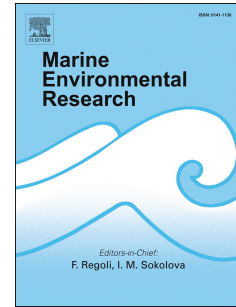


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Marta Seoane, Carmen Rioboo, Concepción Herrero, Ángeles Cid



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Toxicity induced by three antibiotics commonly used in aquaculture on the marine microalga *Tetraselmis suecica* (Kylin) Butch.

Marta Seoane, Carmen Rioboo, Concepción Herrero, Ángeles Cid *

Laboratorio de Microbiología
Facultad de Ciencias
Universidad de A Coruña
Campus de A Zapateira s/n
15071 A Coruña, SPAIN

* Corresponding author

Tel.: +34 981 167 000. E-mail address: angeles.cid@udc.es (Á. Cid).

Abstract

Aquaculture facilities are a potential source of antibiotics to the aquatic ecosystems. The presence of these compounds in the environment may have deleterious effects on non-target aquatic organisms such as microalgae, which are often used as biological indicators of pollution. Therefore, the toxicity induced by chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC), three antibiotics widely used in aquaculture, on the marine microalga *Tetraselmis suecica* was evaluated. Growth inhibition and physiological and biochemical parameters were analysed. All three antibiotics inhibited growth of *T. suecica* with 96 h IC₅₀ values of 11.16, 9.03 and 17.25 mg L⁻¹ for CHL, FLO and OTC, respectively. After 24 hours of exposure no effects on growth were observed and cell viability was also unaffected, whereas a decrease in esterase activity, related with cell vitality, was observed at the higher concentrations assayed. Photosynthesis related parameters such as chlorophyll *a* cellular content and autofluorescence were also altered after 24 hours of antibiotics addition. It can be concluded that *T. suecica* was sensitive to the three antibiotics tested.

Keywords: antibiotics, cell activity, cell viability, growth inhibition, microalga, photosynthesis, toxicity.

1. Introduction

The anthropogenic activity is a constant threat to the stability of aquatic ecosystems, since they are a sink for many chemicals that can have a direct effect on the aquatic organisms.

Aquaculture is a growing industry in response to the dramatic global population growth and the increasing demand for food. As a result of the activities in the aquaculture facilities, effluents containing a complex mixture of chemical compounds as disinfectants, antifouling substances, anesthetics or antibiotics are generated and these compounds may reach aquatic ecosystems.

Infectious diseases are the main cause of the economic losses in aquaculture and have become a limiting factor for its development (Blanco et al., 2004). Therefore, the use of antibiotics has been essential to prevent the spread of pathogenic bacteria. Antibacterial agents are used as prophylactic and therapeutic tools to prevent or combat pathogens and their overuse may cause several adverse effects for the human and animal health and for the environment (Cabello, 2006). Medicated feeds are the main route of drug administration in fish because of their low cost and their easy use, but a considerable portion of the administered food may be not eaten or absorbed by fish (Burka et al., 1997). In mollusc hatcheries, antibiotics are usually applied directly into the water. In both cases, these substances, eventually reach the environment and this may result in adverse ecological effects (Carballeira et al., 2012). In addition, antibiotics are molecules commonly used in both human and veterinary medicine and, in the last years, they have been considered emerging environmental micropollutants (Aminov, 2010; Kümmerer, 2009).

Although recorded environmental levels of antibiotics are usually low in waters (at ng L^{-1} to $\mu\text{g L}^{-1}$) (Gulkowska et al., 2007; Isidori et al., 2005; Xu et al., 2007) these drugs are considered “pseudopersistent” contaminants due to their continued release into the environment and their permanent presence (Hernando et al., 2006). The presence of antibiotics in the environment may also have deleterious effects on non-target aquatic organisms such as microalgae. In certain studies, using aquatic organisms of different trophic levels, it has been found that the toxicity of antibiotics is, in general, higher to cyanobacteria, probably due to their prokaryotic nature, than to unicellular eukaryotic primary producers as microalgae (González-Pleiter et al., 2013; Halling-Sørensen, 2000). Among eukaryotic organisms, multicellular species are in general less sensitive than unicellular microorganisms (Ferreira et al., 2007; Migliore et al., 1997; Wollenberger et al., 2000). Microalgae play a very important role in the aquatic ecosystems because they are the primary producers and any effect on them could affect higher trophic levels (Campanella et al., 2001; Rioboo et al., 2007). Microalgae have been recommended as test organisms because of their ecological relevance, sensitivity and because they are easily cultivated in the laboratory. For these reasons, these organisms are commonly used as biological indicators of pollution in ecotoxicological studies (McCormick and Cairns, 1994; Prado et al., 2009b).

The response of microalgae to a toxic substance is typically measured using population-based parameters, such as specific growth rate, biomass, pigment content or chlorophyll *a* fluorescence (Couderchet and Vernet, 2003; DeLorenzo and Fleming, 2007; Geoffroy et al., 2007; Nestler et al., 2012). The development of markers based on the physiological response of living organisms can accelerate the delivery of biological data and analyse the risk associated with environmental release of potentially polluting compounds. In this regard, several studies have shown that flow cytometry (FCM) is an effective tool in toxicological research (Cid et al., 1996a; Franklin et al., 2001; Franqueira et al., 1999; Prado et al., 2012a; Prado et al., 2012b; Rioboo et al., 2002).

FCM is an alternative to the standard algal population based endpoints, since it allows a rapid and quantitative measurement of individual algal cells responses to toxic stress. Using this technique, simultaneous measurements of multiple cellular parameters are made separately on each cell within the suspension, near *in vivo* conditions, and not just as average values for the whole population (Prado et al., 2009a).

The aim of the present study was to evaluate the potential toxic effect of three antibiotics, chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC), on the marine microalga *Tetraselmis suecica*. This species has a wide distribution along the Galician coast and is frequently used in aquaculture as food in the early larval stages of mollusks, fish and crustaceans (Fábregas et al., 2001). The antibiotics used in this study were selected also based on their use in aquaculture. All of them are broad-spectrum antibiotics widely used in aquacultural practice as antimicrobial agents to control diseases. CHL has been commonly used in hatcheries to control microbial growth in larval cultures (Uriarte et al., 2001). OTC and FLO antibiotics are frequently used for the treatment of the major bacterial pathologies affecting Spanish fish farming like vibriosis, redmouth disease, furunculosis or pasteurellosis (Blanco et al., 2004). Their mechanism of action is related with the inhibition of protein synthesis. CHL and FLO are antibiotics that bind to the 50S subunit of bacterial ribosomes, preventing the transfer of amino acids to extending peptide chains and subsequent protein formation (Marconi et al., 1990). OTC belongs to the tetracycline antibacterial group. This antibiotic binds to the 30S ribosomal subunit and blocks the A site, preventing the binding of amino-acyl tRNAs, thus blocking the first step of the elongation phase (Chopra & Roberts, 2001).

We hypothesize that the presence of these antibiotics may cause alterations on non-target organisms and could have harmful effects on the environment. To demonstrate this, microalgal cells were exposed to different concentrations of each drug. Growth inhibition, a classical parameter in ecotoxicological studies, was determined during 96 h. Other physiological and biochemical parameters were analysed after 24 h to detect early alterations in the microalgal cell physiology. These parameters were relative cell size changes, cell viability and activity and chlorophyll *a* autofluorescence and cellular content.

2. Materials and methods

2.1. Microalgal cultures

The marine microalgal species used in the present work, *Tetraselmis suecica* (Kylin) Butch (*Prasynophyceae*), was obtained from the Culture Centre of Algae and Protozoa (Cambridge, U. K.) and was isolated from Dr. Fábregas, University of Santiago (Fábregas, 1982).

T. suecica was maintained in filtered (pore size: 5 µm) and autoclaved (121 °C, 20 min) seawater enriched with Algal-1 medium (Herrero et al., 1991), at 18 ± 1 °C, illuminated with $68.25 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ with a dark:light cycle of 12:12 h and continuous aeration with filtered atmospheric air (Millipore Millex FG filters of 0.20 µm).

Batch cultures were carried out to evaluate the potential toxic effects of antibiotics on the microalga *T. suecica*. Toxicity tests were conducted in Kimax glass tubes containing 45 mL of culture. The medium and culture conditions were the same as the used for maintaining the algae. Cells from a 3-day-old culture, growing in a logarithmic phase, were used as inoculum for all experiments. Initial cell density for each experiment was 5×10^4 cells mL⁻¹. All cultures were carried out in triplicate, being antibiotics tested in three independent experiments and each one was carried out with three biological

replicates. Since a change in the pH of the culture could modify the toxicity of the antibiotics or alter the growth of the microalga, it was ensured that pH did not change significantly by daily measurements. Registered pH values were between 8.4 and 8.6.

2.2. Chemicals

The antibiotics were purchased from Sigma Aldrich. The purity of each antibiotic was higher than 95%. Stock solutions were made in distilled and sterilized water and then were filtered (pore size: 0.20 μm). These solutions were freshly prepared before each experiment. Four concentrations were tested for each test substance (2.5, 5.0, 7.5 and 10 mg L^{-1}). This range covers the concentrations used in aquaculture for the three antibiotics. In addition to these, cultures without antibiotic were also included as a control.

2.3. Flow cytometric determinations

FCM analysis of *T. suecica* cells was performed in a Gallios flow cytometer (Beckman Coulter Inc.) equipped with an argon-ion excitation laser (488 nm), detectors of forward (FS) and side (SS) light scatter and four fluorescence detectors corresponding to four different wavelength intervals: 505–550 nm (FL1), 550–600 nm (FL2), 600–645 nm (FL3) and > 645 nm (FL4). To exclude non-algal particles, chlorophyll-*a* fluorescence was used, and red fluorescence histograms (> 645 nm) were used as a gate.

2.3.1. Cell density

Cellular density was determined every 24 hours during the 96 hours of the test for each treatment and for the controls. Each of the three biological replicates was sampled once. Growth of microalgal cultures was measured by counting daily culture aliquots in the flow cytometer using a suspension of fluorochrome-containing microspheres for its calibration (Flow Count Fluorospheres, Beckman Coulter Inc.).

Growth rates (μ), expressed as day^{-1} , were calculated using the following equation:

$$\mu = [\ln(N_t) - \ln(N_0)] / \ln 2 (t-t_0)$$

where N_t is the cell density at time t and N_0 is the cell density at time 0.

Growth inhibition was determined by comparing the cell density of the treated cultures with the cell density of the control cultures (zero inhibition).

2.3.2. Relative cell size

Cultures were analysed by flow cytometry to study potential alterations in cell size after 24 h of exposure to each pollutant. Forward light scatter (FS) is related to cell size or volume and FS intensity increases with the increase of cell cross-sectional area (Mullaney et al., 1969). At least, 5×10^3 cells for sample were analysed and data were presented as FS arbitrary units (a.u.).

2.3.3. Cell viability

Cell viability was estimated using the fluorescent emission of cells stained with propidium iodide (PI). PI is a fluorescent dye that intercalates with double stranded nucleic acids to produce red fluorescence when excited by blue light. Because of its polarity, it is unable to pass through intact cell membranes; however, when a cell dies the integrity of the cell membrane fails, and PI is able to enter and stain the nucleic acids. In this way, PI can be used to discriminate between viable non-fluorescent cells (PI-) and non-viable fluorescent cells (PI+) (Cid et al., 1996b; Prado et al., 2009a).

PI-derived fluorescence was detected in FL3 detector (600 – 645 nm) in a logarithmic scale. This assay was performed following the method described by Prado et al. (2009a)

using aliquots of 5×10^3 cells mL^{-1} . At least 10^4 cells were analysed per culture. Results were obtained as the percentage of viable cells versus the total amount of analysed cells.

2.3.4. Metabolic activity

Metabolic activity was studied using a fluorescein diacetate-based cell esterase activity assay, which has been reported as a sensitive and rapid technique to assess phytoplankton metabolic activity (Jochem, 2000; Peperzak and Brussaard, 2011). Fluorescein diacetate (FDA) is a non-polar, non-fluorescent lipophilic molecule that freely diffuses across cell membranes. Inside the cell, non-specific esterases break the FDA molecule into one brightly fluorescing fluorescein and two acetates. Due to its high polarity, the fluorescein is trapped within cells exhibiting intact plasma membranes. Therefore fluorescence intensity will increase depending on the metabolic activity of those esterases.

Since fluorescein is accumulated by active cells, metabolic activity can be measured by means of the fluorescent intensity signal emitted by cells, which is proportional to the amount of accumulated fluorescein and the time elapsed (Prado et al., 2009a). Comparing any differences in the fluorescence emitted by metabolically active cells (FDA+), it is possible to detect changes in cell activity.

FDA-derived fluorescence was detected in FL1 detector (505 – 550 nm) in a logarithmic scale. This assay was performed following the method described by Prado et al. (2009a) using aliquots of 5×10^3 cells mL^{-1} and the FDA at a final concentration of $0.4 \mu\text{g mL}^{-1}$. At least 10^4 cells were analysed per culture. Results are expressed as the mean fluorescence value (a.u.) of metabolically active cells population (FDA+) that is related with the cell activity.

2.3.5. Chlorophyll *a* autofluorescence

T. suecica presents natural fluorescence due to the presence of chlorophyll *a* and other accessory pigments. Chlorophyll *a* is excited by blue light at 488 nm and emits fluorescence in the range of 680-720 nm (red fluorescence) which is collected by the FL4 detector. Therefore this parameter is studied directly from cell suspensions without using any fluorochrome. A minimum of 5×10^3 cells were analysed and the mean fluorescence value (a.u.) of the cell population was calculated.

2.4. Spectrophotometric determination of chlorophyll *a*

The chlorophyll *a* content of *T. suecica* was analysed by spectrophotometry. Cells were collected by centrifugation of culture aliquots at 8000 g and 4 °C for 20 min. Pigments were extracted in 90% acetone in the dark at 4 °C during 24 hours. Then, samples were centrifuged again. The supernatant, which contains cell pigments, was decanted off to read the absorbances and the pellet was discarded. Absorbances were read at 664 and 647 nm on the spectrophotometer. The chlorophyll *a* concentration was calculated using the following equation (Jeffrey and Humphrey, 1975):

$$\text{Chlorophyll } a = 11.93 A_{664} - 1.93 A_{647}$$

where Chlorophyll *a* represents the concentration in $\mu\text{g mL}^{-1}$; A_{664} and A_{647} are the absorbances read at 664 and 647 nm respectively.

2.5. Data analysis

Mean and standard deviation values of the three biological replicates were calculated for each treatment and for the control. In all cases, data are given as mean values \pm standard deviation of the mean.

To determine significant differences among test concentrations, data were statistically analysed by an overall one-way analysis of variance (ANOVA) using IBM SPSS Statistic 21.0.0 software. A p -value < 0.05 was considered statistically significant. When significant differences were observed, means were compared using the multiple-range Duncan test at a level of significance of 0.05 ($p < 0.05$).

IC₅₀ values were calculated using linear regression analysis of the logarithm of antibiotic concentration versus percentage of inhibition.

3. Results

3.1. Growth

In general, the antibiotics assayed showed inhibitory effects on the growth of *Tetraselmis suecica* (Fig. 1). The presence of the antibiotics tested in the culture medium significantly ($p < 0.05$) affected cell division of *T. suecica* and this negative effect on growth was concentration-dependent ($p < 0.05$). However, in the case of OTC growth was slightly enhanced at the lowest concentration assayed (2.5 mg L⁻¹) and no significant differences were found between control and cultures exposed to 5 mg L⁻¹.

After 96 h of exposure to antibiotics, cultures exhibited growth rates (μ) lower than control, and these growth rates decreased as the antibiotic concentration increased (Table 1). Statistical analysis showed a significant reduction ($p < 0.05$) in growth at concentrations equal or higher than 2.5 mg L⁻¹ for CHL; 5 mg L⁻¹ for FLO and 7.5 mg L⁻¹ for OTC (Table 1). The highest concentration assayed (10 mg L⁻¹) of CHL and FLO caused the maximum decrease in growth rate detected in this assay with 36.8 and 36.6 % less than the control, respectively; whereas the highest concentration of OTC caused the minimum decrease detected in growth rate, with 23.9 % less than the control. IC₅₀ values of CHL, FLO and OTC determined after 96 h were 11.16, 9.03 and 17.25 mg L⁻¹ respectively. Based on the IC₅₀ values obtained, the antibiotics can be ranked according to toxicity level as follows: FLO > CHL > OTC.

3.2. Relative cell size

After 24 h of exposure, the presence of the antibiotics tested in culture medium caused a decrease in the FS signal, related to a decrease in the cell volume of *T. suecica*. Relative cells size significantly ($p < 0.05$) decreased as antibiotic concentration increased (Fig. 2). At the highest concentration tested (10 mg L⁻¹), CHL and OTC substantially reduced relative cell size with respect to control cells, but the most drastic decrease in relative cell size was detected with FLO.

3.3. Cell viability

Cell viability in *T. suecica* cultures assayed by FCM was not significantly ($p < 0.05$) affected by the addition of these three antibiotics tested to the medium. None of the antibiotics assayed, even at the highest concentration tested, affected cell viability of *T. suecica*, that remained close to 100% for all antibiotic concentrations assayed (data not shown). Cell membranes were not altered and therefore, cells remained viable throughout the duration of the test.

3.4. Metabolic activity

After 24 h of exposure, the cell activity was significantly affected by antibiotics in a concentration-dependent way, except for CHL ($p < 0.05$). The lowest concentration assayed of FLO significantly ($p < 0.05$) reduced the cell activity of *T. suecica*, whereas the highest concentrations of OTC (7.5 mg L⁻¹ and 10 mg L⁻¹) showed a significant ($p <$

0.05) reduction in cell activity. Furthermore, a non-significant ($p < 0.05$) elevation of activity at 2.5 mg L^{-1} of OTC was detected (Fig. 3).

3.5. Chlorophyll *a*

Since differences in cellular volume of the different treatments were observed, chlorophyll *a* autofluorescence is expressed corrected by relative cell size (ratio FL4/FS), thereby avoiding autofluorescence variability due to differences in relative cell volume (Fig. 4). After 24 h, cultures exposed to the antibiotics showed a significant ($p < 0.05$) increase in the values of the autofluorescence. As drug concentration increased, the intensity of the fluorescence also increased. All three antibiotics enhanced the intensity of the autofluorescence emitted, but the most relevant increase was obtained in the presence of FLO and CHL. Chlorophyll *a* content was also affected by the presence of antibiotics in the culture medium. All antibiotic treatments significantly ($p < 0.05$) reduced pigment cell content compared to control cultures, except for the OTC at 2.5 mg L^{-1} (Fig. 5). At the highest concentration assayed, the amount of chlorophyll *a* per cell was reduced in a 45 % in the presence of CHL, 22 % for FLO and 35 % for OTC relative to the control cultures.

4. Discussion

In the last years, pharmaceuticals have been receiving an increasing attention regarding their potential harmful effects on the environment (Halling-Sørensen et al., 1998). In particular, antibiotics are of particular concern for marine ecosystems since they are extensively used in aquaculture and they also may reach the environment by other ways such as urban and industrial effluents (Migliore et al., 1997; Wollenberger et al., 2000). The three antibiotics assayed in this study alter the growth of the microalgae *Tetraselmis suecica*. The IC_{50} values (96 h) obtained for the three antibiotics are close to the values reported for other microalgal species in the literature. In this way, De Orte et al. (2013) found 96 h OTC IC_{50} values of 1.73 and 6.43 mg L^{-1} to *Phaeodactylum tricornutum* and *Isochrysis galbana*, respectively. In the study of Lai et al. (2009) the 96 h IC_{50} of FLO to *I. galbana* was 8.00 mg L^{-1} and the 96 h IC_{50} of CHL to *Tetraselmis chuii* was 4.00 mg L^{-1} . These results suggest that microalgae, despite being non-target organisms, are strongly affected by antibiotics.

Obtained results show that *T. suecica* is significantly affected by the presence of these antibiotics in the medium. Therefore, in the case of CHL, a better control or government regulation and law enforcement should be exercised about its illegal use in aquaculture. Despite being banned in many countries, its broad spectrum of activity, ready availability and low cost attracts some producers (Huang et al., 2006). For the OTC, the IC_{50} value obtained was higher than for the other two antibiotics, therefore, this compound seems less harmful for this species than the others tested. FLO is an antibacterial agent especially developed for veterinary use, being applied even against bacteria that have developed resistance to other antibacterial drugs and it is less persistent in the aquatic environment than OTC. In addition, although it is a structural CHL analogue, FLO has a broader spectrum of activity and has no toxic effects in humans (Xu et al., 2005); because of this, the use of this drug has increased in recent years. However, in this study FLO significantly inhibited the growth of the marine microalgae *T. suecica*, showing much higher toxicity than the two other antibiotics assayed. Therefore, the use of this antibiotic should be more carefully monitored to reduce the potential contamination risk of the receiving waters.

Since cultures were not axenic, alterations observed on growth in the presence of antibiotics could also be due to the lack of some growth factors or vitamins synthesized

by associated bacteria, which could be affected. In the case of OTC, the stimulation of growth at the lowest concentration tested could be explained by a phenomenon of hormesis. Although the biochemical mechanism by which hormesis works is not well understood, it is possible that low doses of the drug might activate the repair mechanisms of the cell.

Except for growth, the remaining parameters were evaluated after 24 h of antibiotic exposure, when growth was not significantly affected (Fig. 1).

The presence of a proportion of smaller cells in cultures with antibiotics may be explained by the inhibition of growth detected (Fig. 2). Active cells enlarged before dividing because of the increase in cellular constituents. But cells exposed to antibiotics did not increase their volume as fast as the control cultures because they had their growth inhibited.

Toxic effects of these pollutants on microalgae are generally evaluated using phytotoxicity tests based on growth inhibition, a population-based parameter. However, physiological cellular endpoints could allow early detection of cell stress and elucidate underlying toxicity mechanisms (Prado et al., 2009a). In this regard, the measurement of cell viability and enzyme inhibition in microalgae are rapid and sensitive indicators of environmental stress (Blaise and Ménard, 1998). Results obtained with the FDA test provide additional information to that obtained with the PI test. Although both assay procedures can distinguish between cells with an intact plasma membrane (viable) and cells with a damaged membrane (not viable), the FDA assay also includes those cells with intact plasma membrane, but with altered esterase activity.

Several studies have shown that some toxic agents such as copper or some pesticides may damage the integrity of microalgal membranes causing a decrease in the percentage of viable cells (Cid et al., 1996b; Lage et al. 2001; Prado et al., 2009a). Reactive oxygen species (ROS) generated by these agents have been linked to the origin of the damage to cell membranes. It has also been found that exposure to low concentrations of these agents stimulate cell esterase activity since these enzymes are essential for the replacement of phospholipids in cell membranes (Franklin et al., 2001). However, in this study, an opposite effect was detected. On one hand, cell viability of the cells of *T. suecica* analysed by CMF using PI was not affected by the presence of antibiotics in the culture medium and on the other hand, the FDA test detected a decrease in metabolic activity (Fig. 3). This could be explained because chloroplasts and mitochondria have their origin in primitive bacteria and these organelles may be the target of the antibiotics in microalgae, so these drugs could alter cell physiological state, but not its integrity or viability (Ebringer, 1972; Nicolas, 1981; Liu et al., 2012).

Regarding cell chlorophyll *a* content, a significant decrease was observed in cultures exposed to antibiotics (Fig. 5). Hendry and Price (1993) reported the loss of chlorophyll associated with environmental stress. Several studies have shown that some compounds such as carbamazepine can strongly inhibit the synthesis of chlorophyll (Tsiaka et al., 2013). This inhibitory effect is probably due to the interference of these pollutants with the chlorophyll synthesis (Zhang et al., 2012). Furthermore, the decrease in the cellular content of pigments could be caused by the enhancement of oxidative stress-related effects (Tsiaka et al., 2013), since these are one of the most important causes of degradation of pigments in plants.

Although the green alga is a non-target organism, the observed toxicity exerted by some antibiotics could be, at least in part, due to the prokaryote-like elements of chloroplast metabolism and reproduction, which makes these plastids potential antibiotic targets (McFadden and Roos, 1999; González-Pleiter et al., 2013). Due to the similarities between the ribosomal RNA of bacteria and chloroplasts, chloroplast ribosomes are

sensitive to many antibiotics (Gray, 1992). Proteins of the photosynthetic apparatus are synthesized in chloroplasts (Mayfield et al., 1995), thus damage to chloroplast ribosomes would cause harmful effects to photosynthetic organisms, affecting the chlorophyll synthesis (Liu et al., 2012). In the same way, antibiotics may cause similar effects on the mitochondria, as some antibiotics such as chloramphenicol, inhibits mitochondrial protein synthesis in eukaryotic cells (McKee et al., 2006; Yunis, 1989). Mitochondrial ribosomes are involved in the synthesis of proteins implicated in electron transport, which is essential for energy metabolism and cellular respiration process, therefore the study of these parameters in microalgal cells exposed to antibiotics would also be interesting.

Changes in chlorophyll fluorescence have been used to study the physiological state of microalgal cells under several stress factors (González-Barreiro et al., 2004; Chalifour et al., 2009; Hadjoudja et al., 2009). Although treated cells decreased in size and in chlorophyll *a* content, an increase in chlorophyll *a* fluorescence was detected. The increase of the autofluorescence could be explained by a blockage of the electron transport chain at the PS II level (Cid et al., 1995), indicating an inhibitory effect localised on the oxidant side, probably due to inactivation of some PS II reaction centres as postulated by Samson and Papovic (1988) in their study of phytotoxicity of heavy metals and pesticides in the microalga *Dunaliella tertiolecta*. It has also been reported by Singh and Singh (1987) and by Murthy et al. (1990) in cells exposed to the action of copper and mercury, respectively.

Taken into account the obtained results, chloramphenicol, florphenicol and oxytetracycline provoke adverse effects on the marine microalgae *Tetraselmis suecica*.

5. Conclusions

The three antibiotics tested caused significant alterations on the microalga and the most affected parameters were those related to photosynthesis and growth.

Before growth was affected, cellular content and autofluorescence of chlorophyll *a* of the microalga were altered by the presence of antibiotics in the environment, and this may adversely affect the photosynthetic rate. This effect could be due to the cyanobacterial nature of chloroplasts which makes these plastids potential antibiotic targets.

Flow cytometry provides additional information to the ecotoxicological studies, which are often based on growth inhibition assays. It can be concluded that cellular physiological parameters studied by flow cytometry can allow us to detect early alterations that classical studies of growth inhibition do not detect.

Further studies about the toxicity of the mixtures of antibiotics should be carried out to detect possible interactions (synergism or antagonism) among these compounds in the environment.

References

- Aminov, R.I., 2010. A brief history of the antibiotic era: lessons learned and challenges for the future. *Front. Microbiol.* 1, 134.
- Blaise, C. and Ménard, L., 1998. A micro-algal solid phase test to assess the toxic potential of freshwater sediments. *Water Qual. Res. J. Can.* 33, 133-151.
- Blanco, M.M., Liébana, P., Gibello, A., Alcalá, C., Fernández-Garayzábal, J.F., Domínguez, L., 2004. Principales patologías bacterianas en la piscicultura española. *REDVET – Revista Electrónica de Veterinaria*. Available on:

- <http://www.veterinaria.org/revistas/redvet/n020204.html> Viewed: 4 September 2013.
- Burka, J.F., Hammell, K.L., Horsberg, T.E., Johnson, G.R., Rainnie, D.J., Speare, D.J., 1997. Drugs in salmonid aquaculture - A review. *J. Vet. Pharmacol. Ther.* 20, 333-349.
- Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ. Microbiol.* 8(7), 1137-1144.
- Campanella, L., Cubadda, F., Sammartino, M. ., Saoncella, A., 2001. An algal biosensor for the monitoring of water toxicity in estuarine environments. *Wat. Res.* 35(1), 69-76.
- Carballeira, C., Ramos-Gómez, J., Martín-Díaz, M.L., DelValls, T.A., Carballeira, A., 2012. Designing an Integrated environmental monitoring plan for land-based marine fish farms located at exposed and hard bottom coastal areas. *J. Environ. Monitor.* 14, 1305-1316.
- Chalifour, A., Spear, P.A., Boily, M.H., DeBlois, C., Giroux, I., Dassylva, N., Juneau, P., 2009. Assessment of toxic effects of pesticide extracts on different green algal species by using chlorophyll *a* fluorescence. *Toxicol. Environ. Chem.* 91, 1315-1329.
- Chopra, I. and Roberts, M., 2001. Tetracycline antibiotics: mode of actions, applications, molecular biology and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65(2), 232-260.
- Cid, A., Fidalgo, P., Herrero, C., Abalde, J., 1996b. Toxic action of copper on the membrane system of a marine diatom measured by flow cytometry. *Cytometry* 25(1), 32-36.
- Cid, A., Herrero, C., Abalde, J., 1996a. Functional analysis of phytoplankton by flow cytometry: A study of the effect of copper on a marine diatom. *Sci. Mar.* 60(1), 303-308.
- Cid, A., Herrero, C., Torres, E., Abalde, J., 1995. Copper toxicity on the marine microalga *Phaeodactylum tricornutum*: effects on photosynthesis and related parameters. *Aquat. Toxicol.* 31, 165-174.
- Couderchet, M. and Vernet, G., 2003. Pigments as biomarkers of exposure to the vineyard herbicide flazasulfuron in freshwater algae. *Ecotoxicol. Environ. Saf.* 55, 271-277.
- De Orte, M.R., Carballeira, C., Viana, I.G., Carballeira, A., 2013. Assessing the toxicity of chemical compounds associated with marine land-based fish farms: The use of mini-scale microalgal toxicity test. *Chem. Ecol.* 29(6), 554-563.
- DeLorenzo, M.E., and Fleming, J., 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. *Arch. Environ. Contam. Toxicol.* 54(2), 203-10.
- Ebringer, L. 1972. Are plastids derived from prokaryotic microorganisms? Action of antibiotics on chloroplasts of *Euglena gracilis*. *J. Gen. Microbiol.* 71(1), 35-52.
- Fábregas J., Otero, A., Domínguez, A., Patiño, M., 2001. Growth rate of the microalga *Tetraselmis suecica* changes the biochemical composition of *Artemia* species. *J. Mar. Biotechnol.* 3, 256-263.
- Fábregas, J., 1982. Las microalgas como eslabón de infraestructura en la Microbiología Marina: aislamiento, caracterización, ciclo celular y aprovechamiento tecnológico. Tesis Doctoral. Universidad de Santiago de Compostela.
- Ferreira, C.S.G., Nunes, B.A., Henriques-Almeida, J.M.M., Guilhermino, L., 2007. Acute toxicity of oxytetracycline and florphenicol to the microalgae *Tetraselmis*

- chuii* and to the crustacean *Artemia parthenogenetica*. *Ecotox. Environ. Safe.* 67, 452-458.
- Franklin, N.M., Stauber, J.L., Lim, R.P., 2001. Development of flow cytometry-based algal bioassays for assessing toxicity of copper in natural waters. *Environ. Toxicol. Chem.* 13, 160-170.
- Franqueira, D., Cid, A., Torres, E., Orosa, M., Herrero, C., 1999. A comparison of the relative sensitivity of structural and functional cellular responses in the alga *Chlamydomonas eugametos* exposed to the herbicide paraquat. *Arch. Environ. Contam. Toxicol.* 36, 264–269.
- Geoffroy, L., Gilbin, R., Simon, O., Floriani, M., Adam, C., Pradines, C., Cournac, L., Garnier-Laplace, J., 2007. Effect of selenate on growth and photosynthesis of *Chlamydomonas reinhardtii*. *Aquat. Toxicol.* 83(2), 149–158.
- González-Barreiro, O., Rioboo, C., Cid, A., Herrero, C., 2004. Atrazine induced chlorosis in *Synechococcus elongatus* cells. *Arch. Environ. Contam. Toxicol.* 46, 301-307.
- González-Pleiter, M., Gonzalo S., Rodea-Palomares I., Leganés F., Rosal R., Boltes K., Marco E., Fernández-Piñas, F., 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: Implications for environmental risk assessment. *Water Res.* 47, 2050-2064.
- Gray, M.W., 1992. The endosymbiont hypothesis revisited. *Int. Rev. Cytol.* 141, 233-357.
- Gulkowska, A, He, Y., So, M.K., Yeung, L.W.Y., Leung, H.W., Giesy, J.P., Lam, P.K.S., Martin, M., Richardson, B.J., 2007. The occurrence of selected antibiotics in Hong Kong coastal waters. *Mar. Pollut. Bull.* 54(8), 1287–1293.
- Hadjoudja, S., Vignoles, C., Delucht, V., Lenain, J.F., Le Jeune, A.H., Baudu, M., 2009. Short term copper toxicity on *Microcystis aeruginosa* and *Chlorella vulgaris* using flow cytometry. *Aquat. Toxicol.* 94: 255-264.
- Halling-Sørensen, B., 2000. Algal toxicity of antibacterial agents used in intensive farming. *Chemosphere* 40, 731-739.
- Halling-Sørensen, B., Nielsen, S.N., Lanzky, P.F., Ingerslev, F., Lühøft, H.C.H., Jørgensen, S.E., 1998. Occurrence, fate and effects of pharmaceutical substance in the environment - a review. *Chemosphere* 36, 357–393.
- Hektoen, H., Berge, J.A., Hormazabal, V., Yndestad, M., 1995. Persistence of antibacterial agents in marine-sediments. *Aquaculture* 133(3-4), 175-184.
- Hendry, G.A.F. and Price, A.H., 1993. Stress indicators: chlorophylls and carotenoids. In: Hendry, G.A.F. y Grime, J.P. (Eds.), *Methods in Comparative Plant Ecology*. Chapman & Hall, London, pp. 148-152.
- Hernando, M.D., Mezcuca, M., Fernández-Alba, A.R., Barceló, D., 2006. Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. *Talanta* 69(2), 334-342.
- Herrero, C., Cid, A., Fábregas, J., Abalde, J., 1991. Yields in biomass and chemical constituent of four commercially important marine microalgae with different culture media. *Aquacult. Eng.* 10(2), 99-110.
- Huang, Z.Y., Sun, M.Y., Li, S., Huang, G.L., 2006. Pharmacokinetics of chloramphenicol in carp (*Cyprinus carpio* L.) after drug administration. *Aquacult. Res.* 37, 1540-1545.
- Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., Parrella, A., 2005. Toxic and genotoxic evaluation of six antibiotics on non-target organisms. *Sci. Total Environ.* 346, 87-98.

- Jeffrey, S.W. and Humphrey, G.F., 1975. New spectrophotometric equations for determining chlorophylls *a*, *b*, *c1* and *c2* in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pfl.* 167, 191-194.
- Jochem, F.J., 2000. Probing the physiological state of phytoplankton at the single-cell level. *Sci. Mar.* 64(2), 183–195.
- Kümmerer, K., 2009. Antibiotics in the aquatic environment – a review - Part I. *Chemosphere* 75, 417-434.
- Lage, O.M., Sansonetty, F., O'Connor, J.-E., Parente, A.M., 2001. Flow cytometric analysis of chronic and acute toxicity of copper (II) on the marine dinoflagellate *Amphidinium carterae*. *Cytometry* 44, 226-235.
- Lai, H.T., Hou, J.H., Su, C.I., Chen, C.L., 2009. Effects of chloramphenicol, florphenicol, and thiamphenicol on growth of algae *Chlorella pyrenoidosa*, *Isochrysis galbana* and *Tetraselmis chuii*. *Ecotox. Environ. Safe.* 72, 329-334.
- Liu, W., Ming, Y., Huang, Z., Li, P., 2012. Impacts of florphenicol on marine diatom *Skeletonema costatum* through photosynthesis inhibition and oxidative damages. *Plant Physiol. Bioch.* 60, 165-170.
- Marconi R., Lodmell, J., Hill W., 1990. Identification of a rRNA / Chloramphenicol interaction site within the peptidyltransferase center of the 50S subunit of the *Escherichia coli* ribosome. *J. Biol. Chem.* 265(14), 7894-7899.
- Mayfield, S.P., Yohn, C.B., Cohen, A., Danon, A., 1995. Regulation of chloroplast gene expression. *Annu. Rev. Plant. Biol.* 46, 147-166.
- McCormick, P.V. and Cairns, J.J., 1994. Algae as indicators of environmental change. *J Appl. Phycol.* 6, 509-526.
- McFadden, G.I. and Roos, D.S., 1999. Apicomplexan plastids as drug targets. *Trends Microbiol.* 7(8), 328-333.
- McKee, E.E., Ferguson, M., Bentley, A.T., Marks, T.A., 2006. Inhibition of mammalian mitochondrial protein synthesis by oxazolidinones. *Antimicrob. Agents Ch.* 50(6), 2042-2049.
- Migliore, L., Civitareale, C., Brambilla, G., DiDelupis, G.D., 1997. Toxicity of several important agricultural antibiotics to *Artemia*. *Water Res.* 31(7), 1801-1806.
- Mullaney, P.F., Van Dilla, M.A., Coulter, J.R., 1969. Cell sizing: a light scattering photometer for rapid volume determination. *Rev. Sci. Instrum.* 40, 1029 - 1034.
- Murthy, S.D.S., Bukhov, N.G., Mohanty, P., 1990. Mercury-induced alterations of chlorophyll *a* fluorescence kinetics in cyanobacteria: multiple effects of mercury on electron transport. *J. Photoch. Photobiol, B.* 6, 373-380.
- Nestler, H., Groh, K. J., Schönenberger, R., Behra, R., Schirmer, K., Eggen, R. I. L., Suter, M. J.-F., 2012. Multiple-endpoint assay provides a detailed mechanistic view of responses to herbicide exposure in *Chlamydomonas reinhardtii*. *Aquat. Toxicol.* 110-111, 214–224.
- Nicolas, P., 1981. Sensitivity of *Euglena gracilis* to chloroplast – inhibiting antibiotics, and properties of antibiotic – resistant mutants. *Plant. Sci.* 22, 309-317.
- Peperzak, L., and Brussaard, C.P.D., 2011. Flow Cytometric Applicability of Fluorescent Vitality Probes on Phytoplankton. *Journal of Phycology*, 47(3), 692–702.
- Prado, R., García, R., Rioboo, C., Herrero, C., Abalde, J., Cid, A., 2009a. Comparison of the sensitivity of different toxicity test endpoints in a microalga exposed to the herbicide paraquat. *Environ. Int.* 35, 240-247.
- Prado, R., Rioboo, C., Herrero, C., Cid, A., 2009b. The herbicide paraquat induces alterations in the elemental and biochemical composition of non-target microalgal species. *Chemosphere* 76, 1440-1444.

- Prado, R., Rioboo, C., Herrero, C., Cid, A., 2012b. Screening acute cytotoxicity biomarkers using a microalga as test organism. *Ecotox. Environ. Saf.* 86, 219–226.
- Prado, R., Rioboo, C., Herrero, C., Suárez-Bregua, P., Cid, A. 2012a. Flow cytometric analysis to evaluate physiological alterations in herbicide-exposed *Chlamydomonas moewusii* cells. *Ecotoxicology* 21, 409–420.
- Rioboo, C., González, O., Herrero, C., Cid, A., 2002. Physiological response of freshwater microalga (*Chlorella vulgaris*) to triazine and phenylurea herbicides. *Aquat. Toxicol.* 59, 225–235.
- Rioboo, C., Prado, R., Herrero, C., Cid, A., 2007. Population growth study of the rotifer *Brachionus* sp. fed with triazine-exposed microalgae. *Aquat. Toxicol.* 83, 247–253.
- Samson, G. and Papovic, R., 1988. Use of algal fluorescence for determination of phytotoxicity of heavy metals and pesticides as environmental pollutants. *Ecotox. Environ. Saf.* 16, 272–278.
- Singh, D.P. and Singh, S.P., 1987. Action of heavy metals on Hill activity and O₂ evolution in *Anacystis nidulans*. *Plant Physiol.* 83, 12–14.
- Tsiaka, P., Tsarpali, V., Ntaikou, I., Kostopoulou, M.N., Lyberatos, G., Dailianis, S., 2013. Carbamazepine-mediated pro-oxidant effects on the unicellular marine algal species *Dunaliella tertiolecta* and the hemocytes of mussel *Mytilus galloprovincialis*. *Ecotoxicology* 22, 1208–1220.
- Uriarte, I., Farias, A., Castilla, J.C., 2001. Effect of antibiotic treatment during larval development of the Chilean scallop *Argopecten purpuratus*. *Aquacult. Eng.* 25, 139–147.
- Wollenberger, L., Halling-Sørensen, B., Kusk, K.O., 2000. Acute and chronic toxicity of veterinary antibiotics to *Daphnia Magna*. *Chemosphere* 40, 723–730.
- Xu, L.W., Liao, C. R., Liu, G.F., 2005. Security about florphenicol used in aquaculture. *J. Fish. Sci. China* 12, 512–518.
- Xu, W., Zhang, G., Zou, S., Li, X., Liu, Y., 2007. Determination of selected antibiotics in the Victoria Harbour and the Pearl River, South China using high-performance liquid chromatography-electrospray ionization tandem mass spectrometry. *Environ. Pollut.* 145(3), 672–679.
- Yunis, A.A., 1989. Chloramphenicol toxicity: 25 years of research. *Am. J. Med.* 87, 44–48.
- Zhang, W., Zhang, M., Lin, K., Sun, W., Xiong, B., Guo, M., Cui, X., Fu, R., 2012. Ecotoxicological effect of carbamazepine on *Scenedesmus obliquus* and *Chlorella pyrenoidosa*. *Environ. Toxicol. Phar.* 33(2), 344–352.

Table and figure captions

Table 1. Growth rate (μ) of *T. suecica* cultures after 96 h of exposure to different concentrations of chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC). Values are the mean (three replicates) \pm standard deviation. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

Figure 1. Growth curves of the cultures of *T. suecica* in the absence (Ct) and in the presence of different concentrations (mg L^{-1}) of chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC). Values are the mean (three replicates) of number of cells per mL in each treatment \pm standard deviation.

Figure 2. Relative cell size of *T. suecica* after 24 hours of exposure to different concentrations of chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC) (mg L^{-1}). Data are given as mean values \pm standard deviation of the FS signal. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

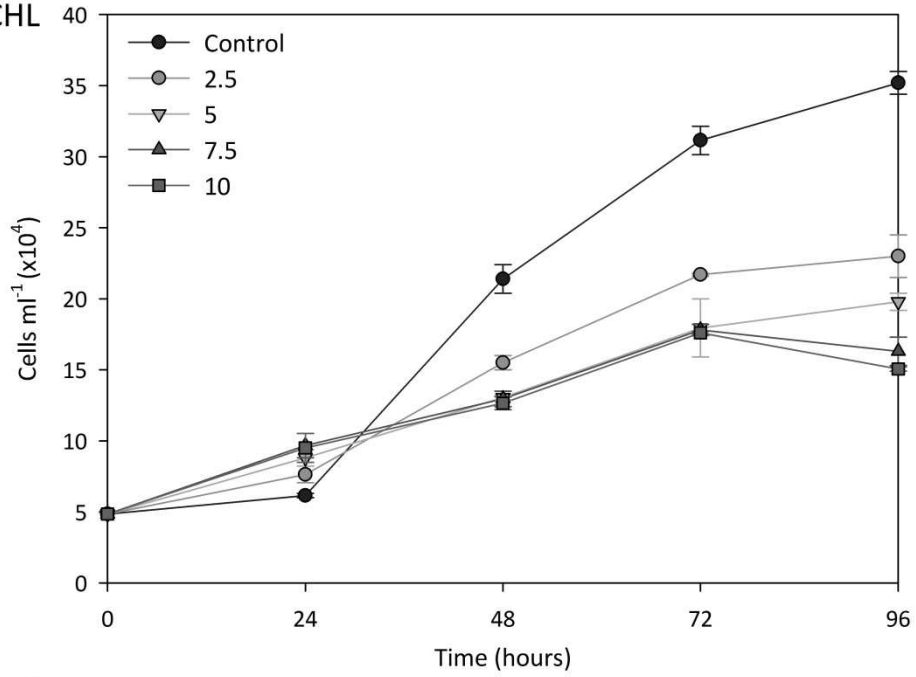
Figure 3. Metabolic activity of *T. suecica* cells after 24 hours of exposure to different concentrations of chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC) (mg L^{-1}). Data are given as mean values \pm standard deviation. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

Figure 4. Chlorophyll *a* autofluorescence expressed as the ratio between the chlorophyll *a* fluorescence and the relative cell size of the cultures of *T. suecica* after 24 hours of exposure to different concentrations (mg L^{-1}) of chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC). Data are given as mean values \pm standard deviation. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

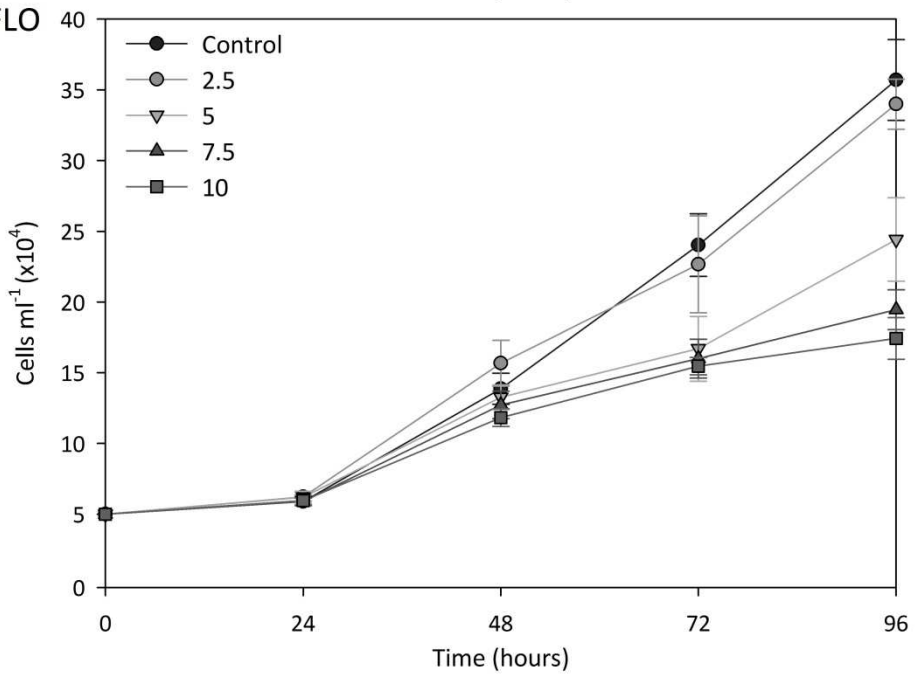
Figure 5. Chlorophyll *a* content of the cultures of *T. suecica* after 24 hours of exposure to different concentrations (mg L^{-1}) of chloramphenicol (CHL), florphenicol (FLO) and oxytetracycline (OTC). Data are given as mean values \pm standard deviation. Significant differences with respect to control at a level of significance of 0.05 ($p < 0.05$) are represented by an asterisk (*).

Antibiotic (mg l ⁻¹)	μ (day ⁻¹)		
	CHL	FLO	OTC
Control	0.68 ± 0.05	0.71 ± 0.03	0.71 ± 0.05
2.5	0.59 ± 0.04*	0.69 ± 0.02	0.74 ± 0.02
5	0.53 ± 0.03*	0.57 ± 0.04*	0.69 ± 0.02
7.5	0.48 ± 0.07*	0.49 ± 0.03*	0.62 ± 0.03*
10	0.43 ± 0.03*	0.45 ± 0.03*	0.54 ± 0.05*

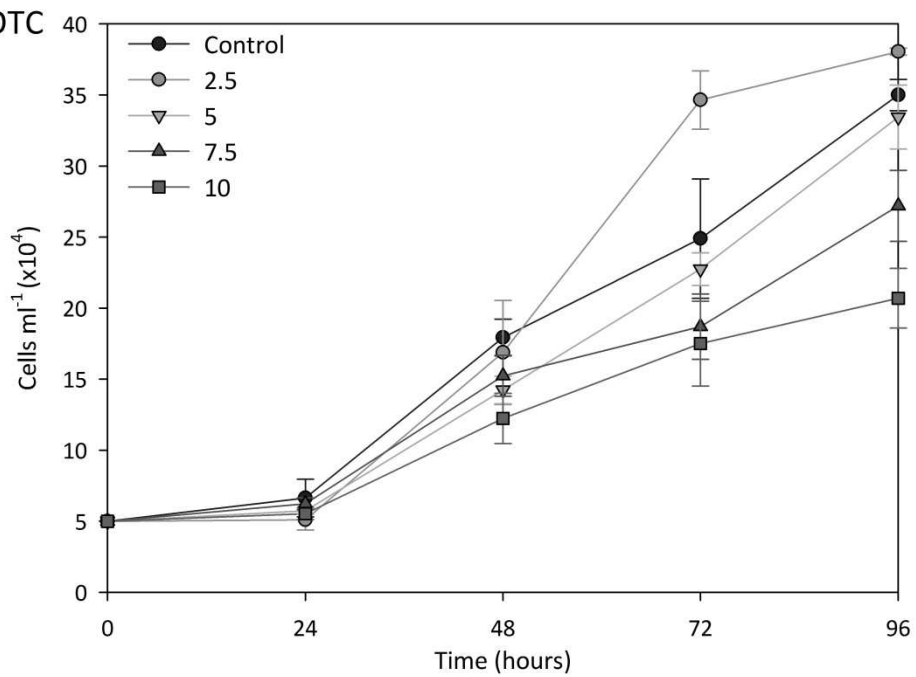
CHL

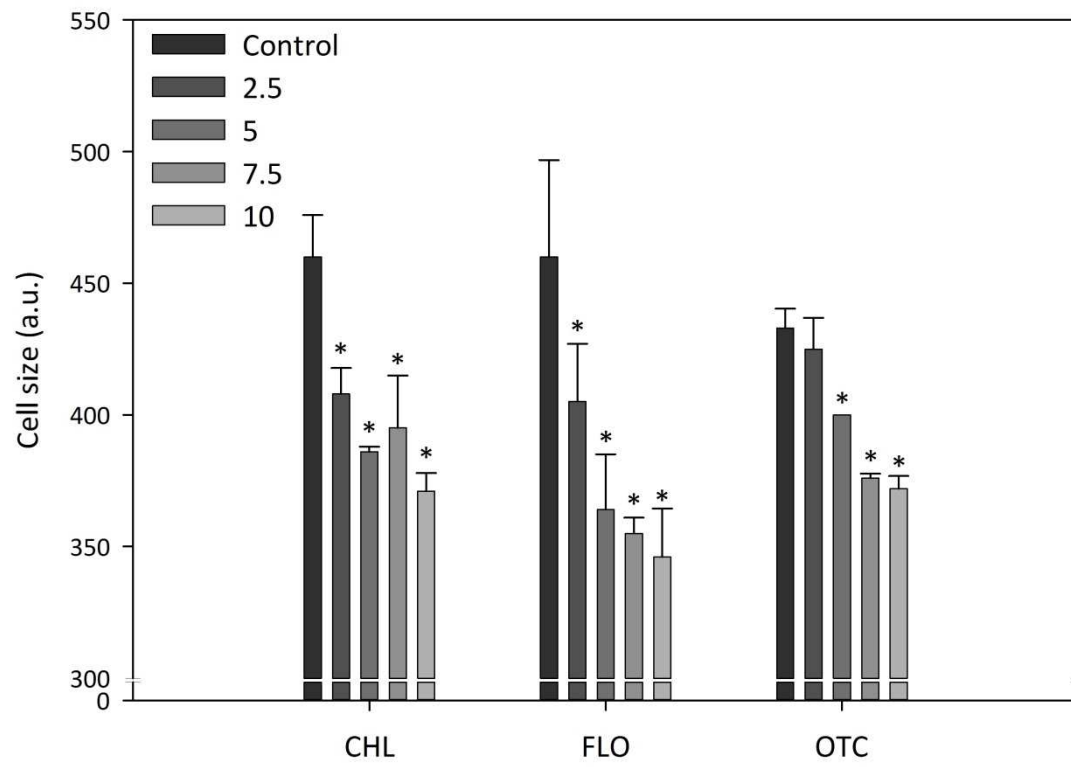


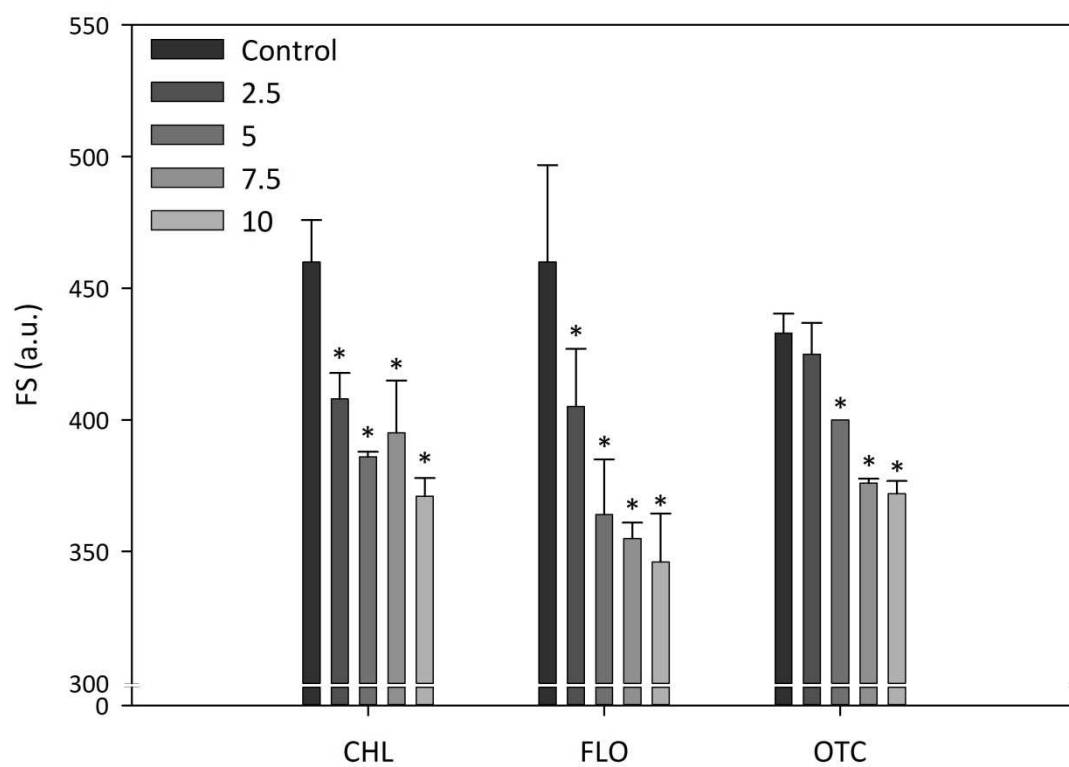
FLO

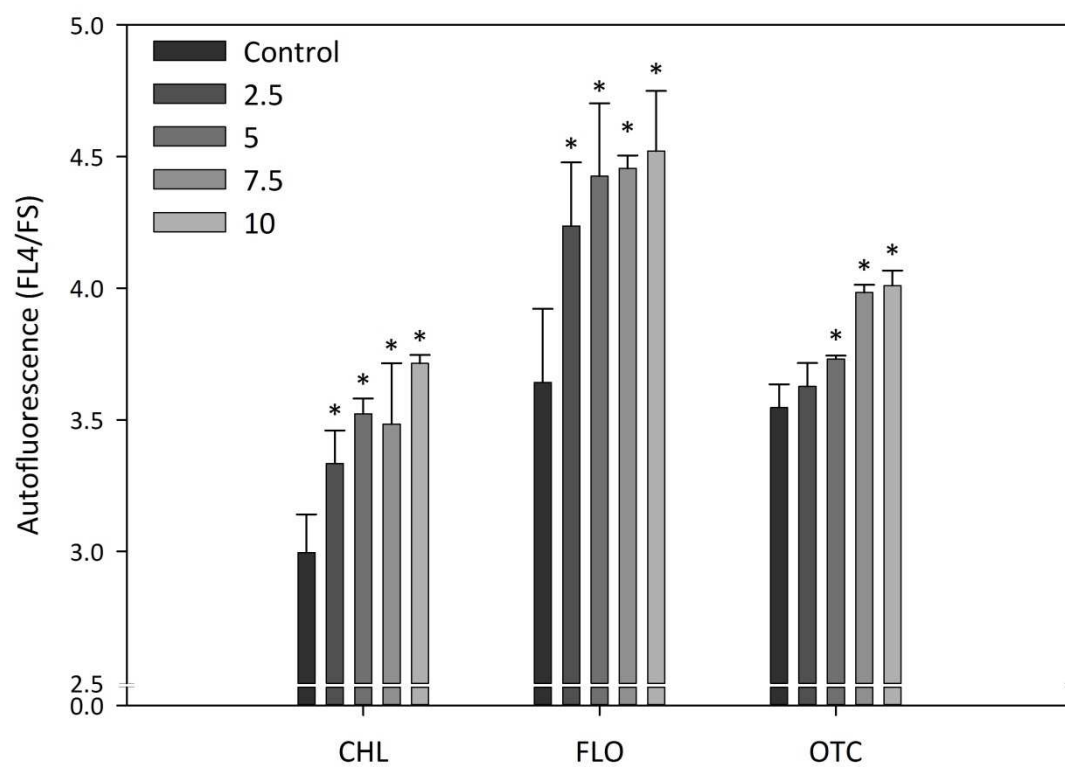


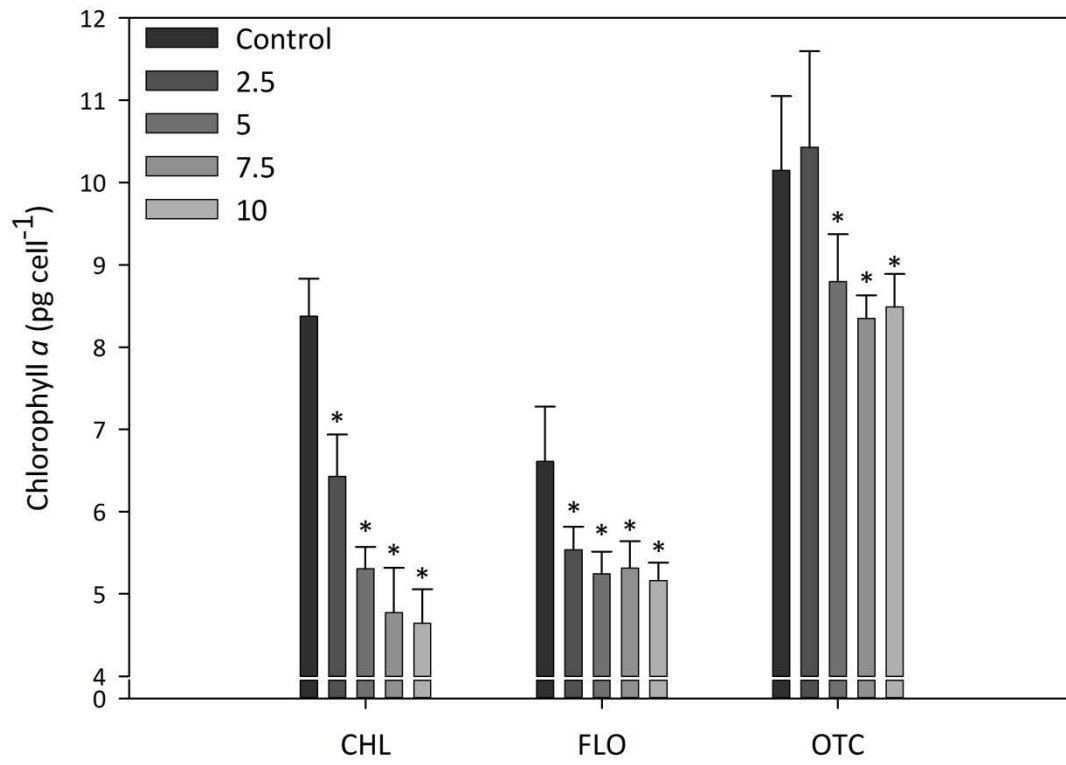
OTC











Highlights

Antibiotics assayed cause adverse effects on the microalgae *Tetraselmis suecica*.

The most affected parameters were those related to photosynthesis and growth.

Based on the IC₅₀ values obtained toxicity can be ranked as FLO > CHL > OTC.

Flow cytometry provides additional and accurate information for this type of studies.