Potential of Cyanobacterium *Spirulina platensis* for Eutrophic Water Restoration

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Abstract

Around 70% of the world is covered with water but only 2.5% of it is freshwater and even less is available for the ecosystem and humanity. The limited available freshwater is facing increasing challenges from water pollutions. Among those, eutrophication is one of the major concerns worldwide. The reason for eutrophication is the presence of excessive amounts of phosphorus and nitrogen in water bodies, which may cause algal blooms and a variety of harms to the aquatic ecosystem in association with algal blooms. Among these two components, phosphorus plays a major role in eutrophication control and recovery, since atmospheric N_2 can be fixed by biological nitrogen fixation (BNF) processes and is therefore of little meaning to control. In this study, we investigated for the first time the potential of using controlled growth of algae and in particular, filamentous cyanobacterium *Spirulina platensis*, for eutrophic water restoration.

This study investigated the algal cell growth, algal product production, and phosphate removal efficiency of cyanobacterium *S. platensis*, which is non-toxic and filamentous, at different phosphate levels in artificial wastewaters and artificial eutrophic waters. Results indicate that *S. platensis* could remove 90.17% of phosphate from artificial wastewaters containing 10 mg/L phosphate in a 16-day cultivation period. When tested for eutrophic water restoration, *S. platensis* was able to convert hypereutrophic, eutrophic, and meso-eutrophic waters to oligotrophic water. It was shown that using 100-micron mesh nylon cloth for harvesting biomass, would be sufficient to keep biomass concentration at 0.30±0.02 g/L or lower. In the meantime, light/dark tests indicate that the dissolved oxygen level would not go below the hypoxic level, i.e., 2 mg/L after a 12-hour dark period at biomass concentration up to 1 g/L. These results indicate that it is possible to use *S. platensis* for both controls of point source discharge and restoration of eutrophic waters.

Keywords: Eutrophication, Eutrophic restoration, *S. platensis*, Hypoxia, Harvesting algae

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Sommaire

Environ 70% du monde est couvert avec de l'eau, mais seulement 2,5% est de l'eau douce et encore moins est disponible pour l'écosystème et l'humanité. La disponibilité limitée de l'eau douce faisant face aux obstacles croissants de la pollution de l'eau et de l'eutrophisation est une des principales préoccupations mondiales. La cause de l'eutrophisation est la concentration excessive de quantité de phosphore et d'azote dans les masses d'eaux, ce qui peut causer des proliférations algales et une variété de dommages à l'écosystème aquatique à travers l'association avec les proliférations algales. Parmi ces deux composantes, le phosphore joue un rôle principal dans le contrôle de l'eutrophisation et le rétablissement puisque le N_2 atmosphérique peut être réparé par des processus de fixation biologique de l'azote et a donc peu d'impact sur le contrôle. Dans cette étude, nous avons examiné pour la première fois le potentiel d'utiliser la croissance contrôlée de l'algue et plus particulièrement des cyanobactéries filamenteuses, *Spirulina platensis* pour la restitution de l'eau eutrophe.

Cette étude a fait une enquête de la croissance des cellules de l'algue, la production des sous-produits de l'algue et l'enlèvement du phosphate par le *S. platensis* à des niveaux de phosphate différents dans des eaux usées artificielles et des eaux eutrophes. Les résultats indiquent que le *S. platensis* peut enlever 90,17% de phosphore des eaux usées artificielles qui contiennent 10 mg/L de phosphate dans une période de culture de 16 jours. Lorsqu'ils ont été testés pour le rétablissement des eaux euphorbes, le *S. platensis* était capable de convertir les eaux hypereutrophes, eutrophes et mésotrophes, et à des eaux oligotrophes. Il a été démontré que l'utilisation des feuilles de maille en nylon 100 microns dans la culture de la biomasse serait suffisante pour garder la concentration de biomasse à 0,30±0,02 g/L ou moins, ce qui est en dessous de la concentration de biomasse nécessaire pour éviter des conditions hypoxiques. Pendant ce temps, les tests clairs-obscurs indiquent que le niveau d'oxygène dissous n'ira pas sous le niveau hypoxique, p. ex. 2 mg/L suite à une période obscure de 12 heures avec une concentration biomasse jusqu’à 1 g/L. Ces résultats indiquent qu’il est possible d’utiliser *S. platensis* pour autant le contrôle du rejet de la source ponctuelle et le rétablissement des eaux eutrophes.

Mots clés : l’eutrophisation, rétablissement eutrophe, *S. platensis*, hypoxie, moissonnage de l’algue

Statement of Originality

All the work and results submitted in this thesis are the product of original work done by the author under the supervision of Dr Christopher Lan at the University of Ottawa in the Department of Chemical and Biological Engineering in partial fulfilment of the requirements for the degree of Master of Applied Science in Chemical Engineering at the University of Ottawa. This work has been presented in:

1) Paper entitled “Eutrophicated water Recovery using Microalgae” at Canadian Society for Chemical Engineering's 70th edition of the Canadian Chemical Engineering Conference held virtually on Oct. 26-30, 2020.

2) Poster with the same title presented at the “Graduate Poster Competition” conducted by the University of Ottawa on Tuesday, March 3, 2020

3) Chapters 3 and 4 will be submitted to peer-reviewed journals after reformatting.

Acknowledgement

I would first like to express my sincere gratitude to Dr Christopher Lan for believing in me and providing me with an opportunity to work under his supervision, for his continuous and constant support and guidance. His insightful feedback and guidance have always helped me in thinking at a higher level and inspired me throughout the research work.

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Abbreviations

AEW – Artificial Eutrophicated Water

S. platensis – Spirulina platensis

DO – Dissolved Oxygen

HAB – Harmful Algal blooms

EPA – Environmental Protection Agency

USA – United States of America

NOAA - National Oceanic and Atmospheric Administration

ATP - Adenosine triphosphate

GLA – Gamma-Linolenic Acid

OHD – Oregon Health Division

WRI – World Resources Institute

GLWQA – Great Lakes Water Quality Agreement

SI – Severity Index

TBP – Total Bioavailable Phosphorus

WHO – World Health Organization?

BNF – Biological Nitrogen Fixation

NASA – National Aeronautics and Space Administration

GRAS – Generally Recognized As Safe

FDA – Food and Drug Administration

SPUR – Specific Phosphate Uptake Rate

OUR – Oxygen Uptake Rate

OPR – Oxygen Production Rate

SROE – Specific Rate of Oxygen Evolution

WW- Waster water

Chl A – Chlorophyll A

Chl B – Chlorophyll B

S. maxima – Spirulina maxima

UTEX – University of Texas

UV-Vis – Ultraviolet-Visible

SMEWW - Standard Methods for the Examination of Water and Wastewater

PBS – Phosphate Buffer Saline

OD – Optical Density

PC – Phycocyanin content

AWW – Artificial Wastewater

AEW – Artificial Eutrophicated Water

Conc. – Concentration

Nomenclature

h – hour

A_{www} – Absorbance at OD www

μ - Specific growth rate (h^{-1})

k_d – Death rate (h^{-1})

t – time (hour)

q_{o2} – Specific rate of oxygen consumption (mg O_2/g of DCW)

Chapter 1: Introduction

1.1 Introduction

Eutrophication has become one of the major concerns around the world. This problem may intensify in the future because of the fast growth of the world population, the modernization of agriculture and the rapid industrialization which have resulted in the tremendous increase of discharge of phosphorus-containing chemicals (e.g., fertilizers) as industrial wastes, sewage discharges, and runoffs from urban areas, construction sites and agricultural lands [1]. Consequently, excessive phosphorus in natural water bodies such as rivers, lakes, bays, and oceans lead to uncontrolled algal blooms in water, which may block sunlight, reduce dissolved oxygen in the water to a hypoxic level which leads to the death of aquatic animals and plants beneath, decrease the drinking water quality and increase water treatment cost. The water quality could be worsened to such an extent that it becomes detrimental to humans as well as animals [2]. Consequently, a eutrophic water restoration plan has to be adopted for affected waters.

Eutrophication can be treated either by chemical treatment or biological treatment. In most cases, the chemical treatment like precipitation and inactivation with alum has been a failure due to high costs and even worse, sometimes chemical treatments may create a toxicity problem in water bodies due to uncontrolled chain reactions [3].Compared to the chemical process, the biological process is non-toxic, which depends on the growth of nontoxic aquatic plants such as microalgae and cyanobacteria to reduce phosphorus in water bodies. The biomass thus produced could be harvested and converted into value-added bioproducts [4].

1.2 Thesis Objectives

This thesis has four main objectives:

1 Demonstrate the potential of *S. platensis* for eutrophic water restoration;

2 Study cell growth kinetics of *S. platensis* and to extract the bioproducts of *S. platensis* in artificial wastewaters and artificial eutrophic waters;

3 Test the effectiveness of nylon mesh screen with different pore size for *S. platensis* harvest

4 Study the dynamics of DO in eutrophic water bodies at a varied biomass concentration

1.3 Thesis organization

This thesis is composed of five chapters. **Chapter 1** briefly gives an introduction to the thesis. **Chapter 2** is a literature review focusing on eutrophication, its impacts, chemical and biological treatment of eutrophic waters, as well as the introduction of *Spirulina platensis*. **Chapter 3** focusses on the experimental methods and results of the eutrophication water treatment using algae research. **Chapter 4** is another research paper that discusses critical biomass concentration in lakes to avoid hypoxic condition. **Chapter 5** presents the conclusions of this thesis and a few knowledge gaps identified for future studies.

Chapter 2: Literature review on Microalgae for Eutrophication Water Restoration

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Abstract

Excessive nutrients like nitrogen and phosphorus in the water body lead to eutrophication, which can cause harmful algal blooms. These algal blooms have several negative effects on the aquatic environment, humans and water quality. The eutrophication can be controlled and mitigated using microalgae since microalgae grow well in nutrient-rich water and they absorb the nutrients and convert them into valuable biomass. This review aims at discussing the eutrophication problem around the world, the cause and negative effects of eutrophication, different methods to treat them including physical and biological methods. The potential of *Spirulina platensis* to treat the eutrophicated water is also addressed in this review to understands the knowledge gap on this research topic.

2.1 Introduction

Eutrophication has become a major problem all around the world. Eutrophication decreases the water quality and recreational values and causes the death of the phytoplankton, fish and other organisms in the affected water bodies. Because of the above-mentioned reasons, eutrophicated lakes have gained recent attention for treatment. Eutrophic waters can be recovered by either chemical or biological processes. In biological processes, algae (which refer to macroalgae, microalgae and cyanobacteria in this thesis), among other aquatic plants, have gained attention recently in water treatment. Among the different types of algae, the prokaryotic cyanobacterium *Spirulina platensis* can be used effectively for eutrophic water recovery because it is non-toxic, fast-growing, easy to harvest since it is filamentous and with known commercial values as a source of human nutrients, nutraceuticals, antioxidants, and pigments. This literature review discusses eutrophication, sources of nutrients, and different methods to restore eutrophic waters with a particular focus on the potential of microalgae in eutrophication mitigation and eutrophic water restoration.

Eutrophication has become a major concern of water pollution in the middle of the 20th century when the algal blooms started to emerge and then grow rapidly in the European and Northern America rivers and lakes[5]. Eutrophication is a natural ageing process of the lake but due to the increased population, industrialization, and modernization of agriculture, the ageing process is accelerated to a rate beyond the capacity of the natural restoration of these water bodies[6] and hence the destruction of the aquatic ecosystem involved.

2.2 Eutrophication

The word eutrophication is derived from "eutrophos", a Greek word meaning enriched or well-nourished. Eutrophication occurs due to the excessive loading of nutrients (mainly phosphorus and nitrogen) into the water bodies from different sources[3]. It can be technically defined as "the enrichment of water by nutrients causing an accelerated growth of algae and higher forms of plant life to produce an undesirable disturbance to the balance of organisms present in the water and the quality of the water concerned" [7, 8].

According to the Canadian framework, the phosphorus trigger ranges have been set for all the lakes and rivers as shown in Table 2-1. They are classified according to the trophic status of the area. Oligotrophic water can be eminently used for all purposes and has excelled water quality, mesotrophic has good water quality and suitable for main purposes, meso-eutrophic has intermediate water quality and some uses may be jeopardized, whereas eutrophic and hyper-eutrophic has bad and very bad quality and unsuitable for main and several uses [9].

Table 2-1 *Total phosphorus trigger ranges according to Canadian framework* [6]

Trophic status	**Trigger Range in Phosphorus (µg/L)**	**Trigger Range in Phosphate (µg/L)**
Ultra- Oligotrophic	<4	<12.26
Oligotrophic	4-10	12.26 - 30.66
Mesotrophic	10-20	30.66 – 61.33
Meso-eutrophic	20-35	61.33 – 107.32
Eutrophic	35-100	107.32 – 306.65
Hyper-eutrophic	>100	>306.65

If the value of total phosphorus reaches above the trigger range then it shows that there is some potential environmental problem and it will lead to investigate several water properties like pH, salinity, nutrient contents, etc. This trigger ranges help us to understand the exact condition of the water bodies and also help us to manage the water bodies in a good way [10].

2.2.1 Sources of nutrients

The main nutrients responsible for eutrophication are phosphorus and nitrogen. The contents of phosphorus and nitrogen can be increased in the water bodies because of the runoffs from agricultural lands, livestock breeding, industries, domestic and human sewage, aquaculture and local household activities [11]. Among these sources, agricultural runoff plays a major role in eutrophication. The increased population force to increase agricultural practices all over the world has a direct impact on the increase in the usage of fertilizers. The major components of fertilizers are nitrogen and phosphorus [12]. When it rains, especially when the storm comes, the runoff from agricultural land is fed to rivers and lakes, which will eventually increase the nutrient content.

Figure 2-1 *Soil and fertilizer runoff from a farm after heavy rain (Credit: National Science Foundation)*

Figure 2-1 shows the agricultural runoff due to heavy rain. Similarly, industrial wastewaters, domestic sewage discharge increases the nutrient content in the surface water bodies and they are considered as point sources. The nutrient sources (nitrogen, phosphorus) are relatively well-controlled in developed countries but still a major contributor in developing countries [13]. In aquaculture, depleting the fish stock by fishing too much has become a major problem. Because of the excess demand for fishes, excessive amounts of feed of high nitrogen and phosphorus contents have been given to the fish, which directly contributes to eutrophication [14]. Worth noting is that the use of phosphorus-containing detergents used to be a major source of phosphorus loading. Although we have replaced them with phosphorus-free detergents nowadays, all the nutrients that have been accumulated in the beds of lakes over decades and decades ago still contribute to the eutrophication problem.

The high nutrient content in water bodies leads to excessive growth of phytoplankton and accumulation of biomass. This increase in phytoplankton in a particular area may increase the amount of fish harvested from that area but only up to a certain point after which the harvesting will start to decrease. Thus, the higher nutrient input increases the fish biomass as well as their preys in water bodies in an advantageous way. Whereas on the other side the increased accumulation of biomass on the surface will decrease light penetration, which results in intruding on the function of macroalgae and aquatic plant growth and their reproduction. The algal organic matter starts to sediment to the bottom layer of the water bodies, where they would decompose and eventually deplete the oxygen to cause the hypoxic condition. Hypoxia is a condition when the dissolved oxygen level is too low for fishes and other aquatic animals to survive, ultimately forcing them to flee the area or start to die [15]. The benchmark of hypoxia is 4 mg/L DO.

Eutrophication can be caused by nutrient inputs from a point source or non-point source. Point sources include industrial outlet, sewage water effluents and fish farms. Whereas non-point sources include runoff from urban areas, agricultural lands, septic tank and constructional site [16,17]. Figure 2-2 shows the schematic representation of the eutrophication process.

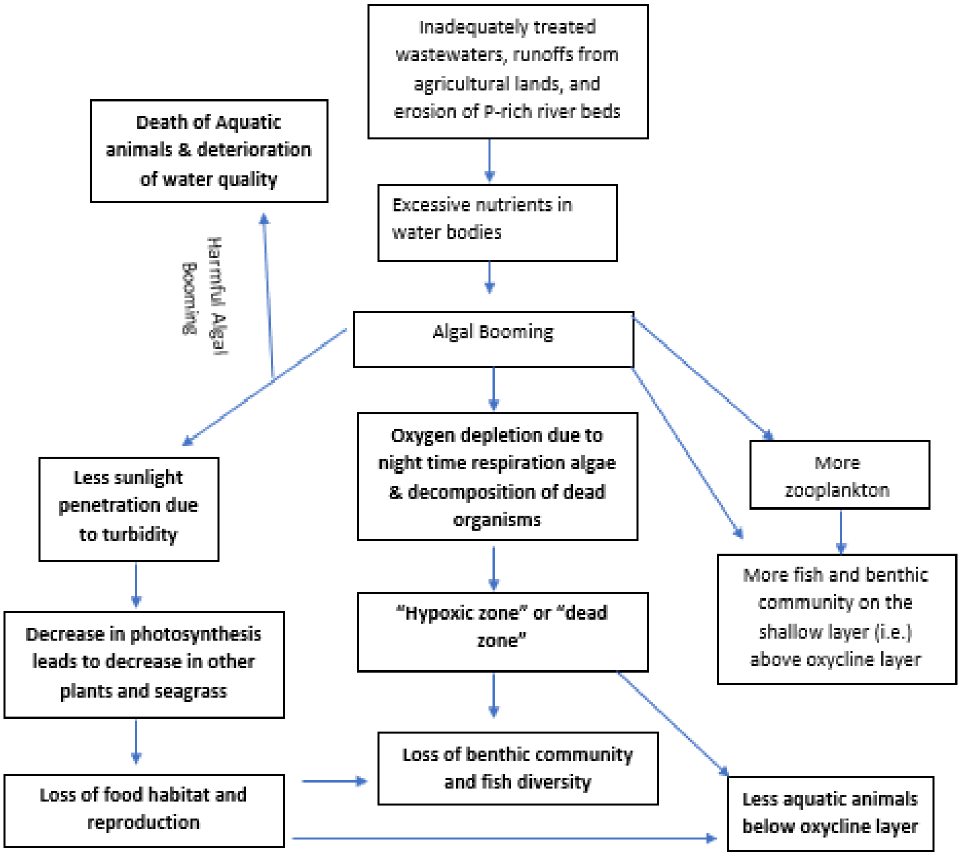

Figure 2-2 *Schematic representation of Eutrophication process modified according to [11]. Harmful effects of eutrophication are highlighted in bold*

2.2.2 Algal Blooms

Algal blooms are defined as the increase or accumulation of cells of one or more phytoplankton species in fresh or in marine water bodies [18]. Algal blooms can occur when excess nutrients are available to the species while other conditions such as temperature and radiation are favourable. The algal bloom can be of different colours like green, red, brown, purple and yellowish-brown depending on the type of algae as well as the environmental condition [19]. The cell density of algal blooms can vary from hundreds to thousands of cells per millilitre depending on the phytoplankton species. The algal bloom can also be classified as "mini-bloom" which is caused by the harmless algae and "harmful algal bloom". The algal bloom can happen due to cyanobacteria, microalgae, as well as macroalgae, where the macroalgal blooms are easily recognizable and hence it is easy to remove [20].

2.3 Negative effects of algal bloom

Algal blooms can have a negative effect not only on the aquatic ecosystem but also on human beings, the environment, economy and industries relying on the affected water system.

2.3.1 Depletion of oxygen – hypoxia

When the algae start to bloom with the availability of excessive nutrients, they may produce a large quantity of oxygen during the daytime but may use up all the dissolved oxygen (DO) in the water during night-time, especially when algae need to consume oxygen for respirational cell maintenance. Oxygen depletion may also be enhanced by the decomposition of dead biomass settled to the bottom of the water. When the DO decreases to a certain level, i.e., 2 mg/L, "hypoxia" happens [21]. As aforementioned, the hypoxic condition will pose a threat to many fishes and aquatic organisms to survive. So, the young fishes and other organisms will leave that particular area which is termed as a "dead zone" [22].

2.3.2 Blockage of light

Algal bloom will form a thick layer on the surface which prevents the light from penetrating the water surface. Sunlight is necessary for various plants and photosynthetic microorganisms, which produce their nutrients and food for their survival. When this sunlight is deprived, aquatic organisms cannot produce enough food and this leads to a reduction in several benthic organisms and various types of aquatic plants [23]. The algal bloom may form a thick blanket of biomass on the surface which reduces the visibility for the fish to swim and also reduces the ability of the fish to find food. This may lead to the death of the fishes or force the fish to leave that particular area [22].

2.3.3 Diminishing water quality

Algal bloom discolours the water body and also diminish the water quality depending on the algal species and pigments. When this water comes to direct exposure to humans or animals it causes various allergic reaction and health issues [22]. Algal blooms will increase the cost of drinking water treatment as well as increase the cost of cleaning the water body. Harmful algal bloom can release a certain type of toxins which is detrimental for other living organisms in the water as well as humans [22].

2.3.4 Harmful Algal bloom (HAB)

Harmful algal blooms can be caused by a diversified group of organisms like toxic cyanobacteria, macroalgae and benthic algae [24]. Cyanobacteria are a blue-green photosynthetic organism, which grows in lake, river, ocean or in pretty much any natural water bodies which often cause harmful algal blooms. Among the different species of cyanobacteria, some are harmless and some are toxic [25,24]. Studies show that around 25%-75% of cyanobacteria are toxic [26]. When a water body is accumulated with excessive nutrients, microalgae will start to grow rapidly. The condition under which cyanobacteria start to produce cyanotoxins is not well understood. Some cyanobacteria which can produce toxins may not produce them under certain conditions but some cyanobacteria produce multiple types of toxins like microcystin, anatoxin, aplysiatoxins, debromoaplysiatoxin, lygbyatoxin, nodularin, etc[27,25]. Among these cyanotoxins, microcystin is the dominant toxin produced by cyanobacteria. These toxins levels will be increased in the water which will reduce the water quality and directly affect the environment as well as local people surrounding the area.

The harmful algal bloom is detrimental to the fishes and other aquatic organisms. Figure 2-3(a) and (b) shows the death of a catfish due to toxic algal bloom on the shore of North Toledo, Ohio. In 2007, according to U.S. Fish and Wildlife Endangered Species database, 139 fish, 70 mussels, 4 crayfish, 23 amphibians and one dragonfly were endangered or facing threat to extinct. Every year the US government is spending around $44 million to prevent aquatic biodiversity from eutrophication. The government loses around $2.2 billion annually due to the damage caused by eutrophication in US freshwaters [28].

Figure 2-3 *(a) and (b) catfish died in the shoreline due to algal bloom in North Toledo, Ohio, USA 22. (c) Fishes died due to algal bloom in Lake Okeechobee (Source: USGS)*

2.3.5 Health Risk of Algal blooms on Humans, Animals and Plants

When the algae start to bloom it smells like fresh grass and later when it reaches its death stage it smells like rotten [29]. During heavy algal blooms, the current water treatment is inadequate to remove the cyanotoxins which will continue to stay in the processed water from the wastewater treatment plant. The reports from the USA and Australia state that the cyanotoxins cause an acute lethal effect when the algal bloom is treated with other chemicals (e.g.) copper sulphate, which leads to breakage of cells and releases more cyanotoxins. These toxins will create problems in human health like abdominal pain, vomiting, nausea, diarrhoea, sore throat, cough, headache, pneumonia and blistering of the mouth. Similarly, these toxins can affect humans while taking shower or through some recreational activities like water-skiing [30].

2.4 Eutrophication- a global environmental problem

Eutrophication has become a major threat to water quality around the world. The World Resources Institute (WRI) has published a map (Figure 2-4), which shows that around 762 coastal areas all over the world were affected by eutrophication or hypoxic condition [31], where hypoxia is a state in which dissolved oxygen decreases with increase in total phosphorus content and biomass concentration [32]. In these affected areas, around 228 sites are experiencing eutrophication symptoms like algal bloom, loss of other aquatic species and creating a negative impact on the coral reefs. Around 479 sites were affected by eutrophication and under hypoxic condition, out of which 55 sites are now improving [31]. The southern part of Asia which includes India and China contributes to around 91% of the world phosphorus fertilizer usage, which is followed by South America at 21% and North America at around 4%. Consequently, Southern Asia ranks top among all the above-mentioned countries in anthropogenic nutrient input, which will result in an adverse negative impact on the environment, one among them is eutrophication. On the positive side, Europe successfully reduced its annual consumption of phosphorus fertilizer by around 17% between the years of 2009-2013 when compared to the 1960s [33].

In the USA, Environmental Protection Agency (EPA) has been trying to restore the water bodies which have been impacted by eutrophication.12 out of 14 rivers and lakes in the USA ecoregions have exceeded the total nitrogen and total phosphorus reference median values.

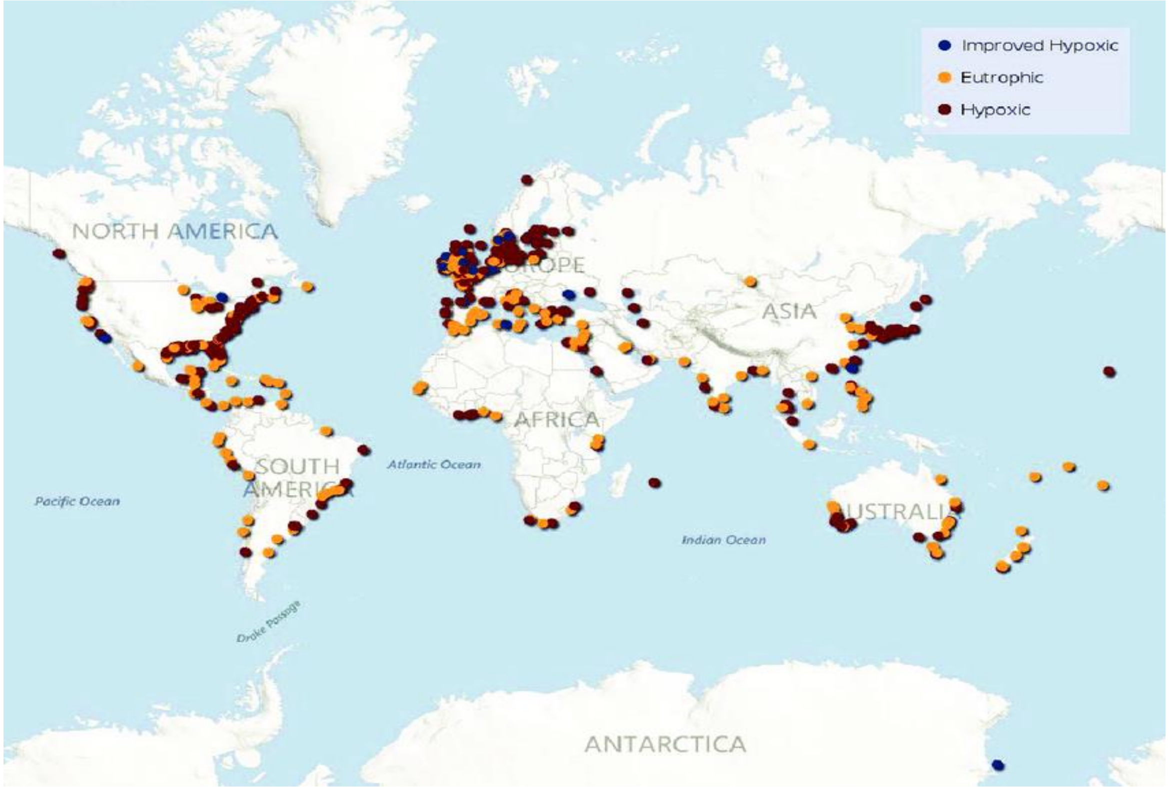

Figure 2-4 *World map showing areas affected with Eutrophication & Hypoxia* [24]

During the summer season of 2016, Lake Okeechobee, the largest freshwater lake of Florida has faced a severe algal bloom which resulted in a massive negative impact on the livelihood of people as well as aquatic organisms, closure of beaches, loss of aquatic organisms, tourist and recreational activities [2]. This algal bloom was spread throughout the state of Florida through Lake Okeechobee to the St. Lucie river and its estuary, dominating the cyanobacterial bloom. The *Microcystin* from the cyanobacterial bloom reduced the water quality to such an extent that the Florida government had to declare a state of emergency to reduce the human activities near the Florida river [34]. Figure2-5(a) shows the aerial view of toxic algal bloom in Lake Okeechobee in July 2016 and Figure 2-5(b) shows the algal bloom of Lake Okeechobee in June 2019 [2].

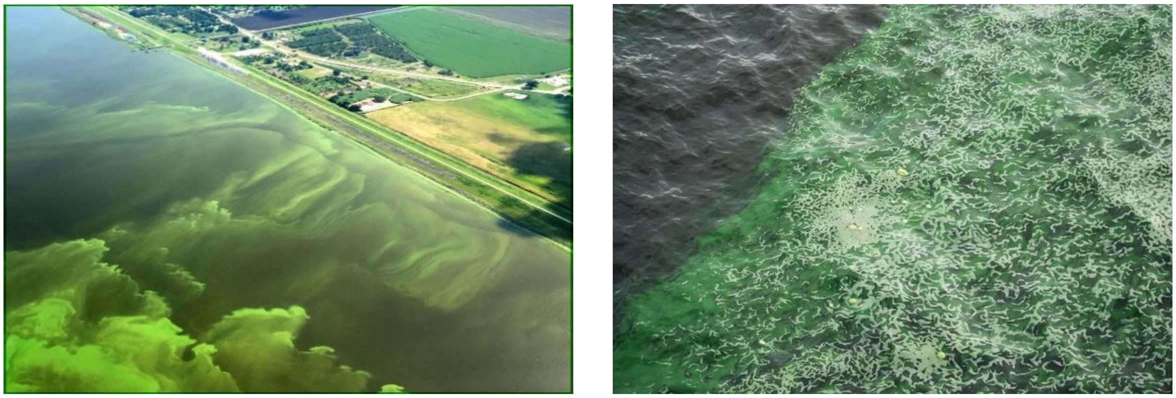

Figure 2-5 *(a) Aerial view of algal bloom in Lake Okeechobee in summer 2016* [2] *(b) Algal bloom in Lake Okeechobee in June 2019 (Photo: XAVIER MASCAREÑAS/TCPALM)*

In China, Qingdao, the major city of Shandong province, was drastically affected by major algal blooms in the Yellow Sea in the years 2013 and 2017. These algal blooms were caused by a common green algal species *Enteromorpha prolifera*, which are non-toxic to humans and animals. However, the blooms created a drastic change to the lives of living organisms that are surviving beneath the blanket of algae in the ocean, leading to the formation of the dead zones in some severely affected regions.

Figure 2-6 *Algal bloom in Qingdao Bay, on July 11, 2019 (PHOTO CREDIT: AFP news agency)*

Similarly, in developing countries like India along with a rapid increase in industrialization, population and urbanization, eutrophication also has become a major problem. According to a survey[35], around 15% of the total India population play a part in discharging phosphorus-containing wastewater effluents into water bodies.

In Europe Arctic waters, Baltic sea, Greater North Sea, Celtic Sea, Bay of Biscay and the Mediterranean Sea are facing an adverse impact of eutrophication. The major source of phosphorus discharges that lead to algal blooming in Europe is the discharges from agricultural lands, animal husbandry, industry, households and also soil erosion [36].

2.5 Eutrophication in Lake Erie - Canada

The University of Alberta researched during 2001-2011 by collecting samples from several lakes and rivers and found that 246 water bodies in Canada have microcystin which is a cyanobacterial toxin [37].

Figure 2-7 *Algal bloom in The Great Lakes in October 2011* [38]

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Among the five Great Lakes of North America, Lake Erie is the fourth-largest lake. Since the 1960’s Lake Erie has been affected by eutrophication because of the external loading of phosphorus. This led to harmful algal blooms in the lake. These blooms at some time create hypoxic condition. As shown in Figure 2-7 among The Great Lakes of Canada, algal blooms can be noticed prominently in Lake Erie.

In the year 1972, the Great Lakes Water Quality Agreement (GLWQA) was signed between the USA and Canada to restore the water quality in Lake Erie. In the year 1980’s around 8 billion USD dollars was spent to reduce the external phosphorus loading to Lake Erie by building sewage water treatment plants. But the investigation shows that even after building sewage water treatment plants, the algal bloom and hypoxic condition increased during the late summer.

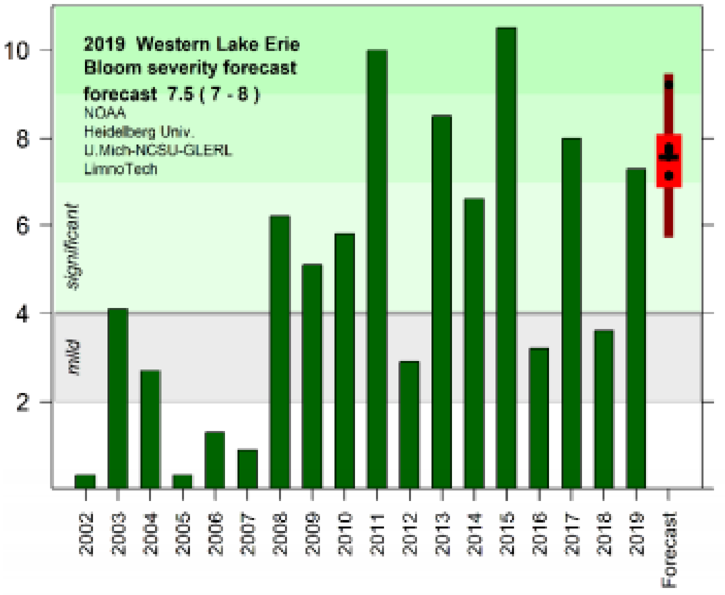

Figure 2-8 *Algal bloom Severity Index (SI) from 2002-2019* [39]

Scientists of the National Oceanic and Atmospheric Administration predicts the severity of algal bloom using an index scale from 1-10 called as severity index (SI) scale. They predicted that the algal bloom in Lake Erie in 2019 will be worse than in 2018 as shown in Figure 2-8 [39]. As predicted, the algal bloom in 2019 has severity index (SI) of 7.3 out of 10 whereas in the year 2018 the SI was 3.6 and in 2017 the SI was 8 [39].

The Heidelberg University has been collecting the Total Bioavailable Phosphorus (TBP) load into Lake Erie through the Maumee River which is located in the northeastern part of Ohio. The data in Figure 2-9 shows that in the year 2019, 472 metric tons of TBP from the Maumee river had been loaded into Lake Erie. Years 2015 and 2011 seem to have a higher load of TBP around approximately 600-700 metric tons and that created severe algal blooms in Lake Erie during that two particular years[39]. Figure 2-10 shows the algae bloom in Lake Erie on July 28th, 2015 which is captured by Landsat 8.

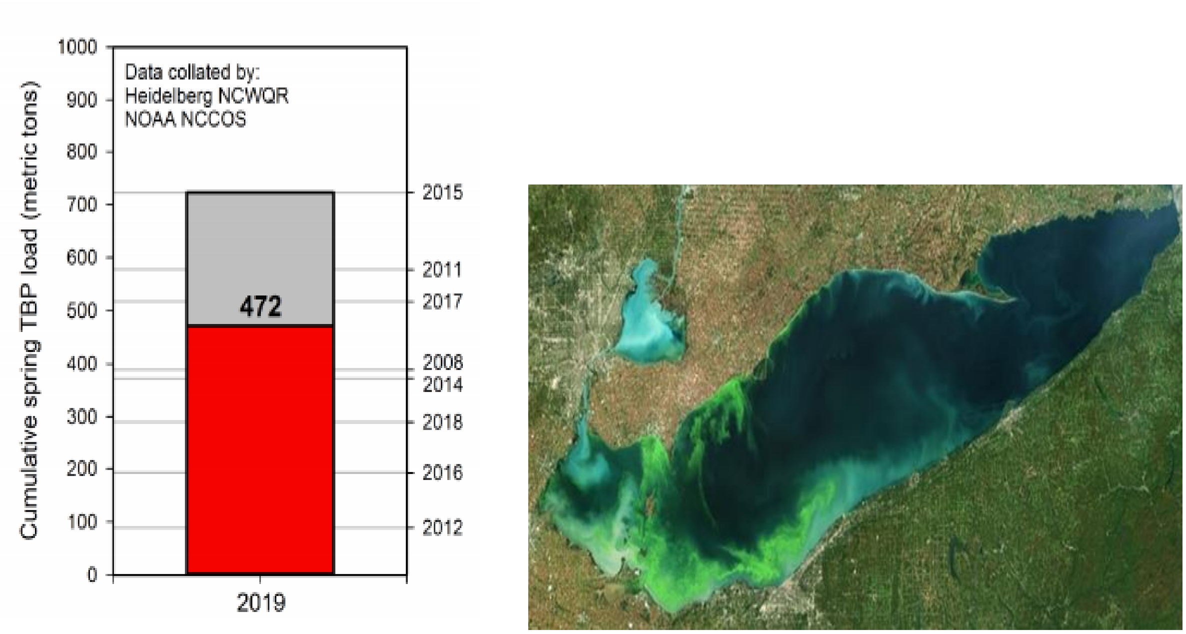

Figure 2-9 *(a)Total Bioavailable Phosphorus (TBP) from the Maumee river for years 2008-2019* [39] *(b) Algal bloom in Lake Erie in 2019 in x-axis compared with other years in y-axis* [40]

The algal bloom in the year 2015 on the western part of Lake Erie was a replicate of the year 2011 bloom which created adverse effects on humans, pets and aquatic organisms. On August 2nd 2014, environmental monitors detected that microcystin toxin level was higher in the water than recommended by the World Health Organization (WHO). The harmful algae bloom created a major impact on water quality consumption and recreational activities which led to the shutdown of the drinking water supply to Ohio and Toledo completely, affecting half a million residents living near the lake. After that, the water treatment department included extra filtering steps like treating water with activated carbon. On August 4, 2014, the Public Water Sources announced that the water is safer to drink [41].

Figure 2-10 Algal bloom around Lake Erie on July 28th, 2015 captured by Landsat 8 [42]

In Lake Erie, microcystin is the dominant toxin produced by algae which cause dizziness, liver damage, numbness and vomiting. It is not just humans who get affected due to algal blooms, even pets are affected when they come in contact with blue-green algae. In the year 2017, during the summer season, the deaths of several dogs near Vitoria Lake in British Columbia are suspected to be due to their contact with the algae. Similarly, in 2018 three dogs died in Canada after being exposed to algal blooms [38].

Figure 2-11 *(a) Children swimming in algal bloomed water at Silverwood lake, California* [43] *(b) Drinking water condition of Lake Erie on August 2nd, 2014* [44]

2.6 Eutrophication mitigation and eutrophic water restoration

2.6.1 Chemical dosing

Nutrients in eutrophic lakes can be treated by chemical dosing, which includes chemical precipitation and chemical inactivation. Chemical precipitation makes the inorganic form of phosphorus in the water column to settle to the bottom of the lake, whereas the chemical inactivation method inactivates the phosphorus settled and prevent its further release [3]. Iron is commonly used in phosphorus precipitation because of its key role in the phosphorus cycle. Iron is recommended to be used in the lakes of low-level pH because during the thermal stratification of the lakes hypolimnetic anoxic condition (which is a severe condition of hypoxia) can occur [45]. On the other hand, slaked lime is injected into the lakes of higher pH up to 9, but the success was short term because the phosphorus precipitation was occurring in the hypolimnion layer, which could be released into the water again when the condition changes [46].

The first chemical precipitation treatment to reduce phosphorus in the lake was done in Sweden during the early 1960s and late 1970s. In the USA, the Horseshoe lake was the first lake to be treated in 1970. Aluminium, Calcium and Iron salts were widely used to treat the drinking water supply over several centuries[47].

Algicides like copper sulphate were used to vanish the algae, but these algicides impacted the non-target organisms rather than the targeted toxic algae. Similarly, in Canada, slaked lime was added to the shallow surface of the lake. Since the precipitation of phosphate happened in the hypolimnion layer but the goal was short term due to the thermal stratification of the lakes[47].

Similarly, Horseshoe lake was treated with alum and there was a positive result but the success was short term because the lake was still receiving external phosphorus loadings [48]. The alum treatment in the hypolimnion layer of Eau Galle Reservoir in Wisconsin made a drastic reduction in the internal loading of phosphorus but the external loading made the phosphorus to increase in the epilimnion layer [49]. Though the alum treatment was cost-effective and can be applied over a large surface area, it cannot compensate for the external phosphorus loadings to the lakes. The alum treatment is effective if the pH is around 6-8 as they form an insoluble $Al(OH)_3$. In the lower pH, the soluble Al^{3+} dominates the insoluble $Al(OH)_3$ which can be toxic to the lake [47]. Whereas if the pH of the lake is higher it will start to release phosphorus back to the lake as well as producing toxic aluminium ions [50]. These chemicals can impact non-targeted organisms and plants.

2.6.2 Biological Process

In the biological process, the usage of microalgae in water treatment has been trending. When microalgae grow, they assimilate nutrients for their cell growth. Heterotrophic organisms such as fish, fungi, and bacteria can break down organic nitrogen into inorganic nitrogen such as nitrate and ammonia, which are the desired form of nitrogen needed for the growth of microalgae. Among these, microalgae prefer ammonia because it uses lesser energy during the assimilation[51].

Algae can grow photo-autotrophically by using light as an energy source to fix carbon dioxide to produce organic matter (e.g., sugars, proteins, etc.) for cell growth. So, they serve a dual purpose in removing the nutrients from the wastewater as well as using the produced biomass for extraction of bio-products. Since phosphorus-limited microalgae uptake more phosphorus than nutrient-rich microalgae, there is a possibility that using phosphorus-limited microalgae in the eutrophicated water will increase the phosphorus assimilation rate.[52]

While algae are efficient consumers of reduced nitrogen such as ammonia and nitrate, many algal species, especially cyanobacteria, can fix molecular nitrogen (i.e., N_2), which is abundant in the atmosphere, for cell growth. This process is called Biological Nitrogen Fixation (BNF). Therefore, it is of little meaning to control the nitrogen to prevent algal blooms. Logically, phosphorus mitigation becomes the primary strategy for eutrophication mitigation and eutrophication water recovery.

2.7 Phosphorus Removal by Microalgae

Phosphorus is an essential element for the growth of microalgae. Algae take in phosphorus in the form of orthophosphate, preferably dihydrogen phosphate ($H_2PO_4^-$) or hydrogen phosphate (HPO_4^{2-}).[53] Biological phosphorus removal is achieved by the microalgae via two different mechanisms,(1) essential phosphorus consumption and (2) luxury phosphate consumption. In the first mechanism, the microalgae cells consume phosphorus mainly for the production of phospholipids, nucleic acids, ATP and nucleotides[52,54]. Algal cells have approximately 1% of phosphorus by dry weight but under certain environmental conditions, algae are triggered to consume more phosphorus for storage [54]. In this mechanism, algae uptake phosphorus and store it in their cells for later growth. This process is called "Luxury uptake of phosphorus"[55]. In this luxury uptake process, phosphorus is stored in the biomass in the form of polyphosphate. This polyphosphate can be stored in two types, i.e., acid-soluble polyphosphate and acid-insoluble polyphosphate. Acid soluble polyphosphate is involved in the metabolism whereas the acid-insoluble polyphosphate is stored to be used when the external phosphorus concentration becomes limiting [55]. When this phosphorus in the environment becomes limiting, algae will use the stored phosphorus which is assimilated by the luxury uptake process. [54]

A research paper reported that in the shallow algae culture, *Chlorophyceae*, which is a type of green algae, has a phosphorus removal efficiency between 78% and 92% with a removal rate of 2.2 mg P L^{-1} day^{-1} and in the deeper culture it showed a phosphorus removal efficiency between 66% and 88% at a removal rate of 1.9 mg P L^{-1} day^{-1} [52]. In another study, experimental results showed that *Chlorella vulgaris* has a removal efficiency of 78%. It reduced the PO_4-P concentration from 7.7 mg L^{-1} to 1.7 mg L^{-1} [56]. *Chlorella kessleri* microalgae showed a low phosphorus removal efficiency of 8-20% with an initial concentration of 10 mg L^{-1} under continuous dark/light cycle [18]. *Chlorella vulgaris* and *Chlamydomonas reinhardtii* under temperate conditions uptake phosphorus in a polyphosphate form which accounts for 53±8 % removal efficiency of biomass phosphorus [57]. Similarly, in aqueous solution, *Chlorella vulgaris* showed 89.90% efficiency when phosphate concentration was 0.25 g/L and 88% when phosphate concentration was 0.45 g/L [58]

2.8 *Spirulina platensis*

Spirulina platensis is a planktonic blue-green alga that shows an enormous population in water bodies that needs a moderate pH to grow. Relatively high pH also suits well for *Spirulin*a, which can inhibit the growth of other algae within the system. To preserve high pH and prevent variability, the culture medium should .have a high level of sodium bicarbonate[59] *S. platensis* has been used as a staple food by the Aztec people since the 16th century. They have high nutrient content like phycocyanin, chlorophyll, carotenoids, protein, essential and non-essential amino acids, gamma-linolenic acid(GLA), natural vitamins and minerals which make them one of the earth's superfoods [60]

Table 2-2 *Nutrient composition of Spirulina with milk and egg*[57]

Nutrients	**10 gm *Spirulina***	**200 ml Milk**	**One Egg**
Protein	6.6 g	6.6 g	6.6 g
Vitamin A	14000 I.U.	248 I.U.	1.050 I.U.
Nicotinic Acid	1.18 mg	0.20 mg	0.04 mg
Riboflavin	0.40 mg	0.38 mg	0.19 mg
Thiamine	0.55 mg	0.01 mg	0.095 mg
Vitamin B12	30.0 mcg	0.28 mcg	2.3 mcg
Iron	5.8 mg	0.40 mg	1.6 mg

During the 60th United Nations General Assembly held on September 13th 2005, a draft resolution was submitted by Burundi and people on the "Use of *Spirulina* to combat hunger and malnutrition and help achieve sustainable development"[61]. The use of *Spirulina* as food became famous when NASA used them as a potential food source for the astronauts in the Moon Mission and planning to use them in future for the Mars Mission. Krutika Desai & Subramanian Sivakami compared the nutrient composition of *Spirulina* with milk and egg as shown in Table 2-2.

2.9 Potential of *Spirulina platensis* for eutrophic water restoration

Spirulina is recognized worldwide in two species form such as *Arthrospira platensis* and *Arthrospira maxima* and they are considered innocuous [62]. In the year 1999, Health Canada conducted broad testing for cyanotoxin content in several blue-green algae out of which none of the 10 samples of *S. platensis* contained microcystin and it was announced that *Spirulina* was microcystin-free [63]. *Spirulina* manufactured by the Cyanotech Corporation has also received GRAS (Generally Recognized As Safe) certificate by FDA (Food and Drug Administration) in the year 1981 [64]. *Spirulina* does not produce toxin by itself but they may be contaminated due to contact with other toxin-producing blue-green algae. Consequently, some samples of *Spirulina* have microcystin content but below the critical limit set by the Oregon Health Department (OHD) [64].

Spirulina shows great ability to remove phosphate. It showed 81.49% of phosphate removal efficiency when 0.45 g/L of phosphate solution was for 8 days [58]. When *Spirulina platensis* is used to treat municipal wastewater it reduced the phosphate concentration from 58.98 mg/L to 34.32 mg/L in 6 days of treatment [65]. Experimental results also show that *Spirulina platensis* showed 100% removal of phosphorus when the phosphorus concentration was lower (10 mg/L) with higher light intensities ($60\ \mu E\ m^{-2}\ s^{-1}$) and showed the lowest removal percentage when phosphorus concentration is higher [66]. So, from the above evidence, we can say that *Spirulina* may have a greater efficiency to remove phosphate from eutrophicated water and effectively reduce the eutrophic water to the mesotrophic or oligotrophic state.

S*pirulina* is a multicellular and filamentous blue-green alga. Because of its filamentous and spiral shape, the *Spirulina* is easy to harvest [67]. Cheap Nylon mesh can be used to separate the biomass from the liquid and based on the requirement *Spirulina* can be harvested with different nylon mesh size. Because of the easy harvesting method, biomass concentration can be controlled very effectively.

Spirulina has several nutrient components like protein, carbohydrates, vitamins, pigments, amino acids, lipids, etc. Microalgae biomass is widely used as a fish meal in several countries. *Spirulina* is fed to fish, shrimp and salmon. *Spirulina* enhanced aquaculture as well as increased the protein digestibility, improved resistance to stress and disease and influenced in early maturation of the fish which leads to shorter breeding periods as well as reduce the period of fish cultivation [68]. *Spirulina platensis* consist of several bioactive components, which can be marketed as high-value commercial products to offset the cost during production.

2.9.1 Biomass

Batch cultivation of algae typically starts with the lag phase when cells are adjusting to the new environment and show no apparent growth, then cells enter the exponential phase and grow at the maximum specific growth rate. For photoautotrophic cultivation of algae, the exponential phase is usually short as light limitation develops quickly with the increase of biomass concentration. This is followed by a transition phase which could be short or long and may exhibit a linear growth period. After the transition phase, cells reach the stationary phase when the apparent growth rate becomes zero [69]. The biomass is usually harvested in the late exponential phase or early stationary phase using appropriate methods. The biomass of *S. platensis* possesses high-value bio components like protein, fatty acids, amino acids, pigments, phenolic acids, polysaccharides, phycocyanin, vitamins, antioxidant components, carotenoids, phycocyanin, etc which can be used in nutraceutical, pharmaceutical applications, biofuel production or in energy conversion technologies[70,66].

2.9.2 Protein

Protein is an essential component and a building block for the human body. As a blue-green alga, *Spirulina platensis* is a complete protein source with low cholesterol and calories. The protein content of *S. platensis* varies from 50-65% of the dry weight, based on the growth and environmental conditions[71]. When the biomass of the microalgae increases, the protein content will also show an increase eventually. Whereas the rich vegetable protein source like soya has 35% of protein content and even the meats like raw beef have only 23% of protein content[72]. Hence the *S. platensis* offers higher protein content compared to all the other protein sources whether it is meat or plant-based protein.

2.9.3 Chlorophyll

S. platensis is known to have a high content of chlorophyll and the extraction of chlorophyll is widely studied because of its anti-oxidant, anti-mutagenic, anti-cancer and anti-obesity properties[73,74]. The chlorophyll cellular content will be high when there is high light intensity. Among the several kinds of chlorophyll, Chlorophyll A and B are the major forms available in cyanobacteria. Among these Chlorophyll A is dominant in *S. platensis*[74]. *Spirulina platensis* contains about 2 weight % of chlorophyll in its biomass, which is ten times higher than recorded in ordinary terrestrial plants. Hence *S. platensis* have 8-10% photosynthetic conversion efficiency compared to land plants which just has 3% conversion efficiency [75].

2.9.4 Phycocyanin

Phycocyanin belongs to phycobiliprotein, a family of proteinous photosynthetic pigments present in some cyanobacteria and microalgae. Among the different types of phycobiliprotein, phycocyanin is most important because of its pharmacological properties. Recently many researchers have signified the anti-oxidant, anti-cancer, anti-inflammatory and hepatoprotective properties of phycocyanin[76]. Phycocyanin is a blue-green pigment that can be used as a colouring agent in food, cosmetic, textile, drug industries that can potentially replace the synthetic colours used in the food and drug industry [77].

2.10 Conclusion

The purpose of this literature review is to help the readers widely understand eutrophication and its negative impacts on humans, animals as well as aquatic organisms. Through this literature review explains eutrophication around the world, the main focus of this review is to concentrate on eutrophication in Lake Erie, Canada and Canadian Standards of Water Quality. This review also elaborates on the non-toxic algae, their application and their biosorption properties to remove nutrients from wastewater. But to study the efficiency of *S. platensis* to treat Artificial Eutrophicated Water (AEW), further lab experiments are needed.

Chapter 3: Potential of *Spirulina platensis* for Eutrophication Mitigation and Eutrophic Water Restoration

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Abstract

The removal of phosphate from Artificial Eutrophicated Water (AEW) by *Spirulina platensis* is studied in this paper. Experiments were conducted at different levels of eutrophication states like hyper-eutrophic, eutrophic and meso-eutrophic at room temperature (23-24 ^{o}C). *S. platensis* showed the capacity to reduce the AEW from the hyper-eutrophic and eutrophic levels to oligotrophic and ultra-oligotrophic levels respectively, after a 16-day growth period of cultivation. During this period, the pH, biomass, protein, phycocyanin and chlorophyll A and B were measured using respective analytical methods.

Keywords: *Spirulina platensis*, eutrophic water, restoration, wastewater

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3.1 Introduction

Water covers a major part of the earth’s surface and plays an essential role in all living beings. However, water pollution has become an increasing concern because of different reasons like the rapid growth of the world population, industrialization, and climatic change.

Eutrophication, which refers to excessive algal blooms in water bodies including ponds, lakes, rivers and oceans, has become a major form of water pollution. When excessive nutrients accumulate in water bodies, cyanobacteria and microalgae (they are collectively referred to as algae hereafter) will start to grow rapidly in seasons with sufficiently high temperature and solar radiation intensity. When algae reach the death phase of their growth, cells will start to rupture and some species may release deadly toxins like microcystin, anatoxin, aplysiatoxins, debromoaplysiatoxin, lygbyatoxin and nodularin [27]. Among these, microcystin is the dominant toxin produced by the algae. The blooming of toxic algae leads to the accumulation of toxins in water and eventually bring them to a level that is harmful to aquatic organisms and humans depending on the affected waters. Algal blooms may also cause other detrimental effects to the aquatic system including hypoxia. While algae produce oxygen during daytime in photosynthesis, they consume oxygen in respiration during night-time for cell maintenance. Furthermore, dead cells would be decomposed, which is a process that consumes a large amount of oxygen and emits unpleasant odours.

Therefore, a few criteria need to be satisfied for an algal species to be considered for the successful implementation of the “control-algal-blooms-using-algae” strategy. These include: 1) the algal species involved must be non-toxic during any physiological stage of its life cycle; 2) they should be able to grow well in water containing phosphate and other nutrients 3) they should be able to grow well at a phosphate level that is commonly found in eutrophic waters and be effective in removing phosphate in such conditions; and 4) it should be easy to harvest the algae to avoid over-accumulation of algal biomass in waters, to prevent algae from entering death phase in water and to recover algal products to offset costs. To this end, cyanobacterium *S. platensis* seems to be a promising candidate.

S. platensis, alias *Arthrospira platensis*, is one of the two commercially important *Spirulina* species (the other one being *S. maxima*) [62]. *Spirulina* does not produce toxin and has been recognized as nontoxic by academic researchers[62] and health authorities around the globe [63]. For instance, the *Spirulina* produced by the Cyanotech Corporation received the GRAS (Generally Recognized As Safe) certificate by FDA (Food and Drug Administration), USA, in 1981 [64].

Spirulina has shown a great phosphate removal efficiency from municipal wastewater, artificial wastewater, hydroponics wastewater, etc which is discussed later in this chapter[58,65,66]. From these experimental results, we can say that *Spirulina* may have a greater efficiency to reduce the phosphorus levels of eutrophic waters to the mesotrophic or oligotrophic state.

S*pirulina* is a blue-green alga, which is multicellular and filamentous. The *Spirulin*a is easy to harvest because of its filamentous and spiral form. [67]. To distinguish the biomass from the medium, cheap nylon mesh can be used and the *Spirulina* can be harvested with different Nylon mesh sizes depending on the requirement. The biomass concentration can be managed very effectively because of the simple harvesting process.

There are many nutrient components in *Spirulina*, such as protein, carbohydrates, vitamins, pigments, amino acids, lipids, etc. In many nations, algae biomass is commonly used as a fish meal. The fish, shrimp and salmon are fed *Spirulina*. The *Spirulina* improved the growth of the fish as well as increased the digestibility of the protein, improved resistance to stress and disease and affected the early maturation of the fish, resulting in shorter breeding times as well as a decrease in the duration of fish cultivation. [68]. *Spirulina platensis* consist of several bioactive components, which can be marketed as high-value commercial products to offset the cost during production.

A key to the success of the controlling-algal-blooms-using-algae strategy is the ability to control the over-accumulation of algal cells in water and sedimentation of algal cells to the bottom. For this purpose, an easy and inexpensive method for alga harvest must be available. When algae reach the death phase, they start to consume lots of oxygen which will deplete the oxygen level in the lake leading to hypoxic condition [78]. To avoid this condition the algae needs to be harvested. Harvesting algae is usually a difficult process because of their unicellular cell structure and a smaller size ranging from 3-30 µm in diameter [79]. However, in the case of *S. platensis*, it is filamentous and spiral-shaped, so it is easy to scoop them out from the water using simple devices such as a nylon mesh. Apart from that, *Spirulina* has gained massive attention due to its nutritive values.

Various papers have studied the efficiency of *S. platensis* to remove phosphate from municipal wastewater, artificial culture or aqueous solution. But this paper concentrates on restoring the eutrophicated water at different phosphate levels using *S. platensis* at low phosphate concentrations (in micrograms), harvesting the *S. platensis* to avoid the hypoxic condition in lakes and to study the nutritive value and high-value bioproducts of *S. platensis* for industrial applications.

This paper investigates the potential of restoring eutrophicated water using a non-toxic cyanobacterium, *S. platensis*. Different sets of Artificial Waste Water (AWW) and Artificial Eutrophicated Water (AEW) were prepared and water restoration was studied.

3.2 Materials and Methods

3.2.1 Organism and inoculum preparation

S. platensis (UTEX LB 2340) was purchased from the UTEX Culture Collection of Algae at The University of Texas at Austin. Inoculum of *S. platensis* was grown in 250 ml flasks containing 25 ml medium.

3.2.2 Medium, Artificial Waste Water (AWW) and Artificial Eutrophic Water (AEW)

The medium for the cultivation of *S. platensis* inoculum was adapted from the Culture Collection of Autotrophic Organisms. The medium composed of 13.61 g $NaHCO_3$,4.03 g Na_2CO_3, 0.50 g K_2HPO_4, 2.5 g $NaNO_3$, 1 g K_2SO_4, 1 g NaCl, 0.2 g MgSO4.7H2O, 0.04 g CaCl2.2H2O, 0.01 g FeSO4.7H2O, 0.08 g $Na_2EDTA.2H_2O$ and 5 ml of trace metal solution. The trace metal solution is composed of 0.5 g $Na_2EDTA.2H_2O$, 0.7 g $FeSO_4.7H_2O$, 1 ml $ZnSO_4.7H_2O$ (1 g/l), 1 ml $MnSO_4.7H_2O$ (2 g/l), 1 ml H_3BO_3 (10 g/l) ,1 ml $Co(NO_3)_2.6H_2O$ (1 g/l), 1 ml $Na_2MoO_4.2H_2O$ (1 g/l) and 1 ml $CuSO_4.5H_2O$ (0.005 g/l). The medium is prepared and sterilized at 121ºC for 20 minutes.

AWW, and AEW were prepared according to the above recipe of the medium except that the concentration of K_2HPO_4 was adjusted according to the required phosphate level as indicated in the text.

3.3 Experimental setup and growth condition

The experimental setup consists of three 500 ml Erlenmeyer flask containing 400 ml of the above-mentioned medium with three different phosphate concentration AWW of 100 mg PO_4^{3-}/L, 50 mg PO_4^{3-}/L, 10 mg PO_4^{3-}/L and AEW of 100 µg PO_4^{3-}/L,250 µg PO_4^{3-}/L, 450 µg PO_4^{3-}/L. Different phosphate concentration is chosen according to Canadian trigger phosphate ranges in different trophic states of water. The initial pH of the solution is 9.2 ± 0.5 and the experiment is carried out in an illumination chamber (model: LI15, manufactured in the USA by Sheldon Manufacturing INC) for 15 days. The temperature of the culture is maintained at 25°C throughout the experiment.

In each flask, 10 ml of *S. platensis* inoculum of biomass density of approximately 0.297 g/L was inoculated into 400 ml of sterile medium. The culture was stirred using a magnetic stirrer at 50 rpm. External CO_2 was not fed into the cultured flask to keep the pH of the medium lower which will facilitate the biotic removal of phosphate rather than the abiotic removal.

3.4 Analytical methods

Phosphate content, pH, biomass, protein content, phycocyanin, chlorophyll A and chlorophyll B were determined in the algae samples. The samples were withdrawn once every two days from the cultivation bottles and centrifuged and washed twice with deionized water before being used for the analysis. The samples were sonicated for 27 minutes with 3 seconds on/off cycle and then the protein, chlorophyll A and chlorophyll B were measured.

3.4.1 Biomass Concentration and pH:

The biomass of the *S. platensis* was determined at an optical density at 700 nm using the GENESYS 10S UV-VIS Spectrophotometer and the pH of the medium is measured using Corning 350 pH meter.

3.4.2 Phosphate Concentration:

The phosphate concentration in the medium was determined using the stannous chloride method adapted from the Standard Methods for Examination of Water and Wastewater which can detect up to 3 µg PO_4^{3-}/L. The sample is centrifuged at 1427 (x g) and filtered using 0.45 µm filter paper to remove the debris. When ammonium molybdate is added to the phosphoric acid solution molybdophosphoric acid is formed which is further reduced to intense blue colour by stannous chloride [80].

3.4.3 Protein Concentration:

The sample was centrifuged at a relative centrifugal force of 1427 (x g) and washed twice with distilled water and sonicated for 20 minutes. The protein concentration of *S. platensis* is monitored using the Bradford assay analysis.

3.4.4 Phycocyanin Concentration:

40 ml of biomass is washed twice with deionized water to reduce the pH to 7.0-8.0. Then the biomass was re-suspended in 40 ml phosphate buffer saline (PBS) at pH 6.8 and homogenised using the vortex mixer. The sample was then put through freezing and thawing cycles for 2 days with 24 hours interval in each cycle. After that, the cell debris was removed by centrifugation at 1427 (x g) for 10 min. The supernatant was the crude extract containing phycocyanin in purple/blue colour. After that, the optical density (OD) of the supernatant was determined spectrometrically at two wavelengths, i.e., 615 nm and 652 nm. The phycocyanin content was determined using the Bennet and Bogorad formula [81 76].

$$\text{PC content} = \frac{OD_{615} - 0.474\,(OD_{652})}{5.34}$$

3.4.5 Chlorophyll Concentration:

The algal samples were washed thrice with deionized water and then sonicated for 20 minutes with 90% acetone for effective extraction of chlorophyll. Then the suspension was centrifuged at 1427 (x g) and the supernatant was subjected to spectrometer determination at OD of 647 nm and 664

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nm. The Chl A and Chl B concentrations were then calculated according to the following equation [82]:

$$\text{Chl A } (\mu g/ml) = 11.93 \text{ x } A_{664} – 1.93 \text{ x } A_{647}$$

$$\text{Chl B } (\mu g/ml) = 20.36 \text{ x } A_{647} – 5.50 \text{ x } A_{664}$$

3.5 Results

3.5.1 Mitigation of phosphate in wastewater from point sources and runoff to prevent eutrophication

Minimalizing phosphorus discharges from point sources such as high phosphorus content wastewaters and effluents of wastewater treatment plants that do not carry out tertiary treatment is the main goal of this part of the experiment. Aiming at the removal of excessive phosphate and nitrogen is an important part of the overall strategy of eutrophication mitigation. To estimate the potential of *S. platensis* for phosphate removal from high-PO_4^{3-} wastewaters, artificial wastewater (AWW) at different concentrations of phosphate, namely 100 mg PO_4^{-3} /L, 50 mg PO_4^{-3} /L, 10 mg PO_4^{-3} /L, were used to cultivate *S. platensis* in cultivation flasks for 16 days. Figure 3-1(a) shows the phosphate removal percentage in AEW by *S. platensis*. Figures 3-1(b) and 3-1(c) show the biomass and pH profiles over the 16-day cultivation period. These results are following the results reported in the literature by another research group, which are summarized in Table 3-1. The AWW of 100 mg PO_4^{-3} /L showed 56.78 % of phosphate removal with a removal rate of 3.54±0.29 mg- PO_4^{-3} L^{-1} day^{-1}, 50 mg PO_4^{-3} /L of AEW showed 77.12 % of phosphate removal with 2.41±0.26 mg- PO_4^{-3} L^{-1} day^{-1} removal rate and 10 mg PO_4^{-3} /L of AEW showed around 90 % of removal with a removal rate of 0.56±0.01 mg- PO_4^{-3} L^{-1} day^{-1} respectively on the 16th day of the experiment as shown in Figure 3-1(a). Table 3-1 compares the capacity of different algal species to remove phosphate in different types of wastewaters.

Table 3-1 *Phosphate removal by different algal species in different kinds of wastewater*

Algal Species	**Type of WW**	**Phosphate content in WW (mg/L)**	**Aeration**	**Phosphate Removal %**	**$PO4^{-3}$ removal rate ($mg\ L^{-1}D^{-1}$)**	**Reference**
Chlorophyceae	Hydroponics effluent	5-9	Yes	78-92	2.2	[52]
Chlorella vulgaris	AWW	450	Yes	88	49.6	[58]
S. platensis	Municipal wastewater	58.98	Yes	41.81	5.72	[65]
S. platensis	AWW	450	Yes	81.49	45	[58]
S. platensis	Culture medium	10	Yes	100	-	[66]
S. platensis	AWW	10-100	None	90.17-56.78	0.48-1.74	This Study
S. platensis	Artificial eutrophic water	0.10-0.45	None	95.70-96.7	0.025-0.005	This Study

Among all the above-mentioned algae in Table 3-1, *S. platensis* showed a great versatility to reduce phosphate in a diversity of different wastewaters containing different concentrations of phosphate.

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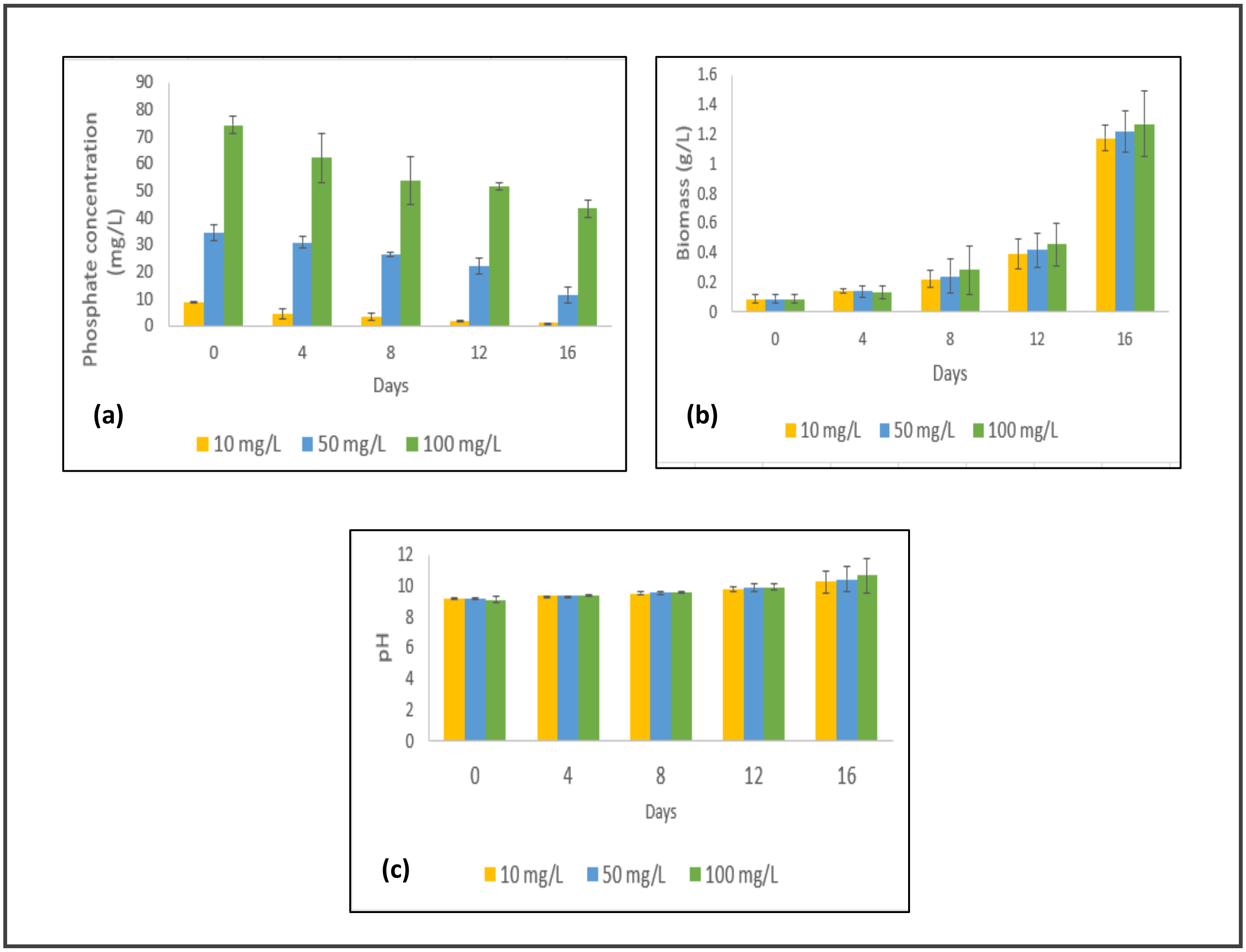

Figure 3-1 *Phosphate concentration (a) biomass concentration of Spirulina (b) and pH of culture (c) in AWW with different phosphate concentration*

3.6 Mitigation of phosphate in hyper-eutrophic, eutrophic and meso-eutrophic water

To estimate the potential of *S. platensis* for the restoration of eutrophic waters, cell cultivation experiments were conducted for 16 days. Figure 3-2(a) shows the phosphate removal by *S. platensis*. Around 96.78 % of phosphate removal was observed when the phosphate concentration was 100 µg PO_4^{-3} /L at a removal rate of 0.006±3.5E-05 mg PO_4^{-3} L^{-1} day^{-1}. As the phosphate concentration increased to 250 and 450 µg PO_4^{-3} /L, phosphate removal was 94.85% and 96.71%

at a removal rate of 0.02±0.0001 µg PO_4^{-3} L^{-1} day^{-1} and 0.03±0.0003 µg PO_4^{-3} L^{-1} day^{-1} respectively.

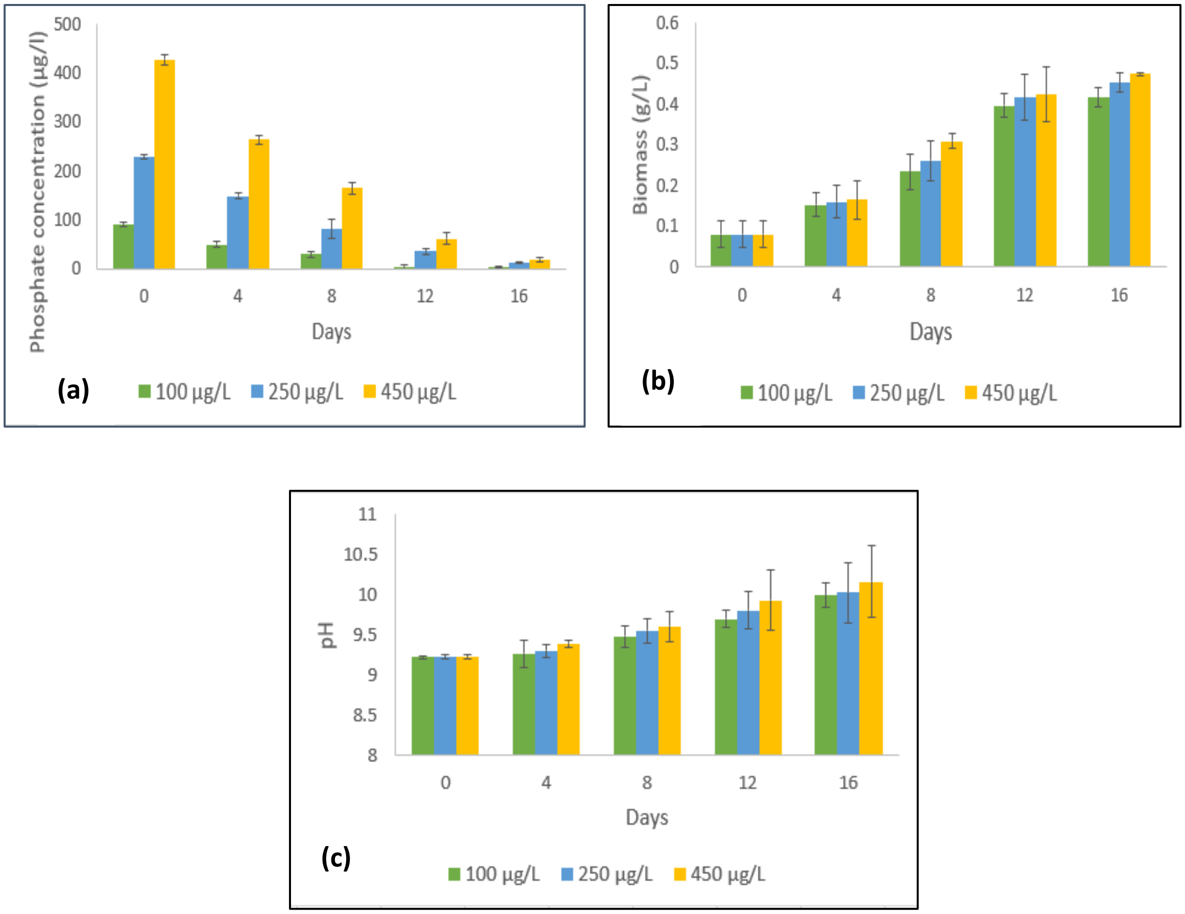

Figure 3-2 *Phosphate removal from hyper-eutrophic (450 µg PO_4^3 /L), eutrophic (250 µg PO_4^3/L) and meso-eutrophic water (100 µg PO_4^{3-}/L) by S. platensis: (a) Residual phosphate concentration, (b) Biomass concentration and (c) pH for 16 days of experiment*

Figure 3-1(c) and Figure 3-2(c) show the pH change of the Artificial Waste Water (AWW) and Artificial Eutrophic Water (AEW) respectively for 16 days during the growth of *S. platensis*. The pH shows a positive trend with an increase in phosphate concentration. As shown in Figure 3-1(c) the pH started to increase from the 4th day of cultivation and it reached 10.66 for AWW of 100 mg

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PO_4^{-3}/L, 10.43 for 50 mg PO_4^{-3}/ L and 10.26 for 10 mg PO_4^{-3}/L. As shown in Figure 3-2(c), the AEW with 100, 250 and 450 µg PO_4^{-3}/ L showed a lesser pH when compared to the above AWW experimental values. Among those, the AEW with 450 µg PO_4^{-3}/ L had a higher pH than 250 and 100 µg PO_4^{-3}/ L and 100 µg PO_4^{-3}/ L showed the least pH.

Figure 3-1(b) depicts the biomass concentration for 10, 50 and 100 mg PO_4^{-3}/L. The biomass production showed a correlation with the pH trend and phosphate concentration. Figure 3-2(b) shows the biomass production graph of *S. platensis* at different phosphate concentrations. The biomass concentration was 0.42 g/L for 100 µg PO_4^{-3}/L, 0.45 g/L for 250 µg PO_4^{-3}/L and 0.47 g/L for 450 µg PO_4^{-3}/L. These data seem to suggest that phosphate was not limiting when it was 450 or 250 µg PO_4^{-3}/L but was limiting when it was 100 µg PO_4^{-3}/L.

3.7 Growth kinetics of *S. platensis* in AWW and AEW

When algae start to grow, they undergo the lag phase, exponential phase, linear phase and death phase. Figure 3-3 represents the growth kinetics of *Spirulina* in (a) AWW and (b) AEW.

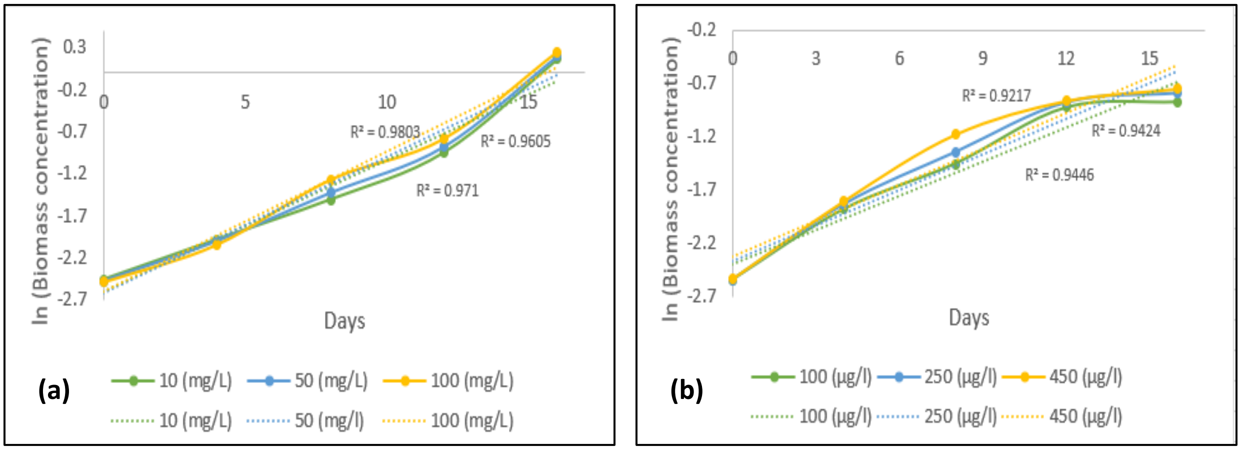

Figure 3-3 Logarithmic growth phase of Spirulina in (a) AWW and (b) AEW

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Table 3-2 illustrates final biomass concentration along with a percentage of phosphate removal and change in phosphate concentration from initial to the final concentration in AWW and AEW.

Table 3-2 *Cell growth and % phosphate removal of S. platensis*

Initial PO_4^{3-} concentration (mg/L)	**Final PO_4^{3-} concentration (mg/L)**	**% of PO_4^{3-} Removal**	**Rate of PO_4^{3-} removal (mg $L^{-1}D^{-1}$)**	**Final Biomass concentration (g/L)**
100	43.21±3.27	56.78±4.62	3.54±0.29	1.27±0.23
50	11.43±2.89	77.12±8.17	2.41±0.26	1.22±0.13
10	0.98±0.15	90.17±2.12	0.56±0.01	1.17±0.08
0.45	0.019±0.004	95.71±1.25	0.03±0.0003	0.47±0.07
0.25	0.013±0.002	94.85±1.13	0.02±0.0001	0.45±0.02
0.10	0.003±0.0004	96.78±0.56	0.006±3.5E-05	0.42±0.01

3.8 Specific Phosphate Uptake Rate (SPUR)

Specific Phosphate Uptake Rate (SPUR) is the amount of phosphate assimilated by algae per grams of cell concentration.

$$SPUR = \frac{(\Delta P/\Delta t)}{x}$$

Where ΔP is change in phosphate concentration (mg/L or µg/L), Δt is change in time (day) and x is biomass concentration (g/L).

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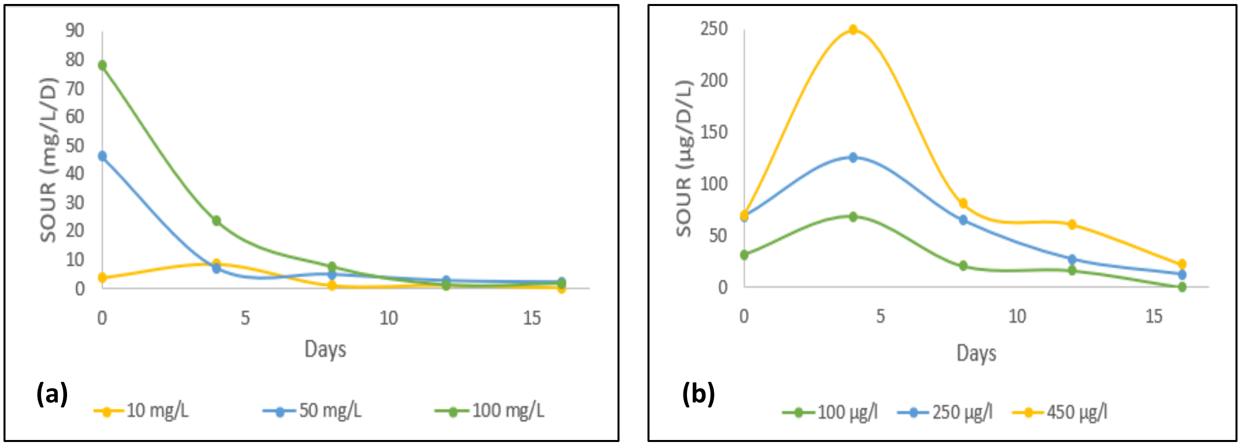

Figure 3-4 *(a) SPUR of Spirulina in AWW (b) SPUR of Spirulina in AEW*

Figure 14 shows the Specific Phosphate Uptake Rate of *Spirulina* in both AWW and AEW.

3.9 Value-added bioproducts of *S. platensis*

3.9.1 Chlorophyll A and Chlorophyll B

Figures 3-5 (a) and (b) depict a Chl-A and Chl-b, of *S. platensis* growing in AWW and Figures 3-5 (c) and (d) illustrate the same for AEW at different initial phosphate concentration.

Chlorophyll extracted from algae can be used as food colouring agents, antioxidant agents and antimutagenic agents[73]. Chl-a and Chl-b play different roles in the photosynthetic process. Chl-a is a key pigment involved in the photosynthetic process whereas Chl-b is an accessory pigment that collects energy during the photosynthetic process and gives it to Chl-a [83].

Figure 3-6 (a) and (b) depicts protein and phycocyanin concentration of *S. platensis* in the treatment of artificial wastewater and Figure 3-6 (c) and (d) depicts the same for artificial eutrophication water.

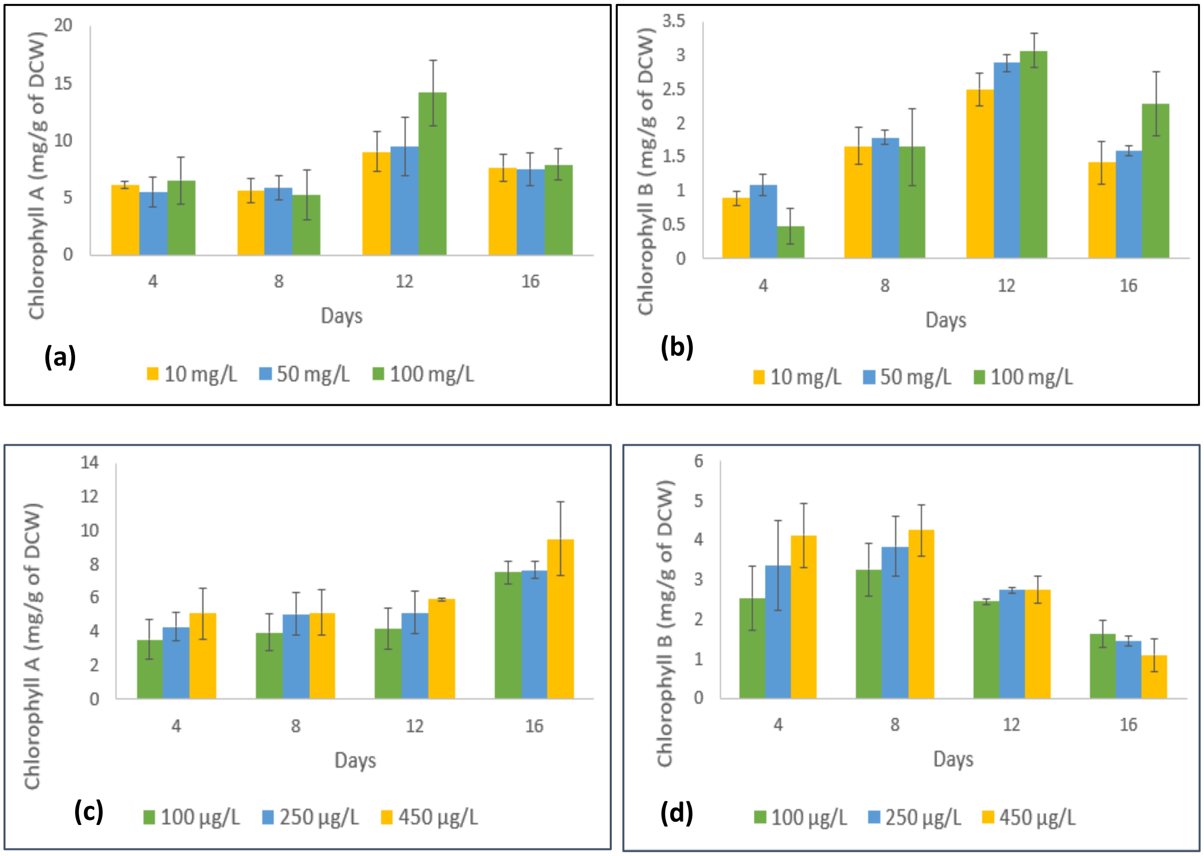

Figure 3-5 *Time courses of cell contents of Chlorophyll A of AWW(a); Chlorophyll B of AWW (b), Chlorophyll A of AEW (c) and Chlorophyll B of AEW(d) of S. platensis at varied phosphate concentration.*

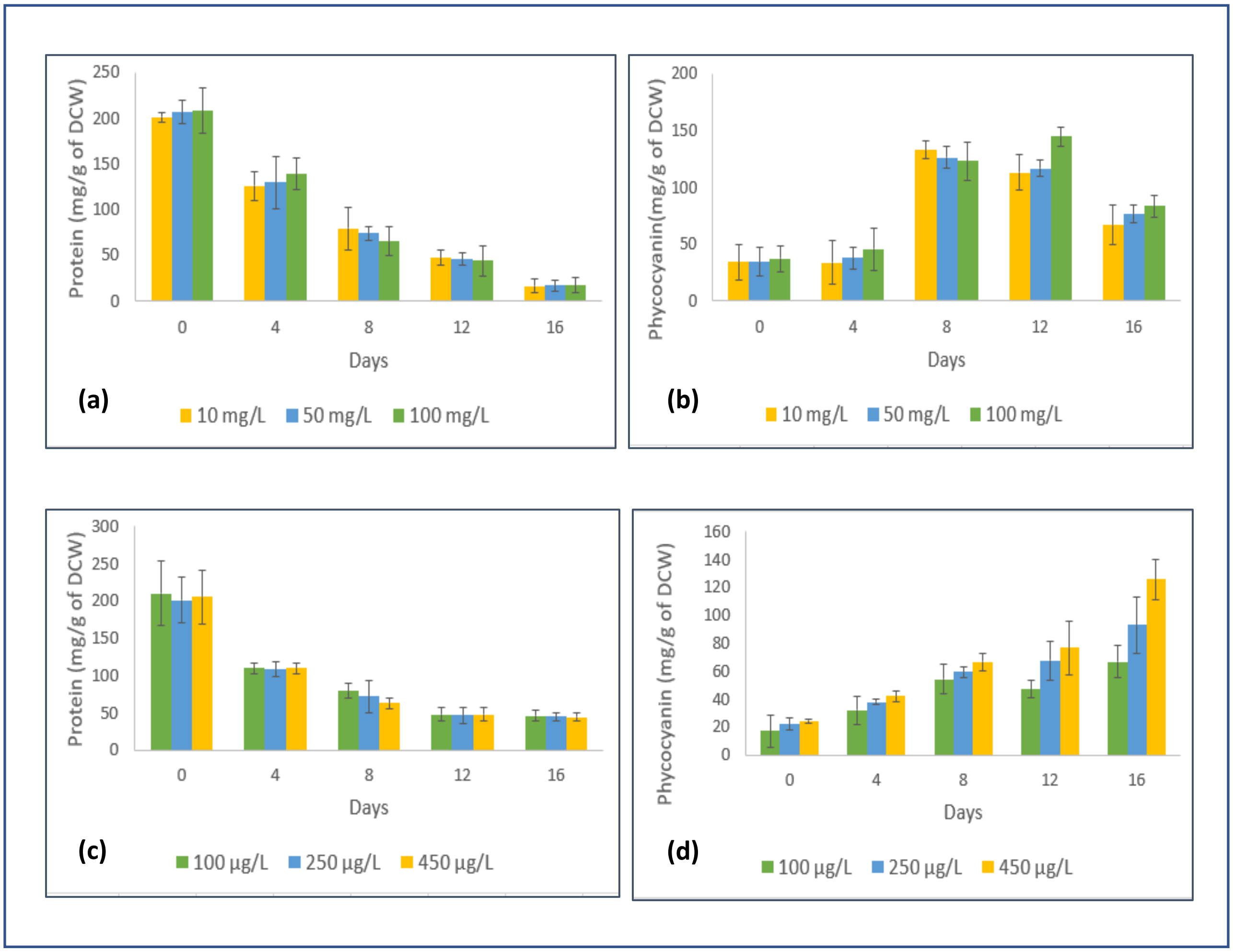

Figure 3-6 *Time courses of cell contents of Protein and Phycocyanin of AWW(a) and (b); Protein and Phycocyanin of AEW (C) and (d) of S. platensis at varied phosphate concentration*

3.9.2 Protein and Phycocyanin

Figure 3-6 (a) and (b) show the protein and phycocyanin concentration of *S. platensis* in AWW and Figure 3-6 (c) and (d) illustrate protein and phycocyanin concentration of *S. platensis* in AEW respectively. Protein is a major component of *S. platensis*, which is present in abundance. The phycocyanin is a complex protein and an accessory photosynthetic pigment of *Spirulina.* The protein extracted from *Spirulina* has been used as a staple diet for humans as well as animals [84].

While the phosphate content decreased with cultivation time, the cellular cell content of phycocyanin and protein increased with time.

3.10 Harvesting the Biomass from the culture of varied biomass concentration

Before harvesting the algae biomass, the culture was observed under a haemocytometer to find the growth of algae cells at different phosphate concentration.

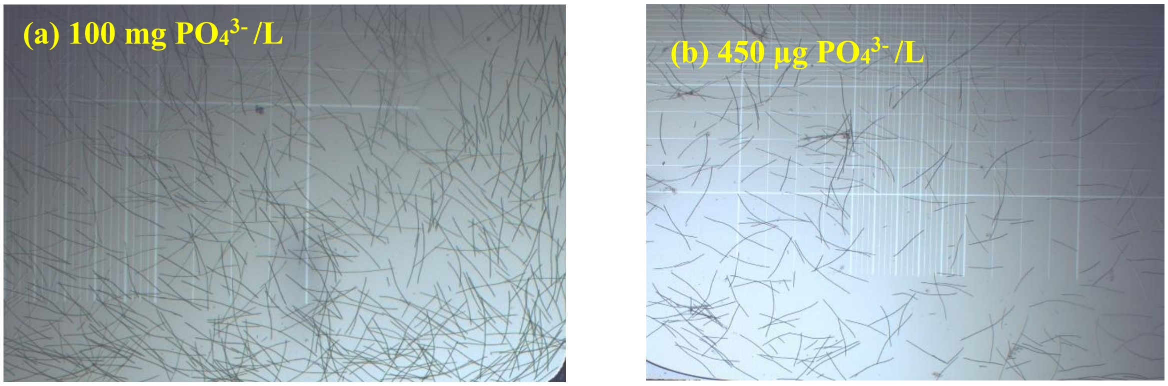

Figure 3-7 *Haemocytometer image of Spirulina platensis Biomass at different phosphate concentration (a)100 mg /L and (b) 450 µg /L taken at 40X magnification*

Figure 3-7(a) shows the micrograph taken at 40× magnification of *S. platensis* for 100 mg PO_4^{3-}/L of AWW and Figure 3-7(b) shows the micrographs of cells grown in 450 µg PO_4^{3-}/L of AEW.

From the micrographs it can be observed clearly that the biomass concentration in 100 mg PO_4^{3-}/L was denser with a biomass concentration of 1.26 g/L compared to 450 µg PO_4^{3-} /L with a biomass concentration of 0.473 g/L Since the biomass was lesser in the later solution, it will not form any hypoxic condition in the water and harvesting the biomass will not be much productive for bioproduct extraction. So, there was no point in harvesting the solution with a biomass concentration of 0.473 g/L Hence, we are going to harvest the solution which has 1.26 g/L of biomass concentration.

Considering the uneven distribution of biomass in the lakes, culture in AWW with 100 mg PO_4^{3-}/L was harvested at different dilution ratios of 2 times, 5 times and 10 times dilution and filtered using different size of nylon mesh screen of choice.

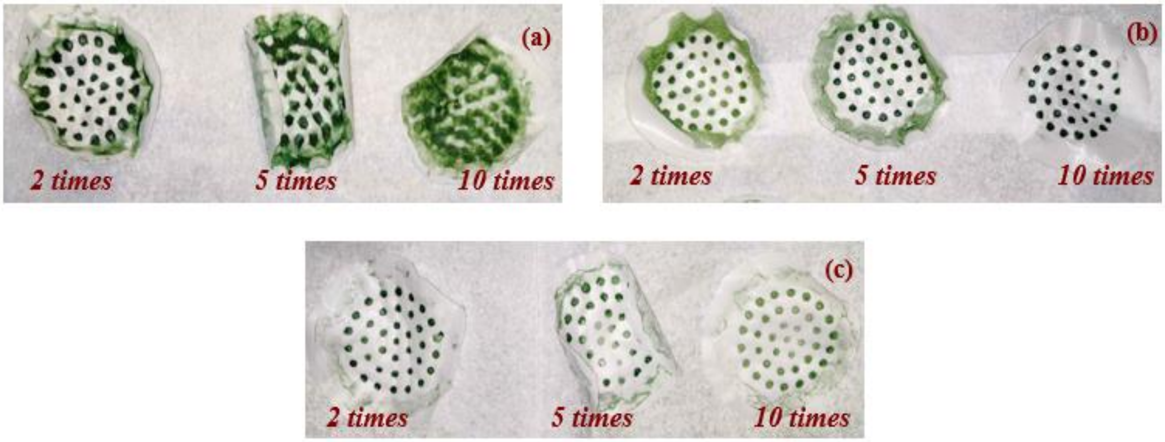

Figure 3-8 *Image showing Spirulina platensis harvested in different size mesh screen (a) 10 micron (b) 50 micron (c) 100 micron from 100 mg* PO_4^{3-} */L of AEW*

Figure 3-8 shows the harvesting image of *S. platensis* 100 mg PO_4^{3-} /L AWW with a biomass concentration of 1.26 g/L, using a different mesh screen size of 100-micron, 50 micron and 10 microns at a different dilution rate of 2 times, 5 times and 10 times.

Table 3-3 summarizes the residual biomass concentration of *S. platensis* after filtration using nylon filtration clothes of three different mesh sizes, i.e., 10, 50 and 100-microns with different initial biomass concentrations.

Similarly, the experiment was conducted in a bioreactor at the same condition used for cultivation flask experiments, to achieve a maximum biomass concentration of 1.00±0.04 g/L. After the 12th day of the experiment, the culture was harvested using only a 100-micron size of nylon mesh due to the higher biomass concentration. The biomass was harvested in two ways namely (a) without any dilution (b) with 2 times dilution rate. Table 3-3 shows the residual biomass concentration of the undiluted sample and diluted sample.

Table 3-3 *Efficiency of harvest using 10-micron, 50-micron and 100-micron nylon mesh at different concentrations of S. platensis grown in medium containing 100 mg* PO_4^{3-} *- /L and in a bioreactor*

	10 Micron mesh		**50 Micron mesh**		**100 Micron mesh**	
Initial Biomass Conc (g/L)	**Residual Biomass Conc (g/L)**	**% Biomass Recovery**	**Residual Biomass Conc (g/L)**	**% Biomass Recovery**	**Residual Biomass Conc (g/L)**	**% Biomass Recovery**
0.69±0.05	0.24±0.06	65.21	0.23±0.07	66.67	0.36±0.07	47.82
0.32±0.06	0.12±0.05	62.50	0.23±0.06	28.12	0.28±0.03	12.50
0.19±0.06	0.09±0.04	52.63	0.11±0.04	42.10	0.16±0.06	15.78
Bioreactor Experiment						
1.00±0.04	-	-	-	-	0.30±0.02	70
0.49±0.004	-	-	-	-	0.16±0.01	67.83

It was clear that the residual biomass concentration decreased with the decrease of cloth mesh size when compared to the similar initial biomass concentration, which is expected. Data also show that the residual biomass concentration increased with the initial biomass concentration when compared with the filtration clothes of the same mesh size, which is again expected. It is worth noting that the residual biomass concentration was 0.24±0.06 g/L when 10-micron cloth was used to filter 0.69±0.05 g/L of initial biomass concentration and when 100-micron mesh cloth was used, the residual biomass was 0.30±0.02 when the initial biomass concentration was 1.00±0.04. Therefore, the 10-micron filtration cloth and 100- micron cloth can be used for harvesting *S. platensis* to control the biomass concentration below a critical point to prevent hypoxia from happening based on the initial biomass concentration.

3.11 Discussion

3.11.1 Phosphate removal

Algae have great potential to remove phosphate. This phosphate removal process can be abiotic and biotic. The abiotic process involves the chemical precipitation of phosphate [85]. The abiotic process will take place if the pH values increases or by the enhanced photosynthetic process [66]. In biotic phosphate removal, cells assimilate phosphate for the biosynthesis of cellular materials such as ATP, nucleic acids, amino acids, phospholipids, nucleotides, biochemical precursors, etc [65]. Many algal species have great storage capacity for phosphorus, with phosphorus in the form of intracellular polyphosphate. This polyphosphate will be used for the growth when the external phosphate concentration is limiting [54]. The biotic process takes place during the growth phase of the algae, whereas the abiotic process of phosphate removal will dominate only when the biomass of the algae reaches threshold level [4, 86]. Since *S. platensis* was harvested before it reached the threshold level in this study, the phosphate removal was likely predominantly biotic rather than abiotic. There might be also a possibility that biomass production is increased due to the biological assimilation of phosphate [57].

From the results, it is seen that the phosphate concentration has a strong relationship with phosphate removal. Since the phosphorus is assimilated by *S. platensis* in the form of phosphate, the phosphate removal is expected to decrease with the increase in phosphate concentration [4] but 100% removal is not reached during the 16 days of the experiment as shown in Table 3-2 which might be due to the limitation of CO_2 since no aeration was provided during algal growth to replicate the natural condition in lakes and to prevent the drastic increase in pH.

3.11.2 Mitigation of phosphate discharge from point sources

Table 3-4 shows the result of phosphate removal by *S. platensis* in AWW. According to the table, the % phosphate removal was highest when the phosphate concentration was lower and vice versa. Similarly, the biomass was higher for the highest phosphate concentration.

Table 3-4 *Phosphate removal by Spirulina platensis from artificial wastewater*

Initial PO_4^{3-}/L concentration (mg/L)	**Final PO_4^{3-}/L concentration (mg/L)**	**% Removal of phosphate**	**Final Biomass (g/L)**
100	43.21±3.27	56.78±4.62	1.26±0.23
50	11.43±2.89	77.12±8.17	1.22±0.13
10	0.982±0.15	90.17±2.12	1.17±0.08

The substantial phosphate content in wastewater is primarily attributable to the use of detergents, agricultural runoffs, sewage treatment and industrial wastewater treatment. where only 3 to 4 mg/L of phosphorus was detected in treated municipal wastewater on predetermined days, compared to the concentrations of 10 to 20 mg/L recorded more recently[65]. Commonly in Canada, the phosphate concentration in wastewater treatment plant effluent should not surpass a monthly average concentration of 0.02-1 mg/L depending on the provinces[87].In this study, the algae removed 56.78% of phosphate when the initial phosphate concentration was 100 mg/L with a final biomass concentration of 1.26 g/L on the 16th day of the experiment and the 50 mg/L of AWW showed around 77.12% of phosphate removal. Likewise, 10 mg PO_4^{3-}/L of AWW removed around 90.17% of phosphate and maintained the phosphate concentration of AWW as per the standards.

3.11.3 Eutrophic water recovery from hyper-eutrophic, eutrophic and meso-eutrophic water

Table 3-5 depicts the phosphate percentage removal and compares the AEW with trophic states of lakes according to Canadian guidelines.

***Table 3-5** Comparing the phosphate removal results with trophic states of lakes*

Initial PO_4^{3-}/L concentration (µg/L)	**Final PO_4^{3-}/L concentration (µg/L)**	**% Removal**	**Final Biomass (g/L)**	**Initial Trophic status**	**Final Trophic status**
450	19.29±0.004	95.71±1.25	0.473±0.07	Hyper-Eutrophic	Oligotrophic
250	12.86±0.002	94.85±1.13	0.452±0.02	Eutrophic	Ultra-Oligotrophic
100	3.21±0.0004	96.78±1.25	0.416±0.01	Meso-Eutrophic	Ultra-Oligotrophic

When the experiment was conducted with an AEW containing 450 µg PO_4^{3-} /L which has higher phosphate than the threshold value for hypereutrophic condition as per Canadian standard (>306.65 µg PO_4^{3-} /L), *S. platensis* removed 95.71% phosphate and reduced the trophic status from hypereutrophic state to oligotrophic. Similarly, *S. platensis* showed 94.85% removal of phosphate from 250 µg PO_4^{3-}/L AEW and reduced the state from eutrophic state to ultra-oligotrophic and showed around 96.78 % removal of phosphate in 100 µg PO_4^{3-}/L AEW and reduced the water from meso-eutrophic to ultra-oligotrophic. From Table 3-5, it can be seen that the *S. platensis* has a greater efficiency to restore the eutrophicated water by reducing them from a eutrophication state to oligotrophic status which has a better drinking water quality as well as no algal blooming.

From Table 3-2, we could notice that the higher initial phosphate concentration has more cell growth and removed phosphate at a higher removal rate of 3.54 ± 0.29 mg L^{-1} D^{-1}. As the initial phosphate concentration reduced the rate of phosphate removal reduced with a decrease in biomass growth. So, the phosphate removal rate is proportional to the initial phosphate concentration.

3.11.4 pH

The lab-scale experiments were carried at a constant temperature of 25ºC. Both the AWW and AEW had a higher concentration of sodium bicarbonate and sodium carbonate, so the initial pH values were in the range of 9.2 ± 0.5. But, due to the photoautotrophic growth of algae, there was a progressive alkalinisation of the AEW and AWW due to CO_2 uptake. This was the reason behind an increase in the pH value up-to pH 11 [4].

3.11.5 Biomass production

From Figure 3-1 (b) and Figure 3-2 (b), it is seen that the biomass production is correlated with phosphate removal but the biomass production is lesser due to the limiting factor of carbon dioxide. The biomass concentration of AWW with 100 mg PO_4^{3-}/L, 50 mg PO_4^{3-}/L and 10 mg PO_4^{3-}/L is higher and has a faster growth when compared to AEW with 450, 250 and 100 µg PO_4^{3-}/L.

In both AWW and AEW the biomass concentration was increasing with a decrease in phosphate concentration. The reason behind this is phosphate act as an essential nutrient for algae growth by producing nucleic acid, biomolecules, protein, sugar phosphate and other metabolites. The algae can accustom to a low phosphate environment, but when more phosphate is available, they tend to take them in and store it as polyphosphate in larger amounts. That the reason why phosphate concentration decreases when biomass concentration increased.

The algae growth will not stop in the medium when the phosphate is exhausted because the *S. platensis* can uncouple the nutrient uptake and continues to grow using stored nitrogen [88] and phosphorus sources [85]. Since we aim to reduce the phosphate concentration in the eutrophicated water, the biomass was grown for 16 days and the cells were harvested before the cells rupture and give the phosphate back into the medium. Then the harvested biomass is used for the extraction of several bioproducts.

But we assume that it is difficult to predict the biomass yield in a closed flask experiment to the biomass yield in the open lakes and rivers. So further study is needed to understand the correlation between them.

3.12 Growth kinetics of *Spirulina* and Specific Phosphate Uptake Rate of *Spirulina* in AWW and AEW

In a batch culture of algae, cell growth could be divided into the lag phase, exponential phase, linear growth phase, transition phase, stationary phase, and death phase. When cells are inoculated into a fresh medium, algal cells may experience a period when no increase of cell number is observed, which is called the lag phase. In the lag phase, cells are adjusting to the new environment and may grow in cell size but not in cell number. At the end of the lag phase, the cells of algae have adapted to the new environment and start to rapidly multiply themselves and increase the cell number and biomass concentration exponentially with time. The average cell size and cell composition are constant in the experimental phase. The exponential growth of cell is characterized by a straight line on a semilogarithmic plot of ln (biomass concentration) versus time. From Figure 3-3, we could notice that the *Spirulina* growth showed a straight line in the semilogarithmic graphs which can prove that the cells are in exponential phase in both AWW and AEW until the 16th day. During this period balanced growth is obtained which means all the components of the cell grows at the same rate. That might the reason why the initial phosphate concentration reduced linearly whereas the biomass growth was slower during the 4th and 8th day in Figure 3-1 and 3-2.

From Figure 3-4 (a) we could notice that in AWW the higher initial phosphate concentration of 100 mg/L showed a higher phosphate uptake rate with the increase in biomass concentration whereas the lowest initial phosphate concentrate shows the lowest uptake rate. Similarly, in Figure 3-4(b) in AEW, 450 µg/L solution showed the highest phosphate uptake rate during the 4th and 8th day and decreased with an increase in the cultivation period. When the cells are during the initial stage of growth, they need more phosphate to synthesis essential components for their cell growth. That might be the reason why the SPUR was higher during the first 8 days and decreased over the cultivation period.

3.13 Harvesting the Biomass

Spirulina has high-value biomass which contains the highest amount of protein, chlorophyll and phycocyanin pigments which have excellent antioxidant properties, essential amino acids, polysaccharides and trace metal elements [89]. The biomass is used to produce various foods for animals, humans, in aquaculture and also used in the pharmaceutical industry [90].

When we consider a lake, biomass production is affected by several reasons like water temperature, wind speed, wind direction, light intensity, nutrients available in that particular region, etc. When the algae grow over a longer period they will reach a nutrient exhaustion phase where the algae will uncouple the nutrient uptake like phosphorus assimilation for growth [85]. On the other hand, when the biomass concentration increases on the surface it will cause blockage of light inside the lake and also reduce the dissolved oxygen concentration in the water which causes a detrimental situation for other aquatic plants and organisms. This particular problem with low DO is called as "hypoxic condition". This might lead to an increase in phosphate concentration in the water. Based on this, the assumption has been made that the biomass of *S. platensis* is not evenly distributed on the surface of the lake and needs to be harvested. So, the nylon mesh of 100-micron, 50-micron and 10-micron mesh size is used to harvest the samples. Similarly, the samples are diluted by 10 times dilution, 5 times dilution and 2 times dilution and filtered using the three different sizes of nylon mesh.

The 100-micron mesh is having a large pore size, so there is a chance that the biomass will pass easily through the filter. But this can be avoided by using a tighter mesh filter of 50-micron and 10-micron. The main aim of this part of the experiment is to harvest around 70% of the biomass and leaving behind 30% of biomass back in the lake which can be used as a seed for the next batch of growth. From Table 3-3, it can be seen that when the 10-micron mesh is used for harvesting the 2 times diluted sample the biomass of sample before filtration was 0.69 ±0.05 g/L and biomass of sample after filtration was 0.24 ±0.06 g/L which shows that around 65% of the biomass has been harvested and left only 35% of biomass as a seed for the growth of next batch.

When the experiment was conducted with the biomass from the bioreactor, 1g/L of biomass concentration was obtained after 12 days of the experiment. Table 3-3 shows the biomass from bioreactor showed a biomass removal of exactly 70% and has left 30% of the biomass in the filtrate when 100-micron mesh screen was used. This leads to the conclusion that cheap nylon mesh can be used to harvest the *S. platensis* effectively. So, based on the dilution level of the area, the appropriate mesh size can be chosen for harvesting.

3.14 High-value products of *S. platensis*

3.14.1 Chlorophyll A and Chlorophyll B

Chlorophyll is a green colour pigment found in algae which are used for the photosynthesis process as a photoreceptor. Chlorophyll appears green in colour because it reflects the green wavelength and absorbs all the other colours [91]. The chlorophyll concentration is proportional to the biomass concentration of the medium. When the biomass concentration is higher, more chlorophyll concentration is recorded. The phosphate concentration also impacts the biomass concentration. So, the initial phosphate concentration and biomass concentration has a direct correlation with chlorophyll content.

In both AWW and AEW the chlorophyll concentration started to increase over the cultivation time but decreased after a certain period. This might be due to the shading effect of biomass which reduces the chlorophyll content [92]. The chlorophyll might also degrade when they are exposed to excess light, change in pH, high temperature and oxygen/air conditions [73].

In a eutrophicated lake, increased phosphorus and nitrogen will increase the chlorophyll content. But reducing the N will not have any impact on chlorophyll reduction when the phosphate in the lake is $\leq 306.65\ \mu g\ PO_4^{3-}/L$ [93.] So, the chlorophyll concentration is correlated with the phosphate concentration as well as biomass concentration in the lake.

3.14.2 Protein and Phycocyanin

The protein is the building block of any living cell. From Figure 3-6, it is evident that the protein and phycocyanin concentration of the cell depends on the phosphate concentration. Phycocyanin is a complex protein pigment synthesized by blue-green algae like *Spirulina platensis*. This phycocyanin reflects the blue colour and that’s the reason behind the name of the cyanobacteria as a blue-green algae [94].

When the phosphate concentration is 450 µg PO_4^{3-}/L, the protein content per gram of DCW is slightly higher when compared to 250 µg PO_4^{3-}/L and 100 µg PO_4^{3-}/L of artificial eutrophicated water. Whereas among the artificial wastewater, 100 mg PO_4^{3-}/L showed higher protein content per gram of DCW when compared to 50 mg PO_4^{3-}/L and 10 mg PO_4^{3-}/L. The protein content decreased as the initial phosphate concentration decreased in both AWW and AEW. It is worth to notice that, protein and phycocyanin are more in AEW per gram of DCW when compared to AWW. The reason behind this is, protein has 16% of average nitrogen content in them. Since the biomass concentration of AWW was higher the nitrogen might be a limiting nutrient there which could weaken the synthesis of protein, whereas in the AEW the biomass concentration is much lesser compared to AWW so the nitrogen content might not a limiting factor [95].

In a research paper it has been mentioned that, when *S. platensis* is growing under stressful conditions like phosphate limitation, they change their metabolism to survive under that condition by limiting the protein production and increasing the accumulation of carbohydrates or lipids [96]. Thus, the protein content decline when the initial phosphate concentration is limiting.

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3.15 The algae-for-eutrophication-water-restoration strategy

Since the major environmental hazard in association with water eutrophication is algal blooms, the algae-for-eutrophicated-water-restoration strategy as proposed in this thesis is somewhat counterintuitive. The reasoning behind this strategy is as follows:

1) The harmful algal blooms are caused due to excessive nutrients in the lake and runoff water. By using *Spirulina* which is a non-toxic cyanobacterium, we could remove phosphate concentration from runoff water and eutrophicated water which will ultimately reduce the growth of toxic and unwanted algal species. This will eliminate the root cause of eutrophication.

2) Next the *Spirulina* we used for restoring the lake might cause a hypoxic condition in the lake due to their overgrowth. So, we need to harvest the *Spirulina* at appropriate times to control the algal concentration in water to be below a certain threshold. Since the algae are filamentous and float on the surface of the water body, we could effectively harvest the *Spirulina* using simple devices such as nylon mesh of appropriate mesh size. So, this process will prevent *Spirulina from* causing hypoxia condition by their overgrowth during the water restoration process.

3) The harvested *Spirulina* biomass can be used for other commercial purposes such as human foods, pigments for cometic, food-colouring, nutraceutical and pharmaceutical applications, as well as animal, feeds to offset the costs of algal growth, monitoring, and harvesting.

3.16 Conclusions

It can be seen from experimental results that the *S. platensis* has a great potential to remove phosphate from the artificial eutrophicated water and reduce the trophic state of water from eutrophication to ultra-oligotrophic which is much suitable for drinking. Since the process involved is biotic removal, the research work is environmentally friendly, non-toxic and the biomass can be used for producing bio-products. However, these results need further research work by involving the real lake water or by applying for this research work in a small portion of the eutrophicated lake to study the competitive behaviour of *S. platensis* with other algae and aquatic organisms as well as its behaviour to the exact environmental temperature and conditions.

Chapter 4: Dynamics of dissolved oxygen in an unaerated *Spirulina platensis* culture with light/dark cycle

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Abstract

To successfully implement the algae-for-eutrophic-water-restoration strategy, we must control the algal cell density in water bodies to be below a critical value to prevent hypoxia from occurring at night time, when algae have to consume oxygen through respiration for cell maintenance. This study investigated the dynamics of dissolved oxygen (DO) in unaerated cultures of *Spirulina platensis* with 12-hour/12-hour light/dark cycles to simulate the conditions in a natural water body, e.g., a lake. After testing a large range of concentrations of actively growing *S. platensis* cells, it was estimated that the average specific oxygen evolution rate was 1.18 mg $g^{-1}h^{-1}$ during daytime and the average specific oxygen uptake rate was 0.06 mg $g^{-1}h^{-1}$ during night-time for a biomass concentration up to 1 g/L. The lowest DO level experienced in the entire course of cultivation was 7.15 mg/L, which was greater than the threshold of hypoxia, i.e., 4 mg O_2/L, indicating that it is safe to operate at biomass concentration up to 1 g DCW/L in the tested conditions.

Keywords: dissolved oxygen, hypoxia, oxygen evolution, oxygen consumption

4.1 Introduction

While much efforts have been made, especially in developed economics such as North America and the Europe Union, the problem of eutrophication is still persistent in many regions of these countries. For instance, eutrophication in Lake Erie has been happening for the past 50 years, which creates a poor-quality water ecosystem for the public as well as for aquatic plants and animals. The eutrophicated water may result in excessive growth of eukaryotic algae and prokaryotic bacteria (which are collectively referred to as algae hereafter), i.e., algal blooms when other conditions such as temperature and solar radiation level are favourable. A thick layer of algal cells at the water surface will block sunshine and the transfer of oxygen from the air to water. Some cells, if not harvested timely, may sink to the bottom, which becomes oxygen consumers and then they decay after death. Even if the cells that sink to the water bottom are effectively eliminated or mitigated by appropriate timely harvest, large cell density of algal cells in water may still cause hypoxia during night time, when light is not available and algal cells have to resort to respiration for cell maintenance.

Hypoxia is a term that refers to a condition where the DO of the water bodies reduces to 2-4 mg/L, at which aquatic plants and animals cannot survive. A prolonged hypoxic condition during an algal bloom may create "dead zones", where aquatic plants and animals are dead and the decay of their dead bodies would further deteriorate the hypoxia and the water quality as well. Furthermore, the hypoxic condition may cause the release of the phosphorus from sediments, alias the "internal loading", which will increase the water eutrophication and algal bloom [97].

During algal bloom or when an algae-for-eutrophic-water-restoration approach is implemented, hypoxia may happen during the night time if algal biomass concentration is too high. While algae produce oxygen during daytime through photosynthesis, they consume oxygen for respiration

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during night time for cell maintenance. Therefore, it is important to determine the DO dynamics of algal cultures at different biomass concentrations so that we could control the algal biomass concentration below a limit that is safe.

4.2 Causes for hypoxic condition

The oxygen can deplete either by natural factors or anthropogenic factors. [98] The lakes in the moderate temperature region go through thermal stratification during the summer and fall seasons. The water body will be divided into three distinct layers namely the epilimnion- the topmost layer, metalimnion- the middle layer of the lake and hypolimnion- the bottom layer of the lake. The metalimnion is also called a thermal layer or thermocline which is a thin distinct layer that separates the epilimnion and hypolimnion.[99] When the upper layer of the water gets warmed up, it will induce thermal stratification which restricts the proper mixing of water and hinders the transportation of oxygen to the depths which likely increases the hypoxic condition in the lake. [98]

Humans are producing more and more phosphorus and nitrogen through agricultural products, industries, a household which will persistently increase the algal bloom in water causing eutrophication. This will affect both the supply as well as uptake of oxygen. The phytoplankton microorganism growth will increase the oxygen concentration in water at the initial stages of photosynthetic activity but later when they start to degrade and decompose it will eventually decrease the oxygen concentration in the water. [98, 15]

4.3 Effects of Hypoxic condition

Oxygenated water is required for all aquatic plants and animals to survive. But when the algae grow excessively, oxygen is getting consumed by the algae during night time, when light is not available for biosynthesis, causing the aforementioned hypoxia. Mobile animals or fishes can sometimes survive this oxygen depletion but whereas young fishes become intolerant to this hypoxic condition which results in a fish kill.[100]

Fish-eating birds like heron, otters, sea eagle, etc as well as humans cannot survive in the hypoxic condition where there is no fish This will lead to habitat loss, long term ecological changes and economic loses.[100]

When the algae die and decompose, they consume oxygen by creating a hypoxic condition where any of the aquatic organisms cannot survive by forming a “dead zone”. These areas can kill fish and benthic organisms such as crabs, snails and clams. [2]

Another problem of bottom layer hypoxic condition is internal regeneration of nutrients which is a primary cause of eutrophication. Phosphorus loaded into the lakes will usually oxidize and bound to metals under normal condition, but when the oxygen decreases the water system becomes reducing and release the phosphorus again into the water body which will again stimulate the eutrophication and algal blooms.[32]

4.4 Photosynthesis and Respiration in Eutrophicated lakes

When the algae grow it undergoes photosynthesis process and respiration process. Photosynthesis happens only during the day time where it obtains energy from sunlight and transforms the energy into chemical energy in the form of starch and sugar. This process involves taking up the carbon dioxide from the living environment and it releases the oxygen into the environment which is derived from water molecules [101,102].

Whereas the algae undergo a respiration process during the night time when there is no light. Respiration is a process where oxygen is consumed by the algae and CO_2 is being released. Due to this, algae will face a low oxygen condition during the night when there is no oxygen produced by the photosynthesis process [102,103].

In lakes, oxygen is required for fishes, phytoplankton, benthic organism, seaweed, etc to survive. When a particular lake is eutrophicated and causes an algal bloom, the oxygen in the water should be distributed to the above-mentioned organisms as well as for the growth of algae on the top surface of the lake. When there is an excess bloom more oxygen is consumed by fishes, phytoplankton and other organisms for their survival.[104]

Similarly, when these algae start to die, the bacteria will consume more oxygen to decompose the algae. This can further reduce the oxygen concentration in the lakes below the sustainable levels and this can be detrimental for all aquatic organisms [104,105]

4.5 Materials and methods

4.5.1 Organism and inoculum preparation

Spirulina platensis (UTEX LB 2340) was purchased from the Culture Collection of Algae at The University of Texas at Austin. Inoculum of *S. platensis* was grown in 250 ml flasks containing 25 ml of the medium.

4.5.2 Medium and artificial eutrophic water (AEW)

The medium for the cultivation of *S. platensis* inoculum was adapted from the Culture Collection of Autotrophic Organisms. The medium composed of 13.61 g $NaHCO_3$,4.03 g Na_2CO_3, 0.50 g K_2HPO_4, 2.5 g $NaNO_3$, 1 g K_2SO_4, 1 g NaCl, 0.2 g MgSO4.7H2O, 0.04 g CaCl2.2H2O, 0.01 g FeSO4.7H2O, 0.08 g $Na_2EDTA.2H_2O$ and 5 ml of trace metal solution. The trace metal solution is composed of 0.5 g $Na_2EDTA.2H_2O$, 0.7 g $FeSO_4.7H_2O$, 1 ml $ZnSO_4.7H_2O$ (1 g/l), 1 ml $MnSO_4.7H_2O$ (2 g/l), 1 ml H_3BO_3 (10 g/l) ,1 ml $Co(NO_3)_2.6H_2O$ (1 g/l), 1 ml $Na_2MoO_4.2H_2O$ (1

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g/l) and 1 ml $CuSO_4.5H_2O$ (0.005 g/l). The medium is prepared and sterilized at 121ºC for 20 minutes.

AEW was prepared according to the above recipe of the medium except that the concentration of K_2HPO_4 was adjusted according to the required phosphate level as indicated in the text.

4.5.3 Experimental Setup and experimental condition

The experiment was conducted in a Bioflo 110 fermentation reactor which has a capacity of 2.5 L and a working capacity of 2.0 L as shown in Figure 4-1. The reactor has an input gas control, pH sensor, agitation controller, dissolved oxygen sensor and temperature controller.

Figure 4-1 *Experimental setup of a Bioreactor*

Experiments were conducted in 12-hour light/12-hour dark cycles. The samples were taken every twelve hours using a syringe filter to check the biomass concentration. In the meanwhile, the dO_2 and pH were recorded automatically every 15 minutes to check the change in dO_2 and pH concerning the biomass concentration. The experiment is carried out using an AEW containing 450 µg PO_4^{3-}/L. The seed of the algae is inoculated at two different concentrations namely 2% and 7%. From that critical biomass, concentration to avoid hypoxic condition is determined.

4.5.4 Analytical methods

The biomass concentration of the culture was measured at a wavelength of 700 nm in a gap of 12 hours cycle which is before the light off (considering as sunset) and before light on (analogy to dawn time). The Dissolved Oxygen (DO) and pH are also measured every 15 minutes using the probe attached to the bioreactor.

4.5.4.1 Net growth rate and maintenance rate of microalgae

When the algae are growing, part of the bioenergy it manages to gain has to be utilized for cell maintenance. All the bioenergy to be utilized by cells for cell growth or cell maintenance has to be first assimilated into the cell to become part of the cells. In this sense, cell maintenance is a process utilizing cellular materials for cell survival.

The specific maintenance is defined as the rate of utilization of cellular materials (e.g., sugars and lipids) for cell maintenance. In the case of algae, bioenergy is obtained from the photosynthetic process, which fixes CO_2 to produce organic molecules such as sugar in cells. During night time, since light is not available for photosynthesis, cell maintenance through respiration would cause the loss of biomass [106]. μ_{max} is the maximum growth rate of algae and it occurs mainly between the lag phase and exponential phase [44]. The net growth rate of microalgae during day time and the maintenance rate of the night time was determined from experimental data using the following equation:

$$\mu_{\text{net}} = \frac{\frac{\text{Biomass at the end of day period} - \text{Biomass at the start of day period}}{\text{Biomass at the start of day period}}}{0.5}$$

$$\mathrm{k_d} = \frac{\frac{\text{Biomass at the start of night period} - \text{Biomass at the end of the night period}}{\text{Biomass at the end of the night period}}}{0.5}$$

Both the μ and $\mathrm{k_d}$ are divided by 0.5 because of 12/12-day-night cycle.

4.5.4.2 Oxygen uptake rate (OUR) and Oxygen Evolution Rate (OER)

Oxygen uptake rate is the measure of the oxygen consumption rate of algae during the night time.

Oxygen update rate (OUR) can be calculated from the formula,

$$\text{OUR} = \frac{\text{Dissolved oxygen at } \mathrm{t_2} - \text{Dissolved oxygen at } \mathrm{t_1}}{\mathrm{t_2} - \mathrm{t_1}}$$

Where t_1 is the time when the night period started and t_2 is the time when the night period ended.

Oxygen evolution rate is a measure of oxygen evolved or produced by the algae during the day time

$$\text{OER} = \frac{\text{Dissolved oxygen at } \mathrm{t_4} - \text{Dissolved oxygen at } \mathrm{t_3}}{\mathrm{t_4} - \mathrm{t_3}}$$

Where t_4 is the time when the day period ended and t_3 is the time when the day period started.

4.5.4.3 The specific rate of oxygen consumption (q_{O2}) and Specific rate of oxygen evolution (SROE)

The specific rate of oxygen consumption is defined as oxygen consumption or respiration rate and is represented as milligrams of oxygen consumed per gram of dry cell weight [102]. This parameter gives intrinsic information about the oxygen requirement for the organism for growth and product synthesis[107].

$$q_{o2} = \frac{\text{Oxygen Uptake Rate } (\frac{\text{mg}}{\text{L}})}{\text{Biomass } (\frac{\text{g}}{\text{L}})}$$

Where biomass concentration is in g/L and q_{o2} is in mg O_2/g dw cells h

Algal cells start to evolve oxygen when the light is illuminated [108]. SROE is a measure of oxygen evolved per gram of algae biomass. It can be represented as below:

$$\text{SROE} = \frac{\text{Oxygen Evolution Rate } \left(\frac{\text{mg}}{\text{L}}\right)}{\text{Biomass} \left(\frac{\text{g}}{\text{L}}\right)}$$

4.6 Results

4.6.1 Dissolved oxygen vs biomass profile

To determine the critical biomass concentration of *Spirulina* to avoid the hypoxic condition, the experiments were conducted at two different inoculum sizes, i.e., 2% and 7%. The profile of dissolved oxygen and biomass of *S. platensis* is measured in the bioreactor and the graph is shown in Figure 4-2 and Figure 4-3.

Figure 4-2 presents the profiles of biomass and DO vs days for 2% inoculum and Figure 4-3 shows the profile of biomass and DO vs days for 7% inoculum. The dissolved oxygen was measured every 15 minutes to see the change of DO with biomass concentration. The experiment continued for 11 days, after which biomass concentration started to decrease.

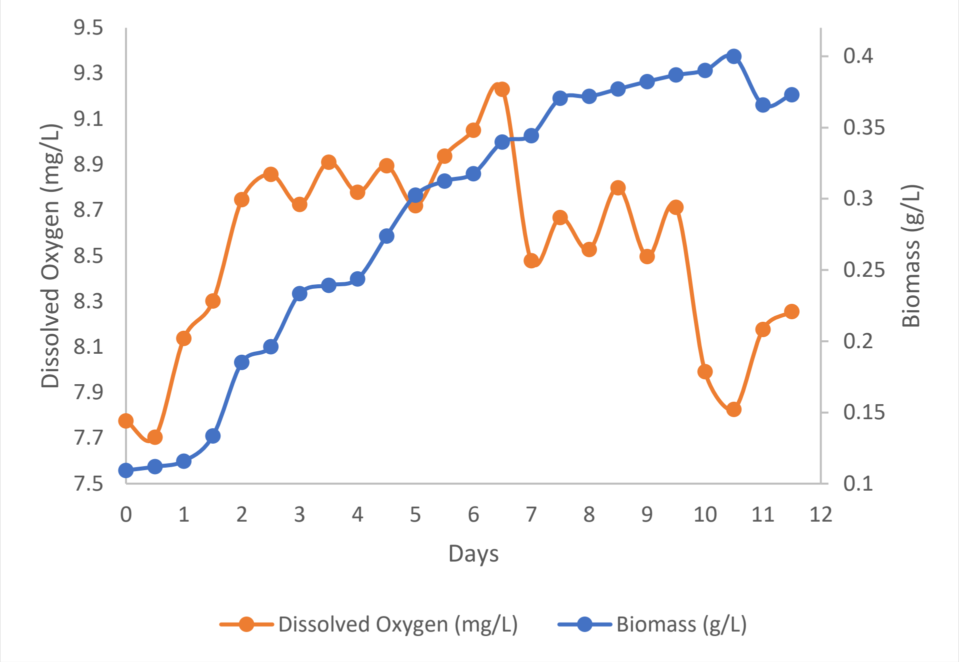

Figure 4-2 *Dissolved Oxygen (mg/L) and Biomass (g/L) profiles of culture with 2% inoculum*

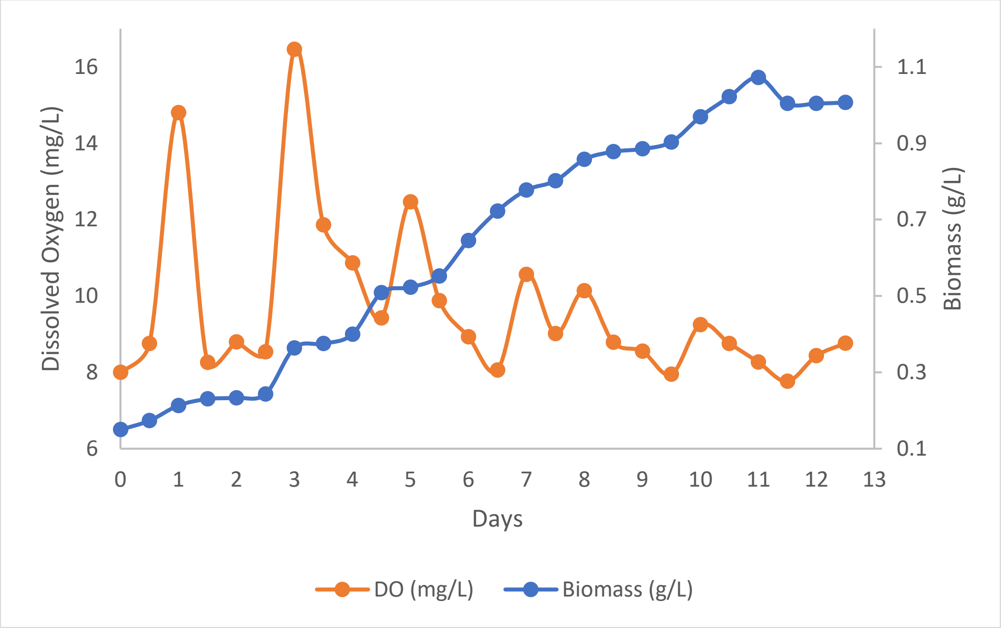

Figure 4-3 *Dissolved Oxygen (mg/L) vs Biomass (g/L) profile for 7% inoculum experiment*

In Figure 4-3, the biomass concentration started to decline after the 12th day so the experiment was terminated on the 12th day.

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4.6.2 Biomass and pH profile

A lake needs a high pH to start a eutrophic condition. Once the eutrophication is initiated, the biomass produced by the algal blooms will also increase the pH. So, it is also necessary to monitor the pH profile along with the DO and biomass. Figure 4-4(a) and (b) show the pH profile of *Spirulina* at different inoculum sizes of 2% and 7% respectively.

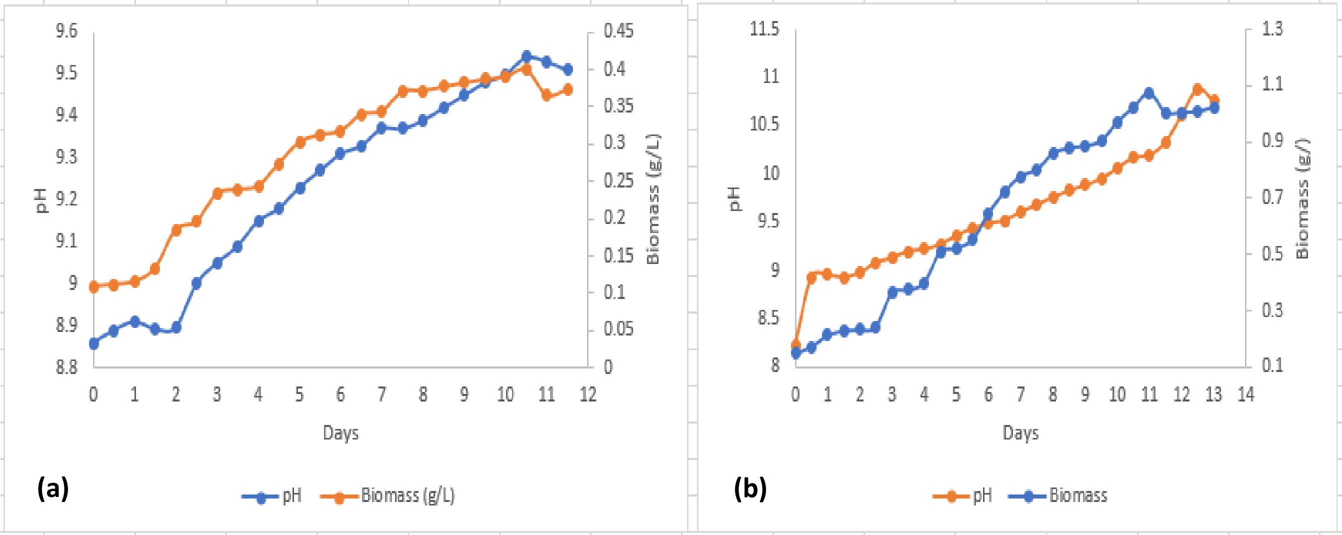

Figure 4-4 *pH vs Biomass (g/L) profile for (a) 2% inoculum experiment (b) 7% inoculum experiment*

Similarly, the Oxygen Uptake Rate (OUR) and Oxygen Evolution Rate (OER) of the two independent experiments were measured for 12 hours day cycle and 12 hours night cycle. The OER is measured during the day time and OUR is measured during the night time. Figure 4-5 shows a comparison graph of OER and OUR of *S. platensis* at 2% inoculum experiment and 7% inoculum experiment.

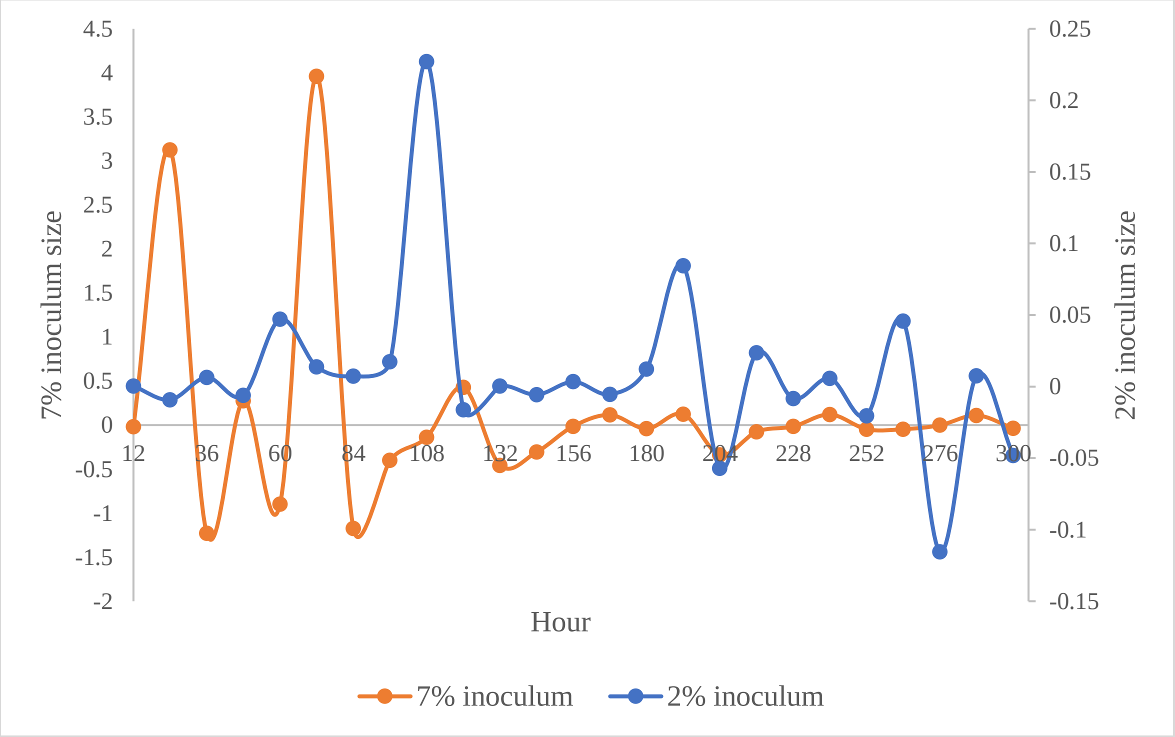

Figure 4-5 *Comparison of Oxygen Production Rate (OER) and Oxygen Uptake Rate (OUR) for 2% inoculum and 7% inoculum experiment*

4.6.3 Net growth rate and maintenance rate of microalgae

Figure 4-6 shows the results of the net growth rate of *Spirulina* during the daytime, which is measured for 11 days experiment and Figure 4-7 shows the maintenance rate of microalgae during the night time measured during 11 days of the experiment. During both the experiments the μ_{max} and k_d are low during the initial growth days of microalgae and as the cultivation time increased, the growth rate and maintenance rate also increased. After a certain time, both started to decrease due to their change in the growth phase.

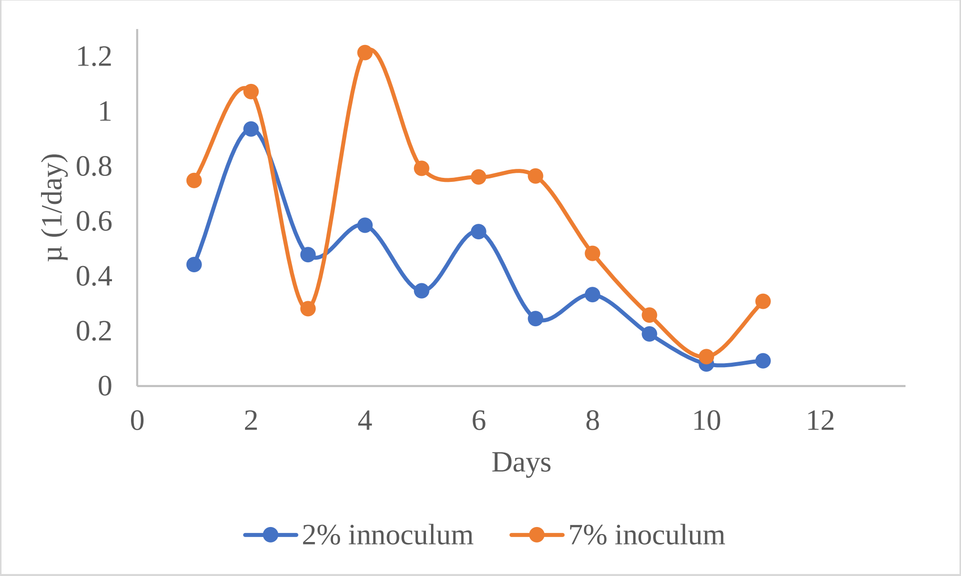

Figure 4-6 *Net growth rate of Spirulina*

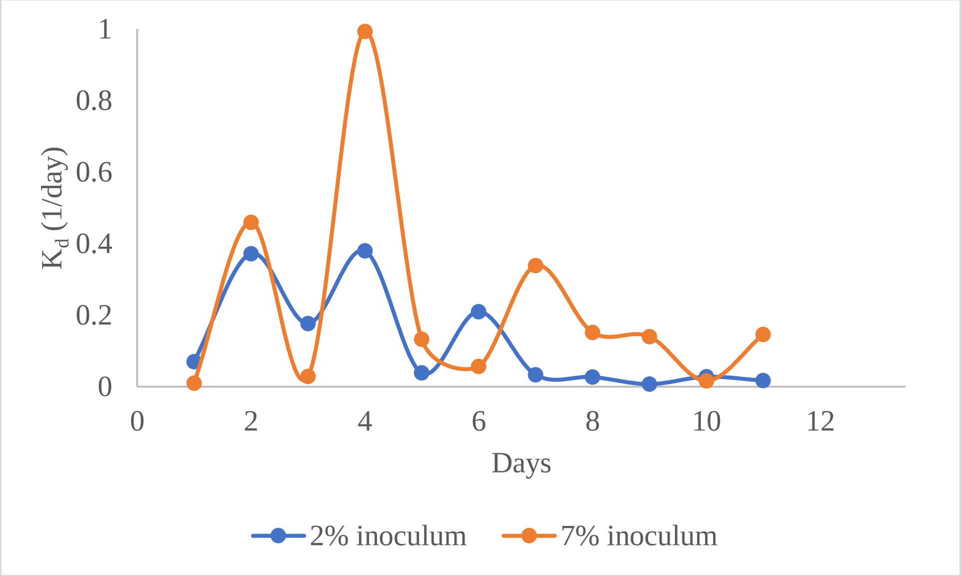

Figure 4-7 *Maintenance rate of Spirulina during night time*

4.6.4 Specific Oxygen Uptake Rate (q_{o2}) and Specific Oxygen Evolution Rate (SOER)

The specific oxygen uptake rate (q_{o2}) and Specific Oxygen Evolution Rate (SOER) of *S. platensis* are measured in the exponential phase and linear phase for both the experiments to compare the results of oxygen uptake concerning the biomass production.

Table 4-1 *Comparison of specific oxygen uptake rate for 2% inoculum and 7% inoculum experiment*

Growth phase	**2% inoculum experiment**		**7% inoculum experiment**	
	q_{o2} **mg $g^{-1}h^{-1}$**	**SOER** **mg $g^{-1}h^{-1}$**	**q_{o2}** **mg $g^{-1}h^{-1}$**	**SOER** **mg $g^{-1}h^{-1}$**
Exponential phase	0.01	0.21	0.65	1.18
Linear phase	0.08	0.07	0.07	0.06

The first 5 days are considered as an exponential phase and from the 5th to 10th day it is a linear phase. So, the q_{o2} and SOER value is calculated as an average of 1st to 5th day for exponential phase and from 5th day to 10th day for linear phase. The values are shown in Table 4-1.

4.7 Discussion

4.7.1 Dissolved Oxygen vs Biomass profile

The dissolved oxygen concentration increased during the day time and decreased during the night. The change of dissolved oxygen consumption and production throughout the day and night cycle is shown in Figure 4-8. When the light is available the DO will increase due to the photosynthetic activity. During the night time, the light is unavailable so the respiration process takes, place and therefore the dissolved oxygen decreases [109]. That is the reason for ups and downs in the dissolved oxygen values shown in Figures 4-2 and 4-3. The pattern of DO fluctuation observed in a laboratory photobioreactor without aeration is compatible with the fluctuation of DO in Lake Erie[101]. However, the DO levels are higher in the photobioreactor, probably because in the closed photobioreactor oxygen produced in photosynthesis during daytime was mostly accumulated inside the reactor, in both the liquid phase and the gas phase while in an open lake it was dispersed quickly after evolution.

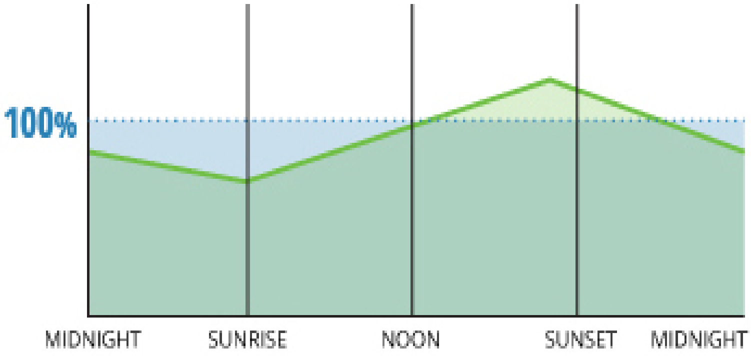

Figure 4-8 *Dissolved Oxygen fluctuation during the day and night cycle.*[101]

From Figure 4-2, it is observed that the final biomass concentration after the 12th day of the experiment was 0.39g/L and the DO never went below the 4 mg/L (hypoxic condition). From Figure 4-3 it is seen that even when the final biomass concentration was 1g/L on the 12th day of the experiment, the dissolved oxygen did not fall below hypoxic condition (4 mg/L). From this, it can be proven that 1g/L of the biomass of *S. platensis* is not a critical biomass concentration to create the hypoxic condition.

4.7.2 Biomass vs pH profile

The pH transition is directly related to the availability and absorption of lake nutrients. When the algae start to grow rapidly, the pH will start to increase gradually along with the increase in biomass because the culture pH in an algal culture is determined by the following balance:

$$H_2CO_3\ (dCO_2+H_2O) \Longleftrightarrow H^+ + HCO_3^- \Longleftrightarrow 2H^+ + CO_3^-$$

When cells are growing quickly at high biomass concentration, dCO_2 is consumed at a rate higher than the maximum rate of CO_2 absorbance. As a result, the concentration of dissolved CO_2, which is equivalent to H_2CO_3, would decrease. The decrease of dCO_2 concentration would cause the above balance moving towards the left, consuming protons and therefore causing culture pH to increase. This explains the strong correlation between pH and biomass concentration in the exponential and linear growth phase.

The high pH of a natural water body such as a lake and river can 'blind' organisms that rely on the perception of dissolved chemical signals for their survival by impairing their chemosensory functions [110]. Consequently, the increased pH could impact the production of plankton in the affected waters adversely [111].

From Figure 4-4(a) it can be observed that the pH of 2% inoculum reached a maximum of 9.5 on the 10th day and started to decrease until the 12th day of cultivation. Similarly, in Figure 4-4(b) the maximum pH of culture with 7% inoculum reached a maximum of 10.8 on the 12th day and it started to decrease on the 13th day.

On the other side, when the algal biomass decomposes, carbon dioxide may also be produced leading to a reduction of the water pH. The acidic pH levels have the potential to adversely affect the production and survival of marine species and the growth of phytoplankton [105]. So, the pH always needs to be maintained in an optimum condition to avoid more basic as well as more acidic condition. To avoid both extreme conditions the algae need to be harvested at an appropriate time as mentioned in the previous **Chapter 2**.

4.7.3 Oxygen Uptake Rate (OUR) and Oxygen Production Rate (OER)

The OER takes place during the day time and OUR takes place during the night time. The OER and OUR values for the first few days in Figure 4-5 for the 2% inoculum experiment are insignificant due to the low biomass concentration. After certain days the OER and OUR follow a regular pattern. Whereas for the 7% inoculum experiment the OER and OUR showed a regular pattern from day one because of higher initial biomass concentration, which was 0.15 g/L on the 0^{th} day. After 48 hours there was a large OER followed by OUR and it could be because the cells were active during the exponential phase.

4.7.4 Net growth rate and maintenance rate of microalgae

As shown in Figure 4-6 and Figure 4-7 the growth rate and maintenance rate of *Spirulina* started to increase with the increase in cultivation time and decreased after a certain period of cultivation. As shown in Figure 4-6 growth rate of the 7% inoculum experiment is higher when compared to the 2% inoculum experiment due to higher initial biomass concentration in the former. When the biomass concentration is higher, more cells will die during the night time for cell maintenance by respiration process and that is the reason why the 7% inoculum experiment showed a higher k_d value when compared to 2% inoculum as shown in Figure 4-7.

Table 4-2 *Maximum growth rate and death rate of microalgae at two different inoculum size*

Inoculum size	**μ_{max}**	**k_d**
2%	0.93	0.12
7%	1.21	0.20

Table 4-2 shows the μ_{max} and k_d for the 2% and 7% inoculum experiment. From this, we can see that higher initial biomass concentration showed higher μ_{max} and k_d values whereas lower initial biomass concertation showed vice versa.

4.7.5 Specific Oxygen Uptake Rate (q_{o2}) and Specific Oxygen Evolution Rate (SOER)

As listed in Table 4-1, the specific oxygen evolution rate for an exponential phase in 7% inoculum experiment is 1.18 mg O_2/g of DCW and for 2% inoculum experiment it is 0.21 mg O_2/g of DCW The SOER of the exponential phase of 7% is around 5 times higher when compared to 2% inoculum. In the linear phase, 2% inoculum and 7% inoculum showed almost similar specific oxygen evolution rate of 0.07 mg O_2/g of DCW.

Similarly, the q_{o2} of the culture with 2% inoculum was 0.01 mg O_2 h^{-1} g^{-1} DCW and the culture with 7% inoculum experiment showed 0.65 mg O_2 h^{-1} g^{-1} DCW during the night time. Since the biomass concentration is higher in the later when compared to the former, the cells might consume more dissolved oxygen during the night time for cell maintenance.

During the exponential phase, the cells are more active when compared to the linear phase so more oxygen is up-taken by the biomass.

4.8 Conclusion

In conclusion, by using a 2% inoculum as well as a 7% inoculum the dissolved oxygen never reached below 4 mg/L. Even when the biomass concentration reached 1.0 g/L, the DO didn’t fall below 6 mg/L. In other words, shall an algae-for-eutrophic-water restoration strategy be implemented in the future, it would be safe to operate at a *S. platensis* biomass concentration of 1.0 g/L or below. Experiments at higher biomass concentration are warranted to obtain the critical biomass concentration at which dO_2 may fall below 4 mg/L, the hypoxic conditions.

Chapter 5: Conclusions and Recommendations

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5.1 Conclusions

The following conclusions are derived from this study.

1. Cyanobacterium *S. platensis* can restore the eutrophicated water by the biological assimilation process. The algae could reduce the trophic status of eutrophicated water from hyper-eutrophic to oligotrophic and from eutrophic and meso-eutrophic to ultra-oligotrophic.

2. Results indicate the phosphate removal was achieved mainly through biotic mechanisms.

3. Harvest experiments indicated that it is possible to control the biomass concentration at 0.39 g/L using nylon clothes of 0.1 mm mesh.

4. Culture pH rose to 10.8 when 7% inoculum was inoculated to the culture and when the biomass concentration was approximately 1.0 g/L.

5. Dissolved oxygen dynamics studies indicated that no hypoxia occurred during 12-hour dark periods for biomass concentrations up to 1.0 g/L.

6. The growth kinetics of *S. platensis* was studied in artificial eutrophicated water at different phosphate concentration. The results indicated that the bioactive components, pH and biomass of *S. platensis* have a strong correlation with the phosphate concentration in water. Higher phosphate concentration showed higher cellular contents of chlorophylls, phycocyanin, total protein, biomass and final pH.

7. The culture DO never reach below 4 mg/L in the light/dark cultivation experiments when the highest biomass concentration was 1.0 g/L.

8. The oxygen production rate, oxygen uptake rate and specific oxygen uptake rate of *S. platensis* at two different inoculum sizes were studied which proved that the higher biomass concentration will have high OUR and qo_2 when compared to lower biomass concentration.

Overall, these results indicate that *S. platensis* has the potential to be applied for removing phosphate from wastewaters and to reduce the phosphate concentration in eutrophicated water to oligo-eutrophic level without creating the hypoxic condition. The biomass could be conveniently harvested using a nylon cloth of 0.1 mm mesh to keep the residual biomass concentration at around 0.39 g/L when the initial biomass concertation is1.0 g/L.

5.2 Recommendation

The following are some recommendations for future studies,

1. Studies on the dissolved oxygen dynamics of cultures with biomass concentration higher than 1.0 g/L and with better deoxygenation mechanism is warranted to determine the critical biomass concentration that sets the upper limit shall *S. platensis* is employed for eutrophic water restoration.

2. Studies should be carried out at light intensities and temperatures mimicking the seasonal changes of the Canadian climate, especially between May to October when algae could grow actively.

3. Phosphate removal by *S. platensis* from the natural eutrophicated water from Lake Erie and/or some other eutrophicated lakes in Canada should be studied.

4. Pilot studies testing the algae for eutrophicated water restoration strategy should be carried in a real eutrophicated lake.

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