A STUDY OF THE EFFECT OF AMPICILLIN ON MICROALGAE (*CHLORELLA VULGARIS AND EUGLENA VIRIDIS*)

By

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CERTIFICATION

This is to certify that this research work was carried out by Giegbefumwen Osarumwense of the Department of Plant Biology and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, Nigeria.

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DEDICATION

This work is dedicated to God Almighty for His guidance, direction, and strength, as well as to my parents and siblings for their support throughout.

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ABSTRACT

A study on the effect of ampicillin on microalgae *Chlorella vulgaris* and *Euglena viridis* was carried out. The experiment was carried out for 14 days using concentration at 0mg/l (control), 10mg/l, 20mg/l, 40mg/l, 60mg/l, 80mg/l, 100mg/l of ampicillin, affected the growth of *Chlorella vulgaris* and *Euglena viridis.* Samples were analysed throughout the experiment for physiochemical parameters using standard methods. The result showed that the different concentration of ampicillin affect the growth of *Chlorella vulgaris* and *Euglena viridis* and was inhibited at 10mg/l, 20mg/l, and 40mg/l treatment. The pH value at the beginning of all the experiment for all the treatment were unstable and around 8.16 and 6.68 but increased gradually at the end of experiment across all treatment.

There was significant difference in the growth of the microalgae to the ampicillin concentration.

INTRODUCTION

Microalgae are microscopic organisms found in both freshwater and marine environments. They are a diverse group of photosynthetic organisms, meaning they use sunlight to produce energy. Microalgae are an important component of the aquatic ecosystem, as they form the base of the food chain and produce a significant portion of Earth's oxygen. They are found in various aquatic environments, including oceans, lakes, rivers, and even moist terrestrial habitats. Microalgae are tiny, often single-celled organisms that require a microscope to be seen (Richmond., and Hu,. 2013). They are among the oldest forms of life on Earth, dating back billions of years, and have played a crucial role in shaping the planet’s ecosystems. As primary producers, microalgae convert sunlight, carbon dioxide, and nutrients into energy through photosynthesis, forming the base of aquatic food chains. They are a vital source of oxygen, contributing significantly to global oxygen production, and play an essential role in carbon sequestration by capturing and storing atmospheric carbon dioxide. Some microalgae produce bioactive compounds, including peptides, lipids, and secondary metabolites, that exhibit antimicrobial activity. These compounds can act against bacteria, fungi, and viruses, offering a potential alternative to traditional antibiotics. Microalgae-derived compounds often work through mechanisms distinct from traditional antibiotics, potentially overcoming resistance.

Antibiotics

Antibiotics were invented almost 90 years ago, and since then they have revolutionized human medicine. Today, antibiotics play a crucial role in the management of effective diseases, and they are consumed extensively in human and vertinary medicine and aquaculture. In addition to therapeutic applications, antibiotics are used for non-therapeutic purposes, for example to promote the growth of cattle, poultry, and hogs (Sarmah *et al.*, 2006, Kummerer, 2009). The use of antibiotics as growth-promoters was prohibited in the EU in 2006, but they are still used in other parts of the world such as China and India (Ronquillo and Hernandez, 2017). Due to their extensive consumption, antibiotics are ubiquitous and they have been detected in various ecosystems from terrestrial to aquatic environments (Yang and Carlson, 2003, kummerer, 2009, Martinez, 2009, Leung *et al*., 2012, Alygizakis *et al*., 2016). Fundamentally, antibiotics were designed to be effective towards micro-organisms, due to which they are likely the most antibiotic-sensitive group of organisms, making them of particular interest (Brandt *et al*., 2015). Antibiotics like ampicillin are biodegradable and can break down in environment through microbial action, particularly by a bacteria that can degrade organic compounds. Their toxic properties toward micro-organisms can remain even at trace levels (Kummerer *et al*., 2000, Brown *et al*., 2006).Prokaryotes are likely the most sensitive environmental organisms to antibiotics because antimicrobial agents are efficient inhibitors of bacterial growth (Martinez, 2009, Brandt., 2015). Also, the presence of the antibiotic residues in the aquatic ecosystem is dependent on the pharmacokinetic profile of antibiotics.

Aquatic Ecosystem

Understanding antibiotic degradation in the environment is essential, given the fact that the impact of antibiotic residues on aquatic and terrestrial ecosystems is still not fully understood. There are a number of mechanisms of antibiotic degradation in the environment, which can be biotic, i.e. biodegradation by bacteria and fungi (microbial degradation) and/or non-biotic: hydrolysis, photolysis, oxidation and reduction, depending on the physicochemical properties, environmental conditions (temperature, light, etc). Antibiotics are considered to be "persistent or pseudo-persistent substances because their entry rate into the environment is higher than the elimination rate" and comprise heterogeneous compounds, with various functional groups, responsible for very different physicochemical properties and behaviors in the ecosystem . Also, the presence of the antibiotic residues in the environment is dependent on the pharmacokinetic profile of antibiotics. The biological activity of antibiotics in different environmental matrices depends on their bioavailable fraction and interaction with environmental conditions such as pH, organic carbon content, type of soil, water content and type of organism etc. Therefore, to provide information about the bioavailable fractions and their effects on the environment represents a necessity and a major challenge especially regarding analytical determinations. In this case, there could be a potential gap between analytical results obtained in the lab and the bioavailable fraction in the environment. The growing awareness of the potential for antibiotic residues to damage aquatic organisms has led to the placement of some antibiotic compounds (amoxicillin and ciprofloxacin) on the European Union (EU)" Watch List" of emerging water pollutants in 2015, followed by the addition of a few more antibiotics (erythromycin, clarithromycin and azithromycin) to this list in 2018. However, our main focus is on ampicillin, and how it affects the aquatic ecosystem.

Ampicillin

Ampicillin is used to treat certain infections that are caused by bacteria such as meningitis (infection of the membranes that surround the brain and spinal cord), and infections of the throat, sinuses, lungs, reproductive organs, urinary tract, and gastrointestinal tract (medlineplus.gov). It is extensively used in agriculture and medicine against bacterial infection and increasing animal growth but the undesirable residues in foodstuffs of ampicillin leads to numerous health problems like breathing difficulties, seizures and allergic reaction (pmc.ncbi.nlm.nih.gov).

However, the rate and extent of the biodegradation of ampicillin can vary depending on environmental conditions such as temperature, pH, and the presence of specific micro-organisms.

Aim

The objective of the study were to :

1. Evaluate the response growth of the microalgae *Chlorella vulgaris* and *Euglena viridis* to different concentrations of ampicillin.
2. Determine the percentage inhibition or stimulation of the test microalgae to ampicillin.
3. Physicochemical parameters of (pH,EC and TDS) of the culture throughout the experiment.

LITERATURE REVIEW

Ampicillin is a penicillin beta-lactam antibiotic used in the treatment of bacterial infections caused by susceptible, usually gram-positive, organisms. The name "penicillin" can either refer to several variants of penicillin available, or to the group of antibiotics derived from the penicillins. Ampicillin has *in vitro* activity against gram-positive and gram-negative aerobic and anaerobic bacteria. The bactericidal activity of Ampicillin results from the inhibition of cell wall synthesis and is mediated through Ampicillin binding to penicillin binding proteins (PBPs). Ampicillin is stable against hydrolysis by a variety of beta-lactamases, including penicillinases, and cephalosporinases and extended spectrum beta-lactamases (https://go.drugbank.com/drugs/DB00415) . Antibiotics have been widely used for therapeutic purposes in human and veterinary medicine and also as animal growth promoters. The global consumption of antibiotics increased by 65% from 2000 to 2015 (Klein *et al*., 2018). Many antibiotics are not completely metabolized in the human and animal bodies, and through excretion, parent compounds could be recoverable up to 50–90% in urine and fecal matter (Halling-Sorensen *et al*., 2002; Jelic *et al*., 2011). Eventually, the parent compounds, their transformation products, and their metabolites can reach wastewater treatment plants (WWTPs) from residences, hospitals, and manufacturing sites, or indirectly through runoffs and stormwater (Michael *et al*., 2013; Rizzo *et al*., 2013). This literature review summarizes existing research on the impact of ampicillin on microalgae , focusing on impact on aquatic ecosystem, evaluate growth inhibition, impacts on both individual and combined growth of microalga *CHLORELLA VULGARIS* and *EUGLENA VIRIDIS*, effects on growth and response.

IMPACTS ON AQUATIC ECOSYSTEM

Antibiotics like ampicillin have been detected in aquatic environments such as lakes, rivers, water reservoirs, wastewater treatment plants influent and effluent, groundwater, and even drinking water, even though drinking water was treated. As was mentioned before, the antibiotics found in the aquatic ecosystem, come from domestic, hospitals, the pharmaceutical industry, aquaculture, and agricultural activities. The presence of antibiotics in the aquatic environment is a serious concern because it may accelerate the proliferation of antibiotic-resistant pathogens, through genetic mutations and resistance vectors with high transfer rate between pathogens, thus lowering the therapeutic effect of antibiotics. In addition, antimicrobial resistance can be transferred between different organisms throughout the food chain. According to the World Health Organization , antimicrobial resistance is a significant challenge to global human and animal health, food safety, and development today, with the perspective of aggravation in the upcoming years, if adequate measures are not implemented. It was determined that detected levels of antibiotics in WWTP effluent samples exhibit an impact on the environment, especially in microbial communities in aquatic systems causing antibiotic resistance development (https://pmc.ncbi.nlm.nih.gov/articles/PMC8423433/) Long-term alteration of the bacterial community composition may lead to variation in biogeochemical cycling and aquatic ecosystems. For example, anoxic environments promote harmful algal blooms (Ding and He, 2010; van der Grinten *et al*., 2010; Janecko *et al*., 2016; Roose-Amsaleg and Laverman, 2016; Xiong *et al*, 2019). These findings suggests that if ampicillin disrupts these bacterial communities , nutrient cycling maybe altered, leading to an environment that is favorable for algablooming.

MICROALGAL GROWTH

concentration of antibiotics may enhance the growth of Low concentration of antibiotics (ampicillin) may enhance the growth of microalgae. while high concentration of antibiotics may inhibit the growth of microalgae (*Cheng et al., 2017*). Use of antibiotics in aquaculture is a common practice in Vietnam to treat or prevent infections, and to promote growth of the batches. The utilization of intensive farming method, which facilitates the development of several bacterial diseases, has consequently led to an increase in antimicrobial uses. This topic has been the focus of previous reviews about antibiotics in the aquatic environment of Vietnam (Thuy *et al*., 2011, Rico *et al*., 2013). Pharmaceutical residues have been reported to distort the growth of phytoplanktons. It was reported by (https://www.sciencedirect.com/science/article/pii/S2468227622001958#bib0097) that streptomycin prevented the growth of blue-green algal species at concentrations (0.05 to 0.93 mg/L). Likewise, *Scenedesmus obliquus* and *Chlorella vulgaris* were found to have grown luxuriously in streptomycin concentrations of 0.66 mg/L. From findings this can be improved on by gradually exposing cyanobacteria to low doses of streptomycin may allow them to develop resistance over time.

MATERIALS AND METHODS

Study area

This study was carried out in the University of Benin, Benin City, Edo State, Nigeria. The experiment were carried out in the Limnology and phycology Laboratory of Department of Plant Biology and Biotechnology.

Microalgae test

Chlorella vulgaris and *Euglena Viridis* were selected for the study. These algae were collected from gutters around Benin city.

Isolation of pure culture

Cultures of the test microalgae were obtained by isolating desired alga and inoculating into growth medium. Algal cultures were obtained after a series of subcultures microscopic examination of the cultures was then carried out to confirm the algal species in the samples prior to use.

Botany of test organisms

Chlorella vulgaris is a specie of single-celled photosynthetic microalgae. The range in size from 2 to 10µm and are spherical in shape. It is commonly found in fresh waterbodies such as rivers, lakes and ponds, and grows best in sunny conditions.

Taxonomic classification

Kingdom - Protista

Division - Chlorophyta

Class - Trebouxiophyceae

Order - Chlorellales

Family - Chlorellaceae

Genus - Chlorella

Species - *Chlorella vulgaris*

Euglena specie

Euglena is a type of euglenoid. Euglenoids are unicellular microorganisms, that have a flexible body. They possess the characteristic features of plants and animals. Euglena has plastids and performs photosynthesis in light, but moves around in search of food using its flagellum at night. There are around 1000 species of Euglena found. They are found in freshwater, saltwater, marshes and also in moist soil.

Domain - Eukaryota

Phylum - Euglenozoa

Class - Euglenida

Clade - Euglenophyceae

Order - Euglenales

Family - Euglenaceae

Genus - Euglena

Species - *Euglena viridis*

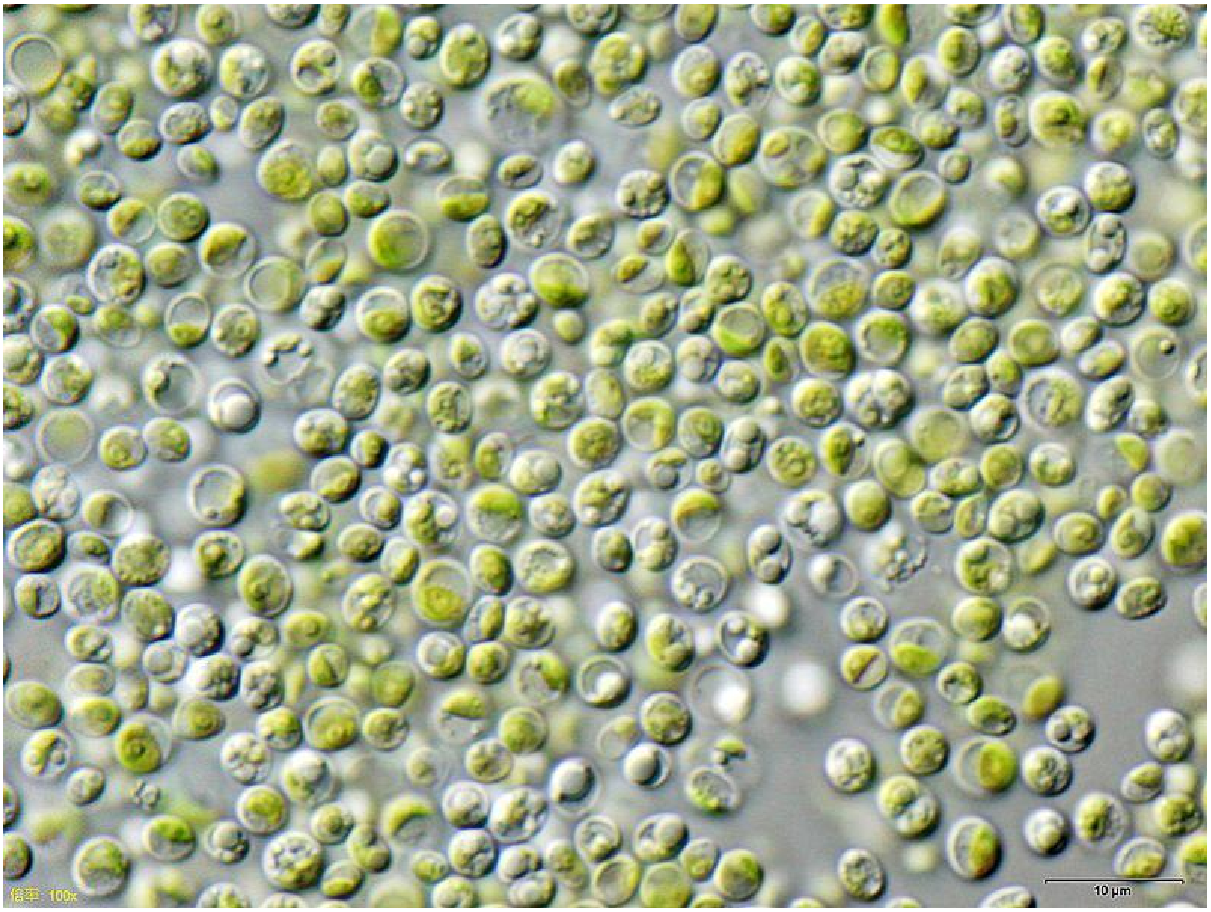

Plate 1: ***Chlorella vulgaris***

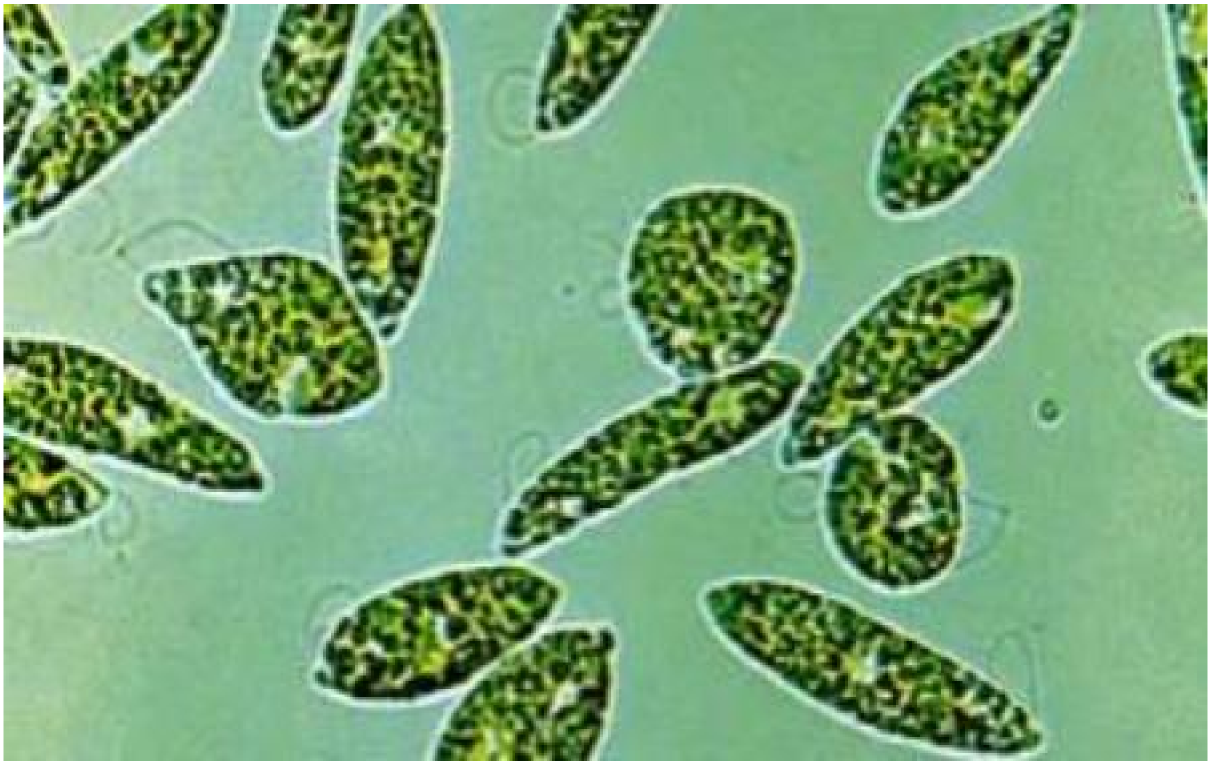

Plate 2: ***Euglena Viridis***

Preparation of culture media

Chu's No.10 modified medium was used in combination with the algae. The steps for the preparation are shown below.

A stock solution was made using 100ml of distilled water and salts (micronutrients) as shown in table below. The prepared solution was autoclaved and allowed to cool before use.

Macronutrient stock

Salts/Nutrients	g/100ml
$Cacl_2.2H_2O$	3.67
$MgSO_4.7H_2O$	3.69

$NaHCO_3$	**1.26**
K_2HPO_4	**0.87**
$NaNO_3$	**8.50**
$NaSi0_3$	**2.84**

Iron stock was prepared by adding 3.35g of citric acid (C6H8O7.2H2O) and 50ml of distilled water followed by the addition of 3.5g of ferric citrate (FeC6H5O3.5H2O). The volume was then brought up to 100ml final volume and autoclaved before use.

Iron stock

Nutrients	**g/100ml**
Citric acid ($C_6H_8O_7.2H_2O$)	**3.35**
Ferric citrate ($FeC_6H_5O_3.5H_2O$)	**3.35**

The salts specified in the table were each dissolved in one litre of distilled water to create salt solutions according to the concentrations listed in the table.

Trace element stock

Salts/Nutrients	**mg/100ml**
$CuSO_4.5H_2O$	**19.6**
$ZnSO_4$.7H2O	**44**

$CoCl_2.6H_2O$	**20**
$MnCl_2.4H_2O$	**36**
$NaMO_4.2H_2O$	**12.6**
H_3BO_3	**618.4**

Vitamin stock was prepared by the combination of 0.004g of B12, thiamine and biotin were each dissolved in 100ml of distilled water.

Vitamin stock

Nutrients.	**g/100ml**
Cyanocobalamin (B_{12})	**0.004**
Thiamine	**0.004**
Biotin	**0.004**

For the final preparation, 10ml each of each salt from macronutrient, trace element, iron and Nutrients were combined and made up to 10L final volume.

Culture vessel

Bottles of volume 350ml were used for the trials. Brand new bottles were purchased, washed with detergents, rinsed with distilled water and then dried at room temperature before being used. The various concentrations of microalgae were labelled appropriately.

Source of ampicillin

Ampicillin was purchase from a local commercial outlet, a pharmacy store in Benin City.

Table Preparation of Ampicillin Treatments

Ampicillin Concentration	**Volume of** growth medium **(ml)**	**Volume of Ampicillin stock (ml)**
0mg/l**(control)**	**900**	**0**
100mg/l	**855**	**45**
20mg/l	**810**	**90**
40mg/l	**675**	**225**
60mg/l	**450**	**450**
80mg/l	**225**	**675**
100mg/l	**0**	**900**

Plate 3 : ***Chlorella vulgaris*** **at Day 2**

Plate 4 : *Chlorella vulgaris* at Day 10

Plate 5 : *Euglena viridis* at Day 2

CHAPTER THREE

RESULTS

3.1 Growth response of *chlorella vulgaris* to ampicillin

Fron the experiment carried out, on the two algal species *Chlorella vulgaris* and *Euglena viridis, the growth response was measured* using a spectrophotometer for a period of two weeks (8 days). Statistically, one way ANOVA revealed that there were significant differences ($p<0.5$) in the growth response of *Chlorella vulgaris* across different concentrations of ampicillin throughout the experiment.

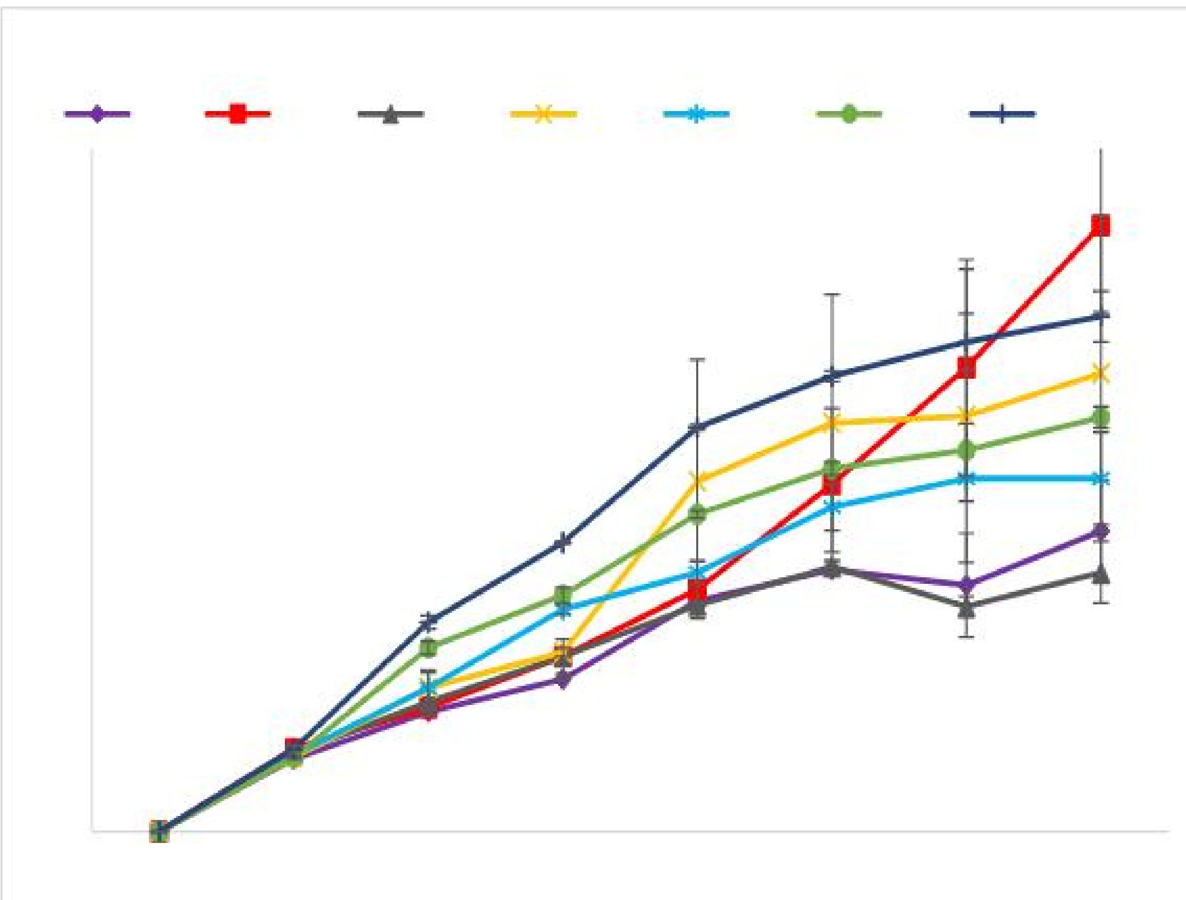

Figure 1 : Growth response of *chlorella vulgaris* to Ampicillin

Figure2: growth response of *chorella vulgaris* to Ampicillin. Statistically, one-way ANOVA revealed that there were significant differences ($p<0.05$) in the growth response of Chlorella vulgaris across different concentrations of ampicillin throughout the experiment.

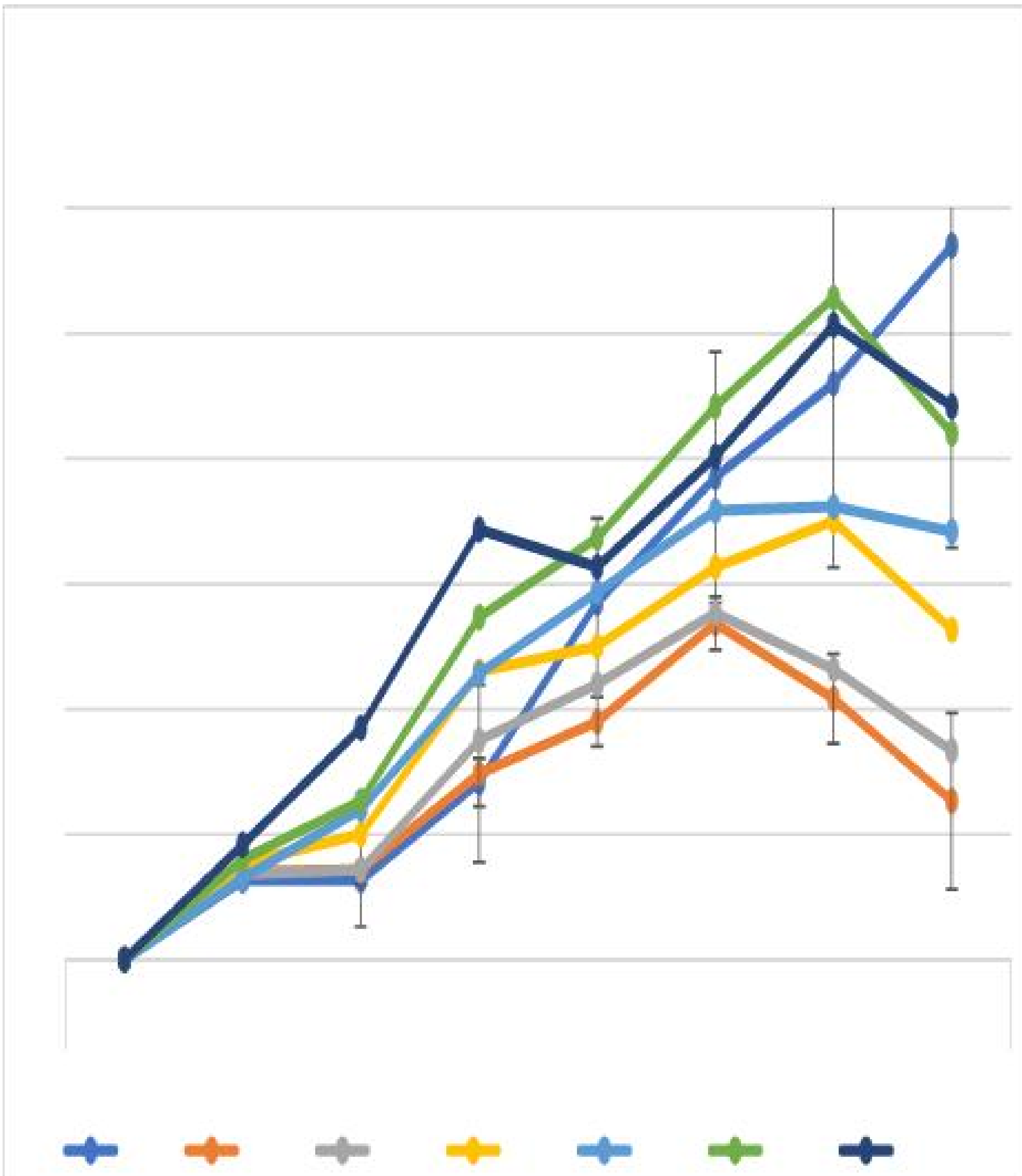

Figure 2: Growth response of Chlorella vulgaris to Ampicillin

Figure 3 : pH of different concentrations of Ampicillin on *chlorella vulgaris* . Statistically, one-way ANOVA showed that there were significant differences ($p < 0.05$) between Ampicillin pH levels across each day of *chlorella vulgaris* growth.

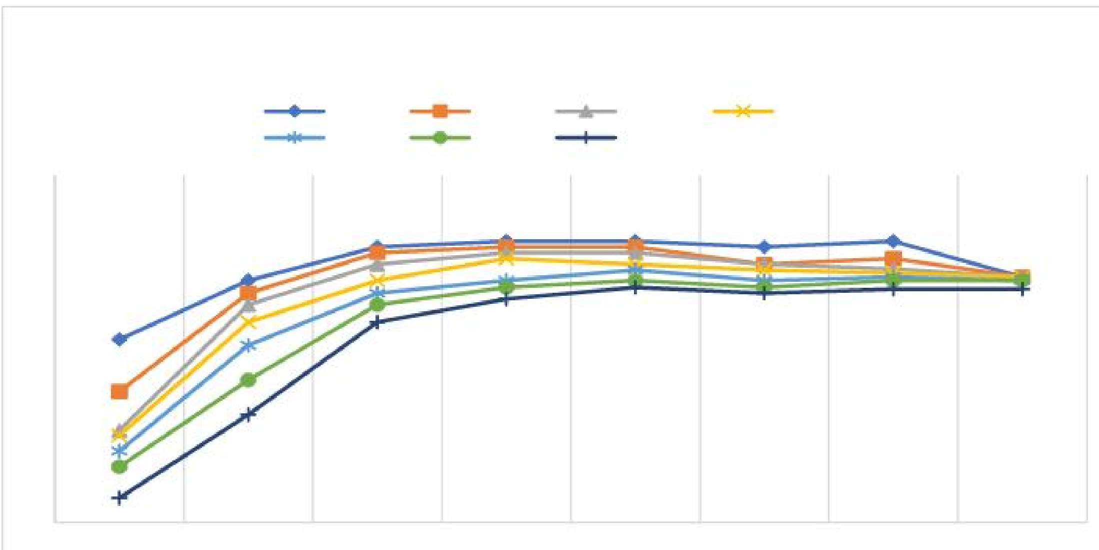

Figure 3 : pH of different concentrations of Ampicillin on *Chlorella vulgaris*

Figure 4 : Total dissolved solids of different concentrations of Ampicillin on *chlorella vulgaris* Statistically, one-way ANOVA showed that there were significant differences between Ampicillin total dissolved solid levels across each day of *Chlorella vulgaris* growth.

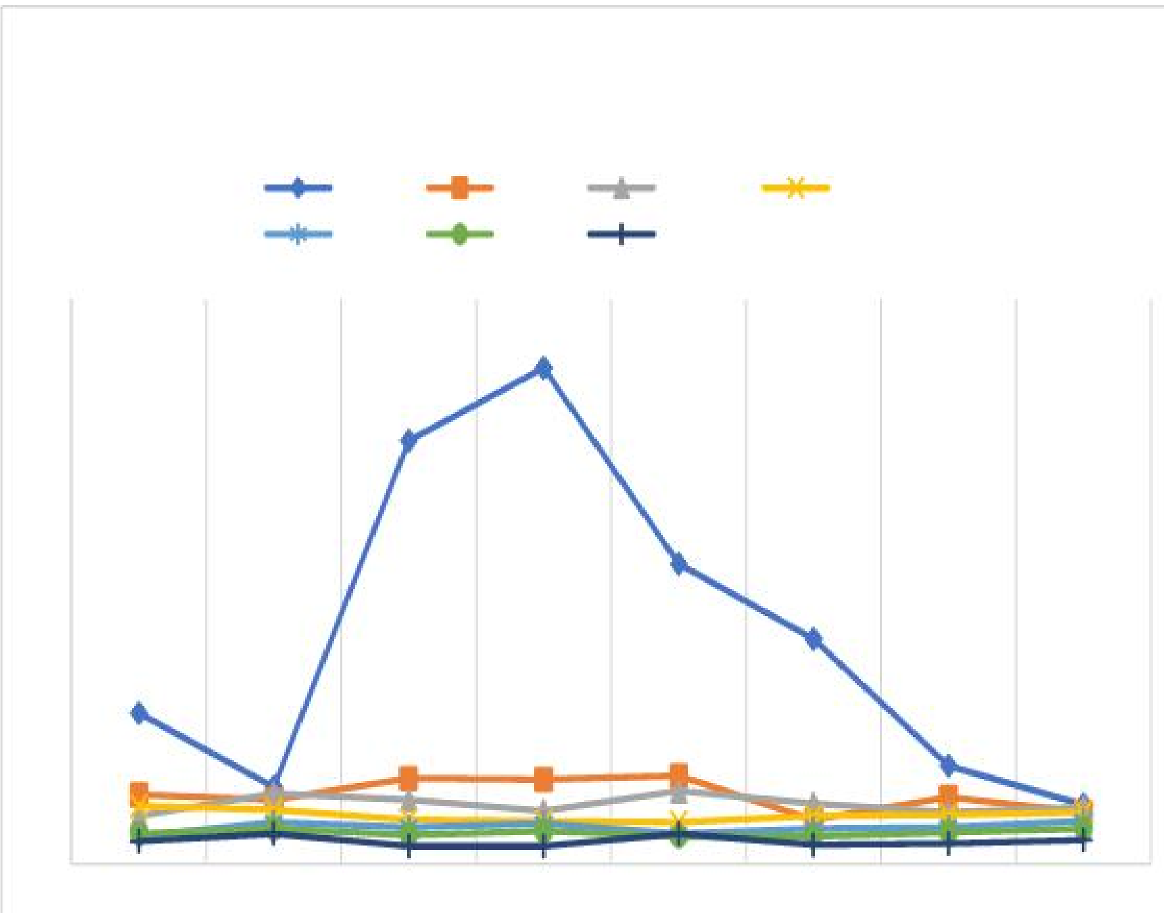

Figure 4 : Total Dissolved Solids of different concentrations of Ampicillin on *Chlorella vulgaris*

Figure 5 : Conductivity of different concentrations of Ampicillin on *Chlorella vulgaris* Statistically, one-way ANOVA showed that there were significant differences ($p < 0.05$) in Ampicillin conductivity levels across each day of *Chlorella vulgaris* growth.

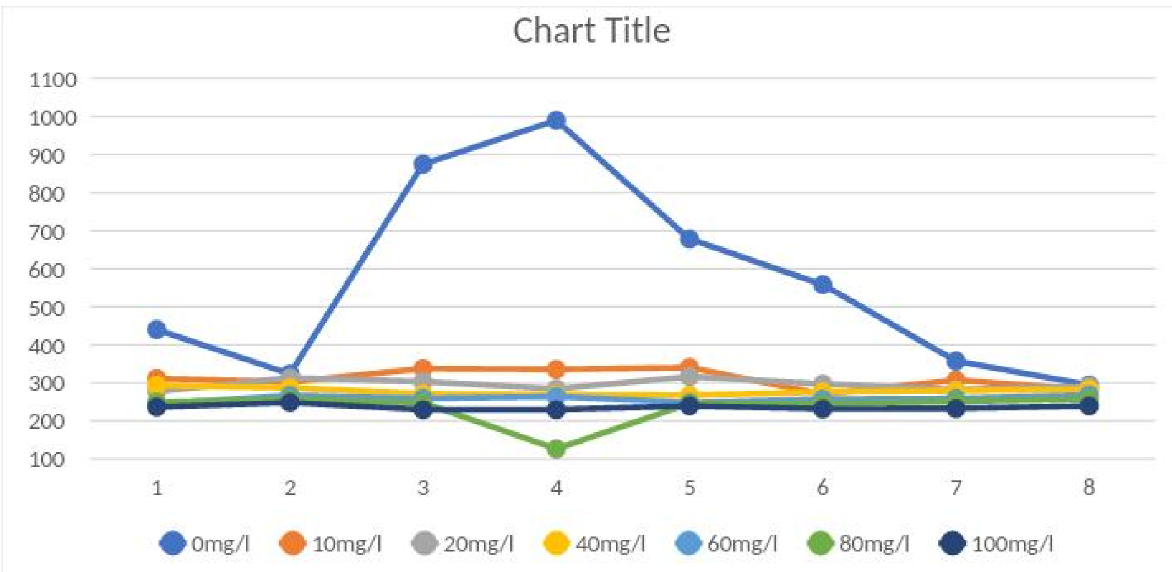

Figure 5 : Conductivity of different concentrations of Ampicillin on *Chlorella vulgaris*

Percentage inhibition of *Chlorella vulgaris* and *Euglena viridis*

This figure showed a stimulatory effect of *Euglena viridis* at 10mg/l of ampicillin, 20mg/l displayed an inhibitory response. Subsequently, an increasing stimulatory response was recorded at higher concentrations (60mg/l, 80mg/l, 100mg/l).

For *Chlorella vulgaris*, it showed a significant stimulatory response at 0mg/l, 10mg/l, 60mg/l, and 100mg/l with the least stimulatory at 20mg/l. There was an increased inhibitory effect at 80mg/l concentration.

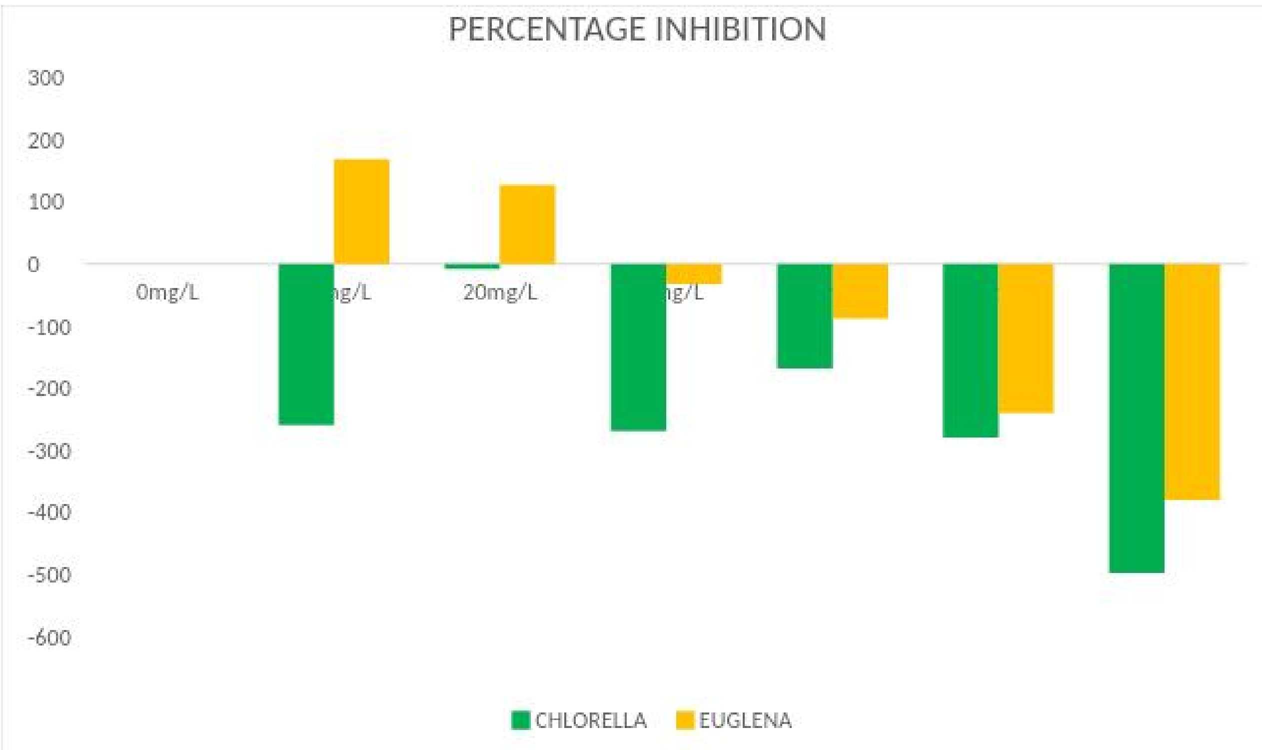

Figure 6 : Percentage inhibition of *Chlorella vulgaris* and *Euglena viridis*

CHAPTER FOUR

DISCUSSION

The effects of Ampicillin on the growth of microalgae *Chorella vulgaris and Euglena viridis* was carried out in this study. According to the study's findings, the maximum growth of *Chlorella vulgaris* occurred at a control treatment at Day 14 and was followed by 10mg/l treatment at that same day. This is consistent with the findings of (Abdel-Raouf *et al,.* 2012), who noted that *Chorella vulgaris* had its greatest growth at all treatment concentrations between day 12 and 14.

The control treatment had the highest growth response for *Chlorella vulgaris* at day 12 and 14, followed by the 10mg/l treatment at days 12 and 14. This is also consistent with the findings of (Abdel-Raouf *et al.,* 2012), who noted that day 12 to day 14 had the greatest growth of *Chlorella vulgaris* for all treatment concentrations.

Chlorella vulgaris showed the greatest percentage of inhibition at 10mg/l treatment, followed by 40mg/l treatment. 0mg/l represents the lowest percentage of inhibition. This is in line with the findings of the study by Zhou *et al.* (2019) demonstrated that exposure to high concentrations of ampicillin (≥50 mg/L) led to a significant decline in the growth rate of *Chlorella vulgaris*, suggesting toxicity at elevated levels.

The highest pH for *Chlorella vulgaris,* followed by 0mg/l treatment, on day 6 , 0mg/l of the treatment was given before 100mg/l of it. This is consistent with a study of Konyab *et al.* (2013), which stated that, *Chlorella marina* may increase wastewater pH from 2.85 to 7.42.

Euglena viridis highest pH was recorded at 0mg/l treatment followed by 10mg/l treatment at day 8 and at 10mg/l treatment followed by 20mg/l treatment at Day 8. According to Konyab *et al.* (2013) research, the pH range of wastewater with *Chlamydonomas* is 3.50 to 6.01. Water's pH is controlled by carbon dioxide, and algae development can cause it to reach limitations values of up to pH 9. In all of the treatments utilised, the pH was mildly acidic at the start of the experiment but became severely acidic at the end.

Chlorella vulgaris maximum TDS occurred in 0mg/l treatment followed by 10mg/l treatment at day 8 and in 0mg/l treatment followed by 10mg/l treatment at day 8 . According to Konyab *et al.* (2015). TDS of wastewater treated with *Chlorella marina* ranges from 141 to 321 mg/l. According to Hadiyanto *et al.* (2013), high TDS can cause cellular damage in living things, inhibit photosynthesis and cause an increase in the temperature of the water.

At day 0, 0mg/l treatment produced the highest TDS for *Euglena viridis,* followed by 10mg/l treatment. On day 14, 0mg/l of the treatment was given before 10mg/l of it. This is consistent with the findings of Idris *et al.* (2018), who suggested that the TDS ranged from 115 to 468 mg/l for wastewater treated with *Chlamydonomas*. Residential runoff, mountain water that is rich in clay, point source water pollution discharge from industrial or sewage treatment plants, etc, are the main sources of TDS in water bodies.

At day 0, 0mg/l treatment followed by 10mg/l treatment produced the highest Conductivity for *Chlorella vulgaris,* while at day *14,* 0mg/l treatment followed by 10mg/l treatment produced the highest Conductivity. The ability of a solution to conduct electricity is measured by its Conductivity, which is directly related to the total amount of dissolved solids in the solution.

At day 0, 0mg/l treatment followed by 10mg/l treatment produced the highest Conductivity for *Euglena viridis,* while at day 14, 0mg/l treatment followed by 10mg/l treatment produced the highest Conductivity. Since Conductivity is closely related to temperature, the increased Conductivity report was the result of the rising water temperature.

The growth response of recorded for *Euglena viridis* was higher than that of *Chlorella vulgaris* at the same concentrations of ampicillin, indicating that higher concentrations of ampicillin were required by *Chlorella vulgaris* to stimulate the same amount of growth that a lower concentration would stimulate in *Euglena viridis*.

Microalgae can bind ampicillin to their cell surfaces, reducing its toxicity and enabling normal metabolic processes. Certain microalgae possess enzymes that stimulates the breakdown of ampicillin that stimulate the growth of microalgae (pmc.ncbi.nlm.nih.gov).

Microalgae interact with ampicillin through various mechanisms, including bioadsorption, bioaccumulation, and biodegradation. *Chorella pyrenoidosa* has been studied to effectively adsorb and accumulate antibiotics thereby reducing their concentration in waterbodies (frontiersin.org).

Microalgae such as *Chlorella sp*., *Dictyostelium sp*., and *Scenedesmus obliquus* have been shown to effectively degrade antibiotics (Chen *et al*., 2020; Chen *et al*., 2020; Yang *et al*., 2020; Aydin *et al*., 2022). Nevertheless, microalgae have specific preferences for antibiotic types, and different microalgae have different cell hydrophobicity, which affect the degradation of antibiotics (Bai and Acharya, 2016; Xiong et al., 2017). Therefore, recent studies have focused on the consortia of microalgae and bacteria or other microalgae to improve the removal efficiency of antibiotics (Rodrigues *et al*., 2020; Rodrigues *et al*., 2021; Wang *et al*., 2022). However, whether the determinant of antibiotic removal efficiency of microalgae consortia is higher than for keystone species needs further studies.

CONCLUSION

The study shows that Ampicillin inhibited the growth of microalgae (*Chlorella vulgaris* and *Euglena viridis*). The study shows that Ampicillin can have significant effects on microalgae, altering their growth rate by exhibiting inhibitory effect on the microalgae. This study showed that the test microalgae (*Chlorella vulgaris* and *Euglena viridis)* shows the importance of monitoring antibiotic contamination in water bodies to ensure ecological balance.

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