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Cytotoxicity of degradation products of emerging microcontaminants in freshwater microalga *Raphidocelis subcapitata*

Citotoxicidad de los productos de degradación de microcontaminantes emergentes sobre la microalga dulceacuícola *Raphidocelis subcapitata*

Citotoxicidade dos produtos de degradación de microcontaminantes emerxentes sobre a microalga doceacuícola *Raphidocelis subcapitata*

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A mi abuela,

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List of abbreviations

- ANOVA: Analysis of Variance
- AOP: Advanced Oxidative Process
- DHR123: Dihydrorhodamine 123
- $DiBAC_4(3)$: Bis-(1,3-Dibarbituric acid)-trimethine oxanol
- EC: Emerging Contaminants
- EC_{10}: Effective Concentration of 10%
- FCM: Flow Cytometry
- FDA: Fluorescein Diacetate
- FL: Fluorescence
- FSC: Forward Scattered
- HPLC-MS: High Performance Liquid Chromatography and Mass Spectrum
- PABA: Para-aminobenzoic Acid
- PAH: Polyaromatic Hydrocarbon
- PAM: Pulse Amplitude Modulation
- PI: Propidium Iodide
- PLSMX: Photolyzed SMX
- PP: Photoproducts
- PSII: Photosystem II (PSII)
- REDOX: Reduction-Oxidation
- ROS: Reactive Oxygen Species
- SMX: Sulfamethoxazole
- SSC: Side Scattered

Abstract/Resumo/Resumen

Emerging contaminants (EC), such as pharmaceuticals, pose a potential risk in aquatic environments since their presence in water media can induce toxic effects onto the environment's biota. This kind of compounds, not completely degraded after their use/application, can easily reach different water bodies, like lakes or oceans, given that many water treatment systems cannot efficiently eliminate them nowadays. Thus, it is of great concern the potential negative effect these compounds can cause on representative organisms of these environments, such as microalgae, given their essential role on the trophic net as primary producers. In recent years, advance oxidation processes (AOP) have been researched as promising mechanisms of remediation and elimination of these emerging contaminants from aquatic environments.

In the present study several experiments were carried out in order to test the possible toxic effect of the antibiotic and emerging contaminant sulfamethoxazole (SMX) on the freshwater microalga *Raphidocelis subcapitata*. For that, several parameters, like proliferation, viability, vitality, photosystem II's quantum yield, oxidative stress, and potential of cytoplasmic and mitochondrial membrane were monitored through flow cytometry (FCM). The effective concentration of SMX that induces a 10% inhibition on the growth of the microalga was calculated (EC_{10}). Furthermore, the toxicity of photolyzed SMX (PLSMX), obtained after exposure of SMX solutions at their EC_{10} to UV light, was also studied by monitoring of the same parameters.

The results obtained demonstrated that exposure to both compounds, pure and photolyzed, significantly induces ($p<0.05$) great levels of cells with low vitality. Nevertheless, PLSMX also induces total cell growth inhibition and the decrease of daughter cells percentage. Further, the cells also show a loss in cell viability and a decrease on the quantum yield of their photosystem II. Moreover, *R. subcapitata* exposed to PLSMX also exhibited enhanced levels of intercellular reactive oxygen species (ROS) production and great values of cells with both cytoplasmic and mitochondrial depolarized membranes.

From the results obtained it can be concluded that PLSMX showed a greater toxic effect on the studied microalga compared to pure SMX. This has great ecological relevance, since remediation of SMX by AOP could affect the aquatic environments in a more toxic manner compared to pure SMX, scenario aggravated by the fact that microalgae are the fundamental primary producers of these environments. Thus, further research on SMX remediation is needed.

Key words: Emerging contaminant (EC), cytotoxicity, effective concentration, advanced oxidation process (AOP), flow cytometry (FCM), antibiotic, sulfamethoxazole (SMX), reactive oxygen species (ROS)

Os contaminantes emerxentes (EC), coma os medicamentos, supoñen un potencial risco nos medios acuáticos dado que a súa presencia nestes ambientes pode inducir efectos tóxicos na biota do medio. Este tipo de compostos, os cales non se degradan completamente despois do seu uso/aplicación, poden alcanzar facilmente diferentes masas de auga, coma lagos ou océanos, dado que a día de hoxe os sistemas de tratado de auga non as poden eliminar de maneira eficiente. Polo tanto, é de elevada preocupación o potencial efecto negativo que estes compostos poden causar nos organismos representativos destes medios, coma as microalgas, dado o seu papel esencial na rede trófica coma produtores primarios. En anos recentes, os procesos de oxidación avanzada (AOP) foron investigados como mecanismos prometedores de remediación e eliminación destes contaminantes emerxentes en medios acuáticos.

Neste estudo varios experimentos foro levados a cabo para comprobar o posible efecto tóxico do antibiótico e contaminante emerxente sulfametoxazol (SMX) na microalga de auga doce *Raphidocelis subcapitata*. Para iso, varios parámetros, coma a proliferación celular, a viabilidade, a vitalidade, o rendemento cuántico do fotosistema II, o estrés oxidativo, e o potencial das membranas citoplasmática e mitocondrial foron monitorizados por citometría de fluxo (FCM). A concentración efectiva de SMX que induce unha inhibición do 10% no crecemento da microalga foi calculada (EC_{10}). Por outra parte, a toxicidade de SMX fotolizado (PLSMX), obtido logo da exposición de solucións de SMX na súa EC_{10} con luz UV, tamén foi estudiada por monitorización dos mesmos parámetros.

Os resultados obtidos demostraron que a exposición de *R. subcapitata* a ambos compostos, puro e fotolizado, induce significativamente ($p<0.05$) elevados niveis de células con baixa actividade metabólica. Sen embargo, PLSMX tamén induce a inhibición total do crecemento celular e a redución da porcentaxe de células fillas. Ademais, as células tamén mostran unha perda da viabilidade celular e unha redución no rendemento cuántico do fotosistema II. Adicionalmente, *R. subcapitata* exposta a PLSMX tamén exhibiu niveles elevados de produción intracelular de especies reactivas de osíxeno (ROS) e de células con membrana citoplasmática e mitocondrial despolarizadas.

Cos resultados obtidos pódese concluír que PLSMX mostrou un maior efecto tóxico na microalga estudiada en comparación con SMX puro. Isto é de gran relevancia ecolóxica, dado que a remediación de SMX con AOP pode afectar aos medios acuáticos dunha maneira máis tóxica ca SMX puro, escenario agravado polo feito de que as microalgas son os produtores primarios fundamentais destes medios. Polo tanto, maior investigación na remediación de SMX é necesaria.

Palabras chave: Contaminante emerxente (EC), citotoxicidade, concentración efectiva, procesos de oxidación avanzada (AOP), citometría de fluxo (FCM), antibiótico, sulfametoxazol (SMX), especies reactivas de osíxeno (ROS)

Los contaminantes emergentes (EC), como los fármacos, suponen un potencial riesgo en los ecosistemas acuáticos dado que su presencia en estos ambientes puede inducir efectos tóxicos en la biota del medio. Este tipo de compuestos, los cuales no se degradan completamente después de su uso/aplicación, pueden alcanzar fácilmente diferentes masas de agua, como lagos u océanos, dado que a día de hoy los sistemas de tratamiento de aguas no los pueden eliminar de manera eficiente. Por lo tanto, es de elevada preocupación el potencial efecto negativo que estos compuestos pueden causar en los organismos representativos de estos medios, como las microalgas, dado su papel esencial en la red trófica como productores primarios. En años recientes, los procesos de oxidación avanzada (AOP) han sido investigados como mecanismos prometedores de remediación y eliminación de estos contaminantes emergentes en medios acuáticos.

En este estudio varios experimentos fueron llevados a cabo para comprobar el posible efecto tóxico del antibiótico y contaminante emergente sulfametoxazol (SMX) en la microalga de agua dulce *Raphidocelis subcapitata*. Para ello, varios parámetros, como la proliferación celular, la viabilidad, la vitalidad, el rendimiento cuántico del fotosistema II, el estrés oxidativo, o el potencial de las membranas citoplasmática y mitocondrial fueron monitorizados por citometría de flujo (FCM)., La concentración efectiva de SMX que induce una inhibición del 10% en el crecimiento de la microalga fue calculada (EC_{10}). Por otra parte, la toxicidad de SMX fotolizado (PLSMX), obtenido luego de la exposición de soluciones de SMX en su EC_{10} con luz UV, también fue estudiada por monitorización de los mismos parámetros.

Los resultados obtenidos demostraron que la exposición de *R. subcapitata* a ambos compuestos, puro y fotolizado, induce significativamente ($p<0.05$) elevados niveles de células con baja vitalidad. Sin embargo, PLSMX también induce la inhibición total del crecimiento y la reducción del porcentaje de células hijas. Además, las células también muestran una pérdida de viabilidad celular y una reducción en su rendimiento cuántico del fotosistema II. Adicionalmente, *R. subcapitata* expuesta a PLSMX también exhibió niveles elevados de producción intracelular de especies reactivas de oxígeno (ROS) y de células con membrana citoplasmática y mitocondrial despolarizadas.

Con los resultados obtenidos se puede concluir que PLSXM mostró un mayor efecto tóxico en la microalga estudiada en comparación con SMX puro. Esto es de gran relevancia ecológica, dado que la remediación de SMX con AOP puede afectar a los medios acuáticos de una manera más tóxica que el SMX puro, escenario agravado por el hecho de que las microalgas son los productores primarios fundamentales de estos medios. Por lo tanto, mayor investigación en la remediación de SMX es necesaria.

Palabras clave: Contaminante emergente (EC), citotoxicidad, concentración efectiva, procesos de oxidación avanzada (AOP), citometría de flujo (FCM), antibiótico, sulfametoxazol (SMX), especies reactivas de oxígeno (ROS)

Introduction

Recently, the application of synthetic chemical products has become crucial in our everyday life. However, the poor biodegradability of the components of these products is a relevant drawback for their use. An increasing interest has been growing over the treatment, degradation, remediation, and elimination of these compounds, known as emerging contaminants (EC) **[1]**. Under the term EC several compounds can be found, such as personal care products, pesticides, herbicides, nanoparticles, endocrine disruptors, disinfection by-products, organometallic compounds, and several toxins **[2]**. Given their widespread use in healthcare, agriculture, and sanitation, ECs and their metabolites are being found in a wide variety of ecosystems in concentrations that range from ppt to ppb **[3]**. Besides their purpose, ECs have been found to bioaccumulate in macroinvertebrates and other relevant aquatic organisms in the trophic web **[4]**. Despite the potential environmental and human health risk, most ECs are not being restricted under any of the current water quality programs and are not commonly supervised in the environment **[5]**. Thus, it is of primary concern the determination of their ecotoxicological effects and their precise concentrations in different media to upgrade the current environmental legislation **[6]**.

Pharmaceuticals are one of the most relevant groups among ECs due to their intense application and constant inputs to aquatic environments **[7]**. This is mainly due to the fact that these compounds are not efficiently applied or completely metabolized by the organism, with the excess being eliminated in excretions **[8]**. Many pharmaceuticals, like the antibiotics sulfamethoxazole, erythromycin, and clarithromycin, have been found at ecological risk concentrations in coastal lagoon water in Spain **[9]**. Given their ubiquity and their optimized design towards entering biological membranes, the chance of these compounds on producing negative effects on non-target aquatic organisms, like microalgae, is potential and concerning.

In this dissertation, the studies were focused on an antibiotic: sulfamethoxazole (SMX). Sulfamethoxazole is a sulfonamide bacteriostatic antibiotic applied for bacterial urinary infections, bronchitis, and prostatitis, commonly used in combination with trimethoprim. It is a structural analog of para-aminobenzoic acid (PABA). Thus, SMX competes with PABA to bind to dihydropteroate synthetase and inhibits the dihydrofolic acid synthesis, which interferes with folic acid synthesis in susceptible bacteria. Folic acid is an essential metabolite for DNA and aminoacid synthesis. Hence, SMX is considered a bacteriostatic antibiotic since it inhibits bacterial growth **[10]**. Excretion of SMX is primarily by the kidneys through both glomerular filtration and tubular secretion. The average concentration of total sulfonamide retrieved in urine from 0 to 72 hours after a single dose is up to 84,5%. From this, 30% is free sulfamethoxazole **[11]**. Although this compound is poorly soluble in water (<1 mg/mL at 25ºC), it has a very low vapor pressure (6.93×10^{-8} mmHg at 25ºC), making this compound not readily volatile. In fact, this compound has been recently proposed as a candidate substance for the third EU Watch List of emerging pollutants in aquatic environments **[12]**.

SMX is seen as a potential ecotoxicological threat, thus the optimization of methods that allow its rapid and efficient degradation should be researched and taken in consideration. One of the latest and most promising alternative treatment processes researched for the removal of ECs are Advanced Oxidative Processes (AOPs), term coined for the first time by William H. Glaze *et al.* in 1987 **[13]**. They are based on the use of powerful hydroxyl or sulfate radicals as major oxidizing agents. Although AOPs are not usually employed for the disinfection of wastewater, given that they have a very short half-life (close to

microseconds), these highly oxidizing agents can easily degrade recalcitrant organic pollutants and eliminate inorganic contaminants in wastewater. Thus, AOPs are mostly applied for the destruction of ECs and their transformation into less or non-harmful metabolites/products **[14]**. Actually, AOPs have been demonstrated to successfully eliminate an extensive variety of ECs, like trichloroethylene, several pesticides, and polyaromatic hydrocarbons (PAHs) through their mineralization **[15]**. Nevertheless, the most relevant obstacles that AOPs show on their application are their high cost and, more importantly, the undetermined toxicity of the photoproducts (PPs) obtained. Hydroxyl radicals are non-selective, creating many numerous PPs from the AOPs. It is crucial to understand the chemical nature of the PPs, given that these can be more harmful to the environment and humans' health than the pure compound **[16]**. However, most of the water quality programs currently applied in the EU are based on chemical analysis, which only provide information about concentration and nature of the compounds found in the masses of water analyzed **[5]**. Hence, these reports do not detect unidentified products nor degradation products like those obtained from AOPs. They can neither provide information about the potential toxicological damages that the presence of these compounds can induce in organisms in aquatic environments **[17]**.

The development of bioassays with model organisms has become a main scientific goal in toxicology, not only to study the toxicity of aquatic contaminants, but it can also be a powerful tool to stablish the efficiency of the degradation processes of these contaminants **[18]**. Hence, the water quality programs should be combined with bioassay analysis, which can provide relevant information about the effect of the exposure of these compounds to representative organisms of aquatic environments. One of the most widely used environmental quality indicators are microalgae, the main primary productors in aquatic environments. The well-being of these microorganisms is crucial for the environment since they are the foundation of the aquatic trophic net **[19]**. Based on this, microalgae have started to be widely applied as model organisms for toxicity bioassays of many compounds, particularly EC and EC degradation products **[7], [18], [20]**.

The most widely analyzed parameters in toxicity bioassays with microalga are the growth rate and the photosystem II's quantum yield **[21]**. Nevertheless, these two parameters are very limited upon explaining the biochemical interaction between the EC and the microalga as well as the physiological processes induced in the cell. Hence, research on fast-response biomarkers that allow the detection of alterations in the cell after a short exposure to the contaminant is needed. These parameters are usually related to structural changes, metabolic alterations, or even cell dynamics.

Among the different biochemical alterations that microalgae can undergo on contaminant exposure, the production of reactive oxygen species (ROS) is one of the most reported and studied one. ROS are usually produced in the cells as a response to the presence of different contaminants, leading to relevant toxic effects in the cells. When the cellular antioxidant system is unable to control the production of ROS, the reduction-oxidation (REDOX) equilibrium is destabilized, and oxidative stress is produced **[22]**. Research on ROS production as a potential toxic mechanism in EC is relevant since its excess can cause lipid peroxidation, DNA damage, and even attach to cell surfaces, thus disrupting the cell membrane and interacting with essential metabolic systems **[23], [24]**.

Also, membranes are one of the most sensitive parts of the cell, being fundamental for its correct functioning: transport of nutrients, cell signaling, etc. The study of any alteration at this subcellular level, like its potential, can be a good biomarker for the toxic effects by

contaminant exposure. Particularly, the analysis of mitochondrial membrane potential can be relevant since it is directly related to the functioning of this organelle, and therefore, related to alterations in the respiratory metabolism and energy production **[25], [26]**.

Finally, as a consequence of the toxic effects induced by exposure to contaminants, organisms can also see their vitality affected. This can be observed as alterations in their metabolic activity. Furthermore, exposure to contaminants can also compromise their viability **[27], [28]**.

In the present work, the green microalga *Raphidocelis subcapitata* was applied for the toxicity bioassays with the purpose of studying the potential toxicity of the antibiotic SMX and its photoproducts. Formerly known as *Selenastrum capricornutum*, this microalga has been previously and widely used in standardized toxicity bioassays. This freshwater microalga has been studied as a possible bioindicator for the toxicity of many substances, like metallic nanoparticles, organometallic compounds, antibiotics, graphene oxide, and most importantly, to photoproducts of ECs obtained through AOPs **[29], [30].**

Objectives

The main objective of this dissertation is to study the cytotoxicity of the antibiotic SMX on the microalga *Raphidocelis subcapitata*. The cytotoxicity of its photoproducts, obtained through AOP experiments with UV light, is also studied.

Thus, first, the effective concentration of 10% (EC_{10}) will be calculated through bioassays of the pure compound with *Raphidocelis subcapitata*. Then, photodegradation of solutions of the calculated concentration of EC_{10} of SMX will be carried out. Finally, these samples will be used for the correspondent bioassays. Therefore, culture growth, cell proliferation and viability, vitality, photosynthetic yield affectation, intracellular ROS production, and changes in the potential of both the cytoplasmatic and mitochondrial membranes were analyzed.

Materials and Methods

Raphidocelis subcapitata *Korshikov*: *description of the microalga species*

In this study, the microorganism used for toxicity bioassays was the microalga *Raphidocelis subcapitata* (***Figure 1***). The strain applied (CCAP 278/4) was obtained from the Culture Collection of Algae and Protozoa of Dunstaffnage Marine Laboratory (Scotland, UK). It is a green freshwater microalga, which belongs to the Chlorophyta Phylum, Chlorophyceae Class, Sphaeropleales Order and Selenastraceae Family **[31]**.

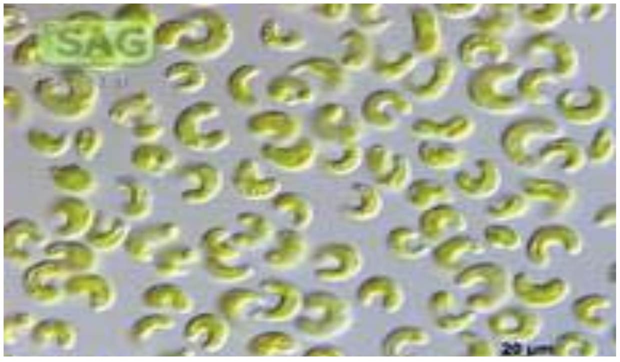

Figure 1.- Raphidocelis subcapitata *culture* ***[32]****.*

This microalga, formerly known as *Selenastrum capricornutum* and *Pseudokirchneriella subcapitata*, has a curved and twisted, sickle-like shape, which reminds of a crescent moon. The cells are usually presented in solitary form, though they can also form small colonies embedded in irregular and structureless mucilage. Its length ranges between 8 and 14 μm, while they are between 2 to 3 μm wide. This species reproduces asexually by autospores released by cell wall rupture **[33]**.

R. subcapitata is a microalga usually applied as a model organism for toxicological bioassays. In particular, this species is recommended for this kind of bioassays by the OECD, since they possess greater growth rates and sensitivity towards several substances than other algae species **[34]**.

Culture conditions

The cultures of this microalga were carried out in Goldmedium-Freshwater Species (GM-FWS) medium (AQUALGAE SL). The stock cultures were kept in 500 mL Pyrex bottles with 250 mL of culture and an initial cell density of $2x10^5$ cell/mL and placed in a culture chamber at 18 ± 1 ºC. The cultures were aerated at a constant flux of 10 L/min of atmospheric air through an insufflator and filtered through *Millipore FG* filters of 0.22 μm diameter pores prior its entrance into the cultures. The light applied, of 70 μmol of photon $m^{-2}s^{-1}$, is applied by fluorescent tubes (Philips TLD) of 36W. The 24h photoperiod applied was of 12h:12h light:darkness in order to keep the circadian cycle of the cells. All the material applied in the experiments and the culture medium were sterilized with autoclave prior its use at 120 ºC for 20 min.

Chemicals and experimental setup

The emerging contaminant tested was the antibiotic sulfamethoxazole (SMX; CAS No. 144930-01-8; 4-amino-N-(5-methyl-1,2-oxazol-3-yl)benzenesulfonamide). SMX (***Figure 2***), with molecular formula $C_{10}H_{11}N_3O_3S$ and molecular weight 253.28 g/mol, is a sulfonamide bacteriostatic antibiotic that inhibits the enzyme dihydropteroate synthase, preventing the formation of dihydropteroic acid. This acid is a precursor of folic acid, required for bacterial growth **[35]**.

For the bioassay tests, an analytical standard chemical of SMX obtained from Sigma-Aldrich was used, with purity greater than 95%. Before each experiment, concentrated stock solutions of this compound were freshly prepared by dissolving the pure compound in Milli-Q water with 24h stirring. Exposures were performed in triplicates in glass tubes filled with 40 mL of culture under the same culture conditions as stock cultures. Cells in mid-logarithmic growth phase were used as inoculum and initial cell density was adjusted to $2x10^5$ cells/mL. Cultures without SMX were always included as controls.

Figure 2.- *Chemical structure of sulfamethoxazole*

In order to detect the possible toxicity undergone into *R. subcapitata* by action of SMX and its photoproducts, several 3h and 48h bioassays were carried out.

This study was linked to a Chemistry project that proposes a state of the art method to eliminate the forementioned emerging contaminant from drinkable water. This method, as mentioned in the **Introduction** section, is based on AOPs, which use powerful oxidizing radical agents to degrade and/or eliminate compounds from solution. Although promising, the main drawback on the application of radicals is due to the undetermined toxicity of their photoproducts (PPs). Thus, the study here presented tries to elucidate whether the photoproducts obtained through photolysis of SMX are more or less toxic than the pure compound.

For this purpose, several toxicity assays of SMX on the growth of *R. subcapitata* were carried out in order to determine the concentration of antibiotic that induces a reduction of 10% on the growth of this microalga after a specific time of exposure (effective concentration of 10%, EC_{10}). The assayed concentrations of this study were 1, 2, 4, 6, and 8 ppm of SMX. After 48h of exposure, the cell density of each assay was determined, and the growth rate (μ) calculated, as later seen in **Cell density and proliferation analysis**. The value of EC_{10}, in base of the culture growth rates values obtained after 48h of exposure was calculated using the *CompuSyn* software, resulting in a value of 0.8 ppm of SMX (see the growth section in **Results & Discussion**).

Therefore, after calculating the EC_{10} of SMX, photolyzed SMX (PLSMX) solutions of this concentration were obtained. For the AOP applied, 250 mL solutions of SMX at the EC_{10} calculated were irradiated with UV light (254 nm) in an UV reactor. 200 mL samples were taken from this solution after 10 min of irradiation and used for the consequent bioassays. EC_{10} was chosen in order to fully photolyze the contaminant and assure that only photoproducts are present in the bioassays.

The photoproducts obtained from SMX photolysis (UV light, 254 nm) were characterized with High Performance Liquid Chromatography and Mass Spectrum (HPLC-MS) in *Laboratorio de Química Física 1 (Universidade da Coruña)*. These photoproducts are shown in ***Table 4*** from **Annex**. Through the HPLC chromatograms, it was proved that the PLSMX solutions contained no pure SMX in solution, meaning that SMX was completely degraded during its photolysis processing. No peak at a retention time close to 9.267 min (corresponding to SMX) was observed; hence it can be assumed that no SMX was present in those cultures incubated with PLSMX solutions, and that the effects seen on these cultures were solely due to PLSMX.

Contaminant Removal Analysis

Parallel assays to the forementioned in the **Chemicals and experimental setup** were performed in order to study the removal of the SMX from cultures by *R. subcapitata*.

For this, control cultures, cultures exposed to the EC_{10}, and cultures exposed to the photolyzed EC_{10} were prepared. In addition, solutions of the pure contaminant without microalga were also prepared and incubated in the culture chamber at the same conditions as the other assays. Samples of 1.5 mL from all the assays were taken at t=0h, 24h, and 48h. These samples were analyzed through HPLC, and their chromatograms were recorded. To avoid interferences during the HPLC measurements, those containing microalga were previously centrifuged (4000 rpm, 4 ºC, 5 min) and the supernatant filtered

with *Millipore* teflon filters of 0.2 mm pore size. The contaminant was monitored in the chromatograms by their retention time. In order to study whether the contaminants were being removed from culture medium, the areas of the correspondent samples without microalga were compared to those with microalga, assuming the difference to be the amount of compound removed by the cells. Hence, the removal was as percentage, calculated with the following equation:

$$Removal\ \% = \frac{Area\ of\ sample\ without\ microalga - Area\ of\ sample\ with\ microalga}{Area\ of\ sample\ with\ microalga} * 100$$

Flow Cytometry Analysis

Flow cytometry (FCM) is a powerful technique that allows the study of a great number of properties through fast multi-parametric analysis of single cells in solution. This technology uses lasers to produce scattered and fluorescent light signals, which are read by several detectors. The properties and functions of individual cells can be analyzed based of these fluorescent and light scattering characteristics. Several fluorescent compounds are used in flow cytometry for the determination of different cell biomarkers, like viability dyes, fluorescent expression proteins, etc. **[28]**.

For the FCM analysis, a *CytoFLEX* (Beckam Coulter) flow cytometer with blue laser (488 nm) was applied. The detectors available in this equipment are the forward scattered light (FSC) for cell size, side scattered light (SSC) for cell complexity, FL1 (505-545 nm, green fluorescence), FL2 (560-590 nm, yellow fluorescence), FL3 (605-635 nm, orange fluorescence), and FL4 (660-700 nm, red fluorescence). The detection is performed by avalanche photodiodes. An excitation laser at 488 nm was used as the light source in all assays, and at least 10,000 cells per sample were analyzed. The microalgal population was characterized in the cytometer by its size (FSC detector) and chlorophyll *a* content (red autofluorescence, FL4 detector). The analysis of the data obtained is carried out with *Kaluza Flow Cytometry Analysis V.1.1* software (Beckman Coulter Inc.)

For each analysis, aliquots of each culture were taken, and their cell density was adjusted to 1.5×10^5 cells/mL in phosphate buffer at pH 7.4. Each aliquot was incubated with the desired fluorochrome in darkness, following the pertinent steps later explained in ***Table 5*** from **Annex**, and several parameters (viability, vitality, intracellular ROS level, cytoplasmatic membrane potential, mitochondrial membrane potential) were measured at 3h and 48h, unless specified otherwise.

- Cell density and proliferation analysis

The cell density of aliquots of known volume of each assay was measured after 48h of SMX exposure by cell count in the flow cytometer. With the cell density count results, the growth rate was calculated with the following equation, where N_t is the cell density at time *t* and N_0 is cell density at t_0, expressed in days. Furthermore, both daughter and mother cell subpopulations are monitored based on their cell size and their chlorophyll content.

$$\mu = \frac{Ln\ N_t - Ln\ N_0}{Ln2(t - t_0)}$$

- Analysis of cell viability with Propidium Iodide (PI)

To study the viability of the *R. subcapitata* microalgae, propidium iodide (PI), a red-fluorescent nuclear and chromosome stain, was applied. PI is not permeant to live cells; thus, it is useful for dead cells counting in a specific population. PI binds to DNA by intercalating between the bases with virtual no preference. In aqueous solution, this dye has an excitation/emission maximum of 493/636 nm. Once the dye is binded to the DNA, its quantum yield is enhanced 20 to 30 times, and the fluorescence excitation maximum is shifted 30-40 nm to the red, while the fluorescence emission maximum is shifted 15 nm to the blue. Thus, the resulting fluorescence emission maxima is 617 nm, analyzed through FCM by the FL3 detector (605-635 nm) **[28].**

- Analysis of vitality with Fluorescein Diacetate (FDA)

To study the vitality of *R. subcapitata*, fluorescein diacetate (FDA) was applied. This compound is a lipophilic non-fluorescent esterase substrate that can serve as a vitality probe that measures both enzymatic activity, required to activate its fluorescence, and cell-membrane integrity, required for intracellular retention of their fluorescent product. Once FDA enters the cell, its acetate residues are eliminated by non-specific esterases, yielding fluorescein, a hydrophilic fluorescent product, proportionally to this enzyme's activity. Fluorescein, due to its polarity, is retained in the plasma membrane. Given the increase of fluorescein inside the cell due to enzymatic activity, fluorescence will increase over time depending on the metabolic activity of the cells, and thus, on its vitality. Thus, this increase of fluorescence indicates the well-being of the plasmatic membrane and/or the correct esterase activity of the cells. In the present study, since FDA hydrolyzation and fluorescein release is recorded (see **Results & Discussion**), it is possible to monitor those vital, metabolically active cells as FDA +. Therefore, the greater the fluorescence, the greater the metabolic activity and the cell vitality. The assays were analyzed through FCM by the FL1 detector (505-545 nm) **[28]**.

- Analysis of the alterations in the membrane potential
  - *Cytoplasmatic membrane potential with DiBAC$_4$(3)*

To study the depolarization of the cytoplasmatic membrane of *R. subcapitata* after its exposure to the contaminants, DiBAC$_4$(3) was applied. This indicator, also known as Bis-(1,3-Dibarbituric acid)-trimethine oxanol, is a slow-response, potential-sensitive anionic dye that can enter depolarized cells. Here, it binds to intracellular proteins or membrane lipids, and as a consequence, its fluorescence is enhanced, and its spectra shifts towards green. DiBAC$_4$(3) has an excitation maximum of 493 nm and an emission maximum of 516 nm, which is analyzed through FCM by the FL1 detector (505-545 nm). The entrance of this dye inside the cell is favored with increasing depolarization of the cell membrane, followed by an increase in the fluorescence. Then, as seen with FDA, the greater the fluorescence of DiBAC$_4$(3)-incubated cells, the greater the depolarization. On the contrary, the hyperpolarization of the membrane is seen as a decrease in fluorescence since the cytoplasm turns more electronegative than the external media. The anionic nature of this dye prevents it from entering the mitochondria given its overall negative charge **[28].**

  - *Mitochondrial membrane potential with JC-1*

To study the depolarization the mitochondrial membrane of *R. subcapitata* after its exposure to the contaminants, JC-1 was applied. This dye, based on cationic carbocyanine, has potential-dependent accumulation in the mitochondria. In normal conditions, this dye

enters the mitochondria in greater concentrations, forming clusters (called J aggregates) that have an emission maximum of 590 nm (orange). With a depolarized mitochondria membrane, JC-1 shows low concentrations inside the mitochondria and is present as a monomer that produces a green fluorescence (emission maximum of 525 nm). Having two emission maxima depending on the aggregation state of the dye, these fluorescence phenomena are monitored through the FL1 detector (505-545 nm) and the FL3 detector (605-635 nm). Hence, mitochondrial depolarization is indicated by a decrease in the orange/green fluorescence intensity ratio **[28].**

- Analysis of intracellular ROS levels with dihydrorhodamine 123 (DHR123)

To study the intracellular ROS levels undergone by the contaminants onto *R. subcapitata*, the dye dihydrorhodamine 123 (DHR123) was applied. This fluorochrome is an uncharged and nonfluorescent ROS indicator that can cross the plasmatic membrane. Its oxidation to the cationic form occurs inside the cell and localizes in the mitochondria and exhibits green fluorescence (536 nm). This fluorescence emission is analyzed through FCM by the FL1 detector (505-545 nm) **[28].**

Analysis of the photosynthetic yield with Pulse Amplitude Modulation (PAM)

The change in photosynthetic activity of *R. subcapitata* after 3h and 48h of contaminants exposure was monitored through fluorimetry with Pulse Amplitude Modulation (PAM), using an AquaPen-C-AP-C100 fluorimeter as a measure of the efficiency of photosystem II (PSII). This device measures the fluorescence of microalgal and bacterial pigments. It is equipped with a cuvette reader, a blue LED light source (455 nm) for chlorophyl excitation, and a red LED light source (620 nm) for phycobilin excitation.

For this monitoring, 3 mL samples of each assay were kept in darkness for about 30 min in order to allow the opening of all the reaction centers of PSII. Next, the samples are placed in the cuvette and into the device. The samples are illuminated with the LED lights, and the photosystems start to catch the light through the antenna complexes of the plastid chlorophyll. The photosystems, when working at optimum levels, do not absorb all the receiving light, which is reemitted as fluorescence. This fluorescence is detected, measured, and recalculated as quantum yield (QY) by the device. A great value of QY indicates a low value of fluorescence under saturation of light, and thus, a great photosynthetic yield. Hence, microalgae in optimum conditions show great values of QY, while those stressed cells show lower QY values. The results are expressed as the QY values obtained for each assay.

Statistical analysis of the data

Results were expressed as percentage of each measured parameter compared to the values obtained for the control assays. Then, mean and standard deviation (SD) values of the three replicates were determined for each treatment and for control cultures. These data were statistically analyzed using SPSS Statistics software (version 26.0, SPSS, IBM). For each experiment, the hypothesis that the concentration of the tested compound does not affect the parameter was statistically analyzed using a one-way analysis of variance (ANOVA). When the hypothesis was rejected, Tukey´s post hoc test was used to analyze how each concentration of SMX affects the parameter studied and to group the different treatments, considering a significance level of 0.05 ($p<0.05$).

Graphs were created with the advanced graphics package SigmaPlot version 14.0.

Results & Discussion

SMX and photolyzed SMX (PLSMX) exposure inhibits growth in R. subcapitata

The results obtained from this study indicate that the cell growth of *R. subcapitata* is significantly affected ($p<0.05$) by the presence of SMX in culture medium (***Table 1***) at all assayed concentrations of the contaminant and 48h of exposure.

Table 1.- *R. subcapitata* growth rates after 48h of incubation to 0 (control), 1, 2, 4, 6, and 8 ppm of SMX.

Sample (ppm)	Growth rate (day^{-1}) at 48h
Control	0.711 ± 0.027^{a}
1	0.156 ± 0.017^{bc}
2	0.273 ± 0.023^{b}
4	0.144 ± 0.015^{bc}
6	0.074 ± 0.022^{c}
8	0.048 ± 0.034^{c}

The different letters (a, b, c) indicate significative differences ($p<0.05$) among the treatments according to the Tukey *post hoc* test. Letter a indicates no significant differences between treatment and control.

The growth rates data shows a notable inhibition with increasing concentration of SMX. 8 ppm-exposed cultures showed the lowest growth rate after 48h of treatment, with 0.048 ± 0.034 day^{-1}. These values drift away from the control growth rate of 0.711 ± 0.027 day^{-1} after 48h of incubation. Similar decrease in growth rates is also observed in Eguchi *et al.,* 2004 **[36]**. Their results showed that, for 1.5 ppm of SMX on *R. subcapitata*, the growth rate inhibition, expressed as % of the control after 48h of incubation, was *ca*. 30%, which are comparable to the results seen in this study.

This phenomenon was also observed in other species of microalgae, like *Scenedesmus obliquus, Chlorella vulgaris*, or *Synechococcus leopolensis*, where a decrease in the growth rates at 1 ppm of SMX and greater concentrations was also reported. Other sulfonamide antibiotics, like sulfamethazine, also produce the same concentration-depending effect on *Scenedesmus obliquus* **[37]**, matching the results observed in the present study.

Moreover, the photolysis of SMX solutions at the EC_{10} value previously determined was carried out. For that, 0.8 ppm solutions of SMX were radiated with UV light (254 nm), as described in the **Materials and Methods** section.

The results obtained from the toxicity bioassays of PLSMX on *R. subcapitata* (***Table 2***) demonstrated that the photoproducts obtained from the photolysis of SMX affect significantly ($p<0.05$), arresting the growth of the microalga.

Table 2.- Growth rate of *R. subcapitata* in presence of 0 ppm (control) and 0.8 ppm of SMX (0.8), and 0.8 ppm of PLSMX (PL 0.8) after 48h of incubation.

Sample (ppm)	Growth rate (day^{-1}) at 48h
Control	1.077 ± 0.063^{a}
0.8	0.966 ± 0.014^{a}
PL 0.8	0.000 ± 0.092^{b}

The different letters (a, b) indicate significative differences ($p<0.05$) among the treatments according to the Tukey *post hoc* test. Letter a indicates no significant differences between treatment and control.

From the inhibition growth rate values, obtained after 48h of exposure with respect to control ones, 0.8 ppm of PLSMX (100.5%) in comparation to that value obtained for 0.8 ppm of SMX (10.3%), the photoproducts of SMX affect the growth of *R. subcapitata* in a more severe manner than the pure compound.

Similar results have been observed for other organisms, like the bacteria *Vibrio fischeri* and the crustacean *Daphnia magna*, which suffer an inhibition on their growth of 49% and 100%, respectively, after an exposure of 48h to PLSMX **[20]**. Therefore, it can be assumed that the photoproducts of PLSMX produce a more acute effect on the growth of different organisms compared to pure SMX.

Of course, the toxicity of the PLSMX photoproducts rely directly on the biochemical nature of the products themselves, and consequently, on the main compound itself. For example, the photolysis photoproducts of triclosan, a broad-spectrum antimicrobial, increased its EC_{50} level by 11% and its EC_{80} by 29% on *R. subcapitata* after UV photodegradation **[29]**. Something similar was reported with glyphosate, an active herbicide ingredient, which increases its EC_{50} level by *ca*. 269% on the same microalgae after UV-C photodegradation **[30]**. Thus, in these two studies, the toxicity of the photoproducts was reduced compared to the pure contaminants, in contrast with the increased toxicity observed in this study for PLSMX respect pure SMX.

Removal of SMX from culture medium by R. subcapitata *is effective*

Since the effect of exposure to 0.8 ppm of both SMX and PLSMX on the growth of *R. subcapitata* was observed on the previous section, the potential removal of the pure contaminant from culture medium by the microalga was tested. For that, samples of each assay were analyzed by HPLC, as defined in the **Contaminant Removal Analysis** section.

The results from the removal of SMX, shown in ***Table 3***, demonstrated that SMX was successfully being removed from culture medium by *R. subcapitata*.

Table 3.- Removal percentage of SMX by *R. subcapitata* after the intervals of 0 to 24h and 24h to 48h of exposure

Sample (ppm)	Removal (%) from 0 to 24h	Removal (%) from 24 to 48h
0.8	26.4 ± 5.6	8.5 ± 10.8

Furthermore, the results prove that during the first 24h of incubation, *R. subcapitata* assimilates more SMX from solution than the following 24h. This removal phenomenon was also seen in other microalgae (*Scenedesmus obliquus*) which is able to remove the 29% of a 0.2 ppm SMX solution after 11 days of exposure **[37]**. This can be compared to the total of *ca*. 35% removal from the 0.8 ppm SMX solution after 48h of exposure on the present study.

The removal of SMX, along with trimethoprim, and triclosan, was also studied with the green algae *Nannochloropsis* sp. 100% and 68% of the initial concentrations of both trimethoprim and sulfamethoxazole remained in solution after 14d of incubation, being also not detected in the microalgal cells. In the case of triclosan, only 28% of the initial concentration was detected immediately after the incubation began **[38]**. These results further prove that the removal effect of a given contaminant depends on the nature of both the contaminant and the organism assayed.

PLSMX exposure decreases the cell proliferation and the viability in R. subcapitata

It was observed in previous sections that both SMX and PLSMX present an inhibiting effect on the growth rates of *R. subcapitata*. In order to further characterize this effect, cell proliferation of *R. subcapitata* after exposure to 0.8 ppm of both SMX and PLSMX was studied. ***Figure 3*** displays the % of mother and daughter cells after 3h and 48h of incubation in the different treatments. The values obtained for the cultures exposed to pure SMX did not show significant differences respect those obtained for the control assay ($p<0.05$), keeping similar % of mother and daughter cells. On the contrary, PLSMX affects the microalgal proliferation significantly ($p<0.05$), inducing an increase in the subpopulation of mother cells (94.6 ± 0.2 %) and a decrease in daughter cells (5.9 ± 0.2%) just after 3h of incubation, which is also seen after 48h of incubation.

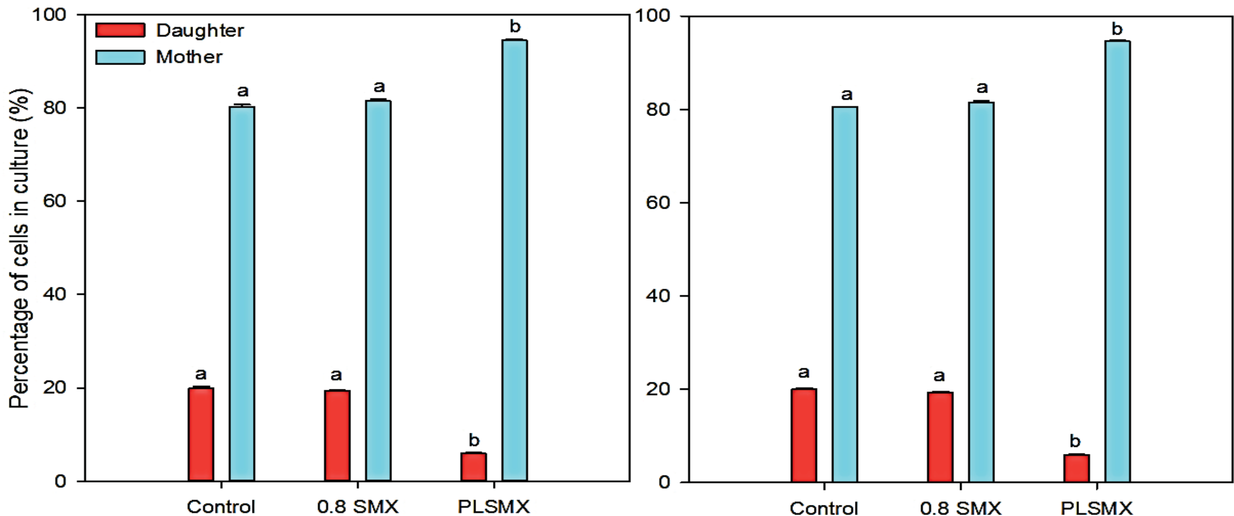

Figure 3.- *Percentage of daughter and mother cells of the control, 0.8 SMX, and 0.8 ppm PLSMX assays after 3h (left) and 48h (right) of exposure. The different letters (a, b) indicate significative differences (p<0.05) among the treatments according to the Tukey* post hoc *test. Letter a indicates no significant differences between treatment and control.*

Furthermore, in order to further understand the effect of this compounds on the microalga studied, several biochemical and physiological biomarkers were carried out. One of them is cell viability, determined through PI staining by FCM. This compound is membrane impermeant and generally excluded from viable cells. Thus, PI is usually applied to identify non-viable cells in a population **[28]**.

The results displayed in ***Figure 4*** showed that SMX at the calculated EC_{10} had no significative effect ($p<0.05$) on the viability of *R. subcapitata* at 3h nor 48h of exposure compared to the control results. The retained cytoplasmatic membrane integrity of *R. subcapitata* after exposure to other emerging contaminants similar to SMX, like erythromycin, has been previously demonstrated. The viability of this microalga after 72h exposure to 0.2 ppm of erythromycin has been demonstrated to be >99%, meaning that virtually all cells kept the integrity of their cytoplasmatic membrane **[39]**.

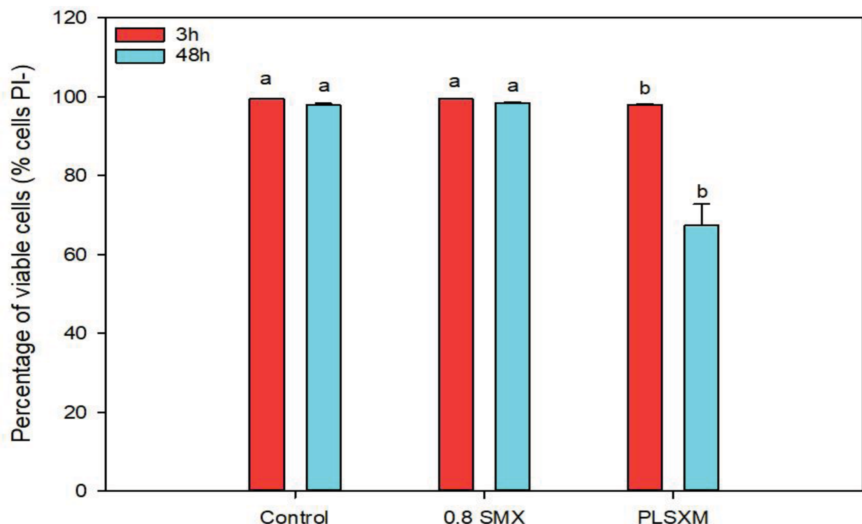

Figure 4.- *Percentage of viable cells (% PI-) in the control, 0.8 ppm SMX, and 0.8 ppm PLSMX assays after 3h and 48h of exposure. The different letters (a, b) indicate significative differences (p<0.05) among the treatments according to the Tukey* post hoc *test. Letter a indicates no significant differences between treatment and control.*

Nevertheless, PLSMX shows a significant effect ($p<0.05$) on the viability of the microalga after only 3h of exposure, showing the greatest effect after 48h of incubation with a viability of 67.3 ± 5.5 %. Thus, the PLSMX photoproducts induce a notable decrease in the viability of *R. subcapitata.*

PLSMX exposure decreases the vitality and QY in R. subcapitata

The study of different enzymatic activities in microalgae are usually applied in toxicity bioassays given that they are very fast and sensitive response biomarkers **[40]**. In this study, the unspecific esterase enzymatic activity was monitored as a general metabolic activity biomarker in *R. subcapitata* since this parameter has demonstrated being able to detect cytotoxicity effects in other microalgal species only few hours after exposure to several contaminants **[41]**. In this study, a gradient upon this activity was observed. Thus, microalgal populations were divided in three different subpopulations: cells with low vitality, intermedium vitality, and high vitality.

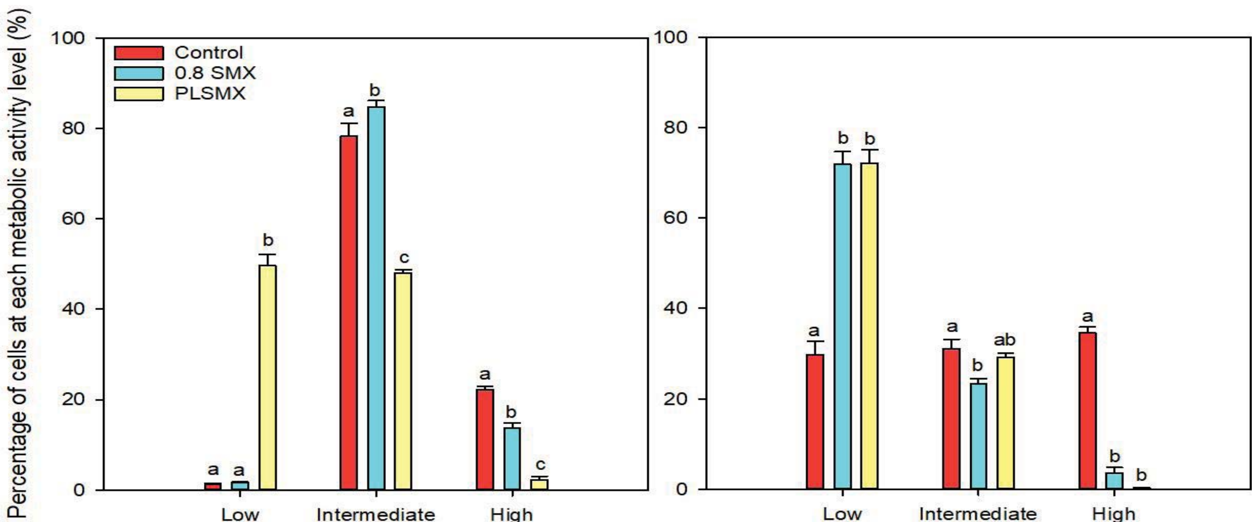

Figure 5.- *Percentage of cells with low, intermediate, and high metabolic activity of the control, 0.8 SMX, and 0.8 ppm PLSMX assays. Exposure time: 3h (left) and 48h (right). The different letters (a, b, c) indicate significative differences (p<0.05) among the treatments according to the Tukey* post hoc *test. Letter a indicates no significant differences between treatment and control.*

The results displayed in ***Figure 5*** demonstrated that both SMX and PLSMX affect the vitality of *R. subcapitata*. PLSMX affected significantly ($p<0.05$) the vitality of the microalga after just 3h of exposure, observed as a significative increase in the low activity cell population and a decrease in the intermediate activity subpopulation compared to the control assays. On the other side, SMX showed a significant effect ($p<0.05$) on the vitality of the microalga after 48h of exposure in a similar manner compared to PLSMX, observed as a significant increase in the low activity subpopulation at the expense of the decrease of the high activity subpopulation.

In previous studies, the negative effect of SMX on the vitality of other microalga, like *Microcystis aeruginosa*, was demonstrated, presenting a vitality loss of 50% after an exposure of 4d to 0.125 ppm of SMX **[42]**. Other studies also researched on the toxicity effect of other biocides, like atrazine, pentachlorophenol, and malathion on *R. subcapitata*, observing affectation of the cells' vitality **[43]**.

The results observed for the metabolic activity of *R. subcapitata* after its exposure to pure SMX and PLSMX can be related to the effect of these contaminants on the photosynthesis activity of the microalga. Several studies have already proved the elevated sensitivity of PSII against different stressful conditions, like exposure to contaminants **[44]**, high temperatures **[45]**, or high radiation **[45]**.

In order to measure the photosynthetic yield of the PSII, cultures exposed to 0.8 ppm SMX and 0.8 ppm PLSMX after 3h and 48h were analyzed through PAM. ***Figure 6*** displays the data obtained as the total QY values obtained from each assay after 3h and 48h of exposure.

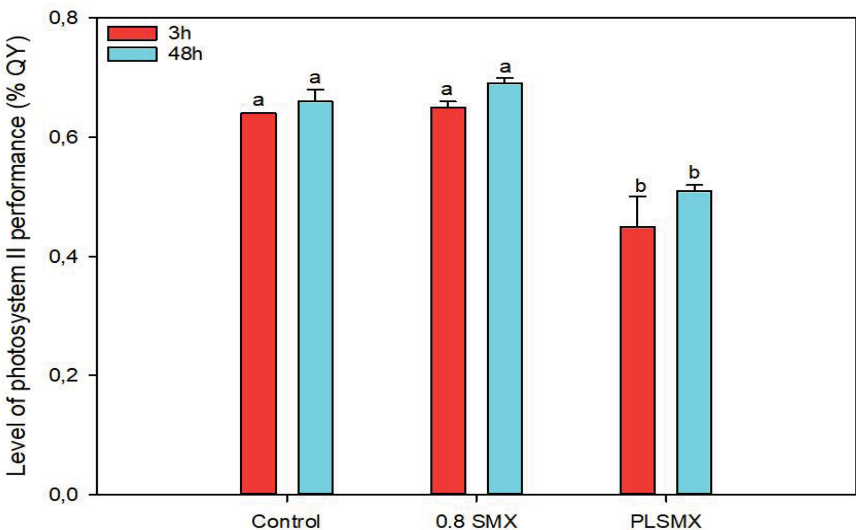

Figure 6.- *Level of photosystem II performance (% QY) of the control, 0.8 SMX, and PLSXM assays after 3h and 48h of incubation. The different letters (a, b) indicate significative differences (p<0.05) among the treatments according to the Tukey* post hoc *test. Letter a indicates no significant differences between treatment and control.*

While exposure to SMX does not induces a significant variation (p<0.05) on QY of *R. subcapitata* cells compared to the control values, exposure to PLSMX provoked an acute effect (p<0.05) on the photosynthetic yield of *R. subcapitata*, decreasing it by *ca.* 30% after 3h of incubation and *ca.* 22% after 48h. Interestingly, the decrease in metabolic activity for PLSMX observed as an increase in the microalgae population with a low vitality level (***Figure 5***) and this QY decrease reported here, both after 48h, could be in closed connection.

It has been previously observed that *R. subcapitata* exhibits a decrease in QY values when exposed to >1 ppm of SMX, and other antibiotics like erythromycin and ciprofloxacin. These compounds could inhibit many photosynthetic-related processes in a concentration-dependent manner **[47]**. The combinate effect of fipronil and 2,4-D mixtures on *R. subcapitata* has also been researched, showing that these mixtures, found in natural ecosystems, induce changes in photosynthetic activities **[48]**. Furthermore, the work of Esperanza *et al.,* 2017 (**[49]**) reported the inhibition in photosynthetic activity of atrazine on *Chlamydomonas reinhardtii.* Thus, as observed before, the inhibition of photosynthesis by many different contaminants can interrupt the fixation of CO_2 from atmosphere by microalgae. Taking in mind that microalgae are the major CO_2 fixators on the planet, many serious consequences on climate change can be expected.

PLSMX exposure induces the formation of ROS in R. subcapitata

Many recent studies have demonstrated that the production of intracellular ROS, in the form of O^{2-}, OH^{-}, and H_2O_2, is a very sensitive parameter, relevant to be analyzed in toxicity bioassays **[50], [51], [52]**. In this study, DHR123 was applied in order to infer the formation of ROS in the different assays carried out. The results in ***Figure 7*** are shown as total percentage of DHR+ cells, i.e., total percentage of cells with great intracellular ROS concentration. Low percentages of cells with high values of intracellular ROS in the microalgae are seen after exposure to pure SMX, registered as slightly lower values of cell population with high values of intercellular ROS compared to those of the control after 3h of exposure, reaching similar levels to the control after 48h, and displaying no significant effect (p<0.05). On the other side, PLSMX exposure significantly induces (p<0.05) the formation

of ROS in *R. subcapitata* after 3h of exposure. The levels of cell population with high values of intercellular ROS increase after 48h of exposure, up to 24.9 ± 1.9%.

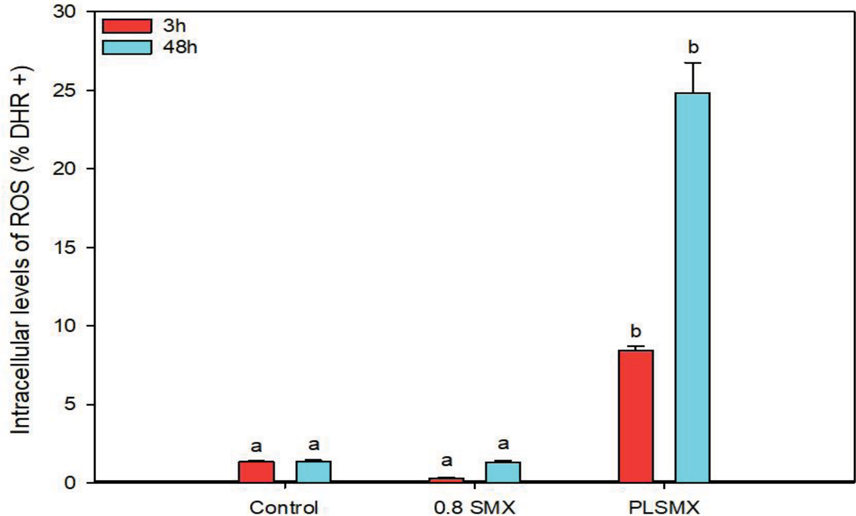

Figure 7.- *Intracellular levels of ROS (% DHR +) of the control, 0.8 SMX, and PLSMX assays after 3h and 48h of exposure. The different letters (a, b) indicate significative differences (p<0.05) among the treatments according to the Tukey* post hoc *test. Letter a indicates no significant differences between treatment and control.*

The sensitivity of *R. subcapitata* towards production of ROS has been previously studied with other antibiotics, like erythromycin, which induces a 50% increase of ROS in this microalga **[51]**. Other compounds, like graphene oxide, also induce ROS production in *R. subcapitata*, provoking oxidative damage and disruption of the cell membrane by lipid peroxidation **[52]**. However, the low levels of ROS induced by SMX match other studies on the antioxidant system of *R. subcapitata* under exposure to SMX, like the study of Nie et al., 2013 (**[53]**). In this study, the results indicate that the toxicity of SMX on the photosynthetic apparatus is reduced by the xanthophyll cycle and GST activity, thanks to their mechanism of antioxidation action, which also matches the results obtained from the QY of the microalgae after exposure to SMX (***Figure 6***). As in the present study, Nie *et al*. concluded that the chronic toxicity could not be neglected, and further investigations are needed.

Furthermore, it has been previously studied that many antibiotics are photosynthesis inhibitors, since they block the electron transport chain in PSII (**[54]**), and hence induce the formation of ROS from O_2 **[55]**. Thus, when the intracellular ROS levels start to increase, several antioxidant mechanisms act in order to regulate and/or remove the ROS.

PLSMX exposure provokes a depolarizing effect on both the cytoplasmatic and the mitochondrial membrane

As in the case of the vitality FCM analysis, the cytoplasmatic membrane potential also demonstrated a gradation, displaying polarized, depolarized, and greatly depolarized cell populations.

The results obtained from the SMX and PLSMX assays are displayed in ***Figure 8***. The control cultures showed great values of polarized cells (88.9 ± 2.1%) and low values of depolarized cells (9.6 ± 1.3%) after 3h, which do not vary after 48h. Both the SMX and PLSMX showed significant similar values compared to the values observed for the control assay (p<0.05) of depolarized and polarized cells after 3h of exposure. Regarding to the

greatly depolarized subpopulation, only those cells exposed to SMX and PLSMX presented greatly depolarized cells after 3h of incubation, though with low values (3.3 ± 0.2% and 1.5 ± 0.1%, respectively). After 48h of exposure, cells exposed to SXM showed significant similar values (p<0.05) of depolarization compared to the control assays. However, PLSMX showed a significant decrease (p<0.05) in polarized cells (down to 63.0 ± 5.9%) and a consequent increase in the depolarized population (up to 31.1 ± 2.6%) and the greatly depolarized cell population (up to 3.9 ± 1.0%). In conclusion, while exposure to SMX does not seem to induce the depolarization of the cytoplasmatic membrane on *R. subcapitata* for its similar behavior to the control culture, PLSMX does seem to induce the depolarization on the cytoplasmic membrane of microalgae after 48h of exposure.

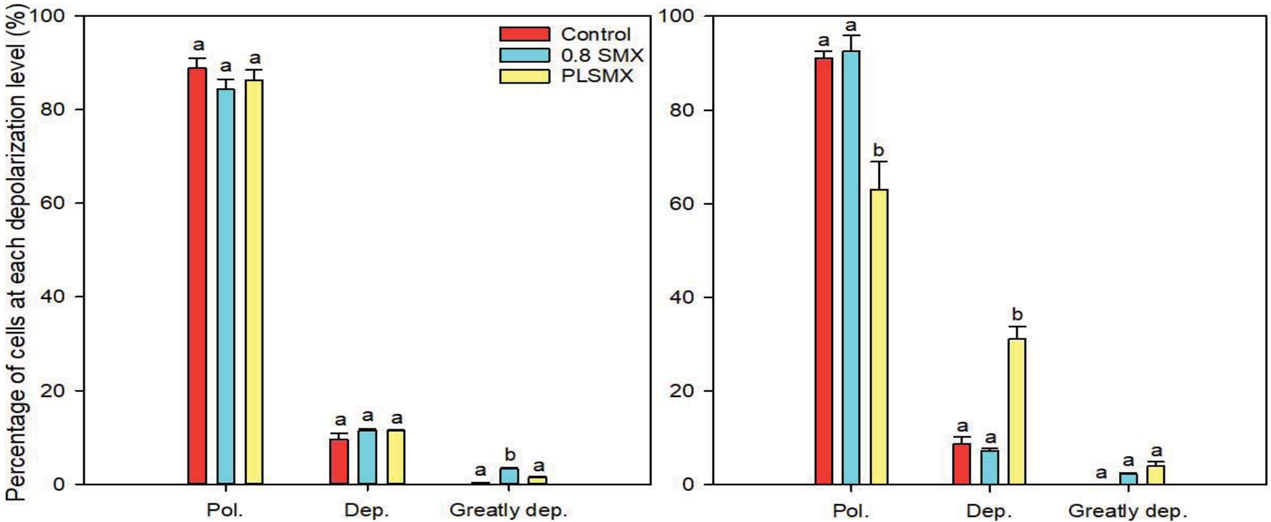

Figure 8.- *Percentage of cells at each depolarization level (polarization, depolarization, great depolarization) of the control, 0.8 SMX, and PLSMX assays after 3h and 48h of exposure. The different letters (a, b) indicate significative differences (p<0.05) among the treatments according to the Tukey* post hoc *test. Letter a indicates no significant differences between treatment and control.*

On the other side, the depolarization of the mitochondrial membrane was carried out with the JC-1 dye. Results are shown in ***Figure 9*** as JC-1 – %, displaying the subpopulation of cells with depolarized mitochondrial membrane at each assayed condition. After the analysis of these results, a significant (p<0.05) increased percentage of cells with depolarized mitochondrial membrane is observed after just 3h of exposure to PLSMX compared to the control sample, with a value of 97.1 ± 1.0 %. This value is kept after 48h of exposure (97.6 ± 1.3%). In the case of SMX, this compound showed values significantly lower (p<0.05) than those from the control samples after 3h, but significantly similar (p<0.05) to the control after 48h of exposure.

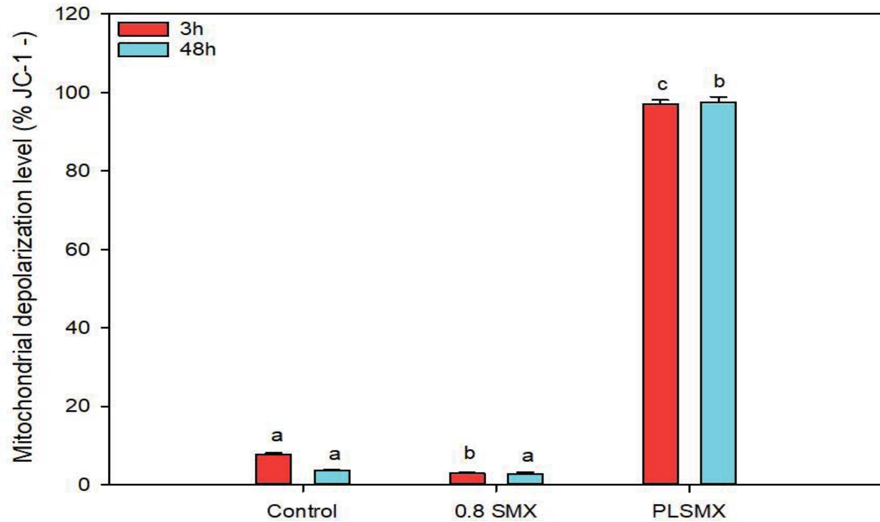

Figure 9.- *Depolarization of the mitochondrial membrane (% JC-1 -) of the control, 0.8 SMX, and PLSMX assays after 3h and 48h of exposure. The different letters (a, b, c) indicate significative differences (p<0.05) among the treatments according to the Tukey* post hoc *test. Letter a indicates no significant differences between treatment and control.*

The increase in ROS levels (***Figure 7***) could be associated with the increase of cells with cytoplasmic and mitochondrial depolarized membranes (***Figure 8*** and ***Figure 9***). Those cells exposed to PLSMX suffer the highest depolarization of their cytoplasmic and mitochondrial membranes, while also experiencing the greatest levels of intracellular ROS (***Figure 7***). Thus, the effects observed of PLSMX on ROS levels, cell membranes potentials, and, finally, loss of vitality and cell death could be related.

Conclusions

The concentration of SMX that induces a growth inhibition of the 10% (EC_{10}) in *Raphidocelis subcapitata* was calculated as 0.8 ppm. At this concentration, SMX significantly induces low metabolic activity in the studied microalga, being this the only parameter affected.

In the case of the photoproducts of SMX obtained after its UV photolysis at its EC_{10} concentration (PLSMX), these induce total cell growth inhibition, decrease of cell proliferation, loss of cell viability, higher levels of cells with low vitality, decrease on photosystem II`s quantum yield, increased levels of intercellular ROS production, and greater values of cells with depolarized cytoplasmic and mitochondrial membranes in *R. subcapitata*.

Among the different parameters studied, vitality, oxidative stress, and mitochondrial membrane potential show the greatest affection in *R. subcapitata* after exposure to PLSMX, proving that PLSMX has a greater toxic effect on *R. subcapitata* than pure SMX.

Conclusións

A concentración de SMX que induce unha redución no crecemento do 10% (EC_{10}) en *Raphidocelis subcapitata* foi calculada como 0.8 ppm. Nesta concentración, SMX induce de maneira significativa valores de actividade metabólica baixos na microalga estudada, sendo este o único parámetro afectado.

No caso dos fotoprodutos de SMX obtidos trala súa fotólise UV na súa concentración de EC_{10} (PLSMX), estes inducen a inhibición total de crecemento celular, a redución na porcentaxe de células fillas e o aumento da porcentaxe de células nai, diminución na viabilidade celular, valores maiores de células con actividade metabólica baixa, o descenso no rendemento cuántico do fotosistema II, o aumento dos valores de produción de ROS intracelular, e valores maiores de células con membranas citoplasmáticas e mitocondriais despolarizadas en *R. subcapitata*.

De entre os diferentes parámetros estudados, a viabilidade, o estrés oxidativo, e o potencial de membrana mitocondrial mostran os maiores valores de afectación en *R. subcapitata* despois da súa exposición a PLSMX, demostrando así que PLSMX ten un efecto tóxico maior sobre *R. subcapitata* que o SMX puro.

Conclusiones

La concentración de SMX que induce una reducción en el crecimiento del 10% (EC_{10}) en *Raphidocelis subcapitata* fue calculada como 0.8 ppm. A esta concentración, SMX induce de manera significativa valores de actividad metabólica bajos en la microalga estudiada, siendo este el único parámetro afectado.

En el caso de los fotoproductos de SMX obtenidos tras su fotólisis UV en su concentración de EC_{10} (PLSMX), estos inducen la inhibición total del crecimiento celular, la reducción en el porcentaje de células hijas y el aumento del porcentaje de células madre, disminución en la viabilidad celular, valores mayores de células con actividad metabólica baja, el descenso en el rendimiento cuántico del fotosistema II, el aumento de los valores de producción de ROS intracelular, y valores mayores de células con membranas citoplasmáticas y mitocondriales despolarizadas en *R. subcapitata*.

De entre los diferentes parámetros estudiados, la viabilidad, el estrés oxidativo, y el potencial de membrana mitocondrial muestran los mayores valores de afectación en *R. subcapitata* después de su exposición a PLSMX, demostrando así que PLSMX tiene un efecto tóxico mayor sobre *R. subcapitata* que el SMX puro.

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Annex

Table 4.- Structures, molecular formula, and molecular weight of SMX

	Molecular structure	Molecular formula	Molecular weight (g/mol)
1		$C_{10}H_{11}N_3O_3S$	253.28
2		$C_6H_7NO_2S$	157.19
3		$C_4H_6N_2O$	98.11
4		$C_6H_7NO_4S$	189.19
5		$C_7H_9N_3O_3S$	215.23
6		$C_{10}H_{13}N_3O_5S$	287.29
7		$C_4H_8N_2O_3$	132.12
8		$C_6H_8N_2O_2S$	172.20

(1), 4-hydrosulfonylaniline (2), 5-methylisoxazol-3-amine (3), 4-aminobenzenesulfonic acid (4), 4-amino-N-carbamoylbenzenesulfonamide (5), 4-amino-N-(4,5-dihydroxy-5-methyl-4,5-dihydroisoxazol

Table 5.- Applications and mode of action of the different fluorochromes applied in FCM, with their specific FCM detector, final concentration and incubation time (min) in darkness

Fluorochrome	Applications	Expression of results	FCM detector	Final concentration	Incubation time (min)	References
PI	Viability	The results are presented as viable cells (PI-) respect the total analyzed.	FL3	5 μg mL^{-1}	10	**[28], this work**
FDA	Esterase activity	The results are presented as the percentage of metabolic active cells (FDA+) respect the total analyzed.	FL1	0.25 μg mL^{-1}	15	**[28], this work**
$DiBAC_4(3)$	Cytoplasmic membrane potential	Results were expressed as the percentage of depolarized cells ($DiBAC_4(3)$ +) respect the total analyzed.	FL1	2.5 μg mL^{-1}	15	**[28], this work**
DHR 123	Intracellular levels of superoxide anion	Results were expressed as the percentage of cells with high intracellular levels of superoxide anion (DHR +) respect the total analyzed.	FL1	10 μg mL^{-1}	40	**[28], this work**
JC-1	Mitochondrial membrane potential	Results were expressed as the percentage of depolarized cells with (JC-1-) respect the total analyzed.	FL3/FL1	1 μg mL^{-1}	15	**[28], this work**