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Toxicity of Epoxiconazole to the Marine Diatom *Chaetoceros calcitrans*: Influence of Growth Conditions and Algal Development Stage

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Abstract The triazole fungicide epoxiconazole is extensively used to control fungi on crops and may present some potential risk from runoff on coastal ecosystems located close to agricultural areas. Phytotoxicity assessments were conducted on the marine diatom *Chaetoceros calcitrans* using both the active ingredient and its formulated product (Opus). The 3-day EC_{50} using cell count was 2.31 mg/L for epoxiconazole active ingredient and 2.9 μg/L for epoxiconazole-formulated. The fungicide produced an increase of cellular volume, pigment (chlorophylls *a*, *c*, and carotenoids) content, ATP synthesis, and rates of photosynthesis and respiration. Progressive algal cell recovery from epoxiconazole effects occurred after 3 days, with the increasing cell density. Differences in cell age, light, and nutrient composition induced changes in epoxiconazole sensitivity. Since these parameters affect cellular division rates, the cellular density is an important parameter in toxicity tests.

Keywords Age cell · *Chaetoceros calcitrans* · Culture conditions · Epoxiconazole · Growth · Physiological parameters

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1 Introduction

Epoxiconazole (CAS registry number 133855-98-8), a systemic triazole fungicide, is commonly used in the world to control fungi and to regulate plant growth in a number of economically important crops such as cereals and fruits like grapes (Ammermann et al. 1992; Schöfl and Zinkernagel 1997; Bertelsen et al. 2001). This broad-spectrum fungicide inhibits the cytochrome P450-mediated C14 demethylase reaction, crucial to the formation of ergosterol, component of fungal cell membrane (Akers et al. 1990; Kwok and Loeffler 1993).

Several studies report on the physiological effects of epoxiconazole in higher plants; however, most of them are related to its use in agriculture (Benton and Cobb 1997; Wu and Von Tiedemann 2001; Percival and

Noviss 2008). Epoxiconazole is very persistent in soil and aquatic sediment (Bromilow et al. 1999; Tomlin 1999; Lin et al. 2001; AGRITOX 2010; Passeport et al. 2011) and could lead to accumulation. Following repeated applications, it enters adjacent freshwater ecosystems from spraying, leaching, and runoff, making its way to estuarine and marine areas. As a consequence, the introduction of this xenobiotic pollutant into the marine environment may lead to serious noxious effects on nontarget aquatic organisms, including phytoplankton.

The potential threat of epoxiconazole on aquatic ecosystems has raised considerable concern, and the toxicity of epoxiconazole on fish, Daphnia, freshwater algae (AGRITOX 2010), and oyster (Stachowski-Haberkorn et al. 2008) has been studied. Moreover, epoxiconazole may interact synergically with an herbicide (Stachowski-Haberkorn et al. 2008) or a pyrethroid insecticide towards *Daphnia magna* (Nørgaard and Cedergreen 2010).

Phytoplankton is dominated by microalgae which play an important role in the equilibrium of marine ecosystems, being at the basal level of food webs and the starting point of trophic transfer (Zeitzschel 1978). In a recent report, Baird and DeLorenzo (2010) have shown that *Dunaliella tertiolecta* was especially susceptible to four conazole fungicides. Up to now, data on toxicity effects of epoxiconazole on marine microalgae are relatively sparse, only a genotoxic effect on the dinoflagellate *Karenia mikimotoi* (Akcha et al. 2008) and changes in phytoplankton communities (Stachowski-Haberkorn et al. 2009) have been reported. Because microalgae are highly sensitive even to low doses of toxic chemicals, the selected algal species is the diatom *Chaetoceros calcitrans*, representative of the field communities of concern in Tunisia.

This study provides insight on the sublethal effects of epoxiconazole alone and its formulation to the physiology of a nontarget algal cell.

2 Material and Methods

2.1 Microalgal Culture

C. calcitrans was obtained from Ifremer (Brest, France) and was maintained in a f/2 medium (Guillard and Ryther 1962). The inoculum (0.2×10^4 to 10^4 cells/mL) was taken from the logarithmic phase and cultures were grown in 1-L Pyrex glass bottles containing 500 mL of culture medium.

Two culture mediums were used in toxicity tests: f/2 and pre-winter Gabès medium. NaH_2PO_4 (4.4 μM) and 8.9 μM $NaNO_3$ was added to filtered seawater; these nutrient enrichment conditions were determined in accordance with in situ pre-winter measurements taken in the Gulf of Gabès (Tunisia). After autoclaving, sterilized vitamins, $NaSiO_3$, Fe-ethylenediaminetetracetic acid, and metal traces according to Guillard and Ryther (1962) were added. Non-axenic microalgal cultures were maintained at 20±2 °C and, depending on experiment, under either a 16:8-h light/dark cycle or a continuous lighting with 60 $\mu mol m^{-2} s^{-1}$ of photosynthetically active radiations (PAR), measured with a light meter (model Li 250, LiCor).

2.2 Pesticide

Epoxiconazole (1-[[(2S,3S)-3-(2-chlorophenyl)-2-(4-fluorophenyl)oxiran-2-yl]methyl]-1,2,4-triazole; CAS no 133855-98-8) was purchased from Dr Ehrenstorfer GmbH, Germany. A stock solution was prepared by dissolving epoxiconazole in dimethyl sulfoxide (DMSO).

A commercial formulation of epoxiconazole (Opus; 125 g active molecule per kg) (BASF AGRO SAS) was also tested. A stock solution was prepared by dissolving 10 mg per liter of seawater and sterilized by filtration through a 0.22-μm membrane (Millipore).

The effective epoxiconazole concentration was checked in subsamples. Analyses were performed at the "IDHESA, Brest", using high performance liquid chromatography coupled with a triple quadripole mass spectrometer. The samples were stored frozen in previously burnt glass bottles until use, which was no more than 1 month.

2.3 Algal Toxicity Tests

Spherical flasks of 250 mL volume were used for keeping 100 mL of test medium volume. A range of five concentrations of epoxiconazole was tested and two types of control: flasks with DMSO (active molecule exposure) or without DMSO (formulated product exposure). Three replicates test flasks were used for each treatment and all experiments were performed in triplicate.

To assess the effect of development stage, assays were performed in pre-winter medium culture, under continuous light, and epoxiconazole was added at different times of algae growth: at the beginning (day 0), after 1 (day 1), 2 (day 2), and 3 (day 3) days of growth.

2.4 Growth Measurement

Counting of desired initial cellular concentrations and daily counting during the tests were performed using a Malassez counting chamber, following the standard procedure ISO 10253 (AFNOR 1998). The EC_{50} (concentration required to cause a 50 % reduction in growth) were calculated 3 days after algae inoculation. For each culture, the average specific growth rate was calculated after log transformation according to the ISO 10253, and the Excel Macro REGTOX was applied to the growth rate to obtain the EC_{50} according to Vindimian et al. (1983) and Vindimian (2009).

The cell volume was determined from the length measures made by the microscope Zeiss Axiovert 135. Algae, stained with Lugol, were fixed with polylysine on a slide for microscopic observation. The cell volume was estimated using the Visilog software (version 6).

2.5 Measurement of Pigments

To assure sufficient biomass, measurement of pigments was realized after 3-day culture. Diatom cells were harvested by filtration through a 0.45-μm GV membrane filter (Millipore) and pigments were extracted with 100 % methanol at 65 °C during 20 min. After cooling, the methanolic solution was centrifuged (2,500×*g*, 10 min) and its absorbance was measured at 470, 510, 630, and 664 nm (spectrophotometer Hitachi, U 2000). Concentrations of chlorophylls (*a* and *c*) and total carotenoids were determined according to the methods of Jeffrey and Humphrey (1975) and Gala and Giesy (1993), respectively.

2.6 Photosynthetic and Respiration Rates

Gross photosynthesis and dark respiration were determined as O_2 exchanges at 20 °C using a Clark-type electrode (YSI 53 Oxygen Monitor). Photosynthesis was measured under a 140-$\mu mol\,m^{-2}\,s^{-1}$ PAR.

2.7 Determination of ATP

The ATP content was determined as previously described (Hourmant et al. 2009), using an ATP bioluminescent assay kit (PerkinElmer®). One hundred microliters of the algal suspension was injected directly in a well of a 96-well plate and added with 50 μL cell lysis solution. The plates were covered with precut acetate tapes (Dynatech®) and agitated for 5 min (700 rpm). After addition of 50 μL luciferin–luciferase, plates were shaked for 5 min and incubated in the dark (10 min). Luminescence measurements were performed at the luminometer (Victor, Perkin Elmer®) using a 10-s integration time. The luminescence data (relative illumination units) were converted using a calibration curve obtained with ATP dissolved in sterile seawater.

2.8 Statistical Analyses

All experiments were conducted three or more times, data were statistically analyzed by a one-way analysis of variance and, when differences observed were significant, means were compared by the Duncan multiple range test. Significant differences at a level of significance of 0.05 ($p<0.05$) are represented by an asterisk. Data are given as means values±standard errors of the means.

3 Results

3.1 Effect of Epoxiconazole and Formulated Product (Opus) on Growth

The growth of *C. calcitrans* in pre-winter Gabès medium was much lower than in f/2 medium under both conditions of light (continuous or 16 h photoperiod), reaching a stationary phase at day 3 (Figs. 1 and 2). The growth rate, evaluated after 72 h, was maximal under continuous illumination (0.065/h) and minimal with a 16 h photoperiod (0.011/h) (Table 1).

Whatever the conditions of culture, addition at day 0 of Opus caused an immediate inhibition of the growth that increased with the fungicide concentration until 3 days (Figs. 1 and 2). However, this inhibition was clearly more marked when the algae were grown in pre-winter Gabès medium. Thus, in this medium, 2.3 μg/L Opus inhibitions were of 76 and 91 % compared to 62 and 80 % in f/2 medium, respectively under continuous light (Fig. 1) and a 16-h photoperiod (Fig. 2). From the fourth day of culture, the inhibition attenuated progressively over time but remained significant at day 7, being in f/2 medium of 27 % (Fig. 1a) and 48 % (Fig. 2a) and in pre-winter medium with continuous light of 50 % (Fig. 2a); a total extinction of the inhibitory effect was observed under the 16-h photoperiod (Fig. 2b).

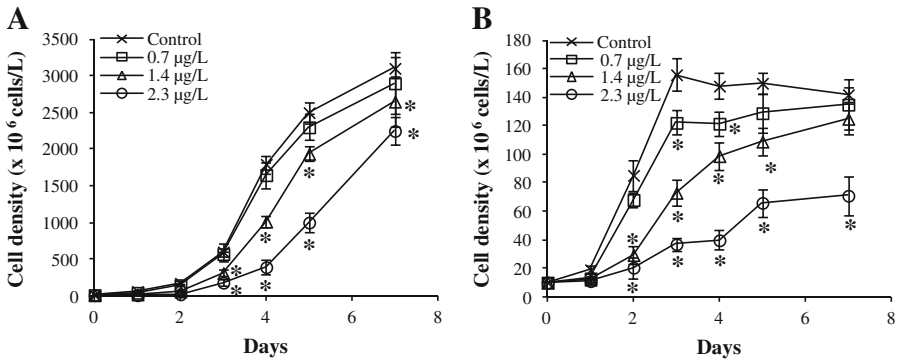

Fig. 1 Growth curves for *C. calcitrans* when exposed to different concentrations of Opus under continuous light. **a** f/2 medium; **b** pre-winter Gabès conditions. *p <0.05 indicates a value significantly different

The 3-day EC_{50} values obtained with Opus were estimated to be, under continuous light, 4.47 and 2.9 µg/L, respectively with f/2 and pre-winter media, whereas it was 3.38 and 2.63 µg/L with a 16-h photoperiod (Table 2). After a 3-day exposure to pure epoxiconazole under continuous illumination, the calculated EC_{50} values were 4.900 and 2.310 µg/L, respectively, for f/2 and pre-winter media (Table 2).

Exposure to the 2.88 µg/L epoxiconazole-formulated product Opus increased significantly the cell volume (Fig. 3); this increase was detected at days 2, 3, and 4 (70, 47, and 45 %), respectively, relative to control (p<0.05) (100 %).

3.2 Influence of Algal Development Stage on Opus Toxicity

Figure 4 shows the growth curves for *C. calcitrans* when 2.3 µg/L Opus was added at different times of the culture. Exposure to the fungicide after 1 day of culture produced an inhibition slightly lower than the exposure from day 0. The addition of the fungicide after 2 or 3 days of culture, produced 24 h later a reduction of the cell density and the growth stopped.

3.3 Effect of Opus on Pigment Content

The pigment alterations produced by Opus in *C. calcitrans* cultured in the pre-winter medium for 3 days are summarized in Table 3. A significant increase in chlorophylls and carotenoids occurred when the fungicide was added at day 0 and, to a lesser extent, when added at day 1. A tendency of stimulation was still observed when Opus was added at day 2.

3.4 Effect of Opus on Photosynthetic and Respiration Rates

The time responses of gas exchanges in *C. calcitrans* grown in pre-winter medium and continuous illumination are shown in Fig. 5. In control cultures, after a large increase in photosynthesis occur after 2 days (Fig. 5a), the rate returned to its initial value and then declined. The same tendency was observed for the respiration rate (Fig. 5b). Opus (2.3 µg/L) added at day 0 increased significantly gas exchanges. The stimulation of photosynthesis, +25 % after the 2-day

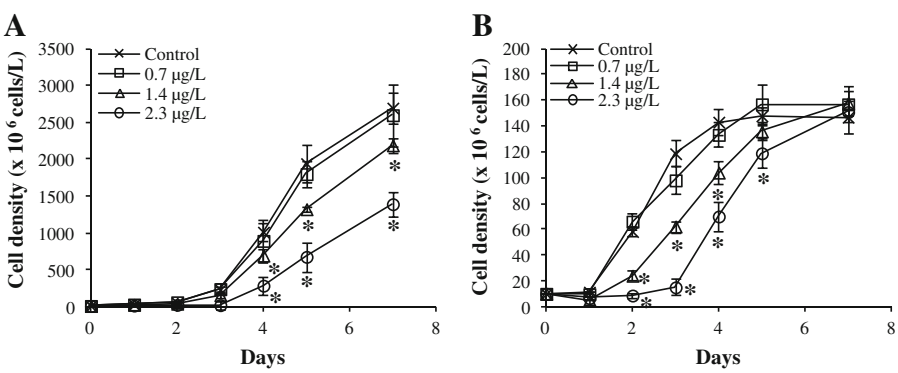

Fig. 2 Growth curves for *C. calcitrans* when exposed to different concentrations of Opus under a 16 h photoperiod. **a** f/2 medium; **b** pre-winter Gabès conditions. *p <0.05 indicates a value significantly different

Table 1 Growth rate (μ/h ± SE) of *C. calcitrans* measured after 3 days in the different culture conditions

	Continuous light	16-h photoperiod
f/2 medium	0.065±0.0012	0.045±0.011
Gabès medium	0.037±0.0049	0.011±0.0008

exposure, significantly increased afterwards. The respiration stimulation, maximal after 2 days of incubation (+140 %), tends to attenuate with time. When added after 2 days of culture, Opus produced, 1 day later, a significant increase of photosynthesis (+40 %) and respiration (+45 %) that was still observed after the 2-day exposure.

3.5 Effect of Opus on ATP Content

In control culture, a peak in ATP production was observed after 1 day of culture which decreased gradually afterwards (Fig. 6). Exposure to 2.3 μg/L Opus at the start of the culture maintained the peak value for 2 days and delayed the decline in ATP amount, giving rise to a significant stimulation of the ATP, maximal at day 4 (+253 %). When Opus was added after 1 day of culture, it produced, 2 days later, a significant increase of the ATP content (+109 %) that slowed down later on. Added after 2 or 3 days of culture, the fungicide produced, 1 day later, a slight stimulation (+37–35 %) of the ATP content, disappearing later on.

4 Discussion

The sensitivity of *C. calcitrans* to epoxiconazole and its formulation (Opus) was examined, and the influence of the nutrient level, light photoperiod, and cell age was studied. Whatever the conditions of growth, an immediate progressive reduction of cell density was produced by increasing epoxiconazole concentration. However, after a 3-day inhibitory growth period, a progressive extinction of the inhibitory effect of fungicide was observed.

Table 2 EC_{50} (in micrograms per liter) (±SE) of epoxiconazole obtained after 3 days growth of *C. calcitrans* grown in different culture conditions

		Continuous light	16 h photoperiod
f/2 medium	Opus	4.47±0.4	3.38±0.29
	Epoxiconazole	4 900±460	–
Gabès medium	Opus	2.9±0.24	2.63±0.29
	Epoxiconazole	2310±180	–

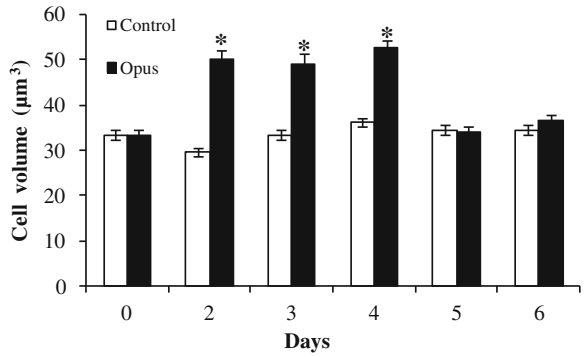

Fig. 3 Influence of 2.88 μg/L Opus on the cell volume of *C. calcitrans* grown in a pre-winter Gabès medium under continuous light. Results expressed as cubic micrometer±SE. *$p<0.05$ indicates a value significantly different

Under continuous light, with a f/2 medium or with a pre-winter Gabès medium, the 3-day EC_{50} for the formulated product (respectively 4.47 and 2.9 μg/L) were in both cases 3 orders of magnitude below the pure compound's EC_{50} (respectively 4.9 and 2.31 mg/L). These results questioned the use of ecotoxicological data obtained solely using active molecules of pesticides rather the complete formulation because it is the formulation that goes to the marine ecosystem. As shown by several authors, surfactants and so-called inert components can contribute to the overall toxicity of the formulation either by exerting toxic activity of the formulation

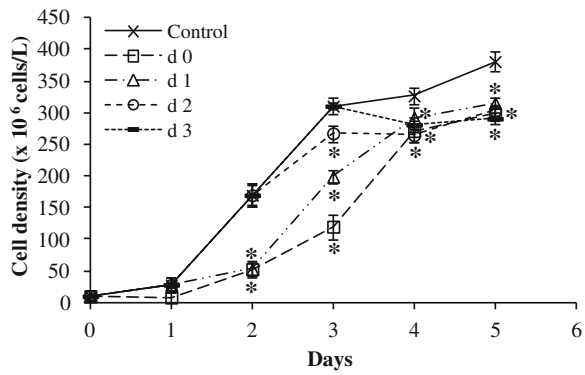

Fig. 4 Growth curves for *C. calcitrans* in pre-winter Gabès medium when exposed to 2.3 μg/L Opus, added at different stages of development (*d* = day). Cultures under continuous illumination. *$p<0.05$ indicates a value significantly different

Table 3 Effect of 2.16 μg/L Opus added at different stages of growth on the pigment content of *C. calcitrans* measured after 3 days of culture under continuous illumination. Results expressed as nanogram per million cells ± SE, value in parentheses indicates the percentage relative to the control

	Chlorophyll *a*	Chlorophyll *c*	Carotenoids
Control	70.83±8.67	25.06±5.42	53.15±3.08
+ Epoxi. at day 0	97.35±9.21 (137 %)*	37.30±3.81 (149 %)*	68.44±4.32 (129 %)*
+ Epoxi. at day 1	91.26±6.43 (129 %)*	33.90±3.54 (135 %)*	64.26±4.87 (121 %)*
+ Epoxi. at day 2	74.48±7.62 (105 %)	27.60±4.22 (110 %)	57.71±3.90 (108 %)

*$p<0.05$, value significantly different

on their own or by interacting with the active ingredient (Caux et al. 1996; Oakes and Pollak 2000; Martin Tsui and Chu 2003; Hourmant et al. 2009; Pereira et al. 2009; Lipok et al. 2010). The EC_{50} of the pure molecule are within expected values reported in the literature about toxic effects of other triazole fungicides. Baird and DeLorenzo (2010) obtained for the Chlorophyceae *D. tertiolecta* 96-h EC_{50} ranging between 0.91 to 5.98 mg/L with hexaconazole, propiconazole, triadimenol, and triadimefon. In contrast, the 72-h EC_{50} value for *Pseudokirchneriella subcapitata* (>10 mg/L) with epoxiconazole suggests a lower toxicity towards freshwater algae (AGRITOX 2010).

The growth rate and the inhibitory effect of epoxiconazole are dependent on nutrient level and light intensity. Thus, under optimal growth conditions (f/2 medium, continuous light), maximal values were obtained for the growth rate (0.065/h) and the 72-h EC_{50} value for the formulated product (4.47 μg/L). By contrast, minimal values were obtained under a 16-h photoperiod with the pre-winter Gabès medium, being for μ—0.011/h and EC_{50}—2.63 μg/L. When the cell density is high, less femtomoles epoxiconazole per cell are available and this could explain the higher toxic effect of the fungicide when the growth was limited either by light or by nutrient concentration. This is in agreement with data about Cr reported by Sbrilli et al. (2003).

As shown on growth curves, after a 3-day epoxiconazole exposure, the cells of *C. calcitrans* try to increase the growth rate, after a strong inhibitory growth period. Metabolism of epoxiconazole in plants as well as in soil has been shown to be limited (Bromilow et al. 1999; Lin et al. 2001; Buerge et al. 2006; EFSA 2008; Passeport et al. 2011). The increasing cell density occurring with time decreases the amount of epoxiconazole available per cell and allows the alleviation of inhibition to take place. Moreover, the lower inhibition displayed by epoxiconazole added during the exponential growth confirms that cellular density is a very important parameter in toxicity. These results are in accordance with data about Cu reported by Moreno-Garrido et al. (2000). However, the differences in susceptibility to this sterol-inhibiting fungicide might also result from the changes in lipid composition with growth stage, as suggested by Tuckey et al. (2002).

Growth rate is often closely related to energy production. Interestingly, while growth inhibition was clearly observed, epoxiconazole (added at day 0 or 1) increases the oxygen exchanges and delays the decline in ATP that occurs in the control after day 1.

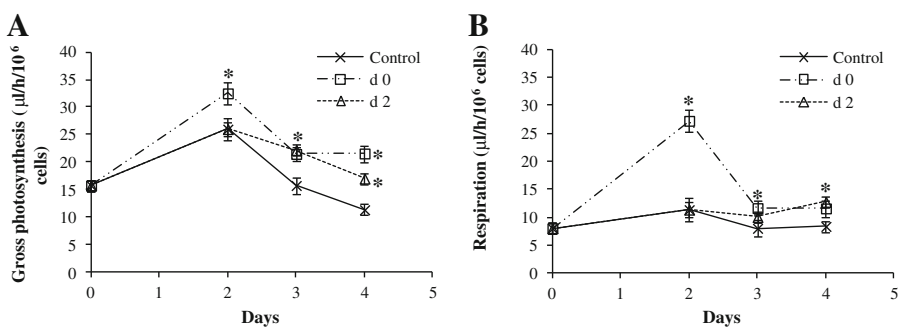

Fig. 5 Oxygen exchanges obtained in cultures of *C. calcitrans* exposed to 2.3 μg/L of Opus added at the beginning (*d 0*) or after 2 days (*d 2*) of culture in pre-winter Gabès medium under continuous illumination. **a** photosynthesis ; **b** respiration. *$p<0.05$ indicates a value significantly different

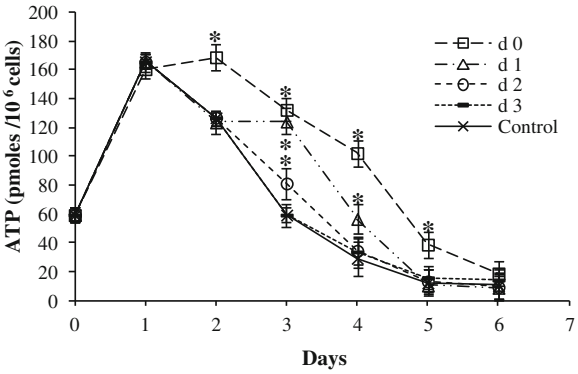

Fig. 6 Influence of 2.3 μg/L Opus added at different stages of growth on the ATP content of *C. calcitrans* cultivated in pre-winter Gabès medium under continuous illumination (*d* = day). **p*<0.05 indicates a value significantly different

This may suggest that ATP produced was not use to cope with growth requirements and, as a consequence, accumulates within the cell.

The pigment content was significantly increased by epoxiconazole, added at the start of the culture or 1 day later, and may explain the higher photosynthesis activity observed. Examples of herbicide-induced increase of algal chlorophyll content have been reported in freshwater (Mayer and Jensen 1995; Rioboo et al. 2002) and marine microalgae (Hourmant et al. 2009). The mechanism of toxicity is therefore probably not an interference with formation or maintenance of pigments but with other essential process. In many higher plants, a reduction of growth by triazole compounds has also been shown, accompanied by increases of pigments and photosynthesis (Percival and Noviss 2008; Jaleel et al. 2008 and references therein) but a reduction of in vitro thylacoid activity has been reported in *Galium aparine*, although chlorophyll was increased on a unit area basis (Benton and Cobb 1997).

Cell volume of *C. calcitrans* was increased by epoxiconazole and this may be related to the growth inhibition and therefore influence the remaining parameters, pigment and ATP content. Previous studies indicate that algal cell volume is highly correlated with toxicity (Tang et al. 1998; Rioboo et al. 2002; Hourmant et al. 2009) and the swelling of *D. tertiolecta* exposed to conazole fungicides likely attributable to altered membrane structure resulting from a large increase of total lipid content (Baird and DeLorenzo 2010).

Our results suggest that epoxiconazole, known as an inhibitor of ergosterol biosynthesis, could be affecting the mechanisms of cell division, but not the production of new material for the construction of new cells. Consequently, the reduction of growth rate might be caused by a prolongation of the cell cycle, cellular density being an important parameter in the sensitivity to the fungicide.

5 Conclusions

Exposure of *C. calcitrans* to the sterol biosynthesis inhibiting fungicide epoxiconazole reduces the growth rates while increasing the cell volume, and toxicity of the formulation was markedly higher. This fungicide increases pigment content and thereby photosynthesis and ATP production. When the growth rate was increased by continuous illumination and nutrient level, the sensitivity of *C. calcitrans* to epoxiconazole was decreased. A progressive algal cell recovery from epoxiconazole effects occurred after 3 days, with the increasing cell density. When the toxic was given during the exponential phase, its effects were weakened. These results suggest that cellular density is an important parameter in toxicity tests.

Based on these results, one can say that nontarget marine microalgae may be affected by a fungicide. Because algae serve as primary producers within marine ecosystems, they could influence organisms that feed in the food chain.

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