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Antibiotics choice: procedure for establishment of *Conticribra weissflogii* and *Isochrysis galbana* cultures

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Abstract. For both phytoplankton species *Conticribra weissflogii* and *Isochrysis galbana* the antibiotics treatments 0.63 g L^{-1} penicillin G + 0.25 g L^{-1} streptomycin and 0.32 g L^{-1} penicillin G + 0.83 g L^{-1} streptomycin resulted in cell growth enhancement compared to control. We suggest the application of these treatments to improve marine microalga cultures.

Key words: algae, bacteria, marine culture, microbiology, phytoplankton

Resumo: Escolha de antibióticos: procedimento para estabelecimento de cultivos de *Conticribra weissflogii* e *Isochrysis galbana*. Para ambas as espécies de fitoplâncton, *Conticribra weissflogii* e *Isochrysis galbana*, os tratamentos de antibióticos 0,63 g L^{-1} de penicilina G + 0,25 g L^{-1} de estreptomicina e 0,32 g L^{-1} de penicilina G + 0,83 g L^{-1} estreptomicina resultaram em um aumento do crescimento celular comparado ao controle. Sugerimos a aplicação destes tratamentos para beneficiar cultivos de microalgas marinhas.

Palavras-chave: algas, bactérias, cultivo marinho, microbiologia, fitoplâncton

The establishment of microalgae cultures is required in different fields, such as biology and ecology, involving genetic, cytology, morphology, physiology, toxicology and taxonomy studies, in studies focused on phytoplankton-bacteria interactions (hypothesis testing) or even to enhance or treat organisms used as food in aquaculture (Shishlyannikov *et al.* 2011, Molina-Cárdenas *et al.* 2016). Cultures at high densities in combination with dead cells and high loads of organic matter, however, stimulate the selection and growth of opportunistic bacteria (Olafsen 2003, Vadstein *et al.* 1993, Agostini *et al.* 2018). In cultures of microalgae, bacteria benefit from dissolved organic matter excreted by algae (*i.e.*, exudates) and actively compete for the same resources needed for growth and survival of phytoplankton (Hamdan & Jonas 2007, Han *et al.* 2016). In this way, the use of specific prokaryotes inhibitors as antibiotics may be a valuable method to improve microalgae growth, as well as for the study of microalgae-bacteria interactions (Trottet *et al.* 2011). Many studies employed antibiotics in microalgae cultures (Spencer 1952, Droop 1967, Hamdan & Jonas 2007, Pringault *et al.* 2007, Molina-Cárdenas *et al.* 2016), however, most of them did not present any data evaluating the effects of these substances on the species of interest. Moreover, just because a particular antibiotic benefits a particular species, does not mean that this same substance will have the same effect on a different species, even considering that they are taxonomically close (Windler *et al.* 2012). Therefore, this study aimed to evaluate the effects of different antibiotic combinations on cultures of *Conticribra weissflogii* (Bacillariophyceae) (Grunow) Stachura-Suchoples

and Williams, 2009 and *Isochrysis galbana* (Prymnesiophyceae) Parke 1949 in initial cultures, evaluating their effects on growth and discuss their suitability for routine procedures to intensify microalgae growth in laboratory.

This experiment was conducted at the Laboratory of Zooplankton of the Federal University of Rio Grande during seven days (168 h) and used 24 flasks (200 mL previously autoclaved) for each microalga. Each flask contained 100 mL of culture medium and represented eight treatments with three replications each, of a single strain of each species (Table I). The experiment was conducted in initial cultures (first seven days), because they were not nutrient limited.

The antibiotics treatments (Table I) chosen have already been successfully applied in planktonic cultures, however, with other objectives, such as the hypothesis test involving the role of bacteria in the community (Yetka & Wiebe 1974, Sherr *et al*. 1986, Pringault *et al*. 2007) and the improvement of invertebrate larvae cultures (Bojerevic 1966, Tighe-Ford *et al*. 1970). In microalgae cultures, only the antibiotic treatments of Spencer (1952) and Droop *et al*. (1967) had already been tested, although for different species.

In this study we decided to test combinations of antibiotics instead of using a single substance, because when used in combination, antibiotics ensure the broad spectrum of bacterial inhibition, being more efficient than individual antimicrobials (Trottet *et al*. 2011, Agostini *et al*. 2016, 2018, Lopes *et al*. 2018). Both microalgae species were cultivated in F/2 culture medium (Guillard 1975) at temperatures of 25 ± 1 °C, using a photoperiod of 12:12 (L:D) with artificial light (70 µmol photons $s^{-1}m^{-2}$) in a DBO incubator (Marconi 403) with salinities of 30 and 28, for *C. weissflogii* and *I. galbana*, respectively. The initial concentrations of *C. weissflogii* and *I. galbana* used in the experiment were 1,406 cells mL^{-1} and 188 cells mL^{-1}, respectively. The initial concentrations were obtained by dilution of 400 mL of the strain of each species in 2 L of the respective culture medium. For the test, the same culture conditions were applied, except that the photoperiod was changed to 14 L:10 D to intensify cellular growth. Because no aerators were used, the vials were manually shaken every six hours to prevent cell sedimentation.

To check for changes in cells density, 2 mL samples of each culture were removed daily after manual homogenization. The collected material was deposited in Eppendorf vials and fixed with lugol (1 %) (Throndsen 1978). Cell counts were made in a Neubauer chamber using an optical microscope (Olympus CH2) with a 40× final magnification. Cell density, specific growth rates (µ), doubling per day (k) and doubling time ($Time_2$) estimation followed Wood *et al*. (2005).

General Linear Model (GLM) analysis was performed for each species, using the software R (R Development Core Team 2016). The model used was adapted to a Poisson distribution with "log" link function. Post-hoc Tukey tests followed the analyses. After the experiments, the culture medium with antibiotics were stored in sealed containers and disposed by SANIPLAN©, an Engineering and Environmental Services company.

The results indicate the resistance of *Conticribra weissflogii* and *Isochrysis galbana* to most of the antibiotic combinations and concentrations tested (see the growth rate and doubling time between the control and the treatments for both species in Electronic Supplementary Material - ESM Table I). For *C. weissflogii*, the greatest cell growth occurred in treatment T5 (5250±819 cells mL^{-1}) at 168 h, followed by T2 (5072±668 cells mL^{-1}) and T7 (4792±496 cells mL^{-1}), respectively, with 22, 18 and 12 % more cells than the control (4292±235 cells mL^{-1}) (Fig. 1).

Table I. Summary of treatments tested for *Conticribra weissflogii* and *Isochysis galbana* cultures.

Abbreviation	Treatments
Control	no antibiotics
T1	0.1 g L^{-1} of penicillin G potassium + 0.1 g L^{-1} of streptomycin sulphate[1]
T2	0.015 g L^{-1} of penicillin G potassium + 0.025 g L^{-1} of streptomycin sulphate[2]
T3	0.05 g L^{-1} of neomycin sulphate[3]
T4	0.5 g L^{-1} of penicillin G potassium + 0.5 g L^{-1} of streptomycin sulphate + 0.1 g L^{-1} of chloramphenicol[4]
T5	0.63 g L^{-1} of penicillin G potassium + 0.25 g L^{-1} of streptomycin sulphate[5]
T6	0.2 g L^{-1} of vancomycin sulphate + 0.001 g L^{-1} of penicillin G potassium[6]
T7	0.32 g L^{-1} penicillin G potassium + 0.83 g L^{-1} of streptomycin sulphate[7]

[1]Pringault *et al.* (2007); [2]Tighe-Ford *et al*. (1970); [3]Yetka & Wiebe (1974); [4]modified of Droop (1967); [5]Bojerevic (1966); [6]Sherr *et al*. (1986); [7]Spencer (1952)

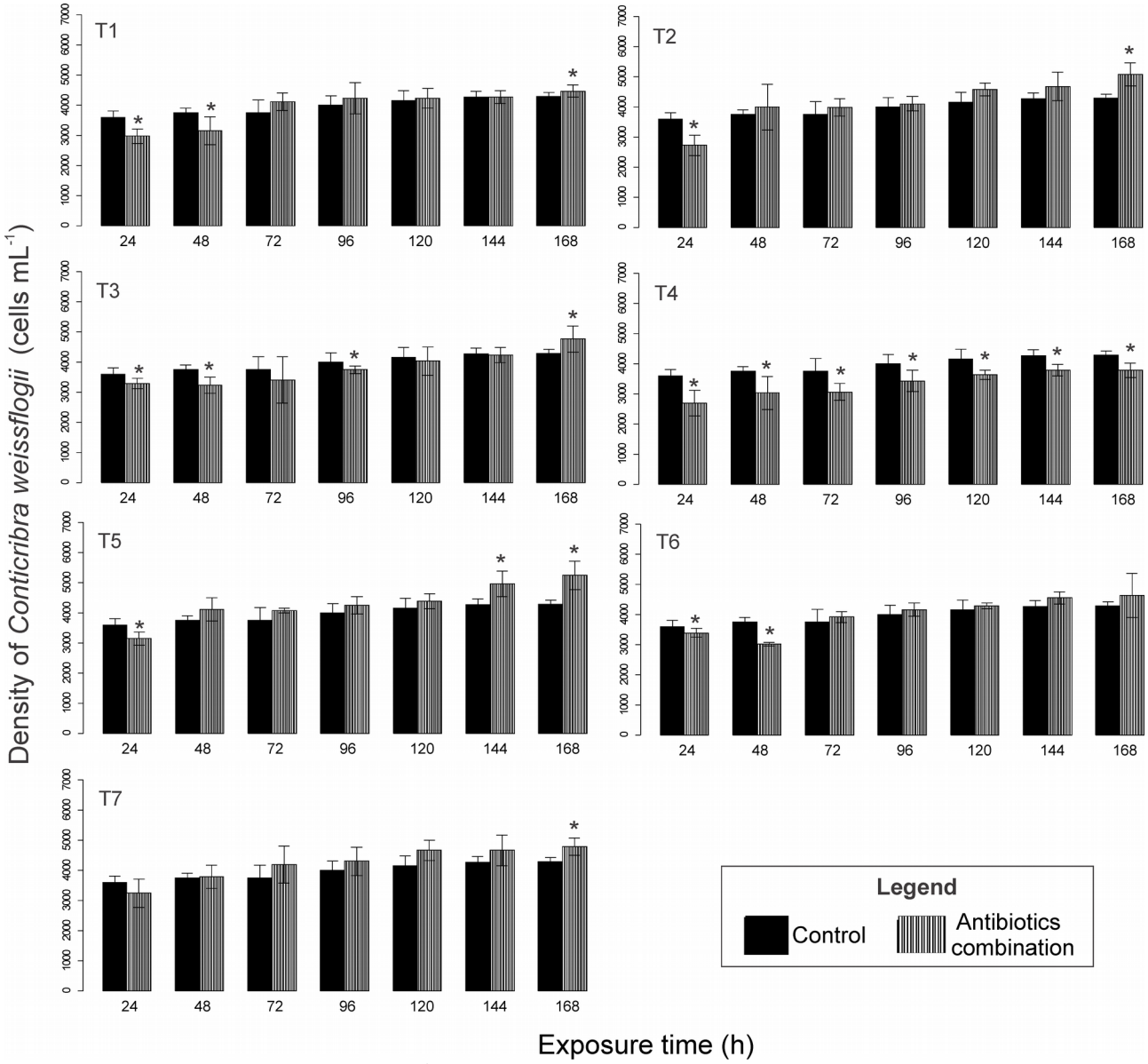

Figure 1. *Conticribra weissflogii* (cells mL^{-1}) densities in the control and each antibiotic treatment at different exposure times. T1) 0.1 g L^{-1} of penicillin G potassium + 0.1 g L^{-1} of streptomycin sulphate, T2) 0.015 g L^{-1} of penicillin G potassium + 0.025 g L^{-1} of streptomycin sulphate, T3) 0.05 g L^{-1} of neomycin sulphate, T4) 0.5 g L^{-1} of penicillin G potassium + 0.5 g L^{-1} of streptomycin sulphate + 0.1 g L^{-1} of chloramphenicol, T5) 0.63 g L^{-1} of penicillin G potassium + 0.25 g L^{-1} of streptomycin sulphate, T6) 0.2 g L^{-1} of vancomycin sulphate + 0.001 g L^{-1} of penicillin G potassium, and T7) 0.32 g L^{-1} of penicillin G potassium + 0.83 g L^{-1} of streptomycin sulphate. The vertical bars denote standard errors and * denotes = significant results (p<0.05) compared to control.

For *I. galbana*, the T6 (3000±691 cells mL^{-1}), T5 (2677±734 cells mL^{-1}) and T7 (2542±488 cells mL^{-1}) treatments showed the highest average cell densities at the end of the experiment with 64, 46 and 39 % more than the control (1833±237 cells mL^{-1}), respectively (Figure 2). Statistical differences among the control and the antibiotics treatment for both species are shown in ESM Table II.

Regarding to variations in cells densities, it was possible to observe the continuous growth of cells over time in all treatments, with the number of cells at the end of the experiment being between 2.5 and 4 times higher for *C. weissflogii* and between 7 and 16 times higher for *I. galbana*, when compared to the values at the beginning of the experiment. It was observed that the yield percentage (cell growth) in relation to the exposure time for all treatments between 0 to 24 hours resulted in the highest percentage of cell growth, being 53 % for *C. weissflogii* and 62 % for *I. galbana*.

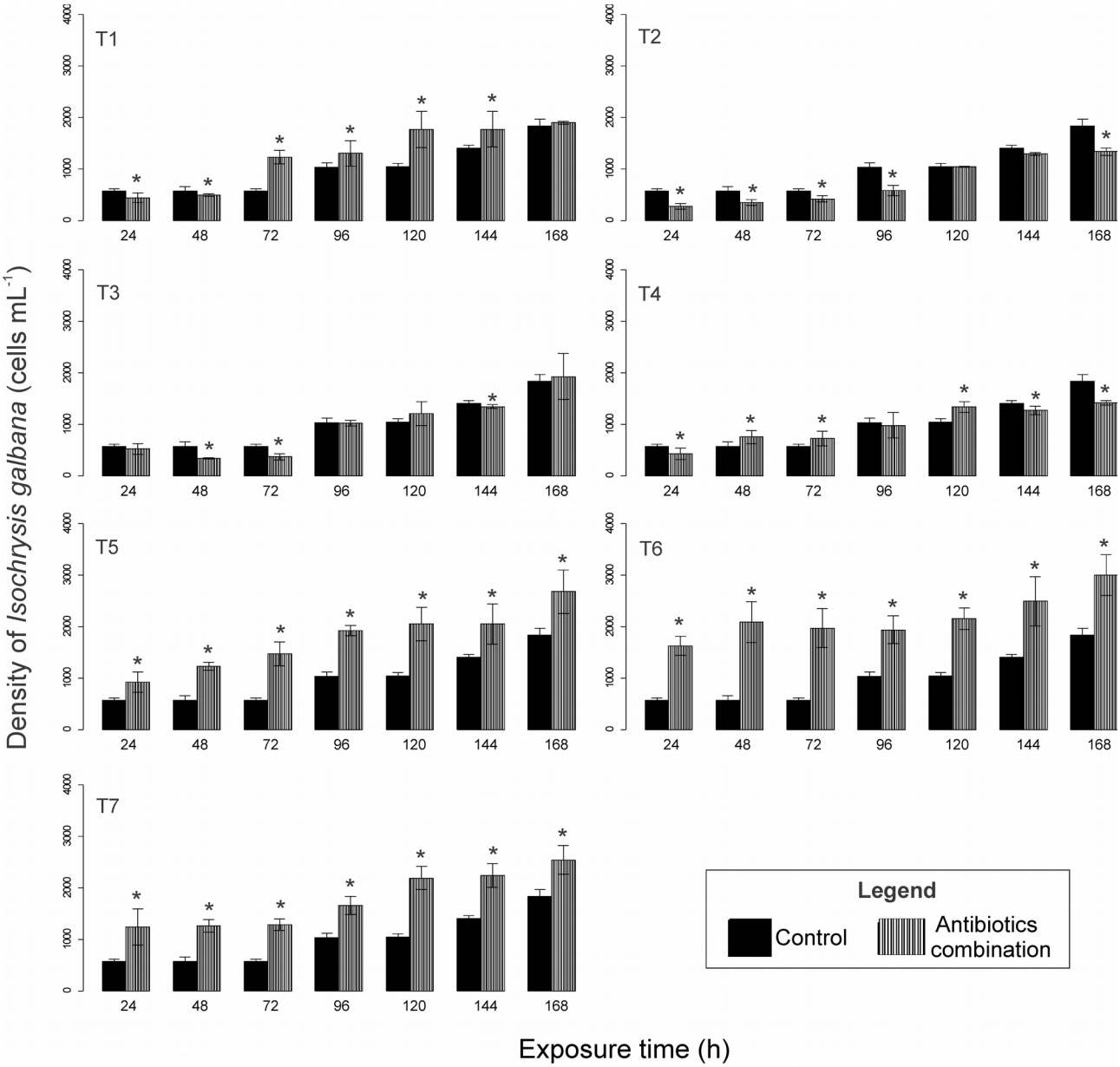

Figure 2. *Isochrysis galbana* (cells mL^{-1}) densities in the control and each antibiotic treatment at different exposure times. T1) 0.1 g L^{-1} of penicillin G potassium + 0.1 g L^{-1} of streptomycin sulphate, T2) 0.015 g L^{-1} of penicillin G potassium + 0.025 g L^{-1} of streptomycin sulphate, T3) 0.05 g L^{-1} of neomycin sulphate, T4) 0.5 g L^{-1} of penicillin G potassium + 0.5 g L^{-1} of streptomycin sulphate + 0.1 g L^{-1} of chloramphenicol, T5) 0.63 g L^{-1} of penicillin G potassium + 0.25 g L^{-1} of streptomycin sulphate, T6) 0.2 g L^{-1} of vancomycin sulphate + 0.001 g L^{-1} of penicillin G potassium, and T7) 0.32 g L^{-1} of penicillin G potassium + 0.83 g L^{-1} of streptomycin sulphate. The bars denote standard errors and * denotes = significant results (p<0.05) compared to control.

In this study, some aspects must be considered about the different responses of *Isochrysis galbana* and *Conticribra weissflogii* microalgae to the same treatments. Regarding to cell density, it seems that treatment T2 was detrimental to *I. galbana* and beneficial to *C. weissflogii* because it significantly inhibited the growth of *I. galbana* by 27 % and improved the growth of *C. weissflogii* by 18 % when compared with the control. Most algae can tolerate higher concentrations of antibiotics than bacteria during short exposure times (Lai *et al.* 2009, Windler *et al.* 2012). However, microalgae differ in their sensitivity, and closely related species can have very different tolerances (Droop 1967, Windler *et al.* 2012). So, before using antibiotics in cultures of planktonic organisms, a test with the species of interest is recommended.

It was also observed that the T4 treatment had a final cell growth 12 % lower than the control for *C. weissflogii* and 23 % lower for *I. galbana*. This lower cell density could be related to the presence of chloramphenicol. Cottrell & Suttle (1993), Youn & Hur (2007), and Lai *et al.* (2009) determined the extent of antibiotics' effects on microalgal cultures and observed that lethal effects were common when chloramphenicol was employed. On the other hand, Campa-Córdova *et al.* (2006) found that chloramphenicol and erythromycin, when used individually in cultures, did not affect the growth of *I. galbana*.

Another important aspect of the results was the beneficial effect observed on the cell growth of *C. weissflogii* and *I. galbana* especially in the T5 and T6 treatments respectively. Cultures of microalgae free of bacteria excludes competition between both groups of microorganisms, boosting microalgae growth (Molina-Cárdenas *et al.* 2016).

Based on those results it was noteworthy that the T5 treatment was latter applied to cultures of *Thalassiosira* sp., *Chaetoceros muelleri* Lemmermann 1898, *C. calcitrans* (Paulsen) Takano 1968, *Asterionellopsis guyunusae* Luddington, and *Anaulus* cf. *australis* Drebes and Schulz held at the Laboratory of Marine Phytoplankton and Microorganisms of the Federal University of Rio Grande, resulting in bacteria inhibition from cultures and an increase of microalgae growth rate (Lucélia Borges and Andréa Franco pers. comm). This combination had been previously used to improve Porifera larvae cultures (Bojerevic 1966), being this the first time that it is applied in microalgae cultures.

It was observed that the use of the combination of penicillin + vancomycin or vancomycin + neomycin, similar to treatment T6, ensured the complete inhibition of bacterioplankton, yet it did not cause any adverse effects in the species of interest (Sherr *et al.* 1986; Azma *et al.* 2006), corroborating the findings of the present work for both species.

The highest percentage of cell growth observed between 0 and 24 hours could be explained by the antibiotics half-life, that is short in aquatic systems (Agostini *et al.* 2016, 2018, Lopes *et al.* 2018). Maybe an antibiotic replacement after 24 hours could ensure microalgae culture improvement for a longer period.

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