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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 coastal and groundwater. 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, based on PSII inhibition, were: *Gonyaulax spinifera* > *Fibrocapsa japonica* > *Lingulodinium polyedrum* while the most resistant were 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 *G. spinifera*, were exposed, under different temperature conditions (15, 20 and 25 °C), to 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 was 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 $\mu g\,L^{-1}$ 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 exposure *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.

1. Introduction

The Northern Adriatic Sea is characterized by shallow waters (70 m maximum depth) and significant freshwater input coming mainly 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

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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 exceed acceptable 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 commonly detected herbicides in Italian rivers and ground waters (Sbrilli et al., 2005). The half-life of TBA 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 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) who 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 concentrated on 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 polysaccharide production, were examined in two flagellates (*Prorocentrum minimum* and *G. 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 simulating the herbicide effects on primary producers (Fiori, 2013).

2. Materials and methods

2.1. Phytoplankton cultures

The species used were the Dinophyceae: *P. minimum* (Pavillard) Schiller, *P. micans* Ehrenberg, *P. reticulatum* (Claparède and Lachmann), *A. lusitanicum* Balech, *S. trochoidea* (Stein) Loeblich III, *L. polyedrum* Stein (Dodge), *G. spinifera* (Claparède et Lachmann) Diesing and the Raphidophyceae *H. akashiwo* (Y. Hada) Y. Hada ex Y. Hara & M. Chihara and *F. 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 most affected by the above 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, *P. minimum* (chosen from the most tolerant species) and *G. spinifera* (among 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 length of time.

Growth curves were monitored through cell counting made daily in settling chambers following Utermohl's method (Hasle, 1978). All experiments were run in duplicate; 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