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Phytoplankton Response to Environmental Variables and Organic Pollutants. Laboratory Cultures and Numerical Simulations Experiments

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To Andrea

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Introduction

1. Phytoplankton

The prefix “phyto” comes from the Greek word for plant, phytos. “Plankton” derives from another Greek word meaning wanderer. Hence phytoplankton refers to organisms that wander in the surface waters of lakes, rivers and oceans. Phytoplankton, planktonic unicellular algae, play an important role in aquatic ecosystem as the most important primary producers. Through the process of photosynthesis, inorganic nutrients are converted into new organic compounds, and energy is transferred to higher trophic levels of the food web. Despite its microscopic size, phytoplankton are responsible for about half of the global primary production, driving essential biogeochemical cycles, exporting massive amounts of carbon to deep waters and sediments in the open ocean and strongly influencing the water – atmosphere gas exchanges (Rost et al., 2008).

Phytoplankton have a vast range of sizes and forms, both as single cells and as colonies, and are found in the water column where irradiance levels support photosynthesis at a rate sufficient for net production. Seasonal algal blooms (AB) are recurrent in the annual cycles of plankton. At medium latitudes AB are recurrent during spring and autumn as results of physical processes, such as the warming and shallowing of the surface mixed layer by thermal stratification during spring, and its breakdown by convective mixing during the autumn. Blooms are also a regular feature of the upwelling regions of low latitudes (Assmy and Smetacek, 2009). Unusual algal blooms have also been reported usually connected to special environmental or anthropogenic conditions/events.

Microalgae have a density greater than water and thus have the tendency to sink. This vertical movement in the water column is not necessarily disadvantageous, it allows cells to encounter

deeper nutrient-rich water and may also be a mechanism for avoiding predators and high, potentially damaging, levels of irradiance at the water surface. Some algal species possess flagella and are motile. Many others, as diatoms and coccoid forms of various divisions, are non-motile. To avoid losses due to sinking, these species have evolved different strategies to control their location in the water column. Most cytoplasmic components have densities higher than water (1.027 g ml^{-1} in sea water), for example the silica walls of diatoms have a density of 2.6 g ml^{-1}. Only lipids are less dense than water (0.86 g ml^{-1}) and may contribute to the reduction of cell density. Ionic regulation, in which heavier ions are replaced to lighter ones, may have some effects in reducing cell density. Cyanophyceae possess flotation devices termed gas vacuoles, which consist of a number of closely packed cylinders, or vesicles. When cells are grown under low irradiance they produce gas vacuoles, but when cells are exposed to high light intensity the vacuolation decreases (South and Whittik, 1987).

The ability of phytoplankton species to become dominant and form blooms in natural environments is largely determined by their ability to outcompete for limiting resources. Phytoplankton cells have developed various strategies to acquire the necessary elements for their growth and reproduction. Most algal groups are photoautotrophs, using inorganic nutrients and sunlight for photosynthesis to acquire energy. Some species can use organic nutrients, either by assimilating dissolved organic matter (DOM), i.e. osmotrophy, or by ingesting other cells as particulate organic matter (POM), i.e. phagotrophy. The ability of some algal species to combine both nutrient strategies, photoautotrophy and heterotrophy, is defined mixotrophy. This mechanism can allow algal growth, also in environments where resources and environmental conditions are limiting (Barsanti and Gualtieri, 2006).

The growth rate is the net rate of change in number of cells or algal biomass and represents the balance between additions due to reproduction and losses due to various source of mortality or export. Loss processes are all those factors that remove or displace phytoplankton such as: physiological mortality, infection by various pathogens, sinking of cells out of the euphotic zone (sedimentation), and grazing by zooplankton (predation). Grazing is one of the main losses process for phytoplankton. To avoid ingestion, phytoplankton exhibit morphological features such as horns, spines and other protrusions, or form colonies and chains (Tillmann, 2004). Algal motility may also be an effective defense against grazers. The production of toxins may deter or directly kill predators (Landsberg, 2002). Furthermore, some species are able to withstand digestion as they possess features such as gelatinous sheaths and resting cysts (Porter, 1973; Montresor et al., 2003; DeMott et al., 2010).

2. Factors affecting phytoplankton growth

2.1 Environmental factors

The presence of a particular algae species in a given water body depends on a number of complex, spatial and temporal interactions among environmental factors affecting growth rate, behavior characteristics of the alga and the activities of other organisms. Furthermore phytoplankton species differ in their tolerance to environmental factors.

2.1.1 Temperature

The third major environmental factor to influence phytoplankton growth is temperature, which affects chemical reactions (metabolic rates) and thus all processes of an organism. Phytoplankton organisms increase the growth rate with the increasing temperature up to some temperature level after which growth rate declines. The optimal temperature for growth of many marine algae is in the rage 18 – 25 °C, although cold-water species generally have lower optima, i.e. some Antarctic diatoms have temperature optima of 4-6 °C.

The thermal effect is usually described with the Q_{10} equation (1), which is the ratio between the metabolic rate at a given temperature t and the metabolic rate at t + 10 (Fig. 1). The Q_{10} ratio for enzymatically controlled reactions vary between 1.1 and 5.3 with the average of 2.0. Thus, within the tolerances of the organism's enzymes, the metabolic rate will double with every 10 °C rise in temperature (Dawes, 1988).

$$Q_{10} = \frac{R_{T+10}}{R_T} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}} \quad (1)$$

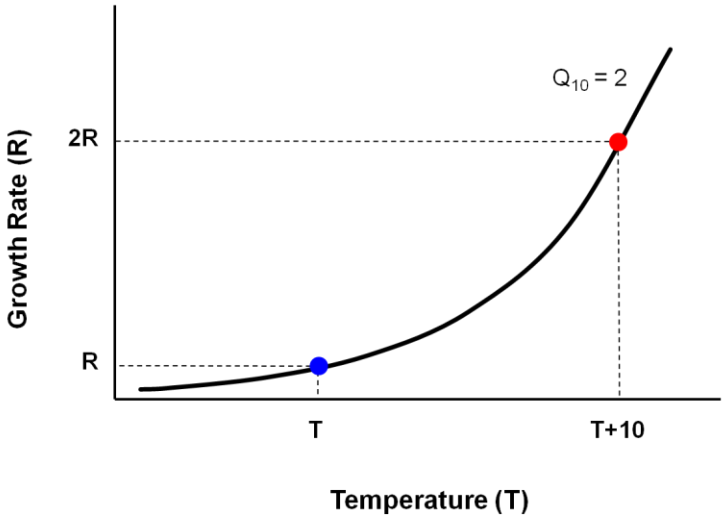

Fig. 1 Effect of temperature (T) on the metabolic rate using the Q_{10} equation.

2.1.2 Light

Light, which must be considered in term of photoperiod and quality (wavelength) as well as intensity, is one of the main factor controlling algal growth, due to its involvement in photosynthesis process.

The response of the photosynthetic rate to light intensity is plotted in P (photosynthetic rate) versus I (irradiance) curves (Fig. 2). At low light intensities the photosynthetic rate is limited by the light reactions of photosynthesis. The photosynthetic rate increases linearly with light intensity until it approaches a plateau at saturing light intensities, reaching the maximum photosynthetic rate (P_{max}). Very high light intensities are often inhibitory. The light intensity (I_k) at which the initial slope and P_{max} intersect is a convenient reference point on the curve and is usually taken as the intensity at which light saturation occurs (Graham and Wilcox, 2000).

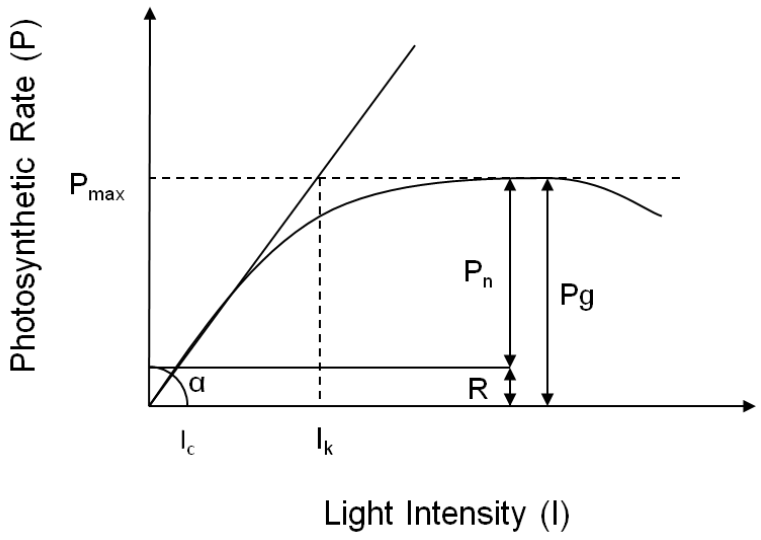

Fig. 2 Light saturation curve of photosynthesis. P_{max} = the maximum rate of photosynthesis; I_c = the compensation light intensity; I_k = the light intensity at which saturation occurs; R = respiration; P_n = net photosynthesis; and P_g = gross photosynthesis.

Light is often a limiting factor because it fluctuates in both space (depth and latitude) and time (daily and seasonally). Phytoplankton cells modify their photosynthetic response to ambient light intensity to maximize their growth potential under various light conditions. This response, called sun-shade or light-shade adaptation, may be accomplished by modifying the light reactions (changing the initial slope) and/or the dark reaction (P_{max}). Thus, a cell which is near the bottom of the euphotic zone will grow slowly due to light limiting conditions, but will make the most of what light energy is available by producing more light capturing system. Typical shade adaptation, as observed under culture conditions, involves between 2-10 times increase in chlorophyll content per cell with a resultant steeper initial slope and lower I_k. At

high light intensities plenty of light is available and cell growth is limited by the rate at which carbon is fixed, which depends on cell metabolism. As a result cells present less chlorophyll content. An example is given in Fig. 3. The diatom Skeletonema marinoi was grown at constant temperature conditions (15°C) and exposed to three light intensities (30, 75 and 125 µmol photons m^{-2} s^{-1}), 12:12 L:D cycle, to recreate the attenuation of the light irradiation through the water column (Fiori et al., in prep).

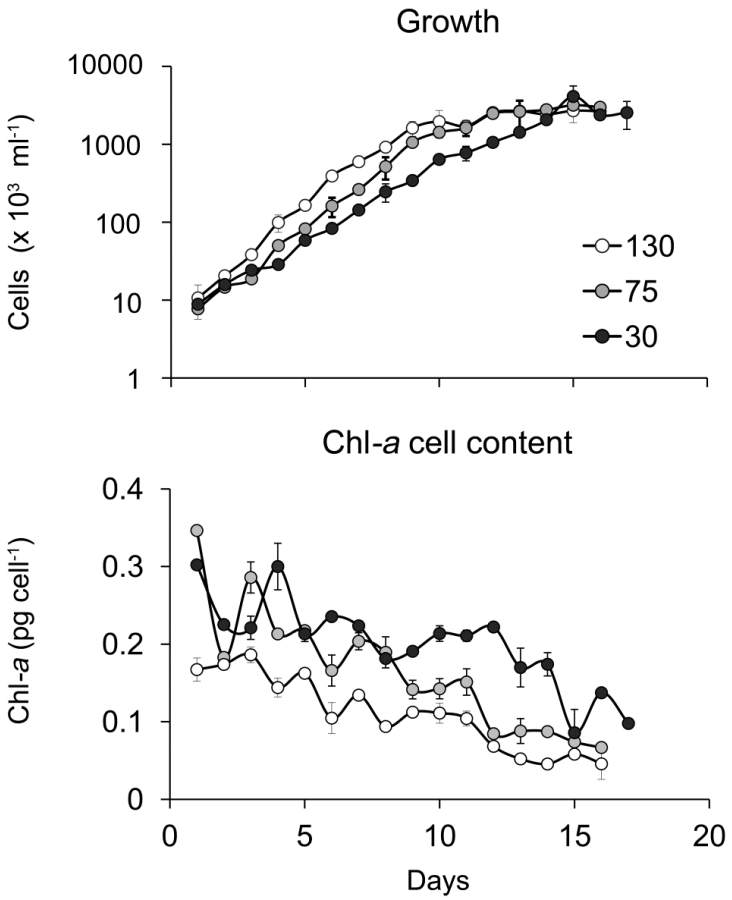

Fig. 3 Skeletonema marinoi cell densities at three light conditions (30, 75 and 130 µmol photons m^{-2} s^{-1}) and chlorophyll-a (Chl-a) cell content.

2.1.3 Nutrients

Nutrients are essential elements for phytoplankton growth. Algae require a variety of both inorganic and organic nutrients. Phosphates and nitrates (and silicates for diatoms) are taken up in the greatest abundance and are termed macronutrients. In addition to carbon, hydrogen and oxygen, algae use some additional elements to grow and reproduce. Most of these nutrients are usually present in sufficient amounts, relative to the alga's needs, so as not to be potential limiting factors for growth. The concentrations of nitrogen and phosphorous however are often

low enough to limit phytoplankton growth in surface waters. Dynamics of phytoplankton are primarily correlated with spatial and temporal fluxes of major nutrient ions such as N, P and Si. Redfield (1958) determined that marine phytoplankton growing at their maximum growth rate possessed a characteristic ratio of major nutrient ions of 106 C:16 N:1 P. Diatoms and other algae that require silica, should have a ion ration of 106 C:16 Si:16 N:1 P.

2.1.4 Salinity

Dissolved inorganic salt in water my affect phytoplankton growth as a function of their elemental composition or as a function of their osmotic activity.

Some species from coastal habitats can grow well in salinities ranging from fresh water to full strength sea water (35‰) or above, whereas other species are less tolerant, requiring water with more limited salinity ranges (e.g. 4 - 20‰).

2.1.5 Water movements

Oceans are turbulent environments. The interplay of wind action and solar heating with tides and the rotation of earth creates many different type of water motions. Turbulence affect populations of phytoplankton by concentrating or dispersing patches of phytoplankton. Species of algae differ in their tolerance of turbidity (Fogg, 1991; Willen, 1991) and these difference may affect competitive interactions and the formations of blooms.

2.2 Anthropogenic factors

The biodiversity of marine organisms is at risk due to direct anthropogenic activities as: fishing, pollution, habitat alteration, introduction of exotic species and indirect effects as global climate change (Committee of Biological Diversity, 1995). Singly or in combination, such human activities can change energy-flow patterns and alter the structure of marine communities.

Marine pollution is a consequence of human pressure on the marine environment, arising either when the concentrations of naturally occurring substances are increased, or when unnatural synthetic compounds are released into the environment. The large urbanization and several economic sectors such as tourism, agriculture, shipping and sewage play an important role in the discharge of contaminants into coastal marine environments.

The most heavily impacted marine phytoplankton community have been estuarine and nearshore coastal system, which are close to human habitation and agricultural areas. Pollution effects on algae may either inhibit or stimulate growth.

2.2.1 Eutrophication

The excessive increase in available nitrogen, phosphorus and carbon through human activities causes an enhancement of algal production, which can lead to conspicuous, and sometimes toxic, algal blooms and possibly to changes in species composition and seasonality. Eutrophication is a process linked to the massive algal growth which physically impede waterways and increase oxygen demand during the decay phase. The effects of eutrophication are many and include: decreases in dissolved oxygen that can reach anaerobic levels, shifts in species and reduction in species diversity, degradation of the habitat from decreased light transmittance due to increases in phytoplankton growth. This process can disrupt the marine habitat, damaging the planktonic and benthic phauna and lead to serious economic losses.

2.2.2 Organic pollutants

Agricultural and industrial runoff into rivers and streams contribute to the introduction of organic pollutants, such as pesticides, herbicides, PCBs and other antifouling compounds, in estuaries and coastal ecosystems. Although these substances are toxic, they usually occur in such low concentrations in seawater that they are not taken into much consideration. Even so, interactions between different pollutants can enhance their toxic effects and biomagnifications of these compounds can cause their accumulation in food chains. The effects of sub-lethal pollution are much less obvious but are probably of greater ecological significance in the long term. Furthermore, some of these compounds, i.e. DDT and PCBs, are very resistant to degradation and persist for long periods in the environment causing long-term effects.

Triazinic herbicides form a wide group of compounds used for pre- and post- emergence weed control. Photosystem II (PSII) inhibitors, triazinic herbicides act by competing with plastoquinone at the QB binding site of the D1 protein in the PSII reaction centre, thereby inhibiting energy transfer (Oettmeier, 1992).

A fraction of herbicides applied in agriculture is washed into natural water, due to precipitations, and may enter diverse estuarine and coastal communities through freshwater streams. These compounds behave differently under different natural conditions (soil, water, air) according to their physical properties, methods of application and environmental conditions. Photodegradation is one of the major transformation processes affecting the fate of herbicide. However, triazinic herbicides have been shown to be very persistent in the soil and water (Navarro et al., 2004). Accumulation and/or transformation in the aquatic ecosystems and possible biomagnifications constitute a real risk to human health, wildlife and environment (Stevens and Sumner, 1991; Navarro et al., 2000). Application of some of the herbicides has

been restricted by law in most developed countries (Ware, 1986). In Europe, in an attempt to protect the quality of potable and surface water a priority list of pesticides has been compiled (ECD, 1998). The ECD Directive on the Quality of Water Intended for Human Consumption sets a maximum admissible concentration of 0.1 µg L^{-1} per individual pesticide and 0.5 µg L^{-1} for the sum of all pesticides in water samples. However, the peak concentration of some restricted use PSII herbicides has been reported to exceed the fixed concentration limits (Solomon et al., 1996; Carafa et al., 2007) (Table 1).

ng/l	LOD	Sacca di Goro					Adriatic Sea					EQS	% Samples above EQS
		Average	St. dev	Max	Min	Median	Average	St. dev	Max	Min	Median		
Atrazine	0.8	4.57	1.55	8.18	2.35	4.18	3.75	1.68	5.93	1.27	3.90	50	0
Atrazine-desethyl-2-hydroxy (OH-DEA)	1.1	3.35	3.88	14.97	bdl	1.31	3.11	3.73	8.89	bdl	2.12		
Atrazine-desisopropyl (DIA)	2.3	bdl	2.20	11.21	bdl	bdl	bdl	1.21	3.43	bdl	bdl		
Atrazine-2-hydroxy (OH-Atrazine)	1.1	65.51	71.36	193.17	bdl	32.11	39.42	42.74	113.02	bdl	25.48		
Atrazine-desethyl (DEA)	0.2	0.60	1.19	4.10	bdl	bdl	0.27	0.67	1.63	bdl	bdl		
Simazine	0.8	6.11	5.66	24.13	1.45	3.01	6.18	9.70	25.96	1.83	2.10	10	25
Simazine-2-hydroxy (OH-Simazine)	1.1	58.44	51.32	151.15	bdl	52.16	55.67	50.63	124.48	4.98	40.11		
Cyanazine	0.2	bdl	0.11	0.47	bdl	bdl	bdl	0.12	0.28	bdl	bdl		
Chloridazon	0.2	15.27	28.14	101.48	bdl	1.50	9.55	16.18	40.59	bdl	1.04		
Metamitron	0.2	1.91	1.71	7.33	bdl	1.66	1.09	1.10	2.55	bdl	0.73		
Terbuthylazine	0.2	66.30	128.74	694.32	0.35	5.32	52.11	92.82	234.50	0.57	4.96		
Terbuthylazine-2-hydroxy (OH-TER)	0.4	12.85	23.77	106.83	bdl	5.03	3.23	3.73	8.91	bdl	2.05		
Terbuthylazine-desethyl (DE-TER)	0.2	14.23	18.00	102.40	1.75	6.38	11.35	14.99	41.44	2.43	5.47		
Terbuthylazine-desethyl-2-hydroxy (OH-DE-TER)	1.1	5.62	13.73	65.57	bdl	bdl	1.29	1.51	3.51	bdl	bdl		
Metolachlor	0.2	16.11	33.05	171.53	bdl	bdl	12.96	23.86	59.29	bdl	bdl		
2-ethyl-6-methylaniline	1.1	2.48	2.45	7.43	bdl	2.21	1.81	1.26	3.40	bdl	1.98		
Alachlor	0.2	2.58	4.95	20.86	bdl	bdl	2.38	4.88	12.20	bdl	bdl	30	0
2,6-Diethylanilline	1.1	4.97	3.95	18.70	bdl	3.58	2.30	1.90	5.66	bdl	1.87		
Molinate	0.6	20.35	32.35	121.13	0.68	5.38	30.65	70.97	175.51	0.82	1.51		
Chlorfenviphos	1.1	1.61	8.96	55.01	bdl	bdl	bdl	bdl	bdl	bdl	bdl	2	5.5
Prometryn	0.2	0.28	0.72	3.41	bdl	bdl	bdl	0.10	0.23	bdl	bdl		
Terbutryn	0.2	0.70	1.16	5.59	bdl	0.33	0.21	0.07	0.32	bdl	bdl		
Tribenuron-methyl	1.1	1.24	1.65	5.68	bdl	bdl	1.85	2.17	4.68	bdl	1.17		
Pendimethalin	0.6	bdl	bdl	bdl	bdl	bdl	bdl	0.29	0.72	bdl	bdl		
Diuron	0.2	9.96	7.64	40.78	bdl	8.41	6.65	9.19	25.32	1.65	3.04	10	44.4
Isoproturon	0.2	1.24	5.15	32.08	bdl	0.33	0.26	0.22	0.53	bdl	bdl	10	2.7
Linuron	0.2	1.72	4.94	30.79	bdl	0.75	0.42	0.43	1.08	bdl	0.32	100	0
Chlorpyrifos	1.9	bdl	3.15	19.41	bdl	bdl	bdl	bdl	bdl	bdl	bdl	1	5.5
3,5,6-Trichloro-2-pyridinol	2.3	1.57	2.06	8.34	bdl	bdl	bdl	2.25	4.90	bdl	bdl		

bdl: below detection limit; EQS proposed by the Italian law (Decreto 6 novembre 2003, n. 367).

Table 1 Limit of detection (LOD), range, mean, standard deviation and median values of herbicide concentrations determined in the upper water column of the Sacca di Goro lagoon during one year (Carafa et al., 2007)

Atrazine was the most widely used triazinic herbicide in the world because of its low cost and effectiveness as a control of annual broadleaf weeds and grass plants (Graymore et al., 2001). Even though atrazine has been banned in Italy for more than 10 years, this compound and its metabolites are still detectable in water samples (Fig. 4).

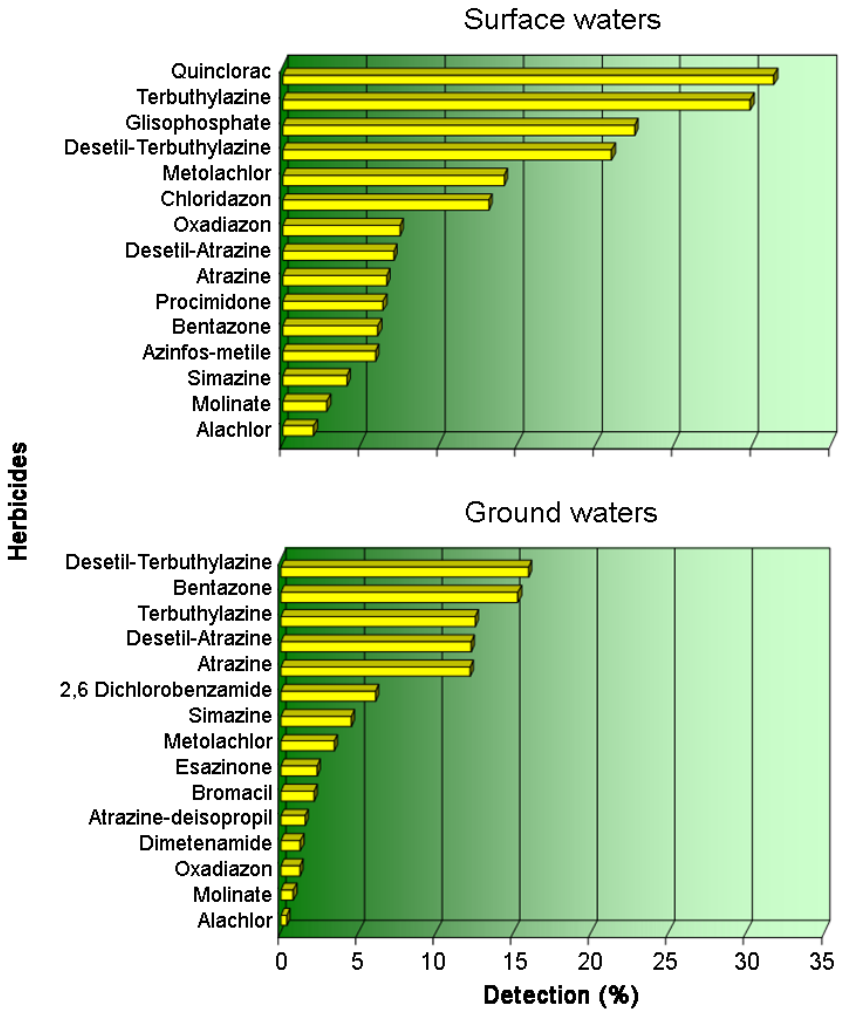

Fig. 4 Percentage of detection of different herbicides in Italian surface and ground waters (monitoring study carried out by ISPRA, Istituto Superiore di Protezione e Ricerca Ambientale, in 2010)

In Italy, atrazine has been substituted by terbuthylazine, which is now an emergent contaminant in surface and ground water, with its degradation product desetil-terbuthylazine (Sbrilli et al., 2005; Boldrin et al., 2005; Carafa et al., 2007; Toulpakis et al., 2005) (Fig. 5).

Many studies have examined the effects of triazinic herbicides on non-target species as phytoplankton (Huber, 1993; Solomon et al., 1996; DeLorenzo et al., 2001; Dorigo et al., 2004a,b). Algal responses to triazinic herbicides is reported to vary widely, depending on concentrations used, duration of exposure and algal species tested.

Intensive agriculture, supported by the massive use of herbicides, pesticides and compounds with biocidal activity, is a significant cause of the biodiversity crisis (Tilman, 1999). Depending on which species or groups are affected and in what manner, variations have the potential to alter productivity and to cause feedback on biogeochemical cycles in many ways. Investigating the differential capacity of the response of phytoplankton to increasing amounts of different herbicides has become a key issue in understanding further the future repercussions on the functioning of marine ecosystems.

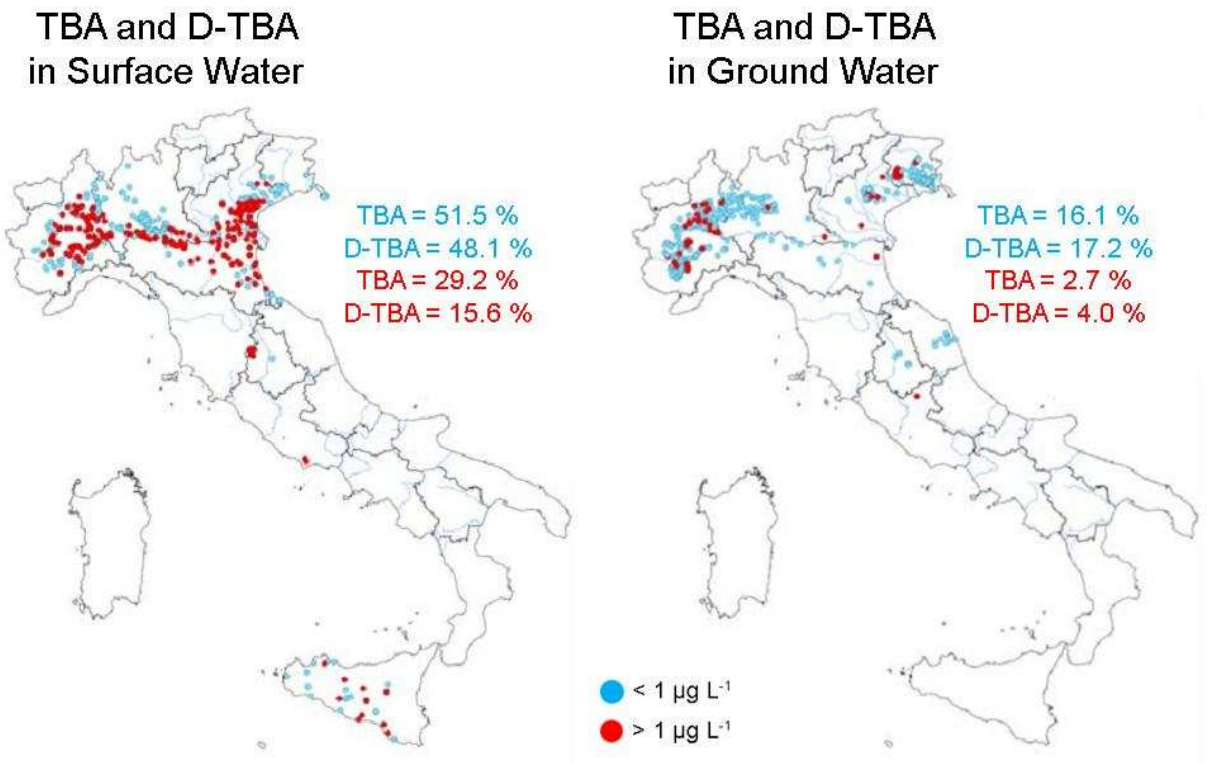

Fig. 5 Percentage of detection of Terbuthylazine (TBA) and its degradation product (D-TBA) in Italian surface and ground waters (monitoring study carried out by ISPRA, Istituto Superiore di Protezione e Ricerca Ambientale, in 2010)

2.2.3 Heavy metals

Release of heavy metal may be another consequence of discharging waste water effluents. Metals such as mercury, cadmium, lead, arsenic, copper and chromium occur in industrial and domestic waste and they can contaminate sea water and coastal areas. Heavy metals are normal constituents in seawater at low concentrations and, in some cases, are required by marine plants in trace amounts. However these compounds cause harmful effects when present in bigger amounts.

2.2.4 Oil spills

Shipping is an important activity affecting the marine environment, with some of the busiest shipping lanes in the world found in the North Sea, Baltic Sea and Mediterranean Sea. Despite mandatory global and regional regulations against discharges of oil and litter from ships, such discharges nevertheless represent a source of chronic impact from shipping. Major concerns include illegal discharges of oil and waste, atmospheric emissions of nitrogen oxide (NO_x), sulphur dioxide (SO_2), particulates and CO_2, noise, and accidental discharges of hazardous substances. There is also the risk of accidents, which can be particularly serious if oil tankers are involved.

Crude oil is a mixture of about 10,000 types of hydrocarbons, with about 20% being paraffins (aliphatics), 55% naphthenes (cycloalkanes), and 20% aromatics (benzenes). The last contains many toxic compounds. Major problems can occur when massive amounts of oil are suddenly released. The physiological effects of oil include the disruption of cellular membranes and effects on intracellular components.

3. Use of algae

Phytoplankton play an important role in aquatic ecosystem as the most important primary producers. Algae serve as human food source, either directly or indirectly, by supporting aquaculture of shrimps and fish or used as fertilizers.

Algae are also beneficial as producers of chemical derivates, i.e. various types of algae have proved to be sources of compounds with antibiotic and anti-cancer activity.

Various phytoplankton species are used as biomonitors. Bioassay are conducted in laboratories and algal responses are used to estimate the effects of physical and chemical agents in the environment. Biomonitors provide early warning of possible environmental deterioration, and my provide sensitive measures of pollution. One common use of algal cultures is as biomonitors in the detection, in natural waters of algal nutrients or substances that are toxic to algae. Algae, in fact, are more sensitive than animals to some pollutants, including detergents, textile-manufacturing effluents, dyes and especially herbicides.

The decay-resistant silica walls of diatoms which form layered sediments in lake and ocean sediments are used in paleoclimatological studies. Similarly, the calcified scales of coccolithophorid algae and decay-resistant cysts of dinoflagellates persist in ocean sediment for millions of years, forming records used to deduce past environmental change.

More recently algae have begun to play important roles also in biotechnology. Since some of our fossil fuel is derived from algae, efforts have been made to identify microalgae that produce relatively large amount of lipids for possible mass cultivation and extraction of useful hydrocarbons.

Through the use and development of modern laboratory and cultivation techniques, algae offer abundant opportunities for present and future utilization (Graham and Wilcox, 2000).

4. Harmful Algal Blooms (HABs)

When phytoplankton species reach high density levels they can became a nuisance, e.g. by creating oxygen depletion during decay or produce toxins affecting other organisms (Granéli et al., 1989).

Harmful algal blooms (HABs) occurrence increased worldwide during recent decades, with negative impacts on both ecosystem and economies (Anderson et al., 2002). Although human pressures, such as pollution and eutrophication, have been identified as the main causes for the increase, climate change has also been suggested because of its associated effect, i.e. increase in warm temperature. In addition, the accidental introduction of alien HABs into new environments, i.e. through ballast water discharge, enhance the global expansion of HABs (reviewed by Hallegraeff, 2010).

Harmful algae are characterized by their ability to be toxic, i.e. they produce harmful secondary metabolites, or have properties that are deemed as harmful for humans and/or other organisms. Of the approximately 5000 known algal species (Sournia, 1995; Landsberg et al., 2002), roughly 300 species form harmful blooms deleterious to the aquatic ecosystem and 80 species have the ability to produce toxins (Hallegraeff, 2003). Toxicity and other negative effects caused by harmful algae are not limited to a single algal class, 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.

Some algal toxins can be transported and accumulated to higher trophic levels of the food chain contaminating marine fauna from benthic invertebrates to pelagic fish and even mammals and marine birds (Fig. 6). For example, species of the dinoflagellates genus Alexandrium are well known for their production of paralytic shellfish poisoning (PSP) toxins, which accumulate in mussels (Lilly et al., 2002). Reports of harmful algal blooms, associated to human illness or damage to aquaculture operations are receiving increased attention in the media and scientific literature. In fact with increased problems of overfishing of coastal waters, more and more countries are looking towards aquaculture as an alternative.

Some microalgae produce and release into the environment organic compounds that negatively affect the growth of other organisms (allelopathy). Many allelochemical compounds are secondary metabolites with hemolytic capacity causing damages to cell membranes of other phytoplankton species, their grazers, or even fish gills, i.e. Prymnesium parvum toxins (Igarashi et al., 1998). Other allelophatic species (Heterosigma akashiwo and Alexandrium spp.) are toxic to their protistan grazers through exudates (Hansen, 1989; Tillmann and John, 2002; Tillmann 2004).

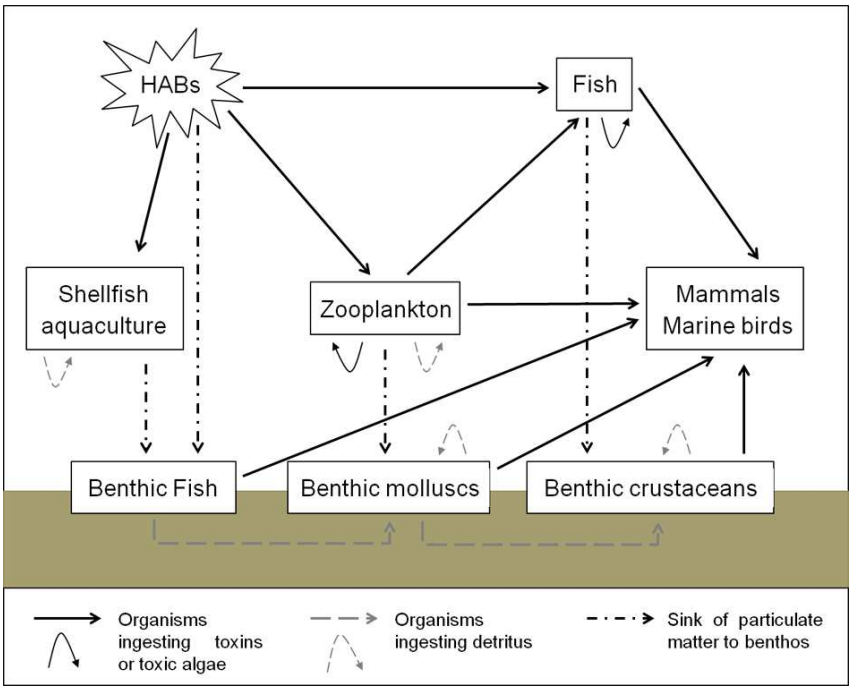

Fig. 6 Predominant pathways of phycotoxin trophic transfer, including organisms ingesting toxins or toxic algae, ingestion of detritus and sinking of particulate matter to the benthos.

Abiotic factors are known to influence toxins and allelopathic compound production, it is thus important to determine which factors govern the growth and toxicity of phytoplankton. There is a wide variation, not only between algal groups but also species and strains, with respect to their toxin content in response to different environmental conditions. In general, changes in toxin content are associated with unbalanced physiology conditions (Anderson, 1994; Flynn et al., 1996). The toxicity of P. parvum was reported to increase in nutrient limiting conditions (Johansson and Granéli, 1999) and to be pH-dependent: a change of pH from 8 to 9 increased toxicity to fish (Shilo and Achner, 1953; Ulitzur and Shilo, 1964). Phosphorous deficiency has been shown to increase PSP toxin levels 3 to 4 times in Alexandrium tamarense in comparison to nutrient sufficient or N-deficient cells (Cembella, 1998). Low temperature decreased the growth rate of the PSP-producing dinoflagellates Alexandrium catenella and Gymnodinium catenatum, whereas it increased toxin content per cell (Ogata et al., 1989, cited in Granéli et al., 1998).

Harmful algal blooms can have severe impacts on public health and fisheries economics with significant ecological consequences. Commercial oysters and mussels along the Atlantic and Pacific coasts are exposed to paralytic, diarrhetic and amnesic shellfish poisoning (Oshima, 1982; Yasumoto, 1982; Anderson, 2002; Landsberg, 2002). Fish-killing HABs have become major threats to aquaculture industries in Asian countries (Chen 1993; Dickman, 2001; Kim, 2005).

Nonetheless, predictive models and mitigation technique are not developed enough to secure the safety of seafood and to minimize the impacts of HABs on commercial fisheries.

4.1 Mitigation techniques

A variety of mitigation strategies have been developed to directly or indirectly affect the size of HABs population or its impact. These technique can be classified into two categories: precautionary impact preventions and bloom controls (Kim, 2005).

4.1.1 Precautionary impact preventions

Precautionary impact prevention includes HAB monitoring, prediction and emergent action. The role of monitoring is to detect HABs and their associated toxins in algae, shellfish and fish. Prediction involves more scientific approaches based on the oceanography and ecology. Accurate forecasting of the timing and transport pathway of HABs can help to have a better understanding of the events in order to take emergency actions. Some predictive models have already been developed for some dinoflagellate blooms (Eilertsen and Wyatt, 1998; Allen, 2004).

4.1.2 Bloom controls

A variety of chemicals, flocculants biological and physical techniques have been used in attempts to direct control HABs.

- Biological control - Implicate the use of grazers as a top-down control (Kim et al., 1999) and bacteria, viruses and parasites to apply a bottom-up control (Nagasaki et al., 2005).
- Physical control involves removing of the harmful algae cells using physical treatments such as isolation, ultrasonic disruption and electrolyzation of sea water.
- Chemical control - numerous chemical compounds, such as copper sulphate (Rounsefell and Evans, 1958), cysteine compounds (Jenkinson and Arzul, 2001), sodium hypochlorite (Kim et al., 2000), ozone (Rosenthal, 1981) and magnesium hydroxide (Maeda et al., 2005), have been applied to control HABs blooms. However most of the chemical control techniques are not species specific and may damage co-occurring species and/or other organisms, i.e. copper is lethal to sensitive marine organisms.
- Flocculant clays – Non-chemical strategy which involves the treatment of blooms with flocculant clays. This process removes HA cells from the water column by sedimentation. Clay minerals include kaolinite, illite and montmorillonite. Clay mitigation methods have been widely applied in many countries, such as Korea (Kim, 1987), Japan (Shirota, 1989), China (Yu et al., 1994) and USA (Sengco et a., 2000). However, ecotoxicological effects of clays on the benthic ecosystems are not clear. Environmental risk assessment should be carefully implemented to clarify chronic impacts of clays on marine organisms.

- Nutrients/Eutrophication - Eutrophication is a major cause of the initiation of HABs (Granéli et al., 1989). Therefore, reduction of terrestrial nutrients is one of the ways to reduce the outbreaks of HABs (SCOR/GEOHAB, 1998). Changes in nutrients (N:P:Si) ratios influence species composition (Smayda and Borkman 2005) and beneficial species might be encouraged.
- Modification of water circulation - In some semi-closed areas, HABs linked to restricted circulation can be reduced by changing circulation of water masses to optimize flushing of nutrient-rich water (SCOR/GEOHAB, 1998). Aeration can be applied in mitigating small-scale blooms in fish farms by braking down stratification.

The goal of management and mitigation of HABs is to secure public health and to protect aquaculture procedures against economic losses. These goals can be accomplished by direct control of HABs and reducing terrestrial pollutants in order to reduce eutrophication that leads to frequent HABs. Real time monitoring and prediction is the first precautionary action to be implemented to minimize damage caused by HABs. Ballast water has long been recognized as a major vector for the introduction of non-indigenous and harmful species. Invasions of exotic HAB species are causing significant ecological and economic damage in various parts of the World. Restriction on ballast water discharges should be considered, as well as the manner in which live fish and shelfish are transported and dispersed.

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Aims

The aims of this thesis are (1) to study the effects anthropogenic factors, mainly herbicides, on different phytoplankton species, that play an important role in the marine ecosystem. Some species studied were selected as abundant primary producers with worldwide distribution, while other species are known to contribute to HABs; (2) to study the effects on algal growth of the triazinic herbicide terbuthylazine, one of the most detected herbicide in surface and ground water; (3) to determine phytoplankton physiological responses to terbuthylazine exposition; (4) to evaluate the possible interaction between terbuthylazine effects and environmental factors, as increasing temperature conditions; (5) to reproduce experimental data of algal growth obtained in laboratory experiments, using a numerical biogeochemical model; (6) to simulate the effect of the herbicide terbuthylazine on algal growth at increasing temperature conditions.

The chapters of this thesis address the following topics:

- Chapter 1 describes the effect of the herbicide terbuthylazine on different flagellates species (Prorocentrum minimum, Prorocentrum micans, Protoceratium reticulatum, Alexandrium lusitanicum, Scrippsiella trochoidea, Lingulodinium polyedrum, Gonyaulax spinifera, Heterosigma akashiwo and Fibrocapsa japonica) characteristic of the Adriatic sea. The study is focused on the effect of terbuthylazine on Prorocentrum minimum and Gonyaulax spinifera growth at increasing temperature conditions, and their physiological responses.

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- Chapter 2 reports the effects of different herbicides: metolachlor, simazine and tebuthylazine, and its degradation product desetil-terbuthylazine, on the diatom Skeletonema marinoi. The effect of terbuthylazine Skeletonema marinoi growth and physiology was studied at increasing temperature conditions.

Manuscript to be submitted

- Chapter 3 numerical modelling of the experimental data collected during laboratory experiments shown in Chapters 1 and 2. The aim is to devise (and incorporate into a simplified version of a biogeochemical model) a numerical algorithm and parameterization describing the effect of terbuthylazine on the species grown in cultures.

Manuscript to be submitted

- Chapter 4 reports a study on the effect of light intensities on growth, toxicity and mixotrophy of the ichtyotoxic species Prymnesium parvum. The aim of this work is to test the possible use of high light exposition as an environmental friendly mitigation technique

The work was conducted at the marine ecology department of Linnaeus University (Kalmar, Sweden)

Submitted revised version - Harmful Algae

- Chapter 5 presents a study on the effect of emamectin benzoate on the feeding and reproduction of Calanus finmarchicus. The aim of this work was to study the synergic effect of the pollutant emamectin with other anthropogenic stressors, such as oil pollution and induced phytoplankton blooms.

The work was conducted during a mesocosm experiment (MESOAQUA, http://mesoaqua.eu/) (Bergen, Norway)

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Chapter 1

Combined effects of the herbicide terbuthylazine and temperature on different flagellates from the Northern Adriatic Sea

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Abstract

The triazinic herbicide terbuthylazine (TBA) is becoming an emergent contaminant in Italian rivers and in costal and groundwaters. A preliminary analysis of the sensitivity of marine flagellates to TBA was performed by monitoring the photosynthetic efficiency of nine species (belonging to the Dinophyceae or Raphidophyceae class) isolated from the Adriatic Sea. Different sensitivity levels for each flagellate were observed and the most sensitive microalgae, in order of PSII inhibition, were: G. spinifera > F. japonica > L. polyedrum while the most resistant where two species belonging to the Prorocentrum genus. Then the response of two microalgae to drivers, such as temperature and terbuthylazine, applied in combination was also investigated. Two potentially toxic flagellates, Prorocentrum minimum and Gonyaulax spinifera, were exposed, under different temperature conditions (15, 20 e 25 °C), at TBA concentrations that did not completely affect PSII. For both flagellates, effects of TBA on algal growth, measured through cell density and carbon analysis, as well as on the photosynthetic activity are reported. All parameters analyzed showed a negative effect of TBA from the exponential phase. TBA effect on algal growth were significantly enhanced at the optimal temperature conditions (20 and 25 °C), while no difference between control and herbicide treatments were detected for G. spinifera grown at 15 °C, which represented a stress condition for this species. The maximum inhibition of photosynthetic efficiency was found at 20 °C for both organisms. Both flagellates increased cell carbon and nitrogen content in herbicide treatments compared to the control, except G. spinifera grown at 15 °C. Chlorophyll-a production was increased only in G. spinifera exposed to 5 µg L^{-1} of TBA and the effect was enhanced with the increase of temperature. Herbicide-induced variations in cellular components determined changes in cellular carbon:nitrogen (C:N) and chlorophyll:carbon (Chl:C) ratios. The C:N ratio decreased in both species, while only G. spinifera showed an increase in the Chl:C ratio at all temperature conditions. In response to TBA exposition G. spinifera increased extracellular polysaccharides release at 20 and 25 °C, while no difference was reported for P. minimum. Changes in nutrient uptake rates were also observed for P. minimum. Nitrate and phosphate uptake significantly increased in the presence of TBA and this response was enhanced at 25 °C, while nitrate uptake increased in G. spinifera only when grown at 25 °C. As for growth rates, the observed changes in intracellular component contents increased at optimal temperature conditions. In this work it is shown that temperature conditions can have an important role on the effect of terbuthylazine on algal growth and on the physiological responses of different species. Furthermore, the algal resistance and recovery can be dependent on nutrient availability.

Keywords: terbuthylazine, flagellate, photoinhibition, physiology, growth, Adriatic Sea, raphidophyte, fluorometry, C:N ratio, chlorophyll, polysaccharide, nutrient uptake

1. Introduction

The Northern Adriatic Sea is characterized by shallow waters (70 m maximum depth) and significant freshwater input coming in the main from Italy's longest river, the Po, which flows through one of the most productive and intensively farmed regions of the country and represents a primary source of nutrients as well as pollutants for the Adriatic (Provini et al., 1992). As a consequence this area is characterized by high productivity, particularly close to the Po Delta,

where phytoplankton blooms are frequently observed (Bernardi Aubry et al., 2004; Revelante and Gilmartin, 1992). During winter and spring, diatom species are predominant, while dinoflagellates and nanoflagellates are characteristic of the summer periods. The latter two are present in significant abundance in June and July along the northern Italian coasts (Vollenweider et al., 1992). Although valuable for its primary production, this area has also been subjected to recurring cases of harmful algal blooms (HABs) with phenomena of "red tides", mucilage accumulation and shellfish contamination with different kinds of toxins, all affecting shellfish farming and/or the tourist and fishery industries (Pistocchi et al., 2012).

Numerous studies have highlighted the increasing frequency of pesticide pollution, due to agricultural activity, in lakes and rivers with a high predominance of herbicides (Galassi et al., 1992) that often exceeded concentrations set by Italian and European legislation (Strandberg and Scott-Fordsmand, 2002). The herbicide terbuthylazine (TBA) has been widely used for decades in crop farming as a weed control, mainly in the early spring. Because of its percolation through the soil, after heavy rain, TBA is one of the most detected herbicides in Italian rivers and ground waters (Sbrilli et al., 2005). The TBA half-life was measured in river, ground and sea water samples from Murcia (South-East of Spain) incubated under different laboratory conditions by Navarro et al. (2004), who found a range of between 76 and 331 days. In recent years TBA has become an emergent contaminant in the Po River and consequently in the Northern Adriatic Sea along with its degradation product desethyl-terbuthylazine and a number of other compounds (Boldrin et al., 2005; Carafa et al., 2007; Touloupakis et al., 2005). During seasonal samplings (2004 - 2005) from different stations of the Sacca di Goro coastal lagoon and in the Northern Adriatic Sea, TBA was reported as the most frequently detected herbicide, reaching a maximum concentration of 694.32 ng L^{-1} in the Sacca di Goro Lagoon and 234.50 ng L^{-1} in the Adriatic Sea (Carafa et al., 2007). Carafa et al. (2007) also showed that the occurrence of TBA is evident within a few months following its application and indeed seasonal variations of the herbicide can be clearly seen in the water column exhibiting spring peaks. These results are in line with those of Vianello et al. (2005) which measured very high TBA concentrations during the first rainfall events after treatments. The migration of herbicides from farmland, by runoff to surface water and by leaching to groundwater, can lead to toxic effects on non-target organisms. Because of their physiological similarities with the intended target organisms, phytoplankton organisms are particularly exposed to herbicide toxicity (DeLorenzo et al., 2001; Dorigo et al., 2004a, 2004b).

In this study, a preliminary TBA phytotoxicity assessment was conducted in the laboratory on nine microalgae species belonging to different classes of phytoflagellates and representative of the Adriatic Sea phytoplankton population. This was done to observe differences in sensitivity among primary producers and harmful species. In particular, we have taken into account two dinoflagellate species belonging to the Prorocentrum genus, i.e. P. micans and the potentially toxic P. minimum (Taylor et al., 2003), because of their worldwide distribution and common presence in the Adriatic Sea. The Raphidophyceae Fibrocapsa japonica and Heterosigma akashiwo were chosen as species causing frequent red tide phenomena (Pistocchi et al., 2012) and Scrippsiella trochoidea as the cause of non-toxic massive blooms in the Adriatic Sea (Vollenweider et al., 1992). The yessotoxin producers and often recurrent species Protoceratium reticulatum, Lingulodinium polyedrum and Gonyaulax spinifera have also been studied and finally Alexandrium lusitanicum which was at times present in the Adriatic Sea and has been responsible for mussel contamination with PSP ("paralytic shellfish poisoning") toxins in amounts exceeding the regulatory limit (Pistocchi et al., 2012). A further aim of this study was also to determine how TBA sensitivity and algal cellular responses may be modified by temperature. In order to do this different parameters, such as growth, cell chlorophyll, carbon and nitrogen content, macronutrient (nitrate and phosphate) uptake, photosynthetic efficiency and polysaccharides production, were examined in two flagellates (Prorocentrum minimum and Gonyaulax spinifera) exposed to TBA and grown at three different temperatures. The experimental design was also planned in order to use the data in a biogeochemical model, aimed at simulate the herbicide effects on primary producers (Fiori, 2013).

2. Materials and Methods

2.1 Phytoplankton cultures

The species used were the Dinophyceae: Prorocentrum minimum (Pavillard) Schiller, Prorocentrum micans Ehrenberg, Protoceratium reticulatum (Claparède and Lachmann), Alexandrium lusitanicum Balech, Scrippsiella

trochoidea (Stein) Loeblich III, Lingulodinium polyedrum Stein (Dodge), Gonyaulax spinifera (Claparède et Lachmann) Diesing and the Raphidophyceae Heterosigma akashiwo (Y.Hada) Y.Hada ex Y.Hara & M.Chihara and Fibrocapsa japonica Toriumi and Takano, all isolated from the Adriatic Sea. The cultures were maintained in sterilized f/10 medium (salinity 35) at 20 °C under a 16:8 h L:D cycle (ca. 74 µmol photons m^{-2} s^{-1} from cool white lamps). For experimental work batch cultures were grown in a modified f/2 medium containing 116 µM N-NO_3 and 7.2 µM P-PO_4 (N:P = 16). Cultures were kept at a light intensity of 140±8 µmol photons m^{-2} s^{-1}, 12:12 h L:D cycle, and constant temperature conditions. Before the experiments started, cultures were allowed to acclimate to nutrients, light and temperature conditions by subculturing them for at least 2 weeks. The sea water used for the medium was collected at an oligotrophic site 20 Km off the coast of Cesenatico and aged in the dark for at least 2 months. Before use it was filtered (GF/C Whatman) (when it was necessary the salinity was adjusted to 35 with distilled water) and sterilized at 120 °C for 20 min.

2.2 Herbicide solution

10 mg of TBA (Chem Service, 99.5% purity) was dissolved in 10 mL methanol to obtain a final concentration of 1000 µg L^{-1}. This solution was directly applied to the cultures. The final solvent concentration in the culture medium never exceeded 0.002%.

2.3 Experimental design

In a preliminary experiment all nine microalgae species, grown in 250 mL volumetric flasks, were exposed to two TBA concentrations (25 and 50 µg L^{-1}) at 20 °C under constant light intensity of 140±8 µmol photons m^{-2} s^{-1}. For each species controls with and without methanol (0.001 and 0.002%) were run at the same environmental conditions.

Then three species (F. japonica, L. polyedrum, and G. spinifera) which were mostly affected by the above indicated concentrations, were exposed to four lower concentrations of the pollutant (1, 5, 10, 15 µg L^{-1}). In both experiments the herbicide effect was followed daily by measuring photosystem II efficiency with a PAM fluorometer (see below).

In order to investigate the influence of TBA on algal growth and cellular changes more accurately, two potentially toxic flagellates, Prorocentrum minimum (chosen from the most tolerant species) and Gonyaulax spinifera (from the most sensitive species) were grown under different sublethal levels of TBA. P. minimum was exposed to 30 µg L^{-1}, while G. spinifera was exposed to 1 and 5 µg L^{-1} TBA.

The latter cultures were grown in 1.8 L medium, by using 2 L volumetric flasks, under three temperature conditions (15, 20 and 25 °C) obtained in a thermostated chamber. The experiment ended when each culture reached the stationary phase. Thus due to the slowing effect of temperature and herbicide on growth, different cultures grew for different time length.

Growth curves were monitored through cell countings made daily in settling chambers following Utermohl's method (Hasle, 1978). All experiments were run in duplicates; the large volume of the flasks did not allow for a higher number of replicates in the limited space of the chamber, as exposure to equal light was required. The culture volume daily withdrawn for sampling was not replaced. However, the remaining volume was never less than 30% of the initial volume as, except for the first few days, the sampled volume could be reduced up to 10-15 mL. This allowed duplicate samplings to be performed during most of the experiment. Experiments with culture sets of smaller volumes were performed separately in order to confirm the main measured parameters, such as the maximum quantum yield achieved, the percentage of growth rate inhibition and the maximum carbon and chlorophyll content per cell (data not shown).

2.4 Pulse Amplitude Modulation (PAM) fluorimetry

The effective quantum Yield of PSII was measured daily through a Pulse Amplitude Modulation fluorometer (101-PAM connected to a PDA-100 data acquisition system, H. Walz, Effeltrich, Germany) provided with a high power LED Lamp Control unit HPL-C and LED-Array–Cone HPL-470 to supply saturating pulses, US-L665 and 102-FR to supply far red light and measuring light, respectively. 3 mL algal samples were analyzed in cuvettes (10 x 10 mm) mounted on an optical unit ED-101US/M. After a 20 min dark adaptation, continuous actinic light of about the same intensity (145±2 µmol photons m^{-2} s^{-1}) as the algae were exposed to during the experiment, was applied for 5 min. This time was previously optimized, and showed that the yields were stabilized. The operational quantum yield, Φ'_M, was obtained by the ratio:

$$\Phi'_M = (F'_m - F) / F'_m = Fv'/F'm$$

Where F'_m is the maximum fluorescence after a saturation pulse (> 3000 µmol photons m^{-2} s^{-1} for

0.8 s) and F is the steady-state fluorescence (Genty et al., 1989).

2.5 Carbon and Nitrogen analysis

Carbon (C) and nitrogen (N) cell contents were measured from samples, collected daily for short experiments and every 2-3 days for long lasting ones, using a CHNS elemental analyzer (ThermoFisher, Flash 2000). Volumes from 10 to 100 mL of algal cultures in duplicate were filtered on Whatman glass microfiber filters (GF/F). The filters were dried at 450 °C in a muffle furnace for 4 hours before use.

2.6 Chlorophyll-a analysis

Chlorophyll analysis were performed by collecting cells, from 10 to 100 mL of algal cultures, on cellulose filters (Millipore, pore size 0.45 µm). The pigments were extract in 15 mL of 90% acetone solution. Chlorophyll-a concentration in the supernatant was analyzed spectrophotometrically according to Strickland and Parson (1972).

2.7 Polysaccharides analysis

Extracellular carbohydrates were determined from 15 mL filtered culture medium (Millipore, pore size 3.0 µm). It was precipitated with 2 volumes of cold ethanol and centrifuged at 3,670 x g. The extraction was performed according to Myklestad and Haug (1972). Briefly, 1 mL 80% H_2SO_4 was added to the pellets and incubated for 20 h at 20 °C. The suspension was then diluted with distilled water, and 1 mL used for polysaccharide analysis following the phenol-sulphuric acid method (Dubois et al., 1956).

2.8 Nutrient analysis (Nitrate, Phosphate)

Nutrient ($N\text{-}NO_3$ and $P\text{-}PO_4$) concentrations in the medium were analyzed in 10-50 mL of the filtrate collected for chlorophyll-a and CHN analysis (0.45 µm or GF/F) and diluted when necessary. Nitrates were reduced to nitrites in reduction columns filled with amalgamated cadmium and analyzed spectrophotometrically (543 nm), according to Morris and Riley (1963). To determine phosphate concentrations in the medium, samples were analyzed spectrophotometrically (885 nm) according to Murphy and Riley (1962).

2.9 Calculation

Specific growth rates (µ, d^{-1}) were 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 times t_0 and t_1.

Variations of chlorophyll:carbon (Chl:C) values from those measured in the controls were expressed as a percentage (BIAS%) and calculated as:

$$BIAS\% = \frac{1}{N}\sum\left(\frac{R_t - R_c}{R_c}\right) * 100$$

R_t represents the Chl:C ratio measured in terbuthylazine treatments, while R_c is the ratio measured in the controls. N is the number of observations.

The nutrient ($N\text{-}NO_3$ and $P\text{-}PO_4$) uptake (U) was calculated according to Lim et al. (2006). Nitrate (nmol $N\text{-}NO_3^-$ $cell^{-1}$ d^{-1}) and phosphate (nmol $P\text{-}PO_4$ $cell^{-1}$ d^{-1}) uptake rates were calculated from the residual nutrient concentrations in the medium (C) and the change in cell densities (ϒ) over a period of time ($\Delta t = t_1 - t_0$), when the depletion of nutrients was linear. Δt therefore differs depending on algal species and temperature conditions: between day 0 (t_0) and day 10 – 18 (t_1) for Gonyaulax spinifera and between day 0 (t_0) and day 4 – 8 (t_1) for Prorocentrum minimum.

$$U = -\frac{C_1 - C_0}{\gamma \Delta t}$$

$$\gamma = \frac{N_1 - N_0}{\ln N_1 - \ln N_0}$$

C_0 and C_1 are the nutrient concentrations (µM) at the time t_0 and t_1; and N_0 and N_1 are cell density (cell mL^{-1}) values in the same days.

2.10 Statistical analysis

Statistical analyses were performed with MATLAB R2011a. Lilliefor's test was used to check normal distribution, while Bartlett's test was used to test homogeneity of variances.

Two-way analysis of variance (ANOVA) was conducted on photoinhibition values measured for the different marine flagellates.

Differences in growth rate (d^{-1}) and nutrient uptake (nmol $cell^{-1}$ d^{-1}) among all treatments and temperature conditions were tested by two-way analysis of variance (ANOVA). One-way ANOVA was also conducted when there was an interaction between the two factors studied. Consequently Tukey's honestly significant difference (HSD) was performed to evaluate differences between conditions (P. minimum and G. spinifera).

ANOVA repeated measures were used to underline differences overtime (day 0, 6, 12, 15-16) in chlorophyll-a cell content (G. spinifera), using the SPSS statistical program.

3. Results

3.1 Flagellates sensitivity to TBA assay

Due to the specific effect of TBA on the photosystem II, in the present study, the inhibition of the effective quantum yield of PSII (Fv'/Fm') has been used to determine the toxicity of TBA on different flagellate species of the Northern Adriatic Sea. Monocultures of nine flagellate species were exposed to two herbicide concentrations (25 and 50 µg L^{-1}).

The effect of TBA is reported in Fig. 1 and given as a percentage of photosynthetic efficiency inhibition in respect to that measured in the control. Yield measurements were performed daily and the highest values for each species are reported. Our results show that sensitivity to TBA varied between the different flagellates. All the species, with the exception of P. minimum, were significantly ($F = 178.7$, $p < 0.05$) affected by both concentrations of TBA with a percentage of PSII inhibition ranging between 18 and 95%. Significant differences ($F = 12.19$, $p < 0.05$) were also detected between the effect of the two different concentrations of TBA (25 and 50 µg L^{-1}). Multi-comparison tests highlighted significant differences in the sensibility to TBA between Prorocentrum species (P. minimum and P. micans) and the other flagellates.

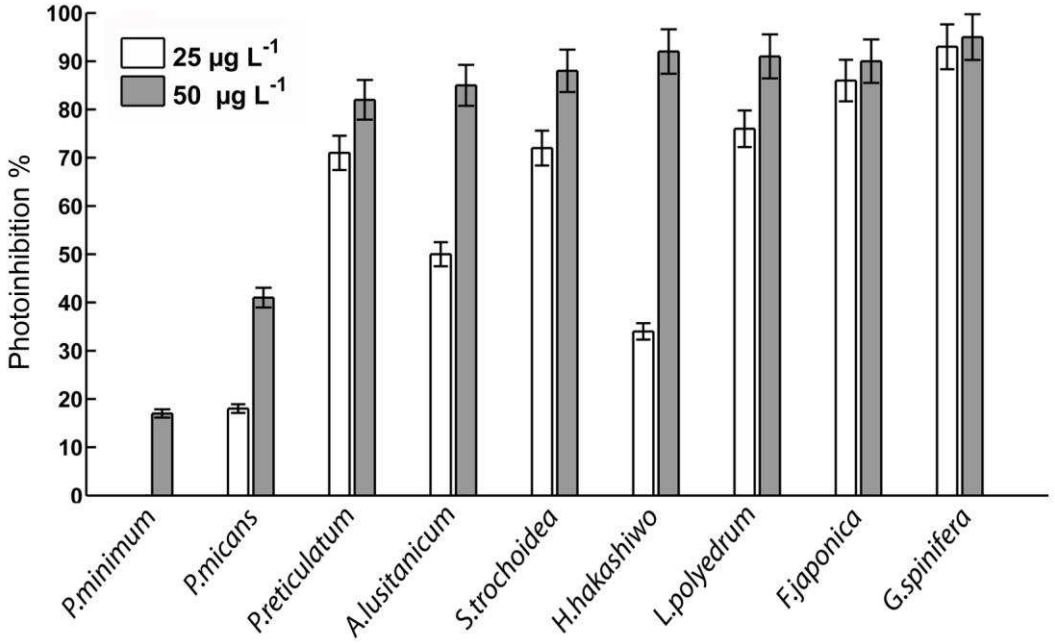

Fig. 1 Effective quantum yield inhibition, as percentage respect to that measured in controls, for 9 species of flagellates (P. minimum, P. micans, P. reticulatum, A. lusitanicum, S. trochoidea, H. hakashiwo, L. polyedrum, F. japonica and G. spinifera) exposed to two concentrations of terbuthylazine (25, 50 µg L^{-1}).

In fact, for Prorocentrum spp. the effect on photosynthetic activity was the lowest reported and P. minimum was the only species not affected by exposure to 25 µg L^{-1} TBA. Only at the herbicide concentration of 25 µg L^{-1}, were similarities found between P. micans and H. hakashiwo. The other species displayed variable effects in response to the presence of this TBA concentration. H. akashiwo and A. lusitanicum were more resistant to 25 µg L^{-1} TBA than the other species. The most sensitive flagellates were L. polyedrum, F. japonica and G. spinifera showing a photoinhibition higher than 75% when grown in the presence of 25 µg L^{-1} TBA. However, no significant differences ($p > 0.05$) in sensitivity to TBA were observed between these three species, P. reticulatum, A. lusitanicum and S. trochoidea. The most sensitive species (L. polyedrum, F. japonica and G. spinifera) were chosen to be subsequently exposed to four lower concentrations (1, 5, 10 and 15 µg L^{-1}) (Fig. 2). For all the concentrations tested the effect of TBA on photosynthetic activity was significantly higher in Gonyaulax spinifera than in L. polyedrum ($F = 14.6$, $p < 0.05$).

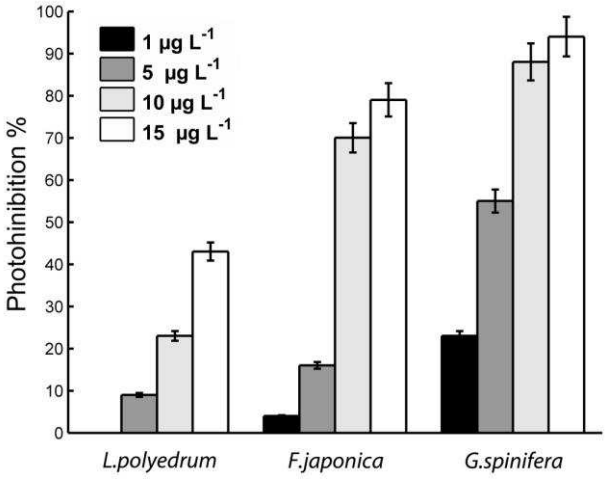

Fig. 2 Effective quantum yield inhibition, as percentage respect to that measured in controls, for the dinoflagellates L. polyedrum and G. spinifera and for the raphidophycean F. japonica exposed to different concentrations of terbuthylazine (1, 5, 10, 15 µg L^{-1}).

No difference was observed between F. japonica and G. spinifera sensitivities. However, the strongest effect of TBA on photosynthetic activity was observed in Gonyaulax spinifera with a photoinhibition of 23 ± 1% measured in the presence of 1 µg L^{-1} TBA. Both F. japonica and G. spinifera PSII efficiency was affected by the exposure to the lowest concentration of 1 µg L^{-1}, while for L. polyedrum photoinhibition started from the concentration of 5 µg L^{-1}.
No significant differences were observed between controls and those with added methanol concentrations (0.001 and 0.002 %) for all phytoplankton species tested (data not shown).

3.2 Effect of TBA on Prorocentrum minimum and Gonyaulax spinifera grown at different temperatures
The effect of TBA on algal growth, intracellular components and macronutrient (nitrate and phosphate) uptake was investigated in more detail for the two flagellates P. minimum and G. spinifera, under different growth temperatures (15, 20 and 25 °C). Since G. spinifera was the most sensitive microalgae in the experiment described above, it was exposed to two low concentrations of TBA (1 and 5 µg L^{-1}). Since the threshold concentration for observing a photo-inhibitory effect on P. minimum appeared to be set between 25 and 50 µg L^{-1} TBA, it was exposed to the concentration of 30 µg L^{-1}.

3.2.1 Growth and photosynthetic efficiency
The growth pattern was followed by carrying out both cell counts and carbon analysis; parallel measurements of the PSII quantum yield, both maximum and effective, were performed. Growth curves and effective quantum yield (Fv'/Fm') obtained at the three temperature conditions are reported in Fig. 3 and Fig. 4 for P. minimum and G. spinifera, respectively. Growth curves based on carbon were not reported since the pattern obtained was identical to that resulting from cell counts.
The inhibition pattern of TBA on PSII was enhanced compared to that obtained through growth or carbon measurement. Although no significant inhibitory effects of TBA were reported on P. minimum growth, the effective quantum yield efficiency was inhibited at all the temperature conditions. The effect of TBA on quantum yield was higher at 20 than at 15 and 25 °C. In addition, at 20 °C the effect on photosynthetic activity and cell growth was observed earlier than in the cultures grown at the other two temperatures (Fig. 3). PSII efficiency generally increased until the cultures were in the exponential growth phase and decreased as soon as the nutrients were depleted (data not shown) and cultures entered the stationary growth phase. This pattern was similar to what was described for different species, where increasing values during the exponential growth, were attributed to acclimation to decreasing light availability due to shelf-shading (Yang et al., 2011).
The effect of the herbicide on G. spinifera was evident only at the two highest temperatures (20 and 25°C), whereas at 15 °C the cultures exposed to the two TBA concentrations did not differ from the control (Fig. 4). Generally the lowest TBA concentration tested (1 µg L^{-1}) had limited effects on both growth and PSII efficiency while in presence of 5 µg L^{-1} TBA the effect was quite pronounced. As for P. minimum, the strongest effect of TBA on effective quantum yield was observed at 20 °C.

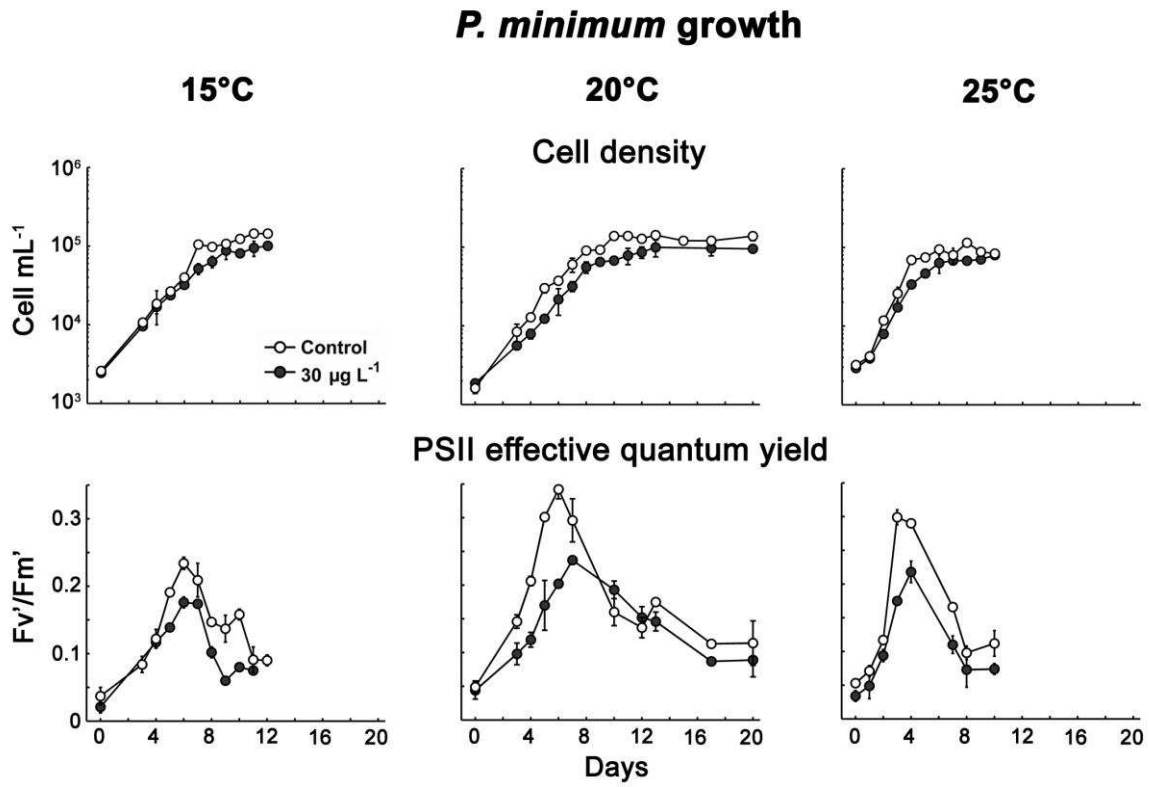

Fig. 3 P. minimum growth curves and PSII Effective Quantum Yields (Fv'/Fm') obtained in controls and in cultures added with 30 µg L^{-1} of terbuthylazine and measured at 3 temperature conditions (15, 20 and 25 °C).

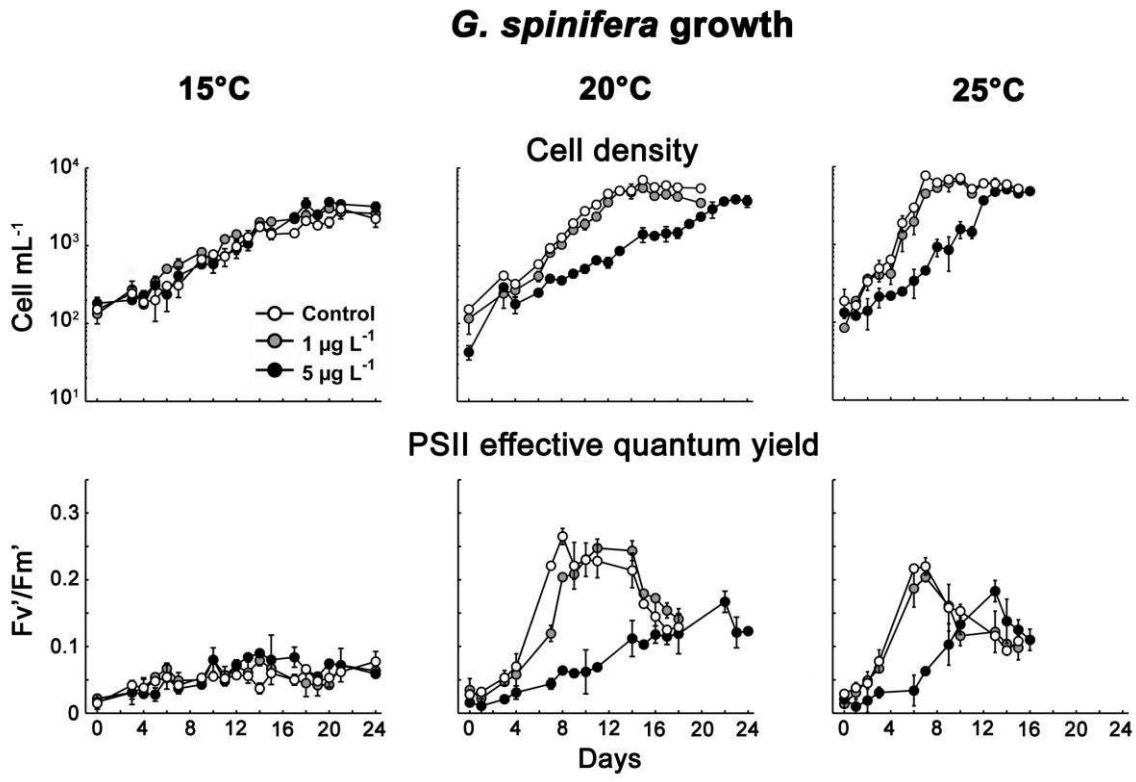

Fig. 4 G. spinifera growth curves and PSII Effective Quantum Yields (Fv'/Fm') obtained in controls and in cultures added with 1 and 5 µg L^{-1} of terbuthylazine and measured at 3 temperature conditions (15, 20 and 25 °C).

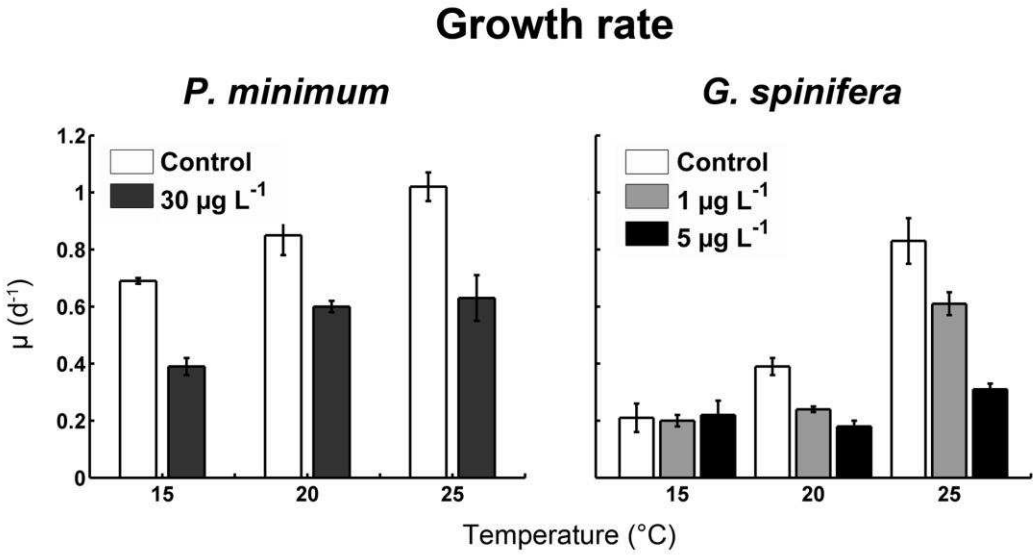

Fig. 5 P. minimum and G. spinifera growth rates in controls and terbuthylazine treatments (P. minimum = 30 µg L^{-1}; G. spinifera = 1 and 5 µg L^{-1}) at different temperature conditions (15, 20 and 25 °C).

P. minimum reached a higher growth rate than G. spinifera at all temperature conditions (Fig. 5). Growth rates were positively correlated with temperature for both flagellates. In particular, P. minimum growth was significantly lower ($F = 34.4$, $p < 0.05$) at 15 °C than at high temperature conditions (20 and 25 °C), while G. spinifera growth rate at 25 °C was significantly higher ($F = 149.7$, $p < 0.05$) compared to the other temperature conditions (15 and 20 °C). When cultures were exposed to TBA, the growth rate was significantly reduced (P. minimum: $F = 121.9$, $p < 0.05$; G. spinifera: $F = 56.2$, $p < 0.05$), with the exception of G. spinifera cultured at 15 °C, where no significant difference between treatments was observed. At the other two temperature conditions (20 and 25 °C) the growth rate decreased with increasing concentration of the herbicide. Our results show that high temperatures significantly enhanced TBA effect on both flagellates, showing the highest effect on growth rate at 25 °C. Furthermore, an interaction factor was found between temperature and TBA effect on G. spinifera growth rate when conducting a two-way ANOVA ($F = 24.3$, $p < 0.05$), demonstrating that changes in temperature conditions can affect the sensitivity of this flagellate to the herbicide.

3.2.2 Intracellular carbon, nitrogen and chlorophyll contents

Carbon, nitrogen and chlorophyll-a cell content (pg $cell^{-1}$) for all treatments and temperature conditions are reported in Figs. 6 and 7. In P. minimum (Fig. 6) the cellular carbon content of cultures exposed to TBA was higher than that of the control in the first days of growth at all temperature conditions, whereas it did not differ from the control after 4 days. Nitrogen content in the presence of herbicide showed a similar but prolonged pattern. No significant difference in chlorophyll-a cell content was observed between the control and herbicide treatments at all temperature conditions tested. Higher concentrations of carbon, nitrogen and chlorophyll-a were observed in G. spinifera cells exposed to 5 µg L^{-1} of TBA, while at the lowest concentration (1 µg L^{-1}) no significant variation in cell components was detected compared to the control (Fig. 7). Increasing temperature conditions also influenced the response of G. spinifera to TBA exposure in terms of intracellular components. Variations in intracellular components were observed at high temperature conditions (20 and 25 °C), while for G. spinifera grown at 15 °C no difference in carbon and nitrogen content was detected, compared to the control. Repeated ANOVA measurements showed a significant ($F = 6.68$; $p < 005$) increase in chlorophyll cell content in cultures exposed to 5 µg L^{-1} of TBA, at all temperature conditions after 6 -12 days of growth.

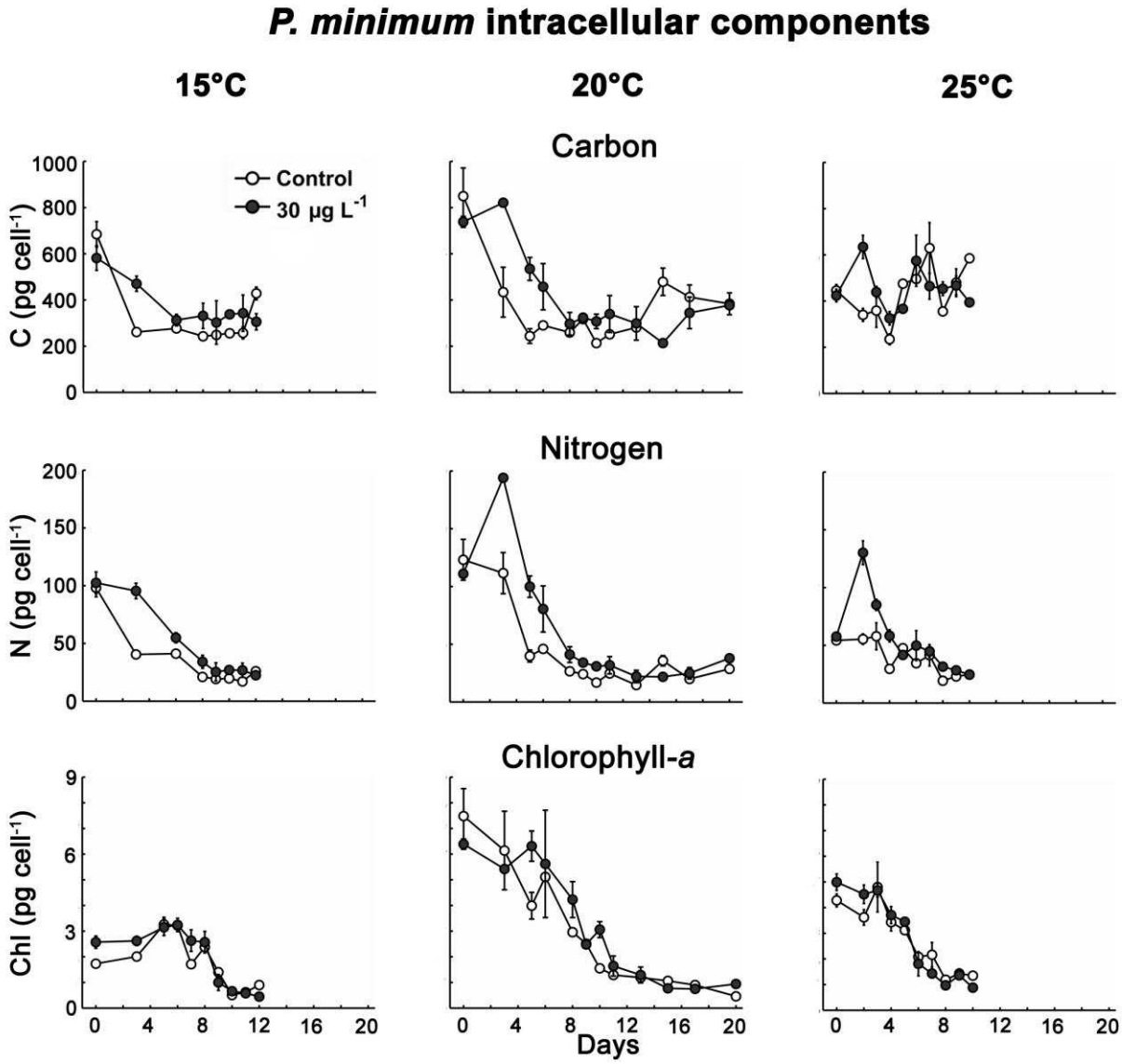

Fig. 6 P. minimum intracellular components (carbon (C), nitrogen (N), chlorophyll-a (Chl)) (expressed as pg cell^{-1}) in controls and terbuthylazine treatments (30 µg L^{-1}) at three temperature conditions (15, 20 and 25 °C).

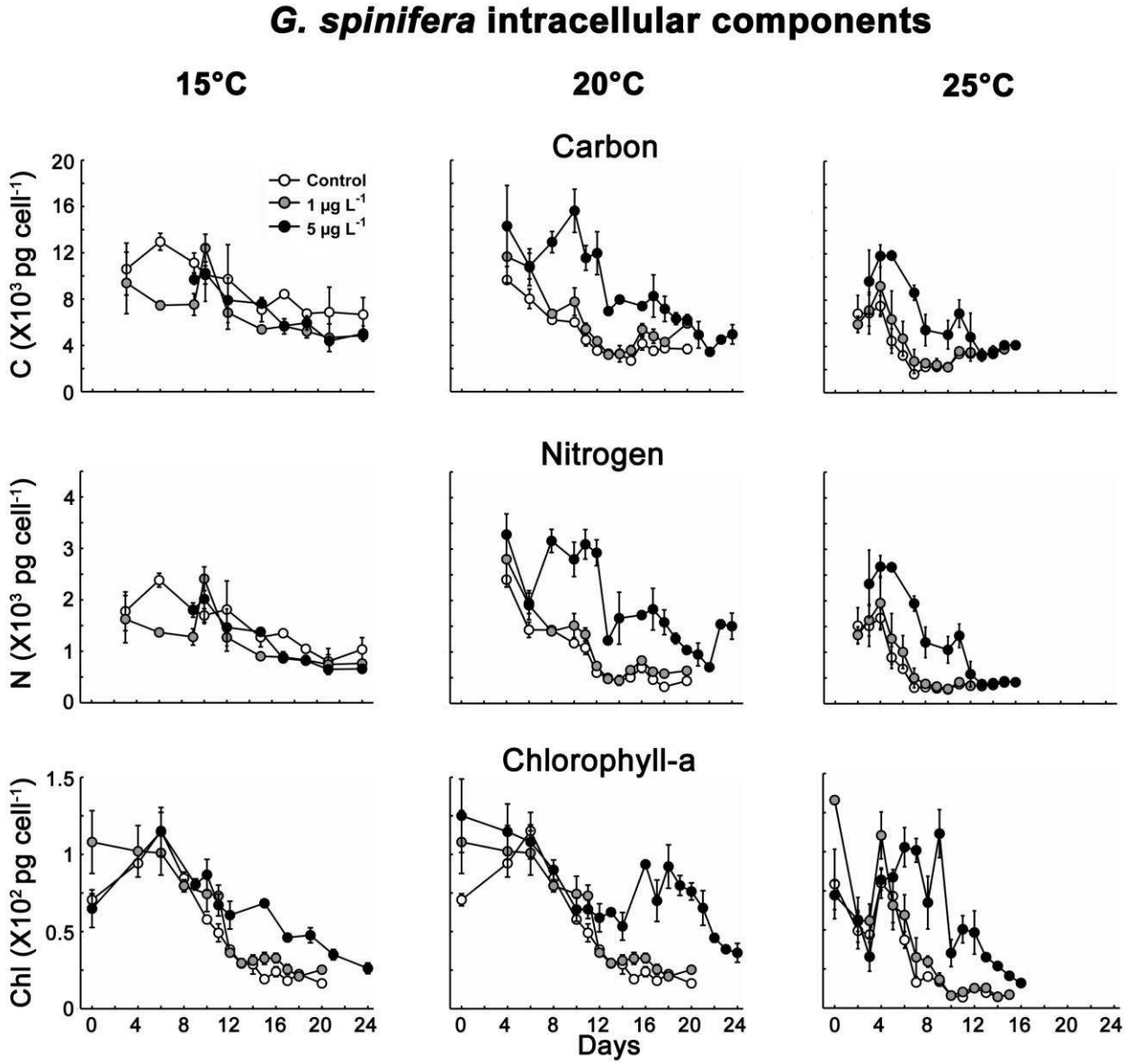

Fig. 7 G. spinifera intracellular components (carbon (C), nitrogen (N), chlorophyll-a (Chl)) (expressed as pg cell^{-1}) in controls and terbuthylazine treatments (P. minimum = 30 µg L^{-1}; G. spinifera = 1 and 5 µg L^{-1}) at three temperature conditions (15, 20 and 25 °C).

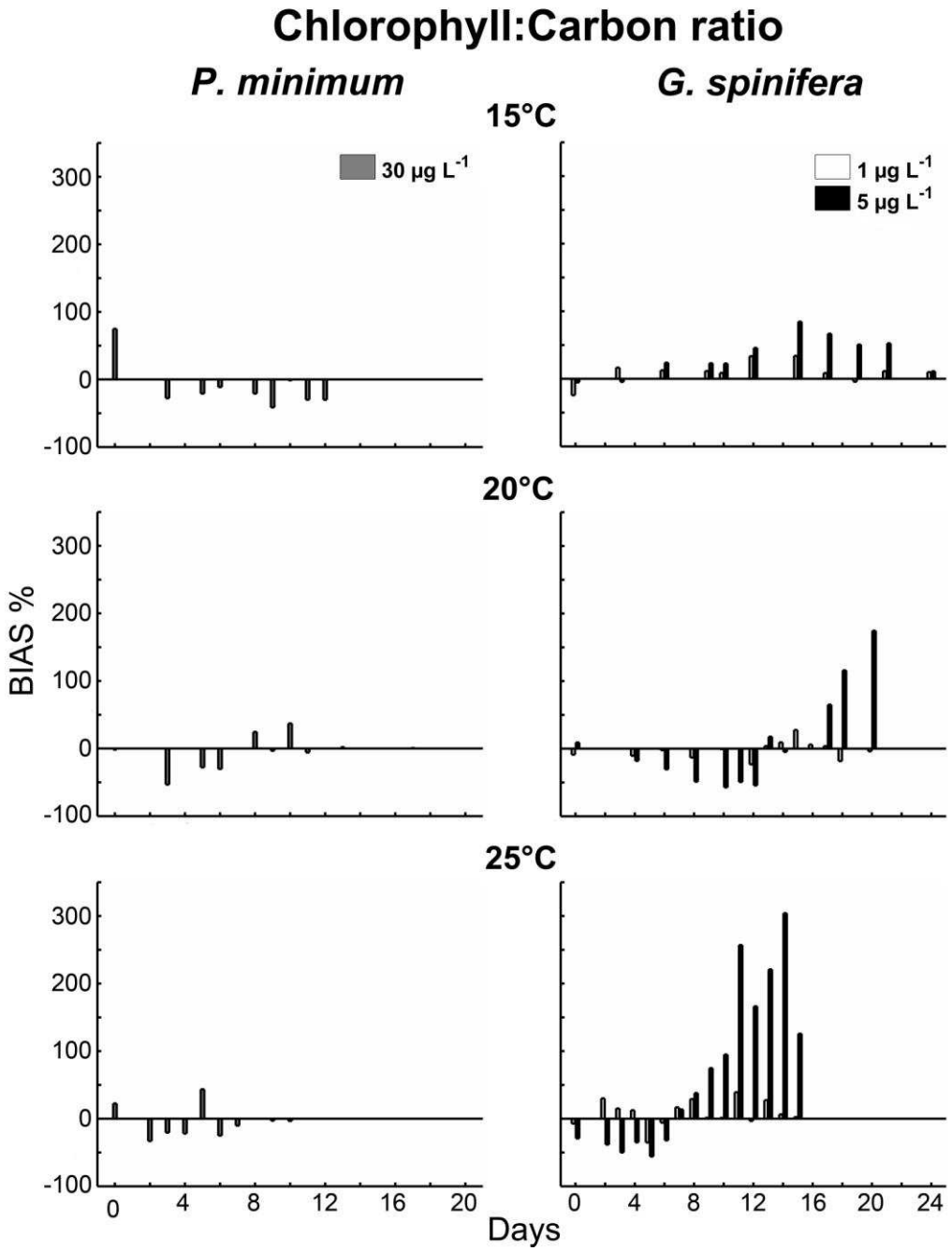

Fig. 8 BIAS% of the chlorophyll:carbon ratio for P. minimum and G. spinifera exposed to different terbuthylazine concentrations (P. minimum = 30 µg L^{-1}; G. spinifera = 1, 5 µg L^{-1}) and temperature conditions (15, 20 and 25 °C).

Moreover, temperature had significant (F = 6.89; p < 005) effects on chlorophyll cell content and differences were reported between 25°C and the other two temperature conditions (15 and 20 °C). Chlorophyll cell content was positively correlated with temperature: at 15 and 20°C (day 15) it was, respectively, 1 and 2.9 times higher in TBA (5 µg L^{-1}) treatments than in the control and at the highest temperature (25°C) it increased by up to 5.6 times compared to the control.

Herbicide-induced variations in cellular components determined changes in cellular carbon:nitrogen (C:N) and chlorophyll:carbon (Chl:C) ratios. In both flagellates the C:N ratio decreased in the presence of TBA (not shown), while different results for the two species were observed for the Chl:C ratios, reported in Figure 8 as BIAS%. BIAS% represents a positive or negative variation of Chl:C ratio between cultures exposed to TBA and the control, the latter being represented as the X axes in the figures. P.

minimum grown in presence of TBA showed values of this ratio close to those of the control at all temperature conditions. G. spinifera cultures exposed to herbicide showed a positive variation in Chl:C ratio that increased with increasing herbicide concentrations. The positive values of BIAS% indicate a higher Chl:C ratio compared to the control, due to an increase of Chl production. Moreover, the increase in the Chl:C ratio was positively correlated with temperature and exceeded the value measured in the control by 300% when G. spinifera was exposed to the highest TBA concentration at 25°C.

3.2.3 Extracellular polysaccharides

Extracellular polysaccharides were measured at intervals during growth. The amount measured in the P. minimum culture medium was in the range of 16 ± 0.4 - 24 ± 2.8 pg cell^{-1} in the stationary phase at all the temperatures examined (data not shown). No difference in polysaccharide release was observed for this species in the presence of TBA. G. spinifera produced a higher amount of extracellular polysaccharides than P. minimum (range: 160 ± 24 – 380 ± 28 pg cell^{-1}). Polysaccharide release increased in G. spinifera exposed to 5 µg L^{-1} TBA at high temperature conditions. Polysaccharide content in particular was 4.7 and 5.4 times higher than that of the control, at 20 °C (day 16) and 25 °C (day 10) respectively. No difference between the control and the treated cultures was observed at 15 °C (data not shown).

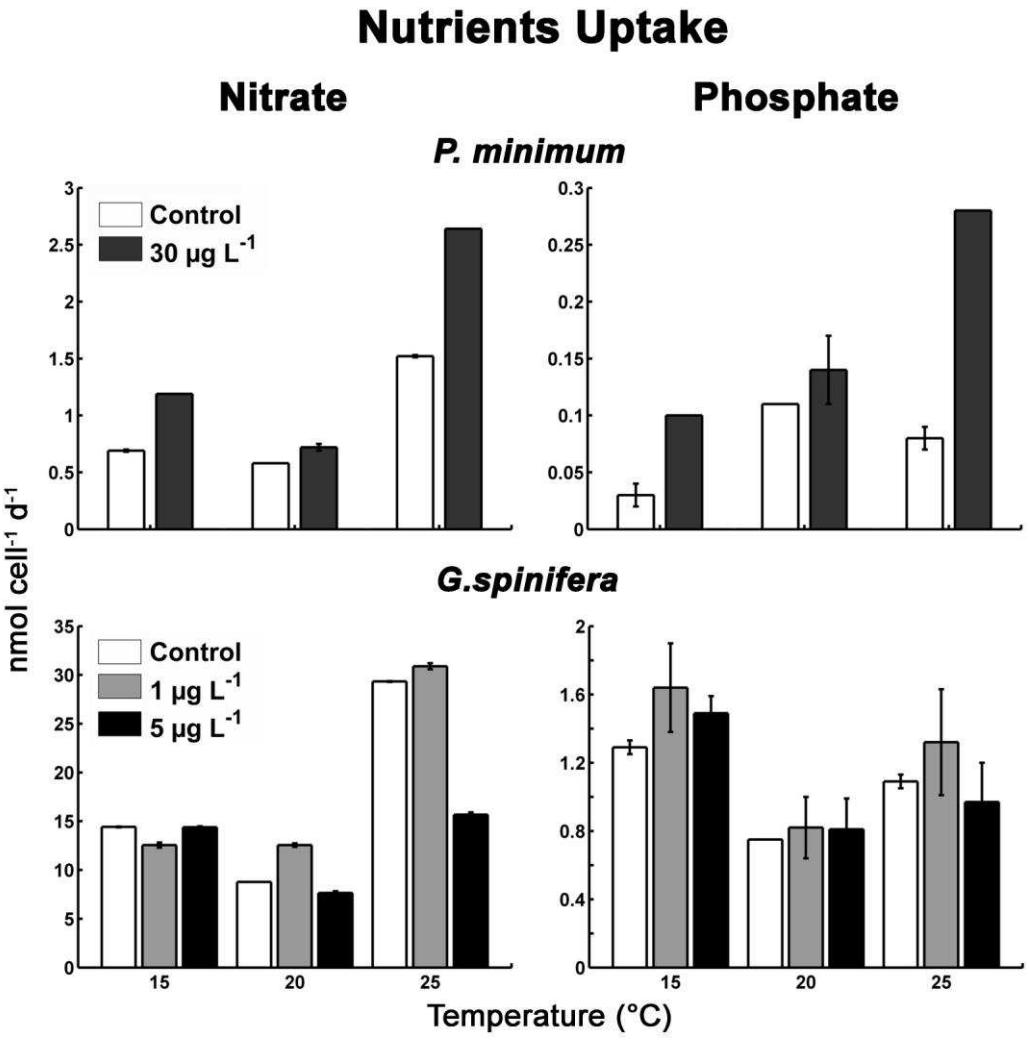

Fig. 9 Nutrients (N-NO_3 and P-PO_4) uptake (nmol cell^{-1} day^{-1}) for P. minimum and G. spinifera exposed to different terbuthylazine concentrations (P. minimum = 30 µg/L; G. spinifera = 1 and 5 µg/L) and temperature conditions (15, 20 and 25°C).

3.2.4 Macronutrient uptake

Nitrate and phosphate uptake rates were calculated from consumption measurements, monitored daily throughout the growth, and are reported in Fig. 9. An effect of TBA on nutrient uptake was observed for P. minimum (nitrate: $F = 173.4$, $p < 0.05$; phosphate: $F = 88.4$, $p < 0.05$). In the herbicide treatments both nitrate and phosphate uptakes were significantly enhanced at 15 and 25 °C. Statistical analysis underlined an interaction factor between temperature and TBA effects on nutrient uptake (nitrate: $F = 18.9$, $p < 0.05$; phosphate: $F = 55.1$, $p < 0.05$). The strongest herbicide effect on nutrient uptake was observed when P. minimum was grown at 25 °C and significant differences from the other temperature conditions (15 and 20 °C) were observed with multi-comparison procedures for both nitrate ($F = 106.5$, $p < 0.05$) and phosphate ($F = 67$, $p < 0.05$) uptake. G. spinifera did not show any significant difference in phosphate uptake, while for nitrate an interaction between temperature and TBA effects was observed ($F = 5.4$ $p < 0.05$). No difference in nitrate uptake was detected at 15 and 20 °C, while at the highest temperature (25 °C) the uptake significantly increased in the control and 1 µg L^{-1} TBA treatment compared to the other temperature conditions ($F = 5.4$, $p < 0.05$). At 25 °C cultures exposed to 5 µg L^{-1} TBA showed a significantly lower nitrate uptake than that measured in the control and the 1 µg L^{-1} treatment ($F = 7.3$, $p < 0.05$). Comparing the two flagellate species, we observed that P. minimum consumed all the nutrients available faster than G. spinifera. In particular, all the nutrients were taken up by P. minimum cells in 8 - 12 days of growth depending on temperature conditions, while in G. spinifera cultures the nutrients were detected for up to 8 - 12 days at the lowest concentration of TBA (1 µg L^{-1}) and up to 20 days when G. spinifera was exposed to 5 µg L^{-1} of herbicide (data not shown). However, the nutrient uptake rates (Fig. 9) calculated for the two species were 10 times greater in G. spinifera than in P. minimum. These high levels were due to the different cell volumes of the two species. In fact G. spinifera cell size is reported to be between 44 - 54 µm long and between 36 - 46 µm wide (Riccardi et al., 2009), while P. minimum is around 20 µm long and slightly smaller in width (Grzebyk and Berland, 1996; Heil et al., 2005). Cellular volumes calculated according to Hillebrand et al. (1999), were about 10 times smaller for P. minimum compared to G. spinifera.

4. Discussion

The purpose of this work was to study the sensitivity to TBA of the flagellate community of the Adriatic Sea, among which there are several harmful species and to investigate the changes in carbon and nutrient fluxes in response to the exposure to herbicides and increasing temperature conditions. In this study we have demonstrated that various flagellates (of the Dinophyceae and Raphidophyceae classes) typical of the plankton community of the Adriatic Sea are sensitive to low levels of the triazinic herbicide (TBA). Sensitivity and algal response to TBA exposure vary widely depending on the species and strain tested as was previously demonstrated for different classes (Bèrard and Benninghoff, 2001; DeLorenzo et al., 2004; Hoagland et al., 1993; Ma and Chen, 2005). Prorocentrum species were particularly tolerant to TBA, while Gonyaulax spinifera and Fibrocapsa japonica were the most sensitive flagellates, being slightly affected by the lowest concentration used in this study of 1 µg L^{-1}. A strong effect of TBA was observed on photosynthetic activity, measured using PAM fluorometry. As we know from available literature, the inhibition of PSII in phytoplankton organisms can be estimated using Pulse Amplitude Modulation (PAM) fluorometry which measures the absorbed light energy that cannot be used to drive electron transport and is dissipated as fluorescence. This fluorescence is then used to derive the effective Quantum Yield (Y(II)), a parameter proportional to the photosynthetic efficiency of PSII (Genty et al., 1989; Schreiber, 1986). Triazinic herbicides are known to be PSII inhibitors, acting biochemically by displacing a plastoquinone (Q_B) from its binding site in the D1 protein of photosystem II (PSII) (Hull, 1967). It thus proved to be a good method for the preliminary investigation of algal sensitivity to these herbicide classes, showing inhibitory effects from the first days of exposure, as previously reported (Choi et al., 2012).

With regard to the two species studied in more detail in this work (Prorocentrum minimum and G. spinifera), we observed that the growth rates were significantly reduced in herbicide treatments compared to the controls, as observed for other phytoplankton species by DeLorenzo et al. (2004) and Rioboo et al. (2002) albeit at a different level. Our results show that TBA effect on algal growth depends not only on herbicide concentrations but also on other factors, such as exposure times and temperature conditions, as reported in the literature (Guasch et al., 1997; Herman et al., 1986;

Mayasich et al., 1987; Stay et al., 1989). Gonyaulax spinifera cultures showed a prolonged lag phase when exposed to the highest TBA concentration (5 µg L^{-1}), reaching the stationary phase later than the control. After an inhibitory growth period, the growth rate in G. spinifera increased, in line with Rioboo et al. (2002) who reported the same pattern on Chlorella vulgaris. We also demonstrated that the effect of TBA is influenced by environment temperature conditions and by optimal growth values. In our experiments, the cosmopolitan species P. minimum was able to grow at all temperatures tested and herbicide effects on algal growth were observed under all conditions. The TBA effect increased at high temperature conditions (20 and 25 °C) where P. minimum achieved the highest cell densities (20 °C) and growth rates (25 °C). G. spinifera was able to efficiently grow only at high temperatures (20 and 25 °C), showing the maximum growth rate and cell numbers at 25 °C, while at 15 °C growth rates and photosynthetic activities were significantly reduced. Both herbicide concentrations tested (1 and 5 µg L^{-1}) showed an effect on algal growth, but no significant differences between control and herbicide treatments were observed at 15 °C. In fact, statistical analyses highlighted an interaction factor between temperature and herbicide effect on the growth rates. A common pattern was observed for both flagellates: the herbicide affected algal growth at all temperature conditions where the algae were able to grow. On the contrary the cyanobacterium Microcystis aeruginosa was greatly affected by the herbicide atrazine at 15 °C, a temperature that is suboptimal for growth (Chalifour and Juneau, 2011).

An algal response to TBA exposure was also observed in intracellular content (carbon, nitrogen, chlorophyll) and their ratios (carbon:nitrogen (C:N), chlorophyll:carbon (Chl:C)). Both flagellates increased the carbon and nitrogen content per cell, when exposed to TBA, especially in the first days of growth. No difference in nitrogen and carbon content was reported for G. spinifera grown at 15 °C. As reported by Rioboo et al. (2002), the C:N ratio significantly decreased in cultures exposed to TBA. Due to photosynthesis inhibition, the cells' exposure to herbicide would not have enough energy for cellular division. This could be the reason why carbon and nitrogen content increased per cell, although algal productivity (mg C L^{-1}) was significantly reduced, as observed in other studies (Bérard et al., 1999; DeLorenzo et al., 2004; Weiner et al., 2007). P. minimum did not show significant differences in cellular chlorophyll content between control and herbicide treatments, as was also found by DeLorenzo et al. (2004) for cryptophytes. When exposed to the highest TBA concentration (5 µg L^{-1}) G. spinifera showed an increase in Chl cell content at all temperature conditions. Examples of triazine-induced increases of the algal chlorophyll content were reported for Selenastrum capricornutum (Mayer et al., 1998), Chlorella vulgaris (Rioboo et al., 2002) and Amphydinium operculatum (DeLorenzo et al., 2004).This was interpreted as a tolerance mechanism (Francois and Robinson, 1990). In fact, responses such as the synthesis of thylakoid components are considered to be a general adaptation response to situations in which electron transport rate is strongly limited for photosynthesis (Behra et al. 1999). If the response induced by herbicide is similar to that observed during photoacclimation under low irradiance, the synthesis of chlorophyll-protein complexes is followed by an increase in the synthesis of reaction centers (Falkowski and Raven, 1996). Thus, the more photosynthetic targets available, the more atrazine would be required to block it, as suggested by DeLorenzo et al. (2004). An increased Chl:C ratio was observed for G. spinifera tested at both herbicide concentrations. Furthermore, this ratio was shown to increase with temperature. We can conclude for both species that temperature influenced the effects of TBA not only on algal growth but also in cellular components. Indeed, according to what was reported previously for growth rate, we found that changes in intracellular components were enhanced at temperatures optimal for the algae.

A previously unreported response to TBA exposure was observed for P. minimum concerning nutrient uptake. Both nitrate and phosphate uptake was significantly enhanced in the presence of the herbicide. Temperature also had an influence on nutrient uptake in presence of TBA and an interaction was observed between the two factors. The maximum nitrate and phosphate uptake was reached in herbicide treatments at the highest temperature (25 °C). G. spinifera did not show any difference in phosphate uptake, whereas nitrate uptake was increased only at 25 °C. The increase in nutrient uptake can allow the cells to synthesize intracellular components (i.e. proteins, chlorophyll, DNA, RNA) to be used in cellular division. This is probably a strategic response to TBA exposure used by P. minimum.

Not only the direct effects on algal growth, due to photoinhibition and decreases in cell densities, but also the indirect effects, such as increases in nutrient uptake, can be a selective factor in the phytoplankton community. In fact, nutrients

essential for algal growth are known to be limiting elements in a marine environment. In the presence of TBA, species that increase the nutrient uptake (i.e. P. minimum) can survive and adapt faster to the herbicide exposure, while other species might not have enough nutrients to supplement their growth. On the other hand, species that are highly inhibited by TBA have a better chance of surviving if they can find enough nitrogen to sustain the chlorophyll increase needed for a higher synthesis of PSII units. Thus the availability of N in the medium G. spinifera to synthesize Chl to counteract the herbicide action.

Other algal responses such as the increment in polysaccharide production, observed in G. spinifera following TBA exposure at 20 and 25 °C, could be interpreted as a resistance mechanism similar to what has been observed for different pollutants such as metals (Pistocchi et al., 1997).

More studies are required on the effect of TBA on marine phytoplankton species to understand the possible impact on the primary producers. Physiological responses should also be studied in order to understand different responses to TBA exposure, since the growth strategies of different species can lead to other indirect effects. However, environmental factors should also be taken into account. As demonstrated in this work, temperature conditions can play an important role on the effect of TBA on algal growth, and on the physiological responses of different species. The interaction of the herbicide effects with other environmental factors and the possible compound effects of different pollutants should also be studied in order to evaluate the real impact of this herbicide on the environment.

5. Conclusions

PSII inhibitors can act as a selective anthropogenic factor on algal assemblages since the sensitivity to this herbicide class is species-specific, as previously observed. In this paper we have also demonstrated that marine flagellates of the Adriatic Sea were affected differently by TBA exposure. This aspect can be rather important, especially since some toxic and/or bloom forming species were observed to be among the most resistant organisms, thus being able to outcompete other species. In addition, different survival strategies and adaptation processes adopted by different species (P. minimum and G. spinifera) were revealed, as well as the importance of the trophic conditions of the area in influencing the possibility of cellular recovery from the inhibitory effect of certain pollutants. Consequently, the use and an ongoing presence of TBA in coastal areas can have a major impact on the marine ecosystem, altering the diversity of the flagellate community and leading to possible changes in other components of the trophic web (i.e. grazers).

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Chapter 2

Effect of temperature on the sensitivity of the diatom Skeletonema marinoi (Bacillariophyceae) to herbicides

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1. Introduction

Diatoms are distributed globally and comprise the most abundant marine phytoplankton species. A dominant component of phytoplankton spring blooms, diatoms are at the base of the marine food chain providing the bulk of the food sustaining top consumers and fisheries. They significantly contribute to the global carbon cycle (Treguer et al., 1995) and are estimated to produce 20 to 25 % of world net primary production (Werner, 1977). In the Adriatic Sea diatoms represent the main component of the phytoplankton, especially in the late winter/early spring period (Acri et al., 2000; Bernardi Aubry et al., 2006; Bernardi Aubry et al., 2004).

The Northern Adriatic Sea is one of the most productive regions of the Mediterranean Sea; however phytoplankton biomass and production are highly variable in both space and time, since the dynamics of phytoplankton in coastal systems become more complex in areas subject to emission of large quantities of fresh water (Degobbis and Gilmartin, 1990). In particular, the total plankton biomass shows a strong decreasing gradient from the mouth of the Po River to the east, as well as from the Northern to the Southern Adriatic (Socal et al., 1992; Zavatarelli et al., 1998; Socal et al., 2002). The general trend of phytoplankton over the years shows that the main limiting factors for the growth of phytoplankton in the Adriatic coastal systems are represented not only by irradiance and temperature, but also by the strong influence of meteorological factors (Sangiorgi et al. 2005).

The North East area of Italy is an intensively farmed zone, mainly formed by the alluvional Po valley, where the use of herbicides has increased dramatically during the last two decades due to the intensive agriculture. Herbicide residues reach the aquatic environment through direct run-off and their concentrations follow a clear seasonal pattern (Vianello et al., 2005; Carafa et al., 2007). This particular area is subject to strong anthropogenic pressure as a consequence of the Po River influence and because of its shallow waters. Triazinic compounds, as simazine (SIM), terbuthylazine (TBA) and its degradation product desetil-terbuthylazine (D-TBA), are the most detected herbicide in this area, showing high concentration levels during spring periods (Boldrin et al., 2005; Sbrilli et al., 2005; Toulpakis et al., 2005; Kostantinou et al., 2006; Carafa et al., 2007). However other herbicide compounds are also detected, among which metolachlor (MET) also reaches high concentrations (Sbrilli et al., 2005; Vianello et al., 2005; Carafa et al., 2007). The increase in the concentration levels of TBA, and consequently of D-TBA, is related to the use of this herbicide as a substitute for atrazine, which is a banned compound in Italy for agriculture use (Tomlin, 1995). Navarro et al. (2004) reported a study on the persistence in the environment of triazinic herbicides. According to his results SIM was most readily degraded (with a half life of 29 - 49 days), while TBA was the most persistence showing longest half life (76 - 331 days).

Triazinic herbicides inhibit photosynthesis via blockage of the electron transport in the photosystem II (PSII) (Oettmeier, 1992; Lamoureux, 1998), while metolachlor (MET) is known to affect several physiological processes in cells, such as the lipid-protein synthesis thus indirectly inhibiting respiration and photosynthesis (Chesters et al., 1989).

In marine environments adverse effect of herbicides on non-target plants, as primary producers, can affect the structure of the ecosystem, resulting in oxygen depletion and decreased primary productivity; alterations of species composition in algal communities (DeLorenzo et al., 2001; Dorigo et al., 2004a,b; Coor and Kuckekorn, 2006) can also occur due to the different sensitivity among algal species (Fiori et al., in press). In addition it was observed that environmental variables, such as temperature and nutrients, can influence algal growth in the presence of herbicides as well as cellular responses adopted to counteract their toxic effects (Fiori et al., in press).
In this paper the effects of different herbicides on diatom carbon and chlorophyll content, growth and photosynthetic efficiency were studied. Skeletonema marinoi was selected as a test organism since it is the most abundant diatom in the Adriatic Sea. In addition this species is very similar to S. costatum, which has a worldwide distribution. It was exposed to herbicides (terbuthylazine (TBA), metolachlor (MET) and simazine (SIM)) and to TBA degradation product (desetil-terbuthylazine (D-TBA)) commonly detected in Italian surface and ground waters. In order to evaluate the interaction between temperature and herbicide effects, S. marinoi was grown at three temperature conditions (15, 20 and 25°C) and treated with different concentrations of TBA (1, 5, 10, 20 and 30 µg L^{-1}).

2. Materials and Methods

2.1 Phytoplankton cultures

Cultures of Skeletonema marinoi Sarno et Zingone (CCMP2497) were maintained in sterilized f/10 medium (salinity 35 psu) at 20 °C under a 16:8 h L:D cycle (74 µmol photons m^{-2} s^{-1} from cool white lamps). For experimental work, batch cultures were grown in a modified f/2 medium containing 116 µM N-NO_3 and 7.2 µM P-PO_4 (N:P = 16). Cultures were kept at a light intensity of 140 µmol photons m^{-2} s^{-1}, 12:12 h L:D cycle, and constant temperature conditions. Before the experiments started, cultures were allowed to acclimate to nutrients, light and temperature conditions by subculturing them for at least 2 weeks. Medium was prepared using filtered (GF/C Whatman) and sterilized (at 120 °C for 20 min) aged sea water (salinity 35 psu).

2.2 Herbicide solutions

For each herbicide (TBA, D-TBA, SIM and MET) 10 mg of the chemical compounds were dissolved in 10 ml of methanol. These solutions (1000 µg L^{-1}) were directly applied to the cultures at time 0 h. The final methanol concentration in the culture medium never exceeded 0.002%.

2.3 Experimental design

2.3.1 Experiment 1

The diatom Skeletonema marinoi (500 mL) was exposed to 3 concentrations (5, 15 and 30 µg L^{-1}) of different herbicides (TBA, MET and SIM) and a degradation product (D-TBA). The experiment was conducted in triplicate under constant light intensity (140 µmol photons m^{-2} s^{-1}) and temperature (20 °C) conditions.
A control was also run at two methanol concentrations (0.001 and 0.002%). Algal growth was followed daily as cell turbidity and PSII efficiency. Carbon and chlorophyll production was measured at the end of the experiment (day 9).

2.3.2 Experiment 2

S. marinoi cultures were exposed to different concentrations, from environmental to sub-lethal levels (1, 5, 10, 20 and 30 µg L^{-1}), of the most toxic herbicide (TBA) detected in experiment 1. Cultures were grown in 4 L volumetric flasks (3.5 L medium) under constant light intensity (140 µmol photons m^{-2} s^{-1}) and three temperature conditions (15, 20 and 25 °C) in a thermostat chamber. Each treatment was run in duplicate. The experiment ended when each culture reached the stationary phase. Different cultures grew for different lengths of time, due to temperature and herbicide effects. Carbon and chlorophyll content and nutrient (nitrate and phosphate) consumption were analyzed during the course of all the experiments. The culture volume daily withdrawn (5-100 mL) for sampling was not replaced. However, the remaining volume was never less than 30% of the initial volume.

2.4 Turbidity and Cell counting

Cell turbidity was recorded daily during experiment 1. Fresh samples (2 mL) were analyzed spectrophotometrically (UV/VIS Jasco 7800) at a wave length of 750 nm. Preliminary tests showed very high correspondence between cell counting and turbidity measurements.
Growth curves in experiment 2 were monitored every day with cell counting made in settling chambers following Utermohl's method (Hasle, 1978).

2.5 Pulse Amplitude Modulation (PAM) fluorometry

In experiment 1 and 2 the effective quantum Yield of PSII was measured every day through a Pulse

Amplitude Modulation fluorometer (101-PAM connected to a PDA-100 data acquisition system, H. Walz, Effeltrich, Germany) (Fiori et al., accepted).

2.6 Carbon and Nitrogen analysis

Carbon (C) and nitrogen (N) contents were measured at the end of experiment 1 (day 9), and every day in experiment 2. Volumes from 15 to 100 mL of algal cultures were filtered on Whatman glass microfiber filters (GF/F). The filters were dried at 450 °C in a muffle furnace for 4 hours before use. Analysis were conducted using a CHNS elemental analyzer (ThermoFisher, Flash 2000).

2.7 Chlorophyll analysis

Chlorophyll-a analysis were performed by collecting cells, from 15 to 100 mL of algal cultures, on cellulose filters (Millipore, pore size 0.45 µm). The pigments were extract in 15 mL of 90% acetone solution. Chlorophyll-a concentration (Chl) in the supernatant was analyzed spectrophotometrically according to Strickland and Parson (1972). Chl content was measured at day 9 in experiment 1, and every day in experiment 2.

2.8 Nutrient analysis (Nitrate, Phosphate)

Nitrate ($N\text{-}NO_3$) and phosphate ($P\text{-}PO_4$) concentrations in the medium were analyzed every two days in samples of filtered algal cultures (0.45 µm) from experiment 2 (only 1 replicate). Nitrates were reduced to nitrites in reduction columns filled with amalgamated cadmium and analyzed spectrophotometrically (543 nm), according to Morris and Riley (1963). Analyses of phosphate were conducted spectrophotometrically (885 nm) according to Murphy and Riley (1962).

2.9 Calculation

Cell densities data from experiment 2 were used to calculated specific growth rates (μ, d^{-1}) as:

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

Where N_0 and N_1 are cell density values at times t_0 and t_1.

Variations of the chlorophyll:carbon (Chl:C) ratio between TBA treatments and the controls are reported as bias, expressed in percentage (BIAS%), and calculated as:

$$BIAS\% = \frac{1}{N}\sum\left(\frac{R_t - R_c}{R_c}\right) * 100$$

R_t represents the Chl:C ratio measured in TBA treatments, while R_c is the ratio measured in the controls. N is the number of observations.

2.10 Statistical analysis

All statistical analyses were conducted using SPSS statistics program. Repeated measures ANOVA and Bonferroni multi-comparison test were applied on turbidity and photosynthetic efficiency data, collected in experiment 1. Herbicide effects on carbon and chlorophyll production, measured at day 9, were analyzed with 2-way-ANOVA and Bonferroni multi comparison test.

Differences in S. marinoi carbon and chlorophyll content, the Chl:C and C:N ratios were evaluated in experiment 2. Due to the discrepancies in the duration of experiments, repeated measures ANOVA was conducted until day 11.

3. Results

3.1 Experiment 1

The effects of different herbicides (TBA, MET and SIM) and a degradation product (D-TBA) on Skeletonema marinoi growth (cell turbidity) and PSII efficiency were examined daily during the experiment (Fig. 1). All pollutants tested significantly affected S. marinoi cell growth ($F = 65.5$, $p < 0.05$) and PSII efficiency ($F = 35.91$, $p < 0.05$). TBA treatments showed the highest inhibition on both parameters studied, and multi comparison tests underlined similarities between D-TBA, MET and SIM effects. In the presence of the herbicides (TBA, MET and SIM) S. marinoi growth and PSII efficiency significantly decreased at all concentrations tested (5, 15 and 30 µg L^{-1}) and the inhibition was enhanced with the increase of the concentrations applied. Only high concentrations (15 and 30 µg L^{-1}) of TBA degradation product (D-TBA) affected the parameters studied, while the lowest concentration (5 µg L^{-1}) did not differ from the control ($p > 0.05$). Effects on photosynthetic activity could be observed earlier than on cell turbidity. Strong inhibition of PSII was already reported at day 2 in cultures exposed to high concentrations (15 and 30 µg L^{-1}) of TBA. All the other pollutants affected PSII efficiency from day 5. Conversely, cell turbidity was affected only in the last 3 days of the experiment in all herbicide treatments.

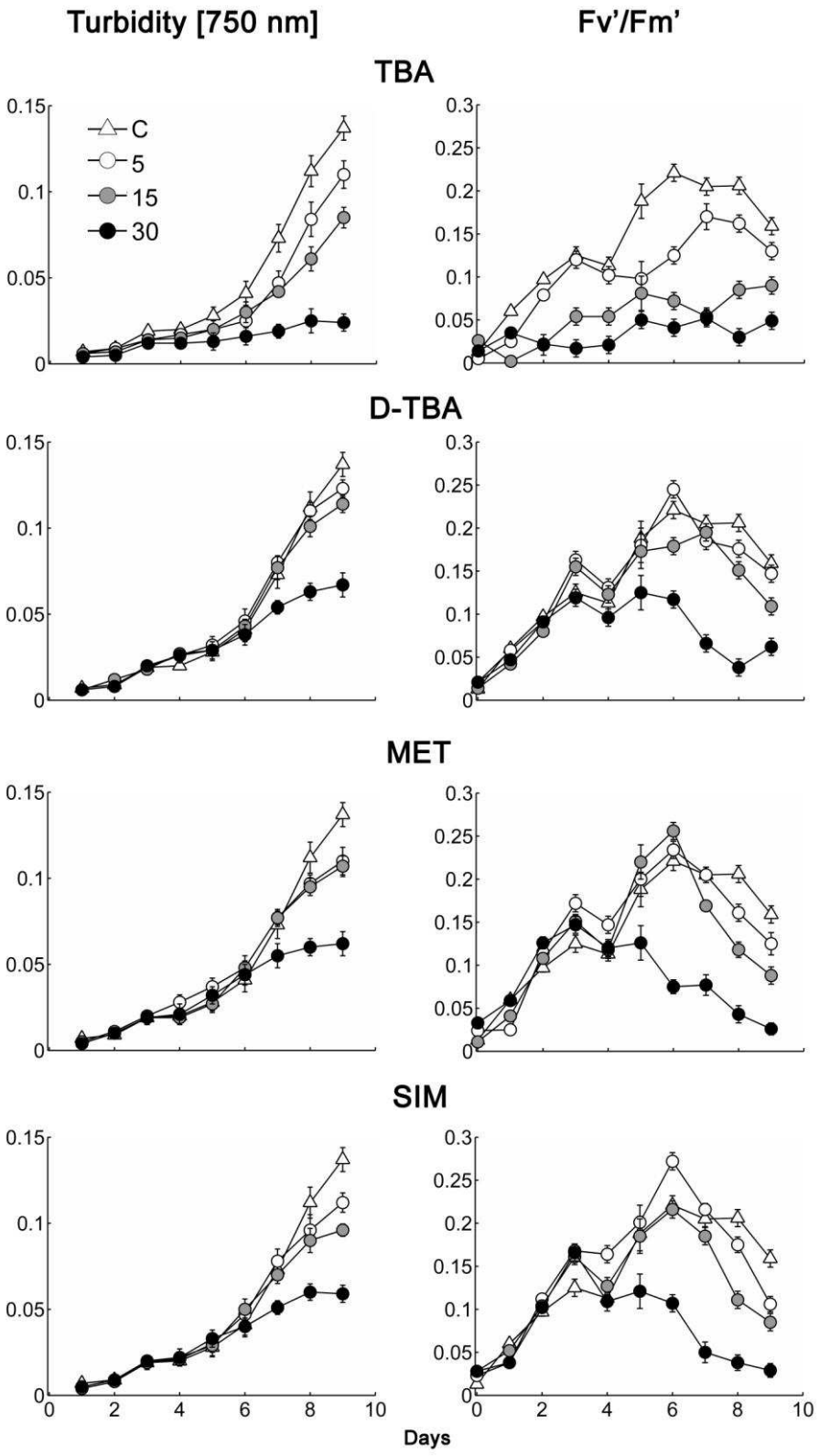

Fig. 1 S. marinoi growth, as cells turbidity (750 nm) and photosynthetic efficiency (Fv'/Fm'), in the controls and at three concentrations (5, 15 and 30 µg L^{-1}) of different herbicides (terbuthylazine (TBA), desetil-terbuthylazine (D-TBA), metolacholr (MET) and simazine (SIM)).

At the end of the experiment (day 9), carbon (C) and chlorophyll (Chl) contents of the cultures were measured in all treatments and utilized to determine the chlorophyll:carbon (Chl:C) ratio (Fig. 2). Chl:C ratio significantly ($F = 706.18$, $p < 0.05$) differed from the control in the herbicide treatments, except for cultures exposed to 5 µg L^{-1} of TBA and MET ($p > 0.05$). The Chl:C ratio reached the maximum level in cultures exposed to 15 µg L^{-1} TBA, due to both an increase of Chl content and a decrease in carbon production (data not shown). When S. marinoi was exposed at 15 µg L^{-1} of the other pollutants (D-TBA, MET and SIM) the Chl:C ratio was significantly lower than that of the control and it was significantly reduced in cultures exposed to 30 µg L^{-1} in all herbicide and D-TBA treatments.

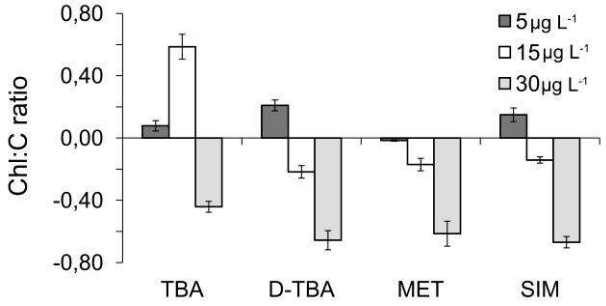

Fig. 2 Changes in S. marinoi chlorophyll:carbon (Chl:C) ratio exposed at three concentrations (5, 15 and 30 µg L^{-1}) of different herbicide treatments (terbuthylazine (TBA), desetil-terbuthylazine (D-TBA), metolacholr (MET) and simazine (SIM)) compared to the control, at day 9.

3.2 Experiment 2

Skeletonema marinoi was exposed to five concentrations of TBA and algal growth was measured daily as carbon content, C mg m^{-3} (Fig. 3). Algal growth was strongly inhibited in TBA treatments and effects were already reported during the first 11 days of the experiments. Carbon content significantly ($F = 731.46$; $p < 0.05$) decreased from the concentration of 10 µg L^{-1} of TBA, while no significant differences were reported between the controls and low concentrations (1 and 5 µg L^{-1}) at all the temperatures tested. Repeated measures ANOVA underlined significant ($F = 1066.47$; $p < 0.05$) differences between the three temperature conditions. The herbicide effects were enhanced with the increase of temperature: cultures exposed to 20 µg L^{-1} of TBA started to grow approximately 6 days later when kept at 25 °C compared to the cultures grown at 15 and 20 °C; at the concentration of 30 µg L^{-1} the diatom was able to grow only at the lowest temperature (15 °C). The growth curves based on carbon content were confirmed by cell counts performed every day (data not shown).

S. marinoi growth rates (µ, d^{-1}), based on cell counts, significantly ($F = 216.97$, $p < 0.05$) decreased as TBA concentrations were risen (Fig. 4). Herbicide effects on cell divisions were reported at lower concentrations compared to carbon content. Differences from the controls were reported from the concentration of 1 and 5 µg L^{-1}, respectively at 25 and 20 °C, while at 15 °C growth rates were significantly reduced only in cultures exposed at 20 ad 30 µg L^{-1}. Temperature significantly ($F = 54.88$, $p < 0.05$) affected growth rates in controls and TBA treatments. Higher growth rates were observed at 20 °C compared to 25 and 15 °C for controls and cultures exposed to low TBA concentrations (1and 5 µg L^{-1}). At 10 and 20 µg L^{-1} of TBA growth rates were not significantly different between 15 and 20 °C, while at 30 µg L^{-1} only cultures kept at 15°C were able to grow.

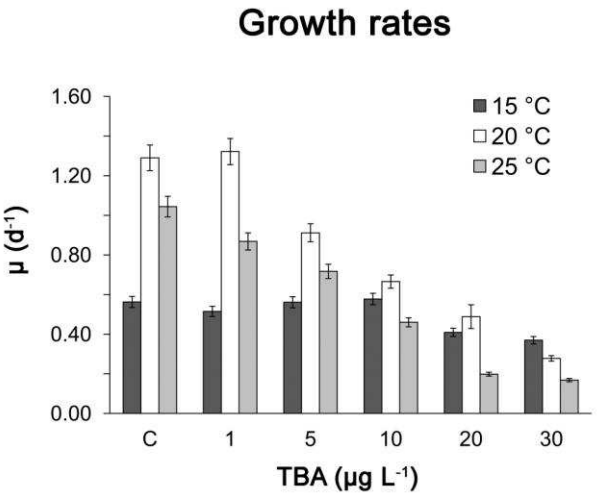

Fig. 4 S. marinoi growth rates measured in the controls and in the TBA treatments (1, 5, 10, 20 and 30 µg L^{-1}) at three temperature conditions (15, 20 and 25 °C).

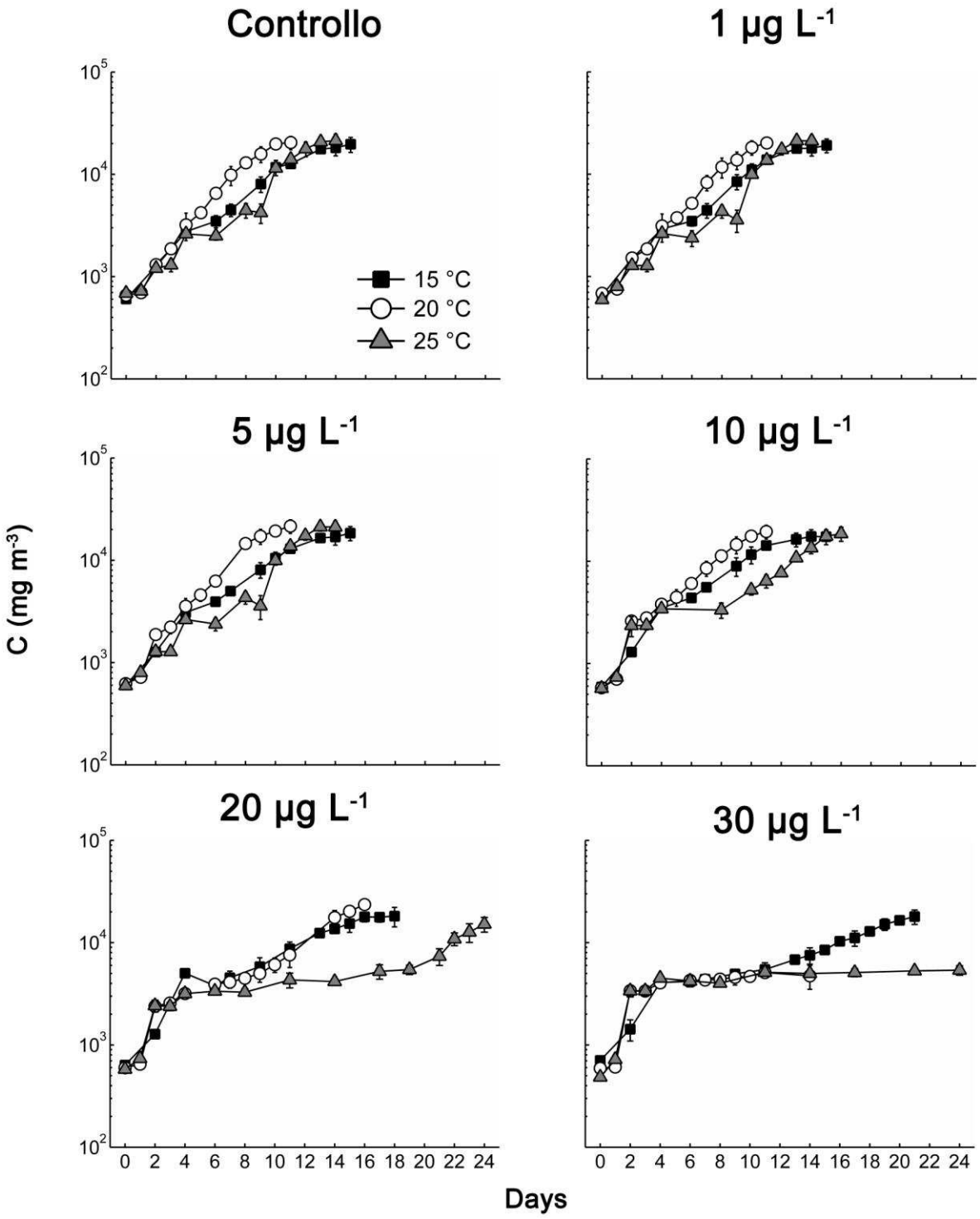

Fig. 3 S. marinoi growth, as carbon content (mg m^{-3}), exposed to five concentrations of TBA (1, 5, 10, 20 and 30 µg L^{-1}) and grown at three temperature conditions (15, 20 and 25 °C).

Effects of TBA were observed earlier on PSII efficiency than on algal growth (Fig. 5). Photosynthetic efficiency was inhibited from the lowest concentration tested (1 µg L^{-1}), and gradually decreased with the exposition to higher TBA concentrations (5, 10, 20 and 30 µg L^{-1}). Moreover the photoinhibition was stronger at high temperature conditions (20 and 25 °C), as reported for carbon production and cell density.

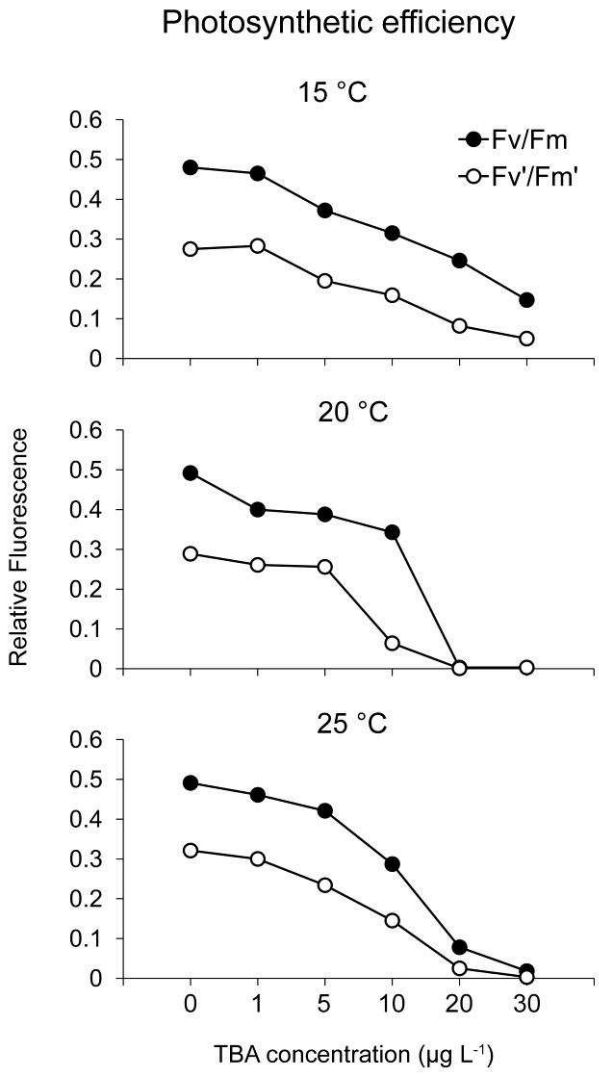

Fig. 5 Skeletonema marinoi PSII Maximum (Fv/Fm) and Effective Quantum Yields (Fv'/Fm') obtained in controls (0 µg L^{-1}) and in TBA treatments (1, 5, 10, 20 and 30 µg L^{-1}), measured at three temperature conditions (15, 20 and 25 °C).

At all temperature conditions chlorophyll content (mg m^{-3}) was significantly ($F = 147.38$, $p < 0.05$) higher in cultures at 5 and 10 µg L^{-1} of TBA compared to the control (Fig. 6), despite the lower cell concentrations. Temperature strongly influenced the effect of the herbicide on Chl content and significant ($F = 181.11$, $p < 0.05$) differences were observed between the temperatures studied (15, 20 and 25 °C). In cultures treated with 20 µg L^{-1} of herbicide Chl significantly increased only at 15 and 20 °C. When S. marinoi was exposed at 30 µg L^{-1}, Chl was significantly lower than that of the controls at high temperatures (20 and 25 °C), while at 15 °C Chl production was enhanced.

Variations in Chl:C and C:N ratios were also monitored during the experiments for all the conditions studied (Fig. 7, 8). For both parameters studied statistical analyses underlined significant (Chl:C, $F = 3.39$, $p < 0.05$; C:N, $F = 12.16$, $p < 0.05$) differences between the control and cultures exposed to TBA during the first 11 days of the experiments. Furthermore temperature strongly affected these ratios and significant (Chl:C, $F = 11.08$, $p < 0.05$; C:N, $F = 5.65$, $p < 0.05$) differences between the three conditions were observed.

At 15 °C the Chl:C ratio (Fig. 7) was increased in TBA treatments compared to the control, showing the maximum value of bias at 30 µg L^{-1} of TBA (+ 272.54 %). At 20 °C the Chl:C ratio was higher in cultures exposed to 10 and 20 µg L^{-1} of TBA (+90.72 % and + 284.03 %) than in the control. An increase in the Chl:C ratio was reported at 25 °C only at 5 µg L^{-1} of TBA (+39.03 %), while at higher concentrations (10, 20 and 30 µg L^{-1}) this ratio was lower than in the controls. However an increase in Chl:C ratio was reported in cultures exposed to 20 µg L^{-1} of TBA, from day 11 reaching levels similar to those of the control.

The C:N ratio (Fig. 8) decreased in cultures exposed to high TBA concentrations (10, 20 and 30 µg L^{-1}) and this effect was enhanced at high temperature conditions. No differences from the controls were reported at lower TBA concentrations (1 and 5 µg L^{-1}) at all temperature conditions. At 15 and 20°C no significant differences in the C:N ratio were observed between cultures exposed to 10 µg L^{-1} of TBA and the control while for those grown at 25°C lower values were observed starting from this concentration. Herbicide treatments (20 and 30 µg L^{-1}) grown at 20 and 25°C showed significant lower C:N ratios compared to the control. However, at 20 °C C:N ratio increased in cultures exposed to 20 µg L^{-1} of TBA after 12 days, reaching values comparable to the control.

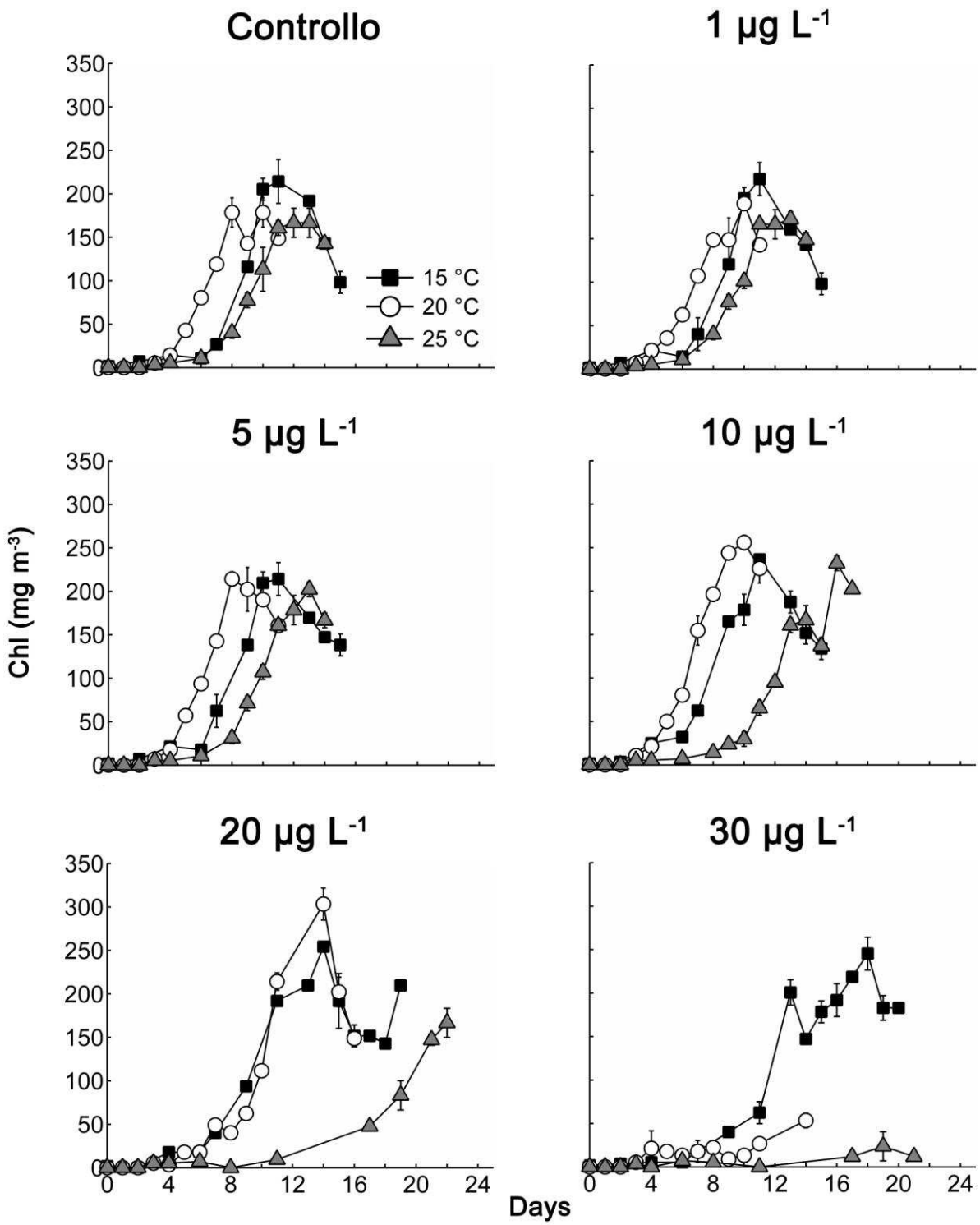

Fig. 6 Chlorophyll-a (Chl) content (mg m^{-3}) measured in S. marinoi cultures exposed to five concentrations of TBA (1, 5, 10, 20 and 30 µg L^{-1}) at three temperature conditions (15, 20 and 25 °C).

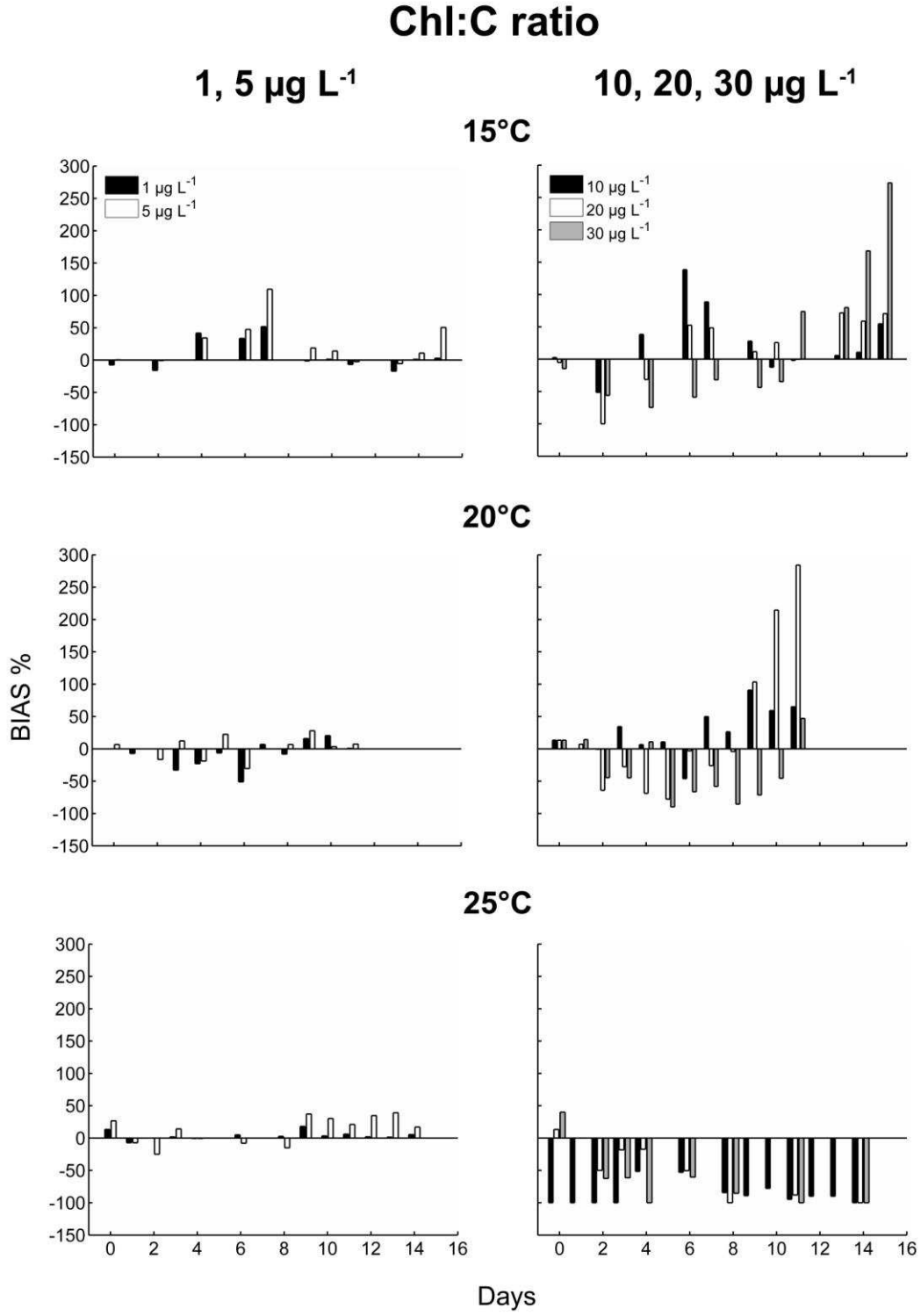

Fig. 7 Changes in the Chlorophyll:Carbon (Chl:C) ratio between the TBA treatments (1, 5, 10, 20 and 30 µg L^{-1}) and the controls, express as bias in percentage, at three temperature conditions (15, 20 and 25 °C)

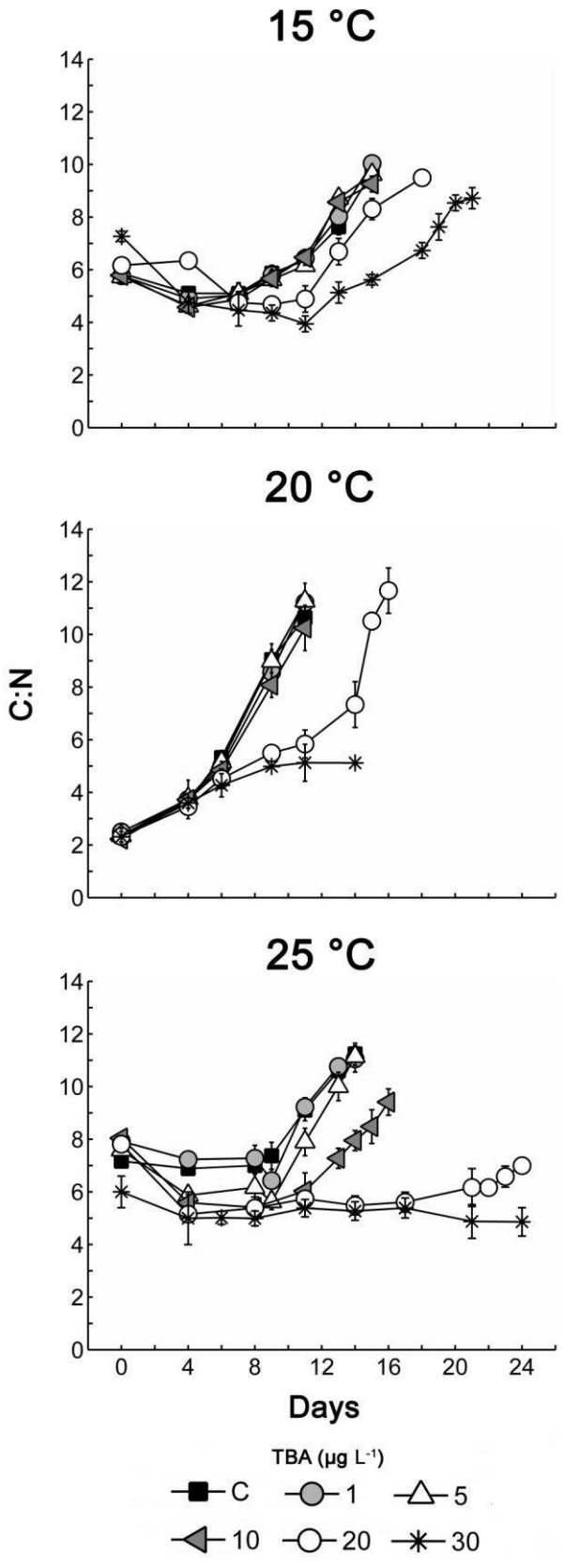

Fig. 8 Carbon:Nitrogen (C:N) ratio in the controls and TBA treatments (1, 5, 10, 20 and 30 µg L^{-1}) at three temperature conditions (15, 20 and 25 °C)

Nutrient consumption curves fell as the growth curves rose (Fig. 9). Nitrates were exhausted as the cultures entered the stationary phase and consumption was lower in cultures treated with high concentration of TBA (20 and 30 µg L^{-1}) which had reduced growth rates. On the contrary, phosphate consumption was faster in TBA treatments compared to the controls at all temperature conditions.

4. Discussion and Conclusions

Our study demonstrates that the diatom Skeletonema marinoi is sensitive to some of the most detected herbicides (TBA, SIM and MET) and degradation products (D-TBA) in Italian surface and ground waters.

Effects on cell growth and photosynthetic efficiency were observed for all pollutants tested. Furthermore PSII efficiency was inhibited earlier (day 2-5) than algal growth (day 7), and the effect was higher, attesting that photosynthetic efficiency is a more sensitive parameter to triazinic herbicides than algal growth. These results are in line with those of Macedo et al. (2008), who exposed Skeletonema costatum to the herbicide bentazon. In previous studies (Fiori et al., in press) we demonstrated that photosynthetic activity could be used as a valid parameter to assess earlier the toxicity of the triazinic herbicide TBA towards flagellate species.

TBA determined the highest inhibition on S. marinoi growth and PSII efficiency compared to the other herbicides (MET and SIM) at all concentrations tested (5, 15 and 30 µg L^{-1}). Moreover, S. marinoi was more sensitive to TBA than to its degradation product (D-TBA), which affected the diatom growth only at the concentration of 15 µg L^{-1} and higher. No information is reported in the literature regarding the effect of D-TBA on marine phytoplankton. Thus, it is important to take into account for future evaluation of the TBA effect on phytoplankton community the decrease in toxicity reported after TBA degradation, although TBA was shown to be very persistent in the environment (Navarro et al., 2004).

S. marinoi cultures exposed to 30 µg L^{-1} of the pollutants tested (TBA, SIM, MET and D-TBA) showed significantly lower chlorophyll:carbon (Chl:C) ratios compared to the control, due to a decrease in carbon but mainly in chlorophyll content of the cultures.

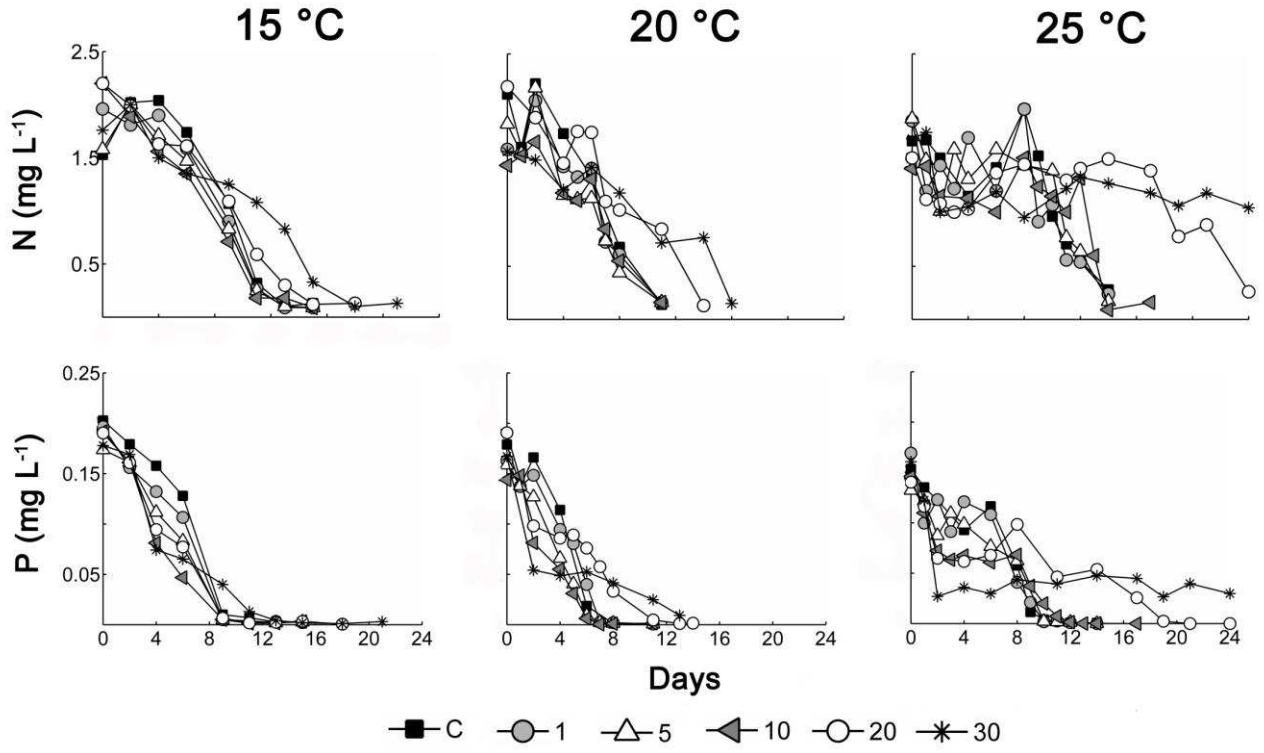

Fig. 9 Nutrients (Nitrates and Phosphates) consumption (mg L^{-1}) in the control and in TBA treatments (1, 5, 10, 20 and 30 L^{-1}).

Debenest et al. (2009) reported an inhibition in biomass (measured as chlorophyll concentration) when freshwater periphytic diatoms were treated with 30 µg L^{-1} of isoproturon and s-metolachlor.

In our study the exposition to triazinic compounds (TBA, SIM and D-TBA) stimulated the Chl production at the lowest concentration (5 µg L^{-1}) tested. At higher concentration (15 µg L^{-1}) the Chl:C ratio significantly increased only in TBA treatments. Positive variation of the Chl:C ratio compared to the control were reported at non-lethal TBA concentrations also for the flagellate Gonyaulax spinifera (Fiori et al., in press).

Due to the strongest effect of TBA on S. marinoi growth and PSII efficiency compared to the other pollutants, this algal species was exposed to TBA, from environmental to sub-lethal concentrations (1, 5, 10, 20 and 30 µg L^{-1}), at three temperature conditions (15, 20 and 25 °C).

As observed for other algal species (DeLorenzo et al., 2004; Rioboo et al., 2002; Fiori et al., in press) S. marinoi growth rates were significantly reduced in presence of TBA.

Furthermore, algal response to TBA exposition was strongly influenced by temperature conditions. High temperatures (20 and 25 °C) significantly enhanced the effect of TBA on the diatom growth and photosynthetic efficiency, as already reported for the flagellates P. minimum and G. spinifera (Fiori et al., in press). At 25 and 20°C growth rates significantly decreased starting from the concentration of 1 and 5 µg L^{-1} of TBA respectively, while at 15 °C significant effects were reported only at 20 and 30 µg L^{-1}. During the experiment a prolonged exponential growth phase was observed in cultures exposed to high concentrations of TBA (10, 20 and 30 µg L^{-1}) as reported for G. spinifera (Fiori et al., in press) and Chlorella vulgaris (Rioboo et al., 2002). As reported in experiment 1, PSII inhibition was observed at lower TBA concentrations compared to those affecting algal growth; i.e. at 15 °C effects on photosynthetic activity were reported from 5 µg L^{-1}. In this study it was also demonstrated that carbon and chlorophyll concentrations in the cultures were significantly affected by TBA exposition. In cultures treated with TBA Chl content was higher than in the controls from the concentration of 5 µg L^{-1}. This presumably represents an algal response to TBA, which acts on PSII, to compensate activity losses. This effect has already been reported in the literature for other algal species (Mayer et al.,

1998; Rioboo et al., 2002; DeLorenzo et al., 2004, Fiori et al., in press). However, as observed in experiment 1, Chl content decreased in cultures exposed to the highest concentration (30 µg L^{-1}), at high temperatures (20 and 25 °C) but not at 15 °C. TBA induced a decrease in carbon content from the concentration of 10 µg L^{-1} and as for the other parameters studied the effect was enhanced at high temperatures (20 and 25°C).

The described trends for carbon and chlorophyll concentrations correspond to variations of the Chl:C ratios in TBA treatments. The Chl:C ratio was higher in cultures treated with high TBA concentrations (10, 20 and 30 µg L^{-1}) than in the control, attesting that this species relies on the synthesis of new chlorophyll/protein complexes in order to counteract the decreased photosynthetic electron transport rate. A similar pattern was reported for different species (Rioboo et al., 2002, DeLorenzo et al., 2004) including the marine dinoflagellate G. spinifera exposed to lower TBA concentrations (Fiori et al., in press). However in S. marinoi, at high temperature (25 °C) and in the presence of 20 and 30 µg L^{-1} TBA, both nitrate uptake and chlorophyll synthesis were greatly reduced. This led to a C:N ratio significantly lower compared to the control, as also observed by Rioboo et al., (2002) for Chlorella vulgaris.

At these high TBA concentrations S. marinoi increased the phosphates uptake during the first days of growth. This response to TBA exposition was also observed for the flagellates P. minimum (Fiori et al., in press) when grown at high temperatures. The increase in phosphate uptake can be interpreted as a response aimed at the acquisition of high ATP levels to be used in cellular responses, however the overall cellular metabolism was altered and cell divisions were greatly inhibited.

The effect of triazinic herbicides on algal growth and intracellular components was already reported to vary depending on algal species (DeLorenzo et al., 2004; Ma and Chen, 2005). However marine species are less studied, although they have a fundamental role in the marine food chain.

The diatom tested represents an important primary producer in the Adriatic Sea reaching the highest cell numbers mainly at the end of winter, when temperature is below 20 °C (Bernardi Aubry, 2006). S. marinoi was quite resistant to TBA exposition at 15 °C, being affected only at the highest concentration tested. Diatom community species were in fact reported to be more resistant to s-triazine herbicides than other phytoplankton species (Berard and Pelte, 1996; Beraard et al., 2003 Guash et al., 1998; Debenest et al., 2009) although the effect of temperature is not widely discussed in the literature. Nevertheless, in the Adriatic Sea the presence of this species is reported also during summer months when temperature conditions are higher than 15 °C (Bernardi Aubry, 2004, 2006). At 20 and 25 °C a significant increase in S. marinoi sensitivity to TBA was reported, with effects on growth and PSII efficiency from the lowest concentrations tested. During summer months usually dinoflagellates are also present in the study area and harmful species such as Prorocentrum minimum displayed a much lower sensitivity to TBA (Fiori et al., in press).

Therefore, it is important to consider possible additional factors, i.e. temperature, that can enhance the herbicide effects. The interaction between pollution and global warming could thus lead to a shift in phytoplankton composition, with a possible decrease in the number of important species for primary production as Skeletonema marinoi.

6. Acknowledgements

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Chapter 3

Modelling of the effects of the herbicide terbuthylazine on phytoplankton

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1. Introduction

Coastal marine ecosystems have complex physiological-biological dynamics and provide "goods and services" (Costanza et al., 1997) relevant for socio-economical systems. The non-linear dynamics of the ecosystem components make the modeling a challenging effort. Superposed this natural complexity is the anthropogenic pressure, the effects of which are often poorly known. The lack of specific information constrains model improvement and parameterization. As a result, there is an increasing need to better understand the effects of anthropogenic factors on marine ecosystem and their interactions with the environmental stressors, to develop ecosystem models able to assess the effect of anthropogenic impacts in a complex system.

This work is focusing on the modelisation of algal responses to the exposure to the herbicide terbuthylazine (TBA). TBA belongs to the triazine compounds family and is one of the most abundant herbicides found in Italian rivers and ground waters. The occurrence of TBA in coastal marine ecosystems and its effects on phytoplankton communities are described in detail in Chapter 1 (section 1) and 2 (section 1).

The aims of this study are: (1) to establish a generic model description of the effect of TBA on three algal species: Skeletonema marinoi, Prorocentrum minimum and Gonyaulax spinifera, based on the results obtained from laboratory experiments (Chapter 1 and 2); (2) simulate algal response to increasing TBA concentrations and at concurrently increasing temperature conditions.

2. Materials and Methods

2.1 Experimental data

The experimental laboratory data, discussed in Chapter 1 and 2, constitute the observational base for the modeling effort. Three phytoplankton species typical of the northern Adriatic Sea were selected: the diatom Skeletonema marinoi, and the flagellates Prorocentrum minimum and Gonyaulax spinifera. Laboratory cultures of the above species were carried out according to the experimental design schematized in Fig. 1. Cultures were performed at the Redfield N:P ratio, at constant Photosintetically Active Radiation, PAR (143 µE m^{-2} s^{-1}), and at different temperatures (15, 20 and 25 °C), in order to mimic the Adriatic seasonal thermal variability. Cultures were exposed to different TBA concentrations depending on the species tested, from environmental to inhibiting concentrations (as described in Chapters 1 and 2). S. marinoi was tested at 1, 5, 10, 20 and 30 µg l^{-1} of TBA; Prorocentrum minimum at 30 µg l^{-1} because it showed a strong resistance to this pollutant; and Gonyaulax spinifera was exposed to 1 and 5 µg l^{-1} of TBA because it was the most sensitive species tested. Control cultures were also run for each species without TBA exposure (0 µg l^{-1} in Fig.1).

Phytoplankton carbon (C) and chlorophyll-a (Chl) concentrations were measured daily. Nutrients (phosphate and nitrates) levels were monitored during the experiments. All these measurements were also taken at the start of the experiments to provide the model initial conditions.

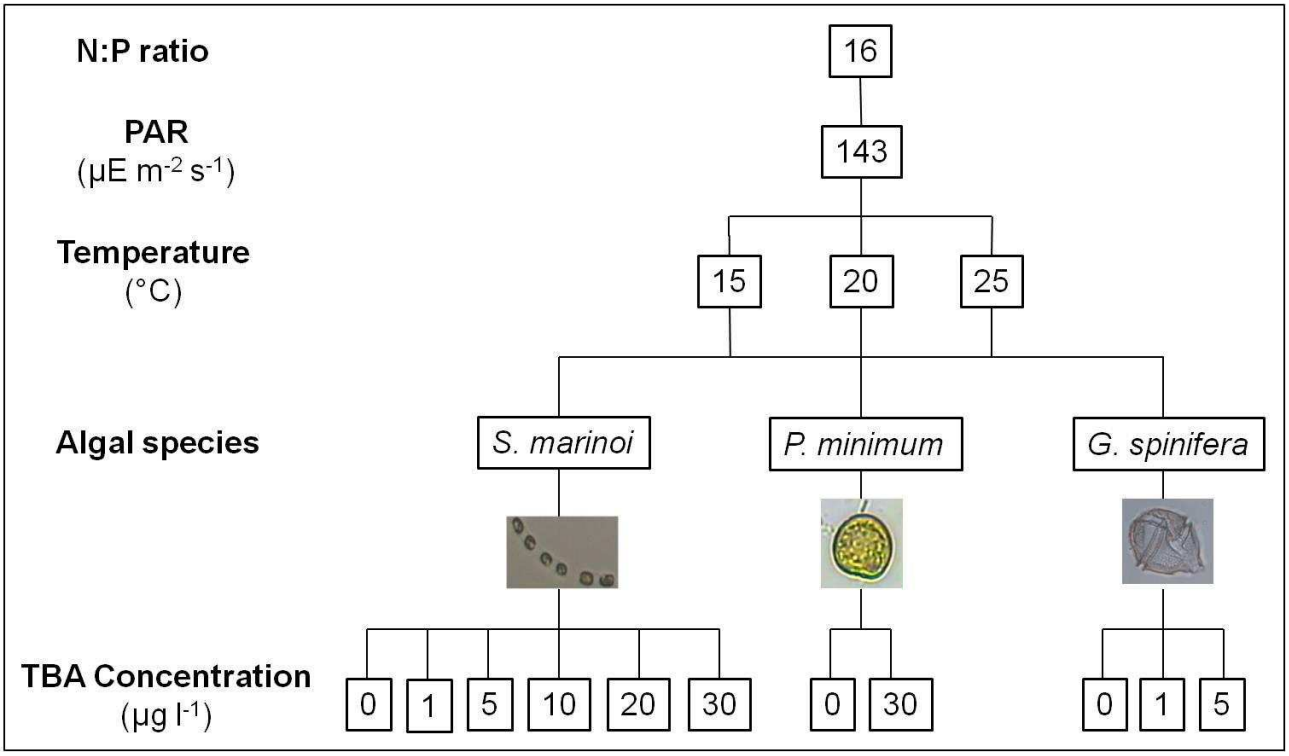

Fig. 1 Experimental design scheme. Three phytoplanktonic species, the diatom Skeletonema marinoi, and the two flagellates Prorocentrum minimum and Gonyaulax spinifera, were exposed to different concentration of the herbicide terbuthylazine (TBA), at three temperature conditions (15, 20 and 25 °C). Controls were run for each species (TBA = 0 µg l^{-1}).

2.2 The biogeochemical model

The model used in this study is the "zero" dimensional (ØD) version of the Biogeochemical Flux Model (BFM, Vichi et al., 2006). BFM (Fig. 2) is a complex lower trophic level marine biogeochemical model. It is a biomass based model, designed to simulate the main marine biogeochemical fluxes through the description of the ecological functions of producers, decomposers and consumers and their specific trophic interactions in terms of basic elements (carbon, nitrogen, phosphorous, silicon and oxygen) flows. The model biological constituents are organized into Chemical functional Families (CFF) and Living Functional Groups (LFG) (Fig. 3). CFFs are divided in organic (living and non-living) and inorganic compounds, and they are measured in equivalents of major chemical elements or in molecular weight units. LFGs are designed as a standard organism (Fig. 4), whose biomass is composed of living CFFs and interact with non-living CFFs through physiological (e.g. photosynthesis) and ecological processes (e.g. predation). Each LFG is mathematically expressed by a multi-dimensional array that contains the concentrations of the living CFF based upon biogeochemical elements. The complete CFF listing and description is given in Table 1.

A BFM simplification was adopted in order to reproduce laboratory cultures of Chapters 1 and 2: only phytoplankton groups ($P_i^{(n)}$) were considered. A schematic view of the simplified model is given in Fig. 5.

The phytoplankton functional groups considered are: diatoms ($P_i^{(1)}$, 20 – 200 µm), autotrophic nanoflagellates ($P_i^{(2)}$, 2 - 20 µm) and large "non-diatom" phytoplankton ($P_i^{(4)}$, 20 – 200 µm). In particular Skeletonema marinoi is representative of the diatom group, Prorocentrum minimum is related to the nanoflagellates and Gonyaulax spinifera is considered as large phytoplankton species. Diatoms are the main source of biogenic silica in the model and differ from the other groups because their growth can be limited by dissolved silicate (Si). All the three phytoplankton groups are described with the same equation and differentiated by different values of the physiological parameters, which are listed in Table 2 for each functional group.

For a generic phytoplankton state variable P there are five living CFFs that describe the biomass (C, N, P, Si and Chl) and thus for each group we have 4 or 5 equations (Vichi et al., 2007).

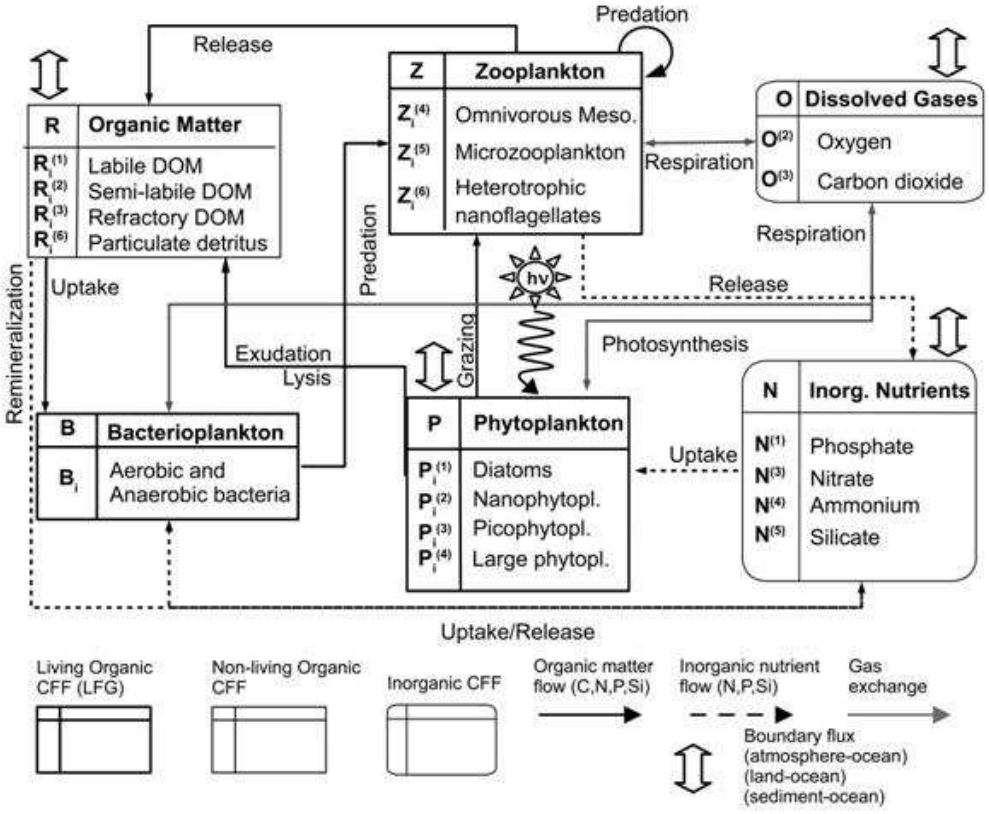

Fig. 2 General overview of the matter fluxes between the BFM state variables. Square boxes represent the model functional groups exchanging Carbon (C), Nitrogen (N), Phosphorus (P), Silicon (Si) and Oxygen (O). Organic matter (C, N, P, Si) flows are indicated by solid black arrows; N, P and Si nutrient uptake/remineralisation flows are represented by the dashed black arrows. Solid grey arrows mark the gas C (Carbon dioxide) and O flows. Purely biochemical processes are indicated by the dashed grey arrows. Small double arrows above the boxes mark boundary (water-atmosphere and water–sediment) flow. See Table 1 for state variables description.

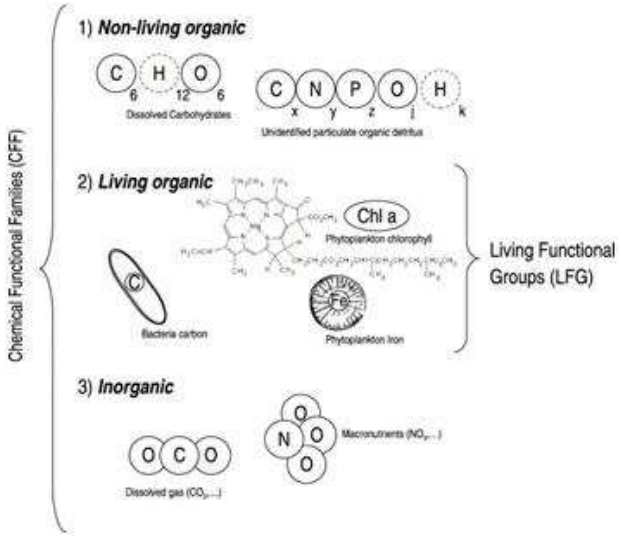

Fig. 3 Chemical functional families (CFF) expressed in term of basic biogeochemical elements. Living organic CFFs are the basis for the modeling of Living functional Groups (LFGs) (Vichi et al., 2007).

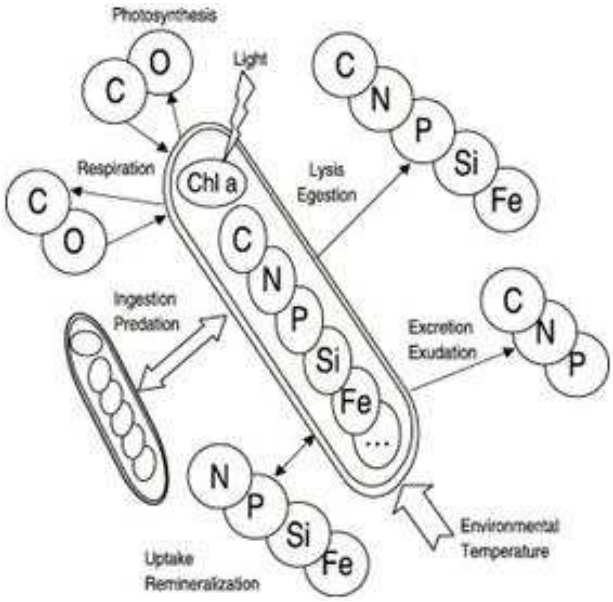

Fig. 4 Scheme of the standard organism, which is a prototype of any Living Functional Group (LFG), and the physiological/trophic relationships among the Chemical Functional Families (CFF) and major environmental forcing (Vichi et al., 2007).

Variable	Type	Components	# of CFFs	Description	Reference
$N^{(1)}$	IO	P	1	Phosphate (mmol P m^{-3})	Baretta et al., 1995
$N^{(3)}$	IO	N	1	Nitrate (mmol N m^{-3})	"
$N^{(4)}$	IO	N	1	Ammonium (mmol N m^{-3})	"
$N^{(5)}$	IO	Si	1	Silicate (mmol Si m^{-3})	"
$N^{(6)}$	IO	R	1	Reduction equivalents, HS^- (mmol S m^{-3})	Vichi et al., 2004
$N^{(7)}$	IO	Fe	1	Dissolved iron (μmol Fe m^{-3})	Vichi et al., 2007
$O^{(2)}$	IO	O	1	Dissolved oxygen (mmol O_2 m^{-3})	Baretta et al., 1995
$O^{(3)}$	IO	C	1	Carbon dioxide (mg C m^{-3})	–
$P_i^{(1)}$	LO	C N P Si Fe Chl	6	Diatoms (mg C m^{-3}, mmol N–P–Si m^{-3}, μmol Fe m^{-3} and mg Chl-a m^{-3})	Varela et al., 1995; Ebenhöh et al., 1997; Baretta-Bekker et al., 1997; Vichi et al., 2007
$P_i^{(2)}$	LO	C N P Fe Chl	5	Flagellates (")	"
$P_i^{(3)}$	LO	C N P Fe Chl	5	Picophytoplankton (")	"
B_i	LO	C N P	3	Pelagic bacteria (")	Baretta-Bekker et al., 1995; Baretta-Bekker et al., 1997
$Z_i^{(4)}$	LO	C N P	3	Omnivorous mesozooplankton (")	Broekhuizen et al., 1995; Vichi et al., 2007
$Z_i^{(5)}$	LO	C N P	3	Microzooplankton (")	Baretta-Bekker et al., 1995; Baretta-Bekker et al., 1997; Vichi et al., 2007
$Z_i^{(6)}$	LO	C N P	3	Heterotrophic Flagellates (")	"
$R_i^{(1)}$	NO	C N P	3	Dissolved organic detritus (")	Baretta et al., 1995 and Vichi et al., 2003a
$R_i^{(6)}$	NO	C N P Si Fe	5	Particulate organic detritus (")	"

Type legend: IO = Inorganic; LO = Living organic; NO = Non-living organic. The subscript i indicates the basic components (if any) of the CFF, e.g. $P_i^{(1)} \equiv (P_c^{(1)}; P_n^{(1)}; P_p^{(1)}; P_s^{(1)}; P_l^{(1)}; P_f^{(1)})$.

Table 1 List of the Chemical Functional Family state variables (CFF, for a total of 44 prognostic equations) for the pelagic model and references to the original publications

Symbol	S. marinoi	P. minimum	G. spinifera	Description
T_{ref}	20	25	25	Reference temperature for f^T computation (Q_{10} based) (°C)
r_{0_p}	1.80	1.02	0.83	Maximum specific photosynthetic rate at T_{ref} temperature (d^{-1})
Q_{10_p}	2.00	2.00	2.00	Characteristic Q_{10} coefficient
$h^S_{P(1)}$	0.30	-	-	Half saturation value for Si-limitation (mmol Si m^{-3})
b_P	0.01	0.01	0.05	Basal specific respiration rate (d^{-1})
γ_P	0.10	0.025	0.05	Activity respiration gpp fraction (-)
β_P	0.05	0.025	0.15	Excreted fraction of primary production (-)
d_{0_p}	0.01	0.01	0.01	Maximum specific lysis rate (d^{-1})
a_1	$2.50\ 10^{-3}$	$2.50\ 10^{-3}$	$2.50\ 10^{-3}$	Specific affinity constant for P ($mg\ C^{-1}\ d^{-1}$)
a_3	$1.00\ 10^{-3}$	$1.00\ 10^{-3}$	$2.50\ 10^{-3}$	Specific affinity constant for N-NO_3 and N-NH_4 ($mgC^{-1}\ d^{-1}$)
$n_P^{min}, n_P^{opt}, n_P^{max}$	$1.26\ 10^{-2}$ × (0.3, 1, 2)	$1.26\ 10^{-2}$ × (0.3, 1, 2)	$1.26\ 10^{-2}$ × (0.3, 1, 2)	Minimum, optimal and maximum nitrogen quota (mmol N mgC^{-1})
$p_P^{min}, p_P^{opt}, p_P^{max}$	$7.86\ 10^{-4}$ × (0.25, 1, 2)	$7.86\ 10^{-4}$ × (0.25, 1, 2)	$7.86\ 10^{-4}$ × (0.5, 1, 2)	Minimum, optimal and maximum phosphorus quota (mmol P mgC^{-1})
α^0_{chl}	$0.70\ 10^{-5}$	$0.70\ 10^{-5}$	$6.80\ 10^{-6}$	Maximum light utilization coefficient (mgC mg chl^{-1} µE $m^{-2}\ s^{-1}$)
θ^0_{chl}	0.05	0.07	0.05	Maximum chl:C quotum (mg chl mg C^{-1})
δ	0.50	0.10	0.90	Empirical parameter for chl losses (d^{-1})
$f^{n,p}_{lim}$	0.90	0.70	0.90	Nutrient stress threshold for chl losses (-)

Table 2 Symbols, standard values and description of the phytoplankton parameters. S. marinoi = diatoms; P. minimum = autotrophic nanoflagellates; G. spinifera = large "non-diatom" phytoplankton

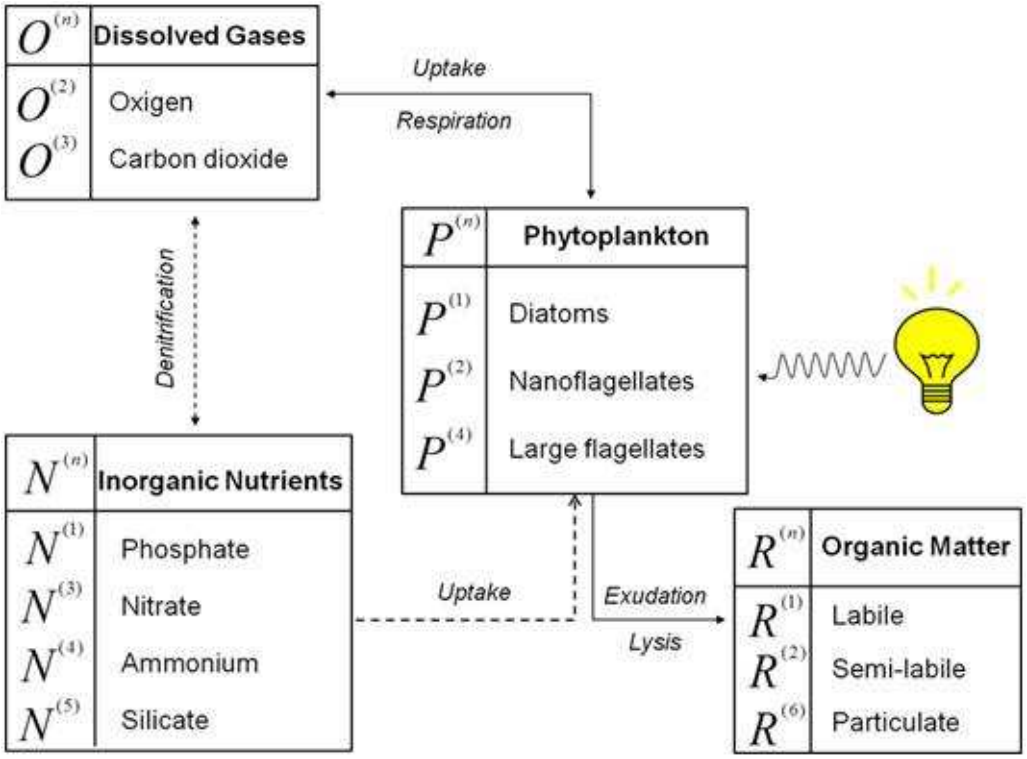

Fig. 5 Schematic representation of the Biogeochemical Fluxes Model (BFM) simplification used in this study, in order to simulate the laboratory experiments. See Table 1 for state variables description and caption of Fig. 2 for the flow definition.

The differential equations describing the CFF's dynamics are listed below.
The superscripts are the abbreviations indicating the processes which determine the variation (spelled in Table 3). The subscripts are the CFF state variables involved in the process (Table 1). All the parameters appearing in the equations below are listed and spelled out in Table 2. As mentioned above, the main model characteristics are described in detail in Vichi et al., 2006. We provide a detailed description of the equation describing the carbon ad chlorophyll dynamics. Since some modifications (with respect to the original formulations) were introduced based on the laboratory experiment.

Abbreviation	Comment
gpp	Gross primary production
rsp	Respiration
prd	Predation
rel	Biological release: egestion, excretion
exu	Exudation
lys	Lysis
syn	Biochemical synthesis
nit/denit	Nitrification, denitrification
scv	Scavenging
rmn	Biochemical remineralization
upt	Uptake

Table 3 List of all the abbreviations used to indicate the physiological and ecological processes

Carbon dynamics:

$$\frac{\partial P_c}{\partial t} = \left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{gpp} - \left.\frac{\partial P_c}{\partial t}\right|_{R_c^2}^{exu} - \left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{rsp} - \left.\frac{\partial P_c}{\partial t}\right|_{R_c^{(1,2,6)}}^{lys} \quad (1)$$

Where:

$$\left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{gpp} = f^T f^E f^s r^0 P_c \quad (2)$$

Equation (2) describes the Gross Primary Production (gpp) rate of change of phytoplankton carbon P_c due to photosynthesis. The "f" terms indicate the functions providing temperature (f^T; Q_{10} based), light (f^E; Platt et al., 1980) and nutrients (nitrate, phosphate and silicate, $f^{n,p,s}$; based on Liebig principle) environmental regulation. The term r^0 represent the maximum specific photosynthetic growth rates, which were obtained from the experimental data (see Chapter 1 and 2), and are listed in Table 2.

$$\left.\frac{\partial P_c}{\partial t}\right|_{R^{(2)}}^{exu} = \left[\beta + \left(1-\beta\right)\left(1-f^{n,p}\right)\right]\left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{gpp} \quad (3)$$

$$\left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{resp} = \left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{Arsp} + \left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{Brsp} \quad (4)$$

$$\left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{Arep} = \gamma\left(\left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{gpp} - \left.\frac{\partial P_c}{\partial t}\right|_{R_c^{(2)}}^{exu}\right) \quad (5)$$

$$\left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{Brsp} = f^T b_p P^C \quad (6)$$

$$\left.\frac{\partial P_c}{\partial t}\right|_{R_C^{(1,2,6)}}^{lys} = \frac{1}{f^{p,n} + h^{p,n}} d^0 P_C \quad (7)$$

Chlorophyll dynamics:

Synthesis of chlorophyll is essentially based on Geider et al. (1996, 1997).

$$\frac{\partial P_{chl}}{\partial t} = \left.\frac{\partial P_{chl}}{\partial t}\right|_{chl}^{syn} - \left.\frac{\partial P_{chl}}{\partial t}\right|_{chl}^{loss} \quad (8)$$

The equation (8) was modified in this study by introducing a synthesis reduction term, due to activity respiration $\left(-\left.\frac{\partial P_c}{\partial t}\right|^{Arsp}\right)$ and exudation $\left(\left.\frac{\partial P_c}{\partial t}\right|^{exu}\right)$.

$$\left.\frac{\partial P_{chl}}{\partial t}\right|_{chl}^{syn} = \rho_{chl}\left(\left.\frac{\partial P_c}{\partial t}\right|^{gpp} - \left.\frac{\partial P_c}{\partial t}\right|^{Arsp} - \left.\frac{\partial P_c}{\partial t}\right|^{exu}\right) \quad (9)$$

With the dynamic Chl:C ratio:

$$\rho_{chl} = \theta^0_{chl}\frac{f^{n,p} f^E r^0 P_c}{\alpha^0_{chl} E_{PAR} P_{chl}} \quad (10)$$

Where θ^0_{chl} is the maximum potential Chl:C ratio.

The losses of Chl are defined as follow:

$$\left.\frac{\partial P_{chl}}{\partial t}\right|_{chl}^{loss} = P_{chl}\left[\frac{1}{P_c}\left(\left.\frac{\partial P_c}{\partial t}\right|^{lys} + \left.\frac{\partial P_c}{\partial t}\right|^{Brsp}\right) + \delta \max\left(0, f_{\lim}^{n,p} - f^{n,p}\right)\right] \quad (11)$$

in order to introduce chlorophyll losses depending on phytoplankton lysis, respiration and on nutrient limitation. The former term was suggested by the results of phytoplankton cultures described in Chapters 1 and 2. δ is an empirical parameter tuned, for each phytoplankton functional group, on the basis of the laboratory cultures. $f_{\lim}^{n,p}$ is the nutrient limitation threshold value.

Nutrient dynamics:

The nutrient dynamics were implemented without modifications as described in Vichi et al. (2006).

$$\frac{\partial P_n}{\partial t} = \sum_{i=3,4} \left.\frac{\partial P_n}{\partial t}\right|_{N^{(i)}}^{upt} - \left.\frac{\partial P_n}{\partial t}\right|_{R_n^{(1,6)}}^{lys} \quad (12)$$

$$\frac{\partial P_p}{\partial t} = \left.\frac{\partial P_p}{\partial t}\right|_{N^{(1)}}^{upt} - \sum_{j=1,6} \left.\frac{\partial P_p}{\partial t}\right|_{R_p^{(6)}}^{lys} \quad (13)$$

$$\frac{\partial P_s}{\partial t} = \left.\frac{\partial P_s}{\partial t}\right|_{N^{(5)}}^{upt} - \left.\frac{\partial P_s}{\partial t}\right|_{R_s^{(6)}}^{lys} \quad (14)$$

If $P_s = P_s^{(1)}$ otherwise $\frac{\partial P_s}{\partial t} = 0$

2.3 Herbicide parameterization

The parameterization of TBA effects (f^h) was introduced in the equation of Gross Primary Production (gpp).

$$\left.\frac{\partial P_c}{\partial t}\right|_{O^{(3)}}^{gpp} = f^h f^T f^E f^s r^0 P_c \quad (15)$$

Therefore, the newly introduced regulating factor accounts for the TBA limitation on gpp. The laboratory experiments indicated that phytoplankton production is limited by increasing TBA concentrations (C_{TBA}) and that the effects are magnified by increasing temperature conditions (see Chapter 1 and 2). From the laboratory experiment we estimated a inhibiting TBA concentration for each species studied (C_{TBA}^i) and a temperature condition (T_{TBA}^i) at which the inhibition is fully developed. The values adopted for C_{TBA}^i and T_{TBA}^i are given in Table 4.

Therefore, the f^h term took the form:

$$f^h = \min\left(1, \frac{C_{TBA}}{C_{TBA}^i}\right) \min\left(1, \frac{f^T}{f_{TBA}^{Ti}}\right) \quad (16)$$

Where f_{TBA}^{Ti} is the Q_{10} based limiting factor at T_{TBA}^i temperature.

	G. spinifera	S. marinoi	P. minimum
$C_{TBA}^i (\mu g L^{-1})$	25	30	70
$T_{TBA}^i (°C)$	25	25	25

Table 4 Values of the inhibiting TBA concentration (C_{TBA}), and of temperature conditions (T_{TBA}^i) at which the inhibition is fully developed, measured for each species studied (G. spinifera, S. marinoi, P. minimum).

2.4 Model - Data comparison

Simulation results were qualitatively and quantitatively compared with the results of laboratory cultures.
The quantitative comparison were carried out computing the percentage bias (BIAS):

$$BIAS = \frac{1}{N}\sum\left(\frac{V_P - V_O}{V_O}\right) * 100 \quad (17)$$

and the associated Root Mean Square Error (RMSE)

$$RMSE = \sqrt{\frac{1}{N}\sum\left(\frac{V_P - V_O}{V_O}\right)^2} * 100 \quad (18)$$

V_P and V_O indicate the simulation result and the culture observation respectively. N is the number of observations. Positive or negative values of the BIAS indicate an under- or overestimation of the simulations with respect to the observations.
BIAS was also calculated to compare the chlorophyll:carbon (Chl:C) ratio simulated in the cultures exposed to TBA with the Chl:C ratio simulated in the controls.

3. Results

3.1 Skeletonema marinoi

A qualitative comparison between control model simulations and experimental data at the three temperature conditions (15, 20 and 25 °C) is reported in Fig. 6. The model is able to reproduce the temporal trend of the diatom growth at 15 and 20 °C, in terms of both carbon (C) biomass and chlorophyll (Chl) content. In the laboratory experiment conduced at 25°C, S. marinoi cultures started to grow at day 8 - 10, a few days later than the cultures kept at lower temperatures (15 and 20 °C). On the contrary the simulated dynamics at 25 °C show a early algal growth during the first days (started at day 2 - 3), greatly overestimating the experimental data.
The quantitative comparison (Fig. 7) between simulations and experimental data of carbon and chlorophyll content in the controls (0 µg L^{-1} of TBA) shows a general model overestimation for both parameters. At 15 and 20 °C the overestimation, calculated with percentage bias, is lower than 5%, while the maximum overestimation is reported at 25 °C (C: + 2.62%; Chl: + 7.08%). Despite this overestimation reported at 25 °C in the first days of the experiment, the maximum carbon production simulated was in the same order of magnitude of the value measured in laboratory (Fig. 6).
Temporal model simulations and experimental data (C and Chl) of the diatom monocultures treated with three concentrations of TBA (5, 20 and 30 µg l^{-1}) are represented in Fig. 8, 9 and 10. Control simulations and laboratory data are also reported in the figures to show a more immediate comparison between the different treatments. The parameterization of TBA effect on gross primary production (gpp) is able to reproduce a decrease in both carbon and chlorophyll, and these effects are enhanced with the increase of herbicide concentrations (Fig. 8, 9 and 10). In the laboratory experiments the increasing temperature was enhancing the TBA effect (see Chapter 2). S. marinoi growth in particular was completely inhibited when kept at 25 °C and exposed to high TBA concentrations (20 and 30 µg l^{-1}). Similarly the model simulations are characterized by an increase of the TBA effect at 20 °C: cultures exposed to TBA (5, 20 and 30 µg l^{-1}) show a weak exponential phase extended in time, with low production levels at 30 µg l^{-1} of the pollutant (Fig. 8, 9 and 10). At 25 °C in presence of high TBA concentrations (20 and 30 µg l^{-1}) the model simulations strongly anticipate algal growth, as observed for the controls (Fig. 6).

From a quantitatively point of view (Fig. 7), all the experiments involving the TBA (1, 5, 10, 20 and 30 µg l^{-1}) exposure, indicated a model underestimation at 15 and 20°C, while a overestimation of the experimental data was reported at 25°C. Bias values never exceed the - 5% and + 6% in cultures exposed to low TBA concentrations (1, 5, and 10 µg l^{-1}) at all temperature conditions tested (15, 20 and 25°C). When S. marinoi cells were treated with high TBA concentrations (20 and 30 µg l^{-1}) bias values were < 6 % at 15 and 20°C, while the overestimation increased at 25°C, reaching the maximum bias values of + 18.7% and + 67.3%, respectively for carbon and chlorophyll.

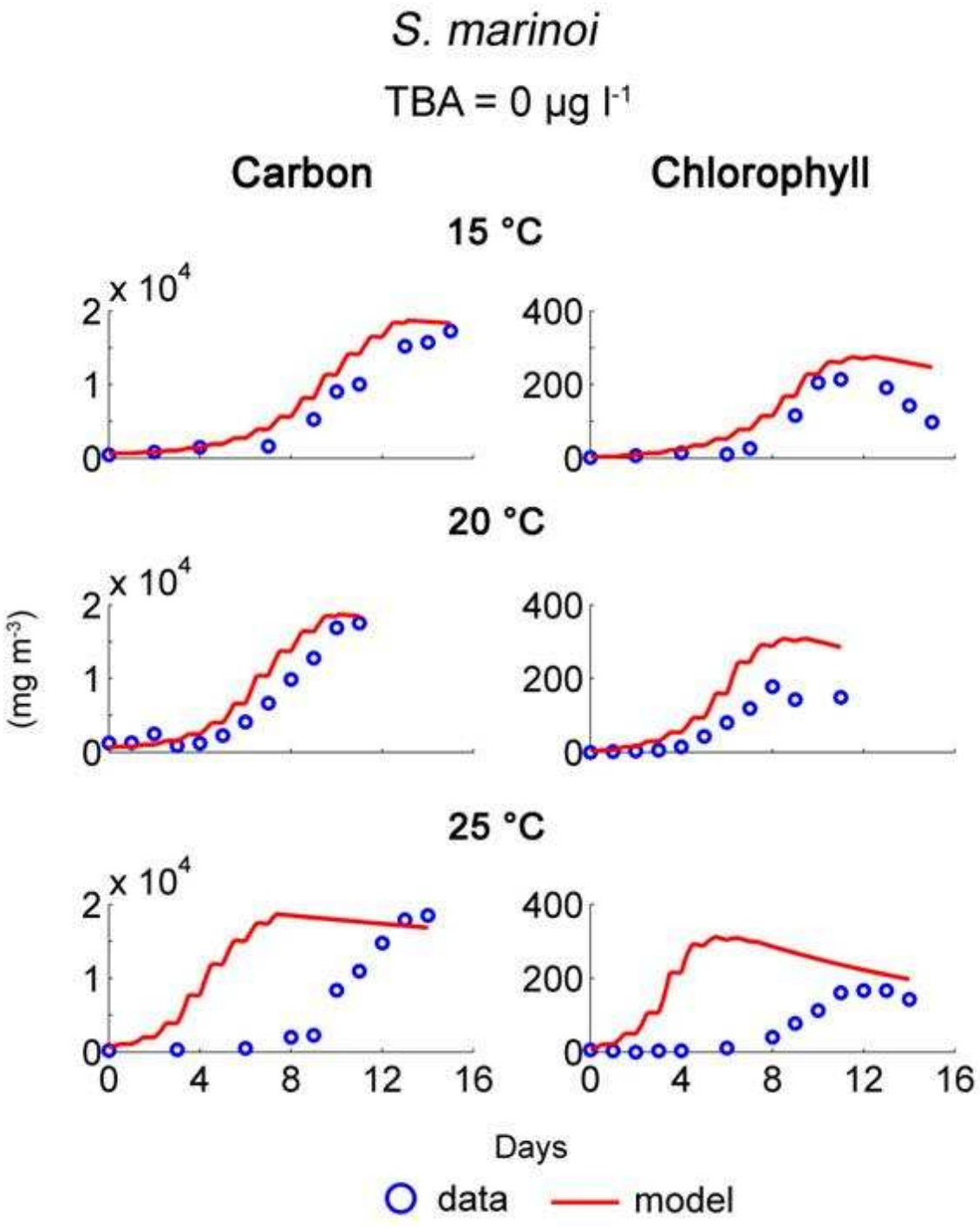

Fig.6 Model simulations and experimental data (carbon and chlorophyll) for the control of Skeletonema marinoi at the three temperature conditions tested (15, 20 and 25 °C).

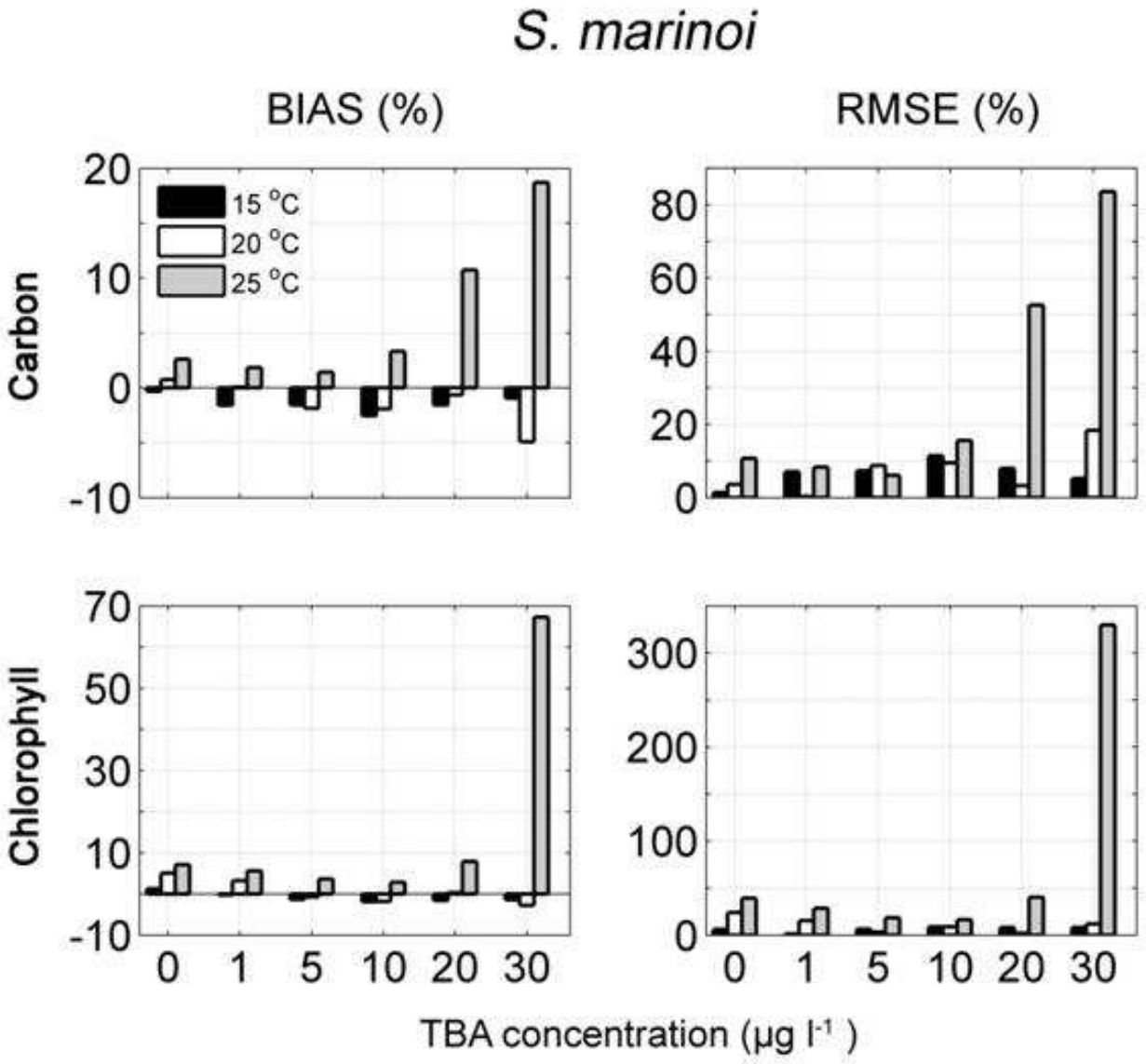

Fig.7 Percentage bias (BIAS %) calculated for carbon and chlorophyll production of Skeletonema marinoi controls (TBA = 0 µg l^{-1}) and monocultures tested at different TBA concentrations (1, 5, 10, 20 and 30 µg l^{-1}).

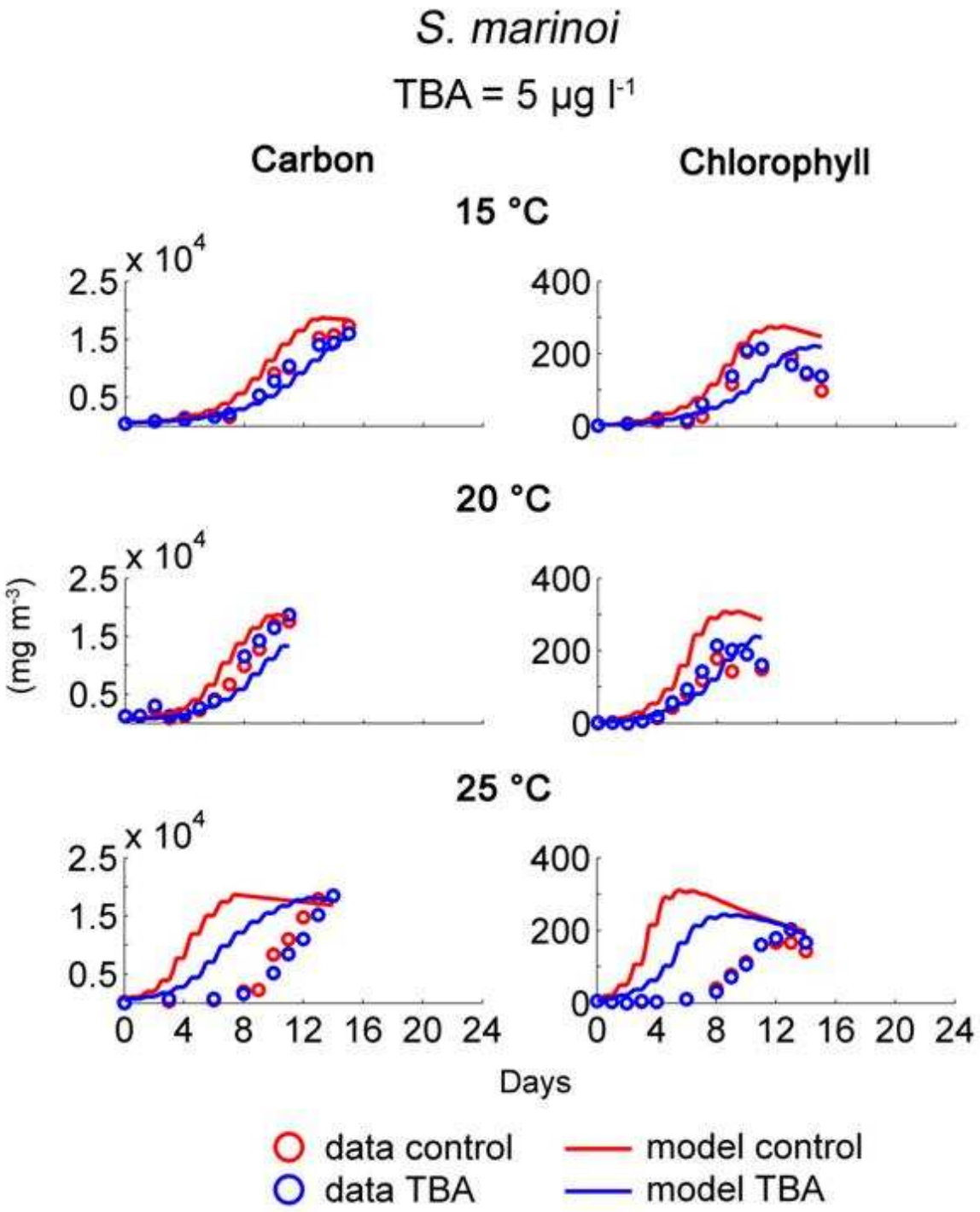

Fig.8 Model simulations and experimental data (carbon and chlorophyll) for Skeletonema marinoi controls (red) and monocultures exposed to 5 µg l^{-1} of TBA (blue), at three temperature conditions (15, 20 and 25 °C).

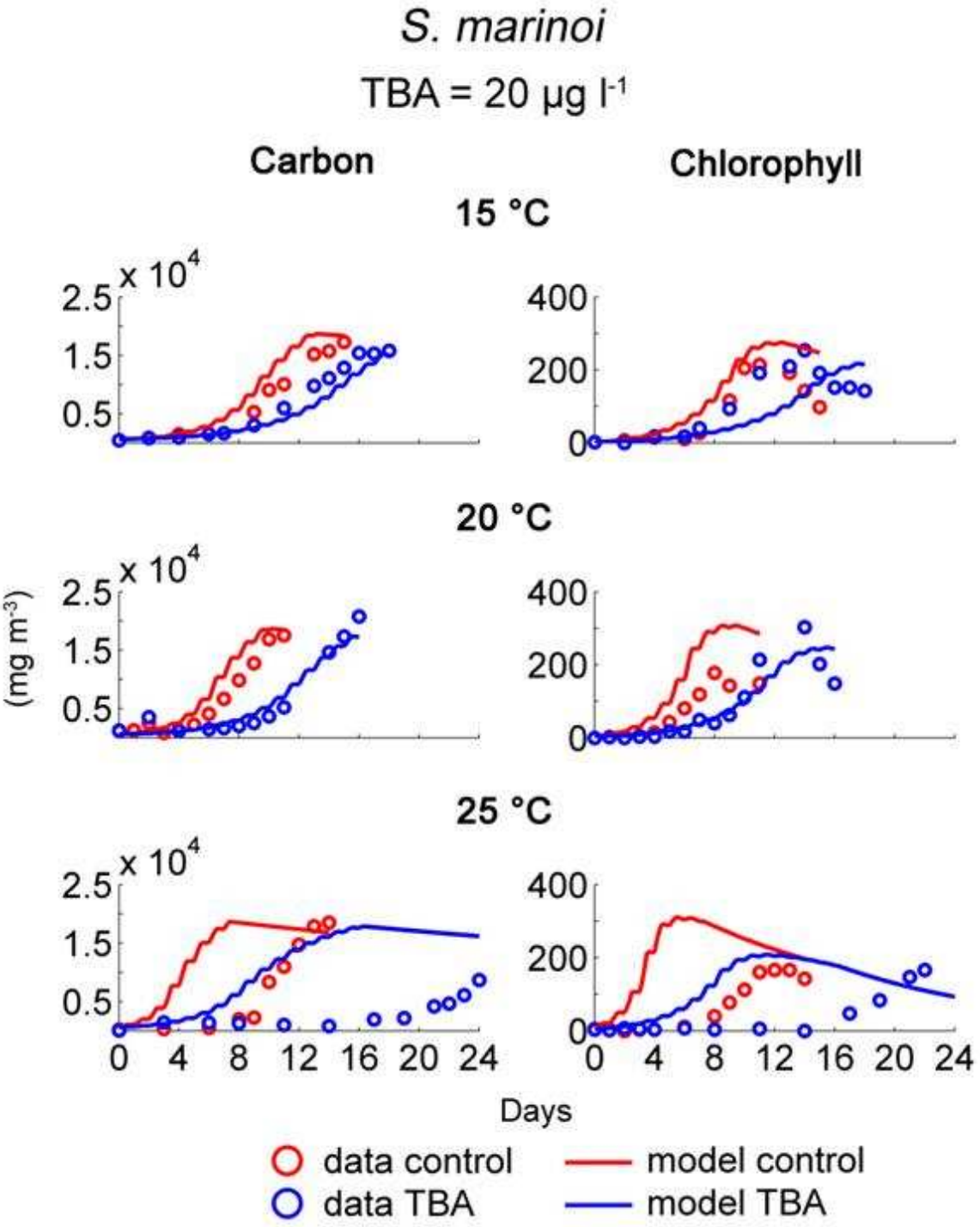

Fig.9 Model simulations and experimental data (carbon and chlorophyll) for Skeletonema marinoi controls (red) and monocultures exposed to20 µg l^{-1} of TBA (blue), at three temperature conditions (15, 20 and 25 °C).

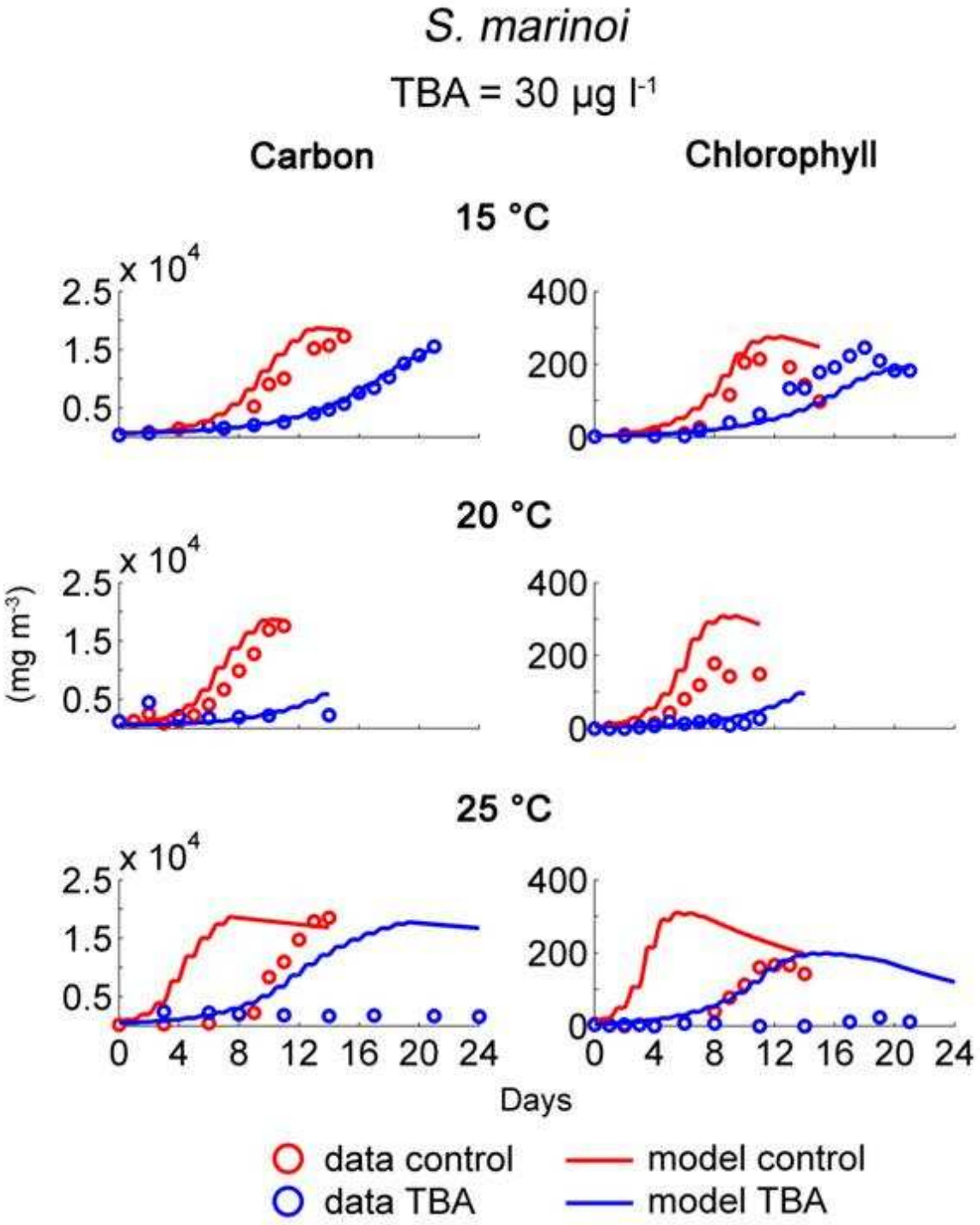

Fig.10 Model simulations and experimental data (carbon and chlorophyll) for Skeletonema marinoi controls (red) and monocultures exposed to 30 µg l^{-1} of TBA (blue), at three temperature conditions (15, 20 and 25 °C).

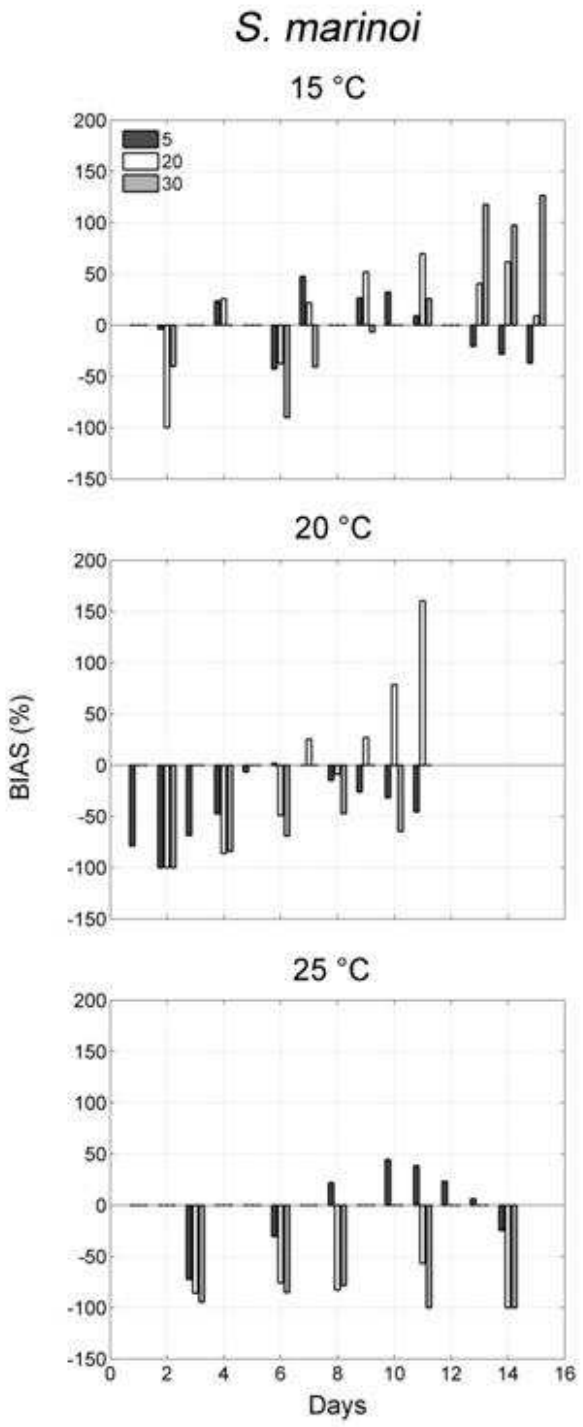

Fig.11 Temporal evolution of the percentage changes in Chl:C ratio in Skeletonema marinoi cultures exposed to different concentrations of TBA (5,20 and 30 µg l^{-1}). The percentage changes are computed from the difference between the values obtained in the exposure and in the control simulations.

Laboratory cultures exposed to TBA showed an increase in the carbon:chlorophyll (C:Chl) ratios compared to the controls (see Fig. 7 in chapter 2). The model is able to reproduce the changes in C:Chl ratios in cultures exposed to TBA (Fig. 11), observed in experimental data. At 15 °C simulations reported a higher Chl:C ratio in TBA treatments (5, 20 and 30 µg l^{-1}) compared to the Chl:C ratio simulated in the control. The differences in the simulated Chl:C ratios between the control and the TBA treatments increased at high TBA concentrations (20 and 30 µg l^{-1}). At 20 °C, the Chl:C ratio was lower in cultures exposed to 5 and 20 µg l^{-1} of TBA compared to the control values. An increase of this ratio was reproduced only at 30 µg l^{-1} of the pollutant. At the highest temperature condition (25 °C) the model simulates lower Chl:C ratio at high TBA concentrations (20 and 30 µg l^{-1}) compared to the control, while Chl:C ratio increased at 5µg l^{-1} of TBA.

3.2 Prorocentrum minimum

The model is able to qualitatively reproduce the temporal trend of algal growth, measured as carbon production, at all temperature conditions tested (Fig. 12). As observed in the laboratory experiments, at 15 and 20°C the simulations reproduce an increase in chlorophyll content from day 2 to day 8 - 9, when P. minimum reaches the maximum values, followed by a decrease of Chl content. At the highest temperature condition tested (25°C) the model reproduces a longer exponential phase of the Chl curve (day 0 - 6) compared to the experimental data (day 0 - 4), resulting in an overestimation of the maximum Chl value and a delay in the decay of Chl.

Quantitative comparison (Fig. 13) shows a slight underestimation of carbon production (respectively -3 % and -2.9 %) at 15 and 25 °C and a slight overestimation of chlorophyll content (respectively +2.3 % and +1.9 %). At 20 °C the model is able to reproduce quantitatively the algal growth, with bias values < 1 %.

The simulations conducted in the presence of the herbicide followed the trend of the experimental data. As observed in the laboratory (Chapter 1), the simulated algal growth decreases in the presence of 30 µg l^{-1} TBA (Fig. 14). The model is able to reproduce an increase of TBA effects at high temperature conditions (20 and 25 °C), as reported in the laboratory experiments.

The underestimation of carbon production and the overestimation of chlorophyll content observed in the control slightly increased in the TBA

treatments, especially at 25°C (C: -3.24; chl: +2.77) (Fig. 13).
In the laboratory experiments the Chl:C ratio measured in TBA (30 µg l^{-1}) treatments did not significantly differ from the controls, at all temperature conditions (see Fig. 8 in Chapter 1). On the contrary, the simulated Chl:C ratio increased in cultures exposed to TBA at all temperature conditions (Fig. 15), due to the underestimation of carbon production.

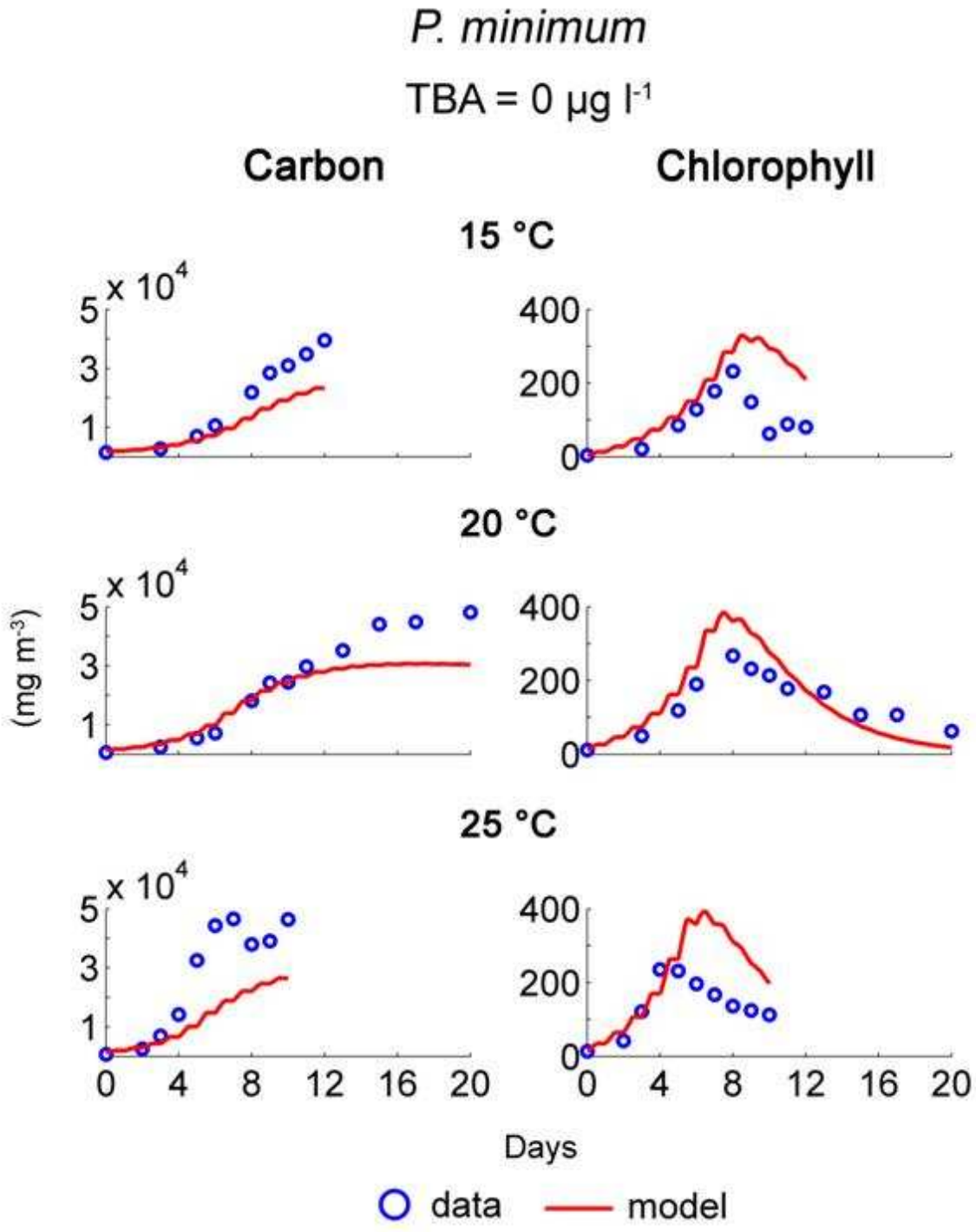

Fig.12 Model simulations and experimental data (carbon and chlorophyll) for the control of Prorocentrum minimum at the three temperature conditions tested (15, 20 and 25 °C).

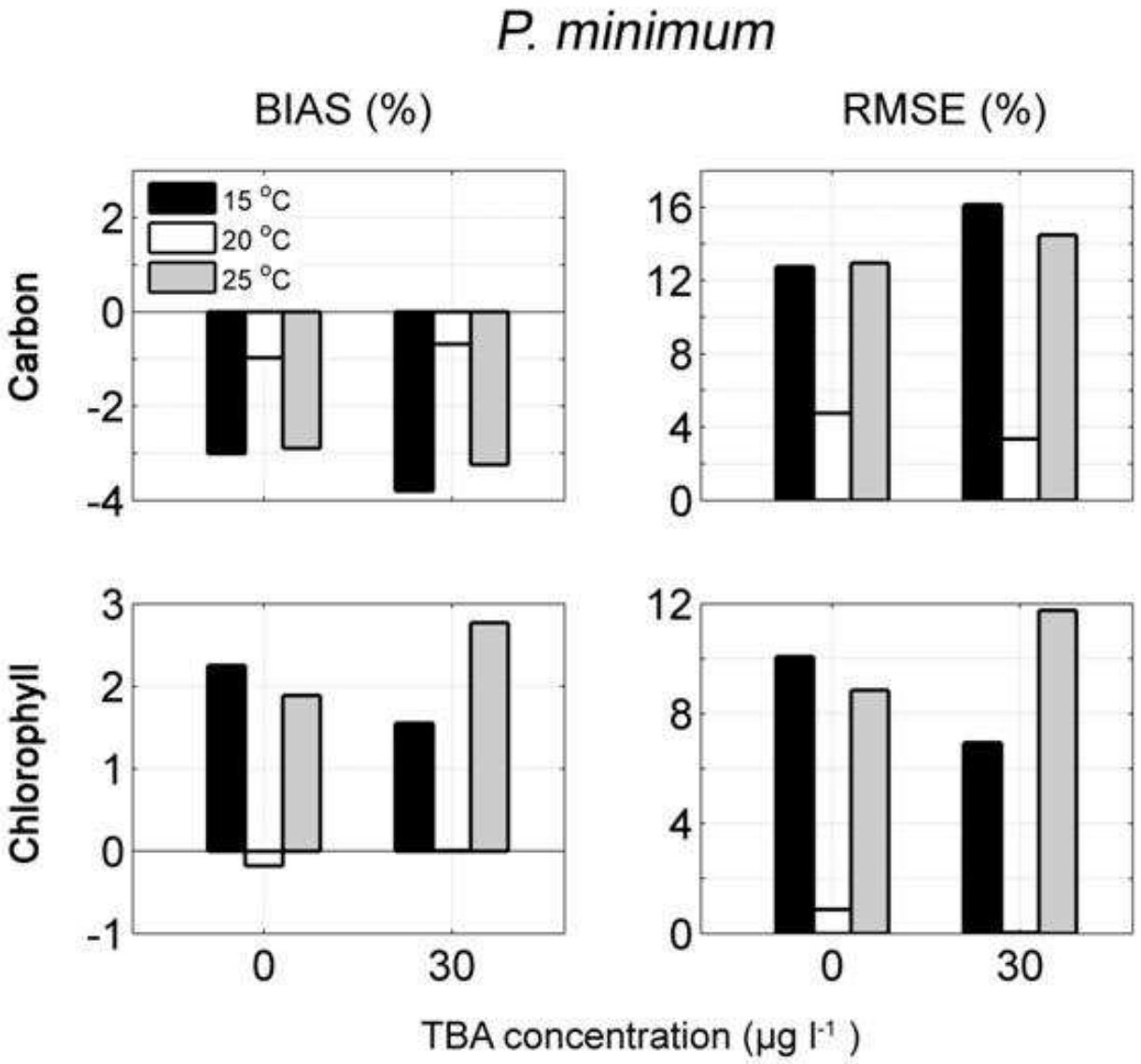

Fig. 13 Percentage bias (BIAS %) calculated for carbon and chlorophyll production of Prorocentrum minimum controls (TBA = 0 µg l^{-1}) and monocultures tested at 30 µg l^{-1} of TBA.

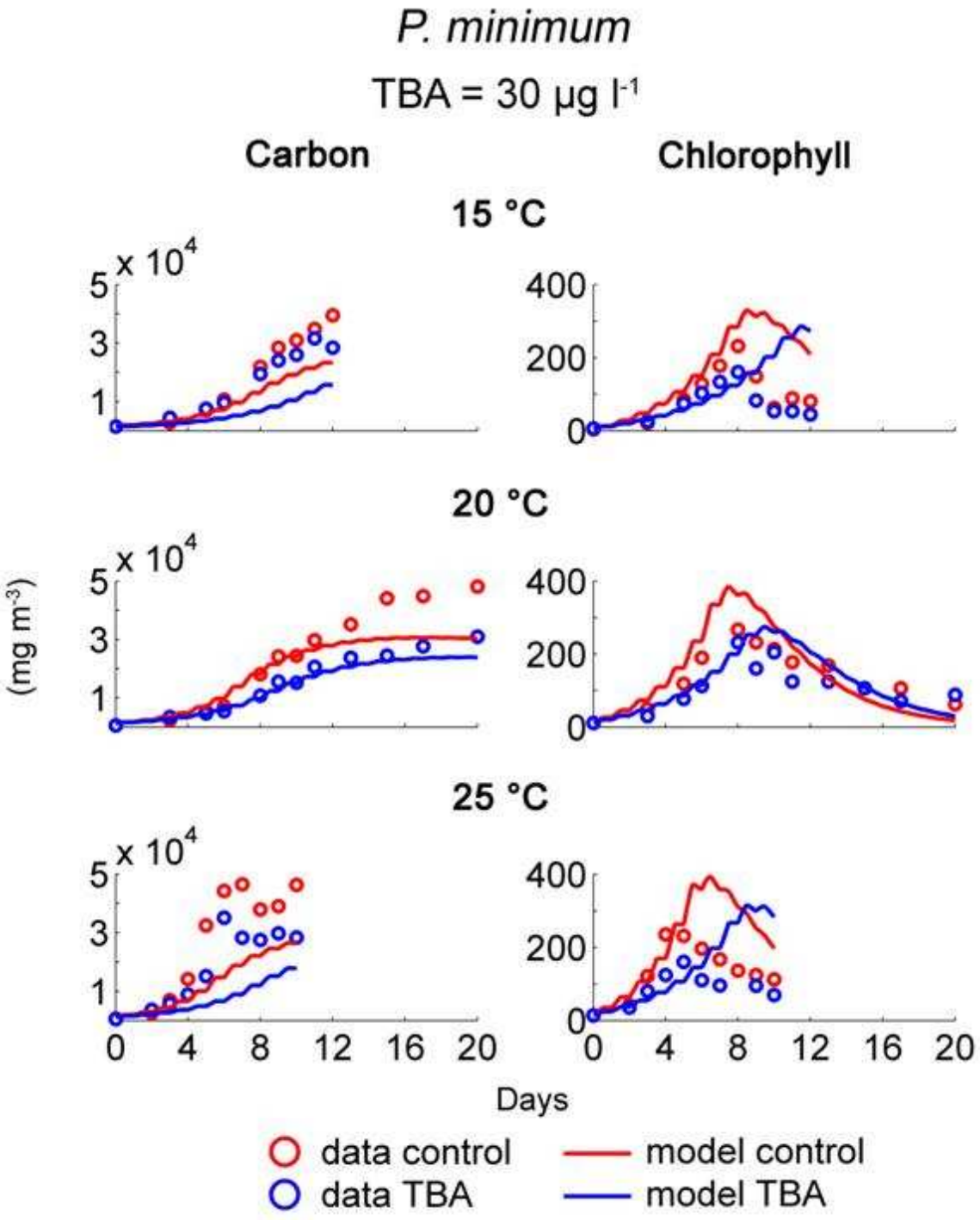

Fig. 14 Model simulations and experimental data (carbon and chlorophyll) for Prorocentrum minimum controls (red) and monocultures exposed to 30 µg l^{-1} of TBA (blue), at three temperature conditions (15, 20 and 25 °C).

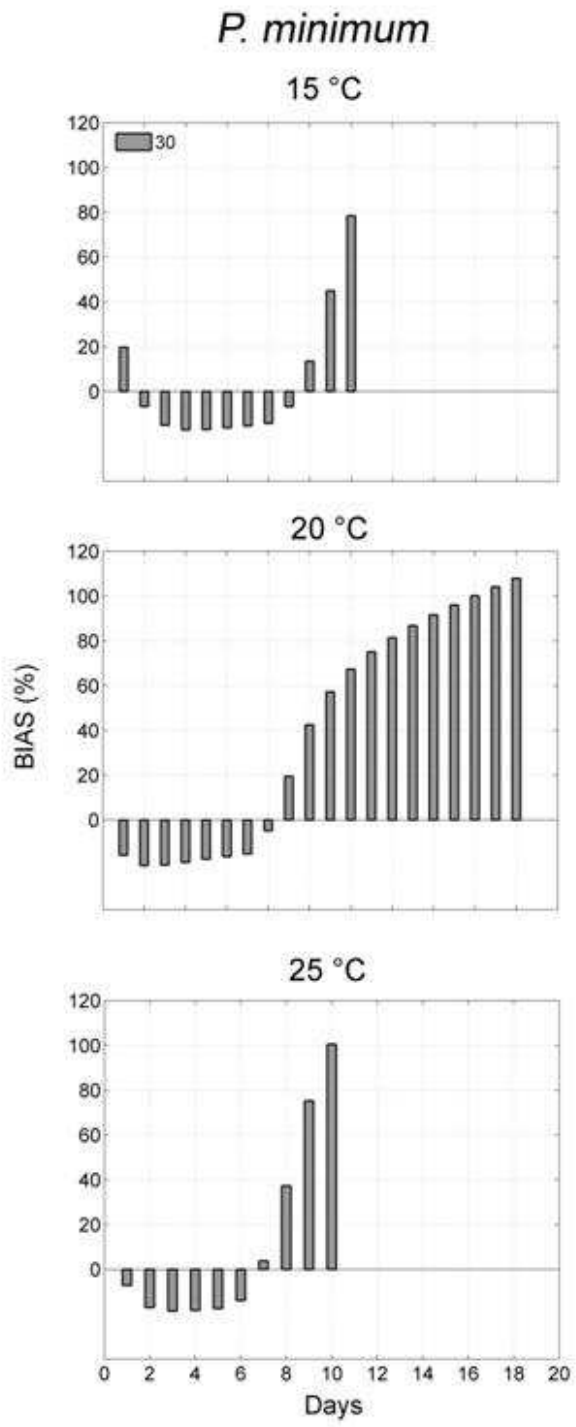

Fig. 15 Temporal evolution of the percentage changes in Chl:C ratio in Prorocentrum minimum cultures exposed to 30 µg $l^{-1}$of TBA. The percentage changes are computed from the difference between the values obtained in the exposure and in the control simulations.

3.3 Gonyaulax spinifera

Carbon production was qualitatively well simulated for this phytoplankton species, at all the temperatures studied (15, 20 and 25 °C) (Fig. 16). At the temperature condition of 15°C the model simulates an increase in Chl content from day 4, conversely to what was observed in the laboratory cultures. This temperature is a limiting condition for this species, which is found in the Adriatic Sea during the summer period. G. spinifera showed lower production and growth rates at 15 °C compared to higher temperatures conditions (20 and 25 °C) (Chapter 1). At higher temperature (20 °C) the chlorophyll curve is well simulated, with a progressive increase until the maximum chl value is reached at day 12 and a consequent decay of Chl. At 25°C the model reproduces a slower and prolonged exponential phase (until day 10) for Chl, while laboratory data registered a rapid increase of the Chl content between day 3 and 5.

From a quantitatively point of view the simulations slightly differ from the experimental data (Fig. 17). The maximum overestimations of C and Chl content, +0.71 and +3.15% respectively, were reported for the simulations conducted at 15 °C. At the temperature condition of 20 °C the model underestimates both C and Chl content. An underestimation of carbon and an overestimation of chlorophyll were reported at 25 °C, due to a delay of the simulation of algal growth compared to the laboratory experiments.

When G. spinifera was exposed to 5 µg L^{-1} of TBA the model was able to reproduce a decrease in algal growth compared to the control, and a weak exponential phase extended in time (Fig. 18). The effect of TBA is simulated to increase at high temperatures, as reported in laboratory cultures (Chapter 1). The model is able to qualitatively reproduce the temporal trend of carbon production at all temperature conditions. At 20 °C the model anticipated the chlorophyll dynamic, reaching the maximum Chl value at day 16, while in the laboratory the maximum Chl content was registered at day 21. At the other temperature conditions (15 and 25°C) the simulations follow the temporal trend of the experimental data. However at 25 °C the model is not able to reproduce a decay after day 12, as shown from the laboratory data.

The quantitatively comparison shows slight differences between model simulations and experimental data measured in TBA treatments (Fig. 17). Regarding chlorophyll, the percentage bias values decrease in TBA treatments compared to the controls. At the concentration of 5 µg l^{-1} of TBA, the model slightly underestimated carbon production at all temperature conditions, up to -1.5

% at 25 °C, while chlorophyll content was slightly overestimated at 15 and 25 °C. At 1 µg l^{-1} of TBA model simulations reproduced a similar quantitative pattern of the control.

The model simulated an increase of the Chl:C ratio in presence of TBA (1 and 5 µg l^{-1}) compared to the control (Fig. 19). In particular this ratio increased with the increase of TBA concentrations, as reported in laboratory cultures (See Fig. 8 in Chapter 1).

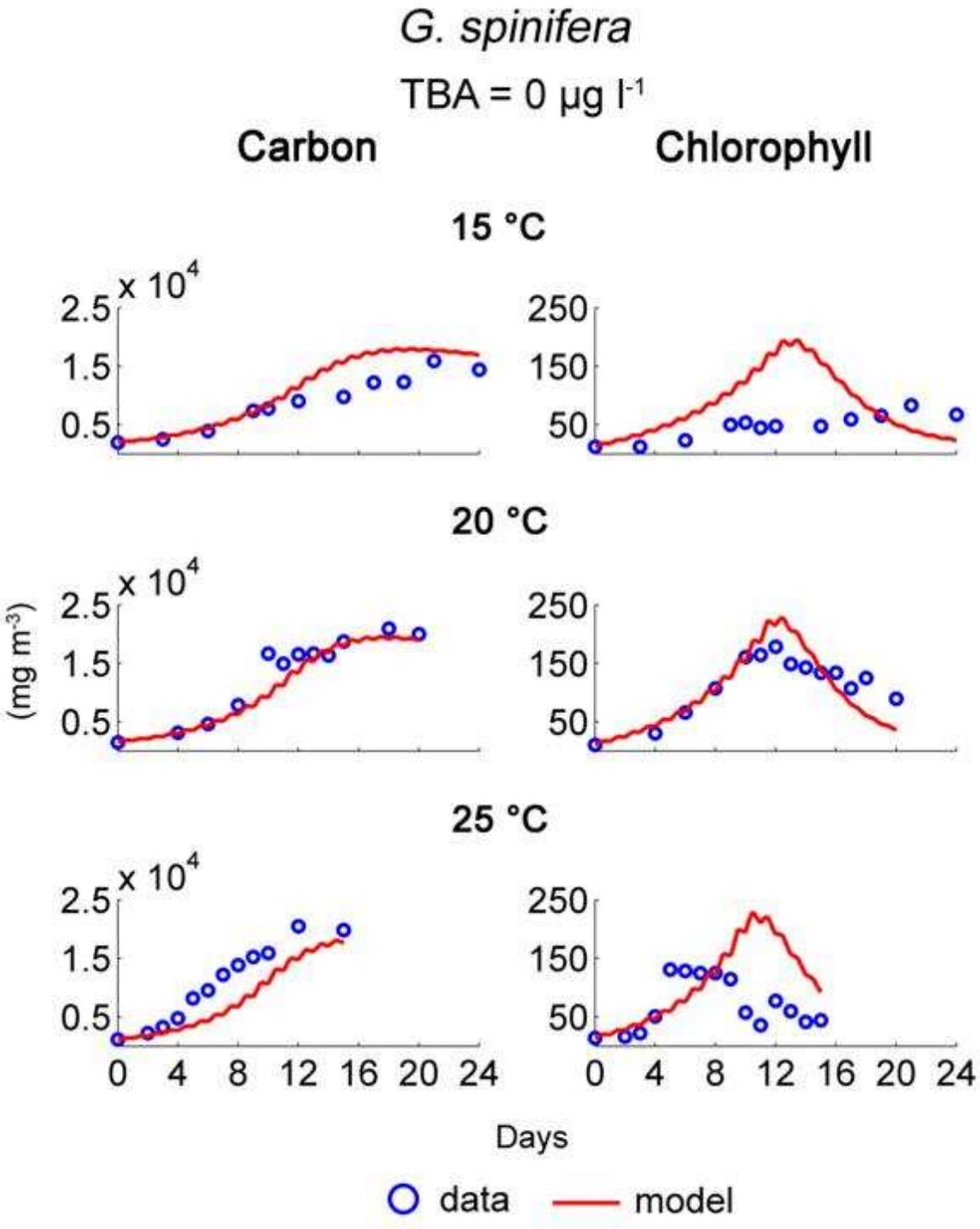

Fig.16 Model simulations and experimental data (carbon and chlorophyll) for Gonyaulax spinifera controls (red) and monocultures exposed to 5 µg l^{-1} of TBA (blue), at three temperature conditions (15, 20 and 25 °C).

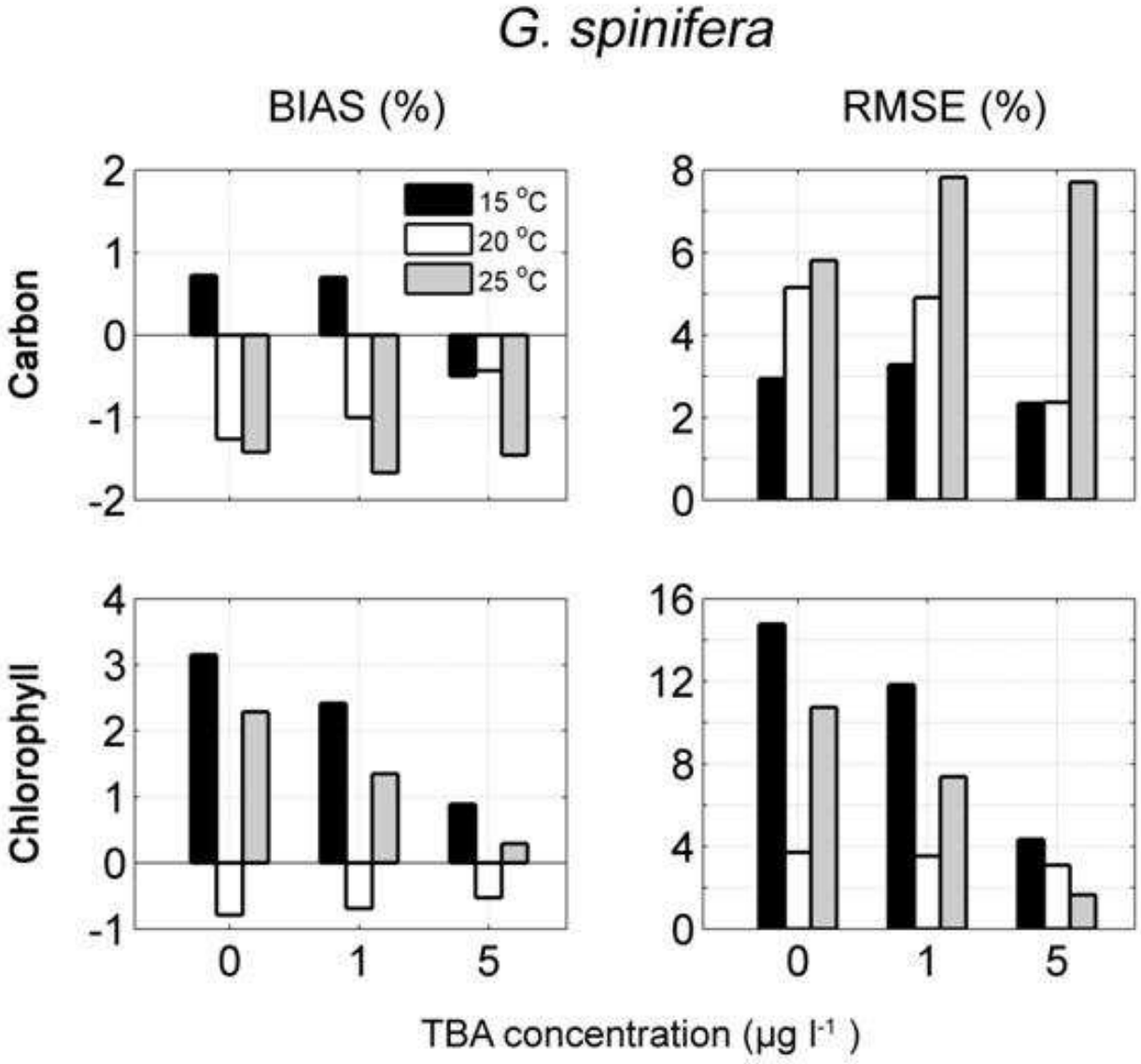

Fig. 17 Percentage bias (BIAS %) calculated for carbon and chlorophyll production of Gonyaulax spinifera controls (TBA = 0 µg l^{-1}) and monocultures tested at different TBA concentrations (1 and 5 µg l^{-1}).

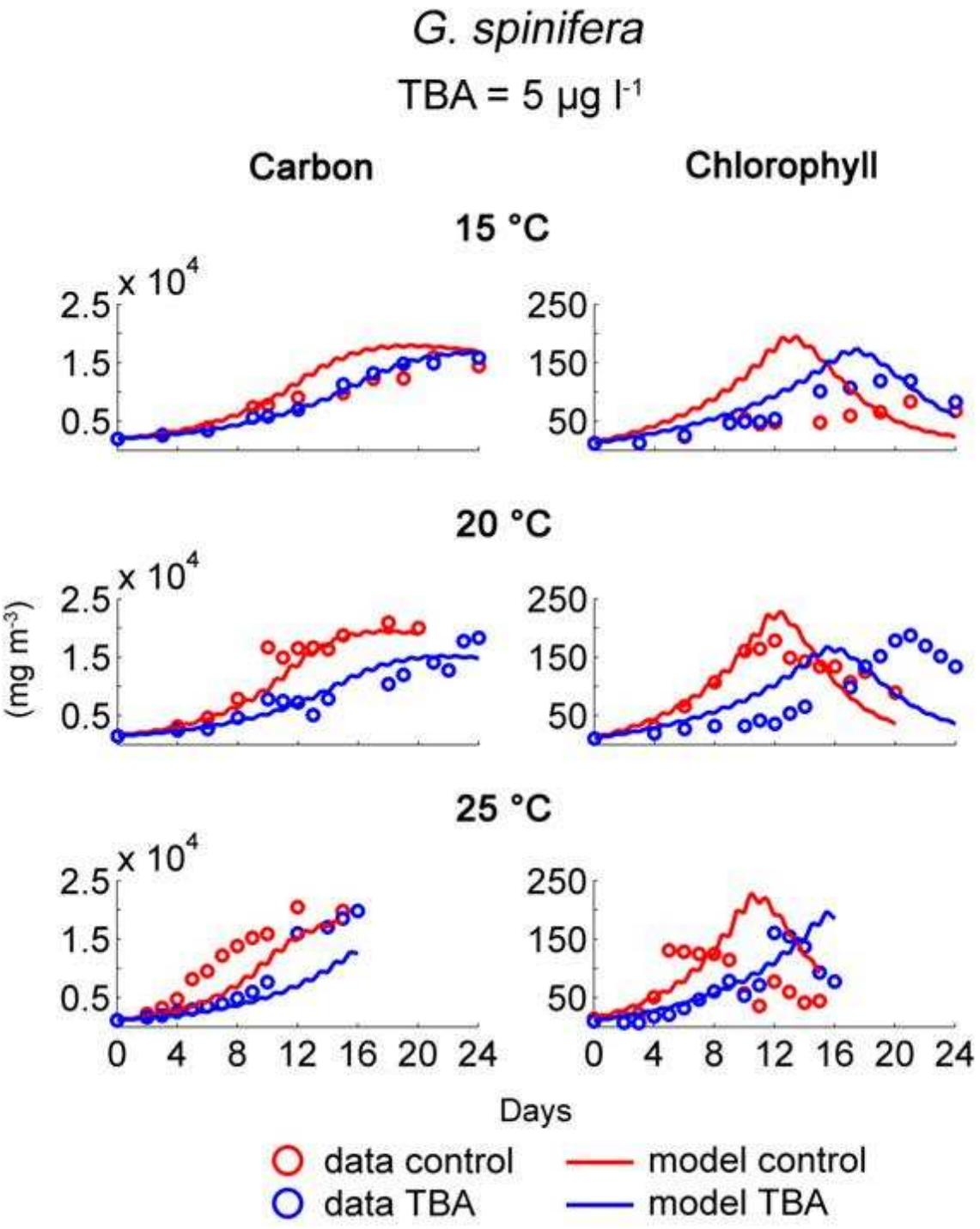

Fig. 18 Model simulations and experimental data for Gonyaulax spinifera monocultures exposed to 5 µg L^{-1} of TBA at the three temperature conditions tested (15, 20 and 25 °C).

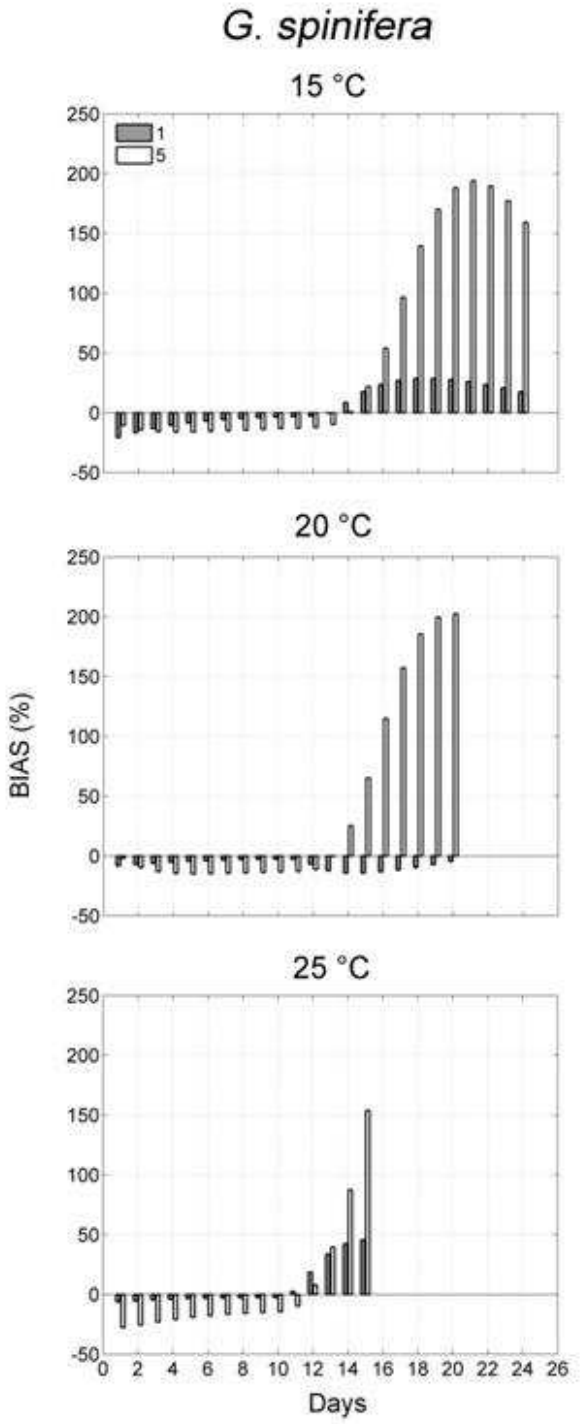

Fig. 19 Temporal evolution of the percentage changes in Chl:C ratio in Gonyaulax spinifera cultures exposed to different concentrations of TBA (1 and 5 µg l^{-1}). The percentage changes are computed from the difference between the values obtained in the exposure and in the control simulations.

4. Discussion and Conclusions

4.1 Controls

Before discussing the simulations of laboratory cultures exposed to TBA, it is necessary to considerer the quality of the control experiments, in order to understand how accurate the TBA parameterization is.

The control simulations carried out for all three phytoplankton species considered, indicated that the model parameterization of the carbon and chlorophyll dynamics, is in general able to capture the temporal evolution of the laboratory cultures. However, in the previous section it has been stressed that the simulations of S. marinoi are characterized by a decrease in the accuracy of the model as temperature increases. Fig. 6 indicates that at 25 °C S. marinoi simulations significantly deviate from the laboratory data, by developing an exponential phase much earlier than the occurrence in the laboratory cultures. Diatoms are known to grow at temperatures lower than 25°C (Bernardi Aubry et al., 2006; Bernardi Aubry et al., 2004). However, the marked deviations occurring at higher temperatures seems to indicate an inadequate representation of the Q_{10} based formulation of the temperature regulating factor f^{T}.

A sensitivity analysis with the Q_{10} parameter (not shown) did not yield any improvement but on the contrary, a deterioration of the 15 and 20°C simulation quality.

The behavior of the regulating f^{T} factor with increasing temperatures seems characterized by a marked linearity that is (apparently) not obtained with a Q_{10} based formulation. This point has been raised by Montagnes et al., (2003) and the results we obtained with the S. marinoi simulations seems to indicate the need for some in depth work on the issue.

The P. minimum and G. spinifera control simulations (Fig. 12 and 16 respectively), on the contrary, indicate a tendency towards carbon underestimation with increasing temperature, while the chlorophyll evolution (with the notable exception of G. spinifera grown at 15 °C) appears very similar to that observed during the growing phase. The decreasing phase, corresponding in general with the nutrient depletion, is not yet satisfactory despite the introduction of the chlorophyll loss term (equation 11). This term is dependent on nutrient limitation conditions, and was developed on completely empirical

considerations to mitigate the almost absent chlorophyll decrease (corresponding to nutrient depletion) in the original chlorophyll synthesis equation. Despite the use of this "ad-hoc" term simulated concentrations still show the tendency to "overshoot" the observed maximum value and to exhibit a slower chlorophyll concentration decrease. These two discrepancies appear enhanced by increasing temperatures and this shows the need for greater attention to be paid to the temperature regulating factor.

4.2 Herbicide parameterization

The simulations involving TBA exposure are affected by the discrepancies described above for the control experiments. However, the parameterization of the effect of TBA $\left(f^{h} \right)$ on gross primary production (gpp, see equation 15) is able to reproduce the effects on algal growth observed in the laboratory experiments for all the species studied. In fact, the model simulates a decrease in both carbon and chlorophyll content with TBA concentration increases. Furthermore, this effect is enhanced with the increase of temperature (see for instance Fig. 18).

In laboratory experiments changes in the chlorophyll:carbon (Chl:C) ratio were measured in S. marinoi and G. spinifera monocultures exposed to TBA compared to the respective control values, while for P. minimum no differences were reported between controls and TBA treatments.

The simulated temporal variations in the Chl:C ratio for S. marinoi and G. spinifera are in very good qualitative agreement with the observations. Regarding the simulated P. minimum Chl:C ratio, quantitative differences still persist; but it seems that at least part of them are due to the parameterization of primary production at higher temperatures rather than to specific TBA parameterization.

Finally, we think that the parameterization of the TBA effect on phytoplankton species studied is a sound starting point for additional work aimed at the analysis of the pollutants impact on the marine ecosystem lower trophic levels. Any further studies, however, will have to consider the improvement of the simulation of the phytoplankton dynamics also in absence of the impact of the pollutant. The parameterization of the role of temperature in particular appears the most urgent factor to be considered.

6. Acknowledgements

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Chapter 4

Influence of light on growth, toxicity and mixotrophy of Prymnesium parvum

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Abstract

The haptophyte Prymnesium parvum has a worldwide distribution, with a dramatic increase in blooms in recent years. P. parvum blooms are often associated with massive fish kills that have a devastating impact on ecosystems and fish farms with subsequent economic loss. P. parvum is a mixotrophic organism, utilizing organic dissolved substances and particles to support its photosynthetic growth. The ability of P. parvum to produce toxic compounds means it can inhibit growth or kill other phytoplankton species to acquire essential substances. These mechanisms come into play when environmental conditions are not optimal for P. parvum growth. We report results on growth, toxicity and mixotrophy from experiments where P. parvum cells were grown as monocultures or together with Rhodomonas salina as mixed cultures and exposed to various light conditions: dark, 100, 700, 2000 µmol photons m^{-2} s^{-1}. The results showed that P. parvum growth is affected at light intensity of 700 µmol photons m^{-2} s^{-1} and the cells stop growing when exposed to irradiance above this value. An inverse relationship between intracellular toxicity, SnEq $cell^{-1}$, and light intensity was observed, i.e. lower light irradiation induced greater intracellular toxicity. However R. salina cells in mixed cultures were detected only during the first 3 hours at 700 and 2000 µmol photons m^{-2} s^{-1}. We therefore suggest that in these conditions P. parvum cells were photolysed and toxins released in the medium caused an increase in the extracellular toxicity in the initial hours of the experiment. Phagotrophy was observed in all these conditions. P. parvum reached significantly higher cell densities when growing together with R. salina than in monocultures, while intracellular toxicity significantly decreased in the mixed cultures. The negative effect of high irradiation was attenuated in the presence of prey.

Keywords: Prymnesium parvum, irradiance, mixotrophy, growth, toxicity

1. Introduction

The haptophyta Prymnesium parvum has been responsible throughout the world for toxic blooms, which are often associated with massive fish kills and great ecological impacts and economic losses as a consequence (Granéli et al., 1993; Edvardsen and Paasche, 1998; Morohashi et al., 2001; Brooks et al., 2011). Some of the toxic compounds that P. parvum produce were first described by Igarashi et al. (1995) who fully identified two toxins, prymnesins 1 and 2, as polyoxy-polyene-polyethers. More recent studies have found several other cytotoxic, hemolytic, neurotoxic or ichthyotoxic compounds, but most of them are not yet chemically identified (Henrikson et al., 2010; Manning and La Claire II, 2010; Schug et al., 2010). Since the majority of the P. parvum toxins are unidentified various bioassays must be used to determine toxicity (Freitag et al. 2011). However, differences have often been observed between, for example, hemolytic activity and toxic

effects on other phytoplankton species or fish when aliquots of one extract were used for the assays (Schug et al., 2010; Freitag et al., 2011). It is therefore hard to compare different studies. Furthermore, intracellular toxin concentrations can be estimated with a hemolytic assay, whereas different bioassays must be used to determine any extracellular toxins (Freitag et al., 2011).

The production of Prymnesium toxins is enhanced when cells grow under stress conditions, such as nutrient deficiency, sub-optimal temperature, salinity, and light conditions (Reich and Parnas, 1962; Granéli and Flynn, 2006; Roelke et al., 2011; Granéli et al., 2012). Allelopathy, i.e. the release of chemical compounds with negative effects on co-occurring species, can give a phytoplankton species a competitive advantage when, for example, abiotic factors limit its growth (reviewed by Legrand et al., 2003. Granéli and Salomon, 2010). Although the actual relevance of phytoplankton allelopathy in nature has been challenged (Lewis, 1986; Jonsson et al., 2009; Remmel and Hambright, 2012), several studies have shown that P. parvum have a toxic effect on other algal species and grazers. Prymnesium is for instance capable of immobilizing, of inhibiting growth and/or of killing co-occurring plankton species (Fistarol et al., 2003; Granéli and Johansson, 2003a,b; Legrand et al., 2003; Skovgaard and Hansen, 2003; Skovgaard et al., 2003; Tillmann, 2003; Uronen et al., 2007; Ianora et al., 2011). The prey can vary remarkably in size from small bacteria to large autotrophic and heterotrophic dinoflagellates (Nygaard and Tobiesen, 1993; Tillmann, 1998; Legrand et al., 2001). Furthermore, since P. parvum is a mixotrophic organism it can utilize organic particles (phagotrophy to acquire macronutrients) (Caron et al., 1993; Arenovski et al., 1995; Legrand et al., 2001), or dissolved organic substances (osmotrophy) to support its photosynthetic growth (Lindehoff et al., 2009; Lindehoff et al., 2011). If light is not sufficient for CO_2 fixation, phagotrophy can supplement or even substitute photosynthesis (Rahat and Jahan, 1965).

Much research has been carried out and practical strategies have been developed in an effort to control P. parvum blooms (Barkoh and Fries, 2010; Barkoh et al., 2010). However, some of the proposed mitigation methods involve toxic chemicals such as copper sulfate (Reichenbach-Klinke, 1973; Guo et al., 1996) or using ammonium sulphate and liquid ammonia (Shilo and Shilo, 1962; Sarig, 1971). Nutrient manipulation, e.g. nitrogen and phosphorous enrichment (Guo et al., 1996; Kurten et al., 2011), has also been shown to reduce toxicity. These chemical compounds must often be added to the environment at high concentrations and they therefore have a negative effect on non-target organisms. Other water treatment methods, such as ozonation of aquaculture water (Colt and Tomasso, 2001) have also been considered. However, ozone is also toxic to fish and other aquatic life (Summerfelt et al., 2001). Research is therefore being carried out to develop more environmentally friendly mitigation techniques. Light intensity is one of the main factors that directly influence P. parvum growth and toxicity (Granéli and Salomon, 2010; Manning et al., 2010; Granéli et al., 2012;). Reich and Parnas (1962) demonstrated that cultures under constant illumination did not produce ichthyotoxins. Recently, Freitag et al. (2011) studied P. parvum toxicity after 2 h exposition to a series of environmental "shocks", including high light exposition (700 µmol photons m^{-2} s^{-1}). They observed an increase in intracellular toxicity, but the toxic effect on Rhodomonas salina cells on the other hand decreased. James et al. (2011) demonstrated that acute toxicity to fish is completely removed when cell-free filtrates were exposed to natural sunlight for 2 h.

Since mitigation methods for Harmful Algal Blooms (HABs) needs to be environmentally friendly, high light intensities is a technique worth considering. However, more studies are needed to understand the effects of extremely high light on P. parvum growth and toxicity, which might help to evaluate if high light exposition can be used as an efficient method to reduce negative effects of P. parvum on aquatic life. The aims of this work were to study (1) the effect of different light intensities on Rhodomonas salina growth, and on Prymnesium parvum growth and toxicity; (2) since light at 700 µmol photons m^{-2} s^{-1} reduces only extracellular toxins (Freitag et al., 2011), whether an even higher intensity (2000 µmol photons m^{-2} s^{-1}) would affect P. parvum intra- and extra-cellular toxin concentrations; (3) if extremely high light exposition has a negative effect on P. parvum toxicity, whether the availability of prey (i.e. R. salina) under high light intensities would decrease even further the toxin production by P. parvum.

2. Materials and Methods

2.1 Phytoplankton cultures

Non-axenic monocultures of Rhodomonas salina and Prymnesium parvum, obtained from The Kalmar Algae Collection (KAC 30 and KAC 39, Linnaeus University, Kalmar, Sweden), were grown as batch cultures in 10 l bottles. 7 l modified f/2 medium was used for R. salina and 7 l modified f/10 medium for P. parvum (Guillard and Ryther, 1962). Culture of R. salina was grown in nutrient sufficient conditions at Redfield N:P ratio of 16:1 (f/2: N = 580 µM and P = 36.3 µM) to maintain this alga in healthy conditions; while P. parvum culture was maintained in P-limiting conditions with an N:P ratio of 20:1 (N = 116 µM, and P = 5.8 µM) to stimulate the production of toxins (Johansson and Granéli, 1999). All cultures were grown at a light intensity of 100 µmol photons m^{-2} s^{-1}, 16:8 light-dark (L:D) cycle, and at 20 °C. Culture media were prepared with filtered (Whatman GF/C) and autoclaved aged Baltic Sea water (salinity 7 psu) adjusted to pH 8 - 8.4. The cells were allowed to acclimatize for 6 weeks while being maintained in exponential growth before the start of the experiment.

2.2 Experimental design

The experiment started when the stock cultures had reached high cell densities (P. parvum 7.76 x 10^5 cells ml^{-1}, and R. salina 4.20 x 10^5 cells ml^{-1}). Three treatments were used for the experiment, i.e. P. parvum monoculture, R. salina monoculture and a mixed culture of both microalgae, where P. parvum cells were at twice the abundance of R. salina to induce phagotrophy. Initial cell densities for the mixed cultures were achieved by mixing 150 ml of P. parvum with 150 ml of R. salina (mixed culture). Monocultures were prepared by diluting 150 ml of P. parvum and R. salina stock cultures with 150 ml of P deficient f medium and f/2 medium respectively to obtain the same initial cell densities as that used in the mixed cultures. Thus, initial culture volumes of 300 ml from each treatment in 500 ml glass bottles (Schott Duran), in triplicate, were exposed to three light conditions: 100, 700, 2000 µmol photons m^{-2} s^{-1} irradiation (daylight lamps, Osram Powerstar HQI-E 250 WD) and one in continuous darkness (Fig.1). The experiment lasted 4 days, with controlled laboratory conditions regarding temperature (20 °C), salinity (7 psu) and light with a 16:8 light-dark cycle. The experiment started 6 hours after the onset of the light period. Flow cytometry was used to quantify the change in the number of cells of each species throughout the experiment.

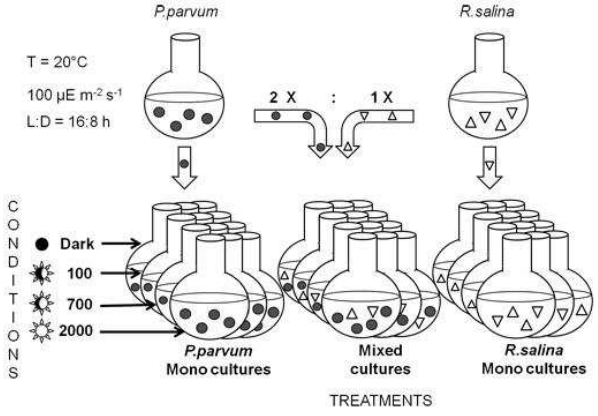

Fig. 1 Schematic representation of the experimental set-up used to test the effect of light on P. parvum toxicity in mono cultures and in the presence of prey in mixed cultures.

All treatments were also monitored with light and epifluorescence microscopy for the first two days. The first cell counts were done at time 0.5 h, and since microscope observations and flow cytometry results showed fast changes in phycoerythrin content and cell densities in mixed cultures, successive countings were done every hour for the first 10 hours and then daily for 4 days. Cell densities were measured directly in the stock cultures (time 0 h) before the experiment started to calculate the initial ratio between the two species. 10 ml sub-samples from P. parvum monocultures and mixed cultures were collected for toxin analysis. P. parvum toxins were analyzed as hemolytic activity and expressed as SnEq $cell^{-1}$ (see below) in samples withdrawn from time 0.5 h to 92 h in all the treatments. Hemolytic activity at time 0 h, i.e. in the stock culture, was also measured.

2.3 Cell counts

Prymnesium parvum and Rhodomonas salina cell densities were estimated in all the treatments from 1 ml fresh samples using flow cytometric analysis (FACSCalibur flow cytometer, Becton Dickinson). Fluorescent reference beads (BD Trucount™) were used to calibrate the instrument.

A series of logical gates were used to distinguish healthy R. salina and P. parvum cells, and also to separate the two species in the mixed cultures. Signals of phycoerythrin (FL2 – Relative orange fluorescence) and/or chlorophyll-a (FL3 – Relative red fluorescence) cell contents were used to count R. salina in stock monoculture before the experiment started. The same gates were used to recognize and count healthy R. salina in both mono and mixed cultures during the experiment. P. parvum counting was based on chlorophyll-a (FL3 – Relative red fluorescence) cell content measured

in P. parvum initial stock monoculture. However, P. parvum cells gained high signal of orange fluorescence (FL2) after the phagotrophic ingestions of one or more R. salina cells, which was also confirmed from microscopic observation. The high phycoerythrin signal emitted from P. parvum cells in mixed cultures was therefore also considered when determining P. parvum abundance in these treatments.

Maximum specific growth rates (day^{-1}) of P. parvum and R. salina were calculated according to the equation:

$y = \ln (N_2 - N_1) / (t_2 - t_1)$

in which N_1 and N_2 are total cell concentrations at time, t_1 and t_2, respectively.

2.4 Microscopic observation

Fluorescence emitted by live cells was observed with an epifluorescence microscope equipped with a blue filter (Olympus U-MNB: λex 470 to 490 nm, λm > 550 nm). The same technique was also used to investigate phagotrophy in mixed cultures. Pictures were taken with an Olympus DP50 digital camera coupled to a microscope at 400X magnification in automatic exposure mode.

2.5 Hemolytic test

Prymnesium parvum intracellular toxicity was determined as hemolytic activity of a cell methanol extract (Igarashi et al., 1998). Subsamples of 10 ml drawn from the cultures were centrifuged for 7 min at 4000 rpm (Universal 16) to obtain a cell-pellet. After centrifugation the supernatants were carefully removed with a pipette without disturbing the cell pellets and 5 ml were transferred to clean polystyrene tubes. The toxins in the cell pellets were extracted in 2 ml of 99% methanol for 30 min in the dark. Both cell extracts and the supernatants were stored at -20 °C prior to analysis.

The hemolytic test was performed using 5% horse erythrocytes and the hemolytic activity was determined measuring the absorbance at 540 nm with a plate reader (BMG FLUOstar) after 1 hour of incubation. The tests were carried out in duplicate. A standard hemolytic curve based on increasing concentrations of saponin (Sigma S-2149) in an isotonic phosphate buffer was used as the reference curve. Hemolytic activity in methanol was also determined, following the same procedure, to exclude the possible toxic effect of methanol. Hemolytic activity of P. parvum cells was calculated relative to saponin hemolytic activity and expressed as saponin nano-equivalents per cell (SnEq $cell^{-1}$). The equivalent cell concentration that causes 50% hemolysis of blood cells (HE_{50}) was determined by a linear fit (50% hemolysis = a + bx * log cell concentration) between the logarithm of cell concentration (determined flow cytometrically, see above) and the percentage hemolysis. The same fit was used to determine the HE_{50} for saponin (Saponin HE_{50}). The hemolytic activity of P. parvum as SnEq $cell^{-1}$ was calculated by dividing the Saponin HE_{50} by the HE_{50} of P. parvum. The supernatants were also tested for hemolytic activity without further sample preparation.

2.6 Statistical analysis

MATLAB R2011a was used for all the statistical analysis and results were regarded as significant when $p < 0.05$. Data were checked for normal distribution with Lilliefor's test, and for homogeneity with Bartlett's test.

Differences in maximum cell densities (Cells ml^{-1}), maximum specific growth rates and maximum toxin concentrations (SnEq $cell^{-1}$) among all treatments and light intensity conditions were tested using two-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD). Whenever an interaction between the two factors studied was revealed, 1-way ANOVA was applied to each treatment.

3. Results

3.1 Growth

P. parvum cell density decreased in monocultures exposed to high light intensity (700 µmol photons m^{-2} s^{-1}), and cells were totally photolysed within 4 h exposition to 2000 µmol photons m^{-2} s^{-1} (Fig. 2).

When exposed to 700 µmol photons m^{-2} s^{-1}, P. parvum cell densities decreased in monocultures while there were no significant differences ($p > 0.05$) from the initial cell densities in mixed cultures. At 2000 µmol photons m^{-2} s^{-1} P. parvum survival was longer in mixed cultures (up to 10 – 26 h) than in monocultures (4 h). P. parvum reached the highest cell densities, in both mono- ($7.46 \pm 0.32 \times 10^5$ cells ml^{-1}) and mixed ($8.42 \pm 0.47 \times 10^5$ cells ml^{-1}) cultures, when exposed to 100 µmol photons m^{-2} s^{-1}, and these cell densities were significantly higher than those obtained under the other conditions ($F = 192.01$, $p < 0.05$ for monocultures; and $F = 50.52$, $p < 0.05$ for mixed cultures). Statistical analysis of the maximum specific growth rates showed the same results for mixed cultures (1.06 ± 0.04 d^{-1}) ($F = 95.27$, $p < 0.05$), while the maximum growth rates registered in monocultures at 100 µmol photons m^{-2} s^{-1} (0.81 ± 0.09 d^{-1}), even if slightly higher, did not differ significantly ($p > 0.05$) from those in the dark conditions (0.60 ± 0.05 d^{-1}).

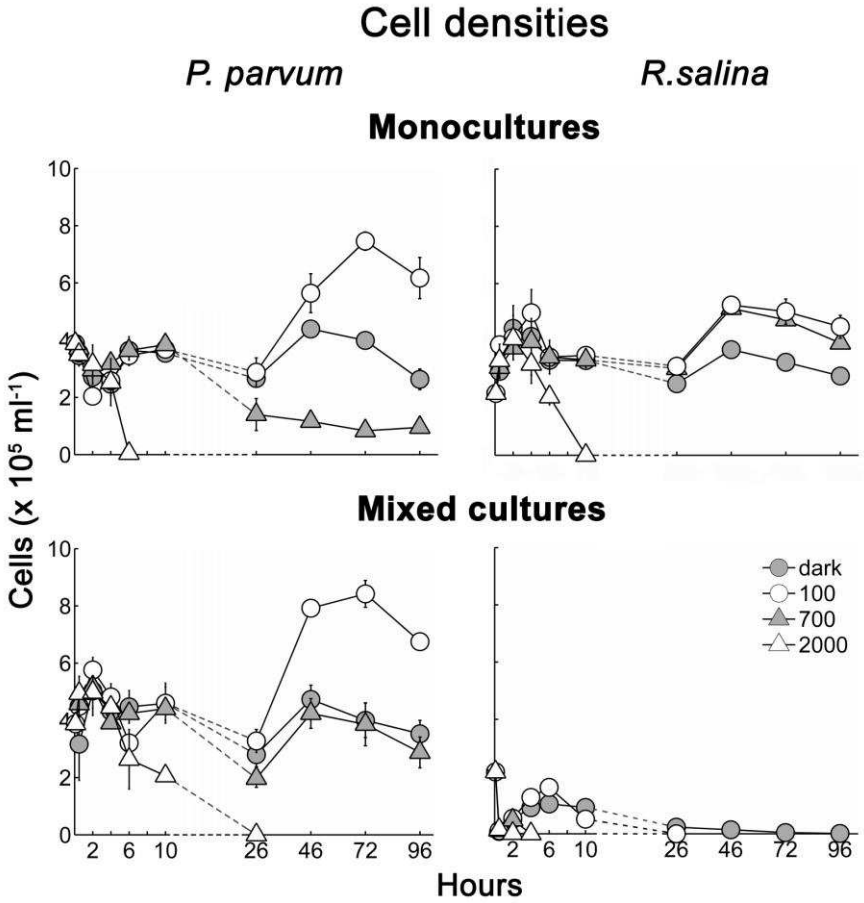

Fig. 2 Growth of mono (P. parvum and R. salina) and mixed cultures, exposed to four light conditions: dark, 100, 700 and 2000 µmol photons m^{-2} s^{-1}.

Both maximum cell densities and maximum specific growth rates were significantly higher in mixed than in monocultures in all light conditions ($F = 53.5$, $p < 0.05$ for maximum cell densities; and $F = 172.6$, $p < 0.05$ for maximum specific growth rates). In darkness P. parvum maximum cell densities were higher in mixed cultures than in monocultures, however this difference was not significant ($p > 0.05$). An interaction term was also observed between treatments and conditions ($F=8.5$, $p < 0.05$ for maximum cell densities; and $F = 47.7$, $p < 0.05$ for maximum specific growth rates), which underlined that the presence of prey influences the effect of light on P. parvum growth.

R. salina was more resistant than P. parvum to high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}). Neither species was able to grow at the highest light intensity studied (2000 µmol photons m^{-2} s^{-1}), however, R. salina lasted longer (10 – 26 h) than P. parvum. R. salina monocultures reached the highest cell densities (5.25 ± 0.16 and 5.13 ± 0.21 x 10^5 cells ml^{-1}) and maximum specific growth rates (0.63 ± 0.07 and 0.63 ± 0.08 d^{-1}) at 100 and 700 µmol photons m^{-2} s^{-1} (Fig. 2). Significant differences in maximum cell densities ($F = 50.54$, $p < 0.05$) and maximum specific growth rates ($F = 7.82$, $p < 0.05$) were observed between conditions of 100 and 700 µmol photons m^{-2} $s^{-1}$and conditions of dark and 2000 µmol photons m^{-2} s^{-1}.

R. salina cell densities based on) phycoerythrin (FL2) and chlorophyll-a (FL3) concentrations were also monitored throughout the experiment (Fig. 3). Cells grown in monocultures presented both pigment cell concentrations in the same range as those found in healthy R. salina cells at the beginning of the experiment. This corresponds to a red epifluorescence signal in observations of the cells under the microscope (Fig. 4- R. salina monocultures).

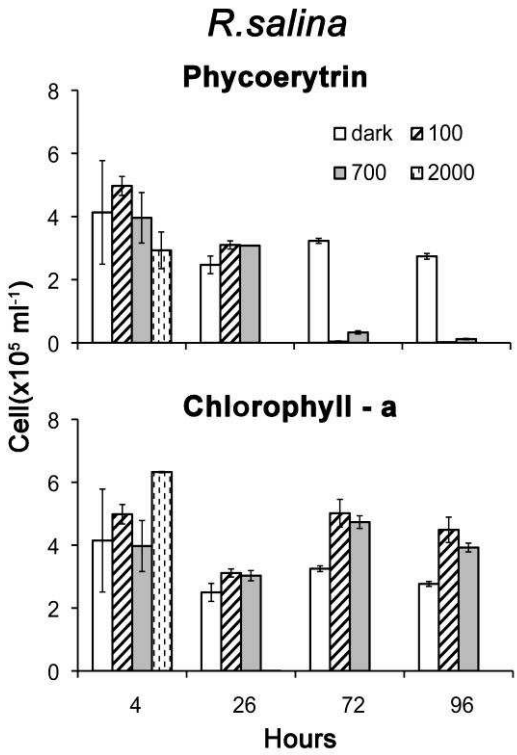

Fig. 3 R. salina monoculture cell densities based on phycoerythrin (FL2) and chlorophyll-a (FL3) contents measured under different light conditions: dark, 100, 700, 2000 µmol photons m^{-2} s^{-1}.

However, as R. salina cell densities decreased after 4 hours exposure to 2000 µmol photons m^{-2} s^{-1} (Fig. 3), a green epifluorescence signal was emitted instead (Fig. 4- R. salina monocultures).

None of these pigments were detected at time 26 h, which indicated that cells were totally destroyed. In the irradiance conditions of 100 and 700 µmol photons m^{-2} s^{-1} chlorophyll-a signals were detected up to 96 h which represented the end of the experiment, while phycoerythrin content decreased over time, reaching values close to zero after 72 h. Only cultures kept in darkness contained both pigments throughout the experiment (Fig. 3).

As soon as the two algae were mixed together, R. salina cells were lysed and rapidly attacked by several P. parvum cells which formed large aggregates around the prey before ingesting it. A high concentration of phycoerythrin inside P. parvum cells, after the cells had ingested R. salina, was observed when using epifluorescence microscopy (Fig. 4-mixed cultures).

When exposed to high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}), R. salina completely disappeared after only 2-3 h, while at 100 µmol photons m^{-2} s^{-1} and in dark conditions R. salina survived up to 46 h and 96 h, respectively (Fig. 2).

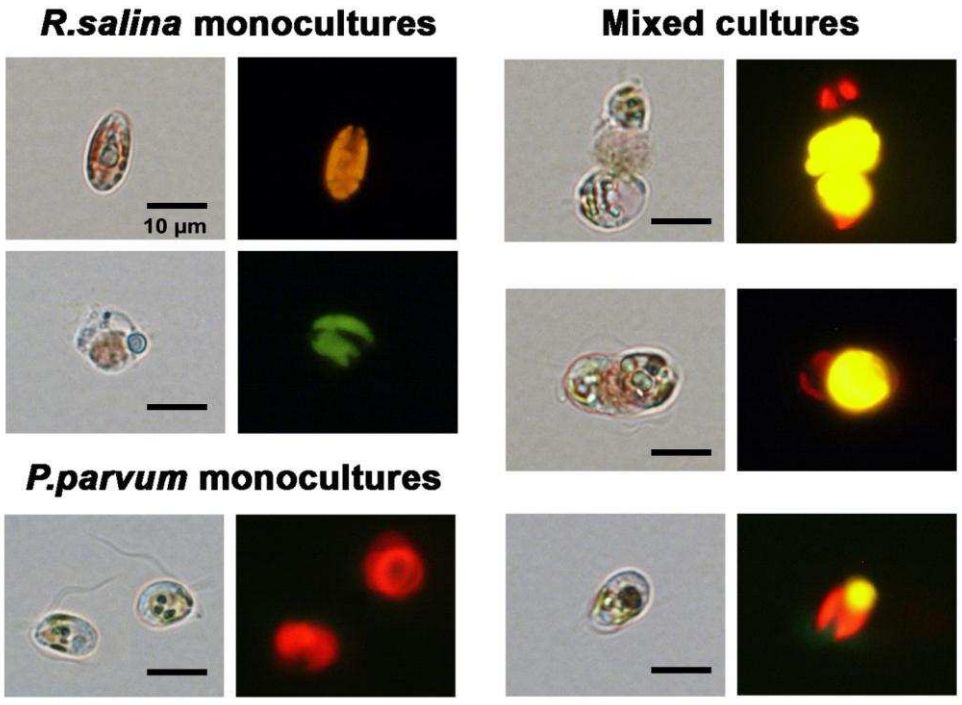

Fig. 4 Light microscope and epifluorescence micrographs of Rhodomonas salina (up: healthy cell with phycoeritryns; below: cell with low phycoeritryn level showing green epifluorescence signal), Prymnesium parvum monoculture and mixed cultures (up and middle: P. parvum cell eating R. salina; below: P. parvum cell with parts of R. salina inside, showing a high phycoeritryn signal). Photos: Christina Esplund.

It is easier to observe the pattern described above and to compare mono and mixed cultures when looking at the percentage changes in R. salina and P. parvum maximum cell densities normalized to initial cell densities (Fig. 5). P. parvum achieved higher values of density in mixed cultures than in monocultures in all conditions studied; for example an increase in cell densities of 116 ± 6.3 % was found in the mixed cultures grown at 100 µmol photons m^{-2} s^{-1}. When exposed to high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}), P. parvum cell densities were reduced (up to -1.7 ± 0.7 % and -10.3 ± 3.1 %) in monocultures, while they increased (respectively 32.5 ± 4.8 % and 27.5 ± 6.0 %) in mixed cultures.

R. salina maximum cell densities increased with increasing light exposition in monocultures, reaching the maximum value (133.2 ± 6.9 %) at 700 µmol photons m^{-2} s^{-1} irradiation. A decrease in R. salina cell numbers was observed in the mixed culture in all light and dark conditions.

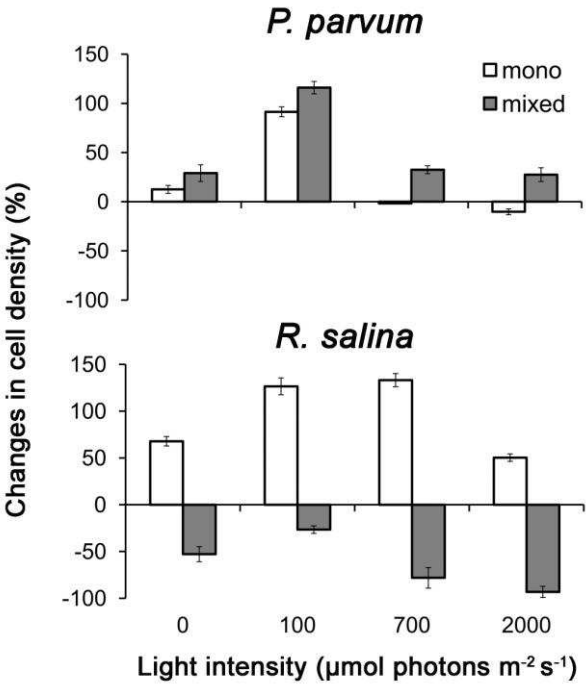

Fig. 5 Changes in P. parvum and R. salina maximum cell densities normalized with the first sampled cell densities (0.5 h), at different light conditions (0, 100, 700 and 2000 µmol photons m^{-2} s^{-1}). Cell densities at time 0.5 h are represented as x - axes. Positive and negative variations from the x - axes represent the increase and the decrease in cells numbers from the initial cell densities respectively.

3.2 Toxicity

Toxicity of the P. parvum stock culture, i.e. before the experiment started (time 0 h), expressed as hemolytic activity, was 0.17 SnEq $cell^{-1}$. All monocultures reached significantly higher maximum toxicity levels ($F = 1172.2$, $p < 0.05$) than mixed cultures (Fig. 6).

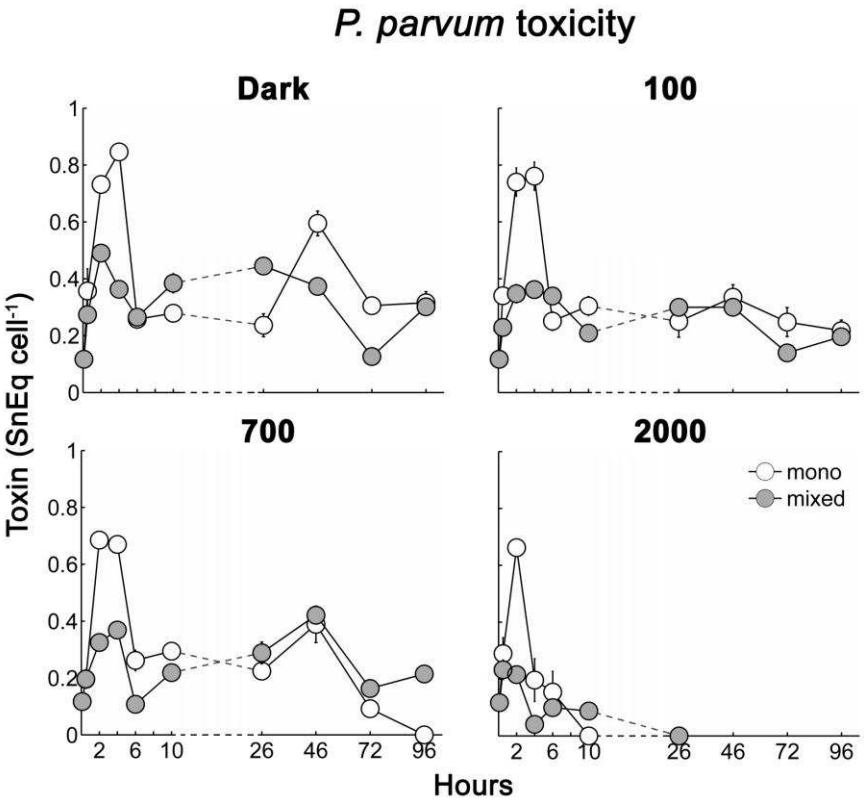

Fig. 6 P. parvum cellular toxicity (SnEq $cell^{-1}$) measured in mono and mixed cultures grown at different light conditions:
dark, 100, 700, 2000 µmol photons m^{-2} s^{-1}.

Moreover, a rapid increase in monoculture toxicity was detected during the first 2 – 4 h. The highest toxicity values were achieved when monocultures were kept in continuous darkness (0.85 ± 0.03 SnEq cell^{-1}) and these levels were significantly ($F = 36.9$, $p < 0.05$) higher than those obtained in light conditions (100, 700 and 2000 µmol photons m^{-2} s^{-1}). Cells grown as monocultures at 100 µmol photons m^{-2} s^{-1} were more toxic than cells exposed to higher light intensities (700 and 2000 µmol photons m^{-2} s^{-1}). Hemolytic responses lasted until the end of the experiment in monocultures at 100 µmol photons m^{-2} s^{-1}, while intracellular toxicity was completely ameliorated when monocultures were irradiated at 2000 µmol photons m^{-2} s^{-1} within 10 h, and at 700 µmol photons m^{-2} s^{-1} at time 96 h. No differences ($p > 0.05$) in the maximum cell toxicities were observed between monocultures exposed to 700 and 2000 µmol photons m^{-2} s^{-1}. When grown in mixed cultures P. parvum reached significantly ($F = 53.1$, $p < 0.05$) higher toxicity in darkness than in light conditions, the same pattern found for monocultures. Maximum cell toxicity levels did not differ ($p > 0.05$) between mixed cultures at 100 and 700 µmol photons m^{-2} s^{-1}. When P. parvum was exposed to 2000 µmol photons m^{-2} s^{-1}, it was able to survive longer in mixed cultures (up to 10-26 h) than in monocultures, showing low hemolytic activity until 10-26 h. Statistical analyses (2-way ANOVA) applied to maximum toxin concentration data, indicated an interaction between treatments (mono and mixed cultures) and light conditions ($F = 15.77$, $p < 0.05$), as was also the case for the maximum cell densities and growth rates.

Hemolytic responses were not detected in supernatant samples. However, when mixed cultures were exposed to high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}) a decrease in R. salina cell densities testified that there had been an increase in extracellular toxicity. In these conditions R. salina cell densities decreased - 83.8 ± 27.5 and - 99.7 ± 14.9 % (respectively at 700 and 2000 µmol photons m^{-2} s^{-1}) compared to the initial cell density, at time 2 h (Fig. 7). In monocultures R. salina cell densities were more than 50 % higher than the initial cell densities in all conditions (dark: 50.5 ± 3.9; 100 µmol photons m^{-2} s^{-1}: 58.6 ± 4.6; 700 µmol photons m^{-2} s^{-1}: 51.6 ± 4.0) until time 10 h, except at 2000 µmol photons m^{-2} s^{-1} where cell densities were 7.2 ± 0.9 % lower than the initial values.

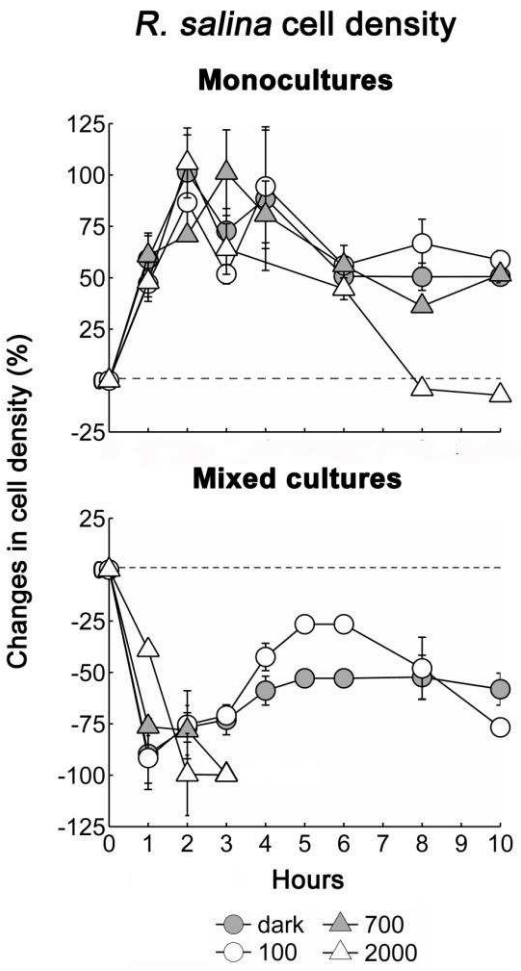

Fig. 7 Growth of R. salina in mono and mixed cultures, exposed to 4 light conditions: dark, 100, 700 and 2000 µmol photons m^{-2} s^{-1}, during the first 10 h of the experiment.

4. Discussion

4.1 Growth

In the conditions tested (dark, 100, 700 and 2000 µmol photons m^{-2} s^{-1}), the light intensity of 100 µmol photons m^{-2} s^{-1} represented the optimal growth condition for P. parvum, both when growing as mono- and as mixed cultures. P. parvum is inhibited by high light irradiances (700 µmol photons m^{-2} s^{-1} and above this level). When cultures were exposed to 700 and 2000 µmol photons m^{-2} s^{-1} P. parvum cells stopped growing and were photolysed. In particular, at 2000 µmol photons m^{-2} s^{-1} the cells were totally destroyed during the first 26 hours. Rhodomonas salina on the other hand grew well in monocultures at 100 and 700 µmol photons m^{-2} s^{-1}, and its survival time at 2000 µmol photons m^{-2} s^{-1} was longer than for P. parvum.

In mixed cultures P. parvum used its toxic compounds to paralyze and capture R. salina cells,

which were rapidly phagocytated. This has also been reported in a number of published studies (Skovgaard and Hansen, 2003; Tillmann, 2003; Granéli, 2006; Uronen et al., 2007; Bowers et al., 2010). Phagotrophy was observed at all light intensities, confirming that it represents a permanent nutritional adaptation for P. parvum even under optimal conditions, as reported by Carvalho and Granéli (2010) and Granéli et al. (2012). Moreover, our results show that P. parvum achieved higher cell densities and growth rates in mixed cultures than in monocultures. As observed in other studies, the ingestion of R. salina contributed to the mixotrophic growth of P. parvum (Legrand et al., 2001; Fistarol et al., 2003; Granéli and Johansson, 2003a; Tillmann, 2003; Lindehoff et al., 2009; Brutemark and Granèli, 2011). However, the significant increase in P. parvum cell densities in mixed cultures could also have been favored by the availability of nutrients due to the mixing with R. salina, that was grown in nutrient enriched medium. Furthermore, we observed that P. parvum was only able to grow at 700 µmol photons m^{-2} s^{-1} when in mixed cultures, and survival was longer in mixed cultures than in monocultures when the cells were exposed to 2000 µmol photons m^{-2} s^{-1}. Another possible explanation is the shading effect between R. salina and P. parvum cells, as the total cell density in mixed cultures was higher (approximately 1/3 more, total cell density in mixed cultures: 11.96 x 10^5 cells ml^{-1}) than in the monocultures (P. parvum cell density: 7.76 x 10^5 cells ml^{-1}) at the beginning of the experiment (time 0 h). However, the shading effect due to R.salina cells is plausible only during the first 2 hours of the experiment. At high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}) R. salina was rapidly phagocytated by P. parvum. At time 2 h R. salina cell densities had already decreased by up to - 83.8 ± 27.5 and - 99.7 ± 14.9 % compared to the initial cell density. After 3 h only P. parvum cells were detected in mixed cultures exposed to high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}).

4.2 Toxicity

In our experiment, the light condition under which the maximum cell densities were reported (100 µmol photons m^{-2} s^{-1}) did not coincide with that for toxin production (darkness). The same pattern has also been reported for other toxic species, such as Microcystis aeruginosa (Van der Westhyizen and Eloff, 1985); Alexandrium tamarensis (Ogata et al., 1987) and Heterosigma akashiwo (Ono et al., 2000), which showed an increase in toxin production when grown in non-optimal light conditions. Furthermore, toxicity of these species has also been reported to be greatly influenced by light conditions (Ogata et al., 1987; Utkilen and Gjølme, 1992; Ono et al., 2000). Just like P. parvum, Microcystis aeruginosa has been reported to decrease its toxicity when exposed to irradiance higher than 40 µE m^{-2} s^{-1} (Utkilen and Gjølme, 1992).

P. parvum reached the maximum intracellular toxicity levels when grown in monocultures in dark conditions. When cultures were exposed to high light intensity (700 and 2000 µmol photons m^{-2} s^{-1}) intracellular toxicity significantly decreased and no hemolytic activity was detected after 6 hours exposition to 2000 µmol photons m^{-2} s^{-1}. However, even at high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}) we found a rapid increase in hemolytic activity during the first hours of the experiment in the monocultures. Similar results were reported by Freitag et al. (2011), i.e. an increase in P. parvum intracellular toxicity when cells were adapted to 90 µmol photons m^{-2} s^{-1} and then shocked for 2 h at 700 µmol photons m^{-2} s^{-1}. P. parvum cells kept in mixed cultures presented lower toxicity than cells grown in monocultures. As suggested by Legrand et al. (2001), prey can supply the nutrients that are lacking in the water and thereby allow P. parvum to grow in conditions that should be limiting.

In our study, discrepancies between hemolytic activities and extracellular toxicity were observed, as is reported in the scientific literature elsewhere (Schug et al., 2010; Freitag et al., 2011). Contrary to hemolytic activity, extracellular toxicity significantly increased in mixed cultures exposed to high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}). Prymnesium toxins are reported to be released when there is cell-to-cell contact with the prey, and also due to stress when growth conditions are not optimal (Remmel and Hambright, 2012), as in our case when high light intensity induced stress. R. salina disappeared faster (within only 3 hours) in mixed cultures exposed to high light exposition (700 and 2000 µmol photons m^{-2} s^{-1}) than in cultures grown at low light (dark and 100 µmol photons m^{-2} s^{-1}) intensities, where it lasted until the end of the experiment. At high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}) toxins were released in the medium due to photolysis of P. parvum cells. This process caused an increase of extracellular toxicity that rapidly killed R. salina during the first hours of the experiment, before the released toxins were degraded by the high light intensity. The process of photo-degradation of toxins requires a certain period of time, not yet established. Previous studies demonstrated different periods of time required to degrade P. parvum

toxins: Parnas et al. (1962) reported a complete photo-inactivation of P. parvum toxins within 90 min when exposed to continuous illumination (400-520 nm and UV light 255 nm); Freitag et al. (2011) observed a decrease in extracellular toxicity (measured as lysed Rhodomonas baltica cells) after P. parvum cells were exposed to 700 µmol photons m^{-2} s^{-1} for 2 h; James et al. (2011) found that toxicity to fish can be completely ameliorated when cell-free filtrate was exposed to natural sunlight (2-8 h). However, there are no studies reported in the literature concerning the changes in P. parvum extracellular toxicity during the first hours of exposition to high light levels. In the studies cited above (Freitag et al., 2011; James et al., 2011) the bioassays, with phytoplankton and fish, were conducted after P. parvum was treated for a certain period of time with high light. In our study we exposed R. salina to P. parvum in mixed cultures from the beginning of the experiment (time 0 h) when the exposition to different dark and light (100, 700 and 2000 µmol photons m^{-2} s^{-1}) conditions started. There are difficulties of comparison with other studies as R. salina was exposed to P. parvum cells in mixed cultures, and not to the filtrate. P. parvum cells can produce toxins continuously, while in the filtrates toxins are progressively photolysed (Fistarol et al. 2003, Granéli and Salomon 2010).

Although high light intensities inhibit P. parvum growth and reduced its hemolytic responses, we conclude that extracellular toxicity is significantly enhanced at light intensity of 700 µmol photons m^{-2} s^{-1} and above this level, causing the death of the co-occurring phytoplankton species.

5. Conclusions

High light intensity exposition (700 µmol photons m^{-2} s^{-1} and above this level) can be used as an efficient method of controlling Prymnesium parvum growth and of reducing its hemolytic activity. However, in mixed cultures the effect of high irradiation on P. parvum growth was significantly reduced, in particular at 700 µmol photons m^{-2} s^{-1} where P. parvum was able to grow when in mixed cultures. Therefore, in natural conditions the presence of prey or the availability of nutrients plays an important role not only in the survival of P. parvum even in stressful light conditions but also in decreasing the cells toxicity. Extreme high light intensity (2000 µmol photons m^{-2} s^{-1}) seems to be required in order to efficiently reduce P. parvum growth and cell toxicity. Only when exposed to 2000 µmol photons m^{-2} s^{-1} was intracellular toxicity completely ameliorated within 26 h in both mono and mixed cultures. However, even at high light intensities (700 and 2000 µmol photons m^{-2} s^{-1}) our results show an increase in both intracellular and extracellular toxicity in the first hours of the experiment. Therefore, high light irradiation cannot be implemented as mitigation method.

More information about the P. parvum effect on other co-occurring species during high light irradiation is required regarding the use of high light exposition as a mitigation method in fish farms, with particular attention paid to the first hour of exposition when both intracellular and extracellular toxicity rapidly increases.

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Chapter 5

Emamectin inhibits Calanus finmarchicus feeding and reproduction

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Abstract

Reproduction (egg production) and feeding (fecal pellet production) of the calanoid copepod Calanus finmarchicus was assessed during a mesocosm experiment with a marine pelagic community. The aim of the mesocosm experiment was to investigate possible interactions between a toxic substance, emamectin (EMA), oil and diatoms bloom. EMA is an antiparasitic pharmaceutical used in aquaculture, known to be particularly toxic to some crustaceans. EMA was added alone and in combination with mineral oil in different mesocosms, in which diatom or non-diatom blooms were generated by fertilizing a natural plankton community from a coastal area in western Norway.
Mature females of C. finmarchicus were incubated in water collected daily from each mesocosm and their eggs and fecal pellets production were recorded during seven days. Reproduction and feeding were dramatically reduced in all females exposed to EMA. Egg and fecal pellet production dropped to 0 after 48 hours of incubation and at an EMA concentration of 18 ng L^{-1}. There was no significant modulation of the effects by the addition of oil and/or diatoms, but any interactions were presumably overshadowed by the strong effect exerted by EMA on C. finmarchicus.

Keywords: Toxicity; Zooplankton; Oil; Emamectin; reproduction; feeding; mesocosm.

1. Introduction

Aquaculture may affect marine ecosystems in several ways: inputs of nutrients and the presence of pharmaceuticals are the two most important pressures fish farms exert in coastal waters. In 2011 there were 1464 fish farm along the coasts of Norway and of these about 70% were salmon farms (Norwegian Directorate of Fisheries, 2012). Farmed salmon are most commonly grown in coastal areas inside large, floating cages which allow the release of nutrients and chemicals to the marine environment (Burridge et al. 2010). Farmed salmon are susceptible to epidemics of parasitic diseases. Sea lice are ectoparasitic copepods which infect many species of fish and have become a serious problem for the salmon aquaculture industry (Roth et al. 1993). The most effective methods to limit sea lice epidemics in aquaculture have in the past entailed the use of chemical antiparasitic substances. They can be administrated to the fish or by bath treatment or by in-feed treatment. Compounds used in in-feed treatments will ultimately to some extent be released to the aquatic environment. They can be distributed through the marine ecosystem in the vicinity of salmon farms in the form of unconsumed food waste and through fecal pellets from treated fish (Willis and Ling 2003). Anti-lice agents are not species specific and may therefore inadvertently affect non-target organisms in surrounding areas (Burridge et al. 2010). Emamectin benzoate (EMA) is the active ingredient of the antiparasitic treatment Slice®. It is administrated to salmonids in feed and remains active for several months after digestion

and assimilation (McHenery and Mackie 1999). EMA has been shown to be highly toxic also for non-parasitic copepods. Non-parasitic copepods are the dominant crustaceans in marine plankton biomass and play a critical role in marine food webs as energy transfer from the primary production to higher trophic levels (Verity and Smetacek 1996). However. Willis and Ling (2003) tested the toxic effect of EMA on different life stages of four copepod species and found that the concentration causing copepod mortality was several orders of magnitude higher than the highest concentration found in the vicinity of fish cages. This was further confirmed by a field study (Willis et al. 2005), leading to the conclusion that the use of EMA for sea lice control would be unlikely to affect natural copepod populations.

When a toxicant is released to the marine environment, however, its effect will not only depend on its concentration, but also on modulation by other biotic and abiotic factors such as e.g. phytoplankton blooms, the presence of other sources of organic carbon and/or interaction with other contaminants. A phytoplankton bloom, which may be stimulated by nutrients released from the fish farm, may act as "sponges" to mop up organic and many inorganic contaminants in the water column. Therefore, for filter-feeding copepods, the association of EMA with phytoplankton cells may actually increase exposure as both food and water will contain the toxin. For carnivorous copepods this process would decrease water-borne exposure, but increase exposure through food exposure. Oil spills are ubiquitous in coastal waters and the presence of oil micelles or droplets in the water column would be expected to modulate EMA bioavailability for copepods. Oil may however also represent an energy source for bacteria (Bailey et al. 1973), which are linked to copepods through microzooplankton. Copepods feeding on microzooplankton could therefore accumulate EMA to higher concentrations than would be expected from bioconcentration from water only.

The objective of this study was to clarify whether EMA would affect copepod reproduction and feeding, alone or in the presence of oil or water from a phytoplankton bloom. Test solutions came from a mesocosm experiment. As model organism we chose Calanus finmarchicus, a key species in Atlantic and in the Norwegian waters, constituting up to 70% of mesozooplankton biomass during the summer period (Tande 1991; Planque and Batten 2000). C. finmarchicus mature females were incubated in in laboratory in water collected daily from each mesocosm for seven consecutive days. Egg production, egg hatching success and fecal pellet production were compared between the different treatments.

2. Materials and Methods

C. finmarchicus reproduction and feeding was studied during a mesocosm experiment conducted between the 2nd and 12th of June 2010 at the Espeland Marine Biological Field Station by the Raunefjord, close to Bergen, Western Norway. For a general description of the location see http://www.uib.no/bio/en/research/infrastructure-at-bio/espeland-marine-biological-station. In the present experiment eighteen land-based tanks (average water volume ca. 2.4 m^3; inner diameter 1.5 m, average water depth 1.4 m) were filled with unfiltered seawater collected outside the field station at ~12 m depth. Water was collected with a pump specially designed to minimally damage live plankton. To ensure that each mesocosm was as similar as possible, the individual mesocosms were filled sequentially, a third at a time, such that the process was staggered. The eighteen tanks were placed in three large tanks with a continuous flow of seawater that functioned as water baths to keep the temperature stable. Temperature in the tanks varied between 10 and 14°C during the experimental period. A gentle bubbling system ensured that the water in the mesocosms was continuously circulated to avoid stratification.

Mesocosms were manipulated to yield six different treatments: control (Ctr), diatom (Si), oil (Oil), emamectin (EMA), diatom and emamectin (Si+EMA) and oil and emamectin (Oil+EMA). There were three replicated tanks for each treatment. All treatments except Ctr were fertilized with $NaNO_3$ and KH_2PO_4 to initial concentrations of 1.36×10^{-6} ng L^{-1} and $7.0\ 10^{-4}$ ng L^{-1} respectively. In the Si treatments, diatoms bloom was stimulated adding 1.22×10^{-6} ng L^{-1} of Na_2O_3Si. Oil treatments received a dose of 6.1×10^{-6} ng L^{-1} of pure mineral oil. (M3516, Sigma-Aldrich®; mostly alkenes i.e. ~5 mg C · L^{-1}). EMA treatments received 100 ng L^{-1} of emamectin benzoate (PESTANAL®) (CAS 119791-41-2; QP54A A06) every second day from Day 0 until Day 8 for a total of five additions.

Depth-integrated water samples (0–1.4 m) of all 18 mesocosms were taken daily around 08h00 by using tube samplers and pouring the water into plastic carboys. Sampling was organized as to minimize contamination risk and separate tubes were used for Oil and EMA treatments.

EMA concentration was estimated at day 1, 5 and 9 of the experiment. 0.3 L of water was filtered through methanol washed GF/F filters following filtration through methanol washed 0.2 µm Poretics

Polycarbonat filters. Solid phase extraction (SPE) was thereafter performed on the filtered water samples using Strata-X 33 µm Polymeric Reversed Phase (Phenomenex®)). Filters and SPE-columns were stored at -20°C until double extraction of the filter associated EMA using methanol and acetone (1:9) and SPE elution with 2 x 6 mL methanol into silanised glass tubes. This was followed by liquid chromatography tandem mass spectrometry (LC/MS/MS) analysis.

Copepods were collected one nautical mile from the mesocosm location with an oblique tow from 50 to 0 m depth using a 330 µm mesh size, 1 m diameter net with a 4 L non-filtering cod-end. At the same location ≈ 10 L of water were collected from 5 m depth using a Kemmerer water sampler. Water was collected inside a carboy. Copepods and water were transferred within 30 minutes to a temperature controlled room maintained at the mesocosms water temperature (10°C) and with a 17:7 hour light:dark regime. These laboratory conditions were maintained constant for the rest of the experiment. Mature females of Calanus finmarchicus were sorted with a wide-mouth pipette under a dissecting microscope. Different Calanus species may be present in this area at this time of year. C. finmarchicus was identified and distinguished from potentially co-ocurring C. helgolandicus based on the shape of the spermathecae according to Williams (1972). Mature females of C. finmarchicus were placed into a 5 L beaker filled with the water collected in fjord. Approximately 2 L of water were daily removed from the beaker and replaced with the water collected previously collected in the fjord.

After three days, 18 females were placed individually in 400 mL cylinders with a 500 µm bottom mesh to prevent egg cannibalism. The cylinders were kept in 500 mL polyethylene beakers filled with water daily collected from each of the 18 mesocosms. After each 24-h incubation period, the cylinders with the females were transferred to new beakers with freshly collected mesocosm water. Eggs and fecal pellets were collected by carefully pouring the content of each beaker into a submerged 40-µm filter and then back washing the filter into a 6-well tissue plate. Eggs were immediately counted under a dissecting microscope and incubated in the same mesocosm water and at the same laboratory conditions, for 48 h to allow hatching. After 48 h non-hatched eggs and nauplii were fixed in 4% formaldehyde. The number of hatched nauplii was counted under a dissecting microscope one week after the end of the experiment in order to quantify hatching success. Fecal pellets were immediately counted and measured under a dissecting microscope. Fecal pellet volume was estimated assuming a cylindrical shape and it was used as a proxy for C. finmarchicus daily feeding (Nejstgaard et al. 2001).

After seven days copepods were removed from the mesocosm water and incubated for 48 h in EMA-free filtered seawater.

Data are reported as mean values between the three replicated of each treatment ± standard deviation (SD) or ± standard error of the mean (SEM) for the analysis of EMA concentration. Distributions built with the mean values of egg hatching success and with the cumulative number of eggs and fecal volume produced for each treatment during the whole experiment, were compared using ANOVA followed by Dunnett's test for comparisons versus the control. Data were checked for equal variance and were log-transformed when the normality condition was not satisfied.

3. Results

EMA concentrations were higher at the end than at the beginning of the experiment (Table I). On Day 1 after the first dose of 100 ng EMA·L^{-1}, concentrations varied between 4.3 and 10.2 ng EMA·L^{-1} in the EMA + Oil and EMA treatments respectively.

Day	Treatment	Mean ± SEM (ng L^{-1})
1	EMA	10.2 ± 3.7
	EMA + Oil	4.3 ± 1.1
	EMA + Si	6.9 ± 1.8
5	EMA	22.7 ± 3.6
	EMA + Oil	24.5 ± 6.4
	EMA + Si	26.8 ± 4.5
9	EMA	23.0 ± 0.6
	EMA + Oil	16.0 ± 0.6
	EMA + Si	39.7 ± 12.0

Table I. Emamectin concentrations in the water on the indicated days.

At day 9, after the addition of 5 x 100 ng·L^{-1} EMA, EMA + Oil treatment had a concentration of 16.0 ± 0.6 ng·L^{-1} EMA, while the highest EMA concentration was found in the EMA + Si treatments (39.7 ± 12.0 ng·L^{-1}) (Table I).

EMA had a rapid and dramatic effect on copepod physiology. Following 24 h of incubation C. finmarchicus drastically changed its behaviour in all EMA treatments. Copepods were lying alive, but motionless, on the bottom of the experimental beakers and after the second day they stopped producing eggs and fecal pellets (Figures 1A and 3A).

In all EMA treatments, C. finmarchicus egg production decreased from 44-58 eggs $female^{-1}$ to 3-14 eggs $female^{-1}$ already after one day of incubation and it dropped to zero at day 3 remaining unvaried throughout the experiment (Figure 1A). In the Si and Oil treatments, the cumulative egg production was not significantly different from the Ctr treatment (Dunett $p > 0.05$). During the entire experiment, C. finmarchicus females produced in total 426 ± 186 eggs in the Ctr treatment, compared to 388 ± 103 and 432 ± 196 eggs $female^{-1}$ in the Si and Oil treatments, respectively (Figure 1B).

EMA toxic effect on egg production was reflected in the egg hatching success (Figure 2A-B). No significant differences (ANOVA $p = 0.26$) between the treatments were observed for eggs laid during the first day. In all EMA treatments hatching success ranged between 59% and 72% but then decreased to 0% at day 2 in the EMA+Si treatment and at day 3 both in EMA and in EMA+Oil treatments (Figure 2A). No differences were also observed between the non-EMA treatments for which the mean hatching success ranged between 57% and 68% over the whole experiment (Figure 2B).

As mentioned above feeding activity was also strongly and rapidly affected by EMA. Already after 24 h, all C. finmarchicus incubated in the EMA decreased their fecal pellets production which dropped to zero after the second day of incubation (Figure 3A). No differences were observed in feeding activity between the no-EMA treatments. The cumulative fecal volume produced in the diatom and oil treatments were similar to the control (Dunett's > 0.05) (Figure 3B).

All EMA affected copepods remained immobile throughout the experimental period and this was also evident after a recovery period of 48 h in EMA-free filtered seawater. Within the time frame of this study, the effect of EMA was irreversible.

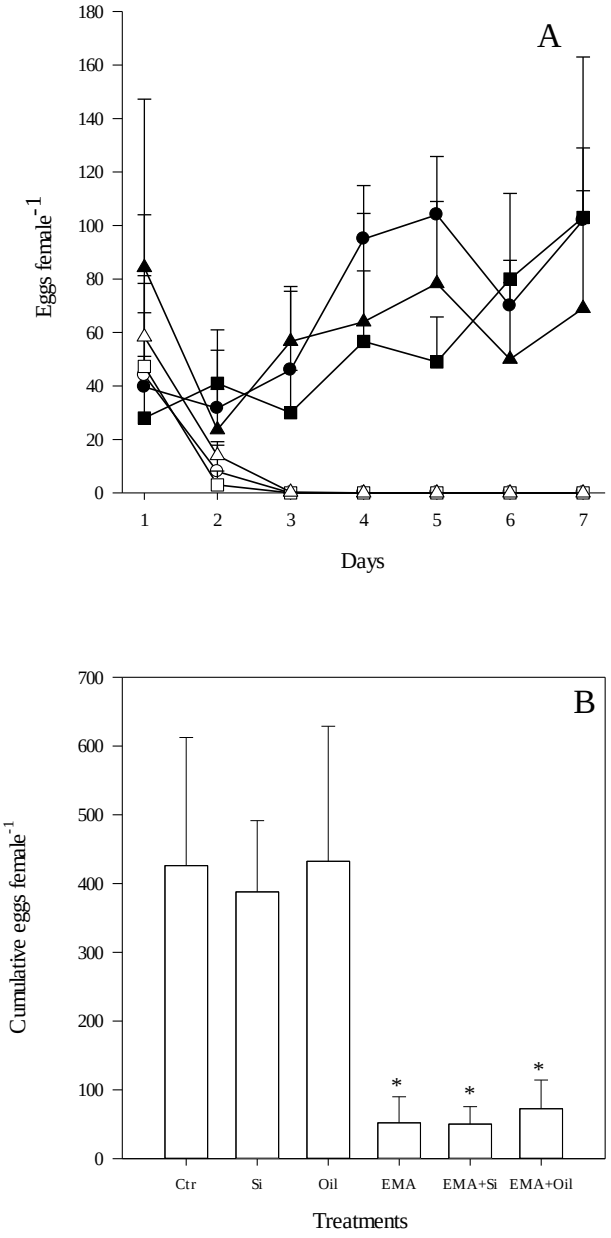

Figure 1: **A)** Mean (+SD) daily egg production of Calanus finmarchicus females for each treatment. ▲ control; ■ diatoms; ● oil; EMA; □ EMA + diatoms; ○EMA + oil. **B)** Mean (+SD) of the cumulative number of eggs produced per C. finmarchicus females over the whole experiment. Dunett's method was used after ANOVA for multiple comparison versus the control treatment: * $p = 0.05$.

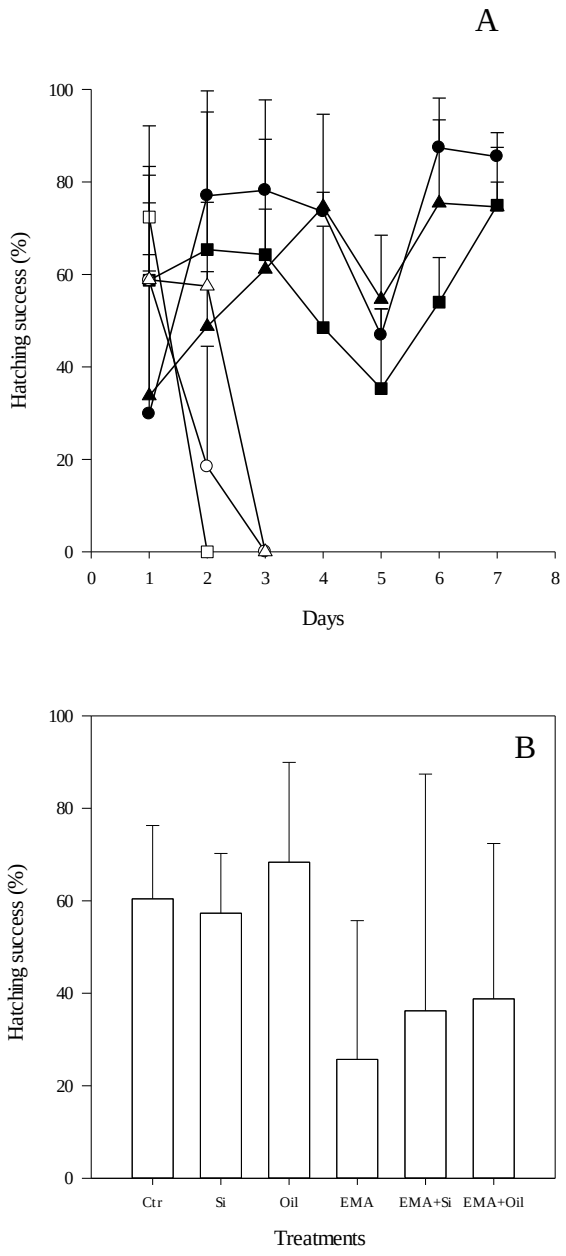

Figure 2: **A)** Mean (+ SD) daily egg hatching success (as %) for each treatment. See Figure 1 for a description of the symbols. **B)** Mean (+SD) daily egg hatching success (as %).

4. Discussion

The interruption of the mobility, reproduction and feeding of C. finmarchicus observed in this study were clearly due to the toxicity of EMA. Thus, copepod performances in both oil and diatom treatments did not differ from the control. Because EMA affected C. finmarchicus so strongly, it was not possible to observe modulation of EMA toxicity as a result of interactions with oil or higher density of diatoms. Based on earlier observations (Connor et al. 1994; Willis and Ling 2003) it was unexpected that the concentrations of EMA used would have such dramatic effects. EMA was added to mesocosms every second day throughout the experiment in an attempt to maintain the concentration of the substance at the same level and the initial concentration was 18 ng. L^{-1}. In an earlier study, 96-h LC50, for the mysidacean shrimp Mysidopsis bahia was 40 ng L^{-1} (Connor et al. 1994). In a 7-day study on the effects of EMA on the reproduction of the copepod Acartia clausi, Willis and Ling (2003) did not observe any significant reduction in egg production (no-observed-effect-concentration, NOEC) at an EMA concentration of 50 ng L^{-1} over the experimental period. They only observed a significant decrease in A. clausi egg production at 158 ng L^{-1}. In addition, EMA effects were more dramatic in the current study than the observations by Willis and Ling (2003). As reported above, C. finmarchicus were completely immobile with no feeding activity and eggs production, while in Willis and Ling (2003) A. clausi continued to produce eggs (ca. 30 eggs $female^{-1}$) even at an EMA concentration of 500 ng/L, and there were no signs of immobility in that study.

The above differences were presumably due to different EMA tolerance of the two copepod species. Acartia spp. would however have been expected to be more sensitive to EMA than C. finmarchicus since its physiology is characterized by low energy storage capacity which results in rapid responses to toxins and changes in food availability (Kleppel et al. 1998). In contrast, C. finmarchicus is a species with a slower response to changes in the food environment because its metabolism can be fuelled by its lipid reserves (Båmstedt et al. 1999) and is therefore considered also more resistant to toxins.

Another possible explanation for the discrepancy between this study and that of Willis and Ling (Willis and Ling 2003) are different experimental

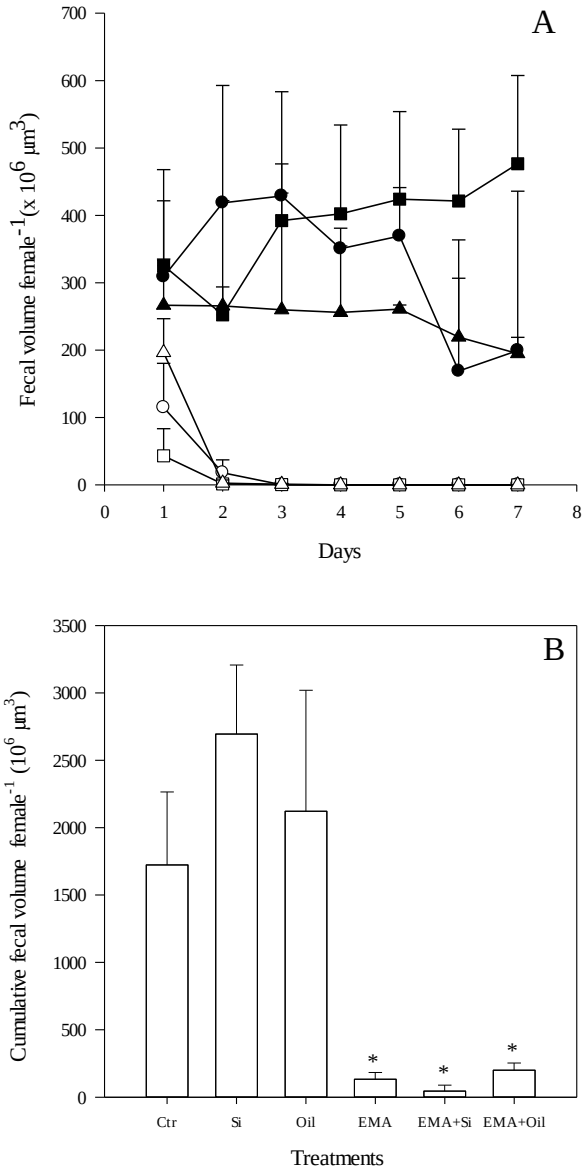

Figure 3: **A)** Mean (+ SD) daily fecal volume produced by Calanus finmarchicus females for each treatment. See Figure 1 for a description of the symbols. **B)** Mean (+SD) of the cumulative fecal volume produced by C. finmarchicus females over the whole experiment. Dunett's method was used after ANOVA for multiple comparison versus the control treatment: * p = 0.05.

conditions. Water temperature was higher (14°C) in the Willis and Ling (Willis and Ling 2003) experiment compared with our study (10°C). In addition, copepods from a natural population were used in the current study and they were fed the plankton ocurring in the mesocosms. Willis and Ling (Willis and Ling 2003) used laboratory cultures of copepods, which were fed cultured phytoplankton. The culture medium used, Walne's medium (Walne 1970), contains EDTA, which may have affected the bioavailability of EMA. To avoid possible effects of growth media, phytoplankton cells should be filtered and re-suspended in chemical-free water to avoid bias due to chemical interactions.

Finally, the higher sensitivity to EMA observed for C .finmarchicus compared to Acartia clausi (Willis and Ling 2003) could have been due to the general condition of the copepods, i.e. that the organisms used in the current study were stressed and more susceptible to EMA. There was however no sign of stress for Calanus in the other exposures.

Experimental conditions appear to have an important role in determining the effect of EMA in C. finmarchicus physiology. More studies are necessary to determine EMA LOEC values for this important species and the test should be performed in conditions as close as possible to the natural ones. For this scope, mesocosms experiments represent a useful tool.

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