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 EC50 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

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, fruits, grapes (Ammermann *et al.*, 1992; Schöfl & 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 & 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 & Cobb, 1997; Wu & Von Tiedemann, 2001; Percival & 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 run-off, 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 non-target 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 *D. magna* (Nørgaard & 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 & 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 non-target algal cell.

2. Material and methods

2.1. Microalgal culture

Chaetoceros calcitrans was obtained from Ifremer (Brest, France) and was maintained in a f/2 medium (Guillard & 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. $4.4 \mu\text{M}$ NaH_2PO_4 and $8.9 \mu\text{M}$ NaNO_3 was added to filtered sea water; 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-ethylene diamine tetracetic acid (Fe-EDTA) and metal traces according to Guillard & Ryther (1962) were

added. Non-axenic microalgal cultures were maintained at 20 ± 2 °C and, depending on experiment, under either a 16 h : 8 h light/dark cycle or a continuous lighting with $60 \mu\text{mol m}^{-2}\text{.sec}^{-1}$ 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 $0.22 \mu\text{m}$ membrane (Millipore).

The effective epoxiconazole concentration was checked in sub-samples. 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 one 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 (d 0), after one (d 1), two (d 2) and three (d 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 poly-lysine 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 $0.45 \mu\text{m}$ 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 & Humphrey (1975) and Gala & Giesy (1993) respectively.

2.6. Photosynthetic and respiration rates

Gross photosynthesis and dark respiration were determined as O₂ exchanges at 20 °C using a Clark-type electrode (YSI 53 Oxygen Monitor). Photosynthesis was measured under a 140 µmol m⁻² sec⁻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 (Perkin Elmer®). 100 µl of the algal suspension were injected directly in a well of a 96-well plate and added with 50 µl cell lysis solution. The plates were covered with pre-cut acetate tapes (Dynatech®) and agitated for 5 min (700 rpm). After addition of 50 µl luciferin-luciferase, plates were shaken 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 (ANOVA) and, when differences observed were significant, means were compared by the multiple range Duncan 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 the both conditions of light (continuous or 16 h photoperiod), reaching a stationary phase at day 3 (Figures 1 and 2).

Figure 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. * indicates a value significantly different at $p < 0.05$.

Figure 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. * indicates a value significantly different at $p < 0.05$.

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).

Table 1. Growth rate ($\mu/h \pm SE$) 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

Whatever the conditions of culture, addition at d 0 of Opus, caused an immediate inhibition of the growth that increased with the fungicide concentration until 3 days (Figures 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 of 76 % and 91 % compared to 62 % and 80 % in f/2 medium, respectively under continuous light (Figure 1) and a 16 h photoperiod (Figure 2). From the fourth day of culture, the inhibition attenuated progressively over time but remained significant at d 7, being in f/2 medium of 27 % (Figure 1A) and 48 % (Figure 2A) and, in pre-winter medium with continuous light, of 50 % (Figure 2A); a total extinction of the inhibitory effect was observed under the 16 h photoperiod (Figure 2B).

The 3 day EC₅₀ 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 EC₅₀ values were 4 900 and 2 310 µg/L, respectively, for f/2 and pre-winter media (Table 2).

Table 2. EC₅₀ (µg/L) (± 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	-

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

Figure 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 µm³ ± SE. * indicates a value significantly different at $p < 0.05$.

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 were 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 d 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.

Figure 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. * indicates a value significantly different at $p < 0.05$.

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 d 0 and, to a lesser extent, when added at d 1. A tendency of stimulation was still observed when Opus was added at d 2.

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 ng / million cells $\pm$ SE, value in parentheses indicates the percentage relative to the control. * indicates a value significantly different at $p < 0.05$.

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

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 Figure 5.

Figure 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 two days (d 2) of culture in pre-winter Gabès medium under continuous illumination. (A) photosynthesis ; (B) respiration. * indicates a value significantly different at $p < 0.05$.

In control cultures, after a large increase in photosynthesis occurring after 2 days, (Figure 5A), the rate returned to its initial value and then declined. The same tendency was observed for the respiration rate (Figure 5B). Opus (2.3 µg/L) added at d 0, increased significantly gas exchanges. The stimulation of photosynthesis, + 25 % after the 2-day 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 (Figure 6).

Figure 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). * indicates a value significantly different at $p < 0.05$.

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.

Under continuous light, with a f/2 medium or with a pre-winter Gabès medium, the 3 day EC₅₀ for the formulated product (respectively 4.47 and 2.9 µg/L) were in both cases three orders of magnitude below the pure compound's EC₅₀ (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 on their own, or by interacting with the active ingredient (Caux *et al.*, 1996; Oakes & Pollak, 2000; Martin Tsui & Chu, 2003; Hourmant *et al.*, 2009; Pereira *et al.*, 2009; Lipok *et al.*, 2010). The EC₅₀ of the pure molecule are within expected values reported in the literature about toxic effects of other triazole fungicides. Baird & DeLorenzo (2010) obtained for the chlorophyceae *Dunaliella tertiolecta* 96 h EC₅₀ ranging between 0.91 to 5.98 mg/L with hexaconazole, propiconazole, triadimenol and triadimefon. In contrast, the 72 h EC₅₀ 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 EC₅₀ 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 EC₅₀ : 2.63 µg/L. When the cell density is high, less fmoles 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. 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 & 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 & 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 & 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 *Dunaliella tertiolecta* exposed to conazole fungicides likely attributable to altered membrane structure resulting from a large increase of total lipid content (Baird & 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 a non-target marine microalgae may be affected by a fungicide. Because algae serve as primary producers within marine ecosystems, they could be influence organisms that feed in the food chain.

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