fig. 3.3), which is indicative of an impaired metabolism. Extracts of different algae (max concentrations: *Scrippsiella* sp. 1.26 mg mL^{-1}, *P. tricornutum* 1.26 mg mL^{-1}, *P. reticulatum* 1.04 mg mL^{-1}) were tested in order to have a quantitative measurement of the effects exerted on bacteria by various microalgal extracts (Fig. 3.3). EC_{50} values obtained showed a strong bioluminescence inhibition testing *F. japonica* extract, while the other microalgae decreased bacteria metabolic activity only at higher extract concentrations. These results are reported in Table 3.6, where the EC_{50} values are expressed either as μg extract or as corresponding cell concentration. The result didn't

change even considering the great difference in cell sizes and calculating the EC_{50} on a biovolume basis (data not shown).

Fig. 3.3 - Relationship between the concentration of different algal extracts and the bioluminescence of *Vibrio fischeri*, after a 15 min exposure.

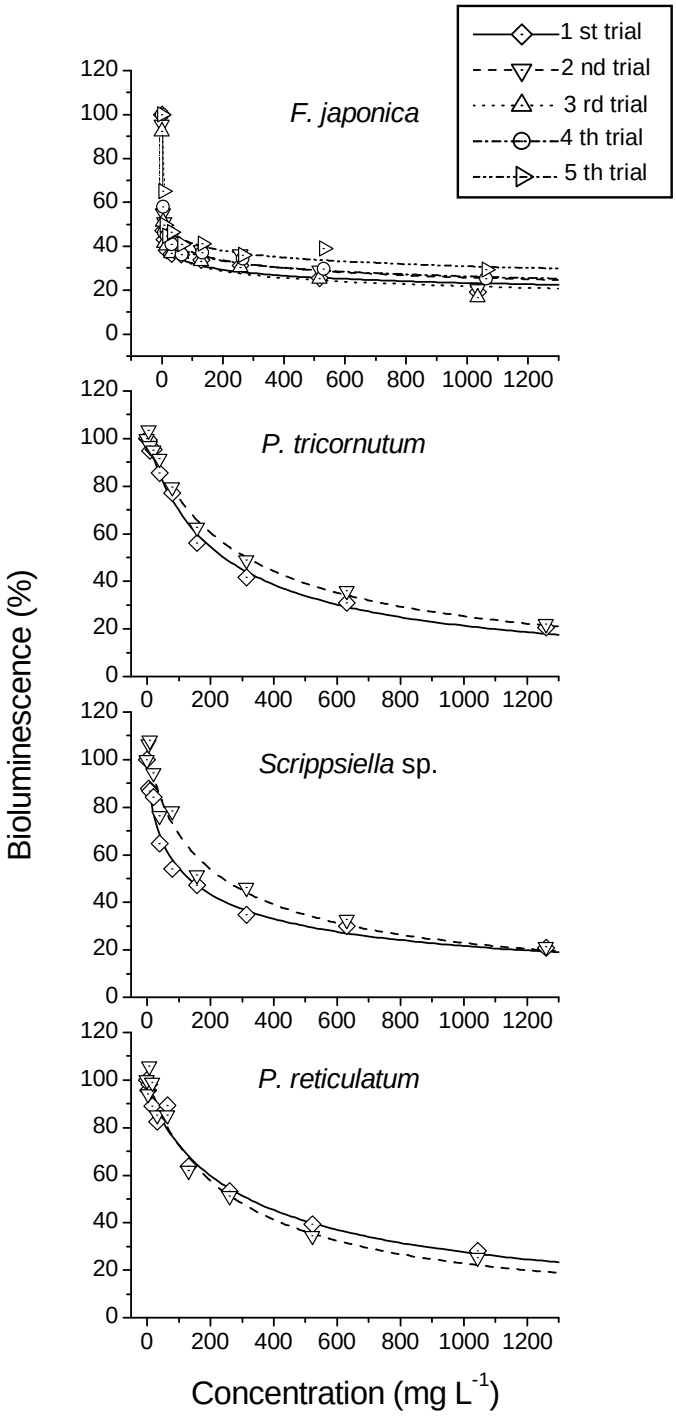
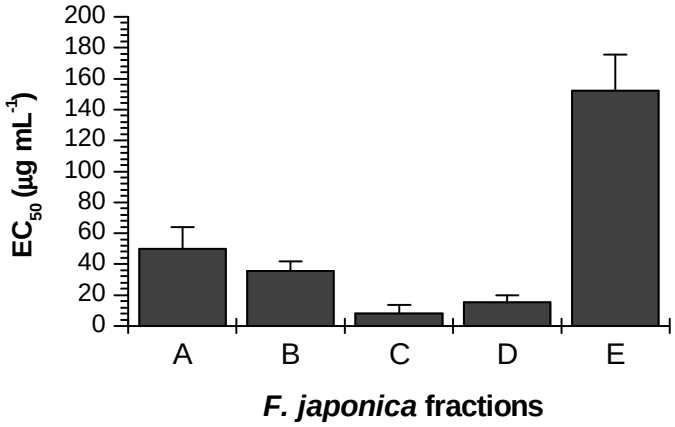


Table 3.6 - The 50% effect concentrations (EC_{50}) of different algal samples expressed either as extract or cell concentrations for *Vibrio fischeri* in a 15 min inhibition of bioluminescence test. The reported values are means ± SE of two to five independent trials. Values refer to mL extract.

	EC_{50}	
	extract (μg mL^{-1})	**concentration (10^3 cell mL^{-1})**
F. japonica	9.5 ± 6.2	1.03 ± 0.67
***Scrippsiella* sp.**	164.0 ± 52.9	42.88 ± 13.83
P. tricornutum	264.1 ± 43.9	1520.87 ± 228.63
P. reticulatum	293.4 ± 44.1	51.20 ± 8.51

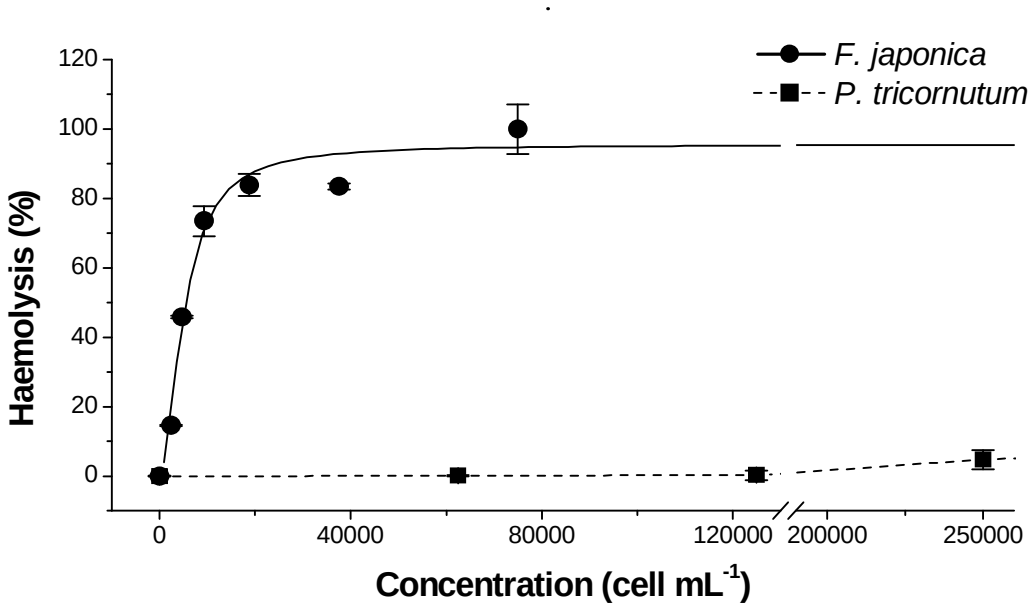
Fig. 3.4 - The 50% effect concentrations (EC_{50}) of different algal fractions for *Vibrio fischeri* in a 15 min inhibition of bioluminescence test. The reported values are means ± SE of two trials.



3.3.3. Haemolysis of *Cyprinus carpio* erythrocytes

The ruptured cell suspension of *F. japonica* (max concentration: 75 x 10^3 cells mL^{-1} ELA buffer) produced haemolytic activity for *C. carpio* erythrocytes. The percentage of haemolysis increased with cell density (Fig. 3.5), giving an EC_{50} value of 5.19 ± 0.59 x 10^3 cells mL^{-1}. *P. tricornutum* cells showed no haemolytic activity even at a high cell density (max concentration: 2.50 x 10^5 cells mL^{-1} ELA buffer), reaching a maximum haemolysis percentage of 13.5%. Results were not different even considering the biovolumes of the two microalgae. Saponin standard was used to conduct the assay with a chemically defined haemolytic agent and the obtained EC_{50} value was 1.42 ± 0.03 μg mL^{-1}.

Fig. 3.5 - Haemolytic activity of the ruptured cell suspensions of *Fibrocapsa japonica* and *Phaeodactylum tricornutum.*



3.3.4. Effect of Dicentrarchus labrax exposition to *Fibrocapsa japonica*

Fish mortality

In the first week of exposure, no sea bass or sea bream mortalities occurred in the tanks where fish were exposed to *F. japonica* culture. In successive experiments sea bass was exposed to about 50 x 10^3 cells mL^{-1} for a longer period during which algal cell number did not change, as evidenced through daily counting (data not shown). After 8-9 days fish started to show abnormal behaviour and inability to maintain a correct position in the water column, then at day 10 began to die, and by the 11^{th} day all the sea basses were dead. Dead fish were immediately put at -80°C. In the tanks with control algae (max concentrations: *P. tricornutum* 3.51 x 10^6 cells mL^{-1}, *S. marinoi* 1.70 x 10^6 cells mL^{-1}) and in those with 44 and 88 µM H_2O_2 they were all alive and maintained a normal behaviour till the end of the experiment (Fig. 3.6). An observation made under the light microscope of the gills of fish exposed to *F. japonica* culture revealed that gill tissue was damaged and covered with mucus substances, which might had interfered with O_2 transfer (Fig. 3.7). Fish were also exposed to *K. brevis* (max concentration: 1.17 x 10^3 cells mL^{-1}), in order to evaluate the sea bass resistance to a microalga which produces brevetoxins. After 5 h from the beginning of the exposure, fish in the tanks with *K. brevis* culture began to die and 21 h later they were all dead.

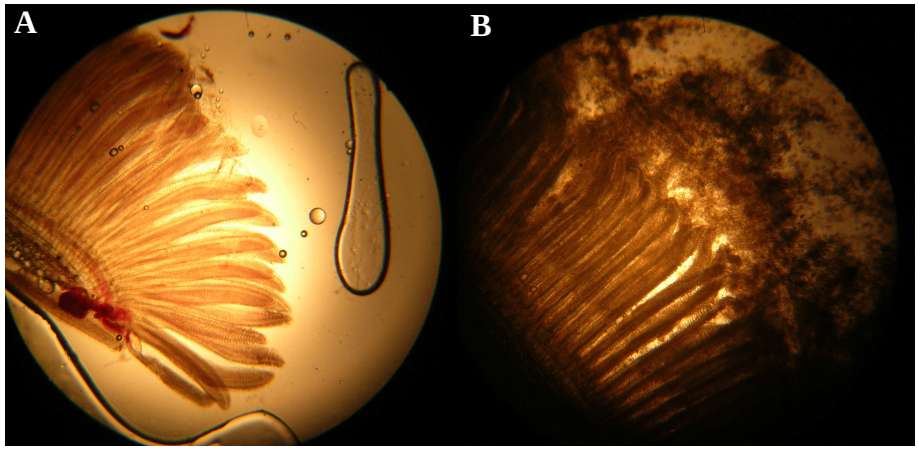
Fig. 3.6 - Toxicity of different microalgae and H_2O_2 for the fish (*Dicentrarchus labrax*). Results refer to a single experiment and the cell concentrations used are indicated. Time is expressed as hours (h) and days (d).

	Concentration cell mL^{-1}	Concentration μM	tank	n° dead fish / n° tested fish
S. marinoi	1 695 553		1 2	0/4 0/4
P. tricornutum	3 513 013		1 2	0/4 0/4
F. japonica	49 622		1 2	3/4 4/4 4/4
K. brevis	1 174		1 2	2/4 4/4 1/4 4/4
H_2O_2		44 - 88	1 2	0/4 0/4

0 5h 21h 1d 5d 9d 10d 11d 12d 13d 14d 15d 16d

time

Fig. 3.7 – Gills of the controls (A) and of fish exposed to *F. japonica* culture (B) observed under the light microscope. Fish tested with the Raphidophyceae revealed that gill tissue was damaged and covered with mucus substances.

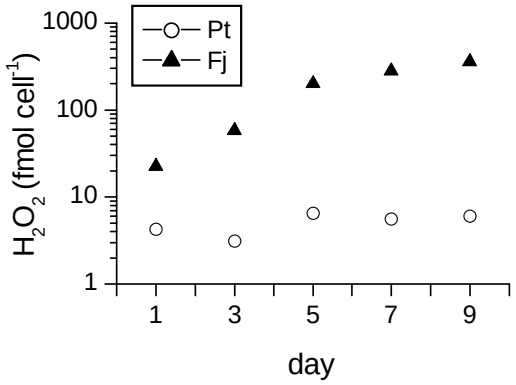


ROS analysis

ROS analysis revealed an increase in H_2O_2 produced by *F. japonica* in the tanks where the fish assays were conducted, while no effects were observed in tanks containing *P. tricornutum* (Fig. 3.8). The initial H_2O_2 concentration measured in *F. japonica* medium was 1.15 µM, which corresponded to 22.60 fmol cell^{-1} (day 1) and reached a value of 18.39 µM, corresponding to 361.75 fmol cell^{-1}, at day 9 (data not shown), when the maximum H_2O_2 concentration was measured (Fig. 3.8). Nine days represented the period after which the fish began to die and, on this day, values measured in the tanks were about 16-fold and 1.4-fold

higher than on day 1 in the tanks with *F. japonica* and *P. tricornutum*, respectively. ANOVA analysis showed that the differences observed for the two microalgae were significant ($P<0.001$). The H_2O_2 concentration in *P. tricornutum* medium was rather constant during the assays, with values of 5.64-7.93 μM at day 1 and 9, respectively, which corresponded to 4.27 and 6.01 fmol $cell^{-1}$.

Fig. 3.8 - Concentration of H_2O_2 during the sea bass test in the tanks where fish were exposed to *Fibrocapsa japonica* (Fj) and in the control tank with *Phaeodactylum tricornutum* (Pt). Each value is expressed as log10 of the concentration detected.



Fish gills analysis

Quantification of MDA, which is a marker for oxidative damage, was performed in the fish gills. The MDA values ranged from 55.97 to 82.43 μmol MDA kg^{-1}, while in the controls the mean value reported was 5.10 μmol MDA kg^{-1}. Results of statistical analysis indicated that the MDA content in the gills of the exposed fish was significantly different (ANOVA, $P<0.05$) from that of the controls.

3.4. Chemical characterization

3.4.1. Brevetoxin analyses

The search for brevetoxins was carried out by both NMR and LC-MS analyses. ^{1}H-NMR (600 MHz, $CDCl_3$) spectrum of the PbTx-2 standard showed some characteristic peaks, in particular δ 9.52 (s, 1 H, CHO), 5.78-5.75 (m, 2 H, CH=CH), 5.73 (s, 1 H, =CH), 4.26 (d, 1 H, OCHC=), 2.66 (bs, 1 H, OH). These peaks were not evidenced in any of the spectra realized with *F. japonica* extracts and fractions.

Table 3.7 – Fatty acid composition (expressed per cell) by GC-MS analysis of *F. japonica* and *P. tricornutum* extract and extracellular medium.

	Cellular fatty acids		**Extracellular fatty acids**	
Fatty acid	***F. japonica***	***P. tricornutum***	***F. japonica***	***P. tricornutum***
	(pg/cell)		**(fg/cell)**	
12:0	nd	nd	3.70	0.04
14:0	22.50	0.090	7.70	0.09
16:0	16.64	0.118	35.55	0.36
16:1n-7	1.00	0.252	nd	nd
16:1isomer	1.35	0.019	nd	nd
16:2	nd	0.046	nd	nd
18:0	0.93	0.005	13.57	0.15
18:1n-9	2.98	0.005	1.66	0.05
18:1n-7	3.36	nd	nd	nd
18:2n-6	1.10	0.005	nd	nd
18:3n-6 (γ)	0.89	nd	nd	nd
18:3n-3 (α)	1.80	nd	nd	nd
18:4n-3	7.94	nd	nd	nd
20:4n-6	2.51	nd	nd	nd
20:5n-3	13.33	0.259	nd	nd
22:6n-3	nd	nd	nd	nd
Tot PUFAs	27.57	0.309	nd	nd
Tot	76.34	0.799	62.19	0.68

Chemical names and notations: dodecanoic acid (12:0, lauric acid); tetradecanoic acid (14:0, myristic acid); hexadecanoic acid (16:0, palmitic acid); 9-hexadecenoic acid (16:1n-7, palmitoleic acid); hexadecenoic acid (16:1); 9,12-hexadecadienoic acid (16:2n-4); octadecanoic acid (18:0, stearic acid); 9-octadecenoic acid (18:1n-9, oleic acid); 11-octadecenoic acid (18:1n-7, vaccenic acid); 9,12-octadecadienoic acid (18:2n-6, linoleic acid, LA); 6,9,12-octadecatrienoic acid (18:3n-6, γ-linolenic acid, GLA); 9,12,15-octadecatrienoic acid (18:3n-3, α-linolenic acid, ALA); 6,9,12,15-octadecatetraenoic acid (18:4n-3, stearidonic acid, OTA); 5,8,11,14-eicosatetraenoic acid (20:4n-6, arachidonic acid, AA); 5,8,11,14,17-eicosapentaenoic acid (20:5n-3, EPA); 4,7,10,13,16,19-docosahexaenoic acid (22:6n-3, DHA).

nd = not detected

The LC-MS ion chromatogram of *K. brevis* extracts revealed the presence of a peak (RT 24.029) with a product ion spectrum of m/z 895.8 (MH^+) corresponding to PbTx-2, that is the most widespread algal brevetoxin. At the same retention time no peak was found in the ion chromatogram of *F. japonica* extracts and fractions. In any case, no peak characterized by the presence of ion m/z 895.8 could be detected.

3.4.2. Analysis of fatty acid composition of *Fibrocapsa japonica* and *Phaeodactylum tricornutum* cell extract and extracellular culture medium

The cellular fatty acid profile of both *F. japonica* and *P. tricornutum*, determined by GC-MS analysis, is presented in Table 3.7. In *F. japonica*, four major components were found, the level of each being 10.40–29.48% of the total fatty acids. These four components were the saturated fatty acids 14:0, 16:0 and the polyunsaturated acids 18:4n-3 and 20:5n-3. The minor components represented 1.16–4.40% of the total fatty acids and consisted mainly in unsaturated fatty acids.

P. tricornutum is a diatom known for its high content in PUFAs, in particular 20:5n-3; it was never found to affect other organisms and is widely used as food in aquaculture. Therefore, in this study it was used as control, and analyzed to compare its fatty acid profile with that of *F. japonica*. The fatty acid profile of *P. tricornutum* revealed the expected high amount of 20:5n-3 (32.41%), while the presence of PUFAs with 18 carbon atoms was low or below the detection limit. Data are expressed as pg/cell thus emphasizing the largely different fatty acid content of the two algae; by taking into account the 50-fold smaller biovolume of *P. tricornutum*, the 20:5n-3 content per biovolume unit of the two algae is quite similar while the total PUFAs amount is two-fold higher in *F. japonica*.

The two species were compared also in their extracellular fatty acid profile which is presented in Table 3.7. Only five fatty acids were found in the medium of *F. japonica*; they were the saturated acids 12:0, 14:0, 16:0, 18:0, and the mono-unsaturated 18:1n-9, the latter representing 2.67% of the total fatty acids. Almost the same profile was obtained for the *P. tricornutum* extracellular medium. None of the PUFAs detected within the cells was found in either of the extracellular media.

3.4.3. Analysis of *Fibrocapsa japonica* fractions

The fifty-four TMS-fractions obtained by chromatographic separation of the algal extract were combined, as described in the experimental section, in five final fractions (A-E) which

were used for subsequent toxicity assays. The composition of each fraction is reported in Table 3.3. Two samples contained mostly PUFAs, in particular, fraction B had about 8% of 18:4n-3, while fraction C contained 18.5% of PUFAs with 18 carbon atoms, 6.4% of 20:4n-6 (AA) and a high amount (36.8%) of 20:5n-3 (EPA). In the other fractions (A, D, E) sugars, saturated fatty acids or β-sitosterol were present.

3.5. Development of LC-MS methods for Brevetoxins (BTXs)

Microalgae belonging to the Raphidophyceae, *Fibrocapsa japonica*, *Heterosigma akashiwo* and *Chattonella* spp., have been documented as ichthyotoxic and causing massive fish mortality events mostly in the Seto Inland Sea of Japan (Iwasaki, 1971; Okaichi, 1972; 1989; Toriumi and Takano, 1973). Since the production of brevetoxins is one of the major factors which had been proposed for the ichthyotoxicity of these raphidophytes (Khan et al., 1996), together with reactive oxygen species (ROS) (Oda et al., 1997, Marshall et al., 2003), haemolytic compounds such as PUFAs (Fu et al., 2004a), and mucocyst ejection (de Boer et al., 2004), in this thesis the presence in algal extracts of these raphidophytes of some of the known brevetoxins was investigated. To achive this aim, rapid and sensitive methods to analyze brevetoxins in algal extracts were developed. This work was performed in the laboratory of the Dr. Michael Quilliam at the National Research Council, Institute for Marine Biosciences (NRC-CNRC), in Halifax (Canada).

3.5.1. Bioassay and chemical analytical methods for the toxin determination

Methods for the determination of toxins may be divided into assay and chemical analytical methods. In assay methods, the measured signal is either a specific response to a single toxin structure or an integration of responses to several structures in a group. In order to use the assay result for evaluating seafood safety, it is most useful if the response correlates with overall toxicity. On the other hand, in a chemical analytical method, signals corresponding to individual toxin structures are measured. Most analytical methods are based on chromatography which allows the separation and detection of several toxins in one analysis. Calculation of individual toxin concentrations requires accurate standards to calibrate the responses, and evaluation of seafood safety further requires specific toxicity data.

The most common assay to check for the presence of toxins is the mouse bioassay (MBA). For over 50 years, this assay has been used successfully in inspection programs to monitor for PSP toxins, which are easily extracted into an acidic aqueous solution suitable for direct

injection (Stephenson et al., 1955; AOAC International, 2000). One of the problems with the method is that the inherent variability can exceed ±20%, compared to most chemical techniques that have uncertainties less than 10%. Major disadvantages of using the MBA for the detection of toxins are the lack of specificity (no differentiation between the various components of a group), subjectivity of death time and maintenance of laboratory animals. Additionally, the MBA is time consuming, does not provide appropriate detection limits for some toxins (e.g., yessotoxins, okadaic acid and DTX esters, azaspiracids) and may give 'false positive' results because of interferences by other compounds (Combes, 2003). Notably, hydrophilic toxins belonging to the amnesic and paralytic shellfish poisoning groups, free fatty acids (Lawrence et al., 1994) and bioactive spiroimines (e.g., spirolides and gymnodimines; Gill et al., 2003) have been shown to be toxic to mice. Within the EU, it is a requirement to take all steps to refine, reduce and replace (3R's), the use of animals used in bioassays and scientific experiments under Directive 86/609/EEC. To this aim, the European Centre for the Validation of Alternative Methods (ECVAM) met to consider the 3R's approach to shellfish toxin testing. The results of the deliberations of the working group are available (Hess et al., 2006); it was suggested that immediate possibilities for implementation of the 3R's include the use of liquid chromatography–mass spectrometry (LC–MS) methods for the detection of toxins.

Finally, since several countries have banned animal bioassays due to protests by animal rights groups, there is considerable pressure to develop alternative methods.

A number of alternative assay methods have been developed (Van Dolah et al., 2001). These include in vitro cell toxicity assays, receptor protein assays, and immunological assays. Despite their potential for speed, high sensitivity and low cost, there are some limitations associated with such methods. Since receptor assays are usually based on radioactive tracers, they are restricted to specially equipped laboratories. Immunoassays generally cannot be used for the precise quantitative analysis of samples containing families of toxins that have variable levels of individual toxin. Antibodies for such assays are initially developed for single toxins but can have varying degrees of cross-reactivity towards toxins of similar structure. Although assays are excellent for screening out negative samples, it is generally recognised that positive results should still be confirmed by chemical methods.

Chemical methods of analysis have the potential for sensitive, precise and fully automated quantization of known toxins, as well as confirmation of identity. Methods based on chromatographic and spectroscopic techniques are particularly well suited for the identification of new toxins. It should be noted that there are many challenges to overcome

before it is possible to fully implement any chemical method into comprehensive monitoring programs and research studies.

All members of a toxin class must be well characterised in terms of structure, and although considerable information on the structures of toxins that can contaminate shellfish has been accumulated, the situation becomes more complicated with the frequent discovery of new toxin analogues and even new toxin classes. In addition, toxins produced by a particular microalgal species may be transformed in shellfish to metabolites, some toxic and others non-toxic. This is certainly a complicating factor in the development of methods and indicates a need to continue research on the identification of new toxins. Accurate and readily available calibration standards are required for each of the various toxins. This has always been a major stumbling block in shellfish toxin research and monitoring, and much more effort has to be put into the development of reference materials and standards. The specific toxicity of individual toxins must be determined to allow calculations of overall toxic potential of samples. Risk assessment studies also need to be conducted to establish allowable levels of toxins in seafood. Finally, robust methods must be developed and validated for various shellfish tissues. They must provide adequate detection limits (μg kg^{-1} levels) and accurate quantitation of all individual toxin analogues within a toxin class.

Many specific analytical methods have already been developed for individual toxins or toxin groups. Although there are some difficulties with the technique (i.e. most of the toxins do not possess a chromophore for sensitive UV absorbance or fluorescence detection) liquid chromatography has proven to be the most valuable instrumental analytical tool for toxins because it is well suited to the analysis of polar, non-volatile compounds. It provides excellent quantitative precision and is easily automated.

3.5.2. Liquid chromatography–mass spectrometry

Since the 1970s the mass spectrometer not only provided molecular mass and structural information, but could also act as a very sensitive and selective detector for quantitative analysis of complex mixtures. Through the 1980s, many efforts were made to achieve the same success obtained with the combination of gas chromatography and mass spectrometry (GC–MS) also with the combination of LC and MS. Early LC–MS interfaces such as thermospray ionisation and continuous-flow fast atom bombardment were only partially successful. The former was only applicable to thermally stable molecules with medium polarity, while the latter was very difficult to implement on a routine basis.

In 1984, a breakthrough came with the development of electrospray ionisation (ESI) (Yamashita and Fenn, 1984), which is well suited to compounds of widely ranging polarities and is easily implemented. Immediate success was achieved with the application of LC–ESI-MS to marine toxins using the first commercial mass spectrometer from SCIEX in 1989 (Quilliam et al., 1989). Due to its high sensitivity, specificity and selectivity, LC–MS quickly became the preferred method for the confirmatory analysis of all known toxins (Quilliam, 1998; Quilliam et al., 2001). Recently, effort has been directed towards finding mobile phases and columns that would be suitable for the simultaneous separation and detection of a wide range of toxins, in both plankton and shellfish samples (Quilliam et al., 2001). One of the most important factors for achieving success in electrospray LC–MS is the choice of mobile phase. Parameters that affect sensitivity include pH, ionic strength, type of buffer, and percentage of the organic solvent. A volatile buffer with a low ionic strength (ideally <10 mM) is desirable to prevent a build-up of salts on the sampling orifice of the MS. In addition, a higher percentage of organic solvent tends to give higher sensitivity with most compounds. Suitable mobile phases include aqueous methanol or acetonitrile doped with formic acid, acetic acid, trifluoroacetic acid (TFA), the ammonium salts of these acids, or ammonium hydroxide. TFA has been very popular because it is easy to use and produces a low pH that facilitates protonation of most toxins. The latter can be important for the chromatography of acidic and basic toxins on silica-based supports, as interactions with free silanol sites are minimized. However, it has been observed that TFA forms strong ion pairs with amines and this leads to suppression of ionization and reduced sensitivity. Also, TFA cannot be used if negative ion work is planned on the same day, as it gives a very strong signal due to the CF_3COO^- anion which persists in the source for a long time.

3.5.3. Brevetoxins

Brevetoxins (BTXs) are cyclic trans-fused polyether toxins produced by the red tide dinoflagellate *Karenia brevis* (Van Dolah, 2000; Pierce et al., 2005). These toxins are broadly defined as belonging to a lipid soluble group of cyclic polyether compounds with a molecular weight around 900 amu (Fig. 3.9). Many analogs of brevetoxin have been identified in cultures of *K. brevis*, seawater blooms as well as metabolites in shellfish and other marine life (e.g. Abraham et al., 2006). Most of these analogs were reputed to be biosynthetic products of either BTX1 or BTX2 as precursors (Baden et al., 2005). Typically in marine waters the dominant brevetoxin is BTX2 with lesser amounts of BTX1 and BTX3, whereas in marine

aerosols BTX3 dominates (Pierce et al., 2005). Recent work by Tester et al. (2008) has shown though as a *K. brevis* bloom ages there is a relative increase in the BTX3 compared to the BTX2 homologues.

These compounds are responsible for massive fish kills, shellfish contamination (NSP), and respiratory, eye and skin irritations in humans and mammals exposed to contaminated seawater and aerosols in Florida and the Gulf of Mexico. Available methods for their detection include bioassays, immunoassays, thin layer chromatography and LC. Since brevetoxins have weak chromophores, LC with UV detection (at 215 nm) provides low sensitivity and poor selectivity.

Hua et al. (1996) reported that electrospray LC-MS is a suitable technique for analysis of these toxins. Under the conditions used (aqueous methanol mobile phase), the positive ion spectra were dominated by sodiated molecules ($[M+Na]^+$), as well as dimers ($[2M+Na]^+$) and trimers ($[3M+2Na]^{2+}$), which is a reflection of the ionophoric nature of these molecules. Quilliam et al. (unpublished) have investigated the ion spray LC-MS of the brevetoxins, using an acidified (0.1% TFA) aqueous methanol mobile phase to promote protonation.

It has been already established on-line liquid chromatography (LC)/electrospray ionization (ESI)-mass spectrometry as a powerful analytical tool to provide relatively rapid quantitative and structural information concerning brevetoxins, with high sensitivity and low sample quantity requirements. The strength of the polyether backbone, however, has two major implications for ESI-MS/MS analyses. First, the polyether structure has a very high affinity for sodium cations that result in the initial generation of abundant $(M+Na)^+$ ions; second, when attempting to produce collision-induced dissociations, at least two bonds of the polycyclic backbone must be broken in order to obtain backbone fragmentations. Thus, it is difficult to obtain useful product ions in detectable abundances from brevetoxin $(M+Na)^+$ precursor ions during tandem mass spectrometry experiments.

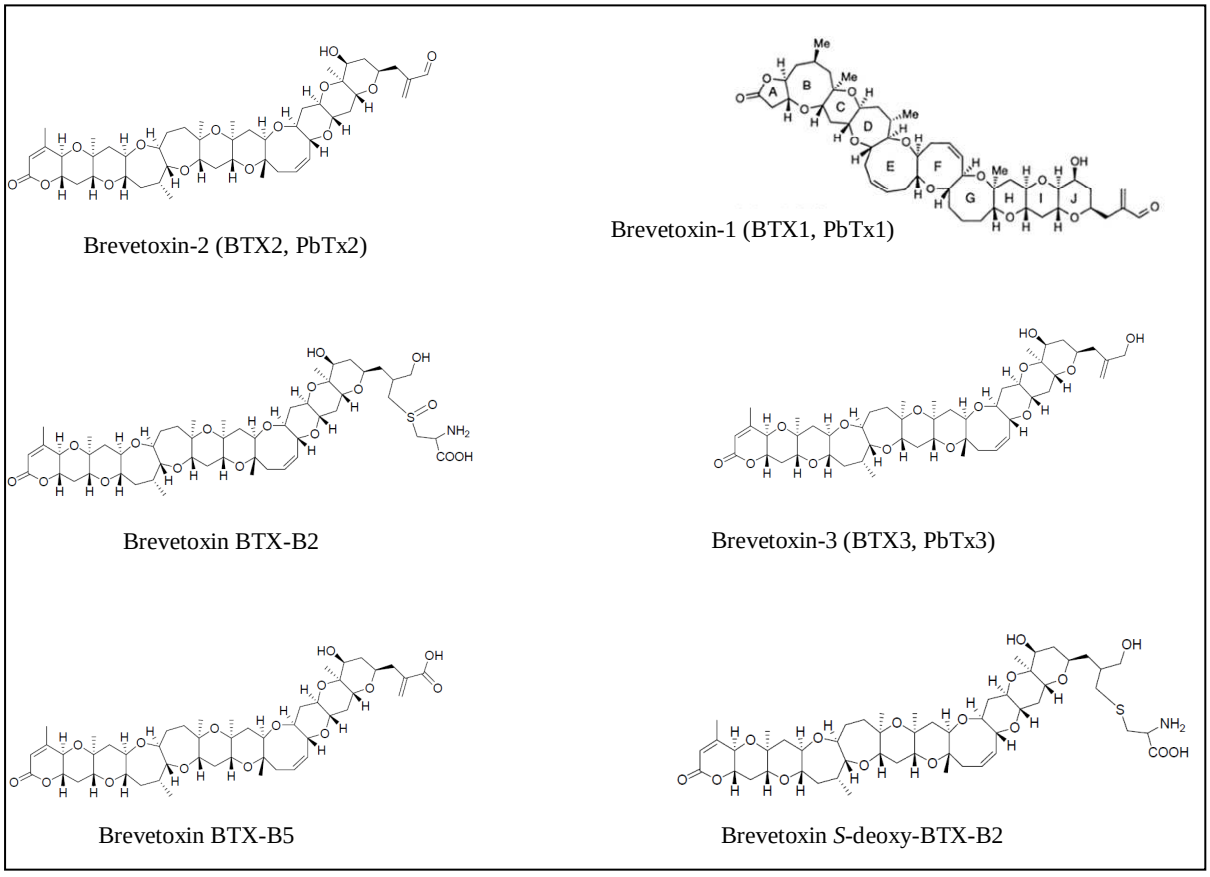
The work presented in this thesis was focused on the development of rapid LC-MS/MS methods to analyse brevetoxins in algal extracts. In particular, the different aims were: I) to develop a rapid isocratic method to analysed BTX1, BTX2, and BTX3 in algal samples (e.i *Karenia brevis* extracts); II) to develop a gradient method to quantify BTX1, BTX2, BTX3, BTX-B2, deoxyBTX-B2, and BTX-B5, which was then apply to algal samples obtained from cultures of *Karenia brevis* and raphidiphytes (*Fibrocapsa japonica, Chattonella subsalsa and Heterosigma akashiwo*) from the Adriatic Sea; III) to develop a method to analyse hand-picked *K. brevis* cells by micro-column liquid chromatography-tandem mass spectrometry.

3.5.4. Experimental section

Chemicals

Analytical grade acetonitrile (ACN), methanol (MeOH), and formic acid (FA) were purchased from Caledon (Georgetown, Canada). The brevetoxins (BTXs) certified reference material, such as CSRM-BTX1 (3.0 µg mL^{-1}), CSRM-BTX2 (6.6 µg mL^{-1}), CSRM-BTX3 (3.6 µg mL^{-1}), CSRM-BTX-B2 (5. µg mL^{-1}), CSRM-S-deoxyBTX-B2 (5.0 µg mL^{-1}), CSRM-BTX-B5 (4.3 µg mL^{-1}), was provided by the National Research Council, Institute for Marine Biosciences (NRC-CNRC, Halifax, Canada). Water was distilled and further purified using a Milli-Q purification system (Millipore, Billerica, MA, USA) and seawater at salinity 35 was filtered using 0.22 µm cartridge filters (Harmsco, North Palm Beach, FL, USA).

Fig. 3.9 – Structures of the brevetoxins used for this study



Algal cultures

A strain of *Karenia brevis* (CCMP2281) from the Gulf of Mexico (USA) isolated in 1999 was used in this study. For the experiment, 1000 mL Erlenmeyer flasks containing 550 mL of sterilized f/2-Si medium (Guillard, 1975) at salinity of 35, were prepared in duplicate.

The culture flasks were illuminated at 105-110 μmol photons m^{-2} s^{-1} from daylight type cool white lamps (16:8 h light-dark period), with temperature controlled at 20°C. Every 2-3 days 10 mL culture were collected from each flask, centrifuged at 4000 x *g* for 5 min and the algal pellet so obtained was kept at -80°C. Cell counts were made in settling chambers following Utermohl's method (Hasle, 1978).

Raphidophyceae *Fibrocapsa japonica, Chattonella subsalsa* and *Heterosigma akashiwo* cells were isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from blooms which occurred in 2006 in the Adriatic Sea (Marche, Italy). After initial growth in microplates, cells were kept in sterile Erlenmeyer flasks sealed with cotton at 20°C, during a 16:8 h light–dark period at about 100–110 μmol photons m^{-2} s^{-1} from cool white lamps (McLachlan, 1973).

These strains were cultured in f/2-Si medium (Guillard, 1975) made with natural seawater, adjusted to a salinity of 35 through the addition of distilled water, and kept under the temperature and the light conditions described above.

For the experiment, 4 Erlenmeyer flasks containing 1500 mL culture were used for each species and collected at the end of the stationary phase (total volume: 6000 mL for each species) centrifuging at 5000 x *g* for 10 min. Algal pellets were kept at -80°C.

BTXs extraction

Algal pellets of *K. brevis, F. japonica, C. subsalsa and H. akashiwo* were extracted using a methanol solution. Algal pellets were added to 1 mL (for *K. brevis*) or 2 mL (for Raphidophyceae) 100% MeOH and sonicated for 2 min in pulse mode, while cooling in an ice bath. Then the mixture was added to a same volume (1 or 2 mL) 60% MeOH, centrifuged at 5000 x *g* for 15 min, and the supernatant was decanted. The extract was adjusted to a final volume of 1.5 or 3 mL for *K. brevis* or the Raphidophyceae, respectively.

LC–MS analysis

The LC–MS analysis was carried out using an Agilent 1100 series LC system coupled to an API-4000 triple quadrupole (Applied Biosystems, Foster City, CA, USA) mass spectrometer, equipped with a Turbo V electrospray ion source (MDS Analytical Technologies, Concord, Canada). Nitrogen nebulizer gas was set at 50 (GS1, GS2) and heated at 275 °C. A higher temperature can give somewhat increased sensitivity, but was not used to avoid magnification of matrix effects (unpublished data). BTXs were detected using selected reaction monitoring (SRM) with the ion source in positive mode.

BTXs quantitation and limit of detection (LOD) measurements were achieved with BTXs calibration solutions (10-2500 ng mL^{-1}) prepared in 80% MeOH. LOD was measured statistically by the formula: LOD= [(3 SD)/b]; where "SD" is the standard deviation of repeated runs of the minimum measurable BTX concentration in a standard solution (n = 5) and "b" is the slope of the calibration curve. The best transition (bold, in Table 1) was used for LOD determination.

3.5.5. Optimization of instrumental parameters

In preliminary experiments, instrumental parameters and chromatographic conditions were optimized. These parameters included source temperature (60-550°C), declustering potential (DP = 20-90 V), collision energy (CE = 15-60 eV), mobile phase composition (acidic and neutral), flow rate, and column temperature. Different columns (50 mm X 2 mm i.d.) were used in the development of the LC-MS methods for BTXs:

- Synergi Fusion RP, 2 μm;
- Thermo Hypersil-BDS-C8, 3 μm;
- Phenomenex LUNA-C18, 3 μm.

In preliminary experiments the mass spectrometer with ESI interface was operated either in positive and negative mode and reported as best transitions those shown in Table 3.8 for each compound. ESI^+ was subsequently selected as it resulted the best mode to analyze all the BTXs. The monitored selected reaction monitoring (SRM) transitions as well as the optimazed declustering potential (DP) and collision energy (CE) are reported in Table 1: *m*/*z* 884.6→221.1, *m*/*z* 884.6→403.4 and *m*/*z* 884.6→850.5 for BTX1; *m*/*z* 912.6→877.5, *m*/*z* 912.6→473.6 and *m*/*z* 912.6→319.3 for BTX2; *m*/*z* 897.6→725.5, *m*/*z* 897.6→303.4 and *m*/*z* 897.6→249.3 for BTX3; *m*/*z* 1034.7→929.6, *m*/*z* 1034.7→403.4 and *m*/*z* 1034.7→249.3 for BTX-B2; *m*/*z* 1018.7→929.7, *m*/*z* 1018.7→473.5 and *m*/*z* 1018.7→403.4 for deoxyBTX-B2; and *m*/*z* 928.6→299.1, *m*/*z* 928.6→473.5 and *m*/*z* 928.6→403.4 for BTX-B5.

An abundant $[M+NH_4]^+$ ion dominated the spectrum of BTX1 and BTX2, while $[M+H]^+$ resulted the predominant ion in the spectra of all the other BTXs. Fragment ions were characteristic of these compounds and in accordance with the fragmentation reported for these reference standards.

The retention that compounds experience on reversed phase HPLC columns is governed by their lipophilic properties and by the presence of polar or ionic groups, which can interact with the stationary phase. Moreover, the pH of the mobile phase is an important parameter

which can significantly influence the amount of interaction between the toxins and the stationary phase.

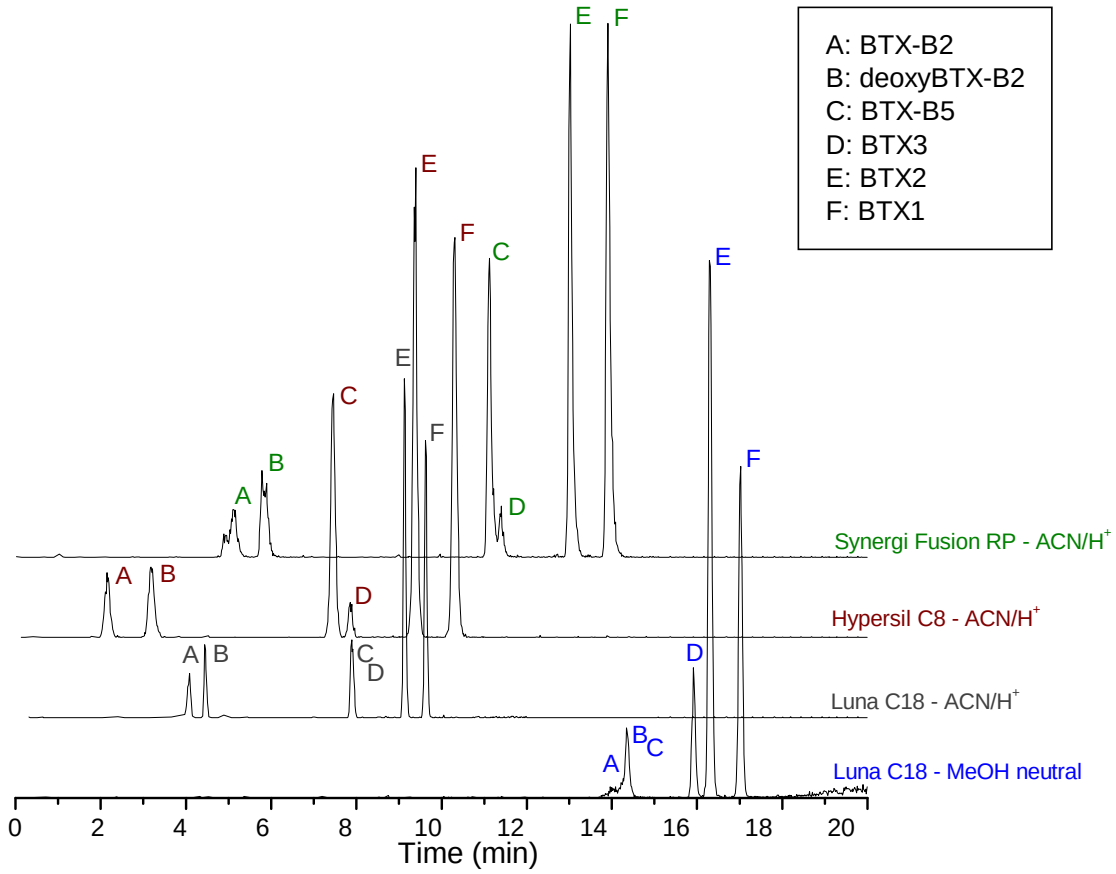
Table 3.8 – MS/MS conditions used for the multiple reaction monitoring (MRM) acquisition to detect brevetoxins (BTXs). Bold refer to the best transition, which was used for LOD determination.

Analyte	ESI mode		Transition	CE (eV)	DP (V)
		[M+NH4]+			
BTX1	ESI+	884.6	**884.6/221.1**	**35**	**60**
			884.6/403.7	35	60
			884.6/850.5	30	60
		[M+NH4]+			
BTX2	ESI+	912.7	**912.6/877.5**	**35**	**50**
			912.6/473.6	45	50
			912.6/319.3	45	50
		[M+H]+			
BTX3	ESI+	897.6	**897.6/725.5**	**40**	**90**
			897.6/303.4	35	90
			897.6/249.3	40	90
		[M+H]+			
BTX-B2	ESI+	1034.7	**1034.7/929.6**	**55**	**90**
			1034.7/403.4	55	90
			1034.7/249.3	70	90
		[M-H]-			
	ESI-	1032.7	**1032.7/897.5**	**65**	**90**
			1032.7/721.5	70	80
			1032.7/525.4	70	90
		[M+H]+			
deoxyBTX-B2	ESI+	1018.7	**1018.7/929.7**	**50**	**90**
			1018.7/473.5	60	90
			1018.7/403.4	60	90
		[M-H]-			
	ESI-	1016.7	**1016.7/929.4**	**60**	**70**
			1016.7/739.5	65	80
			1032.7/525.4	75	90
		[M+H]+			
BTX-B5	ESI+	911.7	**928.6/299.1**	**50**	**40**
			928.6/473.5	50	40
			928.6/403.4	50	40
		[M-H]-			
	ESI-	910.6	**910.5/739.5***	**60**	**90**
			910.6/461.4	70	70
			910.5/525.4	65	90

Therefore, in initial experiments, the three columns were compared using all the six reference BTX standards (BTX1, BTX2, BTX3, BTX-B2, deoxyBTX-B2, and BTX-B5) under acidic and neutral mobile phase conditions, in particular these were:

- Acetonitrile (ACN) with 2 mM ammonium formate (HCOONH4) and 50 mM formic acid (HCOOH);
- Methanol (MeOH) with 5 mM ammonium formate (HCOONH4) and 2.5% isopropanol (IPA);
- Acetonitrile (ACN) with 5 mM ammonium formate (HCOONH4).

Fig. 3.10 – Separation of brevetoxins (BTXs) standards using the Synergi Fusion RP, the Hypersil C8 and the Luna C18 columns under acidic LC conditions; and the Luna C18 column under neutral conditions.



As for the mobile phase, ACN/H^+ resulted in a better separation of the different brevetoxins (Fig. 3.10), which eluted already in the first part of the chromatogram. Under acidic conditions a slightly improved peak shape was obtained for all the BTXs, especially with the LUNA-C18 as column, and this is in accordance with the methods described far often for the

separation of BTXs (Roth et al., 2007; Twiner et al., 2007), where a mobile phase containing acetic acid is used.

The survey of different stationary phase columns (50 mm X 2 mm i.d.) revealed that those packed with 3 μm Hypersil-BDS-C8 and LUNA-C18 are capable of a better resolution in term of separation and peak shape. Although a better sensitivity was obtained with the Hypersil-BDS-C8 column, LUNA-C18 was selected due to the best peak shape, in particular peaks appeared narrower and more symmetric.

Using Sinergi Fusion RP column, overlapping of toxins occurred. For all the tested toxins the peak width was better on the LUNA-C18 column with the acidic mobile phase; thus, it can be concluded that both this column and the acidic mobile phase contributed to a better peak shape.

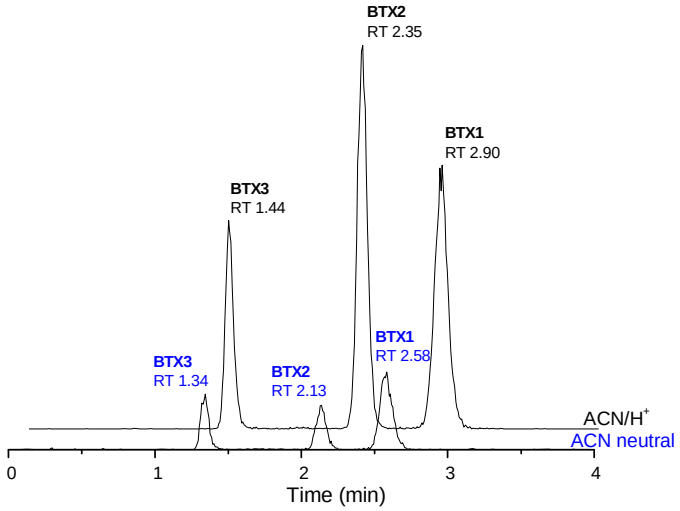
LC-ESI-MS-MS analyses conditions applied in the development of the different LC-MS methods for BTXs were therefore based on the results of these preliminary experiments.

3.5.6. Isocratic rapid method for BTX1, BTX2, BTX3

LC-MS conditions: BTXs were separated by an Agilent 1100 LC series using the 3 μm Phenomenex LUNA-C18 column (50 X 2 mm i.d.). The mobile phase was based on aqueous acetonitrile (ACN) with 2 mM ammonium formate and 50 mM formic acid (acidic mobile phase). Additionally, the column was tested for a neutral mobile phase system which consisted in 5 mM ammonium formate. After preliminary experiments in which different percentage of ACN were used, the selected method resulted in a 75:25 acetonitrile:water mobile phase for 4 min. Source and column temperatures were 350 and 25°C, respectively. The flow rate was set at 0.3 mL min^{-1}, and 3 μl of sample were injected. BTXs were detected using an API-4000 triple quadrupole (Applied Biosystems, Foster City, CA, USA) mass spectrometer equipped with a Turbo V electrospray ion source (MDS Analytical Technologies, Concord, Canada) in selected reaction monitoring (SRM), with the ion source in positive mode. Structural identification, detection and quantification were carried using the SRM transitions and conditions previously described (Table 3.8). An external calibration curve was obtained using 6 concentrations (10-2500 ng mL^{-1}) of the BTXs standard solution (BTX1, BTX2 and BTX3). Each sample was analyzed in triplicate and the concentrations were reported as mean values.

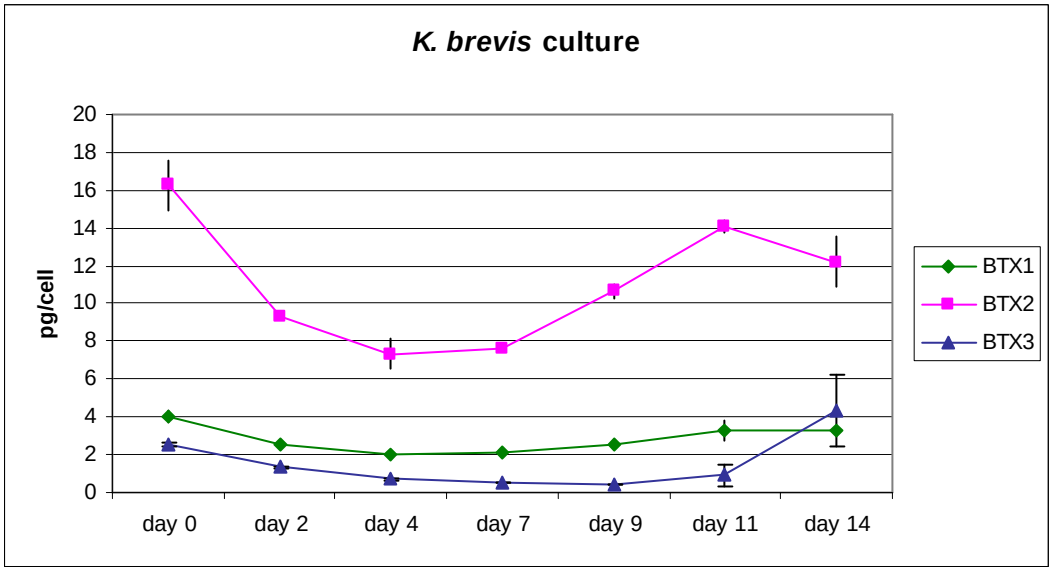
Results: The isocratic method resulted a rapid and sensitive method to detect and quantify BTX1, BTX2, BTX3 in algal extracts. Since these brevetoxins in the preliminary experiments reported good peak shape and separation using either the neutral and acidic mobile phases, the two different conditions were compared, using the LUNA-C18 as column (Fig. 3.11).
ACN/H^+ resulted in a better sensitivity for all the different brevetoxins, which reported higher peak intensities and better linearity in the calibration curves. Using the acidic mobile phase R^2 were in fact 1, 0.9941 and 0.9998 for BTX1, BTX2 and BTX3, respectively; while values in the range 0.9834-0.9917 were found with the neutral mobile phase. The LOD of BTXs with the acidic method was 15, 43 and 27 pg on-column for BTX1, BTX2 and BTX3, respectively.

Fig. 3.11 – Separation of brevetoxins (BTX1, BTX2 and BTX3) standards using the Luna C18 column under acidic and neutral LC conditions.



This method was subsequently applied to analyze algal extracts of *K. brevis* (Fig. 3.12). The experiment was performed growing two different cultures for 14 days; every 2-3 days pellet were collected and extracted and BTX1, BTX2, BTX3 produced during the growth were analyzed. It has to be noted that the high toxin concentration per cell at the beginning of the experiment is probably due to the fact that for the inoculum a culture with cells in the stationary phase was used, therefore cellular toxin content was high. The BTXs pattern was similar for the two replicates, being the BTX2 the major toxin, followed by BTX1, as previously reported for *K. brevis* (Pierce et al., 2005). However, as *K. brevis* culture aged there was a relative increase in the BTX3 compared to the BTX2, probably due to degradation of BTX2, which formed BTX3. This last finding had been already observed during *K. brevis* blooms (Tester et al., 2008).

Fig. 3.12 – Brevetoxins concentration (pg cell^{-1}) in *Karenia brevis* cultures during the growth. Two different replicate cultures were used for the experiment.



3.5.7. Gradient method for BTX1, BTX2, BTX3, BTX-B2, deoxyBTX-B2 and BTX-B5

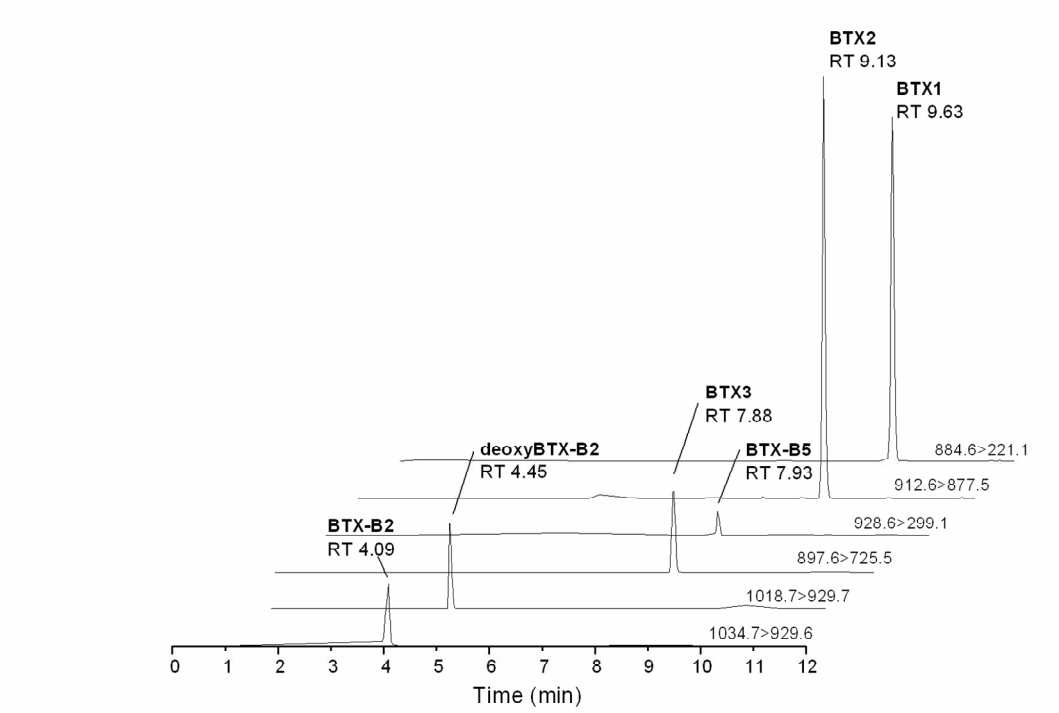
LC-MS conditions: BTXs were separated by an Agilent 1100 LC series using the 3 µm Phenomenex LUNA-C18 column (50 X 2 mm i.d.). The mobile phase was based on aqueous acetonitrile (ACN) with 2 mM ammonium formate and 50 mM formic acid. A gradient elution was used: 35-100% ACN over 10 min, and hold for 2 min. Source and column temperatures were 275 and 40°C, respectively. The flow rate was set at 0.3 mL min^{-1}, and 3 µl of sample were injected. BTXs were detected using an API-4000 triple quadrupole (Applied Biosystems, Foster City, CA, USA) mass spectrometer equipped with a Turbo V electrospray ion source (MDS Analytical Technologies, Concord, Canada) in selected reaction monitoring (SRM) with the ion source in positive mode. Structural identification, detection and quantification were carried using the SRM transitions and conditions previously described (Table 3.8). An external calibration curve was obtained using 6 concentrations (10-2500 ng mL^{-1}) of the BTXs standard solution (BTX1, BTX2, BTX3, BTX-B2, deoxyBTX-B2 and BTX-B5). Each sample was analyzed in triplicate and the concentrations were reported as mean values.

Results: The gradient method developed using an acidic mobile phase and LUNA-C18 column provided a good baseline separation of the six brevetoxins (Fig. 3.13). It resulted sensitive and in a good linearity of all the BTXs standards, with R^2 values 0.9940-0.9996.

LOD of BTX1, BTX3 and BTX-B2 was in the range 31-44 pg on-column; it slightly increased for BTX2 (67 pg on-column) and reached a maximum value of 115 and 126 pg on-column for BTX-B5 and deoxyBTX-B2, respectively.

Algal extracts of the Raphydophyceae *F. japonica*, *H. akashivo and C. subsalsa* were analyzed using this method, to check for the presence of some of the most common brevetoxins found in algal samples and their metabolites. None of these toxins was found in the analyzed algal extracts, leading to the conclusion that these species don't produce any of these known brevetoxins, especially in considered that the method resulted sensitive and that the algal extracts had been obtained from large cultures (6000 mL).

Fig. 3.13 - Separation of brevetoxins (BTXs) standards using the Luna C18 column under acidic LC conditions.



3.5.8. Analysis of hand-picked *K. brevis* cells by micro-column liquid chromatography-tandem mass spectrometry

The method involves micro-pipette sampling of cells, transfer to the cup of a micro filter, removal of seawater, extraction of cells with methanol, and direct LC-MS/MS analysis. A column switching system is used that allows a large volume injection and cleanup. Toxins are

trapped on an LUNA-C18 cartridge and are then back-flushed onto a microbore LC column coupled with the MS (Fig. 3.14).

Sample Extraction: Millex-GV4 filters (0.22 µm) (Millipore) were soaked in methanol (MeOH) overnight, followed by a final flush with 500 µL MeOH and then air drying. Before use, each filter was filled with 100 µL MeOH for 5 min, followed by centrifugation at 2000 x *g* for 5 min, and then a rinse with 50 µL of filtered (0.22 µm) seawater, followed by centrifugation at 2000 x *g* for 5 min, was performed.

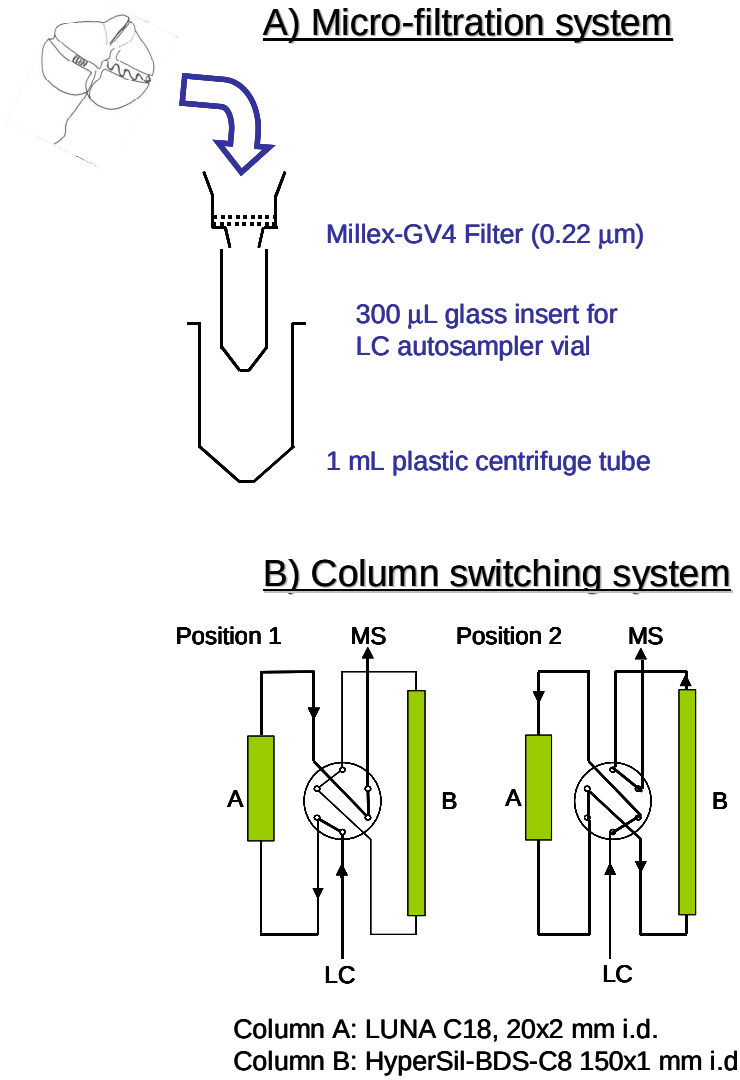
As shown in Fig. 3.14A, cells were selected and pooled by micropipette isolation and transferred to the cleaned Millex-GV4 filters. Excess seawater from the cell(s) was removed by centrifuging for 2 min at 2000 x *g*; the filtrate was then discarded.

To each filter 50 µL of 100% MeOH were added; after standing for 5 min, filters were centrifuged at 4000 x *g* for 5 min to collect the extracts in a 300 µL micro-insert for LC autosampler vials. Then, 25 µL of 40% MeOH were added to each filter; after standing for 5 min, centrifugation at 4000 x *g* for 5 min was performed and the total extract was collected in the 300 µL micro-insert. The used filters were discarded and the inserts containing the extracts were transferred to LC vials, tightly capped, and stored at -20°C until LC-MS analysis.

LC-MS conditions: BTXs were separated by an Agilent 1100 binary LC, using a LUNA-C18 cartridge (20 x 2 mm i.d.) and a Hypersil-BDS-C8 column (150 x 1 mm i.d.) connected to a switching valve as shown in Fig. 3.14B. Mobile phases consisted in water (A) and 95% acetonitrile (B), both with 50mM formic acid and 2mM ammonium formate. Equilibration was obtained using a 75 µl/min flow for 15 min at 10% B in position 2. 50 µL of each sample were injected and the flow continued at 10% B in position 1 (flow through LUNA-C18 cartridge only) for 2 min at 0.2 mL/min. A gradient elution was used: the valve was switched to position 2 (both columns, back-flushing from the LUNA-C18 cartridge onto the Hypersil column) at 75 µl/min, then the gradient increased from 10 to 30% B over 0.5 min and then from 30% B to 100% B over 10 min, hold at 100% for 18 min. BTXs were detected using an API-4000 triple quadrupole (Applied Biosystems, Foster City, CA, USA) mass spectrometer equipped with a Turbo V electrospray ion source (MDS Analytical Technologies, Concord, Canada) in selected reaction monitoring (SRM) with the ion source in positive mode. Structural identification, detection and quantification were carried using the SRM transitions and conditions previously described (Table 3.8).

BTXs quantitation and LOD measurements were achieved with BTXs calibration solutions (10-2500 ng mL^{-1}) prepared in 80% MeOH. Each sample was analyzed in triplicate and the concentrations were reported as mean values.

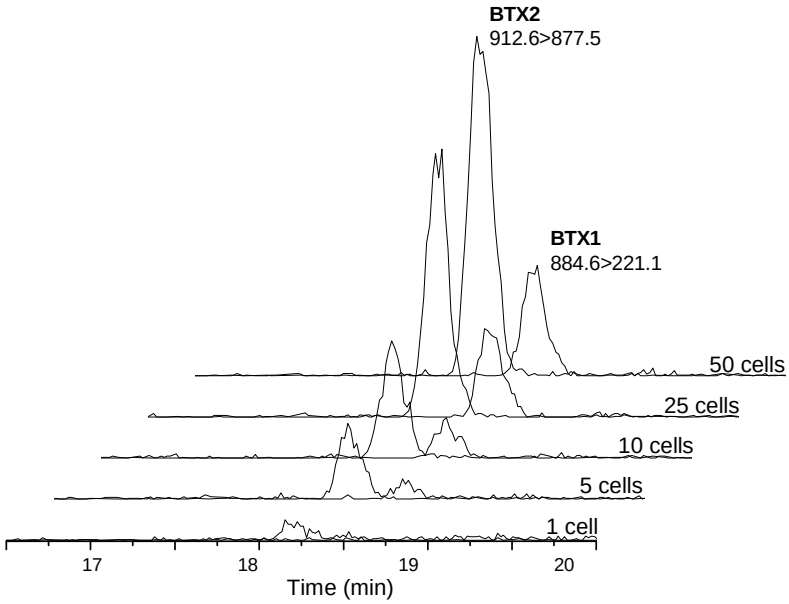
Fig. 3.14 – A) Micro-filtration system used to extract picked cell samples, and B) Column switching system used for analysis.



Results: The method proposed is based upon a micro sampling and extraction procedure coupled with micro-column liquid chromatography-tandem mass spectrometry and demonstrated the analysis of single or multiple hand-picked plankton cells. In fact, the toxin concentration and profile present in plankton can vary considerably between different geographical areas and even within one region and between seasons due to the presence of

different species and strains; moreover, variations in toxin concentration could also be due to differences in the cell biovolume or within a population toxin content variability.

Fig. 3.15 - Analysis of 1, 5, 10, 25 and 50 individually picked cells of *Karenia brevis* sampled from a culture. In each case, only 2/3 of the extract was injected (50 μL from 75 μL). It is interesting to note that the BTX1 and BTX2 concentration increased proportionally from 1 cell to 50 cells samples.

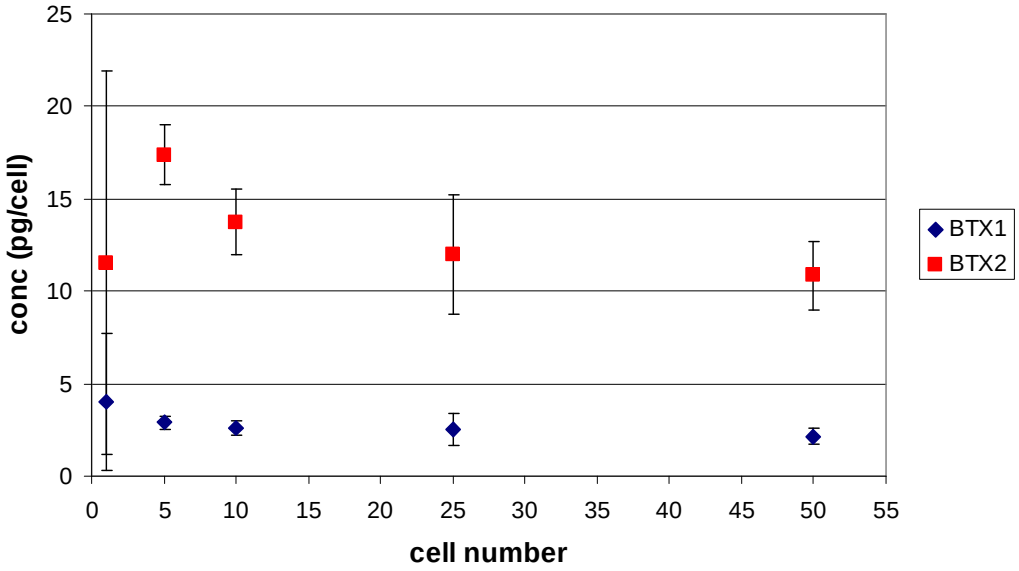


Traditional methods of analysis require substantial sample sizes (e.g., millions of cells), making it difficult to attribute the presence of toxins or variations in toxin profiles to individual species.

This method resulted in a good linearity of the BTX1, BTX2 and BTX3 standard solutions, which reported R^2 in the range 0.9950-0.9977 and LOD values of 15, 45 and 25 pg on-column, respectively.

It was subsequently used to analyzed the brevetoxin content in *K. brevis* samples obtained hand-picking 1 to 50 cells. As shown in Fig. 3.15, this system was able to detect BTXs in extracts obtained from 1-5 cells of *K. brevis* and resulted in a good correlation of the toxin content with the number of extracted cells (Fig. 3.16). Although toxin content variability was quite high for single cell samples, probably due also to the natural variability of the cells in the toxin production, it decreased considerably already for 5 cells samples.

Fig. 3.16 – Analysis of a series of extracts prepared from cells hand-picked from a *Karenia brevis* culture (CCMP2281). In each case, only 2/3 of the extract was injected (50 µL from 75 µL).



3.5.9. Conclusion – LC-MS methods for BTXs

Development in the use of LC-MS has progressed rapidly, since it has been considered a good alternative to the MBA, offering greater selectivity, sensitivity, reliability and rapidity (Hess et al., 2003; Ciminiello et al., 2002). In recent years much effort has been put in the development of LC-MS/MS methods that are dedicated to either detect the specific classes of marine lipophilic toxins or detect as much as possible different marine lipophilic toxins in a multi-toxin method. In the literature, several methods for the separation of okadaic acid, its derivatives dinophysistoxins, yessotoxins, domoic acid, spirolides, pectenotoxins and azaspiracids have been described (Quilliam, 2003). BTXs are lipophilic toxins produced by the dinoflagellate *K. brevis* and are rapidly metabolized to cysteine conjugates when accumulated in shellfish (Roth et al., 2007). Due to the presence of the ionic amino acid group, brevetoxins elute in the same region of the chromatogram as okadaic acid and yessotoxins and are best analyzed in ESI^+; therefore, it is not possible to analyze them simultaneously with the other marine lipophilic toxins without polarity switching (Gerssen et al., 2009).

The LC-MS methods for BTXs described here allows the determination of brevetoxins in algal extracts or hand-picked plankton cells from cultures or from the field. The use of an acidic mobile phase and LUNA-C18 column substantially improved the separation of these compounds and gave good linearity of the standard solutions and good LOD values. These

LC-MS/MS methods resulted rapid, specific and sensitive, being able to separate and detect the major BTXs and metabolites found in environmental samples. An accurate determination of the toxin profile and individual toxin concentrations within a single cell was also possible, and result in a good correlation between the number of extracted cells and BTXs total content.

3.6. Conclusion – *F. japonica*

The appearance of *F. japonica* in north European waters during the 1990s caused concern because of the reported ichthyotoxicity of various species belonging to the Raphidophyceae class (Iwasaki, 1971; Okaichi, 1972; 1989; Toriumi and Takano, 1973). In this thesis the toxicity of Adriatic strains was evaluated by considering all the compounds previously postulated as involved in the toxic mechanism, such as brevetoxins, fatty acids and ROS to understand their role in toxicity. Several hypotheses have in fact been proposed for the toxic mechanism of the raphidophytes, especially for the species *Chattonella antiqua* and *Chattonella marina*, which have been studied more extensively. Recently a number of studies were also performed on *F. japonica* strains from the USA, Japan, Australia, New Zealand, the Netherlands, Germany, and France in order to characterize their growth and toxicity features (Khan et al., 1996; Fu et al., 2003; de Boer et al., 2004; van Rijssel et al., 2008; Guidi-Rontani et al., 2010); nevertheless the toxic mechanism of this raphidophyte is still unclear.

The chemical analyses of cultured Adriatic strains excluded the presence of known brevetoxins, in accordance with the results obtained in the ichthyotoxic assays with *D. labrax*, which highlighted the difference between the delayed effects of *F. japonica* and the rapid ichthyotoxic effects of *K. brevis*, a brevetoxin-producing microalga.

The cellular fatty acid profile evidenced a high amount of PUFAs, including the three haemolytic ones 18:4n-3 (OTA), 20:4n-6 (AA), and 20:5n-3 (EPA), found in a German strain and called fibrocapsins Fj1, Fj2, and Fj3 (Fu et al., 2004a). These compounds were confirmed to be haemolytic (de Boer et al., 2009) and to be toxic for crustaceans and bacteria (Fu et al., 2003; Jüttner, 2001).

Fatty acid composition and quantification of our strain showed values in accordance with those reported for Japanese and New Zealand strains (Marshall et al., 2002; Mostaert et al., 1998; Cho et al., 1999b), except for a lower amount of arachidonic acid (20:4n-6). So far, all the results obtained in several strains reveal a high variability in the composition of *F. japonica* depending on the isolate (summarized in Table 3.9). In fact, the total amount of PUFAs has been reported in one case to vary between 14 and 49 pg $cell^{-1}$ (Marshall et al.,

2002), in another between 150 and 350 pg cell^{-1}, and reaching the highest value of 450 pg cell^{-1} in a German strain (de Boer et al., 2009).

Table 3.9 - Summary of the fatty acid composition (%) of several *F. japonica* isolates.

	% of total fatty acids						
	present study	Marshall et al. 2002		Cho et al. 1999		Mostaert et al., 1998	
Fatty acid	**FJAP0603**	**FJJp**	**FJNZ**	**NZFj**	**JFj**	**N136**	**N560**
12:0							
14:0	29.48	18.5	20.1			27.1	24.9
16:0	21.79	7.6	10.7	30.4	30.7	11.0	10.5
16:1n-7	1.31	2.6	1.7	4.7	5.8	2.4	4.9
16:1isomer (n-13)	1.77	1.2	1.0			1.8	2.1
16:1isomer						0.9	0.8
16:2							
16:4n-3				0.4	0.1		
18:0	1.22	1.6	0.7	4.3	6.1		
18:1n-9	3.91	7.4	6.3	16.3	24.0	5.9	6.9
18:1n-7	4.40	0.5	5.7				
18:2n-6	1.44	2.9	3.1	1.3	1.7	4.6	5.9
18:3n-6 (γ)	1.16	1.3	0.8			3.2	2.5
18:3n-3 (α)	2.36			1.6	1.5	3.3	5.9
18:4n-3	10.40	26.6	12.0	7.7	6.5	15.2	12.4
18:5n-3		0.0	0.0	0.5	0.2		
20:2n-6				0.2	0.2		
20:4n-6	3.29	4.4	7.2			6.9	6.8
20:4n-3		0.7	1.1	7.4	5.4		
20:5n-3	17.47	17.4	24.5	20.9	12.3	16.5	15.0
22:4n-6		0.3	1.7				
22:5n-6		0.6	0.1				
22:5n-3		0.0	0.0				
22:6n-3		0.4	0.2	1.4	1.9	1.0	1.0
Tot PUFAs	36.12	54.5	50.7				
Tot PUFAs (pg/cell)	27.57						
Tot (pg/cell)	76.34	25	97				

The fatty acids quantification through GC-MS analysis led us to calculate the PUFA concentrations in live *F. japonica* cells to which *Artemia* nauplii were exposed during the reported toxicological assays and to establish that they rarely reached toxic levels. The maximum *F. japonica* cell densities tested during the short-term assays contained a PUFA amount of 8.8 μg mL^{-1}, a level proved to be non-toxic by present tests with various fatty acid standards (Table 3.4). Regarding the diatom *P. tricornutum*, which was never reported as toxic, even at a cell concentration of 10^6 cells mL^{-1}, none of the cell densities tested with *Artemia* nauplii resulted in an amount of PUFAs sufficient to cause mortality, as the highest cellular concentration used in the experiments corresponded to 1.2 μg mL^{-1}. Death occurred only when *Artemia* were exposed to high *F. japonica* cell concentrations (long-term assay), probably because the nauplii could accumulate fatty acids, thus becoming lethal when a high amount of cells per single organism was tested. Nevertheless, Cho et al. (1999b) reported the absence of a correlation between the amount of PUFAs and the mortality of *Artemia*.

The solid phase extraction (SPE) of the Adriatic *F. japonica* extract resulted in a high concentration of PUFAs in two main fractions, B and C, which caused 100% mortality of *Artemia* nauplii. These fractions had PUFA amounts of about 63 μg mL^{-1} (B) and 311 μg mL^{-1} (C), levels comparable with those of the fatty acid standards which were used for the *Artemia* test, and which were also found to cause nauplii mortality. The toxicity of these fractions was also confirmed by the inhibition of *V. fischeri* bioluminescence. None of the PUFAs was instead detected in *F. japonica* extracellular medium, thus justifying the lack of effects for *Artemia* (Table 3.5).

The various toxicological assays showed rather different results according to the organism complexity. The effect of cell extracts on *V. fischeri* bioluminescence was high compared with that exerted by other microalgae. These included a strain of *P. reticulatum* which produces yessotoxins (YTX), compounds which evidently do not interfere with the bioluminescence emission of *V. fischeri*. An effect of *F. japonica* on *V. fischeri* bioluminescence was also found in a German strain (van Rijssel et al., 2008), even if less strong than that observed in this study. In that case, the effect was caused by live cells at concentrations far lower than those present in our extracts and it was thought to be caused by substances which were extracellularly released.

The assay with *C. carpio* erythrocytes showed that *F. japonica* had haemolytic effects, as increasing cell concentrations caused the lysis of up to 100% erythrocytes. The reported EC_{50}

values were in accordance with the range found by de Boer et al. (2009) and were relatively low. This result could be indicative of a marked ichthyotoxic potency, an aspect not supported by the fish assays which evidenced a rather delayed effect of the Adriatic *F. japonica* with mortality occurring only after 9-10 days of exposure.

The significant increase of H_2O_2 obtained in the tanks where sea basses were exposed to *F. japonica* was also relevant. This indicates that the presence of fish stimulates H_2O_2 production by algal cells and supports the hypothesis of H_2O_2 involvement in *F. japonica* toxicity. Kim et al. (1999) reported lipid peroxidation of gill tissue in *Cochlodinium polykrikoides*-exposed flatfish and suggested that ROS were one of the factors inducing fish mortality, through reduction of the capability for oxygen transfer in gills. They proposed that when *C. polykrikoides* passed through the fish gill lamella, gill cells were attacked by the ROS generated by algal cells, resulting in an oxidative damage. In the present work, the concentration of H_2O_2 measured in the *F. japonica* tanks were not considerably higher than in those with the non-toxic diatom *P. tricornutum* and since the compound alone was not toxic for the fish, even when added at high concentrations, this result confirms that other factors are likely to be involved in the toxic mechanism, as already suggested for *Chattonella marina* (Marshall et al., 2003; Woo et al., 2006).

The presence of cellular PUFAs in high amounts could substantiate the hypothesis of a combined effect of these compounds and ROS. Thus, to evaluate the possible involvement of oxidative stress in mortality of sea basses, MDA levels on the gills of the exposed fish were determined. MDA is the major aldehyde formed upon breakdown of lipid hydroperoxides and is still the most commonly applied assay for oxidative stress (Seljeskos et al., 2006); in fact, ROS can react with the double bonds of PUFAs to yield lipid hydroperoxides and one of the major secondary oxidation products is MDA. The average MDA concentrations measured in the gills of fish exposed to *F. japonica* were 13-14-fold higher than those of the controls, in contrast to the results obtained by Woo et al. (2006) in goldlined seabream exposed to *Chattonella marina* for a period up to 6 h. We had also observed that fish exposed to *F. japonica* for 5 days without reporting mortality, then being kept in seawater for 2-4 weeks and exposed for the second time to *F. japonica*, died within a shorter time period (6-8 days) (data not shown). This fact leads us to postulate the involvement in fish mortality of a permanent and accumulating damage, such as the one caused by organ impairment, but to exclude an acute effect such as the one resulting, for example, from red blood haemolysis.

In conclusion, *F. japonica* contains a high amount of PUFAs per single cell; it was already known that these cells produce ROS, and this study confirms that the presence of predators

stimulates H_2O_2 production. As we have shown that PUFAs are not released into the extracellular medium by live cells, we can assume that *F. japonica* cells, which easily cling to fish gills due to their high polysaccharide production, can release in loco both ROS and, as a result of the easy cell breakage, PUFAs. Then they could react in the gills by producing lipid hydroperoxides as testified by the MDA increase; the resulting oxidative damage, possibly associated with haemolytic effects, increases with the number of cells that passes through the gills and accumulates with time, eventually causing fish death.

Although the exact molecular sequence of events leading to crustacean and fish death is not definitively proved, this study on *F. japonica* shows that the Adriatic strains can be harmful to crustaceans as well as to higher level organisms. It was also ascertained that a high cell density and a long exposition time are necessary to cause severe damage on the fish gills or death for crustaceans, thus justifying the absence of fish kill events during the frequent and dense bloom episodes along the Italian coasts.

4. The dinoflagellate *Ostreopsis* cf. *ovata*

Since the end of the1990s massive blooms of *Ostreopsis* cf. *ovata* (Fukuyo, 1981) have been reported in a number of coastal areas of temperate regions with increasing frequency, intensity and distribution. The occurrence of benthic biocenosis suffering and occasional human distress have been described since the earliest bloom events (Bianco et al., 2007; Simoni et al., 2004). Mortality of benthic organisms and human health problems broke out in summer 2005 with an extensive phenomenon along the Ligurian coast, where hundreds of people exposed to marine aerosol during recreational activities required medical cares due to symptoms which mainly affected the respiratory apparatus. This event occurred concurrently to a massive *O.* cf. *ovata* bloom (Mangialajo et al., 2008) and the symptoms in humans disappeared when the bloom intensity decreased (Durando et al., 2007). *Ostreopsis* species were found to produce palytoxin-like compounds, particularly *O. siamensis* and *O. mascarenensis* reported the ostreocin-D (Usami et al., 1995; Ukena et al., 2001) and mascarenotoxins (Lenoir et al., 2004), respectively.

The toxins produced by *O.* cf. *ovata* have been initially identified as a putative palytoxin (pPLTX) in small amount and ovatoxin-a (OVTX-a) as the major toxin (Ciminiello et al., 2008; Guerrini et al., 2010); recently the presence of putative PLTX and OVTX-a was confirmed and the occurrence in the algal extract of four new palytoxin-like compounds, OVTX-b, -c, -d, and -e, was highlighted (Ciminiello et al., 2010). After the 2005 event, studies on *Ostreopsis* spp. have greatly increased and a more careful monitoring of benthic biocenosis have been conducted, evidencing widespread *Ostreopsis* spp. proliferations in the Mediterranean areas, including several Italian regions (Mangialajo et al., 2008; Monti et al., 2007; Totti et al., 2010). It is difficult to establish if *O.* cf. *ovata* is really a species of recent introduction in the Mediterranean area; however, the massive cell proliferations represent a new phenomenon since the brownish mucilaginous film covering all benthic substrates and the associated presence of high cell numbers in the overlying water column, both typical of *O.* cf. *ovata* blooms (Aligizaki and Nikolaidis, 2006), have been reported to occur only recently in Mediterranean basin.

Table 4.1 - Summary of data on the presence of *Ostreopsis* species from several tropical and temperate areas and the concurrently measured environmental conditions. Data not clearly mentioned in the text, when possible, have been extrapolated from tables or figures, and refer to temperature and salinity values of *Ostreopsis* spp. proliferation and to the nutrients range measured in the studied sites.

Algal species	Origin	Temperature (°C)	Salinity (psu)	Nutrients			Reference
				N (μM)	P (μM)	Si (μM)	
Ostreopsis ovata	Mediterranean, Tyrrhenian Sea	28-29					Tognetto et al., 1995
Ostreopsis sp.	Mediterranean, Tyrrhenian Sea	24					Bianco et al., 2006
Ostreopsis ovata	Mediterranean, Tyrrhenian Sea	> 22					Simoni et al., 2003
Ostreopsis ovata	Mediterranean, Ligurian Sea	25					Ciminiello et al., 2006
Ostreopsis ovata	Mediterranean, Ligurian Sea	> 26	38.0-38.2				Mangialajo et al., 2008
Ostreopsis ovata	Mediterranean, Ligurian Sea						Granéli et al., 2008
Ostreopsis cfr. ovata	Mediterranean, western Adriatic Sea	27-29	35.8-36.3				Ingarao et al., 2009
Ostreopsis ovata	Mediterranean, northern Adriatic Sea	16.8-21.8					Totti et al., 2010
Ostreopsis cfr. ovata	Mediterranean, northern Adriatic Sea	20.6 (Croatia) 22.4 (Italy)	37.8 (Italy) 38.6 (Croatia)				Monti et al., 2007
Ostreopsis ovata	Mediterranean, southern Adriatic Sea		36.75-38.44				Ungaro et al., 2005
Ostreopsis sp. (= *siamensis*)	Mediterranean, Strait of Messina	< 25					Gangemi et al., 2006
Ostreopsis sp. (= *siamensis*)	NW Mediterranean, Catalan Sea	24-26 (1997), 18-20 (1998)	37.2-38.1	0.76-7.74 (DIN)	0.11-0.86 (PO_4^{3-})	0.17-4.51 (silicate)	Vila et al., 2001
Ostreopsis ovata	Mediterranean, Aegean Sea	18		0-85 (DIN)	0.5-6.5 (DIP)		Spatharis et al., 2009
O. heptagona and *O. siamensis*	Knight Key (Florida)	25	33				Morton et al., 1992
Ostreopsis lenticularis	northernwestern Cuba			0.2-12.5 (NO_3^-) 0.8-39.9 (NH_4^+) 1.4-52.84 (TIN)	0.005-4.28 (PO_4^{3-}) 0.003-7.78 (TP)		Delgado et al., 2006
Ostreopsis heptagona	Gulf of Mexico	29.5	31	1-1.5 (DIN)*	0.5-2.2 (PO_4^{3-})*		Okolodkov et al., 2007
Ostreopsis sp. 1	Islands of Hawaii	27.6	30.8	0.2-2.5 (NO_3^-+ NO_2^-) 0-0.9 (NH_4^+)	0-0.2 (PO_4^{3-})	0.7-40.9 (silicate)	Parson and Preskit, 2007
Ostreopsis ovata	Islands of Hawaii	25.4	26.7	0.2-2.5 (NO_3^-+ NO_2^-) 0-0.9 (NH_4^+)	0-0.2 (PO_4^{3-})	0.7-40.9 (silicate)	Parson and Preskit, 2007
Ostreopsis lenticularis	Caribbean Sea, Puerto Rico	> 28					Ballantine et al., 1988
Ostreopsis siamensis	New Zealand	20		1.6-3.8 (TN)	0.33-1.10 (PO_4^{3-})		Shears and Ross, 2009
Ostreopsis siamensis	East coast Tasmania	> 20	> 30				Pearce et al., 2001

* expressed in mg/L as reported in the paper

Cells are in fact easily resuspended in the water column, causing a decrease of their abundances in the benthic environment (Totti et al., 2010; Vila et al., 2001), and the important role of hydrodynamism in the bloom development and decline has been hence highlighted (Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008). Moreover, higher concentrations of *Ostreopsis* spp. are usually recorded during warmer periods, characterized by high temperature and salinity and low hydrodinamism; therefore the environmental conditions appear to be one of the main determining factors in the proliferation of these species. Table 4.1 summarizes the environmental parameters (e.g. temperature, salinity and nutrients) recorded in situ during *Ostreopsis* spp. field studies.

Blooms of toxic dinoflagellates usually occur with a seasonal trend; the cell toxin content, consisting in a balance between synthesis and excretion, can be highly influenced by variables such as population growth phase, environment physical and chemical parameters, interaction with bacteria and mechanical stress. Until now a few studies have been performed on the effects of environmental conditions on the growth and toxin production of *O.* cf. *ovata* as well as of other epiphytic dinoflagellates including the genera *Gambierdiscus*, *Prorocentrum*, *Coolia*, and *Amphidinium* (Chinain et al., 2010; Morton et al., 1992; Vanucci et al., 2010), which usually constitute an assemblage with *Ostreopsis* spp.

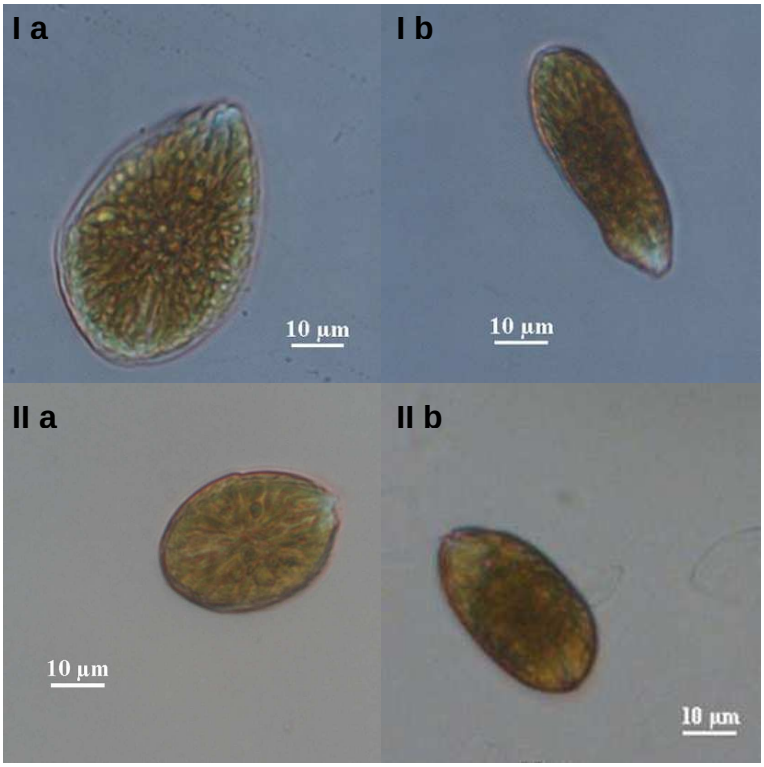
4.1. Toxin production during growth

The evaluation of *O.* cf. *ovata* growth profile was conducted using cultures of two Italian isolates collected along Adriatic and Tyrrhenian coasts of Italy (Guerrini et al., 2010, Fig. 4.1). The two strains reported similar growth patterns, in fact both cultures stayed in the exponential phase for about 10 days and then entered the stationary phase, which lasted up to 28-30 days. Growth rates were initially measured as 0.37 and 0.32 day^{-1} for the Adriatic and the Tyrrhenian strain, respectively (Guerrini et al., 2010); however, subsequent measurements made at different conditions reported higher values up to 0.49 day^{-1} for the Adriatic strain (section 4.2 this thesis). Cell numbers of cultures grown at the optimum conditions could reach values around 13,000-16,000 cells per ml in the stationary phase. Similarly, growth rates of *O. heptagona* and *O. siamensis* from tropical areas were in the range of 0.25-0.50 div day^{-1} (Morton et al., 1992), corresponding to 0.17-0.35 day^{-1} and being therefore slightly lower than those obtained for *O.* cf. *ovata*. The measured growth rates for *Ostreopsis* spp. were thus higher than those reported for cultured *Prorocentrum lima*, which were around 0.22 day^{-1} (Vanucci et al., 2010 and references therein), although cell yields for this species were higher, up to 33,000 per ml.

Both *O. ovata* and *P. lima* share the fact that toxin production increases during the progression of growth, in contrast to what observed for some toxic planktonic species, such as *Pyrodinium bahamense* and *Alexandrium* spp., where the highest toxin level was found during the exponential phase (Gedaria et al., 2007; Navarro et al., 2006). The quantification of the toxin released in the medium by *O. ovata* evidenced that this amount was rather low during the exponential growth phase and increased during the stationary phase, leading to an extracellular toxin content per litre which is three- to nine-fold higher than that measured during the exponential phase (Guerrini et al., 2010). This amount was presumably due to toxin accumulation during the growth, as well as to an increasing number of broken cells; therefore as a natural bloom aged, the toxin content in the water could be enhanced, posing an increasing threat to public health and toxic effects towards marine organisms or people inhaling the aerosol.

The toxin profiles appeared alike in the different Italian strains analysed so far, although toxin levels displayed a certain variability (Ciminiello et al., 2008; Guerrini et al., 2010), in accordance with the results found also in others dinoflagellates (e.g. Errera et al., 2010; Guerrini et al., 2007; Lim and Ogata, 2005).

Fig. 4.1 - Light micrographs of *Ostreopsis ovata* cells from a culture sample of I) Adriatic strain and II) Tyrrhenian strain in (a) dorsal or (b) anteroposterior view (from Guerrini et al., 2010).



4.2. Effect of environmental parameters

4.2.1. Influence of temperature and salinity on the Adriatic *Ostreopsis* cf. *ovata* growth and evaluation of toxin content through HR LC-MS and biological assays

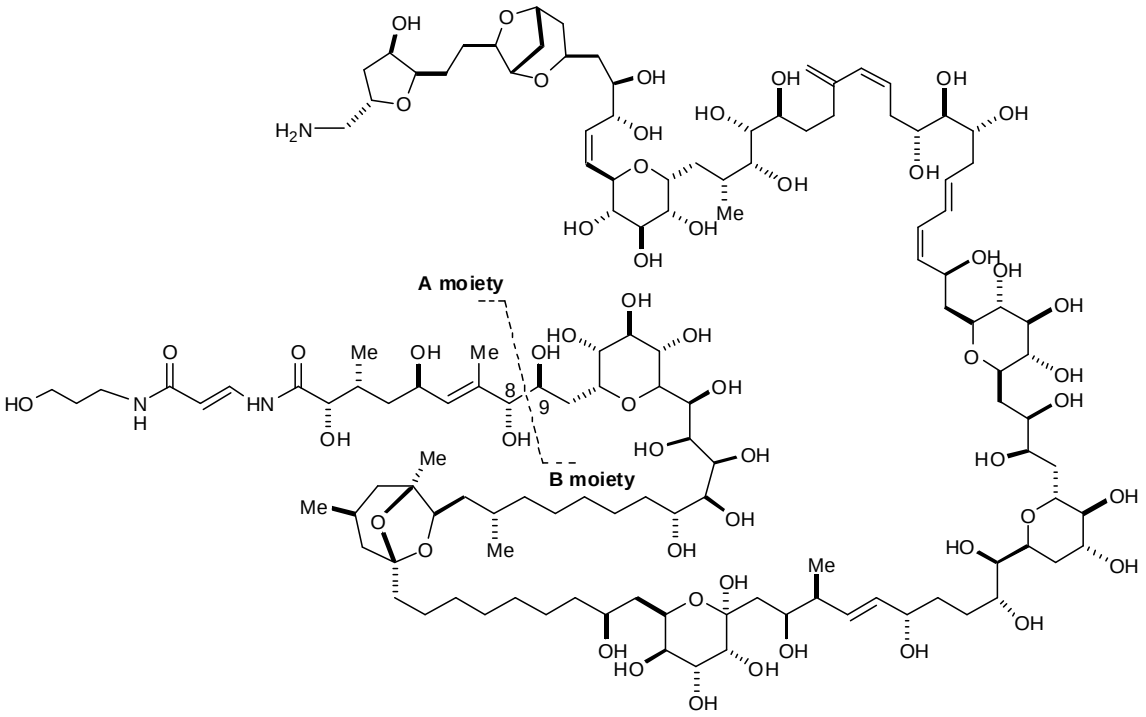
Massive blooms of the benthic dinoflagellates *Ostreopsis* spp. are reported worldwide in many tropical and temperate regions (Faust et al., 1996; Vila et al., 2001; Aligizaki and Nikolaidis, 2006; Chang et al., 2000; Rhodes et al., 2000; Ciminiello et al., 2008). In the Mediterranean Sea, blooms of *O.* cf. *ovata* and *O. siamensis* have been reported since the late '70s (Taylor, 1979; Abboud-Abi Saab, 1989) but, in the last decade, they have become increasingly frequent and resulted in relevant benthic biocenosis sufferings and human health problems.

Ostreopsis species, typically, proliferate in shallow and sheltered waters, with low hydrodynamism; they form a rusty-brown coloured mucilaginous film, which covers reefs, rocks (Bottalico et al., 2002), and soft sediments (Vila et al., 2001) as well as seaweeds (Vila et al., 2001; Bottalico et al., 2002; Aligizaki and Nikolaidis, 2006; Totti et al., 2010), marine angiosperms, and invertebrates (Bianco et al., 2007; Totti et al., 2007). The whole of the above evidence suggests that the presence of *Ostreopsis* spp. in coastal waters may pose a real threat to coastal food web and fishery (Aligizaki et al., 2008). However, the effects on marine organisms and ecosystem dynamics remain still unknown, although mortality of several marine organisms, in particular sea urchins, which lost their spines during blooms of *O.* cf. *ovata* or *O. siamensis*, was reported (Granéli et al., 2008; Sansoni et al., 2003; Shears and Ross, 2009).

Ostreopsis species were supposed to produce palytoxin (or its analogues) (Fig. 4.2) (Taniyama et al., 2003), one of the most potent marine toxin so far known, which acts on the Na^+/K^+ pump converting it into an ionic channel and causing the subsequent depletion of the K^+ ions (Habermann, 1989). This hypothesis was later supported by identification of putative palytoxin as the causative toxin of human poisonings occurred during *O. siamensis* blooms (Onuma et al., 1999) and, most importantly, by identification of some palytoxin-like compounds from various *Ostreopsis* spp. Particularly, ostreocin-D was isolated from *O. siamensis* and structurally elucidated by NMR (Usami et al., 1995; Ukena et al., 2001) while mascarenotoxins were identified, basing only on MS evidence, as palytoxin-like compounds from *O. mascarenensis* (Lenoir et al., 2004). Putative palytoxin and ovatoxin-a were detected in field and cultured samples of *O.* cf. *ovata*, collected along the Ligurian coasts (Italy) (Ciminiello et al., 2006, 2008) as well as in *O.* cf. *ovata* cultures from the Adriatic and

Tyrrhenian Sea (Guerrini et al., 2010) by liquid chromatography-mass spectrometry (LC-MS). Recently, several new ovatoxins, namely ovatoxin-b, -c, -d, and -e, were also detected in an Adriatic *O.* cf. *ovata* culture through an in-depth high resolution (HR) LC-MS investigation (Ciminiello et al., 2010).

Fig. 4.2 - Structure of palytoxin. Molecular formulae (M) of ovatoxins, elemental composition of their relevant A and B moieties and most abundant peaks of $[M+2H-H_2O]^{2+}$ and $[M+2H+K]^{3+}$ ion clusters for each compound.



Toxin	Rt (min)	M	A moiety	B moiety	$[M+2H-H_2O]^{2+}$	$[M+2H+K]^{3+}$
Palytoxin	10.78	$C_{129}H_{223}N_3O_{54}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{48}$	1331.7436	906.8167
Ovatoxin-a	11.45	$C_{129}H_{223}N_3O_{52}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{46}$	1315.7498	896.1572
Ovatoxin-b	11.28	$C_{131}H_{227}N_3O_{53}$	$C_{18}H_{32}N_2O_7$	$C_{113}H_{195}NO_{46}$	1337.7623	910.8318
Ovatoxin-c	10.90	$C_{131}H_{227}N_3O_{54}$	$C_{18}H_{32}N_2O_7$	$C_{113}H_{195}NO_{47}$	1345.7584	916.1628
Ovatoxin-d	11.07	$C_{129}H_{223}N_3O_{53}$	$C_{16}H_{28}N_2O_6$	$C_{113}H_{195}NO_{47}$	1323.7456	901.4884
Ovatoxin-e	11.07	$C_{129}H_{223}N_3O_{53}$	$C_{16}H_{28}N_2O_7$	$C_{113}H_{195}NO_{46}$	1323.7456	901.4884

Currently, *O.* cf. *ovata* blooms occur each year from June to late October at several sites of the Italian coastlines, characterized by different environmental conditions, such as seawater temperature in the range 18-30°C and salinity in the range 30-39 (Pistocchi et al., 2011).

However, *O.* cf. *ovata* has never been detected in the Northwestern Adriatic sea, at sites located close to the Po river delta, where peculiar salinity conditions occur, thus suggesting that some environmental conditions play a key role in influencing *O.* cf. *ovata* growth and/or its geographical dispersal.

Several authors indicated seawater temperature as an important factor affecting cell proliferation (Tognetto et al., 1995; Sansoni et al., 2003; Simoni et al., 2004; Aligizaki and Nikolaidis, 2006; Mangialajo et al., 2008). In most studies (as reviewed by Pistocchi et al., 2011), high temperature values (24-29°C) were associated with the increase of *Ostreopsis* cell number in seawater; however, in Adriatic (Totti et al., 2010) and Catalan Seas (Vila et al., 2001) such positive correlation was not observed. Recently, the influence of temperature on *O.* cf. *ovata* growth and toxicity has been also reported by Granéli et al. (2011) using a Tyrrhenian isolate from the Ligurian coast: the highest toxicity was found in cultures grown at 20°C, whereas the highest algal biomass was recorded at 30°C.

In the present study, we report on in-depth investigation of the effect of some environmental parameters on the growth and toxicity of *O.* cf. *ovata*. An Adriatic *O.* cf. *ovata* isolate, whose growth and toxin profile had been previously characterized at 20°C and salinity 36 during the exponential and stationary phases (Guerrini et al., 2010), was employed. Cultures were grown at different temperature (20, 25 and 30°C) and salinity values (26, 32, 36 and 40); HR LC-MS analyses were carried out to determine their toxin profile, including the recently found ovatoxins (Ciminiello et al., 2010), and to evaluate the total toxin amount released in the extra-cellular medium during the stationary growth phase.

A further object of the present study was to compare the total toxin content of algal extracts measured by HR LC-MS with the results obtained through the haemolysis assay (Riobó et al., 2008), with the aim of gaining information on the accuracy of the haemolytic test, a rapid and very sensitive biological assay widely employed for palytoxins detection (Riobó et al., 2011).

Finally, the toxicity of *O.* cf. *ovata* cultures on crustaceans and fish was also investigated using *Artemia* sp. assay and the ichthyotoxicity test with juvenile sea basses (*Dicentrarchus labrax*) (IRSA-CNR, 2003) to evaluate the potential *O.* cf. *ovata* impact on the ecosystem.

Experimental section

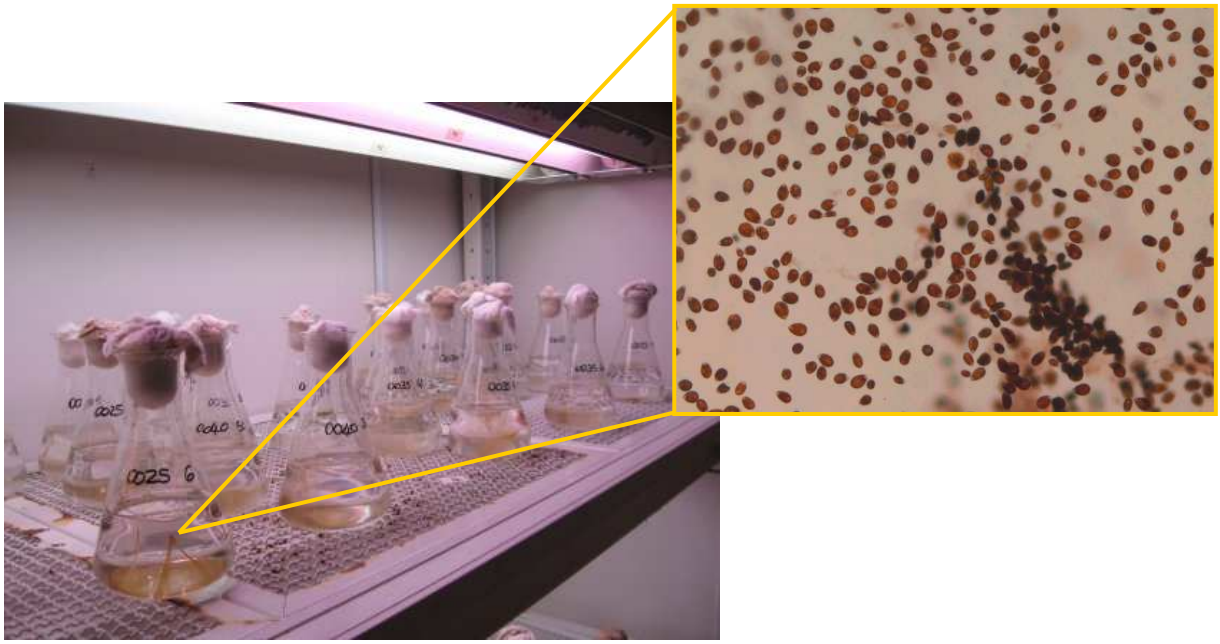
Cultures of Ostreopsis cf. ovata

O. cf. *ovata* was isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from water samples collected along the Adriatic coasts of Italy (Marche region, Numana sampling site, strain OOAN0601) in October 2006, in proximity of the seaweeds *Cystoseira* sp. and

Alcidium corallinum. After an initial growth in microplates, cells were cultured at 20°C under a 16:8 h L:D cycle from cool white lamp in natural seawater, at salinity 36, adding macronutrients at a five-fold diluted f/2 concentration (Guillard, 1975) and selenium. In order to evaluate the effect of environmental parameters on growth and toxicity of *O.* cf. *ovata*, temperature and salinity experiments were carried out. In the salinity experiment, cultures (at 20°C) were established at salinity 26, 32, 36 and 40 in a thermostatic room, maintaining light irradiance at 100-110 µmol m^{-2} s^{-1}. Salinity levels 26, 32 and 36 were obtained by diluting seawater (salinity 38) with deionized water, while salinity 40 was obtained by evaporation of the seawater. In the temperature experiment, cultures (salinity 36) were established at 20, 25 and 30°C in water baths kept in the same thermostatic room, thus light irradiance slightly decreased to 90 µmol m^{-2} s^{-1}. *Phaeodactylum tricornutum* (strain PTN0301 from the North Sea, Holland) was cultured using f/2 medium under the same conditions and used in the experiments either for comparisons or control.

Both temperature and salinity experiments were carried out by using, for each condition, 2 series of batch cultures that were used for evaluation one of growth profile and the other of toxin content.

Fig. 4.3 – *Ostreopsis* cf. *ovata* cultures. In focus are shown cells aggregates which characterize the growth of this species.



Evaluation of growth profile

Since evaluation of growth profile of *O.* cf. *ovata* in batch cultures was complicated by the presence of mucous aggregates (Fig. 4.3), the sampling method developed by Guerrini et al. (2010) was used for counting. For each temperature/salinity level, 15 Erlenmeyer flasks containing 200 ml of culture were grown in parallel (Fig. 4.3); every other day, two out of the initial flasks were treated with HCl to a final concentration of 4 mM. Acid addition dissolved mucous aggregates and homogenous sampling could be performed. After counting, the two acidified flasks were discarded.

Cell counts were made following Utermöhl method (Hasle, 1978) and specific growth rate (μ, day^{-1}) was calculated using the following equation:

$$\mu = \frac{\ln N_1 - \ln N_0}{t_1 - t_0}$$

where N_0 and N_1 are cell density values at time t_0 and t_1.

Calculation of cell volume was made with the assumption of ellipsoid shape using the following equation (Sun and Liu, 2003):

$$V = (\pi/6)*a*b*c$$

where a = dorsoventral diameter (length), b = width, c = mean anteriorposterior diameter (height).

Evaluation of toxin content

For each temperature and salinity level, a set of four culturing flasks was set up. Due to the available equipment, salinity experiment was carried out in a thermostatic room by using 1,500 mL flasks, while temperature experiment was carried out by locating 800 mL flasks in water baths. Cell counting was carried out on one out of the four flasks as described above. Five replicate countings were performed in one of the four flasks of each treatment and used to determine the cell density and to express toxin content on a per cell basis. Culture collection was carried out during the late stationary growth phase by gravity filtration through GF/F Whatman (0.7 μm) filters at day 21^{st} and 25^{th} for the salinity and temperature experiment, respectively. Cell pellets and growth media for each temperature/salinity level were provided for chemical analysis.

Chemical analysis

Chemicals

All organic solvents were of distilled-in-glass grade (Carlo Erba, Milan, Italy). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). Acetic acid (Laboratory grade) was purchased from Carlo Erba. Analytical standard of palytoxin was purchased from Wako Chemicals GmbH (Neuss, Germany).

Extraction

Cell pellets and growth media for each temperature/salinity level were separately extracted. Each pellet sample was added of 9 mL of a methanol/water (1:1, v/v) solution and sonicated for 30 min in pulse mode, while cooling in ice bath. The mixture was centrifuged at 3000 x *g* for 30 min, the supernatant was decanted and the pellet was washed twice with 9 mL of methanol/water (1:1, v/v). The extracts were combined and the volume was adjusted to 30 mL with extracting solvent. The obtained mixture was analyzed directly by HR LC-MS (5µl injected). Each growth medium was extracted five times with an equal volume of butanol. The butanol layer was evaporated to dryness, dissolved in 5 mL of methanol/water (1:1, v/v) and analyzed directly by HR LC-MS (5 µl injected). Recovery percentage of the above extraction procedures were estimated to be 98% and 75% for the pellet and growth medium extracts, respectively (Ciminiello et al., 2006).

High Resolution liquid chromatography-mass spectrometry (HR LC-MS)

High resolution (HR) LC-MS experiments were carried out on an Agilent 1100 LC binary system (Palo Alto, CA, USA) coupled to a hybrid linear ion trap LTQ Orbitrap XL^{TM} Fourier Transform MS (FTMS) equipped with an ESI ION MAX^{TM} source (Thermo-Fisher, San Josè, CA, USA). Chromatographic separation was accomplished by using a 3 µm gemini C18 (150 × 2.00 mm) column (Phenomenex, Torrance, CA, USA) maintained at room temperature and eluted at 0.2 mL min^{-1} with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid. A slow gradient elution was used: 20-50% B over 20 min, 50-80% B over 10 min, 80-100% B in 1 min, and hold 5 min. This gradient system allowed a sufficient chromatographic separation of most palytoxin-like compounds (Fig. 4.2).

HR full MS experiments (positive ions) were acquired in the range *m/z* 800-1400 at a resolving power of 15,000. The following source settings were used in all HR LC-MS experiments: a spray voltage of 4 kV, a capillary temperature of 290°C, a capillary voltage of

22 V, a sheath gas and an auxiliary gas flow of 35 and 1 (arbitrary units). The tube lens voltage was set at 110 V.

Due to commercial availability of the only palytoxin standard, quantitative determination of putative palytoxin, ovatoxin-a,-b,-c,-d, and -e in the extracts was carried out by using a calibration curve (triplicate injection) of palytoxin standards at four levels of concentration (25, 12.5, 6.25, and 3.13 ng mL^{-1}) and assuming that their molar responses were similar to that of palytoxin. Extracted ion chromatograms (XIC) for palytoxin and each ovatoxins were obtained by selecting the most abundant ion peaks of both $[M+2H-H_2O]^{2+}$ and $[M+2H+K]^{3+}$ ion clusters (Fig. 4.2). A mass tolerance of 5 ppm was used.

Toxicity assays

Artemia sp. assay

The assay was carried out according to the short-term test of the IRSA-CNR (2003) method, consisting in a 24 h exposure of *Artemia* sp. to the potentially toxic sample. 10 nauplii were incubated in 1 mL of sample in a glass tube for 24 h. Initially, aliquots of a culture grown at 20°C and salinity 36, containing five increasing concentrations of live cells, lysed cells, algal extracts and growth media, were tested in triplicate. Live cell aliquots were sampled during the stationary phase of the culture. Lysed cell aliquots were obtained by sonicating 10 mL of the culture for 3 min. Algal extracts were obtained as reported above and diluted (1:100 to 1:10,000) with seawater. A palytoxin stock solution (12.5 μg mL^{-1}) in methanol/water (1:1, v/v) was diluted with seawater and tested in the concentration range 500-10,000 pg mL^{-1}. Growth medium aliquots were obtained by filtering 50 mL of the culture through GF/F Whatman (0.7 μm) filters.

Toxicity of *O.* cf. *ovata* cultures grown at different temperature/salinity conditions was evaluated by *Artemia* sp assay, using only live cells. Five different concentration levels of each sample, obtained through dilution with seawater, were tested in triplicate. Toxic effects on *Artemia* sp. of sample exposure were evaluated after 24 h and dead organisms were counted. Seawater samples, methanol/water (1:1, v/v) solution (diluted 1:100, v/v with sea-water) and f/2 medium at the investigated salinity levels (diluted 1:5 with seawater) were used as control. EC_{50} values were calculated as reported below

Haemolytic assay

Haemolytic assay was carried out following the procedure proposed by Bignami (1993) and modified by Riobó et al. (2008). The test is based on photometrical determination of

haemoglobin released from sheep erythrocytes following exposure to haemolytic compounds. Sheep blood was kindly provided by the Department of Veterinary Public Health and Animal Pathology (University of Bologna). Erythrocytes were separated from plasma by centrifugation (400 x *g* at 10°C for 10 minutes) and washed twice with a solution containing phosphate buffered saline (PBS) 0.01 M, pH 7.4, bovine serum albumin (BSA), calcium chloride ($CaCl_2$ $2H_2O$) 1 mM and boric acid (H_3BO_3) 1 mM. Finally, the erythrocytes solution was diluted with PBS at a final concentration of 1.7 x 10^8 red cells mL^{-1}.

According to the reported method (Riobó et al., 2008), two blood solutions, one added of ouabain (2.5 μM) and one ouabain-free, were prepared to a final concentration of 1.7 x 10^7 erythrocytes. 1 mL of each blood solution was mixed with 1 mL of the sample diluted in PBS (either pellet extract or palytoxin standard previously dissolved in methanol/water (1:1, v/v)) and incubated at 25°C for 20 h. After the incubation, samples were centrifuged at 400 *x g* for 10 min and the supernatant absorptions were measured at 405 nm. Two replicates of algal extract at different concentration levels, control solutions for blanks (PBS buffer and methanol/water (1:1, v/v) in PBS) and total haemolysis sample were prepared in each experiment. Palytoxin standard at seven concentration levels (4-196 pg mL^{-1}) was used for generating calibration curve. The haemolytic effects of the algal extracts were expressed either on cell basis (cell mL^{-1}) and on toxin content basis (pg mL^{-1}). EC_{50} values obtained testing the palytoxin standard and the algal extracts were calculated as reported below. Stock solutions of the algal extracts and palytoxin standard used in the haemolytic assay were quantified by HR LC-MS.

Fish bioassay

Sea bass (*Dicentrarchus labrax*) employed in the assay were collected from the hatchery Valle Ca' Zuliani (Pila di Porto Tolle, Rovigo, Italy). After the transfer, they were kept in a 60-70 L aquarium, aerated by a small dispenser (Hailea) and kept at room temperature and salinity 36 for one month. For the experiments, 2 L aerated tanks containing algal culture were used. Three juveniles (5.0 ± 1.0 g) were put into each tank, kept at 20 °C, during a 16:8 h light–dark period and observed for 4 days. Two replicates of *O.* cf. *ovata* culture grown for 4-6 days at 20°C and salinity 36 were tested at three concentration levels. An equal volume of *Phaeodactylum tricornutum* culture was used as control. Fish were considered dead when gill opercular movements ceased.

Data analysis

The 50% effect concentration (EC_{50}) of each sample for the *Artemia* sp. and haemolytic assays was estimated by fitting the experimental concentration-response curves to a logistic model:

$$y = \frac{a}{1+\left(\frac{x}{EC_{50}}\right)^{b}}$$

where: y = endpoint value; x = substance concentration; a = expected endpoint value in absence of toxic effect; b = slope parameter. The parameters of the equation, including the EC_{50}, were estimated using the non-linear regression procedures implemented in Statistica (Statsoft, Tulsa, OK, USA). An independent estimate of EC_{50} was obtained for each of the experiments.

Differences in cell biovolume, EC_{50} value, and toxins concentration among the samples were tested by using the multivariate analysis-of-variance (ANOVA) test, using Statistica (StatSoft, Tulsa, OK, USA) software. Whenever a significant difference for the main effect was observed ($P<0.05$), a Newman-Keuls test was also performed.

Results

Batch cultures of an Adriatic strain of *O.* cf. *ovata*, collected along the Marche coasts of Italy (Numana sampling site) in October 2006, were established in order to evaluate the effect of salinity and temperature on algal growth and toxin profile. Particularly, in the temperature experiment, cultures were set at 20, 25 and 30°C by maintaining salinity at 36 and light irradiance at 90 μmol m^{-2} s^{-1}, while in the salinity experiment cultures were established at salinity 26, 32, 36 and 40, by maintaining temperature at 20°C and light irradiance at 100-110 μmol m^{-2} s^{-1}.

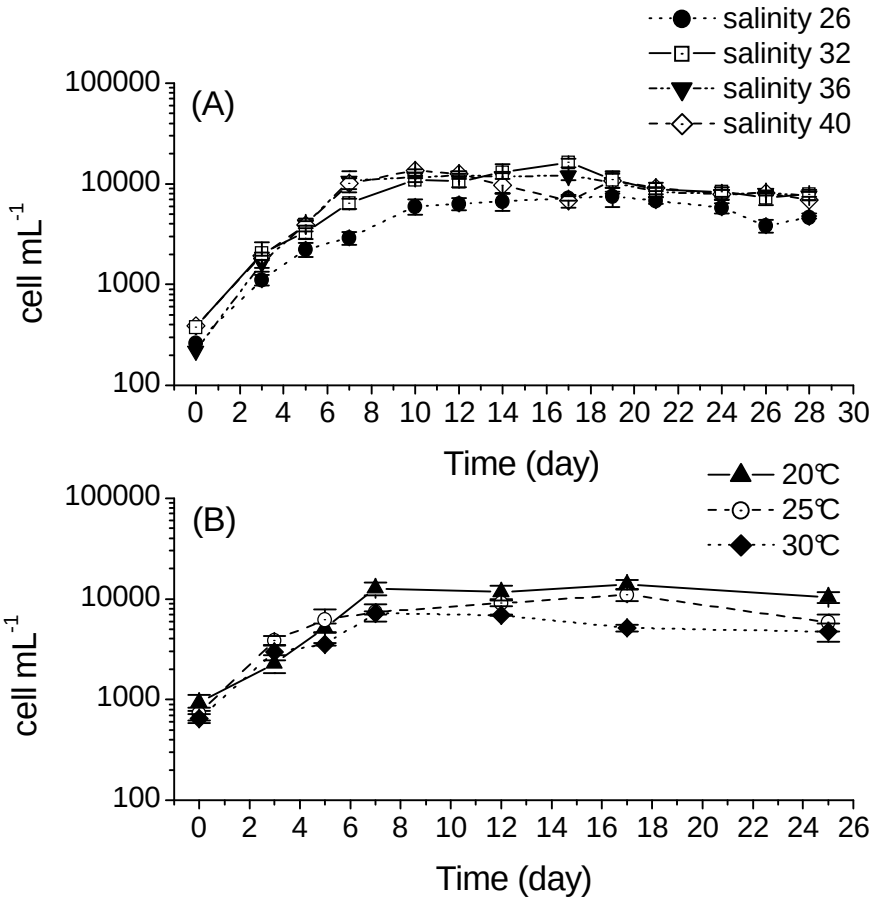
Growth pattern and cell volume

The growth profile of *O.* cf. *ovata* cultures exposed to different salinity and temperature values was analyzed by measuring the cell density every 2-3 days from the beginning of the exponential phase to the end of the stationary phase (Fig. 4.4A and 4.4B). Under the different growth conditions, *O.* cf. *ovata* growth rates in the range 0.34-0.49 day^{-1} were observed.

As for the temperature experiment, despite during the first 5 days cells grew better at 25°C, at the end of the exponential phase the maximum growth rate of 0.49 day^{-1} was recorded at 20°C, followed by 0.43 and 0.34 day^{-1} at 25 and 30°C, respectively. As for the salinity

experiment (carried out at 20°C) growth rate was not significantly affected by the salt concentration (0.43-0.47 day^{-1}) (ANOVA, P>0.05).

Fig. 4.4 - Growth pattern of *O.* cf. *ovata* cultures exposed to different salinity (A) and temperature (B) conditions.



In the stationary phase, the maximum density was 13,000-16,000 cell mL^{-1} at 20°C and intermediate salinities (32 and 36), while the cell yield dropped to 7,500 cell mL^{-1} both at salinity 26 (temperature 20°C; Fig. 4.4A) and temperature 30°C (salinity 36; Fig. 4.4B).

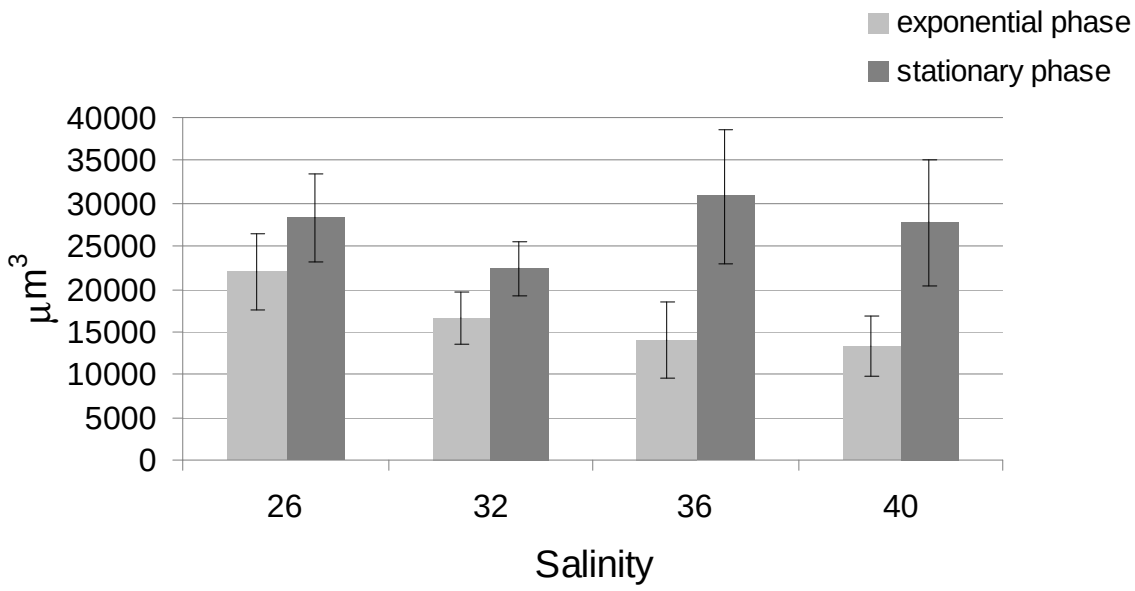
In the course of the experiments, we noticed that the culture volume played a key role on the final cell yield: decreasing cell densities were obtained as culture volumes increased from 200 mL to 800 mL up to 1500 mL.

Another aspect we considered in the salinity and temperature experiments was the cell biometric measurement. It is to be noted that *O.* cf. *ovata* cells appeared highly different both in size and in shape, within each cell culture; therefore, a statistically significant cell number ($n \geq 50$) was used for estimating the mean biovolumes which are reported in Fig. 4.5. In both

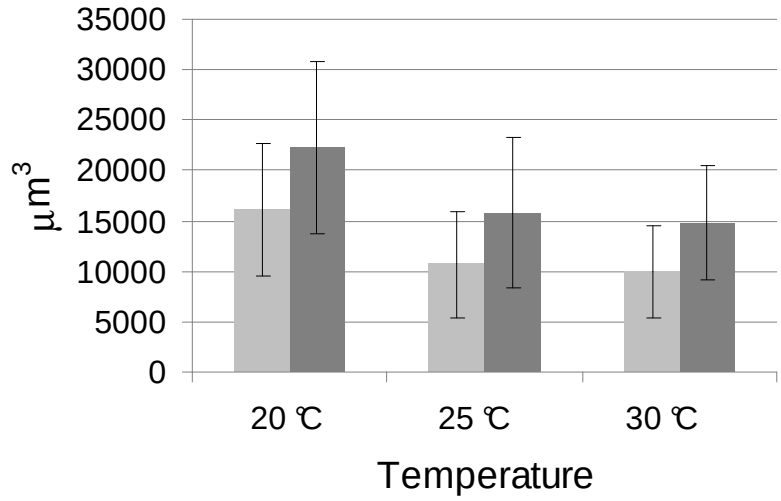
salinity and temperature experiments, a significant difference was observed between cell volumes measured in the exponential and stationary phases (ANOVA, P<0.001).

Fig. 4.5 - Cell volumes of *O.* cf. *ovata* cells exposed to different salinity (A) and temperature (B) conditions.

(A)



(B)



In the salinity experiment, the highest difference among biovolumes was observed in the exponential phase where a mean value of 22,000 μm^3 was reached at the lowest salinity (26) and resulted significantly higher (Post-hoc SNK test, P<0.001) than those observed at 36 and 40 (14,000 and 13,000 μm^3, respectively). An intermediate biovolume mean value was observed at salinity 32 (17,000 μm^3). In the stationary phase, cells were more homogenous in

size, with cell volumes in the range 28,000-30,000 μm^3; however, the value reported at salinity 32 (22,400 μm^3) resulted significantly lower than those observed at the other salinity levels (Post-hoc SNK test, $P<0.001$).

In the temperature experiment, in both growth phases cell volumes decreased as temperature increased, with a maximum biovolume of 22,000 μm^3 being reached at 20°C (stationary phase), which was significantly higher than biovolumes measured at 25 and 30°C (Post-hoc SNK test, $P<0.001$).

Determination of toxin content by HR LC-MS

Cell pellets and growth media of *O.* cf. *ovata* cultures grown at the different temperature and salinity values were collected during the late stationary growth phase. Samples were separately extracted as reported in the experimental and the crude extracts were used to evaluate the toxin profile. HR LC-MS experiments were acquired in full MS mode by using an LC method which allowed chromatographic separation of the major components of the toxin profile. The spectra were acquired in the mass range *m/z* 800-1400 where each palytoxin-like compound (Fig. 4.2) produces bi-charged ions due to $[M+H+K]^{2+}$, $[M+H+Na]^{2+}$, and $[M+2H]^{2+}$, tri-charged ions due to $[M+2H+K]^{3+}$ and $[M+2H+Na]^{3+}$, and a number of ions due to multiple water losses from the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions.

The presence of putative palytoxin and of all the ovatoxins (ovatoxin-a, -b, -c, -d, and -e) recently identified in *O.* cf. *ovata* (Ciminiello et al., 2008, 2010) was highlighted in all the analyzed samples. With the purpose of gaining information on the relative abundance of individual compounds, extracted ion chromatograms (XIC) were obtained by summing the most abundant peaks of both $[M+2H-H_2O]^{2+}$ and $[M+2H+K]^{3+}$ ion clusters for each compound (Fig. 4.2). Due to lack of standards for ovatoxins, quantitative analyses were carried out basing on the tentative assumption that they present the same molar response as palytoxin. These appeared reasonable since ovatoxins present elemental formulae similar to that of palytoxin.

Concentrations of putative palytoxin and ovatoxins in all the analyzed extracts were extrapolated from a calibration curve of palytoxin standard injected under the same conditions and the obtained results were corrected basing on recovery percentages of the extraction procedures, namely 98% and 75% (Ciminiello et al., 2006) for pellet and growth medium extracts, respectively.

Significant differences were observed in the total toxin content of different algal extracts (ANOVA, P<0.001), whereas relative abundance of individual toxins were quite similar: ovatoxin-a was by far the major component of the toxin profile (47-56% of the total toxin content; Post-hoc SNK test, P<0.001), followed by ovatoxin-b (24-27%), ovatoxin-d and -e (15-18%), ovatoxin-c (4-8%) and putative palytoxin (0.5-3%) on the basis of their decreasing relative abundance.

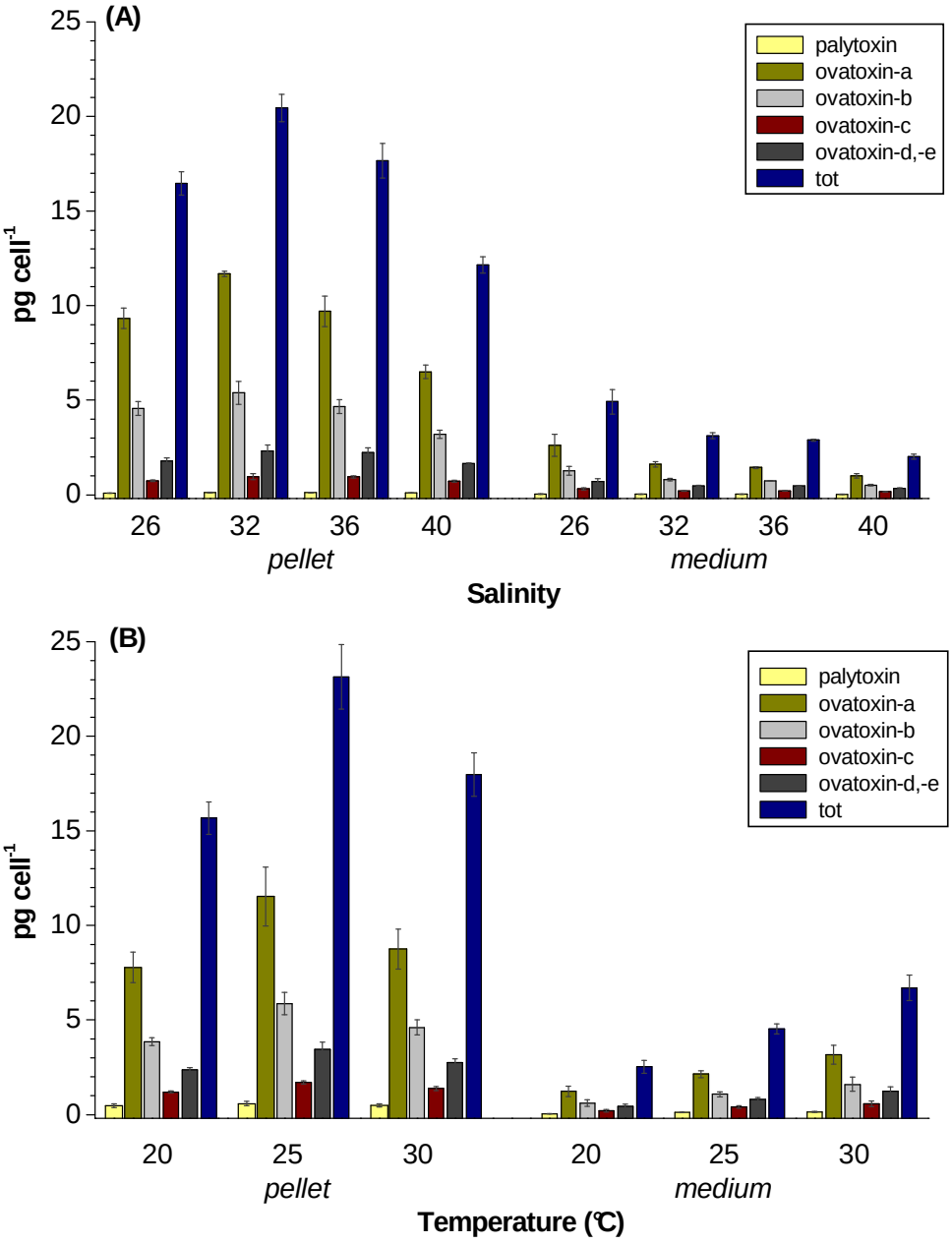
Total toxin content of pellet and medium extracts expressed as μg L^{-1} culture in both salinity and temperature experiments are reported in Table 4.2. Toxin contents were significantly higher in the cell pellet then in the corresponding culture medium (Post-hoc SNK test, P<0.001), resulting in relatively low release percentages (13-16%) in most of the growth conditions applied; however, the release increased up to 23 and 27% under the most unfavourable growth conditions, namely salinity 26 (temperature 20°C) and temperature 30°C (salinity 36), respectively.

Table 4.2 – Total toxin content (putative palytoxin, ovatoxin-a, -b, -c, -d, and -e) of *O.* cf. *ovata* culture pellet and medium extracts, measured by HR LC-MS in both salinity and temperature experiment. Data are expressed as μg per Liter of culture (μg L^{-1}). Cell density (cell L^{-1}) and extracellular release(%) are also reported.

		Total toxin content (ug L^{-1})			
	cell L^{-1}	pellet	medium	total	extracellular release (%)
Salinity					
26	3,450,333	57	17	74	23
32	4,646,333	95	14	109	13
36	4,281,333	76	12	88	14
40	5,619,000	68	11	80	14
Temperature					
20℃	9,869,587	155	25	180	14
25℃	5,581,677	129	25	154	16
30℃	4,493,377	81	30	111	27

Total and individual toxin contents on a per cell basis (pg $cell^{-1}$), are reported in Fig. 4.6A and 4.6B for salinity and temperature experiments, respectively. Small differences in total toxin content were observed between the experiments, that should have provided similar results, namely the cultures grown at temperature 20°C and salinity 36. Such differences could be likely due to the slightly different growth conditions, among which the difference in light intensity and in culture volume could have played a major role.

Fig. 4.6 – Total and individual toxin contents of putative palytoxin, ovatoxin-a, -b, -c, -d, and -e of *O.* cf. *ovata* cultures grown under different salinity (A) and temperature (B) conditions. HR LC-MS measurements (pg cell^{-1}) were carried out for both pellet and medium extracts at the end of stationary growth phase.



In the salinity experiment, total toxin content reached the highest value in the culture grown at 32 (20 pg cell^{-1}) and the lowest at 40 (12 pg cell^{-1}).

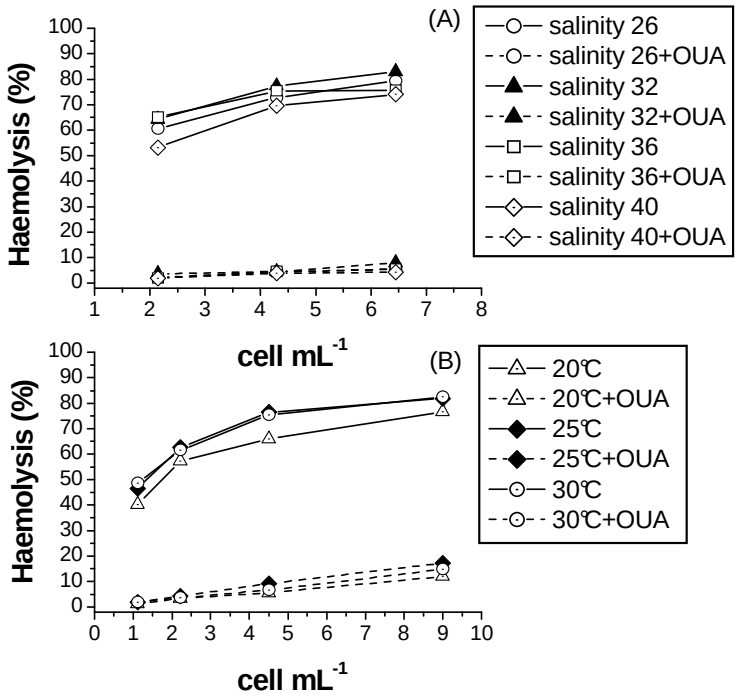
As for the temperature experiment, *O.* cf. *ovata* grown at 25°C was found to have a total toxin content of 23 pg cell^{-1}, while cultures grown at 20 and 30°C produced 16 and 18 pg cell^{-1},

respectively. This last finding apparently is not in agreement with the maximum concentration (μg L^{-1}) observed at 20°C (salinity 36), which was indeed affected by the high cell yield of the culture (Table 4.2). Particularly, culture grown at 20°C showed a cell density almost two-fold higher than the others.

Haemolytic assay

Palytoxin converts Na^+/K^+ pump into a non-selective cation channel, causing cell lysis; ouabain and other cardiac glycosides are used as indicators for the site of action since these compounds are specific ligands for the Na^+/K^+-ATPase.

Fig. 4.7 - Haemolytic activity of *O.* cf. *ovata* extracts grown at different salinity (A) and temperature (B) conditions on sheep erythrocytes in absence (solid lines) and in presence (dashed lines) of ouabain (OUA). Data are expressed as haemolysis percentage (%) versus cell number mL^{-1} assay (cell mL^{-1}).



The haemolytic assay proposed by Riobó et al. (2008) is a rapid and sensitive method to determine palytoxin content and it was applied to culture extracts of O. cf. ovata grown under different salinity and temperature conditions.

All the O. cf. ovata culture extracts investigated in the present study were tested by haemolytic assay and the results, expressed as haemolysis percentage versus cell number

present in 1 mL of assay solution (cell mL^{-1}), are reported in Fig. 4.7. All showed a strong delayed haemolysis of sheep erythrocytes, which was specifically inhibited by ouabain, even at concentrations corresponding to very low cell densities; however, a percentage of not-specific haemolysis was left over even in the presence of ouabain as shown in Fig. 4.7 (dotted lines).

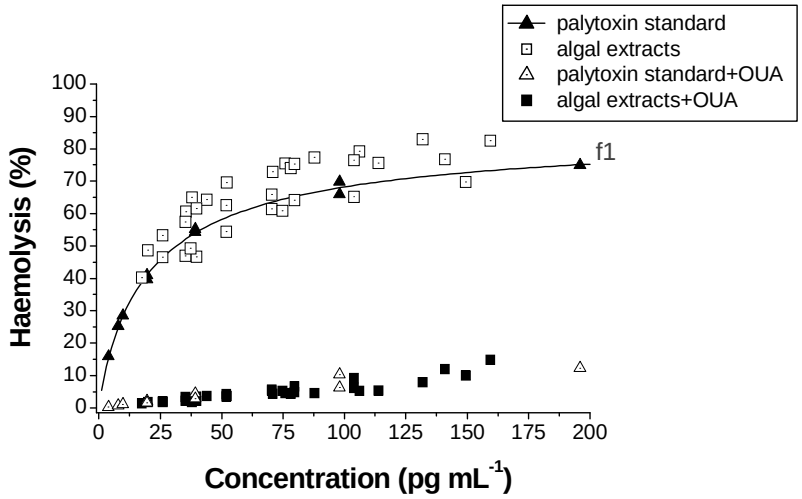
The haemolytic activity of cultures grown at different salinity levels (Fig. 4.7A) followed a pattern similar to that measured by HR LC-MS; particularly the highest haemolysis (83%) was observed for the culture grown at salinity 32 (total toxin content = 20 pg $cell^{-1}$) followed by cultures grown at salinity 26 (haemolysis 79%, total toxin content = 16 pg $cell^{-1}$), 36 (haemolysis 76%, total toxin content = 18 pg $cell^{-1}$), and 40 (haemolysis 74%, total toxin content = 12 pg $cell^{-1}$).

In the temperature experiment (Fig. 4.7B) cells grown at 20°C reported the lowest haemolytic activity (haemolysis 76%, total toxin content = 16 pg $cell^{-1}$) in agreement with HR LC-MS results, while an 82% haemolytic effect was observed for both cultures grown at 25 and 30°C, despite the slightly different toxin content of 23 and 18 pg $cell^{-1}$, respectively. All the above data were consistent with HR LC-MS results expressed as pg $cell^{-1}$.

A comparison of the results of the haemolytic assay with the quantitative results achieved by HR LC-MS could provide useful information about the haemolytic activity of ovatoxins in comparison with that of palytoxin. To this aim, the haemolytic activity of the algal extracts from salinity and temperature experiments was also expressed as haemolysis percentage versus concentration of pg total toxin contained in 1 mL assay solution (pg mL^{-1}) as measured by HR LC-MS. These data are compared in Fig. 4.8 with those obtained for the haemolytic activity of palytoxin standard tested at seven different concentrations. This clearly suggests that the haemolytic activity of the overall ovatoxins is quite similar to that of palytoxin standard.

Values obtained for the palytoxin standard were interpolated using a non-linear estimation curve, described by the reported equation (f1, Fig. 4.8). The resulting EC_{50} values for palytoxin standard and algal extracts were not significantly different (ANOVA, $P>0.05$), being 22 ± 2 and 25 ± 8 pg mL^{-1}, respectively.

Fig. 4.8 - Haemolytic activity of *O.* cf. *ovata* extracts and palytoxin standard on sheep erythrocytes in absence and in presence of ouabain (OUA). Data are expressed as haemolysis percentage (%) versus concentration of palytoxin equivalent per mL of assay (pg mL^{-1}). Equation f1: non-linear estimation curve obtained for the palytoxin standard.



	Function (f1)
PLTX standard	y = (85.5819)-(85.5819)/(1+(x/(21.473))^(log(9)/(log((254.169)/(21.473)))))

Artemia sp. assay

Artemia sp. assays were carried out using both live and lysed cells of *O.* cf. *ovata* cultures as well as the algal extract and the growth medium of a culture grown at 20°C and salinity 36.

O. cf. *ovata* live cells induced rapid and high mortality of *Artemia* sp. nauplii, even at low cell concentrations. From the EC_{50} values of all the samples calculated at 24 h (Table 4.3), cell toxicity appeared relevant and significantly different (ANOVA, $P<0.001$): the growth medium resulted significantly less toxic than the live cells (Post-hoc SNK test, $P<0.001$), with an EC_{50} value of 720 cell mL^{-1} versus 8 cell mL^{-1}, respectively. This result confirmed the presence of small amounts of toxins released in the growth medium. The lysed cells induced a similar mortality as the algal extract, as evidenced by the comparable EC_{50} values (Post-hoc SNK test, $P>0.05$).

EC_{50} values of all the tested *O.* cf. *ovata* samples were expressed also as pg of toxins per mL assay (pg mL^{-1}) (Table 4.3), basing on the total toxin contents measured by HR LC-MS. A palytoxin standard at five levels of concentrations (500-10,000 pg mL^{-1}) was also tested; it

presented an EC_{50} value of 4606 pg mL^{-1}, which was significantly higher (Post-hoc SNK test, $P<0.001$) than that of the algal extract (1146 pg mL^{-1}).

Table 4.3 – The 50% mortality on *Artemia* nauplii (EC_{50}) is expressed both as cells of *O.* cf. *ovata* per mL (cell mL^{-1}) and total toxin content per mL (pg mL^{-1}) and it is reported for *O.* cf. *ovata* live and lysed cells, extract, and growth medium. EC_{50} measured for palytoxin standard is reported as pg mL^{-1}. Each value is the mean of three replicates ± standard error.

	EC_{50} (cell mL^{-1})	**EC_{50} (pg mL^{-1})**
***O.* cf. *ovata* live cells**	8 ± 5	115 ± 72
***O.* cf. *ovata* lysed cells**	96 ± 6	1376 ± 86
***O.* cf. *ovata* extract**	80 ± 7	1146 ± 272
***O.* cf. *ovata* medium**	720 ± 54	1822 ± 137
palytoxin standard		4606 ± 781

Administration of *O.* cf. *ovata* live cells, grown at different salinity and temperature conditions, to *Artemia* sp. resulted in no significant differences among the measured EC_{50} values (ANOVA, $P>0.05$) (Table 4.4); however, the lowest EC_{50} values were measured for live cells grown at 25°C (EC_{50} = 6 cell mL^{-1}), and for those grown at salinity 32 (EC_{50} = 9 cell mL^{-1}) in the temperature and salinity experiment, respectively. This appears in good agreement with total toxin contents (pg $cell^{-1}$) measured by HR LC-MS (Fig. 4.8).

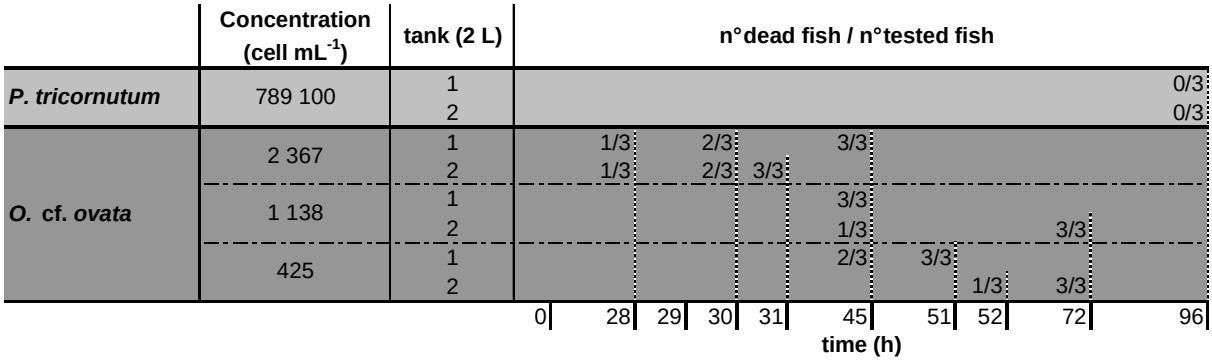
Table 4.4 - The 50% mortality on *Artemia* nauplii (EC_{50}) of *O.* cf. *ovata* live cells grown at different salinity and temperature conditions, expressed as cell mL^{-1}. Each value is the mean of three replicates ± standard error.

	EC_{50} (cell mL^{-1})
Salinity	
26	17 ± 3
32	9 ± 2
36	24 ± 8
40	17 ± 4
Temperature	
20℃	11 ± 3
25℃	6 ± 2
30℃	14 ± 1

Fish bioassay

Fig. 4.9 shows the results of the ichthyotoxic assay performed with different concentrations of *O.* cf. *ovata* live cells. Sea bass mortality occurred after 1 day of exposure, only at the highest *O.* cf. *ovata* cell density (2367 cells mL^{-1}). After 45 h from the beginning of the assay, even fish exposed to lower algal concentrations (1138 and 425 cells mL^{-1}) began to die and they were all dead after 72 h. Fish exposed to the diatom *Phaeodactylum tricornutum* (789,100 cells mL^{-1}) used as control survived and behaved normally till the end of the experiment (96 h).

Fig. 4.9 - Toxicity of different concentrations of *O.* cf. *ovata* cells on fish (*Dicentrarchus labrax*). *Phaeodactylum tricornutum* was used as control and was tested at the reported cell density. Time is expressed as hours (h).



Discussion

Several field surveys indicated that environmental conditions play a major role in determining *Ostreopsis* species proliferation (Pistocchi et al., 2011). Since very few laboratory studies on the effects of environmental parameters on growth and toxicity of *Ostreopsis* isolates were reported (Granéli et al., 2011; Ashton et al., 2003; Morton et al., 1992), a detailed study was carried out on an Adriatic strain of *O.* cf. *ovata* grown at different temperature and salinity conditions.

Growth and cell size pattern

The Adriatic *O.* cf. *ovata* strain showed to be tolerant to salinity variation in the range 26-40. Very similar growth rates and yields were observed within the tested salinity range, with the lowest growth yield being recorded at salinity 26. This is in good agreement with field measurements performed during Mediterranean *O.* cf. *ovata* blooms (Totti et al., 2010; Monti et al., 2007) as well as with results of a survey of epiphytic dinoflagellates along the Hawaiian

coast, (Parsons and Preskitt, 2007) in which *O.* cf. *ovata* was the only dinoflagellate to be negatively correlated with salinity.

In the temperature experiment, the analyzed Adriatic *O.* cf. *ovata* strain reached the highest growth yield at 20°C, whereas the lowest yield was recorded at 30°C. Our results are in good agreement with field surveys in the Adriatic Sea, where *O.* cf. *ovata* proliferation occurs from the end of August to October, when water temperature is about 20-22°C (Totti et al., 2010; Monti et al., 2007). On the contrary, Granéli et al. (2011) indicated, for a Tyrrhenian *O.* cf. *ovata* strain, that high water temperatures (26-30°C) increased both growth rate and yield; this is consistent with the field surveys reporting *O.* cf. *ovata* blooms in the Tyrrhenian Sea in the middle of the summer. Our results and those observed by Granéli et al. (2010) agree in highlighting that Adriatic and Tyrrhenian strains are differently affected by environmental temperature.

As for the morphometric characters, in both salinity and temperature experiments, a certain cell size variability was observed; however, the cell volumes reported under the different growth conditions didn't show a specific pattern, particularly in the stationary growth phase. A marked variability in the biovolumes of *O.* cf. *ovata* cells from the same culture had been already observed (Guerrini et al., 2010), and it is in agreement with field observations (Aligizaki and Nikolaidis, 2006; Bianco et al., 2007).

Toxin profile

Putative palytoxin and all the ovatoxins so far known (Ciminiello et al., 2010) were detected in *O.* cf. *ovata* extracts. In the cultures grown under different conditions relative abundance of individual toxins was similar, with ovatoxin-a and putative palytoxin being the major and the minor component of the toxin profile, respectively.

The highest total toxin content on a per cell basis (pg $cell^{-1}$) was recorded in cultures grown at 25°C, while the highest total toxin concentration on a per liter basis was recorded at 20°C, namely under conditions that induced the highest growth yield. A reverse correlation between growth and toxin production has been reported also by Granéli et al. (2011), as found also for other dinoflagellates (Etheridge and Roesler, 2005; Errera et al., 2010). As for the salinity experiment the highest total toxin content (pg $cell^{-1}$) was measured at salinity 32, while it decreased at lower and higher salinity values, as previously observed for the dinoflagellate *Protoceratium reticulatum* (Guerrini et al., 2007). However, no clear correlation between growth and toxin content was observed in the salinity experiment.

The extracellular release increased as the temperature increased, with the maximum 27% value being observed at 30°C, the most unfavourable growth condition in the temperature experiment. This suggests that high temperatures favour cell lysis, leading to toxins being released in the growth medium. Similarly, in the salinity experiment, the highest release was also measured at the most unfavourable growth condition (26); comparable results were obtained for *P. reticulatum* (Guerrini et al., 2007) and this could represent a response of the cells to the osmotic stress.

Haemolysis results in comparison with HR LC-MS data

The haemolytic assay was reported as a rapid, easy and sensitive method to determine palytoxin by Riobó et al. (2008). In our study, it was successfully applied to the analyses of *O.* cf. *ovata* extracts in order to gain information about the haemolytic activity of ovatoxins. The haemolytic assay resulted highly reproducible even among separate set of experiments and using different blood samples.

The haemolytic activity was tested by using *O.* cf. *ovata* extracts obtained from cultures set up at different growth conditions. The obtained data showed a good correlation between haemolysis percentage and the total toxin content measured through HR LC-MS. This is the first time that a cross check between biological assay and chemical analysis was applied to palytoxin-like compounds. Useful information was obtained from haemolytic tests after pretreatment with ouabain. They showed that ovatoxins behave similarly to palytoxin, suggesting a common mechanism of action, which involves a binding to the Na^+/ K^+ pump.

The haemolytic activity of all the *O.* cf. *ovata* extracts was found to be very similar to that of palytoxin, as confirmed also by the similar EC_{50} values. These data suggested that ovatoxins, which represent the major components of the *O.* cf. *ovata* extracts (99.5-97%), have a similar haemolytic effect as palytoxin standard. It has to be noted that, in our analyses, the total activity of ovatoxins was measured and it has still to be ascertained whether individual components of the ovatoxin profile present different haemolytic activity. This will be possible when each ovatoxin will be isolated as a pure compound and used to evaluate its haemolytic activity.

In conclusion, the haemolytic assay appears a good method for preliminary quantification of the whole of palytoxin-like compounds in algal extracts: equation (f1, Fig. 4.8) obtained from the haemolysis curve, indicating the total haemolysis, can be a powerful tool to evaluate total toxin concentration of algal extracts, especially in laboratories where LC-MS is not available.

However, some drawbacks of this assay are represented by the interference of other possibly co-occurring haemolytic compounds and its inability to define toxin profile.

Toxicity for crustacean and fish

The toxicological assays revealed a marked toxicity of compounds produced by *O.* cf. *ovata* on *Artemia* nauplii and juvenile sea basses.

In the *Artemia* sp. assay performed with *O.* cf. *ovata* live cells the death of nauplii was observed even at very low cell densities and the relevant EC_{50} value was significantly lower than those obtained for *O.* cf. *ovata* lysed cells, algal extract, and growth medium (Table 4.3). The difference in EC_{50} values of *O.* cf. *ovata* live cells versus both *O.* cf. *ovata* lysed cells and algal extract can be related to a different toxin uptake by the *Artemia* sp. nauplii: live cells were actually ingested by nauplii whereas either lysed cells or algal extract were assumed through filtration. Thus, this latter mechanism of toxin uptake seems to be less powerful than ingestion. This suggests that herbivorous fish, that feeds on seaweeds where the benthic dinoflagellates proliferates, is the most exposed to *O.* cf. *ovata* toxicity.

The high EC_{50} value of the *O.* cf. *ovata* growth medium also deserves consideration. It can be related to the low toxin extracellular release emerging by HR LC-MS data (Table 4.2). Despite apparently low toxicity of *O.* cf. *ovata* growth medium on *Artemi*a nauplii, a long lasting bloom could be anyway hazardous to marine crustaceans, particularly considering that cell lyses and toxin extracellular release increase at the end of the stationary phase reached at the end of the bloom.

Unlike the haemolytic assay, the *Artemia* sp. assay was not able to detect the different toxin contents of *O.* cf. *ovata* cultures grown at different salinity and temperature conditions. This could be due to the extreme sensitivity of *Artemia* sp. nauplii to *O.* cf. *ovata* live cells (EC_{50} values ranging from 6 to 24 cell mL^{-1}), which has not been observed for any other harmful algae so far (Pezzolesi et al., 2010). Thus, *Artemia* sp. assay is not able to catch relatively small differences among different samples and, therefore, it cannot be used for quantitative purposes.

In the ichthyotoxic assay, sea basses exposed to *O.* cf. *ovata* live cells died within a few days despite they are known not to feed on microalgal cells. This mortality could be attributed to an haemolytic effect of palytoxin-like compounds on the gills, where Na^+/K^+ ATPase activity is high in the juvenile stage of sea basses (Varsamos et al., 2004). However, we cannot exclude an effect due to accidental ingestion of algal cells, which were contained in the surrounding water at high density.

4.2.2. Effect of temperature on the toxicity and growth of different O. cf. ovata strains

Experimental section

Cultures of Ostreopsis cf. ovata

O. cf. ovata cells were isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from water samples collected along the Adriatic (Bari, Puglia; strain OOAB0801) and Tyrrhenian (Latina, Lazio; strain OOTL0707) coasts of Italy in 2008 and 2007, respectively. Cultures were established at salinity 36 adding macronutrients at a five-fold diluted f/2 (Guillard, 1975) concentrations. In order to evaluate the effect of temperature on growth and toxicity of diffferent *O.* cf. *ovata* strains, cultures were prepared at 20, 25 and 30°C in water baths, under a 16:8 h L:D cycle from cool white lamp, maintaining light irradiance at 90 μmol m^{-2} s^{-1}(see section 4.2.1).

As previoulsy described (section 4.2.1), to evaluate the growth profile, 15 Erlenmeyer flasks containing 200 ml of culture were grown in parallel for each condition; every other day, two out of the initial flasks were treated with HCl to a final concentration of 4 mM and discarded after the counting. Cell counts were made following Utermöhl method (Hasle, 1978), while calculation of cell volume was made with the assumption of ellipsoid shape using the following equation (Sun and Liu, 2003).

For each temperature condition, a set of four 800 mL culturing flasks was set up for the evaluation of toxin content. Cell counting was carried out on one out of the four flasks as described above. Culture collections were carried out during either the exponential and stationary growth phases by gravity filtration through GF/F Whatman (0.7 μm) filters at day 9^{th} and 22^{th}, respectively. Cell pellets for each condition were provided for chemical analysis.

Chemical analysis

HR LC-MS analyses of palytoxin-like compunds were performed as reported in section 4.2.1.

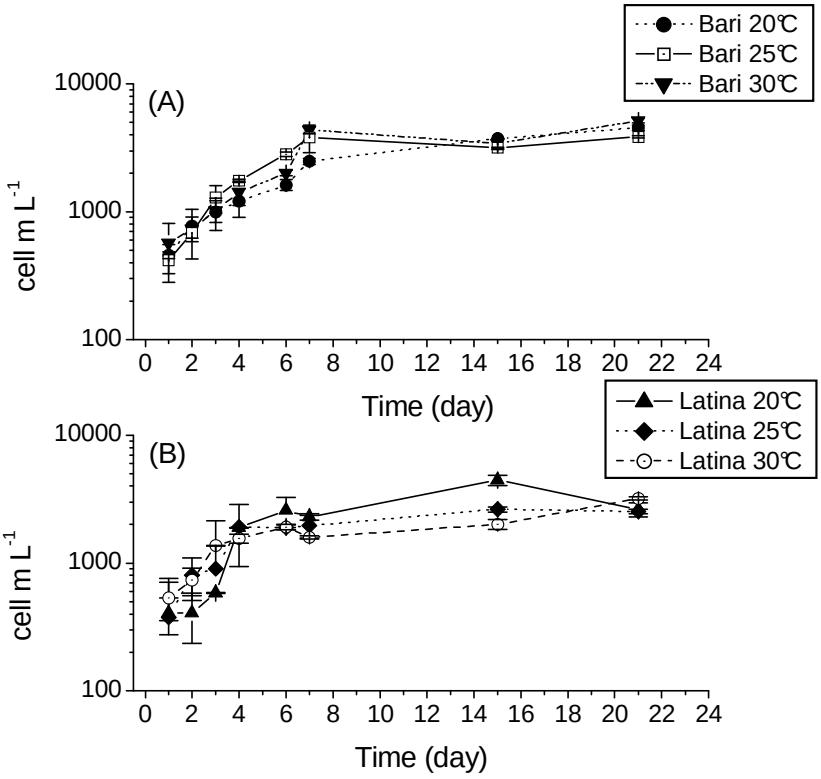
Results

Batch cultures of an Adriatic and Tyrrhenian strain of *O.* cf. *ovata* were established in order to evaluate the effect of temperature on algal growth and toxin profile. Particularly, cultures were set at 20, 25 and 30°C by maintaining salinity at 36 and light irradiance at 90 μmol m^{-2} s^{-1}, as previously performed using another Adriatic strain from Ancona. This study allows to compare the effect of temperature on different *O.* cf. *ovata* strains to evaluate how this environmental parameter affects the cell proliferation in these areas.

Growth pattern

The growth profile of *O.* cf. *ovata* cultures exposed to different temperature values was analyzed by measuring the cell density every 2-3 days from the beginning of the exponential phase to the end of the stationary phase (Fig. 4.10A and 4.10B). Under the different growth conditions, *O.* cf. *ovata* growth rates in the range 0.30-0.45 day^{-1} were observed.

Fig. 4.10 - Growth pattern of *O.* cf. *ovata* cultures exposed to different growth temperatures. (A) Adriatic strain (OOAB0801) and (B) Tyrrhenian strain (OOTL0707).



Temperature affected cell growth of both strains; however differences between the two isolates were obsereved. Final cell yield of the Adriatic strain was only slightly affected by the various temperature, and reached values of about 4000-5000 cell mL^{-1}; while relevant differences were reported for the Tyrrhenian strain. In fact, a maximum cell density of about 4500 cell mL^{-1} was reported at 20°C, whereas lower values were reached at the highest temperatures (2600 and 3200 cell mL^{-1} at 25 and 30°C, respectively).

Both strains reported a better growth rate at 25°C (ANOVA, $P<0.05$), being 0.41 and 0.45 day^{-1} for the Adriatic and Tyrrhenian strain, respectively; while the lowest growth rates were observed at the highest tested temperature (30°C), namely 0.30 and 0.32 day^{-1}, respectively.

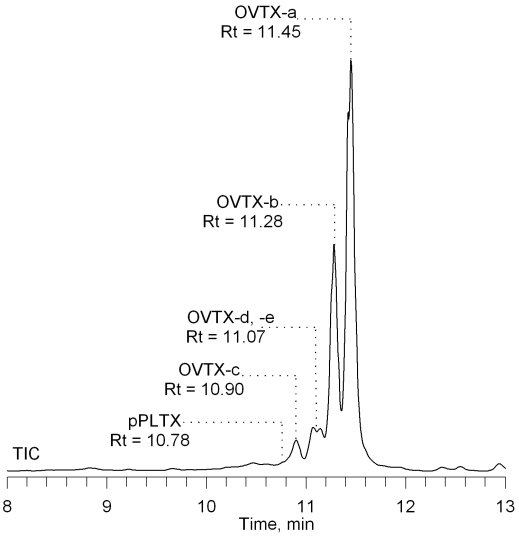
Temperature of 20°C showed intermediate growth rates for both isolates, resulting in 0.32 and 0.39 day^{-1} for the Adriatic and Tyrrhenian strain, respectively.

Determination of toxin content by HR LC-MS

Cell pellets of *O.* cf. *ovata* cultures grown at the different temperature values were collected during the exponential and stationary growth phases. HR LC-MS experiments were acquired in full MS mode by using an LC method which allowed chromatographic separation of the major components of the toxin profile (Fig. 4.11). The spectra were acquired in the mass range *m/z* 800-1400 where each palytoxin-like compound produces bi-charged ions due to $[M+H+K]^{2+}$, $[M+H+Na]^{2+}$, and $[M+2H]^{2+}$, tri-charged ions due to $[M+2H+K]^{3+}$ and $[M+2H+Na]^{3+}$, and a number of ions due to multiple water losses from the $[M+2H]^{2+}$ and $[M+3H]^{3+}$ ions, as reported in the experimental.

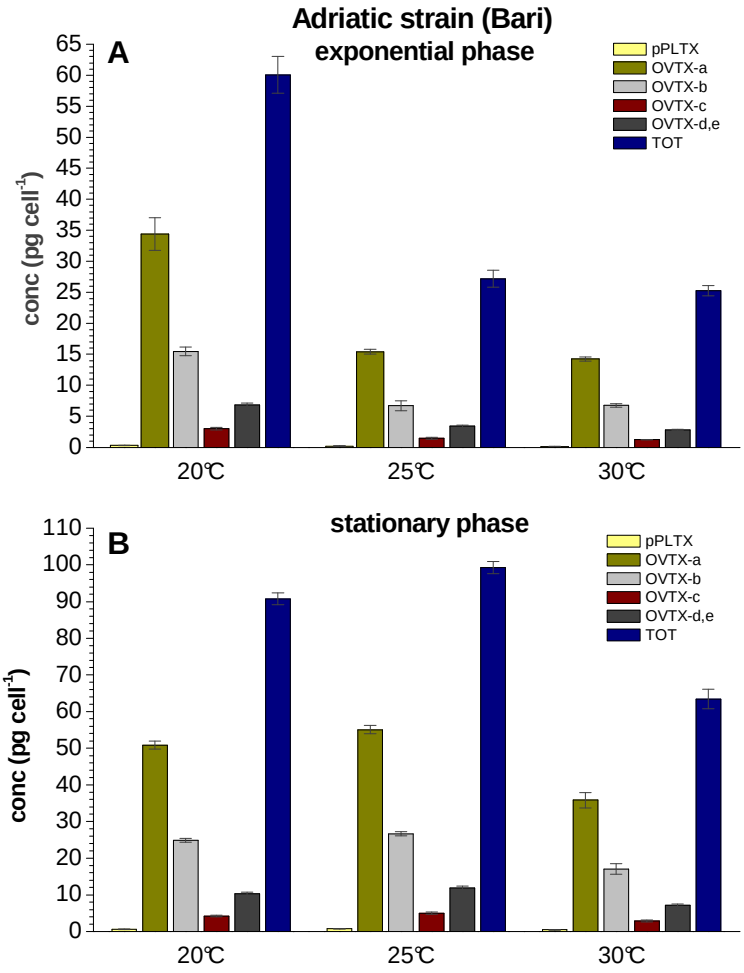
The presence of putative palytoxin and of all the ovatoxins (ovatoxin-a, -b, -c, -d, and -e) recently identified in *O.* cf. *ovata* (Ciminiello et al., 2008, 2010) and found in the Adriatic strain from Ancona (section 4.2.1) was highlighted in all the analyzed samples (Fig. 4.12 and 4.13).

Fig. 4.11 - Total ion chromatogram (TIC) of the *O. ovata* culture extract containing putative PLTX, OVTX-a, -b, -c, -d, and-e. LC-MS analysis was carried out in full MS positive ion mode in the mass range 800-1400 amu, by using the chromatographic conditions reported in the experimental (from Ciminiello et al., 2010).



Significant differences were observed in the total toxin content of different algal extracts (ANOVA, P<0.001), whereas relative abundance of individual toxins were quite similar: ovatoxin-a was by far the major component of the toxin profile (55-57% of the total toxin content; Post-hoc SNK test, P<0.001), followed by ovatoxin-b (25-28%), ovatoxin-d and -e (11-13%), ovatoxin-c (4-6%) and putative palytoxin (0.5-0.7%) on the basis of their decreasing relative abundance.

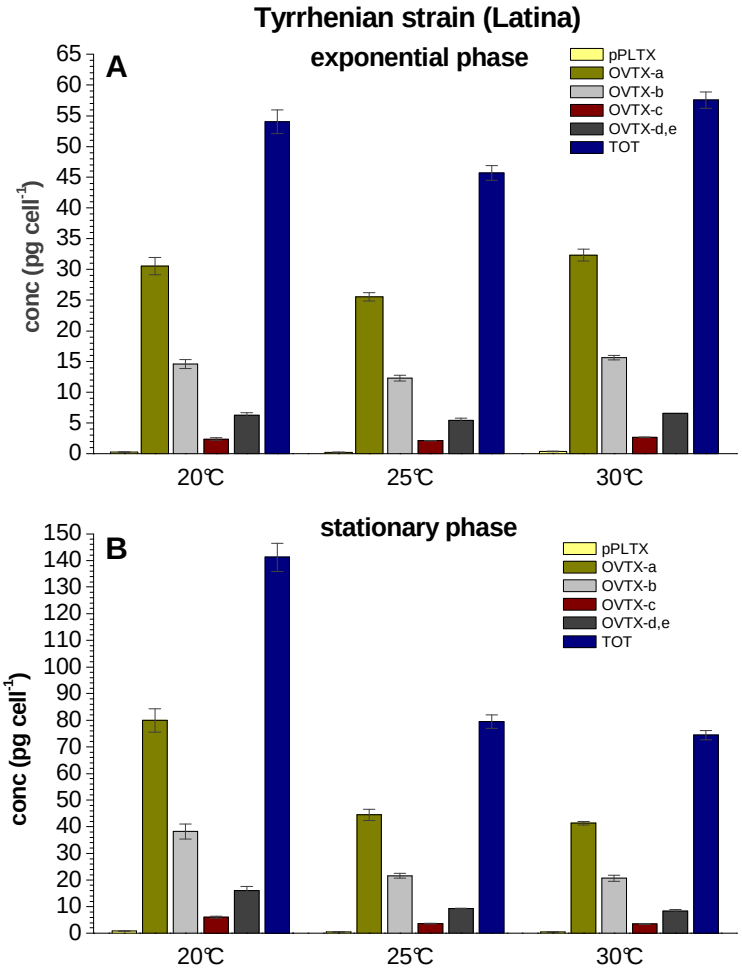
Fig. 4.12 - Total and individual toxin contents of putative palytoxin, ovatoxin-a, -b, -c, -d, and -e of the Adriatic *O.* cf. *ovata* strain grown under different temperatures, in the exponential (A) and stationary (B) phase. HR LC-MS measurements (pg cell^{-1}) were carried out for cell pellet extracts.



Total toxin content of pellet extracts expressed as μg L^{-1} culture in both strains are reported in Table 4.5. Toxin contents were significantly different in the stationary than in the corresponding exponential phase (Post-hoc SNK test, P<0.001), being about 2 to 4-fold higher at the end of the growth. Total content expressed as μg L^{-1} culture was higher in the

Adriatic strain than in the Tyrrhenian one and the highest value was detected at 20°C either for both isolates and growth phases (270 and 242 µg L^{-1} culture for the Adriatic and Tyrrhenian strain, respectively). At the other two temperatures (25 and 30°C) not significant differences were reported in the expoenetial phases, whereas values significantly changed in the stationary phase (ANOVA, $P<0.05$): 25 and 30°C resulted in a higher toxin content for the Adriatic (251 µg L^{-1} culture) and Tyrrhenian (156 µg L^{-1} culture) strain, respectively.

Fig. 4.13 - Total and individual toxin contents of putative palytoxin, ovatoxin-a, -b, -c, -d, and -e of the Tyrrhehnian *O.* cf. *ovata* strain grown under different temperatures, in the exponential (A) and stationary (B) phase. HR LC-MS measurements (pg $cell^{-1}$) were carried out for cell pellet extracts.



Total and individual toxin contents on a per cell basis (pg $cell^{-1}$), are reported in Fig. 4.12 and 4.13 for the Adriatic and Tyrrhenian strain, respectively. Differences in total toxin content were observed between the strains: the strain from Bari was more toxic at all the different growth temperatures besides at 20°C in the stationary phase, where a maximum value of 130

pg cell^{-1} was reported for the strain from Latina (Fig. 4.13). Toxin expressed on a cell basis resulted in a different toxicity pattern, in fact in the Adriatic strain the highest toxin content was observed at 25°C in the stationary phase (99 pg cell^{-1}), followed by 20°C (91 pg cell^{-1}) and 30°C (63 pg cell^{-1}), while in the Tyrrhenian strain the maximum toxin amount found at 20°C (130 pg cell^{-1}) was followed by 25°C (79 pg cell^{-1}) and 30°C (74 pg cell^{-1}).

Table 4.5 – Total toxin content (pPLTX and OVTX-a,b,c,d,e) in the cell pellets of the two *Ostreopsis* cf. *ovata* strains (OOAB0801 and OOTL0707) grown at the different temperatures, measured in both exponential and stationary phases. Data are expressed as μg L^{-1} culture.

	Adriatic strain (Bari) conc (μg/L)		**Tyrrhenian strain (Latina) conc (μg/L)**	
	exp. phase	stat. phase	exp. phase	stat. phase
20℃	97.4	270.3	63.0	242.2
25℃	68.0	250.7	58.9	131.8
30℃	71.9	214.3	59.9	155.9

Conclusion

Ostreopsis cf. *ovata* proliferation in the Italian coasts is characterized by differences in the environmental paraters, especially in temperature values (Pistocchi et al., 2011). To evaluate the effect of temperature on the growth and toxicity of this dinoflagellate, various strains isolated in recent years in the Italian costal waters, have been cultured and compared.

Growth pattern

Temperature resulted to affect *O.* cf. *ovata* growth, as the analyzed Adriatic and Tyrrhenian strains reported the highest growth rates at 25°C, whereas the lowest rates was recorded at 30°C. These results differed slighltly from those obtained using another Adriatic isolate from Ancona (see section 4.2.1), which showed a better growth rate at 20°C. However, our results are in good agreement with field surveys in the Adriatic and Tyrrhenian Seas, where *O.* cf. *ovata* proliferation occurs in different period of the year: in July and August blooms are present in the Tyrrenian Sea (Tognetto et al., 1995; Simoni et al., 2004) and southern Adriatic Sea (Ungaro et al. 2005), when water temperature is around 24-28°C; while blooms are usually detected in the northern Adriatic Sea near Ancona from the end of August to October, with water temperature about 20-22°C (Totti et al., 2010; Monti et al., 2007). Despite Granéli et al. (2011) indicated, for a Tyrrhenian *O.* cf. *ovata* strain, that high water temperatures (26-

30°C) increased both growth rate and yield, our survey showed that the growth yield of a different Tyrrhenian strain (from Latina) was higher at 20°C.
However, these results agree in highlighting one more time that Adriatic and Tyrrhenian strains are differently affected by environmental temperature.

Toxin profile

Putative palytoxin and all the ovatoxins so far known (Ciminiello et al., 2010) were detected in all *O.* cf. *ovata* extracts. In the cultures grown under different conditions relative abundance of individual toxins was similar, with ovatoxin-a and putative palytoxin being the major and the minor component of the toxin profile, respectively.
The total toxin content of the strains from Bari and Latina on a per cell basis (pg $cell^{-1}$) was significantly higher (ANOVA, $P<0.01$) than that detected for the Adriatic strain from Ancona, which reported values in the range 12-23 pg $cell^{-1}$. These strains have a toxin content about 5 to 6-fold higher and even when toxin were expressed on a culture volume basis toxicity resulted significantly higher (ANOVA, $P<0.01$). A reverse correlation between growth and toxin production, as reported also by Granéli et al. (2011), was found also for these strains: the highest total toxin content (μg L^{-1}) was measured at temperature 20°C in both strains, while growth rates were higher at 25°C. However, the correlation between growth and toxin content is still not clear, depsite it had been already reported for other dinoflagellates (Etheridge and Roesler, 2005; Errera et al., 2010; Guerrini et al., 2007).
These highlighted differences in the toxin production may have important implication in *O.* cf. *ovata* proliferation, as blooms in these coastal areas (southern Adriatic and Tyrrhenian Sea) may cause more relevant toxic effects on the ecosystem and human health problems.

4.2.3. Changes of *O.* cf. *ovata* palytoxin-like compounds under N- and P- limitation

It is well recognized that environmental factors such as temperature, salinity, light, and nutrient unbalanced conditions affect both growth and toxicity of many planktonic toxic dinoflagellates (Morton et al., 1992; Granéli and Flynn, 2006); while the role of the different environmental conditions favouring the onset of benthic harmful dinoflagellates has been less investigated (Morton et al., 1992; Van Dolah, 2000; Chateau-Degat et al., 2005; Yasumoto et al., 1977).
Up to now, worldwide surveys including Mediterranean area, suggest that temperature, hydrodynamism, wave action, and substrate typology display major roles for *Ostreopsis* spp. growth (Totti et al., 2010; Pistocchi et al., 2011) as for other epibenthic dinoflagellates typical

of ciguatera areas (Morton et al., 1992; Van Dolah, 2000; Chateau-Degat et al., 2005; Yasumoto et al., 1977), while links with salinity and nutrient availability are less clear.

N:P supply is a topic of particular concern for the development of nuisance algal blooms with or without enhancement through eutrophication (Flynn, 2002). In field situations, changes in the nutrient N:P supply ratio are to be expected both for cells exposed to tidal or river flows (Flynn, 2002). Indeed changes in nutritional status due to eutrophication of the coastal waters (Nixon, 1995), together with increasing surface water temperature and water column stability, favour the occurrence and proliferation of a selected group of phytoplankton (Balode et al. 1998; Anderson et al., 2002; Parsons et al., 2007; Hallegraeff, 1993; Heisler et., 2008; Collos et al., 2004). Decreases in N/P ratios due to phosphorus loading have been related to harmful algal bloom events (Hodgkiss and Ho, 1997), including many planktonic toxic dinoflagellates of the genus *Alexandrium* (Bechemin et al., 1999).

Most studies agree that nitrogen or phosphorus limitation reduces growth of toxic dinoflagellates (Gallardo Rodríguez et al., 2007; Shi et al., 2005; Siu et al., 1997; Wang and Hsieh, 2002). Nevertheless, changes of the nutrient pool affect not only the growth of the organisms but also their biochemical composition. In laboratory controlled setting, cellular toxin of some paralytic shellfish toxins (PSTs) producing species are induced under P-limited or high N/P ratio conditions (Boyer et al., 1987; Anderson et al., 1990; Bechemin et al., 1999), while other studies showed that an increase of cellular toxin is due to simultaneous N and P limitations (Flynn et al., 1994; John and Flynn, 2000). The difference might be due to ecotypic variation in ecophysiological adaptation in the environment from where they originated. Anyhow, P-stress has been often associated with the development of major toxicity in flagellates (Granéli et al., 1998; John and Flynn, 2000) planktonic dinoflagellate (Frangopulos et al., 2004; Guerrini et al., 2007), but also in benthic dinoflagellates such as *Prorocentrum lima.* For some harmful species like *P. lima* (Tomas and Baden, 1993), *Prymnesium parvum* (Johansson and Granéli, 1999) and *Chrysochromulina polylepis* (Dahl et al., 2005; Johansson and Granéli, 1999) phosphorus limitation increases toxicity through stimulation of toxin production.

With regard to *Ostreopsis* cf. *ovata* and more generally to Ostreopsidaceae the role of nutrients and/or nutrient unbalanced conditions on *Ostreopsis* spp. growth and toxicity is almost unknown. Very limited information on relationships between field nutrient concentrations and *Ostreopsis* abundances deal with some environmental surveys carried out for assessing and monitoring the presence and abundance of the epibenthic dinoflagellates which typically co-occur in ciguatera endemic regions. In some of these studies, although

some evidence appears on the linkage between nutrient concentrations and benthic dinoflagellates abundances, the results based on correlation analyses are controversial (Vila et al., 2001; Delgado et al., 2006; Parsons and Preskitt, 2007; Okolodkov et al., 2007; Shears and Ross 2009; Armi et al., 2010).

So far, information on laboratory experiments assessing effects of nutrient unbalanced conditions on *O.* cf. *ovata* growth and toxin production are almost lacking (Granéli et al., 2008).

The aim of this work was to investigate how nutrient limited conditions affect *O.* cf. *ovata* cell growth and toxin content. To do this, a *O.* cf. *ovata* strain isolated from the Adriatic Sea (OOAN0601) was grown under 1/50-nitrogen limited and 1/50-phosphorus limited conditions with respect to control nutrient conditions (five-fold diluted f/2 medium plus selenium; N/P ratio: 16).

Experimental section

Cultures of Ostreopsis cf. ovata

O. cf. *ovata* was isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from water samples collected along the Adriatic coasts of Italy (Marche region, Numana sampling site, strain OOAN0601) in October 2006 (see section 4.2.1). Cultures were established at salinity 36 and 20°C, in a thermostatic room under a 16:8 h L:D cycle from cool white lamp, maintaining light irradiance at 100-110 $\mu mol\ m^{-2}\ s^{-1}$. In order to evaluate the effect of nutrient concentrations on growth and toxicity of *O.* cf. *ovata*, cultures were prepared adding macronutrients at a five-fold diluted (for control), and twenty five-fold diluted f/2 (Guillard, 1975) concentrations for N- and P- depletion conditions.

As previoulsy descrive (section 4.2.1), to evaluate the growth profile, 15 Erlenmeyer flasks containing 200 ml of culture were grown in parallel for each condition; every other day, two out of the initial flasks were treated with HCl to a final concentration of 4 mM and discarded after the counting. Cell counts were made following Utermöhl method (Hasle, 1978), while calculation of cell volume was made with the assumption of ellipsoid shape using the following equation (Sun and Liu, 2003).

For each nutrient condition, a set of four 800 mL culturing flasks was set up for the evaluation of toxin content. Cell counting was carried out on one out of the four flasks as described above. Culture collections were carried out during either the exponential and stationary growth phases by gravity filtration through GF/F Whatman (0.7 μm) filters at day 9^{th} and 22^{th},

respectively. Cell pellets and growth media for each condition were provided for chemical analysis.

Chemical analysis

HR LC-MS analyses of palytoxin-like compunds were performed as reported in section 4.2.1.

Nutrient analysis

Nitrate and phosphate analyses were performed on filtered culture medium aliquots (Millipore cellulose filters, pore size 0.45 mm) and analysed spectrophotometrically according to Strickland and Parsons (1972).

Results

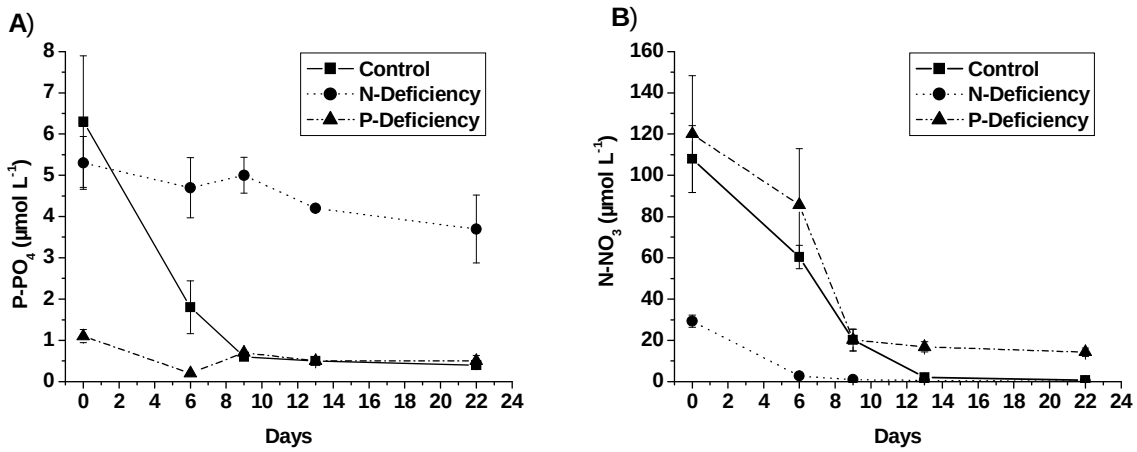
Nutrient concentrations

Figures 4.14 shows nutrient concentration changes during *O.* cf. *ovata* cell growth under control, N-nitrogen and P-phosphorous limited conditions (N:P ratio: 17, 5, 105, respectively). Under control conditions (actual initial concentrations: N = 108 and P = 6.35 µM, respectively; N:P ratio = 17), both N-nitrogen and P-phosphorous were rapidly taken up leading to a N:P ratio of about 30 from day 6 to day 9, when N and P concentrations (20.18 and 0.65 µM, respectively) were about 5-fold and 10-fold lower than initial concentrations. During stationary phase nutrient concentrations decreased further (i.e. N = 2.14 and P = 0.48 µM, respectively, 13th day), especially N concentration, and the N:P ratio dropped to values <5. Finally, in late stationary phase (i.e. 22nd day) N:P ratio was <2 (N = 0.73 and P = 0.40 µM).

Under N-limited conditions (initial concentrations: N = 29.29, P = 5.29 µM, respectively; N:P ratio= 5.54) N concentration decreased rapidly within the first 6 days (N = 2.68 µM; 10-fold lower than N initial concentration) and by the end of the exponential phase (6-9th day) N and P concentrations were <1 and >4.5 µM, respectively with a N:P ratio <1 (N:P ratio = 0.17); thus, most of the phosphate was available in late stationary phase (22nd day), whereas N was mostly depleted (0.2 and 3.7 µM, for N and P, respectively).

Under P-limited conditions (initial concentrations: N = 120 and P = 1.14 µM; N:P ratio = 105), from late exponential phase to late stationary phase (i.e. from 9th from 22nd day) both N and P concentrations changed slightly (N = 20, P = 0.69 µM at 9th day, and N = 14, P = 0.45 µM at 22nd day) resulting in a almost constant N:P ratio of about 30 (29 and 32, 9th and 22nd day, respectively).

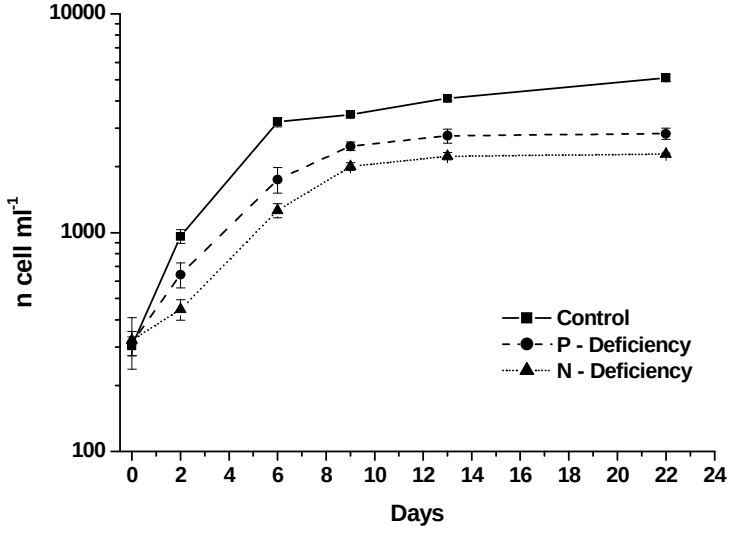
Fig. 4.14 - Nitrogen (NO_3^-) and phosphorus (PO_4^{3-}) concentrations measured in *O.* cf. *ovata* cultures grown under different nutrient conditions. Bars indicate standard deviations.



Cell growth and cell size

Growth curves of *Ostreopsis* cf. *ovata* under control N- and P-limited conditions are shown in Figure 4.15. Initial cell densities were on average, 300-330 cells mL^{-1} and the exponential growth phase lasted till 6-9th day.

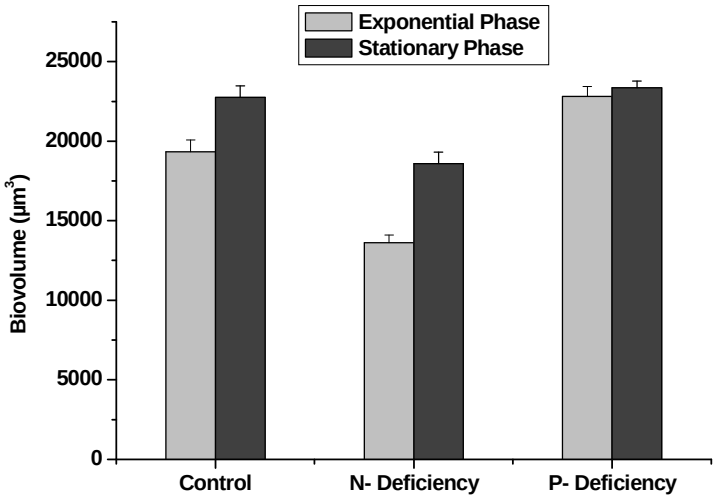
Fig. 4.15 - Growth pattern of *O.* cf. *ovata* cultures exposed to different nutrient concentrations.



During the early exponential phase (i.e. from 1st to 6th day) the mean growth rate (µ) was significantly lower under nutrient limited conditions comparing to control (µ= 0.40 ± 0.013, 0.29 ± 0.034, and 0.24 ± 0.055, day^{-1}, for control, N- and P-treatment, respectively; ANOVA,

both P<0.001), whereas, no significant difference was found between N- and P-limited treatments (P>0.05).

Fig. 4.16 - Cell volumes of *O.* cf. *ovata* cells exposed to different nutrient conditions.



As a result, by the end of the exponential phase (6-9th day) under both nutrient limited conditions cell densities were significantly lower than in the control conditions (1.9- and 1.5-fold lower for N- and P-treatments, respectively; ANOVA, both P≤0.05). Finally, in the stationary phase (22nd), cell yields under N-limited and P-limited treatments attained to a 2.2-fold and 1.8-fold lower values with respect to control yield (mean cell yield: 4.57 x 10^3, 2.31 x 10^3, and 2.95 x 10^3 cells mL^{-1}, for control, N-, and P- limited conditions, respectively; ANOVA, P<0.001). N-limited cell yield was also significantly lower than P-limited yield (ANOVA, P<0.01).

Under all tested conditions and over the growth cycle *O.* cf. *ovata* cell cultures were composed by two cell size classes, a small one (i.e. class 1; cell volume range: 5728-10,008 μm^3, control in the exponential phase and P-limited treatment in the stationary phase, respectively) and a large one (i.e. class 2; cell volume range: 20,280-30,232 μm^3, N-limited treatment in the exponential phase and P-limited treatment in the stationary phase, respectively). As a general trend, under all conditions, both cell size classes showed an increasing volume from exponential to stationary phase (Fig. 4.16) which was significant under N-limited treatment for both classes, and under control conditions for class 1 (ANOVA, all P<0.01) but not for P-limited treatment. The highest cell volumes were found under P-limited conditions over the growth cycle with significant higher values for class 1 when compared to control (ANOVA, P<0.01 and P<0.05, exponential and stationary phase,

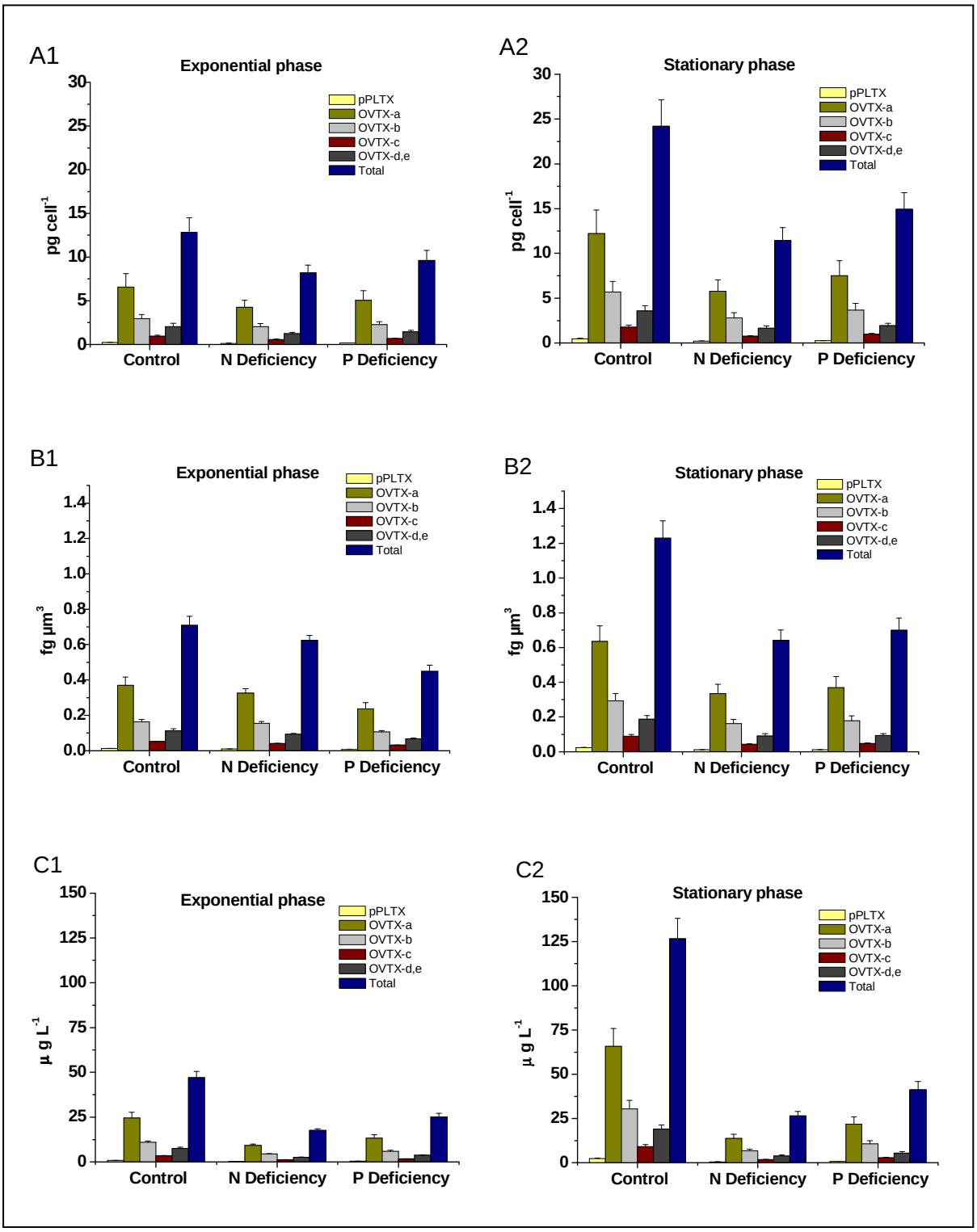
respectively), and for both classes when compared to N-limited conditions (ANOVA, $P<0.05$, class 1 in both phases; ANOVA, $P<0.01$, class 2 in the exponential phase).

The relative contribution of the two size classes to the total cell abundance and cell volume showed different temporal trend under the different nutrient conditions (data not shown). More in deep, under control conditions cells were mostly of class 2 and their relative contribution to the total cell abundance increased slightly and not significantly from the exponential to the stationary phase (67 and 70%, respectively). A similar high contribution of class 2 cells to cell abundance was found under P-limited condition, although this contribution decreased slightly from the exponential to the stationary phase (73 and 66% of total cells, in exponential and stationary phase, respectively). Differently, under N-limited conditions class 2 cells represented 52 and 54% of the total cell numbers in the exponential and in the stationary phase, respectively. So that, the recorded changes in cell size composition and cell class volume returned into: 1) a significant lower mean cell volume under N-limitation over the growth cycle when compared to both control (29 and 18% lower in the exponential and stationary phase, respectively) and P-limited conditions (40 and 20% lower in the exponential and stationary phase, respectively; ANOVA, all $P<0.001$); 2) a significant higher cell volume under P-limitation in the exponential phase when compared to control conditions (19%; ANOVA, $P<0.001$) (2% in stationary phase); 3) a significant increase of the mean cell volume from exponential to stationary phase under control and N-limited conditions (ANOVA, $P<0.001$), but not under P-limitation (ANOVA, $P>0.05$).

Toxin concentrations

As general, under all tested conditions (either control, N- or P- deficiency) *O.* cf. *ovata* showed the same qualitative toxin profile producing putative palytoxin (pPLTX), ovatoxin-a (OVTX-a), and the recently identified palytoxin-like compounds OVTX-b, OVTX-c, OVTX-d, and OVTX-e (Fig. 4.17). The relative contribution of each toxin to the total toxin content varied slightly throughout the experiments with OVTX-a and OVTX-b as major contributors (accounting for 52-53% and 23-26%, respectively), followed by OVTX-d plus OVTX-e (13-15%), and OVTX-c (6.6-7.4%); whereas pPLTX contribution was less than 2%. Moreover, under all tested conditions, toxin concentrations (either expressed on per cell or on cell volume basis) showed an increasing trend from the exponential to the stationary growth phase. Nevertheless, toxins content of cells grown at different nutrient conditions changed differently over cell growth cycle.

Fig. 4.17 – Total and individual toxin contents of putative palytoxin, ovatoxin-a, -b, -c, -d, and -e of the Adriatric *O.* cf. *ovata* strain grown under different nutrient conditions, in the exponential (1) and stationary (2) phase. HR LC-MS measurements were carried out for cell pellet extracts and espressed as content per cell (pg cell^{-1}) (A), per biovolume, (fg µm^{3}) (B) and per culture volume (µg L^{-1}) (C).



More in details, under control conditions, all toxins on a cell basis, showed significantly higher concentrations (close to 2-fold) in the stationary growth phase compared to the exponential phase (ANOVA, all $P<0.01$; Fig. 4.17A).
Control toxin concentrations expressed on cell volume basis ranged from 0.012 to 0.564 fg μm^{-3} (pPLTX in the exponential and OVTX-a in the stationary phase, respectively; Fig. 4.17B), showing the same significant increasing trend (up to 1.6-fold).

N-limited condition: comparison between growth phases. Under N-limited condition, concentration of all toxins on a cell basis also increased (about 1.35-fold for all toxins except for pPLTX with a 2.1-fold) over the growth cycle (Fig. 4.17A); however, it resulted not significantly (ANOVA, all $P>0.05$). The relative increase of each toxin concentration was reduced when expressed on cell volume basis (from 0.97-fold to 1.13, OVTX-d plus OVTX-e and pPLTX, respectively; ANOVA, all $P>0.05$, Fig. 4.17B) due to significant higher cell volume in the stationary phase with respect to the exponential phase.

N-limited condition: comparison between control and N-treatment. Under N-limitation all toxins showed lower concentrations (on a cell basis) with respect to control conditions over the growth cycle (Fig. 4.17A). Nevertheless, in the exponential phase, toxin concentrations were significantly lower only for pPLTX and OVTX-c compared to control (ANOVA, $P<0.05$, and $P<0.01$, respectively). These significant differences still persist when values were referred to cell volume (ANOVA, $P<0.01$ for both pPLTX and OVTX-c) regardless the significant smaller cell volume found under N-limitation than in control conditions all over the growth cycle (29% and 18% smaller than control, exponential and stationary phase, respectively). Whereas, over stationary phase, all toxins concentration on cell basis, were significantly lower with respect to control conditions (ANOVA, $P<0.01$ for OVTX-a, OVTX-b, and total toxins; and $P<0.001$ for pPLTX, OVTX-c, and OVTX-d plus OVTX-e) with a decrease exceeding 50% for all toxins (range: 51-57% lower than control, OVTX-a and OVTX-c, respectively). In details, the highest reduction was observed for OVTX-c concentration (57% lower than control conditions) followed by pPLTX (55%), whereas OVTX-a, OVTX-b showed almost the same decrease with respect to control (i.e. 51 and 53%, respectively). When comparison was performed on cell volume basis all toxin concentrations showed similar pattern and were still significant lower than under control conditions (ANOVA, all $P<0.01$), although the relative decrease was reduced (range: 40 to 47% lower than control, OVTX-b and OVTX-c, respectively).

P-limited condition: comparison between growth phases. Under P-limitation all toxin concentrations on a cell basis (Fig. 4.17A) increased over the growth cycle, with significant higher values in the stationary phase for pPLTX, OVTX-a, OVTX-b, and OVTX-c, compared to exponential phase (ANOVA, $P<0.05$). Toxin concentration on a cell volume basis (Fig. 4.17B) showed the same results and statistical pattern (exponential phase range: 0.007–0.234 fg μm^{-3}, pPLTX and OVTX-a, respectively; stationary phase range: 0.010–0.330 fg μm^{-3}, pPLTX and OVTX-a, respectively; ANOVA, $P<0.01$ for pPLTX and OVTX-c; $P<0.05$ for OVTX-b), however accompanied with a reduced increasing range (i.e. from 1.25-fold to 1.53-fold, for OVTX-d plus OVTX-e and OVTX-b, respectively).

P-limited condition: comparison between control and P-treatment. Under P-limitation, in the exponential phase, all toxins showed lower concentrations than those found under control conditions, nevertheless differences were not significant (ANOVA, $P>0.05$). When data were expressed on cell volume basis (Fig. 4.17B), pPLTX, OVTX-c, OVTX-d plus OVTX-e showed significant lower values than in the control (ANOVA, $P<0.05$), very likely due to a significant higher cell volume (20% higher). In the stationary phase, toxin values on cell basis were significant lower when compared to control conditions; the highest decrease was observed for pPLTX (49% lower than control; ANOVA, $P<0.001$) followed by OVTX-d plus OVTX-e, (46 % lower than control; ANOVA, $P<0.01$), OVTX-c (44% lower than control, ANOVA, $P<0.001$), OVTX-a (39%, ANOVA, $P>0.05$) OVTX-b (36%, ANOVA, $P<0.05$), total toxin ($P<0.05$). When toxin concentrations were expressed on a cell volume basis, all toxins presented the same significant decreasing trend found (range: 38-49%, OVTX and pPLTX, respectively) with respect to control (ANOVA, all $P<0.01$).

Comparison between N-limited and P-limited conditions. When comparing toxin concentrations (on per cell basis) in both exponential and stationary growth phases (Fig. 4.17A), N- and P-deficient conditions no significant differences were found for all toxins, except for OVTX-c at stationary phase (ANOVA, $P<0.001$); in fact OVTX-c concentrations showed significant lower values (22% less) under N-treatment when compared with P-treatment (0.76 and 0.98 pg $cell^{-1}$, respectively). In the exponential phase when comparing toxin concentration expressed on a cell volume basis (Fig. 4.17B), pPLTX, OVTX-c, OVTX-d plus OVTX-e were significant lower under P-limitation than under N-limitation (ANOVA, $P<0.05$ and $P<0.01$ for pPLTX, OVTXd,e, and OVTX-c, respectively). By contrast, in the

stationary phase toxin concentration did not show any difference between N- and P-treatment (ANOVA, all P>0.05).

Conclusion

The results reported in this study show that nutrient unbalanced conditions affects *O.* cf. *ovata* cell growth rate, biovolume, yield, and toxin amounts. While a cell growth limitation was expected, a toxin content decrease was more surprisingly.

Cell growth and nutrients

Results reported in this study show that both N- and P- applied limited conditions affected *O. ovata* cell growth already in the early exponential phase due to a significant reduction of the mean growth rate (mean value 0.26 day-^{1}) compared to that one found under balanced nutrient conditions (0.40 day-^{1}) that was within the range of values reported for Mediterranean *O.* cf. *ovata* strains (section 4.2.1 this thesis; Guerrini et al., 2010; Granéli et al., 2010) and other worldwide distributed *O*. spp. strains (Pearce et al., 2001).

Therefore, both nutrient limited conditions caused significant lower final cell yields with respect to control conditions with more severe effects under N-limitation.

O. cf. *ovata* growth pattern was clearly dependent on nutrient dynamics; under control conditions (N:P ratio = 16) phosphorous uptake rates (data not shown) were high during the exponential phase while nitrogen maintained relative high uptake rates also during the early stationary phase. Accordingly, under N-limitation (N:P = 5), nitrogen was exhausted by the end of the exponential phase reaching a N:P ratio <1. On the other hand, under P-limitation (N:P = 100) most phosphorous was uptake during the first few days of growth (day 2), reporting in the exponential phase (day 6) a N:P ratio of about 400; thereafter, phosphorous concentration slightly changed, leading to a nearly steady N:P ratio of about 30.

Interestingly, *O.* cf. *ovata* growth was affected more severely by nitrogen than phosphorous limitation and this in accordance with recent results reported by Vidyarathna and Granéli (2010). Whereas, this result may be apparently in contrast with findings for some toxic planktonic dinoflagellates including diarrheic shellfish poisoning (DSP) species (e.g. *Protoceratium reticulatum*; Guerrini et al., 2007; Gallardo Rodríguez et al., 2009), and paralytic shellfish poisoning (PSP) (e.g. *Alexandrium minutum*, Lim et al., 2005) (Granéli and Flynn, 2006 and references therein), but also for some benthic dinoflagellates such as *P. lima* (Varkitzi et al., 2010; Vanucci et al., 2010), as P was found to affect mainly their growth.

It has to be noted that N and P concentrations *per se* are also important factors to be considered when dealing with the effect of nutrient on dinoflagellate cell growth. Indeed, the

initial nutrient amounts interfere with the dynamics of nutrient cell uptake, storing and nutrient intracellular ratio, and ultimately on timing of nutrient limitation status. For instance, the complex mechanisms underlying P uptake, its transport and assimilation interactions within the cell depend on species cell eco-physiology and on internal nutrient ratio which is the effective ratio (Flynn, 2002). As such, John and Flynn (2000) showed that in the absence of phosphate, growth and carbon fixation of *Alexandrium* species continue for several generations, and phosphate may only be limited under N:P ratios which are significantly higher than Redfield ratio.

In our P-limited conditions, at the end of the exponential phase, residual P concentrations were still available (about 0.4 µM) accompanied by appreciable N concentrations; nevertheless, as mentioned above, cells experienced strong P-limitation during early exponential phase preventing cell algal division.

Nutrient limited conditions also affected *O.* cf. *ovata* cell size; particularly, P stress resulted in an increase of cell biovolume. The percentage of large cells recorded at the stationary phase under P-depleted condition was similar to that one found under control condition at the end of the exponential phase when cell experienced a similar N/P ratio (31). This behaviour was also observed in other species of both planktonic (e.g. Latasa and Berdalet 1994; John and Flynn 2000; Lim et al. 2005) and benthic dinoflagellates (Vanucci et al., 2010; Varkitzi et al., 2010). John and Flynn (2000) suggesting that the increase in cell biovolume is due to the arrest of cells in the G1 phase (Vaulot et al. 1996) without undergoing cell division, while other non-P compounds continued to be synthesized.

Toxins

Under all tested conditions *O.* cf. *ovata* Adriatic strain showed the same qualitative toxin profile producing pPLTX, OVTX-a, OVTX-b, OVTX-c, OVTX-d, and OVTX-e, with OVTX-a being the major contributor to the total toxin content (>50%). Moreover, under all tested conditions, toxin concentrations increased from exponential to stationary growth phase; however, this increase was significantly reduced under both N- and P-nutrient limited conditions. The major effect was found under nitrogen limitation with a decrease of final total toxin content of 51% and 39.6% for N-and P-limitation, respectively, when compared to control conditions. Increasing toxin production during the growth was consistent with previous results on different *O.* cf. *ovata* strains (Guerrini et al., 2010) and other toxic benthic dinoflagellates such as *P. lima* (Vanucci et al., 2010; Varkitzi et al., 2010; McLachlan et al.,

1994; Sohet et al., 1995). Whereas, the decreasing toxin trend induced by unbalance nutrients appears quite different from the results found for *P. lima* and many other toxic dinoflagellates. In this study, N-deficiency affected toxin production with major effects under stationary growth phase, although pPLTX and OVTX-c amounts were significantly affected also during exponential phase. Moreover, comparison with control conditions also indicates that under N-limitation the lower toxin amounts is due to a factual decrease in toxin production as under N-limitation cell volume was significantly smaller than under control conditions all over the growth cycle.

Conversely to the observations under N-limitation, P-limited conditions did not affect cell toxin production during the exponential phase at the end of which N:P ratio was about 30. The lower toxic concentrations found for some toxins when expressed on cell volume basis are very likely ascribable to the significant higher cell volume found under P-limitation when compared to control (20% higher than control) due to inhibition of cell division. This indicates that during exponential phase P-limitation affects cell yield whereas cell biochemical pathways involved in qualitative and quantitative production of toxin did not appear to be significantly affected.

As *O.* cf. *ovata* toxins are N containing compounds, our results agree with the results reported for species producing N-rich toxins; in fact N-rich PSP-toxins are synthesized during N-upshock and P-stress (which gives relatively high N-status) and not during N-downshock (Flynn et al., 1994; Granéli et al., 1998; Granéli and Flynn 2006 and references therein). In the tentative to minimize batch experimental artefacts as possible. N and P concentrations reported during *Ostreopsis* spp. surveys worldwide may be taken into consideration and reported a high N-limitation (nitrate range: 0.2–12.5 µM; phosphate range: 0.005–4.28 µM; Pistocchi et al., 2011). In this study we used low concentrations for both nutrients and a Redfield ratio for control. In our conditions, neither N-upshock and P-stress which gives relatively high N-status were experienced by *O.* cf. *ovata*.

4.3. Toxin characterization of Italian *O.* cf. *ovata* strains

Since *O.* cf. *ovata* cells present different growth and toxicity characteristics in the different coastal areas, several strains isolated from field water samples collected along the Italian coasts in recent years were analyzed for their toxin content and their toxin production was compared.

An important issue in the ovatoxins field is represented by lacking of certified reference standards of palytoxin and its analogues; they are difficult to be obtained in acceptable amounts until a large scale culturing of *Ostreopsis* spp. as well as efficient isolation procedures of the produced toxins will be developed. Currently, quantitation is carried out basing on the tentative assumption that palytoxin-like compounds show the same molar response as palytoxin itself, but even limited structural features in large molecules as palytoxins could significantly impact their ionization efficiency (Ciminiello et al., 2011).
Therefore, this work was also done with the aim to select one strain, with the highest toxin production efficency, to be used for large scale culturing in order to isolate ovatoxins and characterize their structures.

Experimental section

Cultures of Ostreopsis cf. ovata

O. cf. *ovata* strains isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from water samples collected along the Adriatic and Tyrrhenian coasts of Italy in 2006, 2007 and 2008 were cultured. Adriatic strains were: OOAN0601 (from Numana, Marche region, isolated in 2006); OOAN0709 (from Numana, Marche region, isolated in 2007); OOAN0816 (from Numana, Marche region, isolated in 2008); and OOAB0801 (from Bari, Puglia region, isolated in 2008); the Tyrrenian strain was OOTL0707 (from Latina, Lazio region, isolated in 2007). Cultures were established at salinity of 36 and temperature of 20°C, in a thermostatic room under a 16:8 h L:D cycle from cool white lamp, maintaining light irradiance at 100-110 μmol m^{-2} s^{-1}. In order to evaluate the toxin content of *O.* cf. *ovata* strains, cultures were prepared adding macronutrients at a five-fold diluted f/2 (Guillard, 1975) concentrations with selenium.
For each isolate, two 400 mL culturing flasks were set up for the evaluation of toxin content, and culture collections were carried out during the late stationary growth phases (day 25) by gravity filtration through GF/F Whatman (0.7 μm) filters. Cell pellets for each condition were provided for chemical analysis.

Chemical analysis

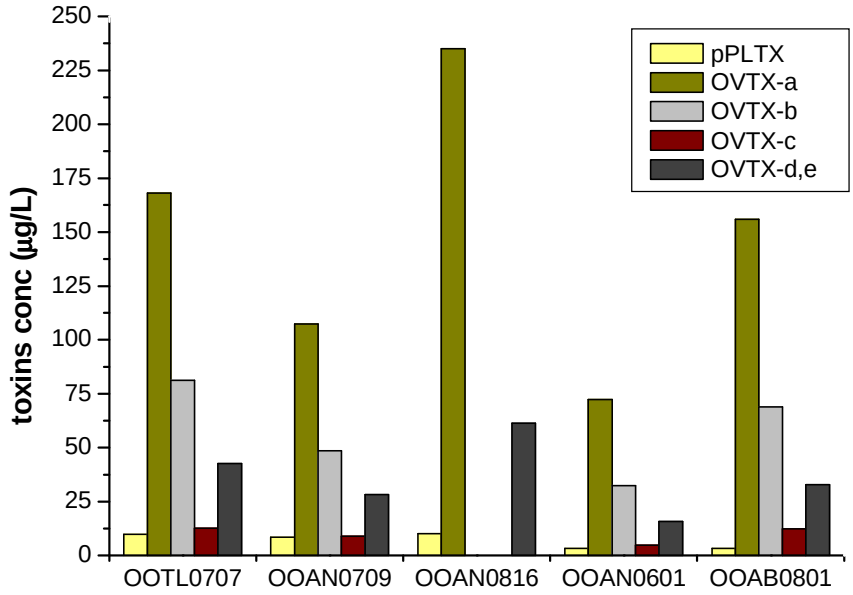
HR LC-MS analyses of palytoxin-like compunds were performed as reported in section 4.2.1.

Results and discussion

Cell pellets of several *O.* cf. *ovata* cutures grown at the same conditions (temperature 20°C and salinity 36) were collected during the late stationary growth phase. Samples were separately extracted as reported in the experimental and the crude extracts were used to evaluate the toxin profile. HR LC-MS experiments were acquired in full MS mode by using an LC method which allowed chromatographic separation of the major components of the toxin profile (see sections 4.2, this thesis). Due to lack of standards for ovatoxins, quantitative analyses were carried out basing on the tentative assumption that they present the same molar response as palytoxin.

The presence of putative palytoxin and of all the ovatoxins (ovatoxin-a, -b, -c, -d, and -e) recently identified in *O.* cf. *ovata* (Ciminiello et al., 2008, 2010) was highlighted in four of the five analyzed samples. The absence of ovatoxin-b and -c in one (OOAN0816) of the *O.* cf. ovata strains was remarked for the first time and was confirmed also by subsequently analyses of algal extracts of the same strain grown at different conditions (data not shown).

Fig. 4.18 – Individual toxin contents of putative palytoxin, ovatoxin-a, -b, -c, -d, and -e of several Adriatic and Tyrrhenian *O.* cf. *ovata* strains in the exponential stationary phase. HR LC-MS measurements were carried out for cell pellet extracts and espressed as toxin content per colture volume (μg L^{-1}).



Significant differences were observed in the total toxin content of the different algal extracts (ANOVA, $P<0.001$), whereas relative abundance of individual toxins were quite similar in all the strains besides the Adriatic *O.* cf. *ovata* starin isolated in 2008 (OOAB0816), which

reported the anomaly in the toxin profile: ovatoxin-a was by far the major component of the toxin profile (52-54% of the total toxin content; Post-hoc SNK test, $P<0.001$), followed by ovatoxin-b (25-26%), ovatoxin-d and -e (13-15%), ovatoxin-c (5-6%) and putative palytoxin (1-2%) on the basis of their decreasing relative abundance. These amounts were in accordance with previous data, as reported in section 4.2. The strain OOAN0816 resulted in a higher amount of ovatoxin-a (about 76%) than the others strains, and the other detected toxins were ovatoxin-d and -e (22%), and putative palytoxin (2%).

Total toxin content expressed as $\mu g\ L^{-1}$ culture are reported in Fig. 4.18. The highest toxin content was reported in the cell pellet of the Tyrrhenian strain OOTL0707 (314 $\mu g\ L^{-1}$), followed by the Adriatic OOAN0816 (307 $\mu g\ L^{-1}$) and OOAB0801 (273 $\mu g\ L^{-1}$); while significantly (Post-hoc SNK test, $P<0.001$) lower amount were found in the two Adriatic strains OOAN0709 and OOAN0601, being 201 and 129 $\mu g\ L^{-1}$, respectively.

These results confirmed once again the existing differences among *O.* cf. *ovata* strains, in term of toxin production (Guerrini et al., 2010; section 4.2.2) and reported for the first time the absence in the toxin profile of two out of the six known palytoxin-like compounds in one strain of this dinoflagellate.

5. Polyketide synthase (PKS) enzymes

The majority of dinoflagellate toxins that adversely affect human health are polyether compounds (Fig. 5.1), synthesized by complex enzymes known as polyketide synthase (PKS) enzymes. Polyketides are often highly oxygenated compounds and include macrolides, polyethers, polyols and aromatics. The construction of dinoflagellate polyketides, is achieved by the polyketide pathway and probably involves a polyketide synthase (PKS) with some additional functional segments (Shimizu, 2003).

Similar to many other dinoflagellates, *O.* cf. *ovata* is a prolific producer of secondary metabolites; in particular, *Ostreopsis* spp. were proposed to be the biogenetic origin of palytoxin (Taniyama et al., 2003). Palytoxin is a large, very complex molecule with a long polyhydroxylated and partially unsaturated aliphatic backbone, containing 64 chiral centers (Kan et al., 2001). Palytoxin has both lipophilic and hydrophilic regions and is referred to as a super-carbon-chain compound, since it has the longest chain of continuous carbon atoms in any known natural product (Wang, 2008). Besides putative palytoxin, new compounds, namely ovatoxins, have been recently isolated from different clones of *O.* cf. *ovata* organism through an in-depth high resolution (HR) Liquid Cromatography-Mass Spectrometry (LC-MS) investigation (Ciminiello et al., 2010). Their structures are still unknown however, they present elemental formulae similar to that of palytoxin.

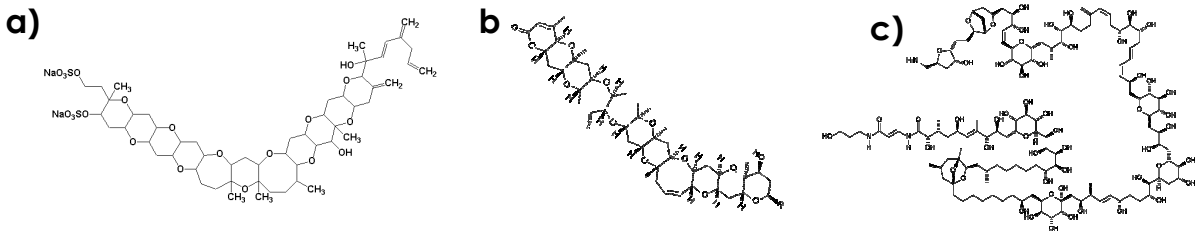
Along the Mediterranean coasts, *Ostreopsis* spp. are generally present in association with other dinoflagellate species, namely *Coolia monotis* and *Prorocentrum* spp. *C. monotis* is quite common in temperate and tropical waters (Steideinger and Tangen, 1997) where it has been frequently detected at low concentrations during warmer periods. *C. monotis* has been reported to produce a toxin, named cooliatoxin, that is believed to be structurally related to yessotoxin based upon the molecular weight, which corresponds to a monosulfated yessotoxin, and the sympoms induced in mice however, its structure is still uncharacterized (Holmes et al., 1995).

Polyketides are synthesized through the sequential addition of carbon building blocks mediated by a set of coordinated catalytic sites that make up polyketide synthase enzymes (PKSs) (Staunton et al., 2001).

Polyketide biosynthesis is analogous to fatty acid biosynthesis and involves similar catalytic sites; the condensation reaction between carboxylic acid building blocks is performed by a •-ketoacyl synthase (KS), and the •-keto group may be reduced by successive ketoreduction by

a ketoreductase (KR), dehydration by a dehydratase (DH), and enoyl reduction by an enoyl reductase (ER) following each chain elongation step.

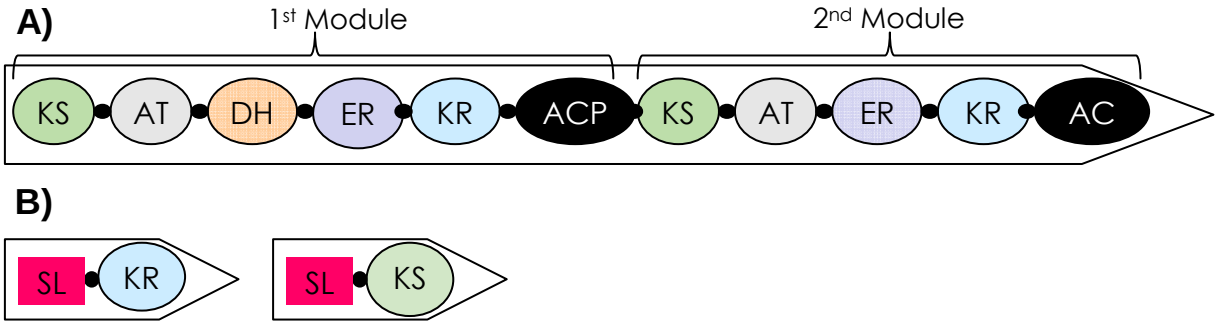
Fig. 5.1 - Chemical structure for some dinoflagellate toxins: (a) yessotoxin (related to *Coolia monotis* toxin), (b) brevetoxin (produced by *Karenia brevis*), (c) palytoxin (produced by *Ostreopsis* spp.)



The growing carbon chain resides on a phosphopantetheine "arm" on the acyl carrier protein (ACP) that "swings" the growing chain in proximity to catalytic sites, while the acyl transferase (AT) brings additional "extender" units to be added to the growing chain. Polyketides are released from the PKS complex by a thioesterase (TE), and post-PKS modifications create the final polyketide structure.

PKS enzymes have classically been categorized as type I-III. Type I PKSs are large multifunctional proteins with multiple active sites on a single polypepetide. Iterative Type I PKSs are found in fungi and are analogous to vertebrate fatty acid synthase (FAS). These complexes are multidomain proteins used repeatedly for each chain elongation. Modular, or processive, Type I PKSs are also multifunctional proteins, but each protein has multiple active site domains organized into modules. Each module catalyzes one chain extension reaction and programmed reduction based on which active site domains are present in the module. Type II PKSs build aromatic polyketides found in bacteria, and unlike Type I PKSs, type II PKSs are organized as complexes of smaller mono-functional proteins where each catalytic domain is located on a separate peptide, a structure similar to bacterial FAS. Also unique to the type II PKSs is the CLF (chain length factor) domain, or KS•, that is part of the minimal PKS required for type II polyketide biosynthesis (Rawlings, 1997). Type III PKS are typically associated with chalcone synthases (CHS) and the stilbene synthases (STS) found exclusively in higher plants, but Type III PKS has also recently been identified in several bacteria (Gross et al., 2006). Type III PKSs are smaller PKSs (40-47kD) and are involved in flavonoid biosynthesis in plants and melanin in bacteria (Funa et al., 1999; Gross et al., 2006; Hopwood, 1997; Khosla et al., 1999).

Fig. 5.2 - A) Type I PKS: a large multifunctional enzyme with several functional or "module" domains within a single protein. B) PKS transcript structure in *K. brevis* with a single domain on a trans-spliced, polyadenylated message (modified from Monroe and Van Dolah, 2008).



Little information exists regarding protist PKSs, especially in dinoflagellates. The first protist PKS gene was identified in the apicomplexan *Cryptosporidium parvum*, which is the closest relative of dinoflagellates to have a fully sequenced genome (Zhu et al., 2000, 2002). The *C. parvum* PKS gene is an intronless 40 kb open reading frame that encodes for a single 13,000 amino acid polypeptide. The polypeptide contains 29 domains including an N-terminal loading unit, 7 modules for chain extension and modifications, and a terminator unit (Zhu et al., 2002). Protist PKS have also been recently identified in the genomes of three chlorophytes, *Ostreococcus taurii*, *O. lucimarinus*, and *Chlamydomonas reinhardtii*, and the haptophyte, *Emiliania huxleyi* (John et al., 2007). Within the compact genome of two *Ostreococcus sp.*, three large PKS genes were identified that comprise 1.5% of the total genome. One long open reading frame with type I PKS domains is present in the *E. huxleyi* genome (John et al., 2007).

The previous identification of protist PKS relied on full genome sequencing, but due to the large genomes of dinoflagellates (e.g. $1x10^{11}$ bp in *K. brevis*, Van Dolah et al., 2007), the production of genome sequences is currently intractable. Despite these impediments, a PKS gene has been identified in *Amphidinium*, a dinoflagellate that produces the polyketide amphidinolide (Kubota et al., 2006). Within the 36,000 base pair clone, six open reading frames were identified that had sequence similarity to several PKS domains (KS, AT, DH, KR, ACP, and TE) spanning 5625 base pairs. As for *K. brevis* PKSs, degenerate PCR primers have been used to identify type I PKS KS domains in 7 dinoflagellate species, including 2 encoded by *K. brevis* (Snyder et al., 2003, 2005). More recently, transcripts with sequence similarity to type I PKS were identified in *K. brevis* through screening of cDNA libraries to *K. brevis* (Monroe and Van Dolah, 2008). Although the full-length PKS transcripts are phylogenetically most similar to type I PKSs from other protists, bacteria, and fungi, they

contain only single catalytic domains, a structure more similar to type II PKSs. Thus, *K. brevis* appears to possess type I PKSs that are unique from other known PKSs. *In silico* translation of *K. brevis* PKS full-length transcripts previously described predicts PKS proteins to be between 50 and 100kDa. Monroe et al. (2010) developed peptide polyclonal antibodies from *in silico* translated KS and KR transcripts to confirm their predicted protein sizes, study their expression, and determine their cellular localization. Several putative PKS genes encoding ketosynthase (KS), ketoreductase (KR), and both acyl carrier protein (ACP) and KS domains were identified from *K. brevis* (Monroe and Van Dolah, 2008), with domains residing on separate polypeptides, more similar to Type II PKS (Fig. 5.2B) while sequence similarity was closest to type I (Fig. 5.2A). Their altered expression in a non-toxic isolate of *K. brevis* suggested their involvement in brevetoxin biosynthesis (Monroe et al., 2010); however, evidence for their function in toxin biosynthesis was limited to correlation. Since PKS and FAS share a long evolutionary history, it can be difficult to distinguish between PKSs and FASs solely on sequence information. The only evidence to suggest these PKSs are involved in brevetoxin biosynthesis was their presence in *K. brevis* and their absence from dinoflagellate species that do not produce brevetoxins, as determined by gene specific PCR. Four PKS transcripts were determined to be *K. brevis*-specific, KB1008, KB2006, KB5299, and KB5361, while the remaining four transcripts were identified in other dinoflagellate species. From this data it was hypothesized that the four PKSs found in other dinoflagellates might be involved in processes conserved among dinoflagellate species, like fatty acid synthesis, while the four *K. brevis*-specific sequences could be involved in brevetoxin biosynthesis.

Since no information exists on PKS proteins of other toxic dinoflagellates, in the current work antibodies developed against *K. brevis* PKS proteins were used to probe for the expression and intracellular localization of PKS domains in the two PKS producing dinoflagellates (*Ostreopsis ovata, Coolia monotis*), and a raphidophyte (*Fibrocapsa japonica*) which causes blooms in the Adriatic Sea and is known to produce high concentrations of free fatty acids (FFA) (Pezzolesi et al., 2010). For the PKS localization within the cells, chloroplasts were isolated from the algal cells, based on the results previously ottained in *K. brevis*. This work was completed at NOAA (Charleston, SC, USA) in the laboratory of the Dr. Frances Van Dolah, with the aim to investigate the role of the identified PKSs in toxin biosynthesis.

5.1. Experimental section

5.1.1. Algal cultures

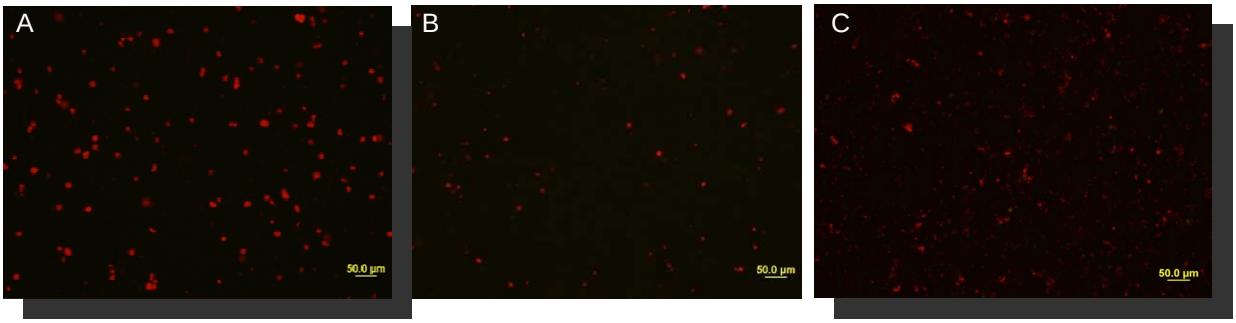
O. cf. *ovata* (strains OOAB0801 and OOTL0707) were isolated by the capillary pipette method (Hoshaw and Rosowski, 1973) from water samples collected along Adriatic (Bari, Puglia) and Tyrrhenian (Latina, Lazio) coasts of Italy in 2008 and 2007, respectively. *C. monotis* (strain CMAB0801) was isolated along the Adriatic (Bari, Puglia) in 2008, while *F. japonica* (strain FJAP0603) was isolated in 2006 in Adriatic Sea near Palombina (Marche, Italy). After an initial growth in microplates, cells were cultured at 20°C under a 16:8 h L:D cycle from cool white lamp in natural seawater at salinity 36, adding macronutrients at a five-fold diluted f/2-Si for *O.* cf. *ovata* and *C. monotis* and at a f/2-Si medium (Guillard, 1975) for *F. japonica*. Cultures were made with natural seawater, adjusted to a salinity of 36 through the addition of distilled water, and kept under the temperature and the light conditions described above.

The experiment was carried out by growing 9000 mL of culture for each strain, from which triplicate 2000 mL aliquots were used for 'chloroplast' isolation and triplicate 1000 mL aliquots were used for 'whole cell' extracts. Cultures were established at salinity of 36 and temperature of 25°C in a thermostatic room, maintaining light irradiance at 100-110 μmol m^{-2} s^{-1}. At the end of the exponential phase (day 8) cultures were centrifuged at 2400 x *g* (3600 rpm) for 20 min and supernatant was discarded. Whole cell samples were put at -80°C, while chloroplast samples were used immediately for the subcellular fractionation.

5.1.2. Subcellular fractionation

Chloroplasts were isolated by a modification of the method of Laatsch et al (2004). *O.* cf. *ovata*, *C. monotis* and *F. japonica* cells were harvested by centrifugation at 2400 x *g* (3600 rpm) for 20 min. Cell pellets were resuspended in chloroplast isolation buffer (CIB) consisting of 50 mM Tris, pH 7.5, 1 mM $MgCl_2$, 10 mM EDTA, 0.25% PVP, and 0.4 M mannitol and homogenized in a Dounce homogenizer on ice until chloroplasts free of nuclei (Fig. 5.3) were apparent by epifluorescent microscopic inspection of a Dapi stained sample. The homogenate was then layered onto a gradient of 4 mL 2.2 M sucrose and 12 mL 1.6 M sucrose, then centrifuged at 14,000 x *g* (28,000 rpm) for 30 min at 4°C in an ultracentrifuge equipped with a swinging bucket rotor. The plastid band was removed, washed with 24 mL isolation buffer, and then harvested by centrifugation at 12,000 x *g* for 10 min at 4°C. The chloroplast pellet was then stored at -80°C until use.

Fig. 5.3 – Red fluorescence of chloroplasts in *Fibrocapsa japonica* (A), *Ostreopsis ovata* (B), and *Coolia monotis* (C) cells.



5.1.3. Antibody Development

Custom peptide polyclonal antibodies were made against *in silico* translation of *K. brevis* KS domain (KB2006; GenBank Accession No. EF410007), ACP/KS domain (KB1008; GenBank Accession No. EF410006) and KR domain (KB5299; Genbank Accession No. EF410009) full length transcripts by ProSci Incorporated (Poway, CA), as described in Monroe and Van Dolah (2008).

5.1.4. Protein isolation and western blotting

Total protein was isolated from *O.* cf. *ovata*, *C. monotis* and *F. jpaonica* cultures using TriReagent (Molecular Research Center, Cincinnati, PH) according to manufacturer's instructions with some modifications. In brief, whole cell or chloroplast pellets were resuspended in 1mL or 0.5 mL of TriReagent, respectively, and sonicated on ice with short pulses of 15-20 sec for a total of 2 min. Phases were separated using 0.2 mL (or 0.1 mL) chloroform. RNA was removed from the aqueous phase and precipitated with isopropanol for further purification. DNA was precipitated from the organic phase using 0.3 mL (or 0.15 mL) ethanol and then proteins were precipitated from the supernatant using 3 mL (or 1.5 mL) isopropanol, washed in 95% ethanol, and the protein pellets resuspended in protein sample buffer containing 30 mM Tris, 2 M thiourea, 7 M urea, and 4% (w/v) CHAPS.

Protein concentration was determined using a BioRad (Hercules, CA) Bradford protein assay, and 10 µg of protein were run on a NuPAGE Novex 4-12% Bis-Tris gel from Invitrogen. Proteins were transferred to polyvinyl difluoride (PVDF) membranes (0.45 µm), and blots were stained with Ponceau stain (Sigma, St. Louis, MO) to examine total protein transferred to the blot. Membranes were blocked with 5% nonfat milk in Tris-buffered Saline (TBS) for 1

hour, and blots were then incubated with appropriate primary antibody dilutions (KB2006: 1:5000, KB5299: 1:500, KB1008: 1:1000) in 5% nonfat milk in TBS overnight at 4°C. Antibody specificity was determined by incubating the antibody with 100-fold excess (by weight) of the peptide used to generate the antibody for 1 hr prior to membrane immunoblotting at the same dilutions indicated above. After 3 washes for 10 min each were done in TBS with 0.1% Tween 20 (TBST), membranes were incubated with an ECL HRP-linked anti-rabbit (1:2000) (GE Healthcare, Buckinghamshire, UK), washed twice with TBST (10 min each) and once with TBS (5 min) and finally developed using Pierce West Pico chemiluminescent substrate (Rockford, IL). Images were taken using a SynGene G:box imaging system and SynGene GeneSnap software, and densitometry was calculated using the SynGene GeneTools software (Frederick, MD).

5.1.5. Evaluation of toxin and chlorophyll content

For toxin and chlorophyll-a quantification in *O.* cf. *ovata* 'whole cell' and 'chloroplast' samples, 6000 mL culture was grown. At the end of the exponential phase, 500 mL and 50 mL of culture, each in duplicate, were centrifuged at 4000 rpm for 15 min to use for 'whole cell' toxin and chlorophyll extraction, respectively. Algal pellets were stored at -80°C until use. 'Chloroplast' samples were obtained by centrifuging 2450 mL culture in duplicate at 4000 rpm for 15 min, and algal pellet obtained were used for the subcellular fractionation, as described above (see section 5.1.2).
Cell counts were made following Utermöhl method (Hasle, 1978).

Chemicals

All organic solvents were of distilled-in-glass grade (Carlo Erba, Milan, Italy). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA, USA). Acetic acid (Laboratory grade) was purchased from Carlo Erba. Analytical standard of palytoxin was purchased from Wako Chemicals GmbH (Neuss, Germany).

Extraction

Whole cell and chloroplast pellets were separately extracted, adding 9 or 1 mL of a methanol/water (1:1, v/v) solution, respectively, and sonicating for 30 min in pulse mode, while cooling in an ice bath. The mixture was centrifuged at 3000 x *g* for 30 min, the supernatant was decanted and the pellet was washed twice with 9 or 1 mL of methanol/water

(1:1, v/v). The extracts were combined and the volume was adjusted to 30 or 3 mL with the methanol/water (1:1, v/v) solution for whole cell and chloroplast samples, respectively. The obtained mixture was analyzed directly by HR LC-MS (5 μL injected). Recovery percentage of the above extraction procedures was estimated to be 98% (Ciminiello et al., 2006).

High Resolution Liquid chromatography-mass spectrometry (HR LC-MS)

High resolution (HR)LC-MS experiments were carried out on an Agilent 1100 LC binary system (Palo Alto, CA, USA) coupled to a hybrid linear ion trap LTQ Orbitrap XL^{TM} Fourier Transform MS (FTMS) equipped with an ESI ION MAX^{TM} source (Thermo-Fisher, San Josè, CA, USA). Chromatographic separation was accomplished by using a 3 μm gemini C18 (150 × 2.00 mm) column (Phenomenex, Torrance, CA, USA) maintained at room temperature and eluted at 0.2 mL/min with water (eluent A) and 95% acetonitrile/water (eluent B), both containing 30 mM acetic acid. A slow gradient elution was used: 20-50% B over 20 min, 50-80% B over 10 min, 80-100% B in 1 min, and hold 5 min. This gradient system allowed a sufficient chromatographic separation of most palytoxin-like compounds.

HR full MS experiments (positive ions) were acquired in the range *m/z* 800-1400 at a resolving power of 15,000. The following source settings were used in all HR LC-MS experiments: a spray voltage of 4 kV, a capillary temperature of 290°C, a capillary voltage of 22 V, a sheath gas and an auxiliary gas flow of 35 and 1 (arbitrary units). The tube lens voltage was set at 110 V.

Due to commercial availability of the only palytoxin standard, quantitative determination of putative palytoxin, ovatoxin-a,-b,-c,-d, and -e in the extracts was carried out by using a calibration curve (triplicate injection) of palytoxin standards at four levels of concentration (25, 12.5, 6.25, and 3.13 ng mL^{-1}) and assuming that their molar responses were similar to that of palytoxin. Extracted ion chromatograms (XIC) for palytoxin and each ovatoxins were obtained by selecting the most abundant ion peaks of both $[M+2H-H_2O]^{2+}$ and $[M+2H+K]^{3+}$ ion clusters. A mass tolerance of 5 ppm was used.

5.1.6. Chlorophyll analysis

Chlorophyll a measurements have historically provided a useful estimate of algal biomass. Chlorophyll analyses were performed by collecting cells (50 mL of algal culture and 1/10 of chloroplast sample obtained from the subcellular fractionation) with centrifugation. Supernatant was removed and pellets put at -80°C. For the extraction, 90% acetone was used

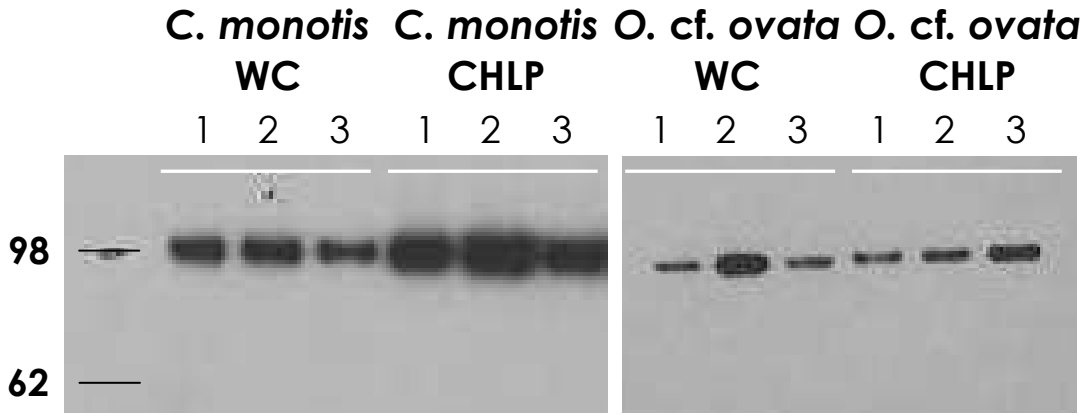
and samples were analyzed spectrophotometrically according to Strickland and Parsons (1972).

5.2. Results

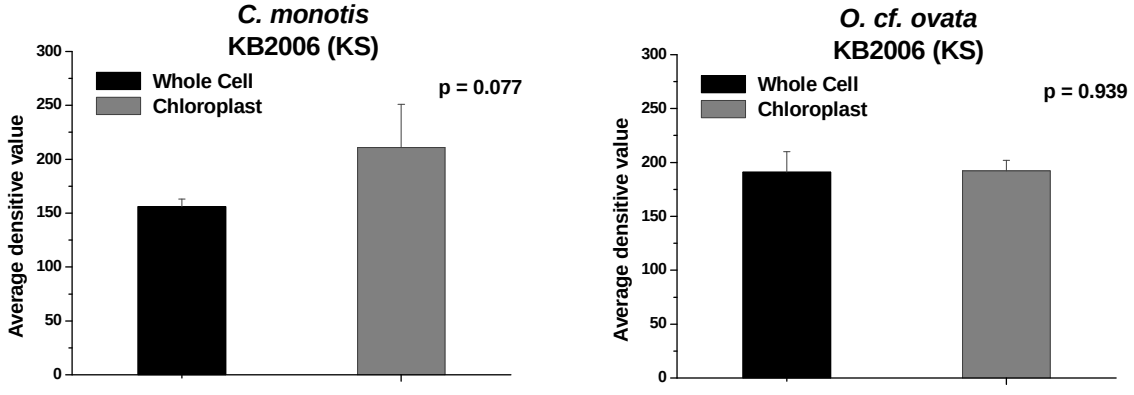
5.2.1. PKS espression in whole cell and chloroplast samples

Fig. 5.4 - Western blot analysis of Ketosynthase domain (KS) KB2006 expression in whole cell (WC) and chloroplast (CHLP). Ten micrograms of protein from whole cells and isolated chloroplasts in triplicates were separated on a 4%–12% gel (A). Average densitive values are shown (B). Results refer to the strain OOAB0801 of *O.* cf. *ovata.*

A)



B)

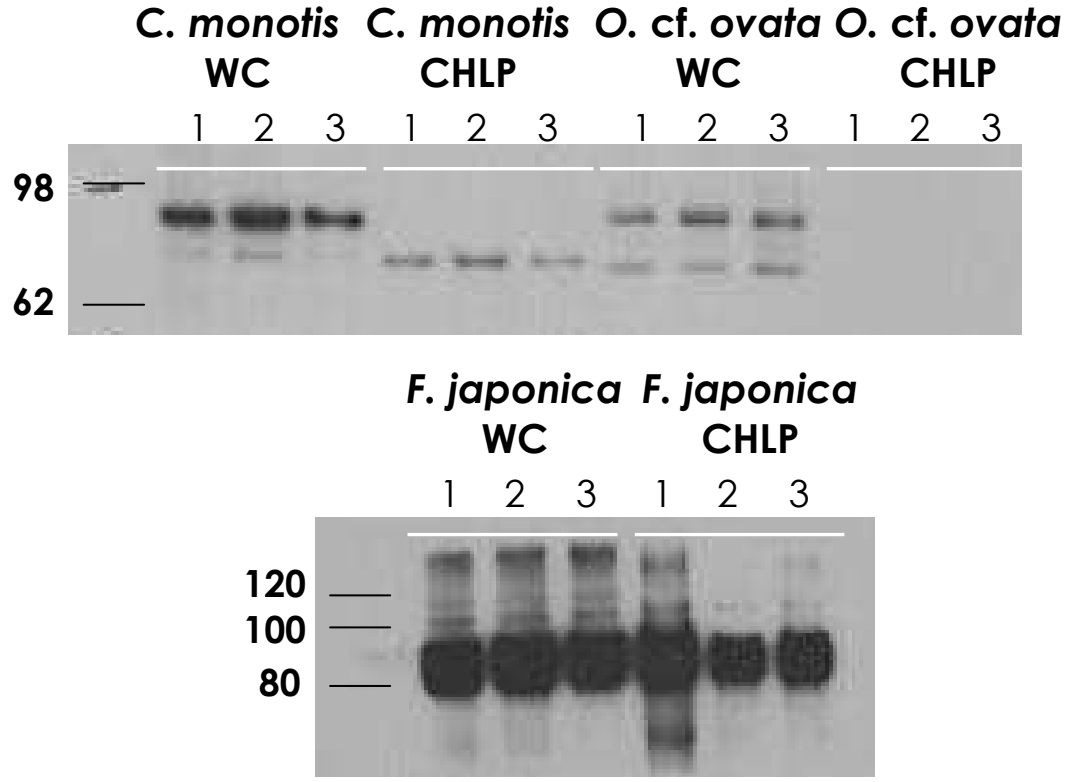


Western blot analysis with anti-KB2006 (KS) identified a band at about 100 kDa in both strains of the dinoflagellates *O.* cf. *ovata* and in *C. monotis*, in accordance with the band found in *K. brevis* and in good agreement with the predicted 101 kDa *in silico* translation data

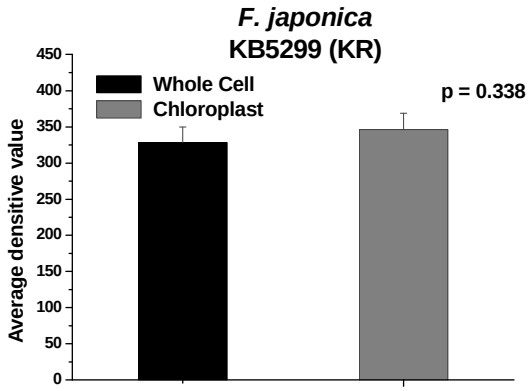
(Fig. 5.4A). No bands were identified in *F. japonica* samples. KS protein was expressed equivalently in whole cell and chloroplast extracts in the two benthic dinoflagellates (Fig. 5.4B) as confirmed by the densitometry.

Fig. 5.5 - Western blot analysis of Ketoreductase domain (KR) KB5299 expression in whole cell (WC) and chloroplast (CHLP). Ten micrograms of protein from whole cells and isolated chloroplasts in triplicates were separated on a 4%–12% gel (A). Average densitive values are shown (B). Results refer to the strain OOAB0801 of *O.* cf. *ovata.*

A)



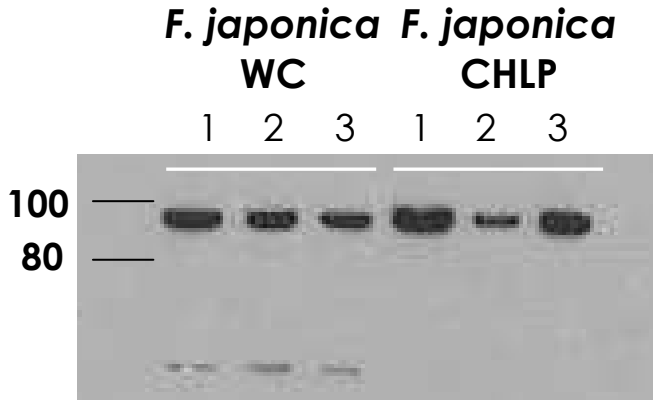
B)



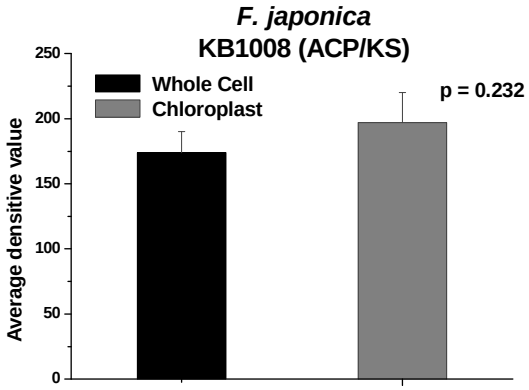
The second antibody anti-KB5299 (KR) identified a protein about 90 kDa, larger than the predicted 56 kDa and even different from the about 40 kDa protein found in *K. brevis*. Proteins cross-reactive with the anti-KR antibody, but larger than in *K. brevis*, were expressed in whole cell but not in the chloroplast extracts in the dinoflagellates.

Fig. 5.6 - Western blot analysis of acyl carrier protein (ACP)/KS domain protein KB1008 expression in whole cell (WC) and chloroplast (CHLP). Ten micrograms of protein from whole cells and isolated chloroplasts in triplicates were separated on a 4%–12% gel.

A)



B)

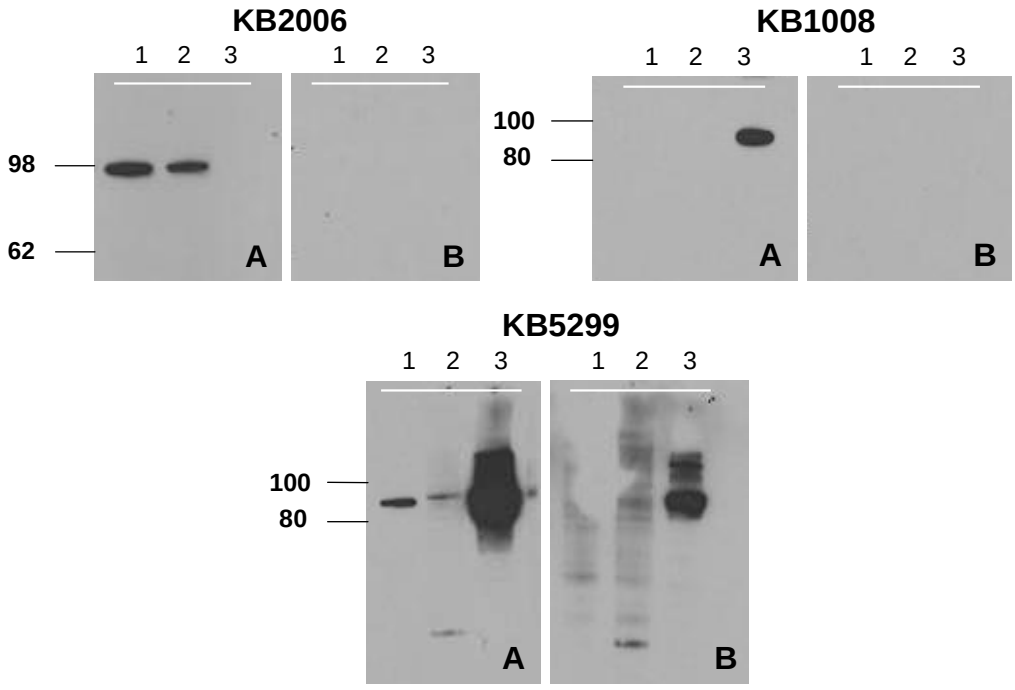


However, a band of approximately 75 kDa was present in chloroplast of *C. monotis*. It has to be noted that no bands were found in the strain OOTL0707 of *O.* cf. *ovata*, resulting in a difference between the expression of PKS proteins in the two isolates of this dinoflagellate, which were used in the study. A protein of similar size was expressed equivalently in whole cell and chloroplast extracts in the raphidophyte (Fig. 5.5A), reporting strong bands.

Densitometry showed no significant difference between these bands (Fig. 5.5B).

The third antibody anti-KB1008 (ACP/KS) identified a protein about 95 kDa, which was expressed equivalently in whole cell and chloroplast extracts in the raphidophyte (Fig. 5.6), but not in the other two dinoflagellates.

Fig. 5.7 – Polyketide synthase (PKS) proteins in the dinoflagellates *O.* cf. *ovata*, strain OOAB0801 (1) and *C. monotis* (2) and in the Raphidophyte *F. japonica* (3). Western blot analysis of PKS proteins using peptide polyclonal anti-KS against *K. brevis* KS domain protein KB2006 (A) and peptide-blocked anti-KS demonstrating specificity for the 100 kDa band (B); anti-KR against *K. brevis* KR domain protein KB2599 (A) and peptide-blocked anti-KR demonstrating specificity for the about 90 kDa band (B); and anti–ACP/KS against *K. brevis* ACP/KS domain protein KB1008 (A) and peptide-blocked anti-ACP/KS demonstrating specificity for the about 95 kDa band (B).



To summarize, the expression of the PKS proteins was compared in protein extracts from the isolated chloroplasts and whole cells by Western blotting with the KS domain (KB2006), KR domain (KB5299), and ACP/KS PKS antibodies. PKS showed prominent bands in the chloroplast (Fig. 5.4, 5.5, 5.6) almost in all cases, besides for the KR5299 which was absent in the chloroplast of the dinoflagellates.

In fact, negative controls with peptide-blocked antibodies demonstrate the specificity of the bands, as reported in Fig. 5.7 for the three PKS proteins in all the algal species. The 75 kDa bands found in *C. monotis* chloroplasts resulted no specific.

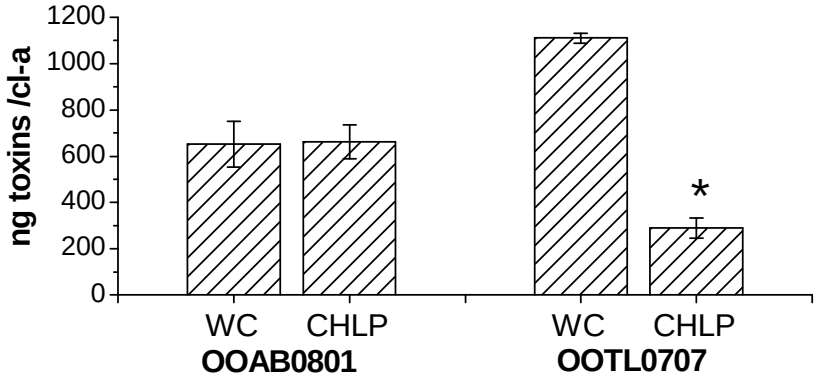
5.2.2. Determination of toxin content and localization by HR LC-MS

Culture and chloroplast pellets of *O.* cf. *ovata* were collected during the late exponential growth phase. Samples were separately extracted as reported in the experimental and the crude extracts were used to evaluate the toxin profile. HR LC-MS experiments were acquired in full MS mode by using an LC method which allowed chromatographic separation of the major components of the toxin profile. The presence of putative palytoxin and of all the ovatoxins (ovatoxin-a, -b, -c, -d, and -e) recently identified in *O.* cf. *ovata* (Ciminiello et al., 2008, 2010) was highlighted in all the analyzed samples.

With the purpose of gaining information on the toxin localization in the cells, total toxin content was expressed on the basis on the chlorophyll a concentration. Spectrometric determination of chlorophyll-a content is in fact a method for the algal biomass evaluation, therefore after evaluation of pg cl-a $cell^{-1}$ in the culture utilized for chloroplast isolation, it provides an estimation of the number of cells corresponding to the chloroplast sample. Due to lack of standards for ovatoxins, quantitative analyses were carried out basing on the tentative assumption that they present the same molar response as palytoxin. Concentrations of putative palytoxin and ovatoxins were extrapolated from a calibration curve of palytoxin standard injected under the same conditions and the obtained results were corrected basing on recovery percentages of the extraction procedures, namely 98% (Ciminiello et al., 2006) for pellet extracts.

Total toxin content expressed as ng per chlorophyll a (ng cl-a^{-1}, Fig. 5.8) reported no significant differences between the culture and CHLP extracts (ANOVA, $P>0.05$) of OOAB0801 *O.* cf. *ovata*, whereas it resulted significantly different (ANOVA, $P<0.001$) in the OOTL0707 *O.* cf. *ovata* strain. The toxin content in the CHLP of the Tyrrhenian strain resulted about 25% of the total content in the cell, thus suggesting a partial localization of the palytoxin-like compounds in this plastid.

Fig. 5.8 – Total toxin content (pPLTX and OVTX-a,b,c,d,e) in the two *O.* cf. *ovata* strains, expressed on the basis of chlorophyll a concentration. Samples are algal (WC) and chloroplast (CHLP) pellets, obtained as reported in the experimental (n=3).



* significantly different (ANOVA, P<0.001)

5.3. Conclusion - PKS

This study identifies PKS proteins in the benthic dinoflagellate *O.* cf. *ovata* and *C. monotis* and in the raphidophyte *F. japonica* for the first time. Genes with sequence similarity to Type I PKS have previously been identified in *K. brevis* (Snyder et al., 2003, 2005; Monroe and Van Dolah, 2008; Monroe et al., 2010), where full-length PKS transcripts predicted proteins smaller than typical Type I PKSs that encode only single catalytic domains (Monroe and Van Dolah, 2008).

The current study confirms the presence of PKS proteins predicted by the *in silico* translation of the transcripts found in *K. brevis* also in other species. Western blotting identified a 100 kDa band cross-reactive to a peptide antibody to the KS domain (KB2006), in close agreement to the result obtained in *K. brevis*. A protein band detected by the antibody specific to the KR domain (KB5299) is larger (about 95 kDa) than predicted by *in silico* translation (56 kDa) and *K. brevis* protein (about 40 kDa). By comparison with the *K. brevis* proteins, Type I PKS proteins typically encode polypeptides containing multiple catalytic domains and, in the case of modular PKSs, multiple modules. It appears that even the PKS proteins found in these species are more similar in size to Type II PKSs, as previously found for *K. brevis* (Monroe et al., 2010) where PKS are multi-protein complexes made up of monofunctional proteins each containing a single catalytic domain.

Although all species, including the raphidophyte expressed proteins cross-reactive with one or more *K. brevis* antibodies, different protein sizes were reported likely due to differences in

species. *C. monotis* and *F. japonica* from the Adriatic Sea have never reported the production of toxins; however, the raphidophyte is known to produce high concentrations of free fatty acids (FFA). Thus, these results lead hypothesize that either (1) these proteins are FAS or (2) single PKS units could be cobbled together to form complexes that synthesize different polyketide compounds and/or fatty acids in different species (Sieber et al., 2002).

Immunolocalization studies and subcellular fractionation of chloroplasts suggested chloroplast localization of both KS and KR proteins in *K. brevis* (Monroe et al., 2010). KR appeared to be chloroplast specific since the density of the Western blotting bands was similar to that of whole-cell protein lysates whereas, the KS domain protein appears to occur in other compartment(s) more prominently than the chloroplast, based on the relative intensity of the chloroplast band and that in the whole cell. The chloroplast localization of *K. brevis* PKSs was consistent with the localization of another dinoflagellate polyketide toxin, okadaic acid (OA), in *Prorocentrum lima* (Zhou and Fritz, 1994; Barbier et al., 1999). Cyanobacterial toxins microcystin and nodularin are similarly strongly associated with the photosynthetic apparatus, with 70% of labeled microcystin observed in the thylakoid, especially the outer portion of the thylakoid (Shi et al., 1995; Young et al., 2005).

The identification of *O.* cf. *ovata* PKSs and the localization of the palytoxin-like compounds produced by this dinoflagellate in a similar location (chloroplast) as that observed for other dinoflagellate and cyanobacterial toxins provides some indication that these proteins may be involved in polyketide biosynthesis. However, their potential function as fatty acid synthases cannot be ruled out, as plant fatty acid biosynthesis also occurs within chloroplasts, albeit in the stroma. This last hypothesis is also supported by the fact that even in the other two species, and in particular in *F. japonica*, PKS proteins were present. PKSs are structurally and functionally similar to FASs and likely originated when an early ancestor of extant prokaryotes and eukaryotes evolved a simple condensing enzyme to make other cellular functions more efficient (Hopwood, 1997). From this primitive enzyme, a PKS is thought to have evolved through the addition of an ACP and AT, and further additions of a reductive cycle converted the primitive PKS to an FAS. PKSs subsequently evolved distinct and diverse pathways through mutations, recombinations, and gene duplications, while the simple FAS were retained because fatty acids had become essential components of the cell.

It is important to point out that the real biological origin of palytoxin-like compounds remains controversial. Palytoxin content in *Palythoa* spp. in fact significantly varies among species, population of the same species and even seasonally (Moore et al., 1982) and, besides algae, sporadic occurrence of palytoxins had been detected also in crabs (Yasumoto et al., 1986),

and fish (Fukui et al., 1987). Therefore, many have defended the assumption that this class of toxins are indeed produced by microorganisms. The occurrence of palytoxins in such a large number of even biogenetically distant marine organisms could reasonably imply a symbiotic relationship of the above organisms with bacteria (Katikou, 2008). This is also consistent with experimental observation that showed haemolytic activity of some *Pseudomonas* (Carballeira et al., 1998), *Brevibacterium, Acinetobacter* and *Bacillus cereus* extracts and others proving that *Vibrio* spp. and *Aeromonas* spp. were producers of molecules antigenically related to palytoxin (Frolova et al., 2000a,b). The co-isolation of symbiontic bacteria in the chloroplast samples can not be excluded, because the small size of the chloroplasts in *C. monotis* and *O.* cf. *ovata* (about 1-3 μm) could potentially allow for comigration of larger bacteria to a similar density in the sucrose gradient. Therefore even the origin of the palytoxin-like compounds detected in these extracts remains uncertain.

Monroe et al. (2010) compared PKS transcript expression and protein abundance in the ‘‘non-toxic’’ strain and its parental, toxic *K. brevis* (Wilson) isolate. By both microarray and qPCR analysis, PKS transcript levels were not significantly different in the ‘‘nontoxic’’ cultures compared to toxin-producing cultures. This was not surprising since many other functions in *K. brevis* are known to be regulated post-transcriptionally (Van Dolah, 2009; Brunelle et al., in prep.). Therefore, more insight might be provided by assessing their expression at the protein level. In fact, the KS domain was expressed at lower levels in the non-toxic isolate, suggesting it may be involved in brevetoxin biosynthesis. However, KB5299 (the KR domain) was more abundant in ‘‘non-toxic’’ cultures.

An interesting finding from the current study is that KR domain was present only in one of the two *O.* cf. *ovata* strains used, confirming the previous results found in *K. brevis* which indicated as KR protein may be not involved in the same cellular process as the KS proteins recognized by the antibodies used in this study (Monroe et al., 2010). Additionally, since this strain (OOTL0707) is the one which showed only about 25% of the toxins being localized in the chloroplast it could be interesting to investigate more deeply the role of KR protein in polyketide biosynthesis.

The previous studies on *K. brevis* suggested the roles for polyketides in photosynthetic processes in algae (Monroe et al., 2008; 2010), as reported also for okadaic acid (OA) (Zhou and Fritz, 1994). Moreover, both the association of microcystins with the thylakoid and the increase in microcystin transcripts in response to light (Kaebernick et al., 2000) led to the hypothesis that microcystins may also have a functional role in photosynthesis and other light-related processes. The localization of PKSs and *O.* cf. *ovata* toxins in the chloroplast

may also suggest a functional role of palytoxin-like compounds in chloroplast processes or suggest that these toxins may be confined in these plastids to avoid damages to the cytoskeleton.

This study provides evidence of the presence of PKS proteins in other dinoflagellates and a raphidophyte, and their localization in the chloroplast. Further studies are needed to clarify the role that these enzymes may have in the toxin biosythesis.

6. General conclusions

The main goal of the present thesis was to study some harmful algal species which cause blooms in Italian coastal waters, leading to consequences for human health, coastal ecosystem, fishery and tourism.

In particular, in the first part of this thesis the toxicity of Adriatic strains of the raphidophyte *Fibrocapsa japonica* was investigated. Despite several hypotheses have been proposed for the toxic mechanism of the raphidophytes, especially for the species *Chattonella antiqua* and *C. marina*, which have been studied more extensively, just a few studies on the toxic effects of these species for different organisms were reported. Moreover, a careful reading of the literature evidenced as any ichthyotoxic events reported worldwide can be linked to *F. japonica* blooms. Although recently several studies were performed on *F. japonica* strains from the USA, Japan, Australia, New Zealand, the Netherlands, Germany, and France in order to characterize their growth and toxicity features, the work reported in this thesis results one of the first investigation on the toxic effects of *F. japonica* for different organisms, such as bacteria, crustaceans and fish. Mortality effects, together with haemolysis of fish erythrocytes, probably due to the relatively high amount of PUFAs produced by this species, were observed. Mortality for fish, however, was reported only at a high cell density and after a long exposition period (9-10 days); moreover a significant increase of H_2O_2 obtained in the tanks where sea basses were exposed to *F. japonica* was also relevant. This result may justify the absence of ichthyotoxic events in the Italian coasts, despite *F. japonica* blooms detected in these areas were characterized by high cell densities. This work reports also a first complete characterization of the fatty acids produced and extracellularly released by the Adriatic *F. japonica*, and results were also compared with the fatty acid profile of other strains. The absence of known brevetoxins in *F. japonica* algal extracts was also highlighted, leading to the hypothesis that the toxicity of *F. japonica* may be due to a synergic effect of PUFAs and ROS.

Another microalgae that was studied in this thesis is the benthic dinoflagellate *Ostreopsis* cf. *ovata*. This species was investigated with the aim to investigate the effect of environmental parameters on its growth and toxicity. *O.* cf. *ovata*, in fact, shows different blooming periods along the Italian coasts and even the reported toxic effects are variable. The results of this work confirmed the high variability in the growth dynamic and toxin content of several Italian strains which were isolated in recent years along the Adriatic and Tyrrhenian Seas. Moreover,

the effects of temperature and salinity on the behaviour of the different isolates are in good agreement with the results obtained from field surveys, which evidence as the environmental parameters are important factors modulating *O.* cf. *ovata* proliferation. Another relevant result that was highlighted is the anomaly in the production of palytoxin-like compounds reported by one of the studied isolate, in particular the one isolated in 2008 in Ancona (Adriatic Sea). Only this strain reported the absence of two (ovatoxin-b and –c) of the five ovatoxins so far known in the toxin profile and a different relative abundance of the other toxins.

The last aspect that was studied in this thesis regards the toxin biosythesis. In fact, toxins produced (palytoxin-like compounds) or supposed to be produced (brevetoxin-like compounds) by *O.* cf. *ovata* and *F. japonica*, respectively, are polyketides, which are highly oxygenated compounds synthesized by complex enzymes known as polyketide synthase (PKS) enzymes. These enzymes are multi-domain complexes that structurally and functionally resemble the fatty acid synthases (FASs). This work reports the first study of PKS proteins in the dinoflagellates *O.* cf. *ovata*, *C. monotis* and in the raphidophyte *F. japonica*. For the first time some PKSs were identified in these species, confirming the presence of PKS proteins predicted by the *in silico* translation of the transcripts found in *K. brevis* also in other species. The identification of *O.* cf. *ovata* PKSs and the localization of the palytoxin-like compounds produced by this dinoflagellate in a similar location (chloroplast) as that observed for other dinoflagellate and cyanobacterial toxins provides some indication that these proteins may be involved in polyketide biosynthesis. However, their potential function as fatty acid synthases cannot be ruled out, as plant fatty acid synthesis also occurs within chloroplasts. This last hypothesis is also supported by the fact that in all the investigated species, and in particular in *F. japonica*, PKS proteins were present. Therefore, these results provide an important contribution to the study of the polyketides and of the involvement of PKS proteins in the toxin biosynthesis.

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Abbreviations

AA 5,8,11,14-eicosatetraenoic acid

ACN Acetonitrile

ACP Acyl carrier protein

ASP Amnesic shellfish poisoning

AT Acyl transferase

AZP Azaspiracid shellfish poisoning

BHT 2,6-Di-*tert*-butyl-4-methylphenol

BSA Bovine serum albumin

BSTFA-TMCS *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane

BTXs Brevetoxins

CE Collision energy

CHLP Chloroplast

CIB Chloroplast isolation buffer

DA Domoic acid

DH Dehydratase

DON Dissolved organic nitrogen

DP Declustering potential

DSP Diarrhoetic shellfish poisoning

DTXs Dinophysistoxins

EC_{50} 50% effect concentration

ELA Erythrocyte lysis assay

EPA 5,8,11,14,17-eicosapentaenoic acid

ER Enoyl reductase

ESI Electrospray ionisation

FA Formic acid

FASs Fatty acid synthases

FFA Free fatty acids

GC-MS Gas chromatography-mass spectrometry

HABs Harmful algal blooms

HR High resolution

KR Ketoreductase

KS Ketoacyl synthase

LC-MS Liquid chromatography-mass spectrometry

LOD Limit of detection

MBA Mouse bioassay

MDA Malondialdehyde

MeOH Methanol

MRM Multiple reaction monitoring

NMR Nuclear magnetic resonance

NSP Neurotoxic shellfish poisoning

OA Okadaic acid

OTA 6,9,12,15-octadecatetraenoic acid

OUA Ouabain

OVTX Ovatoxin

PBS Phosphate buffered saline

PKSs Polyketide synthases

PP2A Protein phosphatise 2A

pPLTX Putative palytoxin

PSP Paralytic shellfish poisoning

PTXs Pectenotoxins

PUFAs Polyunsaturated fatty acids

PVDF Polyvinyl difluoride

RF Response factors

ROS Reactive oxygen species

SNK Student-Newman-Keuls test

SPE Solid phase extraction

SRM Selected reaction monitoring

STXs Saxitoxins

TBA 2-thiobarbituric acid

TBS Tris-buffered saline

TCA Trichloroacetic acid

TE Thioesterase

TFA Trifluoroacetic acid

THF Tetrahydrofuran

TTEP Tetraethoxypropane

WC Whole cell

XIC Extracted ion chromatograms

YTXs Yessotoxins

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