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Sensitivity of freshwater and marine green algae to three compounds of emerging concern

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Abstract

In this study, the toxicity of three compounds of emerging concern (CEC) belonging to different classes [metolachlor (herbicide), erythromycin (antibiotic) and triclosan (antiseptic)], were evaluated and compared using the freshwater alga *Pseudokirchneriella subcapitata* and the marine alga *Dunaliella tertiolecta*. Toxicity assays were performed by exposing algal cells, in exponential phase of growth, to the toxicants for 72 h (*P. subcapitata*) or 96 h (*D. tertiolecta*). The toxicant concentrations that induced an inhibition of 50% of algal growth (EC_{50}) of *P. subcapitata* or *D. tertiolecta* were 118 and 11.3×10^3 μg L^{-1} for metolachlor (MTC), 38 and 5.75×10^3 μg L^{-1} for erythromycin (ERT) and 27.1 and 93 μg L^{-1} for triclosan (TCS), respectively. Based on these EC_{50} values, it was possible to hierarchize (decreasing order) the toxicity of the CEC studied: TCS > ERT > MTC. The EC_{50} values achieved for *P. subcapitata* were between 3.4- and 151-fold lower than those observed with *D. tertiolecta*, which demonstrated the higher sensitivity of the freshwater alga comparatively to the marine alga. All 72 h-EC_{10} or 72 h-EC_{50} values determined in this study with *P. subcapitata* are within the concentration range of these pollutants described in the literature, in ground and surface waters, which underlines the significance of this alga in the ecotoxicity assessment of freshwaters.

Keywords Chlorophyta · *Dunaliella tertiolecta* · Erythromycin · Metolachlor · *Pseudokirchneriella subcapitata* · Toxicity · Triclosan

Introduction

Many organic pollutants, namely alkyl phenols, flame retardants, personal care products, pharmaceuticals, steroids and pesticides, are being introduced in the environment as result of human activities. These compounds are generally referred as contaminants of emerging concern (CEC). According to the US Environmental Protection Agency (US-EPA), CEC are "chemicals and other substances that have no regulatory standard, have been recently 'discovered' in natural streams (often because of improved analytical chemistry detection levels), and potentially cause deleterious effects in aquatic life at environmental relevant concentrations" (US-EPA 2008).

In fact, environmental monitoring has detected CEC in surface waters, ground waters and drinking waters (Boxall 2012). The major sources of CEC in aquatic systems are wastewater treatment plant effluents, aquaculture, animal husbandry, horticulture and leaching from soils and/or waste disposal (Gerecke et al. 2002; Gaw et al. 2014). The most frequently detected CEC include some pesticides (agricultural herbicides), antibiotics and non-prescription personal care products (Rykowska and Wasiak 2015; Fairbairn et al. 2016; Manamsa et al. 2016).

Pesticides are used to combat pests. Nevertheless, 98–99.9% of insecticides and 95% of herbicides attain other destination than its main targets, namely air, water and soil (Miller 2004). Metolachlor is a popular herbicide, belonging to the chloroacetanilide family, being used in the control of certain weed species on agricultural crops. This pesticide has a large potential to contaminate ground waters due to its mobility and persistence in soil (Rivard 2003). Metolachlor is frequently found

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in surface and ground waters usually associated with agricultural areas and can reach a concentration in the order of 100 μg L^{-1}.

Antibiotics have been used, in the last decades, in human and veterinary medicine and in aquaculture. Thus, a variety of antibiotics, namely fluoroquinolones, macrolides, sulphonamides and tetracyclines, have been detected in effluents and in natural waters at levels from ng L^{-1} to μg L^{-1} (Hoa et al. 2011; Gonzalez-Pleiter et al. 2013; Liang et al. 2013). Erythromycin, a macrolide, is one of the most common antibiotics found in surface waters, being widely used in human medicine and in animal production (Alexy and Kümmerer 2006). Considering the environmental risk, macrolides can be considered one of the most harmful antibiotics for aquatic systems (Isidori et al. 2005).

Personal care products, consumption of which is increasing continuously, contain a series of chemicals (such as, fragrances, UV-blockers and antiseptics) that are released in aquatic systems. Triclosan is an antiseptic agent largely used in personal care products like soap, toothpaste, deodorant and cosmetics. It has also other industrial applications namely in adhesives, plastics (toys, toothbrushes), textiles and rubber (Dhillon et al. 2015). Due to its extended use, triclosan has been found in surface, ground and drinking water, at concentrations ranging from ng L^{-1} to μg L^{-1} (Dhillon et al. 2015). Additionally, due to its physicochemical properties, triclosan is considered bioaccumulative and persistent in the environment and can also react to form dioxins (Dhillon et al. 2015).

Many studies have been detecting the presence of CEC in aquatic systems, namely in freshwaters (Rykowska and Wasiak 2015; Fairbairn et al. 2016; Manamsa et al. 2016). However, due to their large number their impact in aquatic organisms has not been deeply investigated. Furthermore, knowledge of the occurrence, distribution and toxic effect of CEC in marine environment is even more limited. The negative impacts of these compounds in aquatic organisms and, as a consequence, in all food chains, led to the need to study the effect of these compounds in the different aquatic systems.

Phytoplankton form the base of most aquatic food chains. Therefore, changes in the phytoplankton will have a huge impact on all aquatic ecosystems. The green algae *Pseudokirchneriella subcapitata* and *Dunaliella tertiolecta* have been widely utilised for evaluation of pollutant toxicity and environmental assessment. *Pseudokirchneriella subcapitata* is common in freshwaters, can be easily cultured in the laboratory, is quite sensitive to a large variety of toxic substances (Janssen and Heijerick 2003; Cho et al. 2009) and is a test organism recommended by the standard methods of the USA (US-EPA 2012) and the European Union (OECD 2011). *Dunaliella tertiolecta* has the ability to grow in severe environments (extreme saline conditions) and lacks a rigid cell wall (Borowitzka and Siva 2007), eliminating a potential barrier to the passage of pollutants into the cell, thus making it a good option on the evaluation of toxicity of compounds present in marine waters (Nikookar et al. 2005; Sacan et al. 2007; Manzo et al. 2013).

In the present study, it was our objective to compare the susceptibility of *P. subcapitata* and *D. tertiolecta* to three compounds of emerging concern: metolachlor (herbicide), erythromycin (antibiotic), and triclosan (antiseptic). In addition, the toxicity of the three compounds was hierarchised considering the concentration of the toxicants that induced an inhibition of 50% of algal growth (EC_{50}). The environmental relevance of these algae in the assessment of ecotoxicity of freshwaters and marine waters, taking into account the concentration of these pollutants (described in the literature) in the environment is also discussed.

Material and methods

Strain, media and culture conditions

The algae *Pseudokirchneriella subcapitata* (strain 278/4) and *Dunaliella tertiolecta* (strain 19/6B), used in this study, were purchased from the Culture Collection of Algae and Protozoa (CCAP), UK.

Pseudokirchneriella subcapitata was preserved in OECD medium with 20 g L^{-1} agar (Merck) at 4 °C in the dark. Stock solutions for culture medium preparation were made and stored according to the Organisation for Economic Co-operation and Development (OECD) guidelines (OECD 2011). Starter cultures, pre-cultures, and cultures with an initial cell concentration of ~5×10^4 cells mL^{-1} were prepared as previously described by Machado and Soares (2012) and incubated at 25 °C on an orbital shaker at 100 rpm under continuous "cool white" fluorescent light (fluorescent lamps with a colour temperature of 4300 K) with an intensity of 54 μmol photons m^{-2} s^{-1} at the surface of the flask.

Dunaliella tertiolecta was cultured in Guillard's (f/2) enriched seawater medium. Artificial seawater was prepared as described by US-EPA (US-EPA 2002) and enriched with f/2 medium (Guillard and Ryther 1962), with minor modifications: final concentration of ethylenediaminetetraacetic acid (EDTA) disodium salt dehydrate and iron (III) chloride hexahydrate of 0.08 and 0.1 mg L^{-1}, respectively. The concentrations of these two components were adjusted to be similar to those of the OECD medium used for the growth of *P. subcapitata*. Then, the pH was adjusted to 8.0 and sterilised by filtration. In order to ensure *D. tertiolecta* axenic cultures, the alga was pre-cultured in 20 mL of f/2 enriched seawater medium containing an extra amount of sodium chloride (2 mol L^{-1}), in 100-mL Erlenmeyer flasks. Cultures were prepared in 100 mL of f/2 enriched seawater medium (with 0.36 mol L^{-1} sodium chloride), in 250 mL Erlenmeyer flasks. Pre-cultures and cultures were inoculated with 5×10^4 cells mL^{-1} and incubated at 25 °C, for 96 h (4 days), on an orbital shaker at 100 rpm, under continuous "cool white" fluorescent light (fluorescent lamps with a colour temperature of

4300 K), with an intensity of 54 μmol photons m^{-2} s^{-1} at the surface of the flask.

Algal cell concentration was determined using an automated cell counter (TC10, Bio-Rad).

Chemicals

Metolachlor (97.6%) (PESTANAL, analytical standard, Ref: 36163), erythromycin (Ref: E5389) and triclosan (≥ 97%) (Ref: 72779) were from Sigma-Aldrich. The metolachlor stock solution (11.8 g L^{-1}) was prepared in deionised water. The stock solutions of erythromycin (12.6 g L^{-1}) and triclosan (1.0 g L^{-1}) were prepared in dimethyl sulfoxide (DMSO). Toxicity assays for erythromycin and triclosan presented a DMSO concentration no higher than 0.007% for *P. subcapitata* and 0.5% for *D. tertiolecta*. Control experiments showed that, at these concentrations, the solvent DMSO did not affect the growth of *P. subcapitata* or *D. tertiolecta* (Fig. S1, Supplementary Material).

Growth curves

Cultures in exponential phase of growth (48 h for *P. subcapitata* and 96 h for *D. tertiolecta*) were centrifuged at 2500×*g* (*P. subcapitata*) or 300×*g* (*D. tertiolecta*) for 5 min and resuspended in the respective medium. Growth curves were performed in 250 mL Erlenmeyer flasks containing 100 mL of OECD medium (*P. subcapitata*) or f/2 enriched seawater medium (*D. tertiolecta*) with an initial cell concentration of ~ 5×10^4 cells mL^{-1} and incubated under the conditions described above. At defined intervals of time (given in the Fig. 1), samples were withdrawn and cell number determined as described above.

The specific growth rate (μ) and the generation time (doubling time) (g) was calculated as previously described (Machado and Soares 2014).

Toxicity assays

Toxicity assays were performed individually with nominal concentrations of each CEC arranged in a geometrical series. For *P. subcapitata*, the following concentration ranges were tested: 16–400 μg L^{-1} metolachlor; 2.2–460 μg L^{-1} erythromycin and 3.7–72 μg L^{-1} triclosan. The alga *D. tertiolecta* was exposed to the toxicants in the following concentration ranges: 460–46 × 10^3 μg L^{-1} metolachlor, 4 × 10^3–63 × 10^3 μg L^{-1} erythromycin and 10–1 × 10^3 μg L^{-1} of triclosan. As control, algal cells were inoculated in the same media but without the toxic. One-hundred millilitre Erlenmeyer flasks containing OECD medium or f/2 enriched seawater medium and various toxic concentrations (geometrical series) were inoculated with 5×10^4 cells mL^{-1} of *P. subcapitata* or *D. tertiolecta* from cultures of 48 h or 72 h, respectively, in a final volume of 40 mL. Cultures were incubated as described above. After incubation, cell concentration was determined. The algal growth (yield) was used as endpoint; it was calculated considering the cell concentration at the end of the assay minus the starting cell concentration.

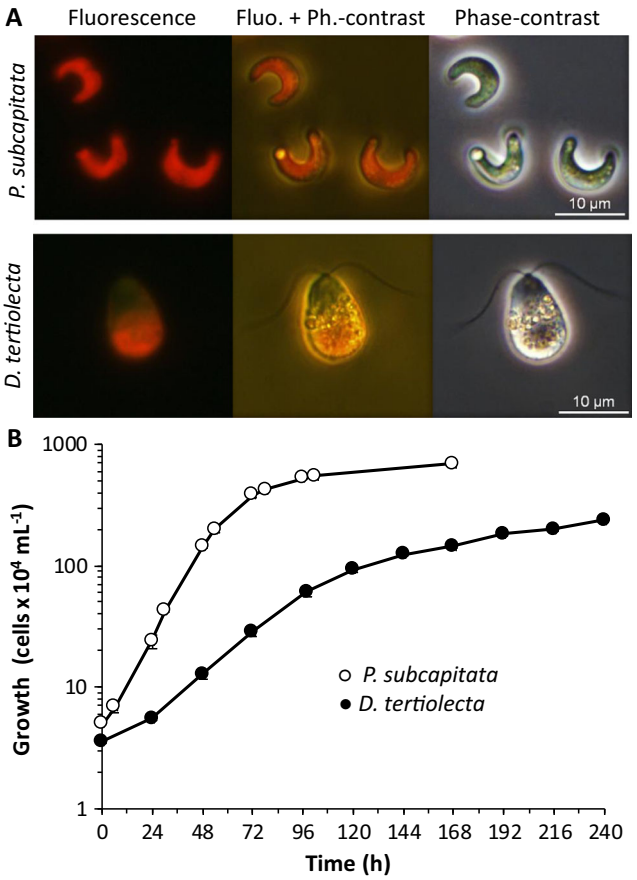

Fig. 1 Morphology and growth of the algae *P. subcapitata* and *D. tertiolecta*. **a** Microphotographs illustrative of the algal cells. In fluorescence and fluorescence plus contrast-phase photos, it can be observed the typical auto fluorescence of algal cells. **b** Algal growth. Cells in the exponential phase of growth were inoculated at 5×10^4 cells mL^{-1} in OECD medium (*P. subcapitata*) or in seawater-enriched f/2 medium (*D. tertiolecta*) and incubated at 25 °C on an orbital shaker at 100 rpm under continuous "cool white" fluorescent light. These experiments were performed two times in triplicate ($n = 6$). The standard deviations (error bars) were calculated with 95% confidence limits; where no error bars are shown they are within the points

Toxicity was expressed as EC_{10}, EC_{25}, EC_{50}, EC_{75} and EC_{90}, which represent the concentration of each chemical that caused 10, 25, 50, 75 and 90% of growth inhibition, respectively, when compared with the control (cells not exposed to toxics) after an exposure of 72 h for *P. subcapitata* or 96 h for *D. tertiolecta*. EC values were calculated by the linear interpolation using the software TOXCALC version 5.0.32 (Tidepool Scientific Software).

Microscopy

Algal cells were observed using an epifluorescence microscope equipped with a HBO-100 mercury lamp and a filter set I3 from Leica. The images were acquired with a Leica DC 300F camera,

using a N plan × 100 objective, and were processed using Leica IM 50-Image manager software. Cells of *D. tertiolecta* were previously fixed with 1% (*v*/*v*) glutaraldehyde at 4 °C.

Reproducibility of the results and statistical analysis

Data presented are the mean values of two independent experiments carried out in duplicate for toxicity tests and in triplicate for growth kinetics. Results were expressed as the mean ± standard deviation, presented with 95% confidence limits.

Results

Growth profile of freshwater and marine algae

In the present work, two unicellular algae were used: *P. subcapitata*, immobile, with a sickle-like shape (Fig. 1a), commonly present in freshwater environments (Guiry and Guiry 2017) and *D. tertiolecta*, motile (presenting flagella) with ovoid shape (Fig. 1a), which are common in marine habitats (Borowitzka and Siva 2007). The typical autofluorescence exhibited by algal cells, due to the presence of chlorophyll *a*, can be observed as shown in Fig. 1a.

The susceptibility of algal cells to toxicants is influenced by its physiological status, which, in turn, is dependent on their growth phase (Machado and Soares 2013). In order to know, in detail, the growth profile of *D. tertiolecta*, the evolution of cell concentration of this alga was followed over the time. Since the two algae under study will be exposed to the toxicants in the same cultural conditions (except culture medium), namely, light, agitation speed, and temperature, for comparative purposes, the growth of *P. subcapitata* was also followed.

When inoculated in f/2 seawater enriched medium, at 5×10^4 cells mL^{-1}, *D. tertiolecta* grew exponentially for about 96–120 h (4–5 days), with a specific growth rate of 0.029 h^{-1}, which corresponded to a doubling (generation) time of 24 h; this alga reached the stationary phase after 144 h (6 days) (Fig. 1b). The alga *P. subcapitata* grown exponentially for 48–72 h (2–3 days), with a specific growth rate of 0.071 h^{-1} and reached the stationary phase after 96 h (4 days) (Fig. 1b). Under the culture conditions used, *P. subcapitata*, in OECD medium, displayed a faster growth than *D. tertiolecta* and presented a doubling time of 10 h.

In order to ensure that the algal cells are in exponential phase of growth and taking into account the growth curves presented (Fig. 1b), the inoculum for the toxicity tests should be conducted with pre-cultures up to 3 days (72 h) for *P. subcapitata* and up to 5 days (120 h) for *D. tertiolecta*.

In the toxicity assays described below, in the case of *P. subcapitata*, we used a 72-h period for the toxicity test, which is in agreement with the OECD recommendation (OECD 2011). For *D. tertiolecta*, the time of the toxicity assay was expanded to 96 h, due to the slower growth of this alga (Fig. 1b).

Evaluation of the toxicity of metolachlor, erythromycin and triclosan, using *P. subcapitata* and *D. tertiolecta*

For assessing the toxic effects of CEC, algal cells were exposed to a series of geometric concentrations of the herbicide metolachlor (MTC), the antibiotic erythromycin (ERT) and the antiseptic triclosan (TCS). The dose-responses curves, expressed as the percentage of growth inhibition (yield) versus toxicant concentration, were plotted (Fig. 2).

TCS and ERT inhibited the growth of *P. subcapitata* even at very low concentrations (Fig. 2). For these two compounds, a 10% of growth inhibition (72 h-EC_{10} values), comparatively to the control (cells non-exposed to toxicants), was observed for 3.0 ± 0.5 μg L^{-1} TCS and 5 ± 4 μg L^{-1} ERT (Table 1). The 72 h-EC_{10} value of MTC (45 ± 3 μg L^{-1}) was approximately ten times higher than those observed for ERT and TCS (Table 1). However, this herbicide was also very toxic to *P. subcapitata* since it inhibited growth at ppb level (μg L^{-1}) (Fig. 2). Using the 72 h-EC_{50} values as the criterion for ranking the toxicity of the CEC, the decreasing order of toxicity, evaluated using *P. subcapitata*, is TCS > ERT > MTC (Table 1).

The evaluation of toxicity for the three CEC, using *D. tertiolecta*, considering the 96 h-EC_{50} values, revealed the same decreasing order of toxicity observed with *P. subcapitata* (Table 1). However, *P. subcapitata* is much more sensitive to toxicants than *D. tertiolecta*. For instance, for MTC, *D. tertiolecta* had 96 h-EC values from 91 to 132 times higher than 72 h-EC values observed with the freshwater alga *P. subcapitata* (Table 1). In the case of ERT, 96 h-EC values for *D. tertiolecta* were between 127 and 376-fold higher than those observed for *P. subcapitata*. In other words, it was observed that *P. subcapitata* was sensitive to MTC and ERT at ppb level (μg L^{-1}), while *D. tertiolecta* was sensitive to these toxicants at ppm level (mg L^{-1}). Within the three CEC tested, *D. tertiolecta* is more sensitive to TCS (96 h-EC values at μg L^{-1} level) (Fig. 2). For TCS, *D. tertiolecta* displayed similar EC values, or in the same order of magnitude, as those of *P. subcapitata* (Table 1).

A detailed comparison of the toxic impact (expressed by the respective EC_{10}, EC_{25}, EC_{50}, EC_{75} and EC_{90} values) of MTC, ERT and TCS over the two algae tested is presented in Table 1. The pictorial representation of the toxicity of MTC, ERT and TCS to *P. subcapitata* and *D. tertiolecta*, based on their EC_{50} values, is presented in Fig. 3; in this figure, it can be clearly observed the higher sensitivity of *P. subcapitata*, compared to *D. tertiolecta*, to the three CEC under evaluation.

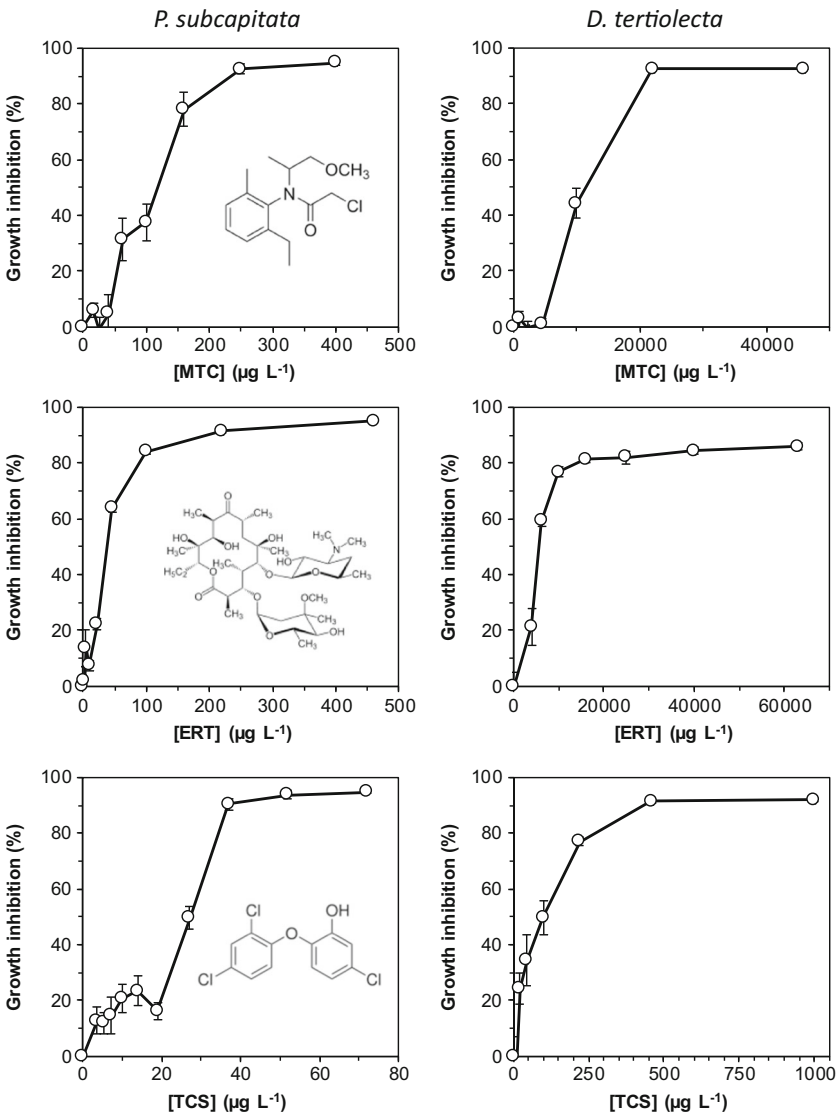

Fig. 2 Dose-response curves for *P. subcapitata* and *D. tertiolecta*. Algal cells (left panel: *P. subcapitata*; right panel: *D. tertiolecta*) were exposed to metolachlor (MTC), erythromycin (ERT) or triclosan (TCS). Insets: chemical structure of the toxicants. These experiments were performed two times in duplicate ($n = 4$). The standard deviations (error bars) were calculated with 95% confidence limits; where no error bars are shown they are within the points

Discussion

Over the last decade, many organic compounds have been detected in aquatic environments, not only as result of improved analytical methods for its determination but also due to its increasing frequency and concentration. They are considered compounds of emerging concern (CEC) mainly due to the fact they may cause serious effects on non-target organisms.

The regulation for these substances has in consideration its occurrence, environmental fate and toxicity. Nevertheless, as CEC represent a wide range of chemicals for which limited data are available, most of CEC do not have regulatory status. Therefore, it is essential to determine the concentration of each CEC that can have a toxic effect, particularly in aquatic organisms. In the case of marine environments, data about the occurrence of CEC is very scarce (Bedoux et al. 2012; Zhang et al. 2013; Gaw et al. 2014; Brumovský et al. 2016) and there are few ecotoxicological studies about the impact of CEC in marine and estuarine organisms when compared with freshwater ones. Additionally, several researches substituted ecotoxicity tests with saltwater organisms by data extrapolated from freshwater organisms, namely on risk assessment determination (Leung et al. 2001; Wheeler et al. 2002, 2014). Thus, some care should be taken on the use of these data, since a chemical can have different modes of action in different species.

In this study, we evaluated the toxicity of three compounds of emerging concern: metolachlor (herbicide), erythromycin (antibiotic) and triclosan (antiseptic), to the green algae (chlorophytes) *P. subcapitata* and *D. tertiolecta*. Toxicity assays performed for MTC resulted in a 72 h-EC_{50} of 118 μg L^{-1} for *P. subcapitata* (Table 1). This value is close to the 96 h-EC_{50} described for *P. subcapitata* by Juneau et al. (2001), using as endpoint the chlorophyll autofluorescence (Table 2). The EC_{50} values described in the literature for

Table 1 Effect concentration (EC) values for metolachlor, erythromycin and triclosan, evaluated using the alga *P. subcapitata* or *D. tertiolecta*

	EC (μg L^{-1})				
	10	25	50	75	90
Metolachlor					
P. subcapitata	45 ± 3	57 ± 3	118 ± 4	156 ± 6	235 ± 6
D. tertiolecta	5.62×10^3 ± 0.14×10^3	7.51×10^3 ± 0.21×10^3	11.3×10^3 ± 0.5×10^3	17.6×10^3 ± 0.2×10^3	21.4×10^3 ± 0.1×10^3
Erythromycin					
P. subcapitata	5 ± 4	24 ± 1	38 ± 1	76 ± 2	197 ± 4
D. tertiolecta	1.88×10^3 ± 0.41×10^3	4.23×10^3 ± 0.25×10^3	5.75×10^3 ± 0.09×10^3	9.62×10^3 ± 0.22×10^3	$>63 \times 10^3$
Triclosan					
P. subcapitata	3.0 ± 0.5	20.3 ± 0.3	27.1 ± 0.4	33.2 ± 0.2	37 ± 1
D. tertiolecta	14 ± 1	21 ± 2	93 ± 10	207 ± 4	456 ± 4

EC_{10}, EC_{25}, EC_{50}, EC_{75} and EC_{90} values represent the toxicant concentration that induces an inhibition of 10, 25, 50, 75 or 90%, respectively, of algal growth, after 72 h (*P. subcapitata*) or 96 h (*D. tertiolecta*), when compared with the control (cells not exposed to toxicant). The mean values were obtained from two experiences performed in duplicate ($n = 4$). Standard deviations are presented with 95% confidence limits

MTC varies widely (among 44 and 5.51×10^3 μg L^{-1}) (Table 2). Some of these variations can be associated with the operating conditions used, namely initial cell density, physiological conditions of algal cells, time of exposure to toxicants, culture medium, temperature, agitation, light intensity and wavelength and toxicity endpoint used.

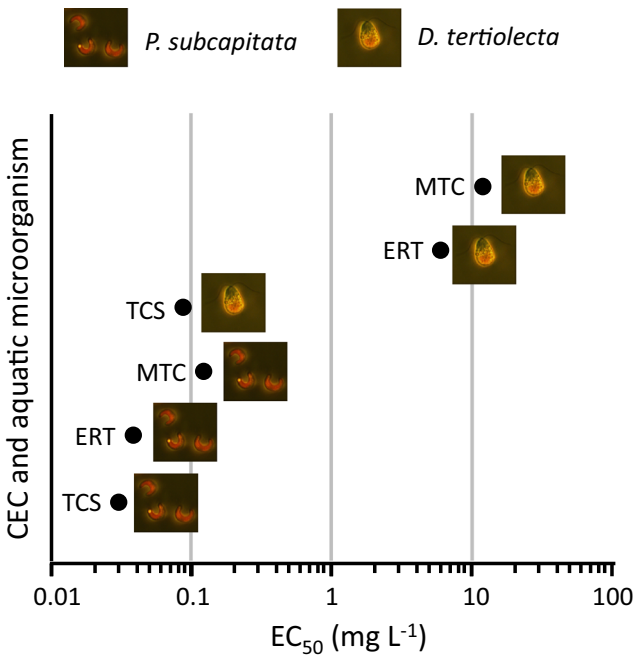

Fig. 3 Pictorial representation of the toxicity of metolachlor (MTC), erythromycin (ERT) and triclosan (TCS) to *P. subcapitata* and *D. tertiolecta*. The position in the graphic reflects their sensitivity to the toxicant, based on their EC_{50} value

In the case of *D. tertiolecta*, for MTC, a 96 h-EC_{50} of 11.3×10^3 μg L^{-1} was observed (Table 1). However, in the literature, a 120 h-EC_{50} of 1.47×10^3 μg L^{-1}, for MTC (Table 2) is described, using *D. tertiolecta* (Thakkar et al. 2013); this value is almost 8 times lower than the one that we obtained. The absence of indication of the initial cell concentration used by the authors limits the direct comparison between the value here obtained and the value described. A survey in the literature regarding the use of different marine microorganisms in the evaluation of MTC toxicity revealed that EC_{50} values also vary widely according to the alga used. Thus, a higher EC_{50} value (lower sensitivity to MTC than *D. tertiolecta*) was described for the marine chlorophyte alga *Tetraselmis suecica* (72 h-EC_{50} of 21.3×10^3 μg L^{-1}) (Ebenezer and Ki 2013). However, different marine algae displayed higher sensitivity than *D. tertiolecta* to MTC. This is the case of the marine diatoms *Skeletonema costatum* (120 h-EC_{50} of 61 μg L^{-1}) and *Navicula pelliculosa* (120 h-EC_{50} of 380 μg L^{-1}) (Erickson and Turner 2002), the dinoflagellate *Prorocentrum minimum* and the diatom *Ditylum brightwellii* with 72 h-EC_{50} of 73 and 423 μg L^{-1}, respectively (Ebenezer and Ki 2013). The different EC_{50} values observed for the marine algae may reflect the different sensitivity of the test species as well as different cultural conditions used, namely the time of exposure to toxicant.

MTC is frequently detected in surface and ground waters in the North of America and in Europe with maximum values occurring during high rain events, after pesticide application (Vallotton et al. 2008). For instance, in Lake Erie St. Clair Basin (USA), MTC was detected in 90% of 315 samples collected from 10 streams from March of 1996 to February of

Table 2 Example of toxicity studies described in the literature for metolachlor, erythromycin and triclosan, using the alga *P. subcapitata* or *D. tertiolecta*

Toxic	Test organism	Toxicity (EC_{50}, μg L^{-1})	Cell density (cells mL^{-1})	Endpoint	Time of exposure (h)	Reference
Metolachlor						
	P. subcapitata	72	1×10^4	Growth yield	72	Sbrilli et al. (2005)
		44.3	$5–10 \times 10^3$	Chl fluorescence	72	Souissi et al. (2013)
		98.0	3×10^4	Growth rate	72	Pérez et al. (2011)
		5.51×10^3	5×10^4	Growth yield	96	Ma et al. (2006)
		116	1×10^4	Chl fluorescence	96	Juneau et al. (2001)
		84	2×10^4	Chl fluorescence	96	Fairchild et al. (1998)
		77	2×10^4	Chl fluorescence	96	Fairchild et al. (1997)
		55.5	2×10^4	Growth yield	96	St. Laurent et al. (1992)
	D. tertiolecta	1.47 × 103	n.d.	Growth yield	120	Thakkar et al. (2013)
Erythromycin						
	P. subcapitata	20	1×10^4	Growth yield	72	Isidori et al. (2005)
		36.6	1×10^4	Growth yield	72	Eguchi et al. (2004)
		350	n.d.	Chl fluorescence	72	Gonzalez-Pleiter et al. (2013)
Triclosan						
	P. subcapitata	0.53	1×10^4	Growth yield	72	Yang et al. (2008)
		4.46	1×10^4	Growth yield	96	Orvos et al. (2002)
	D. tertiolecta	3.55	5×10^4	Growth yield	96	DeLorenzo and Fleming (2008)

Growth rate—biomass increase per unit of time. *Growth yield*—biomass at the end of the assay minus the starting biomass

Chl chlorophyll, *n.d.* not defined, *EC_{50}* toxicant concentration that induces an inhibition of 50% of algal growth, when compared with the control (cells not exposed to toxicant)

1998; a maximum of 78 μg L^{-1} MTC was observed (Frey 2001). In 14 states of the USA, MTC is present in surface waters samples at a maximum concentration of 138 μg L^{-1} (Rivard 2003). The values of MTC described above are in the range of the 72 h-EC_{50} value (118 μg L^{-1}) obtained for *P. subcapitata*, which underline the significance of this algal species in the assessment of MTC ecotoxicity in freshwater environments. The movement of organic pollutants in the aquatic environment can also present a serious threat to marine ecosystems. MTC has been detected in marine environment (Skagerrak, North Sea) at a very low concentration (1.49 ng L^{-1}) (Brumovský et al. 2016). This value of MTC found in coastal waters is several orders of magnitude lower than 96 h-EC_{50} values obtained in the present study and in the studies described in the literature (reported above) with marine algae.

The 72 h-EC_{50} value obtained in this study for ERT using *P. subcapitata* was 38 μg L^{-1} which is in the range described in the literature (Table 2), being very close to the value described by Eguchi et al. (2004). Nonetheless, in the literature, a 72 h-EC_{50} value is given which is approximately ten times higher, when chlorophyll fluorescence was used as toxicity endpoint (Gonzalez-Pleiter et al. 2013). These data suggest that growth inhibition is a more sensitive endpoint for the evaluation of erythromycin toxicity in *P. subcapitata*. A 96 h-EC_{50} of 5.75×10^3 μg L^{-1} was obtained for ERT, using *D. tertiolecta* (Table 1). This EC_{50} value is more than 150 times higher than the one for *P. subcapitata*. Comparing the EC_{50} values of the algae used in this study, *P. subcapitata* showed a higher sensitivity to erythromycin and metolachlor than *D. tertiolecta* (Table 1).

Erythromycin is one of most common antibiotics used in health care (substitute of penicillin) and also in aquaculture (economic antibiotic) resulting in residual levels in surface waters (Xue et al. 2013). For instance, in the USA, 139 streams presented a level of ERT of 1.7 μg L^{-1} (Kolpin et al. 2002b), while in a river of Madrid (Spain), the presence of ERT at a concentration of 3.98 μg L^{-1} has been detected (Rodríguez-Gil et al. 2010). These concentrations of ERT found in freshwaters are within the range of the 72 h-EC_{10} value obtained for *P. subcapitata* (5 ± 4 μg L^{-1}) (Table 1), which again emphasises the importance of this alga in the evaluation of toxicity in freshwater environments. Additionally, erythromycin and other antibiotics have been detected at significant distances from their sources. Antibiotics could be transported to marine environments via river inputs and sewage treatment plants (Zhang et al. 2013). In this context, ERT has been detected in Victoria Harbour (South China Sea) at 1.9 μg L^{-1} (Minh et al. 2009). However, this concentration of ERT is much lower than the 96 h-EC_{10} determined with *D. tertiolecta* (Table 1). As far as we know, the toxicity of erythromycin in marine microalgae has not been evaluated, making the present work the first study with marine green microalgae.

For TCS, the EC_{50} values here determined were of 27.1 μg L^{-1} for *P. subcapitata* and 93 μg L^{-1} for *D. tertiolecta.* These values are higher than those described in the literature (Table 2), but in the same order of magnitude. Concentrations of TCS up to 5.4 μg L^{-1} have been measured in effluents of municipal sewage treatment plants (Savannah, Georgia, USA) (Kumar et al. 2010). Kolpin et al. (2002a) reported the presence of TCS in aquatic environment at a concentration of 2.3 μg L^{-1}. In Tamiraparani, Kaveri and Vellar rivers (India), TCS reached the concentration of 5.16 μg L^{-1} (Ramaswamy et al. 2011), which is higher than the 72 h-EC_{10} value (3.0 μg L^{-1}) determined using the freshwater alga *P. subcapitata* (Table 1). This means that *P. subcapitata* growth is inhibited under environmentally relevant concentrations of TCS. Regarding marine levels of TCS, this compound has been detected at 6.9, 13.6 and 29.0 ng L^{-1} in European, North American and Asian waters, respectively (Bedoux et al. 2012). These levels of TCS found in marine waters are much lower than 96 h-EC values found in this work (Table 1) and in the literature (Table 2) using *D. tertiolecta*. A 96 h-$EC_{50} > 66$ μg L^{-1} was reported for the marine *Skeletonema costatum* (Orvos et al. 2002) which is also a high value when compared with marine water concentration of TCS.

The different level of toxicity of each CEC studied, as well as the different sensitivity of the two algae to a given toxicant, may be due to different (1) cellular targets of each toxicant, (2) culture medium composition and culture conditions used for the growth the algae species and (3) homeostatic mechanisms presented by algae to cope with each toxicant. Thus, metolachlor is a typical herbicide (broad-leaved grassy weeds) used in the USA that acts as a growth inhibitor by preventing chlorophyll, protein, fatty acid, lipid, isoprenoid and flavonoid synthesis in the target plants (Rivard 2003). Metolachlor exposure also has several effects on non-target organisms, such as growth and photosynthesis inhibition in algae (Deng et al. 2015). It has been reported that the growth inhibition of the alga *Scenedesmus acutus* induced by metolachlor was directly correlated with the effect of the compound on fatty acid synthesis (Couderchet et al. 1999). Erythromycin, a macrolide antibiotic, inhibits bacterial protein synthesis by binding to 50S ribosomal subunit. Although it is expected that erythromycin has as target prokaryotic organisms (bacteria), eukaryotic organisms such as algae can also be adversely affected due to the presence of prokaryotic-like ribosomes in their chloroplasts and mitochondria (Vannini et al. 2011). Thus, Liu et al. (2011) reported that erythromycin inhibited the growth of *Selenastrum capricornutum* as consequence of photosynthesis perturbation due to protein biosynthesis inhibition. Triclosan is a common antimicrobial agent that blocks the active site of the enzyme enoyl-acyl carrier-protein reductase avoiding the bacterial fatty acid synthesis necessary for cell membrane building (Bedoux et al. 2012). Triclosan exhibits multiple target sites in different algal species (Franz et al. 2008). The higher sensitivity to the toxicants presented by *P. subcapitata* (EC_{50} values were 3.4–151-fold lower than those observed with *D. tertiolecta*) can also be due to the pH of algal media (pH 7.5 for *P. subcapitata* and pH 8.0 for *D. tertiolecta*). pH can affect the ionisation level of the compounds which, in turn, can affect their level of toxicity (Guo et al. 2016). The highest pH of the culture medium used for marine species promotes the ionisation of weak acids like erythromycin (pK_a 8.88) and triclosan (pK_a 7.9) reducing their bioavailability and uptake into algal cells (Halling-Sorensen 2000). This fact can explain, at least partially, the lower toxicity of these compounds on the marine alga *D. tertiolecta*. The available information regarding the toxicity mechanisms of the three compounds studied over the two green algae species used are very scarce. The improvement of the knowledge about these mechanisms deserves further attention. In this context, studies are currently underway to elucidate the mode of action of these compounds on algae.

In conclusion, using the green algae *P. subcapitata* and *D. tertiolecta* and considering the respective EC_{50} values, it was possible to hierarchise the toxicity of the three compounds of emerging concern studied. The decreasing order of toxicity of these compounds is TCS > ERT > MTC. The differential toxicity of the studied CEC to the green algae can be attributed to distinct targets and mechanisms of action in algal cells. In addition, for the same alga, EC_{50} values can vary by a factor higher than 10, which can result from different protocols, culture media and operational conditions used in EC determinations. Therefore, toxic concentrations cannot simply be extrapolated from one species to others or from freshwater species to marine ones. The growth of *D. tertiolecta* is inhibited in a dose-dependent way by the CEC studied, which suggests that this marine alga can be used in the evaluation of ecotoxicity of these compounds. All 72 h-EC_{10} or 72 h-EC_{50} values determined, for the three CEC evaluated, using *P. subcapitata*, are within the range of the concentrations of these pollutants detected in surface and ground waters. These results highlight the importance of the use of this alga in the assessment of chronic toxicity of CEC in freshwaters.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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