



Algicide activity of rosmarinic acid on *Phaeodactylum tricornutum*

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ABSTRACT

Rosmarinic acid is a natural phenolic compound commonly found in land and aquatic plant species. Although the antibacterial, antiinflammatory, antimutagenic and antiviral activity of rosmarinic has been already demonstrated, its phytotoxic activity has been poorly investigated nor in land or aquatic ecosystems. Therefore, the effects of this secondary metabolite on growth and physiology of the diatom *Phaeodactylum tricornutum* Bohlin were analyzed in this study. Cell density and chlorophyll fluorescence were recorded along the treatment at different times. After five days, the distribution of polar and neutral lipids was observed by confocal microscopy with the fluorochrome Nile Red and photosynthetic pigments content was quantified by spectrophotometry. The results revealed a strong phytotoxic potential of rosmarinic acid with a decrease on cell density and chlorophyll content, and diminution of neutral lipids, which suggest the effect of rosmarinic acid on *P. tricornutum* plasma membrane permeability and survival.

Keywords: Secondary metabolite, phenolic compound, algicide, membrane permeability, phytotoxicity, microalgae.

Introduction

The well-known persistence and toxicity of synthetic algicides as copper sulfate or diuron (Jančula and Maršálek, 2011) are in the origin of the search of more ecologically friendly natural compounds, which can be used as algicides, herbicides or insecticides. Although there are many organisms with algicidal activity, it is very difficult to isolate and characterize the active compound that is responsible of the biological activity. As an example, the secondary metabolite nostocarboline from the cyanobacteria *Nostoc78.12A* showed an IC_{50} of 2.1 μ M in *Microcystis aeruginosa* PCC 7806, 5.8 μ M in *Synechococcus* PCC 6911 and 29.1 μ M in *Kirchneriella contorta* SAG 11.81 (Blom *et al.*, 2006). As well, ethyl-2-methylacetoacetate, commonly found in *Phragmites australis*, showed inhibitory effects on the growth of *Chlorella pyrenoidosa*, *Microcystis aeruginosa* and *Chlorella vulgaris*. (Li and Hu, 2005) Other natural compounds with algicide activity are 9,10-anthraquinone and derivatives, isoquinoline plant alkaloids (Jančula and Maršálek, 2011) or some toxins as cyanobacterine and fischerellin A and B, which can be found in many cyanobacteria (Srivastava *et al.*, 1998; Jüttner *et al.*, 2001; Berry *et al.*, 2008). However, these are limited examples in the algicidal research.

In the search of new biologically active compounds, it is essential to prospect new molecules with demon-

strated biological activity. Therefore, the effects of rosmarinic acid ((R)-O-(3,4-Dihydroxycinnamoyl)-3-(3,4-dihydroxyphenyl) lactic acid), an ester of caffeic acid and 3,4-dihydroxyphenyl lactic (Scarpati and Oriente, 1958) belonging to the group of the phenolic compounds, have been studied. Rosmarinic acid is commonly found in species of the families Boraginaceae and Lamiaceae (Litvinenko *et al.*, 1975), and it has also been described in other plant families, as in ferns of the Blechnaceae family (Häusler *et al.*, 1992), lower plants as hornworts (Takeda *et al.*, 1990), plants of the family Cannaceae (Petersen *et al.*, 2009), species of the Marantaceae family (Abdullah *et al.*, 2008), the marine monocotyls *Posidonia oceanica* or *Zostera marina* of the family Zosteraceae (Petersen *et al.*, 2009; Ravn *et al.*, 1994) or other aquatic plants of the family Potamogetonaceae. This phenolic compound has multiple biological activities, as astringent, antioxidant, antiinflammatory, antimutagenic or antiviral activities (Petersen and Simmonds, 2003; Tepe *et al.*, 2007; Nakamura *et al.*, 1998; Parnham and Kesseling, 1985). Phenolic compounds, as rosmarinic acid, can provide protection against cancer and contribute to the antioxidant activity of cosmetic products, which use plant species as *Rosmarinus officinalis* and *Sanicula europaea* (Petersen and Simmonds, 2003). This compound shows also a broad range of bactericide and fungicide

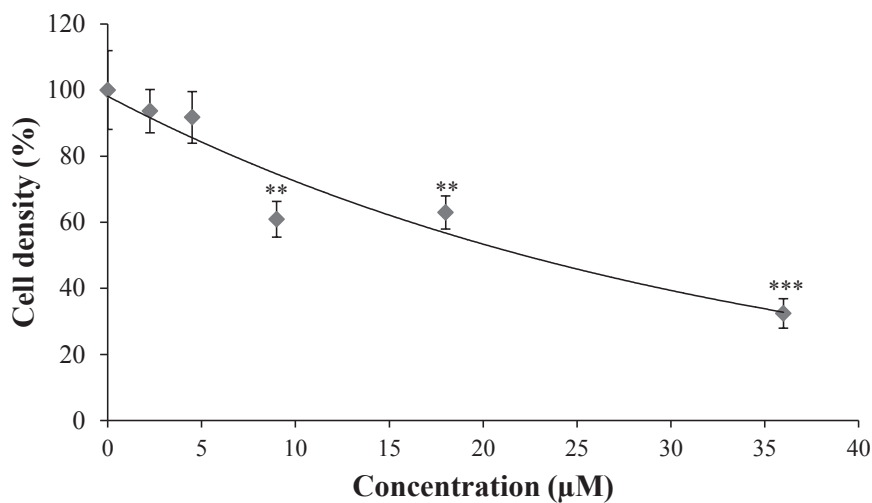


Figure 1. Dose-response curve for cell density of *Phaeodactylum tricornutum* treated with rosmarinic acid (2.25, 4.5, 9, 18 and 36 µM). Data are represented as percentage of the control (R2 = 0.9363). Asterisks indicate statistical differences compared to the control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

activities, and it plays an active role in the regulation of symbiosis and the protection against microorganisms (Bais et al., 2004), i.e. once that basil is damaged by the fungi *Pythium ultimum*, the roots exude high amounts of rosmarinic acid, which inhibits the most part of soil pathogens (Bais et al., 2004).

Algae are commonly used in aquatic toxicity tests due to their easy growth and easy exposure to water soluble compounds. Besides, microalgae are sensitive to many chemicals or contaminants and have a short generation time, which allows the rapid study of the effects of toxins on growth (Choi et al., 2012).

Phaeodactylum tricornutum (Bohlin) (Lewin, 1958), is a diatom (Bacillariophyceae) belonging to the family Phaeodactilaceae. Diatoms represent an important group of eukaryotic algae found in marine and terrestrial ecosystems. These algae play a crucial role in the world primary production, having a key role in the marine food chain, where they are believed to be responsible for up to 25% of world primary production (Scala et al., 2002). These organisms are also of interest as food for the industrial cultivation of aquatic animals. Moreover, many diatoms are used as indicators of water quality (Domergue et al., 2003).

P. tricornutum represents a good biological model because is easy to grow, it has appreciated physiological and genetic characteristics (short generation time, short genome and easy genetic transformation; Scala et al., 2002; Soto et al., 2005) and its genome is fully sequenced (Bowler et al., 2008). This diatom is mainly known because is a potential source for the industrial production of eicosapentaenoic acid (EPA; 20:5^{Δ5,8,11,14,17},

Grima et al., 1996). EPA represents 30% of the fatty acids of *P. tricornutum*, while palmitoleic acid (16:1^{Δ9}), palmitic acid (16:0), hexadecatrienoic acid (16:3^{Δ6,9,12}) and miristic acid (14:0) represent 26, 17, 10 y 15% respectively (Domergue et al., 2003).

Material and methods

Phytotoxic bioassays of rosmarinic acid on microalgae

A dose-response experiment was carried out in order to determine the phytotoxic effects of rosmarinic acid in *Phaeodactylum tricornutum* cells.

The stock culture of the microalgae was obtained from the Marine Science Station of Toralla (ECIMAT, Pontevedra, Spain) and kept as batch culture in the laboratory. Guillard F/2 medium was used as growth medium. The culture medium was prepared in sea-water filtered through 1 µm filter and autoclaved. Nutrients were added in sterile conditions.

Cultures were oxygenated and maintained at 20 ± 1 °C with a photoperiod of 16/8 h light/darkness to allow the growth of *P. tricornutum*. The phytotoxic bioassays were performed for 6 days, being the inoculum of *P. tricornutum* added to fresh culture medium (500 mL per treatment) at day 0. The initial cell concentration was about 50,000 cells mL⁻¹, which was considered a good starting concentration to get the cells in exponential phase still after 6 days of growth. The dose-response curve of treated *P. tricornutum* cells was performed with 2.25, 4.5, 9, 18, 36, 75 and 150 µM rosmarinic acid.

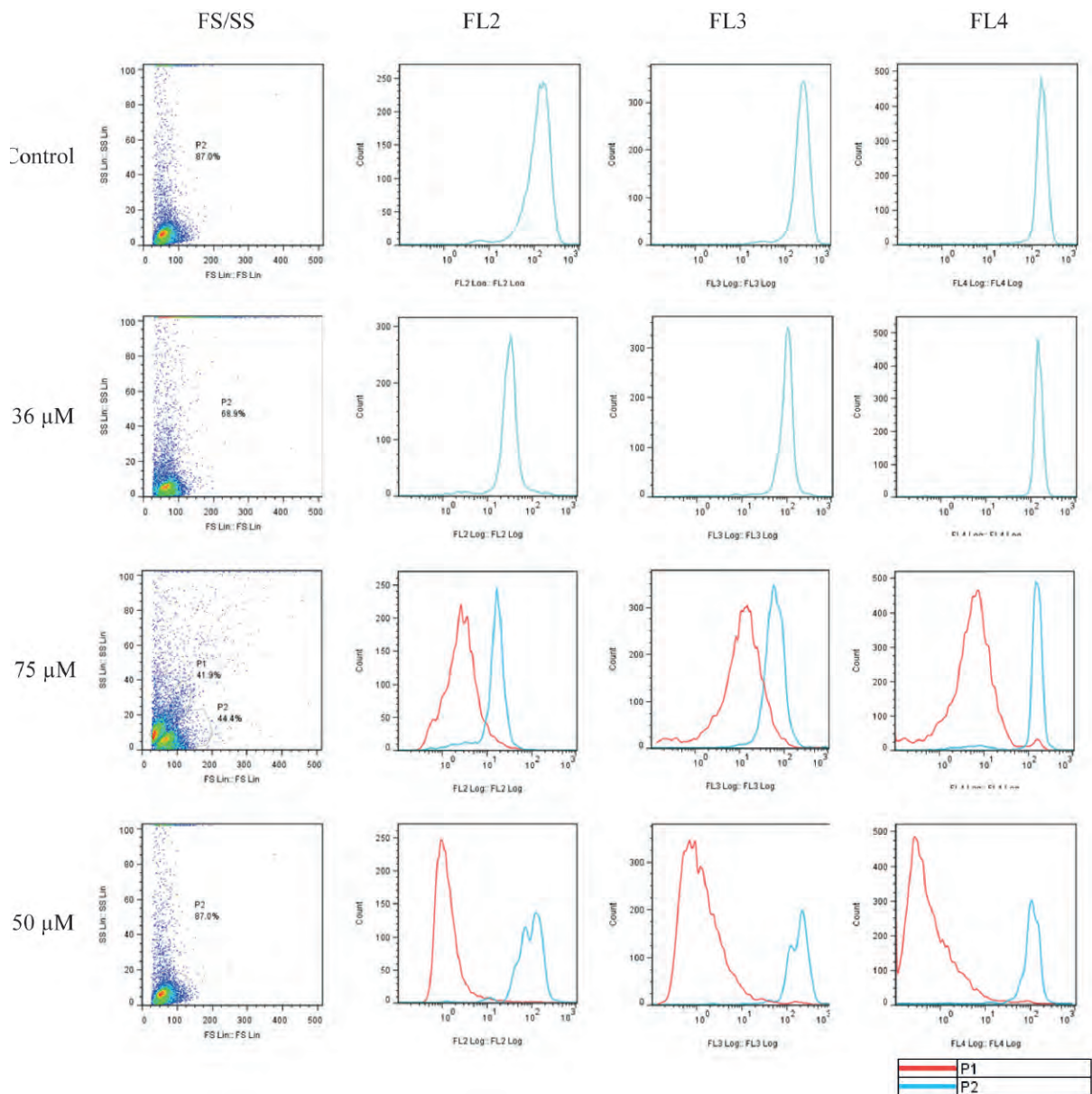


Figure 2. Flow cytometric histograms showing shifts in neural lipid (FL2), polar lipid (FL3) and Chlorophyll *a* (FL4) fluorescences. X and Y histogram axes represent fluorecence (log-transformed voltage units) and number of cells, respectively. Values shown correspond to control and concentrations of 36, 75 and 150 μM of rosmarinic acid.

Cell density was measured with a Neubauer chamber and a light microscope (Nikon ALPHAPHOT-2) 1, 3 and 5 days after treatment. Six complete chambers were counted per assayed concentration and the cell density was calculated ($\text{cel}\cdot\text{mL}^{-1}$). Non-pigmented or lysed cells were not considered for counting. The phytotoxic potential of the compound was determined by calculating the IC_{50} and the IC_{80} (rosmarinic acid concentrations that inhibit the growth of the algal population by 50% or 80%, respectively) of the dose-response curve after 5 days of treatment.

Measurement of chlorophyll a fluorescence by flow cytometry

The measurement of chlorophyll *a* fluorescence, previously used to assess the effect of heavy metals, herbicides, petrochemicals and nutrients on plant metabolism (Ralph et al., 2007), is an adequate technique to evaluate changes in the photosynthetic capacity of algae populations (Farah and Samuelsson, 1992; Samuelsson and Oquist, 1977). Moreover, this parameter is strongly affected by

the physiological status of the algae (Huot et al., 2007).

The chlorophyll *a* fluorescence, the cell size and the complexity were measured on days 1, 3 and 5 of the treatment, using FS and SS detectors, respectively, of FC 500 MPL flow cytometer (Beckman Coulter Instruments) equipped with an Argon laser with emission wavelength of 488 nm. Three samples of each rosmarinic concentration (0, 2.25, 4.5, 9, 18, 36, 75 and 150 μM) were analyzed by flow cytometry. The chlorophyll *a* fluorescence was collected with a 675 nm dichroic filter (FL4), which corresponds to the emission peak of the chlorophyll. About 10,000 cells were analyzed per replicate, using a logarithmic amplification of the fluorescence signal. The results were expressed as fluorescence (log-transformed voltage units).

Detection of polar and neutral lipids by flow cytometry

As previously mentioned, *Phaeodactylum tricornutum* is mainly known as a potential source of EPA (eicosapentaenoic acid), a neutral lipid that is the most abundant fatty acid (30 %) of *P. tricornutum* (Domergue et al., 2003).

The relative abundance of polar and neutral lipids was determined using the solvatochromic fluorochrome Nile Blue A Oxazone (Nile Red; Sigma-Aldrich) that selectively stains cell lipids according to the protocol of Yu et al. (2009), with some modifications. Three replicates of each rosmarinic concentration (0, 2.25, 4.5, 9, 18, 36, 75 and 150 μM) were analyzed by flow cytometry. Staining was performed by adding 25 μL of Nile Red (NR) stock solution in acetone (200 g mL^{-1}) per mL of algae suspension, to get a final concentration of 5 $\mu\text{g mL}^{-1}$. After adding the fluorochrome, the sample was vigorously stirred and incubated for 10 min at room temperature.

Flow cytometry can simultaneously measure the autofluorescence of chlorophyll *a* and the fluorescence of cells stained with a specific staining as NR (de la Jara et al., 2003). When NR is excited at 488 nm with the Argon laser of the FC 500 MPL flow cytometer, it exhibits an intense yellow-gold fluorescence if it is dissolved in neutral lipid, and a red fluorescence if it is dissolved in polar lipids (Alonzo and Mayzaud, 1999).

The optical system of this equipment collects yellow fluorescence emission (575 nm dichroic filter) in the channel FL2 and the red fluorescence (620 nm dichroic filter) in the channel FL3. Over 10,000 cells were analyzed using the logarithmic amplification of the fluorescence signal. Results were expressed as fluorescence (log-transformed voltage units) (Mendoza Guzmán et al., 2010).

Detection of polar and neutral lipids by confocal microscopy

Polar and neutral lipids were detected in 0, 2.25, 4.5, 9, 18, 36, 75 and 150 μM rosmarinic acid treated-cells by using a Leica TCS SP5 confocal microscope (Wetzlar, Germany) with a hybrid detector. Nile red (NR) stained cells were excited at 514 nm for the emission of yellow fluorescence at 580 nm, corresponding to neutral lipids, and at 543 nm for the emission of red fluorescence at 610 nm, corresponding to polar lipids. The emission of *P. tricornutum* chlorophyll between 673 and 678 nm avoided interferences with the detection of neutral or polar lipids.

Statistical analysis

The data were statistically analyzed with SPSS Statistics 19.0 software, testing normality by Kolmogorov-Smirnov and homoscedasticity by Levene's test. The statistical significance of differences among group means was estimated by analysis of variance followed by least significant difference tests in the case of homoscedastic normally distributed data, by Tamhane's T2 test in the case of heteroscedastic normally distributed data, and by the Kruskal-Wallis test in the case of non-normally distributed data.

Fluorescence data were analyzed by student *t* tests.

Results

Phytotoxicity bioassays

Microalgae growth tests were used to measure the toxicity of rosmarinic acid. The treatment with the different rosmarinic acid concentrations resulted in a strong inhibition of algal growth. The dose-response curve obtained with 0, 2.25, 4.5, 9, 18 and 36 μM treated cells showed IC_{50} and IC_{80} values of 22.5 μM and 53 μM respectively (Fig. 1).

Chlorophyll *a* fluorescence and lipid content

The treatment with the highest concentrations of rosmarinic acid, produced an important change in the morphology of the algae population. At concentrations of 75 and 150 μM two different cell populations are founded (P1 and P2) (Fig. 2) with different fluorescence emission peaks in the FL2, FL3 and FL4 channels. One of the populations (P2) was similar to the single population presented in the control and at the lower concentrations of rosmarinic acid. However, P1 showed lower fluorescence values for all the channels

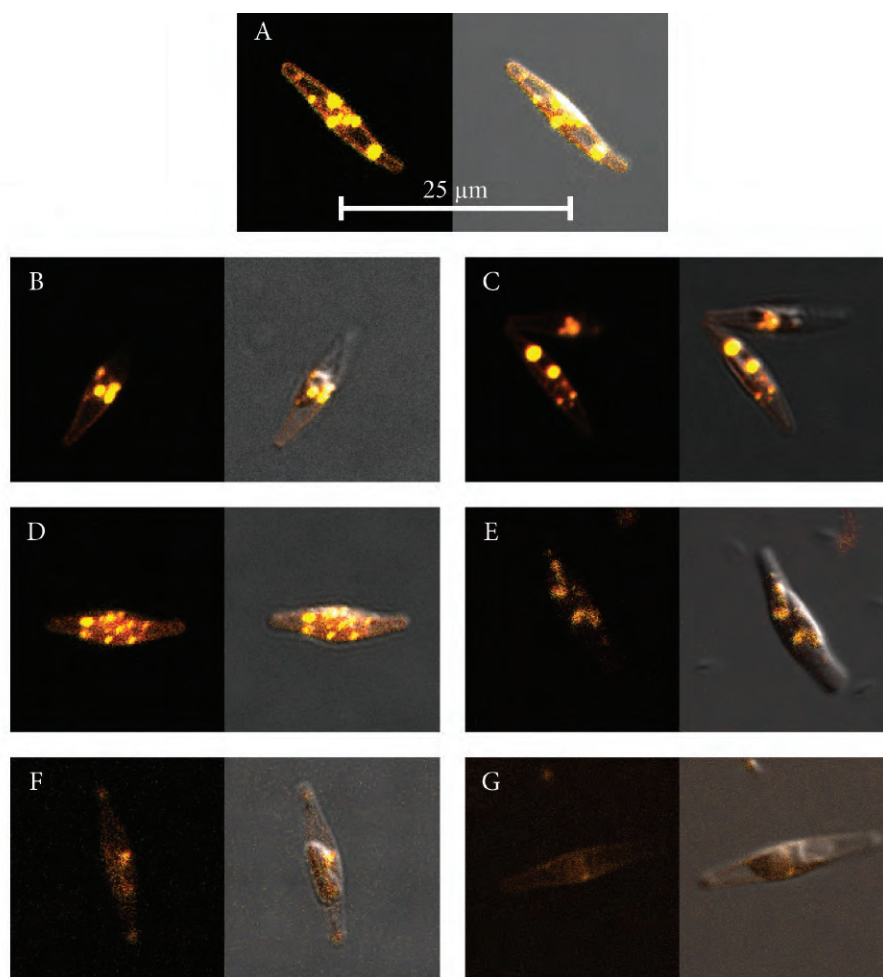


Figure 3. Confocal microscopy images of Nile Red (NR) stained *Phaeodactylum tricornutum* after 6 days of growth in the presence of (A) 0 μM , (B) 2.25 μM , (C) 4.5 μM , (D) 9 μM , (E) 18 μM , (F) 36 μM , and (G) 75 μM rosmarinic acid. Images on the left are overlay images of red and yellow channels while images on the right are brightfield images. Yellow drops indicate neutral lipids while red color corresponds to polar lipids.

which means that the treatment produces a decrease in polar and neutral lipids and in chlorophyll *a* fluorescence.

The strong effects of rosmarinic acid on neutral lipids, detected by flow cytometry, were confirmed by confocal microscopy with Nile Red fluorochrome. Stained cells showed an accumulation of neutral lipids in form of yellow drops (Fig. 3) (Alonzo and Mayzaud, 1999). As low concentrations as 4.5 or 9 μM rosmarinic acid, induced an increase in the number of yellow drops, although higher concentrations showed a decrease in size and number of neutral lipids in form of yellow drops (Fig. 3) and concentrations higher than 18 μM rosmarinic acid showed a strongly decreased staining intensity and lipid drops could not be observed in the samples. Moreover, the typical yellow staining of neutral lipids appears more

dispersed inside and even outside the cells, which could be associated with a release of lipids (Fig. 4). At higher concentrations of this secondary metabolite, the staining intensity is almost zero. These results are in concordance with the cytometer data where a new population of algae with lower fluorescence emission in the channels FL2, FL3 and FL4 appeared at higher concentrations of treatment suggesting that the alga membrane is damaged and there is a release of pigments and lipids out of the cells.

Discussion

The results here obtained show a decrease in the growth of *Phaeodactylum tricornutum* after rosmarinic acid treatment. As already mentioned in the introduction, other compounds, as nostocarboline,

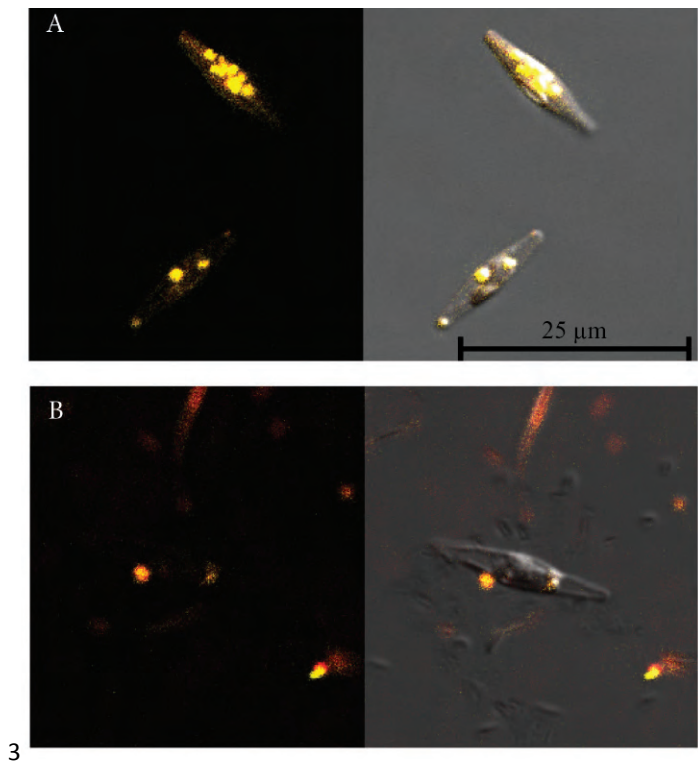


Figure 4. Confocal microscopy images of NR stained *P. tricornutum* after 6 days of growth with (A) 0 μM and (B) 18 μM rosmarinic acid. Images on the left are overlay images of red and yellow channels while images on the right are brightfield images. Yellow drops correspond to neutral lipids in and out of the cell.

cyanobacterine or ethyl-2-methylacetoacetate, have been previously found to show algicide activity, but this is the first time that rosmarinic acid is reported as algicide.

Rosmarinic acid treatment produced a significant decrease in the content of both polar and neutral lipids. Microorganisms, particularly microalgae, are the first organisms affected by chemicals in aquatic ecosystems, as they are in direct contact with the medium from which are only separated by the cell wall and the cytoplasmic membrane, which consists mainly of polar lipids. Cell membranes are selective dynamic barriers that play an essential role in the regulation of biochemical and physiological events, so that any change produced in the medium will produce changes in the membranes of the microorganisms (Cid et al., 1996). Therefore, the altered contents of polar lipids in rosmarinic treated cells suggest altered membrane permeability after the treatment with this phenolic compound.

Furthermore, this secondary metabolite also affected the content of neutral lipids. *P. tricornutum* is a diatom widely known to be a potential source of eicosapentaenoic acid (EPA, 20:5^{Δ5, 8,11,14,17}) for indus-

trial production, as this neutral lipid represents the 30% of total neutral lipids in this species (Domergue et al., 2003). It has been shown that in diatoms, the damage of the membranes causes the release of polyunsaturated fatty acids, particularly of EPA as a defense reaction (Budge and Parrish, 1999; Jüttner, 2001; Pohnert, 2005). Similarly, the mechanical damage caused by osmotic stress caused also the release of fatty acids (Jüttner, 2001; Jüttner and Dürst, 1997). It has been found that most allelochemicals reduce the algal cell membrane integrity and cause a leakage of cytoplasm. For example, salcolin B, extracted from *Hordeum vulgare*, was found to directly attacks the *Mycrocistis aeruginosa* cell membrane causing a significant increase in cell membrane leakage after 5-days exposure (Xiao et al., 2014). Therefore, the effect of rosmarinic acid on the lipid content and the observed leakage of lipids out of the treated cells could be related to a direct damage of rosmarinic acid on the plasma membrane of *P. tricornutum*.

Although chlorophyll *a* fluorescence has been considered to be a good indicator for the toxicity of water soluble compounds (Choi et al., 2012) and it has been previously used to evaluate the effects of heavy met-

Table 1. Neutral lipids (FL2), polar lipids (FL3) and chlorophyll *a* (FL4) fluorescence values (arbitrary units in percentage of the control). The superscripts indicate statistically significant differences among treatments. Shaded cells indicate values with significant differences compared to the control ($p \leq 0.05$).

	Neutral lipids	Polar lipids	Chlorophyll <i>a</i>
Control	100.00 ^{ac}	100.00 ^a	100.00 ^{ab}
2.25 μ M	120.85 ^{abc}	109.17 ^{ab}	100.44 ^{ab}
4.5 μ M	160.00 ^{ab}	138.76 ^b	110.40 ^a
9 μ M	168.72 ^b	125.59 ^{abc}	95.57 ^{abc}
18 μ M	82.21 ^c	83.14 ^{ac}	95.35 ^{abc}
36 μ M	20.59 ^d	44.97 ^d	94.91 ^{abc}
75 μ M	8.03 ^e	15.18 ^d	65.88 ^c
150 μ M	0.97 ^f	0.59 ^e	0.34 ^d

als, herbicides, petrochemicals and nutrients on plant metabolism (Ralph et al., 2007); the treatment caused no change in the *in vivo* chlorophyll *a* fluorescence of *P. tricornutum* cells, hinted that the target is not on the reaction centre of photosynthesis. However at the highest concentration of rosmarinic the chlorophyll *a* fluorescence decreased due to the disintegration of the algal cells.

As it was said before, the release of lipid suggest cell membrane damage, This effect, together with the strong decline in the growth of algae suggests that rosmarinic acid triggers automortality processes in cells of the algae, as at the late stage of the events associated with the automortality, cells lose membrane integrity resulting in a complete disintegration of the algal cells (Kroemer et al., 1995; Naganuma, 1996). It has been demonstrated that non-viable algal cells still possess their photopigments and are capable of photosynthesis but the loss of membrane integrity will accelerate the efflux of small metabolites across the cell wall (Veldhuis et al., 2001).

The strong phytotoxicity of this phenolic compound on the diatom *Phaeodactylum tricornutum*, together with the extensive knowledge of its biosynthesis convert rosmarinic acid in an interesting microalgal growth regulator. A deeper study on their effects at the cellular level and its assay on other algal species, could allow us to better elucidate the mode of action of this compound.

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