Assessing methods for bioprospecting of marine algae for antimicrobial agents

Chirine Issam Kanaan

Thesis for the Master's degree in Pharmacy
45 study points

Department of Pharmaceutical Bioscience, School of Pharmacy
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

May 2019

Assessing methods for bioprospecting of marine algae for antimicrobial agents

Chirine Issam Kanaan

Thesis for Master´s degree in Pharmacy

Department of Pharmaceutical Bioscience, School of Pharmacy

Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

May 2019

Supervisors: Hanne Cecilie Winther-Larsen & Katrine Lekang

2019

Assessing methods for bioprospecting of marine algae for antimicrobial agents

Chirine Issam Kanaan

http://www.duo.uio.no/

Print production: Reprosentralen, University of Oslo

Acknowledgments

The work in the present thesis took place at the Department of Pharmaceutical Biosciences and the Department of Biosciences, Section for Aquatic Biology and Toxicology (AQUA), University of Oslo (UiO).

I owe a deep sense of gratitude, heartfelt indebtedness, and regards to my esteemed guides and supervisors Professor Hanne Cecilie Winther-Larsen and Postdoctoral fellow Katrine Lekang. They consistently allowed this paper to be my own work, and steered me in the right direction whenever i needed it. I am gratefully indebted to them for their support, guidance, and valuable comments on this thesis.

Furthermore, i would also like to acknowledge all people in ZEB who helped me enormously in the lab, particularly Sarah Finke, Agnieszka Wrobel, Mona Gaarder and many more. In addition, i would like to express my sincere gratitude to Rita Amundsen for her wonderful laboratory assistance at Department of Bioscience, Section of Aquatic Biology and Toxicology, UiO.

My family has always been an inspiration for me and the credit of this work goes to them. I really acknowledge my beloved parents and my sister Amani Kanaan, for their unconditional love, help and support. Furthermore, i profoundly acknowledge my husband Ali Alrida Moussa, he helped me greatly through the writing process of this thesis, whose perpetual support and encouragement have backed me up during the hard times of my work. My research wouldn't see the day's light without my family’s constant motivation. I owe them a lot.

Thank you.

Author

Chirine Issam Kanaan

Oslo, May 2019

Abstract

For centuries, infectious diseases had a huge toll on the global mortality and morbidity rates. Antibiotics emergence was fundamental for the process of controlling, preventing and fighting infectious diseases, regardless of their degree of fatality. However, the antibiotic resistance problem is developing and increasing every day, endangering the efficacy of the available antibiotics and threatening the effective treatment of infectious diseases. Antibiotic resistance is considered among the greatest threats to global health in the 21st century. The development and discovery of new antibiotics to combat antibiotic resistance strains is a challenging task today as difficulties in clinical development, scientific bottlenecks, regulatory and economic issues have all hindered the field from gaining enough pace.

Considerable interest in the potential of marine microalgae to produce various bioactive compounds have brought these single cellular organisms into focus. Several methods for assessing the antimicrobial potential of microalgae exist, and there are advantages and disadvantages associated with all of them. Throughout working with this master thesis, four chosen strains of microalgae, namely *Isochrysis galbana*, *Dunaliella tertiolecta* *Nannochloropsis oculate* and *Tetraselmis suecica* were cultured and assessed in terms of potential for antimicrobial compounds, using *Vibrio alginolyticus* as a bacterial test organism. Two methods were used for the mentioned purpose; the co-culturing and the mechanical disruption algal cells methods. Cultures of the microalgae strains *I. galbana* and *D. tertiolecta* showed antibacterial potential against *V. alginolyticus,* as they successfully reduced the bacterial growth when microalgae and bacteria were co-cultivated. Temperature affected antimicrobial activity against *V. alginolyticus,* as these microalgae displayed higher potential for antimicrobial activity when co-cultivated at a temperature of 25 °C than at 20 °C. The co-cultivation experiments also demonstrated that the antimicrobial potential may differ, depending on the algae growth phase. *I. galbana* reduced the growth of *V. alginolyticus* during algal exponential and stationary growth phases, while it increased bacterial growth, during algal death phase. In further experiments, disrupted algal material from all four microalgae was added to cultures of *V. alginolyticus*. The results from these investigations did not demonstrate any significant antimicrobial activity, as the disrupted algae material seemed to stimulate bacterial growth, rather than reducing it. However, a small inhibition zone was observed using the disc diffusion method to test cell-free supernatant from the algal material of *T. suecica*. The obtained

results show that the used methods, the co-culturing and the mechanical disruption algal cells methods, could prove efficient if optimized and adjusted in a correct manner.

Abbreviations

AIDS	Acquired immune deficiency syndrome
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
CFU	Colony forming units
DHF	Dihydrofolic acid
DHFR	Dihydrofolate reductase
DNA	Deoxyribonucleic acid
EM	Extra metabolites
EPA	Eicosapentaenoic acid
EU	European Union
FDA	Food and Drug Administration
G20	Group of Twenty
HIV	Human immunodeficiency virus infection and
HTA	Hexadecatrienoic acid
LPS	Lipopolysaccharide
mg	Milligram
µg	Microgram
MIC	Minimal inhibitory concentration
mL	Milliliter
µl	Microliter
µmol	Micromole
MRSA	Methicillin resistant *S. aureus*
NORCCA	Norwegian Culture Collection of Algae
OD	Optical density
o/n	Overnight

PA	Palmitoleic acid
PABA	Para- aminobenzoic acid
PBPs	Penicillin binding protein
PBS	Phosphate Buffered Saline
PLE	Pressurized liquid extraction
PUA	Polyunsaturated aldehydes
R&D	Research and Development
rRNA	Ribosomal ribonucleic acid
SWE	Subcritical water extraction
TCBS	Thiosulfate-Citrate-Bile Salts-Sucrose agar
THF	Tetrahydrofolate
TLC	Thin-layer chromatography
tRNA	Transfer ribonucleic acid
UK	United Kingdom
USD	United States dollar(s)
USA	United States of America
UV	Ultraviolet
WHO	World Health Organization
xg	Times gravity

Table of Contents

1 Introduction

1.1 Antibiotics

1.1.1 Definition of the term "Antibiotic"

The famous word "Antibiotics" was originally used in the English language as an adjective. No later than 1940s, the Ukrainian-American microbiologist, Selman A. Wekman, who discovered more than 20 antibiotics during his lifetime, championed the use of "Antibiotics" in medical sense as a noun, introduced to describe anti-infectious drugs (Bennett, 2015; Kresge, Simoni, & Hill, 2004). He published the comprehensive definition of antibiotics in 1947, as follows: "*An antibiotic is a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms."* (Waksman, 1947).

This word was accepted worldwide and put in use, both in public and scientific publications immediately. However, the definition of the term antibiotic, has evolved since then and the currently accepted definition is known as "any antimicrobial substance that kills or inhibits the growth of bacteria or other micro-organisms, regardless of its origin". Meaning that compounds may be regarded as antibiotics regardless of whether they are synthetic, semisynthetic, are produced by microorganisms or exist freely in nature (Bennett, 2015; Mohr, 2016).

1.1.2 Discovery and implementation of antibiotics

Prior to the introduction of antibiotics therapy in the twentieth century, infectious diseases were accountable for high morbidity and mortality rates worldwide for centuries. During that time, the humankind witnessed repeated outbreaks and huge epidemics such as smallpox, syphilis, diphtheria, plaque, cholera, typhus, pneumonia, typhoid fever, and tuberculosis, as a result of uncontrolled and often rapid spread of infectious diseases. Such infections were the regulators of our life expectancy, which was no more than 47 years on average, even in industrialized countries (reviewed in Adedeji, 2016; Mohr, 2016; Yoshikawa, 2002). This led to the loss of millions of lives due to lack of knowledge regarding the underlying mechanisms of such diseases.

In the nineteenth century, it was discovered that pathogenic microorganisms are an underlying cause of several diseases as exemplified by the diseases mentioned above (reviewed in Dougherty & Pucci, 2011; Gould, 2016; Saga & Yamaguchi, 2009). This formed a base for the control of infectious diseases that started in the twentieth century (reviewed in Brachman, 2003; Dougherty & Pucci, 2011).

By the 1900s, a dramatic fall in the death toll caused by infectious diseases was noticeably observed in the industrial countries such as the United States primarily due to health and hygiene improvements. Nevertheless, despite the overall positive health progress, the Spanish flu, one of the most severe epidemics in human history, occurred in 1918 (figure 1). This explains the vulnerability of the human race against volatile infectious diseases. However, the continuous decline in infectious diseases was regained and boosted by the introduction of antibiotic treatments.

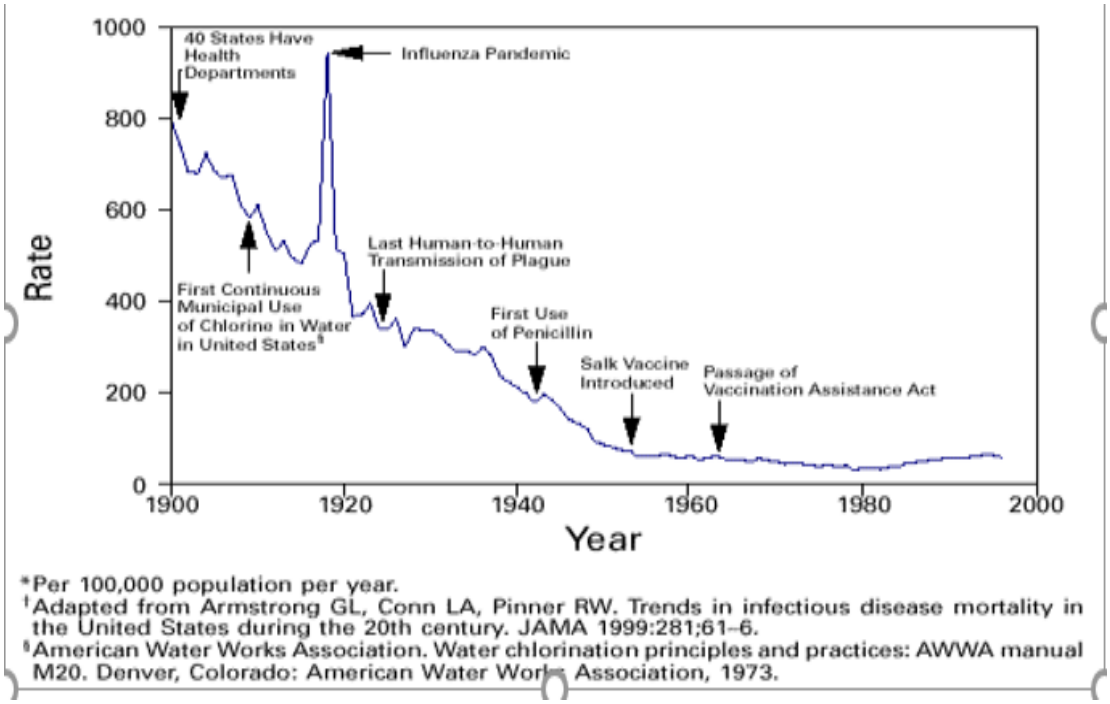


Figure 1 *- Death rates of infectious diseases in the United States during the 20th century (Centers for Disease Control and Prevention, 1999).*

The history of antibiotics discovery and implementation is full of important events that took place long ago. In the 17th century, Antonie Van Leeuwenhoek was the first to observe and

describe single celled organisms, including bacteria, using handcrafted microscope (Smit & Heniger, 1975). This was the first important innovation in microbiology that gave a glimpse into micro objects' world and led the way to the further successful story of antibiotic discovery (reviewed in Mohr, 2016). During the same century John Parkington described the healing effects of mold in his pharmacology book (Parkinson, 1640). Likewise, more scientists and physicians in the 19th century got interested in molds with antimicrobial effect; they investigated the antibacterial effect of the fungus *Penicillium* by successive experiments (Duchesne, 1897; Majno & Joris, 1979; reviewed in Mohr, 2016; reviewed in R. P. Rubin, 2007; Tiberio, 1895; Tyndall, 1876). These experiments were crucial to the later successful discovery and isolation of Penicillium's active substance, later known as penicillin (Mohr, 2016).

Understanding the fundamentals of diseases is the first step towards successful treatments. Overcoming infectious diseases, however, was a dilemma pertaining to elucidating their nature and cause. Robert Koch (1843-1910) was the first who managed to verify the correlation between a specific microbial agent and a disease (Dougherty & Pucci, 2011; Mohr, 2016). He established four postulates, which he used to identify the specific causative agents of tuberculosis (*Mycobacterium tuberculosis*) and cholera (*Vibrio cholera)* (Koch, 1884; Loeffler, 1884). He was awarded the 1905 Nobel Prize as an appreciation of his great work on tuberculosis (Blevins & Bronze, 2010).

The beginning of the modern "antibiotics era" is usually associated with the names, Paul Ehrlich and Alexander Fleming (reviewed in Rustam I. Aminov, 2010). The German physician Paul Ehrlich, in cooperation with the chemist Alferd Bertheim and the bacteriologist Sahachiro Hata, discovered the first synthetic chemotherapeutic agent arsphenamine (Salvarsan) as a treatment for syphilis in 1909 (Ehrlich & Halta, 1910). Enhrlich's discovery of Salvarsan is termed as the first "magic bullet", a term related to his scientific theory of selectively targeting disease-causing microbes (e.g. bacteria) without harming the host body (Witkop, 1999). This theory was applied to find a drug against syphilis in 1904 by a systematic large-scale screening of several hundred synthetic compounds that were tested in syphilis-infected rabbits, until they came across the compound no 606 "Salvarsan" that was effective in treatment of the disease (Rustam I. Aminov, 2010). Salvarsan together with the less toxic Neosalvrasan were the drugs of choice until the arrival of penicillin in the 1940s and other antimicrobial drugs that followed (Mahoney, Arnold, Harris, & Health, 1943). Enhrlich's approach led to the discovery and

development of Prontosil (sulfa drugs), a synthesized drug that was introduced into medicine world in the 1930s (Domagk, 1935).

Although the antimicrobial effect of fungi was first observed by the Italian scientist Vincenzio Tiberio in 1895 (Tiberio, 1895) and the fact that *Pencillium glaucum* possesses antibacterial properties was first presented by Ernest Duchesene in 1897 (Duchesne, 1897), it was Alexander Fleming who utilized this knowledge into effect in the chain of antibiotics discovery. On September 1928, Fleming observed by accident an inhibition of bacterial growth. An uncovered Staphylococcal petri dish, left beside an open window, was contaminated with the fungus *Penicillium notatum* (figure 2) (Fleming, 1929). It was later proven that a compound secreted by this fungus, later named as penicillin, was responsible for this bacterial inhibition. It was then noted that penicillin might have therapeutic value if it could be produced in large quantities (Fleming, 1929). Nevertheless, it was not until 1940, when the two scientists Howard Florey and Ernst Chain improved the isolation and purification of penicillin in quantities sufficient for clinical trials (Abraham et al., 1941; Chain et al., 1940). Their protocol led eventually to Penicillin large-scale production and distribution to treat many different bacterial infections (Rustam I. Aminov, 2010). Fleming's method to screen for antibiotic-producing microorganisms was a great contribution to the antibiotic research history. He observed inhibition zones in lawns of pathogenic bacteria plated on agar plates together with resources tests (Fleming, 1929).

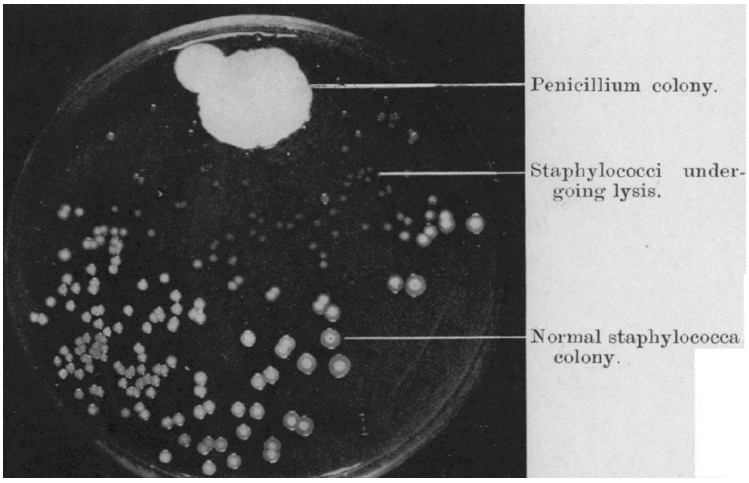


Figure 2 - *This image features colonies of Penicillium that accidentally contaminated a Staphylococcus culture plate and inhibited the nearby bacterial growth (Fleming, 1929).*

The discovery of the first three antimicrobial agents, Salvarsan, Prontosil and Penicillin set up motion for future drug discovery, and the following decades witnessed the discovery and development of a wide variety of new natural and synthetic antibiotics. The pace of antibiotics discovery between the 1950s and 1970s was thereby named the golden era of antibiotics discovery, as most of the antibiotics that are in clinical use today were first characterized (Rustam I. Aminov, 2010). During this period several novel antibiotics were discovered and became the foundation for the therapeutic treatment of infectious diseases, such as aminoglycosides, chloramphenicol, tetracycline, macrolides, glycopeptides, rifamycin, vancomycin, clindamycin, and quinolones, where most of them are derived from microorganisms (Dougherty & Pucci, 2011; Mohr, 2016). The boom in antibiotics discovery during the so-called "golden era", together with the drastic decline in antibiotics discovery are illustrated in the timeline in figure 3.

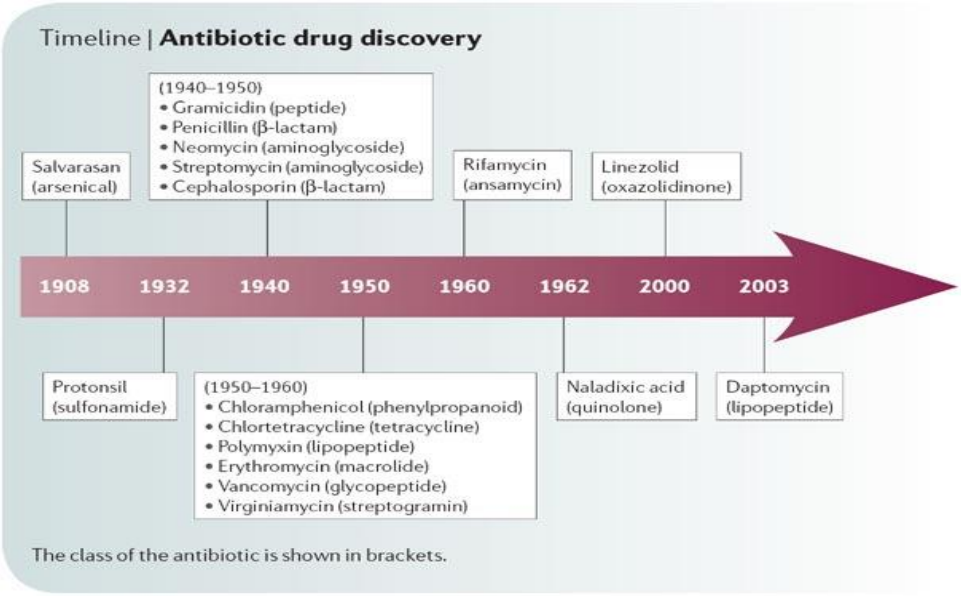


Figure 3 *- Antibiotics discovery timeline (Gerard D. Wright, 2007).*

As a result of this antibiotics revolution during the twentieth century, incidents of infectious diseases began to decline dramatically, and life expectancy increased in an unprecedented manner (Piddock, 2012; Ventola, 2015). Indeed, this is related to a large range of control measures such as vaccine programs, personal hygiene and public health education. However,

discovery and implementations of antibiotics played a vital role (Brachman, 2003). Consequently, the leading causes of death has transitioned from communicable diseases (infectious diseases) to non-communicable diseases (e.g. cardiovascular and cancer diseases), where the infectious diseases were no longer the first cause of death (Adedeji, 2016). As a negative control of this important result, poor and developing countries should be taken into account where infectious diseases still represent the major cause of mortality due to many factors such as poor vaccination coverage, irresponsible antibiotic usage and inadequate hygiene and public health measures (Tagliabue & Rappuoli, 2018).

Since then, less new classes of antibiotics have been introduced, and the discovery rate of antibiotics has seen a rapid downfall. In addition, the emergence of antibiotics resistance that appeared shortly thereafter has posed a huge threat to the world, and it is feared that the "golden era" of antibiotics is coming to an end. Hence, one might say that we are approaching the post-antibiotic era (Alanis, 2005; Jayachandran & Radiology, 2018; Zucca & Savoia, 2010).

1.1.3 Antibiotic mechanisms of action

All antibacterial, antifungal and antivirals are classified as antibiotics. Antimicrobial agents or antibiotics are highly selective toxics, meaning that they can selectively kill the microorganism or inhibit the microbial growth by causing minimal or no toxicity against host mammalian cells (Hugo, Russell, & Denyer, 2011). Antibiotics are classified on the basis of mechanism of action, as shown in figure 4.

Antibiotic action against bacterial cells is broken down into five main mechanisms:

Inhibition of cell wall synthesis: Bacterial cells are surrounded by a cell wall composed of peptidoglycan. The peptidoglycan cellular wall has a vital role in maintaining the shape and mechanical strength of the bacterial cell by preventing osmotic lysis. This structural characteristic is not shared with mammalian cells, confirming the excellent toxic selectivity to bacteria. Peptidoglycan is a macromolecule consisting of sugar (glycan) chains cross-linked by short peptide chains (Hugo et al., 2011). During the cell wall synthesis pathway, peptidoglycan precursors are inserted into the cell wall by the transglycosidase enzymes on the outer face of the cell membrane. In the final stage of peptidoglycan synthesis, the peptide cross-link is reformed between the linear glycan strands assembled by transglycosylation and the existing peptidoglycan in the cell wall. This cross link is executed by transpeptidase enzymes, referred

to as penicillin-binding proteins, to strengthen the cell wall (Hugo et al., 2011). Many antibiotics interfere with the peptidoglycan synthesis process causing weakness in the cell wall that results in burst due to high internal osmotic pressure. Examples include glycopeptides antibiotics (vancomycin, teichoplanin) which bind to the D-alanyl-D-alanine portion on the existing peptidoglycan precursor and thus blocking the transglycosylase enzyme from transferring the growing glycan chain to the existing peptidoglycan precursor (Reynolds, 1989). Another example is Beta-lactam antibiotics (pencillins, cephalosporins, carbapenems, and monobactams) that inhibit transpeptidases enzymes (PBPs) by acting as their alternative substrates instead of D-alanyl D-alanine portion due to the common β-lactam ring, and thus blocking the transpeptidases enzymes from participating in the peptidoglycan assembly which is vital for strengthening of the cell wall (Tipper, 1985).

Inhibitors of protein synthesis: Although the process of protein synthesis is essentially the same in prokaryotic (bacterial) and eukaryotic cells (e.g. mammalian cells), there are many selective agents in this area for antibiotics to act selectively against bacteria. Bacterial ribosomes consist of one 30S and one 50S subunit, and they are smaller than mammalian ribosomes that consist of 40S and 60S subunit (Hugo et al., 2011). Several antibiotics exploit this structural difference and inhibit protein synthesis by targeting 30S and 50S subunit of the bacterial ribosome. Examples include tetracyclines, a class of antibiotics which inhibit protein synthesis by binding the sequences of the 16S rRNA of the 30S ribosomal subunit, thus blocking the ribosome-tRNA interaction (Schnappinger & Hillen, 1996). On the contrary, other antibacterial agents act on 50S subunits in bacteria, such as chloramphenicol that hinder protein synthesis by binding to aminocyl-tRNA in the A site, hence preventing peptidyl transferase from forming a bond with the growing peptide chain on the tRNA in the P-site. Last but not least, macrolides antibiotic that also inhibit protein synthesis selectively by binding to the 50S subunit resulting in blockage of peptide elongation (Hugo et al., 2011).

Inhibition of nucleic acid synthesis: DNA replication and function are essentially the same in prokaryotes and eukaryotes; yet, detailed functioning and properties of the enzymes involved in this process differ. These differences are exploited by several antibiotics for their selective inhibition (Hartmann, Behr, Beissner, Honikel, & Sippel, 1968). For example, fluoroquinolones are a group of antibiotics that target DNA gyrase enzymes required for supercoiling of DNA, and which differ from the eukaryotic topoisomerases II (Wolfson & Hooper, 1985). Other examples are metronidazole that cause bacterial DNA strand breakage by a direct cytotoxic

action (Hugo et al., 2011), and rifamycin that blocks the initiation stage of RNA synthesis by specifically inhibiting bacterial DNA-dependent RNA polymerase (Floss & Yu, 2005).

Alteration of cell membranes: The integrity of the cytoplasmic membrane is vital for the normal functioning of all cells, including bacteria. Therefore, destroying this integrity causes leakage of cytoplasmic contents or loss of metabolic functions associated with the membrane. Most of agents that affect the membrane (e.g. alcohol and bisbiguanides) are used for topical use because they lack selectivity of bacterial membrane (Hugo et al., 2011). Polymyxin E (colistin) has a detergent like activity used for the treatment of serious Gram-negative bacterial infections. Polymyxin E (colistin) binds tightly to lipid A component of LPS in the outer membrane of Gram-negative bacteria and destroy it, penetrate to the cytoplasmic membrane where they bind to phospholipids and abolish6 the membrane integrity (Yahav, Farbman, Leibovici, & Paul, 2012).

Inhibitors of metabolic pathways: Folic acid is important for the synthesis of nucleotides that is vital for cell growth in all living cells. Bacteria, unlike mammalian cells, are unable to take up exogenous folate (dihydropteroic acid), thus they must synthesize it themselves. Folate metabolism pathway is carried out through a serious of reactions involving important enzymes. Dihydrofolic acid (DHF) is synthesized in bacteria from pteridine, para-aminobenzoic acid (PABA) and glutamate, which is then converted to tetrahydrofolic acid (THF) by dihydrofolate reductase enzyme (DHFR). DHFR enzyme in mammalian cells differ structurally from DHFR enzyme in bacterial cells (Hugo et al., 2011). Sulphonamides is a group of antibiotics that possesses a common structural feature with PABA, and so selectively inhibit the enzyme involved in the conversion of para-aminobenzoic acid (PABA) into dihydropropteroic acid (DHF) in bacterial cells. Thus, disturbing the subsequent folate metabolism. Trimethoprim is another example of antibiotics that act similarly to sulfonamides, but by binding selectively to the other bacterial enzyme, DHFR, that is involved in folic acid synthesis (Hitchings, 1973; Sageman, 2015) .

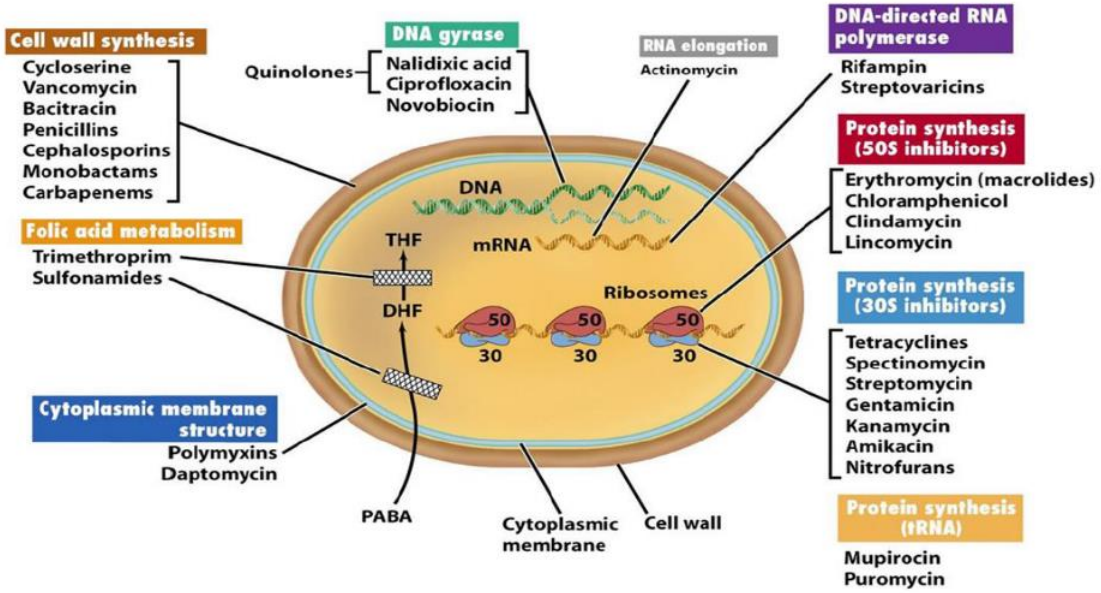


Figure 4 *- A schematic illustration of a typical bacterial cell showing the target sites of the major classes of antibiotics and antimicrobial agents used to treat infections (Madigan & Martinko, 2006).*

1.2 Global challenges with antimicrobial resistant pathogens

1.2.1 Antimicrobial resistance

The innovation and implementation of antibiotics during the twentieth century has greatly contributed to the historical success in the eternal war against pathogenic infectious diseases. Unfortunately, the immense improvement was stalled; mainly because the antibiotic discoveries were unable to keep pace with the rapid emergence of antibiotic resistance in common and uncommon pathogens (Aslam et al., 2018; B. Li & Webster, 2018). Antibiotic resistance is the ability developed by bacteria to withstand and resist the effects of drugs designed to kill them. This ability permits the bacteria to maintain growth and replication even in the presence of therapeutic levels of antibiotics (Zaman et al., 2017). Antibiotic resistance can also be defined using MIC (minimum inhibitory concentration), which is the lowest concentration of an antibiotic required to inhibit the growth of a microorganism. Furthermore, a bacterium is declared resistant if its MIC levels are above the concentration values determined to inhibit the growth of the majority of the other strains of the same species (Gualerzi, Brandi, Fabbretti, &

Pon, 2013). In fact, the rapid spread and worldwide development of resistant pathogens, previously susceptible to antimicrobials, has rendered these original antibiotics largely ineffective (Clardy, Fischbach, & Currie, 2009). Consequently, this became a huge global healthcare challenge for the 21th century (Prestinaci, Pezzotti, & Pantosti, 2015; World Health Organization, 2014).

The development of resistance against the different antimicrobial agents has been known for a long time. In fact, resistance problem was documented even before the usage of antibiotics against infections started (Abraham & Chain, 1940). In 1940, several years before its introduction as a therapeutic agent, penicillin resistance was already noticed when two members of the penicillin discovery group reported that a bacterial enzyme called penicillinase was able to destroy the penicillin by enzymatic degradation (Abraham & Chain, 1940). A mechanism for resistance development was hence identified. Sir Fleming was the first to raise the alarm regarding potential resistance to penicillin when used in small quantities or for short intervals of time during treatment (Fleming, 1945). He conveyed his insight as a cautionary tale when awarded the Nobel Prize for his innovatory work in 1945, as he said: *"The time may come when penicillin can be bought by anyone in the shops. Then there is the danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant".* In addition, Fleming warned in an interview with The New York Times that the inappropriate use of penicillin could lead to the selection of resistant "mutant forms" of *Staphylococcus aureus* which could cause more serious infections (reviewed in Alanis, 2005).

Phylogenetic insights into the evolution of antibiotic-resistant genes, carried by bacterial populations, showed that some of these genes existed long before the antibiotics discovery (Rustam I Aminov & Mackie, 2007). A good example is phylogenetic analysis of β-lactamases enzymes responsible for resistance to β-lactams antibiotics in clones of a metagenomic library derived from 10,000 years old cold-seep sediments of Edison seamount, indicated that the diversity of these enzymes is mostly due to ancient evolution and not recent evolution (Song et al., 2005). The theory of evolution of species and organisms by natural selection mechanism for the purpose of adaption to unfavorable environmental conditions and dangerous agents in order to survive and reproduce was first conveyed in Charles Darwin book "on the origin of species" in 1859 (Darwin, 1968). His theory was used to explain the evolution and spread of antibacterial drug resistance as a result of natural selection of fine mechanisms to overcome the

toxic effects of natural antimicrobials, or as a consequence of heavy selective pressure of antibiotic exposure resulting from the wrong human manipulation of antibiotics (Gualerzi et al., 2013; Hugo et al., 2011).

Bacterial strains use a diversity of complex molecular mechanisms to develop resistance to antimicrobial agents. Most microbial strains are considered intrinsically resistant to naturally produced antimicrobial compounds. However, bacterial strains also have the ability to acquire or develop resistance to antimicrobials they originally were susceptible to (Gualerzi et al., 2013; Munita & Arias, 2016). Intrinsically resistant bacteria exhibit inherited structural and functional characteristics that allow them to overcome and resist the action of naturally produced antimicrobial compounds threatening their survival (Munita & Arias, 2016). Particularly when bacterial species naturally lack the structural or the cellular targets that the antimicrobials must recognize in order to attack them (Gualerzi et al., 2013). As an example, *Pseudomonas aeruginosa* is an intrinsically resistant pathogen to β-lactam antibiotics due to its low permeability of cellular envelopes together with the presence of chromosomally-encoded antibiotic inactivating enzymes or multidrug efflux pumps (Alvarez-Ortega, Wiegand, Olivares, Hancock, & Martínez, 2011). On the other hand, antibiotic resistance is acquired among the susceptible bacteria either as a result of random spontaneous genetic mutations or by acquisition of external genetic material encoding resistance from other bacteria through genetic exchange mechanisms like: conjugation, transduction and transformation (Alanis, 2005; Gualerzi et al., 2013). The acquired resistance development is obtained by the exposure to antibiotics, which induces the selective pressure for the increase and spread of drug resistant pathogens (Roca et al., 2015). Therefore, there are many factors that are paving the way to antibiotic resistance; these factors can be put in two main categories, behavioral or environmental (Larson, 2007). Behavioral factors are defined as irresponsible use and overconsumption of antibiotics among people, lack of infection control, hygiene routines and shortage of new antibiotics development (Larson, 2007). Environmental factors include the continuous uncontrolled agricultural and veterinary extensive use of antibiotics, overuse in fish farming and human overconsumption of antibiotic present in the food chain (McEwen & Fedorka-Cray, 2002; Thanner, Drissner, & Walsh, 2016; Witte, 1998).

The introduction of new antibiotics into widespread clinical use is highly correlated with the emergence of resistant bacteria. Unfortunately, resistance was eventually reported against nearly all different classes of antibiotics that have been developed throughout the years shortly

after their development (figure 5). Infections caused by resistant bacteria to common antibiotics require more complex treatment regimens, as they are much more severe. The first cases of antimicrobial resistance occurred in the late 1930s after the introduction of sulfonamides and in the 1940s after the extensive use of penicillin (Alanis, 2005). Antibiotic resistance was initially a problem exclusively occurring in healthcare institutions settings, however it has recently spread out into the community (Alanis, 2005; Larson, 2007). The first global report on antibiotic resistance released by the WHO was in 2014 (World Health Organization, 2014). WHO stated while publishing the report that "antimicrobial resistance, including antibiotic resistance, globally–reveals that this serious threat is no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country."

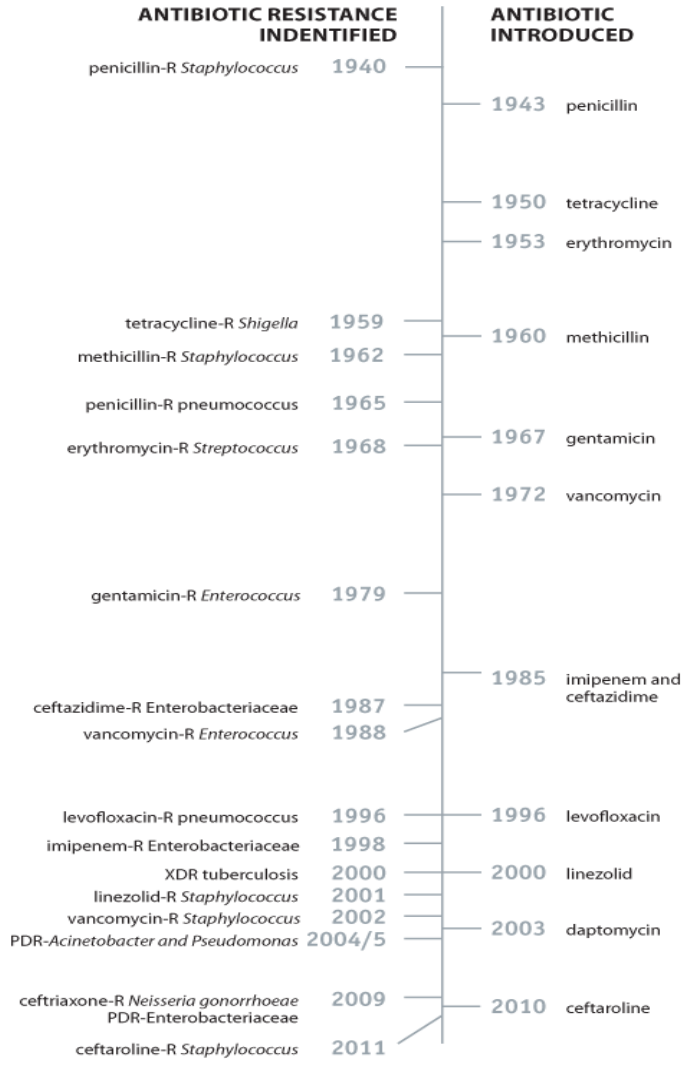


Figure 5 - *Timeline of antibiotic introduction (in the column to the right) compared to antibiotic resistance development (in the column to the left). The sequence of the introduction of antibiotics and development of resistance for the major classes of antibiotics (Centers for Disease Control and Prevention, 2013).*

To make matter worse, bacteria may collect multiple resistance traits over time and acquire resistance to many different families of antibiotics. Multiple antibiotic resistance was detected for the first time when isolating a strain of *Shigella flexneri* resistant to streptomycin, tetracycline, chloramphenicol and sulfonamides in Japan on 1956 (reviewed in Tagliabue & Rappuoli, 2018). Lately, many pathogenic bacteria were discovered to exhibit resistance to several antibiotic classes generating dangerous multi-drug resistant bacterial strains. A few examples of multi-drug resistant bacteria that were detected are *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella* sp, *Escherichia coli*, *Acinetobacter* sp, *Klebsiella* sp, *Streptococcus pneumonia* and *Helicobacter pylori* (Tagliabue & Rappuoli,

2018; Van Duin & Paterson, 2016). Furthermore, there are some cases where bacteria express resistance to nearly all existing antibiotics and are on the verge of turning untreatable (Tagliabue & Rappuoli, 2018). For example, the human pathogen, *Streptococcus pneumoniae,* is a leading cause of bacterial pneumonia, meningitis, sepsis and otitis media in community, and especially among children (Y. Li et al., 2016; K. L. O'Brien et al., 2009). Previously, *S. pneumoniae* was highly vulnerable to penicillin, however today it has developed resistance to penicillin and to multiple antibiotic classes causing much more difficult infections to be treated (Cherazard et al., 2017). In addition, other multi-resistant bacteria particularly Gram-negative bacteria have grasped the attention due to their ability to resist antimicrobial treatments, many of which are located on mobile genetic material that can be transmitted easily to another bacteria, such as *Enterobacteriaceae, Pseudomonas aeruginosa, Acinetobacter baumannii* and others (Exner et al., 2017). On February 27, 2017, WHO published a list of the top global 12 antibiotic resistant "priority pathogens" for new antibiotics and alternative solutions to underline the critical situation and promote research and development of new effective treatments (World Health Organization, 27 February 2017) (figure 6). The published document shows a list of three categories with critical, high and medium priorities depending on the urgency of intervention. This list highlights particularly the multi-resistant Gram-negative bacteria (critical priority) which possess the most serious threat to the world health because of their resistance to a large number of antibiotics including the best currently available antibiotics for treating multi-drug resistant bacteria (carbapenems and third generation cephalosporins). The high and the medium categories include other increasingly drug resistant bacteria that are responsible for more common and severe diseases.

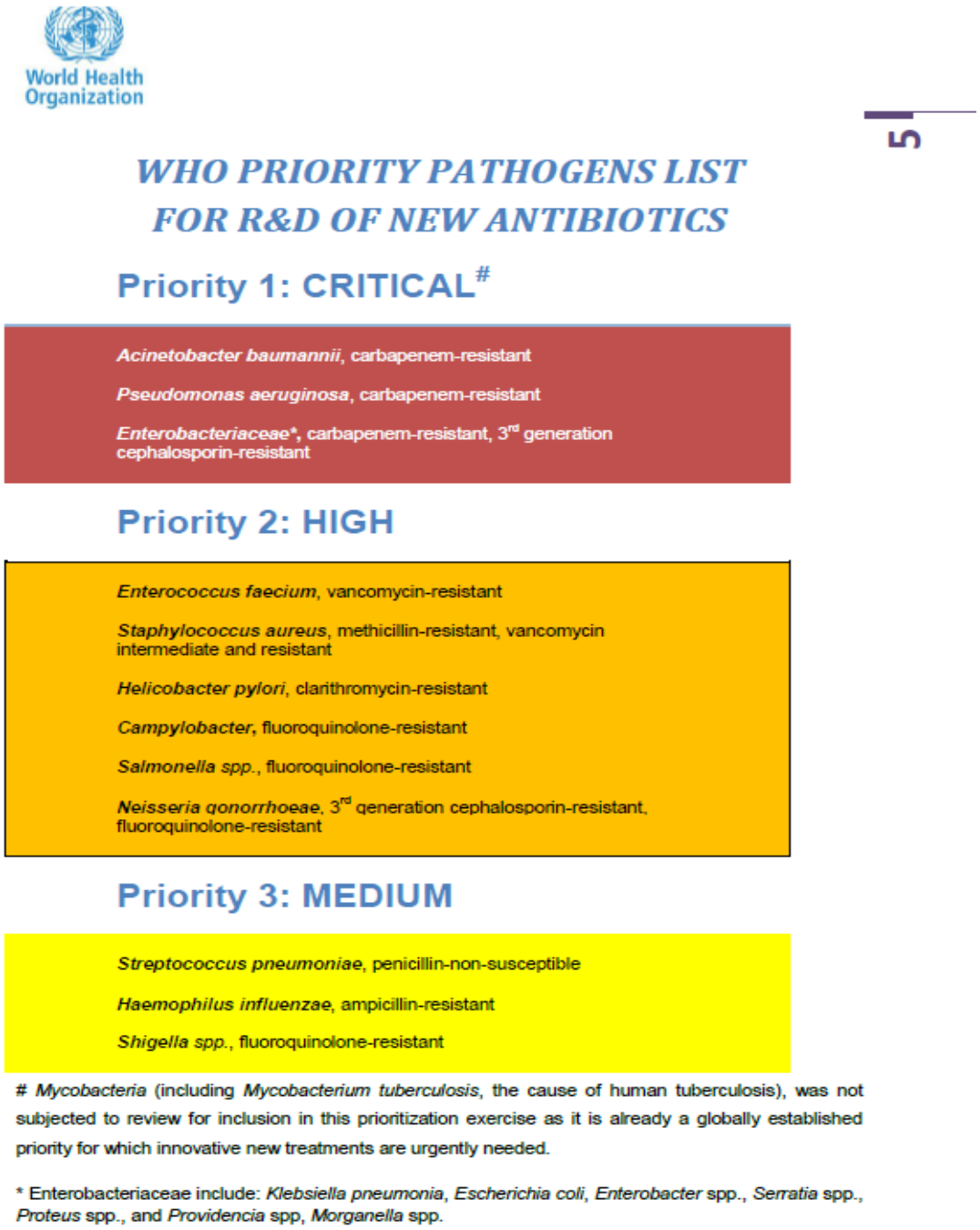


Figure 6 *- WHO`s top 12 priority pathogens list for research and development of new antibiotics (World Health Organization, 27 February 2017).*

1.2.2 Molecular mechanisms of antibiotic resistance

Understanding the mechanisms responsible for the development of antimicrobial resistance in bacterial strains is of high importance. That is to preserve the currently antimicrobial agents and to find new strategies for treatments.

Mechanisms by which bacterial strains withstand the effects of antimicrobials are illustrated in figure 7 and are listed below:

i) Alteration of the target to which the antibiotic interacts.

In this type of antibiotic resistance, the antibiotic target is altered as a result of mutation, substitution, chemical modification and masking of key binding elements. These alterations are attributed to mutations in genes encoding molecular antibiotic targets, thereby leading to a reduction in binding affinity to antibiotics (Gualerzi et al., 2013). A significant example is the MRSA (Methicillin-resistant *S. aureus*) that was documented shortly after the introduction of Methicillin (Jevons, 1961), which is a pathogen resistant to β-lactam antibiotics that act by inhibiting penicillin-binding protein (PBPS) which is involved in the late stages of peptidoglycan biosynthesis. Resistance in MRSA is the result of the expression of *mecA* gene that encodes the penicillin-binding protein 2a (PBP2a), an altered transpeptidase membrane protein that possesses a low affinity to β-lactam antibiotics (Matthews & Tomasz, 1990).

ii) Inactivation of antimicrobial agents via degradation or modification.

The resistance here is a result of destruction or modification of the antibiotics by means of enzymatic reactions. Several enzymes can hydrolyze the chemical bonds in the antibiotics leaving them inactive. Best-known examples are the β-lactamases that cleave the β-lactam ring in cephalosporin and penicillin, esterases that destroy macrolide antibiotics, and epoxidases that are responsible for the destruction of epoxide ring in fosfomycin antibiotic (Gerard D Wright, 2005). Other resistance enzymes modify or inactivate antibiotics chemically, impairing their binding to their respective targets. An example shown by studies for these resistance enzymes is acyltransferases that inactivate aminoglycoside and chloramphenicol antibiotics by adding acetyl groups leading to blockage of their interaction with their targets and

resulting in resistance (Kehrenberg, Schwarz, Cloeckaert, & Doublet, 2004; Gerard D Wright, 2005). Another example is Phosphotransferases that catalyze phosphate transfer which blocks the target binding site in each of aminoglycoside and macrolide (erythromycin) antibiotics (Kono, O'Hara, & Ebisu, 1992; G. Wright & Berghuis, 1998). Another type of enzymes inactivates or modifies antibiotics by means of redox mechanism (Gualerzi et al., 2013). A case which gives an insight to this process is the oxidation of the first and second –generation tetracycline antibiotics that are catalyzed by the redox enzyme TetX which is encoded by a gene found on the conjugative transposons of the obligate anaerobe *Bacteroides fragilis* (Speer, Bedzyk, & Salyers, 1991).

iii) Expulsion of the antimicrobial agents from the cell via general or specific efflux pumps.

Efflux pumps that export antibiotics out of the bacterial cell inhibit the antibiotics from accumulating in high inhibitory concentrations in the cells of the bacteria, thereby preventing their effect. They often act synergistically with other resistance mechanisms generating a great level of resistance to antibiotics. Efflux pumps are encoded by genes and act by utilizing energy to pump out substances (X.-Z. Li & Nikaido, 2009). This type of resistance is witnessed in a variety of bacteria like *E. coli* and *P. aeruginosa* that can possess AcrAB-ToIC and MexAB-OprM complex pumps respectively to get rid of several different and important antibiotic drugs, including fluoroquinolones, chloramphenicol, tetracycline, and macrolides (Phan, Picard, & Broutin, 2015).

iv) Reduced cellular uptake of antimicrobial agents by downregulation or inactivation of the outer membrane channels responsible for cell entry

This strategy for antibiotic resistance is common among Gram-negative bacteria since they possess an outer membrane that can form a permeability barrier for antibiotics. The antibiotic resistance here is intrinsic; however, it can also be developed as a result of changes in the lipid composition of the outer membrane, downregulation of outer membrane porin concentration or by changes in porin channel selectivity. Thus, preventing antibiotics from entry and access to their targets in the bacterial cell. For example, a common mechanism of carbapenem

resistance among *P. aeruginosa* is due to the downregulation in porin expression on its outer membrane (Blair, Webber, Baylay, Ogbolu, & Piddock, 2015).

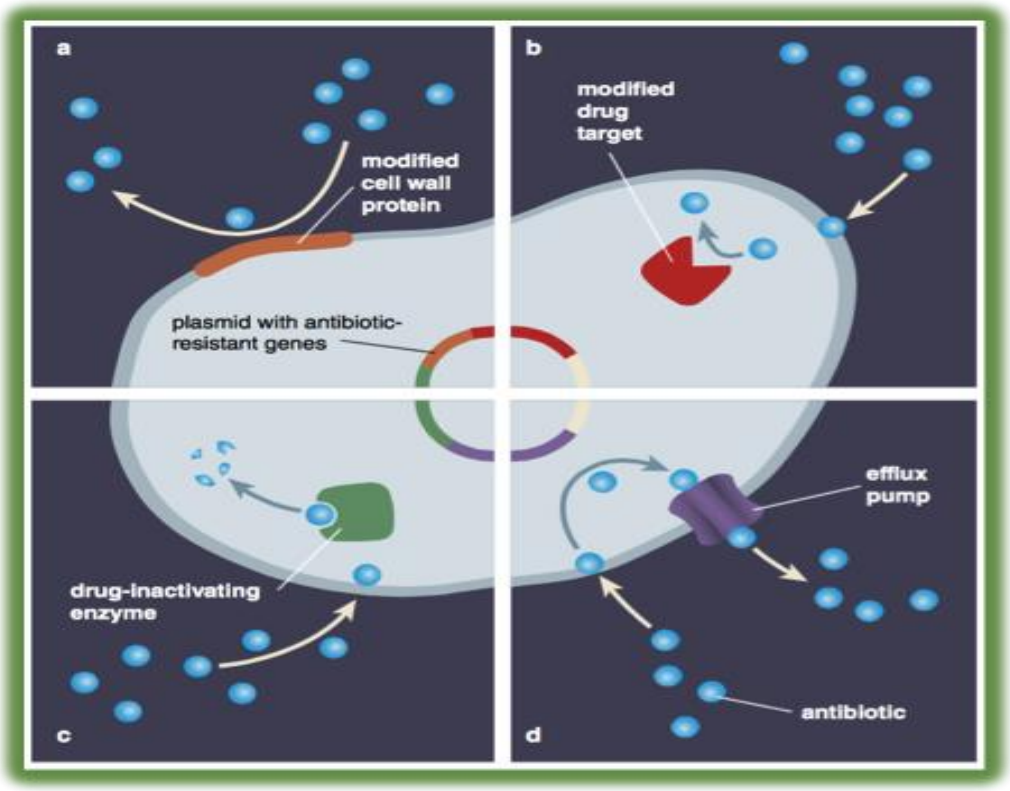


***Figure 7** - The four common mechanisms of antibiotic resistance (Dantas & Sommer, 2014).*

1.2.3 Global approaches to locate and overcome antimicrobial resistance hazards

The world health organization (WHO) has identified antimicrobial resistance (AMR) as one of the greatest threats to human health (World Health Organization, 2019). "Tackling drug-resistant infections globally" a comprehensive review published by a group of experts coordinated by the economist Jim O'Neill and commissioned by the United Kingdom government in May 2016, reported that at least 700,000 people die every year due to common drug resistant bacterial infections, HIV/AIDS**,** tuberculosis and malaria (O'Neil, 2016). It is estimated that AMR can potentially cause 10 million deaths every year starting 2050, greater than the current annual deaths caused by cancer (figure 8). This is estimated to cost the global economy 100 trillion USD annually.

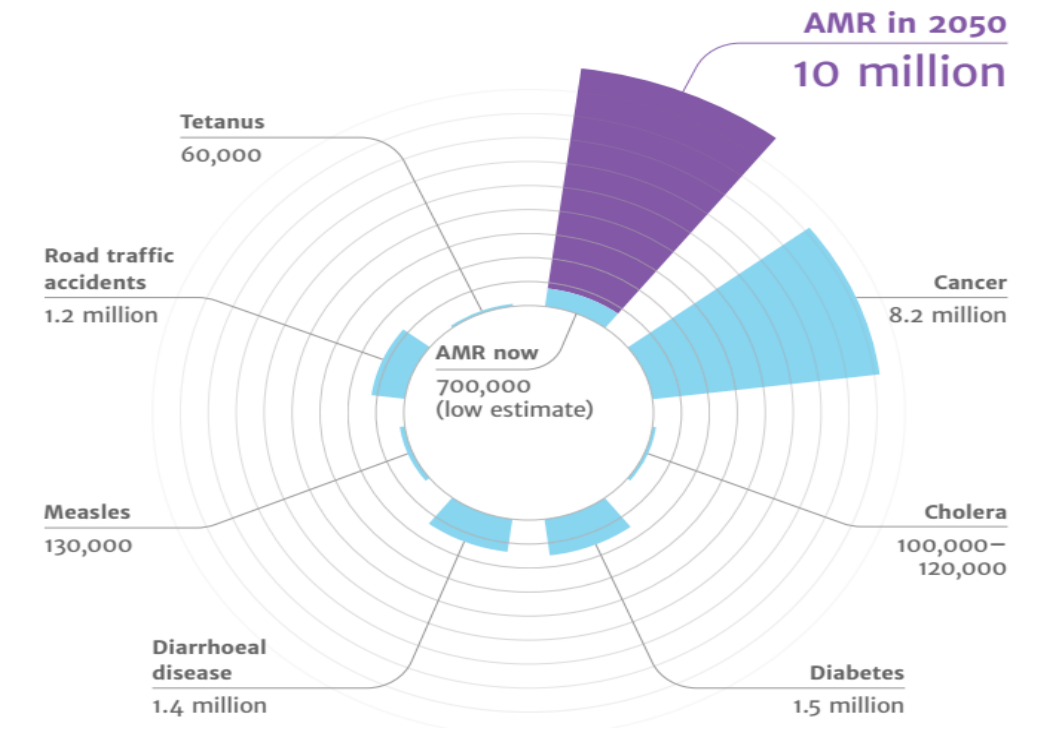


Figure 8 *- Deaths attributable to AMR every year compared to other major causes of death (O'Neil, 2016).*

This previously mentioned review came up with a coordinated set of strategies and recommendations on actions that should be implemented to combat antimicrobial resistance in a global manner such as improvement of public awareness and hygiene, reducing the unnecessary use of antimicrobials in agriculture and among humans, and promoting the development of vaccines to prevent infectious diseases. Among the many recommendations presented in this review, an entire section was devoted for discussing and highlighting the incentives to promote investment for new drugs and to improve existing ones to strictly underline the need for new treatments (O'Neil, 2016). Various calls for new antibiotics to combat the evolving antimicrobial resistance problem were demonstrated by other alarming documents issued by WHO as well as UK and USA governments and the European Union (EU), and by other international organizations like the United Nations General Assembly, the World Bank and the G20 (reviewed in Tagliabue & Rappuoli, 2018).

1.2.3.1 The current situation of the development of new antibiotics

Despite the urgent need for new novel antimicrobial drugs, many pharmaceutical companies lost interest in the development of novel antibiotics (Tacconelli et al., 2017). Instead, efforts are directed towards more controllable chronic diseases such as diabetes and cardiovascular disease that have more promising forecasts for return on investment (Gerard D Wright, 2017). This is probably related to challenges in clinical development, scientific bottlenecks, regulatory and economic issues (S. O'brien, 2015; Tacconelli et al., 2017). The clinical antibiotic trial and development process is generally complex, relatively expensive until approval is obtained, and it is not considered as fruitful business for pharmaceutical industries since it is difficult to recuperate the cost invested in the development after the new antibiotic reaches the market (Gualerzi et al., 2013). This is mainly to the costly development of a drug that is not highly demanded compared to drugs corresponding to chronic diseases as diabetes. In addition, antimicrobial drugs are subject to short interval of time usage and not prescribed for life as other drugs. The whole R&D investments and efforts are at risk of being in vain, as there is always a chance for new emerging resistance to the developed antibiotic (Renwick & Mossialos, 2018; Roca et al., 2015). On the contrary, some companies turned their development efforts to alternative approaches to combat antibiotic resistance such as antibodies, stimulation of host immune response, probiotics, lysins and bacteriophages (Czaplewski et al., 2016; Simpkin, Renwick, Kelly, & Mossialos, 2017). In spite of all the mentioned challenges to match the clinical need of new antibiotics, the development and discovery of antibiotics is and will remain a global priority to face the evolving drug resistant pathogens (Gualerzi et al., 2013; Roca et al., 2015).

Nevertheless, today there is a serious lack of new antibiotics development to address the growing threat of antibiotic resistance. On September 2017, WHO reported by an analysis of the antibacterial clinical development pipeline, the serious lack of novel antibiotics under development to combat the rising threat of antimicrobial resistance (World Health Organization, 2017). In fact, no major new classes of antibiotics have been discovered since the mid-1980s, as the last antibiotics class discovered were liptopeptides (daptomycin) on 1987 that entered to the market in 2003 (Butler & Buss, 2006; Joe et al., 2014). Nearly all antibiotics approved for use in patients today are structural modifications of existing classes of antibiotics, which has been altered in order to improve their safety and potency (The Pew Charitable Trust, 2016).

When it comes to the current situation of the new antibiotics pipeline, The Pew Charitable Trusts have assessed an updated list of antibiotics that are currently in clinical development on March 2019. This list shows that approximately 42 new antibiotics with the potential to treat serious bacterial infections are currently between phases I and III of the clinical pipeline development. However, further investigations showed a slow rate of new antibiotics development with respect to the current need of antibiotics to battle the increasing resistance issue. As the success rates of moving an antibiotic through the different clinical phases shows that only 1 out of 5 infectious disease drugs that reach initial phase of testing in humans will be approval by food and drug administration to be translated into a marketable product (figure 9).

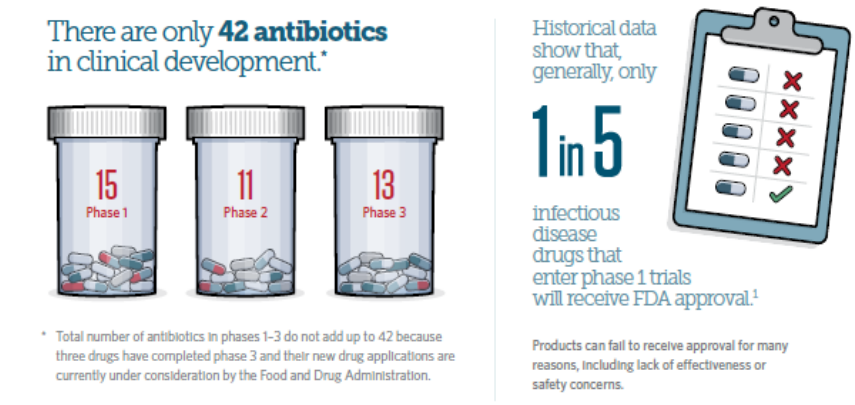


Figure 9 - *The currently clinical development of new antibiotics (The Pew Charitable Trusts, 2019).*

According to this list, many of these antibiotics belong to the natural product-derived antibiotic classes (β-lactam, macrolide, tetracycline, and aminoglycosides). This reveals the mainstay of natural products as significant sources for antimicrobial agents.

1.2.3.2 Natural products: a continuing source for novel antibiotics

Most of the antimicrobial agents that emerged over the past century were originated from microbial natural products (Gualerzi et al., 2013; Sneader, 2005). Therefore, natural sources have historically been an ultimate progenitor of most of the antimicrobials that are in clinical use today (Moloney, 2016). The review by Nerman and Cragg (Newman & Cragg, 2016) showed that 49% of the new drugs launched during the period from 1981 to 2014 have a natural product origin. In addition, a recent analysis of drugs approved by USA's FDA concluded that natural products and their derivatives contribute to more than two-thirds of all antibacterial

agents, and are likely to successfully bridge the antibiotic gap (Patridge, Gareiss, Kinch, & Hoyer, 2016). Nevertheless, the exploration of natural products as a source of new antibiotics has faded in the recent years. Still, the fundamental need of novel antibiotics discoveries has resurrected the interest in natural sources as a route to identify new antibiotics (Moloney, 2016; Silver, 2015).

Recently, many relevant reviews on the role of natural products in modern antibiotic discovery (Butler, 2004; Gyawali & Ibrahim, 2014; Koehn & Carter, 2005; Moloney, 2016; Gerard D Wright, 2017) and natural product-derived compounds in antibacterial clinical trials have been published (Brown, Lister, & May-Dracka, 2014; Butler & Buss, 2006; Moloney, 2016). Natural sources showed a huge potential to antimicrobial discoveries such as natural microbial organisms (Monciardini, Iorio, Maffioli, Sosio, & Donadio, 2014), fungi (Silber, Kramer, Labes, & Tasdemir, 2016), land plants (Gechev et al., 2014; Vandal, Abou-Zaid, Ferroni, & Leduc, 2015), lichens (Yousuf & Choudhary, 2014), seaweeds (Singh, Kumari, & Reddy, 2015), corals (Dobretsov et al., 2015), marine plants (Kiuru et al., 2014), marine algae (Kausalya & Rao, 2015; Shannon & Abu-Ghannam, 2016) and other marine microorganisms (El Amraoui, El Amraoui, Cohen, & Fassouane, 2014).

1.3 Microalgae and effects on bacterial populations

1.3.1 Microalgae

Algae belong to a diverse group of photosynthetic organisms, primarily responsible for oxygen generation in aquatic environments (Matsunaga, Takeyama, Miyashita, & Yokouchi, 2005; Tanaka, Muto, Liang, Yoshino, & Matsunaga, 2015). They are a heterogonous group of organisms with huge variation in size, morphology, life cycle, pigments and metabolism, all which reflect their polyphyletic origins (Graham & Wilcox, 2000; Radmer, 1996; Tanaka et al., 2015). In spite of this algal diversity and nutritional variability, algae are a combination of distantly related groups that have several eco-physiological attributes in common. Most are aquatic organisms found in oceans, lakes, ponds, wetlands, rivers and streams, and act as photosynthetic oxygen producers which metabolize organic compounds. They are typically smaller and less complex than land plants (Graham & Wilcox, 2000). However, there are frequent exceptions to the mentioned features, such as heterotrophic and mixotrophic algae, in

addition to other algae that exist in non-aquatic habitats (Graham & Wilcox, 2000). Algae are broadly classified into the nine major phyla (divisions); *Cyanophyceae, Chlorophyceae, Rhodophyceae, Cryptophyceae, Dinophyceae, Bacillariophyceae, Haptophyceae, Euglenophyceae* or *Prasinophyceae* (Graham & Wilcox, 2000) . They may also be referred to as blue-green algae, green algae, brown algae, or red algae due to the difference in composition and amounts of photosynthetic pigments that give algae their wide variety of colors (Graham & Wilcox, 2000; Tanaka et al., 2015). Algal organisms range greatly in size, and hence they are classified also by their size, as macroalgae that are quite large multicellular organisms (e.g. seaweed that can exceed 50 meters in length), and microalgae which are microscopic unicellular organisms (Graham & Wilcox, 2000). Microalgae has an advantage over macrolagae in experimental and scientific research, as they are easier to handle and more amenable to laboratory cultures (M. A. Borowitzka, 1995). The present thesis considers only the marine microalgae; however, examples from other groups and classes of algae are mentioned when appropriate

Microalgae are small, single cellular organisms, that are only visible with the aid of microscopes (Graham & Wilcox, 2000). They show an immense diversity and exist in different sizes and shapes, as they can appear as individual cells, colonies or extended filaments (Amaro, Guedes, & Malcata, 2011; Graham & Wilcox, 2000). Estimates for the number of microalgae species have been recognized to be between 200,000 and several millions (Norton, Melkonian, & Andersen, 1996). From the ecological point of view, microalgae must exhibit the skill to develop tolerance or defense strategies in order to be able to survive in an environment rich with microbial pathogens (bacteria, viruses and fungi), competitors, parasites and predators (Børsheim, Bratbak, & Heldal, 1990; Cole, 1982; Falaise et al., 2016). As a result, microalgae have adapted to survival in extreme habitats (e.g. hot springs and brine lakes) and under a huge spectrum of environmental stresses (e.g. heat, cold, salinity, osmotic pressure, UV exposure, drought, photo-oxidation and anaerobiosis) (Graham & Wilcox, 2000; Tandeau de Marsac & Houmard, 1993). Consequently, microalgae are commonly distributed throughout the biosphere, and virtually found in any aquatic system (Graham & Wilcox, 2000). It has also been demonstrated that numerous microalgae species originating from different habitats, including freshwater, marine environment and soil, possess potent antimicrobial activity (Cannell, Owsianka, & Walker, 1988; Kellam & Walker, 1989; Pradhan, Das, & Das, 2014; Soltani, Khavari-Nejad, Tabatabaei Yazdi, Shokravi, & Fernandez-Valiente, 2005)

Marine microalgae play a key role in aquatic ecosystems. They possess the intrinsic ability to use solar energy, H_2O and CO_2 to synthesize complex organic compounds (Amaro et al., 2011). About 50 % of global oxygen production is accomplished by marine microalgae (Tanaka et al., 2015), despite the fact that they comprise less than 1 % of plant biomass. These organisms account for the basis of the marine food chains, supporting both microbial and animal plankton (Graham & Wilcox, 2000). They have a substantial metabolic plasticity that confer them the ability to manipulate their different external environmental conditions and stresses, and to survive in ecosystems, densely populated with both pathogens and predators. This allow them to synthesize and produce a variety of compounds and metabolites, originating from different metabolic pathways (Amaro et al., 2011; de Morais, Vaz, de Morais, & Costa, 2015; Encarnação, Pais, Campos, & Burrows, 2015; Lincoln, Strupinski, & Walker, 1991; Shimizu, 1996). Many of these compounds are chemically complex, and are often considered difficult or even impossible to produce by chemical synthesis in a laboratory (M. A. Borowitzka, 1995). Microalga have shown potential applications in various fields of interest such as agriculture, bioenergy, human and animal nutrition, cosmetics, pharmaceuticals, and environmental restoration (reviewed in Barra, Chandrasekaran, Corato, & Brunet, 2014; Raposo, De Morais, & Bernardo de Morais, 2013; Yaakob, Ali, Zainal, Mohamad, & Takriff, 2014)

Since recent decades, marine sources have become the focus of extensive research and exploration efforts within human medicine. As mentioned above, marine organisms are rich sources of biological diverse and novel compounds that have the potential to be used in pharmaceutical industry for therapeutic purposes (Bhatnagar & Kim, 2010; Ely, Supriya, & Naik, 2004; Faulkner, 1986; Kang, Seo, & Park, 2015). Certain marine products have been found to exhibit various antimicrobial effects against a range of harmful pathogens (Bajpai, 2016; Bhatnagar & Kim, 2010; Kang et al., 2015). Among the large spectrum of marine organisms, microalgae represent an exceptional opportunity to discover and develop new novel antimicrobial agents due to their richness in bioactive compounds and secondary metabolites that exhibit antimicrobial features (Amaro et al., 2011; Bajpai, 2016; González-Davis, Ponce-Rivas, Sánchez-Saavedra, Muñoz-Márquez, & Gerwick, 2012; Sánchez-Saavedra, Licea-Navarro, & Bernáldez-Sarabia, 2010).

For most microalgae, the synthesized bioactive metabolites and compounds are subsequently accumulated in the biomass and/or excreted into the medium (Amaro et al., 2011; de Morais et al., 2015). The systematic screening for biologically active principles in microalgae for

therapeutic purposes began in the 1950s (reviewed in Amaro et al., 2011). Screening of extracts and/or extracellular metabolites and compounds, mainly from marine microalgae, have proven to exhibit antibacterial, antifungal and antiviral potential (Amaro et al., 2011; Kellam & Walker, 1989; Lincoln et al., 1991). Bioactive products identified to have the potential for this antimicrobial activity include indoles, terpenes, phenols, fatty acids, polysaccharides, acetogenins, sterols, chlorophyll derivatives and halogenated aliphatic compound (Amaro et al., 2011; M. A. Borowitzka, 1995; de Morais et al., 2015).

1.3.2 Marine microalgae as a potential source of antibacterial agents

Some efforts have been devoted to assess the potential antibacterial activity of numerous marine microalgae, both due to their potential effects in limiting microbial infections in aquaculture and their promising use as sources of natural antimicrobial agents against human pathogens (Falaise et al., 2016). The first isolation of antibacterial compound from microalgae cultures was from *Chlorella*. Here a mixture of fatty acids, Chlorellin, was found to exhibit inhibitory activity against both Gram-negative and Gram-positive bacteria (Pratt et al., 1944). Since then, screening of a range of microalgae for their potential antimicrobial activity has been increasing and various studies have been conducted to identify and characterize chemical compounds responsible for this antibacterial activity.

Large antimicrobial screening studies have been conducted to assess the potential antimicrobial activity of many different microalgae at the same time, and several have found remarkable antibacterial properties in various microalgae (Duff, Bruce, & Antia, 1966; Kellam & Walker, 1989; Ördög et al., 2004). Moreover, most of the antibacterial compounds that were suggested to be responsible for antibacterial activity were detected within the extracts of microalgae. These bioactive compounds can be terpenoids, carbohydrates (Duff et al., 1966), peptides, polysaccharides, alkaloids (M. A. Borowitzka, 1995), chlorophyll derivatives (Bruce, Duff, & Antia, 1967; Jørgensen, 1962) and fatty acids which seem to be most common (Andrew P. Desbois, Mearns-Spragg, & Smith, 2009).

Methanolic extracts of *Chlorococcum* strain HS-101 and *Dunaliella primolecta* was demonstrated to have a strong antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (Ohta et al., 1995), a bacterium that is highly resistant to conventional antibiotics. Analysis of the extracts showed that the active compound contributing to this antimicrobial activity was the unsaturated fatty acids, and the highest

activity was that of γ-linolenic acid (Ohta et al., 1995). Further, cellular lysate of the marine diatom *Phaeodactylum tricornutum* have been reported to display antibacterial activity (Cooper, Battat, Marsot, & Sylvestre, 1983; Duff et al., 1966; Kellam & Walker, 1989). This activity attributed to its polyunsaturated fatty acids, palmitoleic acid (PA), hexadecatrienoic acid (HTA) and eicosapentaenoic acid (EPA), which is active against a range of both Gram-negative and Gram-positive bacteria, including MRSA (Andrew P. Desbois, Lebl, Yan, & Smith, 2008; Andrew P. Desbois et al., 2009). An antimicrobial activity against several microorganisms of high importance for the food industry (*E. coli* and *S. aureus*) has also been demonstrated for ethanol extract from the microalgae *Haematococcus pluvialis* in its red phase (the phase when *H. pluvialis* morphologically changes into red hematocysts without flagella). This has been attributed to its short chain fatty acids, butanoic and methyl lactic acids (Santoyo et al., 2009).

Polyunsaturated aldehydes (PUA), which are probably derived from the polysaturated fatty acids (e.g. EPA), can be generated by some microalgae such as *Skeletonema costatum* and *Thalassiosira rotula* (Wichard et al., 2005). These compounds can possess potential for antibacterial activity. A common example of a highly antibacterial PUA is the decadienal (DD) which displays strong activity against some important Gram-positive and Gram-negative human bacterial pathogens, such as MRSA and *Haemophilus influenzae* (Nostro et al., 2001).

Extracts from various microalgae have shown efficiency against both Gram-positive and Gram-negative bacteria, as well as against *Mycobacterium tuberculosis (Arun, Gupta, & Singh, 2012; Danyal, Mubeen, & Malik, 2013; Prakash & Bhimba, 2005)*. This implies the potential of microalgae to produce compounds with a broad-spectrum activity. However, in some cases, microalgae compounds have shown a lower potential antibacterial activity against Gram-negative bacteria than Gram-positive bacteria (Ghasemi, Moradian, Mohagheghzadeh, Shokravi, & Morowvat, 2007; Kellam & Walker, 1989; Najdenski et al., 2013). This can be explained by the more complex multilayered cell wall structure of Gram-negative bacteria, which makes it hard for the active compounds (e.g. microalgae active compounds) to penetrate them.

In the present thesis, the marine microlagae, *Isochrysis galbana*, *Dunaliella tertiolecta*, *Nannochloropsis oculata* and *Tetraselmis suecica*, were selected for their previously proven antibacterial effect.

The antibacterial activity of *I. galbana* was recognized decades ago. The ability of *I. galbana* to inhibit the growth of *S. aureus, Streptococcus faecalis,* and *Proteus vulgaris* human pathogens was noticed by Duff in his survey of marine microalgae for antimicrobial activity (Duff et al., 1966). These results appeared to be promising for further investigation, as an efficient inhibition of other bacterial pathogens such as *E. coli, Klebsiella pseudomoniae,* and *Salmonella typii* by *I. galbana* extracts also was demonstrated (Srinivasakumar & Rajashekhar, 2009). Lately, it has been found that *I. galbana* possess antibacterial activity against Vibrio bacterial strains like *Vibrio alginolyticus, Vibrio campbellii,* and *Vibrio harveyi* (Bogdanovic, 2018; Kokou, Makridis, Kentouri, & Divanach, 2012; Molina-Cárdenas, Sánchez-Saavedra, & Lizárraga-Partida, 2014). Further analysis of the microalgae extracts and their secondary products, revealed that the antimicrobial activity of *I. galbana* is attributed to its fatty acids content that are abundantly produced in the stationary phase of growth culture (Molina-Cárdenas et al., 2014; Zhu, Lee, & Chao, 1997). In addition, the two derivatives of Chlorophyll *a*, pheophytin *a* and chlorophyllide *a*, were also found to be responsible for the observed antibacterial effects (Bruce et al., 1967).

Microalgae belonging to genus Dunaliella, such as *Dunaliella salina* has been found to produce compounds that exhibit antimicrobial activity against a wide range of bacteria such as *E. coli*, *P. aeruginosa, S. aureus*, *Candida albicans*, *Aspergillus niger* and *K. pneumoniae* (Herrero, Ibáñez, Cifuentes, Reglero, & Santoyo, 2006; J. A. Mendiola et al., 2008; Srinivasakumar & Rajashekhar, 2009). As well as *Dunaliella primolecta* that displayed antibacterial activity against certain bacterial strains (Chang et al., 1993; Ohta et al., 1995). Other microalgae belonging to this genus were hence worthy for further investigations, such as *D. tertiolecta* that attempted a remarkable antibacterial activity against *B. subtilis* (Sánchez-Saavedra et al., 2010), and *P. aeruginosa* (Pane, Cacciola, Giacco, Mariottini, & Coppo, 2015). In addition, the daily supplementation of the brine shrimp *Artemia franciscana* with *D. tertiolecta* was proven to confer a full protection against *Vibrio campbellii* and *Vibrio proteolyticus* (Marques, Thanh, Sorgeloos, & Bossier, 2006), and a reduction in the cell count of *V. alginolyticus* to undetectable levels in co-cultures with *D. tertiolecta* was demonstrated (Bogdanovic, 2018) .

Several studies have documented the antibacterial activity of the green marine microalgae *T. suecica.* Bacterial inhibition of prawn pathogenic strains of Vibrio species including *V. alginolyticus,* was detected by a spray-dried preparation of *T. suecica* (B. Austin & Day, 1990). In addition, *in vitro* experiments of cell-free supernatants and extracts derived from *T. suecica*

have been observed to inhibit the growth of various marine bacterial pathogens (B. Austin, Baudet, & Stobie, 1992). Antibacterial activity against human pathogens, *S. aureus* and *B. subtilis*, was also observed by *T. suecica* extracts (Kellam & Walker, 1989), as well as against *Streptococcus pyogens, Bacillus megaterium* and *Proteus* sp (Bai & Krishnakumar, 2013).

Furthermore, the marine microalgae, *N. oculata*, exhibited antibacterial effect against human bacterial pathogens such as *S. aureus* and *B. subtilis* (Kellam & Walker, 1989). *N. oculata* microalgae culture, like other microalgae cultures (*Chlorella minutissima, Tetraselmis chui, Arthrospira platensis* and *Isochrysis* sp), have shown a growth inhibition of six Vibrio bacterial strains (*V. splendidus, V. parahaemolyticus, V. anguillarum, V. scophthalmi, V. lentus and V. alginolyticus)* when incubating these bacteria in the cultures of microalgae (Kokou et al., 2012).

The biochemical processes of microalgae and their production of bioactive compounds are influenced by many factors such as algal species, growth phases, culture conditions, available nutrients, biotic factors such as pathogens, and environmental factors like light intensity, pH, salinity and temperature (reviewed in M. A. Borowitzka, 1995; Mata, Martins, & Caetano, 2010). The production of the secondary metabolites are typically higher in unfavorable culture conditions (L. J. Borowitzka & Borowitzka, 1989). The antibacterial effects of microalgae are suggested to be found in two types. One is a constitutive effect, by which the antibacterial activity is always existing in algal culture regardless of bacteria presence as in the case where the medium of *Chroococcus turgidus* inhibits the growth of *E. coli*. The second is an induced effect where the antibacterial activity takes place only in presence of bacteria in the surroundings of microalgae e.g the growth of *Escherichia coli* is inhibited in co-culture with *Tetracystis* sp. (Safonova & Reisser, 2005).

During growth, the microalgae cells pass through different phases, exponential, stationary and death phases. The metabolic profile and the production of various bioactive metabolites by microalgae have been demonstrated to vary significantly, depending on these different growth phases (Barofsky, Simonelli, et al., 2009; Barofsky, Vidoudez, & Pohnert, 2009; Vidoudez & Pohnert, 2012). It is assumed that many of the biologically active secondary metabolites, often are most abundant in the stationary phase (M. A. Borowitzka, 1995; Cooper et al., 1983). As an example, testing of extrametabolites (EM) of marine microalgae sampled in different growth phases against reproductive forms of the bacterium *Listeria monocytogenes,* showed that only EM produced by *Skeletonema costatum* in the middle steady-state growth phase demonstrated an antibacterial effect against *L. monocytogenes* (Terekhova, Aizdaicher,

Buzoleva, & Somov, 2009). The production of lipids and fatty acids that are responsible for the antibacterial activity of *I. galbana* was higher in the stationary phase, when cells are not dividing (Lin, Chang, Tsao, & Leu, 2007; Liu & Lin, 2001).

1.3.3 Effects of marine microalgae on bacterial growth

In addition to the inhibitory effect on bacteria, microalgae can influence the growth of marine bacteria in other ways. In some cases, no apparent effect on bacteria is recognized, while in other cases, a promotion of bacterial growth is documented (Kogure, Simidu, & Taga, 1979; Safonova & Reisser, 2005). Various studies have supported the assumption that in some cases the microalgae might be able to stimulate the growth of bacteria. This has been found with *S. costatum*, where the growth of *Flavobacterium* appeared to be stimulated in the co-culture with this alga (Kogure et al., 1979). Another example is when the extrametabolites of *S. costatum* microalgae in the steady-state showed stimulating effects on the reproduction of *L. monocytogenes* bacterium in co-culture (Terekhova et al., 2009). In addition to the cell-free supernatant of *Tetracystis* algal culture exhibiting a significant growth promoting effect on the two bacterial pathogens, *E. coli* and *M. luteus* (Safonova & Reisser, 2005).

Microalgae and Vibrio populations have been found to interact in aquatic environments. A study to observe the dynamics of both Vibrio and microalgae in the Neuse River Estuary (NRE) during the summer of 2004 in North Carolina, USA, suggested that eutrophication leads to algal bloom which in turn increases the fraction of Vibrio attached to algae (Hsieh, Fries, & Noble, 2007). Furthermore, increased growth rates of free-living *V. cholera* has been detected in algal bloom waters (Worden et al., 2006).

1.4 Investigating antimicrobial activity

Owing to the significance of developing new antibiotics to combat the growing threat of antimicrobial resistance, it is of hefty importance to focus on antimicrobial screening and evaluating methods to investigate substances for their potential antimicrobial activity.

1.4.1 Methods for investigating the potential for antimicrobial compounds in microalgae

Several methods for the investigation of microalgae and the potential for antimicrobial compounds exist, while having advantages and limitations associated with all of them.

Extraction of cellular compounds from microalgae cultures, to screen their potential of antimicrobial activity has been used since the investigations of antimicrobial activity in microalgae was initiated (Duff et al., 1966; Kellam & Walker, 1989; Pratt et al., 1944). In this process, crude algal extracts, containing active compounds from marine microalgae, are obtained and separated from raw materials. The procedure commonly progresses by the following steps: 1) microalgae culture is harvested by centrifugation and algal pellets are collected; 2) A solvent is added to the algal pellets to extract the desired active compounds. The solvent penetrates the solid algal pellet and intracellular compounds is further dissolved in the solvent. 3) The culture is filtered to exclude unwanted cellular matter, and crude algal extracts are obtained (Bai & Krishnakumar, 2013; Kellam & Walker, 1989). Many factors can affect the extraction, such as temperature, pressure, extraction duration, and the properties of the selected extraction solvent (e.g. selectivity, polarity) (Zhang, Lin, & Ye, 2018). A variety of organic solvents have been used for extraction of algal biomass such as methanol, hexane, benzene, acetone, dichloromethane and chloroform (Bai & Krishnakumar, 2013; Kellam & Walker, 1989; Pratt et al., 1944). Aqueous solvents such as water have also been used in some cases to extract the active material from microalgae (Naviner, Bergé, Durand, & Le Bris, 1999). This method can give high yields of microalgae biomass, and allow the investigation of several microalgae cultures simultaneously, since several extractions can be performed in parallel. However, the antimicrobial properties that the extraction solvents can carry may give uncertain results. In addition, it has been shown in some studies that the antimicrobial activity of microalgae can depend to some extent on the solvent used for extraction (Bai & Krishnakumar, 2013). Moreover, this method is time and labor consuming, encounters environmental toxicity, fire hazards, and expenses that are involved in the utilization of organic solvents. Other

extraction techniques such as supercritical CO_2, subcritical water extraction (SWE) and pressurized liquid extraction (PLE) have been tested to prepare crude extracts from microalgae for potential antimicrobial activity investigation (Herrero et al., 2006; Jose A Mendiola et al., 2007; Santoyo et al., 2009). These techniques are more environment-friendly as the use of organic solvents is highly limited (Herrero, Mendiola, Plaza, & Ibañez, 2013).

Cohabitation assays of microalgae with bacteria to investigate the potential of antimicrobial compounds in microalgae have been conducted in a range of studies and reported to give positive results (Bogdanovic, 2018; Kokou et al., 2012; Molina-Cárdenas et al., 2014). Using such methods, the antibacterial activity of microalgae is assessed by co-cultivating the algae together with one or several bacterial test strains. The potential effects on bacterial growth is followed by quantifying the bacteria present in the co-cultures over time. This method is relatively easy and requires low costs, but often extensive work efforts and time. A further limitation is that since this method involves living organisms; a range of factors, unknown and difficult to control can influence the results. However, there are also advantages of using living systems, as it makes it possible to include cellular compounds and metabolites which only exists inside the algal cells for a short time, and hence are challenging to obtain by extraction methods.

Another possibility is the mechanical cell disruption techniques. The disintegration and damage of the alga cells leads them to release all intracellular content and products that may possess various effects; e.g. antimicrobial effect. There are no reports that can describe or confirm the use of this method for the aim of investigating the potential of antimicrobial compounds in microalgae. However, it has been tested in bacterial cells for their potential use in industrial applications (Geciova, Bury, & Jelen, 2002), and in algal cells to enhance hydrocarbon and lipid extraction used for biofuels production from algae (S. J. Lee, Yoon, & Oh, 1998; Tsutsumi, Yokomizo, Saito, Matsushita, & Aoki, 2017). This method is easy to apply and is not costly where algal material can be prepared without the use of solvents that may affect the outcome of the experiment. On the contrary, the disadvantage that accompany this method is the involvement of all the substances found in the algae culture, which may affect the results of the experiment.

Methods chosen in this master thesis; the co-culturing and the mechanical disruption algal cells methods, were selected because they appeared to be promising and efficient in terms of application and estimated results.

1.4.2 Methods for testing the effect of potential antimicrobials

Methods available to evaluate the effect of potential antimicrobials of microalgae are listed below.

Agar disk-diffusion method is an assay method that has been in regular use for investigating antimicrobial activity since 1940, as it was first developed for the assay of penicillin (Heatley, 1944). It is a qualitative method that is commonly found in many clinical microbiology laboratories for routine antimicrobial susceptibility testing (Hudzicki, 2009). It was Kirby and his colleague, A.W. Bauer, who first described this technique in 1960s and afterwards it was called Kiry-Bauer disk diffusion test (Bauer, Kirby, Sherris, & Turck, 1966). This method is comparatively used for screening antimicrobial activity in extracts, drugs, plants and other compounds. (Das, Tiwari, & Shrivastava, 2010; Fguira, Fotso, Ameur-Mehdi, Mellouli, & Laatsch, 2005; Konaté et al., 2012; Zaidan et al., 2005). An inoculum of microorganism suspension is first distributed evenly onto the surface of agar plates. After the inoculum has dried, filter paper discs containing the desired test compound and the sought concentration are placed on the agar plate surface. The plates are then incubated under appropriate conditions (Balouiri, Sadiki, & Ibnsouda, 2016; Bauer et al., 1966). The method relies on the diffusion of the test substance from the filter discs into the bacterial cultures on the agar plates. If the test substance exhibit antimicrobial effect, then an inhibition of the microorganism growth and germination can be observed as a zone with no noticeable microorganism growth surrounding the filter papers is formed. The rate of diffusion through the agar is slower than the rate of extraction of the antimicrobial out of the disc, this leads to a high concentration of antimicrobial substance closest to the disc (Hudzicki, 2009). The rate of diffusion of the antimicrobial through the agar is dependent on the diffusion properties, solubility and molecular weight of the antimicrobial compounds (Bauer et al., 1966). Therefore each antimicrobial agent has a unique inhibition zone size (Hudzicki, 2009). The size of the inhibition zone is affected by the depth of the agar, as a larger zone is produced on a thinner layer (Hudzicki, 2009). This method is a qualitative assay, and it is not suitable to determine the minimal inhibitory concentration of antimicrobial agents. In addition, this method cannot be helpful to differentiate bactericidal and bacteriostatic effects. Nonetheless, disk-diffusion examination proposes various advantages over other methods: easiness, low cost, the capability to test large numbers of microorganisms and antimicrobial agents, and simplicity to analyze and interpret the results provided (reviewed in Balouiri et al., 2016).

Agar well diffusion method is a method used to evaluate the antimicrobial activity of drugs (Nathan, Law, Murphy, & MacMillan, 1978). Although it has a similar procedure as the disk-diffusion method, the antimicrobial agents or extract solutions at a desired concentration are introduced into wells punched in the agar plate that is inoculated with microorganism inoculum. The antimicrobial agent diffuses into the agar medium and a readout is made by measuring inhibitions zones similarly to the agar disc diffusion method (reviewed in Balouiri et al., 2016).

Thin-layer chromatography (TLC)–bioautography method: there are three bioautographic techniques (Marston, 2011): 1) agar diffusion in which antimicrobial agents are transferred by diffusion from the chromatograph to an agar plate previously inoculated with the test microorganism. Inhibition zones on agar plate appear in the areas where the antimicrobial agent comes in contact with the agar plate (Meyers & Smith, 1964). 2) Direct bioautography where the developed TLC is sprayed with or dipped in microbial suspension, and then incubated at the appropriate conditions (Hamburger & Cordell, 1987). 3) Agar overlay bioassay where TLC plate is covered with a molten seeded agar medium where the test compounds diffuses into it (Marston, 2011). To sum up, this method is simple, qualitative, and rapid in which it allows the screening of a large number of samples for antimicrobial bioactivity (reviewed in Balouiri et al., 2016).

Dilution method is the most suitable method for determining the minimal inhibitory concentration (MIC), which is the lowest concentration of the tested antimicrobial agent that under suitable conditions inhibits the growth of microorganism. The antimicrobial activity against test microbial is quantified either in broth (broth dilution) or agar (agar dilution) medium. In broth dilution method, bacteria are inoculated in test tubes or in wells in a liquid media in the presence of test substance and then the bacterial count (CFU) is determined by a series of dilutions at regular time intervals. In agar dilution method, on the other hand, the test substance is incorporated at various desired concentrations into an agar plate using serial two-fold dilutions, followed by the inoculation of microbial inoculum on to the agar plate surface. After incubation, the lowest concentration of test substance at which full growth inhibition is observed will be recorded as the MIC (Clinical and Laboratory Standards Institute, 2012).

Antimicrobial gradient method (Etest) is used for direct quantification of antimicrobial susceptibility of microorganisms. In this method, a plastic test strip impregnated with an increasing concentration gradient of the antimicrobial substance from one end to the other is

placed on an agar surface previously inoculated with microbial suspension (Nachnani, Scuteri, Newman, Avanessian, & Lomeli, 1992).

Other methods such as time-kill test (time-kill curve) (Clinical and Laboratory Standards Institute, 1999), ATP bioluminescence assay (Harber & Asscher, 1977) and flow cytofluorometric method (Pore, 1994) are used for further study of the antimicrobial agent`s effects and for a better understanding of the nature of the inhibitory effect. Nevertheless, these methods are not widely used due to the specific equipment required (reveiwed in Balouiri et al., 2016).

2 Aims of the present study

The world oceans occupy more than 70 % of the earth's surface and represent one of the essential natural resources, yet to be fully explored and utilized. Substantial increase in the interest pertaining to the diversity of marine organisms, and the proved potential of marine algae to produce bioactive compounds have brought these single cellular organisms into focus for the development of novel antibiotics to address the rapid increase in antibiotic resistance problem.

The Norwegian Culture Collection of Algae, NORCCA, comprise a unique collection of microalgae from marine and artic waters. The main aim of this thesis is to evaluate two methods for assessing the antimicrobial potential of marine microalgae in the NORCCA collection.

The specific aims are:

- Literature search to identify methods for assessing antimicrobial effects of microalgae.
- Test a method based on mechanical disruption of microalgae to evaluate the potential for antibacterial activity in marine microalgae.
- Test co-cultivation of two marine microalgae species, *Isochrysis galbana* and *Dunaliella tertiolecta*, and the fish pathogenic bacteria *Vibrio alginolyticus* to assess growth inhibiting capability of the two algae, and how it is influenced by temperature.
- Investigate how algae growth phase influence antimicrobial potential.

3 Materials and Methods

3.1 Culturing and quantification of microalgae

3.1.1 Description of microalgae

Four different marine microalgae were used in this project: (i) *Isochrysis galbana*, (ii) *Dunaliella tertiolecta,* (iii) *Tetraselmis suecica,* (iv) *Nannchloropsis oculata.* They are all in the Norwegian Culture Collection of Algae (NORCCA).

(i) I. galbana (figure 10a) is a unicellular flagellate alga that occurs in seawater. It belongs to phylum Haptophyta; class Coccolithophyceae; order Isochrysidales; family Isochrysidaceae and genus Isochrysis. Cells are elongate to sub spherical with a size of 5-6 µm in length, and with flagella of approximately 7 µm in length (Parke, 1949). It is used in aquaculture to feed crustaceans, juvenile fish, and various bivalve larvae in mollusc hatcheries (Godet, Loiseau, Pencreac'h, Ergan, & Hérault, 2010).

(ii) ***D. tertiolecta*** (figure 10b) is a unicellular flagellate marine green alga. It belongs to phylum Chlorophyta, class Chlorophyceae, order Chlamydomonadales, family Dunaliellaceae and genus Dunaliella (Butcher, 1959). The cell is permanently green, ellipsoidal, radially symmetrical, oval or rarely ovate or pyriform. The cell has a length of 5-18 µm (mean 9.412.4 m), a width of 4.514.0 (mean 7.1-8.2 µm), and a flagella of a size 2-2.5 times the cell length. The anterior of the cell is free of the chloroplast and filled with many colorless granules (Guiry, 2019).

iii) ***T. suecica*** (figure 10c) is a unicellular green alga which is found in marine habitats. It belongs to phylum Chlorophyta, class Chlorodendrophyceae, order Chlorodendrales, family Chlorodendraceae, genus Tetraselmis (Butcher, 1959).

(iv) ***N. oculata*** (figure 10d) is a microalga that belongs to phylum Ochrophyta, class Eustigmatophyceae, order Eustigmatales, family Monodopsidaceae, and genus Nannochloropsis (Hibberd, 1981). It is a brackish water species (Guiry, 2019).

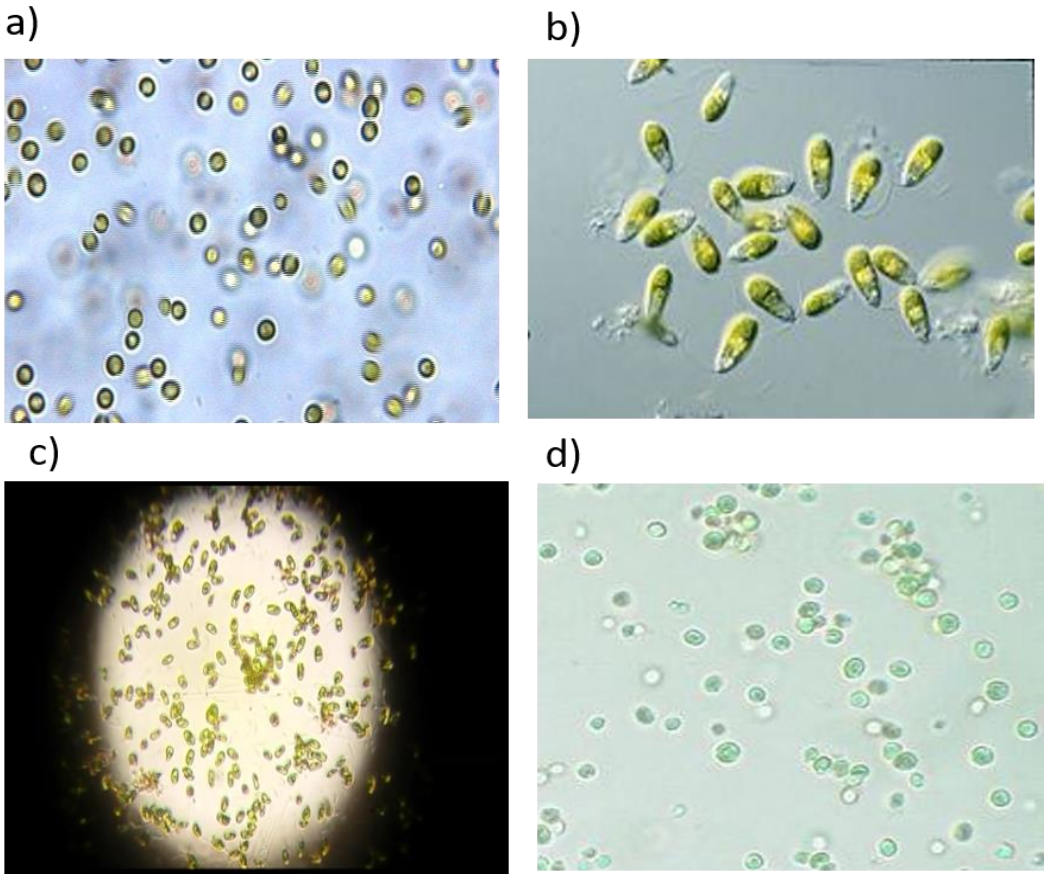


Figure 10 (a-d) - *Light microscopic image of the four microalgae used in this thesis.* ***a)*** *I. galbana x100. CC BY-SA* ***https://alchetron.com/Isochrysis-galbana#****. b) D. tertiolecta photo taken by CSIRO on January 01, 2000, CC-BY- 3.0. c) T. suecica* ***https://alchetron.com/Tetraselmis-suecica*** *CC BY-SA. d) N. oculate (Wageningen University),* ***https://commons.wikimedia.org/wiki/File:15_3klein2.jpg****.*

3.1.2 Culturing microalgae

The four microalgae, (i) *I. galbana, (ii) D. tertiolecta*, (iii) *T. suecica*, and (iv) *N. oculata*, were bought from NORCCA, and maintained at the Section for Aquatic Biology and Toxicology, Department of Biosciences, Blindern, UiO. Culturing the microalgae required artificial conditions of light, temperature and medium composition.

Upon arrival of microalgae stocks, immediate care was required for the maintenance of the algal cultures. From each culture 5 mL were transferred to 100 mL IMR1/2 medium in 250 mL Erlenmeyer flasks. The IMR1/2 medium (modified version by E. Paasche of that described in Eppley et al. 1967, Protocol) (Appendix A) is composed of nutrients and has a salinity which is suitable for a range of marine microalgae. Two replicate cultures were set up for each alga. One of these was used for the experiments, while the second was saved for backup purposes.

All materials and equipment that were to be in contact with the algae cultures, including the IMR1/2 medium, were sterilized in advance, to avoid contamination and introduction of bacteria and other microorganisms. The cultures were sustained in an algae culturing room at the Department of Biosciences, UiO, under 30-50 µmol photons m-2: s-1 light with 12:12 light cycle (12 hours light followed by 12 hours dark), and at a temperature of 16 ℃ for 3 days allowing the algae to grow dense enough to be used as inoculums in larger cultures.

In a routine procedure, the microalgae cultures were renewed every 3-4 weeks in which a portion of the old existing culture is removed and a new fresh IMR1/2 cultural medium is supplied to avoid the inevitable death of the cultures, which have already consumed the nutrient components in the process of reaching their maximum growth.

3.1.3 Quantitative determinations of algal growth and cellular density

The microalgae cultures were quantified by chlorophyll fluorescence measurements and Fuchs-Rosenthal counting chamber in combination with a light microscope.

Measurement of chlorophyll a fluorescence became widespread in 1900s to examine the photosynthetic performance of microalgae cultures (Torzillo, Accolla, Pinzani, & Masojidek, 1996; Vonshak, Torzillo, & Tomaseli, 1994). This is a reliable, sensitive and practical technique, which is used to monitor the photosynthetic potential, and consequently the growth of microalgae cultures. It is based on the re-emission of light at specific wavelengths by the chlorophyll molecules when returned from excited to non-excited status (Maxwell & Johnson, 2000).

The Fuchs-Rosenthal counting chamber is a method used to enumerate algal cells. It is used in combination with a light microscope to quantify cells. The chambers used in these experiments had a depth of 0,2 mm and are ruled with the Fuchs-Rosenthal pattern in which it consists of 16 large squares areas of 1 mm^2 oriented with triple lines. Each area is sub-divided into 16 small squares with 0.25 mm sides and an area of 0.0625 mm^2. A smooth flow of the microalgae culture is filled in one side of a clean microscope-slide base plate of the counting chamber under a cover glass and then placed under the microscope to count the number of cells. When all the 16 squares were counted, a standard formula is used to obtain the concentration of cells.

Total Cells in 1µL sample =

$$\frac{\text{Number of counted cells}}{\text{Total surface counted } (\text{mm}^2) * \text{chamber} - \text{depth}(\text{mm}) * \text{dilution}}$$

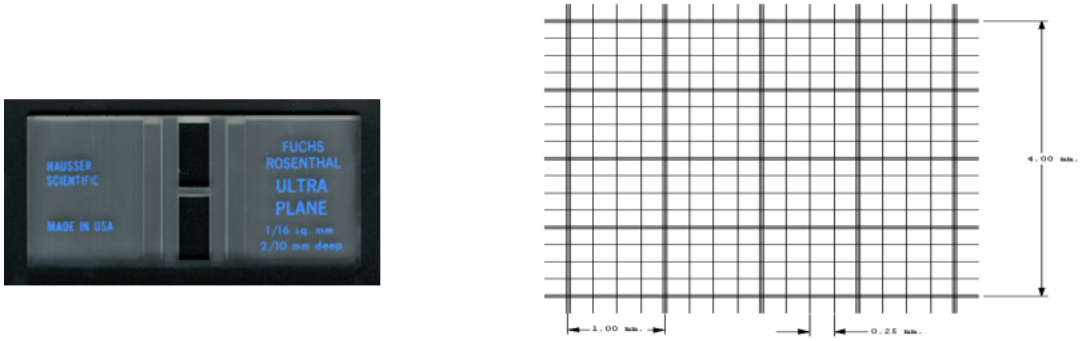


Figure 11 *- Fuchs-Rosenthal Counting chamber.*
Source: http://www.hausserscientific.com/products/fuchs-rosenthal.html

The cultures of *D. tertiolecta*, *I. galbana*, *T. suecica* and *N. oculata* were quantified by chlorophyll fluorescence measurements to monitor their growth phases.

3.2 *Vibrio alginolyticus*

In all experiments, *Vibrio alginolyticus*, was used to test the potential for microalgae antimicrobials. This strain was a kind gift from Prof. Henning Sørum, Faculty of Veterinary Sciences, Norwegian University of Life Sciences, NMBU.

V. alginolyticus, is a highly motile, facultative anaerobic, and halophilic Gram-negative bacterium. It belongs to the Vibrionacea family, and is found in marine water all over the world (Bakeeva, Drachev, Metlina, Skulachev, & Chumakov, 1987; Gjerde & Boe, 1981; Hornstrup & Gahrn-Hansen, 1993; Larsen, Farid, & Dalsgaard, 1981; S. J. Rubin & Tilton, 1975). *V. alginolyticus* prefers temperatures between 15 and 35 °C and a salinity between 5–25‰, consequently concentrations increase during the summer (Janda, Powers, Bryant, & Abbott, 1988).

Human Infections associated with *V. alginolyticus* are ear infections (otitis) and wound infections after exposure to contaminated seawater. However, in rare cases, it develops to cause

sepsis and necrotizing fasciitis , mainly in people with immunodeficiency or with other chronic diseases that become infected by eating raw seafood (Blake, Merson, Weaver, Hollis, & Heublein, 1979; Reilly, Reilly, Smith, & Baker-Austin, 2011; Schmidt, Chmel, & Cobbs, 1979). Drug resistance has been reported for *V. alginolyticus* against commonly used antibiotics like beta lactam antibiotics, lincosamides and sulfonamides (Hornstrup & Gahrn-Hansen, 1993; Hörmansdorfer, Wentges, Neugebaur-Büchler, & Bauer, 2000; Spark, Fried, Perry, & Watkins, 1979). In addition, this bacterium is a major pathogen in aquaculture, as it causes infections in marine shellfish and fish species (B Austin et al., 1993; Burke & Rodgers, 1981; Egidius, 1987; K. K. Lee, Chen, Yu, Yang, & Liu, 1997)

Thiosulfate citrate bile salt sucrose agar (TCBS) is a selective medium established for the isolation of Vibrio bacteria (Kobayashi, Enomoto, & Sakazaki, 1963). This media has a relatively high pH (pH 8.6) which is suitable for Vibrio growth, while it selects against a wide range of other bacteria ("Thiosulphate citrate Bile-salt sucrose (TCBS) agar," 1995). When cultured on TCBS plates, *V. alginolyticus* colonies (2-3 mm in diameter) are yellow. This is due to a color indicator added to the media. *V. alginolyticus* is able to ferment sucrose and reduce pH of the TCBS plates (Lotz, Tamplin, & Rodrick, 1983). This change in acidity leads to the color change of the pH indicators found on TCBC agar plates from blue to yellow (figure 12) (Lotz et al., 1983).

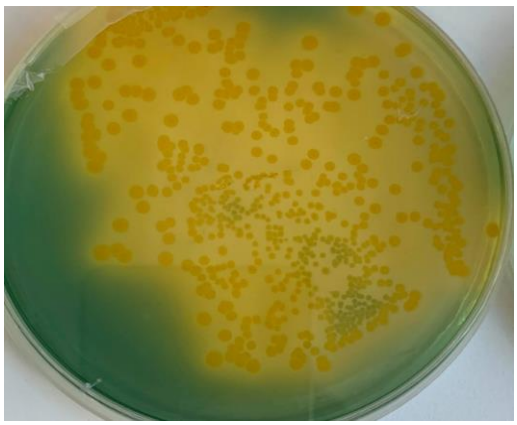


Figure 12 -. Yellow colored (sucrose-fermentation) colonies of *V. alginolyticus* on TCBS agar plate. *Capture: Chirine Issam Kanaan*

V. alginolyticus was cultured in LB liquid media and agar plates (Appendix B), in addition to selective TCBS plates (Appendix C).

Prior to the experiments, bacterial glycerol stocks were made by adding glycerol to a liquid bacterial culture, to a final concentration of 20 %. The stocks were stored at -80 °C. Bacteria were easily recovered from the glycerol stocks by inoculating a small amount of sample material stock on a LB agar plate using a sterile loop. They were cultured either at 37 °C overnight (o/n) or at 20 °C for two days.

Prior to the work of this thesis, two pilot experiments were carried out to investigate the optimal conditions for *V. alginolyticus* growth, and to find out which concentration of *V. alginolyticus* culture can be quantified on TCBS plates.

1. Growth in IMR1/2 medium vs LB medium, and incubation at shaking vs non-shaking conditions.

To test the growth of *V. alginolyticus* in IMR1/2 medium one colony was inoculated in both 6 mL LB liquid medium and 6 mL of IMR1/2 medium. Both tubes were incubated at 37 °C o/n. It has previously been demonstrated that an optical density of 0.05 at 600 nm corresponds to a concentration of 10^6 cells/mL of *V. alginolyticus* culture (Bogdanovic, 2018). The optical density (OD) was measured using a spectrophotometer (Mettler Toledo Gmbh Switzerland). The optical density (OD_{600nm}) was measured after 24 hours in both tubes. Further, in order to assess Vibrio growth under shaking vs non-shaking conditions, 50µL from the 10^6cells /mL Vibrio suspension was transferred into two tubes of 15 mL LB liquid medium, and two tubes of 15 mL IMR1/2 medium supplemented with 150 µl LB medium. One tube from each medium was incubated at 20 °C in a shaking incubator (New Brunswick Scientific Innova 4230, with agitation at 150 rpm), and in a 20 °C non-shaking incubator with 12:12 hours light cycle of 30-50 µmol photons m-2: s-1 for 5 days. OD_{600nm} was measured in all tubes every 24 hours to monitor the growth of Vibrio bacteria.

2. Quantification of low concentrations of *V. alginolyticus* on TCBS agar plates.

V. alginolyticus was cultured on LB agar plate and incubated at 37 °C for 24 hours. One fresh colony was then inoculated in 6 mL LB liquid medium tube and incubated at 37 °C o/n. The Vibrio culture was adjusted to an optical density of 0.05 at 600 nm by diluting with LB liquid medium, to attain a concentration of 10^6 cells/mL. This Vibrio culture was diluted to prepare 10^4 cells/mL and 10^2 cells/mL concentrations. Further, 10 µL from each of the 10^4 cells/mL and 10^2 cells/mL concentrations were inoculated in 5 mL IMR1/2 medium tubes. Vibrio cells

in these tubes were quantified by spreading 100 µL of the cultures on TCBS plates, and incubated at 37 °C o/n.

3.3 Mechanical disruption of algal cells, for assessing effects on *V. alginolyticus* growth

3.3.1 Culturing of microalgae

After 3 days of growth as described in 3.1.2 above, each of *D. tertiolecta* and *I. galbana* were cultured further in three 250 mL replicates, at 16 °C. To set up the replicates, 25 mL of algae culture was inoculated in 225 mL IMR1/2 medium (figure 13). Since these cultures showed little variation, only one replicate was prepared for each of *T. seucica* and *N. oculata*. However, further in the experiment, three replicates were used for each of these algae. *T. seucica* and *N. oculata* cultures were set up by adding 25 mL of inoculum to 225 mL IMR1/2 medium. These cultures were incubated at 19 °C, in order to increase the growth rate. *I. galbana* and *D. tertiolecta* reached the stationary phase at day 21, and *T. suecica* and *N. oculata*, reached stationary phase on day 19.

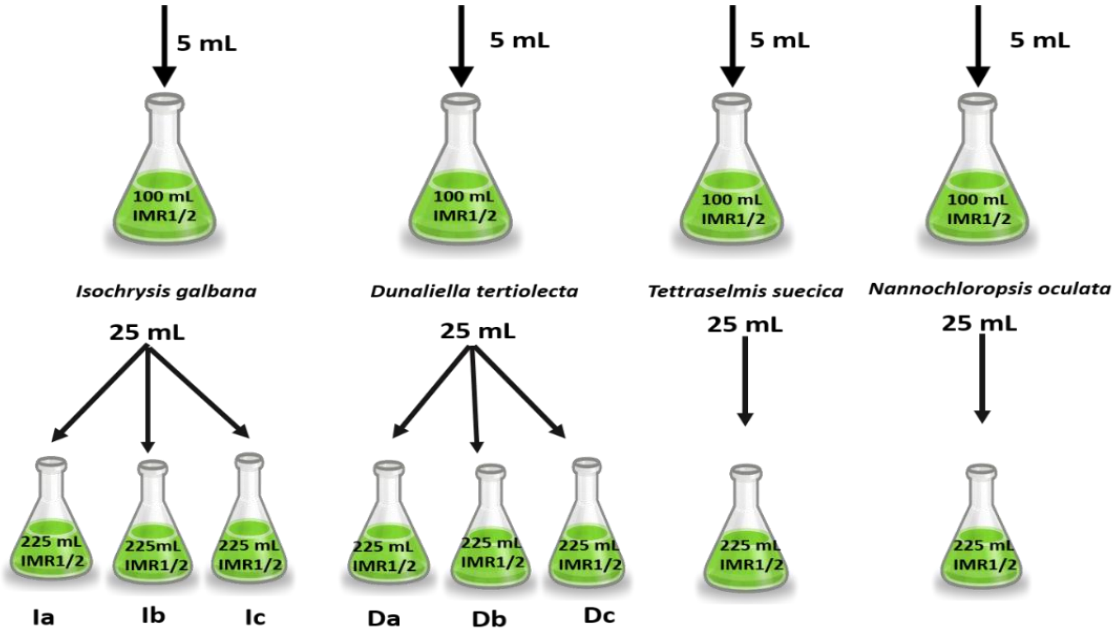


Figure 13 *- Illustration of culturing of (i) I. galbana, (ii) D. tertiolecta, (iii) T. suecica, and (iv) N. oculata. Initially 5 mL preculture was used to inoculate 100 mL of IMRI/2 for all alga. 25 mL was further transferred to inoculate*

one flask of 225 mL IMR1/2 medium for T. suecica and N. oculata and triplicate flasks for I. galbana and D. tertiolecta.

Statistical analysis and graphing

The fluorescence measurements that are summarized in tables 1 and 2 Appendix E, were first formatted in Microsoft excel by plotting fluorescence measurements (relative units) against time (in days) to generate the growth curves for each of *I. galbana* replicates, *D. tertiolecta* replicates, *T. suecica* and *N. oculata* microalgae cultures (figure 2a-d in Appendix F). The mean standard deviation and standard error were calculated for each of *I. galbana* and *D. tertiolecta* cultures from their three replicate measurements to obtain one growth curve, and which will be presented under the results chapter.

3.3.2 Harvesting and processing algal material

The four different microalgae, *I. galbana, D. tertiolecta, T. suecica* and *N. oculata* were cultured as described above in 3.3.1. When reaching the stationary phase, the microalgae were harvested by transferring the cultures into 50 mL centrifuge tubes (Falcon tubes), each containing 40 mL of the content (figure 14). Furthermore, the algae material from *I. galbana, D. tertiolecta, T. suecica and N. oculata* were harvested by centrifuging the tubes for 20 minutes at a speed of 4500 xg (Rotina 420R Centrifuge (Hettich Zentrifugen)).

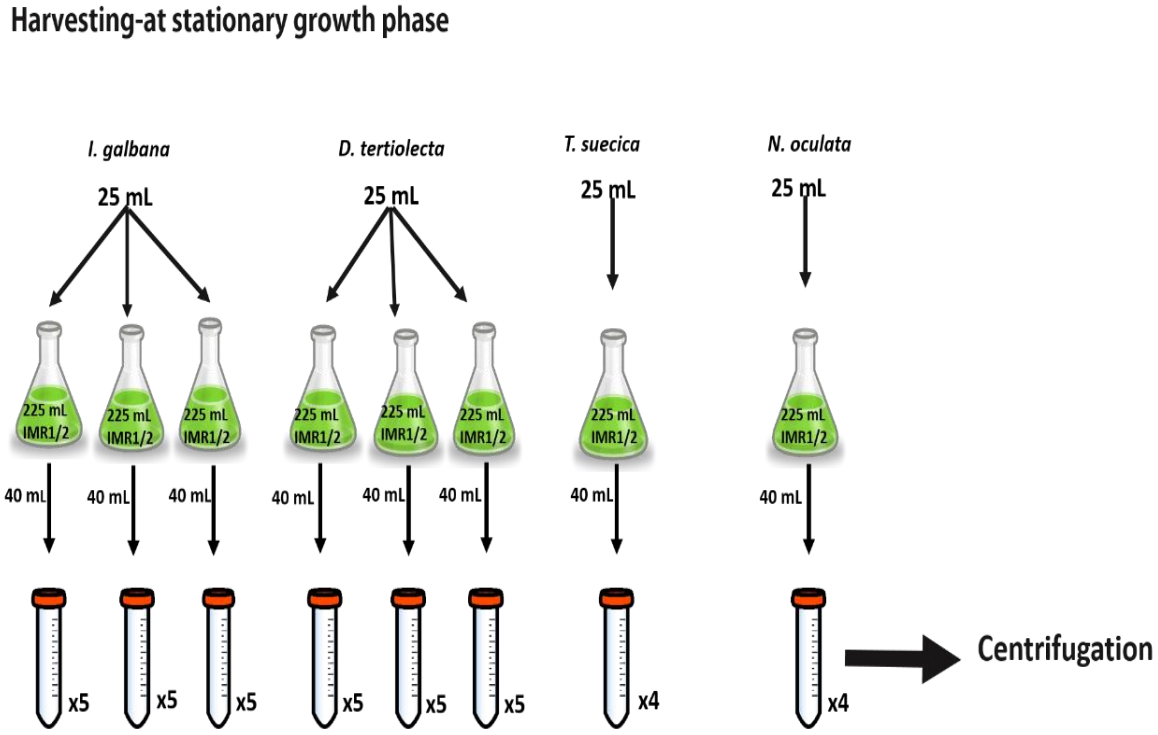


Figure 14 *- Harvesting of I. galbana and D. tertiolecta replicates, T.suecica and N. oculata cultures which is performed by transferring 40 mL of the content from each of I. galbana and D. tertiolecta replicates into five 50 mL size Falcon-tubes, and from each of T. suecica and N. oculate cultures into four 50mL size Falcon-tubes before being centrifuged.*

After centrifuging, the algal material was obtained as a green pellet. The supernatant was removed from the tubes. From each alga, two samples of 13 mL were collected from the cell-free supernatant, transferred to 15 mL Falcon tubes and stored at -20 ℃ until further use. A small amount of the supernatant was retained in the tubes in order to ease pipetting of the algae pellet.

The collected algae pellet was transferred from the Falcon tubes to sterile 1.5 mL microcentrifuge (Eppendorf tubes) containing glass beads (Precellys Lysing Kit (Bertin Technologies)) (figure 15). Further, the algae pellet was disrupted mechanically by a vortex (Minilys homogenizer (Bertin Technologies)), at a maximum speed for 12 minutes. The disrupted algal material was stored at -20℃ until further use.

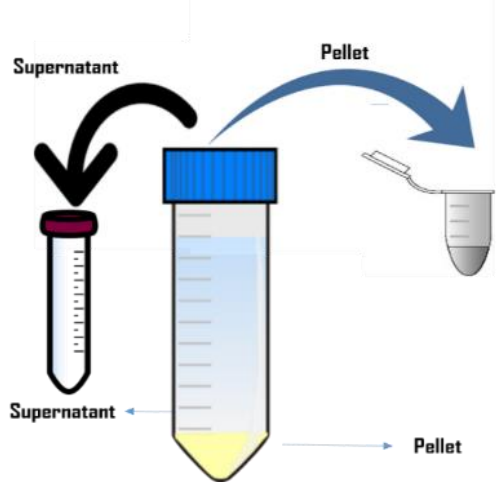


Figure 15 - *After centrifugation, algae pellet from each microalga is collected in microcentrifuge (Eppendorf tubes) containing glass beads, and cell-free supernatant from each microalga is transferred into two 15 mL Falcon-tubes.*

3.3.3 Testing the effects of algal material on *V. alginolyticus* growth

To test the potential for antimicrobial compounds in the four selected marine microalgae, the algal material (3.3.2) were added to *V. alginolyticus* cultured on both solid media (3.3.3.A) and in liquid media (3.3.3.B).

To ensure validity of the experiment, all algae cultures were tested for the presence of Vibrio bacteria prior to the experiment. From each culture, 50 µL were plated on TCBS-petri dishes and incubated at 37 °C for two days. This test confirmed that all the microalgae cultures were free of Vibrio bacteria (figure 3 in Appendix G).

Prior to the experiment, *V. alginolyticus* was cultured from frozen glycerol stocks. Material from the stocks were transferred to a LB-petri dish, using a sterile loop, and incubated at 20 °C for 2 days. 6 mL liquid LB medium was inoculated with one freshly grown colony, using a sterile loop and then incubated at 37 °C o/n.

3.3.3.A Testing the potential for antimicrobial activity of algal material using the disc diffusion method

In this experiment, LB petri dishes were prepared by adding 50 µL of *V. alginolyticus* liquid culture (10^6 cells/mL). The culture was spread evenly using a sterile L-shaped glass spreader. Sterile paper discs (6 mm diameter) were dipped into algal cell-free supernatant, algal disrupted pellet and PBS-buffer (negative control). The paper discs were further placed on the surface of

the *V. alginolyticus* plates using sterile tweezers. Material from each of the three biological replicates of *I. galbana* and *D. tertiolecta* were used in the experiment. For *T. suecica* and *N. oculata*, three technical replicates were used from the algae material. As positive controls, two commercially available discs containing Gentamicin (30 µg) and Ciprofloxacin (5 µg) were placed on the surface of the dishes (figure 16). The plates were left on the bench for 30 minutes to dry prior to incubation at 20 °C for two days.

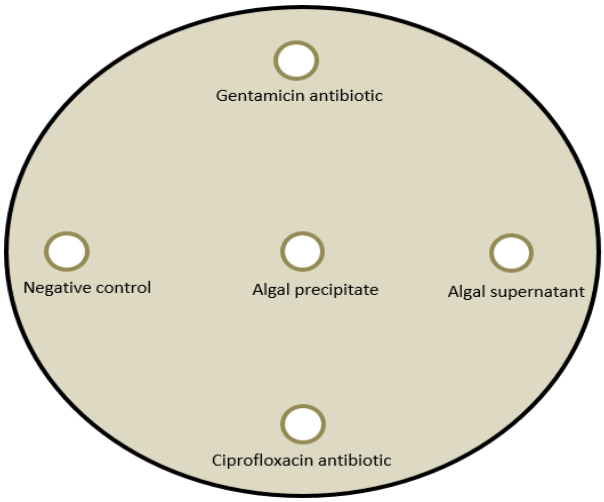


Figure 16 *- The distribution pattern of paper discs on the surface of LB petri dish for the Disc diffusion method.*

3.3.3.B Testing the potential for antimicrobial activity of algae material on liquid cultures of *V. alginolyticus.*

In this experiment, the algal material was added to liquid cultures of *V. alginolyticus*. *V. alginolyticus* was cultured as described in section 3.3.3, but instead of plating the bacteria onto solid media as described in section 3.3.3, *V. alginolyticus* was inoculated in LB liquid medium, and disrupted algae pellet and cell-free supernatant were added to the cultures. A volume of 20 µL of *V. alginolyticus* culture with a concentration of $2x10^8$ cells/mL was inoculated in 3 mL of LB liquid medium in 14 sterile 15 mL Falcon tubes. Further, 100 µL from each of the algae cell-free supernatant and disrupted algal pellet of *D. tertiolecta* and *I. galbana* biological replicates was added to 12 separate *V. alginolyticus* cultures Falcon tubes. Additionally, a positive and a negative control were set up. In the positive control tube, 30 µL of liquid tetracycline antibiotic (12µg/mL) was added, while an untreated *V. alginolyticus* culture was used as a negative control (figure 17). All tubes were incubated in dark conditions (as appropriate for the liquid tetracycline that is sensitive for light), at 20 °C for 48 hours. Bacterial

growth was assessed by spreading 50 µL from all cultures on TCBS plates. Additionally, 1:10 diluted solutions, derived from the positive control, negative control and one of the replicates containing cell-free supernatant and disrupted algal pellet solution from each of *I. galbana* and *D. tretiolecta* were applied on TCBS plates. Finally, all plates were incubated at 20 °C for 48 hours.

Testing the potential for antimicrobial activity of algal material from *N. oculata* and *T. suecica* on liquid cultures of *V. alginolyticus* was performed according to the same procedure described above but at a different time point. Three replicates were made from each of cell-free supernatant and disrupted algal pellet of *N. oculata* and *T. suecica* microalgae (figure 18). All the 14 tubes prepared for testing antimicrobial activity in these two microalgae were incubated at 37 °C for 24 hours**.**

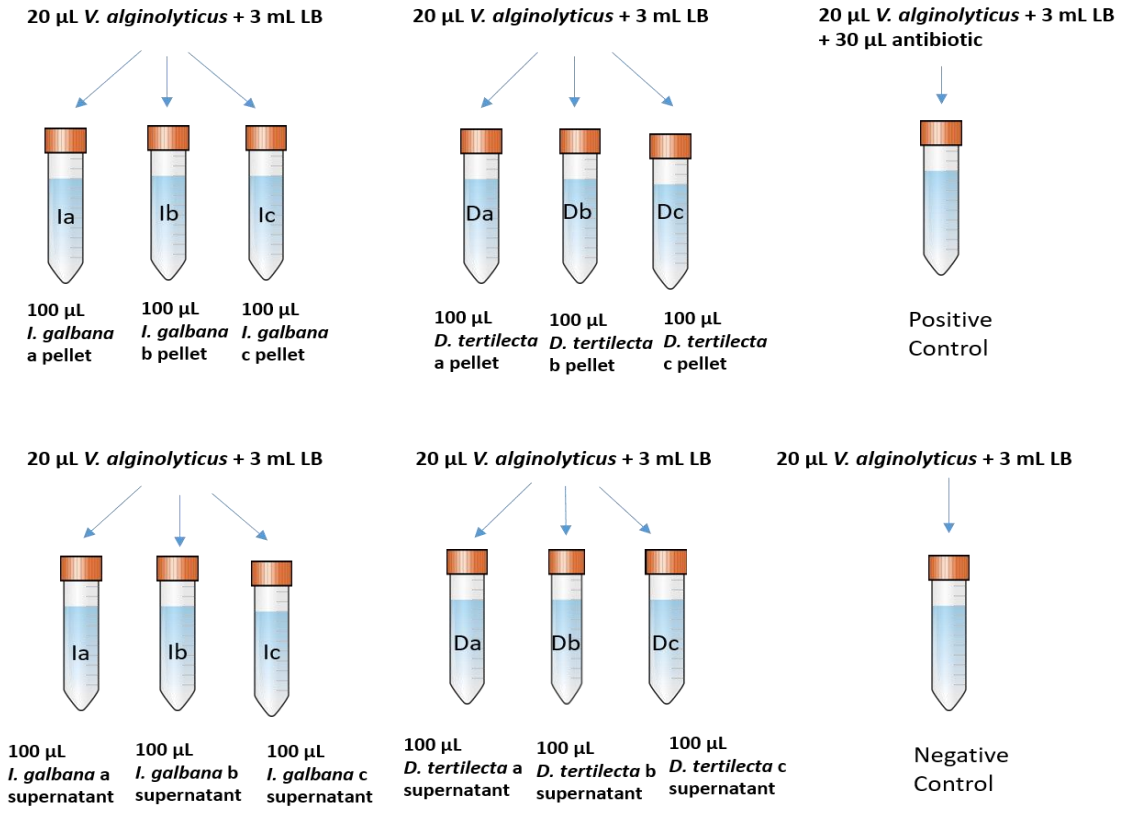


Figure 17 - *Experimental set up for testing antimicrobial activity in cell-free supernatant and disrupted algae pellet material from I. galbana (Ia, Ib, Ic) and D. tertiolecta (Da, Db, Dc). Each alga replicate (n=3) was inoculated with 20 µL of V. alginolyticus suspension in 3 mL LB medium. A positive control was prepared by inoculating 20 µL of V. alginolyticus suspension with 30 µL liquid antibiotic in 3 mL LB medium, and a negative control made up of only 20 µL of V. alginolyticus suspension in 3 mL LB medium.*

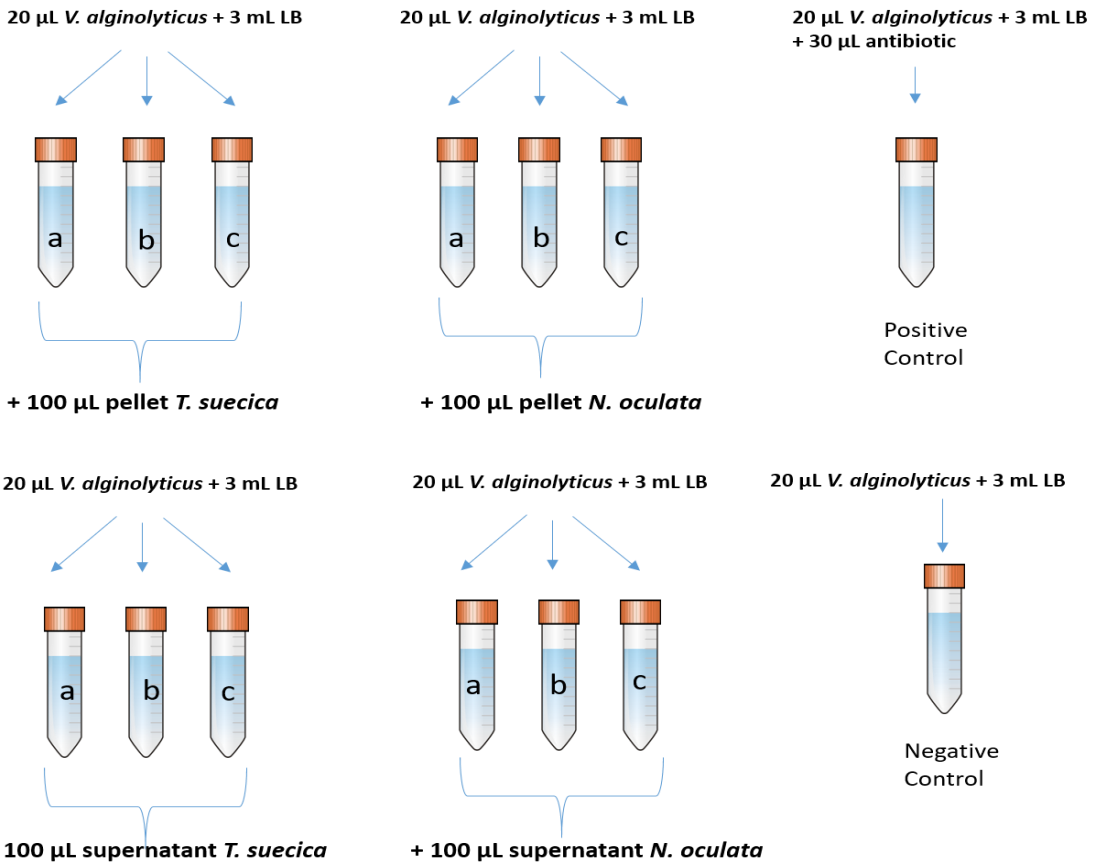


Figure 18 *- Experimental set up for testing antimicrobial activity in cell-free supernatant and disrupted algae pellet material from T. suecica and N. oculata. Three replicates (a, b, c) for each of algae pellet and cell-free supernatant were prepared. Each replicate was inoculated with 20 µL of V. alginolyticus suspension in 3 mL LB medium. A positive control was prepared by inoculating 20 µL of V. alginolyticus suspension with 30 µL liquid antibiotic in 3 mL LB medium, and a negative control made up of only 20 µL of V. alginolyticus suspension in 3 mL LB medium.*

3.4 Co-culturing of microalgae and *V. alginolyticus*

3.4.1 Co-culturing of *V. alginolyticus* and microalgae at different temperatures

Preparation of microalgae cultures

In order to verify the absence of Vibrio bacteria in the microalgae cultures, 30 µL from each of *I. galbana* and *D. tertiolecta* cultures available were spread on TCBS media plates and incubated for 24 hours at 37°C. One culture of each of *I. galbana* and *D. tertiolecta* was used

to prepare three replicates of microalgae-Vibrio cultures for each of the experiments at 20 °C and 25 °C.

Preparation of *V. alginolyticus* culture

For this experiment, growth *V. alginolyticus* was prepared as described above in section 3.3.3. 50 µL of Vibrio-LB liquid culture was then resuspended in 5 mL of IMR1/2 medium and incubated o/n at 37 °C to get a fresh bacteria culture. After 24 hours of incubation, bacterial concentration was adjusted by dilution with sterile IMR1/2 medium, for the co-culturing experiments, to 10^6 cells/mL that corresponds to an optical density of 0.05 at 600 nm. This Vibrio bacterial suspension (10^6 cells/mL) was used to inoculate the replicates of *I. galbana* and *D. tertiolecta* cultures and the *V. alginolyticus* growth control flasks.

Co-culturing

Three replicates of *I. galbana* and *D. tertiolecta* cultures were prepared for each of the 20 °C and 25 °C experiments by transferring 7,5 mL of microalgae cultures into cell culture flasks with 7,5 mL of sterile IMR1/2 medium (figure 19). 15 µL *V. alginolyticus* suspension (10^6 cells/mL) was used to inoculate all the replicates of *I. galbana* and *D. tertiolecta*. In addition, three cell culture flasks containing 15 mL sterile IMR1/2 medium were inoculated also with 15 µL of *V. alginolyticus* suspension of 10^6 cells/mL concentration, acting as a control for *V. alginolyticus* growth. Negative control triplicates were also prepared by filling three culture tissue flasks with 15 mL of sterile IMR1/2 medium.

In the pilot experiment, it appeared that *V. alginolyticus* growth was improved by adding 150 µL liquid LB medium to the IMR1/2 medium (section 3.2), therefore this was added to all flasks in the co-culturing experiment (figure 19). Immediately after the preparation of flasks, the initial concentration of *V. alginolyticus* in the microalgae-Vibrio culture replicates and Vibrio cultural flasks at initial time (T_0) was determined by counting colonies formed on TCBS plates after 37 °C o/n incubation of 10 µL from ten-fold serial dilutions. This count was then expressed in CFU/mL. The microalgae cells at T_0 were also quantified in all the microalgae-Vibrio culture replicates by direct count using Fuchs-Rosenthal hemocytometer in combination with a light microscope.

All cultures were incubated at either 20 °C or 25 °C temperatures for each of the two experiments under the same conditions of 12:12 hours light cycle of 30-50 µmoL photons m-

2: s-1. *V. alginolyticus* colonies were counted on TCBS medium with ten-fold serial dilution from the culture (as shown in figure 20) and incubated at 37 °C, almost every 24 hours for the next 3 weeks. The counts were expressed in CFU/mL. Taking into account the direct relation between microalgae cell growth phases and antimicrobial activity, *I. galbana* and *D. tertiolecta* cell densities in the microalgae-Vibrio cultures were measured by direct count using Fuchs-Rosenthal hemocytometer in order to establish their growth curve throughout the experiment. As a verification of the absence of Vibrio bacteria contamination in IMR1/2 and LB medium, 50 µL from each of the negative control triplicates were spread on TCBS plates by a sterile L-shaped glass spreader, and then incubated at 37 °C o/n.

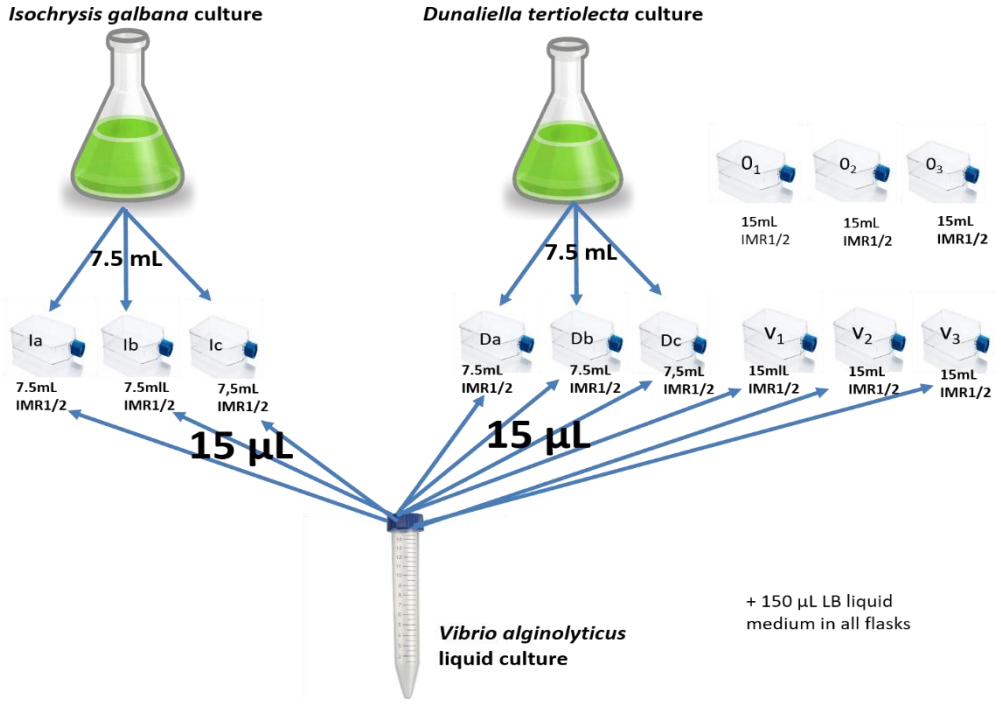


***Figure 19**: Experimental set-up of the co-culturing of V. alginolyticus and each of D. tertiolecta and I. galbana microalgae. The experiment was performed at both 20 °C and 25 °C temperatures. Ia, Ib, and Ic represent the triplicate I. galbana cultures, Da, Db and Dc represent the triplicate D. tertiolecta cultures while V_1, V_2 and V_3 represent the triplicate positive control cultures and O_1, O_2, O_3 represent the triplicate negative control cultures.*

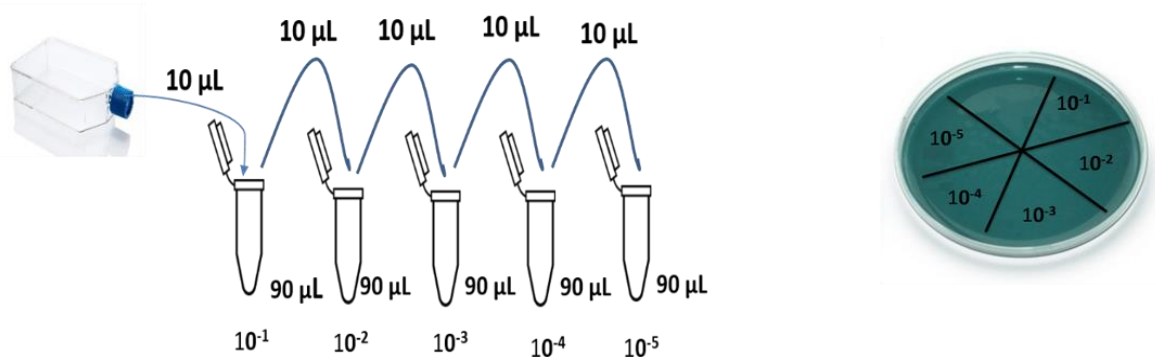


Figure 20 – *V. alginolyticus colonies count in the microalgae-Vibrio and positive control cultural flasks during the 20 °C and 25 °C temperature experiments. The culture is diluted in sterile IMR1/2 medium, and a 10-fold dilution series is prepared. TCBS petri dish is divided in 6 sectors and labeled with the dilutions. In each sector 10 µL from the appropriate dilution is dropped into the surface of the TCBS petri dish.*

Statistical analysis and graphing

The cell densities of *I. galbana* and *D. tertiolecta* in the co-cultures' replicates (cells/mL) were first formatted in Microsoft excel to generate the growth curves for each of *I. galbana* replicates and *D. tertiolecta* replicates in co-cultures over time. In addition, the concentration of *V. alginolyticus* (CFU/mL) in bacterial control replicates and microalgae-Vibrio co-cultures replicates were also plotted first in Microsoft excel against time to generate the growth curves of *V. alginolyticus* in control replicates and microalgae-Vibrio co-cultures replicates respectively (all the raw graphs are presented in Appendix H). The mean, standard deviation and standard errors were calculated to obtain one curve from each triplicate, which were all set up in one figure for each of the 20 °C and 25 °C experiments. These figures will be presented in the results chapter.

3.4.2 Co-culturing *V. alginolyticus* and microalgae in their death phase

In this experiment, *I. galbana* and *D. tertiolecta* microalgae from their death phase of growth culture are co-cultured with *V. alginolyticus* to investigate the potential of growth stimulating effect of dead algal cells on *V. alginolyticus*.

Preparation of microalgae cultures from the death phase

50 mL inoculum from algae cultures of *I. galbana* and *D. tertiolecta* were transferred to 250 mL Erlenmeyer flasks containing 100 mL of IMR1/2 medium. The microalgae cultures were

quantified by direct count using Fuchs-Rosenthal hemocytometer and light microscope to determine their initial concentration at T_0. The content of each *I. galbana* and *D. tertiolecta* culture flask was then distributed into three culture tissue flasks of 15 mL that served as a triplicate for microalgae cultures (a, b, c). All the flasks were placed in the 25 °C incubator with a 12:12 hours light cycle (30-50 µmoL photons m-2: s-1) to grow. The microalgae cultures were quantified almost every day using Fuchs-Rosenthal hemocytometer and light microscope, and their growth phases were determined using excel graphs. The death phase of microalgae cultures was determined out from the growth curve. Prior to inoculation with *V. alginolyticus*, all the microalgae culture tissue flasks were tested for the presence of Vibrio bacteria, by spreading 10 µL of each culture on TCBS plate and left for incubation at 37 °C o/n.

Preparation of Vibrio culture

V. alginolyticus of a concentration 10^6 cells/mL was prepared as described before in section 3.4.1. This bacterial suspension (10^6 cells/mL) was used to inoculate the triplicates of *I. galbana* cultures (Ia, Ib, Ic) and *D. tertiolecta* cultures (Da, Db, Dc) when they had reached the death phase of their culture growth. In addition, it was used to inoculate 15 mL IMR1/2 medium in a culture tissue flask that served as a positive control.

Co-culturing microalgae and *V. alginolyticus*

When the microalgae were observed to reach the death phase of culture growth, *I. galbana* triplicate cultures (Ia, Ib, Ic), *D. tertiolecta* triplicate cultures (Da, Db, Dc), and the positive control, were inoculated with 15 µL *V. alginolyticus* suspension (10^6 cells/mL). A quantity of 150 µL LB liquid medium was also added to all the mentioned flasks. The experimental layout is shown in figure 21. Immediately after the preparation of the flasks, the initial concentration of *V. alginolyticus* in the microalgae-Vibrio cultures and the positive control flask at initial time (T_0) was determined by enumeration on TCBS medium. Vibrio cells were counted on TCBS plates by pipetting 10 µL from ten-fold series dilutions of the culture (as shown in figure 20), and incubated at 37 °C o/n. These counts were then expressed as CFU/mL. All the culture flasks were incubated at 25°C temperature under 12:12 hours light cycle of 30-50 µmoL photons m-2: s-1. Further, *V. alginolyticus* cells in the microalgae-Vibrio cultures and positive control were quantified after 24 and 72 hours of co-culturing.

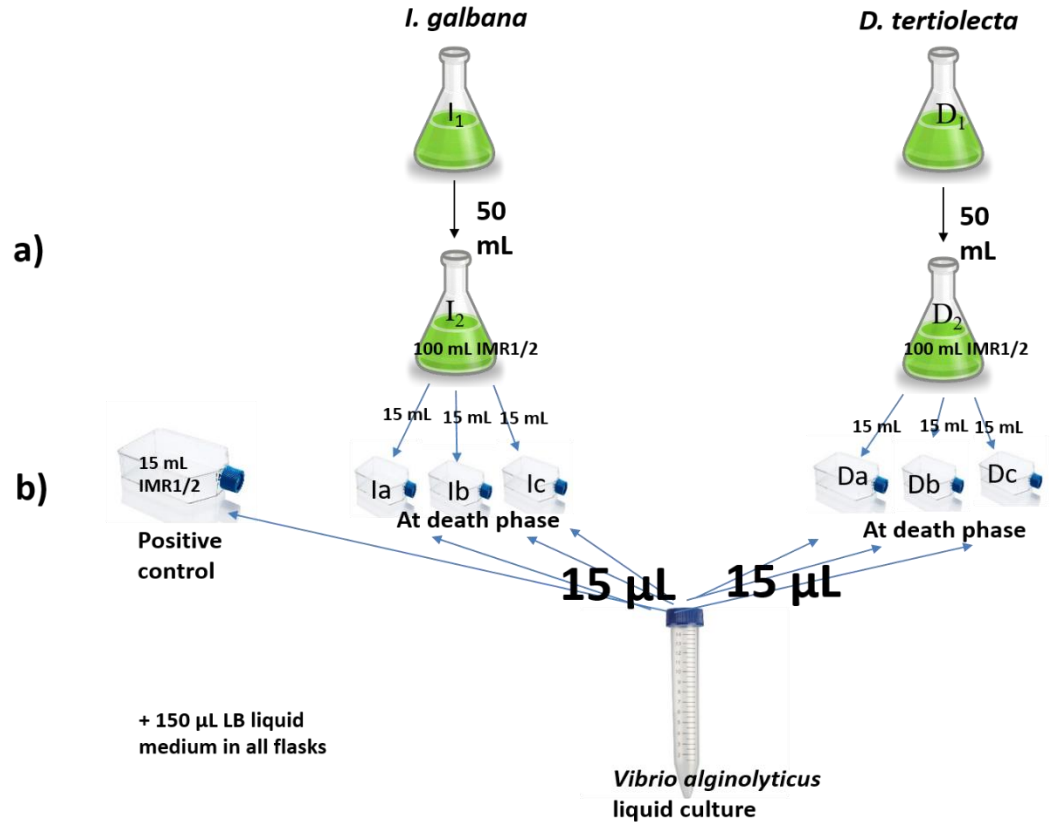


Figure 21 *- Experimental set-up of the co-culturing of V. alginolyticus and microalgae at their death phase. a) Preparation of I. galbana (I_2) and D. tertiolecta (D_2) cultures from microalgae cultures I_1 and D_1 respectively. b) Co-culturing of V. alginolyticus and each of D. tertiolecta and I. galbana microalgae from their death phase. Ia, Ib and Ic represent the triplicate I. galbana-Vibrio cultures, and Da, Db and Dc represent the triplicate D. tertiolecta-Vibrio cultures.*

4 Results

4.1 *Vibrio alginolyticus*

From the pilot experiments carried out to investigate the optimal conditions for *V. aginolyticus* growth, the following was found:

- The addition of LB liquid medium in Vibrio-IMR1/2 cultures is required to obtain a sufficient growth of *V. alginolyticus*, as the growth of bacteria in IMR1/2 medium, added a small amount of LB medium was higher than in IMR1/2 medium alone. When 150 µL LB media was added to 15 mL of IMR1/2 media, bacterial growth was comparable to growth in LB medium.
- Shaking is not essential for the growth of *V. alginolyticus*, as similar growth rates of bacteria cultures were detected with and without shaking.
- No colonies appeared on TCBS plates from the IMR1/2 liquid cultures inoculated with Vibrio bacteria at concentrations of 10^4 cells/mL and 10^2 cells/mL. Thus, the concentration of *V. alginolyticus* suspension chosen to inoculate the microalgae cultures in the further experiments was 10^6 cells/mL.

4.2 Mechanical disruption of algal cells, for assessing effects on *V. alginolyticus* growth

4.2.1 Culturing of microalgae

Since it is described that microalgae often produce antimicrobials compounds at later growth stages (M. A. Borowitzka, 1995), growth of the four microalgae, *Isochrysis galbana*, *Dunaliella tertiolecta*, *Tetraselmis suecica* and *Nannochloropsis oculata* was monitored in order to establish their growth curves. Algal cell density was measured regularly during the culturing period to follow the growth of the microalgae and to detect the optimal time for harvesting, i.e. the moment where the microalgae have reached its maximum growth which is the exact point marking the start of stationary phase and the end of exponential phase.

The microalgae were cultured as described in section 3.3.1. At the early stages of culturing, the growth of algae was noticed by their color change. As the microalgae began to grow, the color

of the cultures transformed gradually as expected from transparent to green for *D. tertiolecta*, *T. suecica* and *N. oculata*, and to yellow color for *I. galbana* (figure 1a-c in appendix D).

As demonstrated by the graphs plotted below (figure 22), the growth rate of *D. tertiolecta* was reduced at day 19 while of *I. galbana* seemed to decrease at day 20. Cultures of *T. suecica* and *N. oculata* appeared to have reduced growth rates on day 18. Both *I. galbana* and *D. tertiolecta* were harvested on day 21. Cultures of *T. suecica* and *N. oculata* were harvested at day 19.

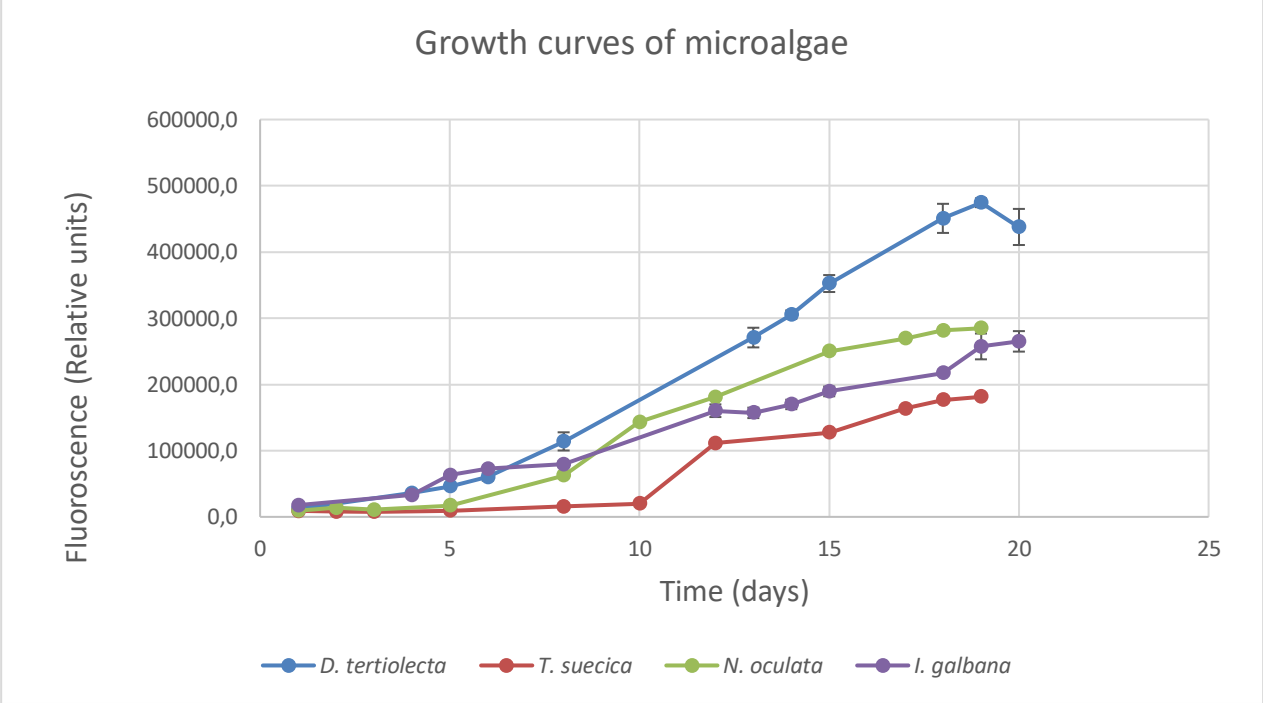


Figure 22 - *Growth curves of I. galbana, D. tertiolecta, N. oculata and T. suecica cultivated in IMR1/2 medium. The growth curves of I. galbana and D. tertiolecta are made of mean values and standard deviations of fluorescence measurements of 3 replicates over time (days), error bars represent standard errors. Growth curve of T. suecica and N. oculata were made by plotting fluorescence measurements (relative units) against time (days).*

4.2.2 Testing the effects of algal material on *V. alginolyticus* growth

4.2.2.A Testing potential for antimicrobial activity of algal material using the disc diffusion method

The disc diffusion assay was used to assess the potential for antimicrobial activity on *V. alginolyticus* in both disrupted algal pellet and the cell-free supernatant obtained from four microalgae: *I. galbana*, *D. tertiolecta*, *T. suecica* and *N. oculata*. As positive controls for antimicrobial activity, paper discs containing commercial antibiotic, Gentamicin and Ciprofloxacin, were used. The plates were left for 2 days of incubation at 20 °C before a readout of inhibition zones was performed.

No inhibition zones were observed around filter discs containing cellular material from neither of the three microalga *I. galbana*, *D. tertiolecta* and *N. oculata* (figure 23a-c); thereby implying the absence of antimicrobial activity or that no antimicrobial active compounds were able to diffuse from the impregnated filter discs.

One replicate of *T. suecica* cell-free supernatant material (C) showed different results from the other replicates (A, B) of supernatant material and from the disrupted algal material (figure 23d-1), where a small inhibition zone (0.5 cm) was observed around the filter disc (figure 23d-2). This implies the presence of antimicrobial activity in this sample. Inhibition zones were formed in all the plates around the two antibiotics control filter-discs, Gentamicin (1) and Ciprofloxacin (2) with a diameter 4 cm and 3 cm respectively (figure 23a-d).

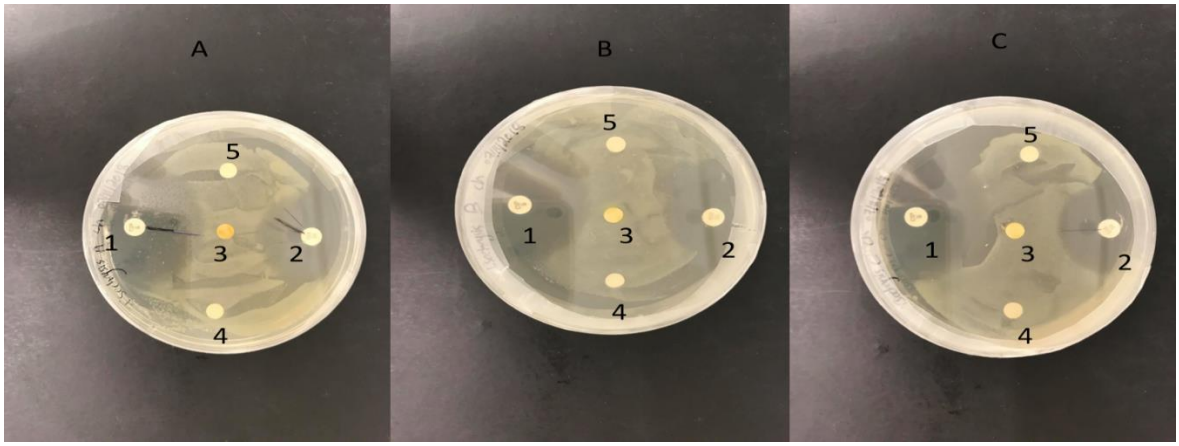


Figure 23a - *Assessing the antimicrobial activity of algal material (supernatant and pellet) from I. galbana replicates (A, B, C) against V. alginolyticus using the disc diffusion method. No inhibition zones were observed around filter discs containing disrupted algae pellet (3), algae cell-free supernatant (4), or PBS (5). Inhibition zones formed only around antibiotics control filter discs, Gentamicin (1) and Ciprofloxacin (2).*

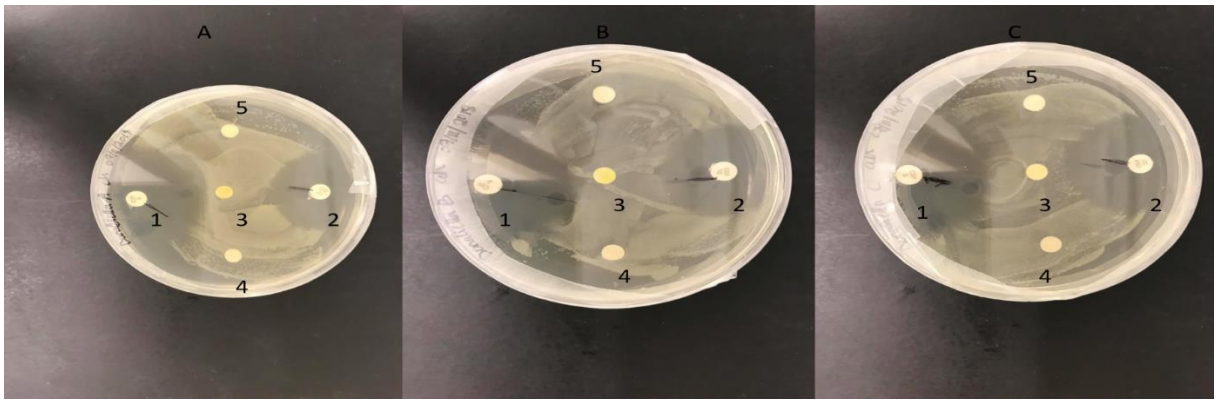


Figure 23b - *By the disc diffusion method the antimicrobial activity of algal material (supernatant and pellet) from D. tertiolecta replicates (A, B, C) was assessed against V. alginolyticus. No inhibition zones were observed around filter discs containing disrupted algae pellet (3), algae cell-free supernatant (4), or PBS (5). Inhibition zones formed only around antibiotics control filter discs, Gentamicin (1) and Ciprofloxacin (2).*

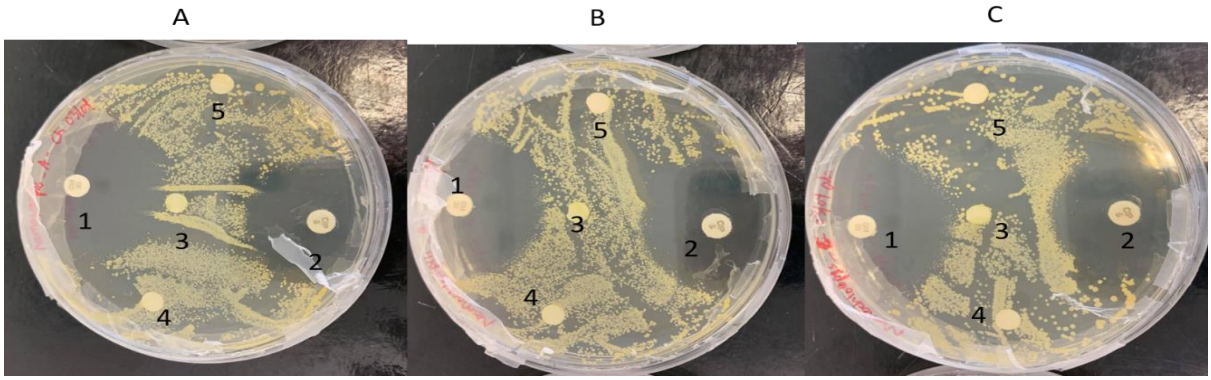
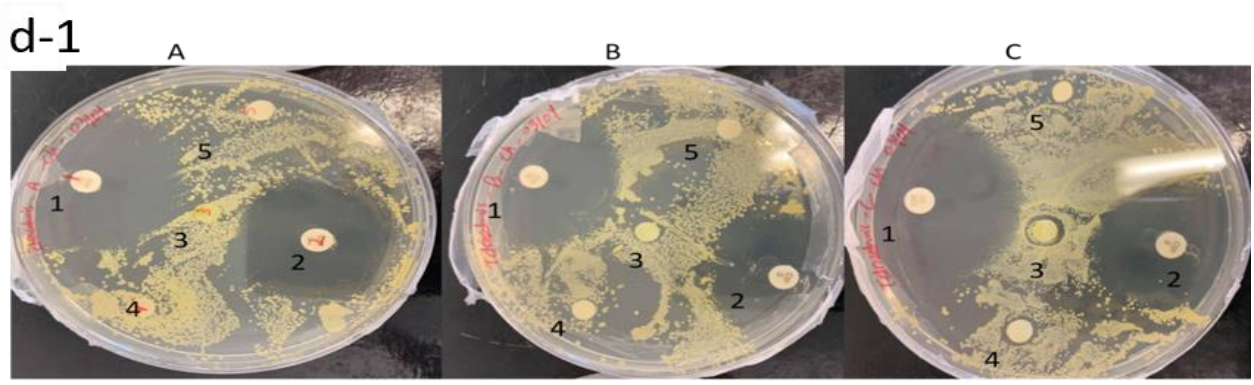


Figure 23c - *Assessing the antimicrobial activity of algal material (supernatant and pellet) from N. oculata against V. alginolyticus using the disc diffusion method. 3 replicates were used (A, B, C). No inhibition zones were observed around filter discs containing disrupted algae pellet (3), algae cell-free supernatant (4), or PBS (5). Inhibition zones formed only around antibiotics control filter discs, Gentamicin (1) and Ciprofloxacin (2).*



d-2) *T. suecica* C

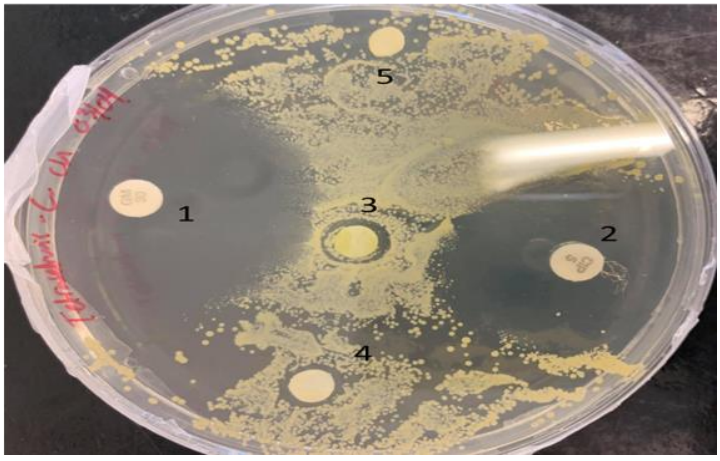


Figure 23d - 1) *Assessing the antimicrobial activity of algal material (supernatant and pellet) from T. suecica against V. alginolyticus using the disc diffusion method. 3 replicates were used (A, B, C). In replicates A&B, no inhibition zones were observed around filter discs containing disrupted algal pellet (3), algae cell-free supernatant (4), or PBS (5), but were only formed around antibiotics control filter discs, Gentamicin (1) and Ciprofloxacin (2). In replicate c, no inhibition zones were observed around filter disc containing algae pellet (3) and PBS (5), but a small inhibition zone was observed around filter disc containing algae cell-free supernatant (4) in addition to the inhibition zones that were formed around antibiotic control filter discs, Gentamicin (1) and Ciprofloxacin (2).* ***- 2)*** *A larger picture of T. suecica replicate C showing the small inhibition zone formed around filter disc containing algae cell-free supernatant (4).*

4.2.2.B Testing the potential for antimicrobial activity of algae material on liquid cultures of *V. alginolyticus.*

The potential antimicrobial activity from the same microalgae was also tested on liquid cultures (described in section 3.3.3.B). Liquid test cultures were plated on TCBS plates to assess the effects of algae material on Vibrio growth. Initially, the number of viable bacteria reflected by the number of colonies (CFU) formed on the petri dishes was supposed to be counted after incubation to indicate the growth of Vibrio bacteria in the tubes. Unfortunately, this method did not result in countable colonies on all dishes. However, the appearance (color and growth extent) made these results comparable. The media in the TCBS petri dishes used in this experiment changes its color from blue to yellow in the presence of Vibrio bacteria (as mentioned in section 3.2). Therefore, the difference in color and bacterial growth were used to describe the growth of *V. alginolyticus* after being exposed to algal material. For each plate, both color and growth extent on a scale from 0 to 5 (table 1) were described. Here, 0 indicates

no growth of *V. alginolyticus* and 5 indicates maximum growth of *V. alginolyticus*, observed in this experiment.

As an example, the plates corresponding to the liquid *V. alginolyticus* culture with the disrupted algal pellet and the cell-free supernatant derived from *I.galbana* replicate a, together with the plates corresponding to negative and positive controls are shown in figure 24. The other plates of the cultures can be seen in figures 4a-e and 5a-e in Appendix G.

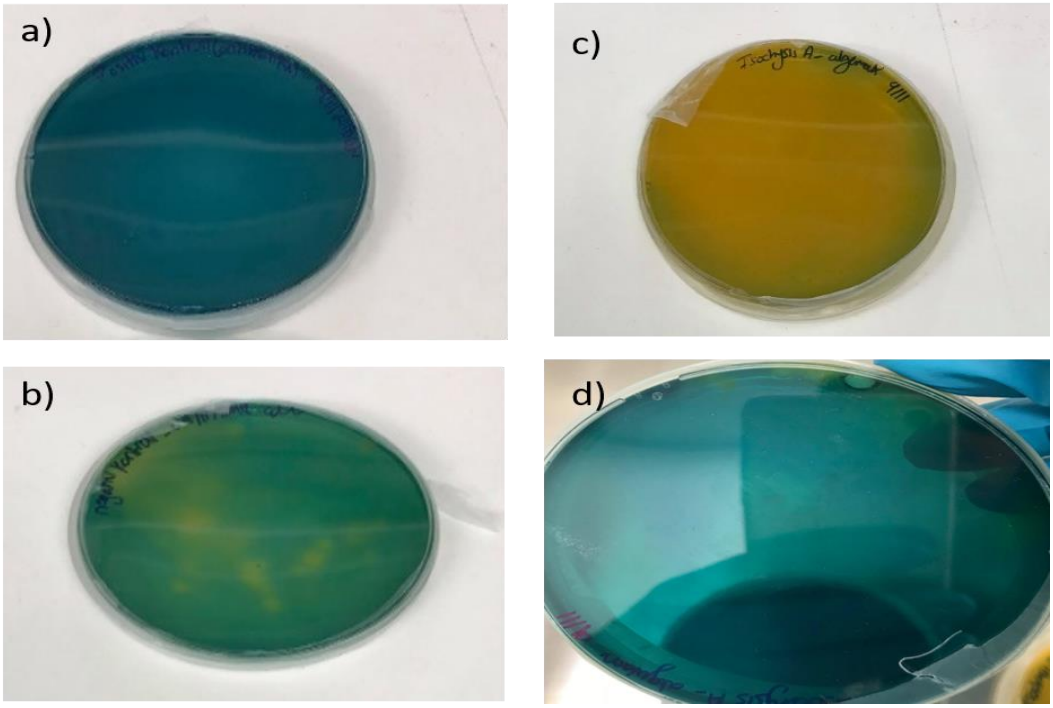


Figure 24 a-d** – Assessing the bacterial growth of V. alginolyticus in the liquid cultures with algal materials by spreading samples on TCBS petri dishes.* ***a) *TCBS petri dish plated with sample from the positive Control containing V. alginolyticus culture supplemented with liquid antibiotic (0 on the growth scale).* ***b)*** *TCBS petri dish plated with a sample from a negative Control containing untreated V. alginolyticus culture (2 on the growth scale).* ***c)*** *TCBS petri dish plated with a sample of disrupted algal pellet derived from I. galbana replicate a in a liquid culture of V. alginolyticus (5 on the growth scale).* ***d)*** *TCBS petri dish plated with a sample of cell-free supernatant derived from I. galbana replicate a in a liquid culture of V. alginolyticus (1 on the growth scale).*

After incubation, the disrupted algal pellet derived from all four microalgae, *I. galbana, D. tertiolecta, T. suecica and N. oculata* appeared to have a positive effect on the growth of *V. alginolyticus* since the plates showed a more yellow color and a better growth extent compared to the negative control treatment. In addition, the cell-free supernatant derived from *D. tertiolecta* seemed to affect the growth of *V. alginolyticus* positively, since the plates recorded

more yellow color and growth extent when compared to the plates of the negative controls. The cell-free supernatant derived from *N. oculata* appeared to have no effect on the growth of *V. alginolyticus,* since the plates looked similar to those of the negative controls. Furthermore, the cell-free supernatant of *I .galbana* (replicate a and b) appeared to have a slightly negative effect on the growth of *V. alginolyticus,* as less growth was registered on these plates compared to the negative control, and in addition, one replicate of the cell-free supernatant of *T. suecica* (c) showed different results from the other replicates and seemed to have a slightly negative effect on the growth of *V. alginolyticus*, as the plates registered less growth compared to the negative controls.

Table 1 - *The growth of V. alginolyticus cultured in a liquid culture with the disrupted algae pellet and the supernatant derived from the replicates D. tertiolecta (a, b, c) and I. galbana (a, b, c) is described by the color transformation of the petri dishes (i.e. blue, yellow, mostly yellow, blue dotted with yellow…) and the estimated growth scale from 0 to 5. 0 indicates no growth of V. alginolyticus and 5 indicates maximum growth of V. alginolyticus. Positive control is a liquid culture of V. alginolyticus supplemented with liquid antibiotic, and the negative control is a liquid culture of V. alginolyticus.*

Sample	Color	Growth
Negative control	Blue dotted with yellow	2
Negative control (1:10 dilution)	Blue dotted with fewer yellow	1
Positive control	blue	0
Positive control (1:10 dilution)	blue	0
I. galbana replicate (a) - disrupted algal pellet	Yellow	5
I. galbana replicate (a) – disrupted algal pellet (1:10 dilution)	Mostly yellow	4
I. galbana replicate (b) – disrupted algal pellet	Yellow	5
I. galbana replicate (c) – disrupted algal pellet	Mostly yellow	4
I. galbana replicate (a) – cell-free supernatant	Blue dotted with yellow	1
I. galbana replicate (a) - cell-free supernatant (1:10 dilution)	Blue	0
I. galbana replicate (b) - cell-free supernatant	Blue dotted with fewer yellow	1
I. galbana replicate (c) – cell-free supernatant	Equally blue and yellow	3

D. tertiolecta replicate (a) – disrupted algal pellet	Mostly yellow	4
D. tertiolecta replicate (a) – disrupted algal pellet (1:10 dilution)	Equally blue and yellow	3
D. tertiolecta replicate (b) – disrupted algal pellet	Mostly yellow	4
D. tertiolecta replicate (c) – disrupted algal pellet	Mostly yellow	4
D. tertiolecta replicate (a) – cell-free supernatant	Mostly yellow	4
D. tertiolecta replicate (a) – cell-free supernatant (1:10 dilution)	Mostly yellow	4
D. tertiolecta replicate (b) – cell-free supernatant	Equally blue and yellow	3
D. tertiolecta replicate (c) – cell-free supernatant	Equally blue and yellow	3

Table 2 - *The growth of V. alginolyticus cultured in a liquid culture with the crushed algae pellet and the cell-free supernatant derived from T. suecica and N. oculata cultures is described by the color transformation of the petri dishes (i.e. blue, yellow, mostly yellow, blue dotted with yellow…) and a growth scale from 0 to 5. 0 indicates no growth of V. alginolyticus and 5 indicates maximum growth of V. alginolyticus. 3 replicates were made for each pellet and cell-free supernatant samples of the two microalgae. Positive control is a liquid culture of V. alginolyticus supplemented with liquid antibiotic, and the negative control is a liquid culture of V. alginolyticus.*

Sample	Color	Growth
Negative control	Mostly yellow	4
Negative control (1:10 dilution)	Blue dotted with fewer yellow	3
Positive control	Blue	0
Positive control (1:10 dilution)	Blue	0
N. oculata replicate (a) – disrupted algal pellet	Mostly Yellow	5
N. oculata replicate (a) – disrupted algal pellet (1:10 dilution)	Mostly yellow	5
N. oculata replicate (b) – disrupted algal pellet	Mostly Yellow	5
N. oculata replicate (c) – disrupted algal pellet	Mostly yellow	5
N. ocualata replicate (a) – cell-free supernatant	Mostly yellow	4
N. oculata replicate (a) – cell-free supernatant (1:10 dilution)	Blue dotted with yellow	3

N. oculata replicate (b) – cell-free supernatant	Mostly yellow	4
N. oculata replicate (c) – cell-free supernatant	Mostly yellow	4
T. suecica replicate (a) – disrupted algal pellet	Mostly yellow	5
T. suecica replicate (a)- disrupted algal pellet (1:10 dilution)	Mostly yellow	4
T. suecica replicate (b) – disrupted algal pellet	Mostly yellow	5
T. suecica replicate (c) – disrupted algal pellet	Mostly yellow	5
T. suecica replicate (a) – cell-free supernatant	Mostly yellow	4
T. suecica replicate (a) – cell-free supernatant (1:10 dilution)	Equally blue and yellow	3
T. suecica replicate (b) – cell-free supernatant	Mostly yellow	4
T. suecica replicate (c) – cell-free supernatant	Blue dotted with fewer yellow	1

4.3 Co-culturing of microalgae and *V. alginolyticus*

The cohabitation of microalgae and Vibrios to assess the potential antimicrobial activity in microalgae have been reported to give positive results in previous experiments, as stated in chapter 1.4.1. Thus, it was of interest to repeat this experiment, yet with certain differences in experimental design. In this assay, two microalgae strains, affiliated with two different taxonomic divisions; *D. tertiolecta* (Chlorophta) and *I. galbana* (Haptophta), were selected and examined with regard to their antibacterial activity against *V. alginolyticus* strains. Both influence of temperature and algae growth phase were investigated.

4.3.1 Co-culturing of *V. alginolyticus* and microalgae at different temperatures

The influence of temperature on antimicrobial potential of the selected algae was investigated by performing co-culturing experiments at two different temperatures; 20 °C and 25 °C. The experiment was performed as described in details in section 3.4.1 and figure 19. Three replicates from each of the two microalgae *I. galbana* and *D. tertiolecta* were inoculated with 15 µL of *V. alginolyticus* suspension at 10^6 cells/mL. Control cultures with only IMR1/2 medium or only *V. alginolyticus* liquid culture were also set up.

V. alginolyticus grew well in positive controls at both temperatures. Mean values of 4×10^4 CFU/mL were registered at the beginning of the assays at both 20 °C and 25 °C (as shown in figure 25 and 26). These values increased to their maximum values, and then decreased subsequently reaching the mean values of 5×10^6 CFU/mL and 5×10^4 CFU/mL at the end of the 20 °C and 25 °C experiments respectively.

The concentration of *V. alginolyticus* increased to their highest values during the first 48 hours in both the co-cultures, and the bacteria control cultures (figure 25 and figure 26). However, the growth rate of *V. alginolyticus* was lower in *I. galbana* and *D. tertiolecta* co-cultures compared to the control cultures. After 48 hours, the *V. alginolyticus* concentration declines in all cultures throughout the rest of the 20 °C and 25 °C experiments. The concentration of Vibrio cells declined faster in the co-cultures, compared to the bacterial control cultures. In the control cultures, the concentration of Vibrio cells decreased relatively slower, and in the end of the experiment, the concentration was 100 times higher compared to the concentrations of co-cultures. A more significant inhibition of *V. alginolyticus* was demonstrated for the *D. tertiolecta* co-cultures compared to *I. galbana* co-cultures. This significance was much more obvious at temperature 25 °C rather than at 20 °C. Over time, a slower but a constant inhibition of bacterial growth, was observed in the *I. galbana* co-cultures.

Although the results from the 20 °C and the 25 °C co-culturing experiments were highly comparable in terms of *V. alginolyticus* growth inhibition, the bacterial inhibition was more distinctive and faster at the temperature of 25 °C than 20 °C. At 25 °C, *V. alginolyticus* in both co-cultures decreased significantly starting from day 2 (48 hours) and became undetectable after 336 and 552 hours respectively. In contrast, at 20 °C, the concentrations of *V. alginolyticus* were almost constant until day 8 (192 hours), declining significantly on day 10 (240 hours) and reaching their lowest values of 4×10^2 CFU/mL and 4×10^3 CFU/mL after 552 hours in each of *D. tertiolecta* and *I. galbana* co-cultures respectively.

The initial concentrations of *I. galbana* and *D. tertiolecta* in the co-cultures were higher in 20 °C temperature experiment than in 25 °C experiment. Cultured at 20 °C, the inhibition of *V. alginolyticus* seemed to be initiated between the end of exponential phase and the beginning of the stationary phase for the microalgae (figure 25). However, at 25 °C, the inhibition appeared to begin during the exponential phase of the microalgae growth culture (figure 26).

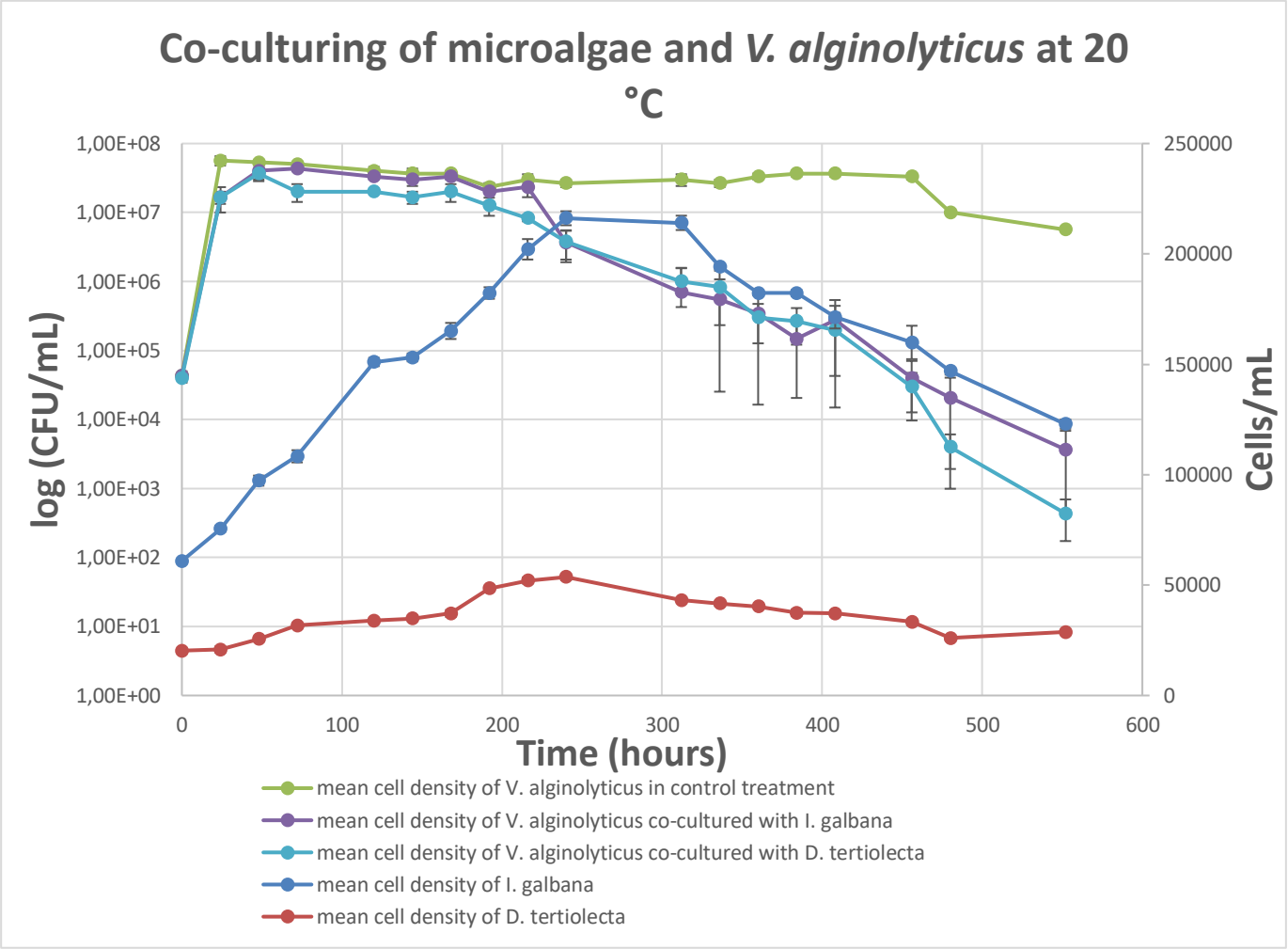


Figure 25 *- Mean values of cell concentration (CFU/mL) (±SE) of V. alginolyticus in control treatments, V. alginolyticus co- cultured with D. tertiolecta microalgae, and V. alginolyticus co-cultured with I. galbana at 20 °C over time (hours) where n=3. The mean cell density (cells/mL) (±SE) of I. galbana and D. tertiolecta during co-culturing over time is also represented (n=3).*

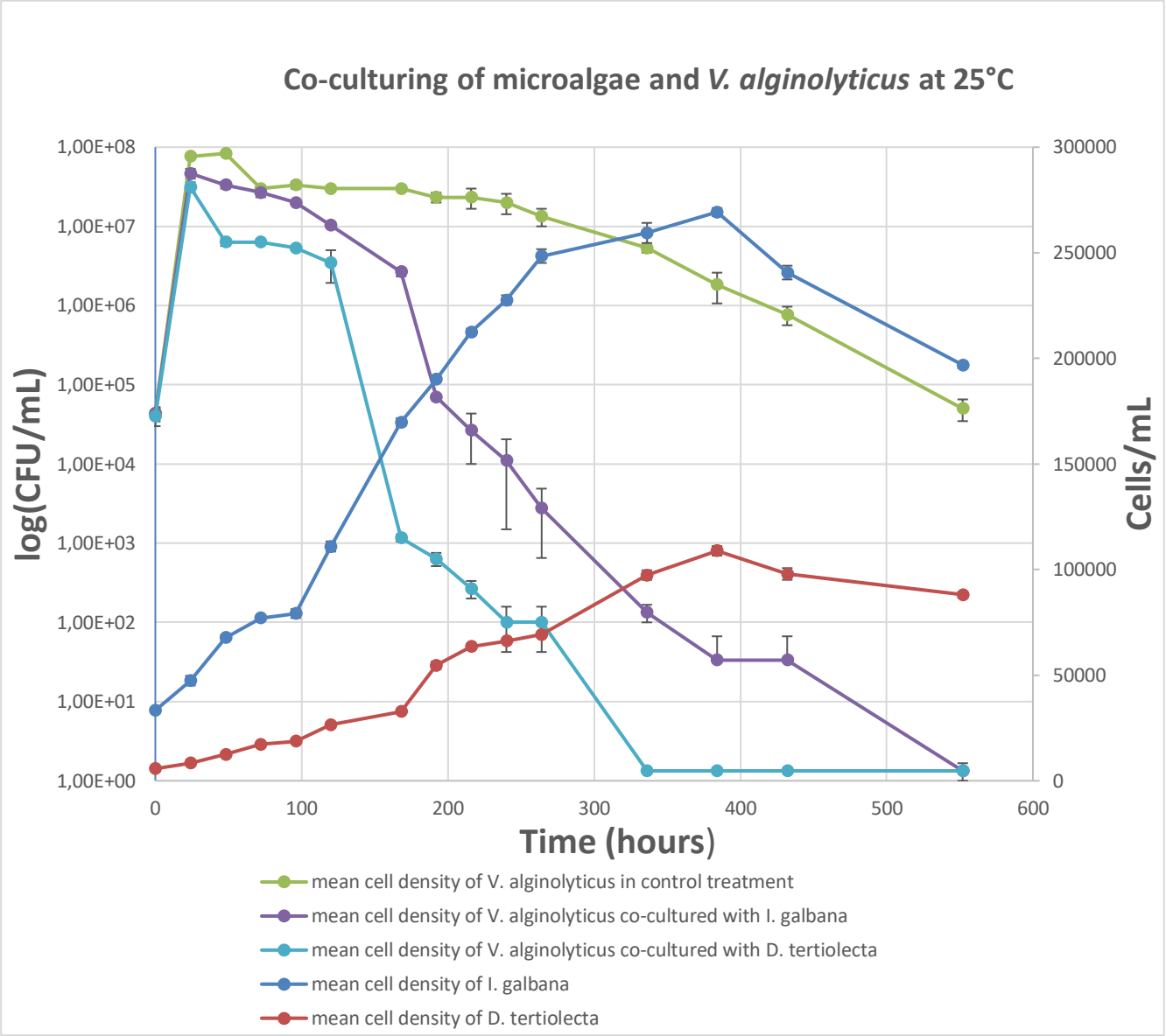


Figure 26 *- Mean values of cell concentration (CFU/mL) (±SE) of V. alginolyticus in control treatments, V. alginolyticus co- cultured with D. tertiolecta microalgae, and V. alginolyticus co-cultured with I. galbana at 25 °C over time (hours) where n=3. The mean cell density (cells/mL) (±SE) of I. galbana and D. tertiolecta during co-culturing over time is also presented (n=3).*

4.3.2 Co-culturing *V. alginolyticus* and microalgae in their death phase

As a stimulation of bacterial growth was detected in the experiment assessing the antimicrobial effect of mechanically disrupted microalgae cells on liquid cultures of *V. alginolyticus* (described in section 4.2.2.B), the effect of *I. galbana* and *D. tertiolecta* in their death phase, was tested on the growth of *V. alginolyticus* in a co-culturing experiment (section 3.4.2).

The primary step was to detect the death phase of microalgae cultures before setting them up with *V. alginolyticus.* The growth curves of the two microalgae, *D. tertiolecta* and *I. galbana* were established and determined (figure 27).

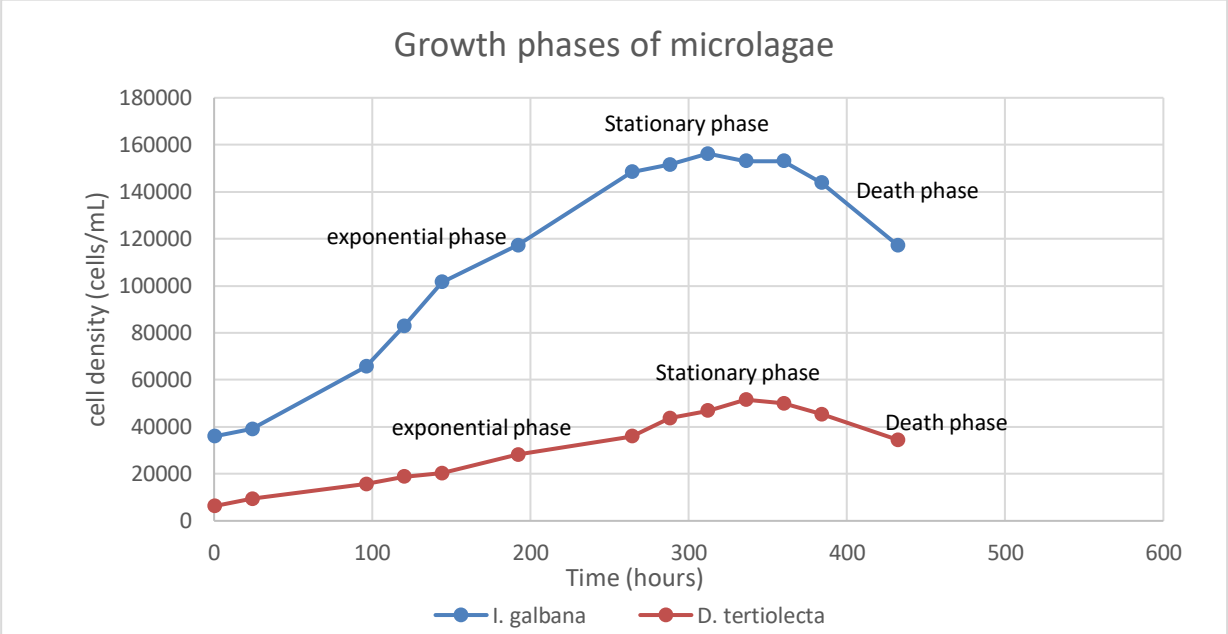


Figure 27 - *Growth phases and growth curves of I. galbana and D. tertiolecta microalgae cultures cultivated in IMR1/2 medium. The growth curve is made by plotting cell density (cells/mL) against time (hours).*

During the death phase of *I. galbana* and *D. tertiolecta* growth cultures, three replicates of each microalgae were inoculated with 15 µL of *V. alginolyticus* suspension at 10^6 cells/mL concentration. A control culture of only *V. alginolyticus* was also set up simultaneously. As more apparent results were demonstrated for the co-cultures cultured at 25 °C compared to 20 °C in the previous experiments (section 4.3.1), the co-cultures and the control culture were all incubated at 25 °C, in addition to 12:12 hours light cycle of 30-50 µmoL photons m-2: s 1, in

this experiment. Concentration of *V. alginolyticus* (CFU/mL) was measured by enumeration on TCBC plates at the moment of inoculation, after 24 hours and after 72 hours (figure 28).

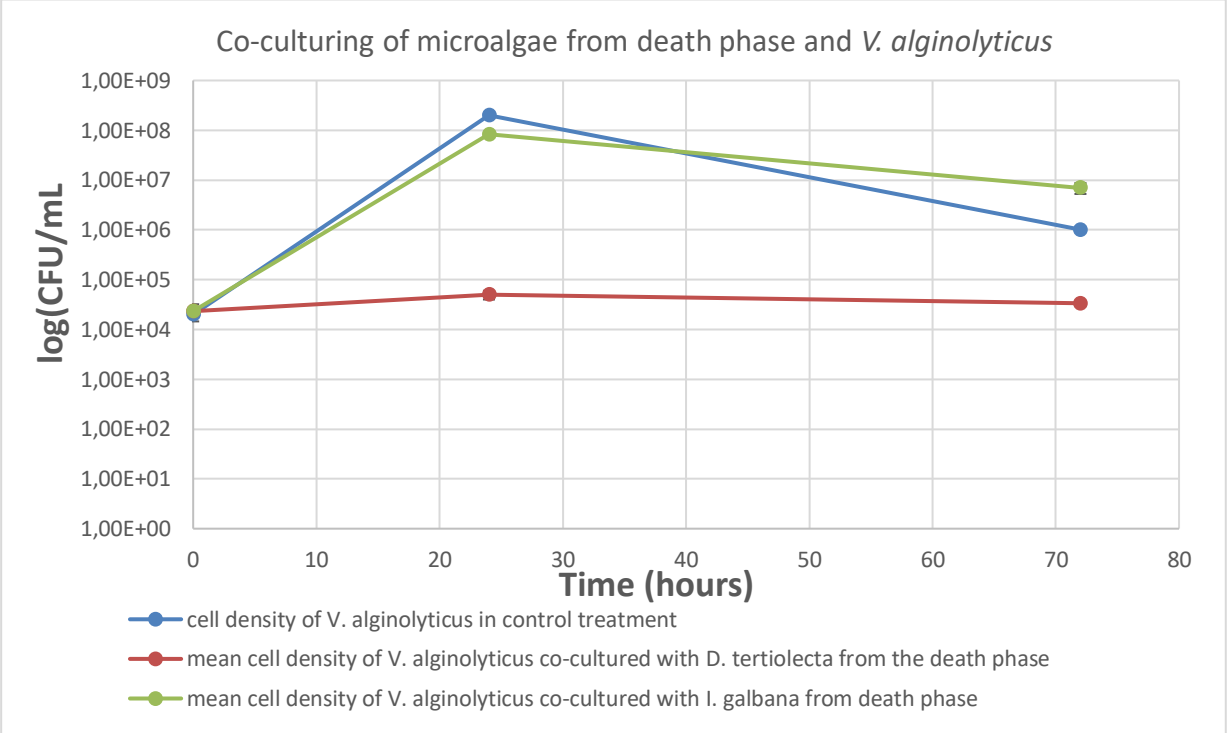


Figure 28 *- The growth of V. alginolyticus in control treatment and in the co-cultures with I. galbana and D. tertiolecta microalgae. The growth curves of V. alginolyticus in the microalgae co-cultures were made by plotting mean values of cell concentration (CFU/mL) (±SE) of V. alginolyticus in the co-cultures with I. galbana and the co-cultures with D. tertiolecta over time (hours) where n=3. The growth curve of V. alginolyticus in control treatment was made by plotting cell concentration (CFU/mL) against time (hours).*

After 24 hours, the highest *V. alginolyticus* concentration values were detected in both control treatment and *I. galbana* co-cultures showing close values, while *D. tertiolecta* co-cultures had a much lower *V. alginolyticus* concentration (figure 28).

As with the control culture, the concentration of *V. alginolyticus* also declined after 72 hours in *I. galbana* co-cultures. However, the bacterial concentration was still ten times higher than that in control culture at final measurement point. Meanwhile, *V. alginolyticus* concentration in *D. tertiolecta* co-cultures decreased slightly, suggesting a slow inhibition of Vibrio growth.

5 Discussion

During this work, two methods for assessing the potential for antimicrobials in microalgae were tested. There were indications of a potential for antimicrobial activity in some of the microalgae included in this thesis. However, it was also observed that bacterial growth could be stimulated in the presence of algal material.

5.1 Methods for investigating antimicrobial effects of microalgae

Investigating microalgae for antimicrobial substances began in the 1950's and was mainly concerned with *in vitro* studies (M. A. Borowitzka, 1995). The majority of these studies were conducted by screening the antimicrobial activity of the crude extracts of microalgae against selected bacterial strains, allowing a greater number of samples to be screened (Duff et al., 1966; Kellam & Walker, 1989; Pratt et al., 1944). Extraction of algal material for determining the potential antimicrobial effects is mostly done using organic solvents, as the bioactivity in microalgae was rarely detected in aqueous extracts (Cannell et al., 1988; Walter & Mahesh, 2000). In addition to the opportunity to screen a massive number of crude extracts simultaneously, this method can give high yield of the bioactive compounds produced by microalgae. However, the extraction technique suffers a number of disadvantages, as uncertain results can be obtained due to the possible antimicrobial properties of the extraction solvents used, or due to the loss of desired compounds with improper extraction solvent selection or by the evaporation of the solvent. Moreover, the solvent may also dissolve unwanted substances or materials, some of which may interfere with the test results. Various organic solvents were reported to provide a high efficacy in extracting antimicrobial compounds, however the antibacterial activity of a microalgae culture can vary according to the extraction solvent used (Cannell et al., 1988; Kellam & Walker, 1989; Krishnika, Bhanupriya, & Nair, 2011; Venkatesan, Karthikayen, Periyanayagi, Sasikala, & Balasubramanian, 2007).

In the present study, two different methods were chosen to assess potential antibacterial activity in microalgae; the co-culturing and the mechanical disruption algal cells methods. Co-culturing microalgae and bacteria to investigate antimicrobial activity has been used in several studies and appeared to be promising (Bogdanovic, 2018; Kokou et al., 2012; Molina-Cárdenas et al., 2014). By this method, both the microalgae culture content and the responses that may develop

as a result of direct interaction of microalgae living cells with bacteria are preserved. The substances we risk losing during chemical extraction are preserved by this method. The methodological approach is cheap and simple; however, it is time consuming and sometimes difficult to control as various natural biological processes and other factors may regulate and adjust the experiment progression. The general results obtained by co-culturing microalgae and bacteria were positive and similar to those obtained by the previous co-culturing studies, thus indicating the efficacy of this method to investigate antibacterial activity in microalgae. However, several factors such as cultural conditions (temperature, medium and light) may affect the method`s outcomes and need to be investigated further in order to control the overall experiment.

To my knowledge, no published data on the use of cellular disruption techniques for investigating the potential antibacterial activity in microalgae exists. However, disruption techniques have been used in microalgae to increase the yield of their intracellular products for other objectives such as biofuel production (Günerken et al., 2015; Halim, Harun, Danquah, & Webley, 2012; A. K. Lee, Lewis, & Ashman, 2012). By disintegrating the cellular structure of microalgae organisms, intracellular products become more readily available for extraction and testing. The microalgae in the present study were disrupted mechanically by bead beating technique. This method is fast, simple and allow the screening of a large group of algae materials from many different microalgae species. However, the results obtained were surprising since the bacterial growth appeared to be boosted by the disrupted cells originating from all selected microalgae species. As the same effects were not observed during co-culturing of *I. galbana* or *D. tertiolecta* together with *Vibrio alginolyticus*, more research is required in order to understand this growth stimulating effect. The disruption technique in combination with disc diffusion or liquid culture sensitivity tests, may provide a rapid alternative to assess antimicrobial effects of microalgae. However, some of the metabolites and cellular compounds produced by the algae, including potential antimicrobial compounds, are commonly released from the cells and into the algae surroundings. These compounds are too small to be included in the fraction collected by centrifugation of the algae cultures, hence will be present in the supernatant in a low final concentration. In order to detect an inhibiting effect of such compounds, their potency must be high, due to their low concentration. Despite challenges during sensitivity, the cellular disruption technique may be proven valuable as a high throughput screening method to assess the antimicrobial potential of many microalgae organisms simultaneously, if optimized further.

5.2 Potential antimicrobial compounds in selected microalgae

Marine microalgae may hold a potential for the discovery of new antibiotics, as their metabolic products yet remain largely unexplored. These organisms synthesize a broad array of bioactive compounds and secondary metabolites with rich chemical diversity, and which has been demonstrated to inhibit several pathogens (M. A. Borowitzka, 1995; John Faulkner, 2000). The making of these compounds often results from their diverse biosynthetic and metabolic pathways, and the defense strategies they possess enable them to survive in an environment densely populated with threats, including pathogens, competitors, parasites and predators (M. A. Borowitzka, 1995; Cole, 1982; reviewed in Andrew Paul Desbois, 2008; Hughes & Fenical, 2010).

Isochrysis galbana

The antibacterial activity of the marine microalgae *I. galbana* against the fish pathogenic Vibrio *alginolyticus* bacteria have been documented in several studies. Ceres *et al* (Molina-Cárdenas et al., 2014) observed that among all the Vibrio bacterial strains which were tested when co-cultivated with *I. galbana* microalgae, *V. alginolyticus* was the most sensitive to the antibacterial activity of *I. galbana*. The inhibitory effect on *V. alginolyticus* by *I. galbana* was also detected in the master thesis of *Sanja Bogdanovic* (Bogdanovic, 2018). Moreover, axenic *Ishochrysis* sp. cultures were proven to be effective in inhibiting the growth of various Vibrio bacterial strains, including *V. alginolyticus*, when co-cultured with microalgae (Kokou et al., 2012). All these studies come in agreement with the results of the present study. Signs of antibacterial activity against the *V. alginolyticus* were demonstrated in the co-cultures with *I. galbana*, as the number of bacteria at the end of the experimental period was ≥ 1000 times lower than that in control treatment. Furthermore, a possible antibacterial activity was also presented when testing cell-free supernatant of *I. galbana* growth culture on the liquid culture of *V. alginolyticus*. No recovery of bacterial strains was observed in the co-cultures of the present study as well as in the three previous studies mentioned before, thus allowing the consideration that the substances produced by *I. galbana* are bactericidal. Substantial antibacterial activity of *I. galbana* against various human bacterial pathogens were also reported in previous studies (Duff et al., 1966; Rajeev, Prakash, & Bhimba, 2006; Srinivasakumar & Rajashekhar, 2009). Hence, it can be concluded that the antibacterial activity of the microalgae *I. galbana* seems to

be active against both human and aquaculture pathogenic bacteria. It has been detected that *I. galbana* release free short chain fatty acids from its lipid content through enzymatic activity (Andrew P. Desbois & Smith, 2010; Roncarati, Meluzzi, Acciarri, Tallarico, & Meloti, 2004). Microalgae species produce fatty acids as a defense strategy against a variety of threats, including pathogenic bacteria and grazers (Jüttner, 2001). These compounds have been reported to exhibit antibacterial activity against a wide range of Gram-positive and Gram-negative bacteria (Defoirdt, Boon, Sorgeloos, Verstraete, & Bossier, 2007; Andrew P. Desbois & Smith, 2010; Kabara, Swieczkowski, Conley, & Truant, 1972; Shin, Bajpai, Kim, & Kang, 2007). Antibacterial action of *I. galbana* is probably attributed to the substances synthesized and secreted by *I. galbana* cells, and particularly to the short chained fatty acids compounds (Molina-Cárdenas et al., 2014). Chlorophyll a derivatives, pheophytin a and chlorophyllide, were found to be responsible for the antibacterial activity of *I. galbana* in addition to fatty acids (Bruce et al., 1967).

Dunaliella tertiolecta

The antimicrobial activity of the marine microalgae *D. tertiolecta* against *V. alginolyticus* was reported in the master thesis of *Sanja Bogdanovic* (Bogdanovic, 2018). Currently, no other published data exist on the antibacterial activity of *D. tertiolect*a against *V. alginolyticus*. Nevertheless, studies which confirmed that *D. tertiolecta* can act as a biological control agent against other Vibrio bacterial strains such as *Vibrio campbellii* and *V. proteolyticus* (González-Davis et al., 2012; Marques et al., 2006), demonstrate the potential antibacterial activity of this marine microalgae. This antibacterial activity of *D. tertiolecta* against *V. alginolyticus* is supported by the positive results obtained in the co-culturing experiment of this present study, as the number of bacteria declined in the co-culture to low-undetectable values at the end of the experimental period. Extracts from *D. tertiolecta* has shown activity against the human pathogenic bacteria *B. subtilis* (Sánchez-Saavedra et al., 2010), *P. aeruginosa* and *S. aureus* (Pane et al., 2015). *D. tertiolecta* were reported to produce polyphenols like gentisic acid, (+) catechin and (-) epicatechin, in the adaptation course to the environment (Lopez, Rico, Santana-Casiano, Gonzalez, & Gonzalez-Davila, 2015). Since it is known that polyphenols possess antimicrobial activity (Coppo & Marchese, 2014), it can be concluded that the antibacterial activity of *D. tertiolecta* are due to these compounds.

Nannochloropsis oculata

The antibacterial activity of the marine microalgae *N. oculata* was observed in a previous study, by which *Nannchloropsis* sp. limited the growth of various Vibrio species, including *V. alginolyticus* in co-cultures, with no recovery observed (Kokou et al., 2012). However, this antibacterial activity of *N. oculata* microalgae against *V. alginolyticus* was not demonstrated when cell-free supernatant and disrupted algal cells derived from *N. oculata* culture were tested. The difference in results obtained may be attributed to the disparities in the *N. oculata* strains between both studies, as biological activity can vary between strains of the same species. In addition, for an antimicrobial effect to be detected by using this method, the compounds causing the effect should likely be highly potent as the final concentration in the test tubes, or on the filter discs, is commonly low. Additional studies have demonstrated the antibacterial activity of *N. oculata* against human pathogenic bacteria like *S. aureus* and *B. subtilis* (Kellam & Walker, 1989). This antibacterial activity of *N. oculata* is probably related to short chain fatty acids that are reported to be produced by the microalgae (Roncarati et al., 2004), and it has recently been revealed that the fatty acid methyl ester of *N. oculata* possess a potential bacterial inhibition (Surendhiran et al., 2014).

Tetraselmis suecica

Several previous studies documented the antibacterial activity of *T. suecica* against *V. alginolyticus*, in addition to other strains of Vibrio species and fish pathogens (B. Austin et al., 1992; B. Austin & Day, 1990). An *in vivo* study showed a reduction of the number of Vibrio species in broodstock gut, eggs and larvae of the white prawn *Fenneropenaeus indicus* fed with *T. suecica*, thus confirming its antibacterial activity (Regunathan & Wesley, 2004). In the present study, a potential for antimicrobial activity was indicated, as the cell-free supernatant of *T. suecica* showed a slight inhibition on the diffusion agar and on the liquid culture of *V. alginolyticus*. It is worth mentioning that the bacterial inhibition seen on the diffusion agar was exerted by a paper disc that was just dipped in a solution of cell-free supernatant with a high content of water. This bacterial inhibition effect is hence expected to be more obvious if the bioactive compounds and metabolites were filtrated from the cell-free supernatants and obtained in higher concentrations. Crude extracts of *T. suecica* with different solvents were

tested against selected human pathogens, and highest inhibition effects were observed at methanol + chloroform (1:1) extract against *Proteus* sp and *S. pyogens* bacteria, and isopropanol extract against *B. megaterium* bacteria (Bai & Krishnakumar, 2013). *Krishnakumar* identified further important organic volatile compounds as fatty acids and derivatives of methyl ester (1-ethyl butyl 3-hexyl hydroperoxide and methyl heptanate) to be probably related to the antimicrobial activity of *T. suecica* (Bai & Krishnakumar, 2013), as they are known to exhibit valuable therapeutic uses.

5.3 Methodological reflections

5.3.1 Algae species and test strains

As the various studies on numerous types of microalgae revealed, the disparities in antimicrobial activity is highly dependent on the species and even strains of species involved in the experiment (Duff et al., 1966; reviewed in Falaise et al., 2016; Kellam & Walker, 1989). Further, the antimicrobial effect of compounds and metabolites produced by microalgae, may differ based on the bacterial strain involved in the study (Alonso, Grima, Pérez, Sánchez, & Camacho, 1992; Catarina Guedes, Barbosa, Amaro, Pereira, & Xavier Malcata, 2011; Mayer, Rodriguez, Taglialatela-Scafati, & Fusetani, 2013). In the present thesis, both *I. galbana* and *D. tertiolecta* inhibited the bacterial pathogen *V. alginolyticus* in the co-culture experiment, however *V. alginolyticus* was more sensitive to *D. tertiolecta* than *I. galbana*. The same difference in the susceptibility of *V. alginolyticus* to these two microalgae species was also reported by *Sanja* in her master thesis (Bogdanovic, 2018). This can be explained by the different divisions and classes the two microalgae belong to, and thus the different secondary metabolites and bioactive compounds produced by them.

5.3.2 Culturing conditions

Marine microalgae in the sea are exposed to and stimulated by various stresses such as high levels of UV light, oxygen, salinity, osmotic stress, bacterial pathogens, parasites, and predators to produce chemicals and develop defense strategies in order to combat these factors and survive (Andrew Paul Desbois, 2008; Hughes & Fenical, 2010). Several studies have demonstrated that the antibacterial activity of microalgae differ within the same species, as to originating from different environments or habitats (Lustigman, 1988; Pawlik-Skowrońska,

2003). This is due to the secondary metabolites with antibacterial activity that are not produced by microalgae for the purpose of their normal growth or reproduction, but rather as a reaction in response to the environmental conditions they are exposed to (M. A. Borowitzka, 1995; reviewed in Shannon & Abu-Ghannam, 2016). Therefore, we can say that the culturing conditions that we choose for the microalgae in our labs including light, temperature, medium composition, and nutrients, may affect both the quality and quantity of the antimicrobial compounds produced by microalgae.

In the present study, a higher antimicrobial activity of *I. galbana* and *D. tertiolecta* against the same *V. alginolyticus* bacterial strain concentration was obtained in the co-cultures incubated at a higher temperature. This can be attributed to the fact that the growth rate and cell density of microalgae cells can influence the antibacterial activity. Although a higher concentration of microalgae species was added to the cultures at the start of the experiment at temperature 20 °C, the growth rate and the cell density reached at the stationary phase of microalgae cultures at temperature 25 °C were higher. These denser microalgae cultures are likely responsible for the higher antimicrobial activity obtained as they are expected to produce high quantity of secondary metabolites that are known to accumulate at the stationary phase.

This bacterial inhibition effect was not seen when the disrupted algal cells from the stationary phase of *I. galbana* and *D. tertiolecta* cultures incubated at 16 °C were tested against *V. alginolyticus*. This was partly explained by the effectiveness of the mechanical disruption method used to investigate the potential antimicrobial activity in microalgae, as discussed in chapter 5.1. In addition, another factor that could be attributed to the negative results obtained here is the low temperature that was used for culturing the microalgae species before harvesting. Culturing *I. galbana* and *D. tertiolecta* at higher temperatures give larger algal yields and consequently higher quantities of bioactive compounds (Kaplan, Cohen, & Abeliovich, 1986; Sosik & Mitchell, 1994). Additional studies proved that the production of lipids and fatty acids by microalgae species (e.g. *I. galbana*) is likely influenced by various cultural conditions such as light intensity, nutrient concentration and source, pH, growth conditions and temperature (Lin et al., 2007; Liu & Lin, 2001; Tzovenis, De Pauw, & Sorgeloos, 1997). The content of β – carotene in *Dunaliella salina* was demonstrated to be higher particularly when cultural conditions include nutrient imitation, high salinity, high temperature and high light intensities (L. J. Borowitzka & Borowitzka, 1989). It was revealed that in some cases the production of

secondary metabolites is higher in unfavorable conditions, where it was found that the green microalga *Dunaliella sp* isolated from highly polluted water were more active against bacteria than those isolated from less polluted waters (Lustigman, 1988).

Taken together, it can be concluded that optimization of the culture conditions including medium, salinity, light intensity and temperature to produce larger yields of bioactive compounds and metabolites might be essential for a better antibacterial activity in microalgae. Nevertheless, more studies are required to establish the optimal culture conditions for the different microalgae species to attain higher concentrations of antimicrobial metabolites required for a bacterial inhibition effect.

5.3.3 Harvesting

The microalgae cultures in this study were centrifuged to separate the algal biomass from medium. The algal biomasses were disrupted mechanically and tested against *V. alginolyticus* but no activity was demonstrated to any of the four microalgae species. However, among the cell-free supernatant of the four different microalgae cultures, antibacterial activity was detected slightly only in *T. suecica* and *I. galbana* cultures. The cell-free supernatants were obtained by centrifuging the microalgae cultures at their end of exponential growth phase. The centrifugation method has been used to harvest biomass of microalgae cultures. At the stationary phase of culture growth, the bioactive compounds and secondary metabolites are reported to be abundantly found (M. A. Borowitzka, 1995; Ördög et al., 2004). In some cases, the bioactive compounds of microalgae are excreted to the medium rather than being accumulated with the biomass algal cells, where they are known as exometabolites (de Morais et al., 2015). The bioactive compounds and metabolites that are produced by microalgae at the stationary phase are often small molecules that could not sediment with the algal cells at the bottom of tube during centrifugation. In addition, it has been demonstrated by some pilot experiments that some of the bioactive compounds content can be lost in the algal biomass collected with centrifugation method, and that a number of these compounds can be released from the algal cells that are broken when microalgae biomass is recovered by centrifugation (Trung, Huyen, Minh, Trang, & Han, 2016). All these findings can lead us to a conclusion that the antibacterial activity shown in the supernatants of *T. suecica* and *I. galbana* and not in the disrupted algal cells is most likely a consequence of the accumulation of the bioactive

compounds in cell-free supernatants when the algal biomass of *T. suecica* and *I .galbana* were harvested by centrifugation method.

The antibacterial activity of *D. tertiolecta* and *N. oculata* was neither detected in cell-free supernatants nor in disrupted algal cells collected from their cultures. These results are likely attributed to either the absence or the low levels of antimicrobials compounds accumulated in their cultures. This can probably be explained by several reasons such as the cultural conditions of microalgae that may affect the production of bioactive compounds (as discussed above), and the effectiveness of the methods used to demonstrate the antimicrobial activity (chapter 5.1).

5.3.4 Impact of microalgae-bacteria interaction on the antibacterial activity of microalgae

Almost all previous studies of the potential antimicrobial activity in microalgae gave little to no regard to the effect of bacterial pathogen challenge on the production and expression of antibacterial compounds in microalgae. The production of bioactive metabolites and compounds by microalgae is one defense strategy they develop against the threat of microbial pathogens (M. A. Borowitzka, 1995; reviewed in Andrew P. Desbois et al., 2008; Engel, Jensen, & Fenical, 2002; Hughes & Fenical, 2010). This defense strategy is suggested to be either inducible by the presence of bacteria in the vicinity of microalgae or constitutive and always present in microalgae culture (reviewed in Andrew P. Desbois et al., 2008; reviewed in Falaise et al., 2016; Safonova & Reisser, 2005). Fatty acids, one of the best documented antimicrobial compounds produced by microalgae, are shown in some cases to be released in the natural environment as a result of activated defense mechanism against the hazard of grazing predators and pathogenic bacteria (de-Bashan, Bashan, Moreno, Lebsky, & Bustillos, 2002; Jüttner, 2001; Smith, Desbois, & Dyrynda, 2010). This demonstrates the beneficial effect of microalgae-bacteria interactions in enhancing the antibacterial activity of microalgae. Both the constitutive and the inducible production of antimicrobial compounds by some microalgae species have been reported by a few previous studies (Safonova & Reisser, 2005; Terekhova et al., 2009).

Although several co-culture studies have suggested an antimicrobial activity of *I. galbana* against Vibrio bacterial strains (Bogdanovic, 2018; Kokou et al., 2012; Molina-Cárdenas et al., 2014), *Srinivasakumar & Rajashekhar's* study did not notice any inhibitory activity of *I. galbana* crude extracts against Vibrio strains (Srinivasakumar & Rajashekhar, 2009). Similarly,

in the present study, signs of antimicrobial activity in *I. galbana* and *D. tertiolecta* were detected in the microalgae-bacteria co-cultures, but not in the disrupted algal cells. Hence the possibility that the antimicrobial activity of microalgae detected in the present study is induced by bacteria should be added to the reasons mentioned before in response to the difference in results obtained in the present study. However, the impact of microalgae-bacterial pathogens interactions on the production of microalgae and accumulation of bioactive compounds remains a matter subject to further investigations and researches to be fully evaluated.

In several cases, the growth of microalgae has been observed to be enhanced by the presence of bacteria (de-Bashan et al., 2002; Gonzalez & Bashan, 2000; Lebsky, Gonzalez-Bashan, & Bashan, 2001). The knowledge and control of this growth promotion effect of bacteria on microalgae could help improve the algal biomass in microalgae production processes of bioactive compounds (Fuentes et al., 2016). In the study of Ceres *et al.* it was conveyed that both the growth rate and average cell density of *I .galbana* were much higher in the cultures with Vibrio species compared to microalgae culture controls (Molina-Cárdenas et al., 2014). The experiments performed in the present study did not investigate the effect of bacteria on the growth of microalgae. However, if we compare the growth curves of *I. galbana* and *D. tertiolecta* in the co-cultures with bacteria at 25 ℃ (figure 26 chapter 4.3.1) to those of microalgae cultures without bacteria cultured at 25 ℃ (figure 27 chapter 4.3.2), it can be seen that the growth rates and cell densities of microalgae were much higher in the presence of *V. alginolyticus*. Although these variations can be explained by other factors such as the quantity of the medium and the disparities in the growth phases of original cultures, the promotion growth effects generated by bacterial pathogens on microalgae cultures should be further investigated. Nevertheless, aside from all possible factors affecting the growth of microalgae, an enhancement in antimicrobial compounds production in microalgae is usually coupled with algal growth stimulation. This possible growth enhancement effect of bacteria on microalgae warrants much further investigations.

5.3.5 Antibacterial activity assays

One of the methods used in the present study for testing the antibacterial activity in selected microalgae is disc diffusion method. This method has been widely used for screening

microalgae species for their potential antimicrobial activity (Cannell et al., 1988; Kellam & Walker, 1989; Ördög et al., 2004). The disc diffusion method is attractive due to its simplicity and low cost but not suitable for determining MIC. This method relies on the diffusion of the test substance from the filter disc into the bacterial cultures on the agar plate. Therefore, the diameter of inhibition zones formed on the agar is dependent on several factors such as diffusion behavior, solubility and molecular weight of antimicrobial compounds. This diffusion assay is not suited to the nonpolar natural antimicrobial compounds since they will diffuse scarcely and slowly into the culture medium in agar plates (Klančnik, Piskernik, Jeršek, & Možina, 2010). In various studies the antimicrobial activity of microalgae extracts was detected in organic solvents and rarely in aqueous solvents (Cannell et al., 1988; Mudimu et al., 2014; Venkatesan et al., 2007), thus concluding that most of the bioactive compounds in microalgae are hydrophobic. The absence of inhibition zones on the diffusion agar plates when testing the disrupted algal cells and the cell-free supernatants of the four microalgae species against *V. alginolyticus* in the present study does not necessarily mean the absence of bioactivity in microalgae cultures. This can be explained by the hydrophobic nature of the bioactive compounds that prevents their uniform diffusion through the agar medium. Moreover, it is worth mentioning that if antibacterial compounds had to be hydrophilic and present in the cell-free supernatants and disrupted algal cells of the microalgae species, they will may not be present in sufficient concentrations in the discs to have a detrimental effect on bacterial growth, since the supernatants only contained a low concentration of microalgae materials.

5.3.6 Microalgae growth phase

The development and growth stage of microalgae culture is significant for their antimicrobial activity. This was revealed by various studies, in which it was shown that most of the secondary metabolites with antibacterial activity are produced and released by microalgae in their late exponential and early stationary phase of growth (M. A. Borowitzka, 1995; Cooper et al., 1983; Lin et al., 2007; Molina-Cárdenas et al., 2014; Ördög et al., 2004). This is considered a general assumption for all microalgae species. It has been documented that the antimicrobial compounds in *I. galbana* are accumulated in high amounts at their stationary phase (Lin et al., 2007; Molina-Cárdenas et al., 2014; Zhu et al., 1997). However, no particular published data exist on the optimal growth phase for the production of antimicrobials compounds in each of the other selected microalgae for the present study. Therefore, it was assumed that the maximal

yield of bioactive compounds in these microalgae species is obtained by harvesting the cultures in their early stationary phase of growth.

The results of the present study showed that among the microalgae cultures that were tested after harvesting, a potential for bacterial inhibition effect was only detected in the cell-free supernatant of *I. galbana* and *T. suecica* cultures. In addition, the co-culturing experiments indicated that the amount of antibacterial activity in *I. galbana* and *D. tertiolecta* microalgae co-cultured with *V alginolyticus* changed during their cell growth (figure 25 and 26 in section 4.3.1), as the inhibitory effects seemed to initiate in the late exponential phase and stationary phase of growth. These results lead to a conclusion that the antimicrobial compounds in *I. galbana*, *D. tertiolecta* and *T. suecica* are likely to be released into the cultures in late growth phases. Nevertheless, it is uncertain if the variation in results obtained for *D. tertiolecta* in the two studies done, co-culturing and mechanical disruption algal cells, was due to the physiological state of the culture when harvested or due to other factors such as culture conditions (e.g. the different temperatures used, 16 °C before harvesting for mechanical disruption method while 20 and 25 °C for co-culturing experiments) and the different methods used in these studies. The absence of bacterial inhibition against *V. alginolyticus* in *N. oculata* culture in the present study can be explained by various factors, one of which is the absence of antimicrobial compounds that may be found at other growth stages rather than the stationary phase. The growth phases under which the antimicrobial compounds are found in high amounts for every microalgae species should be well determined and evaluated in order to obtain the maximal yield of the useful bioactive compounds. Thus, the antibacterial activity must be monitored at large number of points from the growth phases to determine the optimal stage for antimicrobial compounds production.

The growth promotion effect of microalgae on bacterial growth has been seen in several previous studies (Kogure et al., 1979; Safonova & Reisser, 2005). Increased Vibrio bacterial abundance following algal blooms in marine water during summer was reported several years ago (Hsieh et al., 2007; Worden et al., 2006). The Vibrio outbreaks in marine waters are related to the enhanced food and nutrients availability formed when many algal cells begin to die during algal blooming (as algae grow during blooming conditions, other dies and becomes a food for bacteria) (Worden et al., 2006). In the present study, the mechanical disrupted algal pellet of the four microalgae apparently enhanced the growth of *V. alginolyticus* when tested in liquid culture. Faster and greater bacterial growth was recorded from these cultures on the TCBS agar

plates, compared to those from bacterial culture control. In response to these results, one may assume that the dead algal cells and their disrupted intracellular materials can represent a source for energy and stimulating substances for bacterial growth and proliferation. An additional experiment was done in the present study to investigate further the possible growth stimulating effect of microalgae cultures at their death phase of growth on *V. alginolyticus* bacteria. The microalgae species tested in this experiment were *I. galbana* and *D. tertiolecta*. The results showed a slight stimulated growth of *V. alginolyticus* by *I. galbana* culture, as the number of bacteria in the co-cultures after 72 hours was to a certain extent higher than that in bacterial culture control. This result agrees with the result obtained from testing disrupted algal cells of *I. galbana* against *V. alginolyticus* in liquid culture, and thus reveals a possible growth stimulating effect of the dead algal cells of *I. galbana* on *V. alginolyticus*. However, a slight growth inhibition effect was detected in the co-cultures with *D. tertiolecta* from death phase after 72 hours, which contradicts the result obtained from the disrupted algal cells. This may be due to the amount of dead algal material that were lower than the bioactive compounds produced in the culture.

5.3.7 Other factors

The efficiency of microalgae in inhibiting bacterial growth can be attributed to the presence of microbial flora associated to microalgae cultures. This microbiota is demonstrated in some studies to possess antibacterial factors (Lavilla-Pitogo, Albright, & Paner, 1998; Lio-Po et al., 2005; Makridis, Costa, & Dinis, 2006). However, other studies showed that axenic microalgae were able to produce compounds with potent activity against pathogenic bacteria
(Kokou et al., 2012; Tendencia & dela Peña, 2003). The microalgae used in the present study were not axenic cultures and thus the possible antibacterial effect exerted by microbiota could affect the results obtained from the experiments done. In addition, the oxygen radicals produced by microalgae cells during photosynthetic process are suggested to have an antibacterial activity (Marshall, Ross, Pyecroft, & Hallegraeff, 2005), so that they are likely to affect the delicacy of the results of antibacterial activity investigations in microalgae also. Therefore, these antibacterial mechanisms must be taken into consideration in further studies of antimicrobial activity in microalgae.

6 Future perspectives

- Improve the disruption method for isolating bioactive compounds responsible for antimicrobial activity in microalgae. If optimized this technique would serve as a cost and time-effective method to assess the antimicrobial potential of several algae simultaneously. Factors that may be tested and optimized further are e.g. culturing conditions, the optimal growth phase for harvesting, centrifugation speed and duration, antimicrobial sensitivity assays and the mechanical disruption. It would also be interesting to test the algae material on other bacterial species.

- Further investigations of both the growth stimulating and inhibitory effects of microalgae on bacteria by co-culturing methods. E.g. include other bacterial strains such as the strains that were detected to be affected by microalgae in other studies where other methods were used, test other microalgae species, and further optimization of the culturing conditions that may affect the results obtained from co-culturing method such as temperature, light, available nutrients, and salinity.

References

Abraham, E. P., & Chain, E. (1940). An enzyme from bacteria able to destroy penicillin. *Nature, 146*(3713), 837.

Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., & Florey, H. W. (1941). Further observations on penicillin. *The lancet, 238*(6155), 177-189.

Adedeji, W. A. (2016). The Treasure called antibiotics. *Annals of Ibadan postgraduate medicine, 14*(2), 56-57.

Alanis, A. J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? *Arch Med Res, 36*(6), 697-705. doi:10.1016/j.arcmed.2005.06.009

Alonso, L., Grima, E. M., Pérez, J. S., Sánchez, J. G., & Camacho, F. G. (1992). Fatty acid variation among different isolates of a single strain of Isochrysis galbana. *Phytochemistry, 31*(11), 3901-3904.

Alvarez-Ortega, C., Wiegand, I., Olivares, J., Hancock, R. E., & Martínez, J. L. (2011). The intrinsic resistome of Pseudomonas aeruginosa to β-lactams. *Virulence, 2*(2), 144-146.

Amaro, H. M., Guedes, A. C., & Malcata, F. X. (2011). Antimicrobial activities of microalgae: an invited review. *Science against microbial pathogens: communicating current research and technological advances, 3*, 1272-1284.

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in microbiology, 1*, 134-134. doi:10.3389/fmicb.2010.00134

Aminov, R. I., & Mackie, R. I. (2007). Evolution and ecology of antibiotic resistance genes. *FEMS microbiology letters, 271*(2), 147-161.

Arun, N., Gupta, S., & Singh, D. (2012). Antimicrobial and antioxidant property of commonly found microalgae Spirulina platensis, Nostoc muscorum and Chlorella pyrenoidosa against some pathogenic bacteria and fungi. *International Journal of Pharmaceutical Sciences and Research, 3*(12), 4866.

Aslam, B., Wang, W., Arshad, M. I., Khurshid, M., Muzammil, S., Rasool, M. H., . . . Qamar, M. U. (2018). Antibiotic resistance: a rundown of a global crisis. *Infection and Drug Resistance, 11*, 1645.

Austin, B., Baudet, E., & Stobie, M. (1992). Inhibition of bacterial fish pathogens by Tetraselmis suecica. *Journal of Fish Diseases, 15*(1), 55-61. doi:10.1111/j.1365-2761.1992.tb00636.x

Austin, B., & Day, J. G. (1990). Inhibition of prawn pathogenic Vibrio spp. by a commercial spray-dried preparation of Tetraselmis suecica. *Aquaculture, 90*(3), 389-392. doi:https://doi.org/10.1016/0044-8486(90)90261-K

Austin, B., Stobie, M., Robertson, P., Glass, H., Stark, J., & Mudarris, M. (1993). Vibrio alginolyticus: the cause of gill disease leading to progressive low-level mortalities among juvenile turbot, Scophthalmus maximus L., in a Scottish aquarium. *Journal of Fish Diseases, 16*(3), 277-280.

Bai, V. D. M., & Krishnakumar, S. (2013). Evaluation of antimicrobial metabolites from marine microalgae Tetraselmis suecica using gas chromatography–mass spectrometry (GC–MS) analysis. *Int. J. Pharm. Pharm. Sci, 5*(3), 17-23.

Bajpai, V. K. (2016). Antimicrobial bioactive compounds from marine algae: A mini review.

Bakeeva, L., Drachev, A., Metlina, A., Skulachev, V., & Chumakov, K. (1987). Similarity of Vibrio alginolyticus, V. cholerae and other Vibrio species with respect to the structure of their flagellar apparatus and ribosomal 5S-RNA. *Biokhimiia (Moscow, Russia), 52*(1), 8-14.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for in vitro evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis, 6*(2), 71-79. doi:https://doi.org/10.1016/j.jpha.2015.11.005

Barofsky, A., Simonelli, P., Vidoudez, C., Troedsson, C., Nejstgaard, J. C., Jakobsen, H. H., & Pohnert, G. (2009). Growth phase of the diatom Skeletonema marinoi influences the metabolic profile of the cells and the selective feeding of the copepod Calanus spp. *Journal of Plankton Research, 32*(3), 263-272. doi:10.1093/plankt/fbp121

Barofsky, A., Vidoudez, C., & Pohnert, G. (2009). Metabolic profiling reveals growth stage variability in diatom exudates. *Limnology and Oceanography: Methods, 7*(6), 382-390.

Barra, L., Chandrasekaran, R., Corato, F., & Brunet, C. (2014). The challenge of ecophysiological biodiversity for biotechnological applications of marine microalgae. *Mar Drugs, 12*(3), 1641-1675.

Bauer, A., Kirby, W., Sherris, J. C., & Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American journal of clinical pathology, 45*(4_ts), 493-496.

Bennett, J. W. (2015). What is an Antibiotic. *Antibiotics: Current Innovations and Future Trends; Sánchez, S., Demain, AL, Eds*, 1-18.

Bhatnagar, I., & Kim, S.-K. (2010). Immense essence of excellence: marine microbial bioactive compounds. *Mar Drugs, 8*(10), 2673-2701.

Blair, J. M., Webber, M. A., Baylay, A. J., Ogbolu, D. O., & Piddock, L. J. (2015). Molecular mechanisms of antibiotic resistance. *Nature Reviews Microbiology, 13*(1), 42.

Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G., & Heublein, P. C. (1979). Disease caused by a marine vibrio: clinical characteristics and epidemiology. *New England Journal of Medicine, 300*(1), 1-5.

Blevins, S. M., & Bronze, M. S. (2010). Robert Koch and the 'golden age' of bacteriology. *International Journal of Infectious Diseases, 14*(9), e744-e751. doi:https://doi.org/10.1016/j.ijid.2009.12.003

Bogdanovic, S. (2018). *Establishment of methods for bioprospecting of marine algae for antimicrobial agents.* (Master thesis), University of Oslo,

Borowitzka, L. J., & Borowitzka, M. A. (1989). β-Carotene (Provitamin A) Production with Algae. In E. J. Vandamme (Ed.), *Biotechnology of Vitamins, Pigments and Growth Factors* (pp. 15-26).

Borowitzka, M. A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of applied phycology, 7*(1), 3-15. doi:10.1007/bf00003544

Brachman, P. S. (2003). Infectious diseases—past, present, and future. *International Journal of Epidemiology, 32*(5), 684-686. doi:10.1093/ije/dyg282

Brown, D. G., Lister, T., & May-Dracka, T. L. (2014). New natural products as new leads for antibacterial drug discovery. *Bioorganic & medicinal chemistry letters, 24*(2), 413-418.

Bruce, D. L., Duff, D. C., & Antia, N. J. (1967). The identification of two antibacterial products of the marine planktonic alga Isochrysis galbana. *J Gen Microbiol, 48*(2), 293-298. doi:10.1099/00221287-48-2-293

Burke, J., & Rodgers, L. (1981). Identification of pathogenic bacteria associated with the occurrence of 'red spot'in sea mullet, Mugil cephalus L., in south-eastern Queensland. *Journal of Fish Diseases, 4*(2), 153-159.

Butcher, R. W. (1959). An introductory account of the smaller algae of British coastal waters Part I. Introduction and Chlorophyceae. *Fish Investig, 4*, 1-74.

Butler, M. S. (2004). The role of natural product chemistry in drug discovery. *Journal of natural products, 67*(12), 2141-2153.

Butler, M. S., & Buss, A. D. (2006). Natural products—the future scaffolds for novel antibiotics? *Biochemical pharmacology, 71*(7), 919-929.
Børsheim, K., Bratbak, G., & Heldal, M. (1990). Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. *Appl. Environ. Microbiol., 56*(2), 352-356.
Cannell, R. J., Owsianka, A. M., & Walker, J. M. (1988). Results of a large-scale screening programme to detect antibacterial activity from freshwater algae. *British Phycological Journal, 23*(1), 41-44.
Catarina Guedes, A., Barbosa, C. R., Amaro, H. M., Pereira, C. I., & Xavier Malcata, F. (2011). Microalgal and cyanobacterial cell extracts for use as natural antibacterial additives against food pathogens. *International Journal of Food Science & Technology, 46*(4), 862-870. doi:10.1111/j.1365-2621.2011.02567.x
Centers for Disease Control and Prevention. (1999). *Achievements in public health, 1900-1999: Control of Infectious diseases, MMWR (Morbidity and Mortality Weekly report)*. Retrieved from https://www.cdc.gov/mmwr/PDF/wk/mm4829.pdf
Centers for Disease Control and Prevention. (2013). *Antibiotic Resistance Threats in the United States, 2013* Retrieved from https://www.cdc.gov/drugresistance/pdf/ar-threats-2013-508.pdf
Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., & Sanders, A. G. (1940). Penicillin as a chemotherapeutic agent. *The lancet, 236*(6104), 226-228.
Chang, T., Ohta, S., Ikegami, N., Miyata, H., Kashimoto, T., & Kondo, M. (1993). Antibiotic substances produced by a marine green alga, Dunaliella primolecta. *Bioresour Technol, 44*(2), 149-153. doi:https://doi.org/10.1016/0960-8524(93)90189-I
Cherazard, R., Epstein, M., Doan, T.-L., Salim, T., Bharti, S., & Smith, M. A. (2017). Antimicrobial resistant Streptococcus pneumoniae: prevalence, mechanisms, and clinical implications. *American journal of therapeutics, 24*(3), e361-e369.
Clardy, J., Fischbach, M. A., & Currie, C. R. (2009). The natural history of antibiotics. *Current biology, 19*(11), R437-R441.
Clinical and Laboratory Standards Institute. (1999). Methods for determining bactericidal activity of antimicrobial agents: approved guideline. *NCCLS document M26-A, 19*(18).
Clinical and Laboratory Standards Institute. (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard. *Vol. 32 No. 2*(CLSI document M07-A9).
Cole, J. J. (1982). Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecology and Systematics, 13*(1), 291-314.
Cooper, S., Battat, A., Marsot, P., & Sylvestre, M. (1983). Production of antibacterial activities by two Bacillariophyceae grown in dialysis culture. *Canadian Journal of Microbiology, 29*(3), 338-341.
Coppo, E., & Marchese, A. (2014). Antibacterial activity of polyphenols. *Curr Pharm Biotechnol, 15*(4), 380-390.
Czaplewski, L., Bax, R., Clokie, M., Dawson, M., Fairhead, H., Fischetti, V. A., . . . Harper, D. (2016). Alternatives to antibiotics—a pipeline portfolio review. *The Lancet Infectious Diseases, 16*(2), 239-251.
Dantas, G., & Sommer, M. O. (2014). How to fight back against antibiotic resistance. *Am Sci, 102*(1), 42-51.
Danyal, A., Mubeen, U., & Malik, K. A. (2013). Investigating two native algal species to determine antibiotic susceptibility against some pathogens. *Current Research Journal of Biological Sciences, 5*(2), 70-74.

Darwin, C. (1968). On the origin of species by means of natural selection. 1859. *Murray, London, 502*.
Das, K., Tiwari, R., & Shrivastava, D. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *Journal of medicinal plants research, 4*(2), 104-111.
de-Bashan, L. E., Bashan, Y., Moreno, M., Lebsky, V. K., & Bustillos, J. J. (2002). Increased pigment and lipid content, lipid variety, and cell and population size of the microalgae Chlorella spp. when co-immobilized in alginate beads with the microalgae-growth-promoting bacterium Azospirillum brasilense. *Canadian Journal of Microbiology, 48*(6), 514-521. Retrieved from https://doi.org/10.1139/w02-051. doi:10.1139/w02-051
de Morais, M. G., Vaz, B. d. S., de Morais, E. G., & Costa, J. A. V. (2015). Biologically active metabolites synthesized by microalgae. *BioMed research international, 2015*.
Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., & Bossier, P. (2007). Alternatives to antibiotics to control bacterial infections: luminescent vibriosis in aquaculture as an example. *Trends in Biotechnology, 25*(10), 472-479. doi:https://doi.org/10.1016/j.tibtech.2007.08.001
Desbois, A. P. (2008). *Antibacterial free fatty acids from the marine diatom, Phaeodactylum tricornutum.* (phD thesis), University of St Andrews, Retrieved from http://hdl.handle.net/10023/568
Desbois, A. P., Lebl, T., Yan, L., & Smith, V. J. (2008). Isolation and structural characterisation of two antibacterial free fatty acids from the marine diatom, Phaeodactylum tricornutum. *Applied Microbiology and Biotechnology, 81*(4), 755-764. doi:10.1007/s00253-008-1714-9
Desbois, A. P., Mearns-Spragg, A., & Smith, V. J. (2009). A Fatty Acid from the Diatom Phaeodactylum tricornutum is Antibacterial Against Diverse Bacteria Including Multi-resistant Staphylococcus aureus (MRSA). *Marine Biotechnology, 11*(1), 45-52. doi:10.1007/s10126-008-9118-5
Desbois, A. P., & Smith, V. J. (2010). Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. *Applied Microbiology and Biotechnology, 85*(6), 1629-1642. doi:10.1007/s00253-009-2355-3
Dobretsov, S., Al-Wahaibi, A. S., Lai, D., Al-Sabahi, J., Claereboudt, M., Proksch, P., & Soussi, B. (2015). Inhibition of bacterial fouling by soft coral natural products. *International Biodeterioration & Biodegradation, 98*, 53-58.
Domagk, G. (1935). Ein beitrag zur chemotherapie der bakteriellen infektionen. *DMW-Deutsche Medizinische Wochenschrift, 61*(07), 250-253.
Dougherty, T. J., & Pucci, M. J. (2011). *Antibiotic discovery and development*. doi:https://doi.org/10.1007/978-1-4614-1400-1
Duchesne, E. (1897). *Contribution à l'étude de la concurrence vitale chez les microorganismes: antagonisme entre les moisissures et les microbes*: Alexandre Rey, imprimeur de la faculté de médecine.
Duff, D. a., Bruce, D., & Antia, N. (1966). The antibacterial activity of marine planktonic algae. *Canadian Journal of Microbiology, 12*(5), 877-884.
Egidius, E. (1987). Vibriosis: pathogenicity and pathology. A review. *Aquaculture, 67*(1-2), 15-28.
Ehrlich, P., & Halta, S. (1910). *Die experimentelle chemotherapie der Spirillosen* (Vol. 8): Springer.
El Amraoui, B., El Amraoui, M., Cohen, N., & Fassouane, A. (2014). Antifungal and antibacterial activity of marine microorganisms. *72*(2), 107-111. doi:10.1016/j.pharma.2013.12.001

Ely, R., Supriya, T., & Naik, C. (2004). Antimicrobial activity of marine organisms collected off the coast of South East India. *Journal of experimental marine biology and ecology, 309*(1), 121-127.

Encarnação, T., Pais, A. A. C. C., Campos, M. G., & Burrows, H. D. (2015). Cyanobacteria and microalgae: a renewable source of bioactive compounds and other chemicals. *Science Progress, 98*(2), 145-168. doi:10.3184/003685015X14298590596266

Engel, S., Jensen, P. R., & Fenical, W. (2002). Chemical ecology of marine microbial defense. *J Chem Ecol, 28*(10), 1971-1985.

Exner, M., Bhattacharya, S., Christiansen, B., Gebel, J., Goroncy-Bermes, P., Hartemann, P., . . . Trautmann, M. (2017). Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria? *GMS Hyg Infect Control, 12*, Doc05. doi:10.3205/dgkh000290

Falaise, C., François, C., Travers, M.-A., Morga, B., Haure, J., Tremblay, R., . . . Mouget, J.-L. (2016). Antimicrobial Compounds from Eukaryotic Microalgae against Human Pathogens and Diseases in Aquaculture. *Mar Drugs, 14*(9), 159.

Faulkner, D. (1986). Marine natural products. *Natural Product Reports, 3*, 1-33.

Fguira, L. F.-B., Fotso, S., Ameur-Mehdi, R. B., Mellouli, L., & Laatsch, H. (2005). Purification and structure elucidation of antifungal and antibacterial activities of newly isolated Streptomyces sp. strain US80. *Research in Microbiology, 156*(3), 341-347.

Fleming, A. (1929). On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. *British journal of experimental pathology, 10*(3), 226.

Fleming, A. (1945). Penicillin. Nobel Lecture, December 11, 1945. *Nobel e-museum*.

Floss, H. G., & Yu, T.-W. (2005). Rifamycin Mode of Action, Resistance, and Biosynthesis. *Chem Rev, 105*(2), 621-632. doi:10.1021/cr030112j

Fuentes, J. L., Garbayo, I., Cuaresma, M., Montero, Z., González-Del-Valle, M., & Vílchez, C. (2016). Impact of Microalgae-Bacteria Interactions on the Production of Algal Biomass and Associated Compounds. *Mar Drugs, 14*(5), 100. doi:10.3390/md14050100

Gechev, T. S., Hille, J., Woerdenbag, H. J., Benina, M., Mehterov, N., Toneva, V., . . . Mueller-Roeber, B. (2014). Natural products from resurrection plants: Potential for medical applications. *Biotechnology Advances, 32*(6), 1091-1101.

Geciova, J., Bury, D., & Jelen, P. (2002). Methods for disruption of microbial cells for potential use in the dairy industry—a review. *International Dairy Journal, 12*(6), 541-553. doi:https://doi.org/10.1016/S0958-6946(02)00038-9

Ghasemi, Y., Moradian, A., Mohagheghzadeh, A., Shokravi, S., & Morowvat, M. H. (2007). Antifungal and antibacterial activity of the microalgae collected from paddy fields of Iran: characterization of antimicrobial activity of Chroococcus dispersus. *J. Biol. Sci, 7*, 904-910.

Gjerde, J., & Boe, B. (1981). Isolation and characterization of Vibrio alginolyticus and Vibrio parahaemolyticus from the Norwegian coastal environment. *Acta veterinaria scandinavica, 22*, 331-343.

Godet, S., Loiseau, C., Pencreac'h, G., Ergan, F., & Hérault, J. (2010). Isolation and sequence analysis of a cdna encoding a novel putative esterase from the marine microalga isochrysis galbana (prymnesiophyceae, haptophyta) 1. *Journal of phycology, 46*(4), 679-684.

González-Davis, O., Ponce-Rivas, E., Sánchez-Saavedra, M. D. P., Muñoz-Márquez, M. E., & Gerwick, W. H. (2012). Bioprospection of microalgae and cyanobacteria as biocontrol agents against Vibrio campbellii and their use in white shrimp Litopenaeus

vannamei culture. *Journal of the World Aquaculture Society, 43*(3), 387-399. doi:10.1111/j.1749-7345.2012.00567.x
Gonzalez, L. E., & Bashan, Y. (2000). Increased growth of the microalga chlorella vulgariswhen coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium Azospirillum brasilense. *Appl. Environ. Microbiol., 66*(4), 1527-1531.
Gould, K. (2016). Antibiotics: from prehistory to the present day. *Journal of Antimicrobial Chemotherapy, 71*(3), 572-575. doi:10.1093/jac/dkv484
Graham, L. E., & Wilcox, L. W. (2000). *Algae.* Upper Saddle River, N.J: Prentice Hall.
Gualerzi, C. O., Brandi, L., Fabbretti, A., & Pon, C. L. (2013). *Antibiotics: targets, mechanisms and resistance*. Weinheim: Wiley-VCH.
Guiry, W. (2019). AlgaeBase. World-wide electronic publication, National University of Ireland, Galway. *http://www.algaebase.org*.
Gyawali, R., & Ibrahim, S. A. (2014). Natural products as antimicrobial agents. *Food control, 46*, 412-429.
Günerken, E., D'Hondt, E., Eppink, M. H. M., Garcia-Gonzalez, L., Elst, K., & Wijffels, R. H. (2015). Cell disruption for microalgae biorefineries. *Biotechnology Advances, 33*(2), 243-260. doi:https://doi.org/10.1016/j.biotechadv.2015.01.008
Halim, R., Harun, R., Danquah, M. K., & Webley, P. A. (2012). Microalgal cell disruption for biofuel development. *Applied Energy, 91*(1), 116-121.
Hamburger, M. O., & Cordell, G. A. (1987). A direct bioautographic TLC assay for compounds possessing antibacterial activity. *Journal of natural products, 50*(1), 19-22.
Harber, M. J., & Asscher, A. W. (1977). A new method for antibiotic assay based on measurement of bacterial adenosine triphosphate using the firefly bioluminescence system. *Journal of Antimicrobial Chemotherapy, 3*(1), 35-41. doi:10.1093/jac/3.1.35
Hartmann, G., Behr, W., Beissner, K. A., Honikel, K., & Sippel, A. (1968). Antibiotics as inhibitors of nucleic acid and protein synthesis. *Angewandte Chemie International Edition in English, 7*(9), 693-701.
Heatley, N. G. (1944). A method for the assay of penicillin. *The Biochemical journal, 38*(1), 61-65.
Herrero, M., Ibáñez, E., Cifuentes, A., Reglero, G., & Santoyo, S. (2006). Dunaliella salina microalga pressurized liquid extracts as potential antimicrobials. *J Food Prot, 69*(10), 2471-2477.
Herrero, M., Mendiola, J. A., Plaza, M., & Ibañez, E. (2013). Screening for Bioactive Compounds from Algae. In J. W. Lee (Ed.), *Advanced Biofuels and Bioproducts* (pp. 833-872). New York, NY: Springer New York.
Hibberd, D. (1981). Notes on the taxonomy and nomenclature of the algal classes Eustigmatophyceae and Tribophyceae (synonym Xanthophyceae). *Botanical journal of the linnean society, 82*(2), 93-119.
Hitchings, G. H. (1973). Mechanism of Action of Trimethoprim-Sulfamethoxazole—I. *The Journal of Infectious Diseases, 128*, S433-S436. doi:10.1093/infdis/128.Supplement_3.S433
Hornstrup, M. K., & Gahrn-Hansen, B. (1993). Extraintestinal Infections Caused by Vibrio parahæmolyticus and Vibrio alginolyticus in a Danish County, 1987–1992. *Scandinavian Journal of Infectious Diseases, 25*(6), 735-740. doi:10.3109/00365549309008571
Hsieh, J. L., Fries, J. S., & Noble, R. T. (2007). Vibrio and phytoplankton dynamics during the summer of 2004 in a eutrophying estuary. *Ecological Applications, 17*(sp5), S102-S109.

Hudzicki, J. (2009). Kirby-Bauer disk diffusion susceptibility test protocol.
Hughes, C. C., & Fenical, W. (2010). Antibacterials from the sea. *Chemistry, 16*(42), 12512-12525. doi:10.1002/chem.201001279
Hugo, W. B., Russell, A. D., & Denyer, S. P. (2011). *Hugo and Russell's pharmaceutical microbiology* (8th ed. ed.). Chichester: Wiley-Blackwell.
Hörmansdorfer, S., Wentges, H., Neugebaur-Büchler, K., & Bauer, J. (2000). Isolation of Vibrio alginolyticus from seawater aquaria. *International journal of hygiene and environmental health, 203*(2), 169-175.
Janda, J., Powers, C., Bryant, R., & Abbott, S. (1988). Current perspectives on the epidemiology and pathogenesis of clinically significant Vibrio spp. *Clinical Microbiology Reviews, 1*(3), 245-267.
Jayachandran, S. J. J. o. I. A. o. O. M., & Radiology. (2018). Pre-antibiotics era to post-antibiotic era. *30*(2), 100.
Jevons, M. P. (1961). "Celbenin"-resistant staphylococci. *British medical journal, 1*(5219), 124.
Joe, A., Douglas, F., National Research, C., Division on, E., Life, S., Board on Chemical, S., . . . Chemical Sciences, R. (2014). *Challenges In Overcoming Antibiotic Resistance*: National Academies Press.
John Faulkner, D. (2000). Marine natural products. *Natural Product Reports, 17*(1), 7-55. doi:10.1039/A809395D
Jüttner, F. (2001). Liberation of 5, 8, 11, 14, 17-eicosapentaenoic acid and other polyunsaturated fatty acids from lipids as a grazer defense reaction in epilithic diatom biofilms. *Journal of phycology, 37*(5), 744-755.
Jørgensen, E. G. (1962). Antibiotic substances from cells and culture solutions of unicellular algae with special reference to some chlorophyll derivatives. *Physiologia Plantarum, 15*(3), 530-545.
Kabara, J. J., Swieczkowski, D. M., Conley, A. J., & Truant, J. P. (1972). Fatty acids and derivatives as antimicrobial agents. *Antimicrob Agents Chemother, 2*(1), 23-28.
Kang, H., Seo, C., & Park, Y. (2015). Marine peptides and their anti-infective activities. *Mar Drugs, 13*(1), 618-654.
Kaplan, D., Cohen, Z., & Abeliovich, A. (1986). Optimal growth conditions for Isochrysis galbana. *Biomass, 9*(1), 37-48.
Kausalya, M., & Rao, G. N. (2015). Antimicrobial activity of marine algae. *J. Algal Biomass Utln, 6*(1), 78-87.
Kehrenberg, C., Schwarz, S., Cloeckaert, A., & Doublet, B. (2004). Molecular basis of bacterial resistance to chloramphenicol and florfenicol. *FEMS microbiology reviews, 28*(5), 519-542. doi:10.1016/j.femsre.2004.04.001
Kellam, S. J., & Walker, J. M. (1989). Antibacterial activity from marine microalgae in laboratory culture. *British Phycological Journal, 24*(2), 191-194. Retrieved from https://doi.org/10.1080/00071618900650181. doi:10.1080/00071618900650181
Kiuru, P., Valeria D'Auria, M., Muller, C. D., Tammela, P., Vuorela, H., & Yli-Kauhaluoma, J. (2014). Exploring marine resources for bioactive compounds. *Planta Medica, 80*(14), 1234-1246. doi:10.1055/s-0034-1383001
Klančnik, A., Piskernik, S., Jeršek, B., & Možina, S. S. (2010). Evaluation of diffusion and dilution methods to determine the antibacterial activity of plant extracts. *Journal of microbiological methods, 81*(2), 121-126.
Kobayashi, T., Enomoto, S., & Sakazaki, Y. K. (1963). S. A new selective medium for pathogenic Vibrios: TCBS-Agar. Jap. *Journ. Bact, 18*, 387-391.
Koch, R. (1884). Die Aetiologie der Tuberkulose, Mittheilungen aus dem Laiserlichen Gesundheitsampte. 2: 1–88. *Milestones in Microbiology, 1556*.

Koehn, F. E., & Carter, G. T. (2005). The evolving role of natural products in drug discovery. *Nature reviews Drug discovery, 4*(3), 206.
Kogure, K., Simidu, U., & Taga, N. (1979). Effect of Skeletonema costatum (Grev.) Cleve on the growth of marine bacteria. *Journal of experimental marine biology and ecology, 36*(3), 201-215.
Kokou, F., Makridis, P., Kentouri, M., & Divanach, P. (2012). Antibacterial activity in microalgae cultures. *Aquaculture Research, 43*(10), 1520-1527. doi:10.1111/j.1365-2109.2011.02955.x
Konaté, K., Mavoungou, J. F., Lepengué, A. N., Aworet-Samseny, R. R., Hilou, A., Souza, A., . . . M'Batchi, B. (2012). Antibacterial activity against β-lactamase producing Methicillin and Ampicillin-resistants Staphylococcus aureus: fractional Inhibitory Concentration Index (FICI) determination. *Annals of clinical microbiology and antimicrobials, 11*(1), 18.
Kono, M., O'Hara, K., & Ebisu, T. (1992). Purification and characterization of macrolide 2′-phosphotransferase type II from a strain of Escherichia coli highly resistant to macrolide antibiotics. *FEMS microbiology letters, 97*(1-2), 89-94.
Kresge, N., Simoni, R. D., & Hill, R. L. (2004). Selman Waksman: the father of antibiotics. *Journal of Biological Chemistry, 279*(48), e7-e7.
Krishnika, A., Bhanupriya, P., & Nair, B. B. (2011). Antibacterial activity of eight marine microalgae against a few gram negative bacterial pathogens.
Larsen, J. L., Farid, A. F., & Dalsgaard, I. (1981). A comprehensive study of environmental and human pathogenic Vibrio alginolyticus strains. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene. 1. Abt. Originale. A, Medizinische Mikrobiologie, Infektionskrankheiten und Parasitologie, 251*(2), 213-222.
Larson, E. (2007). Community factors in the development of antibiotic resistance. *Annu. Rev. Public Health, 28*, 435-447.
Lavilla-Pitogo, C., Albright, L., & Paner, M. (1998). *Will microbial manipulation sustain the ecological balance in shrimp (Penaeus monodon) hatcheries?* Paper presented at the Advances in Shrimp Biotechnology: Proceedings to the special session on shrimp biotechnology, 5th Asian Fisheries Forum, 11-14 November 1998, Chiengmai, Thailand.
Lebsky, V. K., Gonzalez-Bashan, L. E., & Bashan, Y. (2001). Ultrastructure of interaction in alginate beads between the microalga Chlorella vulgaris with its natural associative bacterium Phyllobacterium myrsinacearum and with the plant growth-promoting bacterium Azospirillum brasilense. *Canadian Journal of Microbiology, 47*(1), 1-8.
Lee, A. K., Lewis, D. M., & Ashman, P. J. (2012). Disruption of microalgal cells for the extraction of lipids for biofuels: processes and specific energy requirements. *Biomass and bioenergy, 46*, 89-101.
Lee, K. K., Chen, F. R., Yu, S. R., Yang, T. I., & Liu, P. C. (1997). Effects of extracellular products of Vibrio alginolyticus on penaeid prawn plasma components. *Letters in applied microbiology, 25*(2), 98-100.
Lee, S. J., Yoon, B.-D., & Oh, H.-M. (1998). Rapid method for the determination of lipid from the green alga Botryococcus braunii. *Biotechnology Techniques, 12*(7), 553-556. doi:10.1023/a:1008811716448
Li, B., & Webster, T. J. (2018). Bacteria antibiotic resistance: New challenges and opportunities for implant-associated orthopedic infections. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society, 36*(1), 22-32. doi:10.1002/jor.23656
Li, X.-Z., & Nikaido, H. (2009). Efflux-Mediated Drug Resistance in Bacteria. *Drugs, 69*(12), 1555-1623. doi:10.2165/11317030-000000000-00000

Li, Y., Hill, A., Beitelshees, M., Shao, S., Lovell, J. F., Davidson, B. A., . . . Jones, C. H. (2016). Directed vaccination against pneumococcal disease. *Proceedings of the National Academy of Sciences, 113*(25), 6898-6903.

Lin, Y.-H., Chang, F.-L., Tsao, C.-Y., & Leu, J.-Y. (2007). Influence of growth phase and nutrient source on fatty acid composition of Isochrysis galbana CCMP 1324 in a batch photoreactor. *Biochemical Engineering Journal, 37*(2), 166-176.

Lincoln, R. A., Strupinski, K., & Walker, J. M. (1991). Bioactive compounds from algae. *Life Chem Rep, 8*(97), 183.

Lio-Po, G. D., Leaño, E. M., Peñaranda, M. M. D., Villa-Franco, A. U., Sombito, C. D., & Guanzon Jr, N. G. (2005). Anti-luminous Vibrio factors associated with the 'green water'grow-out culture of the tiger shrimp Penaeus monodon. *Aquaculture, 250*(1-2), 1-7.

Liu, C.-P., & Lin, L.-P. (2001). Ultrastructural study and lipid formation of Isochrysis sp. CCMP1324. *Botanical Bulletin of Academia Sinica, 42*.

Loeffler, F. (1884). *Untersuchungen über die Bedeutung der Mikroorganismen für die Entstehung der Diphtherie beim Menschen, bei der Taube und beim Kalbe*.

Lopez, A., Rico, M., Santana-Casiano, J. M., Gonzalez, A. G., & Gonzalez-Davila, M. (2015). Phenolic profile of Dunaliella tertiolecta growing under high levels of copper and iron. *Environ Sci Pollut Res Int, 22*(19), 14820-14828. doi:10.1007/s11356-015-4717-y

Lotz, M. J., Tamplin, M. L., & Rodrick, G. (1983). Thiosulfate-citrate-bile salts-sucrose agar and its selectivity for clinical and marine vibrio organisms. *Annals of Clinical & Laboratory Science, 13*(1), 45-48.

Lustigman, B. (1988). Comparison of antibiotic production from four ecotypes of the marine alga,Dunaliella. *Bulletin of Environmental Contamination and Toxicology, 40*(1), 18-22. Retrieved from https://doi.org/10.1007/BF01689380. doi:10.1007/bf01689380

Madigan, M. T., & Martinko, J. M. (2006). Microorganisms and microbiology. *Brock biology of microorganisms. 11th ed. Upper Saddle River, New Jersey (NJ): Pearson Prentice Hall*, 1-20.

Mahoney, J. F., Arnold, R. C., Harris, A. J. A. J. o. P. H., & Health, t. N. (1943). Penicillin treatment of early syphilis—a preliminary report. *33*(12), 1387-1391.

Majno, G., & Joris, I. (1979). Billroth and Penicillium. *Reviews of infectious diseases*, 880-884.

Makridis, P., Costa, R. A., & Dinis, M. T. (2006). Microbial conditions and antimicrobial activity in cultures of two microalgae species, Tetraselmis chuii and Chlorella minutissima, and effect on bacterial load of enriched Artemia metanauplii. *Aquaculture, 255*(1), 76-81. doi:https://doi.org/10.1016/j.aquaculture.2005.12.010

Marques, A., Thanh, T. H., Sorgeloos, P., & Bossier, P. (2006). Use of microalgae and bacteria to enhance protection of gnotobiotic Artemia against different pathogens. *Aquaculture, 258*(1), 116-126. doi:https://doi.org/10.1016/j.aquaculture.2006.04.021

Marshall, J.-A., Ross, T., Pyecroft, S., & Hallegraeff, G. (2005). Superoxide production by marine microalgae. *Marine Biology, 147*(2), 541-549. doi:10.1007/s00227-005-1597-6

Marston, A. (2011). Thin-layer chromatography with biological detection in phytochemistry. *Journal of Chromatography A, 1218*(19), 2676-2683. doi:https://doi.org/10.1016/j.chroma.2010.12.068

Mata, T. M., Martins, A. A., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews, 14*(1), 217-232. doi:https://doi.org/10.1016/j.rser.2009.07.020

Matsunaga, T., Takeyama, H., Miyashita, H., & Yokouchi, H. (2005). Marine microalgae. *Adv. Biochem. Eng. Biotechnol., 96*, 165-188. doi:10.1007/b135784

Matthews, P., & Tomasz, A. (1990). Insertional inactivation of the mec gene in a transposon mutant of a methicillin-resistant clinical isolate of Staphylococcus aureus. *Antimicrobial agents and Chemotherapy, 34*(9), 1777-1779. doi:10.1128/aac.34.9.1777

Maxwell, K., & Johnson, G. N. (2000). Chlorophyll fluorescence—a practical guide. *Journal of experimental botany, 51*(345), 659-668.

Mayer, A. M., Rodriguez, A. D., Taglialatela-Scafati, O., & Fusetani, N. (2013). Marine pharmacology in 2009-2011: marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action. *Mar Drugs, 11*(7), 2510-2573. doi:10.3390/md11072510

McEwen, S. A., & Fedorka-Cray, P. J. (2002). Antimicrobial use and resistance in animals. *Clinical infectious diseases, 34*, S93-S106.

Mendiola, J. A., Santoyo, S., Cifuentes, A., Reglero, G., Ibanez, E., & Senorans, F. J. (2008). Antimicrobial activity of sub- and supercritical CO2 extracts of the green alga Dunaliella salina. *J Food Prot, 71*(10), 2138-2143.

Mendiola, J. A., Torres, C. F., Toré, A., Martín-Álvarez, P. J., Santoyo, S., Arredondo, B. O., . . . Ibáñez, E. (2007). Use of supercritical CO 2 to obtain extracts with antimicrobial activity from Chaetoceros muelleri microalga. A correlation with their lipidic content. *European Food Research and Technology, 224*(4), 505-510.

Meyers, E., & Smith, D. A. (1964). Bioautography of antibiotic spread-layer chromatograms. *Journal of Chromatography A, 14*, 129-132. doi:https://doi.org/10.1016/S0021-9673(00)86603-0

Mohr, K. I. (2016). History of antibiotics research. *Curr Top Microbiol Immunol, 398*, 237-272. doi:10.1007/82_2016_499

Molina-Cárdenas, C. A., Sánchez-Saavedra, M. d. P., & Lizárraga-Partida, M. L. (2014). Inhibition of pathogenic Vibrio by the microalgae Isochrysis galbana. *Journal of applied phycology, 26*(6), 2347-2355. doi:10.1007/s10811-014-0270-1

Moloney, M. G. (2016). Natural products as a source for novel antibiotics. *Trends in pharmacological sciences, 37*(8), 689-701.

Monciardini, P., Iorio, M., Maffioli, S., Sosio, M., & Donadio, S. (2014). Discovering new bioactive molecules from microbial sources. *Microbial biotechnology, 7*(3), 209-220.

Mudimu, O., Rybalka, N., Bauersachs, T., Born, J., Friedl, T., & Schulz, R. (2014). Biotechnological screening of microalgal and cyanobacterial strains for biogas production and antibacterial and antifungal effects. *Metabolites, 4*(2), 373-393. doi:10.3390/metabo4020373

Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiology spectrum, 4*(2).

Nachnani, S., Scuteri, A., Newman, M. G., Avanessian, A. B., & Lomeli, S. L. (1992). E-test: a new technique for antimicrobial susceptibility testing for periodontal microorganisms. *J Periodontol, 63*(7), 576-583. doi:10.1902/jop.1992.63.7.576

Najdenski, H. M., Gigova, L. G., Iliev, I. I., Pilarski, P. S., Lukavský, J., Tsvetkova, I. V., . . . Kussovski, V. K. (2013). Antibacterial and antifungal activities of selected microalgae and cyanobacteria. *International Journal of Food Science & Technology, 48*(7), 1533-1540. doi:10.1111/ijfs.12122

Nathan, P., Law, E. J., Murphy, D. F., & MacMillan, B. G. (1978). A laboratory method for selection of topical antimicrobial agents to treat infected burn wounds. *Burns, 4*(3), 177-187.

Naviner, M., Bergé, J.-P., Durand, P., & Le Bris, H. (1999). *Antibacterial activity of the marine diatom Skeletonema costatum against aquacultural pathogens* (Vol. 174).

Newman, D. J., & Cragg, G. M. (2016). Natural products as sources of new drugs from 1981 to 2014. *Journal of natural products, 79*(3), 629-661.
Norton, T. A., Melkonian, M., & Andersen, R. A. (1996). Algal biodiversity. *Phycologia, 35*(4), 308-326.
Nostro, A., Trombetta, D., Bisignano, G., Laganà, M. G., Arena, S., Saija, A., . . . Mazzanti, G. (2001). In vitro antibacterial activity of some aliphatic aldehydes from Olea europaea L. *FEMS microbiology letters, 198*(1), 9-13. doi:10.1111/j.1574-6968.2001.tb10611.x
O'Brien, K. L., Wolfson, L. J., Watt, J. P., Henkle, E., Deloria-Knoll, M., McCall, N., . . . Cherian, T. (2009). Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *The lancet, 374*(9693), 893-902.
O'brien, S. (2015). Meeting the societal need for new antibiotics: The challenges for the pharmaceutical industry. *British journal of clinical pharmacology, 79*(2), 168-172.
O'Neil, J. (2016). *Tackling drug-resistant infections globally. (WHO)*. Retrieved from
Ohta, S., Shiomi, Y., Kawashima, A., Aozasa, O., Nakao, T., Nagate, T., . . . Miyata, H. (1995). Antibiotic effect of linolenic acid fromChlorococcum strain HS-101 andDunaliella primolecta on methicillin-resistantStaphylococcus aureus. *Journal of applied phycology, 7*(2), 121-127. Retrieved from https://doi.org/10.1007/BF00693057. doi:10.1007/bf00693057
Pane, G., Cacciola, G., Giacco, E., Mariottini, G. L., & Coppo, E. (2015). Assessment of the Antimicrobial Activity of Algae Extracts on Bacteria Responsible of External Otitis. *Mar Drugs, 13*(10), 6440-6452. doi:10.3390/md13106440
Parke, M. (1949). Studies on marine flagellates. *Journal of the Marine Biological Association of the United Kingdom, 28*(1), 255-286.
Parkinson, J. (1640). *Theatrum botanicum : the theater of plantes. Or, an universall and compleate herball*. London: Printed by Tho. Cotes.
Patridge, E., Gareiss, P., Kinch, M. S., & Hoyer, D. (2016). An analysis of FDA-approved drugs: natural products and their derivatives. *Drug discovery today, 21*(2), 204-207.
Pawlik-Skowrońska, B. (2003). Resistance, accumulation and allocation of zinc in two ecotypes of the green alga Stigeoclonium tenue Kütz. coming from habitats of different heavy metal concentrations. *Aquatic Botany, 75*(3), 189-198. doi:https://doi.org/10.1016/S0304-3770(02)00175-4
Phan, G., Picard, M., & Broutin, I. (2015). Focus on the Outer Membrane Factor OprM, the Forgotten Player from Efflux Pumps Assemblies. *Antibiotics (Basel, Switzerland), 4*(4), 544-566. doi:10.3390/antibiotics4040544
Piddock, L. J. V. (2012). The crisis of no new antibiotics—what is the way forward? *The Lancet Infectious Diseases, 12*(3), 249-253. doi:https://doi.org/10.1016/S1473-3099(11)70316-4
Pore, R. S. (1994). Antibiotic susceptibility testing by flow cytometry. *Journal of Antimicrobial Chemotherapy, 34*(5), 613-627. doi:10.1093/jac/34.5.613
Pradhan, J., Das, S., & Das, B. K. (2014). Antibacterial activity of freshwater microalgae: A review. *African Journal of Pharmacy and Pharmacology, 8*(32), 809-818.
Prakash, S., & Bhimba, B. V. (2005). Pharmaceutical development of novel microalgal compounds for Mdr Mycobacterium tuberculosis.
Pratt, R., Daniels, T., Eiler, J. J., Gunnison, J., Kumler, W., Oneto, J. F., . . . Milner, H. (1944). Chlorellin, an antibacterial substance from Chlorella. *Science (Washington)*, 351-352.
Prestinaci, F., Pezzotti, P., & Pantosti, A. (2015). Antimicrobial resistance: a global multifaceted phenomenon. *Pathogens and Global Health, 109*(7), 309-318. doi:10.1179/2047773215Y.0000000030

Radmer, R. J. (1996). Algal diversity and commercial algal products. *Bioscience, 46*(4), 263-270.
Rajeev, A., Prakash, S., & Bhimba, B. (2006). Antibacterial activity of marine microalgae against multidrug resistant human pathogens. *Journal of Aquaculture in the Tropics*.
Raposo, M. F. d. J., De Morais, R. M. S. C., & Bernardo de Morais, A. M. M. (2013). Bioactivity and Applications of Sulphated Polysaccharides from Marine Microalgae. *Mar Drugs, 11*(1), 233-252.
Regunathan, C., & Wesley, S. (2004). Control of Vibrio spp. in shrimp hatcheries using the green algae Tetraselmis suecica. *Asian Fisheries Science, 17*(1/2), 147-158.
Reilly, G., Reilly, C., Smith, E., & Baker-Austin, C. J. E. (2011). Vibrio alginolyticus-associated wound infection acquired in British waters, Guernsey, July 2011. *16*(42), 19994.
Renwick, M., & Mossialos, E. (2018). What are the economic barriers of antibiotic R&D and how can we overcome them? In: Taylor & Francis.
Reynolds, P. E. (1989). Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *European Journal of Clinical Microbiology and Infectious Diseases, 8*(11), 943-950. doi:10.1007/bf01967563
Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., . . . Vila, J. (2015). The global threat of antimicrobial resistance: science for intervention. *New Microbes New Infect, 6*, 22-29. doi:10.1016/j.nmni.2015.02.007
Roncarati, A., Meluzzi, A., Acciarri, S., Tallarico, N., & Meloti, P. (2004). Fatty Acid Composition of Different Microalgae Strains (Nannochloropsis sp., Nannochloropsis oculata (Droop) Hibberd, Nannochloris atomus Butcher and Isochrysis sp.) According to the Culture Phase and the Carbon Dioxide Concentration. *Journal of the World Aquaculture Society, 35*(3), 401-411. doi:10.1111/j.1749-7345.2004.tb00104.x
Rubin, R. P. (2007). A brief history of great discoveries in pharmacology: in celebration of the centennial anniversary of the founding of the American Society of Pharmacology and Experimental Therapeutics. *Pharmacological reviews, 59*(4), 289-359.
Rubin, S. J., & Tilton, R. C. (1975). Isolation of Vibrio alginolyticus from wound infections. *Journal of clinical microbiology, 2*(6), 556.
Safonova, E., & Reisser, W. (2005). Growth promoting and inhibiting effects of extracellular substances of soil microalgae and cyanobacteria on Escherichia coll and Micrococcus luteus. *Phycological Research, 53*(3), 189-193. doi:10.1111/j.1440-183.2005.00384.x
Saga, T., & Yamaguchi, K. (2009). History of antimicrobial agents and resistant bacteria. In: JMAJ.
Sageman, A. (2015). Antibiotic Resistance Mechanisms, Problems, and Solutions.
Sánchez-Saavedra, M., Licea-Navarro, A., & Bernáldez-Sarabia, J. (2010). Evaluation of the antibacterial activity of different species of phytoplankton. *Revista de Biología Marina y Oceanografía, 45*(3).
Santoyo, S., Rodríguez-Meizoso, I., Cifuentes, A., Jaime, L., Reina, G. G.-B., Señorans, F. J., & Ibáñez, E. (2009). Green processes based on the extraction with pressurized fluids to obtain potent antimicrobials from Haematococcus pluvialis microalgae. *LWT-Food Science and Technology, 42*(7), 1213-1218.
Schmidt, U., Chmel, H., & Cobbs, C. (1979). Vibrio alginolyticus infections in humans. *Journal of clinical microbiology, 10*(5), 666-668.
Schnappinger, D., & Hillen, W. (1996). Tetracyclines: antibiotic action, uptake, and resistance mechanisms. *Archives of Microbiology, 165*(6), 359-369. doi:10.1007/s002030050339

Shannon, E., & Abu-Ghannam, N. (2016). Antibacterial Derivatives of Marine Algae: An Overview of Pharmacological Mechanisms and Applications. *Mar Drugs, 14*(4), 81. doi:10.3390/md14040081

Shimizu, Y. (1996). Microalgal metabolites: a new perspective. *Annual Reviews in Microbiology, 50*(1), 431-465.

Shin, S. Y., Bajpai, V. K., Kim, H. R., & Kang, S. C. (2007). Antibacterial activity of bioconverted eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) against foodborne pathogenic bacteria. *International journal of food microbiology, 113*(2), 233-236.

Silber, J., Kramer, A., Labes, A., & Tasdemir, D. (2016). From Discovery to Production: Biotechnology of Marine Fungi for the Production of New Antibiotics. *Mar Drugs, 14*(7), 137. doi:10.3390/md14070137

Silver, L. L. (2015). Natural products as a source of drug leads to overcome drug resistance. *Future microbiology, 10*(11), 1711-1718.

Simpkin, V. L., Renwick, M. J., Kelly, R., & Mossialos, E. (2017). Incentivising innovation in antibiotic drug discovery and development: Progress, challenges and next steps. *The Journal of antibiotics, 70*(12), 1087.

Singh, R. P., Kumari, P., & Reddy, C. R. K. (2015). Antimicrobial compounds from seaweeds-associated bacteria and fungi. *Applied Microbiology and Biotechnology, 99*(4), 1571-1586. doi:10.1007/s00253-014-6334-y

Smit, P., & Heniger, J. (1975). Antoni van Leeuwenhoek (1632–1723) and the discovery of bacteria. *Antonie van Leeuwenhoek, 41*(1), 217-228. doi:10.1007/bf02565057

Smith, V. J., Desbois, A. P., & Dyrynda, E. A. (2010). Conventional and unconventional antimicrobials from fish, marine invertebrates and micro-algae. *Mar Drugs, 8*(4), 1213-1262.

Sneader, W. (2005). *Drug discovery: a history*: John Wiley & Sons.

Soltani, N., Khavari-Nejad, R., Tabatabaei Yazdi, M., Shokravi, S., & Fernandez-Valiente, E. (2005). Screening of soil cyanobacteria for antifungal and antibacterial activity. *Pharmaceutical biology, 43*(5), 455-459.

Song, J. S., Jeon, J. H., Lee, J. H., Jeong, S. H., Jeong, B. C., Kim, S.-J., . . . Lee, S. H. (2005). Molecular characterization of TEM-type β-lactamases identified in cold-seep sediments of Edison Seamount (south of Lihir Island, Papua New Guinea). *The Journal of Microbiology, 43*(2), 172-178.

Sosik, H. M., & Mitchell, B. G. (1994). Effects of temperature on growth, light absorption, and quantum yield in Dunaliella tertiolecta (Chlorophyceae)1. *Journal of phycology, 30*(5), 833-840. doi:10.1111/j.0022-3646.1994.00833.x

Spark, R. P., Fried, M. L., Perry, C., & Watkins, C. (1979). Vibrio alginolyticus wound infection: case report and review. *Annals of Clinical & Laboratory Science, 9*(2), 133-138.

Speer, B. S., Bedzyk, L., & Salyers, A. A. (1991). Evidence that a novel tetracycline resistance gene found on two Bacteroides transposons encodes an NADP-requiring oxidoreductase. *Journal of bacteriology, 173*(1), 176-183.

Srinivasakumar, K., & Rajashekhar, M. (2009). In vitro studies on bactericidal activity and sensitivity pattern of isolated marine microalgae against selective human bacterial pathogens. *Indian J. Sci. Technol, 2*, 16-23.

Surendhiran, D., Vijay, M., Razack, A., Subramaniyan, T., Shellomith, A. S., & Tamilselvam, K. (2014). A green synthesis of antimicrobial compounds from marine microalgae Nannochloropsis oculata. *Journal of Coastal Life Medicine, 2*(11), 862-859.

Tacconelli, E., Carrara, E., Savoldi, A., Harbarth, S., Mendelson, M., L Monnet, D., . . . Zorzet, A. (2017). *Discovery, research, and development of new antibiotics: The WHO priority list of antibiotic-resistant bacteria and tuberculosis* (Vol. 18).

Tagliabue, A., & Rappuoli, R. (2018). Changing priorities in Vaccinology: antibiotic resistance moving to the top. *Frontiers in immunology, 9*.

Tanaka, T., Muto, M., Liang, Y., Yoshino, T., & Matsunaga, T. (2015). *Marine microalgae*.

Tandeau de Marsac, N., & Houmard, J. (1993). Adaptation of cyanobacteria to environmental stimuli: new steps towards molecular mechanisms. *FEMS microbiology reviews, 10*(1-2), 119-189.

Tendencia, E. A., & dela Peña, M. (2003). Investigation of some components of the greenwater system which makes it effective in the initial control of luminous bacteria. *Aquaculture, 218*(1-4), 115-119.

Terekhova, V. E., Aizdaicher, N. A., Buzoleva, L. S., & Somov, G. P. (2009). Influence of extrametabolites of marine microalgae on the reproduction of the bacterium Listeria monocytogenes. *Russian journal of marine biology, 35*(4), 355-358. doi:10.1134/s1063074009040129

Thanner, S., Drissner, D., & Walsh, F. (2016). Antimicrobial resistance in agriculture. *MBio, 7*(2), e02227-02215.

The Pew Charitable Trust. (2016). *A scientific roadmap for antibiotic discovery*. Retrieved from https://www.pewtrusts.org/~/media/assets/2016/05/ascientificroadmapforantibioticdiscovery.pdf

The Pew Charitable Trusts. (2019). *The Critical Need for New Antibiotics*. Retrieved from https://www.pewtrusts.org/-/media/assets/2019/02/critical-need-for-new-antibiotics-infographic.pdf

. Thiosulphate citrate Bile-salt sucrose (TCBS) agar. (1995). In J. E. L. Corry, G. D. W. Curtis, & R. M. Baird (Eds.), *Progress in Industrial Microbiology* (Vol. 34, pp. 450-452): Elsevier.

Tiberio, V. (1895). Sugli estratti di alcune muffe. *Ann. Ig. Sper, 1*, 91-103.

Tipper, D. J. (1985). Mode of action of β-lactam antibiotics. *Pharmacology & Therapeutics, 27*(1), 1-35. doi:https://doi.org/10.1016/0163-7258(85)90062-2

Torzillo, G., Accolla, P., Pinzani, E., & Masojidek, J. (1996). In situ monitoring of chlorophyll fluorescence to assess the synergistic effect of low temperature and high irradiance stresses inSpirulina cultures grown outdoors in photobioreactors. *Journal of applied phycology, 8*(4-5), 283-291.

Trung, T. S., Huyen, N., Minh, N. C., Trang, T., & Han, N. (2016). Optimization of harvesting of microalgal Thalassiosira pseudonana biomass using chitosan prepared from shrimp shell waste. *Asian J Agri Res, 10*(5), 162-174.

Tsutsumi, S., Yokomizo, M., Saito, Y., Matsushita, Y., & Aoki, H. (2017). Mechanical cell disruption of microalgae for investigating the effects of degree of disruption on hydrocarbon extraction. *Asia-Pacific Journal of Chemical Engineering, 12*(3), 454-467. doi:10.1002/apj.2088

Tyndall, J. (1876). Observations on the Optical Deportment of the Atmosphere in Reference to the Phenomena of Putrefaction and Infection. *British medical journal, 1*(787), 121-124. doi:10.1136/bmj.1.787.121

Tzovenis, I., De Pauw, N., & Sorgeloos, P. (1997). Effect of different light regimes on the docosahexaenoic acid (DHA) content of Isochrysis aff. galbana (clone T-ISO). *Aquaculture International, 5*(6), 489-507. doi:10.1023/a:1018349131522

Van Duin, D., & Paterson, D. L. (2016). Multidrug-Resistant Bacteria in the Community: Trends and Lessons Learned. *Infectious disease clinics of North America, 30*(2), 377-390. doi:10.1016/j.idc.2016.02.004

Vandal, J., Abou-Zaid, M. M., Ferroni, G., & Leduc, L. G. (2015). Antimicrobial activity of natural products from the flora of Northern Ontario, Canada. *Pharmaceutical biology, 53*(6), 800-806.

Venkatesan, R., Karthikayen, R., Periyanayagi, R., Sasikala, V., & Balasubramanian, T. (2007). Antibacterial activity of the marine diatom, Rhizosolenia alata (Brightwell, 1858) against human pathogens. *Research Journal of Microbiology, 2*(1), 98-100.

Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and therapeutics, 40*(4), 277.

Vidoudez, C., & Pohnert, G. (2012). Comparative metabolomics of the diatom Skeletonema marinoi in different growth phases. *Metabolomics, 8*(4), 654-669. doi:10.1007/s11306-011-0356-6

Vonshak, A., Torzillo, G., & Tomaseli, L. (1994). Use of chlorophyll fluorescence to estimate the effect of photoinhibition in outdoor cultures ofSpirulina platensis. *Journal of applied phycology, 6*(1), 31-34.

Waksman, S. A. (1947). What is an antibiotic or an antibiotic substance? *Mycologia, 39*(5), 565-569.

Walter, C., & Mahesh, R. (2000). Antibacterial and antifungal activities of some marine diatoms in culture.

Wichard, T., Poulet, S. A., Halsband-Lenk, C., Albaina, A., Harris, R., Liu, D., & Pohnert, G. (2005). Survey of the Chemical Defence Potential of Diatoms: Screening of Fifty Species for α,β,γ,δ-unsaturated aldehydes. *Journal of Chemical Ecology, 31*(4), 949-958. doi:10.1007/s10886-005-3615-z

Witkop, B. (1999). Paul Ehrlich and His Magic Bullets, Revisited. *Proceedings of the American Philosophical Society, 143*(4), 540-557.

Witte, W. (1998). Medical consequences of antibiotic use in agriculture. In: American Association for the Advancement of Science.

Wolfson, J. S., & Hooper, D. C. (1985). The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity in vitro. *Antimicrobial agents and Chemotherapy, 28*(4), 581.

Worden, A. Z., Seidel, M., Smriga, S., Wick, A., Malfatti, F., Bartlett, D., & Azam, F. (2006). Trophic regulation of Vibrio cholerae in coastal marine waters. *Environmental Microbiology, 8*(1), 21-29.

World Health Organization. (27 February 2017). *Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics*. Retrieved from http://apps.who.int/medicinedocs/documents/s23171en/s23171en.pdf

World Health Organization. (2014). *Antimicrobial resistance: global report on surveillance 2014.* Retrieved from http://www.who.int/iris/handle/10665/112642

World Health Organization. (2017). *Antibacterial agents in clinical development – an analysis of the antibacterial clinical development pipeline, including tuberculosis* Retrieved from http://www.who.int/iris/handle/10665/258965.

World Health Organization. (2019). Ten threats to global health in 2019. Retrieved from https://www.who.int/emergencies/ten-threats-to-global-health-in-2019

Wright, G., & Berghuis, A. (1998). Aminoglycoside antibiotics—structures, functions and resistance, p 27–69. InRosen BP, Mobashery S (ed), Resolving the antibiotic paradox. In: Kluwer Academic Publishers, New York, NY.

Wright, G. D. (2005). Bacterial resistance to antibiotics: enzymatic degradation and modification. *Advanced drug delivery reviews, 57*(10), 1451-1470.

Wright, G. D. (2007). The antibiotic resistome: the nexus of chemical and genetic diversity. *Nature Reviews Microbiology, 5*, 175. doi:10.1038/nrmicro1614

Wright, G. D. (2017). Opportunities for natural products in 21 st century antibiotic discovery. *Natural Product Reports, 34*(7), 694-701.

Yaakob, Z., Ali, E., Zainal, A., Mohamad, M., & Takriff, M. S. (2014). An overview: biomolecules from microalgae for animal feed and aquaculture. *Journal of Biological Research-Thessaloniki, 21*(1), 6.

Yahav, D., Farbman, L., Leibovici, L., & Paul, M. (2012). Colistin: new lessons on an old antibiotic. *Clinical microbiology and infection, 18*(1), 18-29. doi:https://doi.org/10.1111/j.1469-0691.2011.03734.x

Yoshikawa, T. T. (2002). Antimicrobial Resistance and Aging: Beginning of the End of the Antibiotic Era? *Journal of the American Geriatrics Society, 50*(s7), 226-229. doi:10.1046/j.1532-5415.50.7s.2.x

Yousuf, S., & Choudhary, M. I. (2014). Lichens: chemistry and biological activities. In *Studies in natural products chemistry* (Vol. 43, pp. 223-259): Elsevier.

Zaidan, M., Noor Rain, A., Badrul, A., Adlin, A., Norazah, A., & Zakiah, I. (2005). In vitro screening of five local medicinal plants for antibacterial activity using disc diffusion method. *Trop biomed, 22*(2), 165-170.

Zaman, S. B., Hussain, M. A., Nye, R., Mehta, V., Mamun, K. T., & Hossain, N. (2017). A review on antibiotic resistance: alarm bells are ringing. *Cureus, 9*(6).

Zhang, Q.-W., Lin, L.-G., & Ye, W.-C. (2018). Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese medicine, 13*, 20-20. doi:10.1186/s13020-018-0177-x

Zhu, C. J., Lee, Y. K., & Chao, T. M. (1997). Effects of temperature and growth phase on lipid and biochemical composition of Isochrysis galbana TK1. *Journal of applied phycology, 9*(5), 451-457. doi:10.1023/a:1007973319348

Zucca, M., & Savoia, D. (2010). The post-antibiotic era: promising developments in the therapy of infectious diseases. *International journal of biomedical science : IJBS, 6*(2), 77-86.

Ördög, V., Stirk, W. A., Lenobel, R., Bancířová, M., Strnad, M., van Staden, J., . . . Németh, L. (2004). Screening microalgae for some potentially useful agricultural and pharmaceutical secondary metabolites. *Journal of applied phycology, 16*(4), 309-314. doi:10.1023/B:JAPH.0000047789.34883.aa

Appendix A

IMR ½-algal medium

(Epply et al.1967, modified by E. Paasche, University of Oslo). Without silicate and with selenite

Stock solutions (made with distilled water or MiliQ-water):

Nitrate: 5 g KNO_3 to 100 mL

Phosphate: 0,68 g KH_2PO_4 to 100 mL

Trace metal solutions: 6 g Na_2EDTA to 1 litre,

1 g $FeCl_3 \times 6H_2O$

620 mg $MnSO_4 \times H_2O$

250 mg $ZnSO_4 \times 7H_2O$

130 mg $Na_2MoO_4 \times 2H_2O$

4 mg $CoCl_2 \times 6H_2O$

4 mg $Cu_2SO_4 \times 5H_2O$

To avoid precipitation (due to H_4EDTA) adjust this solution to pH 8 with concentrated NaOH.

Vitamin solutions: 10 mg thiamine (B1)

0,1 mg cyanocobolamine (B12)

0,1 mg biotine

To 100 mL MiliQ water

The two latter is added from a more concentrated stock solution due to the small amounts.

This solution should be kept in plastic bottle, preferably at –20 °C

Selenite solution: 2,63 mg Na2 SeO3 x 5H2O

Content in 1L medium of 24salinity (up to 90% seawater is fine):

Seawater (34 PSU)	700 mL
Distilled water	300 mL
Nitrate stock solution	0,5 mL (final concentration 250 µM)
Phosphate stock solution	0,5 mL (final concentration 25 µM)
Trace metal-EDTA stock solution	0,5 mL
Vitamin stock solution	0,5 mL
Selenite stock solution	1 mL (final concentration 10 nM)

All stock solutions are as described by Eppley et al. 1967, J.Exp. Mar. Biol. Ecol.1:

191-208. The stock solutions are prepared with Milli-Q water, they are not autoclaved, and kept in the fridge. The vitamin solution can be prepared from stronger stocks that are kept in the freezer on plastic flasks. We use half of the amounts of stock solutions compared to Eppley et al. (0,5 mL per liter instead of 1mL per liter medium) and call the medium IMR1/2.

For flagellates we drop Si (and HCl) and add Se. The seawater is filtrated (Whatman GF/C) and the medium is autoclaved for 15 min at 120 °C (110 °C is probably sufficient and can be used if you get precipitation at 120 °C).

If distilled water is exchanged with Milli-Q-water make sure that the filter is old and rinsed from the formalin in the new filter.

Appendix B

Preparation of LB (Luria-Bertani) liquid medium for bacterial cell culture:

Component	For 1 L
Bactro-tryptone	10 g
Yeast extract	5 g
Sodium Chloride	5 g

Mix the powders and add distilled water to 960 mL in a sterilized glass

Adjust the PH to 7,5 and fill destilled water up to 1 L

Autoclave to sterilize

Store the liquid culture at romtemperature.

Preparation of LB agar plates for bacterial culture:

Prepared as above but adding 1.5 g per liter of Bactro agar before autoclaving.

Once autoclaved the LB agar and cooled down to 55, pour out the media into petri dishes. The agar will harden soon that it is cool enough.

The plates should be stored upside down in the cold room

Appendix C

TCBS agar (Himedia M189-500G)

Thiosulfate-citrate-bile salts-sucrose (TCBS) agar was originally developed for isolation of *Vibrio spp*. pathogenic for humans. The medium is recommended by the WHO for diagnosis of cholera when isolation of *V. cholera* from fecal materials is the clinical procedure employed. The original formula developed by Nakanishi was subsequently modified by Kobayashy et al. for the selective isolation of pathogenic Vibrio species.

Composition of TCBS Agar
Ingredients per liter of deionized water:*
Sucrose 20.0 g
Dipeptone 10.0 g
Sodium Citrate 10.0 g
Sodium Thiosulfate 10.0 g
Sodium Chloride 10.0 g
Yeast Extract 5.0 g
Oxbile (Oxgall) 5.0 g
Sodium Cholate 3.0 g
Ferric Citrate 1.0 g
Bromothymol Blue 0.04 g
Thymol Blue 0.04 g
Agar 15.0 g

Preparation of TCBS Agar
1. Suspend 88.1 g of dehydrated medium in 1 liter of distilled or deionized water.
2. Slowly bring to boiling, stirring with constant agitation until complete dissolution.
3. **Do not autoclave.**
4. Cool to 50 °C and pour into sterile petri dishes

Appendix D

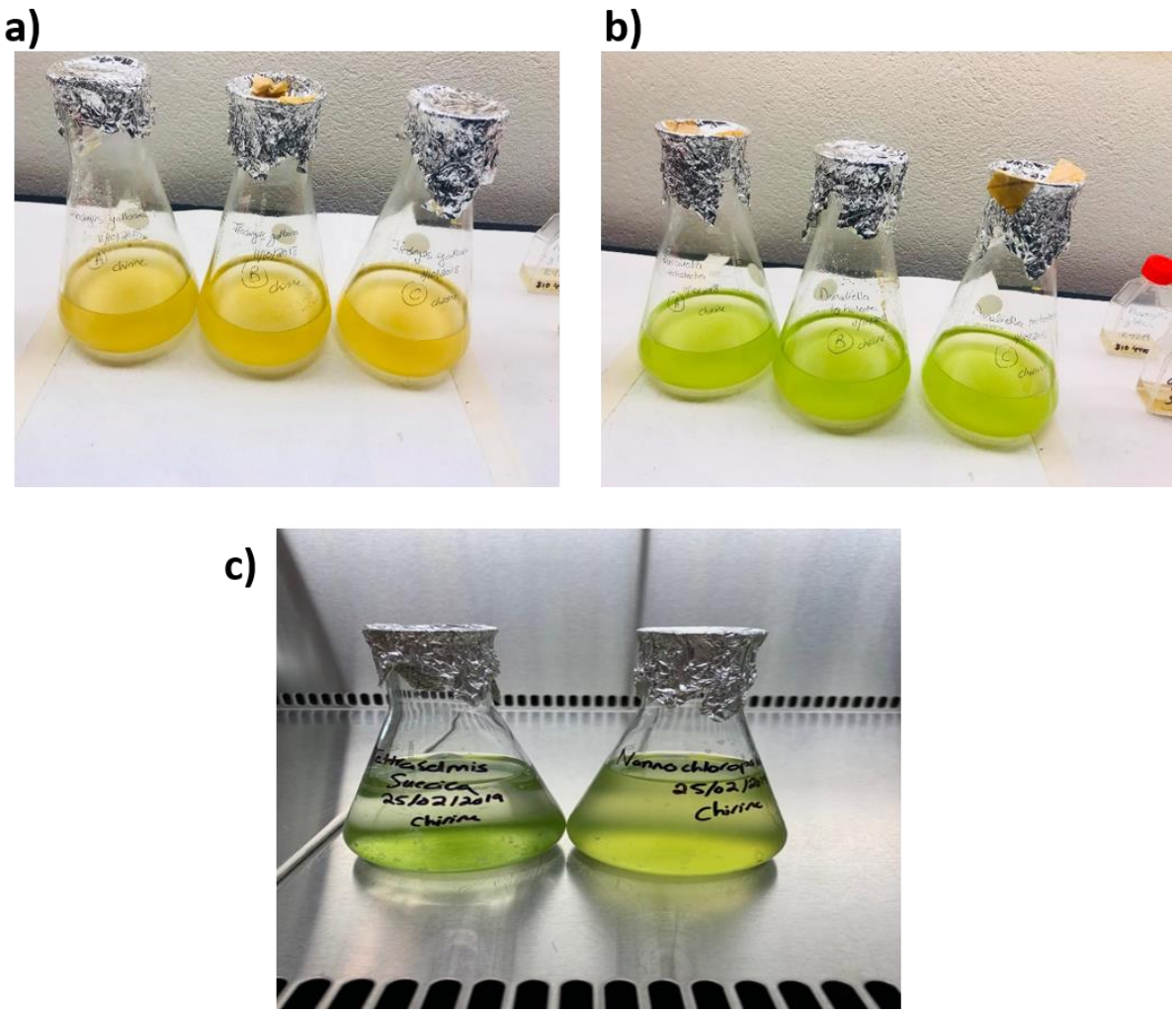


Figure 1a-c) *– culturing of microalgae. a) Transformation of I. galbana triplicate cultures to yellow color at day 13 of culturing. b) Transformation of D. tertiolecta triplicate cultures to green color at day 13 of culturing. c) Transformation of the T. suecica and N. oculata cultures into green color at day 15 of culturing. Pictures taken by Chirine Kanaan.*

Appendix E

Table 1 *- Fluorescence values (relative units) of I. galbana and D. tertiolecta triplicate cultures (a, b, c) read during culturing time from 1 to 20 days.*

Fluorescence (relative units)	Blank liquid (IMR1/2 medium)	*D. tertiolecta* replicate (a)	*D. tertiolecta* replicate (b)	*D. tertiolecta* replicate (c)	*I. galbana* replicate (a)	*I. galbana* replicate (b)	*I. galbana* replicate (c)
Day 1	7376,68	13717	11466,63	10766,16	1682,26	18098,25	18217,66
Day 4	7510,71	34691,30	35200,86	38341,41	31888,01	34003,75	33045,99
Day 5	7497,54	44370,63	44065,62	50076,52	60128,70	66031,63	62331,79
Day 6	7564,59	65898,43	55628,46	58372,09	69467,90	70177,09	78382,21
Day 8	7492,89	106369,21	165704,78	129791,16	73385,3	77217,06	87958,20
Day 13	7838,71	257926,59	254000,29	300374,12	172418,23	146485,82	152214,31
Day 14	7833,20	294104,96	305710,28	316257,68	168229	182877	158283
Day 15	7806,48	376679,56	333339,37	346988,09	178440,95	203338,98	186555,93
Day 18	7856,32	469220,37	476058,78	406952,18	213895,03	222227,23	215296,25
Day 19	7782,43	478626,31	483165,56	461279	295803,96	232356,48	243970,8
Day 20	7653,37	383590,18	459666,18	470069,18	294058,59	241312,71	259689,25

Table 2 - *Fluorescence values (relative units) of T. suecica and N. oculata cultures read during culturing time from 1 to 19 days.*

Fluorescence (relative units)	Blank liquid (IMR1/2 medium)	*T. suecica*	*N. oculata*
Day 1	7356.56	8805.77	9312.92
Day 2	7318.70	7809.00	13453.76
Day 3	7319.71	7534.24	10659.74
Day 5	7254.53	9490.68	17159.48
Day 8	7302.98	15712.71	62326.85
Day 10	7075.46	19711.07	143414.78
Day 12	7145.32	11307.51	180987.60
Day 15	7674.92	127340.9	250064
Day 17	7241.51	163351.17	269574.90
Day 18	7197.17	176592.54	281377.90
Day 19	7272.21	181234.3	281732.5

Appendix F:

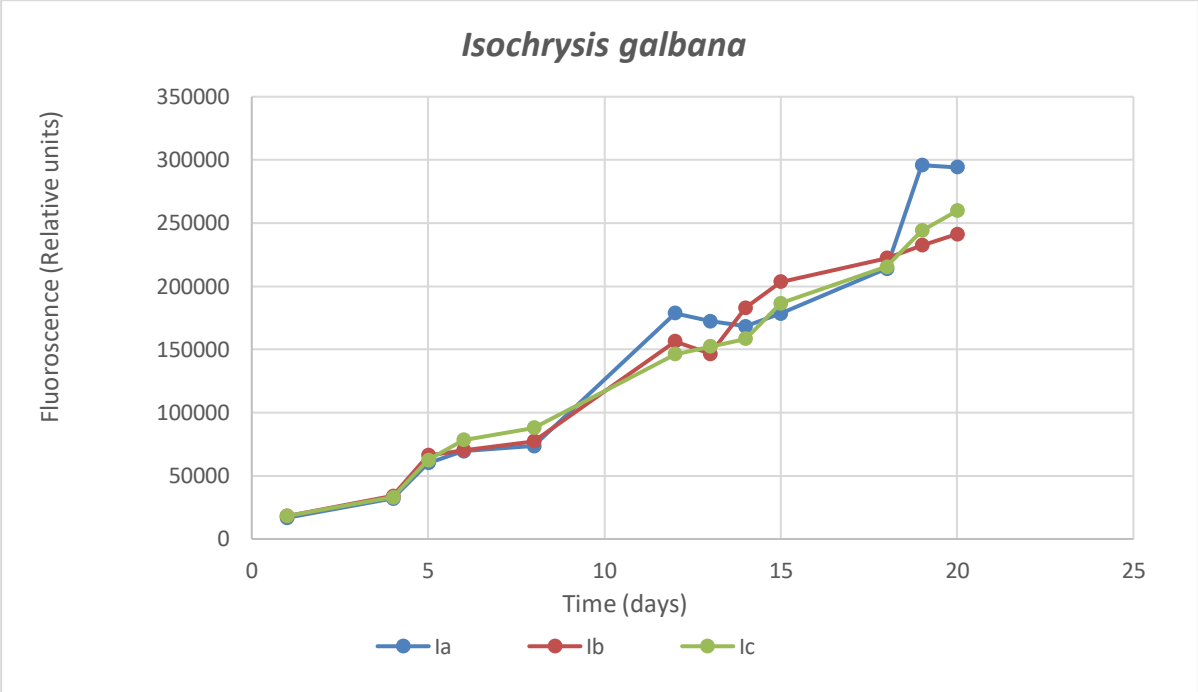


Figure 2a - *Growth curves of 3 replicates of I. galbana (Ia, Ib, Ic) cultivated in IMR1/2 medium. Growth curves were made by plotting fluorescence measurements (relative units) against time (in days).*

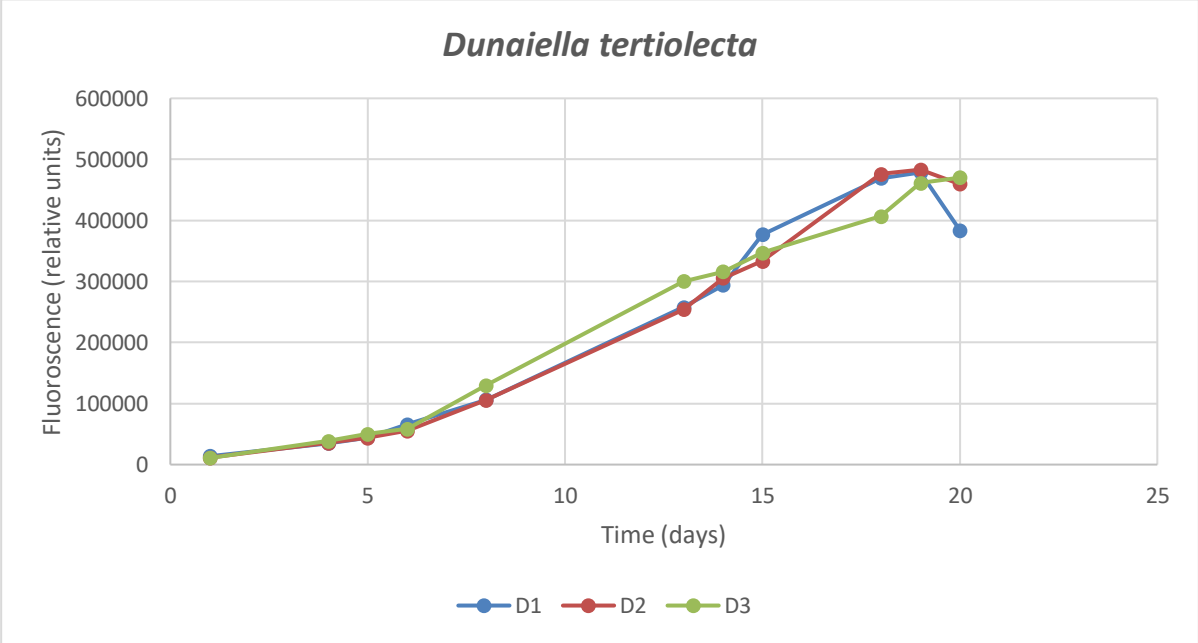


Figure 2b - *Growth curves of 3 replicates of D. tertiolecta (Da, Db, Dc) cultivated in IMR1/2 medium. Growth curves were made by plotting fluorescence measurements (relative units) against time (in days).*

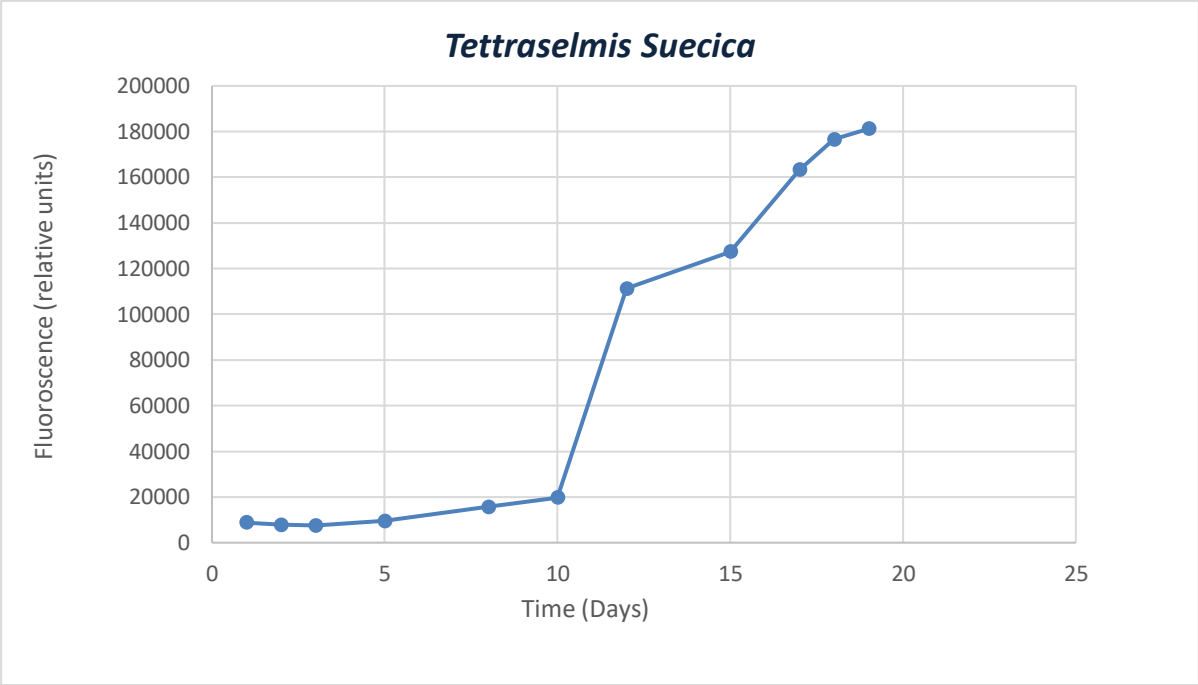


Figure 2c *- Growth curve of T. suecica cultivated in IMR1/2 medium. Growth curve was made by plotting fluorescence measurements (relative units) against time (days).*

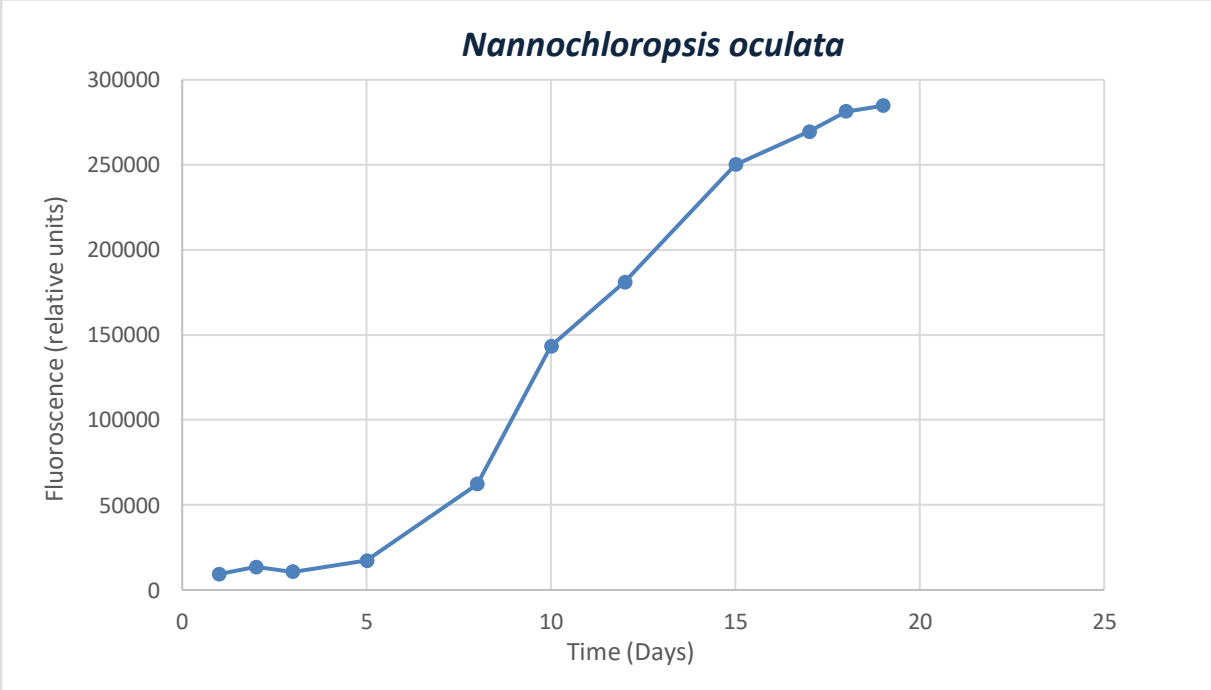


Figure 2d *- Growth curve of N. oculata cultivated in IMR1/2 medium. Growth curve was made by plotting fluorescence measurements (relative units) against time (days).*

Appendix G

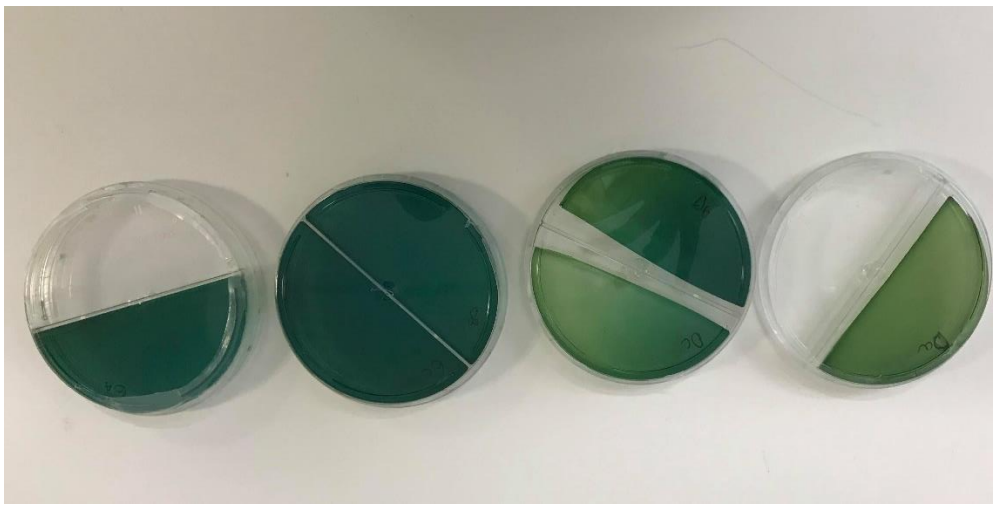


Figure 3 - *Testing the presence of Vibrio bacteria in D. tertiolecta and I. galbana replicates (a,b,c) from experiment 3.3.3 (Testing the effects of algal material on V. alginolyticus growth). No yellow colonies were shown on the TCBS petri-dishes after incubation, indicating the free of Vibrio bacteria in all microalgae cultures.*

Testing each of cell-free supernatant and disrupted algal pellet derived from *I. galbana* and *D. tertiolecta* with *V. alginolyticus* in liquid medium

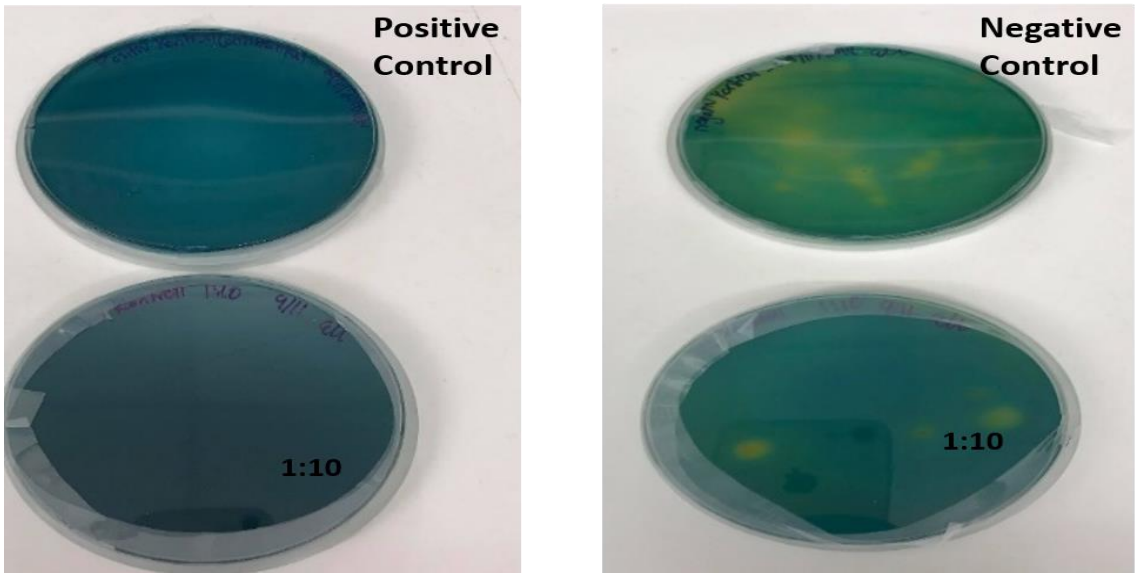


Figure 4a – *Liquid test of positive control (antibiotic with V. alginolyticus) and negative control (untreated V. alginolyticus culture) cultures and their corresponding 1:10 diluted solutions plated on TCBS plates.*

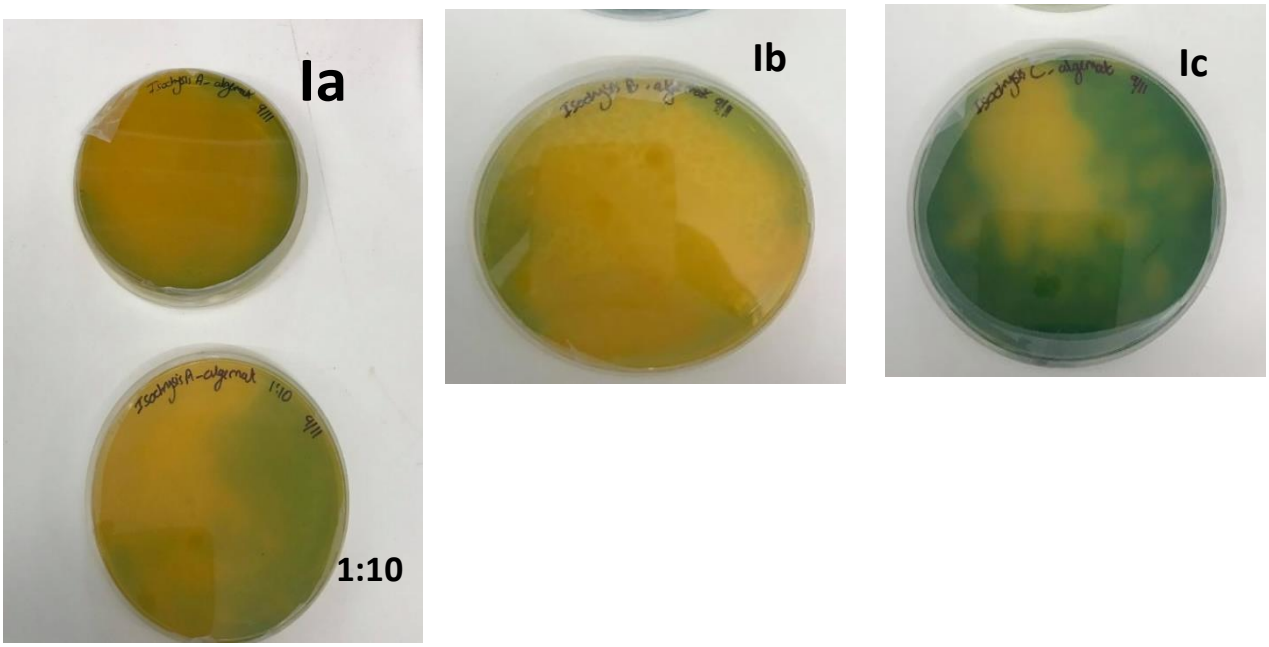


Figure 4b *- Liquid test of solution containing V. alginolyticus with disrupted algal pellet derived from I. galbana replicates (a, b, c) and from the 1:10 diluted solution of replicate a, plated on TCBS petri-dishes.*

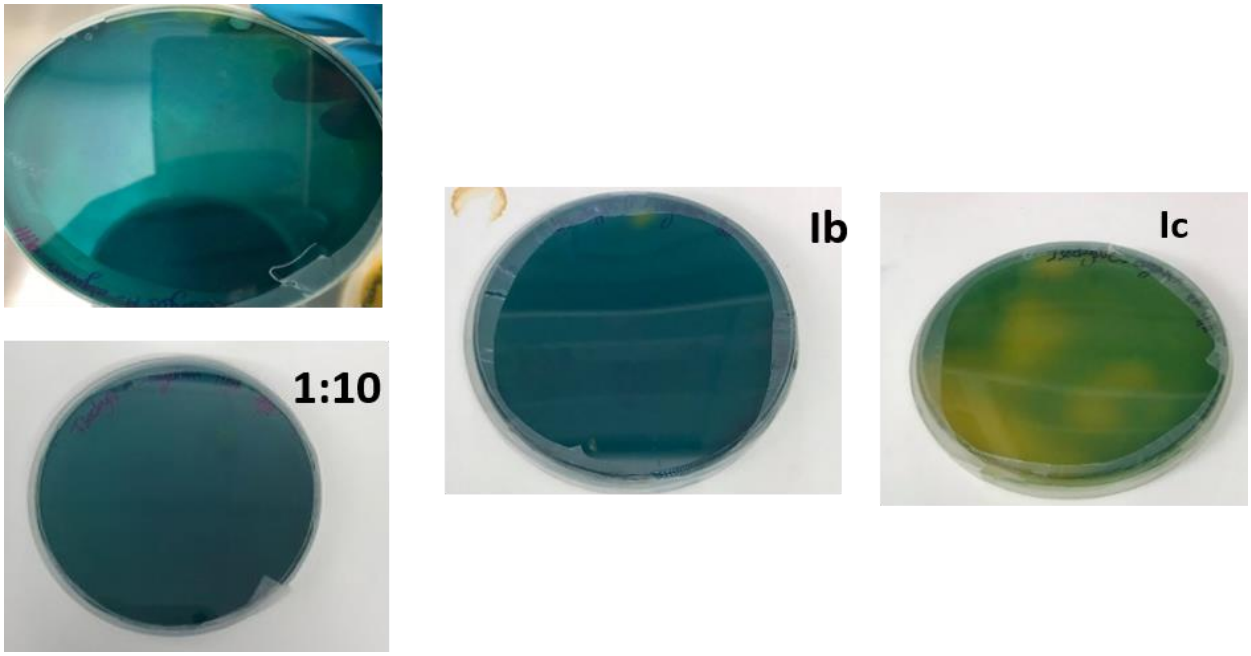


Figure 4c *- Liquid test of solution containing V. alginolyticus with cell-free supernatant derived from I. galbana replicates (a, b, c) and from the 1:10 diluted solution of replicate a, plated on TCBS petri dishes.*

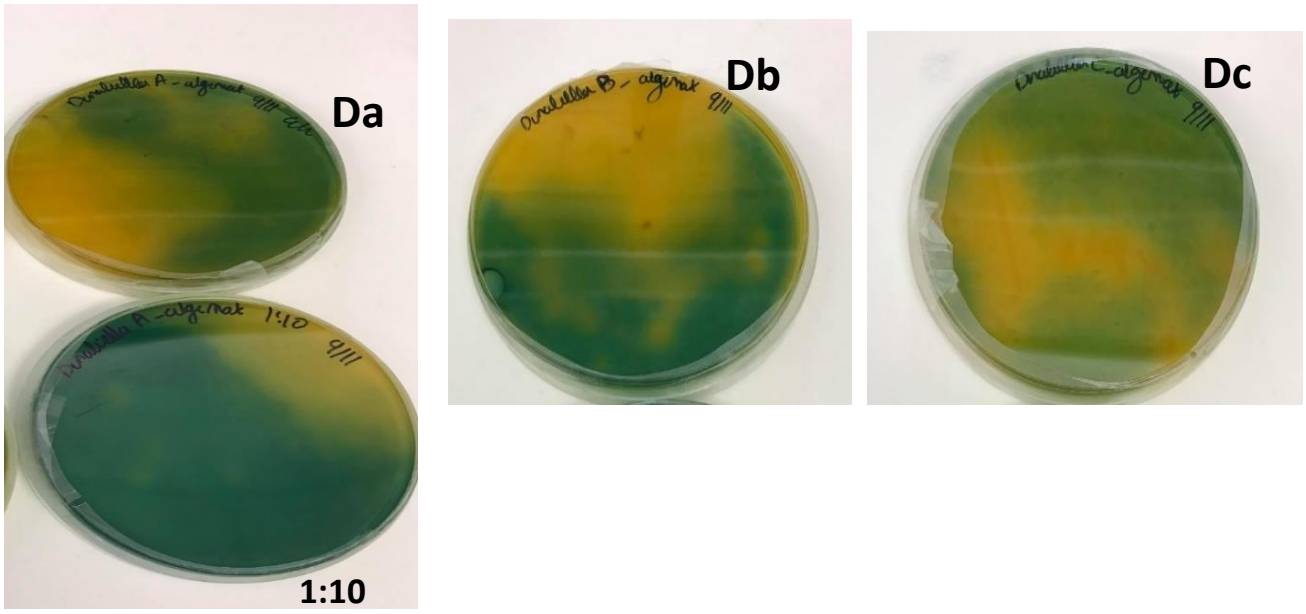


Figure 4d - *Liquid test of solution containing V. alginolyticus with disrupted algal pellet derived from D. tertiolecta replicates (a, b, c) and from the 1:10 diluted solution of replicate a, plated on TCBS petri-dishes.*

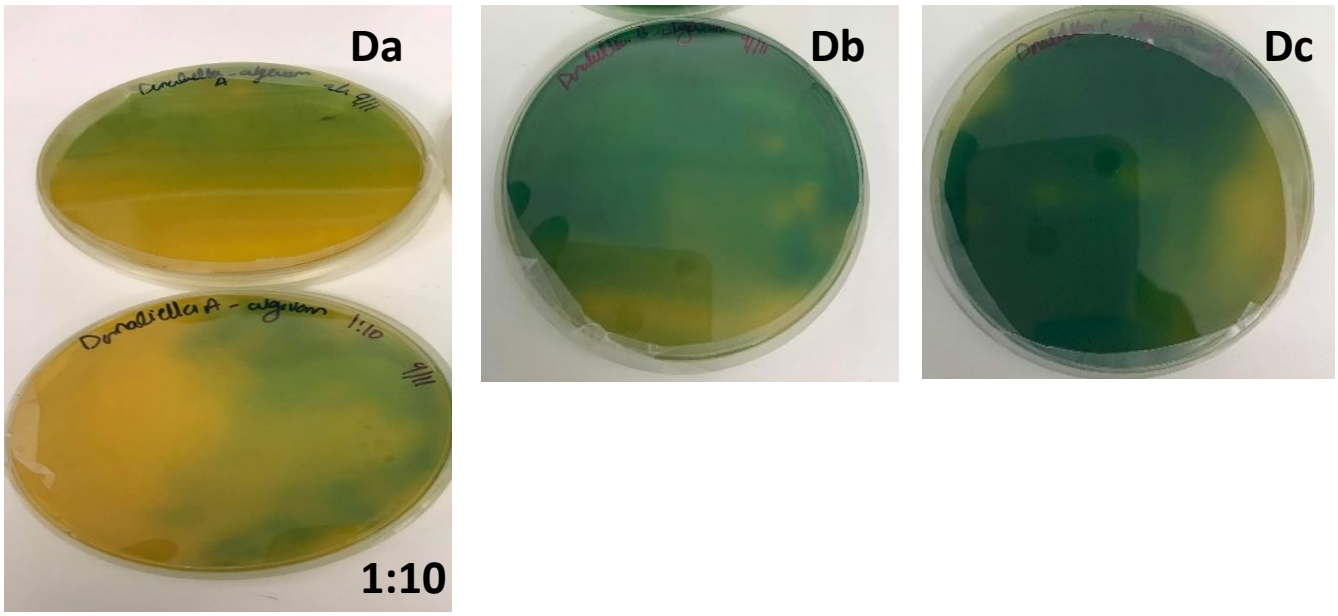


Figure 4e - *Liquid test of solution containing V. alginolyticus with cell-free supernatant derived from D. tertiolecta replicates (a, b, c) and from the 1:10 diluted solution of replicate a, plated on TCBS petri dishes.*

Testing each of cell-free supernatant and disrupted algal pellet derived from *T. suecica* and *N. oculata* with *V. alginolyticus* in liquid medium

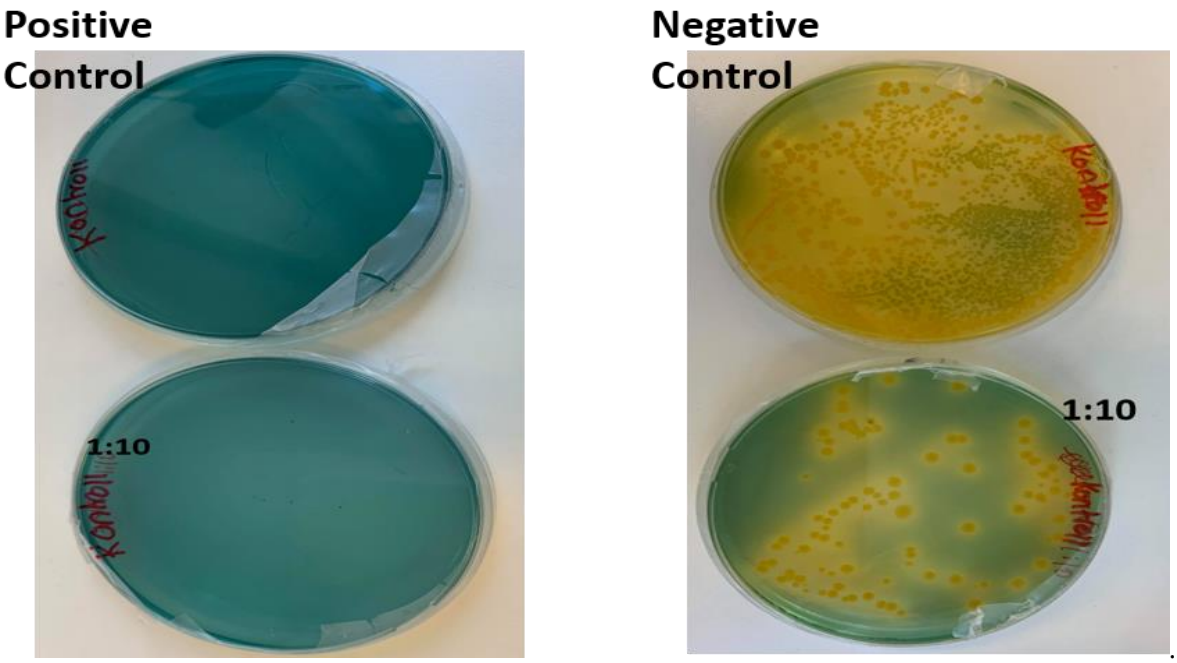


Figure 5a *- Liquid test of positive control (antibiotic with V. alginolyticus) and negative control (untreated V. alginolyticus culture) cultures and their corresponding 1:10 diluted solutions plated on TCBS plates.*

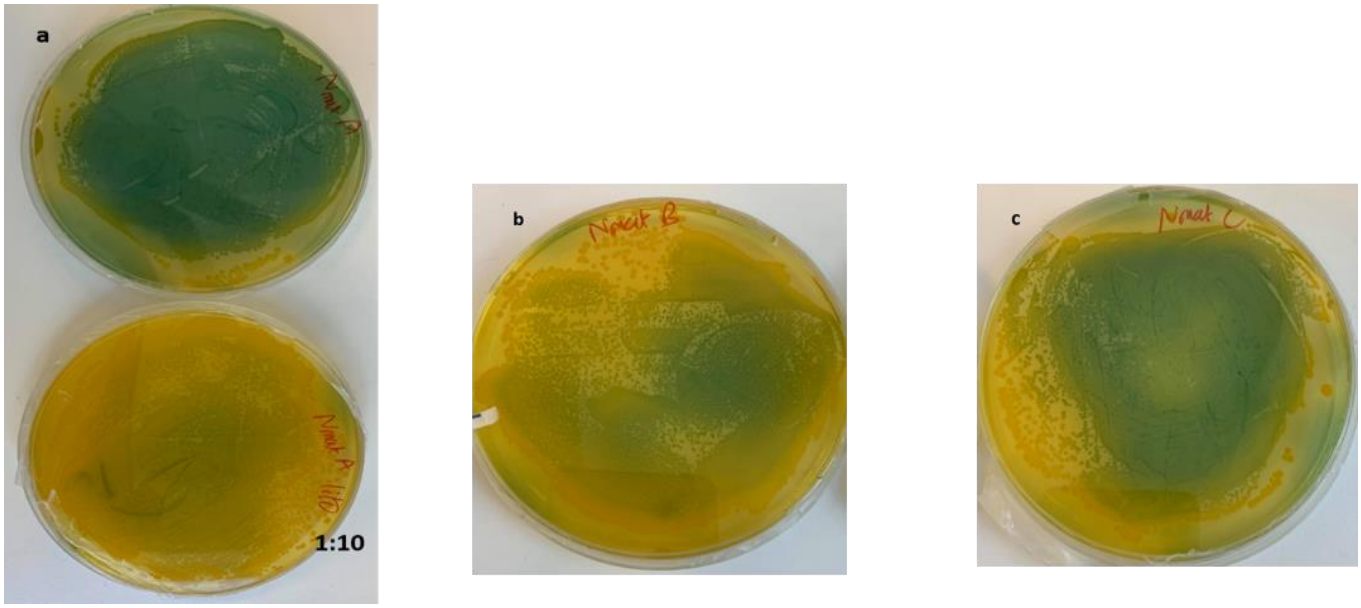


Figure 5b *- Liquid test of three replicates (a, b, c) from a solution containing V. alginolyticus with disrupted algal pellet derived from N. oculata and from the 1:10 diluted solution of replicate a, plated on TCBS petri-dishes.*

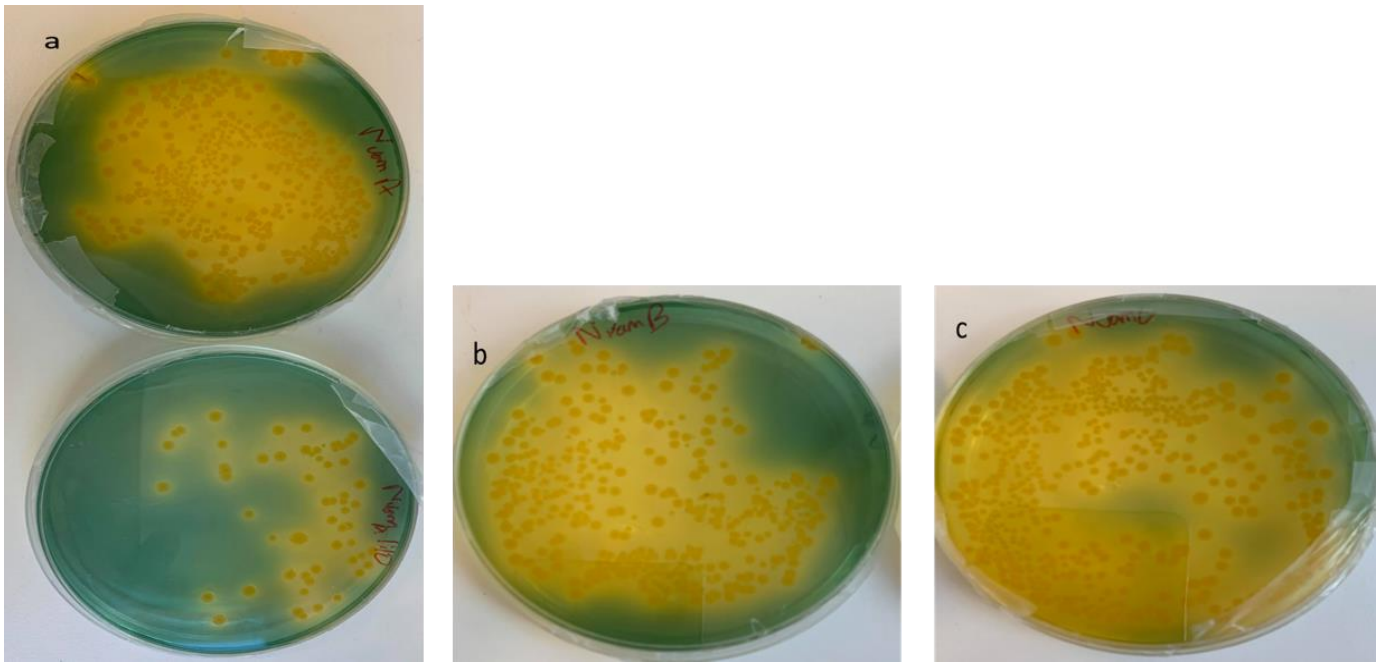


Figure 5c *- Liquid test of three replicates (a, b, c) from a solution containing V. alginolyticus with cell-free supernatant derived from N. oculata and from the 1:10 diluted solution of replicate a, plated on TCBS petri dishes.*

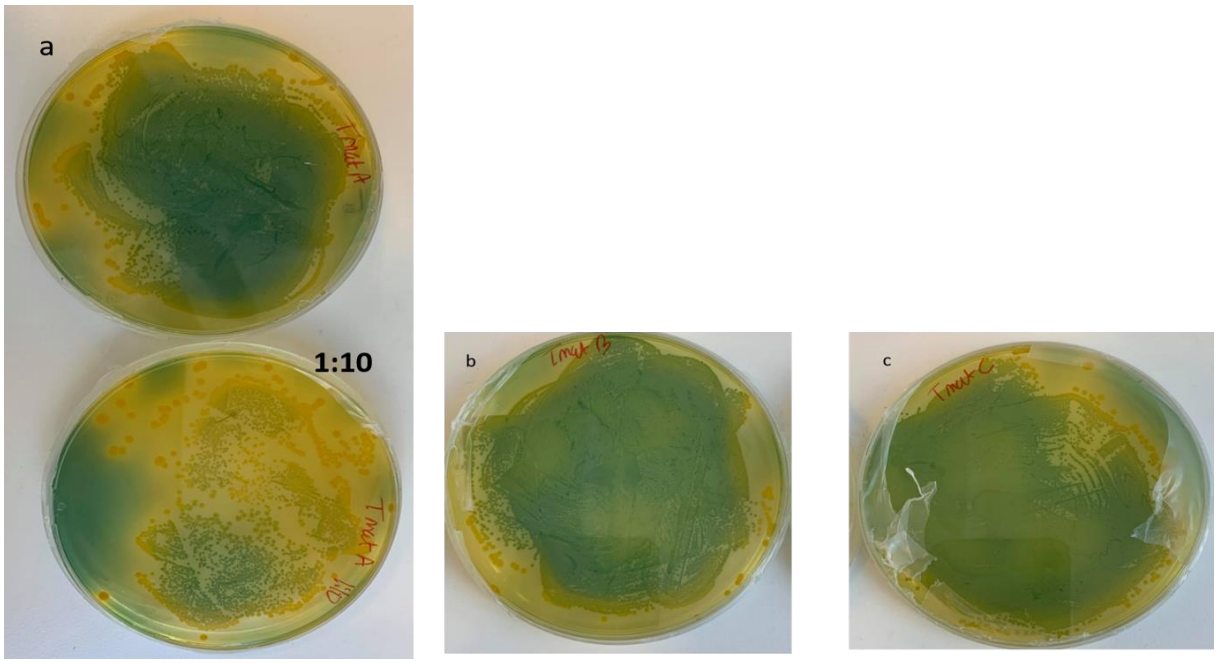


Figure 5d *- Liquid test of three replicates (a, b, c) from a solution containing V. alginolyticus with disrupted algal pellet derived from T. suecica and from the 1:10 diluted solution of replicate a, plated on TCBS petri-dishes.*

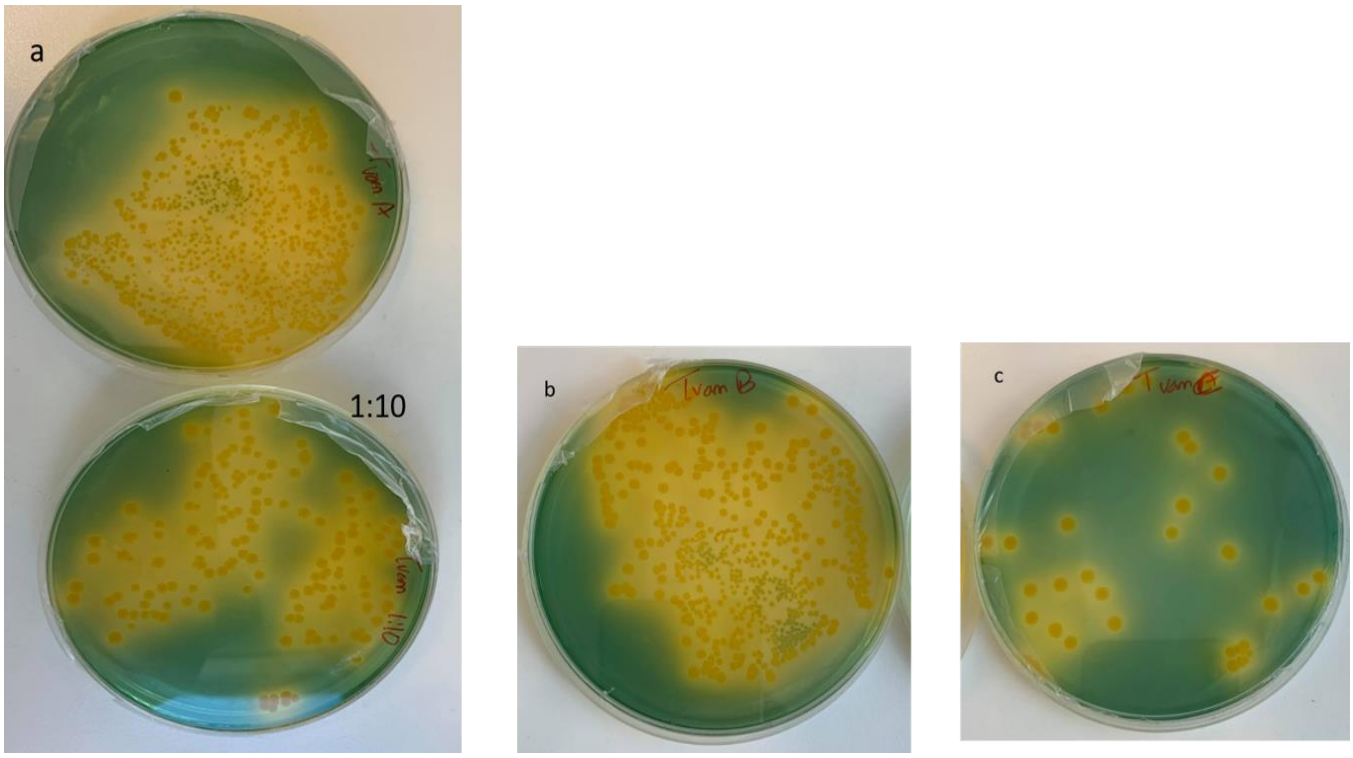


Figure 5e - *Liquid test of three replicates (a, b, c) from solution containing V. alginolyticus with cell-free supernatant derived from T. suecica tertiolecta and from the 1:10 diluted solution of replicate a, plated on TCBS petri dishes.*

Appendix H

Co-culturing of *V. alginolyticus* and each of *I. galbana* and *D. tetiolecta* microalgae at temperature 20 °C

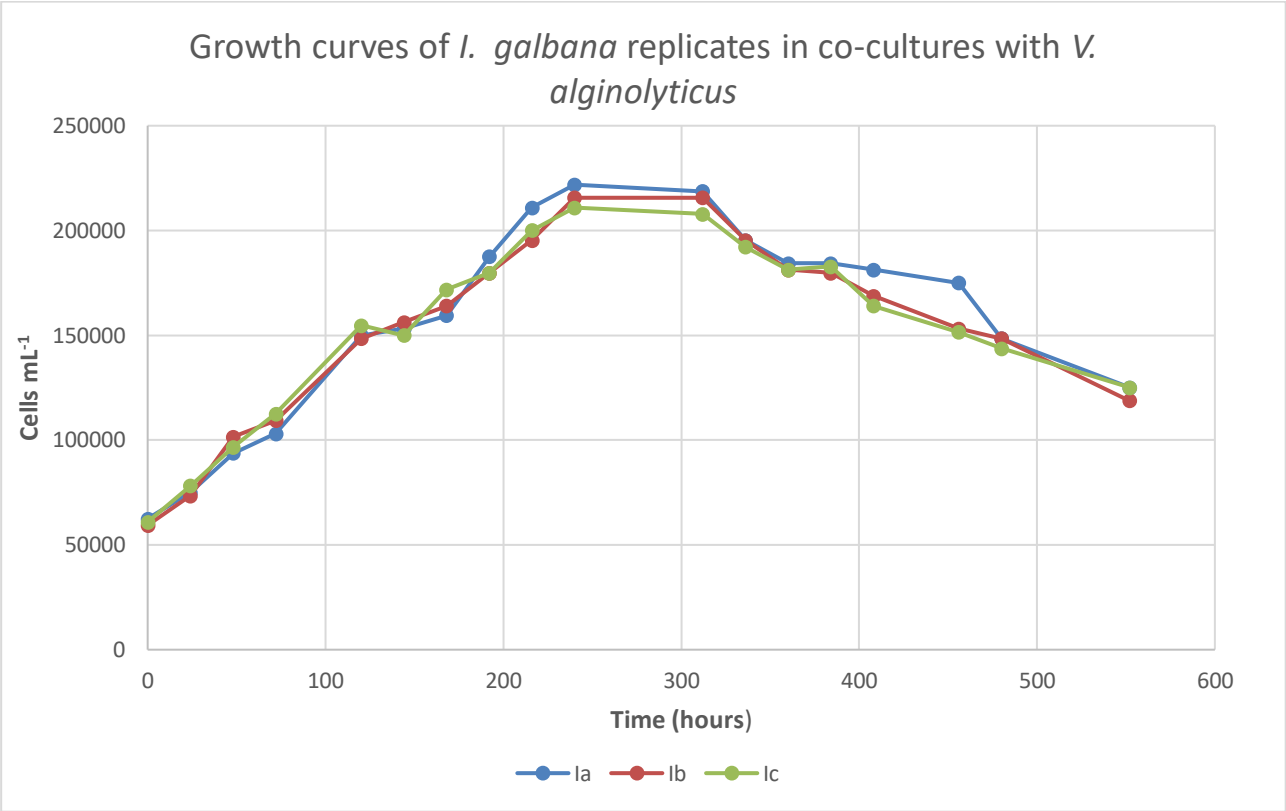


Figure 6a *- Growth curves of I. galbana replicates (a, b, c) in co-cultures med V. alginolyticus at 20 °C. The curves are made by plotting cell densities (cells/mL) over time.*

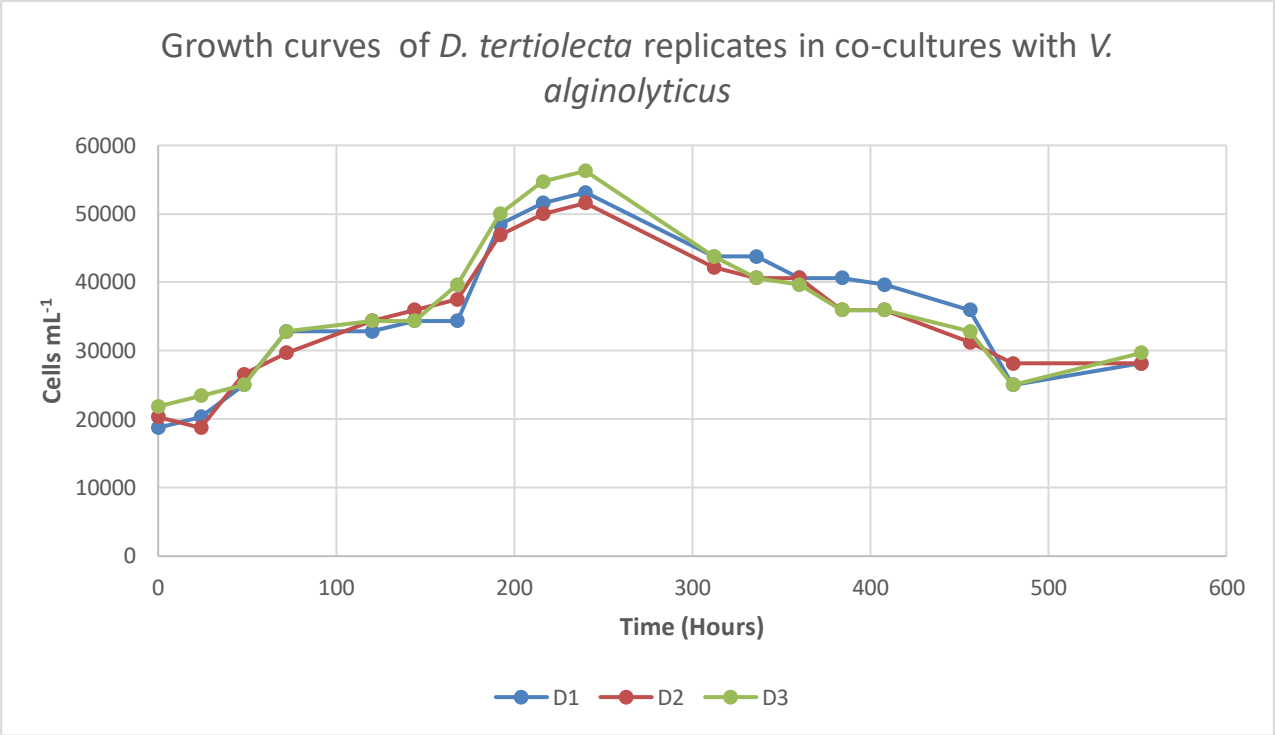


Figure 6b *- Growth curves of D. tertiolecta replicates (a, b, c) in co-cultures with V. alginolyticus at 20 °C. The curves were made by plotting cell densities (cells/mL) over time.*

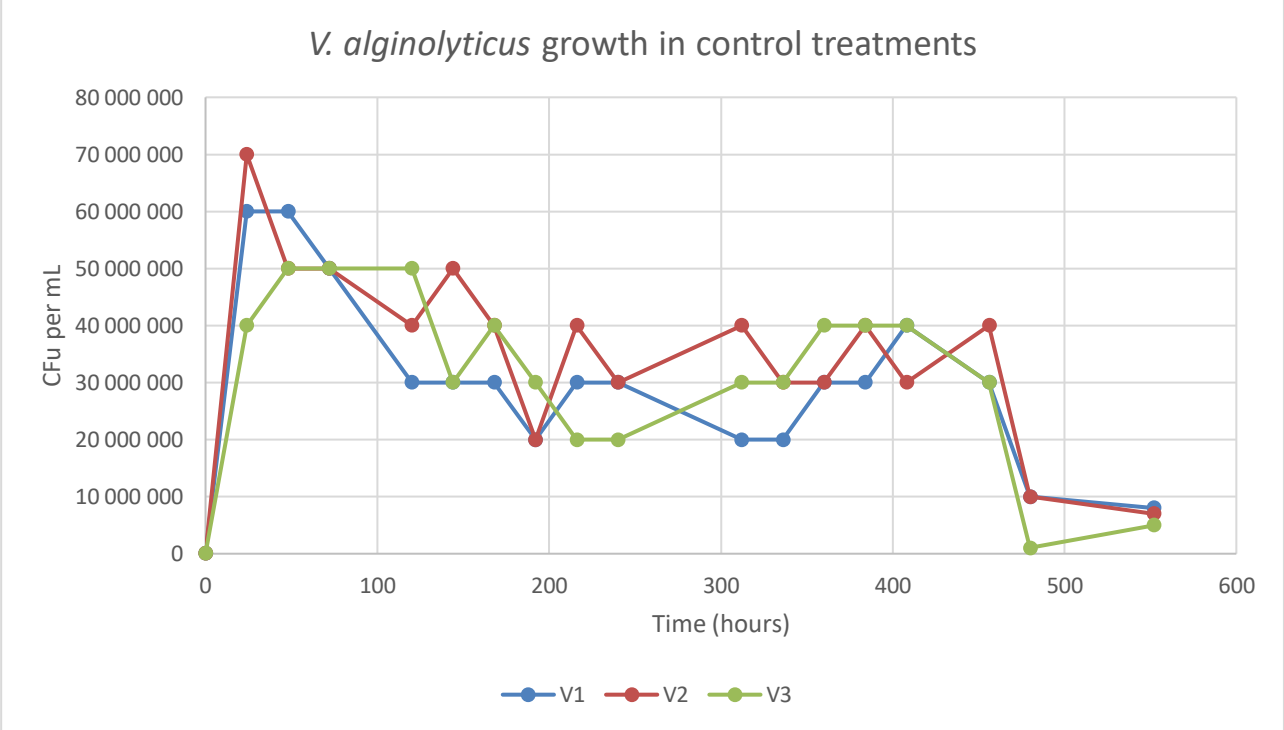


Figure 6c *- Growth curves of V. alginolyticus in 3 replicates (V1, V2, V3) of bacterial control treatments at 20 °C. The curves were made by plotting bacterial concentration (CFU/mL) over time.*

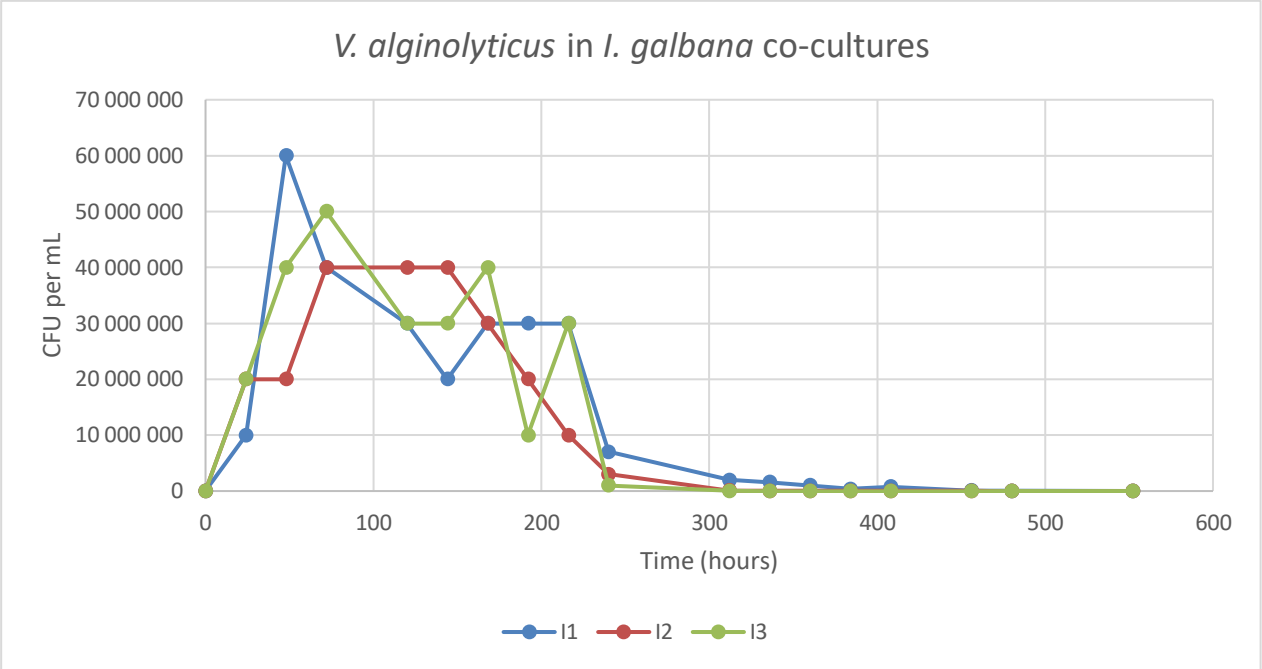


Figure 6d *- Growth curves of V. alginolyticus in 3 replicates of I. galbana- Vibrio co-cultures at 20 °C. The curves were made by plotting bacterial concentration (CFU/mL) over time.*

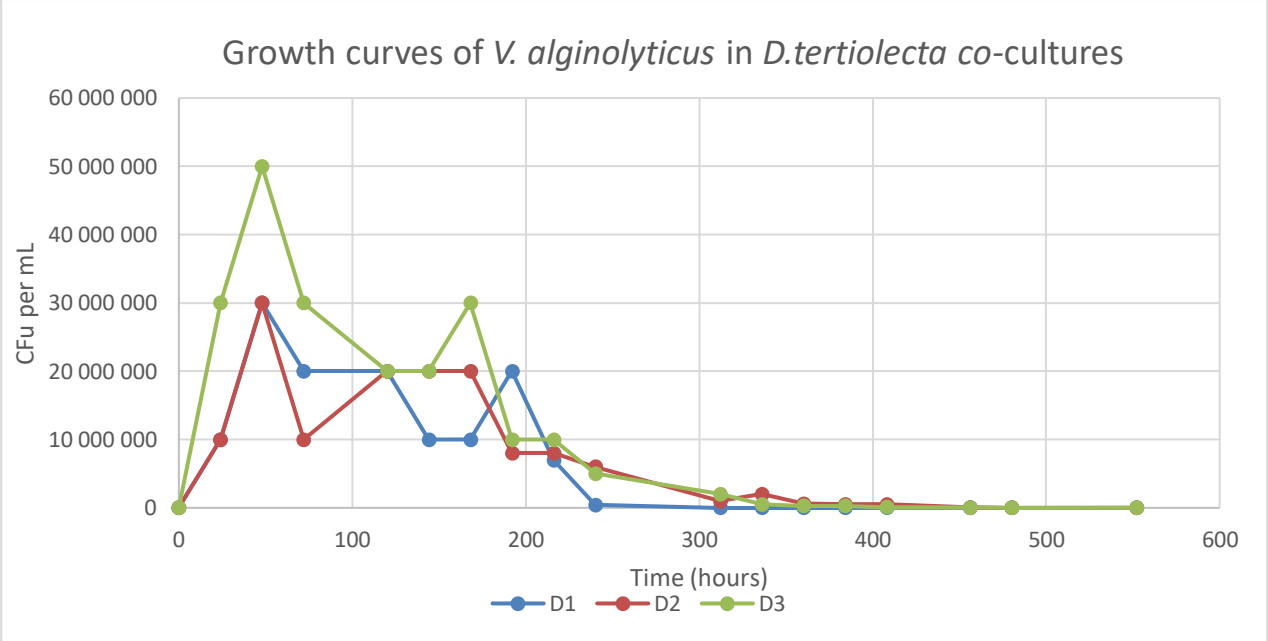


Figure 6e*- Growth curves of V. alginolyticus in 3 replicates of D. tertiolecta-Vibrio co-cultures at 20 °C. The curves were made by plotting bacterial concentration (CFU/mL) over time.*

Co-culturing of *V. alginolyticus* and each of *I. galbana* and *D. tertiolecta* microalgae at temperatures 25 °C

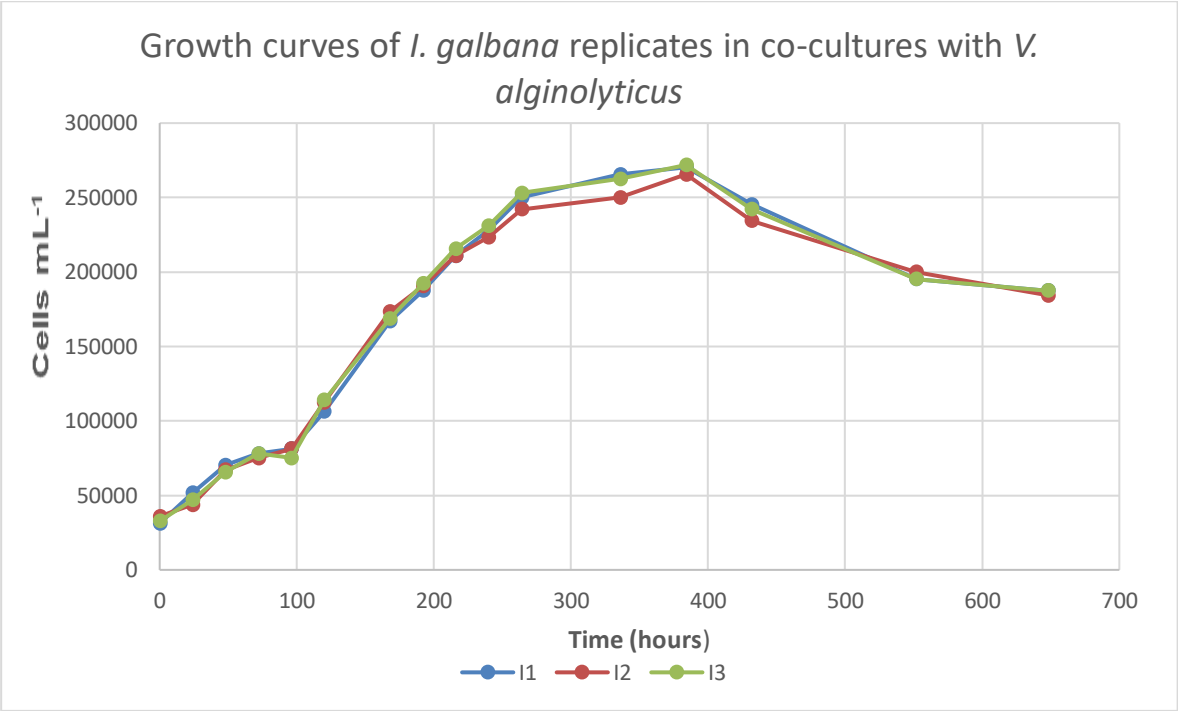


Figure 7a *- Growth curves of I. galbana replicates (a, b, c) in co-cultures with V. alginolyticus at 25 °C. The curves are made by plotting cell densities (cells/mL) over time.*

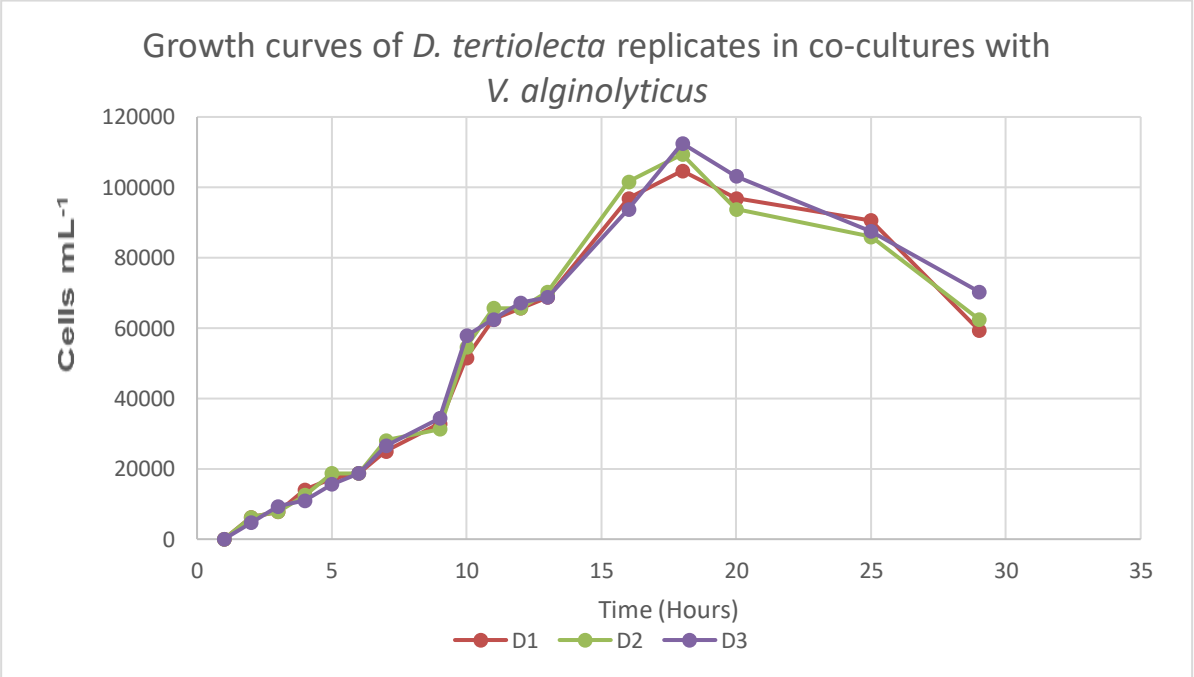


Figure 7b *- Growth curves of D. tertiolecta replicates (a, b, c) in co-cultures with V. alginolyticus at 25 °C. The curves are made by plotting cell densities (cells/mL) over time.*

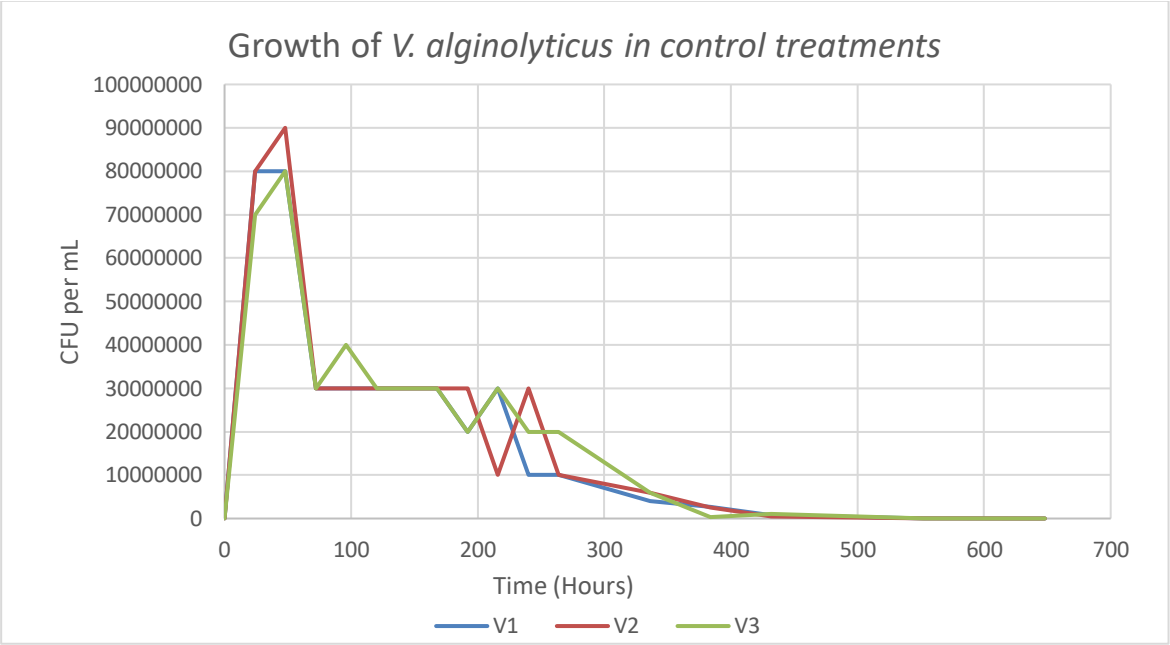


Figure 7c *- Growth curves of V. alginolyticus in 3 replicates (V1, V2, V3) of bacterial control treatments at 25 °C. The curves were made by plotting bacterial concentration (CFU/mL) over time.*

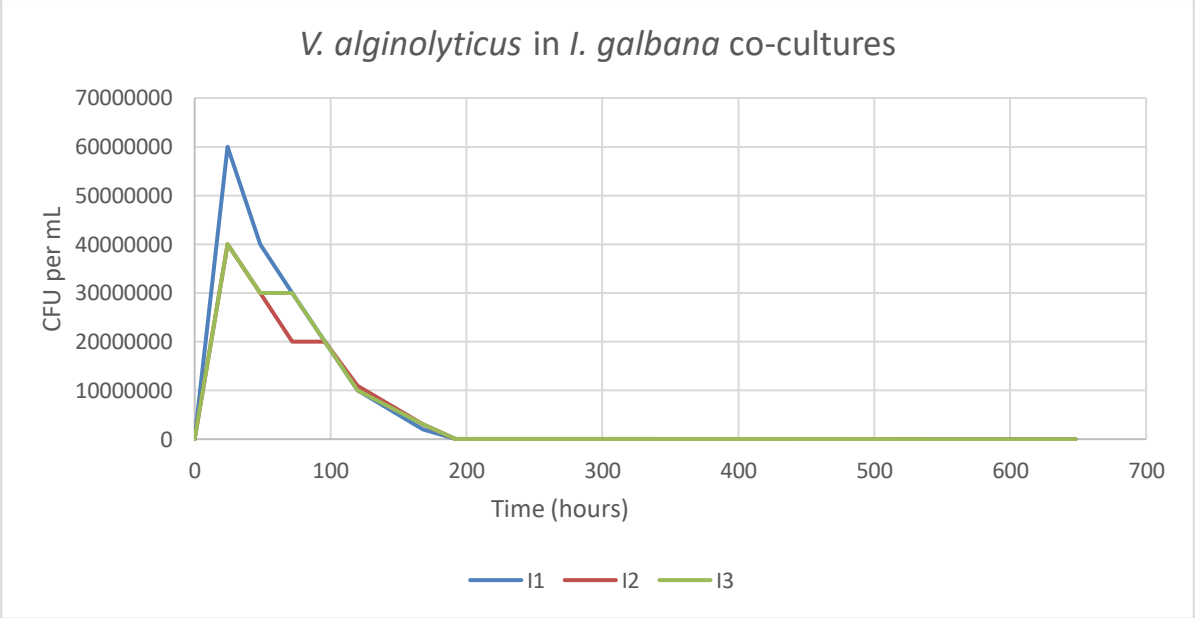


Figure 7d *- Growth curves of V. alginolyticus in 3 replicates of I. galbana-Vibrio co-cultures at 25 °C. The curves were made by plotting bacterial concentration (CFU/mL) over time.*

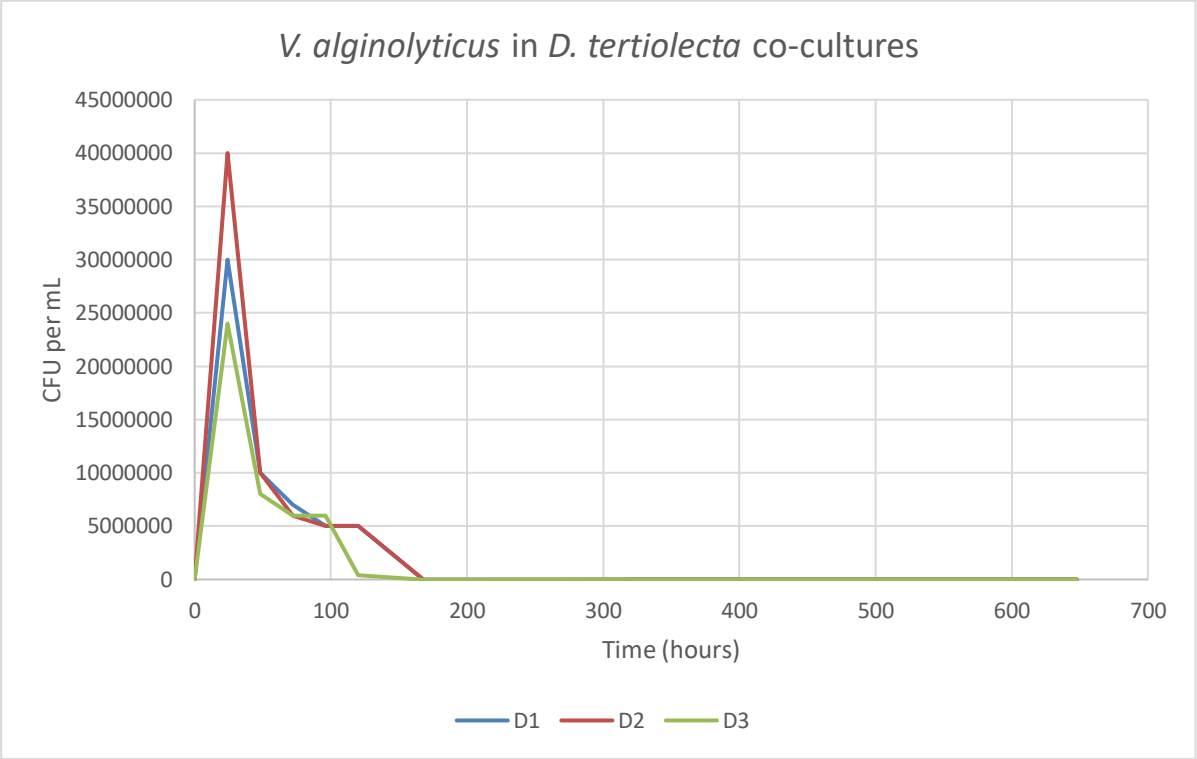


Figure 7e *- Growth curves of V. alginolyticus in 3 replicates of D. tertiolecta-Vibrio co-cultures at 25 °C. The curves were made by plotting bacterial concentration (CFU/mL) over time.*