BIOREMEDIATION OF TANNERY EFFLUENT AND LIPID PRODUCTION BY *CHLORELLA VULGARIS* (BEIJERINCK) UTEX 2714

BY

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ZARIA, NIGERIA.

JANUARY, 2017

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BY

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(MSc/SCI/39325/12-13)

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DECLARATION

I declare that the work in this thesis entitled "**BIOREMEDIATION OF TANNERY EFFLUENT AND LIPID PRODUCTION BY *CHLORELLA VULGARIS* (BEIJERINCK) UTEX 2714**" has been carried out by me in the Department of Biology, Ahmadu Bello University, Zaria, Nigeria. The information derived from the literature has been duly acknowledged in the text and in a list of references provided. No part of this thesis was previously presented for another degree or diploma at this or any Institution.

GAUJE Balli _____ _____

Signature Date

CERTIFICATION

This thesis entitled BIOREMEDIATION OF TANNERY EFFLUENT AND LIPID PRODUCTION BY *CHLORELLA VULGARIS* (BEIJERINCK) UTEX 2714 meets the regulations governing the award of the degree of Master degree in Biology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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DEDICATION

I dedicate this work first to God who have given me life, wisdom and opportunity to undertake this study and to my parents for their prayers and encouragement.

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ABSTRACT

This study was aimed to assess the potential of *Chlorella vulgaris* UTEX 2714 to grow and bio-remediate tannery effluent. Tannery effluent was collected from an effluent stream from Tanneries in Challawa Industrial Estate, Kano. The effluent was sterilised and inoculated with 10 % volume of algal inoculum. Hydrogen potential, conductivity and total dissolved solids were determined using Hanna combo pH/EC meter HI 98129. Phosphate, sulphate, free chlorine and nitrate were determined using HACH DR 2400 spectrophotometer. Standard methods were used to determine alkalinity, dissolved oxygen, biological oxygen demand and chemical oxygen demand titrimetrically. Spectrumlab uv - vis spectrophotometer model 752s was used to determine optical density and dry weight while cell count was achieved using an Improved Neubauer Bright-Line hemocytometer. Cadmium, chromium and lead were determined using Shimadzu Atomic Absorption Spectrophotometer (model AA-6800) while modified Folch procedure was used to extract the lipid from *Chlorella vulgaris*. Fatty acid profile was determined using Shimadzu gas chromatography mass spectrophotometer (model - 2010p). The study determined the effect of various effluent dilutions 0%, 5%, 10%, 25%, 50%, 75%, 100% and Bold basal medium (control) on physicochemical parameter, cell growth and lipid production after bioremediation was determined. There were significant reductions in most physicochemical parameters except for biological oxygen demand with concentration ranging from 0.00mg/l in 100%dilution in day 0 to 131.25mg/l in 75% dilution in day 0. This shows that *Chlorella vulgaris* UTEX 2714 is a good candidate for bioremediation with heavy metal reduction efficiency up to 100%. High dilution of tannery effluent encouraged algal growth while low dilution of tannery effluent showed a long lag phase growth. Lipid production was highest in bold basal medium with 57.25%, SAFA content (%) and PUFA content range from 21.77 to 42.46 and 28.68 to 55.49 respectively. Fatty acid profile shows oleic acid as the most abundant in all the algae cultured in the various dilutions of tannery. *Chlorella vulgaris* is a good candidate for bioremediation for being able to withstand heavy metal toxicity of tannery effluent even in low dilutions, shows good ability to sequester nutrients so as to reduce eutrophication promoting growth and a good feedstock for biodiesel production with lipid yield as high as 50.97% in 10% dilution.

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CHAPTER ONE

1.0 Introduction

1.1 Background of Study

The environment is under increasing pressure from anthropogenic activities all over the world. In the quest to satisfy his needs and aspiration for better living conditions, man generates different kinds of waste that are discharged directly or indirectly into the environment (Ezike *et al*., 2012). Tannery effluent is a by- product of the manufacturing and transformation of hides and skin to leather products. It constitutes an environmental concern as it increases the pollution status of rivers (Midha *et al*., 2008). Tannery waste is generated in huge amounts during the process of tanning by leather industries throughout the world. It is considered one of the most polluted industrial wastes and contains high amounts of metals which are very toxic to soil, plants and animals (Castilloss *et al*., 2007). Corporation/Municipal water treatment facilities in most of the developing countries, at present are not equipped to remove traces of heavy metals (Rajasulochana *et al*., 2009). This exposes every consumer to unknown quantities of pollutants in the water they consume. The problems associated with tannery effluents are their undesirable influence on the biological processes in receiving environmental media, undesirable taste and odour in addition to the high volume of effluent waste generated, which is usually released into the environment usually untreated or with minimal treatment (Rajasulochana *et al*., 2009). The major components of tannery effluent include sulfide, chromium, volatile organic compounds, large quantities of solid waste, and suspended solids like animal hair and trimmings which have been identified as the main causes of eutrophication in natural waters (Thakur, 2006). Less than 5% of the industries in the world have adopted adequate measures for treatment of effluents while most have

neglected it because of its cost implications (Khopkia, 2009; Preethi *et al*., 2009; Prabharathy and Surshendu, 2010; Akpomie, 2013).

Techniques used for decades in removal of pollutants of from industrial waste involves redox reactions, filtration, chemical precipitation, electrochemical treatment, ion exchange, reverse osmosis and evaporation but as it relates to large scale treatment it is not sustainable because of its high cost (Rajasulochana *et al*., 2009). Microalgae require a considerable amount of water for growth, algae is able to grow in different effluent of industry and other origin, therefore using tannery effluent to grow algae is one way to reduce growth cost (Chia *et al*., 2013).

Chlorella vulgaris belongs to division Chlorophyta and class Trebouxiophyceae, and was first isolated by Beijerinck in 1890. *Chlorella* strains have been studied for commercial production of biodiesel because of their high growth rates with some species having the capacity to double their biomass within 24 hours and high yield of lipids (Liu *et al*., 2008; Schenk *et al*., 2008; Liang *et al*., 2009). Kshirsagar (2014) identified the most dominant and pollution tolerant freshwater algal species isolated were *Chlorella vulgaris* Beijerinck and *Scenedesmus quadricauda* (Turpin) Breb. Microalgae are photosynthetic microorganisms which convert sunlight, water and CO_2 to sugars, from which macromolecules such as lipids and triacylglycerides (TAGs) can be obtained. The triacylglycerols (TAGs) are the promising and sustainable feedstock for biodiesel production. Many microalgae have the ability to produce substantial amounts 20-50% of TAGs as a storage lipid under photo-oxidative stress or other adverse environmental conditions (Sakthivel *et al.*, 2011).

Microalgal lipids being the third generation of biofuel have more competitive advantage over agricultural crops and lignocellulosic biomass (which are first and second generation biofuels respectively), due to high photosynthetic efficiency, faster growth, high biomass

productivities (Gouveia *et al*., 2009) and ability to be cultured in small space to produce more biomass (Christi, 2007). Compared to many terrestrial plant-based feedstock, these properties have the potential to produce life cycle savings in land use (including competition with food crops), water consumption, eutrophication, and greenhouse gas (GHG) emissions associated with cultivation and fuel processing (Peccia *et al*., 2013). Further benefit is that microalgae may also be grown on arid lands unsuitable for conventional agriculture, such as desert areas, or in large reservoirs of saline water (Converti *et al*., 2009). The main bottleneck of commercial application of biodiesel is its high cost of production (Behzadi and Farid, 2007). To increase the economic feasibility of biodiesel production from microalgae, it is essential to select microalgal strains that are capable of growing fast and accumulating large amount of lipids. Nascimento *et al.* (2013) reported the highest lipid yields were observed for *Chlorella* at 204.91 mg/ l per day.

This study is designed to provide relevant information relating to the remediation potentials of tannery effluent, its use as medium to support *Chlorella vulgaris* growth and increased lipid generation for biodiesel production.

1.1 Statement of the Problem

Industrialization has long been accepted as a hallmark of civilization. However, the fact remains that industrial emanations have been adversely affecting the environment (Rajasulochana *et al*., 2009). Wet industries like pulp and paper, agro industries, tanneries, distilleries, textile industries are major contributors of effluents (Chandralata *et al*., 2008; Krishna *et al*., 2012) that usually contain very high concentrations of Total Nitrogen and Total Phosphorus as well as toxic metals, that make their treatment expensive (Gasperi *et al*., 2008; Krishna *et al*., 2012; Chia and Musa, 2014) . Due to the absences or ineffectiveness of purification systems, wastewaters poses serious danger,

leading to the transfer and magnification of toxic products in the receiving water bodies with debilitating effect on the ecosystem (Chikere and Okpokwashi, 2004; Bako *et al*, 2008). Tannery waste is generated in huge amounts during the process of tanning by leather industries throughout the world. It is considered one of the most polluted industrial wastes and contains high amounts of metals which are very toxic to plants, animals and soil (Singh *et al*., 2011). The unused and non-usable hides and skins along with the excess process chemicals and water constitute solid and liquid wastes in the tannery. These wastes when untreated affect streams, groundwater, land and sewers in which they are discharged. The discharge of untreated wastewater into water courses affect the physical, chemical and biological characteristics of water and deplete the water bodies of dissolved oxygen (Ladwani *et al*., 2012; Rajiv *et al*., 2012; Subin and Husna, 2013). Tannery wastes are known to reduce germination, growth and yield of grain, wheat and lettuce crops (Castilloss *et al*., 2007; Babyskakila, 2009). It has also been reported that plants take up metals such as chromium from tannery wastes and these become available in roots, shoots, leaves, flowers and fruits with the minimum in fruits (Hussain *et al*., 2010)

The input cost of production medium formulation was identified as a critical factor determining economic viability of the algal biotechnology enterprise (Oswald, 1995). This serves as a major drawback to commercial viable mass algal culture operations, notwithstanding a period of intensive research over several decades.

Fossil fuel and its exploration have a devastating consequence on the physical and biological state of the environment. Therefore, it is necessary to search for renewable, biodegradable and environment-friendly alternative energy sources to reduce the dependence on the crude oil. Carbon neutral renewable liquid fuels are needed to eventually replace the petroleum-based transport fuels that contribute to global warming

(Dharmendra *et al.*, 2014). Owing to their exhaustability and unsustainable environmental impact both generated by their fossil origin, growing attention has emerged about renewable energy, with the aim of diversifying the energy resources to reduce the over utilization of fossil fuel and hence limiting their negative effect (Scarsella, *et al*., 2010).

1.2 Justification

Industrial effluents which are discharged from the textile and tannery industries contains a higher amount of metals especially chromium, copper and cadmium. These effluents released on the environment leads to contamination resulting in series of well documented problems in living organisms (Malarkodi *et al*. 2007).

> "The level of environmental degradation arising from discharges into canals which converges at a point and eventually flows into Challawa River in Kano state is alarming" and "the pollution levels have substantially increased and the waters are coloured showing serious contamination. Indigenes of Kano State especially those living in and around the Challawa and Sharada Industrial Estate, are now living in fear of a possible outbreak of water borne diseases, following the constant pollution of their water sources by tannery operators" (Vanguard, 2013).

The use of tannery effluent as feedstock for *Chlorella vulgaris* growth reduces the hazardous effects of heavy metals in the food web and the less pollution of drinking water sources by taking advantage of the phycoextraction potential of *Chlorella vulgaris*. This option for bioremediation of tannery effluents will greatly reduce the disease burdens associated with effluent generated from this industry.

Media cost remains an important driver in the currently renewed focus on the feasibility and profitability potential of algal biomass in biofuels production (Chisti, 2007; Harun *et*

al., 2010). The particular sensitivity of this factor in production profitability analysis has refocused interest in the use of wastes as possible sources of nutrients and, where possible, the linkage of waste treatment and algal production to achieve reductions in both operational and capital costs (Christensen and Sims, 2011).The growth performance from the various dilutions of tannery effluent will give an indication of which of the dilutions support *Chlorella vulgaris* growth and serve as an alternative medium and will be less expensive to mass culture algae.

Increasing energy access and energy security are seen as key actions for reducing poverty thus contributing to the Millennium Development Goals (FAO, 2014). We depend on various sources of energy like electricity and fuels for industries, automobiles, household activities and many other basic needs. Among these sources, the fossil fuels account for about 88% of primary energy production (Brennan and Owende, 2010). Energy is the driving force of any socio-economic development in any country. Access to modern energy services such as electricity or liquid fuels is a basic requirement to improve living standards (FAO, 2014). Attention in many countries of the world is currently geared towards substituting or blending fossil fuels with biofuel so as to reduce the adverse environmental effect of green house gases (GHG) produced as a result of usage of 100 percent fossil fuel. The lipid produced if in reasonable quantity can serve as a feedstock for the source of sustainable biofuel.

1.3 Aim

To investigate the use of *Chlorella vulgaris* for bioremediation of tannery effluent to reduce its impact to receiving waters, alternative source of media and lipid production

1.4 Objectives

This study is designed to:

1. Determine variation in physicochemical parameters before and after *Chlorella vulgaris* inoculation.
2. Determine the relationship among the physicochemical parameters and its effect on growth parameters and lipid yield.
3. Determine rate of biomass production and cell count of *Chlorella vulgaris* at varying dilutions of tannery effluent.
4. Determine the lipid production of *Chlorella vulgaris* at varying dilutions of tannery effluent.

1.5 Research Hypotheses

1. There is no significant difference in the level of physicochemical parameters before *Chlorella vulgaris* inoculation and after *Chlorella vulgaris* inoculation.
2. There is no relationship among the physicochemical parameters and no effect on the growth and lipid yield.
3. There is no significant difference in biomass production and cell count between varying dilutions of tannery effluent.
4. There is no significant difference in lipid production between varying dilutions of tannery effluent.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bioremediation: Trends and Approaches

Bioremediation involves an accelerated process by which microorganisms (indigenous or introduced) destroy/degrade, contaminants, through microbial metabolism. This process involves biochemical reactions or pathways in an organism that result in activity, growth, and reproduction of that organism. Chemical processes involved in microbial metabolism consist of reactants, contaminants, oxygen, or other electron acceptors, which convert metabolites to well-defined products (Adebowale, 2004) such as carbon dioxide and water. Bioremediation is the technology that uses microorganism activity to remove pollutants which is efficient, less expensive, requires low technique, widely acceptable and can be done on site. Bacteria are generally used for bioremediation, but fungi, algae and plants have also been used (Leung, 2004). Bioremediation technologies are classified into *in situ* and *ex situ*. *In situ* bioremediation involves treating the contaminated material at the site and this is done by either biostimulation or bioaugumentation. Biostimulation is the addition of nutrients, oxygen or other electron donors and acceptors to a contaminated site in order to increase the population or activity of naturally occurring microorganisms available for bioremediation. Bioaugmentation is the addition of microorganisms that biotransform or biodegrade contaminants. The microorganisms added can be a completely new species or more members of a species that already exists at the site (Leung, 2004). *Ex situ* bioremediation involves the removal of the contaminated material to be treated in a contained environment. *Ex situ* bioremediation is achieved by two methods: land farming and bioreactor. Land farming involves soil that is removed and taken to another area where it is spread out in thin layers, then micro organisms are added and it is tilled to aerate. *Ex situ* bioreactor involves the removal of

soil and water and placing them in a reactor where the temperature is set and moisture is controlled, and is mixed with micro organisms to obtain maximum degradation (Kadiri, 2014).The merits of *in situ* bioremediation is that the energy requirement to extract the contaminants is reduced, so there is less exposure of workers. It is also less expensive. It can be performed with little technical ability. However, the demerit of this strategy is the site of bioremediation is not contained, therefore, it is difficult to control conditions and monitor progress. In *ex situ* bioremediation the contained environment allows for easier monitoring and maintaining of conditions and progress, thus making the actual bioremediation process faster. However, the removal of the contaminant from the contaminated site is time consuming, expensive and potentially dangerous. By bringing the contaminants to the surface, the workers and the general public have increased exposure to the toxic material (Leung, 2004). It also requires high technical know- how to perform such operations.

Contaminants especially heavy metals are processed by micro algae in the environment naturally through these biological approaches.

2.1.1 Biosorption

Biosorption is an innovative technology that employs inactive and dead biomass for the removal and recovery of metals from aqueous solutions (McKay *et al.,* 1999a; Romera *et al*., 2007; Kim *et al*., 2008; Sulaymon *et al*., 2013). Biomass from various sources such as bacteria, yeast, algae, fungi and plants have been used to adsorb metal ions from the environment (Kratochvil, 1997; Brady *et al*., 1999; Romera *et al*., 2006; Sulaymon *et al*., 2012; Sulaymon *et al*., 2013). Among the most promising types of biosorbents studied is algal biomass (Figueria *et al*., 2000; Davis *et al*., 2003; Sulaymon *et al*., 2013). Algal biomass has been reported to have a high metal binding capacity. This is due to the presence of polysaccharides, proteins or lipids on the cell wall structure that contain

functional groups such as amino, carboxyl, hydroxyl, sulfate, and others (Hossain *et al*., 2012; Sulaymon *et al*., 2013). These groups have the ability to bind heavy metals by donating an electron pair from these groups to form complexes with the metal ions in solution (Fu and Liu, 2007; Sulaymon *et al*., 2013). The key advantage of microalgal biomass is the wide variety of macro molecules including polysaccharides, proteins, peptides and nucleic acid, which accounts for the majority of metal adsorption by ion exchange mechanism (Goyal *et al*., 2008). The use of the dead cells offers several advantages: (a) metal removal system is not subjected to metal toxicity limitations, (b) there is no requirement for growth media and nutrients, and (c) adsorbed heavy metals can easily be desorbed and the biomass can be reused. Because the fragile biomass of the microalgae is not suitable for use in fixed bed columns (Chu *et al*., 1997; Wilke, 2006)

2.1.2 Bioaccumulation

Heavy metals have the capacity to interfere with a wide spectrum of activities in living organisms. Almost every aspect of their metabolism, growth, and differentiation may be affected. Some prokaryotic and eukaryotic organisms have developed means for rendering toxic heavy metals to innocuous forms. They possess a variety of mechanisms to maintain metal homeostasis and prevent poisoning. According to Mason (2013), metals are transported across biological membranes of aquatic organisms through three transportations mechanisms; Passive diffusion, facilitated transportation and active uptake. Passive diffusion and facilitated transportation are mechanisms employed by algae in moving metals across its cell wall during the biosorption phase. This is achieved by absorption of toxic metal ions to cell-associated materials and/or cell wall components (Kaplan *et al*., 1987; Xue *et al*., 1988; Das *et al*., 2008; De Philippis and Micheletti, 2009; Naja and Volesky, 2011) or secretion of metal-binding organic compounds to the surrounding environment (McKnight & Morel, 1979; Van der Berg *et al*., 1979; Levy *et*

al., 2008). However, specificity is rather low and any metal cation may interact with negatively charged residues of the organic compounds to form complexes. These transportation mechanisms make it possible for dead algal cells to be able to take up heavy metals because their occurrence depends highly on the structure of the algal cell and does not require any form of energy. The second phase is accumulation inside the cell, while the active process used during bioaccumulation phase depends solely on the availability of energy source to facilitate the transportation process (Mason, 2013; Bello, 2015). This is a slow process involving active transport through the cell membrane into the interior and binding to proteins and other intracellular components. It is a metabolism dependent mechanism that is inhibited by low temperatures, absence of an energy source, metabolic inhibitors, and uncouplers. It occurs only in living cells (Wilde and Benemann, 1993) Metal resistance may also result from the ability to cope with high amounts of heavy metals inside tissues to an active process that involves the uptake and accumulation of the metal ions inside the cell. Heavy metals enter microalgae cells via micronutrient transporters (Sunda and Huntsman, 1998 ; Kramer *et al*., 2007; Arunakumara and Zhang, 2008). Once in the cell, heavy metal detoxification may be achieved by binding to specific intracellular compounds and/or transport of the metals to specific cellular compartments (Nagano *et al*., 1984; Heuillit *et al*., 1986; Vymazal, 1987; Perales-Vela *et al*., 2006; Arunakumara and Zhang, 2008).

2.2 Impact of Industrial Effluent on the Environment

Increase in population have contributed to the rapid development of industries such as fertilizer, mining, electroplating, photography, electro- osmosis, iron and steel, pesticides, metallurgy, electrolysis, surface finishing, metal surface treatment and leather so as to meet the growing demand of the populace. Waste products especially effluent from such manufacturing processes are discharged directly or indirectly to the surrounding water

bodies. Pollution threatens the environment when pollutants in industrial waste water are discharged directly without any form of treatment into the environment which are transported and accumulated in the ecosystem leading to a build up in the food chain **(**Pergent *et al*.1999, Priyadarshani *et al*., 2011). Heavy metal pollution is one of the environmental problems due to anthropogenic activities. The resultant effect of the presence of the heavy metals in the environment even in minute quantities is toxic and poses a serious threat to flora and fauna and by extension is detrimental to human life (Kadiri, 2014).

Heavy metals do not decay but can be transformed through sorption, methylation, and complexation, and changes in valence state. These transformations affect the mobility and bioavailability of metals. At low concentrations, metals can serve as important components in life processes, often serving important functions in enzyme productivity. However, above certain threshold concentrations, metals can become toxic to many species (Adebowale, 2004). The increasing load of heavy metals causes imbalance in aquatic ecosystems and the biota growing in such habitats. Recovery of heavy metals from industrial waste streams is becoming increasingly important as society realises the necessity for recycling and conservation of essential metals (Priyadarshani *et al*., 2011). Conventional treatments for removing heavy metals from wastewater include chemical precipitation, ion exchange, reverse osmosis, evaporation, ultra filtration, electro dialysis treatment however, limitations are incomplete metal removal, high energy requirement, uneconomical and generation of toxic sludge (Priyadarshani *et al*., 2011, Araújo *et al*. 2013). There are different forms of metals in the aquatic environment distributed between the sediment and the solution. Metals in the sediment are present in insoluble inorganic complexes as suspended solids or in association with organic colloids. In solution, metals are present as free metallic ions and as organic and inorganic complexes. The equilibrium

among all these metals are interchangeable and dependant on the environmental factors such as temperature, Alkalinity, pH and as well as the biota in the water. Most of the free metal ions, in the aquatic environment are bio-available and are the most toxic; any process that accelerates their transformation into bound forms results in a reduction of toxicity to the biota (Kaplan, 2013)

2.2.1 Chromium

Chromium is a metal of interest because of its widespread application in various industries, such as metallurgical (steel, ferro and non-ferrous alloys) and chemical (pigments, electroplating, tanning, others). Due to industrial run-off, process, large quantities of Cr compounds are discharged in liquid, solid and gaseous wastes into the environment, resulting in adverse biological and ecological effects (Kabata-Pendias and Pendias, 2001; H¬rcsik *et al*., 2006). Chromium exist in varying oxidation states from 0 to VI but chromium (III) and chromium (VI) are the two common oxidation states of chromium in the environment. Cr (III) is the best known form. It displays stability at neutral pH, if the complexation can be neglected. Under redox and pH conditions normally found in natural systems, chromium is removed from the solution as $Cr(OH)_3$, or in the presence of Fe(III) in the form of $(Cr_xFe_{1-x})(OH)_3$ where the x is the mole fraction of chromium (Sass and Rai, 1987; H¬rcsik *et al*., 2006).

Trivalent chromium is considered a micronutrient, essential for the proper function of living organisms with regards to glucose and lipid metabolism (Mertz, 1975; Anderson, 1989, H¬rcsik *et al*., 2006), Cr (VI) forms several oxygen associated species, the relative proportions of which depend on both pH and total chromium (VI) concentration. Within the normal pH range in natural waters CrO_4^{2-}, $HCrO_4^-$ and $Cr_2O_7^{2-}$ oxy anions are the forms generally found and constitute many of the chromium (VI) compounds. They are quite soluble and mobile in the environment (Nieboer and Jusys 1988; H¬rcsik *et al*,

2006). Hexavalent chromium can display toxic effects on biological systems. Cr (VI) crosses cellular membrane and exhibits strong oxidative potential (Kotas *et al*., 2000). Hexavalent form of Cr has been shown to generate hydroxyl radicals from hydrogen peroxide to replace Mg ion from the active site of many enzymes (Vajpayee *et al*., 2000; Ünal *et al*., 2010). Cr phytotoxicity can result in degradation of pigment contents, nutrient balance, enhancement of antioxidant enzymes activity, and induction of oxidative stress in algae (Wong *et al*., 1991; Rai *et al*., 1992; Ünal *et al*., 2010). In the alga *Pseudokirchneriella subcapitata*, Cr (VI) inhibited growth and induced genetic mutations (Labra *et al*, 2007). Cr(VI) also induced the intracellular ROS accumulation in the alga *C. reinhardtii* (Szivak *et al*., 2009). Studies using transmission electron microscopy showed ultra-structural modifications (such as increased vacuolization, condensed cytoplasm and dark precipitation in the cell wall) in cells of the alga *M. denticulata* exposed to Cr(VI) during three weeks (Volland *et al*., 2012). Proteomic analysis revealed that the treatment of *P. subcapitata* alga cells with Cr(VI) altered the expression of proteins associated with the metabolism of amino acids glutamine, arginine and methionine (Vannini *et al*., 2009) and increasing concentration of chromium reduced algal cell density and dry weight (Rodgher, *et al*., 2008).

2.2.2 Lead

Lead is a heavy, low melting, bluish-gray metal that occurs naturally in the Earth's crust (ATSDR, 2007). Lead is not a particularly abundant element, making up only about 0.0013% of the Earth’s crust. Metallic lead, Pb (0), exists in nature. However, it is rarely found naturally as a metal. It is usually found combined with two or more other elements to form lead compounds. Lead exists in three oxidation states: Pb (0) - the elemental form, Pb (II) and Pb (IV) (UNEP, 2010). Lead is one of the most dangerous, important, and potential toxic metal in aquatic ecosystem. Lead enters the environment chiefly via

anthropogenic activities through releases from minning, emission from vehicles, in production of pigment in paints, dyes, and ceramic glazes , iron and steel production, lead-acid-battery manufacturing, and non ferrous (brass and bronze) foundries. and other metals, that make or use lead, lead alloys, or lead compounds (ATSDR, 2007). Pb (II) is the most significant toxin of the heavy metals and its effects are of toxicological and neurotoxical in nature. These include irreversible brain damage. Inorganic forms of lead typically affect the central nervous system, peripheral nervous system, and hematopoietic, renal, gastrointestinal, cardiovascular, and reproductive systems. Organic lead toxicity tends to predominantly affect the central nervous system. The other hazardous effects of lead are visual disturbances, convulsions, loss of cognitive abilities, antisocial behaviours, constipation, anemia, tenderness, nausea, vomiting, severe abdominal pain, anemia, and gradual paralysis in the muscles (Hannachi, 2012). The toxicity levels sensitivity may vary among different micro algae (Soldo and Behra, 2000). It may affect photosynthesis, growth, and enzyme activity and even respiration of micro algae. The tolerance mechanism of micro algae is physiological exclusion of free metal ions due to reduced cell membrane permeability (Brown *et al*., 1988). Chelating to organic complexes is another possible mechanism regulating metal ions detoxification (Gerringa *et al*., 1995). Moreover, it is able to bind strongly to amino acids, enzymes, DNA and RNA and can induce the production of reactive oxygen species (ROS) like the superoxide radical and hydrogen peroxide that can cause severe oxidative damage to plant cells (*e.g.*, by increasing membrane lipid peroxidation and permeability) (Souza *et al*., 2012). Lead can inhibit the synthesis of chlorophyll because it changes the absorption of essential elements such as Mg and Fe (Sunda and Huntsman, 1998; Souza *et al*., 2012). The biological functions of lead in algae are unknown (Pawlik-Skowronska, 2000; Souza *et al*., 2012), but lead is known to have adverse effects on microalgal morphology,

growth and photosynthesis when present at high concentrations (Pawlik-Skowronska, 2002; Souza *et al*., 2012). Baumann *et al*., (2009) showed that for brown algae there was significant variation in Pb concentrations for all seven species of algae examined.

2.2.3 Cadmium

Cadmium in its elemental form is a soft, silver-white metal. It is not usually present in the environment as a pure metal. Cadmium is most often present in nature as complex oxides, sulphides, and carbonates in zinc, lead, and copper ores. It is rarely present in large quantities as chlorides and sulphates. Cadmium exists almost entirely as chloride species ($CdCl^{+}$, $CdCl_2$, $CdCl^{3-}$). Cadmium mobility in aquatic environments are enhanced by low pH, low hardness, low suspended matter levels, high redox potential and low salinity (Government of Canada, 1994). In natural water bioavailability of cadmium is reduced through sorption on suspended particles so that biological responses occur at higher cadmium levels (ECB, 2005).

Concentrations of cadmium in polluted waters ranges from <1.0 to 77 μg/L. Cadmium from polluted soil and water can accumulate in plants and organisms, thus entering the food chain. (ATSDR, 2012). Cd is generally considered a non-essential element. Its toxicity seems to be associated to the high affinity to sulfhydryl groups, the displacement or substitution of essential metal ions (such as Zn) and the modication of the conformation of the bio molecules (Le Faucheur *et al*., 2005). Toxic effects of cadmium on algae include the inhibition of growth (Torres *et al*, 2000; Kallqvist, 2009) and photosynthesis (Singh *et al*., 1993). Cd affects the ultra-structure and photosynthesis of the unicellular freshwater green alga *Micrasterias denticulata* as a consequence of the disturbance of calcium homeostasis probably by displacement of Ca by Cd (Andosch *et al*., 2012). Cadmium induced the production of reactive oxygen species (ROS) (Szivak *et al*., 2009) and activated the transcription of a large number of genes involved in the

oxidative stress defence mechanisms in the alga *Chlamydomonas reinhardtii* (Jamers *et al*., 2013).

2.3 The Tanning Process and its Works

The environment is under increasing pressure from solid and liquid wastes emanating from the leather industry. These are inevitable by-products of the leather manufacturing process that cause significant pollution unless treated in some way prior to discharge.

Tanning is the treatment of hides and skins to preserve and convert them into a stable material (leather) which will not putrefy and is suitable for a wide variety of applications. The figure in appendix I show the steps in the conversion process grouped into beam house and tanyard operations.

Tannery wastewater is highly polluted in terms of biological oxygen demand (BOD), chemical oxygen demand (COD), suspended solids (SS), nitrogen, conductivity, sulphate, sulphide and chromium (Mondal *et al*., 2005) and in most developing countries tannery effluents are discharged directly into sewers or water bodies without treatment (Verheijen *et al*., 1996; Favazzi, 2002). Each tannery effluent is composed of several kinds of waste: soaks, limes, bates, pickles, chrome-, bleach- and fat-liquors, vegetable tan liquors, colouring baths and all kinds of wash waters. All tanning operations are of the batch type and the effluents come on varied time schedules. All these factors make the purification of tannery wastes rather complicated.

2.4 Use of Algae in Bioremediation

Microalgae are very abundant in the natural environment and are well adapted to a range of habitats, such as fresh and sea water, domestic and industrial effluent, salt marshes and constructed wetlands. Algal physiology, biochemistry and photosynthetic apparatus are similar to higher plants, but its growth is very quick. Microalgae are very sensitive to changes in their environment. In response to changes in trace levels of various organic

and inorganic pollutants including heavy metals, they change their overall metabolism. Microalgae are therefore often used as biological sensors for detecting potential toxic effects of heavy metals (Torres *et al*., 2000, Chouteau *et al*., 2004, Durrieu *et al*., 2011, Kaplan, 2013). Microalgal biosensors are found among various algal groups such as chlorophyta, cyanobacteria, and diatoms, (Brayner *et al*., 2011, Kaplan, 2013). Bioremediation using algae presents the opportunity to solve three key issues of environmental pollution by detoxifying pollutants; ensure energy security by yielding lipids for biodiesel and combating global warming by utilising CO_2 for biomass production (Dwivedi, 2012).

The advantage of microalgal bioremediation over bacteria type of bioremediation is that the removal of pollutants under light conditions does not require oxygen. Furthermore, carbon sequestration and aeration takes place as carbondioxide and is used to produce biomass. Fermentation due to action of bacteria produces an offensive smell while in microalgal bioremediation, odour is less of a problem (Priyadarshani *et al*., 2011).

Nutrient enrichment result from agricultural wastewater, runoff, concentrated animal feed operations and tannery containing nitrates and phosphates. These are mostly untreated when discharged into aquatic ecosystem result in eutrophication and harmful algal bloom (Chia, 2015). Studies showed that *Chlorella vulgaris* had an uptake efficiency of 84% and 69.23% of nitrates and phosphates respectively as compared 82.5% and 64.52% in *Synechocystis salina* (Dominic *et al*., 2009). Menke *et al*. (2011) reported that after culture of *Nannochloropsis* sp. for 10 days grown in 75% wastewater, nitrogen and phosphorus was respectively reduced to 80.4% and 72.8%. Costa *et al*. (2004) used the urban secondary sewage as a growing media for *Tetraselmis chuii* cultivation and observed high reductions of nitrite (99.9%) and nitrate (86.40%) for 7 days period. According to Hii *et al*. (2011) cultivation of *Nannochloropsis* sp. in

wastewater removed 33.24 % of nitrates. Similarly, *Nannochloropsis oculata* removed 78.4% of total nitrogen and 92% of nitrates in wastewater from semi – closed recirculation aquaculture system (RAS)

It was observed that *Scenedesmus obliquus* continued bioaccumulating cadmium and zinc with increasing phosphorus in the medium (Dwivedi, 2012). *Spirulina* sp was able to adsorb lead as observed by Chen Hong *et al*. (2005) with a 74% adsorption capacity at the initial stage of 0 – 12 min. Another report stated that a diatom *Phaeodactylum tricornutum* is highly tolerant to cadmium (22.3 mg/L) and has also proved to have high removal capacity (Torres, 1997). *Dunaliella salina* has high tendency for zinc accumulation followed by copper and cobalt, but lowest tendency towards cadmium. Uptake of copper, cobalt and zinc increased with the increasing concentration of metal ions in the medium whereas cadmium concentration had no effect (Shafik, 2008). *Scenedesmus bijuga* and *Oscillatoria quadripunctulata* showed heavy metal removal capacity 37- 50% for copper, 20 - 33% for cobalt, 35 - 100% for lead and 32 - 100% in case of zinc from the sewage and petrochemical industry effluent (Ajayan, 2011). *Chlorella* sp. could efficiently reduce 76% - 96% of cadmium and 78% to 94% of Nickel from the medium within 7 - 28 days when cultured under laboratory condition (Rehman, 2004). Adsorption capacity of heavy metal vary in algae of the same species and also different species of algae (Jin-fen *et al*., 2000). The detoxification mechanisms of heavy metals by algae include systems such as metallothioneins (MTs) and phytochelatins. Alginates cell wall carry functional groups such as amino, carboxyl, hydroxyl and sulfated polysaccharides (Volesky, 2001, El-Sikaily *et al*., 2007).

2.5 Factors that affect Algal Growth and Lipid Production

In cultivating algae, several factors must be considered, and different algae have different requirement, essential factors include water, temperature, light intensity/ duration, pH condition, salinity and nutrients.

2.5.1 Temperature

Water temperature must be in a range that will support the growth of algae. Temperature is the easiest factor that can be controlled in the algae cultivation. It is one of the most important environmental factors that influence algal growth rate, cell size, biochemical composition and nutrient requirements. It is a sensitive factor for algae growth and metabolic processes (Adenan, *et al*., 2013). The optimal temperature for phytoplankton cultures is generally between 20°C and 25°C even though the temperature may vary with the composition of the culture medium. Below optimal growth temperatures, growth rate (μ) increases with increasing temperature, but declines markedly above the species- or strain- specific optimum (Renaud, 2002). The efficiency of carbon and nitrogen utilization decreases at non-optimal temperatures (Darley, 1982, Juneja *et al*, 2013). Most cultured microalgae are capable of tolerating temperatures between 16°C and 27°C. Temperature lower than 16°C will reduce the pace of growth while temperatures higher than 35°C are lethal to a number of species. Temperature may also play a key role in photoinhibition, which invariably will impact algal growth rate to which mechanisms of temperature-dependent photoinhibition have been assumed. These mechanisms are (i) low temperature results in reduced electron transport at a given photon flux rate due to slow rate of CO_2 fixation; (ii) low temperature inhibits the active oxygen species, which results in reducing photoinhibition by protecting PSII; and (iii) low temperature inhibits the synthesis of the D1 protein degraded during photoinhibition, consequently impeding the PSII repair cycle (Vonshak and Torzilla, 2004).

A shift in temperature alters the level of unsaturation of fatty acids in the lipid membrane (Thompson, 1996; Harwood, 2004; Gushina and Harwood, 2009). Intracellular lipid synthesis was found to be inhibited at supra-optimal temperature (32°C); consequently, lipid content decreased to 5% dry weight at 32°C in comparison with 22% at 25°C. *Dunaliella salina* has shown a considerable increase in fatty acid unsaturation in response to decrease in temperature from 30 to 12°C (Lynch, 1982). Similar effects were observed in *Nannochloropsis oculata* and *Chlorella vulgaris*, both of which have an optimum growth temperature of 25°C. Increasing the growth temperature from 20 to 25°C doubled the lipid content (from 7.90% to 14.92%) in *N. oculata*. Increase of temperature from 25 to 30 °C decreased the lipid content in *C. vulgaris* from 14.71% to 5.90% (Converti, 2009).

The decrease in lipid content led to an accumulation of polysaccharides (Kalacheva, 2002; Juneja *et al*., 2013). Starches are synthesized by phosphorylated metabolites in the dark reactions of the photosynthesis cycle using energy-rich phosphate bonds (*i.e.*, ATP) formed in the light reactions. Increased temperature is also reported to impact starch content in the algal cell (Juneja *et al*., 2013). Nakamura and Miyachi (1982) studied the effect of temperature on the starch degradation in *Chlorella vulgaris* grown autotrophically at 20°C. This study reported a significant reduction in starch (17%) with concomitant increase in sucrose (57%), when culture was exposed to 38°C for 10 min after 30 min at 20°C. However, another study with *Chlorella vulgaris* grown autotrophically at 38 °C, reported a reduced degradation of starch at 38°C (Nakamura and Miyachi, 1982a, Juneja *et al*., 2013). Algal culture temperature can be maintained by flowing cold water over the surface of the culture vessel or by controlling the air temperature using air- conditioning units.

2.5.2 Light intensity and photo period

Light is one of the most important factors affecting primary production in streams, but the requirement varies greatly with the culture depth and the density of the algal culture that is at higher depth and cell concentration, the light intensity must be increased to penetrate through the culture. Light influence is much stronger than that of phosphorus, resulting to almost ten-fold increase in benthic microalgae bio-volume over the 10–400 µmol photons m^{-2} d^{-1} range of experimental irradiances (Hill *et al*., 2009). Algal response to light intensity is either by photoadaptation or photoacclimation which leads to changes in cell properties and an increase in photosynthetic efficiency (Dubinsky *et al*., 1995; Juneja *et al*., 2013). Adaptation can occur through multiple mechanisms such as changes in types and quantities of pigments, growth rate, dark respiration rate or the availability of essential fatty acids (F↔bregas *et al*., 2004). Morphological photoacclimation is accompanied by changes in cell volume and the number and density of thylakoid membranes (Berner *et al*., 1989, Juneja *et al*., 2013). Light may be natural or supplied by fluorescent tubes. Despite the intense sunlight, light inhibition by full sunlight was observed only occasionally; the rate of photosynthesis fell progressively with decreasing light (Brock, 1969).

High light intensity may result in photo-inhibition, growth rate of *Dunaliella viridis* decreased to 63% with increase in light intensity from 700 to 1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Gordillo, 1998b, Juneja *et al*., 2013). However, Raikar (2001) reported Rhodophyta cultured in light intensities 10, 40, 80 µmol $m^{-2}s^{-1}$,increased growth rate with increasing light intensity. Morris *et al.* (1974), in a study on the marine diatom, *Phaeodactylum tricornutum*, in low light i.e. 400 lux at the culture surface, led to an increase in the rate of protein synthesis. Low light intensity has been observed to result in higher protein content, while high photon flux density (PFD) results in increased extracellular

polysaccharide content (Iqbal and Zafar, 1993; Juneja *et al*., 2013). Absence of light was observed to increase the total lipid content of the *D. viridis* but reduce triglycerides, free fatty acids, free alcohols and sterols. In *Nannochloropsis* sp., grown under lowlight conditions (35 $\mu E \cdot m^{-2} \cdot s^{-1}$), 40% of the total lipids were found to be galactolipids and 26% were found to be triacylglycerols. In the same system, high light (550 $\mu E \cdot m^{-2} \cdot s^{-1}$) conditions resulted in an increased synthesis of triacylglycerol with a reduction in galactolipid synthesis (Sukenik, 1989; Juneja *et al*., 2013).

Light utilization efficiency may be optimized by prolonging the dark period under conditions of high light intensity. This allows the photosynthesis machinery in the cell to fully utilize captured photons and convert them into chemical energy, thus avoiding the effects of photoinhibiton (Long *et al*., 1994, Juneja *et al*., 2013). Also, overheating due to both natural and artificial illumination should be avoided. The duration of artificial illumination should be minimum 18 hours of light per day, although cultivated phytoplankton develops normally under constant illumination. Significant differences in growth rate were only found when *Chattonella marina* was cultured under light duration of <10 h. i.e. in 10L: 14D and 8L:16D (Liu, 2007).

2.5.3 pH

One of the most important factors in algal cultivation is pH since it determines the solubility and availability of CO_2 and essential nutrients, and because it can have a significant impact on algal metabolism, especially availability of carbon for algal photosynthesis in intensive cultures (Goldman, 1973; AZOV, 1982; Chen, 1994; Juneja *et al*., 2013). The pH range for freshwater algae is between 6.5 and 8.0. For cultured seawater algal species, the pH ranges from 7.0 and 9.0 with the optimum range being 8.2 – 8.7. Hansen (2002) stated that uptake of inorganic carbon by phytoplankton during photosynthesis can increase pH levels in the surrounding water. Changing pH in media

may limit algal growth via metabolic inhibition (Goldman,1982; Juneja *et al*., 2013). Pruder and Bolton (1973) observed that *T. pseudonana* cells adapted to low pH (6.5) had lower growth rate at sub-optimal pH (8.8) (Juneja *et al*., 2013). Normal growth rate was restored after the pH was lowered by addition of HCl. Similar results were reported by Chen and Durbin (1994) where photosynthetic rate and algal growth was minimal at pH 9.0, but carbon uptake rates were enhanced when the pH was lowered to 8.3 (Chen,1994; Juneja *et al*., 2013). Complete culture collapse due to the disruption of many cellular processes can result from failure to maintain an acceptable pH. In the case of high – density algal culture, the addition of carbon dioxide allows to correct for increased pH. Similar to alkaline pH, acidic conditions can alter nutrient uptake (Gensemer, 1993, Juneja *et al*., 2013) or induce metal toxicity (Sunda, 1975; Anderson, 1978; Juneja *et al*., 2013) and thus affect algal growth. The pH of a solution is an important measure for evaluating aquatic toxicity and corrosiveness. The severity of toxicity or corrosion tends to be greater under low-pH or high-pH conditions than at near-neutral pH, because the solubility of many metals can be described as amphoteric, with a greater tendency to dissolve and form cations at low pH or anions at high pH (Langmuir, 1997; Cravotta, 2006) thereby affecting metal speciation and its bioavailability. It was suggested by Goldman (1981) that pH tolerance limits of the algae tested are governed either by chemical influence on the growth medium or by metabolic effects on the cells. Fluctuations of the pH in the medium also have been found to alter the lipid composition of microalgae. Based on morphological observations, alkaline pH inhibited the growth of microalgae, thus diverting the energy to form TAG (Guckert, 1990; Sharma, 2012).The effects of pH on the lipid and FA composition of a *Chlamydomonas* sp. isolated from a volcanic acidic lake was studied. The relative proportion of TAG (as percentage of total lipids) was higher in *Chlamydomonas* sp. grown at pH 1.0 than that in the cells cultivated

at higher pH. The increase in saturation of fatty acids in membrane lipids of *Chlamydomonas* has been suggested to represent an adaptive reaction at low pH to decrease membrane lipid fluidity (Tatsuzawa, 1996; Sharma, 2012)

2.5.4 Aeration/ mixing

Mixing is necessary to prevent sedimentation of the algae, to ensure that all cells of the population are equally exposed to the light and nutrients, to avoid thermal stratification e.g. in outdoor culture and to improve gas exchange between the culture medium and the air (Grobbelaar, 2000). The air is important because it contains carbon source present in the form of carbondioxide (CO_2). CO_2 in the culture medium further buffers the water against pH changes as a result of the CO_2/HCO_3^- balance. Depending on the scale of the culture medium, mixing is achieved by stirring daily with hand (test tube and erlenmeyers), aerating (bags, tanks), paddle wheel and jet pumps in case of ponds (Kadiri, 2014).

2.5.5 Salinity

Algae are ubiquitous i.e. occurring in virtually any habitat so long there is moisture. Aquatic habitat are categorized based on salinity content; fresh water (salinity < 0.5 ‰), Brackish (salinity > 0.5 – 18 ‰) and marine (salinity>18 ‰). Algae differ in their adaptability to salinity and based on their tolerance extent they are grouped as halophilic (salt requiring for optimum growth) and halotolerant (having response mechanism that permits their existence in saline medium). In either case, the algae produce some metabolites to protect from salt injury and also to balance in the surroundings to avoid osmosis (Richmond, 1986; ☑ *et al*.,).

Exposing algae to lower or higher salinity levels than their natural (or adapted) levels can change growth rate and alter composition even though marine phytoplanktons are extremely tolerant to changes in salinity (Kadiri, 2014). Most micro algal species grow

best at salinity that is slightly lower than that of their native habitat, which is obtained by diluting seawater with tap water. Salinities of 20 – 24 ‰ have been found to be optimal (Kadiri, 2014). *Nannochloropsis salina* has been reported to grow fastest between 20 and 40 ‰ (Rodolfi, 2008; Bartley, 2013) and produce the most lipids, as total lipid and percent lipid per unit biomass, at salinities between 30 and 35 ‰ (Abu-Rezq, 1999; Chini Zittelli, 2000; Bartley, 2013). In another study with *Dunaliella tertiolecta*, an increase in intracellular lipids (60% to 67%) and triglyceride concentration (40% to 56%) with an increase in NaCl concentration from 0.5 (freshwater concentration) to 1.0 M was observed (Takagi and Yoshida, 2006). Increasing the NaCl level in cultures of *Botryococcus braunii*, a fresh water alga, showed an increase in growth rate, carbohydrate content, and lipid content. However, the greatest biomass concentration was achieved at the lowest salinity level (Rao *et al*., 2007).

2.5.6 Nutrients

Algae produce energy in the form of biomass from carbon and water. They still require both simple inorganic (phosphorus, nitrogen, silicon, iron etc), four trace metals or micronutrients (zinc, copper, iron, and manganese) and organic (vitamins) nutrients for growth. Algal growth is limited by inorganic nutrient; algae have low requirement levels for vitamins.

2.5.6.1 *Nitrogen*

Nitrogen is an essential constituent of all structural and functional proteins in the algal cells and accounts for 7%–20% of cell dry weight (Hu, 2004). Inorganic nitrogen taken up by algae is rapidly assimilated and undergoes biochemical transformation into active compounds and is recycled within cells to meet changing physiological needs (Vergara *et al*., 1995; Fujita *et al*., 1988). Major effects of nitrogen deficiency in algal culture include the enhanced biosynthesis and accumulation of lipids (Shifrin *et al*., 1981; Thompson,

1996; Wang *et al*., 2009; Converti, *et al*., 2009; Demirbas, 2010; Takagi *et al*., 2000; Stephenson *et al*., 2010) with a corresponding reduction in protein (Fogg, 1956; Holm-Hansen *et al*., 1959; Morris *et al*., 1974; Kilham *et al*., 1996; Lynn *et al*., 2000; Heraud *et al*., 2005). This in turn, results in a higher lipid/protein ratio (Converti *et al*., 2009) unfavourable to growth rate (Li *et al*., 2008). Thus, attempts to increase lipid concentration via nitrogen limitation must be carefully evaluated to ensure high lipid productivity (Metting, 1996). Algae grown in nitrogen-depleted cultures also tend to divert their photosynthetically fixed carbon to carbohydrate synthesis (Hu, 2004) however, the physical significance of this is not clear. Other impacts of reduced nitrogen in culture will lead to decrease oxygen evolution, carbon dioxide fixation, chlorophyll content, and tissue production (Richardson *et al*., 1969; Kolber *et al*., 1988). Holm-Hansen *et al*. (1959) reported an increase in amino acid content of *Chlorella pyrenoidosa* at the expense of sugar phosphates (such as glucose-6-phosphate, fructose-6-phosphate) with addition of ammonium (nitrogen source) to the growing culture. Degradation of phycolbillisomes with nitrogen limitation has been demonstrated in the case of cyanobacteria and red alga (Collier and Grossman, 1992). Phycolbillisomes are the light harvesting antennae of photosystem II in these algae. Photosynthesis continues at a reduced rate, until cell nitrogen falls below a particular species-dependent threshold value. Under nitrogen deficient conditions, *Spiriluna platensis* cells exhibit reduced carbon fixation capacity even under normal to high available CO concentrations (Gordillo *et al*., 1998). Nitrogen starvation also destabilises the enzyme balance of cells, resulting in the synthesis of lipids and a decrease in chlorophyll synthesis, leading to excess carotenoids in the cells (Round, 1984). *Dunaliella* sp. and *Haematococcus pluvialis* were observed to accumulate high amounts of carotenoids, astaxanthin and its acylesters (up to 13% *w/w*), when grown under nitrogen-depleting conditions (Ben-

Amotz and Avron, 1990; Borowitzka *et al*, 1991, Harker *et al*., 1996). Zhekisheva *et al.* (2002) reported that under nitrogen depleting conditions, *Haematococcus pluvialis* produced fatty acids and astaxanthin in a 5:1 ratio. It was suggested that the production of the oleic acid-rich triacylglycerols and the esterification of the astaxanthin, maintain a high content of astaxanthin esters by enabling the oil globules.

2.5.6.2 *Phosphorus*

Phosphorus is an important component required for normal growth and development of algal cells (Hu, 2004). It has been shown that phosphorus, rather than nitrogen, is the primary limiting nutrient for microalgae in many natural environments (Larned, 1998). Phosphorus typically constitutes 1% of dry weight of algae (Borchardt and Azad, 1968), but it may be required in significant excess since not all added phosphate is bioavailable due to formation of complexes with metal ions (Chisti, 2007). Immediate effects of phosphorus limitation include a reduction in the synthesis and regeneration of substrates in the Calvin-Benson cycle and a consequential reduction in the rate of light utilization required for carbon fixation (Barsanti and Gualtieri, 2005). Phosphorus limitation also leads to accumulation of lipids. Total lipid content in *Scenedesmus* sp. was observed to increase from 23% to 53% with a reduction in initial total phosphorus (as phosphate) concentration of 0.1 from 2.0 mg L^{-1} (Li *et al*., 2010). Phosphatidylglycerol (PG), which is one of four major glycerolipids constituting membrane lipids in chloroplasts, was observed to decrease with phosphorus limitation in *Chlamydomonas reinhartdtii* (Sato *et al*., 2000). PG is essential for cell growth, the maintenance of chlorophyll-protein complex levels, and normal structure-function of the PSII complex. Phosphate limitation also reduces the synthesis of n-3 PUFA (Reitan *et al*., 1994). Similar to the effects of nitrogen deficiency, phosphorus starvation reduces chlorophyll *a* and protein content thereby increasing the relative carbohydrate content in algal cells (Healey and Hendzel,

1979; Healey, 1982; Kilham *et al*., 1997). Phosphate deficiency has been demonstrated to result in accumulation of astaxanthin and an overall reduction in cell growth (Kobayashi *et al*., 1993). Collier and Grossman (1992) reported that decrease in cellular phycobilisome under conditions of phosphorus deficiency is due to cell division and the cessation of phycobilisomes synthesis. Theodorou *et al.* (1991) observed that phosphorus starvation in *Selenastrum minutum* reduces respiration rate.

2.5.6.3 *Trace metals*

Trace metals are metals present in algal cells in extremely small quantities (<4 ppm) but that are an essential component of phycophysiology. Iron (Fe), manganese (Mn), cobalt (Co), zinc (Zn), copper (Cu) and nickel (Ni) are the six most important trace metals required by algae for various metabolic functions (Bruland *et al*., 1991). As the aqueous concentration of trace metals is not an indicator of the bioavailability of metals, trace metal availability to algae is highly dependent on speciation i.e. free ion concentration (Parent *et al*., 1996). Deficiencies in trace metals can limit algal growth, whereas excesses or high metal concentrations (above the toxicity threshold) may inhibit growth, impair photosynthesis, deplete antioxidants, and damage the cell membrane. Iron is an important trace metal for normal growth and functioning of photosynthesis and respiration in algae. It acts as redox catalyst in photosynthesis and nitrogen assimilation and mediates electron transport reactions in photosynthetic organisms (Terry and Abadía, 1986). Iron limitation significantly depresses photosynthetic electron transfer, resulting in a reduction in NADPH formation. Reduction in iron decreases the cellular abundance of ferredoxin, which contains Fe, and forces the substitution of flavodoxin, a non-iron functional equivalent, in the cell (Sandmann and Malkin, 1983; McKay *et al*., 1999b; Straus, 2004). Since the catalytic capacity of ferredoxin is much higher than flavodoxin, this can be problematic (Raven, 1984). Iron limitation also reduces cellular chlorophyll

concentration (Greene *et al*, 1992). High concentrations of iron in cultures of *Chlorella vulgaris* were observed to increase the lipid content (Liu *et al*, 2008). Decrease in iron content reduces carotenoid composition (Kobayashi *et al*., 1993; Van Leeuwe and Stefels, 1998). While there are some non-essential metals (e.g., Cd, Pb and Cr), which can inhibit many metabolic processes even in small quantities (Kennish, 1992). There are some essential elements (e.g., Zn and Cu) which when in excess can cause toxicity (Campanella *et al*., 2001). The cell surfaces of algae contain a number of functional groups with high affinity for metal ions and that carry a net negative charge mainly due to carboxylic, sulfhydryl, and phosphatic groups (Crist *et al*., 1990). These groups are binding sites that transport metal ions across the cell membrane and into the cell. Cu, Ni and Fe are metals that are commonly observed to be toxic to the algae, if present in supra-threshold concentration. Cu is one of the most toxic of these metals. Toxic metals can inhibit carbon fixation and delay nutrient uptake (Rai, 1993).

2.6 Algal Lipids Production

The main components of algae cells are proteins, carbohydrates and lipids (Becker, 1994, Sharma, 2012). Microalgae naturally produce lipids as part of the structure of the cell (e.g. in cell membranes and as signalling molecules), and as a storage compound, similar to fat stores in animals and humans (Tsukahara and Sawayama, 2005; Sharma, 2012). Lipids produced by microalgae generally include neutral lipids, polar lipids, wax esters, sterols and hydrocarbons, as well as prenyl derivatives such as tocopherols, carotenoids, terpenes, quinines and pyrrole derivatives such as the chlorophylls. These can be grouped into two categories; storage lipids (non-polar lipids) and structural lipids (polar lipids). Storage lipids are mainly in the form of TAG made of predominately saturated Fatty acids (FA) and some unsaturated FAs which can be transesterified to produce biodiesel (Sharma, 2012). Fatty acids consist of a long unbranched carbon chain. They are

classified according to the number of carbon atoms in the chain and the number of double bonds, for example saturated (no double bonds), monounsaturated (one double bond) or polyunsaturated (more than one double bond). Microalgae commonly contain fatty acids with the number of carbon ranging from C12 to C24, often with C16 and C18 unsaturates. Certain species contain significant amounts of polyunsaturated fatty acids (Griffiths, 2011). Structural lipids typically have a high content of polyunsaturated fatty acids (PUFAs), which are also essential nutrients for aquatic animals and humans. Polar lipids (phospholipids) and sterols are important structural components of cell membranes which act as a selective permeable barrier for cells and organelles. These lipids maintain specific membrane functions, providing the matrix for a wide variety of metabolic processes and participate directly in membrane fusion events. In addition to a structural function, some polar lipids may act as key intermediates (or precursors of intermediates) in cell signaling pathways (e.g., inositol lipids, sphingolipids, oxidative products) and play a role in responding to changes in the environment.

Microalgae contain lipids and fatty acids as membrane components, storage products, metabolites and sources of energy. Of particular importance is the finding that the lipid composition of algae can be altered by the influence of heavy metals (Vavilin *et al*., 1998; Rocchetta, *et al*., 2006; Souza *et al*., 2012). At the same time, it is well known that the oxidation of lipids can occur as a result of oxidative stress, reflecting the production of reactive oxygen/nitrogen species (Pinto *et al*., 2003; Leitão *et al*., 2003; Souza *et al*., 2012). In other experiments (Okamoto *et al*., 2001; Collen *et al*., 2003; Rocchetta *et al*., 2006; Souza *et al*., 2012), it was demonstrated that the levels of polyunsaturated fatty acids (PUFA) are more affected, suffering a greater decrease in the presence of heavy metals. According to Pinto *et al*. (2011), Cd^{2+} was more toxic than Cu^{2+} and greatly reduced the PUFA concentration in *Gracilaria. tenuistipitata*. All algae contain proteins,

carbohydrates, lipids and nuclei acids in varying proportions, and the variations is dependent with the type of algae but the extract table in appendix II show the varying proportion of lipid with (Becker, 1994; Demirbas and Demirbas, 2011).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Sample Collection

Tannery effluent from Mario – Jose, Mahaza, Mahmuda Tanneries in Challawa Industrial Estate, Kano (as shown in figure 3.1) was collected from an effluent stream that flowed to Challawa River. The sampling point was established 10m away from the last discharge point to have a representative sample from the tanneries. A dilute HCl pre – rinsed 10 litres (L) plastic container was dipped into the flowing effluent stream at a depth of 10 cm to collect the tannery effluent and was preserved with ice packs. It was transported to National Research Institute for Chemical Technology, Zaria to determine physicochemical parameters.

3.2 Culturing And Growth Conditions

Chlorella vulgaris UTEX 2714 cultured in modified Bold Basal Medium. Bold's Basal Medium (BBM) with the following composition (g/L) 10ml 25g/l $NaNO_3$, 10ml 2.5g/l $CaCl_2.2H_2O$, 10ml 7.5g/l $MgSO_4.7H_2O$,10ml 7.5g/l K_2HPO_4 ,10ml 17.5g/l KH_2PO_4,10ml 2.5g/l NaCl,1ml 50g/l EDTA,1ml 31g/l KOH, 1ml 4.98g/l $FeSO_4.7_2O$, 1drop H_2SO_4, 1ml 11.42g/l H_3BO_3,1ml 8.82g/l $ZnSO_4.7H_2O$,1ml 1.44g/l $MnCl_2.4H_2O$,1ml 0.71$MoNO_3$, (Gonzalez *et al*., 1997; Andersen, 2005) and 10ml 2.5g/l Na_2CO_3 as carbon source (Chia, 2015). Effluent sample was diluted into 0%, 5%, 10%, 25%, 50%, 75%, 100% concentration sample (Ajayan, 2012) and BBM medium as control was adjusted to pH 7.0 with 0.1 M NaOH and HCl and sterilised in an autoclave at temperature of 121^oC for 15 minutes before inoculation. The culturing was done in the Department of Biological Sciences, Ahmadu Bello University, Zaria.

Sample Collection Area

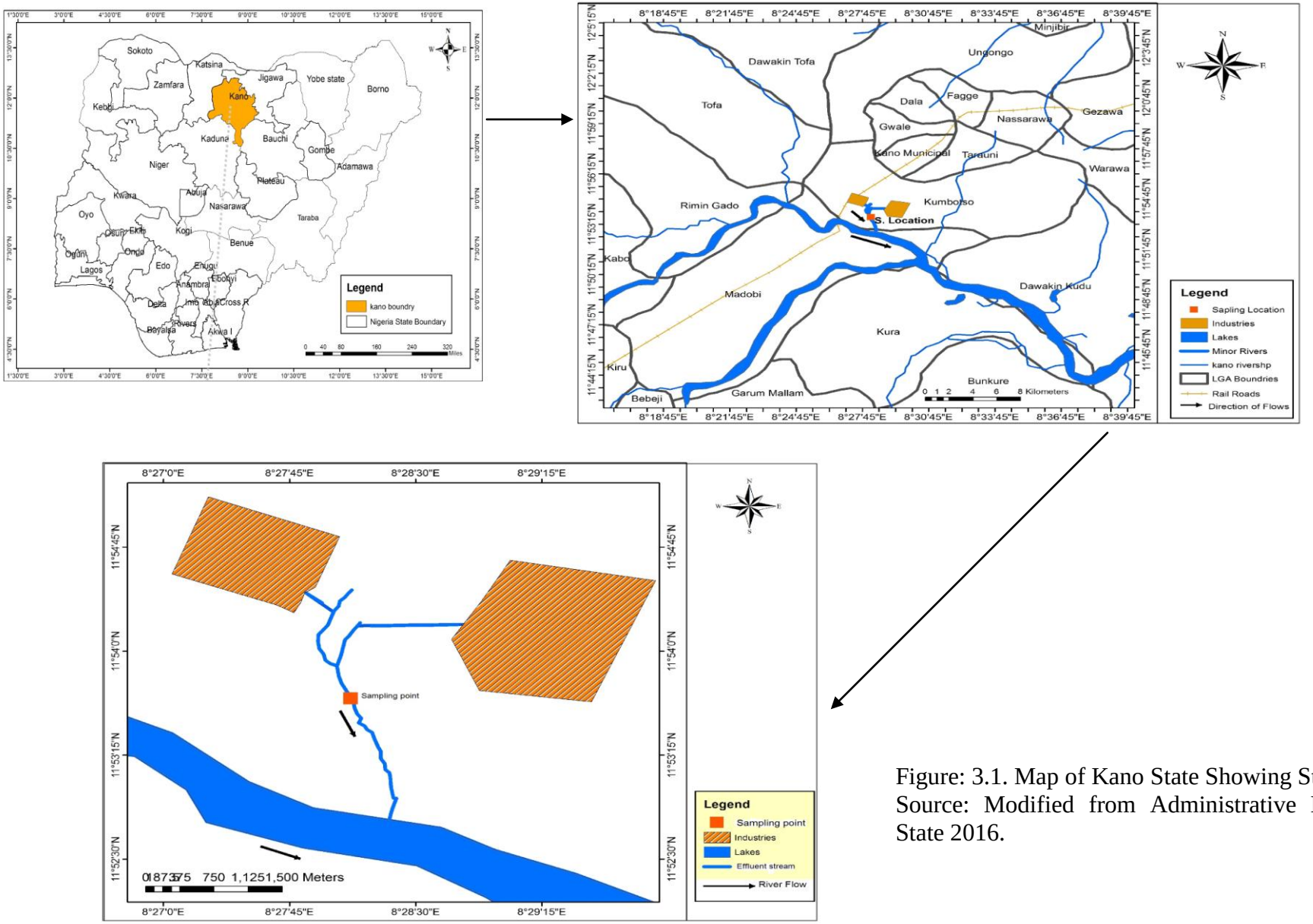


Figure: 3.1. Map of Kano State Showing Study Area
Source: Modified from Administrative Map of Kano State 2016.

The batch experiment was carried out in triplicate using 1 litre conical flasks containing 500 ml of standardized medium and a 10 % volume i.e. 50ml of algal inoculums in the exponential growth phase with cells to a concentration about 1 x 10^6 cell mL^{-1} is shown in plate 3.1. The flasks were kept under temperature mean of 25±2°C. Cells were incubated in a fume cupboard insulated with foil paper with light intensity ranging from (842 – 1565 lux) was provided using cool white daylight fluorescent lamp within a photoperiod of 12:12 h light and dark cycles as seen in plate 3.2. Samples (3 mL) from each test flask were taken and fixed with Lugol's iodine solution to determine the cell count, by counting cells in an Improved Neubauer Bright-Line hemocytometer under Olympus microscope every 48h (Cheesbrough, 2005). Optical Density (OD) was determined at 680 and Dry weight was determined by taking absorbance 750nm and was calculated as follows:

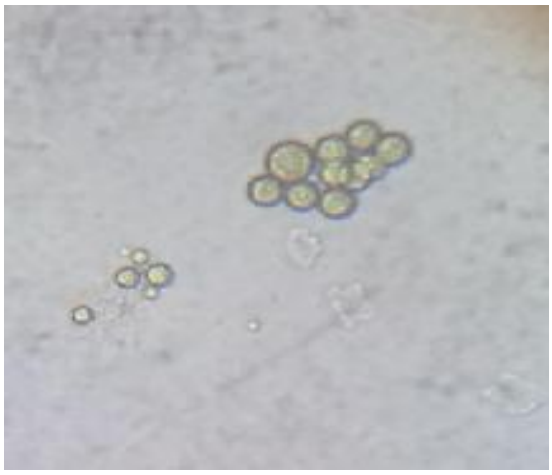
DW = 3.31 + 179.49 x Abs_{750} nm + 617.45Abs^2 (mg/L) (Horvatic *et al*, 2003) using Spectrumlab uv - vis spectrophotometer model 752s.

Cell count per ml and Optical density measurements were plotted against time and used to estimate the growth. Samples are separated from the medium by centrifugation at 3000 rpm for 5 min, using a Gallenkamp laboratory centrifuge.

3.3 Determination of Physicochemical Parameter

3.3.1 pH

The pH was determined using a pH meter (Hanna combo pH/EC meter HI 98129). The meter was calibrated using two buffers (pH 7 and 10) according to the manufacturer's directions. The pH was read directly from the meter according to the manufacturer's instruction (APHA, 2005).



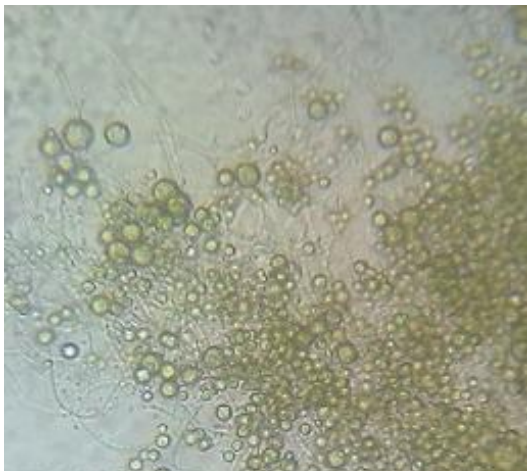


Plate 3.1: *Chlorella vulgaris* cells at x400



Plate 3.2: *Chlorella vulgaris* cutured in 1- litre conical flask

3.4.2 Electrical conductivity (EC)

The electrical conductivity was determined using (Hanna combo pH/EC meter HI 98129 by placing a conductivity probe into the effluent sample in a 250 ml conical flask. The electrical conductance obtained in micro second per centimetre (μs/cm) was recorded directly from the meter after 3 minutes (APHA, 2005).

3.4.3 Total dissolved solid (TDS)

The total dissolved solid was determined using (Hanna combo pH/EC meter HI 98129 by placing a probe into the effluent sample in a 250 ml conical flask. Total dissolved solids was measured in milligram per litre (mg/l) was recorded directly from the meter after 3 minutes (APHA, 2005).

3.4.4 Dissolved oxygen and biochemical oxygen demand

The Modified Winkler- Azide Method was used to analyse tannery effluent samples for dissolved oxygen (DO). Each tannery effluent dilution was collected into two 300ml bottles labeled DO_1 and DO_5, two millilitre (2ml) $MnSO_4$ and two millilitre (2ml) alkali-iodide-azide reagent was added well below the surface of the liquid and stoppered with care to avoid the formation of bubbles. The solution was mixed by inverting the bottle a number of times until a clear supernatant water is obtained. The solution was allowed to settle for about 2 min, 2ml of concentrated H_2SO_4 was added by allowing the acid to run down the neck of the bottle. The bottle was stoppered again. Two hundred millilitre (200ml) was collected in a conical flask, 2ml of starch solution was added and was titrated using 0.0125M Sodium thiosulphate ($Na_2S_2O_3.5H_2O$) solution dropwise until the blue colour disappears to a colourless solution. The other sample labeled DO_5 was kept in an incubator at 20^0C for five days before determination of the dissolved oxygen content.

Therefore dissolved oxygen is calculated: $DO = \frac{16000 \text{ x } M \text{ x } V}{V_1 - V_2}$

M = Molar of sodium thiosulphate, V = volume of sodium thiosulphate, V_1= volume of container, V_2= volume of sample

Biochemical oxygen demand (BOD_5) was determined by the difference of dissolved oxygen DO_1 and DO_5 of samples after incubation at 20ºC for five days. The DO was determined using the Alkaline – Azide modification of Winkler's method as described by Ademoroti (1996). When the iodine is uniformly distributed, 203ml of the sample was taken.. The BOD (mg/l) was obtained by finding the difference between the DO of the first day and fifth day. BOD is calculated: BOD = $\frac{(DO1-DO5)}{\text{Percent dilution}}$

DO_1 = dissolved oxygen in day 1, DO_5 = dissolved oxygen in day 5

3.4.5 Chemical oxygen demand

This was determined using the titrimetric method described by Ademoroti (1996). Twenty millilitres (20ml) of the sample was measured into the refluxing flask and 0.4g mercuric sulphate solution was added and swirled. Ten millilitres (10ml) of 0.021M potassium dichromate was then added to the mixture. Using a dispensing pipette, 30ml of concentrated sulphuric acid containing silver sulphate was also added. The contents in the flask was fitted to a condenser and refluxed for 2 hour. The flask was removed and allowed to cool for approximately 10 minutes. The condenser was washed with distilled water and then the content of the flask was diluted to 100ml. Two drops of Ferroin indicator was added to the content in the flask and the residual dichromate was titrated with standardized ferrous ammonium sulphate. The COD was obtained using the formula:

$$\text{COD mg/l} = \frac{(V_b - V_a) \times 8000 \times M}{\text{volume of sample}}$$

Where,

V_b = average number of ml ferrous ammonium sulphate used in titrating the appropriate blank.

V_a = number of ml ferrous ammonium sulphate used in titrating the sample.

M = Molarity of standard ferrous ammonium sulphate solution.

3.4.6 Total alkalinity

About 50 ml of sample was measured into a clean conical flask. 2 drops of methyl orange was added to the sample. The sample was titrated with standard 0.02M H_2SO_4 until the colour changes from yellow to orange. The volume of acid (V_T) used for titration and the molarity of the acid (M) is used to obtain the total alkalinity using the formula.

$$\text{Total Alkalinity as mg/l } CaCO_3 = \frac{V_T \times M \times 100{,}000}{\text{volume of sample}}$$

3.4.7 Phosphate - phosphorus

A clean sample cell was filled with 25ml of the deionised water and another sample cell was filled 25ml of sample. 1 ml of molybdovanadate reagent was added to each of the sample cell and swirl to mix. The sample cells were allowed to stand for 3minutes. The cell with distilled water was used to blank the spectrophotometer and the prepared sample cell was placed in the cell holder and analysed. Test result in mg/l PO_4^{3-} was displayed, at a wavelength of 880nm using HACH DR2400 portable spectrophotometer (APHA, 2005).

3.4.8 Sulphate

A clean sample cell was filled with 10ml of the sample, the content of sulfaver 4 pillow was added to the sample and stirred. The sample was allowed to stand for five minutes and a second cell was filled with 10ml of sample that was used to blank the spectrophotometer. Within five miuntes the prepared sample cell was placed in the cell

holder and analysed. Test result in mg/l SO_4^{2-} was displayed, at a wavelength of 450nm using HACH DR2400 portable spectrophotometer (APHA, 2005).

3.4.9 Free chlorine

A clean sample cell was filled with 10ml of the sample and was placed in the cell holder to blank the spectrophotometer. A second cell was filled with 10ml of sample, N, N-diphenyl – p phenylenediamine (DPD) pillow powder was added to the second cell and swirled for 20 seconds. One minute of adding the DPD powder, the sample cell is placed in the cell holder and analysed. Test result in mg/l Cl_2 was displayed, at a wavelength of 530nm using HACH DR2400 portable spectrophotometer Free Chlorine Reagent was used to analyse tannery sample (APHA, 2005).

3.4.10 Nitrate - nitrogen

A clean sample cell was filled with 10ml of the sample, the content of nitraver 5 pillow was added to the sample and it is shaken for one minute and allowed to stand for 5 minutes. a second cell was filled with 10ml of sample that was used to blank the spectrophotometer. The prepared sample was placed in the cell holder and analysed. Test result in mg/l NO_3^- was displayed, at a wavelength of 430nm using HACH DR2400 portable spectrophotometer (APHA, 2005).

3.4.11 Elemental analysis

3.4.11.1 *Sample preparation*

The samples was digested according to Standard methods for the examination of water and wastewater, American Public Health Association (APHA, 2005).

Each sample was thoroughly mixed and of the sample 20ml was transferred into a conical flask, 10ml of concentrated nitric acid was added and brought to slow boiling before evaporating on a hot plate to lowest volume (5 – 10ml). Concentrated nitric acid was

added as necessary until digestion was complete as shown by light colour clear solution. The digest was filtered into 50ml volumetric flask and made up to the mark.

3.4.11.2 *Sample analysis*

Chromium, Lead and Cadmium concentration in the digests was determined by Atomic Absorption Spectrophotometry, using Shimadzu Atomic Absorption Spectrophotometer (model AA-6800, Japan) equipped with Zeaman background correction and graphite furnace at National Research Institute for Chemical Technology (NARICT), Zaria-Nigeria. The calibration curve was prepared by running different concentrations of standard solutions. The instrument was set to zero by running the respective reagent blanks. Average values of three replicates were taken for each determination and was subjected to statistical analysis

Reduction efficiency was calculated as

$$\text{R.E} = \frac{C_i - C_e}{C_i} \text{ x } 100$$

3.5 Lipid Content and Fatty Acid Analysis

Total lipids was extracted from a known biomass with a solvent mixture of chloroform, methanol and water (2:1:0.75, by vol.) according to the modified Folch procedure (Christie, 2003).

In the solvent mixture given above containing the algae, homogenize with both sonication and grinding with a steel or teflon rod. After centrifuging at 10000 × g for 2 min, the lower $CHCl_3$ layer was collected. At least three washes of the aqueous phase with 3 ml of $CHCl_3$ were carried out. The lower layer chloroform layer contains your lipid fraction.

Total lipids was determined by drying the sample in the oven at 50 - 60^{o}C and then reweighing the glass vial. The difference between the pre-weighed glass vial (empty)

without the lipid extract and that with lipid extract after drying at 50 - 60^{o}C gives the total lipid concentration. The lipid content was measured gravimetrically and was calculated using the following equation:

Y (%) =W_L /W_{DA}, Where W_L and W_{DA} are the weights of the lipid to be extracted and dry algae biomass, respectively.

The total lipid was used to assay the fatty acid profiles. Fatty acid methyl esters (FAMEs) was prepared by transesterification using 0.4 M KOH – methanol. The FAMEs was analytically verified by gas chromatography using the method of Huang et al. (2012). Briefly, FAMEs was detected using flame ionisation detection after injecting the sample into the Shimadzu 2010 gas chromatograph fitted with the column. The injector mode was split and injection temperature was 250 °C. The column temperature was increased from its initial value of 80 to 200 °C at a rate of 10 °C min, it was held constant for 4 min followed by an increase to 280 °C at a rate of 10 °C min. It was maintained constant for 5 min until all FAMEs was eluted. The eluant gas was helium, and a total flow rate was 6.2 ml min^{-1} at a pressure of 108.0 kPa. Area percentage of peaks was used to quantify fatty acid content. All replicates were pooled together before fatty acid profile was determined.

3.6 Data Analysis

One way analysis of variance (ANOVA) with Tukey's HSD post hoc test was used to test for significant differences among the physicochemical parameter, biomass production, heavy metals obtained through the different dilutions of the tannery effluent. Two way analysis of variance with Tukey's HSD post hoc test was used to test for significant differences of physicochemical parameter among treatment. Relationship between the measured parameters was established using Principal Component Analysis (PCA) and Canonical Component Analysis (CCA). Principal component analysis and Canonical

Component Analysis was done using the PAST version 2.40 for windows statistical software, while ANOVA was done using SPSS version 20 for windows.

CHAPTER FOUR

4.0 RESULTS

4.1 Physicochemical Parameters

4.1.1 pH

The analysis of variance in table 4.1for pH shows that in 25% dilution of day 4 had the lowest pH while the highest was seen in Bold basal medium (BBM) on Day 8. The pH in BBM showed significant increase at $P < 0.05$ from day 0 to day 4, and this significant increase ($P < 0.05$) continued on day 8 then a decrease in pH on day 12 but the changes observed on day 12 was not statistically significant ($P > 0.05$) when compared to day 8. The pH in 0% dilution decrease from day 0 to day 4 then an increase from day 8 to day 12 but no statistical significant ($P > 0.05$) during the days. In 5% dilution, pH reduced significantly from day 0 to day 4 while on day 8 there was a significant increase ($P < 0.05$) and it was maintained on Day12. 10% dilution showed a significant decrease ($P < 0.05$) from day 0 to day 4, day 8 had an increase in pH which was significant this trend continue to day 12. The pH in 25% dilution showed statistical decrease ($P < 0.05$) from day 0 to day 4 and then statistical increase ($P < 0.05$) of day 8 to day 12. There was significant decrease ($P < 0.05$) in 50% dilution from day 0 to day 4 then an increase in pH that was not significant ($P > 0.05$) on day 8 and day 12. The 75% dilution showed significant decrease ($P < 0.05$) from day 0 to day 4, a significant increase($P < 0.05$) on day 8 followed by an increase on day 12 that was not significant ($P > 0.05$) as compared to day 8. A similar result in the 75% dilution was seen in 100% tannery effluent.

4.1.2 Electrical conductivity

The analysis of variance in Table 4.2 for Electrical Conductivity (EC) indicated the lowest EC in 0% dilution on day 0 and the highest EC in 100% effluent on Day 0. This study revealed significant decrease at $P < 0.05$ in BBM from day 0 to day 4 and a further decrease on day 8 and day 12 but these changes were not significant ($P > 0.05$) when

compared to day 4. EC in 0% dilution significantly increased ($P < 0.05$) from day 0 to day 4, the reduction in EC continued on day 8 and day 12 but the reduction on day 8 and day 12 was not statistically different ($P > 0.05$) from day 0. In 5% dilution the EC dropped significantly ($P < 0.05$) from day 0 to day 4, a decrease on day 8 and on day 12 but the variation on day 8 and day 12 was not statistically different ($P > 0.05$) from day 4. The 10%, 25% and 50% dilutions followed the same trend as that of the 5% dilution. The 75% showed a significant decrease ($P < 0.05$) from day zero to day 4, a decrease in EC on day 8 but was not significant ($P > 0.05$) to that day 4 and a significant decrease ($P < 0.05$) on day 12. Observations in 100% effluent showed a significant decrease ($P < 0.05$) of EC from day 0 to day 4, a further decrease on day 8 and day 12 but the changes on day 8 and day 12 do not significantly differ ($P > 0.05$) from day 4.

4.1.3 Total dissolved solid

The analysis of variance for Total Dissolved Solids (TDS) in Table 4.3 was observed to be lowest in 0% dilution on day 0 and the highest in 100% effluent on day 0. BBM showed significant decrease at $P < 0.05$ from day 0 to day 4, a decrease was observed on day 8 and day 12 in TDS but variations on day 8 and day 12 do not significantly differ ($P > 0.05$) from day 4. The 0% dilution had significant increase ($P < 0.05$) on day 4 from day 0, a further reduction in TDS on day 8 and day 12 but the changes observed was not statistically different ($P > 0.05$) from day 4. The 5%, 10%, 25% and 50% dilutions exhibit similar trend with a significant decrease ($P < 0.05$) on day 4 from day 0, a further decrease on day 8 and day 12 but the changes on day 8 and day 12 was not statistically different ($P > 0.05$) from day 4. The 75% dilution showed statistical decrease ($P < 0.05$) from day 0 to day 4, a significant decrease ($P < 0.05$) on day 8 and a significant decrease ($P < 0.05$) on day 12. In the 100% effluent, there was significant decrease ($P < 0.05$) from day 0 to day 4, a decrease was observed on day 8 and day 12 but the changes on day 8 and day 12 was not significantly different ($P > 0.05$) from day 4.

Table 4.1: Effect of *Chlorella vulgaris* on pH at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	7.01±0.01^{c}	8.58±0.46^{b}	10.35±0.31^{a}	10.19±0.23^{a}
0%	7.23±0.04^{a}	7.17±0.22^{a}	7.94±0.37^{a}	8.26±0.11^{a}
5%	8.32±0.05^{a}	6.93±0.04^{b}	7.57±0.37^{ab}	7.57±0.40^{ab}
10%	8.65±0.08^{a}	6.82±0.05^{c}	7.62±0.08^{bc}	8.17±0.41^{ab}
25%	8.70±0.03^{ab}	7.32±0.12^{b}	8.35±0.52^{ab}	9.35±0.34^{a}
50%	8.83±0.02^{a}	7.36±0.15^{b}	8.59±0.32^{a}	8.89±0.30^{a}
75%	8.91±0.01^{a}	7.51±0.07^{c}	8.23±0.11^{b}	8.41±0.09^{b}
100%	8.93±0.01^{a}	7.53±0.24^{c}	8.17±0.08^{b}	8.23±0.08^{b}

Table 4.2: Effect of *Chlorella vulgaris* on Electrical conductivity (µs/cm) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	1752.67±0.88^{a}	821.00±40.84^{b}	756.33±19.27^{b}	714.00±18.19^{b}
0%	0.00±0.00^{b}	213.00±22.07^{a}	195.67±10.81^{b}	183.67±21.73^{b}
5%	467.67±2.33^{a}	328.33±8.41^{b}	321.67±3.76^{b}	281.00±34.21^{b}
10%	886.00±6.93^{a}	444.33±4.26^{b}	434.67±1.45^{b}	413.33±19.19^{b}
25%	1876.00±12.01^{a}	845.67±93.88^{b}	800.33±57.95^{b}	745.67±98.55^{b}
50%	3234.00±2.00^{a}	1388.33±33.84^{b}	1306.67±10.37^{b}	1250.00±16.01^{b}
75%	11076.67±43.72^{a}	1923.67±10.37^{b}	1889.33±20.22^{b}	1690.00±70.92^{c}
100%	14426.67±52.39^{a}	2304.00±16.01^{b}	2276.67±63.56^{b}	2181±71.70^{b}

Table 4.3: Effect of *Chlorella vulgaris* on TDS (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	875.00±0.58^{a}	410.33±20.18^{b}	376.33±10.20^{b}	357.33±9.24^{b}
0%	0.00±0.00^{b}	106.33±10.87^{a}	97.67±5.24^{a}	92.00±10.79^{a}
5%	233.67±1.20^{a}	164.00±4.16^{b}	160.67±2.03^{b}	138.33±11.17^{b}
10%	443.33±3.48^{a}	222.33±1.76^{b}	217.00±0.58^{b}	206.00±8.19^{b}
25%	937.67±5.84^{a}	426.67±45.43^{b}	400.00±29.05^{b}	373.00±49.24^{b}
50%	1599.00±17.52^{a}	692.67±17.90^{b}	675.00±14.42^{b}	653.67±11.10^{b}
75%	5073.33±29.63^{a}	965.33±5.24^{b}	940.67±8.99^{bc}	845.00±35.25^{c}
100%	7233.33±73.11^{a}	1153.33±8.41^{b}	1136.67±30.75^{b}	1089.67±36.15^{b}

Data is represented in mean ± S.E
Analysis was done in triplicate
Means with the same alphabet superscript across row are not significant at ($P > 0.05$)

4.1.4 Total alkalinity

Total alkalinity in table 4.4, was observed to be lowest on day 0 of the 0% dilution and was the highest on day 0 of 100% effluent. Alkalinity in BBM revealed significant increase at $P<0.05$ from day 0 to day 4, an increase on day 8 and day 12. The changes that occurred do not differ significantly ($P>0.05$) from day 4. In 0% dilution, the alkalinity increased steady from day 0 to day 12. The variations in the 0% dilution were not significant statistically ($P>0.05$). In 5% dilution, there was significant decrease ($P < 0.05$) from day 0 to day 4, an increase on day 8 but not significant ($P>0.05$) as compared to day 4 and then a significant increase ($P<0.05$) on day12. Alkalinity in 10% and 25% dilution both decreased from day 0 to day 4, increased on day 8 and day 12. The changes observed were not statistical significant ($P > 0.05$). In 50% and 75% dilution, there was significant decrease ($P < 0.05$) from day 0 to day 4, a significant increase ($P < 0.05$) on day 8 and day 12 but the increase in alkalinity between day 8 and day 12 was not significant ($P >0.05$) during the period of study. In the 100% effluent there was significant decrease ($P < 0.05$) in alkalinity from day 0 to day 4, then an increase in day 8 and day 12 but the increase on day 8 and day 12 did not differ significantly ($P < 0.05$) when compared to day 4.

4.1.5 Phosphate - phosphorus

The phosphate - phosphorus in Table 4.5 was observed to be lowest in 0% dilution on day 0 and highest in 100% effluent on day 0. BBM showed a significant decrease ($P<0.05$) from day 0 to day 4, the decrease continued on day 8 and day 12 but it was not statistically significant ($P>0.05$) as compared to day 4. The 0%, 5% and 10% dilutions showed an increase from day 0 to day 4, a decrease on day 8 and day 12 but these changes in phosphate content throughout the study were not statistically significant ($P>0.05$). Phosphate content in 25% dilution decrease significantly ($P<0.05$) from day 0

to day 4, decreased to day 8 but was not significant (P>0.05) as compared to day 4, day 12 decreased significantly (P<0.05) from day 8. Similarly in the 50% dilution, a significant decrease (P<0.05) in phosphate was observed from day 0 to day 4 and these decrease continued on day 8 and day 12 but do not differ statistically at P > 0.05 when compared to day 4. In the 75% dilution, phosphate content decrease significantly (P<0.05) from day 0 to day 4, decreased significantly (P<0.05) on day 8 and a significant decreased (P<0.05) on day 12. The 100% effluent showed significant decrease (P<0.05) from day 0 to day 4, a decrease on day 8 but do not differ statistically at P>0.05 when compared to day 4 and a significant decrease at P<0.05 on day 12.

4.1.6 Sulphate

The sulphate concentration in table 4.6 was observed to be highest in 100% effluent on day 0 and was lowest in 0% dilution on day 0. In BBM, there was an increase in sulphate content from day 0 to day 4 then a decrease on day 8 and day 12 and does not differ significantly (P>0.05) during the period. The 0% dilution observed a significant increase at P < 0.05 in sulphate content from day 0 to day 4, a significant decrease (P < 0.05) on day 8 and a decrease on day 12 but reduction on day 12 does not differ significantly compared to day 8 (P>0.05). The 5% dilution showed a significant increase (P < 0.05) from day 0 to day 4, and then a significant decrease(P < 0.05) on day 8 and a significant decrease (P < 0.05) on day 12. Changes occurred in the 10% dilution with a significant decrease from day 0 to day 12 which had no significant difference (P > 0.05) during the study period. The 25% dilution showed significant decrease from day 0 to day 4, also a significant decrease on day 8 while day 12 there was decrease in sulphate content but it was not significant as compared to day 8.

Table 4.4: Effect of *Chlorella vulgaris* on Alkalinity (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	82.00±4.16^{b}	233.33±47.02^{a}	288.00±25.17^{a}	300.00±30.55^{a}
0%	9.33±2.40^{a}	76.67±8.82^{a}	193.33±98.38^{a}	286.67±78.39^{a}
5%	48.00±1.15^{b}	86.67±6.67^{ab}	89.67±15.60^{ab}	170.00±45.09^{a}
10%	105.33±12.24^{a}	100.00±0.00^{a}	123.33±29.63^{a}	123.33±3.33^{a}
25%	187.33±2.91^{a}	166.67±3.33^{a}	180.00±20.82^{a}	200.00±5.77^{a}
50%	376.67±22.40^{a}	220.00±50.33^{b}	280.00±0.00^{ab}	286.67±8.82^{ab}
75%	520.67±29.24^{a}	376.67±43.72^{b}	423.33±21.86^{ab}	450.00±5.77^{ab}
100%	864.00±21.20^{a}	353.33±80.07^{b}	493.33±3.33^{b}	500.00±5.77^{b}

Table 4.5: Effect of *Chlorella vulgaris* on phosphate - phosphorus (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	109.67±6.84^{a}	56.67±11.84^{b}	42.67±7.80^{b}	40.33±11.05^{b}
0%	0.13±0.13^{a}	23.33±7.26^{a}	20.00±8.19^{a}	12.00±0.58^{a}
5%	10.00±0.68^{a}	26.67±8.01^{a}	18.33±1.00^{a}	15.00±4.04^{a}
10%	22.00±10.07^{a}	30.00±3.21^{a}	18.00±1.00^{a}	10.00±2.08^{a}
25%	41.67±9.53^{a}	22.67±4.26^{ab}	18.33±5.24^{ab}	12.00±1.53^{b}
50%	71.00±7.23^{a}	41.67±5.33^{b}	26.00±4.73^{b}	15.33±7.33^{b}
75%	114.00±8.72^{a}	91.67±1.76^{ab}	84.00±3.51^{bc}	61.67±8.69^{c}
100%	164.00±23.86^{a}	125.33±10.11^{ab}	104.67±8.69^{ab}	89.67±2.60^{b}

Table 4.6: Effect of *Chlorella vulgaris* on Sulphate (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	100.00±10.00^{a}	170.00±20.82^{a}	150.00±11.55^{a}	103.33±31.80^{a}
0%	0.33±0.33^{b}	190.00±70.00^{a}	120.00±0.00^{ab}	70.00±11.55^{ab}
5%	60.00±5.77^{b}	126.67±23.33^{a}	63.33±8.82^{ab}	60.00±11.55^{b}
10%	106.67±17.64^{a}	123.33±8.82^{a}	103.33±33.33^{a}	96.67±17.84^{a}
25%	250.00±11.55^{a}	190.00±10.00^{ab}	163.33±23.33^{b}	123.33±14.53^{b}
50%	410.00±15.28^{a}	256.67±6.67^{b}	250.00±17.32^{b}	246.67±20.28^{b}
75%	656.67±40.96^{a}	310.00±20.82^{b}	303.33±14.53^{b}	300.33±0.33^{b}
100%	886.67±46.67^{a}	393.33±18.56^{b}	386.67±13.33^{b}	373.33±17.64^{b}

Data is represented in mean ± S.E
Analysis was done in triplicate
Means with the same alphabet superscript across row are not significant at ($P > 0.05$)

Sulphate content in the 50% dilution, 75% dilution and 100% effluent showed a significant decrease from day 0 to day 4 and a further decrease on day 8 and day 12 but the variations observed on day 8 and day 12 was not significantly different from day 4.

4.1.7 Free chlorine

The free chlorine in Table 4.7 had the lowest concentration in BBM on day 0 and highest in 100% effluent on day 0. BBM showed an increase in free chlorine from day 0 to day 4 and a decrease on day 8 and day 12 but there no significant differences ($P>0.05$) during the period of remediation. There was significant increase ($P<0.05$) in the 0% dilution from day 0 to day 4, a decrease on day 8 and a significant decrease the free chlorine content on day 12. The 5% dilution showed significant increase ($P<0.05$) from day 0 to day 4, with a significant decrease ($P<0.05$) on day 8 and a further decrease that was not significant statistically ($P>0.05$) on day 12 when compared to day 8. In the 10% dilution, there was an increase in free chlorine content from day 0 to day 4 and a slight decrease on day 8 and day 12 with no significant difference ($P>0.05$) during the period. There was significant increase ($P<0.05$) in the 25% dilution from day 0 to day 4, a significant reduction ($P<0.05$) on day 8 and day 12. The free chlorine in the 50% dilution increased from day 0 to day 4, with a reduction on day 8 and a decrease on day 12 but no significant difference($P<0.05$) during the period. The changes observed in the 75% dilution and 100% effluent showed a significant decrease ($P<0.05$) from day 0 to day 4, a decrease on day 8 and day 12 with no significant difference ($P>0.05$) when compared to day 4.

4.1.8 Nitrate - nitrogen

In table 4.8 represents the analysis of variance for nitrate - nitrogen which was lowest in 0% dilution on day 0 and highest in BBM on day 0. In BBM, there was significant decrease ($P<0.05$) from day 0 to day 4 followed by a decrease on day 8 and day 12 but

variations observed on day 8 and day 12 was not statistically significant (P>0.05) when compared to day 4. The nitrate- nitrogen content in the 0% dilution increased significantly (P<0.05) from day 0 to day 4, a decrease on day 8 but was not significant (P>0.05) compared to day 4 and a significant decrease (P<0.05) on day 12. Nitrate content in 5% and 10% dilutions increased significantly (P<0.05) from day 0 to day 4 then a significant decrease (P<0.05) on day 8 and a significant decrease (P<0.05) on day 12. The 25% dilution increased significantly (P<0.05) in nitrate from day 0 to day 4 and decreased significantly (P<0.05) on day 8 and remained unchanged on day 12. In the 50% dilution, there was a significant increase (P<0.05) observed from day 0 to day 4, a significant decrease (P<0.05) on day 8 and day 12. Nitrate content observed in the 75% dilution increased significantly (P<0.05) from day 0 to day 4 and a significant decrease (P<0.05) on day 8 and day 12. The 100% effluent significantly increased (P<0.05) in nitrate content from day 0 to day 4, and significantly decreased (P<0.05) on day 8 and day 12.

4.1.9 Dissolved oxygen

The Dissolved Oxygen (DO) in table 4.9 had the lowest concentration in BBM on day 8 and highest in 0% day 0. In BBM, there was significant decrease (P<0.05) from day 0 to day 4 and a significant decrease (P<0.05) continues to day 8, followed with a significant increased (P<0.05) on day 12. In 0% dilution, there was significant decrease (P < 0.05) from day 0 throughout to day 8 with day 4 and day 8 not being statistical different (P>0.05) between them and a significant increase (P<0.05) on day 12. There was a decrease in DO from day 0 to day 4 and later an increase from day 8 to day 12 but there was no significant difference (P>0.05) throughout the days for the period of remediation in 5% dilution.

Table 4.7: Effect of *Chlorella vulgaris* on Free chlorine (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	0.03±0.00^{a}	1.40±0.45^{a}	1.30±0.32^{a}	1.13±0.47^{a}
0%	0.00±0.00^{b}	0.83±0.14^{a}	0.70±0.17^{a}	0.53±0.20^{ab}
5%	0.02±0.01^{b}	1.30±0.46^{a}	1.00±0.15^{ab}	0.70±0.20^{ab}
10%	0.45±0.11^{a}	1.43±0.54^{a}	1.03±0.52^{a}	0.70±0.23^{a}
25%	0.11±0.06^{c}	1.53±0.09^{a}	0.87±0.22^{b}	0.50±0.12^{bc}
50%	1.10±0.30^{a}	1.23±0.47^{a}	0.83±0.44^{a}	0.80±0.23^{a}
75%	6.37±0.58^{a}	1.53±0.84^{b}	1.00±0.12^{b}	0.80±0.17^{b}
100%	7.03±0.33^{a}	1.17±0.22^{b}	0.80±0.46^{b}	0.70±0.10^{b}

Table 4.8: Effect of *Chlorella vulgaris* on nitrate - nitrogen (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	292.67±38.74^{a}	52.67±0.88^{b}	44.33±7.75^{b}	39.00±7.02^{b}
0%	0.10±0.10^{b}	45.67±7.54^{a}	33.67±6.64^{a}	22.33±5.46^{ab}
5%	9.67±1.67^{c}	51.00±2.52^{a}	26.33±4.48^{b}	21.00±3.51^{bc}
10%	12.33±1.76^{c}	58.67±2.60^{a}	25.00±3.06^{b}	16.00±2.89^{bc}
25%	17.67±2.91^{b}	63.67±2.40^{a}	21.67±5.24^{b}	21.67±2.91^{b}
50%	26.67±2.19^{bc}	67.33±0.33^{a}	34.33±2.96^{b}	23.67±2.91^{a}
75%	47.67±2.60^{b}	74.00±0.58^{a}	39.67±14.70^{bc}	27.00±15.51^{c}
100%	55.67±5.21^{ab}	82.00±2.52^{a}	59.33±17.23^{ab}	26.67±3.38^{b}

Table 4.9: Effect of *Chlorella vulgaris* on Dissolved Oxygen (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	4.14±0.48^{ab}	1.57±0.64^{bc}	1.24±0.30^{c}	4.57±0.34^{a}
0%	7.65±0.05^{a}	2.02±0.41^{c}	1.31±0.03^{c}	4.60±0.10^{b}
5%	4.50±0.10^{a}	1.41±0.34^{a}	1.51±0.30^{a}	4.20±0.11^{a}
10%	3.45±0.45^{ab}	2.25±0.71^{ab}	1.44±0.10^{b}	4.47±20.25^{a}
25%	3.78±0.03^{a}	1.51±0.03^{b}	1.41±0.13^{b}	4.33±20.25^{a}
50%	2.38±0.31^{b}	1.35±0.07^{b}	1.58±0.30^{b}	4.20±0.17^{a}
75%	2.05±0.04^{b}	1.61±0.07^{b}	1.61±0.07^{b}	4.50±0.14^{a}
100%	3.86±0.10^{a}	1.51±0.10^{b}	1.51±0.10^{b}	4.43±0.13^{a}

Data is represented in mean±S.E
Analysis was done in triplicate
Means with the same alphabet superscript across row are not significant at ($P > 0.05$)

In the 10% dilution, there was a decrease with no significant difference ($P > 0.05$) on day 0 and day 4 then a significant decrease ($P<0.05$) on day 8 and a significant increase ($P<0.05$) on day 12. In the 25% dilution, there was a significant decrease ($P<0.05$) from day 0 to day 4 then a further decrease to day 8 but it was not statistically significant and on day 12 a significant increase ($P<0.05$) was determined. There was a decrease in the 50 % dilution, from day 0 to day 4, a slight increase on day 8 but was no significant difference ($P>0.05$) then a significant increase ($P<0.05$) on day 12. In the 75% dilution, there was a decrease from day 0, day 4 and remained unchanged on day 8 but not significant different ($P >0.05$) and a significant increase ($P<0.05$) on day 12. The 100% effluent showed a significant decrease ($P<0.05$) from day 0 to day 4 and remained constant through day 8 then with statistical increase ($P<0.05$) on day 12.

4.1.10 Biochemical oxygen demand

The Biological Oxygen Demand (BOD) in table 4.10 had the lowest concentration in 100% dilution on day 0 and highest concentration in 75% dilution on day 0. BOD concentration in all the dilutions i.e. BBM to 100% effluent, increased from day 0 to day 4, then a decrease on day 8 and day 12 but there was no significant difference at $P > 0.05$ during the study period.

4.1.11 Chemical oxygen demand

The Chemical Oxygen Demand (COD) in table 4.11 showed the lowest concentration in 0% dilution on day 0 and highest in 100% on effluent day 0. The COD in BBM reduced from day 0 through day 12 but the variations observed did not differ significantly at $P > 0.05$. In the 0% dilution there was a significant increase ($P<0.05$) on day 4 from day 0, day 8 the COD decreased to day 12, the variations observed on day 8 and day 12 was not significant ($P>0.05$) as compared to day 4. Observations from 5% dilution showed a significant increase ($P<0.05$) on day 4 from day 0, a decrease on day 8 and a decrease in

COD on day 12 but the variations observed on day 8 and day 12 did not differ significantly (P>0.05) as compared to day 4. In the 10% and 25% dilutions, the COD increased from day 0 to day 4, decreased on day 8 and day 12 but the changes observed was not statistically significant (P>0.05). COD in the 50% dilution decreased steady from day 0 to day 12 but the changes observed was not statistically significant (P>0.05). In the 75% dilution, there was a significant decrease (P<0.05) from day 0 to day 4 then further decrease on day 8 and day 12 but the difference observed was not significant (P>0.05) when compared to day 4. The COD in 100% effluent showed a significant decrease at P<0.05 from day zero to day 4, a decrease on day 8 which was not significant at P>0.05 compared to day 4 and a significant decrease (P<0.05) on day 12.

4.1.12 Cadmium

The Cadmium concentration in table 4.12 was observed to be highest in 100% effluent on day 0 and lowest in 0% dilution on day 0. In BBM and 0% dilution, there was minute detection of Cadmium and therefore there was no significant difference at P > 0.05 during the period of study. Cadmium concentration in 5% dilution significantly decrease (P<0.05) from day zero to day 4, then the concentration of cadmium in the sample remained unchanged on day 8 and day 12 but the variations observed was not significant (P > 0.05). In 10% dilution, cadmium concentration reduced from day 0 to day 4 and remained unchanged in day 8 and day 12 but the variations observed was not significant at P > 0.05. The 25%, 50%, 75% dilutions and 100% effluent are similar with the 5% dilution with a significant decrease from day 0 to day 4 then the concentration of cadmium in the sample remained unchanged on day 8 and day 12 but the variations observed was not significant (P > 0.05).

Table 4.10: Effect of *Chlorella vulgaris* on Biological Oxygen Demand (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	48.50±12.50[a]	98.50±2.00[a]	90.00±60.00[a]	86.95±4.55[a]
0%	1.35±0.75[a]	95.25±24.75[a]	79.75±30.25[a]	25.00±5.00[a]
5%	2.25±0.45[a]	97.25±52.25[a]	89.50±1.00[a]	30.75±10.25[a]
10%	11.00±0.8[a]	105.50±45.5[a]	60.75±20.25[a]	30.25±19.75[a]
25%	108.90±2.10[a]	117.25±51.75[a]	90.75±20.25[a]	55.50±24.00[a]
50%	119.50±1.50[a]	129.00±48.00[a]	106.75±13.25[a]	107.75±7.25[a]
75%	131.25±19.25[a]	165.50±76.00[a]	115.50±4.5[a]	109.50±7.50[a]
100%	0.00±0.00[a]	177.25±74.25[a]	129.50±11.50[a]	110.50±10.50[a]

Table 4.11: Effect of *Chlorella vulgaris* on Chemical Oxygen Demand (mg/l) at different dilutions treated tannery effluent

	DAYS			
Dilution	0	4	8	12
BBM	683.33±145.18[a]	553.33±21.86[a]	543.33±36.67[a]	410.00±68.07[a]
0%	0.00±0.00[b]	520.00±32.15[a]	500.00±20.82[a]	480.00±25.16[a]
5%	83.33±37.56[b]	633.33±78.81[a]	470.00±5.77[a]	446.67±14.53[a]
10%	430.00±65.57[a]	523.33±24.04[a]	490.00±5.77[a]	440.00±32.15[a]
25%	363.33±121.70[a]	570.00±26.46[a]	503.33±29.63[a]	490.00±28.87[a]
50%	733.33±90.25[a]	566.67±28.48[a]	545.00±5.00[a]	490.00±0.00[a]
75%	853.33±78.60[a]	566.67±21.86[b]	523.33±23.33[b]	493.33±20.28[b]
100%	970.00±173.21[a]	595.00±45.00[ab]	563.33±32.83[ab]	473.33±8.82[b]

Table 4.12: Effect of *Chlorella vulgaris* on Cadmium (mg/l) at different dilutions treated tannery effluent

	DAYS				
Dilution	0	4	8	12	R.E (%)
BBM	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0
0%	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0
5%	0.21±0.05[a]	0.01±0.00[b]	0.01±0.00[b]	0.01±0.00[b]	95
10%	0.21±0.21[a]	0.01±0.01[a]	0.01±0.00[a]	0.01±0.00[a]	95
25%	0.23±0.05[a]	0.01±0.00[b]	0.01±0.00[b]	0.00±0.00[b]	100
50%	0.33±0.06[a]	0.02±0.01[b]	0.01±0.01[b]	0.01±0.00[b]	97
75%	0.48±0.16[a]	0.01±0.00[b]	0.01±0.00[b]	0.01±0.00[b]	98
100%	0.71±0.30[a]	0.01±0.00[b]	0.01±0.00[b]	0.01±0.00[b]	99

Data is represented in mean±S.E
Analysis was done in triplicate
Means with the same alphabet superscript across row are not significant at (P > 0.05)

4.1.13 Chromium

The Chromium concentration in table 4.13 was observed to be highest in 100% effluent on day 0. The concentration of chromium in BBM to the 75% dilution decreased continuously from day 0 to day 12 except for 0% dilution which was observed the concentration of chromium remains unchanged. The variations observed were not statistically significant at $P > 0.05$. In the 100% effluent, there was a significant decrease ($P<0.05$) from day 0 to day 4, reduced from day 4 to day 8 and remained unchanged on day 12 but the variations observed on day 8 and day 12 was significant ($P>0.05$) as compared to day 4.

4.1.14 Lead

The Lead concentration in table 4.14 was observed to be highest in 100% effluent on day 0. In BBM and 0% dilution, the lead concentrations were minute. In 5% dilution, Lead concentration decreased significantly ($P < 0.05$) from day 0 to day 4 then continue to decreased on day 8 to day 12 but not significant statistically ($P > 0.05$) when compared to day 4. In 10% dilution, the Lead concentration decreased from day 0 to day 4, then decreased on day 8 and day 12 but the variation was not statistically significant ($P > 0.05$). In 25% dilution, there was a continuous decreased in Lead from day 0 to day 12 and the differences were not significant ($P > 0.05$). The variation in 50% dilution showed significant decrease ($P < 0.05$) as observed from day zero to day 4 and later a decrease as seen from day 8 to day 12. A decrease in Lead observed in 75% dilution from day 0 to day 4 was significant and a continuous decrease on day 8 and day 12, the variations in day 4, day 8 and day 12 was no statistically significant ($P > 0.05$). In 100% effluent, there was a significant decrease ($P < 0.05$) from day 0 to day 4, a decreased on day 8 and day 12 with no statistical difference ($P > 0.05$) from day 4 to day 12.

Table 4.13: Effect of *Chlorella vulgaris* on Chromium (mg/l) at different dilutions treated tannery effluent

	DAYS				
Dilution	0	4	8	12	R.E (%)
BBM	0.01±0.00[a]	0.01±0.00[a]	0.00±0.00[b]	0.00±0.00[b]	0
0%	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0
5%	0.08±0.01[a]	0.07±0.01[a]	0.06±0.03[a]	0.04±0.03[a]	50
10%	0.12±0.01[a]	0.07±0.03[a]	0.07±0.01[a]	0.05±0.01[a]	58
25%	0.22±0.12[a]	0.09±0.03[a]	0.08±0.03[a]	0.04±0.02[a]	82
50%	0.23±0.06[a]	0.11±0.05[a]	0.09±0.03[a]	0.08±0.03[a]	65
75%	0.29±0.09[a]	0.09±0.04[a]	0.07±0.02[a]	0.06±0.02[a]	79
100%	0.39±0.12[a]	0.06±0.03[b]	0.05±0.01[b]	0.05±0.01[b]	74

Table 4.14: Effect of *Chlorella vulgaris* on Lead (mg/l) at different dilutions treated tannery effluent

	DAYS				
Dilution	0	4	8	12	R.E (%)
BBM	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0
0%	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0
5%	0.64±0.01[a]	0.19±0.09[b]	0.14±0.12[b]	0.03±0.01[b]	95
10%	0.73±0.00[a]	0.14±0.05[b]	0.13±0.04[b]	0.09±0.06[b]	88
25%	0.74±0.34[a]	0.16±0.06[a]	0.13±0.09[a]	0.11±0.04[a]	85
50%	1.13±1.13[a]	0.19±0.05[b]	0.11±0.05[b]	0.05±0.02[b]	96
75%	1.21±0.07[a]	0.19±0.03[b]	0.11±0.08[b]	0.09±0.04[b]	93
100%	1.59±0.10[a]	0.15±0.03[b]	0.13±0.06[b]	0.10±0.04[b]	94

Data is represented in mean ± S.E
Analysis was done in triplicate
Means with the same alphabet superscript across row are not significant at ($P > 0.05$)

4.1.15 Comparative study among the dilution of tannery

In Table 4.15, analysis of variance for physicochemical and growth parameters in the samples throughout the period of bioremediation. pH was highest in BBM (8.86) and significantly different to 25%, 50%, 75% and 100% dilution but there was no significant difference among 25%, 50%, 75% and 100% dilution. 5% dilution had the lowest pH (7.41) and is not significant different with 0% dilution and 10% dilution.

Electrical conductivity (EC) was highest in 100% dilution (5279.88 µS/cm) and was significantly different to 75% dilution. 75% dilution is significantly different to 50% dilution and 50% dilution was significantly different to BBM and 25% dilution and between BBM and 25% dilution. There was no significantly different between 5% dilution and 10% dilution but significant different to 0% dilution with the lowest EC (152.63 µS/cm).

Total Dissolved Solid (TDS) 100% dilution has the highest TDS (2649.00mg/l) and there was significant differences to 75% dilution. 75% dilution is significantly different to 50% dilution and 50% dilution was significantly different to BBM and 25% dilution and between BBM and 25% dilution. There was no significantly different between 5% dilution and 10% dilution but significant different to 0% dilution with the lowest TDS (76.38mg/l).

Nitrate concentration was highest in BBM (111.00mg/l) and there was significant difference to all the other dilution of tannery therefore, the nitrate concentration among them was not significantly different. The lowest nitrate concentration is in 5% dilution.

Sulphate concentration is highest in 100% dilution (506.25mg/l) and is significantly different with 75% dilution. In 75% dilution is significant difference to 50% dilution and 50% dilution is significant different to 25% dilution and BBM. There is no significant

different among 0% dilution, 5% dilution and 10% dilution with the lowest sulphate concentration is in 5% dilution (82.50 mg/l).

Phosphate concentration is highest in 100% dilution (111.38mg/l) and was not significantly difference to 75% dilution but significantly difference to BBM and 50% dilution. There is no significant difference between 10% dilution and 25% dilution and is comparable to 0% dilution and 5% dilution but it is not significant different between them with 0% dilution having the lowest phosphate concentration 15.00mg/l.

Free Chlorine concentration was highest in 100% dilution (2.45mg/l) and was not significantly different to 75% dilution but significantly different to BBM, 5% dilution, 10% dilution, 25% dilution and 50% dilution. 5% dilution, 10% dilution, 25% dilution is comparable 0% dilution with the lowest concentration (0.41 mg/l).

Dissolved oxygen (DO) was highest in 0% dilution (3.89 mg/l) and is no significant difference to BBM, 10%, and 100% dilutions but significantly different to 5% dilution, 25% dilution, 50% dilution and 75% dilution. 50% dilution has the lowest concentration (2.20 mg/l) but comparable to BBM, 10% and 100% dilutions.

The highest biological oxygen demand (BOD) concentration is in 75% dilution (131.93 mg/l) and the lowest concentration in 0% but there was no significant difference among the various dilutions.

Chemical oxygen Demand (COD) was highest in 100% dilution (696.25mg/l) and it was not significant different to BBM, 50%, 75% and 100% dilutions. There is no significant difference between 10% and 25% dilution and the lowest COD concentration is 0% dilution (366.25mg/l) and was comparable to 5%, 10% and 25% dilutions.

The concentration of Alkalinity was highest in 100% dilution (567.75mg/L $CaCO_3$) and differ significantly to 75%. Alkalinity concentration in 75% dilution is significantly different to 50% dilution and there is no significant difference between BBM and 25%

dilution. There is no significant difference between 5% dilution and 10% dilution with 5% dilution having the lowest alkalinity concentration at 108.50mg/l $CaCO_3$.

Chromium concentration was highest in 100% dilution (0.16mg/l) although it did not differ significantly with 5%, 10%, 25%, 50% and 75% but 5%, 10% 25% and 50% were comparable to 0% dilution and BBM.

Lead concentration in 100% was highest with 0.48mg/L and is not significantly different with 5%, 10%, 25%, 50% and 75% dilutions but significantly different from 0% dilution and BBM. 0% dilution and BBM is comparable to 5%, 10% and 25%.

Cadmium concentration is highest in 100% dilution (0.26mg/l) and is not significantly different to 75% dilution. There is no significant difference among 5%, 10%, 25% and 50% and comparable to BBM and 0% dilution.

The 5% dilution had the highest algal dry weight at 44.18mg/L and was comparable to 0% dilution and BBM. BBM and 0% dilution are statistically comparable to sample 10% and 25% dilutions, and in turn 10% and 25% dilutions were comparable to 50%, 75% and 100% dilutions.

Cell count of *Chlorella vulgaris* was highest in BBM ($95.57x10^6$/ml) and was comparable to 0% dilution. 0% dilution was statistically comparable to 5% and 10% dilutions but differ significantly to 25% dilution. The 25% dilution was statistically comparable to 50% dilution but differ significantly with 75% and 100% dilutions. There was no significant difference between 75% and 100% dilutions but was comparable to 50% dilution.

Lipid Yield was highest in BBM but it was not significantly different in 0% dilution and other dilutions of tannery

Table 4.15: Comparative effect of *Chlorella vulgaris* on physicochemical parameters among treated tannery effluent

	BBM	**0%**	**5%**	**10%**	**25%**	**50%**	**75%**	**100%**
pH	8.86^{a}	7.57^{c}	7.41^{c}	7.57^{c}	8.09^{b}	8.11^{b}	8.23^{b}	8.17^{b}
E C µS/cm	1022.13^{d}	152.63^{f}	360.13^{e}	492.71^{e}	1115.57^{d}	1759.60^{c}	4465.00^{b}	5279.88^{a}
TDS mg/L	510.50^{d}	76.38^{f}	173.88^{e}	246.57^{e}	559.14^{d}	879.40^{c}	2095.57^{b}	2649.00^{a}
Nitrate mg/L	111.00^{a}	24.54^{b}	28.25^{bc}	30.00^{bc}	30.43^{bc}	43.40^{bc}	48.43^{bc}	58.75^{b}
Sulphate mg/L	131.25^{de}	108.88^{e}	82.50^{e}	100.00^{e}	194.29^{d}	278.00^{c}	417.14^{b}	506.25^{a}
Phosphate mg/L	61.13^{b}	15.00^{d}	15.84^{d}	22.86^{cd}	29.00^{cd}	41.00^{bc}	91.43^{a}	111.38^{a}
Free Chlorine mg/L	1.26^{b}	0.40^{c}	0.77^{bc}	0.99^{bc}	0.76^{bc}	1.30^{b}	2.27^{a}	2.45^{a}
DO mg/L	2.88^{ab}	3.89^{a}	2.26^{b}	2.76^{ab}	2.51^{b}	2.20^{b}	2.57^{b}	2.83^{ab}
BOD mg/L	80.99^{a}	50.34^{a}	54.94^{a}	57.83^{a}	95.04^{a}	114.60^{a}	131.93^{a}	104.31^{a}
COD mg/L	592.50^{abc}	366.25^{d}	422.50^{cd}	470.00^{bcd}	458.57^{bcd}	626.00^{ab}	614.29^{ab}	696.25^{a}
Total Alkalinity mg/L$CaCO_3$	247.75^{cd}	136.25^{de}	108.50^{e}	120.29^{e}	187.14^{de}	304.40^{c}	438.57^{b}	567.75^{a}
Eff. Cr mg/L	0.00^{bc}	0.00^{c}	0.07^{abc}	0.06^{abc}	0.08^{abc}	0.12^{abc}	0.13^{ab}	0.16^{a}
Eff. Pb mg/L	0.00^{b}	0.00^{b}	0.28^{ab}	0.22^{ab}	0.32^{ab}	0.34^{a}	0.43^{a}	0.48^{a}
Eff. Cd mg/L	0.00^{c}	0.00^{c}	0.05^{bc}	0.05^{bc}	0.08^{bc}	0.08^{bc}	0.17^{ab}	0.26^{a}
Dry weight mg/L	26.75^{ab}	34.37^{ab}	44.18^{a}	15.51^{bc}	21.00^{bc}	3.22^{c}	0.00^{c}	0.00^{c}
Cell count 10^6/ml	95.57^{a}	77.67^{ab}	56.14^{bc}	49.90^{bc}	41.67^{c}	34.90^{cd}	5.81^{d}	5.38^{d}
Lipid Yield %	57.25^{a}	37.78^{a}	45.00^{a}	50.97^{a}	31.75^{a}	31.34^{a}	28.14^{a}	24.52^{a}

Means with the same alphabet superscript across row are not significant at $P<0.05$

4.1.16 Relationship among physicochemical parameters and dilutions of tannery

The first (69.89%) and the second (17.37%) axes of the principal component analysis (PCA) accounted for 87.26% of the variation in the environmental data. Figure 4.1 revealed the relationship among the physicochemical parameters. The physicochemical parameter such as nitrate - nitrogen, phosphate - phosphorus, free chlorine, total alkalinity, BOD, COD, pH showed a positive correlation ($P< 0.05$) among them and a negative correlation ($P< 0.05$) with 5%, 10%, and 25% dilutions of tannery and TDS, EC, Sulphate, Cd, Cr, Pb, are positively correlated among themselves and with 50%, 75%, and 100% but negatively correlated to DO and 0% dilution and BBM.

The canonical correspondence analysis was observed to put together physicochemical parameters, tannery dilutions, algal growth (cell count and dry weight) and lipid yield in to groups. Less diluted tannery effluent (75% and 100% dilutions) where grouped with BOD, Alkalinity, phosphate, sulphate, free chlorine, TDS, EC, Cr, Cd, and Pb. The physicochemical parameter favour lipid yield but does not favour algal cell count both the positive and negative controls (BBM and 0% dilution) and dissolved oxygen concentration. Algal dry weight favour 5% and 25% dilutions and grouped nitrate, pH and COD to 10% and 50% dilutions (fig. 4.2).

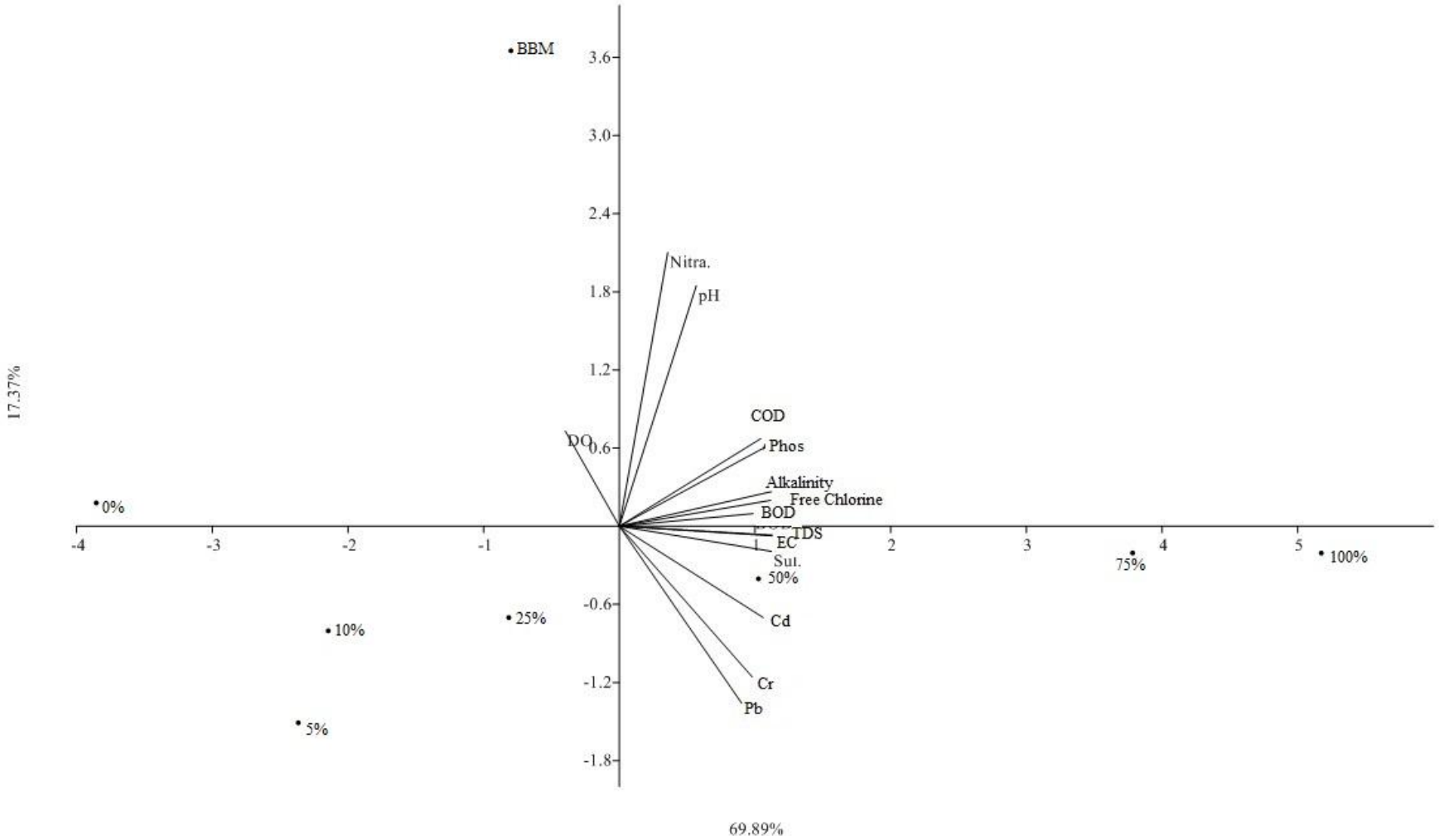


Fig 4.1: The comparative study showing relationship among the physicochemical parameter using PCA biplot analysis

Where BBM - Bold Basal Medium, 0% – distilled water, 5% - 5% effluent , 10% – 10% effluent , 25% – 25% effluent , 50% – 50% effluent , 75% – 75% effluent , 100% – 100% effluent , COD – Chemical Oxygen Demand, BOD – Biological Oxygen Demand, DO – Dissolved Oxygen, EC - Electrical Conductivity, TDS – Total dissolved Solids, Pb – Lead, Cd – Cadmium, Cr – Chromium

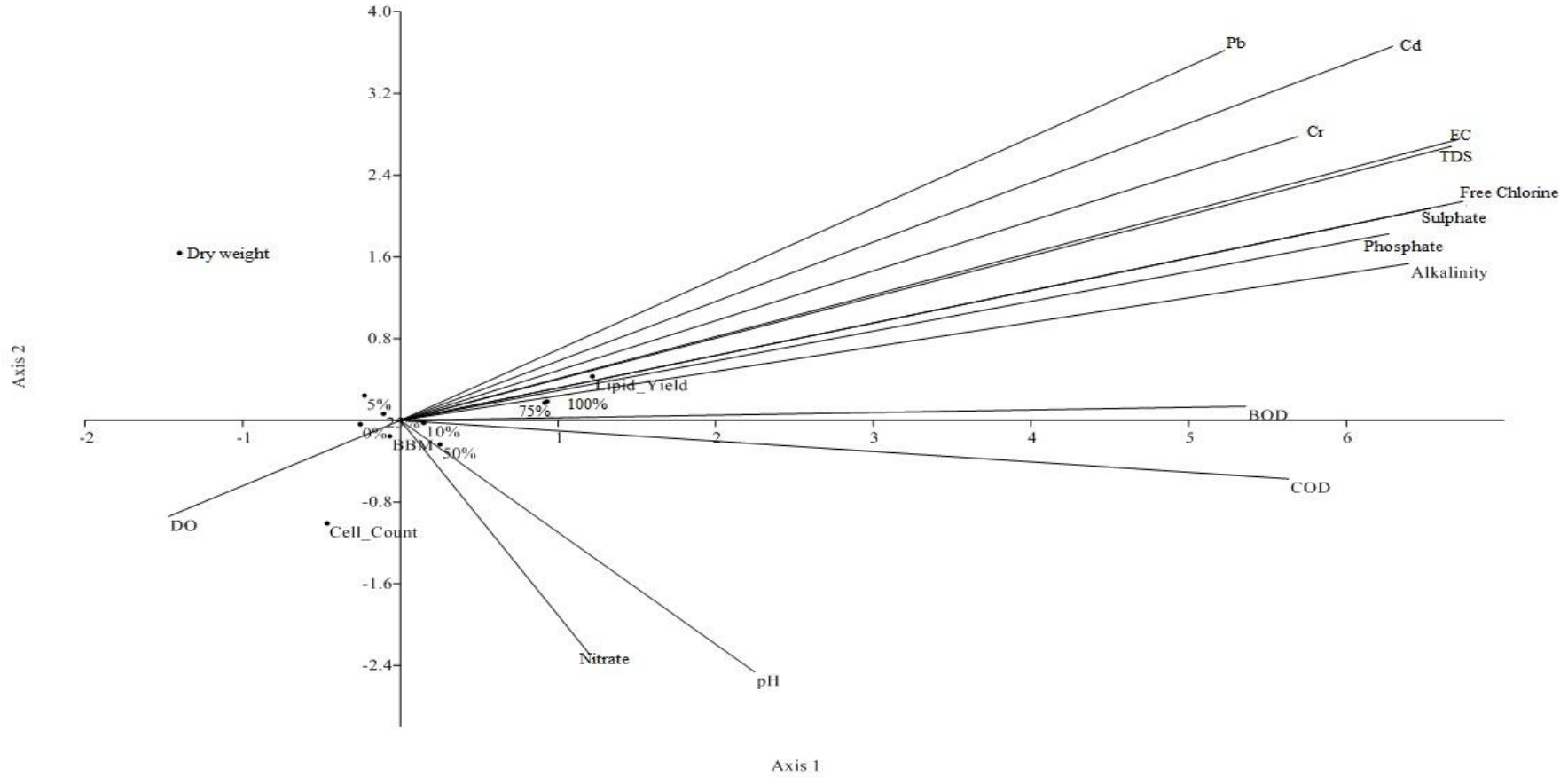


Fig 4.2: Canonical Correspondence Analysis (CCA) triplot for physicochemical parameters, tannery dilutions, growth parameters and Lipid yield

Where BBM - Bold Basal Medium, 0% – distilled water, 5% - 5% effluent , 10% – 10% effluent , 25% – 25% effluent , 50% – 50% effluent , 75% – 75% effluent , 100% – 100% effluent , COD – Chemical Oxygen Demand, BOD – Biological Oxygen Demand, DO – Dissolved Oxygen, EC - Electrical Conductivity, TDS – Total dissolved Solids, Pb – Lead, Cd – Cadmium, Cr – Chromium

4.1.17 Cell count

Figure 4.3 shows the cell count graph for *Chlorella vulgaris* from the analysis of variance. In BBM the highest cell count was observed on day 8 (159.33±67.84) and was not significantly different ($P < 0.05$) from all the days during the period of treatment. In 0% dilution, the highest cell count was observed on day 8 (112.00± 32.87) and was not significantly different ($P < 0.05$) from all the days during the period of treatment. The cell count in 5% dilution was highest on day 14 (99.67±39.35) and was not significantly different ($P < 0.05$) from all the days during the period of treatment. In 10% dilution, highest cell count was observed on day 14 (121.00 ± 21.22) was comparable ($P > 0.05$) to day 12 and day 10 but not significantly different ($P < 0.05$) from day 8 and day 6. The highest cell count in 25% dilution was observed on day 12 (85.00±30.92) and was not significantly different ($P < 0.05$) from all the days during the period of treatment. In the 50% dilution highest cell count was observed on day 14 (69.33 ± 3.38) and comparable ($P > 0.05$) to the cell count on day 10 and day 8. Cell count on day 12 and day 6 was not significant ($P < 0.05$) between them but comparable ($P > 0.05$) to day 4 and day 2. In the 75% and 100% dilutions the highest cell count was observed on day 14 (11.33±3.53 and 8.00±0.57 respectively) but from statistical analysis it was not significantly different ($P < 0.05$) compared to all the other days during the period of remediation.

4.1.18 Optical density

Figure 4.4 shows the optical density graph for *Chlorella vulgaris* from the analysis of variance. In BBM the highest optical density was observed at day 14 (0.33±0.03) and from the statistical analysis shows that the optical density day 14 was not significantly different ($P < 0.05$) from that observed in day 10 and comparable ($P > 0.05$) to the optical density day 12, day 8 and day 6. Optical density on day 4 was not significant ($P < 0.05$) to the observed density on day 2. In the 0% and 5% dilutions the highest optical

density was observed on day 10 (0.33±0.10 and 0.53±0.23 respectively) but from statistical analysis it was not significantly different ($P < 0.05$) compared to all the other days during the period of remediation. In the 10% dilution, highest optical density was observed on day 14 (0.30±0.03) and comparable ($P < 0.05$) to day 10. The optical density of day 12 and day 6 was not significant ($P > 0.05$) between them but comparable ($P < 0.05$) to day 14 and day 2 and significantly different ($P < 0.05$) from day 4 which has the lowest optical density. In the 25% and 50% dilutions, the highest optical density was observed on day 14 (0.35±0.12 and 0.05±0.05 respectively) but from statistical analysis it was not significantly different ($P > 0.05$) compared to all the other days during the period of remediation. During the period of treatment the optical density was 0.00 in the 75% and 100% dilutions because there was no difference between the blank and the samples owing to slow growth of algae.

4.1.19 Dry weight

Figure 4.5 shows the dry weight graph for *Chlorella vulgaris* from the analysis of variance. The highest algal dry weight in BBM was found on day 14 (57.89±10.85mg/L) and from the statistical analysis shows that the dry weight on day 14 was statistically comparable ($P > 0.05$) to day 10. There was no significant difference ($P < 0.05$) in the dry weight on Day 6, day 8 and day 12 but was comparable ($P > 0.05$) to day 2 and day 4. The 0% dilution had the highest dry weight was seen on day 10 (41.98±10.83) but there was no significant difference ($P < 0.05$) on all the days of bioremediation. Similarly in the 5% dilution, the highest algal dry weight was seen on day 14 (66.42±7.32) but there was no significant difference ($P < 0.05$) on all the days of bioremediation. Algal dry weight in the 10% dilution was on day 14 (52.94±9.76) and was comparable to dry weight on day 10 and differs significantly with day 2, day 4, day 6, day 8 and day 12.

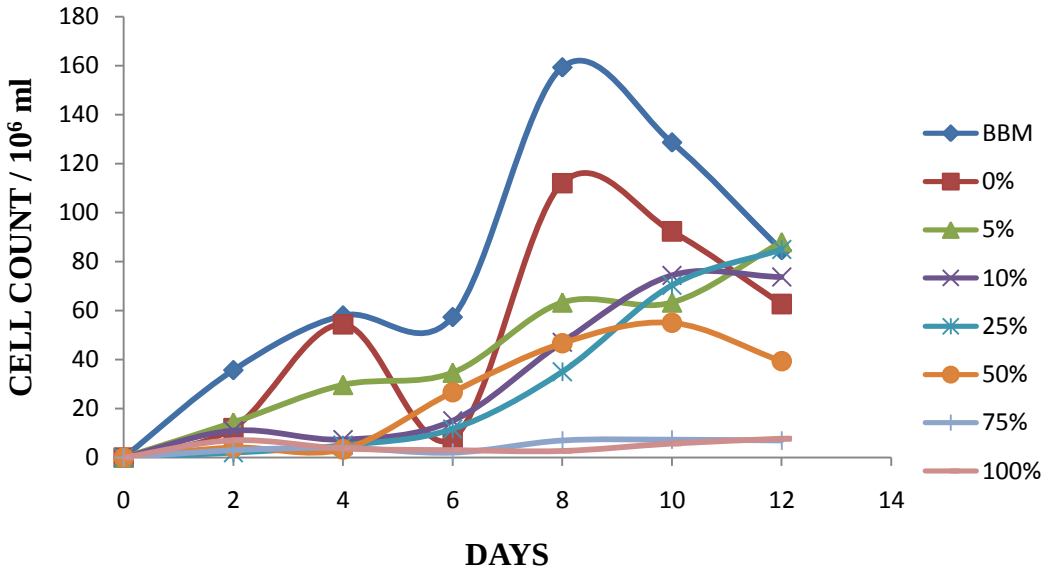


Fig. 4.3: Cell Count (10^6/ml) of *Chlorella vulgaris* cultured in various dilution of Tannery effluent

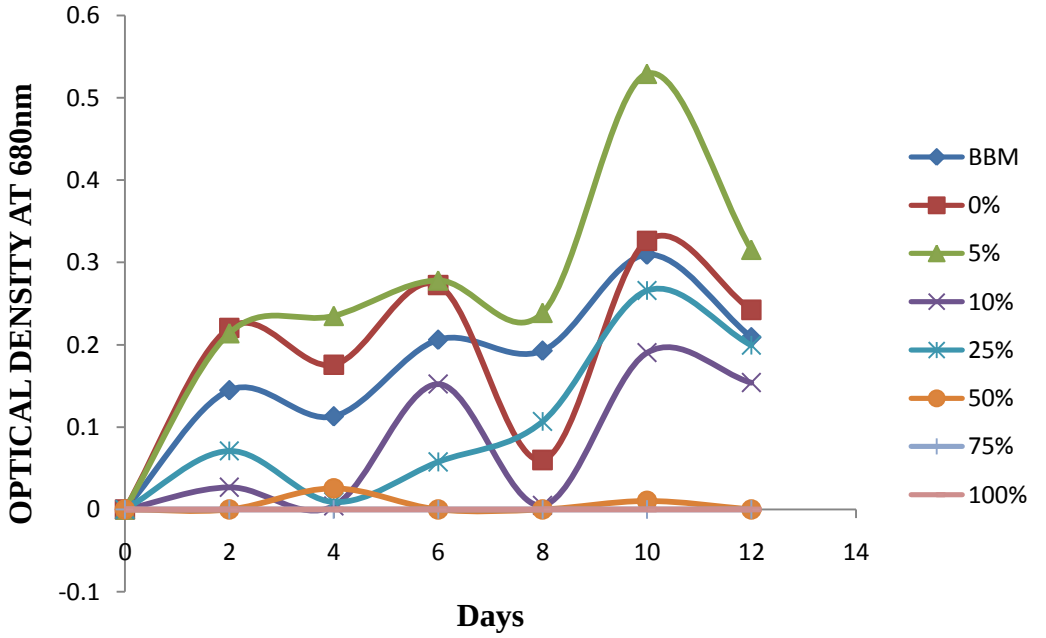


Fig. 4.4: Optical density of *Chlorella vulgaris* cultured in various dilution of tannery effluent

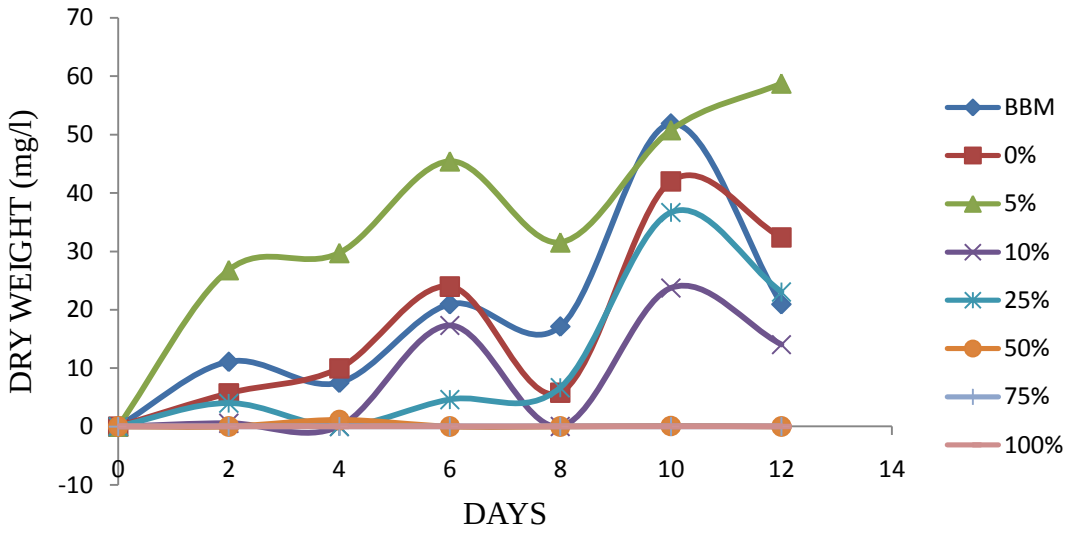


Fig. 4.5: Dry weight (mg/L) of *Chlorella vulgaris* cultured in various dilution of Tannery effluent

The highest Dry weight in the 25% and 50% dilutions were seen on day 14 (72.11±40.94 and 21.35±21.35) but there was no significant difference ($P < 0.05$) on all the days of bioremediation. In the 75% and 100% dilutions, the algal dry weight was very low.

4.1.20 Fatty acid analysis

The table 4.16 shows the various fatty acids found in the total lipid extraction from *Chlorella vulgaris* after bioremediation which was represented in percentages. In BBM, Oleic acid > Hexadecanoic acid > Docosenoic acid > Octadecanoic acid> Octadecadienoic acid > Octadecenoic acid > Pentadecanoic acid. In 0% dilution, Oleic acid > Hexadecanoic acid > Octadecanoic acid > Docosenoic acid > Octadecenoic acid > Pentadecanoic acid > Octadecadienoic acid. In 5% dilution, Oleic acid > Hexadecanoic acid > Octadecanoic acid > Eicosanoic acid > Octadecadienoic acid > Octadecenoic acid > Pentadecanoic acid. In 10% dilution, Oleic acid > Hexadecanoic acid > Octadecanoic acid > Octadecadienoic acid > Octadecenoic acid > Pentadecanoic acid > Docosenoic acid. In continuation of the fatty acid profile, 25% dilution has Oleic acid > Hexadecanoic acid > Octadecanoic acid> Docosenoic acid > Octadecenoic acid > Pentadecanoic acid > Octadecadienoic acid > Decanoic acid. The 50% dilution, Oleic acid > Octadecenoic acid > Hexadecanoic acid > Octadecanoic acid > Docosenoic acid > Octadecadienoic acid > Pentadecanoic acid > Eicosanoic acid. The 75% dilution, Oleic acid > Hexadecanoic acid > Octadecanoic acid > Octadecenoic acid > Eicosanoic acid > Pentadecanoic acid > Octadecadienoic acid. Lastly the 100% dilution, Oleic acid > Hexadecanoic acid > Octadecanoic acid > Docosenoic acid > Octadecenoic acid > Octadecadienoic acid > Pentadecanoic acid. Fatty acid content (%) in the lipid analysis was highest in the 75% dilution with 89.98% and lowest in 10% dilution (62.06). SAFA content (%) and PUFA content range from 21.77 to 42.46 and 28.68 to 55.49.

Table 4.16 Fatty acid Profile of *Chlorella vulgaris* cultured at different dilution of Tannery effluent

Fatty acids	Samples (in area percentage)							
	BBM	0%	5%	10%	25%	50%	75%	100%
Decanoic acid	ND	ND	ND	ND	0.16	ND	ND	ND
Pentadecanoic acid	2.75	2.97	1.32	2.70	3.10	6.23	1.60	1.17
Hexadecanoic acid	11.07	17.70	22.83	16.70	18.11	11.77	26.29	16.23
Octadecadienoic acid	4.12	2.68	3.93	4.66	1.98	9.77	1.01	1.94
Octadecenoic acid	3.75	5.47	2.39	4.34	4.95	13.26	2.27	4.69
Octadecanoic acid	7.95	12.66	12.53	13.98	14.07	11.44	12.74	12.17
Oleic acid	24.61	31.86	37.58	17.65	34.93	22.33	44.24	32.86
Docosenoic acid	9.37	11.83	ND	2.03	7.66	10.13	ND	7.22
Eicosanoic acid	ND	ND	3.42	ND	ND	0.35	1.83	ND
Fatty acid content	63.62	85.17	84	62.06	84.96	85.28	89.98	76.28
SAFA	21.77	33.33	40.10	33.38	35.44	29.79	42.46	29.57
PUFA	41.85	51.84	43.90	28.68	49.52	55.49	47.52	46.71
SAFA/ PUFA	0.52	0.64	0.91	1.16	0.71	0.54	0.89	0.63

Where A – Bold Basal Medium (BBM), B – distilled water, C – 5% effluent, D – 10% effluent, E – 25% effluent, F – 50% effluent, G – 75% effluent, H – 100% effluent, SAFA – Saturated Fatty acid, PUFA – Poly unsaturated Fatty acid

CHAPTER FIVE

5.0 Discussion

The inoculation of algae with 50ml Bold Basal Medium in this study reduced the pH as observed in day 4 then a gradual increase on day 8 and day 12 respectively. The pH in this study changed from neutral to alkaline which was affected by altering carbon dioxide produced due to either respiration or photosynthesis of plants in batch culture. Azov (1982) postulates that increase in pH of algal culture mainly due to decrease in the free carbon dioxide concentration in the medium if bicarbonate is the only source of carbon as in the case of batch culture. The absence of aeration or a constant stirrer is responsible of the limited CO_2 in batch culture making the pH to increase. This agrees with the study of Kotteswari *et al*. (2007) also reported a pH change from 5.62 to 9.82 and Rao *et al*., (2011) Tannery Effluent using *Chlorella vulgaris* in pH changed from 7.6 to 8.0 however disagreed with Mythili and Karthikeyan (2011) where pH of tannery effluent after treatment reduced from 8.7 to 7.2 because of constant aeration. Alkalinity is greatly associated with the rise in pH as a result of utilization of bi- carbonate as the source of carbon and the release of OH^- during photosynthetic activity (Dominic *et al*., 2009). Increased alkalinity serves as buffer in receiving water body as it maintains the pH creating a balance that is beneficial to aquatic organisms which are sensitive to change in pH.

The Electrical conductivity and Total Dissolved Solids measured is an indication of the concentration of dissolved ions in it (Azarpira *et al*., 2014). The increase of EC and TDS in low concentration of tannery dilutions in the 4 day due the addition of BBM and *Chlorella vugaris*. These findings agree with Rao *et al*., (2011) where *Chlorella vulgaris* with a reduction efficiency for EC 21% and TDS 21% respectively. The decrease of EC

and TDS based on a unique mechanism of bio-absorption/adsorption of different types of dissolved ions including various nutrients from wastewater which is used for microalgae growth (Nanda *et al*., 2010). The studies on the nutrient uptake by micro algae shows their ability for wastewater treatment by remove nutrients from polluted water bodies and used to enhance growth. Phosphorus in phosphate form is a macronutrient needed by algae to perform cellular functions and for growth (Yaakob *et al*., 2014). Phosphate reduction in our findings occurs in all the dilutions of tannery and in BBM and 0% dilution that serve as control same as the findings of Ajayan *et al*, (2015). Ahmad *et al*. (2013) reported that *Chlorella vulgaris* reduced phosphate by 99.96% from wastewater and Hammouda *et al*, (2015) *Chlorella* sp significantly reduce phosphorus concentration by 90% with increasing treatment time. The phosphorus, which is used in the algal cells mainly for production of phospholipids, adenosinetriphosphates (ATP) and nucleic acids, gets assimilated as in-organic orthophosphate, preferably as $H_2PO_4^-$ or HPO_4^{2-} (Becker 1994). The initial nitrate concentration was highest in the control i.e. bold basal medium as compared to 100% tannery effluent signifying the use of tannery effluent as an alternative medium will require bioaugmentation so as to sustain algal growth. The findings agrees with similar study by Kshirsagar (2013) with nitrate removal from wastewater at 78.08% and 70.32% when bioremediated with *C. vulgaris* and *S. quadricauda* up to 15^{th} day. Similar findings was found that *Chlorella* sp has preference for ammonia than nitrate but nitrate maintain a higher cell density for longer duration under nitrate than under ammonia (Singh and Dhar, 2007; Hii *et al*., 2011).The uptake and utilization of nitrate by algae species for their growth may be the reason for reduction in nitrate (Gupta *et al*., 2015) Once nitrate is absorbed is reduced to intermediate compounds, such as ammonium and free amino acids which are incorporated into proteins, pigments such chlorophyll, and nuclei acids for cell division (Dortch and Postel,

1989; Rückert, *et al*., 2004). These compounds serve as a long-term nitrogen storage reservoir. The significant reduction of sulphate is observed in day 4 in tannery dilution of 25%, 50%, 75% and 100% and while in dilution of 10% and control there is no significant reduction and 0% and 5% dilution, there was significant increase because of addition of algae and medium. *C. vulgaris* reduces sulphate significantly in dairy waste water from 460mg/l to 31.6mg/l on the 13th day was observed (Sreekanth, 2014).. Most of the sulphate ion culminate are the major source of anion in industrial effluents discharged in receiving water bodies. Bacterial remediation of highly polluted wastewater when dissolved oxygen `is low, sulphate serve as a substitute as electron acceptor to breakdown organic matter which produces hydrogen sulphide (Alaguprathana *et al*, 2015) whereas microalga remediation sulphate reduction is its use as nutrient for algal growth (Azarpira *et al*, 2014). *Chlorella vulgaris* in this study reduced free chlorine which supports the findings of Mutanda *et al*, (2011) that low dosages of free chlorine were found to enhance microalgal growth although one cannot be conclusive because free chlorine in wastewater can be reduced or unavailable by reaction with nitrates or ammonia to form Chloroamines.

Lowering of dissolved oxygen in day 4 and day 8 can be attributed to active cellular respiration, nutrients are converted to biochemical energy such as ATP and oxygen serves as an electron acceptor (Perry *et al*., 2007) while increased biomass and active photosynthesis is responsible for the increase of oxygen on day 12. This agrees with Promya (2008) which reports photosynthesis of the algae may be the reason for increase in DO of municipal wastewater.

BOD across the samples increased above the initial owing to inoculation of algae and medium increases organic matter in the culture. BOD indicates the oxygen required for bacteria to break down decomposable organic matter in water, BOD depletes dissolved

oxygen in the in wastewater. The 100% tannery effluent being too toxic did not support bacteria growth hence BOD concentration is recorded 0.00mg/L. Tannery effluent are characterised with high BOD due to the presence of organic matter contributed by skin and hides of animal (Nanda, 2010). In all the samples, BOD reduction was not significant during the period of treatment which can confirm the findings of Kshirsagar (2013) where *Chlorella vulgaris* in domestic waste water showed the best removal capacity of nitrate and COD while *Scenedesmus quadricauda* showed best result for BOD and phosphate reduction although Sahu (2014) reports *Chlorella vulgaris* in waste water was able to reduce BOD by 70 % in a 21 days treatment period. Metabolism of organic matter by algae is possibly responsible for the BOD reduction. (Azarpira *et al*, 2014). The COD concentration significantly day 0 from initial to day 4 in 0% and 5% dilutions because of the addition of medium and algae at inoculation and a significant decrease was observe only in 75% and 100% dilutions. COD reduction is largely not significant because it is accomplished exclusively by *C. vulgaris* whereas Kshirsagar (2014) findings of a mixed culture *Aspergillus niger* + *Chlorella vulgaris* removed COD at 72.26% or bacterial in vitro cultures reduced COD by 72.25% after a period of 72hours from sewage (Joshi and Sharma, 2002).

Photosynthetic organisms and microalgae produce oxygen that enhances the biological degradation of the organic matter in the wastewaters (Aslan and Kapdan, 2006; Brito *et al*., 2007; Hodaifa *et al*., 2010a, b). BOD and COD reduction can be attributed to the high algal growth rate and intense photosynthetic activity (Ajayan, 2015)

Comparatively, physicochemical parameter and dilution of tannery effluent, Ajayan 2015 reported that 50% dilution not significantly different from the BG-11 medium used to growth algae as an alternative but in contrast our findings show that at certain physico-chemical parameters similar at certain dilutions. In table 4.15 findings show pH in BBM

(control) was significantly different with 0% dilution and all the dilutions of tannery. Alkalinity of BBM is comparable to both sample 25% dilution and 50% dilution. EC and TDS gives an indication that 25% dilution is not significantly different from BBM. Nitrate was highest in BBM and was significantly different from other dilutions. Sulphate was not significantly different from 0% dilution and closely associate with sample 10% dilution while phosphate 50% dilution was closely associated to BBM. DO and BOD in all the dilutions were not significantly different but COD in BBM was comparable to 0% dilution and other dilution of tannery

It was observed the significant rapid removal of metal especially in Cadmium and Lead in four days from inoculation of *Chlorella vulgaris* could suggest metal ion removal can be attributed to biosorption. Physicochemical parameter such as increase in pH and decrease in ionic strength has a direct bearing on adsorption of metals in that increase in pH leads to increasing binding site for at low pH, inactivated cell wall surface becomes positively charged thereby reducing the attraction between metal ions and functional group and vice versa when the pH is high (Hussain, 2009) and decreasing ionic strength favour metal uptake by competing functional group even at fixed pH as reported by Chen (1997). Increase in cell density plays a pivotal role in metal adsorption because it provide more binding sites for metal ion (Bhattacharya *et al*., 2006)

It was observed though with little cells in 75% and 100% dilutions bioremediation takes place to agree with Shehata *et al*. (1980) that increasing concentration of phosphate in the media allows for more accumulation of metal ion. Significant reduction of cadmium and lead ion took place from inoculation of *Chlorella vulgaris* to day 4 however cadmium reduction took place on day four while lead reduction continued to day 12 although when compared to day 4 it was not significant, one can infer that cadmium adsorption was faster than other heavy metals. Cadmium reduction in this study was higher with the

reduction at 95% at 5% and 10% dilutions in 12 days when compared to the findings of Rehman *et al*. (2004) with Cd reduction of 80% in 14 days. Chromium reduction in this study was not significant in the dilutions of tannery effluent except for 100% dilution probably to the basic nature of the tannery effluent because chromium removal favours when the pH is acidic and decreases with increasing pH (Bansal *et al*., 2009). Mohammad *et al*. (2013) reported the maximum adsorption of Chromium was in a pH of 2 and Sivakumar (2015) adsorption of Cr (vi) was optimum with 88.3% at pH of 4. Adsorption of Cr at low pH values can therefore be better adsorbed by the C=O functional group on the adsorbent since oxygen is electronegative and can attract ions that are positively charged (Sharma and Forster, 1994; Olayinka *et al*., 2007). Rafique and Nazir (2013) in their findings reported an adsorption sequence by efficiency of Pb > Ni > Cd > Cu contrary to our findings that the sequence of reduction efficiency was in this order Cd > Pb > Cr. It was also observed in sample E, the highest reduction of Cadmium and Chromium but had the lowest reduction in Lead. One could infer that the binding site for the heavy metals are the same and Cd and Cr are readily adsorbed than Pb. Metal binding proteins present outside of cell membrane attract metal ions exist in solution and assist the transport to cytosol (Kaplan, 2013) which could be responsible for the slow reduction of metal ion observed from day 4 to day 12.

Chlorella vulgaris in 5% dilution of tannery effluent attained the highest optical density of 0.5 higher than the BBM control of an optical density of 0.3 but yet below optical density *Nannochloropsis oculata* (0.9), and *Tetraselmis chuii* (1.0) cultured from waste water from aquaculture as reported by SIrakov (2014). Costa *et al*. (2004) findings showed *Tetraselmis chuii* and *Dunaliella viridis* was cultivated in urban secondary sewage and observed *T. chuii* with a population density of 1.3 at 40% dilution and *D. viridis* at 1.5 at 30% dilution which were higher than control f2 culture medium.

BBM and 0% dilution had the short lag growth period which supports Chia *et al*. (2014a) who studied the impact of indigo dye effluent on *S. quadricauda* shows the highest growth rate of the microalgae was in the control. *Chlorella vulgaris* in various dilutions of tannery show longer lag growth phase because increasing concentration of effluent the organism have to adapt to the new environment before utilizing the available nutrient for growth while 5% dilution which has higher dilution and less effluent concentration had an increasing log growth phase. The high presence of toxicants in 75% and 100% dilutions could be responsible for the little growth despite the high concentration of primary nutrients such as nitrates and phosphates which agree with Ajayan (2015) that high amount of heavy metal content present in the growth medium reduced the growth of microalgae. Cell density, an important biomass parameter varied significantly from medium to medium (Chia *et al*., 2014b) but the control sample A has the highest number in cell count and decreased with increasing effluent concentration i.e. decreasing dilution rate. This clearly affirms the toxicological implication of toxicant in effluent on biomass production and growth measurement of micro algae. The PCA biplot (fig. 4.1) in this study shows there is a negative relationship with dissolved oxygen and heavy metals (Pb, Cd, Cr) and sulphate, EC and TDS which this further confirms that increase in contaminants decrease concentration of dissolved oxygen, there is a positive association with tannery dilutions of 50%, 75% and 100% and heavy metals (Pb, Cd, Cr) and sulphate, EC and TDS which implies the less diluted tannery effluent contain more physicochemical parameters. The CCA triplot in this study as shown in fig 4.2 that in the BBM and 0% dilution has a positive association with dissolved oxygen concentration and cell count which confirms in less polluted aquatic environment supports more algal cell division and the positive relationship with lipid yield and tannery dilutions of 75%, 100% and most physiocochemical parameter except for COD, pH and nitrate which implies

there is more stress in highly polluted aquatic environment therefore reduced algal cell division and cellular metabolism of lipid initiates. COD, pH and nitrate shows a positive association with 10% and 50% tannery dilution and a negative relationship with dry weight.

In table 4.15, Cell count of 0% dilution has a close relationship with BBM and with increase in dilutions of tannery effluent there was a reduction in cell count which is in contrast to the findings of Ajayan (2015) in that there was no significant difference with the cell density in 50% dilulent when compared to the growth in BG11 but there is a possibility of getting a dilution which can serve as an alternative to BBM by extending the period of culturing since tannery dilution had a longer lag growth phase in the 14 day time. In the same vein, 5% tannery effluent dilution suggests that Dry weight was significantly the highest and with a closely association to BBM.

Microalgae have a good advantage as a feedstock for biodiesel as compared to other biomass because of its fast growth and high lipid content. The lipid yield of *Chlorella vulgaris* in 10% dilution is 50.97% close to the control (BBM) with 57.25% higher than 42.10% reported by Selvarajan, *et al*. (2015). Fatty acids have a characteristic bearing on the genus of the algal species especially the type of fatty acid present and the various proportions of fatty acids are indicators if the culture of algae is mixed or mono culture of algae, presences of impurities or presences different organism such as bacteria. In this study, the various proportions of fatty acids the same qualitative composition as those reported for *Chlorella* species (Petkov, 2007). Increase in fatty acid content in the lipid analysis by Rocchetta *et al*. (2006) may be a defense mechanism to counteract oxidative stress which supports our findings with high fatty acid content in low dilutions of tannery i.e. 25% to 100% dilutions or nutrient depleted samples i.e. 0% and 5% dilutions while the control medium (BBM) and 10% dilution where similar and lower which further

proves that with bio-augmentation of nutrients such as nitrogen and phosphorus, 10% dilution would be able to sustain algal culture without causing oxidative stress. Fatty acid content in 75% dilution was highest with 89.98% compared to *Chlorella vulgaris* LC8 with 77.4% (Selvarajan *et al*., 2015). In low polluted medium similar to the control (BBM) and 0% dilution of this study PUFA is higher as compared to the SAFA and increase in SAFA that indicates storage lipids is to adapt to the environment thereby protecting its intracellular membrance structure and cellular functions (Rocchetta *et al*., 2006). The area percentage of oleic acid characterizes it as the major fatty acid in the sample, the highest area percentage observed was in 75% dilution with 44.24%. High amount of oleic acid balances oxidative stability and low temperature properties thereby improves the quality of the biodiesel that will be produced (Liu *et al*., 2011). Choi *et al*. (2011) identified abundance of oleic acid as the result of nitrogen limited conditions.

CHAPTER SIX

6.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

6.1 Summary

Chlorella vulgaris utilizes nutrient and reduces the electrical conductivity and total dissolved solids as well in the various dilutions of tannery. It improves water quality by reducing chemical oxygen demand and biological oxygen demand and increases the dissolved oxygen content by releasing oxygen during photosynthesis. It maintains balance in the medium by increasing the alkalinity serving as a buffer to a change in pH.

Chlorella vulgaris has high reduction efficiency in 25% dilution for cadmium and Chromium and 50% dilution for lead. The sequence of reduction is established in the order Cd > Pb > Cr with Cd having the highest reduction rate.

Cell count of *Chlorella vulgaris* was highest in BBM on day 8, optical density in 5% dilution was highest on day 10 and calculated dry weight was highest in 25% dilution on day 14. Both optical density and calculated dry weight, 5% dilution has the shortest lag phase. Cell count shows a positive relationship with BBM and 0% dilution and a negative relationship with 75% and 100% dilution and cell count showed a negative association with lipid yield.

Chlorella vulgaris the lipid yield was highest in BBM (57.27%) followed by 10% dilution (50.97%). *Chlorella vulgaris* fatty acid content (area %) was highest in 75% dilution with oleic acid highest in the entire samples. *Chlorella vulgaris* fatty acid profile suggests it would be a good feedstock for biodiesel production having high amount of oleic acid which balances oxidative stability and low temperature properties.

6.2 Conclusions

Chlorella vulgaris is a good candidate for bioremediation for being able to withstand toxicity of tannery even in low dilutions and shows good ability to sequester nutrients in water so as to reduce eutrophication promoting growth.

i. *Chlorella vulgaris* is able to significantly reduce high physicochemical parameter before inoculation that is toxic to aquatic life in all dilutions of tannery at the end of the period for bioremediation.

ii. Physicochemical parameter such as pH, nitrate, phosphate, alkalinity, BOD and COD had a positive relationship among them and DO shows a negative relation with increase in heavy metal (Cd, Cr and Pb) concentration and physicochemical parameter such as TDS, EC and sulphate.

iii. Cell count shows a negative relationship 75% and 100% tannery dilution with support more lipid production but a positive relationship with BBM and 0% dilution.

iv. Lipid yield at the end of bioremediation was highest in BBM (control) with 57.25% followed by 10% dilution with 50.97%. PUFA was generally higher than SAFA except for 10% dilution with SAFA was higher than PUFA and oleic acid being the most abundant in all the samples.

6.3 Recommendations

Authority responsible for regulating industrial emission should ensure and enforce that international best practices are carried in treating the effluent before discharge. They should ensure all discharges should be channeled to undergo secondary treatment using microalgae before release into any water body.

To understand the role and growth of algae in various dilution of tannery having observed a long lag phase for either remediating the effluent or finding alternative media to culture algae to reduce the cost, culturing for a period beyond 14 days to make up for time for the algae to adapt to the new environment.

Having determined the fatty acid profile from *Chlorella vulgaris* UTEX 2714 further physicochemical parameters should be analysed such as free fatty acid value, iodine value, saponification value, cetane number to know its suitability for biodiesel.

CONTRIBUTION TO KNOWLEDGE

Title: Bioremediation of tannery effluent and lipid production by *Chlorella vulgaris* (Beijerinck) UTEX 2714

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Programme: MSc. Biology

The contributions to scientific knowledge from this study are as follows:

1. *Chlorella vulgaris* UTEX 2714 maintains balance in the medium by increasing the alkalinity serving as a buffer to a change in pH with the lowest 108.50 mg/l and highest 567.75mg/l across the dilutions
2. Physicochemical parameter such as pH, nitrate, phosphate, alkalinity, BOD and COD had a positive relationship among them and DO shows a negative relation with increase in heavy metal (Cd, Cr and Pb) concentration and physicochemical parameter such as TDS, EC and sulphate.
3. Cell count shows a negative relationship 75% and 100% tannery dilution with support more lipid production but a positive relationship with BBM and 0% dilution.
4. Lipid yield at the end of bioremediation was highest in BBM (control) with 57.25% followed by 10% dilution with 50.97%. PUFA was generally higher than SAFA except for 10% dilution with SAFA was higher than PUFA and oleic acid being the most abundant in all the samples.

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Appendix I

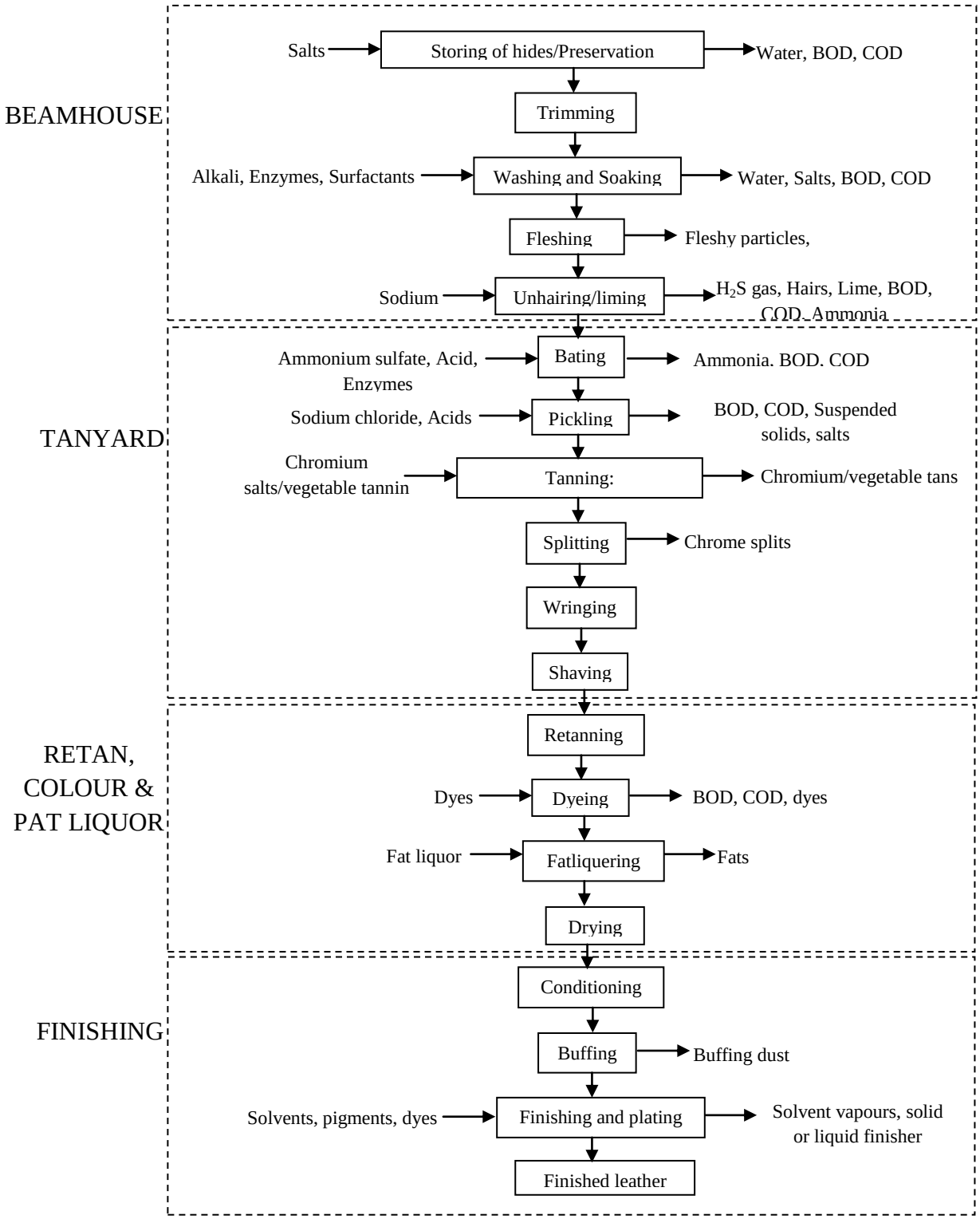


Figure 1: Showing major tanning operations and nature of the aggregated waste generated

Appendix II

Table 1: Percentage lipid content from different types of algae (Becker, 1994; Demirbas and Demirbas, 2011)

Specie of sample	Lipids (%)
Scenedemus obliquus	12 – 14
Scenedemus quadricauda	1.9
Scenedemus dimorphus	16 – 40
Chlamydomonas rheinhardii	21
Chlorella vulgaris	14 – 22
Chlorella pyrenoidosa	2
Spirogyra sp.	11 – 21
Dunalielia bioculata	8
Dunalielia salina	6
Euglena gracilis	14 – 20
Prymnesium parvum	22 – 38
Tetraselmis maculate	3
Porphyridium cruentum	9 – 14
Spirulina platensis	4 – 9
Spirulina maxima	6 – 7
Synechoccus sp.	11
Anabaena cylindrical	4 – 7