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Oxidative stress responses and cellular energy allocation changes in microalgae following exposure to widely used human antibiotics

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ABSTRACT

The individual effect of four human antibiotics on the microalgae *Raphidocelis subcapitata* was investigated following a 120-h exposure. The effects were assessed by analyzing growth, and biochemical parameters related with: 1) antioxidant capacity and oxidative damage by measuring superoxide dismutase (SOD) activity and lipid peroxidation (LPO) levels; and 2) cellular energy allocation (CEA) by quantifying the content in energy reserves, which represents the energy available (*Ea*), and the electron transport system activity that represents a measure of oxygen and cellular energy consumption (*Ec*). Growth yield inhibitory concentrations of sulfamethoxazole (18–30%), clarithromycin (28.7%), ciprofloxacin (28%) and erythromycin (17–39%) were found to elicit a considerable increase in Ec, thereby causing a significant decrease in the CEA. The elevated *Ec* can be a result of the need to respond to oxidative stress occurring under those conditions given the significant increase in SOD activity at these levels. For sulfamethoxazole, erythromycin and ciprofloxacin, the antioxidant responses do not seem to be enough to cope with the reactive oxygen species and prevent oxidative damage, given the elevated LPO levels observed. A stimulatory effect on growth yield was observed (up to 16%) at ciprofloxacin lowest concentration, which highly correlated with the increase in CEA. Based on the no observed effect concentration (NOECs) and/or effective concentration (EC_{10}) results, *Ec*, SOD and CEA were more sensitive than the classical endpoint of growth rate for all the tested antibiotics. By revealing the antibiotic stress effects in *R. subcapitata* at the cellular level, this study suggests CEA as a more reliable indicator of the organisms' physiological status.

1. Introduction

The wide use of antibiotics and their occurrence in the aquatic environment has been recognized as one of the emerging global environmental issues (Hernando et al., 2006). Antibiotics are bioactive molecules with an increasing use in both human and veterinary medicine for the prevention or treatment of microbial infections. It has been reported that the largest number of antibiotics used by humans in most countries consist of β-lactam antibiotics, including the sub-groups of penicillins and cephalosporins followed by sulfonamides, macrolides and fluoroquinolones (Kummerer, 2009; ISD Scotland, 2017). From the antibiotics administered to humans, a large portion (approximately 70%) is excreted unmetabolized into municipal effluents and sewage treatment plants (STPs) as active compounds (Kummerer, 2009). The non-degradability of many antibiotics under aerobic conditions coupled with the inadequacy of STPs to remove them completely allows their entry into the aquatic environment via the sewage system (Halling-Sorensen et al., 1998). Although the recorded environmental levels are usually low, at ng/L to ug/L in waters, they are "pseudopersistent" contaminants due to their continued release into the environment and permanent presence (Hernando et al., 2006).

The major concern of antibiotics, even at low concentrations, is associated with the development of resistance mechanisms by bacteria and its implications in human health (Gullberg et al., 2011). However, their bioactive nature coupled with continuous introduction into different environmental media have raised serious concerns about their toxicity to non-target organisms (Orias and Perrodin, 2013; Johnson et al., 2015; Magdaleno et al., 2015). Microalgae as primary producers play a vital role in oxygen production in the aquatic ecosystems and occupy the lowest trophic level in food webs. Changes in their diversity and abundance could have an indirect but significant effect on the organisms at the higher trophic levels (Li et al., 2006). It has been reported that among river organisms, blue-green algae (cyanobacteria) are the most sensitive (EC_{50} less than 0.1 mg/L) followed by the green algae (EC_{50} between 0.3 and > 1200 mg/L) to the toxic effects of antibiotics (Lai et al., 2009; Gonzalez-Pleiter et al., 2013). The green algae

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are eukaryotes and the cyanobacterial nature of their plastid genome and pathways makes their chloroplast susceptible as potential antibiotic target (McFadden and Roos, 1999). Antibiotic toxicity to green algae could therefore be related to the inhibition and interference of the chloroplast metabolism such as photosynthetic procedures and interrelated protein synthesis, which disturb the function of photosynthetic apparatus and finally affect cell growth (Liu et al., 2011).

This study examined four individual antibiotics (erythromycin (ERY), clarithromycin (CLA), ciprofloxacin (CPX), and sulfamethoxazole (SUF)) selected from a wide range of pharmaceuticals monitored in hospital wastewater in the European Union funded PILLS project (Helwig et al., 2013). Selection was based on hospital contribution, European wide usage and persistence in the environment (Helwig et al., 2013). These antibiotics are used in the treatment of a variety of bacterial infections and CPX inhibits bacterial DNA gyrase and prevent DNA replication, CLA and ERY inhibits protein synthesis by binding to the 23S rRNA molecule of the bacterial ribosome while SUF inhibits bacterial folic acid synthesis (Van der Grinten et al., 2010; Gonzalez-Pleiter et al., 2013; Magdaleno et al., 2015). The studied antibiotics, due to their consumption, discharge, persistence and toxic properties, have been identified as antibiotics of high risk in the aquatic environment of Europe, USA, and Worldwide (Jones et al., 2002; Lienert et al., 2007; Besse and Garric, 2008; UBA, 2010; Hughes et al., 2013; Ortiz de Garcia et al., 2013).

The potential impact of a stressor in ecosystems requires the observation of effects at different levels of biological organization, starting at the molecular level and ending at the population or community level (Lemos et al., 2010; Connon et al., 2012). Many of the studies on the ecotoxic effects of pharmaceuticals are focused on the organismal or higher levels of biological organization and at such levels alone, the mechanisms of toxicity of the drugs are poorly understood and the predictive ability of measurements done is limited (Verslycke et al., 2004b). Over the last decades, biomarkers at suborganismal levels have been considered viable measures of responses to stressors (Huggett et al., 1992; Ferreira Nuno et al., 2015).

Changes in the antioxidant systems and modified macromolecules have served as biomarkers for a variety of xenobiotics (Gil and Pla, 2001). To prevent the damage induced by free radicals (products of cellular metabolism) to cells under oxidative stress, aerobic organisms have developed antioxidant enzyme defences such as superoxide dismutase (SOD). SOD is involved in the reduction of superoxide radical into hydrogen peroxide (H_2O_2) (Van Camp et al., 1994), which readily become broken down by CAT into water and oxygen (De Zwart et al., 1999). Failure of these defences to detoxify excess reactive oxygen species (ROS) can lead to significant oxidative damage including lipid peroxidation (LPO) (Soto et al., 2011).

Other types of biomarkers that have been used successfully are those linked to metabolism and energetics (Verslycke et al., 2004a). According to De Coen et al. (1995), the allocation of specific amounts of energy to basal metabolism, growth, and reproduction in an organism will differ in response to changing environmental conditions and exposure to a pollutant could cause a disturbance in the allocation. Based on this concept, single integrated bioassay such as the cellular energy allocation (CEA) assay was developed as a biomarker tool to evaluate the effects of toxic stress on the metabolic balance or net energy budget of organisms (De Coen et al., 1995; Verslycke et al., 2004a). The difference between available energy reserves (based on the biochemical analysis of total carbohydrate, total lipid, and total protein content) and energy consumption (estimated by measuring the electron transport system activity (ETS) at the mitochondrial level) has been shown to be indicative of an organism's overall condition (De Coen and Janssen, 2003a).

The aim of this study was to link the selected antibiotic effects in green algae at the cellular level to an outcome at the organismal level of organization, for example growth inhibition, elucidating mechanisms of toxicity for these compounds. This study was therefore carried out to investigate the effects of SUF, ERY, CLA, and CPX by assessing antibiotic effects on (1) growth yield and growth rate of the green microalgae, *R. subcapitata*; and (2) biochemical parameters associated with antioxidant capacity (SOD), oxidative damage (LPO) and cellular energy allocation (CEA).

2. Materials and methods

2.1. Microalgal culture and growth inhibition test

Axenic unicellular cultures of *R. subcapitata* (CCAP 278/4) purchased from Culture Collection of Algae and Protozoa (CCAP) were cultivated in 500 mL conical flasks containing sterile Jaworski's Medium (JM). Cultures were maintained at 120 rpm on an orbital shaker in an environmental chamber at 20 ± 1°C under continuous illumination in the range 40–50 μmol m^{-2} s^{-1} of photosynthetic active radiation. To keep the cultures in an exponential growth phase, algae were aseptically transferred to fresh media every 3–4 days.

Stock solutions of the test antibiotics, SUF (CAS no. 723–46-6); ERY hydrate (CAS no. 114-07-8); CLA (CAS no. 81103-11-9) and CPX (CAS no. 85721-33-1) purchased from Sigma with ≥ 95% purity were prepared directly in JM fresh algal medium, immediately before each toxicity test. Sublethal concentrations of each antibiotic were individually tested against *R. subcapitata*: 0, 0.24, 1.97, 3.95 and 13.83 μM of SUF; 0, 8×10^{-3}, 1.19×10^{-2}, 1.7×10^{-2} and 4.08×10^{-2} μM of ERY; 0, 1.3×10^{-3}, 3.3×10^{-3}, 4.6×10^{-3} and 7.3×10^{-3} μM CLA; and 0, 3.02, 6.04, 12.08, and 24.17 μM of CPX. Methanol and hydrochloric acid (HCl) were used as solvents for CLA and CPX respectively. Solvent controls were used in CPX and CLA assays. In the case of CLA, the final concentration of methanol in the assay media was ≤ 0.0005% (v/v) while the final concentration of HCl in the assay media in CPX exposure was ≤ 0.00146% (v/v). Although not shown, both concentrations did not result in any significant effects on the growth of the test organism. The pH of the test media was monitored before and after the tests.

To generate enough intracellular materials for biomarker studies, the bioassays were carried out in 100 mL Erlenmeyer flasks each containing 45 mL of test solution. Tests were performed in accordance with OECD Test Guideline 201 (OECD, 2006) with minor modifications. In each flask, a specified volume of the culture of *R. subcapitata* in exponential growth phase was diluted with a known volume of JM (with or without pharmaceuticals), to obtain equal amounts of initial cell biomass in the range 1.5×10^5 to 3.0×10^5 cells/mL in both the treatments and the control groups. Each concentration of the pharmaceutical and the control was tested using seven replicates (n = 7). The tests were run for 120 h under the same standard conditions used for the inoculum culture. The positions of test flasks were randomized and changed every 24 h for uniform distribution of light (USEPA (United States Environmental Protection Agency), 2002). Cell growth was determined every 24 h using an automatic cell counter (Micro Counter® 1100, Celeromics) in bright field configuration. The pH of the test media in each treatment and the control groups were measured at the commencement and at the end of the experiment and were between 7.35–7.82. To determine the stability of the pharmaceuticals in the test systems, samples were taken at the 0, 48, and 120 h and then stored at -20 °C until further analysis.

2.2. Biochemical biomarkers determination

2.2.1. Cell harvesting, disruption, and enzyme extraction

At the end of exposure period, algal cultures were harvested in 50 mL sterile tubes following centrifugation at 5000 *g* for 10 min. The resultant pellet was then resuspended in 300 μL of 50 mM sodium phosphate buffer (pH 7) containing 1 mM phenylmethylsulfonyl fluoride (PMSF). For homogenization, suspension was transferred to tubes containing 300 μL of 0.42–0.6 mm glass beads (Sigma) and algal