DEVELOPMENT OF ANTI-ALGAL AND ANTI-CYANOBACTERIAL FORMULATIONS FOR LOW-DENSITY POLYETHYLENE

by

Atakan Şurdum Avcı

Submitted to Graduate School of Natural and Applied Sciences

in Partial Fulfillment of the Requirements

for the Degree of Master of Science in

Biotechnology

Yeditepe University

2016

DEVELOPMENT OF ANTI-ALGAL AND ANTI-CYANOBACTERIAL FORMULATIONS FOR LOW-DENSITY POLYETHYLENE

APPROVED BY:

Prof. Dr. Fikrettin Şahin ……

(Thesis Supervisor)

Prof. Dr. Mustafa Özilgen ……

Assoc. Prof. Dr. Hikmet Katırcıoğlu ……

DATE OF APPROVAL: ..../..../2016

ACKNOWLEDGEMENTS

I would like to acknowledge the people who have supported me in three different aspects.

My greatest appreciation, I show to Professor Fikrettin ŞAHİN. With his knowledge in microbiology and biotechnology, he has helped me to channel my energy and efforts to the field that I am more willingly to work on. He has also tried endlessly to motivate me from deviating from the objective to pursue inessential endeavours.

I would like to express my respects to Assoc. Prof. Hikmet KATIRCIOĞLU. She was most kind to spare her time to teach me about algae and the necessary techniques to cultivate and experiment on them, for without her help, the experiments would not be realized. Her devotion and concern for my success were admirable.

Lastly, I would like to express my gratitude to the people who have supported me morally, mainly: my parents Professor Günsel AVCI and Cenk AVCI, for their kind reminders of my objectives and decisions in life; Emine SEZGİN for sharing her vast knowledge in paint industry and lastly all who I can’t name individually for I am sure that the trickle of small contributions from great many people was the result which I claim to be my humble success.

ABSTRACT

DEVELOPMENT OF ANTI-ALGAL AND ANTI-CYANOBACTERIAL FORMULATIONS FOR LOW-DENSITY POLYETHYLENE

One of the problems engineers encounter with machine parts which are in contact with water is photosynthetic microorganisms such as microalgae and cyanobacteria. These microorganisms proliferate thus create fouling in marine environments on vessels or create clogging between machine parts; in both instances reduce machines' efficiency and increase maintenance costs. There are many strategies to control these microorganisms, most widely used ones are biocidal chemical applications, making designs exploiting biomimetics and allowing microbial growth and removal of them through intensive manual labour. The most effective application of them is the use of chemicals either by covering or painting the mentioned surfaces so that it does not allow microbial growth in the immediate vicinity or treating the water reservoir directly. The problem of this application is that it needs to be repeated regularly in order to preserve their efficiencies and high maintenance costs. In order to decrease the costs, these chemicals can be embedded inside the material itself, allowing a slow and controlled release over time thus making the cidal effects last longer. However there The aim of this study is to develop an anti-algal formulation from boron and zinc compounds to produce antifouling polymers. This study especially focuses on creating formulations for machine parts made of low-density polyethylene (LDPE) which can be use as laminating or coating materials. Zinc and boron based compounds, which are known to have anti-algal activities, will be imbued into low density polyethylene and the effects of the chemicals will be tested on a model alga and cyanobacterium, *Chlorella vulgaris* and *Chroococcus sp.* respectively.

ÖZET

DÜŞÜK-YOĞUNLUKLU POLİETİLEN İÇİN ALGLERE VE SİYANOBAKTERİLERE KARŞI FORMÜLASYONLAR GELİŞTİRME

Suyla temas halinde olan makine parçalarında, mühendislerin karşılaştığı sorunlardan en önemlilerinden biri mikroalgler ve siyanobakteriler olarak karşımıza çıkar. Bu mikroorganizmalar ürediklerinde, deniz araçlarının karinelerinde ya da genel olarak su içerisinde bulunan makine parçalarında tortu oluşuma sebep olurlar; bu iki durumda da makinelerin verimi düşer ve bakım masrafları artar. Bu mikroorganizmalara karşı uygulanan stratejilerin başında biyosidal kimyasal uygulamalar, biyomimetikle diğer su canlılarının vücut özelliklerini taklit ederek geliştirilen yöntemler ve tortuların fiziksel olarak temizlenmesi gelir. Bu yöntemlerden en etkilisi kimyasal uygulamalardır; bu uygulamalarda bir yüzey özel bir çeşit boyayla kaplanıp üzerinde bu mikroorganizmaların üremesi engellenir ya da kirlenmiş olan su kaynağı direkt olarak bu kimyasallar ile muamele edilir. Bu tip uygulamaların kötü tarafı, etkilerini kaybetmemeleri için belirli aralıklarla yeniden uygulanmaları gerekmesidir. Bu durum hem iş kaybı hem de bakım maliyetlerinde artışlara yol açmaktadır. Bakım giderlerinin azaltılabilmesi için söz konusu kimyasalların, malzeme içerisine işlenmeleri, yavaş ve kontrollü bir şekilde ortama salınımlarını sağlamak ve sonucunda bu etkilerini uzun süre göstermeleri mümkün olabilir. Bu çalışmada çinko ve bor bileşiklerinden antifouling özellikle formülasyonlar hazırlanıp antifouling özellik taşıyan polimerler geliştirilmeye çalışılacaktır. Çalışma daha çok düşük yoğunluktaki polietilenden (LDPE) imal edilen ve laminasyon ve kaplama gibi alanlarda kullanılacak parçalar üzerinde yoğunlaşmaktadır. Çalışmada kullanılan çinko ve bor bileşiklerinin daha önceden biyosidal etkileri kanıtlanmıştır ve polimerin içine nüfuz ettirildikten sonra bu özelliklerini örnek alg ve siyanobakteri kültürlerinde devam ettirip ettirmedikleri araştırılacaktır. çalışmada örnek alg olarak *Chlorella vulgaris* ve siyanobakteri olarak da *Chroococcus sp.* kullanılacaktır.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS/ABBREVIATIONS

°C	degrees Celsius
g/L	grams per litre
hrs	hours
km^2	kilometers square
L	litre
mL	millilitres
mm	millimetres
rpm	revolutions per minute
sp.	species
µL	microlitres
ATS	Algal turf scrubber
BOREN	Bor Araştırmaları Enstitüsü, National Institute for Boron Studies
CEO	chief executive officer
GİSBİR	Gemi İnşa Sanayicileri Birliği, Turkish Shipbuilders' Association
GUMACC	Gazi University Micro Algae Culture Centre
LDPE	low-density polyethylene
OH	Ohio State
TBT	tributyltin
UK	United Kingdom
US	United States

1. INTRODUCTION

1.1. ALGAE

The term algae, in a broad sense, may refer to any eukaryote or prokaryote in water, of which chlorophyll is the main photosynthetic pigment. Algae include giant water plants as well as unicellular plant-like organisms (Figure 1.1a); a certain portion of which is called "blue-green algae" (Figure 1.1b) however the term cyanobacteria should be used to distinguish these prokaryotes from their eukaryotic counterparts. [1]

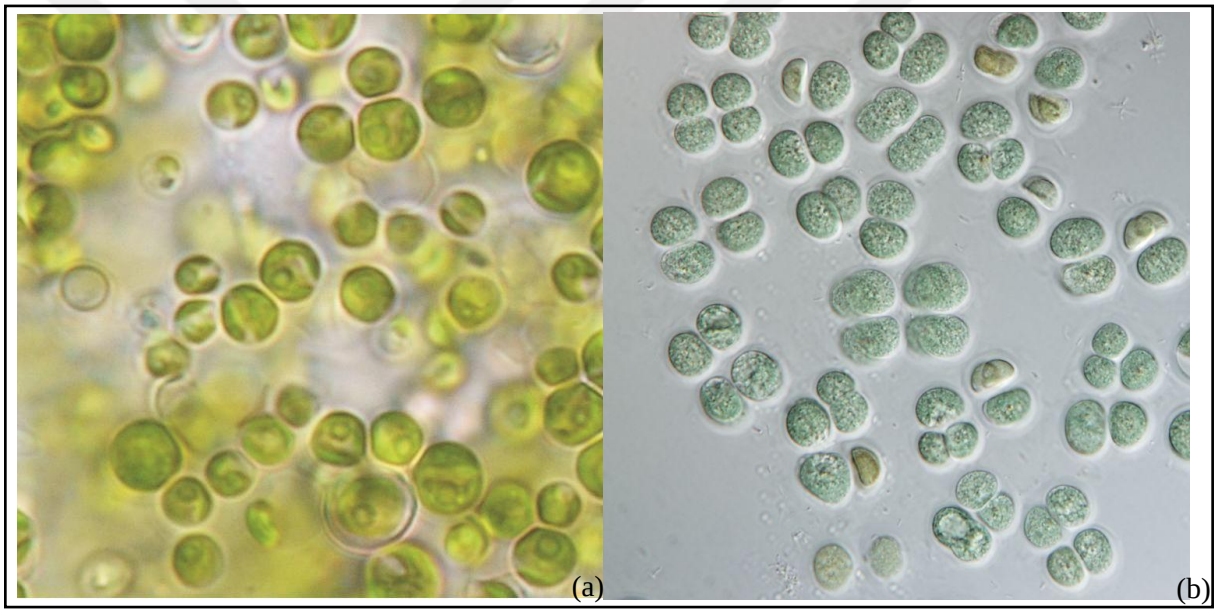

Figure 1.1. Examples for unicellular algae. (a) *Chlorella vulgaris*, a eukaryotic alga (chlorophyta), (b) *Chroococcus minutus*, a prokaryotic cyanobacterium. [2,3]

1.1.1. Prokaryotic Algae – Cyanobacteria

Cyanobacteria may have evolved between 2.1 and 2.7 billion years ago and were responsible for the Great Oxygenation Event. Prior to this event, any free oxygen in the atmosphere was captured in molecules like iron. During about 200 million years leading to the event, cyanobacteria had already produced too much oxygen that iron on Earth's crust was oxidized to iron oxide, along with other greenhouse gases to form carbon dioxide and thus oxygen began to accumulate. This accumulation changed the once reducing

atmosphere to become an oxidizing one and cyanobacteria, through photosynthesis, caused a mass extinction of anaerobic organisms but gave rise to aerobic organisms. [4]

Cyanobacteria, along with other bacteria, were first classified as plants under the phylum Schizophyceae but after Edouard Chatton's study in 1925 they were classified as prokaryotes. [5]

Traditionally were cyanobacteria were classified into five groups based on their morphology: Chroococales, Pleurocapsales, Oscillatoriales, Nostocales and Stigonematalesi given Roman numerals from I to V respectively. Unfortunately, the first three groups are not supported by phylogenetic studies. Moreover, directly quoting the source, the taxa included in the division of "Cyanobacteria" have not been validly published under the Bacteriological Code, Rev. 1990, except for some classes, orders, families and genera. These groups include filamentous and non-filamentous members, the former ones bringing their own set of difficulties during their cultivation. [6]

Cyanobacteria have been a subject of study in many different fields of biotechnology. Their direct use as a renewable energy source via photosynthetic pathway manipulation [7], the use of their biomass to produce biofuels and their use as food dyes or dietary supplements in food industry [8] are the main examples for their biotechnological applications.

1.1.2. Eukaryotic Algae

According to the Theory of Endosymbiosis, around 1.5 billion years ago, chloroplasts of algae and "true plants" were cyanobacteria and were later absorbed into other organisms, forming the chloroplasts inside them. This view is supported by genetic and structural similarities such as having a circular genome and bilayer membrane. The same can also be said for mitochondria. Since virtually all eukaryotes possess mitochondria, the presence of both a mitochondrion and a chloroplast means that endosymbiosis must have occurred at least twice for these algae successor plants. [9] Much later in history, around 500 to 450 million years ago, land plants most likely evolved from shallow water macroalgae. [10]

Under eukaryotes, plant kingdom is divided into three groups: red algae, green algae and land plants. Red algae are mostly multicellular and macroscopic organisms without any flagella or centrioles. They store their energy in a different family of polysaccharides known as the floridean polysaccharides, reproduce sexually and have phycobiliproteins that give them a red colour, hence their name. [11] Green algae can be unicellular or multicellular and may have flagella attached to them. They may enter into symbiosis with other organisms and have chlorophylls a and b in their chloroplasts which give them a bright green colour, hence their name. Green algae are further divided into Chlorophyta and Charophyta which land plants emerged from the latter. [12] The point of focus for this study will be green algae, especially Chlorophyta.

1.1.3. Choice of Organisms for the Study

In this study one species for cyanobacteria and algae was chosen for the experiments: *Chroococcus sp.*, and *Chlorella vulgaris* respectively. The strains were isolated from the freshwaters of Turkey and the cultures were obtained from Gazi University Micro Algae Culture Collection (GUMACC) in Ankara.

The first reason why these species were considered for this study is the fact that both are easy and fast to grow. Therefore, they are also higher in the eutrophication chain. Eutrophication is the response of ecosystems to the presence of additional substances in an aqueous environment. This can result from natural and human causes. A typical example of this is called a bloom. When nutrients like phosphates or nitrates build up in water, this results in an increase in biomass which has its own effects on the ecosystem. [13] In the following chapters, these effects will be discussed in more detail.

Another reason to specifically choose *Chlorella vulgaris* is its current industrial production. *Chlorella* genus is rich in proteins, nutritional value, minerals and vitamins and therefore was considered as an alternative food source. Global food shortage in late 1940's actually turned this idea into a hype at the time but with rising agricultural production efficiency and better land management it lost its attraction. This was also coupled with the high production cost of *Chlorella*. Nowadays, *Chlorella* does not get the same attention as before and is being sold as a dietary supplement. [14]

1.2. CULTIVATION OF ALGAE

1.2.1. Nutritional and Physical Requirements

There are a few requirements for the optimal growth of algae. Like all microorganisms, they need certain building blocks to produce their own macromolecules. These essentials can be provided to them by adding a carbon source, phosphates, nitrates and trace metals. Since these are environmental organisms, the optimal temperature range should be between 18-24°C. Possibly the most important requirement would be light because the main source of carbon for these organisms is carbon dioxide in the air which they later convert into carbohydrates via photosynthesis. This requires an excitation with light in day/night cycles (16/8hrs day/night at 2500-5000lux light intensity). [15]

Among the essential elements zinc, copper and boron should be mentioned. The amounts needed for different kinds of growth media (BG11, Walne and Guillard's) are very small, typically around 10^{-1} grams per litre. [16, 17, 18] Although they are essential for algal survival, excess amounts of them have cidal effects on them, which makes these elements very important for the aim of this study and this will be discussed thoroughly in the following chapters.

1.2.2. Cultivation Methods

Cyanobacteria and microalgae can be assumed as crossovers between microorganisms like bacteria/yeast and plants for argument's sake. From an engineering perspective, a bioreactor has to be designed in such a way that it provides the necessary light to the algae. This way will enable them to proliferate more rapidly than their land plant counterparts and still provide the producer with great quantities of end product, which has made their cultivation a hot topic so far.

1.2.2.1. Small Scale Cultivation

Experimental cultivation of microalgae is similar to regular microbe cultivation. In this study, the culture is inoculated into BG11 medium with a pipette. The main difference from common lab practice for microbe cultivation is the need of a light source with day and night cycles. It is also preferred to provide cultures with air via tubes inserted into the broth but this is not done for cultures smaller than 250mL in volume, whereas for larger or denser cultures, this is a requirement. [19]

1.2.2.2. Large Scale Cultivation

For industrial scale production, there are two approaches. The first one is the closed-loop photobioreactor where the nutrients, air inlet, temperature, light intensity and the mechanical agitation are all closely monitored (Figure 1.2). Despite the gain of a high yield the downside is the cost of this type of production. To decrease the expenses, sunlight can be used as light source for these reactors. To fully absorb the light, the culture has to be spread on a large area rather than left as a bulk as in other cultures. That is why it is quite common to use extensive glass piping to ensure increased surface area. Second type of production is the open pond system where the culture is placed in a big open pool (Figure 1.3). In this type the culture is exposed to air and readily receives the needed carbon dioxide while being mixed with agitators. The light source for this type is sunlight and thus it is subject to natural day/night cycles of the seasons. Major disadvantages of this open pond system are being prone to outside contamination and extra effort that algae need to spend to resist differences in temperature and nutrient levels. Additionally, they will most likely be in competition with other species and thus their biomass might decrease. [20]

1.3. USES OF ALGAE

Cultivation of algae has benefits in many different areas, including food and fuel industry, agricultural and environmental applications.

Figure 1.2. A closed-loop photobioreactor with glass piping. This design allows natural light to be absorbed by most of the culture. With these design and controlled parameters, alga cultures can be rapidly obtained. [21]

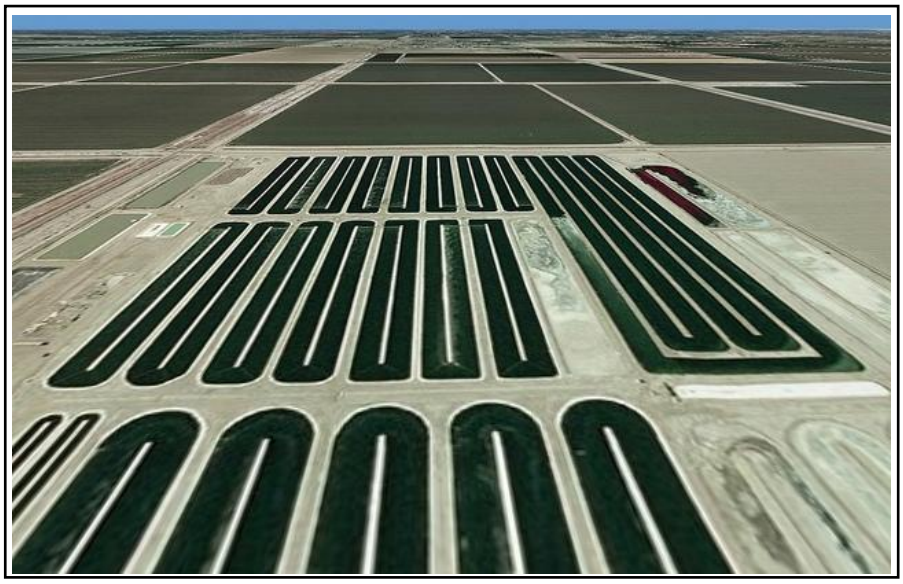

Figure 1.3. An open pond algae cultivation complex. The raceway design is very common in this field and instead of having a very strong agitator in a big circular pond, a paddle just like the ones in old steamboats is used to provide homogeneity and air flow. [22]

1.3.1. Algae as Food

As mentioned earlier, food from algae is not a new concept. Even in the form of seaweed, it has been consumed by many cultures around the world but recently cyanobacteria and microalgae have become available as a source of nutrition after being processed. Current studies showed that they can be a better source of protein than legumes [23], but still are not better than animal products and can be much more costly. [24] Mainly due to economical reasons, microalgae are only sold as nutritional supplements.

1.3.2. Algae in Agriculture

In agriculture particularly in coastal areas, it has been a common practice to use algae as biological fertilizer to restore nutrition to the soil. This application can be specifically done with cultured microalgae but generally seaweeds are preferred for their ease of application. Dried seaweeds or microalgal biomass can also be a very nutritious food source for livestock. [25]

1.3.3. Algae in Environmental Applications

It has been discovered that algae can capture up to 90% of the nitrogen and up to 100% of the phosphorus runoff from wastewaters thus can be utilized to treat them. In fact, this system is called an Algal Turf Scrubber (ATS). In small scale applications, this system can be used to filter aquarium waters. In large scale applications, this system can be used to clean streams, rivers of fertilizers which diffuse from the soil. When a certain algal biomass is reached, the biomass is harvested, it can then be used to fertilize the same fields. Studies have shown that they possess the same efficiency as commercial fertilizers. [26, 27]

1.3.4. Algae in Fuel Production

Among the commercial applications of algae, fuel production became a popular one. In recent years, the fears of global energy and food shortages have led scientists to pursue

more sustainable ways to acquire them. It is believed that with increasing demand, current fossil fuel reserves and agricultural production will not be able to meet our needs in about 100 years. [28] One should note that food production is also dependent on energy where labour, fertilization and pesticide applications all require energy input.

Starting with the Oil Crisis in the 1970's, excess food supply in developed countries is being converted into fuels such as ethanol or biodiesel. Common biofuels are biogas produced from decaying organic matter, bioethanol or biobutanol from carbohydrates such as sugars or starch, and biodiesel from vegetable oils. However, the existing problem for sustainable fuel production by agriculture is that use of arable land or edible agricultural output in fuel production decreases the amount of food available for the total population. This is especially evident when an amount of corn which is enough to feed a person for a whole year is needed to produce enough ethanol to fill one car tank. This situation, therefore, creates a dilemma and it is called "The Food vs. Fuel Debate". [29] This is exactly why cyanobacteria and microalgae become valuable tools to generate bioenergy.

Biofuel production from algae has also been a trend topic since the oil crisis of 1970's and has several advantages to it. While they also release carbon dioxide just like fossil fuels, they release carbon dioxide that was recently captured by photosynthesis whereas burning fossil fuels release carbon dioxide that was captured millions of years ago and had not affected the balance of the atmosphere until their combustions recently. The main advantage of algae comes from their production: since they are cultured in either open or closed systems where a certain volume of operational area can be occupied. In fact when algae are to be cultured for biodiesel, 10 to 23 times more yield in oil per acre can be obtained compared to the next highest oil yielding crop which is oil palm. [30] The Food vs Fuel Debate becomes invalid when algae are used in making fuel because smaller areas of land can be allocated for the production of algae. Moreover, the species which are grown industrially are not suitable for human consumption and therefore are not removed from the food market to create shortages unlike maize or sugarcane.

The disadvantage of biofuel production from algae is the requirement of high capital investment and energy input which is currently ironically obtained by unsustainable means. In addition, biofuels currently need subsidies from governments to compete in market since fossil fuels provide us with cheaper energy. In 2013, then Exxon Mobil Chairman

and CEO claimed that the company was still 25 years away from algae based fuel production. Such a claim from world's lead gasoline and diesel producer should be taken seriously. Still that did not keep the company from spending 600 million US dollars in algae fuel research and development. [31]

1.4. PROBLEMS WITH ALGAE

The question remains why we want to get rid of algae although they can be so beneficial. The problems arising from algae are because of their presence in unwanted places. Algae become problematic in terms of two concepts: formation of algal blooms and biofouling.

1.4.1. Algal Blooms

When adequate organic material is present and coupled with favourable climatic conditions, a phenomenon called an algal bloom can be observed, which is the rapid proliferation and accumulation of microalgae. It can be seen in both marine and freshwater reservoirs. Depending on the species, it can also be called a harmful algal bloom. Harmful effects of these blooms include mass mortalities in marine and freshwater life due to their toxic effects, human illnesses and mortalities through consumption of infected species and contaminated freshwater, mechanical effects on aquatic life and manmade machines and finally depletion of oxygen in water. An example of this can be the proliferation of Microcystis genus of freshwater cyanobacteria which produce a family of 50 neurotoxins called microcystins having high toxicity to liver. [32,33]

There are three types of approaches taken against algal blooms: mechanical/physical, chemical and biological.

1.4.1.1. Mechanical and Physical Approaches

One example of the mechanical methods used against algal blooms is the physical removal of their biomass with a tool, such as a rake which is especially good against filamentous algae. Inert water-soluble dyes can also be added into the water in order to reduce the

ability of algae to absorb the light. Machinery such as centrifuges or filters can be used to separate biomass from the medium completely. Although this method is unsuitable for smaller ponds, it can be considered for reservoirs. Ultrasound is also an alternative because sound waves rupture the gas vesicles inside the cells and destroy cyanobacteria. [34, 35]

1.4.1.2. Chemical Approaches

Chemical approaches against blooms utilize algicides and coagulants. Various compounds have cidal effects on algae, a very common example of which is copper (II) sulphate. Chlorine and lime are also used for these applications and are easily available. These compounds may also indiscriminately harm other organisms or dead cyanobacteria may release their toxins therefore extra care is necessary for their applications. [35] Coagulants form sediments of algae which sink to the bottom of the water. There, without access to nutrients, light and oxygen, algae eventually die. Coagulants include hydrated potassium aluminium sulphate and chitosan. [36,37] A traditional method that has been used against algae for small ponds is the application of barley straw. When exposed to sun and oxygen, barley straw excretes chemicals that have static effects on algae, therefore has to be applied before a potential bloom. The mechanisms behind this are not thoroughly understood yet. [38]

1.4.1.3. Biological Approaches

Biological approaches include creating wetlands or increasing grazing pressure. When wetlands are created with the contaminated water or floating gardens are built on top of the water supply, a competition will arise between the algae and the land plants that were introduced and the amount of available nutrients will decrease, keeping algal population in the balance. One drawback of this approach is that these plants have to be harvested from time to time. The second way is to either introduce more plankton or remove/decrease the number of fish in the water which will again increase the plankton population. These plankton will feed on algae and keep their population in check. [35]

1.4.1.4. Approaches on Marine Algal Blooms

In marine environments chemical treatment against algal blooms will not be feasible since any application need to be massive in scale. Several attempts have been made to remove algae mechanically, like treating them as if they are oil spills. At least parts of the bloom which rise to the surface can be removed from sea water. [35]

Common sense dictates that it is more preferable to prevent algal blooms from happening rather than to treat them, simply by controlling the flow of nitrates and phosphates into these water reservoirs.

Figure 1.4. The result of an algal bloom from Lake Erie, OH, USA.

1.4.2 Biofouling

In global marine logistics business, one of the biggest problems turns out to be hull maintenance. Within minutes of contact with water, organic molecules inside the water bind onto a ship's hull via der Waals interactions, and create a kind of film which then

attracts marine life. The first step of this ladder is made up of marine microorganisms including microalgae, continuing up to macrofoulers such as molluscs, sponges and barnacles. This gradual accumulation of living organisms on wetted surfaces is named biofouling. [39]

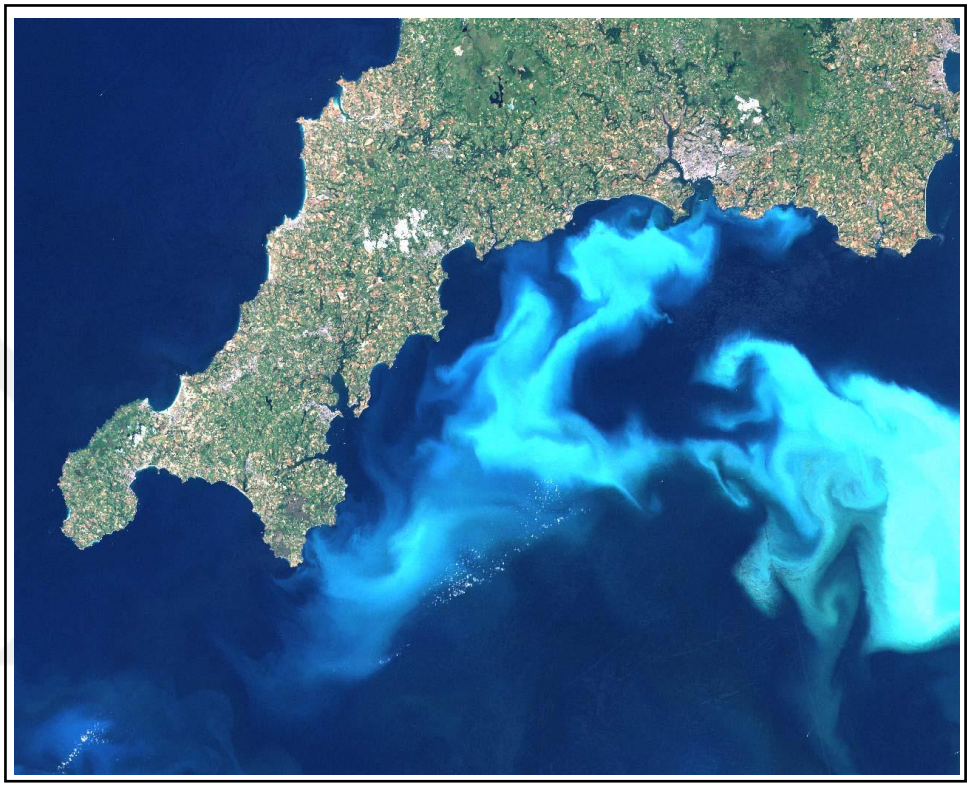

Figure 1.5. An algal bloom just south of the coast of Cornwall, UK from July 24, 1999. [40]

According to a study made by the US Navy, it can result in up to 60% more drag because of increased weight and friction, 10% speed loss, 40% more fuel consumption and an annual expense of 1 Billion US dollars. [41] These figures only take the US Navy into account. Considering the amount of global sea traffic and the number of ships, the costs can be astronomical.

In addition to the shipping industry's hull problems, fouling may also occur in other machine parts which are in contact with water. Algal growth in these instances can result in reduced machine efficiency, increased maintenance costs from machine part deterioration or cleaning of these parts, and end product contaminations. [39]

Ship hulls normally have to be painted without the threat of biofouling. Upon contact with water, surfaces of metal ship hulls immediately begin to be oxidized and given enough time, iron in these hulls completely turn into iron oxides, become flaky and disintegrate. For wooden hulls, water will gradually enter into the fibres of the planks, making the vessel heavier, reducing buoyancy or even causing leaks. Because of these reasons, paint becomes a necessity whether fouling is a problem or not. Rust is so much of a problem in the industry that it is also common for shipping magnates to coat their vessels' hulls with zinc before painting, to prevent rust from spreading if the paint is scratched or lifted. Hull paint can be scratched during ships service for a variety of reasons ranging from flotsam impacts to improper mooring. While it is difficult alone to achieve perfect hull condition without the algae, their presence means that hulls have to be cleaned, sanded and painted again once every one or two years. [43]

Figure 1.6. Biofouling as it is removed from a smaller vessel. This is a very good illustration of biofouling creating a rough surface that hinders the vessels ability to cruise smoothly. [42]

It is not possible to avoid Van der Waals interactions between molecules but algae can be repelled from hull surfaces or can be killed to prevent macrofoulers from accumulating on them. Currently this is achieved in three way: by a series of toxic coating which generally

contain tin, copper and zinc derivatives in their formulae; a series of non-toxic coatings that create a smooth or hydrophobic surface that prevent attachment, and coatings that utilize biomimetics - solutions that mimic other organisms.

1.4.3. Biomimetics

Being under the threat of biofouling, some water organisms themselves develop antifouling measures. Smaller organisms like sponges, corals and sea urchins excrete biocides against microfoulers. These biocides include tannins produced by a vast number of plants which is especially effective when coupled with copper or zinc, nicotinamide which is the amide form of vitamin B3 and the most effective natural biocide bufalin which is 100 times more effective than tributyltin, a synthetic algicide. [44, 45]

1.4.4. Synthetic Biocides

Harvesting natural biocides has cost-related difficulties and that is why mass production of synthetic biocides is preferred over them. Four elements and their uses as synthetic biocides will be discussed further in detail: tin, copper, zinc and boron.

1.4.4.1. Tin

Since tin itself is not toxic, food is preserved in steel cans with tin layers inside. Tin becomes toxic when organotins, compounds with hydrocarbon constituents are formed. One of the best and cheapest antifoulers is tributyltin or TBT, the class of compounds that contain the $(C_4H_9)_3Sn$ group, which are organotins. [46] Starting from 1960's, whole fleets were coated with paints containing TBT. Later it was discovered that TBTs were highly toxic to all marine life and persisted in the environment long after their intended lifetime, which led to their ban from usage on new ships in year 2003 and total global prohibition later in January 2008. [47]

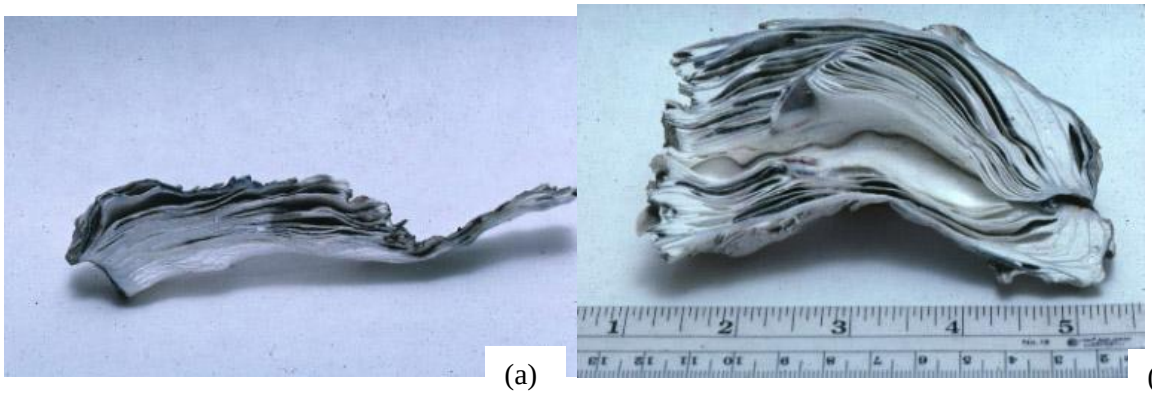

Figure 1.7. Oyster shells (*Crossostria virginica).* (a) An oyster shell in normal conditions, (b) A piece of shell from an oyster exposed to tributyltin. [48]

1.4.4.2. Copper

Copper is another element that needs attention and unlike tin, copper usage on ship hulls is not a new concept. Starting from the 1750's, the British Royal Navy started experimenting with copper coatings, mainly by attaching copper plates around wooden ship hulls and attained various degrees of success. [49] After those experiments, an industrialist from Birmingham, UK called George Frederick Muntz patented and commercialized the Muntz Metal in 1832. His brass alloy had 60% copper, 40% zinc and trace amounts of iron. This alloy provided the same antifouling property as of the copper plates for a third of their price and its effects would last longer. [50] As discussed earlier, copper was also used against algal blooms, especially in the form of copper sulphate. Copper based coatings are currently available in the market mainly for commercial and personal vessels. Due to similar concerns on TBT, copper based coatings are not considered safe and therefore they coatings are banned in Sweden to preserve the already low biodiversity in the Baltic Sea and in the Netherlands particularly on vessels that tread into inland waters. Nevertheless, there is not enough scientific evidence to conclude that negative environmental impacts in marine environment can be completely attributed to copper. [51] Another problem with copper is its price. Copper is a good heat and electric conductor and therefore it is widely used in electronics industry which drives its price up.

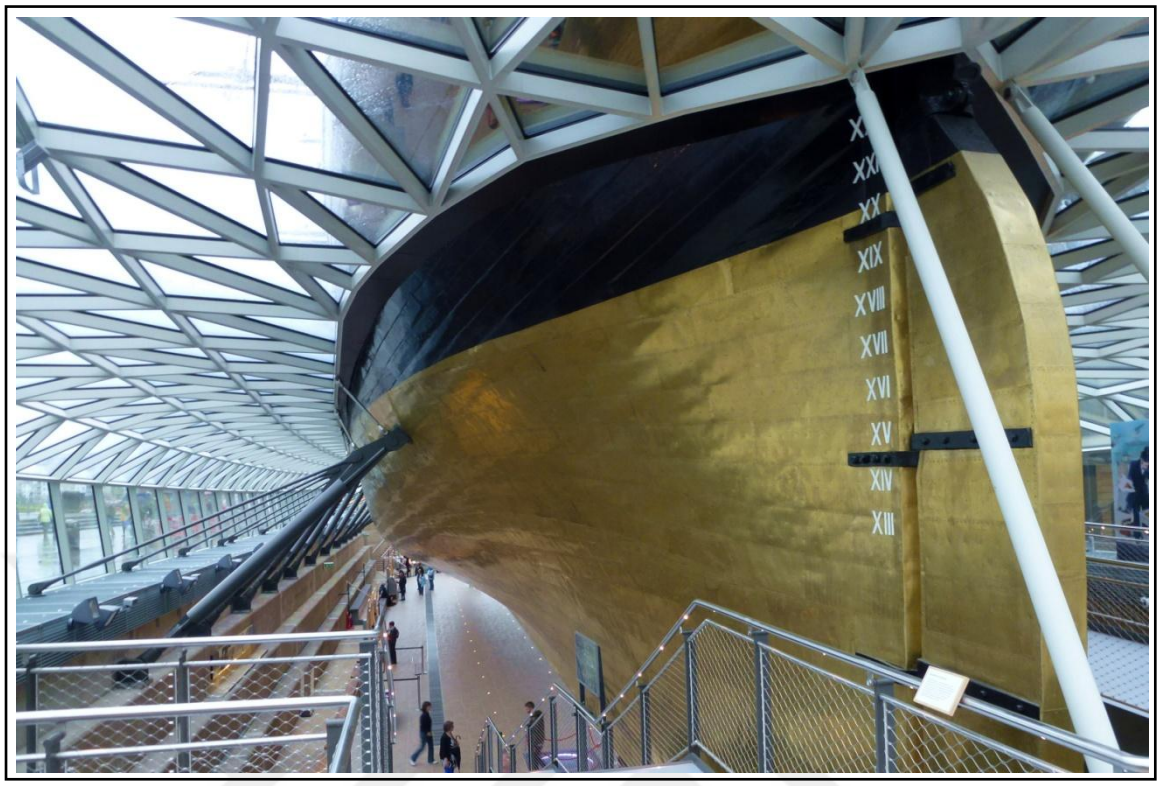

Figure 1.8. Example of the application of Muntz metal. The stern of the famous clipper class vessel “Cutty Sark” with its draft and rudder coated with plates of Muntz metal. [52]

1.4.4.3. Zinc

In the form of brass, zinc has been in use since Early Bronze Age and now that its properties are better understood, it is used in a vast number of areas in industry. As mentioned previously, zinc forms 40% of the Muntz Metal and is a major contributor to the alloy’s antifouling property. Zinc oxide and zinc pyrithione are two important compounds with antimicrobial properties and both are used in various body lotions, creams, deodorants, shampoos. [53] According to one study zinc is effective even in low concentrations. [54] Downside is that zinc compounds are either slightly soluble or not soluble at all in water. This actually prevents surface coatings from being washed away so that they can exhibit their properties longer. Zinc pyrithione has already been used as an antifouling agent in ship hull paints for quite some time after TBT was discovered to be very harmful for marine life. Some questions arise about the environmental fate of zinc pyrithione but still data is insufficient for any conclusion. [55]

1.4.4.4. Boron

Boron is not a common element, either on earth's crust or the solar system because of the kind of nucleosynthesis by which it is formed. [56] However, it is much easier to come by in our country due to its high reserves (72% of world's total boron reserves) and the government's monopoly in its production. Eti Maden İşletmeleri, the government-controlled mining corporation of Turkey currently controls 47% of the global market on boron minerals. [57] Boron and its derivatives are being used in food, glass, fuel industries and have proven to be good biocides. Detergents, cleaning products and bleaches may have boron derivatives in their formula. In fact in joint studies with BOREN, the National Institute for Boron Research and our Biotechnology Department in Yeditepe University, several products have been developed including but not limited to disinfectants, salves, textile products and pesticides. Boron can be available in borax hydrates, mainly sodium tetraborate pentahydreate (also borax pentadyrate) and disodium octaborate tetrahydrate. Unlike zinc compounds they are soluble in water; and both chemicals have been approved to treat algae when dissolved in water. [58]

1.5. STEPS LEADING TO THE CURRENT STUDY

1.5.1. A Preliminary Study

This study is not the first on antifouling in our department. In mid 2013, our department started an unofficial preliminary study with GİSBİR, the Turkish Shipbuilders' Association in Tuzla, Istanbul. Hull paint was mixed with various boron compounds and perlite, and these mixtures were used to coat steel plaques of 25x25x1cm in size, 1cm being the mean thickness of a ship hull. These plaques were supplied by GİSBİR, cut to size and sanded; they were painted according to the checklist provided to us by them and placed into sea where they were left for two months. One side of each plaque was left untouched to serve as negative controls, some plaques only had the original paint without any formulae and some plaques had antifouling coatings on them. Unfortunately only some of the plaques could be recovered due to violent sea conditions. Various degrees of success were obtained

in the experiment; lacking the proper expertise in the field, the local shipwrights were consulted on the results, which they deemed the antifouling effects to be “mediocre”.

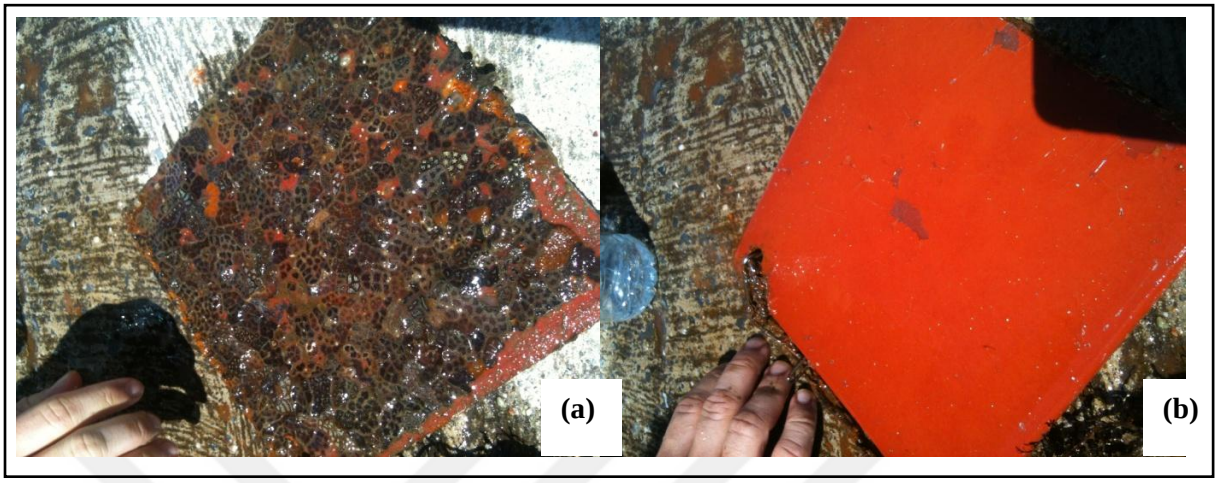

Figure 1.9. Plaques from a preliminary study. (a) One of the plaques from the experiment without the coating, (b) The same plaque’s other side with the prototype antifouling coating. Notice that, some of the paint had come off in the middle and there were some lumps material sticking out on the surface.

1.5.2. Procurement of Machinery

In early 2015, Department of Biotechnology in Yeditepe University obtained an extrusion machine which converts low density polyethylene pellets into either strings or strips. Needless to say, work has started on various antimicrobial formulae and their effectiveness, antialgal formulae turned out to be one of them at the end.

Low-density polyethylene or LDPE is a polymer, started to be produced industrially by Imperial Chemical Industries in Britain in 1939. It is a soft, flexible, colourless, odourless polymer that is used in plastic bags, trays, lids, wraps and packaging; it has varying chemical resistance. [59] Like many other polymers, LDPE is not readily biodegradable and thus accumulates. It is also subject to photodegradation, a process where polymers break into smaller particles when exposed to light but still retain their plastic properties, small enough to be ingested by organisms and to be circulated in the blood stream. LDPE and many other types of plastics find their way to the North Pacific Gyre and form the Great Pacific Garbage Patch, a collection of world’s plastic waste that it is the size of at

least 700.000km^2, about the size of Republic of Turkey, although some estimates are in the range 15.000.000km^2. [60]

1.5.3. Current Study

In this study, several formulations were tested, consisting of zinc pyrithione, zinc oxide and sodium tetraborate pentahydrate. These formulations were separately mixed into LDPE strings and strips.

In early 2015, a joint preliminary study was conducted with Gazi University in Ankara on the effectiveness of these strings. Strings were placed in alga and cyanobacterium broth cultures and their activity was monitored by cell counts; it was soon concluded that the release of the compounds from LDPE was too slow or negligible to affect the population in the broth culture. A second trial was conducted based on Disk Diffusion Essay where a chemical is imbued into a disc and placed on to a spread plate and its effectiveness is measured through the inhibition zone it creates around. Alga and cyanobacterium was spread on agar media, wells were created on them and filled with string pieces. Two weeks after this application, inhibition zones were spotted around the wells. It was concluded that an approach based on the Disk Diffusion Essay could be taken. In order to make the experiment quantifiable, some adjustments were made which will be discussed shortly.

2. MATERIALS

2.1. CHEMICALS

- Distilled water
- Sodium nitrate, Merck
- Potassium nitrate, Sigma-Aldrich
- Magnesium sulphate hepta hydrate, Merck
- Calcium chloride di hydrate, Sigma
- Citric acid, Sigma-Aldrich
- Ferric ammonium citrate, Merck
- Sodium EDTA
- Sodium carbonate, Riedel-de Häen
- Boric acid, Sigma
- Manganese chloride tetra hydrate, Merck
- Zinc sulphate hepta hydrate, Merck
- Sodium molybdate di hydrate, Merck
- Copper sulphate penta hydrate, GPR
- Cobalt nitrate hexa hydrate, Merck
- Agar powder, Sigma
- Zinc pyrithione, Sigma-Aldrich
- Zinc oxide, Sigma-Aldrich
- Sodium tetraborate (borax) pentahydrate, BOREN
- Low-density polyethylene, in pellets, Hanwha Chemical

2.2. EQUIPMENT

- Laminar flow hood, ESCO Class II, Type A2
- Erlenmeyer flasks, 250mL, ISOLAB
- Cotton

- Aluminium foil
- Autoclave, Tuttnauer 5050 ELV
- Centrifuge, eppendorf 5810R
- Petri plates, ISOLAB
- Parafilm
- Plant growth cabin, DigiTech DG12
- Extruder

2.3. ORGANISMS

- *Chlorella vulgaris*
- *Chroococcus sp.*

3. METHOD

3.1. PREPARATION OF BG11 BROTH AND AGAR MEDIA [16]

BG11 is a standard growth medium for cultivation of algae. Unlike most microbiological growth media, it is prepared as a mixture of several different solutions although it is also available commercially. The final concentrations of the compounds is actually quite low, any turbidity or colour may prevent light absorption by algae and decrease growth rate. Several stock solutions are prepared beforehand:

- Sodium nitrate, 150g/L
- Potassium nitrate, 4g/L
- Magnesium sulphate hepta hydrate, 7.5g/L
- Calcium chloride di hydrate, 3.6g/L
- Citric acid, 0.6g/L
- Ferric ammonium citrate, 0.6g/L
- Sodium EDTA, 0.1g/L
- Sodium carbonate, 2g/L
- Trace metal solution
  i. Boric Acid, 2.86g/L
  ii. Manganese chloride tetra hydrate, 1.81g/L
  iii. Zinc sulphate hepta hydrate, 0.22g/L
  iv. Sodium molybdate di hydrate, 0.39g/L
  v. Copper sulphate penta hydrate, 0.079g/L
  vi. Cobalt nitrate hexa hydrate, 0.0494g/L

BG11 medium is normally prepared by mixing 919mL of distilled water with 1mL of trace metal solution and 10mL of the other solutions to a total of 1L; for BG11 agar, 18g of agar powder was added to every litre of BG11 broth. The mixtures are autoclaved afterwards and can be kept at 4°C.

3.2. PREPARATION OF ALGA AND CYANOBACTERIUM CULTURES

- 250mL flasks were filled with 100mL of BG11 broth solution
- Flasks' openings were closed with cotton and covered with aluminium foil
- In the meantime, BG11 Agar solution was also prepared
- Flasks and the agar solution were autoclaved and left to cool afterwards
- Agar solution was distributed into petri dishes
- 1mL of *Chlorella vulgaris* and *Chroococcus sp*. stock cultures were added into the flasks in a laminar flow hood cabin
- Inoculated flasks were left in a plant growth cabin in 21°C temperature and 16-8hrs day/night cycle
- After one week of incubation, contents of these flasks were transferred into 50mL falcon tubes
- These tubes were centrifuged at 10000rpm for 5 minutes, the supernatants were discarded and the pellets were resuspended in 1mL of BG11 broth
- 100µL of these dense mixtures were spread onto BG11 agar plates

3.3. PREPARATION OF LDPE DISKS

- LDPE pellets were loaded into the extrusion machine from point A
- Machine melted the pellets at point B at around 145°C and pushed towards the opening at point C with a screw mechanism,
- The melted polymer was collected by hand until no more comes, this was done to ensure there were no contaminants in the heating chamber
- LDPE pellets and the formulae were loaded into the machine from point A at different runs, they were prepared according to the specifications below:
  i. 5% Zinc pyrithione, 10% zinc oxide, 10% borax pentahydrate, 75% LDPE
  ii. 2.5% Zinc pyrithione, 15% borax pentahydrate, 82.5% LDPE
  iii. 5% Zinc pyrithione, 15% zinc oxide, 80% LDPE
  iv. 3% Zinc pyrithione, 15% zinc oxide, 82% LDPE

v. 15% Zinc oxide, 85% LDPE

Percentages were calculated as weight over weight, all mixtures were mixed by hand.

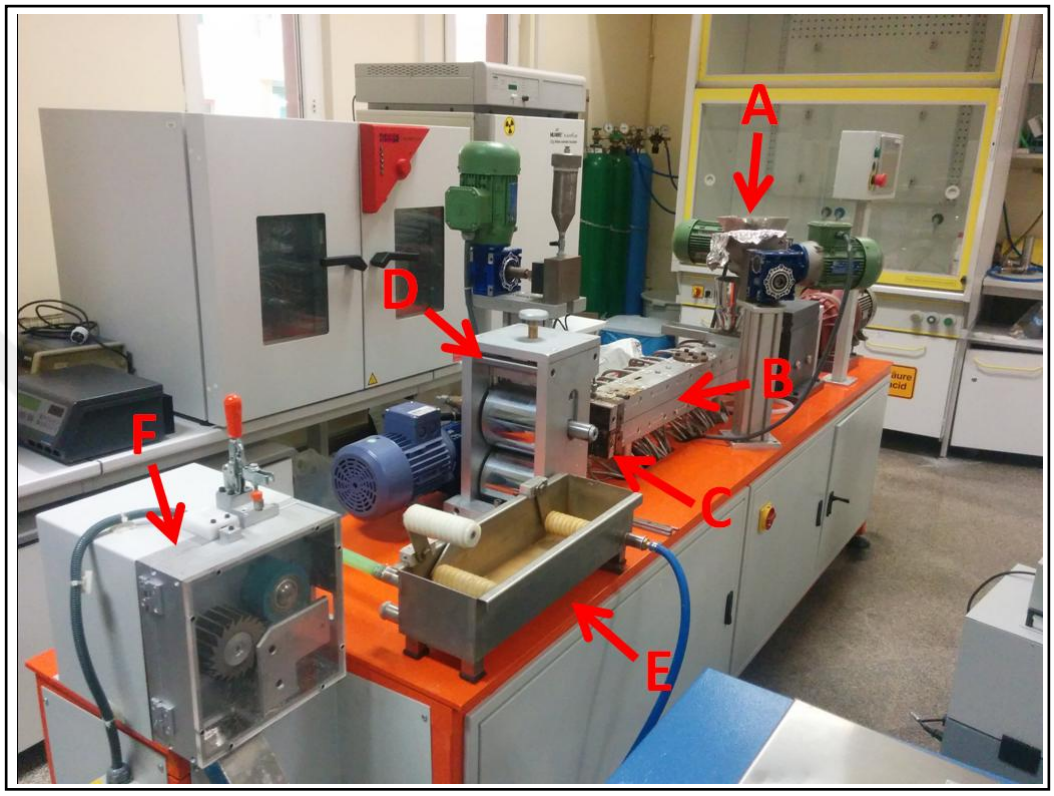

Figure 3.1. Extrusion machine in Biotechnology Department, Yeditepe University.

- For the polymers containing the formulae, each time, the hot polymer was pulled from there which formed a string as it cooled down,
- This string was directed to the water bath at point E which hardened it,
- The cooled string was then directed to the crusher at point F which cut the string into smaller pieces, forming a second set of pellets,
- These pellets were collected from the bottom and were loaded into the machine again at point A for a second run to achieve homogeneity,
- As hot polymer came out of the opening, it was directed to the rollers point D which flattened it out,
- This resulting strip was rolled into a ball manually and marked,

- After polymers were obtained, regular LDPE pellets were loaded into the extrusion machine once again
- The machine was allowed to push out probable contaminants inside with the LDPE pellets to clean it out.
- Uniform holes with 4mm diameters were punched onto the strips with the help of a belt piercer, disks were obtained as a result

3.4. DISK DIFFUSION ASSAY

- Disks obtained from the previous step were then placed onto BG11 agar plates with alga and cyanobacterium spreads from step two
- Petri plates were sealed with Parafilm, marked and placed in a plant growth cabin at 21°C and 16-8hrs day/night cycle
- Zones for *Chlorella vulgaris* were checked after 16 days; zones for *Chroococcus sp.* were checked after 21 days.

4. RESULTS

4.1. RESULTS FOR CHLORELLA VULGARIS

Table 4.1. Zone diameters of the disks on day 16 for *Chlorella vulgaris*, in millimetres

	Disk 1	**Disk 2**	**Disk 3**	**Disk 4**	**Average**	**Standard Dev.**
Pos. Control	0	0	0	0	0	0
Formula 1	8	9	7	7	7,75	0,96
Formula 2	17	16	21	12	16,5	3,7
Formula 3	10	7	7	12	9	2,45
Formula 4	7	0	10	9,5	6,63	4,61
Formula 5	0	10	11	8	7,25	4,99

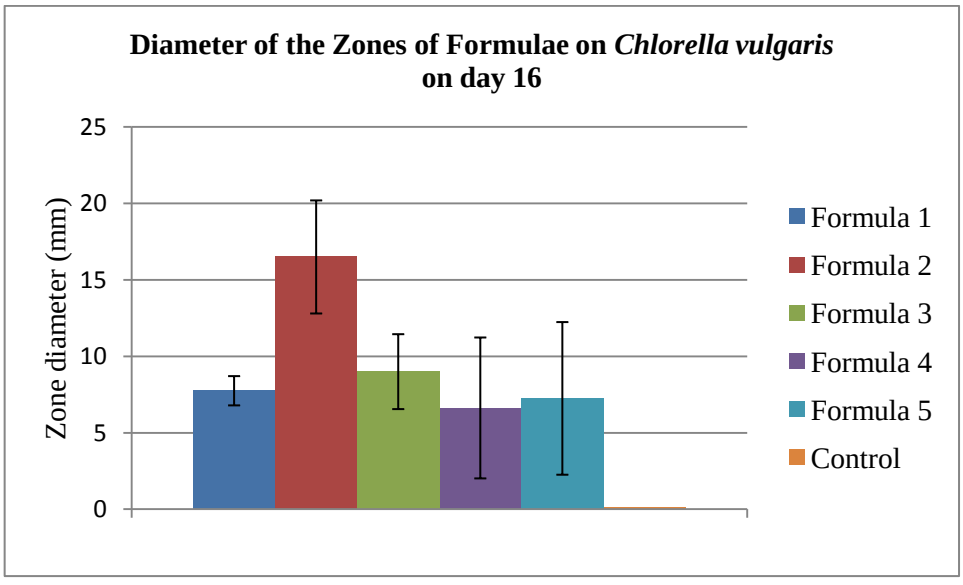

Figure 4.1. Comparative graph of disk zones on *Chlorella vulgaris*

Below are the photographs taken on the 16th day of the experiment from each trial on *Chlorella vulgaris*. The petri plates were marked with both numbers and letters. The numbers signify the formulae: 1 for Formula 1, 2 for Formula 2, 4 for Formula 3 and 5 for

Formula 4. The letters stand for the number of the trial: A for the first one, B for the second and C and D for the third and fourth ones respectively. These trials were arranged in such a manner that in every trial, first four formulae were all tested at the same time. This system doesn't apply for Formula 5; an explanation will be made below. The photographs' contrasts and brightness's were adjusted in order to be perceived better.

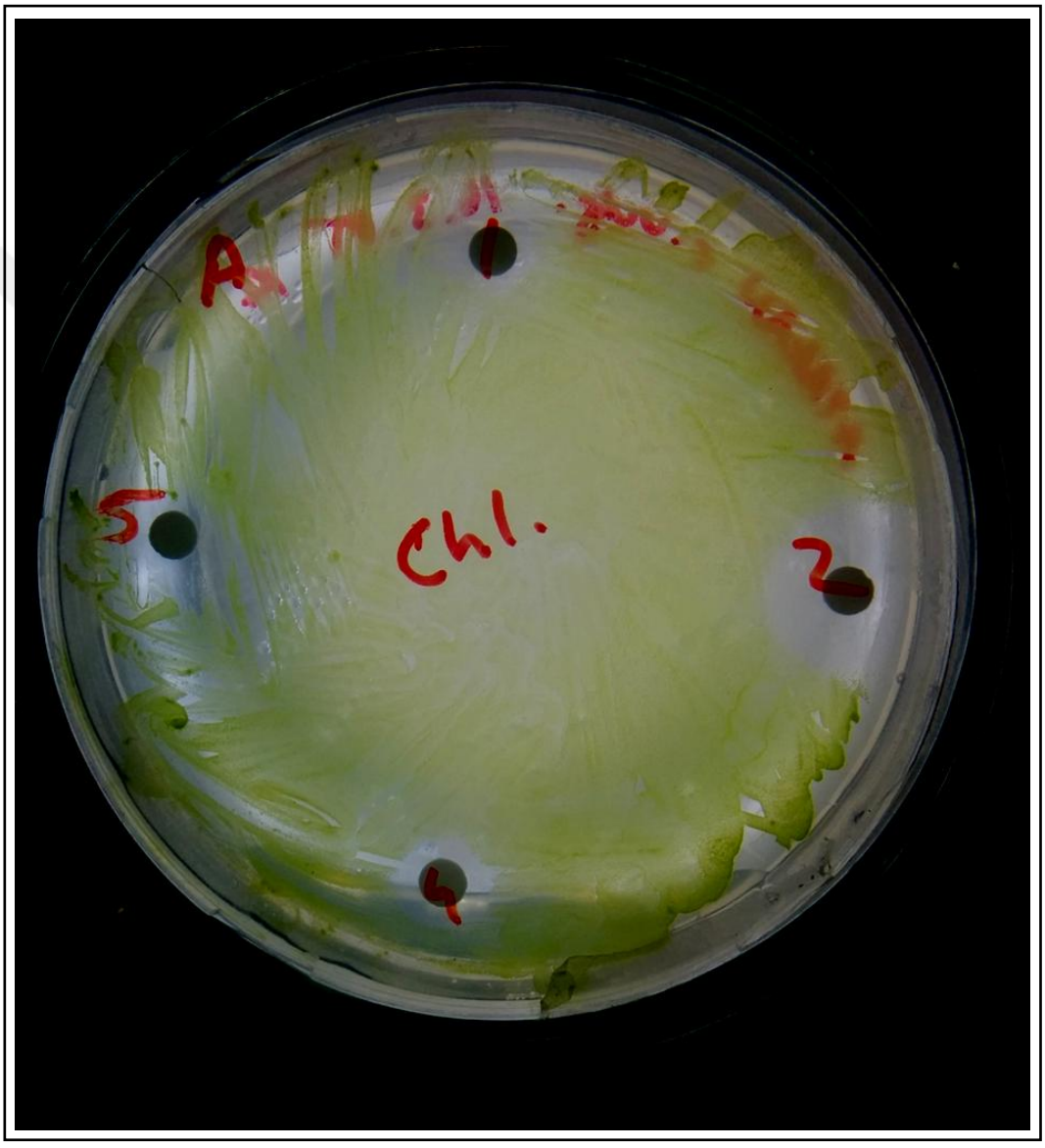

Figure 4.2. First trial of Formulae 1, 2, 3 and 4 on *Chlorella vulgaris*, day 16.

Figure 4.3. Second trial of Formulae 1, 2, 3 and 4 on *Chlorella vulgaris*, day 16.

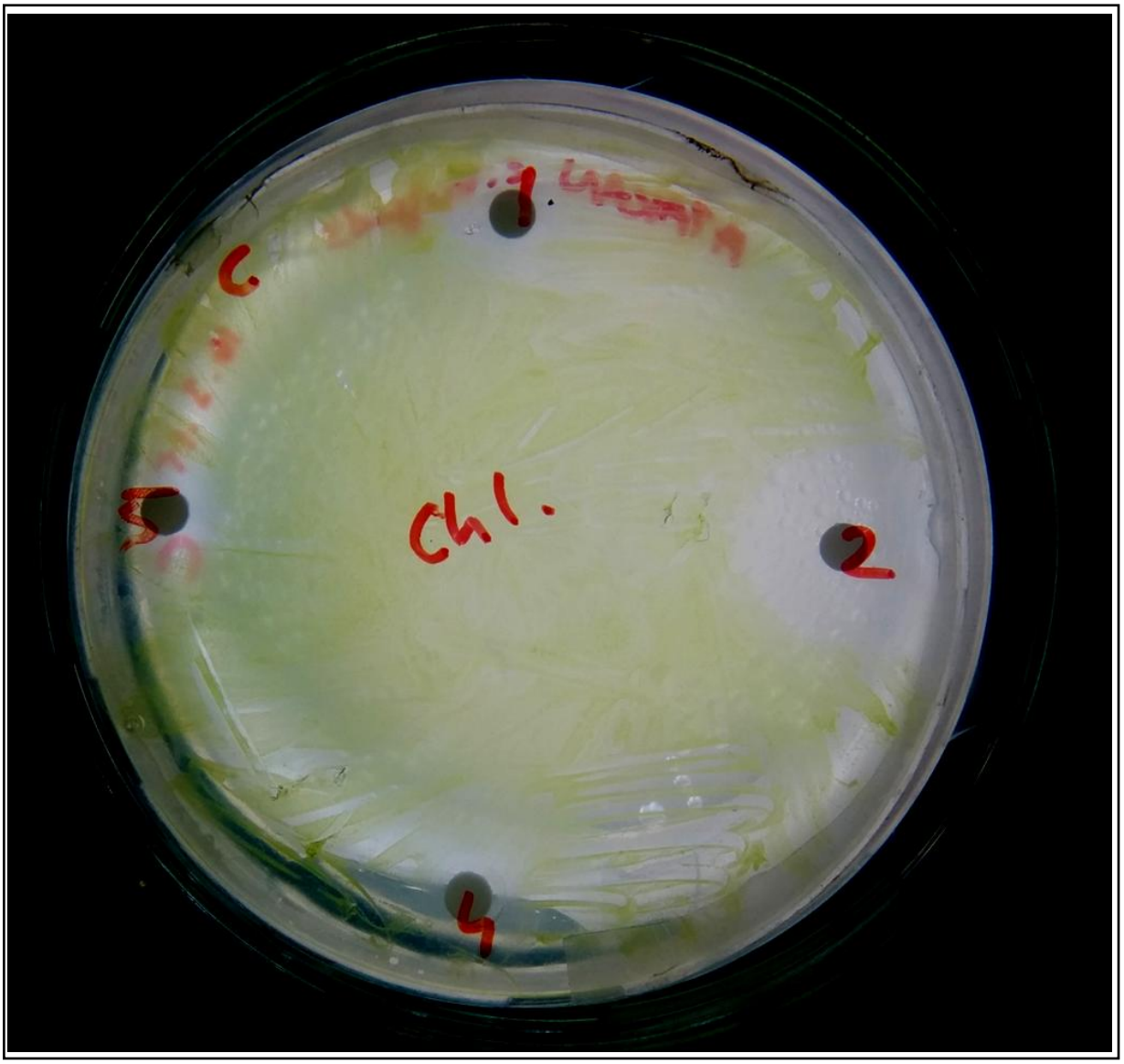

Figure 4.4. Third trial of Formulae 1, 2, 3 and 4 on *Chlorella vulgaris*, day 16.

Figure 4.5. Fourth trial of Formulae 1, 2, 3 and 4 on *Chlorella vulgaris*, day 16.

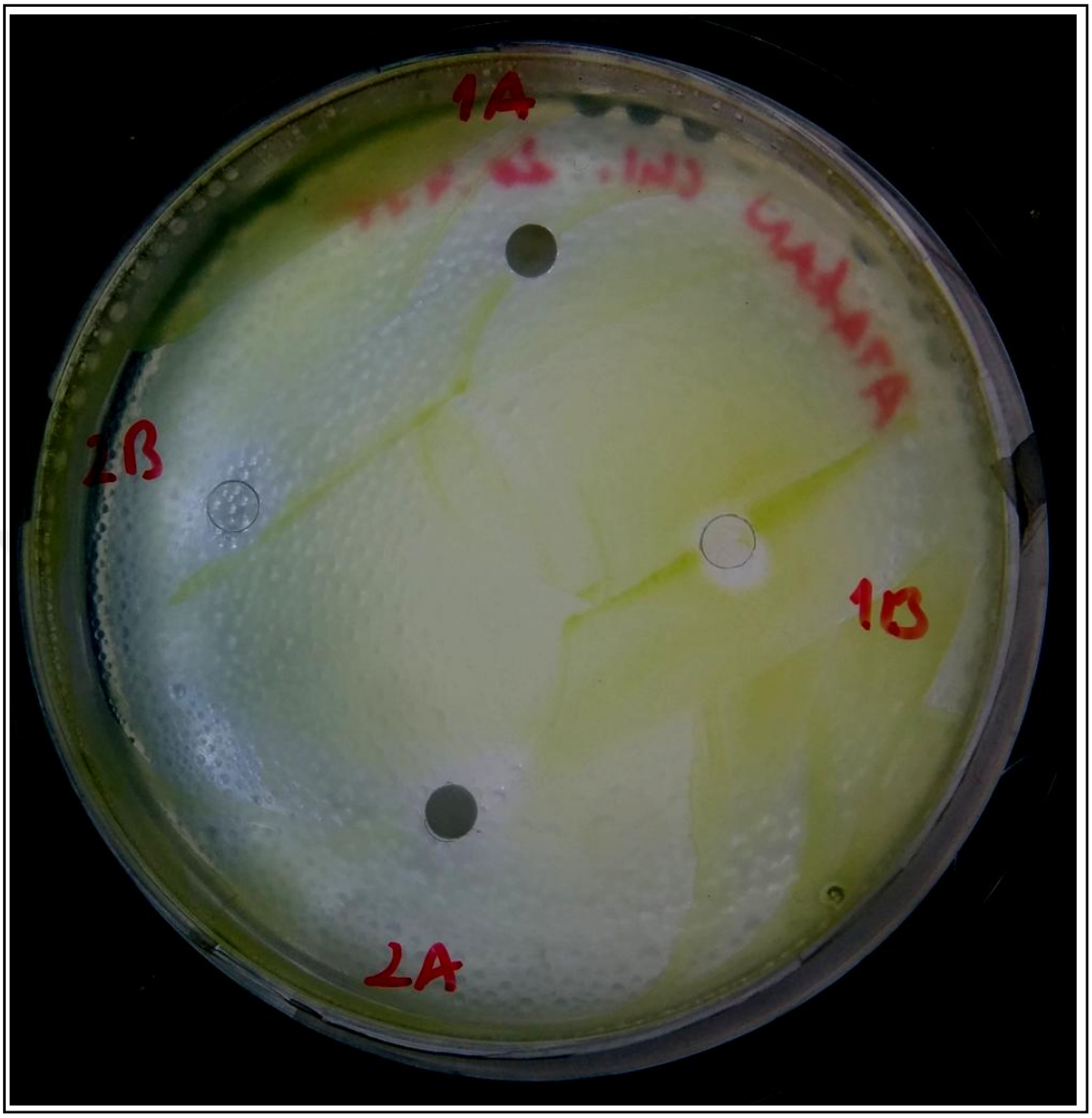

Figure 4.6. First trial of Formulae 5 on *Chlorella vulgaris*, day 16.

For trials on Formula 5, the letter A stands for Formula 5 and the number stands for the trial number; i.e. 1A means Formula 5 – first try. Disks marked with the letter B were also polyethylene disks, meant for another experiment, which wasn't included in this study. The photographs' contrasts and brightness's were adjusted in order to be perceived better.

This set of experiments was conducted some time after the first four, therefore the differences between the markings of the mentioned disks.

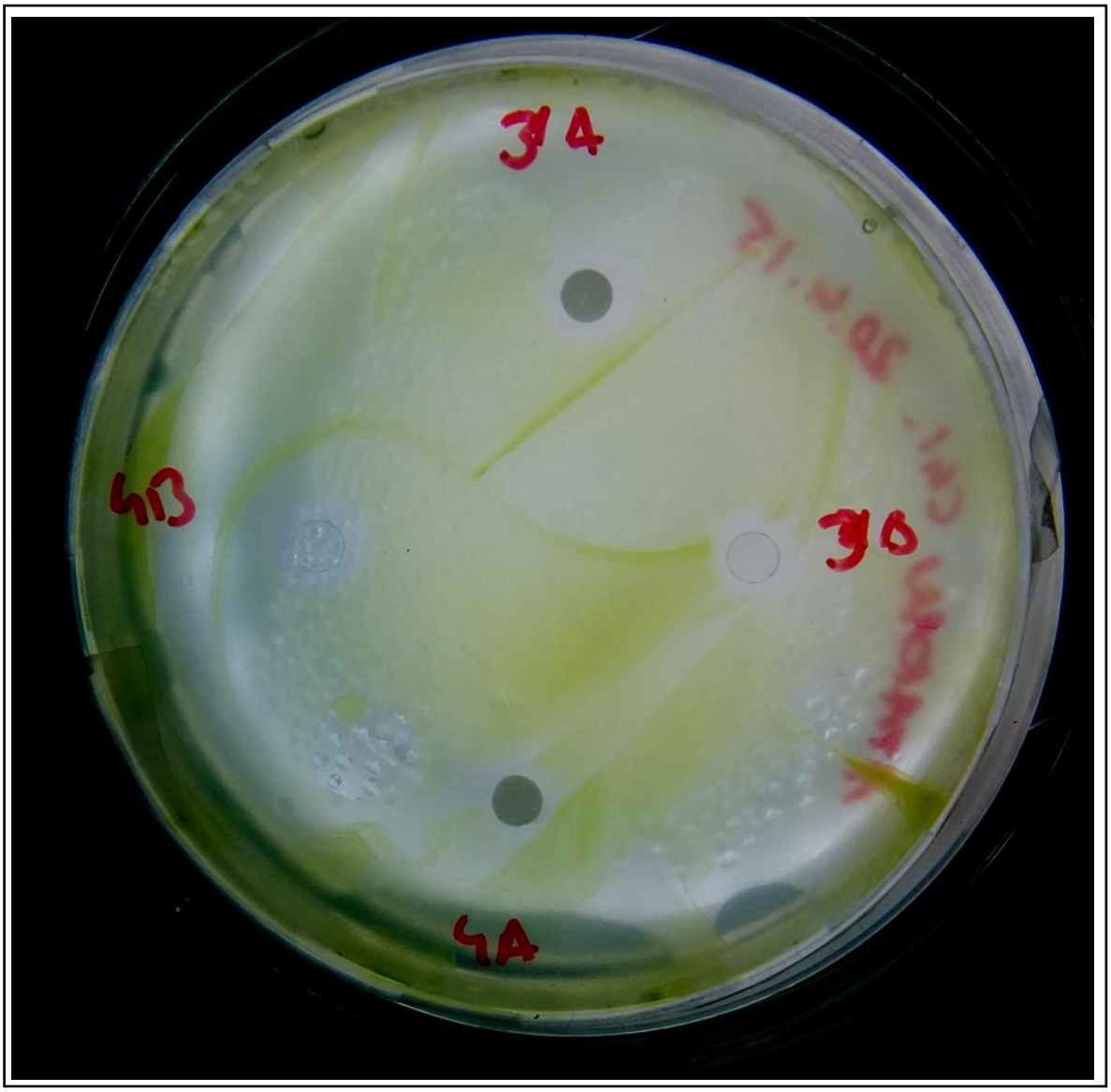

Figure 4.7. Second trial of Formulae 5 on *Chlorella vulgaris*, day 16.

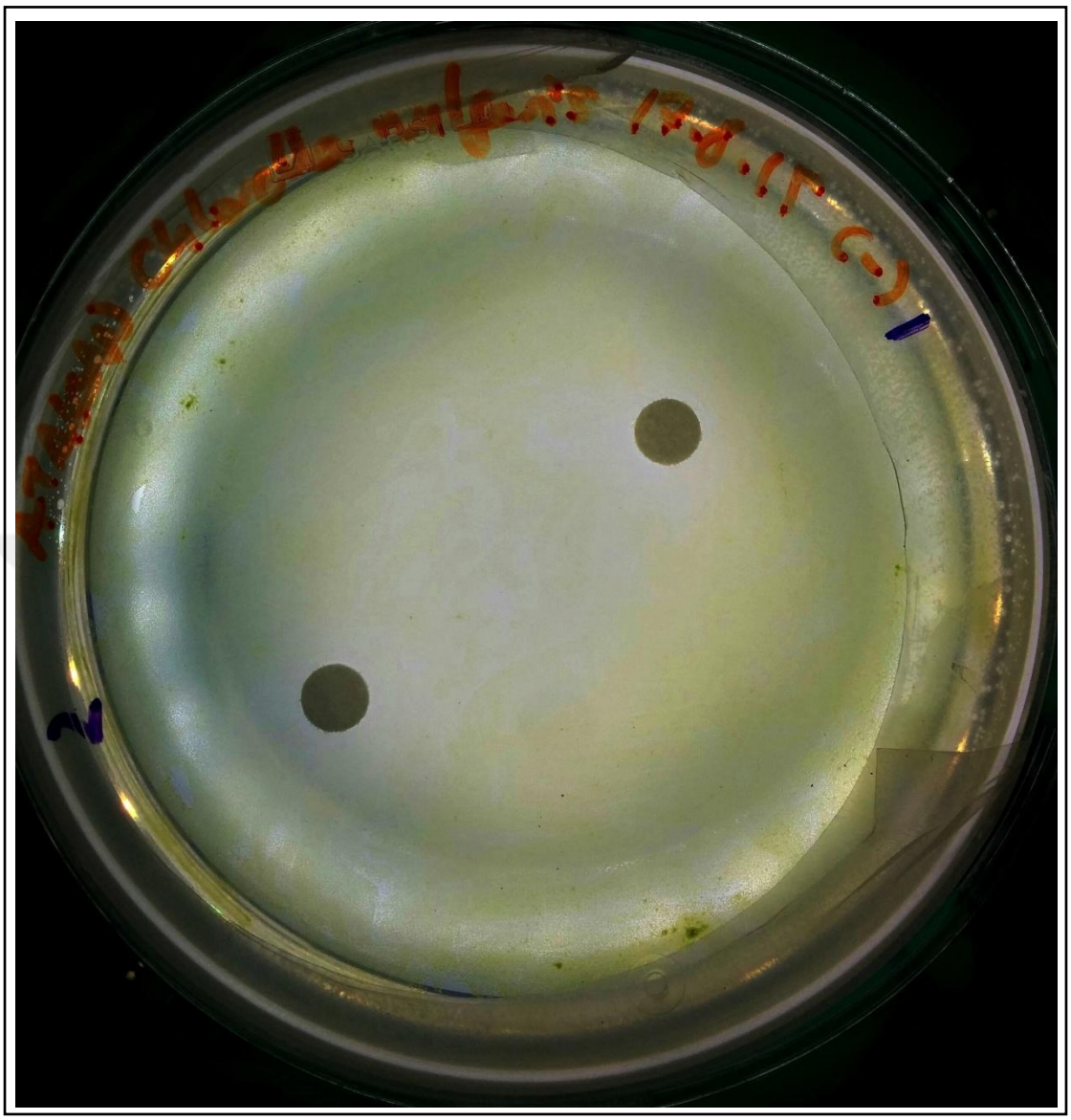

Figure 4.8. First and second trials of the positive control on *Chlorella vulgaris,* day 16

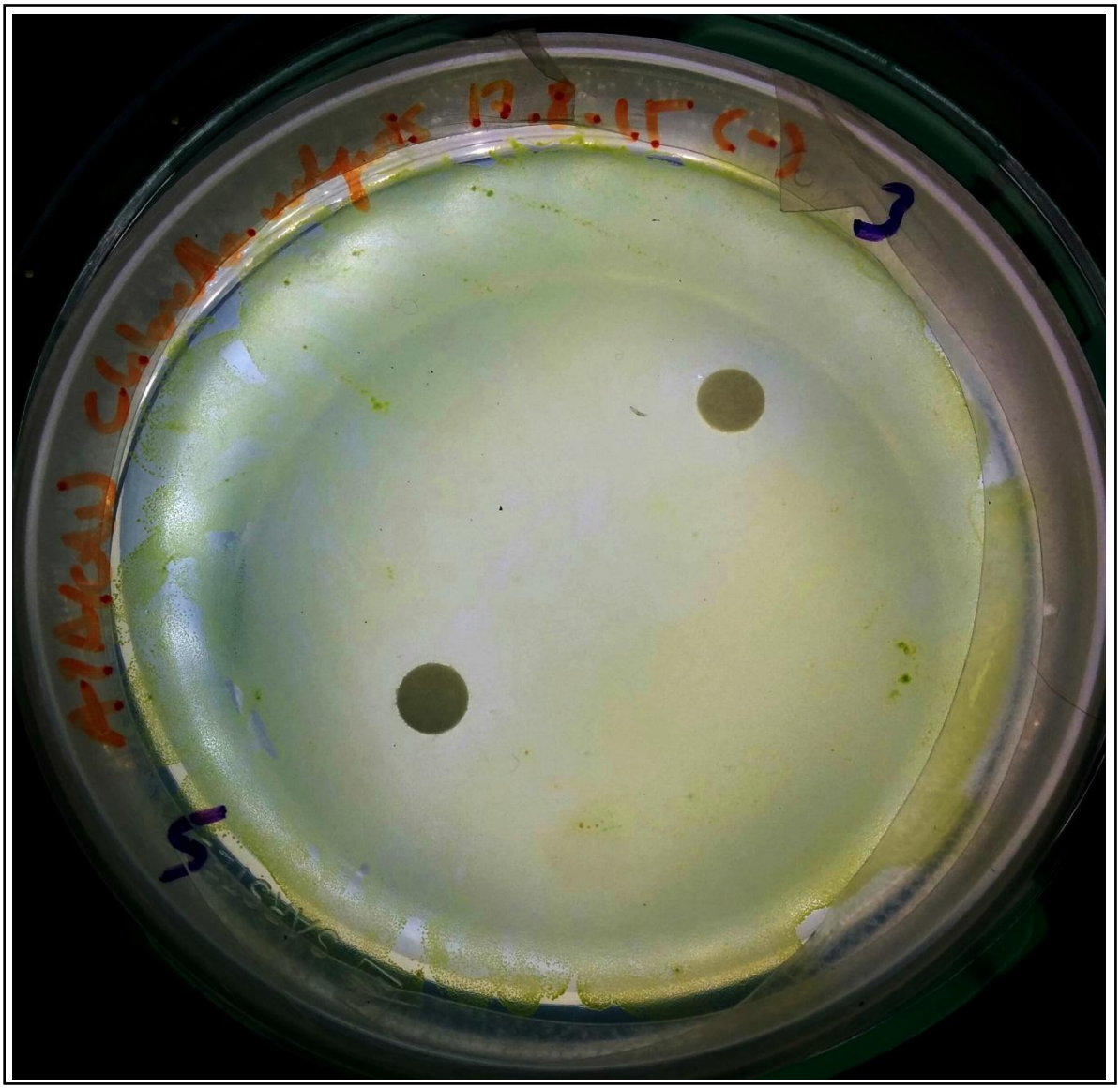

Figure 4.9. Third and fourth trials of the positive control on *Chlorella vulgaris*, day 16

The chemical used as the positive control didn't create a zone around the disk after *Chlorella vulgaris* inoculation. A very faint loss of colour can be spotted around some of the disks but this wasn't enough to conclude on a definite zone diameter. Therefore the said positive control was concluded not to be effective on this organism despite being used as an algicide.

For formulae Number 4 and 5, one disk from each group didn't create a zone around themselves, this will be discussed later; nevertheless when added into the calculations, the error bars turned out to be quite long.

Same thing about the error bars can also be said to Formula number 2, another point which will be discussed later on.

By far the most effective of the formulae was found to be Formula number 2 with an average zone diameter of 16,5mm and a maximum diameter of 21mm. This was followed by numbers 3, 1, 5 and 4 with respective diameters of 9, 7.75, 7.25, 6.63mm. The least effective formula was found to be Formula 4 with an average of 6,63mm; a minimum diameter of 7mm was spotted on several different occasions with Formulae 4, 3 and 5. The reason the average diameter of Formula 4 being smaller than the minimum 7mm was because of the inclusion of 0mm from one of the disks.

4.2. RESULTS FOR CHROOCOCCUS SP.

Table 4.2. Zone diameters of the disks on day 21 for *Chroococcus sp.*, in millimetres.

	Disk 1	**Disk 2**	**Disk 3**	**Disk 4**	**Average**	**Standard Dev.**
Pos. Control	23	20	26	24	23,3	2,5
Formula 1	22	26	28	24	25	2,58
Formula 2	36	39	40	28	35,8	5,44
Formula 3	38	32	32	32	33,5	3
Formula 4	19	22	20	18	19,8	1,71
Formula 5	27	28	38	44	33,5	9,15

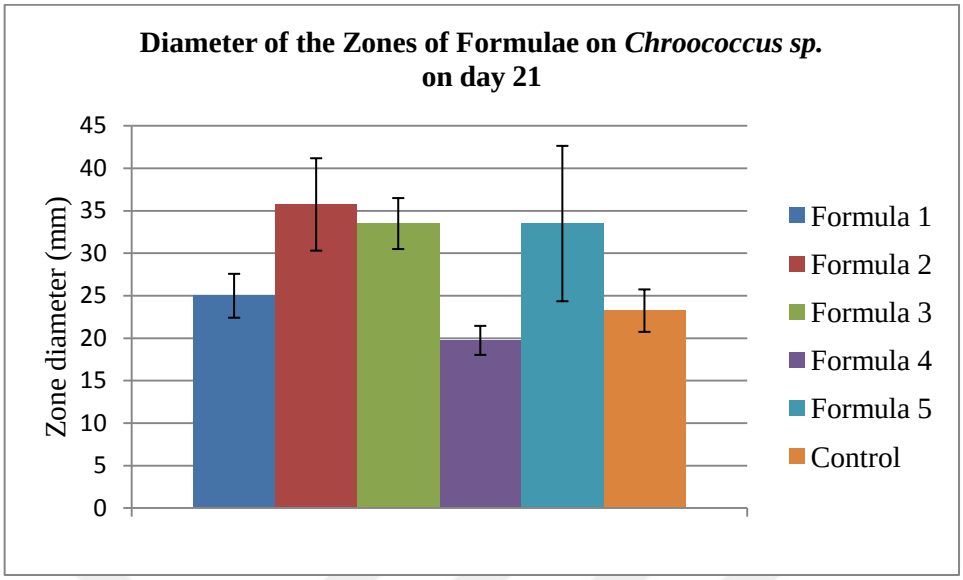

Figure 4.10. Comparative graph of disk zones on *Chroococcus sp.*

Below are the photographs taken on the 21st day of the experiment from each trial on *Chroococcus sp*. Just like for *Chlorella vulgaris,* the petri plates were marked with both numbers and letters. The numbers signify the formulae: 1 for Formula 1, 2 for Formula 2, 4 for Formula 3 and 5 for Formula 4. The letters stand for the number of the trial: A for the first one, B for the second and C and D for the third and fourth ones respectively. These trials were arranged in such a manner that in every trial, first four formulae were all tested at the same time. This system doesn't apply for Formula 5; an explanation will be made below. The photographs' contrasts and brightness's were adjusted in order to be perceived better.

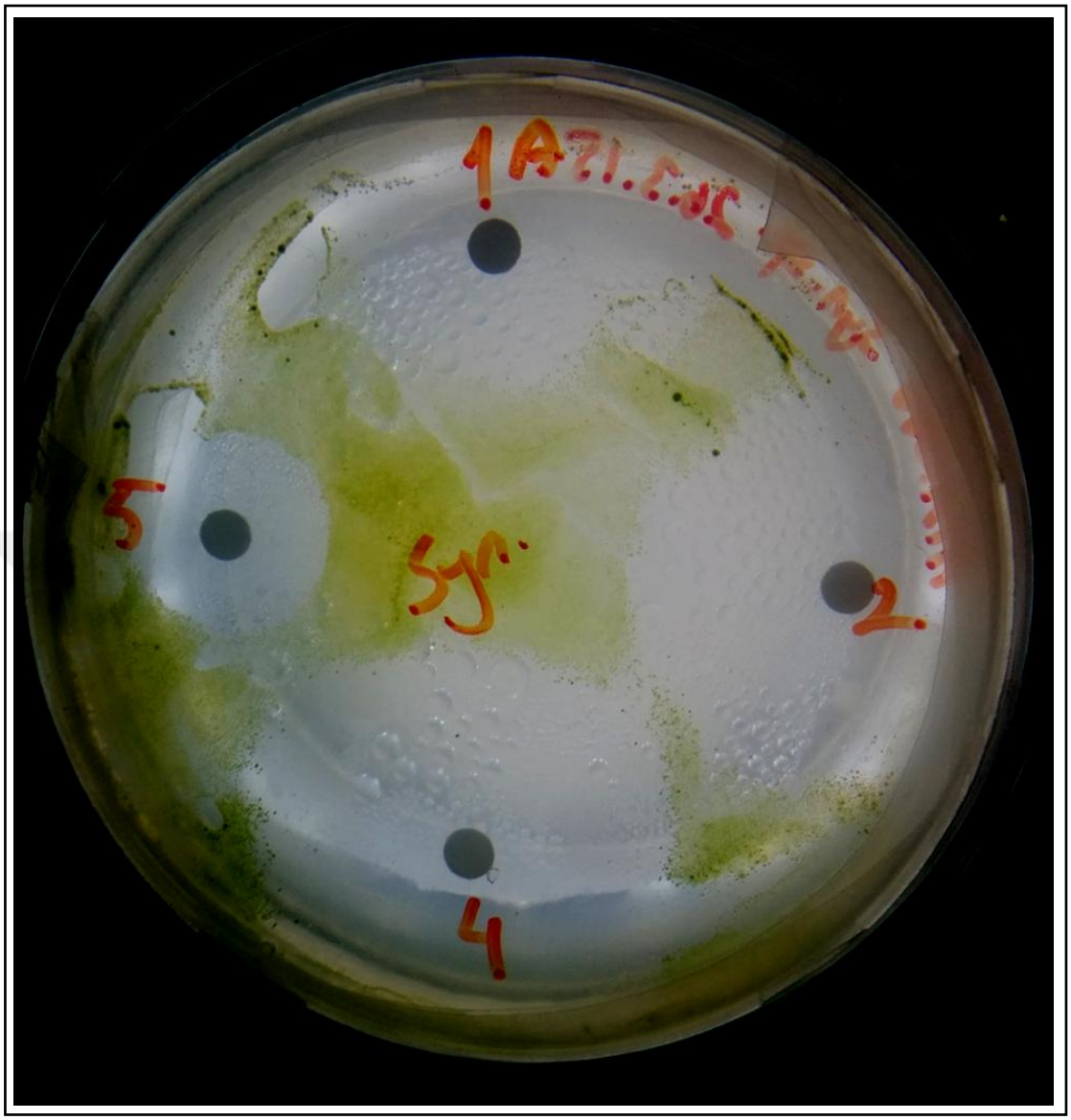

Figure 4.11. First trial of Formulae 1, 2, 3 and 4 on *Chroococcus sp.*, day 21.

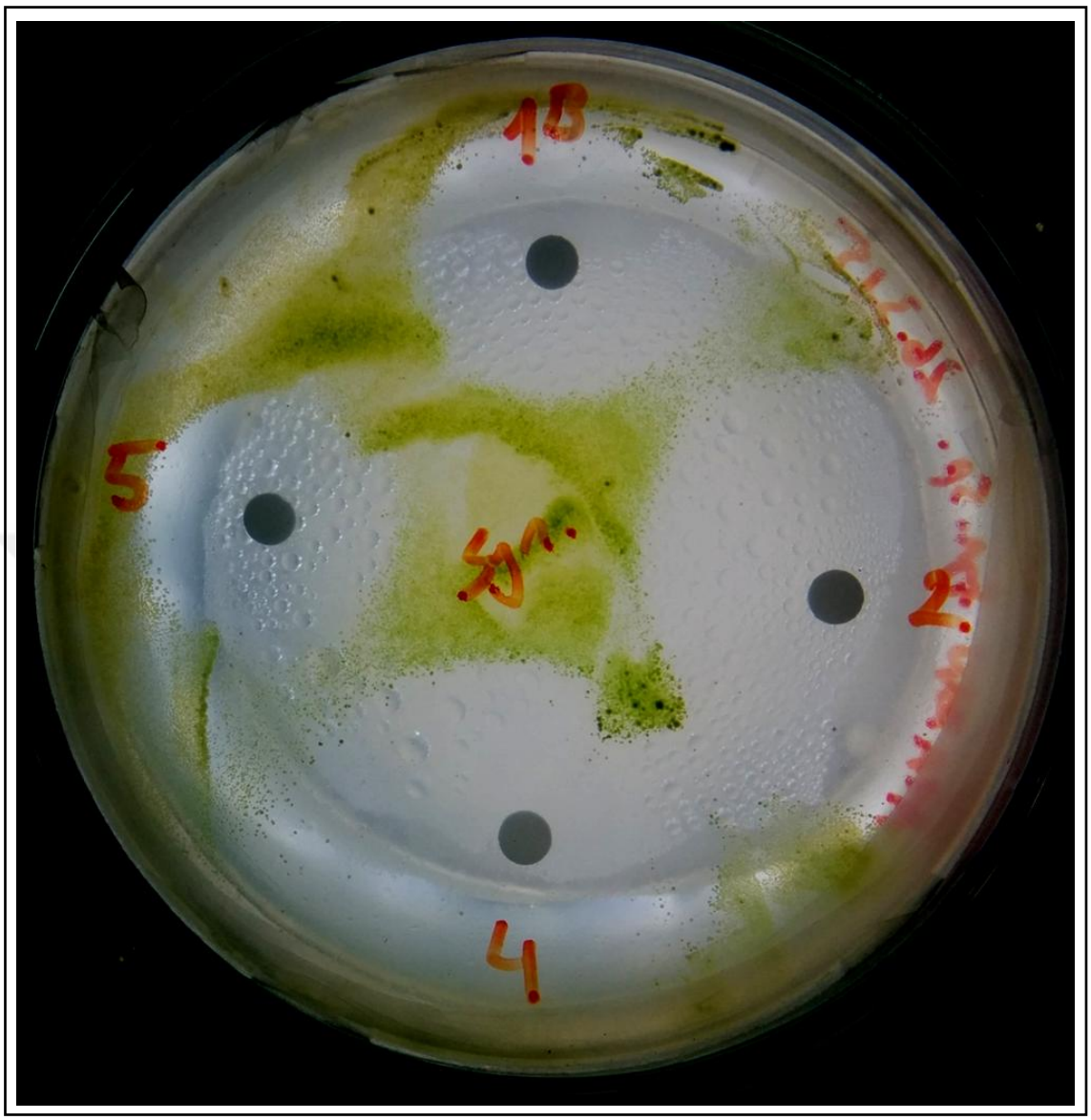

Figure 4.12. Second trial of Formulae 1, 2, 3 and 4 on *Chroococcus sp.*, day 21

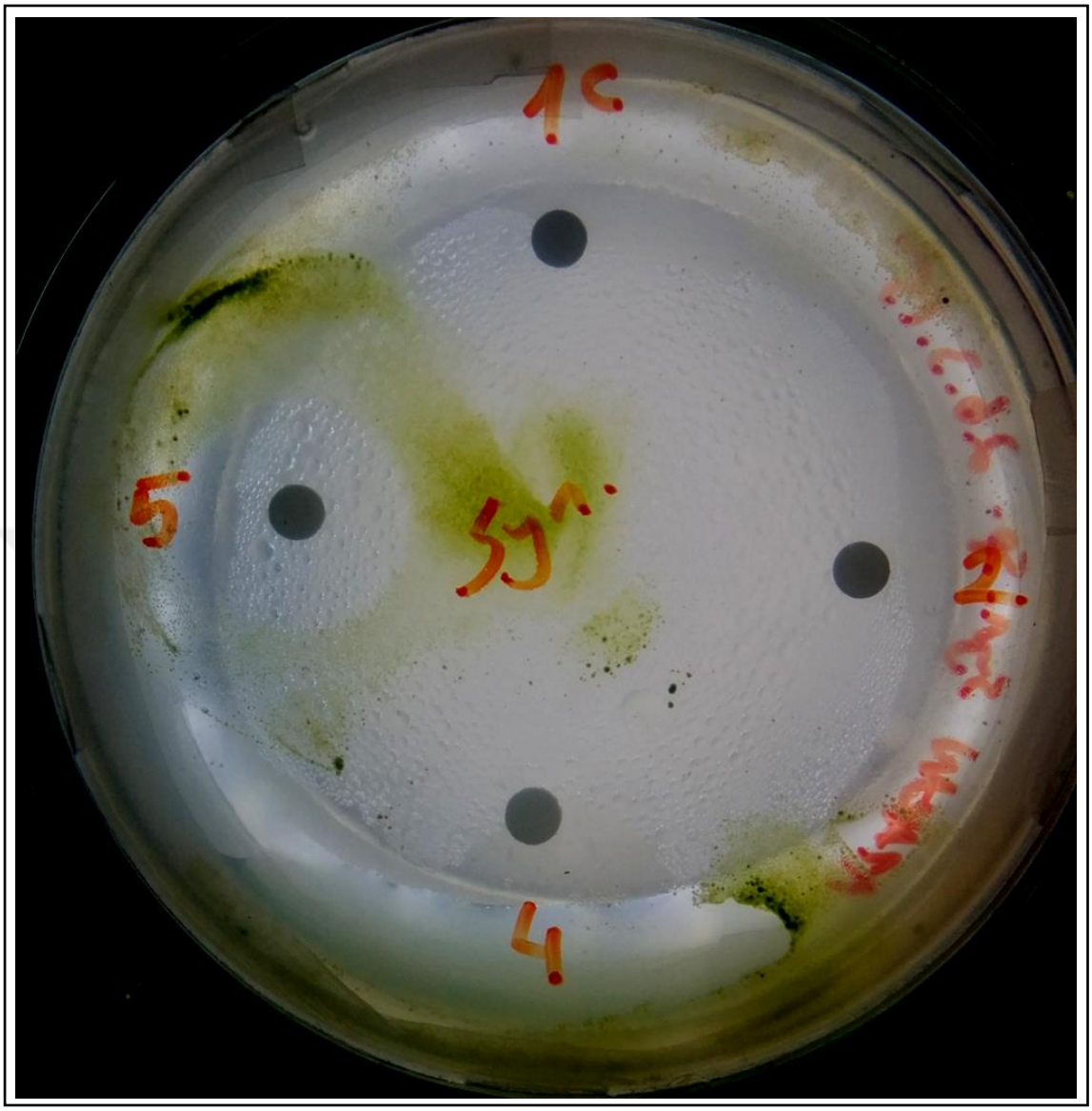

Figure 4.13. Third trial of Formulae 1, 2, 3 and 4 on *Chroococcus sp.*, day 21

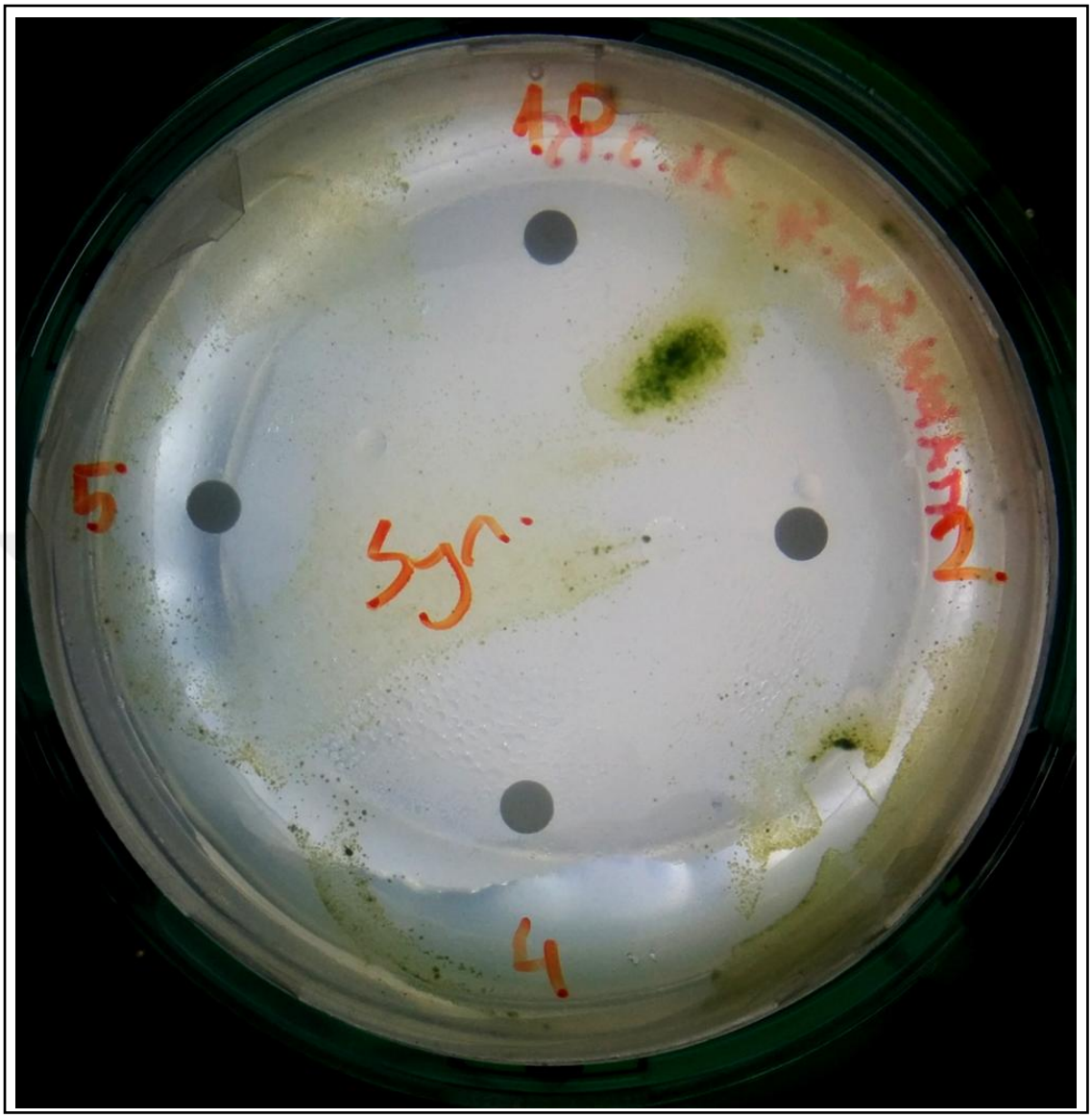

Figure 4.14. Fourth trial of Formulae 1, 2, 3 and 4 on *Chroococcus sp.*, day 21

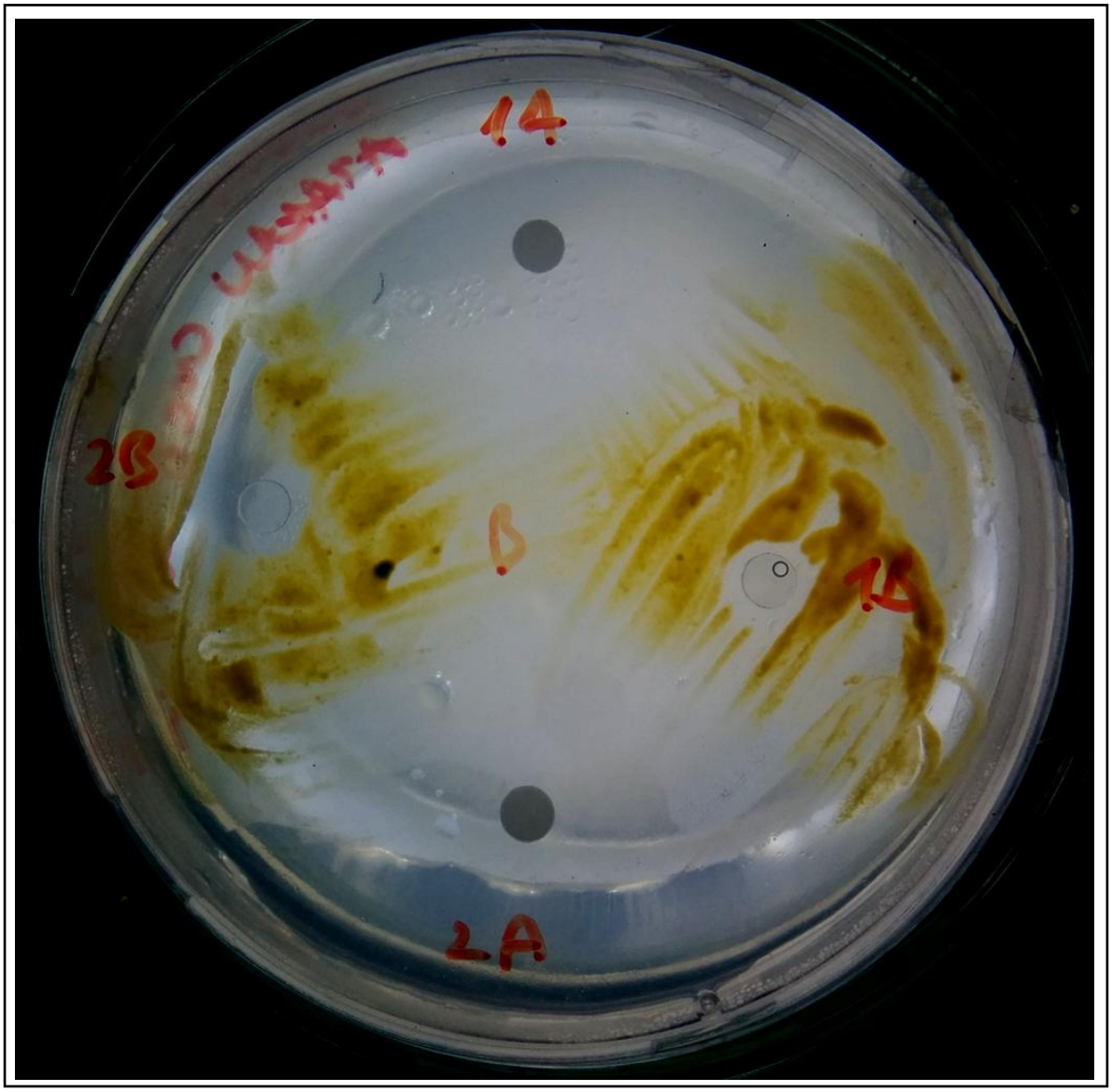

Figure 4.15. First trial of Formulae 5 on *Chroococcus sp.*, day 21.

For trials on Formula 5 the same rule applies from the experiment with *Chlorella vulgaris*, the letter A stands for Formula 5 and the number stands for the trial number; i.e. 1A means Formula 5 – first try. Disks marked with the letter B were also polyethylene disks, meant for another experiment, which wasn't included in this study. The photographs' contrasts and brightness's were adjusted in order to be perceived better.

This set of experiments was conducted some time after the first four, therefore the differences between the markings of the mentioned disks.

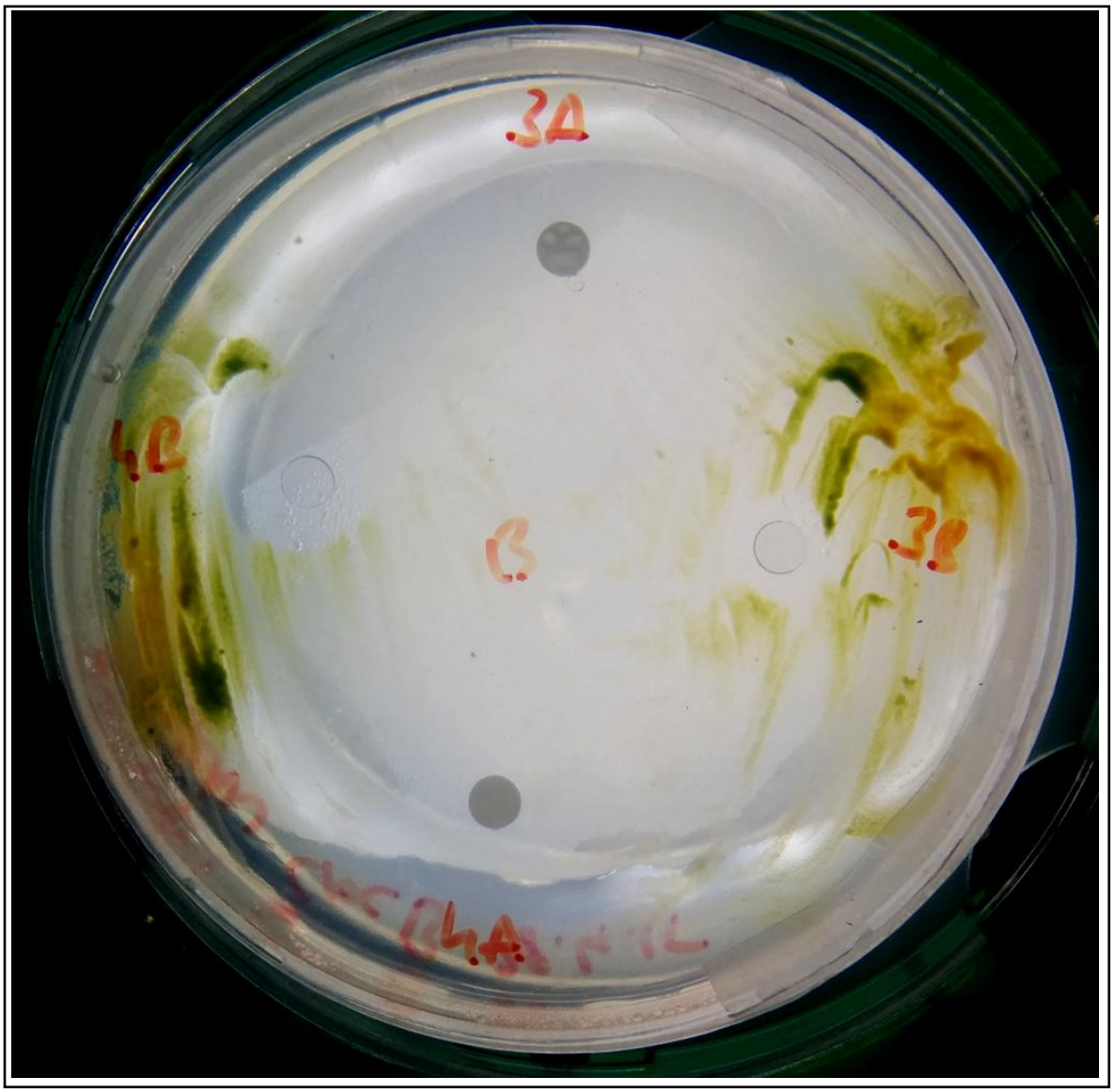

Figure 4.16. Second trial of Formulae 5 on *Chroococcus sp.*, day 21.

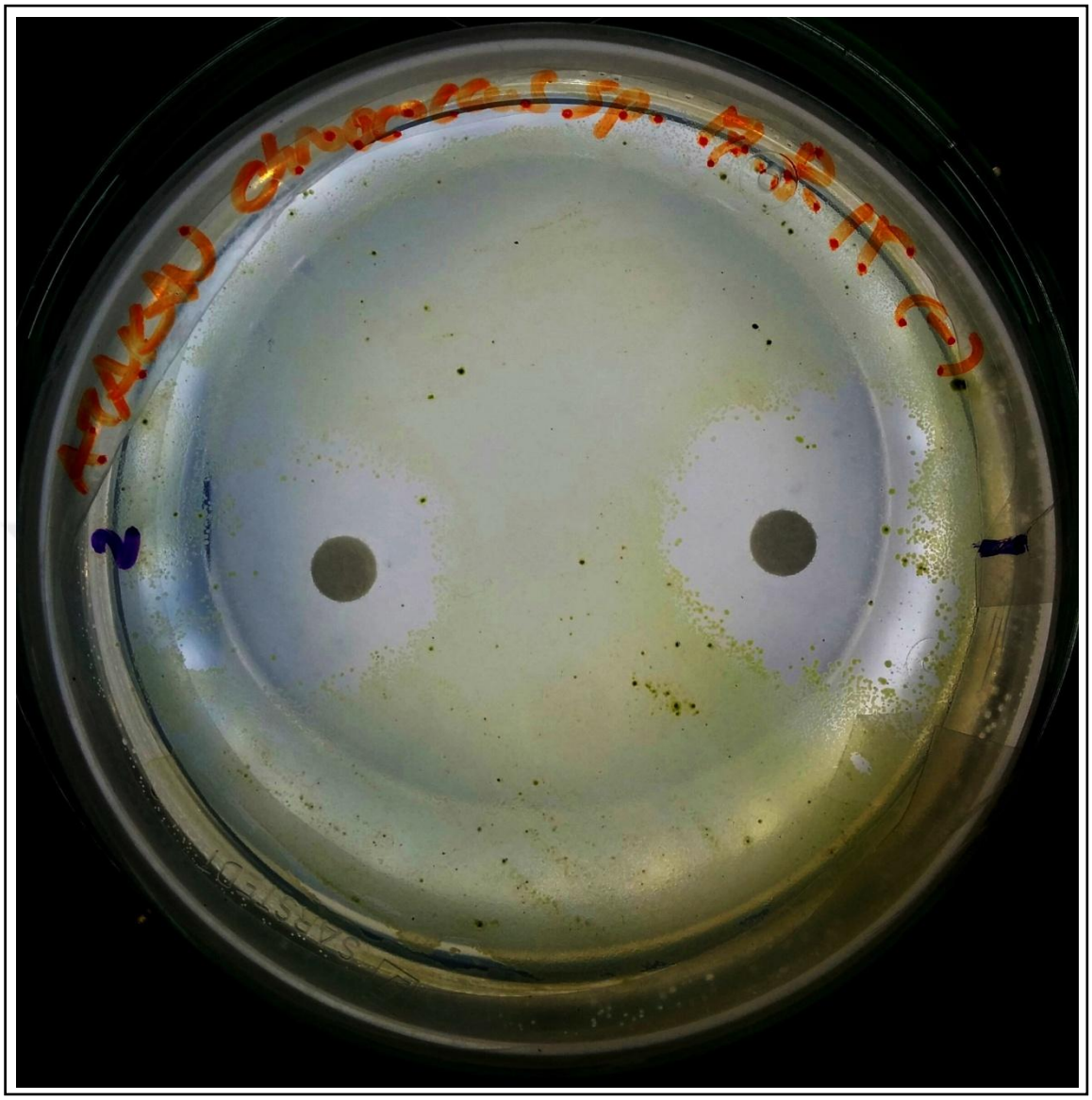

Figure 4.17. First and second trials of the positive control on *Chroococcus sp.*, day 21

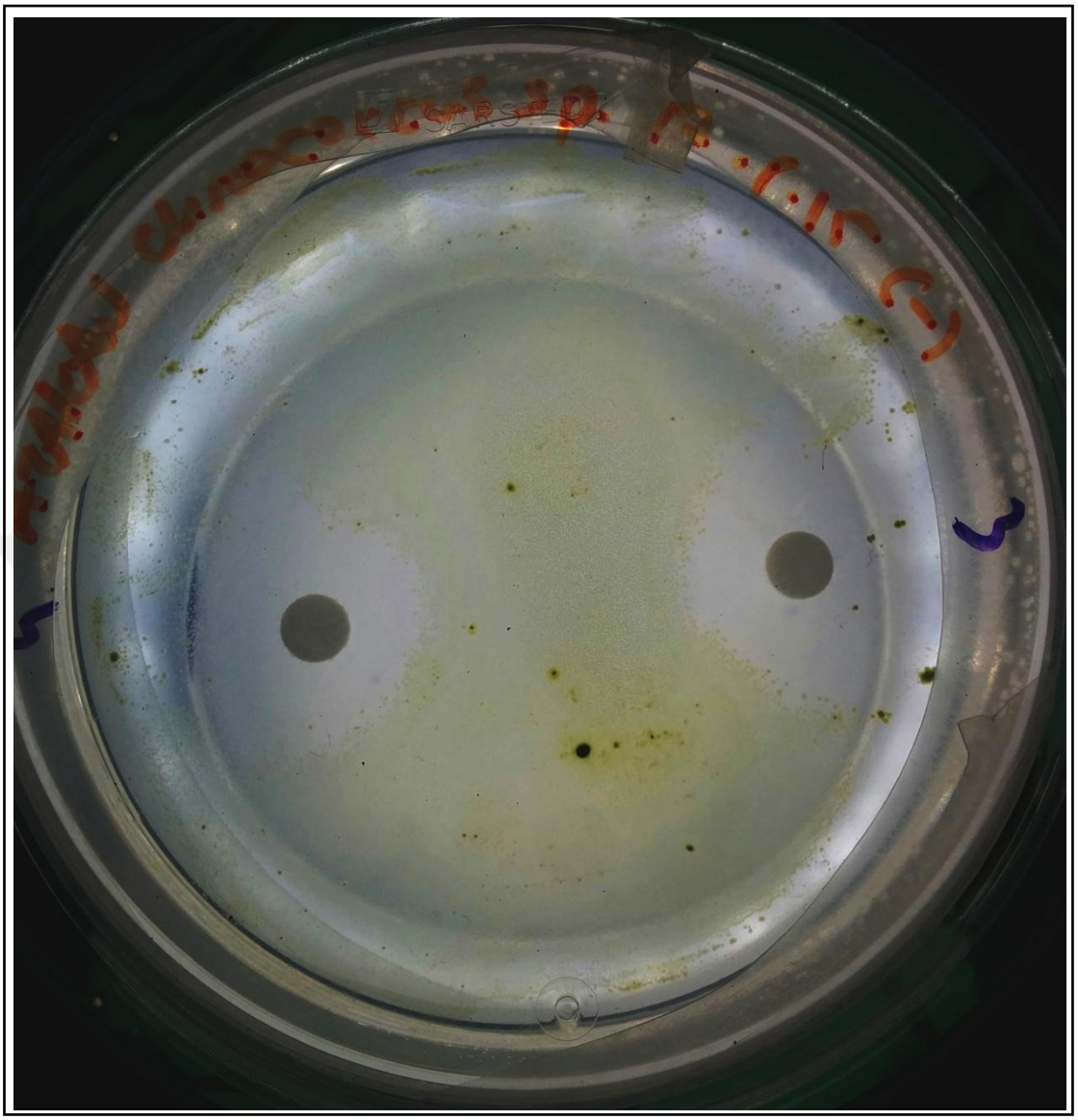

Figure 4.18. Third and fourth trials of the positive control on *Chroococcus sp.*, day 21

As it can be seen from the figure and the table, overall effectivenesses of the formulae were much higher in *Chroococcus sp.* than *Chlorella vulgaris.* Unlike the previous trial, the chemical used for positive control created a zone around the disk therefore created the opportunity for comparison.

Both Formula 2 and 5 had great effectiveness against *Chroococcus sp.* here with Formula 2 having an average diameter of 35,8mm while Formula 5 having 33,5mm; it should be noted that although Formula 2's overall effectiveness was higher, the maximum diameter was recorded in a trial with Formula 5 with 44mm. The wide error bar with Formula 5 will be discussed later on, as with others.

As mentioned just now, the highest effectiveness was observed with Formula number 2 with 35,8mm, followed by a tie between numbers 5 and 3 at 33,5mm and then followed by numbers 1 and 4 with diameters 25 and 19,8mm respectively.

The least effective formula in both trials was found to be Formula number 4 while the most effective formula was found to be number 2.

The influences of the chemicals and other factors will all be discussed in the next section.

5. DISCUSSION

As mentioned earlier in the introduction section, current approach against biofouling is through development of special antifouling paints that inhibit growth on the applied surface. During this experiment, a similar approach was made, by coming up with formulae that once embedded inside a polymer, will inhibit fouling. Formulae were prepared based on previous knowledge of materials with antimicrobial and proven or suspected antialgal properties. These formulae were mixed into polymers and cast together into strings and strips, without chemically imbuing the material to the monomers.

When the results are taken into consideration, it can be concluded that all formulae are effective to some degree, ranging from high to low. These results were expected since zinc pyrithione, zinc oxide and borax pentrahydrate all have proven cidal effects on various life forms. The unexpected were no zones of inhibition as seen with some of the disks. This can be the result of heterogeneity within the disks. Since all formulae were prepared and mixed into the polymer by hand, it is highly likely that a truly homogenous product would not be made. The products were sent into the extrusion machine for a second time to achieve a level of homogeneity but this turned out to be not enough in some cases. It should be noted that the formulae work by their diffusion into the matrix and any barrier inhibiting this will also prevent any success with inhibition; that is, if even a thin layer of LDPE coats a formula microclump, it won't find its way and diffuse properly. Our department's lack of expertise in polymer casting and structure allowed the experiment to be conducted in this manner though the results were still found to be satisfactory.

Another approach to this experiment could be adding the agents to the chemical structure of the monomers but this idea was dropped out due to lack of experience in the field. This approach can't use this model though; a setup has to be prepared in such a way that those disks or surfaces are left in a broth culture to allow algae to create biofouling. Since the chemical structure of the polymer will be changed, no diffusion into the environment will be foreseen.

If the results are to be interpreted, Formula number 2 comes out at the top in both experiments, with the alga and the cyanobacterium. In contrast to others, Formula 2 contains more of borax pentahydrate. This result may indicate that borax pentahydrate was

responsible for the success but this can't be deduced solely by this try only, since the closest composition to the one of Formula 2 was Formula 1's and still Formula 1 had very different amounts of both zinc pyrithione and zinc oxide. Still, it can be claimed that borax pentahydrate was a positive influence on the outcome.

The aim of this study was presented at the beginning as testing compounds with proven anti-algal activity when they are mixed into low-density polyethylene; the aim was met, these compounds are shown to exhibit the same properties when they are inside a polymer cast. A qualitative analysis is not always enough to conclude on the future prospects of these compounds, many more experiments and tries are needed to be done.

The surprise for *Chlorella vulgaris* was that the positive control didn't work on it; this was an unexpected result for a formula which is marketed for its antialgal properties. But since it did create a zone of inhibition for *Chroococcus sp.* and is currently in circulation in the market, it can be said that the formula doesn't work on this specific strain.

Assuming that these formulae will become antifouling products in marine shipping industry, there are two very important areas that needs more exploration. The first one is the minimum effective concentration. This experiment was carried out with only five formulae which differed very much in their content; the objective there was to find possible formulae with anti-algal properties. In a more comprehensive study, many more tries should be made, starting with different weight over weight percentages of the same compound followed by their mixtures; the role of each compound in the formula can only be understood when they are tested separately and at different amounts. Once the effects are understood, the formulae can be tweaked accordingly to come up with the most suitable and economically feasible solution. The lack of time and expertise didn't allow a comprehensive study but with a large time interval and enough resources, such a study can be realized.

The second most important aspect of such a product is its life. Even if such an antifouling product is effective, it has to retain this property for some time. Currently, antifouling paints exhibit their properties for a maximum duration of about two years; hull paint generally has to be renewed after this. This long effective life can be achieved by two

means. When the polymer or the paint is applied in such a way that it forms a scaffold and when this scaffold contains the formula and allows a slow release over time, the antifouling effect can be maintained for a desired amount of time. For paint, this may not be the case because of probable amateur applications but polymers can be prepared beforehand industrially and professionally and applied to the surface. They may even be prepared in slabs or sheets and used to coat the hull with resin. The solution which Muntz came up with with his metal wasn't such a bad idea after all. A second strategy would be to trap the formula or its contents within the chemical structure of the paint. When the compounds are chemically imbued into the paint, it is possible that as long as the chemical bonds aren't disrupted, the antifouling activity will be retained; there might be a problem with this strategy though. Since the inhibition zones were created with the diffusion of the material, it can be safely assumed that the anti-algal property was achieved when the contents of the formulae were made available to the microorganisms, so to speak; if these compounds can't diffuse, the target algae may not give the same reaction.

This experiment, like many others in the world of science, was done in very confined and ideal conditions; the growth medium, day/night cycle, the temperature, were all standardized to allow maximum algal growth. Marine and freshwater environments aren't ideal; they contain countless compounds and organisms. The contents of the formula can react with the surroundings and produce something else, an example to this copper pyrithione which is formed when zinc pyrithione reacts with copper ions in water, copper pyrithione is more stable and considered more toxic to the environment. [55] Another possible outcome would be enhancement of biofouling on the surface; since all of the elements in the formulae are also essential elements to the algae, there could be species of algae or even macrofoulers that would prefer to settle on these abundant resources of essential nutrients. Any product needs to be tested in real life conditions before drawing on conclusions.

Probabilities aside, this experiment proved that an antialgal formulae from zinc and boron based compounds can be made and can be mixed into low-density polyethylene to exhibit this property. Many improvements have to be made but this is a good start.

REFERENCES

1. R. E. Lee. *Phycology*, Cambridge University Press, 2008.

2. Harmonic Arts Botanical Dispensary, "*Chlorella vulgaris"*, https://harmonicarts.ca/2013/04/chlorella-chlorella-vulgaris/ [retrieved 1 October 2015]

3. Culture Collection of Autotrophic Organisms CCALA, "*Chroococcus minutus*", http://ccala.butbn.cas.cz/en/chroococcus-minutus-kuetzing-naegeli [retrieved 1 October 2015]

4. H. D. Holland. The Oxygenation of the Atmosphere and Oceans. *Philosophical Transactions of the Royal Society B,* 361:903–915, 2006.

5. É. Chatton. Pansporella Perplexa. Réflexions sur la Biologie et la Phylogénie des Protozoaires. *Annales des Sciences Naturelles - Zoologie*, 10:1–84, 1925.

6. Jean P. Euzeby, List of Prokaryotic with Standing and Nomenclature, Classification of Cyanobacteria, http://www.bacterio.net/-classifcyano.html [retrieved 1 October 2015]

7. N. Quintana, F. Van der Kooy, M. D. Van de Rhee, G. P. Voshol and R. Verpoorte. Renewable Energy from Cyanobacteria: Energy Production Optimization by Metabolic Pathway Engineering. *Applied Microbiology and Biotechnology*, 91:471–490, 2011.

8. E. Christaki, P. Florou-Paneri and E. Bonos. Microalgae: A Novel Ingredient in Nutrition. *International Journal of Food Sciences and Nutrition*, 8:794–799, 2011.

9. G. I. McFadden. Primary and Secondary Endosymbiosis and the Origin of Plastids. *Journal of Phycology*, 37:951–9, 2001.

10. J. T. Clarke, R. C. M. Warnock and P. C. J. Donoghue. Establishing a Time-scale for Plant Evolution. *New Phytologist*, 192:266–301, 2011.

11. K. M. Cole and R. G. Sheath. *Biology of the Red Algae*, Cambridge University Press, Cambridge, 1990.

12. J. D. Palmer, D. E. Soltis and M. W. Chase. The Plant Tree of Life: An Overview and Some Points of View. *American Journal of Botany*, 91:1437–1445, 2004.

13. D. W. Schindler and J. R. Vallentyne. *Over Fertilization of the World's Freshwaters and Estuaries*, University of Alberta Press, Alberta, 2008.

14. W. Belasco. Algae Burgers for a Hungry World? The Rise and Fall of Chlorella Cuisine. *Technology and Culture*, 38:608-634, 1997.

15. Food and Agriculture Organization of the United Nations, "Manual on the Production and Use of Live Food for Aquaculture", http://www.fao.org/docrep/003/w3732e/w3732e06.htm [retrieved 1 October 2015]

16. R. Rippka, J. Deruelles, J. B. Waterbury, M. Herdman and R. Y. Stanier. Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. *Journal of General Microbiology*, 111:1-61, 1979.

17. P. R. Walne. Studies on the Food Value of Nineteen Genera of Algae to Juvenile Bivalves of the Genera Ostrea, Crassostrea, Mercenaria, and Mytilis. *Journal of Fisheries Investigation*, 26:1¬62, 1970.

18. R. R. L. Guillard and J. H. Ryther. Studies of Marine Planktonic Diatoms. I. Cyclotella Nana Hustedt, and Detonula Confervacea, *Canadian Journal of Microbiology*, 8:229-239, 1962.

19. E. W. Becker. *Microalgae: Biotechnology and Microbiology*, Cambridge University Press, Cambridge, 2008.

20. Y. Chisti. Biodiesel from Microalgae, *Biotechnology Advances*, 25:294–306, 2007

21. LookForDiagnosis, Medical Diagnosis, "Photobioreactors", http://www.lookfordiagnosis.com/mesh_info.php?term=Photobioreactors&lang=1 [retrieved 1 October 2015]

22. Duke University, Nicholas School of the Environment, "Dealing With Microscopic Cows", http://blogs.nicholas.duke.edu/fuelforthought/dealing-with-microscopic-cows/ [retrieved 1 October 2015]

23. Z. Khan, P. Bhadouria and P. S. Bisen. Nutritional and Therapeutic Potential of Spirulina. *Current Pharmaceutical Biotechnology*, 6:373-379, 2005.

24. US National Library of Medicine, "Blue-Green Algae", http://www.nlm.nih.gov/medlineplus/druginfo/natural/923.html [retrieved 1 October 2015]

25. D. J. McHugh. "A Guide to the Seaweed Industry: Food and Agriculture Organization (FAO) of the United Nations Fisheries Technical Paper 441: Other Uses of Seaweeds", http://www.fao.org/docrep/006/y4765e/y4765e0c.htm#bm12 [retrieved 1 October 2015]

26. W. H. Adey. Algal Turf Scrubber, Us Patent #4,333,263, 1980.

27. J. Morrissey, M.S. Jones and V. Harriott. Nutrient Cycling in the Great Barrier Reef Aquarium. *6th International Coral Reef Symposium: Vol. 2: Contributed Papers*, Townsville, 563-568, 1988.

28. British Petrol, "Statistical Review of World Reserves", http://knoema.com/smsfgud/world-reserves-of-fossil-fuels [retrieved 1 October 2015]

29. United Nations Committee of World Food Security, “Biofuels and food security”, http://www.fao.org/fileadmin/user_upload/hlpe/hlpe_documents/HLPE_Reports/HLPE-Report-5_Biofuels_and_food_security.pdf [retrieved 1 October 2015]

30. A. E. Atabani, A. S. Silitongaa, I. A. Badruddina, T. M. I. Mahliaa, H. H. Masjuki and S. Mekhilef. A Comprehensive Review on Biodiesel as an Alternative Energy Resource and its Characteristics. *Renewable and Sustainable Energy Reviews,* 16:2070–2093, 2012.

31. J. Carroll. “Exxon at Least 25 Years Away From Making Fuel From Algae”, Bloomberg Business, http://www.bloomberg.com/news/articles/2013-03-08/exxon-at-least-25-years-away-from-making-fuel-from-algae [retrieved 1 October 2015]

32. Florida Keys National Marine Sanctuary Website, “Phytoplankton Blooms: The Basics”, http://floridakeys.noaa.gov/scisummaries/wqpb.pdf [retrieved 1 October 2015]

33. R. M. Dawson. The Toxicology of Microcystins. *Toxicon,* 36: 953–962, 1998.

34. W. E. Lynch. Controlling Filamentous Algae in Ponds. Ohio State University FACT SHEET, Agriculture and Natural Resources, A-3-09, 2008.

35. United States Environmental Protection Agency, “Control and Treatment of Algal Blooms”, http://www2.epa.gov/nutrient-policy-data/control-and-treatment#what2 [retrieved 1 October 2015]

36. S. D. Lin, R. L. Evans and D. B. Beuscher. State of Illinois Department of Registration and Education Investigation 68, “Algal Removal by Alum Coagulation”, 1978.

37. S. A. Fast, B. Kokabian and V. G. Gude. Chitosan Enhanced Coagulation of Algal Turbid Waters – Comparison Between Rapid Mix and Ultrasound Coagulation Methods. *Chemical Engineering Journal,* 244:403-410, 2014.

38. Penn State Extension, “Barley Straw” http://extension.psu.edu/natural-resources/water/ponds/barley-straw [retrieved 1 October 2015]

39. D. M. Yebra, S. Kiil and K. D. Johansen. Antifouling Technology - Past, Present and Future Steps Toward Efficient and Environmentally Friendly Antifouling Coatings. *Progress in Organic Coatings*, 50:75–104, 2004.

40. Wikipedia, “Algal Bloom”, https://en.wikipedia.org/wiki/Algal_bloom [retrieved 1 October 2015]

41. United Stated Navy Office of Naval Research Corporate Strategic Communications, “New Hull Coatings Cut Fuel Use, Protect Environment” http://www.m2.com/m2/web/story.php/200993B7E129CDB55FA2802575CF0038 13B8 [retrieved 1 October 2015]

42. TransEurope Marinas, “Malahide Marina” http://www.transeuropemarinas.com/malahide-marina/ [retrieved 1 October 2015]

43. A. M. Berendsen. “Ship Painting: Current Practice and Systems in Europe”, Technology Publishing Company, http://www.paintsquare.com/library/articles [retrieved 1 October 2015]

44. N. Bellotti, C. Deya, B. Del Amo and R. Romagnoli. Antifouling Paints with Zinc Tannate. *Industrial and Engineering Chemistry Research*, 49: 3386–3390, 2010.

45. I. Omae. General Aspects of Tin-Free Antifouling Paints. *Chemical Reviews of American Chemical Society*, 103: 3431–3448, 2003.

46. G. G. Graf. *Tin, Tin Alloys, and Tin Compounds*. Ullmann's Encyclopedia of Industrial Chemistry, Wiley-VCH, Weinheim, 2005.

47. B. Antizar-Ladislao. Environmental Levels, Toxicity and Human Exposure to Tributyltin (TBT) Contaminated Marine Environment. A review. *Environment International*, 34:292–308, 2008.

48. University of California Santa Cruz, "Aquatic Toxicology", http://ic.ucsc.edu/~flegal/etox80e/SpecTopics/TBT/ [retrieved 1 October 2015]

49. J. R. Harris. Copper and Shipping in the 18th Century. *The Economic History Review New Series*, 19:550-568, 1966.

50. M. McCarthy. *Ships' Fastenings, From Sewn Boat to Steamship.* Texas A&M University Press, 2005.

51. Copper Antifouling Environmental Programme CAEP, "Copper in antifouling", http://copperantifouling.com/copper/index.html#5 [retrieved 1 October 2015]

52. Copper Development Assocation, "Copper Alloys in Aquaculture", http://copperalliance.org.uk/education-and-careers/education-resources/copper-alloys-in-aquaculture [retrieved 1 October 2015]

53. R. Marks, A. D. Pearse and A. P. Walker. The effects of a Shampoo Containing Zinc Pyrithione on the Control of Dandruff. *British Journal of Dermatology*, 112:415–422, 1985.

54. T. J. McCarthy, J. J. Zeelie and D. J. Krause. The Antimicrobial Action of Zinc Ion/Antioxidant Combinations. *Journal of Clinical Pharmacy and Therapeutics*, 17:51–54, 1992.

55. K. S. Grunnet and I. Dahllöf. Environmental Fate of the Antifouling Compound Zinc Pyrithione in Seawater. *Environmental Toxicology and Chemistry*, 24:3001-3006, 2005.

56. Department of Physics, University of Illinois, “Where Does the Element Boron Come From?” http://van.physics.illinois.edu/qa/listing.php?id=17594 [retrieved 1 October 2015]

57. A. Atabey, “Turkey as the Global Leader in Boron Export and Production”, http://www.easpd.eu/sites/default/files/sites/default/files/EVENTS/Conference2013/presentation_ayfer_atabey.pdf [retrieved 1 October 2015]

58. S. B. Narayan. “Method of Inhibiting Algae Growth on Asphalt Shingles”, US Patent #5,356,664, 1994

59. Dynalon Labware, “Plastic Properties of LDPE” https://www.dynalon.com/PublicStore/pages/LDPE_Properties.aspx [retrieved 1 October 2015]

60. D. Howden and K. Marks. The World's Rubbish Dump: a Tip that Stretches from Hawaii to Japan. http://www.independent.co.uk/environment/green-living/the-worlds-rubbish-dump-a-tip-that-stretches-from-hawaii-to-japan-778016.html [retrieved 1 October 2015]