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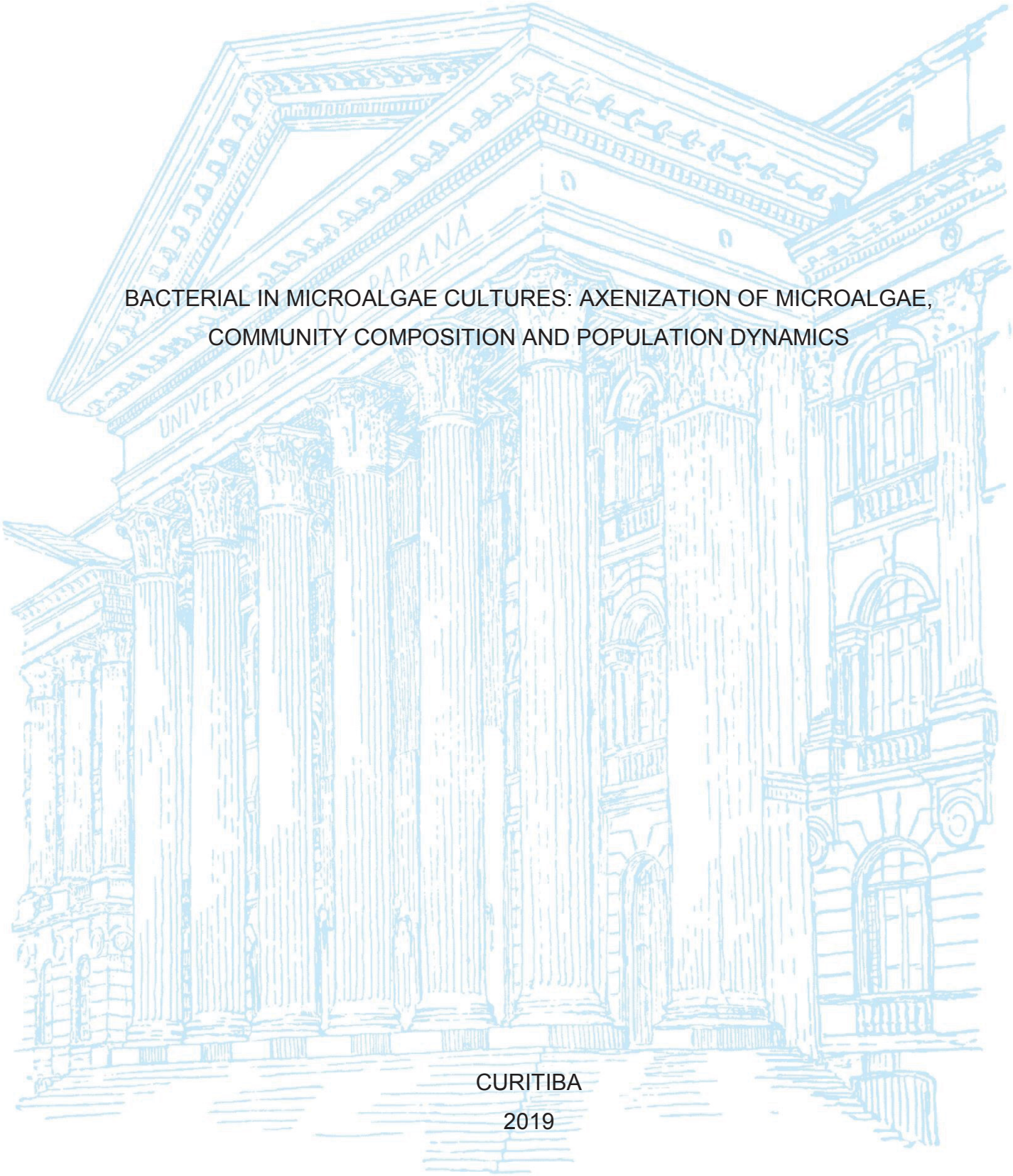

BACTERIAL IN MICROALGAE CULTURES: AXENIZATION OF MICROALGAE, COMMUNITY COMPOSITION AND POPULATION DYNAMICS

CURITIBA

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TERMO DE APROVAÇÃO

Os membros da Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em ENGENHARIA DE BIOPROCESSOS E BIOTECNOLOGIA da Universidade Federal do Paraná foram convocados para realizar a arguição da dissertação de Mestrado de **DENISSE TATIANA MOLINA AULESTIA** intitulada: **"Bacteria in Microalgal Cultures: Axenization of Microalgae, Community Composition and Population Dynamics"**, após terem inquirido a aluna e realizado a avaliação do trabalho, são de parecer pela sua APROVAÇÃO no rito de defesa.

A outorga do título de mestre está sujeita à homologação pelo colegiado, ao atendimento de todas as indicações e correções solicitadas pela banca e ao pleno atendimento das demandas regimentais do Programa de Pós-Graduação.

Curitiba, 09 de Março de 2018.

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To my parents, brothers and nephews. Every achievement is thanks to you.

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First to God for being present in many ways in my life. To my parents, my brothers and nephews, each of you have taught me in many ways to move forward, thanks for the support and always being with me when I need it. To all my family, uncles, cousins, my grandparents, the present and the physically absent. They are always with me and that allows me to continue and continue. To my supervisor Dr. Julio Cesar de Carvalho, for the trust, patience and all the support received during these years. This work would not be possible without all his orientations. To all the technicians and personnel of the Bioprocess Biotechnology Engineering laboratory, thanks for the help, patience, and support. Finally, to all my friends, thanks for the laughs, the coffee, the jokes, Sundays until late in the laboratory. Thank you for always being there when I needed you.

RESUMO

Como parte do controle da contaminação nos cultivos de microalgas foram axenizadas 2 cultivos das microalgas *Neochloris oleoabundans* UTEX LB 1185 e *Dunaliella salina* SAG 84.79, e purificadas (não axenicos) 2 cultivos de cianobactérias *Spirulina platensis* SAG 257.80 e *Spirulina maxima* SAG 84.79. Adicionalmente foram isolados 164 contaminantes procariontes (bactérias) dos quais 14 pertencem ao cultivo de *Neochloris oleabundans* UTEX LB 1185. Partindo dessas 14 bactérias foram realizadas as caracterizações bioquímicas resultando em 5 famílias de bactérias *Bacillaeae* gênero *Bacillus, Rhizobiales, Pseudomonadaceae* gênero *Pseudomona, Streptomycetaceae* gênero *Streptomyces* e *Leoconostocaceae* gênero *Leuconostoc.* Adicionalmente, foram avaliadas as condições iniciais do modelo matemático que avalia a interação entre um contaminante, uma microalga e uma sustância inerte. Finalmente foi avaliada a toxicidade de 5 terpenos usando a microalga *Neochloris oleoabundans* UTEX LB 1185.

Palavras-chave: Microalgas. Toxicidade. Contaminação.

ABSTRACT

As part of the control of contamination in the algae cultures, 2 microalgae culture *Neochloris oleoabundans* UTEX LB 1185 and *Dunaliella salina* SAG 84.79, were axenized, 2 cyanobacteria cultures *Spirulina platensis* SAG 257.80 e *Spirulina maxima* SAG 84.79 were purified. Additionally, 164 prokaryotic contaminat (bacteria) were isolated and 372 were identify using metagenomic assays. 14 contaminate were analyzed using biochemical characteristics resulting in 5 families: *Bacillaeae* genus *Bacillus, Rhizobiales, Pseudomonadaceae* genus *Pseudomona*, *Streptomycetaceae* genus *Streptomyces* e *Leoconostocaceae* genus *Leuconostoc*. In addition, it was evaluated the initial conditions for the mathematical model (prokaryotic contaminate, algae and inert substance). Finally, it was evaluated the toxicity of 5 terpenes using microalgae *Neochloris oleoabundans* UTEX LB 1185

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Keywords: Microalgae. Toxicity. Contamination.

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SUMMARY

1 CHAPTER I CONTROL CONTAMINATION IN MASS CULTURE ALGAE

1.1 ABSTRACT

Microalgae are used for production of different biological products and as a solution for environmental problems. However, and despite its importance, one of the main problems in large scale cultures – the presence of contaminants – is rarely systematically approached. Contamination, or the presence of undesirable organisms in a culture, is deleterious for the culture and frequently leads to culture crashes. To avoid contamination, closed systems can be used; for very large-scale open systems, contamination is unavoidable and remediation procedures are necessary, from physicochemical treatment to addition of biocidal substances. In all cases, early detection and culture monitoring are paramount. This article describes the contamination mechanisms, biological contaminants, and control systems in open and closed cultures, with the purpose to discuss the latest advances and techniques developed in this area. In addition, this article analyzes the complex interactions of algae with other microorganisms that can be expected in cultivation systems.

Keywords: microalgae, contamination, mass cultures, algal parasites, control.

1.2 INTRODUCTION

Microalgae are photosynthetic aquatic microorganisms, which live in saline and freshwater environments. Their main feature is to convert sunlight, water and carbon dioxide into biomass and oxygen. They are commonly used in different industries such as food, pharmaceutical, energy and cosmetic (SPOLAORE et al. 2006; KAN and PAN 2010; HUANG et al. 2014a; REGO et al. 2015b). More than 10^7 tons of algae (micro- and macroalgae) are produced to meet demand of these industries (HALLMANN and HALLMAN 2007). Some of the characteristics for which they are used are: the high photosynthetic efficiency, fast growth, ability of growing in different types of substrates (wastewaters) and their nutritional composition (MA et al. 2016).

Microalgal cultures are usually of low concentration and producing large

amounts of biomass requires equally large volumes. An alternative to do so at low production costs, is the cultivation in open systems (STEPHENSON et al. 2010; McBride et al. 2014; RICHARDSON et al. 2014; MA et al. 2016); nevertheless, this technology is still not fully optimized for all types of algae (MCBRIDE et al. 2014). There are parameters that must be considered in the production of biomass in this kind of systems, for example, culture conditions, contamination control, economic extraction of the products of interest and post-processing of residual biomass (HUANG et al. 2014b).

One of the major problems that algal mass cultures have, similarly to plant crops, is that they can be affected by other organisms (contaminants), which can be considered pests (ABELIOVICH et al. 2005; DAY et al. 2012b; MCBRIDE et al. 2014). These contaminants may impair the development of the cultures, resulting in reduced production, and sometimes complete loss of the desired microorganism population or final products (MESECK 2007; DAY et al. 2012b; MÉNDEZ et al. 2012; FOREHEAD and O'KELLY 2013).

Early detection and control of contaminants is important to avoid significant culture damage and losses. During the last years, techniques have been developed aiming to control the contamination in the mass cultures of algae (MAHAN et al. 2005; KAN and PAN 2010; DAY et al. 2012b; FOREHEAD and O'KELLY 2013; HUANG et al. 2014b; HUANG et al. 2014c; HUANG et al. 2014a). This article reviews relevant aspects for early detection and control of the biological contaminants that may affect mass microalgae algae cultures.

1.3 MICROALGAL MASS CULTURE AND CONTAMINATION

Biological contaminants can be introduced at any stage of microalgae mass cultivation. When the volume of culture increases, this makes control more difficult and sometimes expensive (FOREHEAD and O'KELLY 2013; MOLINA-CÁRDENAS et al. 2016). Most biological contaminants have negative effects on cultures, some of which are summarized in TABLE 1.

TABLE 1. PROBLEMS CAUSED BY THE BIOLOGICAL CONTAMINANTS IN THE MASS CULTURES

Problem	Reference
Reduction of cell density, stress and cellular alteration	(RICHMOND et al. 1987; HUANG et al. 2014c)
Destruction of algae culture - hours after the detection of an aggressive biological contaminant	(FOREHEAD; O'KELLY, 2013; LETCHER et al., 2013; WANG et al., 2013)
Increase in the cost of production, reduction of the production up to 90%, degradation of the product quality	(LINCOLN et al. 1983; ABELIOVICH et al. 2005; MÉNDEZ et al. 2012)

SOURCE: The author (2017).

However, not all "foreign" microorganisms in a culture are deleterious. In fact, several cultures are developed as unialgal cultures, with a tolerable level of contamination; some microalgae cannot be cultivated axenically, presumably because of cofactors produced by a symbiont; and it is even possible to have co-cultures.

1.4 MICROALGAL CULTURE TYPES

Regarding the presence of other microorganisms, microalgal cultures can be classified as axenic, unialgal, mixed and cocultures:

1.4.1 Axenic culture

An *axenic* or pure algal culture contains only one species, i.e., is free from any other kind of microorganisms (ABELIOVICH ET AL., 2005; LEE, 2008). The first report of an axenic algae culture is from the Dutch microbiologist Beijerinck in 1890, who succeeded in isolating C*hlorella* and S*cenedesmus* from samples supposedly free of bacteria (ANDERSON 2005). Axenic cultures are standard in all areas of industrial microbiology, and for some applications, it is indispensable to grow microorganisms in this condition (SPOLAORE et al. 2006; CHO et al. 2013; RAMANAN et al. 2016). An example is the production of bioactive components from cyanobacteria (ARÁOZ et al. 2005; VAZ et al. 2014).

A problem of axenic algal cultures is that they are difficult to obtain and preserve. Their use is essential for the study of metabolism and physiology, because these features vary significantly between axenic and non-axenic cultures (CHOI et al. 2008; CHO et al. 2013; VAZ et al. 2014). Traditional microbiological methods have

been used to separate desired strains from biological contaminants, with limited success. **Erro! Fonte de referência não encontrada.** describes the methods c ommonly used. The isolation or “axenization” success depends not only on the technique chosen, but also on the researcher's experience (CHOI et al. 2008).

TABLE 2. DESCRIPTION OF METHODS FOR OBTAINING AXENIC CULTURES

Method	Reference
Size Selective Screening and Filtration	(RIPPKA ET AL. 1979; ABELIOVICH ET AL. 2005; ANDERSON 2005; CHOI ET AL. 2008)
Differential Centrifugation	(ABELIOVICH et al. 2005; ANDERSON 2005; MOLINA-CÁRDENAS et al. 2016)
Sonication and Vortex	(ANDERSON 2005; GUILLARD 2005; CHO ET AL. 2013)
UV radiation	(LEE and SHEN 2004; ANDERSON 2005; CHOI et al. 2008; CHO et al. 2013; MOLINA-CÁRDENAS et al. 2016)
Antibiotic treatment (penicillin G, gentamycin sulfate, streptomycin, chloramphenicol, cefotaxime, Carbendazin, neomycin, kanamycin, Combination of others)	(LEE and SHEN 2004; ABELIOVICH et al. 2005; ANDERSON 2005; GUILLARD 2005; CHOI et al. 2008; KAN and PAN 2010; MOLINA-CÁRDENAS et al. 2016)
Dilution, agar plates, repeated transfer of cells, phototaxis	(ANDERSON 2005; GUILLARD 2005; MCBRIDE et al. 2014; VAZ et al. 2014)
Gliding motility of cyanobacteria, lysozyme treatment, thermal treatment	(VAZ et al. 2014)
Other agents (phenol, detergents, sodium sulfite, sodium azide, sodium fluoride, chlorinated water or sodium hypochlorite)	(ANDERSON 2005; GUILLARD 2005; CHOI et al. 2008; VAZ et al. 2014)

SOURCE: The author (2017).

Axenic algae cultures are produced and preserved on a laboratory scale. From these cultures, inocula are produced for mass culture – which is, preferably, in closed photobioreactors, where there is a barrier between algae and contaminants. For an axenic batch production system, inoculum preparation and process control are challenging factors, and the associated cost must be justified by the value of the final product. The production in continuous systems is even more sensitive to contamination (MOOIJ et al. 2015).

1.4.2 Unialgal cultures

Unialgal cultures contain only one species of algae but may have different phenotypes or contaminants (e.g. Bacteria). Usually it is free of protozoa and fungi. It was wettstein in 1921 in berlin, who obtained the first unialgal (but not axenic) culture

from a sample with different flagellate algae not grown before (e.g. *Cryptomonas, Synura, Uroglena*) (PREISIG AND ANDERSEN 2005).

Unialgal cultures can be produced in open systems with greater ease, without sterile conditions (WANG et al. 2013; PENG et al. 2015). However, there still is risk of contamination, causing production losses, increasing costs (CARNEY and LANE 2015).

In some cases, microbial interactions may be beneficial, increasing production and yield (RAMANAN et al. 2016). In a study done by (CHO et al. 2015), the presence of growth-promoting bacteria induced an increase in the productivity of biomass and lipids in the microalgae *Chlorella vulgaris* and *Scenedesmus sp.* Interactions are especially interesting for environmental processes, where microbial consortia are common. In a study by ZHOU et al., (2014), it was found that a bacteria-algae consortium reduced the concentration of organic carbon and nitrogen in the wastewater.

Unialgal starter cultures are produced using the same techniques applied for axenization. Normally the methodologies employed are: gravity separation (centrifugation, settling), isolation with use of phototaxis, enrichment cultures, isolation by micropipette, isolation with agar (streaking cells across agar plates, atomized cell spray technique, isolation after dragging through agar), dilution techniques (ANDERSON 2005).

1.4.3 Mixed cultures

Mixed cultures are made up of different, sometimes unknown species. They may be all photosynthetic, or maybe a combination of several microorganisms (e.g. algae and fungi or algae and bacteria) where the combination favors the strains (HESSELTINE and others 1992; GOERS et al. 2014; MAGDOULI et al. 2016). These cultures mimic what can be found in natural water bodies.

Mixed cultures play an important role in environmental and biotechnological process. An example of this type of culture is the production of biomass and energy using opened and closed reactors. The use of consortia has potential in the generation of biomethane (CHINNASAMY et al. 2010). Other applications of mixed culture are: single cell protein (DE-BASHAN et al. 2002; WATANABE et al. 2005), ethanol

production (DOSTALEK and HAGGSTROM 1983), lipid production (YU et al. 2011; BOGEN et al. 2013; YAMANE et al. 2013; CEA-BARCIA et al. 2014).

The main advantages of mixed cultures are the higher production performance, higher rate of growth, obtaining products that cannot be produced axenically, and less risk in the case of contamination (HARRISON 1978; TATE et al. 2013). Some disadvantages include: the difficulty for studying the production process, the definition of the consortium identity, and the need for a period of adaptation of the microorganisms involved in the process. Finally, in case of contamination, its identification will be more problematic (HESSELTINE and others 1992).

1.4.4 Co-cultures

Co-cultures consist in two or more different species of *known* microorganisms. Most studies describe mostly cell-cell interactions between populations (Goers et al. 2014). As with mixed cultures, this system can be very complex – with interactions varying with time, making it difficult to distinguish them from each other without the use of a suitable experimental design. Studies with co-cultures may aid in developing engineered routes for fastidious or hard to axenize microorganisms (Magdouli et al. 2016).

Co-cultures are promising also in industrial production. Some examples are: a fungi co-culture with microalgae, which favors the flocculation of the microalgae avoiding the use of chemical substances and therefore reducing costs (WREDE et al. 2014); a co-culture between yeasts (*Rhodotorula glutinis*) and microalgae (*Chlorella vulgaris)* favoring biomass production and lipid accumulation (CHEIRSILP et al. 2012); the production of hydrogen by microalgae (modified to reduce sulfate) mixed with the bacterium *Rhodospirillium rubrum* (MELIS and MELNICKI 2006; WU et al. 2012); and the production of extracellular polymer substances (EPS) by the combination the cyanobacteria/microalgae and macromycetes (ANGELIS et al. 2012; MAGDOULI et al. 2016).

1.5 POPULATION MONITORING

The evaluation of the population in an algal culture is an essential tool for quality control and detection of contamination. To evaluate the purity of the culture, cell

counting is probably the easiest and fastest method. Some of these techniques can be used in mixed cultures, while others can be used in unialgal cultures (VAN VUUREN, J., PIERTERSE, A., JACOBS, A., & STEYNBERG 1939).

The direct evaluation of the cultures is usually done using counting chambers such as Sedgewick-Rafter (which uses a 50x20x1mm chamber, with an area of 1000 mm^2, and a volume of 1,0 mL). A sample is placed over the top at an oblique angle, the liquid fills the chamber and is left to at rest for a few minutes. The counts are made with 4X or 10X objectives. The counts are carried out in 10 randomly chosen squares, finally determining an average value for each alga present in the sample (FIND 2012). An hemocytometer (e.g. a Neubauer chamber) can also be used for cell counting (BASTIDAS 2016). Small contaminants such as bacteria are difficult to count and identify in low magnification; while high magnifications are incompatible with the focal distance of some chambers.

The gravimetric determination of total biomass is essential for monitoring the kinetics of a cultivation process but does not give much information about the presence of contaminants. Biomass can also be estimated by measuring the concentration of proteins, chlorophyll content or total organic carbon (TOC). Because a microalgal monoculture may have a characteristic absorption spectrum (OOMS et al. 2016), UV-VIS-NIR spectrophotometry which can be used as an indicator of culture concentration and status.

1.6 MICROBIAL INTERACTIONS

Relations developed by algae with other existing microorganisms cover the full range of natural interactions. A typical example is the interaction between algae, bacteria and archaea, which are producers and decomposers, becoming fundamental components of an ecosystem. The study of these interactions is important, but complex due to the difficulty in isolation and maintenance of physiological characteristics (AMIN et al. 2015; CHO et al. 2015; RAMANAN et al. 2016). The most common interactions are mutualism, commensalism and parasitism (MOENNE-LOCCOZ et al. 2011; ZAPALSKI 2011).

1.6.1 Mutualism

Mutualism or symbiosis is an interaction where there is a beneficial interchange between two populations. This interaction is interesting for the development of microorganisms. These interactions have played an important role in the evolutionary process (MOENNE-LOCCOZ et al. 2011), and explain why sometimes it is impossible to axenize a microalgal culture.

Some examples of mutualism are: favoring microalgae growth through the intervention of *Mesorhizobium* and *Azospirillum* bacteria (vice-versa) thought nitrogen fixation and (GONZALEZ 2000; WATANABE et al. 2005; HERNÁNDEZ et al. 2013; CHO et al. 2015) and the interaction between bacteria and algae to produce polysaccharides (GUERRINI et al. 1998). Other examples can be the increase of the cellular concentration (1,4 times) of the microalga *Chlorella vulgaris* ATCC 13482 in presence of the bacterium *Pseudomonas* sp (GUO and TONG 2013). An important case of mutualism is Vitamin B12 for auxotrophs where bacteria supply the vitamin to algae in exchange for fixed carbon (CROFT et al. 2005).

1.6.2 Commensalim

Commensalism is an interaction beneficial to one of the populations, and neutral to the other. A thin line separates mutualism from commensalism and parasitism., and it is often difficult to clearly identify commensalism (MOENNE-LOCCOZ ET AL. 2011; RAMANAN ET AL. 2016).

Currently, it is known that environmental factors determine this interaction; therefore, their growth may be influenced due to nutritional factors as carbon source, n:p ratio, and light intensity. Some studies have indicated that phosphorus-limited algae compete among themselves, sometimes allowing bacterial commensals to outnumber algae (GROVER 2000; ZAPALSKI 2011; RAMANAN et al. 2016).

1.6.3 Parasitism

Many microorganisms can negatively affect the development of microalgae or cyanobacteria. In microalgal cultures, the parasitism is applied to predation, where a population graze on another one. Usually, the main predators that affect the algae culture are protozoans. In natural ecosystems, these microorganisms help in regulating the different populations (MOENNE-LOCCOZ et al. 2011; RAMANAN et al. 2016).

Predation is self-regulated (maintained over time) to an extent, can, severely limit an unialgal culture, and even cause a culture crash, i.e., the destruction of the whole microalgal population for practical purposes. That must do with the population dynamics and can be prevented to an extent by proper management of the culture. A number of important algal parasites have been identified in algal mass culture systems in the last few years and this number is sure to grow as the number of commercial algae ventures increases (LANE and CARNEY 2014). This kind of interaction is manifested between fungi and bacteria associated with algae (CHO et al. 2015).

1.7 TYPES OF CONTAMINANT ORGANISMS

Contamination can be defined as the undesired introduction of microorganisms into culture system (MOOIJ et al. 2015). These represent the greatest limitation in the productivity of mass cultures, particularly in open systems (ABELIOVICH et al. 2005), when the contaminant is a parasite.

These parasites can be classified as: bacterial, fungi, protozoa, rotifers, viruses and other types of algae, different from the strain of interest (NAKAMURA 1976; DE ARAUJO et al. 2000; MORENO-GARRIDO and CAÑAVATE 2000; MAHAN et al. 2005; SHUNYU et al. 2006; VAN WICHELEN et al. 2010; MÉNDEZ et al. 2012; LETCHER et al. 2013; HUANG et al. 2014A; PENG et al. 2015; REGO et al. 2015b; MOLINA-CÁRDENAS et al. 2016; VAN GINKEL et al. 2016).

1.7.1 Fungi

These saprophytic eukaryotic organisms have an enormous diversity and require a lot of nutrients, including carbon and nitrogen for its correct development (SINGH 2006). Most algae grow under autotrophic conditions; however, some require the heterotrophic conditions at certain times of production, increasing the likelihood of contamination with fungi (KAN and PAN 2010). At the other side, microalgal cultures have dissolved organic carbon liberated by the culture itself, that can be used for fungal growth. Algal cultures can be affected by several fungi species, some of not completely identified, making the control process more difficult (CARNEY and LANE 2015).

In freshwater cultures, it is common to find fungi from the order *Chytridomycota* (e.g. *Paraphysoderma sedebokerensis*) (RASCONI et al. 2009). These fungi can

cause the loss of the 90% of the population, or more (HOFFMAN et al. 2008). The parasitic process produce motile dispersing life stages know as zoospores (CARNEY and LANE 2015). This fungi has been found in cultures such as *Scenedesmus, Chlamydomonas,* and *Haematococcus* (SHIN et al. 2001; GERPHAGNON et al. 2013).

Another group of contaminant fungi is the class *Oomycota* (LI et al. 2010). This group includes widespread parasites (also in non-algal cultures), and they can cause losses between 10 and 60% in the seawater industries (GACHON et al. 2009). There are many other fungi that can affect cultures. An example are the group *Labyrinthulids* that in some cases are common found in cultures where the fungi are accompanied by bacteria (KAN and PAN 2010), making difficult to control.

1.7.2 Bacteria

These prokaryote microorganisms are one of the most important biological contaminants and comprise a huge variety of genera. Some of them are known for their pathogenicity, however, they are essential for the metabolic regulation of many algae (MICHAEL T. MADIGAN; et al. 2006; SINGH 2006).

The biological contamination in the cultures by bacteria can be caused by different groups. For that reason, it is often not practical to identify the different species before acting on a process (KAN and PAN 2010). Contamination by bacteria can create different problems, such as, the increment of the susceptibility of the culture to the growth of other pathogens such as *Vibrio;* which reduce the nutritional quality of the biomass, becoming a contamination vector for other cultures (SALVESEN et al. 2000; GOMEZ-GIL et al. 2004). Another example is the *Pseudomonas* bacteria which compete for nutrients, inhibiting algal growth (COLE, 1994).

There are reports of lytic-bacteria such as *Cytophaga* sp., *Myxobacter* sp, *Bacillus* sp., which inhibit algae growth (IMAI et al. 2001; WANG et al. 2013) in cultures such as those of *Scenedesmus, Chlorella, Chlorococcum* and *Ankistrodesmus*. These bacteria can damage the culture in two ways: a direct attack, which depends on the contact between two cells (cell to cell) or an indirect attack through the production of algicidal substances (IMAI et al. 2001; SHUNYU et al. 2006).

1.7.3 Protozoa

These eukaryotic microorganisms, belonging to the kingdom *Protista*, are very diverse and may feed on microalgae cultures. These are heterotrophic protists such as amoebas, flagellates and ciliates) (MORENO-GARRIDO and CAÑAVATE 2000; ma et al. 2016). In the mass culture systems, they are the main biological contaminants, and one of the most difficult to prevent. However, there are a few studies aimed at identifying and controlling them (DAY et al. 2012b; PENG et al. 2015).

Within the group of flagellates, the *Chrysomonadida* order (especially the species *Ochromonas* sp.), are the main contaminant. Cyanobacterial cultures such as those of *Microcystis aeruginosa* and *Oscillatoria* are the main affected. A characteristic of this group is their ability to quickly affect the culture resulting in destruction after a few hours (DRYDEN and WRIGHT 1984).

Ciliates and amoebas are associated with the reduction of the unicellular, colonial or filamentous microalgae or cyanobacteria populations. Usually the amoebae in the trophozoite state crawl in the bottom of tanks (grazing), and many feed through a phagocytic mechanism (GONG et al. 2015). However, there are exceptions: vampyrellid amoebas, for example, puncture the cell wall and membrane of its prey and ingest its cytosolic content (XINYAO et al. 2006; HESS et al. 2012; BERNEY et al. 2013). The massive cultures of *Chlorella* and *Spirulina* are generally the most affected, and the process may lead to a predominance of grazing resistant contaminants. An example is a massive culture of *Chlorella* that was grazed by *Stylonichia* sp.; after five days, the population shifted to dominance by another algae, *Scenedesmus* sp. (ABELIOVICH et al. 2005).

1.7.4 Zooplankton

Massive cultures are susceptible to contamination by zooplankton as well as protozoa (GONG et al. 2015). Together, they are the most challenging biological contaminants in this type of system. Zooplankton includes picoplankton (<2 mm), nanoplankton (2-20 mm), microplankton (20-200 mm), mesoplankton (0.2-20 mm), macroplankton (20-200 mm) and megaplankton (> 200 mm). Rotifer, cladocera and copepods are also possible biological contaminants (FREDERIKSEN et al. 2006; WANG et al. 2013).

The reported rotifers are in the range of 123-292 μm of length and 114-199 μm in width, however, the size may be affected as a function of environmental conditions

such as temperature or feed (HAGIWARA et al. 2001; LAKSHMI, S., KUMAR, R., & RAJENDRAN 2015). The rotifers have two types of mechanisms of feeding, positive and negative mechanism. For the case of the copepods, they can select their mechanism according to the conditions (KLEPPEL et al. 1996).

Zooplankton have a fast growth rate, high pollution capacity, and very high ingestion rates (MORENO-GARRIDO and CAÑAVATE 2000). One of the most studied rotifers is *Brachionus plicatilis*, which has the capacity to consume a total of 12,000 cells of algae per hour, causing significant losses after a few hours (FAWLEY and FAWLEY 2007). Some authors have reported strategies for the control of the pollution caused by this type of contaminant (DE ARAUJO et al. 2000; FISCHER et al. 2012; MÉNDEZ et al. 2012).

1.7.5 Other microalgae

The presence of other algae is inevitable and common in mass cultures (MESECK 2007). In the same way as with other type of contaminates, the problems in the cultures can be that competition for nutrients or light may occur, with the domination of one culture, although it is possible that both species develop to large, stable populations (TWINER et al. 2005).

1.8 **MANAGEMENT STRATEGIES**

Because of the large number of possible contaminants and the lack of information about their biology, there is not a single methodology addressing all contaminations. Each system must be evaluated for development of control techniques; regarding 1) the ways in which the contaminant can enter (carriers); 2) the possible amount (or rate) of entry in the system, and 3) the time to reach a specific threshold (FOREHEAD and O'KELLY 2013).

Currently, the strategies are preventive (avoiding the entry of contaminants) or corrective (trying to control or eliminate an established contamination). Early detection systems are essential to avoid major losses. Methods vary in performance, instrumentation, experience and cost; nevertheless, monitoring methods must be accompanied by physical or chemical control systems.

1.8.1 Microscopy and staining

The simplest and most common technique for the identification of contaminants is microscopy. This methodology allows to perceive and estimate the growth of different polluting species (protozoa, fungi, other algae) (ABELIOVICH et al. 2005). Normally the cells are mounted on slides and covers for observation. These can be examined in light field (bf), dark field (df) and phase contrast (pc) (HOFFMAN et al. 2008). Vital stains may aid in the correct identification of microorganisms. One of the disadvantages of microscopy is that it requires an experienced operator for good results (BENSON 2001).

One of the commonly used methodologies for the detection of contaminants is Calcofluor White Stain, a fluorescent dye that allows the quick detection of fungi and parasitic organisms (CARNEY and LANE 2015). This non-specific fluorochrome binds cellulose and chitin present in the wall of fungi and other microorganisms. The contaminant, stained microorganisms have a bright appearance and may be green or blue, while the others may be fluorescent red or orange (SIGMA-ALDRICH; RONCERO and DURAN 1985).

Some authors use this technique to determine the contaminants present in the samples (RASCONI et al. 2009; ZHOU et al. 2014), however, there are currently new proposals to allow better identification in less time. Researchers such as (GERPHAGNON et al. 2013) combined Calcofluor and SYTOX Green staining methods to assess the degree of chytrid contamination. SYTOX Green is an asymmetric cyanine dye with three positive charges, which excludes living eukaryotes and prokaryotes cells (ROTH et al. 1997).

Another type of staining is based on Congo Red. This is another alternative for the microorganisms whose main component is cellulose. Congo Red is the sodium salt (benzidinediazo-bis-1-naphthylamine-4-sulfonic acid); a diazo dye that is red in alkaline solution and blue in acid solution (CARNEY and LANE 2015; PUBCHEM 2016). Researchers such as (GACHON et al. 2009) used this technique to identify oomycetes contamination.

1.8.2 Flow cytometry

Flow cytometry is a technology that measures and analyzes multiple physical characteristics, usually cells, flowing in a stream through a beam of light (WEAVER 2000). It was developed in the 1960s and 1970s and in 1990s, an automated flow cytometry and microscopy system known as flowcam (fluid imaging technologies) was built. It has a capacity to detect and quantify particles, usually phytoplankton organisms (20-200 µm) (ANDERSON 2005; DAY et al. 2012b; CARNEY and LANE 2015).

This method was used for early detection of different grazing by copepod and toxic dinoflagellates (IDE ET AL. 2008). Flowcam can recognize and identify grazers in very dense cultures and sometimes they can be used semiautomatically. This system has different variety of application; according to (DAY et al. 2012b) The Flowcam recognize twelve different species in a mass culture. This methodology is beneficial in the control of the biological contaminants in the mass culture avoiding loss and damage. Despite the high yield of flow cytometry, its capacity is limited to identify strains with similar phenotypes.

1.8.3 Molecular detection and monitoring

Currently, molecular techniques have been developed that allow the identification and detection of different parasites in algal cultures. Nucleic acid-based methods can be misused if the genes or sequences to be used are not first defined. Genes that encoding ribosome RNA subunits, prokaryotes or eukaryotes, are generally used for identification and taxonomy purposes (FULBRIGHT et al. 2014).

The regions of rRNA that are used for the identification of microorganisms are: small subunit (SSU) rRNA, large subunit (LSU) rRNA genes, and the internal transcribed spacer (ITS). These regions allow the design of PCR primers with high specificity. The SSR gene contains nine hypervariable regions (V1 to 9). Eukaryotes do not have the V6 region, therefore the V4 and V9 regions are used for phylogenetic analyses (NOLAN et al. 2006; CARNEY and LANE 2015).

The polymorphism between the 18S genes of the algae is enough for the differentiation of genera, allowing to identify contamination generated by other microalgae. The techniques are based on the polymerase chain reaction (PCR), have the advantage of allowing identification even with a low concentration of cells (MONIZ et al. 2012). Quantitative PCR (QPRCR) is one of the strategies employed because it

is highly sensitive, in addition to Real-Time PCR (GACHON et al. 2009). However, it is necessary to consider the costs that of these methodologies.

1.9 **PHYSICAL INTERVENTION**

At present, the control of contamination by means of physical methods is one of the main forms of intervention. This generally allows the disinfection of equipment and the handling of inoculums. The drawback is that it is generally useful only at laboratory and pilot scale, being ineffective on a massive scale (HUANG et al. 2014a).

1.9.1 Filtration

This is one of the most common techniques used to eliminate rotifers, however, they must be in adult stage (> 200µm in length). The main problem of this methodology that eggs and rotifers of juvenile age are small and cross the meshes. In order to thoroughly clean a culture, the filtration must be repeated for 3 or 4 days (GONZALEZ-LOPEZ et al. 2013; WANG et al. 2013; HUANG et al. 2014b). For contaminants of similar or smaller size than that of the microalgae being cultivated, filtration is ineffective.

1.9.2 Physical disruption (sonication)

This technique was created with the purpose of controlling contamination in cultures. The conditions should be evaluated according to the size of the microorganism. If organisms have a small size, this methodology presents greater difficulty (CARNEY AND LANE 2015).

Physical processes such as sterilization, ultraviolet light application and pasteurization can be used, however, the development of these is more difficult in massive systems. For this reason, they can be combined with other methodologies in order to obtain the desired results (GONZALEZ-LOPEZ et al. 2013).

1.9.3 Changes in environmental conditions

Light and temperature are variables that determine the development of microalgae; however, these can be a tool to perform culture control. The temperature, for example, is a variable that determines the cycle of reproduction of the rotifer *Brachionus plicatilis*, which is optimal between 10 to 15 °C. Outside this range, the process becomes longer. This can be used to control cultures infested by this contaminant (HAGIWARA et al. 2001).

Another factor that can be manipulated for the benefit of culture control is pH. In (Meseck 2007) the control of contaminating cyanobacteria *Synechococcus sp.*, in cultures of *Tetraselmis chui* was evaluated at a pH close to 7.1, resulting in the reduction of the population of the contaminating cyanobacteria; low pH values, e.g. 3 – 4, can suppress contaminants. Similarly, salinity can inhibit the development of contamination, this is applicable to some microalgae or cyanobacteria, such as *Dunaliella* (POST et al. 1983; MOOIJ et al. 2015; PENG et al. 2015).

1.10 **CHEMICAL INTERVENTION**

This method is considered one of the most feasible because of its effectiveness in the control of contaminants, however, most chemical agents do not show specify to the biological contaminant, but also to microalgae (LINCOLN et al. 1983; WANG et al. 2013; HUANG et al. 2014a; PARK 2014). Determining the appropriate agents and concentrations to perform the control is not a simple task, requiring the determination of the minimum inhibitory concentration for contaminants, the tolerance of the desired microalga, and tests of population dynamics to avoid damages and losses of the cultures.

The cost of chemical control in mass cultures may be high. Sometimes the combination of chemical agents allows better results, reducing time and costs. An example of this is given by (HUANG et al. 2014b), where the combination of the substances Celangulin (CA) and Toosendanin (TSN) (1:9) allowed the extermination of the rotifers *B. plicatilis* from *Chlorella* and *Nannochloropsis* cultures. The cost for elimination was $ 2.00 per ton of microalgal suspension compared to $ 2.84 for TSN alone, a reduction of 29.6%.

Commonly used chemical agents are antibiotics, fungicides, pesticides, salts, aldehydes, peroxides, among others. **Erro! Fonte de referência não encontrada.** shows a brief description of some of these substances, and the concentrations used.

TABLE 3. CHEMICAL SUBSTANCES AND CONCENTRATIONS USED IN CONTAMINANT CONTROL IN MICROALGAL CULTURES

Substance	Microorganism affected	Concentration (ppm)	Reference
Unionized ammonia	Rotifers, Amoebae	0,7-9,8	(DE ARAUJO et al. 2000; HAGIWARA et al. 2001; HUANG et al. 2014a)
Metronidazole	Amoebae	>200	(MORENO-GARRIDO and CAÑAVATE 2000)
Quinine sulfate	Ciliate	12-14	
Formaldehyde	Ciliate	10 -15	
Hydrogen peroxide	Ciliate	150-200	
Trichlorphon	Rotifers	3274-318	(HUANG et al. 2014c; HUANG et al. 2014a; HUANG et al. 2014b)
Pyrethroids	Rotifers	0,30-1,28	
Celangulin	Rotifers	0,175	
Toosendanin	Rotifers	0,002132	
Azadirachtin	Rotifer	18,38	
Vitamin E	*Asplanchna*	100	
Ammonium bicarbonate	Rotifers	100-150	
Rotenone	Rotifers	0,070-0,1380	(VAN GINKEL et al. 2016)
Buprofezin	Rotifer	240-263	
Decamethrin	Rotifer	4,2	(WANG et al. 2013)
Tralocythrin	Rotifer	0,3-0,4	
Ampicillin	Bacteria/ Fungi	500	(KAN and PAN 2010)
Cefotaxime	Bacteria/ Fungi	100	
Carbendazim	Bacteria/ Fungi	40	

Fonte: The author (2017)

Tolerance to different pesticides differs between cyanobacteria and green algae. Currently, these herbicides play an important role in agriculture and due to their characteristics are used in the cultures. Until now, the acute toxicity of a few pesticides, such as cypermethrin, atrazine, promentin, simetin and paraquat, have been studied in some algal species (MISHRA and PANDEY 1989; ABOU-WALY et al. 1991; MA et al. 2002).

Some biocides such as celangulin has acute and chronic toxicity on contaminants such as rotifers. This type of pesticides (derived from plants) targets the digestive system of the contaminant causing death due to cellular damage in the midgut. Currently, they are good candidates for pollution control, despite presenting medium-life problems in addition to rapid degradation at high temperatures or changes in pH (QI et al. 2011).

Alternative substances, such as ammonium, base their toxicity on non-ionized ammonia, with which it is possible to rapidly exterminate most of the contaminants However, when used in *Spirulina* cultures, it is possible to observe damage during growth, limiting its use (YUAN et al. 2011; HUANG et al. 2014b).

Despite the different alternatives for the control of contamination in algae cultures, the use of antibiotics is always an interesting alternative. However, its greatest application is in the initial stages of cultivation (axenization processes), since its efficiency can be affected in large-scale processes (KAN and PAN 2010; Cho et al. 2013; PAREEK and SRIVASTAVA 2013; HAN et al. 2016). However, its usefulness, the main problem is the resistance that many bacterial contaminants can present to different antibiotics (Davies and Davies 2010).

1.11 **RESEARCH CHALLENGES AND OPPORTUNITIES**

Biological contamination in algae cultures is a factor that cannot be avoided. Therefore, it is essential to develop techniques for the timely identification of the presence of different contaminants and thus achieve a significant control. Despite the existence of control mechanisms (physical and chemical), these are not always effective, and sometimes they can become very expensive, especially if the production is executed in open mass systems. For this reason, it is necessary to investigate methodologies specific for each culture, and the study of new substances, which are of natural origin, in order to avoid problems of toxicity in cultures.

1.12 **RESISTANT STRAINS**

The use of substances such as antibiotics or pesticides traces back to several decades, where their purpose was the control of parasites that cause both diseases and losses in agriculture. With the passing of years and the intensive (and sometimes inadequate) use of these substances, the microorganisms adapted through recombination and mutation, creating resistance. (SRIVASTAVA 1970; NAVAJAS-BENITO et al. 2016). However, the use of rational mixtures reduces the possibilities of resistance, increasing efficiency (concentration) with reduction in production costs (AHMAD et al. 2009).

Studies on resistance are mainly developed for bacteria and fungi, due to their importance in the health area, however, in the case of algae, studies indicate that these substances can be toxic even in micro molar concentrations (LOPEZ-RODAS et al. 2001). Resistance of biological contaminants makes the current contamination control

systems to be insufficient, for this reason it is important to find alternatives of handling through novel, preferably natural substances.

1.13 **NOVEL CHEMICAL AGENTS**

The addition of chemicals agents is usually the best solution to the contamination problem. However, these substances can be toxic to algae causing damage to cultures and losses at the industrial level (WANG et al. 2013). Bio-control of pathogens in cultures is one of the alternatives that has gained space in the control through the development of bioherbicides, natural vegetable products (KALIA, A. 2011).

There are few reports on the use of natural agents in algae cultures. However, these substances are being used for pest control in fruit or vegetable crops. An example of this, is the research do by (KONG et al. 2016), where different substances such as cinnamaldehyde, geraniol, carvacrol and cinnamic acid, are evaluated for the control of fungi. Natural chemical agents are generally less polluting, more environmentally friendly, more easily degradable and may comply with regulation in the disposal of the large volumes of wastewater from mass cultures. However, these substances need research and regulation to be used (BAJPAI et al. 2011).

Allelopathy is a biological phenomenon by which an organism can produce one or more substances that can influence the growth, development and reproduction of another microorganism. In the specific case of algae the allelopathy can function as follows: (I) Substances of one algae affect the growth of another algae, (II) Substances segregated by algae to inhibit their own growth, (III) Toxins that affect the growth of other microorganisms, (IV) Algal toxins that can affect the growth of plants (GUTTERMAN 1994; WANG et al. 2013; CARNEY and LANE 2015). Currently there are not enough studies on allelopathy, however, it is known to be a form of control.

A biological alternative is also the possibility of using other types of microorganisms to control biological contaminants. An example of this is the control of small rotifers by larger rotifers such as *Asplanchna* (>700 µm). This can be used as food for the smaller rotifers and the growth of *Asplanchna* rotifers can be controlled by the addition of vitamin E (100 mg/L) (HUANG et al. 2014c).

1.14 **CLOSED AND CONTROLLED BIOREACTORS**

Currently, most industrial algae production system are open ponds. They are advantageous due to their low operating costs, however, this systems are highly susceptible to contamination and therefore have limited biomass production (WANG et al. 2012). The most likely causes for contamination in open systems are: (I) inoculation of contaminated culture or medium (undetected), (II) keeping the culture open for long periods of time and (III) rapid rate of biological contaminant growth (FOREHEAD and O'KELLY 2013).

One alternative to solve this problem is the use of controlled closed systems, taking into consideration the economic impacts that this system can generate. During the last few years, it was tried to obtain high added-value metabolites from microalgae using closed photobioreactors PBRs (OLAIZOLA 2003; MAHMOUD et al. 2016). This kind of systems in the mass culture need considering the conditions for their increment, such as changes in lighting, gas transference and temperature. It is known that with PBRs greater productivities are obtained (UGWU et al. 2008; WANG et al. 2012).

Nowadays, these reactors are widely used, some examples are the commercial cultures of *Chlorella* and *Haematococcus*, these can reach a capacity of up to $25m^3$, occupying a space of $100m^2$. The variables like temperature and pH are controlled from a computer thus guaranteeing the continues and efficient production (OLAIZOLA 2003).

1.15 **OTHER ENHANCEMENTS**

On the development of techniques for control another tool is the use of electric pulses (PEF). This method can produce the waterproofing of biological membranes. The phenomenon is called electroporation, which depends on transmembrane potential (SAULIS 2010; REGO et al. 2015b). One of the problems of this process is that there is not enough information for biological control. However, it allows reducing or avoiding the use of different methods that impact the cultures (ZBINDEN et al. 2013). The system depends on the electric field strength of the pulses and time; in this case, each cell has a specific critical electric field intensity (Eu) and therefore a specific susceptibility time. This methodology could allow the control of early detected biological contaminants, however, the conditions must be established correctly in order

to avoid damage in the cells (GRAHL and MÄRKL 1996; REGO et al. 2015B; REGO et al. 2015a).

Other types of techniques have been developed for the automated identification of contaminants using image processing techniques using mobile phones. The system consists of the capture of the image, segmentation, identification and recognition of the parasite (LAKSHMI, S., KUMAR, R., & RAJENDRAN 2015).

1.16 **CONCLUSION**

The massive algae production has increased significantly with the past of the years and one of the most important problems is the contamination. In the present review, it was analyzed some of the parasites that negatively influence in the development of microalgae and cyanobacteria, with the goal to improve early detection systems and control mechanisms. Some tools have been exposed, but it is necessary to continue researching and developing methodologies to reduce, for example, the use of chemical substances that over time produce toxicity problems.

The feasibility of detection and control is commonly based on the volume of production and the cost of the product. On the mass production, usually is used the open system despite being mostly grazing. For this reason, it is important a correct definition of the culture conditions to exploit the physiology and biology features of the microorganisms (symbiotic process), generating better results.

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2 CHAPTER II BACTERIA IN MICROALGAL CULTURES: AXENIZATION OF MICROALGAE, COMMUNITY COMPOSITION AND POPULATION DYNAMICS

2.1 ABSTRACT

The maintenance of microalgae culture is a fundamental tool in biotechnology and research. However, one of the main problems is contamination. These may be because of bacteria or various populations of microalgae. The present research develops strategies to eliminate bacterial contamination using microbiological techniques (streak plate method) in *Spirulina maxima* SAG 84.79, *Spirulina platensis* SAG 257.80, *Dunaliella salina* SAG 184.80 and *Neochloris oleoabundans* UTEX LB 1185. cultures. Additionally, from these four cultures 372 microorganisms were identified. Of these, 164 bacteria were isolated and maintained on nutritive agar. And 14 were identify to family level using biochemical methodologies. Finally, a mathematical model was established that allows to describe the population dynamics of a microalga in the presence of bacteria and a control substance antimicrobial.

Key words: Microalgae, contamination, bacteria, axenization, isolation

2.2 INTRODUCTION

2.2.1 Algae microorganisms

Phycologists regard any organisms with α-chlorophyll and a thallus not differentiated into roots, steam and leaves to be an alga. This definition includes cyanobacteria, a prokaryotic organism. Therefore, microalgae broadly refers to microscopic algae and photosynthetic bacteria (LEE; SHEN, 2004; ABELIOVICH et al., 2005).

Microalgae traits that can be explored in bioprocesses are its high grow rate, adaptation to extreme conditions (using different kind of substrates), high photosynthetic efficiency (up to ~10% of the total captured solar energy) and nutritional composition (MARTINS ET AL. 2010; HUANG ET AL. 2014B; MA ET AL. 2016).

Microalgae are present in all ecosystems, mainly aquatic and terrestrial, with a wide variety of species. It is estimated that more than 50.000 species, of which only 30,000 have been studied and analyzed (PREISIG AND ANDERSEN 2005).

Nowadays, the commercial production is focused on extremophiles, because the relatively extreme conditions where they thrive guarantee lower contamination. Microalgal biomass can be used in different industries: dietary supplements, pigments biofuel, cosmetics, pharmaceutical (DAY ET AL. 2012A; WANG ET AL. 2013; HUANG ET AL. 2014A). This biomass market has a size of about 5,000 t/year of dry matter and generates a turnover of ca. U.s $ 1,25x10^{9} / year. One example of commercial success is A*rthrospira (Spirulina) platensis,* a filamentous cyanobacterium, with an annual production estimated around 3,000 tons per year (PULZ AND GROSS 2004; CHOI ET AL. 2008).

Microalgal cultures have low concentrations, and different studies have focused on improving cultivation conditions that can be used in large scale systems. Cultivation can be done using open or closed systems and/or under laboratory conditions. Some examples of these systems are: big, shallow ponds, tanks, circular and raceway ponds (ORON et al. 1979; PARMAR et al. 2011; RAWAT et al. 2013). Several parameters must be considered to reach a better yield, for instance: the selection of an appropriate strain, cultivation conditions, contamination control and economic values (TATE et al. 2013).

2.2.2 Contamination problems

One of biggest problems in algae production is the contamination of cultures. This can affect productivity and final yield and increase the production cost, reducing product quality. In order to control contamination, it is important to use an early detection system (LINCOLN et al., 1983; DAY, JOHN G. et al., 2012; MENDEZ et al., 2012).

Generally, contamination affects cultures under open or closed conditions. All this takes place due to the presence of predators, mainly bacteria, fungi, viruses, herbivores such as rotifers, copepods, and sometimes other species of competitive algae (DE ARAUJO ET AL. 2000; MORENO-GARRIDO AND CAÑAVATE 2000; VAZ ET AL. 2014; MOOIJ ET AL. 2015; MA ET AL. 2016; MOLINA-CÁRDENAS ET AL. 2016). Some of these predators have been identified and isolated from various algae

cultures. Examples of contaminants identified to the level of genus and/or species are the rotifers *Brachionus calyciflorus, Brachionus plicatilis* (HUANG ET AL. 2014A), the chytrid *Paraphysoderma sedebokerensis* (HOFFMAN ET AL. 2008), AND THE C*RYPTOMYCOTA AMOEBOAPHELIDIUM PROTOCOCCARUM* (CARNEY AND LANE 2014). Contamination is often described without further identification ("amoeba," "ciliate," or "rotifer") (FOREHEAD AND O'KELLY 2013), and laboratory experiments are restarted from healthy inocula.

In recent years, different process and technologies have been developed to control the presence of different predatory organisms (GRAHL AND MÄRKL 1996; MESECK 2007; DAY ET AL. 2012B; MÉNDEZ ET AL. 2012; WANG ET AL. 2013; HUANG ET AL. 2014A; LAKSHMI, S., KUMAR, R., & RAJENDRAN 2015; PENG ET AL. 2015; VAN GINKEL ET AL. 2016), nevertheless, the efficiency of these depends on several factors, including: detection stage, type of contaminant, production systems and type of control.

2.2.3 Algae culture systems

There are several systems used to cultivate microalgae and cyanobacteria. Their use depends in most cases on the product of interest. Two of the most used systems are: open systems, such as lakes or ponds, and closed and highly controlled systems (PBRs photobioreactors) (JIMENEZ et al., 2003; CAMPO et al., 2007; MARTINS et al., 2010; WANG et al., 2012; ZHENG et al., 2012; GONG et al., 2015; MAHMOUD et al., 2016).

Open systems have large areas and no barriers, reducing capital costs. These are relatively inexpensive compared to closed systems. However open systems are an excellent habitat for a wide variety of contaminants which may be competitors, parasites, or consumers (WANG et al., 2012). In this type of systems, the presence of herbivores in the form of protozoa and zooplankton is common, which can actively consume the microalgae devastating the culture in 2-3 days (RAWAT et al. 2013).

Another important type of contamination that can occur in this type of production systems is the presence of another microalga in the culture. Generally, contamination by competing microalgae is one of the most complex forms in the control of the contamination, since the physical and biological properties of the contaminant are sometimes similar to that of the species being cultivated (MOOIJ ET AL. 2015).

Closed systems, also called photobioreactors (PBRs) can be optimized according to the biological and physiological conditions of the different species of algae to be cultivate. One of the main advantages of these, is that the direct exchange of gases and contaminants between the cells and the atmosphere is limited or is not allowed by the reactor walls. In this case, the risk of contamination is reduced and the control of the contaminant is simpler than in the previous case (MARTINS ET AL. 2010). However, the chance of massive contamination also exists.

2.2.4 Bacteria in axenic cultures and inocula

Microalgae are conserved and traded in agar slants, liquid cultures, and, less often, cryopreserved or lyophilized. Most of these cultures are unialgal, but not axenic (KRAFT 2014), and that is especially true for mass cultures (BOROWITZKA and MOHEIMANI 2013). The contamination can even be beneficial to microalgae, because several species needs cofactors produced by bacteria (HIGGINS et al. 2016). Despite the contamination, microalgae can outcompete bacteria in aerobic conditions amounting to typically to 95 to 98% of the total microbial biomass (PASSARGE et al. 2006; CHO et al. 2017), which means that there are higher counts of bacteria, but low biomass.

2.2.5 Contamination control

For several years, different strategies have been developed with the aim of mitigating the impact of contaminants on algae cultures.

The alternatives for control can be classified into two groups: (i) physical methods and (ii) chemical methods. In addition, we can consider another method that can be used in certain cultures, which is maintaining extreme conditions the whole time - such as high salinity (*Dunaliella salina*), high alkalinity (*Oocystis sp.* and S*pirulina sp.*), or nutritional stress (DE ARAUJO et al. 2000; LEE et al. 2013; MCBRIDE et al. 2014), reducing the risk of contaminants.

2.2.6 Chemical intervention

Several chemicals have been developed over the years to mitigate or eliminate

biological contamination. An example is sodium hypochlorite (20% w / w) and copper sulphate, commonly used in the disinfection of microalgae ponds to prevent contamination (DAY et al. 2012a; HUANG et al. 2014c).

To determinate correctly the substance and concentration to be used is not an easy task. Several parameters must be considered to avoid toxicity problems, since the chemical process is aggressive and in some cases is not the most recommended for some strains (FOREHEAD and O'KELLY 2013; VAN GINKEL et al. 2016). Another important factor that determines the selection of different substances for the control of the pollution is the cost that these will have when doing the massification of the cultures (HUANG et al. 2014c).

Other substances commonly used are: antibiotics, fungicides, pesticides, salts, aldehydes, peroxides and vitamins (ANDERSON 2005; CHOI et al. 2008; KAN and PAN 2010; CHO et al. 2013; VAZ et al. 2014; MOLINA-CÁRDENAS et al. 2016; JONES et al. 2017).

2.2.7 Physical intervention

Physical intervention is one of the main methods to control, reduce or eliminate biological contamination. It can be mainly used in laboratory and pilot scale cultures, however, it is not suitable for mass scale (HUANG et al. 2014c).

Some techniques commonly used are: filtration, physical disruption (sonication, vortexing, or high pressure homogenization), centrifugation, and disinfection of equipment. (RIPPKA et al. 1979; LEE and SHEN 2004; ABELIOVICH et al. 2005; ANDERSON 2005; RAWAT et al. 2013; VAZ et al. 2014; CARNEY and LANE 2015).

2.2.8 Inoculum quality

One of the alternatives for maintaining mass culture quality is, of course, maintaining the purity of seed cultures. Many microalgal culture are poorly conserved by freezing and are routinely maintained as active populations. These cultures are at greater risk of contamination, besides the natural oscillation of the cell counts in non-axenic cultures. It is essential to understand the nature of the contaminants - or symbionts, and eventually to "reisolate" the microalgae of interest from runaway inocula (AMARAL et al. 2013).

2.2.9 Population dynamics in xenic cultures

Because mass cultures can be contaminated but still stable, as xenic or polycultures, one relevant approach is the analysis of the population dynamics. Understanding population evolution and succession may aid in early detection of contamination, as well as the intervention possibilities to avoid culture crash. That involves the establishment of appropriate mathematical models that allows to establish the main interaction mechanisms between each of the organisms present in the system.

A complete model requires thorough physiological information about all the species possibly present in a culture, something usually impractical. A useful alternative for this, is to adopt a predefined model, such as Lotka-Volterra model (ECHEVERRÍA 2013), frequently used to describe the dynamics of biological systems, in which two species interact (one as a predator and the other as prey), and adapt the model to a particular system. In this way, the problem will be reduced to the calculation of the defining parameters of the system, accommodating the interactions between the organisms observed in the system. The main problem with this approach is the difficulty in adopting the model to the specific conditions of the system to be studied.

Another alternative is to construct a more general framework for the system based on specific, but limited characteristics of its constituents and the interactions expected between them, that can model the dynamics of system over time. In this case, the problem will be reduced to the establishment of a discrete-time or continuous-time model using a set of differential equations that describe the rate at which a variable of system changes over time. That approach can also accommodate the inclusion of bioactive substances, which can appear as a population reduction term in the equations. In this work we axenized and cultivated two strains of microalgae, *Neochloris oleoabundans* UTEX 1185 and *Dunaliella salina* SAG 184.80, and two strains of cyanobacteria *Spirulina platensis (Arthrospira*) SAG 257.80 and *Spirulina maxima (Arthrospira)* SAG 84.79. To isolate possible prokaryotic contaminants, present in these four cultures, to determine minimal inhibitory concentrations for these contaminants and develop mathematical model capable of describing the kinetic behaviour of a microalgal culture in the presence of a contaminant population (bacteria) and a control substance.

2.3 **MATERIALS AND METHODS**

2.3.1 Strains and culture conditions

Two cyanophyte strains were tested, *S. platensis* SAG 257.80 *and S. maxima* SAG 84.79 and two chlorophytes, *Neochloris oleoabundans* UTEX *1185* and *Dunaliella salina* SAG *184.80* (fig. 1-4). The cyanophytes were pre-cultivated in 250 mL Zarrouk medium (AIBA and OGAWA 1977; ALGAE 2008) and the chlorophytes in 250 mL BG_{11} and Johnson medium flasks cultures respectively (STANIER et al. 1971; SATHASIVAM and JUNTAWONG 2013) (APPENDIX I, II and III)**.** They were kept in an orbital shaker (120 rpm) at 25°C.

2.3.2 Isolation of contaminating bactéria

To isolate the contaminating bacteria, 100 µl of *Spirulina platensis* SAG 257.80, *Spirulina maxima* SAG 84.79, *Neochloris oleoabundans* UTEX *1185* and *Dunaliella salina* SAG *184.80* were spread on the surface of Nutrient agar (Kasvi, Brazil) and of Zarrouk, BG_{11} and Johnson medium agar plate. Each agar plate was incubated at 25°C, for 1 week with photoperiod 12:12 and an illumination of 60 µE $mol.m^{-1}. s^{-1}$. In addition, based on their morphological characteristics (color and the gross appearance of the colonies on the agar plate) 148 colonies were isolated from the nutrient and Zarrouk plates (CHOI et al. 2008). Additionally, to count the contaminant bacterial, 100 µl aliquots of serially diluted of each strain were also spread on the surface of Nutrient agar. Each test plate was incubated at 25°C for 48 hours. The number of observable colonies were counted on three suitable dilutions, averaged and expressed as CFU (colony forming units).

2.3.3 Biochemical identification of bacterial isolated from *Neochloris oleoabundans* UTEX 1185 cultures

Bacterial colonies with visual different phenotypes were selected for the identification process (LAKATOS et al. 2014). A total of 14 possible isolates were cultivated at 25°C, for 48 hours, with illumination of 60 µE mol.m-1. s-1, in the agars: SIM, Citrate, Methyl Red, Voges Prokauer and Triple Sugar Iron. In addition, tests for

oxidized, catalase and gram stain were performed (BENSON 2001; FERNÁNDEZ OLMOS et al. 2010).

2.4 METAGENOMIC ANALYSES

2.4.1 Total DNA extraction

For each sample, 1 mL of algae culture was centrifuged at 12,000 × g for 1 min. The cell pellet was resuspended in 500 µL Tris-EDTA, homogenized with 10 µL of lysozyme solution 20 mg/mL lysozyme solution (Sigma Aldrich, Arklow, Ireland) and incubated at 30°C for 60 minutes. Then, 50 µL of SDS 10% (w/v) and 10 µL of proteinase K solution 20 mg/mL (Sigma Aldrich, Arklow, Ireland) were added to the lysis solution, followed by homogenization and incubation at 60 ºC during 60 min. 150 µL of phenol-chloroform (25:24) were added, homogenized by inversion, and centrifuged at 12,000xg for 5 min. The supernatant was removed, and the DNA was precipitated with 3x (v/v) absolute ethanol. Pellets were washed with 80% ethanol, dried and resuspended in ultrapure water. Total DNA was quantified with a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, USA).

2.4.2 Illumina high-throughput sequencing

The V4 variable region of the 16S rRNA gene (bases 515 to 806) was amplified from the total DNA extracted samples according to the Illumina 16S metagenomics sequencing library protocol. DNA was amplified with specific primers for the V3 region containing complementary adaptors for Illumina platform (CAPORASO et al. 2012) using KlenTAQ polymerase (Sigma Aldrich, Arklow, Ireland). Bar-coded amplicons were generated by PCR under the following conditions: 95 ºC for 3 min, followed by 18 cycles at 95 ºC for 30s, annealing at 50 ºC for 30s, extension at 68 ºC for 60s, final extension at 68 ºC for 10 min and hold at 10 ºC. Samples were sequenced in the MiSeq platform using the 500 V2 kit, following standard Illumina protocols.

2.4.3 Bioinformatics and data analysis

Data generated by the sequencing went through a rigorous quality system that

involved: (I) identification and removal of sequences containing more than one ambiguous base (N); (II) evaluation of the presence and complementarity of primers and barcodes sequences. Chimeric sequences detection, removal of noises from pre-cluster and taxonomic attribution were also performed using standard parameters of QIIME software package, version 1.9.0. Applying the UCLUST method (EDGAR 2010), sequences presenting identity above 97% were considered the same operational taxonomic units (OTUs) according to the SILVA database (QUAST et al. 2013).

2.5 RE-ISOLATION AND AXENIZATION OF ALGAE CULTURES

The contaminating bacteria in the culture of *Neochloris oleoabundans* UTEX 1185 and *Dunaliella salina* SAG 184.80 were eliminated using streak method on BG_{11} and Johnson solidified medium (1.2% w/v). 100 µL were inoculated on the agar plates. The plates were cultivated with photoperiod 12:12 an illumination of 60 µE $mol.m^{-1}. s^{-1}$ provided by white fluorescent lamps at 25°C. after 7 days, a single green colony was picked and streaked on a new agar plate. The colonies were observed in microscope (Olympus bx-41) to identify remaining contaminations. This process was carried out the sufficient number of times to obtain axenic colonies (KAN and PAN 2010). For *Neochloris oleoabundans* UTEX 1185 this was done five times, and for *Dunaliella salina* SAG 184.80 four times.

For *Spirulina platensis* SAG 257.80 and *Spirulina maxima* SAG 84.79 the methodology was done in two steps. First, a 1:10 dilution was performed on sterile Zarrouk medium. Then, 100 µL of this dilution were inoculated on Zarrouk 2.5% agar plates. The plates were cultivated with photoperiod 12:12 an illumination of 58.59 µe $mol.m^{-1}. s^{-1}$ provided by white fluorescent lamps at 25°C. The light was placed only at one side of the plate so that it could receive the greatest amount of light. After 10 days, the growth of *Spirulina* filaments was observed in the direction of higher intensity of light. Subsequently part of these filaments was transferred to new Zarrouk agar plates (OVANDO 2015).

In the second phase, 2 ml of *Spirulin*a culture were centrifuged for 5 minutes at 6000 rpm, after which the supernatant was removed using a micropipette. Additionally, to the same tube were added 2 ml of sterile distilled water and the centrifugation was repeated. This step was performed four times. Then, the biomass was resuspended in 2 ml of the Zarrouk liquid medium and the tubes were maintained

four hours (CHOI et al. 2008). From these suspensions, all the content was filtrated using glass fiber microfilter (gf-1) of 0,7 µm coupled with a vacuum filter system. The filaments were washed with 2 ml of sodium hypochlorite (1,2 and 3% v/v) for 10, 20, and 30 s and then washed with 5 ml of distilled sterile water. The biomass present on the filter membrane was removed using a transfer loop and streaked onto petri dishes containing Zarrouk solidified medium (1.2 % w/v). These plates were cultivated under the same light and temperature conditions described above for all cultures (Vaz et al. 2014).

2.6 AXENIC CULTURE VERIFICATION

The algae colonies were selected using a transfer loop and then streaked into BG11, Johnson and Zarrouk solidified medium (1.2% w/v agar) containing also 1% added glucose. These plates were incubated at 25°C, for at least 7 days.

2.7 DYNAMICS OF THE BIOLOGICAL SYSTEM

Given the complexity of the system proposed in this research (algae, biological pollutant and a bioactive compound, possibly a terpene), and that most of the models that exist only identify the direct relationships for two variables. It was necessary to establish a model that allowed the definition of a three-way interaction of the variables in a biological system.

2.7.1 Mathematical model

A set of three first-order, non-linear equations was used for describing the concentrations of microalgae, bacteria and a bioactive, antimicrobial agent (FIGURE 1). That is a reasonable assumption for a population of algae under ideal conditions (direct interaction between system constituents, nutrients amount limited, culture perfectly mixed).

FIGURE 1. DIFERENTIAL EQUATION MODEL

$$\frac{\partial C_A}{\partial t} = \alpha C_A + \beta C_A C_B + \gamma C_A C_T + \delta C_A C_B C_T \quad (1)$$

$$\frac{\partial C_B}{\partial t} = \varepsilon C_B + \zeta C_A C_B + \eta C_B C_T + \theta C_A C_B C_T \quad (2)$$

$$\frac{\partial C_T}{\partial t} = \iota C_T + \kappa C_A C_T + \lambda C_B C_T + \mu C_A C_B C_T \quad (3)$$

Source: The author (2017)

In these equations, C is the concentration of a population (A – alga, B – bacteria) or a chemical substance (C), while the Greek letters are proportionality constants. The first equation describes how the microalgal population growth rate ($\delta C_A/\delta_t$) is affected by the population of the microalga (C_A), its interaction with a biological contaminant (C_AC_B), and its interaction with a bioactive substance (C_AC_T).

Similarly, in the second equation, the bacterial population growth rate depends of the contaminant bacterial population interaction with the microalga (C_BC_A) and the bioactive substance (C_BC_T). Finally, the third equation describes the change of bioactive substance concentration, probably degradation, by independent reaction (e.g. oxidation), by interaction with microalgae, (C_AC_T) and with bacteria (C_BC_T). In all equations, the fourth term establishes a triple interaction between all constituents ($C_AC_BC_T$).

The set of equations can describe several situations, from unconstrained microalgal growth to different degrees of interaction, depending on the values of the parameters and initial conditions.

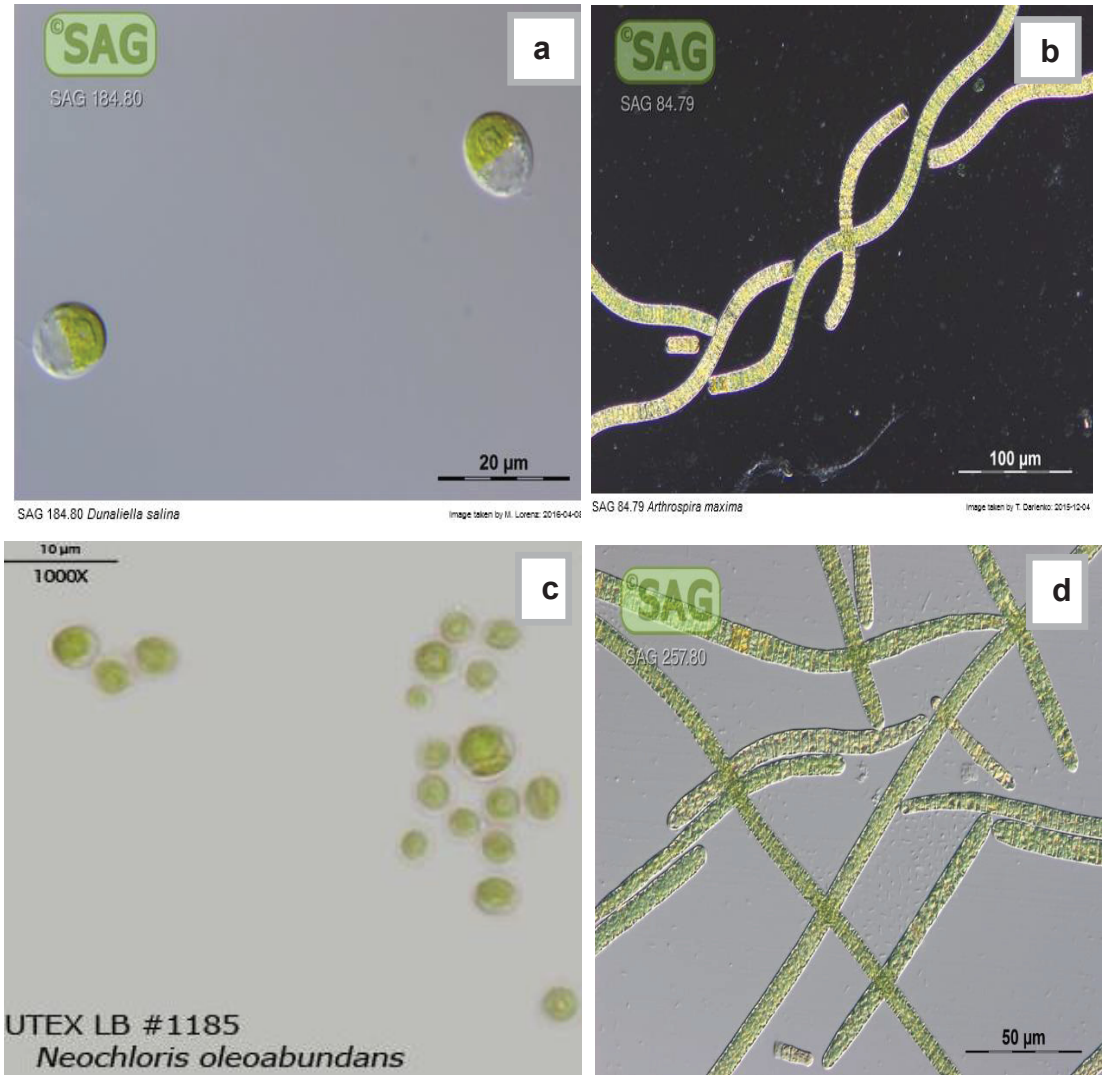
The model was implemented using the software MATLAB, version R2016b using Euler's method. The initial conditions proposed equations are: C_A = 0,1 mg/mL, C_B = 0,001 mg/mL and C_T = 0,05 mg/mL. The results and discussion are presented in (APPENDIX IV).

2.8 RESULTS AND DISCUSSION

2.8.1 Strains and culture conditions

To design a suitable methodology for the purification and axenization of the microorganisms, a study of the biological contaminants presents in the cultures and maintained in the laboratory was carried out. In FIGURE 2a, 2b, 2c, and 2d. We can observe the microalgae and cyanobacteria used in the present work.

FIGURE 2. a) *Neochloris oleoabundans* UTEX LB 1185, b) *Spirulina platensis* SAG 257.80, c) *Spirulina maxima* SAG 84.79, d) *Dunaliella salina* SAG 184.80



Source: The author (2017)

2.8.2 Population and isolation of contaminating bacteria and metagenomic analyses

The population density of contaminating bacteria in the cultures was: *Spirulina platensis* 1,79x10^5 CFU.mL^{-1}, *Spirulina maxima* 6,53x10^5 CFU.mL^{-1}, *Dunaliella salina*

1,74x10^4 and *Neochloris oleoabundans* 5,80x10^5 CFU.mL^{-1}. The size of these populations is lower than that reported by (Choi et al. 2008) for a culture of *Arthrospira platensis* (3.7x10^6 CFU.mL^{-1}) and that reported by (Cho et al. 2013) for cultures of microalgae from environmental samples (6,5x10^7 CFU.mL^{-1}).

This behavior may be due to the culture conditions used. For example, *Spirulina* sp. is a filamentous cyanobacteria that normally requires high levels of carbonate and bicarbonate with alkaline pH values up to 11 (Gami et al. 2011) a condition that is toxic for certain bacteria (Watanabe et al. 2005; Michael T. Madigan; et al. 2006). Selective conditions are also used for the maintenance and production of *Dunaliella salina*, whose cells grow best at 5-10% NaCl, however, concentrations above 12% favors the elimination of predators (BEN-AMOTZ, A., POLLE, J. E. W. & SUBBA RAO 2010).

Bacteria are naturally present in algal cultures and it is now known that they may interact positively or negatively with phytoplankton growth. An example of the positive interaction is promoting growth of microalgae because the release of growth factors, such as vitamins, and benefits the transformation of organic material into more assimilable forms of carbon (COLE 1994; UNNITHAN et al. 2014; MOLINA-CÁRDENAS et al. 2016). On the other hand, some of the negative effects that may occur are: inhibition of algae growth due to the production of algaecides (Seyedsayamdost et al. 2011), induction of cell lysis, competition for nutrients, among others effects (HYENSTRAND et al. 2000; GUO and TONG 2013).

One hundred and forty-eight colonies were isolated from Nutritive agar and Zarrouk agar (four cultures of processed algae) based on the color and morphology of the colonies. For *Dunaliella salina* 25 colonies were isolated, for *Neochloris oleoabundans 14* colonies, for *S. maxima* 60 colonies and for *S. platensis* 56. In total, 164 strains were continuously maintained on Nutritive agar plates (ANEXX V). To evaluate the diversity of contaminants, present in the four initial samples, the High-throughput sequencing by Illumina technique was used. This sequencing allows to identify the microbial species present in a sample (PALLEN et al. 2010; ILLUMINA 2014).

2.8.2.1 Dunaliella salina

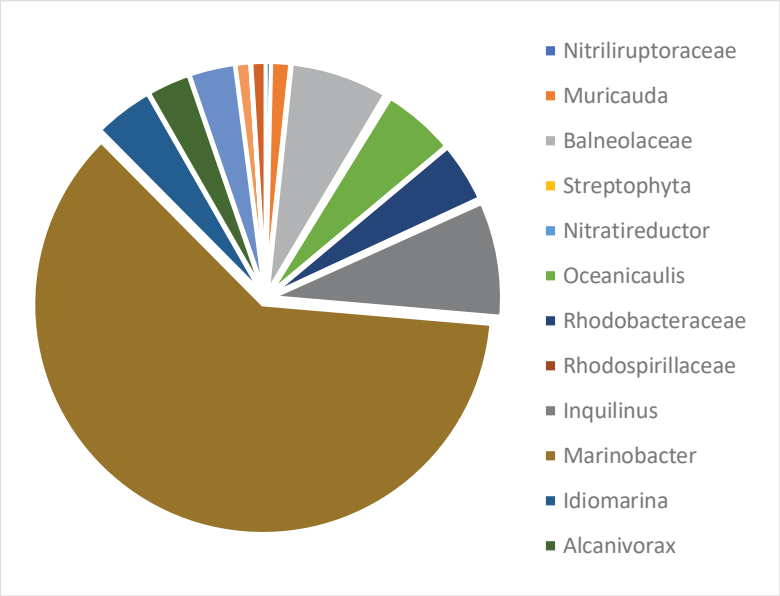
Based on the analyses, a total of 63 microorganisms were identified (at the level of order, family or genus) in the sample of *Dunaliella salina*. These 63 microorganisms correspond to 94.37% of the total identified. The remaining 5.63% were removed from the analysis due to low precision at the time of identification.

Among the bacteria identified, the highest percentage (57.76%) corresponds to *Marinobacter*, a gram negative bacillus (rod-shaped cells), aerobic and mobile by a single polar flagellum (Bowman and Mcmeekin 2015). The presence of this bacterium in the cultures of *Dunaliella* can be due to its ability to grow at 0.5 to 20% of NaCl concentration (i.e. Johnson culture medium). Additionally, its optimal growth temperature range is 25-35°C, temperature at which *Dunaliella salina* is cultivated (SMYTH et al. 2010).

One of the main biotechnological uses of *Marinobacter* at present, is the bioremediation of aliphatic hydrocarbons such as n-hexane (RÖLING et al. 2004; DEPPE et al. 2005; GAUR ed et al. 2012).

FIGURE 3 shows the microorganisms identified with greater abundance in the sample of *Dunaliella salina*. The "other" percentage represents the sum of several identified microorganisms whose presence is minimal in the culture.

FIGURE 3. MICROORGANISMS IDENTIFIED IN CULTURES OF *Dunaliella salina*



Source: The author (2017)

2.8.1.2 Neochloris oleoabundans

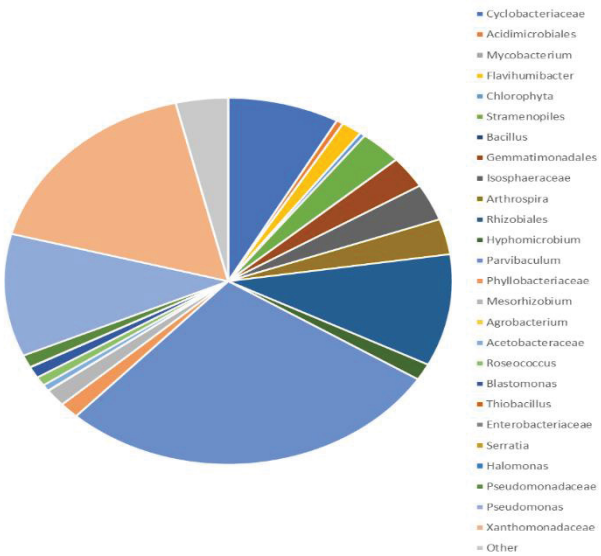
In the case of *Neochloris oleoabundans*, a total of 115 microorganisms were identified at the level of order, family or genus. These microorganisms correspond to 92.67% of the DNA extracted and amplified form the sample, and the reminder 7.32% was eliminated due to lack of identification.

Based on this identification, the contaminants with the greatest abundance are genus *Parvibaculum* (25.83%), order *Xanthomonadaceae* (17%), genus *Pseudomonas* (11%), and genus *Rhizobiales* (10%). This culture has a greater number of biological contaminants in comparison to the culture of *Dunaliella salina*, and no dominant species as observed in the previous case.

In *N. oleoabundans* cultures using BG_{11} as a production medium, interaction of the microalga with different bacteria is reported in the literature (CHO et al. 2013; JONES et al. 2017). Nowadays, it is known that some of these interactions may be favourable for algae. In the case of *Neochloris oleoabundans*, the interaction with the bacterium *Azotobacter vinelandii* allows it to be used as a source of nitrogen in co-cultivation (SANTOS and REIS 2014; VILLA et al. 2014; FUENTES et al. 2016).

FIGURE 4 shows the presence of other microorganisms (order, family, genus) in the culture of *Neochloris oleoabundans*. Again, the microorganisms with the lowest concentration in the culture are represented as label “others” (3.49%).

FIGURE 4. MICROORGANISMS IDENTIFIED IN CULTURES OF *Neochloris oleoabundans*



Source: The author (2017)

2.8.1.3 Spirulina maxima and *Spirulina platensis*

These two cultures are discussed together because they belong to the same genus and are cultivated in the same conditions and -yet, the important differences in the bacterial contaminant populations was observed.

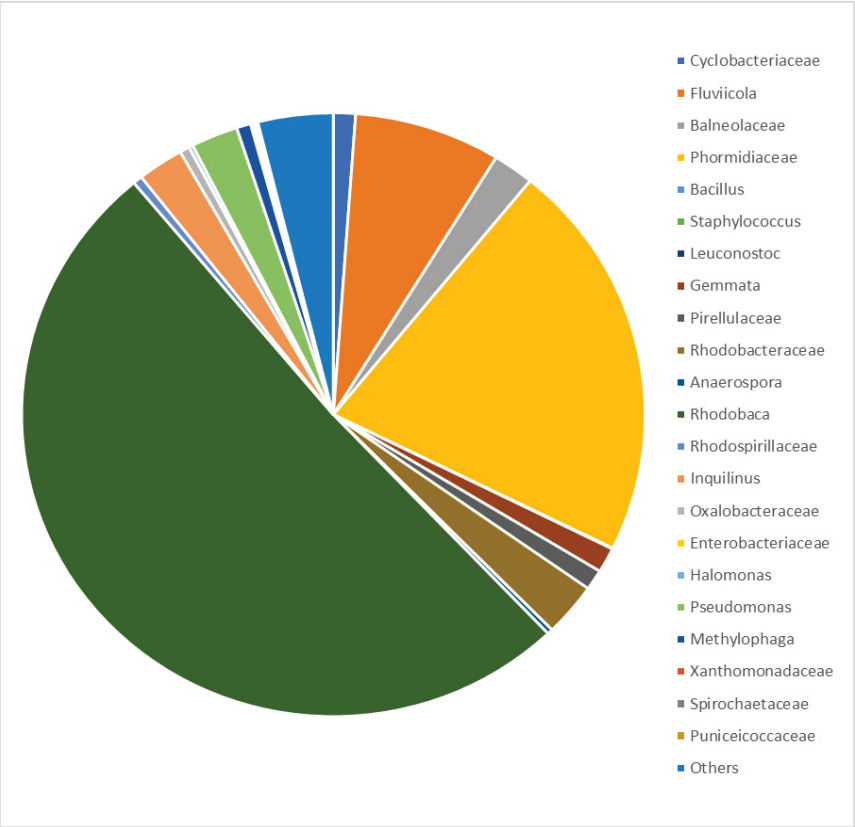
For the cultures of Spirulina maxima and *Spirulina platensis* a total of 101 and 93 biological contaminants were identified respectively. In the culture of *Spirulina maxima*, the 101 contaminants represent 88.16% of the contaminating biomass, whereas for *Spirulina platensis* the 93 contaminants are 85.54% of the contaminating biomass. Similarly, as done in the previous analyses, 11.83% (*S. maxima*) and 14.45% (*S. platensis*) of the information was discarded due to lack of precision in the identification.

For *Spirulina* cultures, the most abundant contaminants are like the chlorophyte contaminants, however, they do not present the same values. The genus *Rhodobaca* is at 44.99% in *S. maximum*, whereas for *S. platensis* it is at 22.98%. The genus *Fluviicola* in *S. maximus* culture is found in 6.71% and in *S. platensis* culture is 9.56%.

In the case of *S. platensis*, we find other biological contaminants in high proportions, some genera are: *Pseudomonas* (4.45%) and *Inquilinus* (3.78%). With a lower presence in *S. maxima* culture, we found the genus *Bacillus* (0.02%), *Pseudomonas* (2,14%), *Sthaphylococcus* (0.0028%). For *S. platensis*, we found genera such as *Halomonas* (1.21%), *Sthaphylococcus* and *Mycobacterium* with (0.0027%). In the work done by (Choi et al. 2008) with *Arthrospira* (*Spirulina*) *platensis*, different species of *Halomonas* and *Sthaphylococcus* were observed, similarly to the present work.

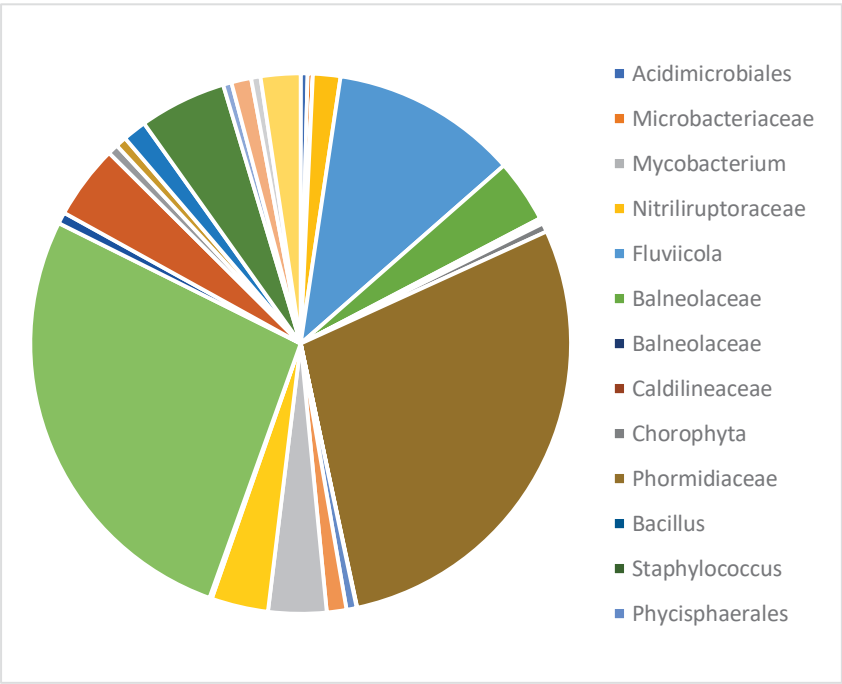
FIGURE 5 and 6 show other identified microorganisms (order, family, genus) for *S. maxima* and *S. platensis* cultures. Again, the microorganisms with the lowest concentration in the cultures are represented with label “others” (3.44% and 2.04%, respectively).

FIGURE 5. MICROORGANISMS IDENTIFIED IN CULTURES OF *Spriulina maxima*



Source: The author (2017)

FIGURE 6. MICROORGANISMS IDENTIFIED IN CULTURES OF *Spriulina platensis*



Source: The author (2017)

Some of the genera above mentioned are common contaminants of different algal cultures. *Pseudomonas*, for example, has been identified along with cultures of *Chlorella vulgaris* (GUO and TONG 2013) and *Oscillatoria* (JONES et al. 2017). Other contaminants identified here have been described as potentiators in certain microalgae cultures. Examples of these are bacteria from the genus *Sphingomonas, Microbacterium, Flavobacterium, and Agrobacterium*, identified in the present study and reported elsewhere (WATANABE et al. 2005; SUBASHCHANDRABOSE et al. 2011; LAKATOS et al. 2014; UNNITHAN et al. 2014; CHO et al. 2015).

The enormous diversity of population in stable, dense microalgal cultures indicate that microalgal inocula may carry a diversity of contaminants, and these contaminants cannot be easily isolated by classical microbiological techniques – the diversity observed in the ribosomal RNA gene analysis is far higher than that obtained in colony isolation.

2.8.3 Population and isolation of contaminating bacteria and metagenomic analyses

Biochemical identification of bacteria isolated from *Neochloris oleoabundans* UTEX LB 1185 culture. It was possible to isolate 14 bacterial strains from *Neochloris oleoabundans* UTEX LB 1185 cultures (C1-C14). TABLE 4 shows the results from Gram stain. From the gram stain it was possible to identify 9 Gram-negative, 6 Gram-positive and 5 Gram-positive cocci.

TABLE 4. GRAM STAIN BACTERIAL ISOLATED FROM *Neochloris oleoabundans*

Strain	**Gram**	
C1	Rod	+
C2	Rod	+
C3	Rod	+
C4	Cocci	+
C5	Cocci	+
C6	Rod	+
C7	Rod	+
C8	Rod	-
C9	Rod	-
C10	Rod	+
C11	Cocci	+
C12	Rod	+
C13	Rod	-
C14	Cocci	+

Source: The author (2017)

Through the physiological and biochemical characteristics, it was possible to classify 5 families of bacteria. *Bacillaceae* (genus *Bacillus*), *Rhizobiales*, *Pseudomonadaceae* (genus *Pseudomonas*), *Streptomycetaceae* (genus *Streptomyces*) and *Leuconostocaceae* (genus *Leuconostoc*). These results correspond to those obtained in the metagenomic analysis **Erro! Fonte de referência não encontrada.**.

TABLE 5. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE ISOLATED BACTERIA FROM *NEOCHLORIS OLEOABUNDANS* UTEX LB 1185 CULTURE

		Biochemical test								
Strain	**Colony aspect**	**Oxidase**	**Catalase**	**Motility**	**Indol**	**H2S**	**Citrate**	**MR**	**VP**	**TSI**
C1	White colony	+	-	+	-	-	+	-	-	R/R
C2	White colony	+	+	-	-	+	+	-	-	R/R
C3	White colony	+	+	-	-	+	+	-	-	R/R
C4	Yellow colony	-	-	+	-	-	-	-	+	A/A
C5	Yellow colony	+	-	-	-	-	-	-	+	A/R
C6	White colony	+	+	-	-	-	+	-	-	R/R
C7	White colony	+	-	-	-	-	-	-	+	R/R
C8	White colony	+	-	-	-	-	-	-	+	R/R
C9	White colony	+	-	-	-	-	-	-	-	R/R
C10	Yellow colony	-	-	-	-	-	-	-	+	A/A
C11	White colony	+	+	-	-	-	+	-	-	R/R
C12	Yellow colony	+	+	-	-	-	+	-	-	A/A
C13	White colony	-	+	+	+	-	+	-	-	R/R
C14	Yellow colony	-	-	+	-	-	-	-	+	A/R

Source: The author (2017)

As described in the section metagenomic results of *Neochloris oleoabuandans*, it is possible to find different types of bacteria associated with microalgae. In the works carried out by (GOECKE et al. 2013; BIONDI et al. 2017; KROHN-MOLT et al. 2017) some of the bacteria reported in the present study were identified. Although many of the relationships between microalgae and bacteria are beneficial in certain cases it is necessary to establish limits on their growth to avoid damage or loss of cultures.

2.8.4 Isolation and axenization of algae culture

The axenic cultures of *Dunaliella salina* were obtained after 4 successive depletion plating's using Jonhson agar plates (1.2% agar), while in the case of

Neochloris oleoabundans were obtained successively 5 successive depletion in BG11 plates (1.2% agar) For the case of *S. platensis and S. maxima*, the positive phototactic property of cyanobacteria was used. This motility allows to obtain separate colonies in solid medium for a subsequent planting until obtaining pure culture (OVANDO 2015) (Appendix VI).

It is known that the process of axenization of algae cultures can be long and expensive, which is why they are not routine activities in laboratories (PAREEK and SRIVASTAVA 2013; FUENTES et al. 2016). At the other side, the benefits of cultivating microalgae with the presence of bacteria have been widely discussed. However, they should be properly analyzed, and the populations known. For example, when the conditions of cultivation are modified, the population ratio between the species will be also altered. This process was observed in cultures of *Chlorella vulgaris* and *Pseudomonas* whose relationship of mutualism changes to competition when culture conditions were modified from autotrophic to heterotrophic (GURUNG et al. 1999; JUNG et al. 2008; GUO and TONG 2013).

Adequate knowledge about genetics and the interactions between these microorganisms, as well as the production of crops with high biotechnology potential in areas such as pharmaceuticals and food, are reasons why the process of axenization is important in laboratories.

2.8.5 Modelling microalgal growth

The model proposed is clearly unable to accommodate all the possible interactions between the populations – and even if it is expanded to include all the species observed, e.g. one equation for each species, the interaction terms would be simply too numerous. However, the analysis of figures in the Appendix V show that for each microalgal culture, more than 75% of the bacterial population belong to ca. 5 species, which gives a manageable number of interactions – 7 equations with 7 terms each, using only pairwise interactions. Still, the prediction of 35 kinetic parameters that are necessarily make the model impractical.

Looking at the other side, the simplest model that could represent the system has only two microorganism populations: one for the microalgal species of interest, and the other of a mixed bacterial population. With this simplification, which includes a bioactive substance, it is possible to simulate situations that occur in contaminated

cultures, e.g. microalgal growth, stability, oscillation or crash, by simply varying the parameters (TABLE 6).

TABLE 6. MORPHOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF THE ISOLATED BACTERIA FROM *NEOCHLORIS OLEOABUNDANS* UTEX LB 1185 CULTURE

Condition	Initial (g/L)	Outcome
Algae concentration	0,01	0,05
Bacterial concentration	0,1	0,01
Bioactive substance concentration	0,05	0,01
Time	20 days	

Source: The author (2017)

Using this approach and having laboratory data for the growth rate of the microalga of interest and representative bacteria in the presence of organic carbon, and the minimal inhibitory concentrations of antimicrobial agents, it might be possible to simulate cultures from field data and predict – and prevent – culture crashes.

2.9 **CONCLUSIONS**

Two microalgae strains, *Dunaliella salina* SAG 1844,80 and *Neochloris oleoabundans* UTEX LB 1185 were axenized. The cyanobacteria *Spirulina maxima* and *platensis* were purified, but final axenization must be confirmed.

A total of 165 bacteria were isolated from the four microalgae cultures *Dunaliella salina* (25), *Neochloris oleoabundans* (14), *S. maxima* (60) and *S. platensis* (56).

With the biochemistry characterization was possible to identify 5 families of bacterias. Bacillaceae (genus *Bacillus*), *Rhizobiales, Pseudomonadaceae* (genus *Pseudomona*), *Streptomycetaceae* (genus Steptomyces) and Leuconostocacea (genus *Leuconostoc*).

From the metagenomics assay were identified in total 372 microorganisms present in the four cultures analyzed. Of these, 63 are found in *Dunaliella salina*, 115 in *Neochloris oleoabundans*, 93 in *S. platensis* and 101 in *S. maxima*.

The biological contaminants with the greatest abundance were for the *Dunaliella salina* culture was the *Marinobacter*, with 57.67% mass of bacteria contaminant; For *Neochloris oleoabundans* was the *Parvibaculum* with 25.83% mass of bacteria contaminant. And in the cases of S. maxima and S. platensis was the *Rhodobaca* with 44.99% mass of bacteria contaminant and 22.98% mass of bacteria contaminant respectively.

A mathematical establishing the direct relations between the constituents of the system has potential to represent the interactions between bacterial and microalgal populations. Defining the precise constants for each bacteria-microalga system essential for adequate modelling.

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3 CHAPTER III LC50S OF NEOCHLORIS OLEOABUNDANS IN RESPONSE TO FIVE TERPENES

3.1 ABSTRACT

Microalgae have great attention as a promising source for sustainable production of fatty acids, carotenoids, vitamins, and biodiesel production. *Neochloris oleoabundans* has recently demonstrated its potential to accumulate relevant amounts of carotenoids, fatty acids. Similarly, to plant crops, the major problems that algal mass cultures is that they can be affected by biological contaminants. This article reviews relevant aspects for early detection and control of the biological contaminants that may affect mass microalgae algae cultures and evaluate the toxicity of 6 terpenes to contamination control of culture of the green algae *Neochloris oleoabundans* through toxicity analysis to obtain the LC_{50}. The LC_{50} for β-pinene and Limonene were 15 and 16 ppm respectively, the value obtained to α-pinene was 65 ppm. Eugenol and Linalool have LC_{50} above 250 ppm. The use of compounds such as α-pinene probably contributes to the "healthy" of the culture of *N. oleoabundans*. In addition, this is a green technology alternative to sanitize the cultures.

Key words: Healthy cultures, Microalgae, Terpenes, Toxicity.

3.2 **INTRODUCTION**

Algae are the vital importance in the primary production of the aquatic ecosystem because they have been considered good indicators of the bioactivity of industrial wastes and they vary in response to a variety of toxic compounds. Therefore, it is important to explore the potential adverse effects of antibiotics and different contaminants on algae to evaluate their risk an aquatic environment (MA et al. 2002; FU et al. 2017).

Microalgae have drawn great attention as a promising source for sustainable production of fatty acids, carotenoids, vitamins, and other compounds of interest (DO NASCIMENTO et al. 2012; DE JESUS RAPOSO et al. 2013). Altogether, secondary

metabolites from microalgae have great potential for industrial development as they include bioactive compounds such as antioxidant, antiviral, antibacterial, antifungal, anti-inflammatory, antitumor, and antimalarial effectors. However, natural products in microalgae remain largely unexplored compared to those in land plants, even though cultivation of microalgae offers many advantages over those of terrestrial plants, e.g., the rapid growth rates, and lack of competition for resources used for food crops, including the use of fresh water and arable lands.

Microalgae cultures are usually of low concentration and producing large amounts of biomass requires equally large volumes. An alternative doing so at low production costs, is the cultivation in open systems (STEPHENSON et al. 2010; McBride et al. 2014; RICHARDSON et al. 2014; MA et al. 2016); nevertheless, this technology is still not fully optimized for all types of algae (BOROWITZKA AND MOHEIMANI 2013; MCBRIDE et al. 2014).

One of the major problems with this algal mass cultures is that they can be affected by other organisms (contaminants) (ABELIOVICH et al. 2005; DAY et al. 2012b; MCBRIDE et al. 2014). These contaminants may impair the development of the cultures, resulting in reduced production, and sometimes complete loss of the desired microorganism population or final products (MESECK 2007; DAY et al. 2012b; MÉNDEZ et al. 2012; FOREHEAD AND O'KELLY 2013).

Early detection and control of contaminants are important to avoid significant culture damage and losses. During the last years, techniques have been developed aiming to control the contamination in the mass cultures of algae (MAHAN et al. 2005; KAN and PAN 2010; DAY et al. 2012b; FOREHEAD AND O'KELLY 2013; HUANG et al. 2014b; HUANG et al. 2014c; HUANG et al. 2014a). This article reviews relevant aspects of early detection and control of the biological contaminants that may affect mass microalgae algae cultures.

The success of microalgae biotechnology depends on the choice of microalgae with relevant properties to specific conditions of cultivation and desired product (PULZ AND GROSS 2004). For this reason, it is very important to evaluate the response of microalgae with the biotechnological potential to contaminants (terpenes), to know which compounds can be used in the contamination control of the culture without affecting the growth of the algae.

The toxicity analysis is the most commonly used methodology for this evaluation; the purpose of this test is to determine the effects of a substance on the

growth of freshwater microalgae and/or cyanobacteria. Exponentially growing test organisms are exposed to the test substance in batch cultures over a period of normally 72 hours. In spite of the relatively brief test duration, effects over several generations can be assessed (MA et al. 2002; OECD 2011).

The system response is the reduction of growth in a series of algal cultures exposed to various concentrations of a test substance. The response is evaluated as a function of the exposure concentration in comparison with the average growth of replicate, unexposed control cultures. For full expression of the system response to toxic effects (optimal sensitivity), the cultures are allowed unrestricted exponential growth under nutrient-sufficient conditions and continuous light for a sufficient period of time to measure the reduction of the specific growth rate (HÖRNSTRÖM 1990; OECD 2011).

Growth and growth inhibition are quantified from measurements of the algal biomass as a function of time. The test endpoint is inhibition of growth, expressed as the logarithmic increase in biomass (average specific growth rate) during the exposure period. From the average specific growth rates recorded in a series of test solutions, the concentration bringing about a specified x % inhibition of growth rate (e.g. 50%) is determined and expressed as the Lethal Media Concentration(Crane and Newman 2000; OECD 2011; Sebaugh 2011).

3.3 **MATERIALS AND METHODS**

3.3.1 Algal strains and culture conditions

The green alga *Neochloris oleoabundans* UTEX 1185 was used as the test organism. Cells of n. *N. oleoabundans* were pre-cultivated in a 250 ml bg11 medium. The culture was massified in 2000-ml Erlenmeyer flask containing 1000-mL (MURRAY et al. 2011). The inoculum was taken in a ratio of 1/3 compared to the medium and the initial concentration of biomass in the medium was standardized to 0.1 g/l (OD640 0.2). The Erlenmeyer were cultivated with photoperiod 12:12 and with an illumination of 60 µe $mol.m^{-1}. s^{-1}$ at 25°C.

3.3.2 Determination of growth parameters

For growth analyses was considered cell number using a Fusch-Rosenthal hemocytometer by microscopy. Optical density was determinate at 640 nm (OD640) with a spectrophotometer (Bioespectro SP-200). The cellular concentration was determinate using dry weight. 15 mL of samples were filtrated using glass fiber microfilter (GF-1) of 0,7 µm (Macharey Nagel, Germany) coupled with a vacuum filter system. The glass fiber microfilter were previously dried and tared, after filtration they were dried in an oven at 105°C for 24 h. The cultures were analyzed every three days (COSTARD et al. 2012; LE CHEVANTON et al. 2013).

Maxima specific growth rate (μ_{max}), and doubling time were obtaining from fitting the experimental data to theorical curves of exponential growth with R2≥0.95 (FIGURE 7 and 8 respectively).

FIGURE 7. MAXIMA SPECIFIC GROWTH RATE

$$Ln\ (DO_2) = \mu_{max} + Ln\ (DO_1)$$

Source: The author (2017)

FIGURE 8. DUPLICATION TIME

$$Td = \frac{Ln_2}{\mu_{max}}$$

Source: The author (2017)

Where, DO_2: Absorbance at the end of the selected time interval; DO_1: biomass concentration at the beginning of the selected time interval.

3.3.3 Toxicity assays

For the toxicity assay, 20-mL aliquots of the BG11 medium containing green algal cells (initial cell concentration 2 x 10^4 /mL) were distributed to sterile 125-mL Erlenmeyer flasks (OECD 2011). The media of *Neochloris oleoabundans* UTEX LB 1185 was treated with various terpenes concentrations ranging from 0 to 1000 mg/L, and incubated for 96 hours, at a temperature 25°C using the same photoperiod and illumination conditions. The growth of algal cells was calculated using a Fusch-Rosenthal hemocytometer by microscopy. Each terpene concentration was replicated

three times. Appropriate control systems containing no terpene were included in each experiment. Control and treated cultures grew under the same conditions of temperature, photoperiod of the stock cultures (HÖRNSTRÖM 1990; HALLING-SØRENSEN 2000; MORENO-GARRIDO AND CAÑAVATE 2000; MA et al. 2002). The LC_{50} and confidence limits were obtained whit the Dose-Response model (RITZ et. al. 2015) using statistics package R Version 1.1.414.

3.4 **RESULTS**

3.4.1 Kinetic parameters

The maximum specific growth rate (μ_{max}), was 0.127 d^{-1} and the doubling time was 5.45 days. These values are lower than those obtained in the work presented by MURRAY et al., (2011). For biomass production, the maximum value reached was 0.468 g / L after 30 days of production. At present there are reports with biomass concentrations higher than the one reported in this study, however, production must be optimized (PRUVOST et al. 2009).

The growth rate of microorganisms can be affected by different factors, i.e. nutrients, temperature, light intensity, light / dark cycle, CO_2 enrichment or others. This can decrease the growth rates of some strains and results in Differences between microalgae.

3.4.2 Toxicity assays

Sufficient supply of nutrients, efficient gas transfer and exchange, and delivery of photosynthetically-active radiation (par) (WANG et al. 2013) are all major challenges during productions of microalgae, which have been the subject in academic and industrial studies. besides these, it was also found by us doe in 2010, microalgal monocultures grown for biofuel and other bioproducts were susceptible to biological pollutants (RODOLFI et al. 2009; MATA et al. 2010; TATE et al. 2013). infection or contamination by biological pollutants could cause the sudden and massive death of microalgal cells, but little attention was paid to this.

The contamination control in microalgal cultures it is a challenge of academic and industrial researchers in the last years, find which are the compounds and the

concentration will be used to have axenic culture without affecting the algae growth it is a critical point. Some researchers are attempted to filter the algae liquid or add drugs to annihilate the biological contaminations, while others focused on changing the environmental conditions to control them (GAIKOWSKI et al. 1999; HUANG et al. 2014a). However, are a few publications suggesting how to use chemicals to inhibit or kill biological pollutants (FISCHER et al. 2012b; FOREHEAD AND O'KELLY 2013; MOLINA-CÁRDENAS et al. 2016; PAREEK AND SRIVASTAVA 2016b). Among these chemicals, pesticides were firstly used to annihilate the zooplankton in the microalgae suspension (DENG et al. 2015).

Although, adding chemicals is one of the options for controlling biological pollutants, it may also damage the growth of target microalgae, the screening of biological drugs which inhibit biological pollutants without damaging the target microalgae is the preferred route (MORENO-GARRIDO AND CAÑAVATE 2000). Li et al. (2006) studies an alcohol extract of *Artemisia annua* L. to inhibit the growth of ciliate. A further issue is that the separation and test of biological drugs still require further investigation.

Biological compounds are an alternative to "axenize" microalgae cultures because they cause less damage to algae and eliminate biological contaminants efficiently. Terpene constituent of essential oils from plants, such as lemon, orange, pine, among others with microbial activity contribute to maintaining cultures "sanitized" (DAYAN et al. 2009; SALEEM 2014; KONG et al. 2016a). The values of LC50 of *Neochloris oleoabundans* UTEX LB 1185 in response to β-pinene, α-pinene, Limonene, Eugenol and Linalool are shown non- TABLE 7.

TABLE 7. LC50 VALUES OF *NEOCHLORIS oleoabundans* IN RESPONSE TO THE TERPENES CITED, AND CONFIDENCE LIMITS 95 %. THE VALUES ARE MEAN ± SE (n=3). THE LC50 ARE OBTAINED WHIT THE DOSE-RESPONSE ANALYSIS ('DRC' MODELS) USING R STATISTICS PACKAGE.

Terpene	**LC_{50} ± SE (ppm)**	**Confidence limits 95%**	
		Lower	**Upper**
α-pinene	17.43 ± 1,85	13.52819	21.32976
β-pinene	65.33 ± 14,83	34.03491	96.62316

Limonene	16.33 ± 2,34	11.40149	21.26189
Eugenol	288.08 ± 192,27	-117.57119	693.72663
Linalool	249.30 ± 159.37	-86.92872	585.53327

Source: The author (2017)

Knowing the values of LC50 can use a correct amount of substances that allow “cleaning” the culture, in this study the values obtained for β-pinene and Limonene are around 16 ppm, while α-pinene the value obtained was 65 ppm. On the other hand, the values obtained for Eugenol and Linalool are above 250 ppm, however, the confidence limits are very wide; which suggests that the maximum concentration used in this study, does not inhibit 50% of the growth of the population of *N. oleoabundans*, for this reason, it is advisable to perform a new test with concentrations above 1000 ppm.

It is important to mention, that in this study could not be performed at higher concentrations because the terpenes Eugenol and Linalool have very low solubility above 1000 ppm, forming microdroplets within the suspension that make the terpene not available in the concentration desired.

Until now, LC50 values have been reported for the control of zooplankton associated with algae cultures, with chemical herbicides (Trichlorphon, Buprofezin), above 250 mg / L (SNELL AND HOFF 1987; SAHA AND KAVIRAJ 2008). which implies the application of very large quantities of herbicides to clean the crops, for this reason, the values obtained in this study seem to indicate that using compounds of natural origin, in low concentrations (<100 ppm) could effectively control the proliferation of microorganisms in large-scale microalgae cultures. The use of compounds such as α-pinene probably contributes to the "axenization" of the culture of *N. oleoabundans*. In addition, they represent an ecologically sustainable alternative that would promote the use of clean technology in this area of research.

3.5 **CONCLUSIONS**

The LC50 values were: α-pinene 17.43 ± 1,85; β-pinene 65.33 ± 14,83; Limonene 16.33 ± 2,34; Eugenol 288.08 ± 192,27; Linalool 249.30 ± 159.37.

The terpene that showed the highest level of toxicity was limonene while the one with the lowest toxicity is Eugenol.

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APPENDIX I – COMPOSITION OF THE JONSHON'S MEDIUM

Stocks	Reagents	Concentration (g/L)
Macronutrients	NaCl	87,7
	$MgCl_2.6H_2O$	1,5
	$MgSO_4.7H_2O$	0,5
	KCl	0,2
	$CaCl_2$	0,2
	KNO_3	1
Fe-solution	$NaHCO_3$	0,043
	KH_2PO_4	0,035
	Na_2EDTA	0,189
	$FeCl_3.6H_2O$	0,244
Trace element solution	H_3BO_3	0,0610
	$(NH_4)_6Mo_7O_{24}.4H_2O$	0,038,
	$CuSO_4.5H_2O$	0,006
	$CoCl_2.2H_2O$	0,0051
	$ZnCl_2$	0,0041
	$MnCl_2.4H_2O$	0,0041

To prepare one liter of culture medium, all macronutrients must be dissolved in 980 mL of distilled water. For the micronutrient and the iron solution 10 mL should be used. To prepare solid agar medium, 1.2-1.5% base Agar should be dissolved, then sterilized and plated.

APPENDIX II – COMPOSITION OF THE BG11 MEDIUM

Stocks	Reagents	Concentration
1	$NaNO_3$	15 (g/L)
2	K_2HPO_4	2,0 (g/500 mL)
	$MgSO_4.7H_2O$	3,75 (g/500 mL)
	$CaCl_2$	1,80 (g/500 mL)
	Citric acid	0,30 (g/500 mL)
	Ammonium ferric citrate Green	0,30 (g/500 mL)
	Na_2EDTA	0,05 (g/500 mL)
	Na_2CO_3	1 (g/500 mL)
3 (Trace metal solution)	H_3BO_3	2,86 (g/L)
	$MnCl_2.4H_2O$	1,81 (g/L)
	$FeCl_3.6H_2O$	0,22 (g/L)
	$ZnSO_4.7H_2O$	0,39 (g/L)
	$CuSO_4.5H_2O$	0,08 (g/L)
	$Co(NO_3)_2.6H_2O$	0,05 (g/L)

To prepare one liter of medium, 100 ml of stock 1, 10 ml of stock 2 and 1 ml of stock 3 should be mixed with 889 ml of distilled water. In the case of agar medium, 1.2-1.5% base agar will be dissolved, then sterilized and plated.

APPENDIX III – COMPOSITION OF ZARROUK MEDIUM

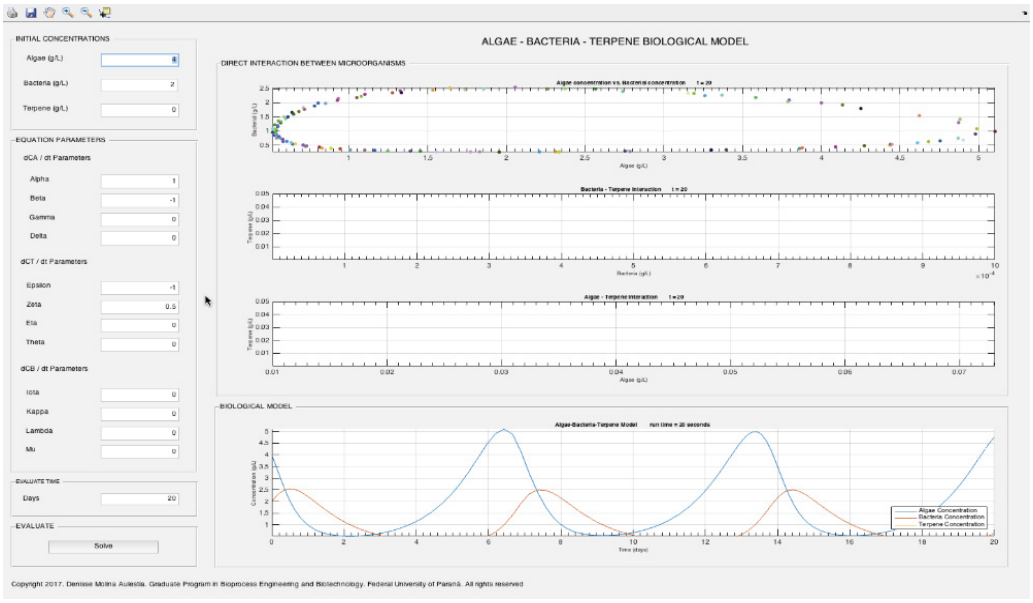
Stocks	Reagents	Concentration
1	$NaNO_3$	2,5
	K_2HPO_4	0,5
	K_2SO_4	1
	NaCl	1
	$MgSO_4.7H_2O$	0,2
	$CaCl_2$	0,04
	$NaHCO_3$	16,8
Fe-solution	Na_2EDTA	0,08
	$FeSO_4.7H_2O$	0,01
Micronutrients	$ZnSO_4.7H_2O$	0,1 (g/100mL)
	$MgSO_4.7H_2O$	0,1 (g/100mL)
	H_3BO_3	0,2 (g/100mL)
	$Co(NO_3)_2.6H_2O$	0,2 (g/100mL)
	$Na_2CO_4.2H_2O$	0,02 (g/100mL)
	$CoSO_4.5H_2O$	0,0005 (g/100mL)
	$FeSO_4.7H_2O$	0,7 (g/100mL)
	EDTA	0,8 (g/100mL)

To prepare one liter of medium, dissolve the reagents in distilled water and add 1 mL of the micronutrient solution. In the case of solid medium preparation, the solutions must be prepared and sterilized separately, including the agar solution (1.2-1.5%) and mixed after cooling.

APPENDIX IV – MATHEMATICAL MODELLING

Two hypothetical situations were interpreted to analyze the functionality of the model. First: consider the prey predator base model. It is observed how populations of prey and predators vary in time. The parameters used were: α = β = γ = δ = 1 (FIGURE 9).

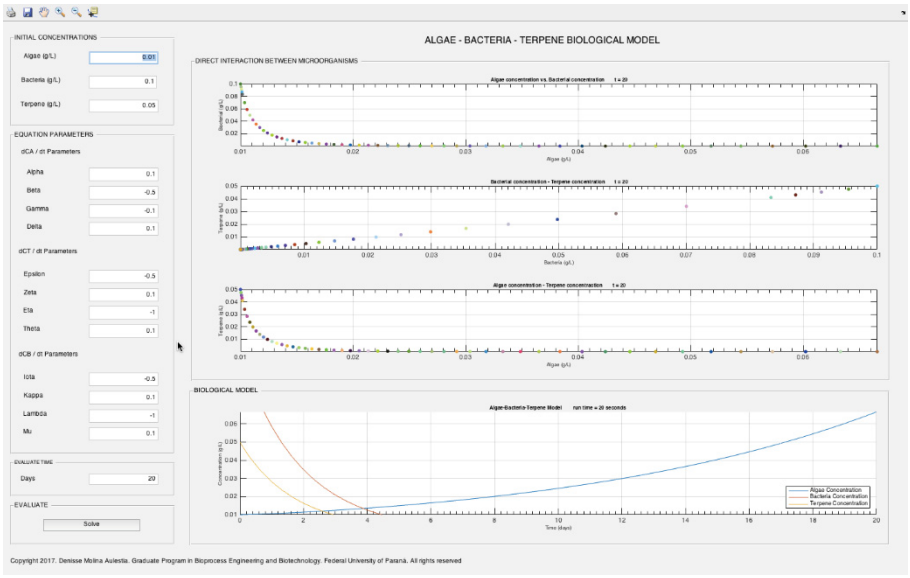
FIGURE 9. PREDATOR PREY MODEL GRAPHICS



Source: The author (2017)

In the second case, all constants and interactions are analyzed. In this case it is possible to observe how the concentration of microalga increases as a function of time and as the antimicrobial agent decreases, besides generating the death of the bacteria. The parameters used were: Algae concentration = 0.01 g / L, bacterium = 0.1 g / L, antimicrobial agent = 0.05 g / L. A = 0.1, β = -0.5, γ = -0.1, δ = 0.1, ζ = 0.1, ε = -0.5, η = -1, θ = 0.1, I = -0.5, κ = 0.1 = λ = -1, μ = 0.1 (FIGURE).

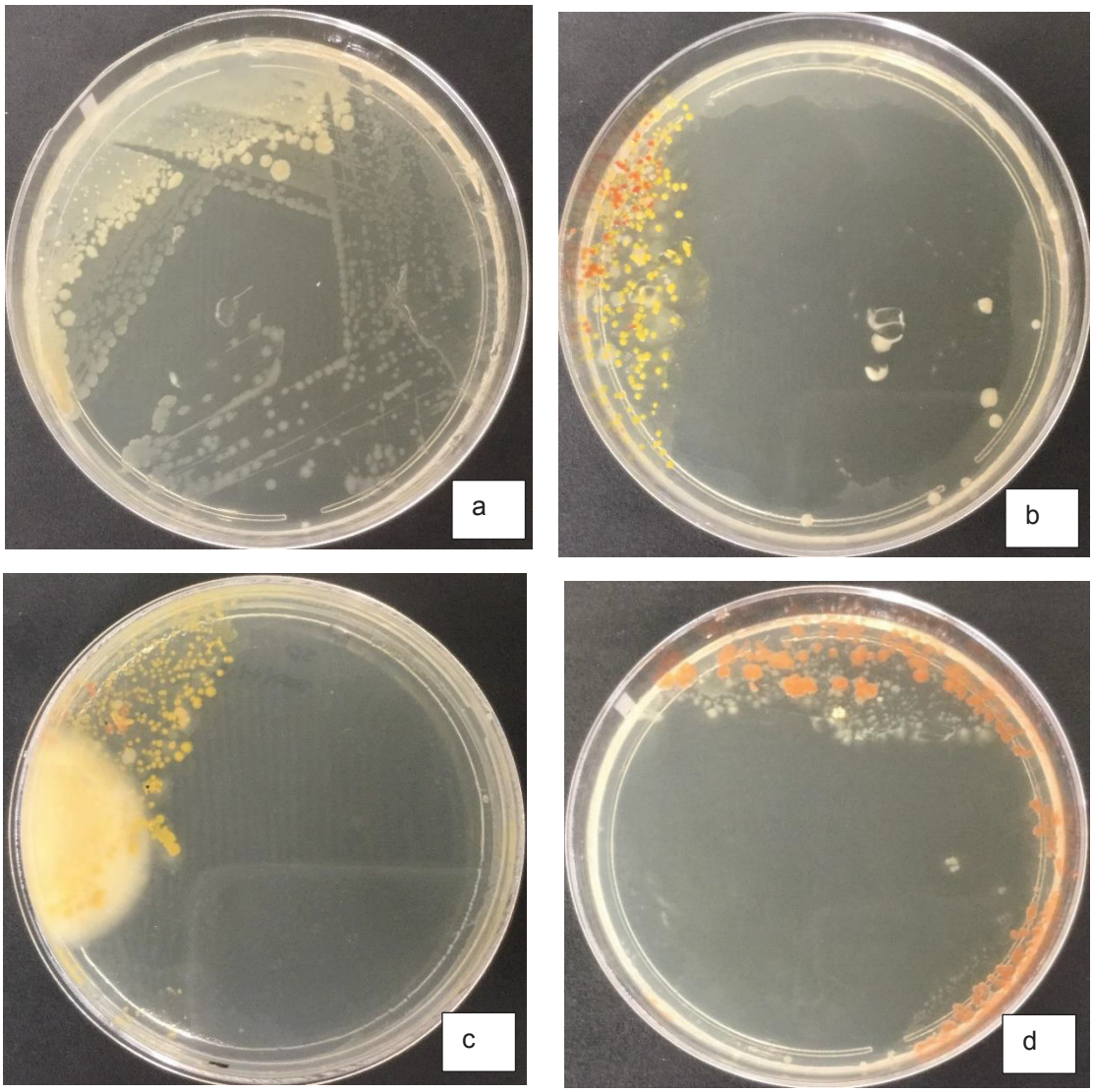
FIGURE 10. SIMULATION OF THE CONCENTRATIONS OF ALGAE, BIOLOGICAL CONTAMINANT AND ANTIMICROBIAL AGENT, USING ALL INTERACTIONS



Source: The author (2017)

APPENDIX V – ORIGINAL SAMPLES OF HEALTHY (BUT NOT AXENIC) MICROALGAL CULTURES AFTER 10 DAYS IN AUTOTROPHIC MEDIA

FIGURE 11. NUTRIENT AGAR PLATE WITH THE FIRST INOCULATION OF a) *Dunaliella salina* SAG 184.80 b) *Spirulina platensis* SAG 257,80, c) *Neochloris oleoabundans* UTEX LB 1185, d) *Spirulina maxima* SAG 84.79



APPENDIX VI – ORIGINAL SAMPLES OF HEALTHY (BUT NOT AXENIC) MICROALGAL ISOLATED AND AXENIC CULTURES

FIGURE 12. PHOTOTACTISM IN CULTURES OF a) *Spirulina platensis*; b) *Spirulina maxima*