ARTICLE

Decreasing of bacterial content in *Isochrysis galbana* cultures by using some antibiotics

Disminución del contenido de bacterias en cultivos de *Isochrysis galbana* utilizando algunos antibióticos

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Resumen.- La obtención de cultivos axénicos es una tarea laboriosa y resultan difíciles de mantener. Los cultivos de microalgas con carga bacteriana reducida pueden ser una alternativa a los cultivos axénicos de microalgas para producir compuestos con potencial biotecnológico y farmacéutico. También éstos pueden ser usados para la criopreservación, y en estudios bioquímicos, fisiológicos, ecológicos y genéticos. El propósito de este estudio fue desarrollar un protocolo para reducir la carga bacteriana en cultivos de *Isochrysis galbana* por medio de lavados por centrifugación y la administración de varios antibióticos (ampicilina, neomicina, kanamicina, cloranfenicol, sulfato G418, estreptomicina y carbencilina), adicionados en varias dosis y combinaciones. Todos los tratamientos fueron realizados por triplicado. La concentración de bacterias heterotróficas y la densidad de células de *I. galbana* fue evaluada diariamente. Se calculó la concentración no letal máxima y la concentración letal (LC$_{50}$). De forma individual, los antibióticos y los lavados por centrifugación no fueron efectivos para reducir la carga bacteriana, pero su combinación removió las bacterias de los cultivos. El máximo de sobrevivencia (84,6 ± 1,4%) y la reducción de la carga bacteriana en *I. galbana* fue efectiva con la combinación de 5 lavados por centrifugación y la administración de un coctel de ampicilina, kanamicina, neomicina, y estreptomicina por 48 h. Los valores de concentración no letal máxima varían entre 75 a 106 µg mL^{-1} y LC$_{50}$ se encontró entre 194 y 332 µg mL^{-1}, por lo anterior, este protocolo resulta ser un método efectivo y rápido para obtener cultivos de *I. galbana* con carga bacteriana baja.

Palabras clave: Carga bacteriana, *Isochrysis galbana*, antibióticos, centrifugación

Abstract.- The axenic microalgae cultures are a difficult task and they are hard to maintain. Microalgae cultures with reduced bacterial load can be an option to axenic microalgae cultures to produce compounds with biotechnological and pharmaceutical potential. Also they can be used for cryopreservation and in biochemical, physiology, ecology and genetic studies. The aim of this study was to develop a protocol to decrease the bacterial load in an *Isochrysis galbana* culture, through washes by centrifugation and the administration of various antibiotics (ampicillin, neomycin, kanamycin, chloramphenicol, sulphate G418, streptomycin, and carbencillin), at several doses and combinations. The concentrations of heterotrophic bacteria and *I. galbana* cell densities were monitored daily. Maximum non-lethal concentration and lethal concentration 50% (LC$_{50}$) were calculated. Individually, antibiotics and washes by centrifugation failed to reduce bacterial load, but their combination removed bacteria from the cultures. Peak survival (84.6 ± 1.4%) and reduction of bacterial load in *I. galbana* cultures were effected with the combination of 5 washes by centrifugation and administration of a cocktail, comprising ampicillin, kanamycin, neomycin, and streptomycin at 48 h. Values of maximum non-lethal concentration varied from 75 to 106 µg mL^{-1} and LC$_{50}$ between 194 and 332 µg mL^{-1}, thus, our protocol is an effective and rapid method of producing *I. galbana* cultures with reduced bacterial load.

Key words: Bacterial load, *Isochrysis galbana*, antibiotics, centrifugation

INTRODUCTION

Much of the search for new chemical compounds with biological activity has been performed using axenic or high purity microalgae cultures (Desbois *et al.* 2008, El-Sheek *et al.* 2008, Cho *et al.* 2013). As a microalgae culture increases in volume, its management becomes more complicated and is harder to keep axenic culture conditions. Therefore, contamination by other microalgae strains, protozoa, and bacteria loads can increase (Guillard 2005, Simonsson 2013). This surge of bacteria load in microalgae cultures causes several problems, increasing the susceptibility of the culture to the growth of other pathogens, such as *Vibrio*; degrading the nutritional quality of the microalgae; and becoming a vector of contamination to secondary cultures, such as zooplankton, larvae, or and organisms that are reared for aquaculture (Salvesen *et al.* 2000,

Gómez-Gil *et al.* 2004, Muller-Feuga *et al.* 2007). These kind of problems can affect *Isochrysis galbana*, which is one of the most widely used in aquaculture species, based on its high nutritional value and content of lipids and polyunsaturated fatty acids, such as docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) (Coutinho *et al.* 2006, van Bergeijk *et al.* 2010). This marine microalgal species is frequently supplied in aquaculture farms as feed for fish larvae, mollusks, and crustaceans (Wikfors & Patterson 1994, Subhash *et al.* 2004) and is used in biotechnology for the synthesis of bioactive compounds (Pulz & Gross 2004, Desbois *et al.* 2008, El-Sheek *et al.* 2008, Chen *et al.* 2012).

Working with high purity *I. galbana* cultures still remains a challenge. There are various physical and chemical methods that can be used to remove bacterial loads from microalgae cultures, such as washes by centrifugation (Makridis *et al.* 2006), Percoll gradients (Ruban *et al.* 2011), ultraviolet radiation (UV) (Chang *et al.* 1985, Munro *et al.* 1999) and growth in selective media (Guillard 2005). The efficiency of these techniques to remove bacteria from cultures is partial and the reduction in bacterial load may take several weeks. Thus, in order to improve the reduction of bacteria, may be necessary apply one of the most common approaches, the use of antibiotics (Cho *et al.* 2002, Vazquez-Martínez *et al.* 2004, Seoane *et al.* 2014). Certain protocols have been developed to reduce the bacteria load in cultures of *I. galbana*, but the results have not been consistent, depending on the mechanisms of action of the antibiotic and the characteristics of the bacterial load in every strain of *I. galbana* (Cho *et al.* 2002, Subhash *et al.* 2004, Youn & Hur 2007). Moreover, the response of cultures to antibiotics is species-specific, and tailored protocols should be developed for each species to obtain axenic microalgae cultures (Bruckner & Kroth 2009). Thus, it is necessary to establish a wide range of protocols with bactericidal activity.

The aim of this study was to propose a protocol to generate a reduced bacterial load of *Isochrysis galbana* culture through centrifugation rinses and the administration of various concentrations, doses, and cocktails of common antibiotics.

MATERIALS AND METHODS

MICROALGAE CULTURE

The *Isochrysis galbana* Parke strain was obtained from the Provasoli-Guillard National Center for Marine Algae and Microbiota, formerly the Center of Culture of Marine Phytoplankton (CCMP) (reference code CCMP 1323). *I. galbana* cells were cultured in 500-mL Erlenmeyer flasks with 300 mL 'f' media (Guillard 1975). The flask was irradiated at 110 µE m^{-2} s^{-1} and kept at 20°C for 10 days to generate a culture in the stationary growth phase.

ANTIBIOTICS

We used the antibiotics carbencillin (Sigma Aldrich), chloramphenicol (Bruluart Laboratories), sulphate G418 (J R Scientific, Inc.), ampicillin (Hormona Laboratories), neomycin (Sigma Aldrich), streptomycin (Sigma Aldrich), and kanamycin (Sigma Aldrich). Chloramphenicol was dissolved in ethanol, and the other antibiotics were dissolved in sterile distilled water. The concentrations and doses of the antibiotics are listed in Table 1.

PURIFICATION OF THE ALGAL STRAIN

The first step to reduce the bacterial load in the *I. galbana* culture was to wash the bacteria by centrifugation. Five-milliliter aliquots of *I. galbana* culture were placed in sterile 15-mL glass culture tubes and centrifuged at 2465 *g* (3500 rpm) and 4°C for 15 min. The supernatant was discarded, and the cell pellet was re-suspended in 5 mL sterile 'f' media. This process was repeated 3 times, and the pellet from the last wash was re-suspended in sterile 'f' media.

BACTERIAL LOAD REDUCTION ASSAYS

After the bacteria were washed by centrifugation, 4 assays were performed to reduce the bacterial load in the *I. galbana* cultures. Details of the antibiotics, concentrations, and doses are shown in Table 1. In the first assay, carbencillin and sulphate G418 were administered individually in single and repeat doses. Maximum non-lethal concentration and the concentration necessary to reduce in 50% the *I. galbana* cell density (LC$_{50}$) were obtained from doses-response curves data and linear regression analysis. Repeat doses in all assays were applied every 24 h for 3 days. A cocktail of these antibiotics was tested in single and repeat doses (Table 1).

In the second assay, higher concentrations of carbencillin and sulphate G418 in repeat doses were evaluated (Table 1). In the third assay, the effect of various concentrations of single-dose chloramphenicol was examined (Table 1). LC$_{50}$ and maximum non-lethal concentration were also calculated for this antibiotic from doses-response data curves and simple linear regression analysis.

The fourth assay was performed using 2 cocktails, in which 5 washes with sterile 'f' media and centrifugation were performed as described above. Cocktail 1 was composed of carbencillin, sulphate G418, and chloramphenicol. Cocktail 2 was a modification of that used by Cho *et al.* (2002), comprising ampicillin, kanamycin, neomycin, and streptomycin. Both cocktails were tested in single and repeat doses (Table 1). The tubes that contained the mixture of cells, 'f' media, and antibiotics were maintained at 20°C and irradiated at 110 µE m^{-2} s^{-1} for all

Table 1. Experimental design to reduce the bacterial load in *Isochrysis galbana* cultures / Diseño experimental para reducir la carga bacteriana en cultivos de *Isochrysis galbana*

Assay	Antibiotics	Concentration (μg mL^{-1})	Doses
1	Carbencillin	150	SD and RD
		200	SD and RD
		250	SD and RD
	Sulphate G 418	100	SD and RD
		125	SD and RD
		150	SD and RD
	Cocktail (Carbencillin + Sulphate G418)	200 of each one	SD and RD
2	Cocktail (Carbencillin + Sulphate G418)	400 Carbencillin + 250 Sulphate G418	RD
3	Chloramphenicol	150	SD
		200	SD
		250	SD
4	Cocktail 1 (Carbencillin + Sulphate G418 + Chloramphenicol)	400 Carbencillin + 250 Sulphate G418 + 200 Chloramphenicol	SD and RD
	Cocktail 2 (Ampicillin+ Kanamycin + Neomycin+ Streptomycin)	250 Ampicillin + 100 Kanamycin + 500 Neomycin + 50 Streptomycin	SD and RD

SD: single dose; RD: repeat doses

assays. Tubes that not were washed by centrifugation or given antibiotics and samples that were only washed by centrifugation were included as controls. All assays were performed in triplicate.

EVALUATION OF MICROALGAE SURVIVAL AND BACTERIAL LOAD

Isochrysis galbana cell density and bacterial load were measured at the beginning (0) and 24, 48, and 72 h after antibiotics were added. The response of *I. galbana* cells to the treatments was evaluated through daily counts with a hemacytometer by using compound microscope. These data were used to calculate the survival of *I. galbana* cells, expressed as percentage. Bacterial load was assessed by counts on the plate, seeding 100 μL of culture in Petri dishes with ZoBell media. Bacterial load was expressed in colony former units per milliliter (CFU mL^{-1}) (Molina-Cárdenas *et al.* 2014).

STATISTICAL ANALYSIS

To analyze the effects of antibiotics on *I. galbana* survival, student´s t-test was applied to initial and final cell densities, and one-way ANOVA was performed using Statistica 7.0 and Sigma Plot 10 software to generate the graphs. Differences in *I. galbana* cell density and survival were considered significant at $P < 0.05$, and for heterotrophic bacteria, differences were significant if they exceeded 4 orders of magnitude.

RESULTS

The initial mean cell densities of *Isochrysis galbana* after being washed by centrifugation were 5.5-6.5 x10^6 cells mL^{-1} but significantly higher in unwashed control groups ($P < 0.05$) (Table 2). None of the antibiotics reduced the concentration of heterotrophic bacteria to undetectable levels individually.

Table 2. Mean and standard deviation values of initial cell densities (ICD), final cell densities (FCD) and cellular survival expressed as percentage of *Isochrysis galbana* / Valores promedio y desviación estándar de la densidad de células inicial (ICD), final (FCD) y la supervivencia celular expresada como porcentaje de *Isochrysis galbana*

Assay	Antibiotic	Concentration (μg mL^{-1})	*I. galbana* ICD (x 10^6)	*I. galbana* FCD (x 10^6)	% Celular survival
1	Carbencillin	NWC control	7.7 ± 0.3 ^{a}	7.1 ± 0.1 ^{a}	91.9 ± 2.2 ^{a}
		WWC control	6.5 ± 0.1 ^{ab}	6.4 ± 0.1 ^{a}	90.0 ± 0.8 ^{a}
		150 SD	6.3 ± 0.3 ^{ab}	5.3 ± 0.1 ^{bc}	75.2 ± 1.7 ^{bc}
		150 RD	5.9 ± 0.3 ^{ab}	6.5 ± 0.7 ^{a}	92.5 ± 2.0 ^{a}
		200 SD	6.1 ± 0.3 ^{ab}	6.5 ± 0.3 ^{b}	71.1 ± 1.7 ^{b}
		200 RD	5.7 ± 0.1 ^{ab}	4.3 ± 0.5 ^{efg}	54.9 ± 8.6 ^{def}
		250 SD	5.8 ± 0.2 ^{ab}	5.4 ± 0.3 ^{def}	60.8 ± 6.7 ^{cd}
		250 RD	6.0 ± 0.3 ^{ab}	4.5 ± 0.1 ^{def}	63.8 ± 2.5 ^{cd}
	Sulphate G 418	100 SD	5.7 ± 0.1 ^{b}	4.8 ± 0.1 ^{bcd}	68.2 ± 1.6 ^{bc}
		100 RD	5.6 ± 0.2 ^{b}	4.6 ± 0.1 ^{cde}	65.8 ± 2.4 ^{c}
		125 SD	5.8 ± 0.4 ^{a}	4.6 ± 0.2 ^{def}	59.7 ± 1.0 ^{cde}
		125 RD	5.5 ± 0.3 ^{b}	3.9 ± 0.1 ^{cde}	64.9 ± 1.8 ^{cd}
		150 SD	5.9 ± 0.1 ^{ab}	4.2 ± 0.2 ^{efg}	55.5 ± 1.5 ^{de}
		150 RD	5.8 ± 0.3 ^{ab}	3.2 ± 0.2 ^{g}	45.4 ± 2.1 ^{f}
	Cocktail	SD	5.7 ± 0.1 ^{b}	4.3 ± 0.2 ^{def}	60.9 ± 3.5 ^{cd}
		RD	5.8 ± 0.4 ^{ab}	3.5 ± 0.1 ^{g}	49.9 ± 0.8 ^{ef}
2	Cocktail	NWC control	7.6 ± 0.3 ^{a}	7.1 ± 0.1 a	91.9 ± 2.2 ^{a}
		WWC control	6.5 ± 0.1 ^{b}	6.4 ± 0.1 ^{b}	90.0 ± 0.8 ^{a}
		RD	7.8 ± 0.2 ^{a}	5.9 ± 0.2 ^{c}	84.0 ± 3.4 ^{b}
3	Chloramphenicol	NWC control	5.9 ± 0.1 ^{ab}	8.1 ± 0.1 ^{a}	100.0 ± 0.0 ^{a}
		WWC control	5.8 ± 0.1 ^{b}	7.5 ± 0.1 ^{b}	92.1 ± 1.5 ^{b}
		150	6.2 ± 0.2 ^{a}	4.3 ± 0.1 ^{eb}	53.8 ± 1.3 ^{d}
		200	6.3 ± 0.1 ^{a}	5.1 ± 0.1 ^{c}	61.6 ± 1.9 ^{c}
		250	6.3 ± 0.1 ^{a}	4.9 ± 0.1 ^{d}	60.8 ± 1.5 ^{c}
4		NWC control	6.5 ± 0.1 ^{a}	8.2 ± 0.1 ^{a}	100.0 ± 0.0 ^{a}
		WWC control	6.2 ± 0.1 ^{a}	7.4 ± 0.4 ^{b}	90.1 ± 3.9 ^{b}
	Cocktail 1	SD	6.0 ± 0.1 ^{b}	4.0 ± 0.1 ^{d}	49.1 ± 0.8 ^{d}
		RD	6.0 ± 0.1 ^{b}	1.8 ± 0.1 ^{e}	22.3 ± 0.9 ^{e}
	Cocktail 2	SD	6.0 ± 0.1 ^{b}	6.9 ± 0.1 ^{b}	84.6 ± 1.4 ^{c}
		RD	6.1 ± 0.1 ^{ab}	6.5 ± 0.2 ^{c}	78.4 ± 2.2 ^{c}

Letters denote significant differences (one-way ANOVA; a>b>c>d). NWC: controls treatments without washes by centrifugation; WWC: Controls treatments with washes by centrifugation; SD: single dose; RD: repeat doses; n= 3
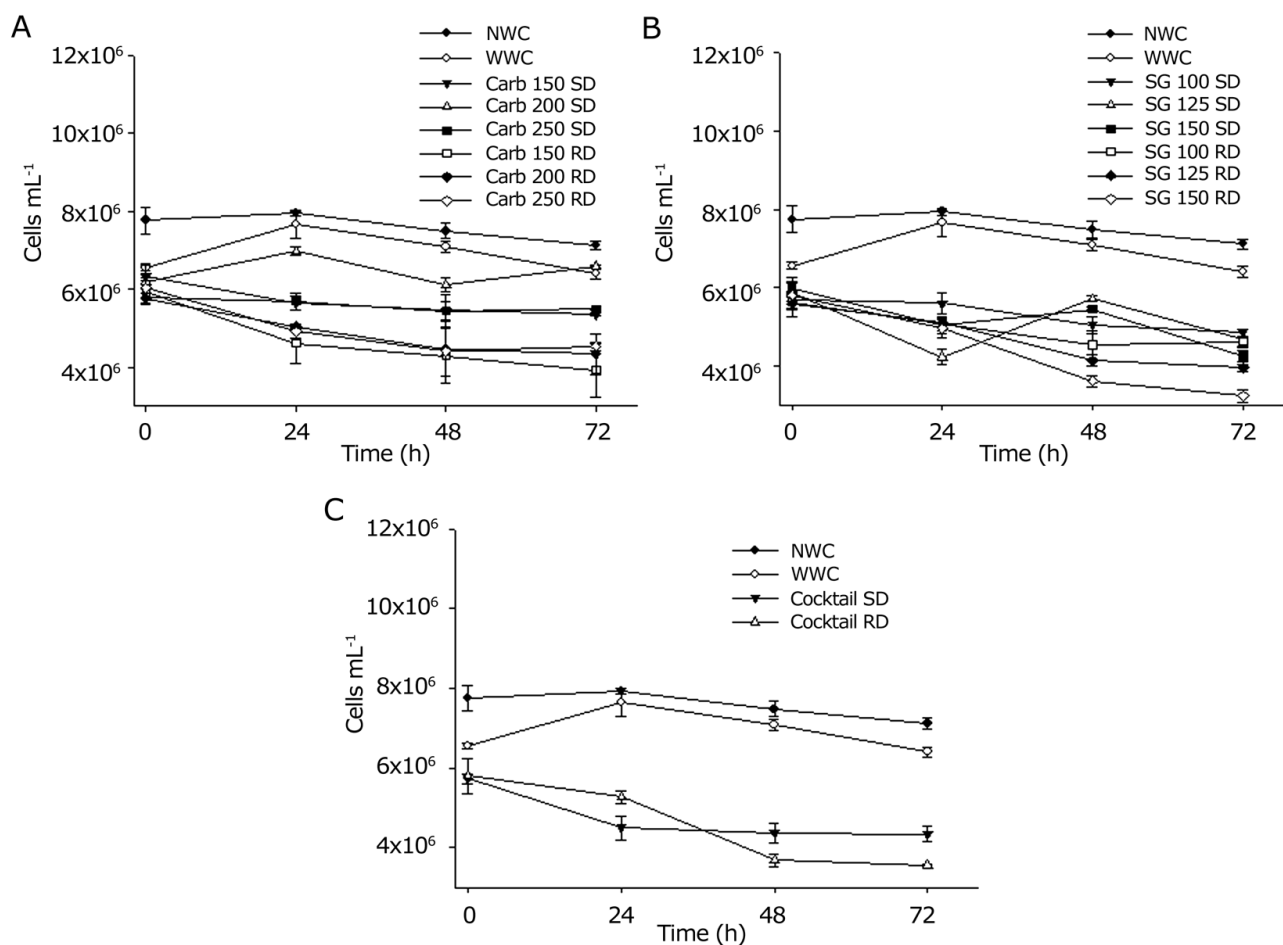
Molina-Cárdenas *et al.*
Reduction of bacterial load in *Isochrysis galbana*

Figure 1. Mean values and standard deviation of *Isochrysis galbana* cell density in assay 1, using 3 concentrations of carbencillin (Carbe) (A) in a single dose (SD) and repeat doses (RD); 3 concentrations of sulphate G418 (S G418) (B) in a single dose (SD) and repeat doses (RD); and a cocktail of 200 µg mL^{-1} carbencillin and 125 µg mL^{-1} sulphate G418 (C) in a single dose (SD) and repeat doses (RD). NWC: control treatments without washes by centrifugation; WWC: control treatments with washes by centrifugation / Valores promedio y desviación estándar de la densidad de células de *Isochrysis galbana* en ensayos usando 3 concentraciones de carbencilina (Carbe) (A) en dosis únicas (SD) y en dosis repetidas (RD); 3 concentraciones de sulfato G418 (S G418) (B) en dosis únicas (SD) y en dosis repetidas (RD); y un coctel de 200 µg mL^{-1} carbencilina and 125 µg mL^{-1} de sulfato G418 (C) en dosis únicas (SD) y en dosis repetida (RD). NWC: tratamiento control sin lavados por centrifugación; WWC: tratamientos control con lavados por centrifugación

However, the bacteria load declined with the cocktails. In the first assay, *I. galbana* cell densities decreased with all antibiotic treatments, bottoming with repeat doses of 150 µg mL^{-1} carbencillin (Fig. 1a) and repeat doses of 150 µg mL^{-1} sulphate G 418 (Fig. 1b); *I. galbana* cell survival rates were 54.9 ± 8.6% and 45.4 ± 2.4%, respectively (Table 2). For carbencillin, the maximum non-lethal concentration was 75 µg mL^{-1} and a LC$_{50}$ of 332 µg mL^{-1}. For sulphate G418, values of 287 µg mL^{-1} and 106 µg mL^{-1} were obtained for LC$_{50}$ and a maximum non-lethal concentration, respectively. The antibacterial effect of carbencillin and sulphate G 418 in this assay was insufficient to obtain a low bacterial load, wherein the concentrations still reached 10^6 CFU mL^{-1} (Figs. 2a-d).

A cocktail of 200 µg mL^{-1} carbencillin and 125 µg mL^{-1} sulphate G418 significantly lowered *I. galbana* cell density at the end of the experiment (Fig. 1c). Greater *I. galbana* cell survival was observed ($P < 0.05$) with single-dose treatments compared with repeat doses of carbencillin and sulphate G 418 (Table 2). In this assay, the bacterial concentration was 10^3 CFU mL^{-1} with treatments in which repeat doses of this cocktail were administered (Fig. 2e). These results prompted us to perform the second assay, doubling the concentration of both antibiotics and administering repeat doses.

In the second assay, we noted that *I. galbana* cell density with antibiotics declined to 5.9 ± 0.2 x10^6 cells mL^{-1} (Fig. 3a). The bacterial load as also decreased significantly, falling
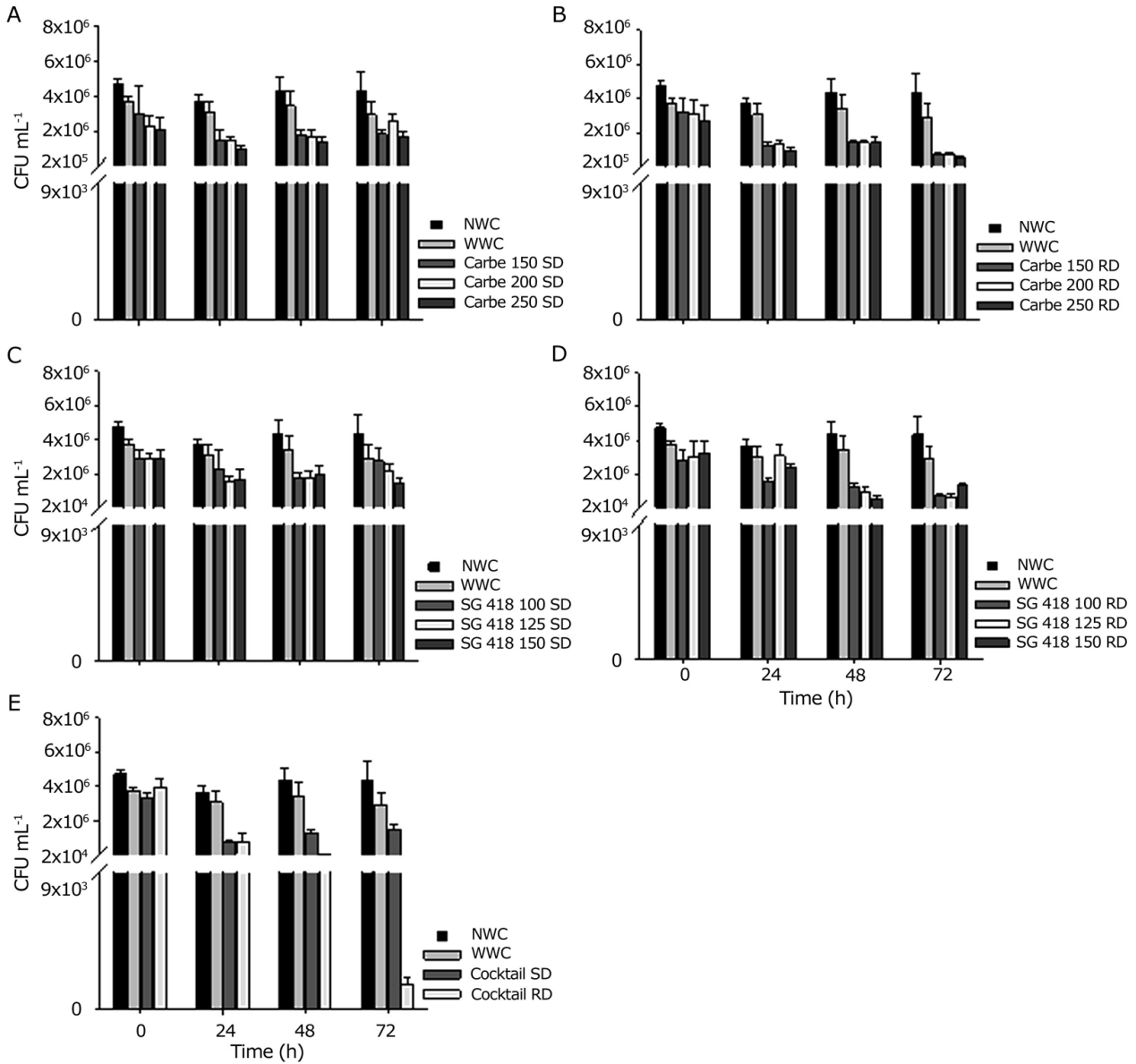
Figure 2. Mean values and standard deviation of concentration of heterotrophic bacteria in cultures of *Isochrysis galbana* in assay 1, using 3 concentrations of carbencillin (Carbe) in a single dose (SD) (A) and repeat doses (RD) (B); 3 concentrations of sulphate G418 (S G418) in a single dose (SD) (C) and repeat doses (RD) (D); and a cocktail of 200 µg mL^{-1} carbencillin and 125 µg mL^{-1} sulphate G418 (E) in a single dose (SD) and repeat doses (RD). NWC: control treatments without washes by centrifugation; WWC: control treatments with washes by centrifugation / Valores promedio y desviación estándar de la concentración de bacterias heterotróficas en cultivos de *Isochrysis galbana* en el ensayo 1, usando 3 concentraciones de carbencilina (Carbe) en dosis única (SD) (A) y en dosis repetidas (RD) (B), 3 concentraciones de sulfato G418 (S G418) en dosis única (SD) (C) y dosis repetidas (RD) (D), y un coctel de 200 µg mL^{-1} carbencilina y 125 µg mL^{-1} sulfato G418 (E) en una sola dosis (SD) y dosis repetidas (RD). NWC: tratamientos control sin lavados por centrifugación; WWC: tratamientos control con lavados por centrifugación
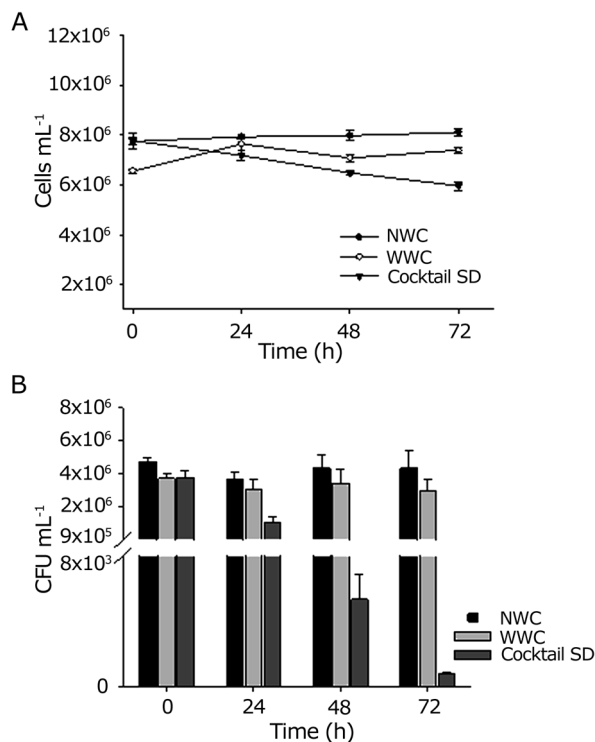
Figure 3. Mean values and standard deviation of *Isochrysis galbana* cell density (A) and concentration of heterotrophic bacteria (B) in the second assay, using a cocktail of 400 μg mL^{-1} carbencillin and 250 μg mL^{-1} sulphate G418 in single doses (SD). NWC: controls without washes by centrifugation; WWC: control treatments with washes by centrifugation / Valores promedio y desviación estándar de células de *Isochrysis galbana* (A) y la concentración de bacterias heterotróficas (B) en el segundo ensayo, usando un coctel de of 400 μg mL^{-1} de carbencilina y 250 μg mL^{-1} de sulfato G418 en dosis única (SD). NWC: tratamiento control sin lavados por centrifugación; WWC: tratamientos control con lavados por centrifugación
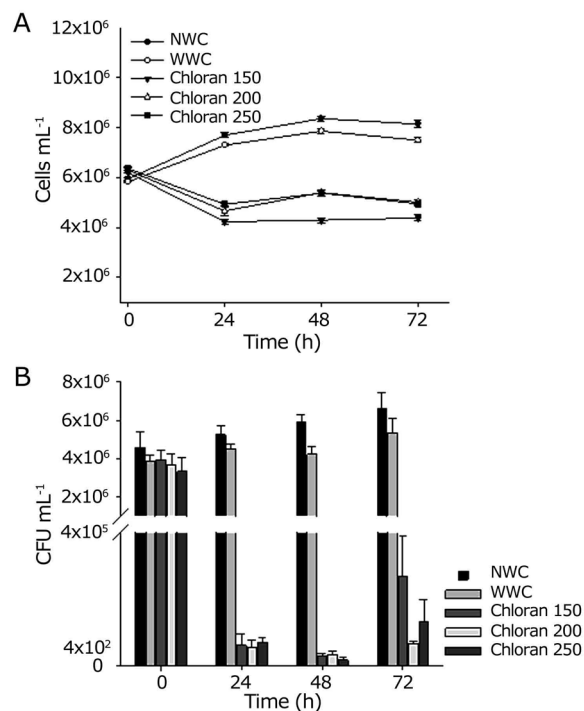

Figure 4. Mean values and standard deviation of *Isochrysis galbana* cell density (A) and concentration of heterotrophic bacteria (B) in assay 3, using three concentrations of chloramphenicol (Chloran) in repeat doses. NWC: control treatments without washes by centrifugation; WWC: control treatments with washes by centrifugation / Valores promedio y desviación estándar de la densidad de células de *Isochrysis galbana* (A) y la concentración de bacterias heterotróficas (B) en el ensayo 3, usando tres concentraciones de cloranfenicol (Chloran) en dosis repetidas. NWC: tratamiento control sin lavados por centrifugación; WWC: tratamientos control con lavados por centrifugación

to as low as 8.0 ± 1.0 x10^2 CFU mL^{-1} (Fig. 3b). In the third assay, 150 and 250 μg mL^{-1} chloramphenicol lowered *I. galbana* cell density (Fig. 4a), and the bacterial load fell to 0.3 ± 0.1 and 0.1 ± 0.1x10^6 CFU mL^{-1}, respectively (Fig. 4b). With 200 μg mL^{-1} chloramphenicol (Table 2), bacterial load decreased to 10^4 CFU mL^{-1}, and the final *I. galbana* cell density was 5.1 ± 0.1 x 10^6 cells mL^{-1}. The LC$_{50}$ for chloramphenicol was 194 μg mL^{-1} and the maximum non-lethal concentration was 23 μg mL^{-1}.

In the fourth assay, *I. galbana* cells were more sensitive to cocktail 1 (Fig. 5a), which effected a cell survival rate of 22.3 ± 0.9% with repeat doses. Both cocktails reduced the bacterial load to 1x10^1 CFU mL^{-1} or undetectable levels single and repeat doses (Fig. 5b). A cocktail of ampicillin, kanamycin, neomycin, and streptomycin was less toxic to *I. galbana* than the other cocktails, effecting densities of 6.9 ± 0.1 cells mL^{-1} with single

doses and 6.5 ± 0.2 x10^6 cells mL^{-1} with repeat doses at the end of the experiment (Table 2).

DISCUSSION

Bacteria are one of the most significant causes of mortalities in organisms that are reared for aquaculture, primarily at the larval stages due the high susceptibility to disease (Anguiano-Beltrán & Searcy-Bernal 2007, Muller-Feuga *et al*. 2007, Simonsson 2013).

Bacteria are naturally present in microalgae cultures and it is known that bacterial populations presents in cultures may interact with phytoplankton cells positively, contributing to a better growth of the cells trough the release of growth promoter factors like vitamins, remineralization of nutrients like phosphorus and nitrate, or transformation of organic material to
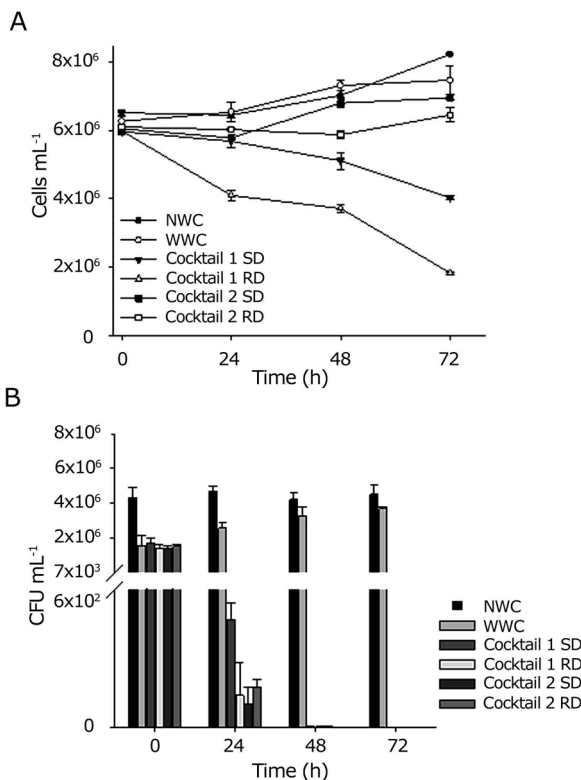
Figure 5. Mean values and standard deviation of *Isochrysis galbana* cell density (A) and concentration of heterotrophic bacteria (B) in assay 4, using cocktail 1 (carbencillin, sulphate G418, and chloramphenicol) and cocktail 2 (ampicillin, kanamycin, neomycin, and streptomycin) in a single dose (SD) and repeat doses (RD). NWC: control treatments without washes by centrifugation; WWC: control treatments with washes by centrifugation / Valores promedio y desviación estándar de la densidad de células de *Isochrysis galbana* (A) y la concentración de bacterias heterotróficas (B) en el ensayo 4, usando el coctel 1 (carbencilina, sulfato G418, cloranfenicol) y coctel 2 (ampicilina, kanamicina, neomicina, y estreptomicina) en dosis única (SD) y dosis repetidas (RD). NWC: tratamiento control sin lavados por centrifugación; WWC: tratamientos control con lavados por centrifugación

more assimilable carbon forms (Sitz & Schmidt 1973, Riquelme & Avendaño 1999, Grossart 1999, Schäfer *et al.* 2002, Grossart *et al.* 2006). Nonetheless, microalgae cultures with low bacterial concentration can be an option to evaluate the response of microalgae cells in bacteria-microalgae interaction studies, for the production of valuable compounds with potential in biotechnology and pharmaceutical areas, and for genetic research of bacteria and microalgae (Pareek & Srivastava 2013). This kind of cultures can be used as microalgae culture stock and in cryopreservation studies, where low bacterial densities are desirable (Andersen 2005). Moreover, it is known that in species such as *Thalassiosira rotula*, high bacterial

concentrations can have a negative effect on microalgae cell densities, especially in stationary growth phase (Grossart *et al.* 2006). Therefore, it is plausible the development of strategies to generate microalgae cultures with reduced bacterial load.

In this study, we performed centrifugation washes and administered antibiotics to obtain *I. galbana* cultures with reduced bacterial loads. Cultures of *I. galbana* that were not washed by centrifugation contained high concentrations of heterotrophic bacteria, whereas tubes that were washed 5 times by centrifugation had lower counts. Our results are consistent with those of Azma *et al.* (2010), concluded that rinsing by centrifugation is an effective method of eliminating bacterial contaminants from *Tetraselmis suecica* cultures. The proper application of centrifugal forces can separate cells from contaminating bacteria, based on their disparate sedimentation rates, as determined by cell size and their tolerance cells to centrifugation.

We found that increasing the number of centrifugation washes with sterile seawater from 3 to 5 lowered the bacterial count in cultures of *I. galbana*. In accordance to our results, Azma *et al.* (2010) concluding that a higher number of washes remove more free contaminants and material that was weakly attached to cells of *Tetraselmis suecica*. Also, Brown & Bischoff (1962) observed that centrifugation is helpful axenizing algae cultures, confirming that at least 5 washes with sterile water was needed to eradicate bacterial cells. Nonetheless, centrifugation alone was insufficient to eliminate all bacteria, which we supplemented with antibiotics as a chemical technique.

Our results demonstrated that individual antibiotics mitigate the bacterial load in *I. galbana* cultures by less than 1 order of magnitude, eliciting concentrations of 10^5 and 10^6 CFU mL^{-1}, regardless of the concentration or number of doses. In the first assay, repeat doses of a cocktail of carbencillin and sulphate G418 affected bacterial counts on the order of 10^3 CFU mL^{-1}, and a better response was observed in the second assay, in which this cocktail was given at twice the concentration and in repeat doses. This response was enhanced in assay 4, in which the bacterial counts were too low or undetectable in some replicates.

These data suggest that washes by centrifugation or the use of individual antibiotics is insufficient to generate an axenic culture, likely due to the variety of bacterial species that is present (Wilkens & Maas 2011). Further, antibiotics are sensitive compounds that might be damaged by microalgae culture conditions, such as light, temperature, and salinity (Hernández-Barrios *et al.* 1995). It is possible that these factors weakened the potency of the antibiotics in this study when used individually and administered in single doses.

Complex mixtures of antibiotics tend to have synergistic effects, usually increasing their toxicity to algae and bacteria (Vazquez-Martínez *et al.* 2004, González-Pleiter *et al.* 2013), likely because each antibiotic has a disparate mechanism and because the activity of an antibiotic might be enhanced by that of another antibiotic in the mixture. The antibiotics that we used have different modes of action. Carbencillin and ampicillin are β-lactams, inhibiting cell wall synthesis (Windler *et al.* 2012), whereas sulphate G418, kanamycin, streptomycin, and neomycin are aminoglucosides that cause mRNA to be misread and protein synthesis to be impaired (Kaufman 2011). Chloramphenicol is a bacteriostatic compound that binds to the 50S subunit of the 70S ribosome and inhibits protein synthesis (Leston *et al.* 2013). Likely, the activities of our antibiotics were complementary, and the combination resulted in low bacterial counts.

In this study, repeat doses of antibiotics helped us obtain low bacterial counts in *I. galbana* cultures. Antibiotics are subject to degradation due to salinity, temperature, light exposure, and the presence of oxygen (Samuelsen 1989, Hernández-Barrios *et al.* 1995). It is possible that our antibiotics deteriorated due to these under *I. galbana* culture conditions; thus, repeated doses can maintain the proper concentration of antibiotics and increase the time during which *I. galbana* cells are exposed to these agents to reduce bacterial load (Cho *et al.* 2002). *I. galbana* cell counts declined in nearly all trials. However, the concentrations and doses that we used failed to kill half of the initial population, except for repeat doses of the carbencillin, sulphate G418, and chloramphenicol cocktail.

The activity of antibiotics against prokaryotic cells is well described, but such data are lacking in algae cells. It is likely that the mechanism of inhibition is unrelated to that of their in bacterial cells, therefore direct effects on chloroplasts and mitochondria cannot be disregarded (González-Pleiter *et al.* 2013). Liu *et al.* (2012) suggested that phenicol antibiotics, such as chloramphenicol and florfenicol, inhibit photosynthetic activity and upregulated reactive oxygen species (ROS). ROS can affect the integrity and permeability of the cell membrane, resulting in cell damage, growth inhibition, and low algal cell viability.

A cocktail of ampicillin, neomycin, streptomycin, and kanamycin was not toxic to *I. galbana* cells. In contrast to the other treatments, this mixture enhanced the growth of *I. galbana* cells. Cho *et al.* (2002) examined this antibiotics and concentrations that were used in assay 4 of our study and 50 µg mL^{-1} gentamycin, obtaining similar results. Liu *et al.* (2012) found that florfenicol concentrations as low as 0.5, 1.0, and 2.0 mg L^{-1} promoted the growth of *Skeletonema costatum*.

I. galbana was highly resistant to antibiotic exposure. Our results of LC$_{50}$ and maximum non-lethal concentrations for carbencillin (betalactamic antibiotic) and chloramphenicol were higher than values reported for other microalgae species. For example, according to Gonçalves-Ferreira *et al.* (2007), 6.06 mg L^{-1} of florfenicol are necessary to inhibit in 50% the growth of *Tetraselmis chuii*, while Lai *et al.* (2009) found that 158 mg L^{-1} of chloramphenicol reduce the growth of *I. galbana* in a 50%. In a study performed by Cho *et al.* (2002) using *I. galbana* cultures, values of LC$_{50}$ of 136 and 100 µg mL^{-1} for chloramphenicol and carbencillin respectively are found toxic for microalgae cells. To the best of our knowledge, there are no reports on the use of sulphate G418, neither to reduce the bacterial load in *I. galbana* culture, nor its toxicity. This antibiotic is an aminoglycoside used in genetic transformations (Chiaiese *et al.* 2011), but rarely used to remove the bacterial load from microalgae cultures. In this study the results showed that *I. galbana* had a high tolerance to the concentrations of sulphate G418, carbencillin and chloramphenicol used in the assays. Microalgae are resistant to certain antibiotics at specific concentrations, differing between species and culture conditions. Qijun & Qunying (2000) reported that penicillin increases the growth of *I. galbana* Parke. Jian-Hang (2004) noted that *Chlorella vulgaris* and *Nannochloropsis occulata* are insensitive to kanamycin, tetracycline, and chloramphenicol. Kvíderová & Henley (2005) found that 25 µg mL^{-1} streptomycin and 50 µg mL^{-1} ampicillin did not affect growth, chlorophyll content, or other photosynthetic parameters in *Dunaliella* sp., whereas these properties were slight affectation in *Picochlorum oklahomensis*. Lai *et al.* (2009) demonstrated that phenolic antibiotics, such as chloramphenicol, florfenicol, and thiamphenicol, inhibit the growth of *Chlorella pyrenoidosa*, *I. galbana*, and *Tetraselmis chuii* at various doses, reporting that the toxicity of antibiotics varies, depending on the antibiotic and the sensitivity of the microalgae species.

Although the use and efficacy of antibiotics in decreasing bacterial loads in microalgae cultures are well known, the response to varying types and concentrations of these compounds can differ, and frequently there is no success because bacterial community is different between microalgae species. Thus, protocols to reduce bacteria counts in microalgae cultures must be species-specific (Kooistra *et al.* 1991, Cho *et al.* 2002). Moreover, most of the methods to decrease bacteria in microalgae cultures are time consuming and involve several steps in isolation, resulting in a tedious and expensive task that frequently may take several weeks depending on the contaminants presents (Cottrell & Suttle 1993, Su *et al.* 2007, Bruckner & Kroth 2009). Our method offers the advantage to obtain low bacterial load in *I. galbana* cultures in less time,

avoiding its culture in solid media and further purification steps. Moreover, we found that after the treatment with antibiotic cocktails here proposed, *I. galbana* cultures maintained low bacterial counts for at least four days, similar result to reported by Campa-Córdova *et al.* (2006); after 10 days, *I. galbana* cultures recovered the initial bacterial load. The strategies and data presented here can be modified to reduce bacterial load in cultures of other microalgae species. This method can be helpful in the aquaculture area for the maintenance of low bacterial content in microalgae strains, for the study of microalgae and bacteria interactions and, for biotechnology, genetic, pharmaceutical and cryopreservation studies. However, antibiotics should be used carefully, in necessary concentrations to reduce bacterial load, and should not be used in excess or as a routine procedure, in order to avoid the surge of resistant bacteria species, which is a major concern in public health and in animal production industry. In conclusion, administering a combination of 5 washes by centrifugation with sterile 'f' media and a cocktail of ampicillin, kanamycin, neomycin, and streptomycin is an effective method of removing bacteria from *I. galbana* cultures. This combination is less toxic to microalgae cells than the combination of carbencillin, sulphate G418, and chloramphenicol. This valuable method can control bacterial loads in *I. galbana* cultures for 3 days and is simple and fast and requires no sophisticated equipment.

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