Marine Environmental Research 101 (2014) 1–7

Contents lists available at ScienceDirect

Marine Environmental Research

journal homepage: www.elsevier.com/locate/marenvrev

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

ARTICLE INFO

Article history:
Received 16 May 2014
Received in revised form
24 July 2014
Accepted 29 July 2014
Available online 7 August 2014

Keywords:
Antibiotics
Cell activity
Cell viability
Growth inhibition
Microalga
Photosynthesis
Toxicity

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 IC_{50} values of 11.16, 9.03 and 17.25 mg L^{-1} for CHL, FLO and OTC, respectively. After 24 h 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 h of antibiotics addition. It can be concluded that *T. suecica* was sensitive to the three antibiotics tested.

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, anaesthetics 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 μg L^{-1}) (Gulkowska et al., 2007; Isidori et al., 2005; Xu et al., 2007) these drugs are considered "pseudopersistant" 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

* Corresponding author. Tel.: +34 981 167 000.
E-mail addresses: angeles.cid@udc.es, cid@udc.es (Á. Cid).

http://dx.doi.org/10.1016/j.marenvres.2014.07.011
0141-1136/

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; De Lorenzo and Fleming, 2008; 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, 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 and 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, *T. 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 μmol photon m^{-2} 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 h during the 96 h 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).